DETECTION AND QUANTITATION OF STAPHYLOCOCCUS AUREUS
DEOXYRIBONUCLEASE IN CHEESE

by

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ABSTRACT

Detection and Quantitation of *Staphylococcus aureus* Deoxyribonuclease in Cheese

by

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A specific method has been developed for the extraction and measurement of staphylococcal nuclease in cheese in which *Staphylococcus aureus* has grown. Ten grams of a cheese sample were homogenized with ninety milliliters of pH ten buffer for three minutes. Ammonium sulfate fractionation was used and a forty to eighty percent fraction was collected and concentrated using ultrafilters. The nuclease activity was determined using a toluidine blue deoxyribonucleic acid agar slide method and a spectophotometric method. The DNA agar slide method was used to compare staphylococcal growth with nuclease production in cheese under varying conditions. When *Staphylococcus aureus* plate counts indicated populations of three to four thousand per milliliter, it was possible to detect nuclease in the cheese sample.

A method has also been developed to detect *Staphylococcus aureus* colonies using DNase agar and toluidine blue, utilizing the heat stability of *Staphylococcus aureus* nuclease.

(89 pages)
INTRODUCTION

Outbreaks of cheese-borne staphylococcal intoxications in people have been reported since 1884 (48). Food poisoning due to ingestion of cheese containing staphylococcal enterotoxin has been a significant public health problem (3, 12, 53, 59, 77, 78, 87, 104, 108, 116, 132, 140, 143, 144, 151, 161, 162). In a survey of a wide variety of cheeses obtained at the consumer level, Mickelsen et al. (108) reported that 70.4 percent contained *Staphylococcus aureus*. When the numbers of *S. aureus* have been high enough to produce food poisonings, manufacturers have had to rely upon limited records and unsubstantiated speculation as to the exact conditions that caused any given outbreak (8, p. 359). Methods for staphylococcal enterotoxin detection are slow and expensive. A more rapid screening method is necessary to aid regulatory officials in epidemiological investigations and to aid manufacturers in the prevention of enterotoxin development in their products (142, 143).

A possible enzymatic means for detecting food contamination by staphylococci is suggested by the fact that nearly all pathogenic staphylococci produce a unique and remarkable heat-stable deoxyribonuclease (1, 47, 72). Lachica, Weiss and Deibel (96) demonstrated better correlation between enterotoxin and deoxyribonuclease production than between enterotoxin and coagulase production. They reported that 96 percent of 283 enterotoxigenic strains examined produced nuclease. This excellent correlation suggests the possibility of employing the heat-stability of this enzyme in diagnostic and
quantitative procedures.

Currently existing techniques involved in the production of fermented dairy products should be evaluated and modified where necessary to improve the protection factors in each process, thereby reducing chances for staphylococcal growth and enterotoxin production. Instead of taking several days for sample preparation for immunological assays, it is now possible to measure staphylococcal nuclease with a minimum amount of extraction and purification. This creates the possibility that a large number of cheese preparations can be economically and possibly automatically evaluated. In addition, it is possible to detect nuclease activity after bacterial cells have lost their viability. Thus, if *S. aureus* had grown in cheese during manufacture or in the cheese milk before manufacture, it would be possible to detect its development.
REVIEW OF STAPHYLOCOCCUS GENUS

History of Genus

Staphylococci were identified in purulent exudate by Pasteur in 1880 (48) and by Ogston in 1881. Although Beaker was the first to grow the organism in pure culture, the classic work of Rosenbach in 1884 laid the foundation for our present knowledge of this group. Ogston is credited with applying the name "staphylococcus" in 1881 because of the typical grapelike clusters of cocci he observed in cultures. Rosenbach, after careful and systematic study, classified the group into albus and aureus forms. By the turn of the century other varieties of staphylococci were studied and named, and their ability to produce toxic substances was recognized.

Gail M. Dack, in his chronicle of staphylococcal food poisoning (48), states that food poisoning by staphylococci predated the scientific discovery of the organism. Dack's accounts of the experiences of V.C. Vaughn and G.M. Sternberg, who independently experimented with cheeses involved in food poisoning incidents, predates its discovery. In 1914 Barber reported the first proven case of staphylococcal enterotoxin in milk (12), and the enterotoxin problem has not yet been resolved. As an example, in 1968 Zehren and Zehren (161) reported a food-poisoning outbreak of enterotoxin in cheese and the subsequent assay of samples from 4.07 million pounds of cheese for enterotoxin A.

In England and Wales staphylococcal food poisoning is less
common than salmonellae and Clostridium perfringens food poisoning. In the United States, however, it continues to be the most common type of food poisoning reported. In 1968, for example, staphylococcal food poisoning accounted for nearly one-third of all incidents reported to the World Health Organization (159).

The risk of poisoning from the ingestion of food containing enterotoxin from staphylococci is well known. The occurrence of more than one immunological type of staphylococcal enterotoxin has been conclusively established by several investigators (15, 16, 21, 22, 23, 27, 32, 34, 49, 65, 130). Classification has been based on precipitin reactions with specific antibodies, and enterotoxins A, B, C, and D are now recognized (17, 20, 21, 23, 32, 34, 45, 130).

Enterotoxin A is often implicated in food poisoning outbreaks (29). Enterotoxin B is seldom associated with food poisonings but is produced along with enterotoxin A by staphylococci from hospital patients suffering from enterocolitis (26, 139). Enterotoxin C is sometimes implicated in food poisoning and seems unique in that it is consistently emetic for monkeys but is not consistently emetic for cats. Enterotoxin D is also associated with food poisoning strains of staphylococci, but many of these also produce enterotoxins A, B, and C (29).

Significant progress has been made in the last few years in the development of assay procedures for enterotoxins and the characterization of growth of staphylococci and enterotoxin production in dairy products. The purification of enterotoxin and the demonstration of antigenicity have made possible the serological detection of enterotoxin in culture supernatants (11, 24, 25, 26, 30, 44, 64, 75, 133,
134, 153, 160) and in foods (5, 6, 10, 17, 18, 31, 33, 44, 48, 53, 55, 59, 68, 73, 74, 77, 80, 87, 106, 108, 118, 119, 124, 132, 140, 141, 144, 149, 151, 161). The serological test is more sensitive than methods involving the feeding of monkeys or parenteral injection into kittens (49, 89, 138). Unsuccessful attempts to assay enterotoxins have been tried utilizing human volunteers (52). There are several immunological methods for detecting enterotoxin including haemagglutination inhibition (88, 109, 127), but the most widely used techniques are those employing gel-diffusion (20, 36, 45). Recently a capillary tube immunological assay has been developed which can detect enterotoxin in five to 40 min depending upon antigen concentrations (67). Comprehensive reviews on the enterotoxins have been reported by Bergdoll (18, p. 1), Casman (29), and Angelotti (8, p. 359).

Cunningham, Catlin, and DeGarilhe (47) were the first to report the occurrence of a calcium activated nuclease in culture media of S. aureus. These authors partially purified this enzyme and studied its action on deoxyribonucleic acid (DNA).

The presence in various organisms and tissues of one or more specific enzymes capable of degrading nucleic acids without affecting their fundamental nucleotide structure has been suspected since the beginning of this century. The search for digestive enzymes for thymus nucleic acid was begun almost immediately after the discovery of nuclease by Kossel in 1894 (94). Iwanoff (94) was the first to demonstrate the presence of enzymes in various molds capable of liquefying thymus nucleic acid. He applied the term "nuclease" to these enzymes. Plenge in 1903 (94) found nucleases to be present in various bacteria, even in bacteria that were not capable of liquefying
gelatin. Both Iwanoff and Plenge were able to show experimentally that the liquefying action was due to nuclease activity and was not due to its capacity to act as a protease. The possible existence of specific enzymes to accomplish special stages of hydrolysis of nucleic acids was suggested by Levene and Medigraceanu in 1911 (94).

Since the discovery of staphylococcal nuclease by Cunningham, Catlin, and DeGarilhe (47) several methods of partial purification have been described (1, 40, 47, 72, 111, 137). The action of micrococal nuclease on DNA has been thoroughly studied (1, 46, 47, 51, 71, 82, 84, 94, 120, 121, 122, 123, 126, 129, 150, 152).

Measurement of nuclease activity is a sensitive means for detection of foods suspected of being contaminated with the metabolic products of S. aureus. The usefulness of the nuclease test as an indicator of suspected contamination is increased by the relative rapidity with which it can be conducted and by the fact that it requires no uncommon reagents or equipment. The specificity of the method and the lack of interference with mixed populations of microorganisms adds to its adaptability to the cheese industry.

Morphology

The staphylococci typically occur as grapelike clusters approximately 0.8 to 1.0 micron in diameter, but individual organisms or entire strains occasionally vary between the extreme limits of 0.2 and 1.2 microns. In smears from purulent exudate the cocci are seen individually, in pairs, in clusters, and even in short chains. The irregular clusters are found characteristically in smears from cultures grown on solid media. In broth cultures short chains and diplococcal
forms occur so frequently that it is often impossible to distinguish between staphylococci and streptococci by their morphology alone. Staphylococci are nonmotile and nonsporulating. Capsules may be formed in very young cultures, but they disappear within a few hours. The cells stain easily with basic aniline dyes but less so with acid dyes. They are gram-positive in a young culture but tend to gram-variability as the culture ages.

Pigment formation occurs in an agitated broth culture but less frequently in a still culture. On solid media, such as Trypticase Soy Agar or Staphylococcus Medium 110, *S. aureus* produces circular, entire, raised, convex colonies that are smooth and characteristically pigmented in shades varying from cream to orange. Nonpigmented colonies, as well as those that are lightly pigmented, often become pigmented or darker in color after a secondary incubation for a few days at room temperature as reported by Chapman (38) and Donnelly, Black, and Lewis (53). Very young colonies of *S. aureus* are colorless, but as growth takes place, a pigment is elaborated which has been classified as lipochrome. It is soluble in alcohol ether, chloroform, and benzol. The staphylococcus originally were separated into species on the basis of pigment production. Thus, the golden staphylococcus was named *S. aureus* and the white was named *S. albus*. The lemon colored staphylococcus was named *S. citreus*. Reimann (125) has demonstrated variability in staphylococci pigment production. The golden yellow staphylococci may give rise to white or translucent, colorless colonies with or without a corresponding change in colony formation and virulence.
Resistance to Adverse Conditions

Staphylococci are among the most resistant of all the non-spore-forming bacteria. Agar slant cultures remain alive at room temperature or in the ice box for months. When dried on threads, paper, or cloth or in purulent exudate, they are viable for six to fourteen weeks. *S. aureus* suspensions of $10^6$ per ml are killed in fifteen min by 2 percent phenol; in ten min by 1 percent bichloride of mercury; in 3 min by 3 percent hydrogen peroxide; and in 1 min by tincture of iodine. Alcohol in 50 to 60 percent solution requires 60 min to kill the organisms. A strain of *S. aureus* which is killed in 10 min but not in five by phenol diluted 1:90 is used by the U.S. Food and Drug Administration as a standard test organism for evaluating other antiseptics. Since they withstand desiccation, drying on porcelain beads is a common method of preserving cultures. Freezing, particularly slow freezing, usually reduces viable members but cocci can withstand frozen storage in foods for long periods (125). Segalove and Dack (131) reported that the lowest temperature at which enterotoxin production has been observed was 18 C. Angelotti, Foter, and Lewis (7) observed that food poisoning strains of *S. aureus* did not multiply in food with internal temperatures at or below 15.8 C.

Staphylococci grow well in the presence of high concentrations of sodium chloride. Koch (36) reported that staphylococci were not inhibited by a concentration of 7.5 percent sodium chloride in solid media. Chapman (36) noted that most bacteria, other than staphylococci, were inhibited on such media and that pathogenic staphylococci grew
more luxuriantly than did nonpathogenic strains. These characteristics and the selective properties of medium containing 7.5 percent sodium chloride make it of particular value for the isolation of staphylococci, especially those suspected of being pathogenic or involved in cases of food poisoning.

Toxins and Enzymes Produced

Staphylococci can produce disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances. In addition to enterotoxins they produce exotoxins, leukocidins, coagulases, hyaluronidases, staphylokinase, proteinases, lipases, and penicillinase (24, 41, 50, 60, 112, 114).

The exotoxins are a filterable, thermolabile mixture that are lethal for animals on injection, causing necrosis of the skin and containing several soluble hemolysins. The alpha hemolysin, a protein of 30,000 molecular weight, dissolves rabbit erythrocytes, damages platelets, and is probably identical with the lethal and dermonecrotic factors of exotoxins (105). Alpha hemolysin also produces a powerful effect on vascular smooth muscle. Beta hemolysin dissolves sheep erythrocytes upon incubation; however, it has little or no effect on rabbit cells. These hemolysins along with gamma and delta hemolysins are antigenically distinct and intravenous injections with concentrated preparations kill rabbits after producing acute necrotic changes (175) in the circulatory system. Exotoxin treated with formalin gives a nonpoisonous but antigenic toxoid which has been used to stimulate antitoxic immunity to staphylococci (42).
The leukocidins are soluble materials which lyse white blood cells of a variety of animal species. The leukocidins are antigenic but more heat-labile than exotoxin (41). The role in pathogenesis is uncertain.

Most staphylococci, pathogenic for man, produce coagulase, an enzyme which clots oxalated or citrated plasma in the presence of a factor contained in many sera. The coagulase reactive factor of serum reacts with coagulase to activate both esterase and clotting activities, in a manner similar to the conversion of prothrombin to thrombin (56). Coagulase may deposit fibrin on the surface of staphylococci, perhaps interfering with their ingestion by phagocytic cells or their destruction within such cells. Elek (57) denies the similarity of coagulase-thrombin and thrombin, because the former was supposed to be inhibited by heparin. The work by Drummond and Tager (56) has demonstrated that the products of fibrinogen clotting by coagulase plasma factor and by thrombin are similar.

Schwabacher (57) found that 93.6 percent of coagulase positive staphylococci produced hyaluronidase. Hyaluronidase is known as a spreading factor; however, it is rapidly neutralized by the products of inflammation. Elek (57) reported that the only effect of this metabolite would be very early in the initial stages of invasion.

About 81 percent of coagulase-positive strains and 30 percent of coagulase-negative staphylococci produce staphylokinase. Staphylokinase activates the plasma plasminogen to the fibrinolytic enzyme plasmin and does not require the plasma proactivator (50). Mueller's phenomenon was readily explained by Quie and Wannamaker (115) as a manifestation of staphylokinase. This phenomenon was described by
Mueller in 1927. According to Quie and Wannamaker (115), aggregations of plasminogen occur in certain areas of the blood agar plates, and the staphylokinase activates the proteolytic enzyme plasmin in areas causing the Mueller's phenomenon.

Forty-seven percent of staphylococci isolated from human lesions and 98 percent from animal lesions produce gelatinase. Elek (57) identified two apparently distinct proteases that they isolated from staphylococci. These proteolytic enzymes may explain the rapid necrosis of tissues, including bone, which is characteristic of staphylococcal infections.

Over 99 percent of the coagulase-positive strains of staphylococci isolated from man are lipolytic. There is no evidence that lipase plays a specific role in the pathogenicity of staphylococci.

Barber and Duper (13) reported an excellent correlation between acid phosphatase activity and pathogenicity as well as between phosphatase activity and coagulase production. Of the strains examined, 41 percent produced phosphatase.

Jay (83) found that 95 percent of the strains he studied produced lysozyme.

Pollock (114) studied the effect of penicillinase produced by staphylococci. This enzyme may appear as either an endocellular or an exocellular product. It acts by opening up the beta-lactam ring of penicillin and, hence, destroys its activity.

Pathology and Pathogenesis

Staphylococcal infections are primarily a disease of man; however, it has been reported in turkeys, dairy cattle, and other animals.
Every tissue and every organ is susceptible to invasion by staphylococci, and the resulting disease is characterized by inflammation, necrosis, osteomyelitis, and abscess formation. The infections vary from mild furuncles on the skin to the almost uniformly fatal pyemias. Coagulase is produced and precipitates fibrinogen around the lesion and within the lymphatics. This results in the formation of a wall which limits the inflammation process and which is reinforced by the accumulation of inflammatory cells and later fibrous tissue. Within the center of the lesion, liquefaction of the necrotic tissue occurs, and the abscess "points" in the direction of least resistance. Drainage of the liquid central necrotic tissue is followed by the slow filling of the cavity with granulation tissue and eventual healing. Staphylococci may be the causitive organism in pneumonia, meningitis, empyema, endocarditis, or sepsis with suppuration in any organ (62).

When penicillin was first introduced in therapy of these infections, only a few strains were found with natural spontaneous resistance; however, Finland (62) reported that penicillin-resistant strains isolated from hospitalized patients and personnel has increased 75 percent in the last 15 years. Each new antibiotic in turn has been hailed as the answer to penicillin resistance, and each in turn has given rise to resistant strains. Bauer, Perry, Kirby, and Seattle (14) and Finland, Jones, Barnes, and Boston (62) reported that strains 80 and 81 are known as hospital epidemic strains. The strains colonize in the nose and on the skin of hospital employees and are taken home by patients who spread them into the community. The spread is accelerated by the promiscuous use of penicillin for
minor infections. Using penicillin in this manner eliminates the penicillin-susceptible strains carried by individuals and permits the recolonization of resistant strains (110, 134).

Staphylococci are members of the normal flora of the human skin, respiratory, and gastrointestinal tracts and are found in varying numbers in air and dust, as well as in water, milk, food, feces, and sewage. The pathogenic capacity of a given strain of staphylococci is the combined effect of extracellular factors and the toxins together with the invasive properties of the strain (146). This varies in pathology from ingestion of pre-formed enterotoxin to staphylococci bacteremia and disseminated abscesses in all organs. The primary habitats of these organisms are the mucous membranes of the nasopharynx and the skin of man and animals. Colonization by \textit{S. aureus} of the nasal passages of man is common, and a large proportion of normal persons carry these organisms in their nose. Williams (154, 155) reported carrier rates in normal adults not associated with hospitals between 30 and 50 percent. The incidence of nasal carriage increased to approximately 60 to 80 percent in patients and working personnel associated with hospitals as reported by Rountree and Barbour (128).

With carrier rate frequencies of the order described it is easy to appreciate why staphylococci are present in foods, particularly those that come in intimate contact with food handlers during processing and preparation. Except for bovine milk and dairy products in which staphylococci are present as a result of shedding from the udder, the single most important source of \textit{S. aureus} in foods is man. Until man is no longer directly involved in preparation and service
of food, staphylococcus will remain a potential food-borne health hazard.

Epidemiology

Dack (48) in his report summarizes the conditions necessary for an outbreak of staphylococcus food poisoning: Contamination of a food with enterotoxin-producing staphylococci, a suitable food in which the organisms can grow, and the keeping of this food for a sufficient time at a temperature compatible for growth. Many different types of foods have been involved in this type of food poisoning. The growth requirements of the staphylococci are easily fulfilled as is evidenced by the many different foods which have been reported.

In recent years bacteriophages have been used to identify strains involved in food poisoning outbreaks and to detect similar strains in food handlers. By this method it has at times been possible to identify tentatively the source of contamination as reported by Williams (156), Wilson and Atkinson (157). Occasionally repeated outbreaks have been traced to the same food handler.

Staphylococci are among the major identified causes of food poisoning outbreaks in the United States. A possible explanation of the high incidence may be related to the enormous numbers of commercially prepared, served, and catered meals consumed by the American people in public establishments. The United States Food Service industries employ about 3.3 million persons in more than 370,000 restaurants, and they hire about 250,000 new employees annually. Most of the United States population has a distorted view of personal hygiene and environmental sanitation, based largely on superficial
training at home and in school, as well as on advertising, news releases, and official publications. The public has been led to believe that the protection afforded by government and industry places the safety of any product offered for sale above reproach. While this view is in large measure justified for some products, it presents an obstacle to acceptance of the need for additional measures. More stress should, therefore, be given to the fact that major abuses occur during preparation, holding and serving of food in restaurants and at home. Meals prepared by amateurs for large groups and by institutional food services seem particularly likely to produce outbreaks of staphylococcal and other food-borne diseases that could be prevented by strict observance of sanitation principles. By comparison, the abuses encountered in commercial food processing operations are relatively infrequent, but they deserve special attention because contamination of such products may subject very large numbers of consumers to the risk of infections.

The vast improvements which have been made in reducing the hazards of food-borne illness in general have not provided the answer to this problem. One of the most important problems of the public health worker is the control of staphylococcus food poisoning. A great practical difficulty is the education of the public. If a food does not smell or taste spoiled, the layman thinks it is safe for eating. The reverse is, unfortunately, often true. In the case of staphylococcal food poisoning, the product may contain sufficient enterotoxin to produce violent illness and yet have no odor, spoilage, or abnormal taste (4, 8, 28, 125).

The control of enterotoxin production resulting in staphylococcus
food poisoning can be accomplished by keeping several factors in mind. First, there must be sufficient contamination of the food with an enterotoxin-producing strain of staphylococcus. Secondly, the food must be a good medium for the growth of the organism and the production of enterotoxin. Thirdly, the food must remain at or near room temperature or above for several hours. Of all these factors, temperature is the most practical to control (4, 8, 28, 48, 125).

Enterotoxins

The risk of poisoning from the ingestion of food containing enterotoxin from staphylococci is well known. Until fairly recently little was known about the nature of staphylococcal enterotoxin, its mode of action, or the methods by which it could be produced and assayed in the laboratory.

The occurrence of more than one immunological type of staphylococcal enterotoxin has been conclusively established and enterotoxins A, B, C, and D are now recognized as outlined on page 4. Recently Gandhi and Richardson (67) developed a capillary tube immunological assay for staphylococcal enterotoxins. They describe a simple assay in which 1 ug of staphylococcal enterotoxins A, B, or D per ml was detected in less than one hr. Interfacial reaction of antisera and enterotoxin solutions in a 1-mm internal diameter capillary tube allowed rapid detection of sera type.

Appreciable levels of enterotoxins are produced only after considerable growth of the staphylococci; usually a population of at least several millions per milliliter or gram must be attained. Therefore, the conditions that favor enterotoxin production are those
most advantageous for growth of staphylococcus. It has been reported that enterotoxin is produced at an appreciable rate at temperatures between 15.6 and 41.6 C, and production is best at 21.1 to 36.1 C (7, 131). Under the best conditions, enterotoxin may become evident within four to six hr. The lower the temperature during growth, the longer it will take to produce enough enterotoxin to cause poisoning. It has been observed that production of enterotoxin by the staphylococci is more likely when competing microorganisms are absent, few, or inhibited. Therefore, a food that had been contaminated with the staphylococci after a heat process would be favorable for enterotoxin production (141). There is evidence that enterotoxin is produced by staphylococci growing in the intestinal tracts of patients when treatment with antibiotics has destroyed or inactivated other competitive bacteria (10, 139).

The type of food evidently has an influence on the amount of enterotoxin produced. The presence of starch and protein in considerable amounts is supposed to encourage enterotoxin production by the staphylococci (85).

An important characteristic of the enterotoxin is its stability toward heat. It has been shown to withstand boiling for 20 to 60 min or even autoclaving, although it gradually loses its potency during such heating. The cooking usually given most foods will not destroy the enterotoxin formed prior to the heat process. Such foods might cause food poisoning, although no live staphylococci could be demonstrated (4, 8, 18, 29, 48).

Comprehensive reviews on the enterotoxins have been given by Bergdoll (18), Dack (48), Casman (29) and Angelotti (8).
**Intoxication**

Although ingestion of staphylococcal enterotoxin is rarely fatal, the symptoms produced are acute and distressing. These include nausea, vomiting, diarrhea, and abdominal pains, generally of no more than one day's duration. The enterotoxin-producing organisms may be introduced into dairy products via the udder of an infected cow or by human carriers or by contaminated equipment. Subsequent incubation or failure to refrigerate properly, may allow growth and consequent enterotoxin production by these bacteria in a food made from infected milk. The presence of enterotoxin may not alter the appearance or taste of the food manufactured from the milk. Pasteurization kills the species, including all the enterotoxin-producing strains, but the heat treatment does not inactivate the food poisoning enterotoxins. Improperly cooled bulk tanks used for storage of raw milk for use in Grade A processing plants have been implicated in food poisoning outbreaks (55).

Following ingestion of food containing the enterotoxin by susceptible individuals, the onset of symptoms is quite rapid and characteristic. The incubation period is usually three to five hr depending on the susceptibility of the individual and dosage consumed. The symptoms are often accompanied by headache and muscular cramps. In severe cases requiring hospitalization, supportive treatment is directed toward relieving shock, preventing dehydration, and controlling vomiting and diarrhea (125).
Deoxyribonuclease

Cunningham, Catlin and DeGarilhe (47) first reported calcium activated nuclease in culture medium of *S. aureus*. These authors partially purified the enzyme and studied its action on DNA.

An enzymatic means for detecting food contaminated by *S. aureus* is suggested by the fact that nearly all pathogenic staphylococci produce the unique and remarkably heat-stable nuclease as outlined on page 5. Detection of this enzyme in foodstuffs would provide firsthand evidence of contamination by *S. aureus*. The suitability for human consumption of any food in which staphylococci has grown is questionable, and a rapid means of identification of such contaminated foods would be very useful in the investigation of food-borne diseases.

Measurement of nuclease activity as an indicator of contamination is increased by the relative rapidity with which it can be conducted and by the fact that it requires no uncommon reagents or equipment. What constitutes a significant level of nuclease activity from a public health standpoint can be estimated by comparison of nuclease activity with corresponding staphylococcal plate counts and enterotoxin production. The specificity of the method and lack of interference even from large mixed populations of microorganisms makes the test adaptable to all types of foods (40).

Several purification procedures have already been reported since the discovery of this enzyme by Cunningham, Catlin and DeGarilhe (47). They purified the enzyme by fractionation with a mixture of ammonium chloride and trichloracetic acid. Dirksen and Dekker (51) utilized precipitation with ammonium sulfate, acetone, and a mixture
of ethanol and calcium chloride. Ohsaka, Mukai, and Lasfowski (111) utilized precipitation with ammonium sulfate, a mixture of ammonium chloride and trichloroacetic acid, ethanol and chromatography, on DEAE-cellulose to obtain a 250-fold purification. Pochon and Privat DeGarilhe (113) utilized ammonium sulfate fractionation followed by chromatography on Amberlite IRC-50 and XE64 to obtain 4,500-fold purification.

Although there has been general agreement as to the absolute requirement for calcium ions, reports of the optimal concentration of the ions have varied from 10 mM (47), 3 mM (46) to 0.1 mM (72). It is now believed that the dependence upon calcium ions varies with pH.

The type of buffer used has little effect on the enzyme activity, and the results obtained with (hydroxymethyl) aminomethane (tris), borate, glycine, and barbital are superimposable. Furthermore, varying the tris or borate concentration from 5 mM to 50 mM causes no change in activity. Addition of NaCl to 0.3 M is also without effect, suggesting that the observed pH and calcium changes are independent of ionic strength effects.

The action of micrococcal nuclease on DNA has been thoroughly studied (46, 47, 51, 71, 72, 111, 120, 121, 122, 123, 126, 129, 150). Exhaustive digestion results in the production of mono and dinucleotides bearing a 3'-monophosphate terminus. Sulkowski and Laskowski (137) postulated that bacterial nuclease first act as an endonuclease up to the formation of tetra and penta nucleotides and then it acts as an exonuclease.

Staphylococcal nuclease is relatively stable in acid pH. The
pH for maximum activity is 0.02 M tris buffer was found to be 8.7 to 8.9 as reported by Hacha and Frederico (72).

Alexander, Heppel, and Hurwitz (1) have confirmed the heat stability of the enzyme. They reported no loss of activity after incubating the enzyme for 20 min at 100 °C at a concentration of 0.15 mg/ml.

Lachica, Weiss, and Deibel (95) working with food poisoning staphylococci reported that 93 percent of the enterotoxigenic strains they studied produced coagulase, and 95 percent produced heat-stable nuclease. They used strains isolated from food-borne outbreaks by Bergdoll and co-workers.

The most widely used method for detecting bacterial nuclease production in cultures involves growth of the organism in an agar medium containing DNA followed by flooding the plate with acid to precipitate intact DNA (45, 84). To detect ribonucleic acid hydrolysis, Lanyi and Lederberg (99) incorporated acridine orange and ribonucleic acid in semi-solid media (100). Other methods reported for nuclease activity are viscometric (47), pH-titrometric (111, 150) and spectral (94, 111).

More recently Lachica, Genigeorgis, and Hoeprich (97) have developed an agar diffusion method for detecting staphylococcal nuclease based on the metachromatic properties of toluidine blue. They report a sensitivity as low as 0.005 μg/ml. The reported sensitivity is based on nuclease zones produced in a series of Brain Heart Infusion dilutions of purified nuclease of S. aureus purchased
No effort was made to correlate the size of zone produced with the number of *S. aureus* organisms present.

Lachica, Genigeorgis and Hoeprich (97) indicated that nuclease activity varies with different strains of micrococcaceae. On the other hand Alexander, Heppel, and Hurwitz (1) reported that among the strains they studied there was little difference in their ability to produce nuclease. A similar conclusion with respect to the production of the enzyme was implied by Cunningham, Catlin, and DeGarilhe (47).

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1Worthington Biochemical Corporation. Freehold, New Jersey 07728, U.S.A.
EXPERIMENTAL

Staphylococcal Deoxyribonuclease Production

The nuclease used in this study was prepared from cultures of S. aureus by a modification of the procedure of Cunningham, Catlin, and DeGarilhe (47).

Nuclease was prepared from strains 13556, 14458, 19095, and 5233235 purchased from the American Type Culture Collection. These strains are known to produce predominately enterotoxins A, B, C, and D, respectively. The strains were inoculated into Bacto Brain Heart Infusion broth. Brain Heart Infusion is especially well suited for the growth of staphylococci or for the preparation of the suspension of the organism used in the production of nuclease. After 48 hr incubation at 37 C the suspensions were centrifuged at 10,000 x g for 15 min to remove the bacterial cells. The cells were autoclaved at 121 C for 15 min and discarded. After measuring, the broth was brought to 40 percent saturation with ammonium sulfate and stirred on a magnetic stirrer for approximately two hr. The solution was centrifuged at 10,000 x g for 15 min, and the precipitate was discarded. The supernatant was brought to 80 percent saturation, and the precipitate was collected in 100 ml of saline and prepared for dialysis. Dialysis tubing was prepared by soaking in distilled water.

2 American Type Culture Collection. 12301 Parklawn Drive, Rockville, Maryland 20852.
The solution containing the precipitate was placed in the dialysis tubing and dialyzed against distilled water for 48 hr. This procedure, utilizing the second ammonium sulfate fractionation, was described by Alexander, Heppel, and Hurwitz (1). After dialysis the solution was collected and frozen as a concentrated stock solution. Preserved in this form, the enzymatic activity remained stable for months.

Purified nuclease used for plotting a standard curve was purchased from Sigma Chemical Company, (Cat. No. 14026). 3

Nuclease is completely inactive in the absence of ionic calcium (47). Since the nuclease produced by the Alexander, Heppel, and Hurwitz (1) method retained some activity when tested without added calcium, it was assumed that the stock solution was slightly contaminated with calcium ions. The addition of 2 uM of ethylenediaminetetraacetic acid (EDTA) per ml of enzyme solution was added to create this calcium, and this eliminated detectable activity. Inasmuch as all the experiments in this study were carried out in the presence of excess calcium, the presence of EDTA did not present a problem.

**DNA Preparations**

Most of the experiments were carried out with highly polymerized calf thymus DNA purchased from Sigma Chemical Company.

Solutions of native DNA were prepared by dissolving the dried fibrous DNA in neutral 0.05 M NaCl by gentle continuous stirring at 5 C. Approximately four days were required to complete this process.

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3 Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178.
The heat denatured DNA used was prepared by dissolving dried fibrous DNA directly in 0.05 M NaCl and heating for 30 min at 100 C. The mixture was immediately cooled by pouring it into a tube held in crushed ice. Inasmuch as some moisture was lost during the heating process, the solution was brought to its original volume. To measure the effects of the denaturation on the DNA, a Beckman DB-G spectrophotometer was used. The spectral increase from the native DNA to the heat denatured DNA at 260 nm was approximately 25 percent. The DNA stock solutions were stored at -20 C.

Staphylococcal Plate Counts

Plate counts were made using Bacto-Staphylococcus Medium No. 110. This medium is selective for the isolation of staphylococci. The selectivity of this medium is based upon its high sodium chloride content. This characteristic makes it of particular value for the isolation of staphylococci, especially those suspected of being pathogenic or involved in cases of food poisonings. Koch (36) reported that staphylococci were not inhibited by a concentration of 7.5 percent sodium chloride in solid media. Chapman (36) noted that most bacteria, other than staphylococci, were inhibited on such media and that pathogenic staphylococci grew more luxuriantly than did nonpathogenic strains. He suggested that 7.5 percent NaCl be added to Bacto-phenol Red Mannitol Agar as a selective isolation medium

4 Beckman Instruments Inc. 2500 Harbor Boulevard, Fullerton, California.

5 Difco Laboratories Inc., Detroit, Michigan 48201.
for staphylococci. Chapman (38) described a culture medium on which food poisoning staphylococci produced a gelatinase test or were "Stone reaction" positive. He pointed out the nonspecificity of a single test and reported that in addition to being "Stone reaction" positive, typical food poisoning staphylococci must produce pigment, coagulate plasma, hemolyze rabbit blood, and ferment mannitol. In addition to being selective for staphylococci due to the high NaCl content, Staphylococcus Medium 110 is well suited for pigment formation, the determination of the fermentation of mannitol, the Stone type gelatinase test, and for the coagulase test. Chapman and Domingo (39) reported that staphylococci incriminated in food poisonings produced an orange pigment, coagulated plasma, fermented mannitol, and gave a positive "Stone reaction" or gelatinase test on Staphylococcus Medium 110 when tested at the time of isolation. Velilla, Faber, and Pelczar (148) suggested the use of both Mannitol Salt Agar and Staphylococcus Medium 110 to insure maximum recovery of coagulase producing staphylococci from suspected bovine mastitis.

Following streaking or smearing of the organism on plates of Staphylococcus Medium 110, the medium is incubated at 30 C for approximately 48 hr or for 43 hr at 37 C. Colonies are first observed for sign of the production of any orange or yellow pigments. The plates were flooded with 5 ml of a saturated solution of ammonium sulfate and incubated for 10 min. Any clear zone developing around the colonies is an indication of gelatinase production.

DNase Agar 6 was used to study nuclease activity. The NaCl

6 Difco Laboratories Inc., Detroit, Michigan 48201.
concentration was increased to 7.5 percent and the solution was
tinted with 8 ml of a 0.01 M toluidine blue. To prepare the modified
medium 42 g of Bacto DNase Test Agar and 74.5 g of NaCl was placed
in a liter flask. Enough de-ionized water was added to the flask
after addition of NaCl and MBola to make the final volume 1.0 liter.
The mixture was heated and continuously stirred until the Agar in the
solution was completely dissolved. To tint the solution 8 ml of
0.01 M toluidine blue solution was added. The DNase toluidine blue
agar was sterilized in an autoclave for 15 min at 15 pounds steam
pressure at 121 C. After autoclaving, the agar was dispensed into
sterile petri plates and allowed to solidify.

Following streaking or smearing of the organisms on plates of
the DNase toluidine blue agar, the medium was incubated at 30 C
for approximately 48 hr or for 43 hr at 37 C. After incubation, the
plates were observed for a clear zone developing around the colonies.
Any clear zone developing in the colonies indicated nuclease production.

**Microslide Agar Preparation For Nuclease Assay**

In an effort to analyze the nuclease activity from cheese and
other food products suspected of contamination by *S. aureus*, the
microslide technique developed by Lachica, Genigeorgis, and Hoeprich
(97) was incorporated. This method enabled assay of extracts from
food samples and cheese, without the growth of the organisms as
required with the plate count method previously described. The
toluidine blue DNA agar mixture used for the assay of enzymatic
activity was prepared as described in Table 1.
Table 1. Toluidine blue DNA agar preparation

<table>
<thead>
<tr>
<th>Materials</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer pH 9.0</td>
<td>0.05M 1 liter</td>
</tr>
<tr>
<td>DNA (Sigma)(^a)</td>
<td>3 g</td>
</tr>
<tr>
<td>Bacto Agar (Difco)(^b)</td>
<td>10 g</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.01 M 1 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>0.1 M 3.0 ml</td>
</tr>
</tbody>
</table>

\(^a\)Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178  
\(^b\)Difco Laboratories Inc., Detroit, Michigan 48201

Before adding the toluidine blue solution, it is essential to heat the mixture until the DNA and agar are completely dissolved. The mixture was divided into 10 ml fractions and stored at room temperature in covered test tubes. The toluidine blue mixture was melted by utilization of a boiling water bath and 3 ml placed on a glass microslide using a 10 ml pipette. After the solution had solidified a number 1 cork borer (4 mm outside diam) was used to make holes in the agar into which the enzyme extracts were measured. After the holes were cut they were cleared with a pasteur pipette connected to an aspirator. It was possible to prepare five or six wells 4 mm in diam on each slide. In measuring the nuclease activity 10 ul of enzyme extract were injected into the wells using a 10 ul Hamilton syringe. Extracts were boiled for 15 min to inactivate any heatable nuclease before injection on to the microslide. Heat stable nuclease was signaled by a bright clear to pink zone of DNA
hydrolysis, discernible after two to four hr of incubation at 37 C. This method is a slight modification of the method described by Lachica, Genigeorgis, and Hoeprich (97) in that the wells they used were larger, and a Hamilton syringe was used instead of an inoculating needle for injection of enzyme extracts.

The toluidine blue mixture is remarkably stable, and sterilization was not necessary even when stored at room temperature for 4 months. Satisfactory results were obtained even when the mixture was subjected to several melting cycles. The stability of the toluidine blue mixture may be attributed to the inhibitory property of toluidine blue towards gram positive bacteria, especially the spore-formers, and the stability of the toluidine blue DNA complex to heat (97). Another important factor is the lack of nutrients for bacteria in the mixture.

In preliminary studies it was observed that the agar was drying out which did not allow the enzyme to diffuse as far as possible. It was necessary to devise a chamber with a high relative humidity which would minimize evaporation. A glass dish measuring 4 x 8 x 4 inches was provided with a plastic cover. A glass plate was cut that would fit inside the dish and the plate was suspended on two 150 ml beakers filled with water. Water was also placed in the bottom of the dish, and two paper towels were used to provide capillary action of the water from the bottom of the dish to the glass plate. Using this method it was possible to incubate the toluidine blue DNA agar microslides for days with no apparent loss of moisture. Slides were placed on the wet paper towel on the glass plate, and the dish
was covered and placed in the incubator at 37 C to provide a constant temperature during incubation.

Spectrophotometric Assay

Another substrate used to analyze for nuclease activity utilizing an ultraviolet spectrophotometer was prepared as outlined in Table 2.

Table 2. Reagents used in spectrophotometric assay preparation

<table>
<thead>
<tr>
<th>Materials</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (Sigma) (^a)</td>
<td>1.5 mg/ml</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>0.15 M</td>
</tr>
<tr>
<td>Glycine buffer pH 8.6</td>
<td>0.17 M</td>
</tr>
<tr>
<td>HClO(_4)</td>
<td>7.0 %</td>
</tr>
<tr>
<td>H(_2)O at 0 C</td>
<td>15.0 ml</td>
</tr>
</tbody>
</table>

\(^a\)Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178

In order to analyze an enzyme extract from a sample of cheese or a food product, 1 ml of the DNA solution and 1 ml of glycine buffer were mixed in a centrifuge tube and mixed with 0.25 ml of the CaCl\(_2\) solution. The mixture was brought to 37± 0.02 C in a water bath. After equilibration 0.375 ml of the enzyme solution was added and the reaction mixture was incubated for 30 min.

The reaction was terminated by adding 2.5 ml of ice-cold 7 percent HClO\(_4\) followed by 15.0 ml of ice-cold water. The mixture was immediately centrifuged for 15 min at 10,000 x g at 4 C. After
centrifugation the supernatant was decanted and prepared for reading at 250 \textmu m in a Beckman DB-G Grating Spectrophotometer.\(^7\)

The blank was prepared in the same way; however, the \(\text{HClO}_4\) was added immediately after adding the enzyme, so that the length of time the unhydrolyzed DNA was exposed to the \(\text{HClO}_4\) in both reaction mixtures and blanks was comparable. The above described method is a modification of the method used by Chesbro and Auborn (41). They indicated in their studies that one unit of nuclease activity was considered to be that amount of enzyme producing an absorbance difference of 1.0 between the reaction mixture and the blank. Furthermore, their study concluded that the production of 0.34 unit of nuclease corresponded to the production of \(9.5 \times 10^{-3}\) \text{ug} of enterotoxin as produced by \textit{S. aureus} strain 234. They indicated that this amount of enterotoxin was an amount likely to be much less than an emetic dose for humans (41).

**Cheese Slurry Preparation**

In an effort to study staphylococcal nuclease production and the effects of hydrogen peroxide, sorbate, pasteurization, and moisture control in cheddar cheese, slurries were prepared according to the method employed by Kristoffersen et al. (92, 93). Curd for the preparation of the slurries was manufactured by a conventional four to five hr cheddar cheese procedure using grade A pasteurized milk (158). The curd was not pressed but was kept at room temperature

\(^7\)Beckman Instruments Inc. 2500 Harbor Boulevard, Fullerton, California.
overnight following salting to allow the lactic acid fermentation to reach completion. Commercial lactic cultures were used for acid production.

Curd slurries were prepared by mixing two parts of fresh curd with one part of a 5.2 percent sterile NaCl solution in a Waring blender (Model 5011) operating at high speed for 3 to 4 min. The homogeneous mixtures were aspirated for 5 min with nitrogen gas, after which additives were incorporated which contribute to flavor development. Glutathione, Na$_3$ citrate, MnCl$_2$, CoCl$_2$, and riboflavin were incorporated into the slurries for this purpose. The slurries were then inoculated with *S. aureus* and incubated at 30°C after treatments with hydrogen peroxide, sorbate, pasteurization, pH 5 adjustment, and moisture control. The incubation continued for 7 days, and the slurries were stirred for 2 min each day to maintain a homogenous mixture.

**Deoxyribonuclease Extraction Methods**

Ten grams of a cheese sample were mixed with 90 ml of glycine-NaOH buffer pH 10 in a Sorvall Omni-Mixer operating at its maximal rated speed for 3 min. With hard cheese it was necessary to cut it into small pieces to facilitate thorough homogenization of the mixture. Samples from the homogenate were adjusted to 40 percent saturation with (NH$_4$)$_2$SO$_4$ calculated on the basis of the total volume

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*Waring Products Division, Dynamics Corporation of America, New Hartford, Conn. U.S.A.*

*Ivan Sorvall, Inc., Newton, Connecticut 00604, U.S.A.*
of the solution. The mixture was stirred continually while the 
(NH₄)₂SO₄ was added slowly to protect against denaturation from the 
(NH₄)₂SO₄. After addition of the amount of (NH₄)₂SO₄ to bring the 
mixture to 40 percent saturation, the mixture was stirred continually 
for 2 hr. The mixture was placed in centrifuge tubes and balanced. 
After balancing, the tubes were centrifuged at 10,000 x g for 15 
min. At this time the precipitate was discarded and the supernatant 
was remeasured, and enough (NH₄)₂SO₄ was added to bring the solution 
to 80 percent saturation. The mixture was again stirred for 2 hr and 
centrifuged as described above. After centrifugation, the super­
natant fluid was discarded and the precipitate was collected in 
0.02 M glycine-NaOH buffer pH 10. The nuclease activity was mea­
sured using both the toluidine blue agar technique and the spectro­
photometric method described above. The method of nuclease analysis 
was applied to establish the degree of correlation between staphy­
lococcal growth and nuclease production in inoculated cheese samples.

Chesbro and Auborn (40) indicated that a trichloroacetic acid 
precipitation step reduced the variability between replicate analyses 
and, in addition, increased the sensitivity of the test by making it 
possible to concentrate the nuclease from relatively large volumes 
of (NH₄)₂SO₄ extracts. To accomplish the precipitation the super­
natant fluid was treated with 0.05 volumes of cold 3.0 M trichloro­
acetic acid and collected by centrifugation at 10,000 x g for 15 min. 
The precipitate was taken up in 5.0 ml of distilled water, transferred 
to a centrifuge tube, and reprecipitated with 0.05 volumes of 3.0 M 
trichloroacetic acid. The precipitate was collected by a second 
centrifugation at 10,000 x g for 15 min, and the supernatant fluid
was discarded. The pH of the precipitate was adjusted to 8.0 to 8.5 with 2.0 N NaOH. The centrifuge tubes were temperature equili-brated in a 37°C water bath, and nuclease was assayed according to the spectrophotometric method described above.

Another method incorporated in this study involved the use of silicic acid columns similar to the method used by Harper (76) in his studies on free fatty acid determination. Silicic acid and cheese were blended together using a mortar and pestle and placed in a 20 x 135 mm column. Using this method, it was possible to elute the fat from the column, using 100 ml of chloroform or hexane. After the fat was extracted the column was then emptied, using forced air and the contents fractionated with \((\text{NH}_4)_2\text{SO}_4\) as described above. The chloroform containing the fat was mixed with equal volumes of de-ionized water and placed on a magnetic stirrer operating at high speed for 5 min. The mixture was then placed in a separatory funnel, and the chloroform was drained off the bottom of the biphasic mixture. The remaining extract was \((\text{NH}_4)_2\text{SO}_4\) fractionated with the extract removed from the column described above. Using this method the efficiency of different extraction chemicals and techniques were assayed. It was found that 10 g of silicic acid blended with 10 g of the cheese or cheese slurry proved a workable combination. Since the \((\text{NH}_4)_2\text{SO}_4\) treatment gave good recovery and was the simplest to perform, it was the method preferred.

**Deoxyribonuclease Collection and Concentration**

Recently a modular system for concentration, purification, and fractionation of solutions and suspensions by selective membrane
separation based on dimensions of solutes has been reported. Diaflo Ultrafilters, type PM-10, size 62 mm,\textsuperscript{10} were purchased from Amicon Corporation and utilized for the recovery of nuclease. The extracts were placed in the reservoir and pressurized to 60 psi using a nitrogen gas cylinder. The solutions were stirred continually with a magnetic stirrer during dialysis. Using this method, it was possible to concentrate 250 ml volumes of extract to 5 ml; and, in addition, it was possible to remove all the \((\text{NH}_4)_2\text{SO}_4\) from the enzyme. After the extract had passed through the membrane the chamber was filled with distilled water and pressure reapplied. The water passing through the chamber and membrane removed almost all residual \((\text{NH}_4)_2\text{SO}_4\). This method proved to be very useful in the recovery and concentration of nuclease.

**Direct Assay For Deoxyribonuclease**

Nuclease activity in food and food products with extremely high staphylococcal counts was assayed directly using the toluidine blue DNA agar method described on page 27. Ten grams of the suspected food was mixed with 90 ml of pH 10 glycine NaOH buffer and blended in a Sorvall\textsuperscript{10} Omni-mixer at high speed for 3 min. It was possible to place a loop of this extract directly on the microslide prepared as described and obtain a positive reaction within 3 to 5 hr.

**Growth of S. aureus Strains**

\textit{S. aureus} strains were grown in Bacto Brain Heart Infusion

\textsuperscript{10}Ivan Sorvall Inc., Newton, Connecticut, 00604, U.S.A.
broth. The medium was prepared by dissolving 37 g of Bacto Brain Heart Infusion in 1000 ml of distilled water. After dissolving the medium 250 ml of broth was placed in a liter flask and sterilized in the autoclave for 15 min at 15 pounds pressure at 121°C. The flasks were inoculated with $10^6$ cells and were incubated at 37°C for different lengths of time depending on the growth desired.

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11 Difco Laboratories Inc., Detroit, Michigan 48201.
RESULTS AND DISCUSSION

Buffer pH For Maximum Nuclease Extraction

From Cheese

At the beginning of this study physiological saline was utilized for extraction of nuclease from cheese and food samples suspected of contamination by *S. aureus*. The results were inconsistent when judged by correlating between the amount of nuclease recovered and the number of *S. aureus* in the sample (41). To establish the most favorable hydrogen ion concentration for maximum nuclease extraction from cheese and other foods, 0.02 M buffers were prepared as outlined in Table 3. The efficiency of different nuclease buffers were compared by injection of a known amount of staphylococcal nuclease activity into cheese preparations and examining the activity recovered. Ten g of cheese inoculated with *S. aureus* nuclease were mixed with 90 ml of 0.02 M buffers. Staphylococcal nuclease had been added to produce a 20 mm diameter zone after incubation for 13 hr on a DNA toluidine blue microslide at 37 C. The mixtures were homogenized for 3 min using a Sorvall\(^{12}\) Omni-mixer operating at its maximum rated speed. The nuclease was prepared according to the method previously described in the experimental section beginning on page 23. The nuclease was extracted from the samples as outlined on page 32. The precipitate collected was placed in dialysis tubing

\(^{12}\)Ivan Sorvall Inc., Newton, Connecticut 00604, U.S.A.
and dialyzed against distilled water overnight to remove the \((\text{NH}_4)_2\text{SO}_4\). Toluidine blue DNA agar was prepared as described on page 27 and the microslides prepared. Ten microliters of the dialyzed extract were injected into the wells using a 10 ul Hamilton syringe, and the slides were incubated for 13 hr at 37 C to allow development of the nuclease zone. The results appear in Table 3.

Table 3. Buffer pH for maximum extraction of nuclease from cheese

<table>
<thead>
<tr>
<th>Buffers</th>
<th>pH</th>
<th>Nuclease zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Phosphate</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Tris HCL</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Tris HCL</td>
<td>9</td>
<td>14</td>
</tr>
<tr>
<td>Glycine NaOH</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Glycine NaOH</td>
<td>11</td>
<td>14</td>
</tr>
</tbody>
</table>

The efficiency of the extraction buffers increased as the pH increased to 10. At pH 12 and above an insoluble precipitate formed, and it was impossible to carry out the experiment. The results indicate that the maximum recovery was obtained using glycine-NaOH 0.02 M buffer pH 10. Staphylococcal nuclease is essentially inactive at pH values below pH 7.5 as reported by Von Hippel and Felsenfeld (150). This is consistent with the results obtained and partially explains the lack of recovery using pH 7.0 physiological saline.
Proteins can be precipitated from solutions in various ways. One of the most important methods is salting out with salts such as ammonium sulfate, sodium sulfate, or magnesium sulfate, since highly concentrated solutions of these salts can be prepared. The salt content of a protein solution is increased stepwise and after each addition the precipitated protein is centrifuged off. In order to find in which (NH₄)₂SO₄ fraction that staphylococcal nuclease was precipitated, five identical samples of cheese containing staphylococcal nuclease were prepared. Ten ug of staphylococcal nuclease were added to produce a 20 mm zone on a toluidine blue DNA agar microslide incubated for 13 hr at 37 C. The microslides were prepared as described on page 27. Ten g of the sample with the nuclease incorporated were homogenized with 90 ml of 0.02 M glycine NaOH buffer pH 10, and the extraction was carried out as explained on page 37. In order to assure that the five samples were identical, one large sample was prepared as described and then this sample was divided into five smaller samples. The amount of (NH₄)₂SO₄ necessary to produce the various fractions per 100 ml of sample is outlined in Table 4. The (NH₄)₂SO₄ was added slowly to avoid denaturation. The fractionation was carried out as described on page 32 of the experimental section. Initially solutions were stirred for different lengths of time up to 6 hr with the (NH₄)₂SO₄; however, it was discovered that there was little difference in the recovery after 2 hr. Each sample was treated in an identical manner, and at each
Table 4. Quantity of ammonium sulfate required to prepare the various fractions and the corresponding nuclease zones recovered

<table>
<thead>
<tr>
<th>Grams (NH$_4$)$_2$SO$_4$ per liter</th>
<th>Fractions</th>
<th>Nuclease Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g)</td>
<td>(%)</td>
<td>(mm)</td>
</tr>
<tr>
<td>114</td>
<td>0-20</td>
<td>* &lt;4.0</td>
</tr>
<tr>
<td>237</td>
<td>20-40</td>
<td>6.0</td>
</tr>
<tr>
<td>369</td>
<td>40-60</td>
<td>16.0</td>
</tr>
<tr>
<td>512</td>
<td>60-80</td>
<td>6.0</td>
</tr>
<tr>
<td>669</td>
<td>80-100</td>
<td>&lt;4.0</td>
</tr>
</tbody>
</table>

*Well diameter is 4 mm \(<4 = \text{No detectable activity.}*

fraction the precipitate was collected and examined for enzyme activity using the toluidine blue DNA agar microslide procedure outlined on page 27. The results of the staphylococcal nuclease activity recovered from the various fractions after dialysis against distilled water overnight are presented in Table 4.

The fractions from 0-40 percent left a large amount of precipitate and a great deal of trubidity in the supernatant. As the percent saturation increased above 40 percent, the precipitate reduced considerably and the supernatant began to clear up. Below 40 percent saturation there was a layer of fat left on top of the supernatant after centrifugation; however, this disappeared as the saturation increased. It appears from Table 4 that most of the staphylococcal nuclease is removed from cheese preparations in the 40-80 percent (NH$_4$)$_2$SO$_4$ fraction. Further studies indicated that a workable procedure was to blend 10 g of the cheese samples with 90 ml of
glycine NaOH pH 10 buffer in a blender. After blending for 3 min
the mixture was placed on a magnetic stirring plate and sufficient
\((\text{NH}_4)_2\text{SO}_4\) was added to bring the solution to 40 percent saturation.
After stirring for 2 hr the solution was centrifuged at 10,000 x g
for 15 min and the precipitate discarded. Most of the extraneous
matter in the sample was removed by this step. When silicic acid
was used as outlined on page 34 and 100 ml of chloroform was used
to remove the fat, the supernatant was crystal clear. It was apparent
that the remaining turbidity in the supernatant after the 40 percent
\((\text{NH}_4)_2\text{SO}_4\) fractionation could be attributed to the fat. The super­
natant was remeasured, and sufficient \((\text{NH}_4)_2\text{SO}_4\) was added to bring
the solution to 80 percent saturation. The sample was then stirred
and centrifuged as described above. The precipitate was collected
and concentrated using pressure dialysis as described on pages 34-35.

This method gave good recovery of staphylococcal nuclease and
was easy to execute (1, 40, 51, 129). With a complex food such as
cheese, it is very difficult to find an extraction method that is
simple to perform and does not require a lot of special chemicals.
In addition, the fat creates problems in that some of the nuclease
activity remains associated with it as was evident by the chloroform
extracts which contained nuclease activity using the method outlined
on page 34. A loss of activity occurred when the methods described
were used to remove the nuclease from the lipid material. Similar
results were encountered with the use of silicic acid columns. It
was concluded that the more extraction procedures engaged in the more
potential was created for loss of activity. The evaluation of the
nuclease activity was based on the total amount of nuclease injected
into the samples and the amount of activity recovered. Inasmuch as all five samples were identical at the beginning of the experiment, it was possible to discern which fraction produced the best recovery of *S. aureus* nuclease.

It was observed from the extraction studies that not all of the nuclease injected into the samples was being recovered. An experiment was designed to determine what amount of nuclease was being recovered and if additional procedures would increase the percent recovery. Experiments involving \((\text{NH}_4)_2\text{SO}_4\) fractionation in conjunction with various solvents were conducted. The procedure for these experiments are outlined on page 34. In using silicic acid columns it was necessary to have the columns completely dry for packing. A minimum of 50 percent silicic acid combined with 50 percent cheese was necessary to facilitate grinding of the cheese sample with the mortar and pestle and even distribution in the column. It was discovered that if the solvents were poured down the side of the column and not directly on the silicic acid and cheese, that a more uniform packing resulted. Air pressure applied at the top of the column increased the flow rate; however, it was apparent that more efficient elution was accomplished by gravity flow. Air pressure did, however, facilitate removal of excess solvents before the column was emptied and prepared for \((\text{NH}_4)_2\text{SO}_4\) fractionation. When chloroform was used to remove fat, some of the protein eluted by the chloroform could be recovered by adding an equal amount of water forming a biphasic solution. The mixture was placed on a magnetic stirring plate and stirred at high speed for 3 min. By placing the solution in a separatory funnel it was possible to remove the
chloroform leaving the water extract. This extract was added to the silicic acid and cheese before \((\text{NH}_4)_2\text{SO}_4\) fractionation.

Instead of using dialysis tubing for the removal of \((\text{NH}_4)_2\text{SO}_4\) and the concentration of the extract, it was observed that ultrafilters in conjunction with pressure dialysis not only did a better job of removing \((\text{NH}_4)_2\text{SO}_4\) but also provided a more concentrated extract. Using dialysis tubing it was very difficult to concentrate the enzyme and to completely remove it from the inside of the tubing. With pressure dialysis and ultrafilters it was possible to concentrate large amounts of extract to 5 ml. This made it possible to wash the ultrafilters as many times as necessary to remove all the \((\text{NH}_4)_2\text{SO}_4\) with little or no loss of enzyme activity. Procedures for use of the ultrafilters are outlined on pages 34-35.

To study the various procedures for the extraction of \textit{S. aureus} nuclease from cheese, a predetermined amount of nuclease known to produce a zone of 20 mm on a toluidine blue DNA agar microslide after 13 hr incubation at 37 C was inoculated in cheese slurry and extracted by the procedures outlined in Table 5. After extraction the extracts were dialyzed using ultrafilters. The recovered nuclease was washed off the filters using 5 ml of glycine-NaOH 0.02 M pH 10 buffer. The activity recovered by the various procedures was assayed using the toluidine blue DNA agar microslide method outlined on page 27. The results appear in Table 5.

Chesbro and Auborn (40) reported a 94.6 percent recovery after \((\text{NH}_4)_2\text{SO}_4\) fractionation in conjunction with two trichloroacetic acid precipitations from high starchy foods. They utilized trichloroacetic acid precipitations instead of ultrafilters for making it possible to
concentrate the nuclease from relatively large volumes of \((\text{NH}_4)_2\text{SO}_4\) extracts.

The amount of activity recoverable as illustrated by this study confirms the findings of Chesbro and Auborn (40) and is related to the type of food being analyzed, but for a given type of food the level of recovery is reproducible. Generally starchy foods give the highest recovery; meats an intermediate recovery; and fat, rich foods the lowest recovery (40).

Table 5. Comparison of extraction procedures for the recovery of nuclease from cheese

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Percent</th>
<th>Recovery Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>0-40</td>
<td>59</td>
</tr>
<tr>
<td>Pressure dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>0-40</td>
<td>56</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>40-80</td>
<td></td>
</tr>
<tr>
<td>Pressure dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silicic acid</td>
<td></td>
<td>47</td>
</tr>
<tr>
<td>Chloroform</td>
<td></td>
<td></td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>0-40</td>
<td></td>
</tr>
<tr>
<td>Silicic acid</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Hexane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4)</td>
<td>0-40</td>
<td></td>
</tr>
</tbody>
</table>

Although the greatest recovery resulted from \((\text{NH}_4)_2\text{SO}_4\) 0-40 percent fraction in conjunction with ultrafiltration, it required 12-14 hr for the supernatant to pass through the membrane. If the supernatant saturation was increased to 80 percent and the precipitate
collected and diluted in distilled water, the solution passed through the membrane in 6 to 8 hr with 60 psi nitrogen pressure. These results again demonstrate that as the number of extraction procedures increase, there is a greater potential loss of enzyme activity.

Nuclease Analysis to Detect *S. aureus* Growth

To study the effects of sodium sorbate, hydrogen peroxide, and pasteurization treatments on *S. aureus* growth and whether or not the effects could be detected using the nuclease assay, cheese slurries were prepared as described on page 31. The treatments include injection of sodium sorbate so that the final concentration was 0.2 percent, addition of hydrogen peroxide to 0.5 percent, and removal by liver catalase after 2 hr and pasteurization for 30 min at 71 C. The control was slurry without any treatment. An identical inoculation of *S. aureus* was injected into all treatments and an aliquot from the control. The results obtained after seven days of incubation appear in Table 6.

Table 6. Nuclease production by *S. aureus* under various growth conditions in cheddar cheese slurries

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Staph. Count</th>
<th>Nuclease *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen Peroxide</td>
<td>$9.8 \times 10^6$</td>
<td>0.03</td>
</tr>
<tr>
<td>Pasteurization</td>
<td>$8.6 \times 10^7$</td>
<td>0.15</td>
</tr>
<tr>
<td>Sodium Sorbate</td>
<td>$5.3 \times 10^3$</td>
<td>0.04</td>
</tr>
<tr>
<td>Staphylococci only</td>
<td>$1.8 \times 10^8$</td>
<td>0.20</td>
</tr>
<tr>
<td>Control</td>
<td>No growth</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Absorbance at 260 nm Chesbro and Auborn (41).
It appears from Table 6 that analysis of nuclease is an excellent method for monitoring the growth of staphylococci under various conditions in cheese. Further work in correlating nuclease production with plate counts indicates that the counts were not consistent with regards to the amount of nuclease produced. This indicates the need for further study of methods for detecting \textit{S. aureus} colonies in making plate counts. The correlation between \textit{S. aureus} plate counts and nuclease production appears in Figure 2 on page 54.

Chesbro and Auborn (41) demonstrated less variation in the enzymatic method of nuclease detection of \textit{S. aureus} than that of the plate count method. They further indicated that they analyzed food samples which exhibited nuclease activity but did not yield \textit{S. aureus} on Trypticase Soy Agar. Crisley, Peeler, and Angelotti (44) demonstrated that the recovery ability of Trypticase Soy Agar decreases when the fraction of \textit{S. aureus} in the total population is small. These results correlate with the work of Chesbro and Auborn (41).

L.W. Jones (1971 unpublished results) has been successful in monitoring the effects of nisin on staphylococci using the nuclease assay. He found that by varying the amounts of nisin in growth media in which \textit{S. aureus} was inoculated and by comparing the total plate count with nuclease production, he was able to demonstrate the effects of nisin on the growth of \textit{S. aureus}. In nonfat dry milk medium the nisin was very effective in controlling the growth of staphylococci; however, when fat was in the media, the nisin became associated with it and lost some of its effectiveness in controlling \textit{S. aureus}. He also was able to detect nuclease directly using the microslide method in reconstituted nonfat dry milk if the \textit{S. aureus}
counts were above $10^5$. After boiling for 15 min to destroy the heat liable nuclease he inoculated the milk directly on the prepared microslide and made observations after incubation. This was accomplished without the extraction of nuclease from the milk or any $(\text{NH}_4)_2\text{SO}_4$ fractionation, or pressure dialysis.

**Toluidine Blue DNase Agar With NaCl For S. aureus Isolations**

Toluidine blue DNase agar (TBDA) was prepared as outlined on pages 26-27 of the experimental section. A 0.01 M toluidine blue stock solution was made by adding 0.3058 g of toluidine blue to 100 ml of distilled water. Eight ml of the 0.01 M toluidine blue solution was used to tint the 7.5 percent NaCl DNase agar.

To compare the ability of the modified agar preparation to detect growth of staphylococci colonies an experiment was designed to compare this medium with Staphylococcal 110 medium. Cheese slurry prepared as outlined on page 31 was inoculated with approximately $10 \times 10^6$ *S. aureus* per g. Samples were collected every 2 hr and plated on both Staphylococcal Medium 110 and TBDA. Ten g of cheese slurry were collected and dilutions made with saline in sterilized test tubes after the slurry had been mixed with 90 ml of saline in a Sorvall Omni-mixer operating at its maximum rated speed for 3 min. Dilutions ranging from 1:1 to 1:1,000,000 were made and 0.1 ml of each dilution was plated in an identical manner on both the Staphylococcal 110 medium and on the TBDA. Total colonies were counted on both sets of plates after incubation for 48 hr at 37 C. After
total counts had been made the TBDA plates were recounted, counting only the nuclease zones present. The results appear in Figure 1. After 48 hr incubation at 37 C the colonies on the Staphylococcus 110 medium were larger in size; however, there were the same number of colonies on both plates. Lachica, Genigeorgis and Hoeprich (97) indicated in their study that toluidine blue was inhibitory toward gram-positive bacteria, especially sporeformers; however, at the concentrations outlined here _S. aureus_ strains grew well and produced large nuclease zones. Micrococci grew on the TBDA; however, no detectable zone was apparent even after incubation for a number of days. The longer the _S. aureus_ were left to incubate, the larger the nuclease zone became. No other organisms that grew on the TBDA produced as large a zone as did _S. aureus_. _S. aureus_ 13566, 19095, 14458, and 523235, known to produce enterotoxins A, B, C, and D, all produce similar zones on the TBDA after incubation. By cutting the nuclease zone out of the TBDA plate and placing it in a test tube in a boiling water bath for 15 min, it would be possible to test for heat stable nuclease using the toluidine blue DNA agar microslide as outlined on page 27. _S. aureus_ is the only organism reported that produces nuclease that retains its activity after 15 min boiling (47). If the toluidine blue DNA agar microslide demonstrated a positive zone after heat treatment, it would be a good indication that the organisms producing the nuclease were _S. aureus_.

It was interesting to note the decrease in _S. aureus_ growth during the incubation time. Recently, Tatini et al. (141) in studies of the production of staphylococcal enterotoxin A in cheddar and colby cheeses indicated that _S. aureus_ did not compete well with
other microorganisms in cheese. They reported that the extent of
*S. aureus* growth in number of generations increase, excluding the
increase without growth, was about five to seven generations in
cheeses of normal starter and over ten in cheeses showing starter
failure. As indicated by Figure 1, as the total number of organisms
increased in the slurry, the number of *S. aureus* began to decrease,
which would indicate that micrococci might be inhibitory in high
concentrations to *S. aureus*.

The TBDA developed is a useful screening technique for detection
and isolation of *S. aureus*. Confirmation can be accomplished by
testing the heat stability of the nuclease in the zones produced
as described above. One disadvantage to this method is the long
incubation period.

Correlation of Plate Counts With
Nuclease Production

To correlate the production of nuclease with *S. aureus* plate
counts, cells were grown in Brain Heart Infusion broth as outlined
on page 35. Cells were grown for various lengths of time and counted
as described on page 25. Nuclease analysis was conducted utilizing
the toluidine blue DNA microslide method as modified and outlined
on page 27. After the nuclease assay and plate counts were underway
the broth was injected into cheese slurry prepared as outlined on
page 31, and the nuclease was extracted and concentrated as outlined
on page 32. The results appear in Figure 2. It appears that as the
*S. aureus* plate counts reach $10^4$ to $10^6$, an increase in the rate of
nuclease production becomes detectable.

Appreciable levels of enterotoxin are produced only after considerable growth of the staphylococci. Usually a population of at least several millions per ml or g must be obtained. With the DNase methods described, it was possible to detect growth of staphylococcus between $3 \times 10^3$ and $5 \times 10^4$ organisms per ml or g of sample. This should be much less than the amount of organisms necessary to produce enough enterotoxin to cause an emetic dose. It should be pointed out that as the nuclease zones on the microslide develop beyond 25 mm, they approach the edge of the slide; thus requiring diluting of the extract for assay. When this condition exists, it appeared that the counts are above $10^7$—a count most likely to produce enterotoxins. These results indicate that this method would be adequate and sensitive enough for screening foods suspected of contamination by S. aureus.
Incubation Time For Maximum Nuclease Zone Development

In an effort to standardize the toluidine blue DNA microslide method and to determine a suitable incubation time that would allow detection of low concentrations of nuclease, an experiment was designed using various concentrations and dilutions of nuclease. Nuclease prepared according to the method described on page 23 was diluted using glycine NaOH 0.02 M buffer pH 10 in dilutions ranging from 1:1 to 1:10,000. Microslides were prepared as outlined on page 27, and wells were cut ranging in size from 10 mm to 1 mm. The dilutions were injected into the well in amounts ranging from 0.01 ml to 1 ul. At the higher concentrations of enzyme and larger inoculum the zones increased rapidly and diffused beyond the edge of the microslide. The intent of this study was to detect the smallest amount of nuclease with the most sensitive method available. An experiment utilizing different incubation times and various sized wells with various inocula was conducted. After numerous experiments it was found that the 4 mm well inoculated with 10 ul of extract provided the best sensitivity at low concentrations of nuclease activity. Inasmuch as most of the assays in this study involved extremely low amounts of nuclease, this was the method of choice. Although Lachica, Genigeorgis, and Hoeprich (87) indicated results of their assays in 3 hr, it was found in this study that at 3 hr some of the zones were not apparent—they developed after longer periods of incubation. In incubating the slides for extended periods it was found that the agar was drying out and not allowing the enzyme to
diffuse as far as possible. An incubation chamber was constructed as outlined on page 29 which allowed incubation for up to seven days with negligible or no dehydration. The chamber was placed in the incubator at 37 C in order to maintain a constant temperature. In an effort to arrive at an incubation time which would allow development of the zone under the conditions described above utilizing the 4 mm well, 10 ul of inoculum of very low concentrations of nuclease dilutions were inoculated on prepared microslides. The nuclease zones were measured at various times during the incubation period, and the results appear in Figure 3. It was apparent that under the conditions stated above that there was little increase in the zone size after 13 hr. It is interesting that microslides prepared under the same conditions and placed in the incubation chamber exhibited little or no change in zone size after 13 hr even after incubation for as long as seven days. It should be emphasized that the long incubation time is only necessary with very low concentrations of nuclease between 0.1 to 1.0 ug. As the amount of nuclease increased, the production of the nuclease zone increased; and with large amounts of nuclease over 100 ug/ml, the zone development occurs in less than 1 hr. Further development of these zones with this amount of S. aureus nuclease allowed diffusion off the edge of the slide as described on page 55.
NUCLEASE ZONE DIAMETER (mm)

INCUBATION TIME (hrs)

DILUTIONS

1:1
1:10
1:100
1:1000
Sensitivity Comparison of Nuclease Assay Methods

Few methods have been described for the detection of \textit{S. aureus} nuclease as outlined on page 21. Most of the methods described have not been adapted or utilized for detection of nuclease from cheese or other foods.

An attempt to develop a method for nuclease detection based on the work of Lachica and Deibel (96) utilizing acridine orange fluorescence was not successful. Their results could not be duplicated in that the relatively complex manipulation necessitated by the use of an ultraviolet light was inconvenient, but more important, the sensitivity of the acridine orange fluorescence was subject to quenching by proteins. This became very apparent in early studies with cheese extracts utilizing the acridine orange deoxyribonuclease agar described by Lachica and Deibel (96) and also in efforts to incorporate a spectrofluorometer in the study. A stock solution containing 1.5 mg/ml of denatured calf thymus DNA was prepared. Various concentrations of acridine orange were mixed with the DNA and measured on an Amicon \textsuperscript{13} spectrofluorometer to establish maximum fluorescence. It was possible to adjust the sensitivity of the fluorometer, and the 1.5 mg/ml DNA solution was a workable concentration. Because of inconsistent results obtained early in the study, it was suspected that an excess amount of acridine orange was interfering with the fluorescence. The solution was eluted through a

\textsuperscript{13}American Instruments Company Inc. 8030 Georgia, Silver Springs, Maryland 20910.
G-50 fine Sephadex column to remove the excess acridine orange from the solution. The DNA was recovered utilizing a spectrophotometer, and the DNA acridine orange solution was used as a substrate to observe if excess acridine orange interfered with the reaction. There was no significant difference in the results obtained. The sample preparations were made similar to the modified method of Chesbro and Auborn (41); however, the DNA acridine orange was used in place of the DNA in an effort to increase the fluorescent sensitivity. After incubation for 30 min and centrifugation, the samples were placed in the spectrofluorometer and excited at 290 nm, and the emission wave length according to this literature was scanned. It was observed that the DNA acridine orange peak was produced at 380 nm. DNA can be excited between 260 nm and 310 nm (99, 100). The excitation at 290 nm gave more sensitivity to the reaction. The inconsistent results obtained from this procedure were difficult to explain until it was discovered that quenching of the fluorescence by interfering proteins was taking place as reported by Lachica and Deibel (96). In theory one should be able to obtain greater sensitivity after conjugation with a fluorescence material such as acridine orange. A possible explanation of the lack of sensitivity is the quenching properties of proteins on the DNA acridine orange complex. The procedures necessary to purify the extracts to eliminate quenching proteins when working with a complex food such as cheese were not considered practical for this work. This was true even after (NH$_4$)$_2$SO$_4$ fractionation.

A slight modification of the method of Lachica, Genigeorgis, and Hoeprich (97) utilizing the metachromatic properties of toluidine
blue was also utilized as outlined on page 27. Instead of using 10 or 12 wells 2 mm in diameter and inoculating with a platinum loop, it was observed that more consistent results were obtained if four to five wells per slide and a Hamilton 10 ul syringe was used for inoculation of extracts. Instead of incubating for 2 to 4 hr at 37 C, it was found that more sensitivity was obtained in a humidity chamber for longer periods of time at 37 C. Toluidine blue in the presence of a polyanion such as agar, causes a shift in wave length of maximum absorbance from 590 nm to 540 nm. DNA causes a slight shift to a higher wave length. When *S. aureus* nuclease acts on the DNA, it causes a shift to a shorter wave length which produces a bright pink colored nuclease zone (97).

A method has been described by Chesbro and Auborn (40) utilizing a spectrophotometer to measure the change in absorbance at 260 nm after the enzyme had incubated for 30 min at 37 C. This method is outlined on pages 30-31. The method outlined is a modification of the method described by Chesbro and Auborn (41). In order to gain more accuracy in measuring solutions for the assay, the amounts were increased fivefold. To assure that all samples were treated in an identical manner, the top of a 10 pound plastic cottage cheese container was prepared for holding test tubes. Holes were cut so the tubes could be pressed in the top of the lid and the lid and tubes would then float in the 37 C water bath. Eight tubes were prepared at a time, and all tubes were treated in an identical manner. This method had the advantage of a short incubation time; however, a centrifugation step was necessary.

One of the objectives of this study was to find a method that
would be adaptable to the cheese industry and could be utilized to assay large amounts of cheese for possible contamination by *S. aureus*. In order to accomplish this goal some kind of automation would be helpful. It was felt that if the sensitivity of the spectophotometer was adequate that the extracts could be examined directly without preparation of agar slides, and the procedure would be more adaptable to an automatic readout system to tabulate the data obtained.

To compare the sensitivity of each method, a sample of *S. aureus* nuclease purchased from Sigma was prepared in amounts ranging from 1,000 ug to 0.01 ug/ml. Each dilution was divided into three identical samples, and the methods described above for nuclease assay were carried out. The results are indicated in Tables 7 and 8. It appears from the results illustrated here that the toluidine blue DNA agar diffusion method is the most sensitive. Where a response could be obtained with 0.01 to 0.1 ug/ml with the microslide method, it required 10.0 ug/ml utilizing the spectophotometer as described by Chesbro and Auborn (41).

### Table 7. Zone sizes produced by 0.01 through 1,000 ug/ml of staphylococcal nuclease following diffusion of 10 ul on toluidine blue DNA agar microslides

<table>
<thead>
<tr>
<th>ug Nuclease</th>
<th>Zone Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>32</td>
</tr>
<tr>
<td>100</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>0.01</td>
<td>slight color change</td>
</tr>
</tbody>
</table>

14 Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178.
Although the microslide method evidently was the most sensitive, it was necessary to incubate for 13 hr to obtain maximum sensitivity. This method would be rather difficult to adapt to automation, and an automatic readout system might be difficult to develop. The spectrophotometric method could be adapted to automation, and a direct readout could be developed. Utilizing AutoAnalyzer systems of instruments for automatic, continuous flow analysis, the optical densities could be measured in a recording spectrophotometer as the samples flow through their respective cuvettes. Samples could be monitored continually for high levels of nuclease, and measures could be developed to eliminate cheese or other food products from being distributed to consumers. This would eliminate the possibilities of contaminated food being distributed to the public; thus creating the potential for food intoxications.

Table 8. Chesbro and Auborn spectrophotometric method utilizing 0.01 through 1,000 ug/ml of staphylococcal nuclease following injection of 10 ul of each dilution and analyzing using the spectrophotometer.

<table>
<thead>
<tr>
<th>ug Nuclease</th>
<th>Absorbance (260 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>1.000</td>
</tr>
<tr>
<td>100</td>
<td>0.150</td>
</tr>
<tr>
<td>10</td>
<td>0.005</td>
</tr>
<tr>
<td>1</td>
<td>0.000</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
</tr>
<tr>
<td>0.01</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Correlation of Zone Diameter With u-Grams Nuclease

To compare the amount of \textit{S. aureus} nuclease required to produce a zone diameter on toluidine blue DNA agar microslides purified Sigma\textsuperscript{15} \textit{S. aureus} nuclease in dilutions ranging from 0.001 to 1,000 ug were prepared. Microslides were prepared as outlined on page 27. Ten microliters of each dilution were inoculated into each microslide well and incubated in the humidity chamber for 13 hr at 37 C. Figure 4 illustrates the results obtained from this experiment. Dilutions were made using 0.02 M glycine NaOH buffer pH 8.6 as recommended by Sigma. Under these conditions it was possible to observe a zone at 0.01 ug/ml. Lachica, Genigeorgis, and Hoeprich (97) were able to detect concentrations as low as 0.005 ug/ml, making dilutions with Brain Heart Infusion. In this study it was observed that below 0.01 ug/ml under the conditions stated above the zones produced were not very pronounced.

Inasmuch as the maximum amount of nuclease recovered in this study from cheese samples was 59 percent, this must be considered when trying to estimate the ug of nuclease present. One must realize that the zone size only represents 59 percent of the nuclease present in the sample. In high starchy foods the zones more closely correlate with the actual amount of nuclease present. (40).

\textsuperscript{15}Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178.
$u$ GRAMS NUCLEASE vs ZONE DIAMETER (mm)
Alexander, Heppel and Hurwitz (1) indicated in their study that there was little variation in the ability of different *S. aureus* strains to produce nuclease. Conversely, Lachica, Genigeorgis, and Hoeprich (97) indicated that there was a difference in the ability of different strains of staphylococci to produce nuclease. An experiment was designed to study nuclease production among *S. aureus* strains 13566, 19095, 14458, and 523235, known to produce enterotoxins A, B, C, and D. The toluidine blue DNA agar microslide method was used to compare the amounts of nuclease produced by each strain. The *S. aureus* strains were grown as described on page 35. The media was prepared for all strains and then divided into four parts to assure identical conditions of growth. Cell suspensions were transferred three times to produce maximum activity, and plate counts were made as described on page 25 to assure that the same number of organisms were inoculated from each strain. Samples were incubated under identical conditions at 37 C and samples collected each hr. To sample for nuclease, 10 ml of the media prepared as outlined on page 35 and containing the *S. aureus* after different periods of growth was collected using a 10 ml pipette. The samples were centrifuged to remove the cells, and the supernatants were placed in a boiling water bath for 15 min to inactivate any heat liable nuclease. The samples were analyzed for nuclease activity utilizing the method described on page 27. The results appear in Figure 5. The boiling for 15 min helped to concentrate the extract. Further studies in an
attempt to quantitate the nuclease required that the volume of the extracts be adjusted to original volume after boiling. It appears from Figure 5 that some strains begin to produce nuclease faster initially; however, they may not produce as much nuclease ultimately. The results indicate that there are differences in the ability of the strains to produce nuclease as reported by Lachica, Genigeorgis, and Hoeprich (97). It is also apparent from this experiment that nuclease can be detected even though the cells are not viable. The heat stability of this enzyme was confirmed by this study (1, 47, 72). Although there is a difference in the production of nuclease as illustrated by Figure 5, the difference appears to be less than 50 ug when correlating the zone diameters produced by the various strains with Figure 4.
NUCLEASE ZONE DIAMETER (mm)

INCUBATION TIME (hrs)

STRAINS
A
B
C
D
**Direct Assay For Deoxyribonuclease**

For food products with extremely high staphylococcal counts, the nuclease activity was assayed directly using the microslide method outlined on page 27. Ten g of a suspected food was mixed with 90 ml of glycine NaOH buffer pH 10 and blended in a Sorvall\textsuperscript{16} Omni-mixer at high speed for 3 min. It was possible to place a loop of this extract directly on the microslide and obtain a positive reaction within 3 hr with high *S. aureus* counts.

An outbreak of staphylococcal poisoning was reported and investigated at Utah State University during this study. A chicken dinner was catered to 60 Moen Hall students for their evening meal. Approximately 3 to 5 hr after ingestion 16 students began to show signs and symptoms of food intoxication, including nausea, vomiting and diarrhea. Food samples of chicken and strawberry cream pie were examined for nuclease and enterotoxin. The cream pies were found to be free from staphylococci and nuclease. The chicken composite tissue was examined for staphylococci and the counts were over $3 \times 10^8$. Upon investigation for nuclease, using the direct technique described above, the activity was very pronounced in less than 3 hr incubation. Upon further investigation it was found that the catering service was short 15 chickens and had carried over 15 chickens from a previous engagement. Apparently these chickens had become contaminated with staphylococci and were effective in causing the staphylococcal intoxication. The nuclease assay was very helpful.

\textsuperscript{16}Ivan Sorvall Inc. Newton, Connecticut 0604 U.S.A.
in the investigation of chicken contaminated with staphylococci. This indicated the usefulness of this method in investigations of food-borne intoxications involving *S. aureus.*
CONCLUSIONS

1. The efficiency of different nuclease extraction buffers were compared, and it was discovered that the maximum recovery was obtained using glycine NaOH buffer 0.02 M pH 10.

2. Different \((\text{NH}_4)_2\text{SO}_4\) fractions were investigated, and it was observed that the nuclease is precipitated in the 40-80 percent saturation fraction. Different methods for the recovery of nuclease from cheese were investigated, and it was discovered that \((\text{NH}_4)_2\text{SO}_4\) fractionation in conjunction with pressure dialysis gave 59 percent recovery of \textit{S. aureus} nuclease.

3. It was possible to detect growth of \textit{S. aureus} under different growth conditions when inoculated into cheese slurries. By analyzing the heat stable nuclease produced in cheddar cheese slurries it was possible to monitor \textit{S. aureus} growth. This was confirmed by the work of L. W. Jones in his study of the effects of nisin on the development of \textit{S. aureus} where he was able to illustrate its effects using the nuclease assay.

4. A toluidine blue DNase agar 7.5 percent NaCl was developed for the detection and possible confirmation of \textit{S. aureus}. Staphylococci growing on this agar produced a wide nuclease zone after incubation for 48 hr at 37 C. This zone can be cut out and placed in a boiling water bath for 15 min which eliminates any heat labile nuclease produced from non \textit{S. aureus} organisms that may have grown in the samples. The extract is then placed on a toluidine blue DNA agar microslide and examined for heat stable nuclease. The
presence of heat stable nuclease would be indicative of \textit{S. aureus} contamination and possible production of enterotoxins. As a result of the inhibitory aspects of the toluidine blue especially toward gram positive spore forming bacteria and the high NaCl content of the media, other nuclease-producing organisms are inhibited, adding to the usefulness of the TBDA media. Instead of performing numerous tests as recommended by Difco\textsuperscript{17}, the orange pigment, coagulase test, fermentation of mannitol and the gelatinase test, it is possible to detect \textit{S. aureus} using the TBDA media and heat stable nuclease analysis. Using the TBDA media it may be possible to monitor the growth of \textit{S. aureus} in cheese slurries.

5. Correlation between plate counts and nuclease production was established utilizing the toluidine blue DNA microslide method for analysis. Using this method it was possible to detect \textit{S. aureus} growth using the microslide method between $3 \times 10^3$ and $5 \times 10^4$ cells per ml or g of sample. This should be much less than the amount necessary to produce enough enterotoxin to cause an emetic dose.

6. Two methods of nuclease assay were compared for sensitivity, adaptability, and usefulness. It was found that the microslide method gave the most sensitivity; however, it required 13 hr incubation, whereas the spectrophotometric method only required a 30 min incubation time. With the microslide method it was possible to detect 0.01 to 0.1 ug/ml nuclease. Using the spectrophotometric method, it required 10 ug/ml. Although the last method demonstrates less sensitivity than the microslide method, it would be more

\textsuperscript{17}Difco Laboratories Inc., Detroit, Michigan 48201.
adaptable to automation and a continuous readout system.

7. Investigation was conducted to determine the incubation time, inoculum, and well size necessary to detect low quantities of nuclease that would represent S. aureus cell numbers in an amount not likely to cause food intoxication. It was discovered that using a 4 mm diameter well and inoculating 10 ul of extract after incubation for 13 hr, it was possible to detect 0.01 to 0.1 ug/ml of nuclease. This amount of nuclease as correlated with S. aureus cells and enterotoxin production is not likely to cause an emetic dose.

8. Correlation was established between nuclease zone diameter and ug of nuclease necessary to produce the zone under the conditions stated above. Dilutions of purified nuclease from Sigma\textsuperscript{18} were made and a standard regression calibration curve established. Using this curve, represented in Figure 4, it is possible to estimate the amount of nuclease in ug by the size of nuclease zone produced.

9. Nuclease production among different S. aureus strains were compared. Although some strains produced nuclease faster to begin with, they may not produce as much totally. Of the strains examined, all produced nuclease ranging between 50 and 100 ug per ml after incubation for 16 hr. It would appear that the sensitivity of the methods studied would detect growth and development of all S. aureus strains examined in this study.

10. A direct assay for nuclease was developed and found successful in the investigation of an outbreak of staphylococcal

\textsuperscript{18}Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63178.
intoxication. When the amount of nuclease produced is between 500 ug and 1,000 ug/ml, a nuclease zone developed on a toluidine blue DNA agar slide in less than 1 hr. By blending the chicken tissues with glycine NaOH buffer pH 10 for 3 min and inoculating the extract directly on the microslide after boiling for 15 min to eliminate heat labile nuclease, a zone developed in less than three hr. This is a great improvement over the eight day procedures outlined for the detection of enterotoxins.

11. Measurement of nuclease activity is a sensitive method for detection of foods contaminated with the metabolic products of S. aureus. In addition to sensitivity, the main advantages of this method are simplicity, rapidity, and also that no uncommon reagents are necessary. The specificity of the method and lack of interference from large mixed populations of microorganisms makes it particularly useful and adaptable to the cheese industry.


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