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Factors affecting the production of thermonuclease by Staphylococcus aureus Z88

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FACTORS AFFECTING THE PRODUCTION OF THERMONUCLEASE BY STAPHYLOCOCCUS AUREUS Z88

Iowa State University PH.D. 1984

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Factors affecting the production of thermonuclease by *Staphylococcus aureus* Z88

by

Deland Jay Myers

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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1984

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DEDICATION

To

Juanita L. Myers

and

Eveadean Myers

Without their love, sacrifice and faith in God, this dissertation would have been in vain. Words cannot express my love and respect for you.

INTRODUCTION

Interest in rapid tests for the detection of micro-organisms in food products has increased during the past decade. The reasons for this are the ease, low cost and speed of the tests. These rapid tests primarily involve the detection of metabolic products made by the micro-organism in the food; i.e., CO_2, acids, proteins, etc.

A rapid test for the presence of *Staphylococcus aureus* in foods is detection of the enzyme thermonuclease. The presence of this protein provides a very good indicator because the enzyme: 1) survives the mild heat processing of food products, 2) has a high correlation with the toxigenicity of *Staphylococcus aureus*, and 3) has physical characteristics that make it specific for *Staphylococcus aureus*.

A number of questions have been raised concerning the various methods used to detect this enzyme. Can any of these tests be used on a daily basis in a quality control laboratory? Can any of these analyses be quantitative as well as qualitative, and what advantage is there in having a quantitative test? A final, very pertinent question is whether thermonuclease will be produced in all food systems and environments in which enterotoxin is produced.

The purpose of this dissertation is an attempt to

answer all or part of the aforementioned questions. The dissertation will address three specific concerns: 1) modification of a test used for detection of the thermonuclease and the limits of that test; i.e., specificity, sensitivity, simplicity and quantitative capability, 2) examination of what effects certain environmental conditions have on the production of thermonuclease in broth culture, and 3) compare two methods for extracting nuclease from a meat product.

LITERATURE REVIEW

The Genus *Staphylococcus*

Staphylococcus is a genus of gram-positive bacteria in the family Micrococcaceae. This genus consists of non-motile cocci that grow in irregular clusters (Baird-Parker, 1974). This genus differs from others in the same family because: 1) it is primarily parasitic, 2) it is faculta-tively anaerobic and produces acid from glucose under anaerobic conditions, 3) the guanine and cytosine content is lower in cells of *Staphylococcus* (30-40%) as compared to the guanine and cytosine content of cells of the other genera in the same family, and 4) the chemical composition of the cell walls differ (Baird-Parker, 1974; Minor and Marth, 1971).

Organisms in the genus *Staphylococcus* grow at an optimum temperature of 35 to 40°C with a temperature growth range of 6.5° to 46°C and at an optimum pH of 7.0 to 7.5 with a pH growth range of 4.2 to 9.3 (Baird-Parker, 1974). These organisms are associated with and usually found on the skin, skin glands, and mucous membranes of warmblooded animals.

Staphylococci require an organic source of nitrogen (amino acids) and two or more vitamins for growth, particu-larly in a synthetic medium (Minor and Marth, 1971).

Staphylococcus aureus

The species of Staphylococcus that is of most concern and is most commonly studied in food microbiology is Staphylococcus aureus. The reason for this is that S. aureus is the only species of this genus implicated in food poisoning outbreaks. This organism, which can be destroyed during the heat processing of foods, produces an enterotoxin that can survive the heat processing of foods. The toxin, when ingested with the food product, causes food poisoning (Minor and Marth, 1972).

Characteristics of S. aureus that distinguish this species from other Staphylococcus species are: 1) the production of coagulase, and 2) the fermentation of mannitol (Baird-Parker, 1974; Minor and Marth, 1971). The organism produces a myriad of extracellular enzymes which are believed to assist the bacterium in infective processes (Baird-Parker, 1974). Enzymes present in this arsenal include proteases, lipases, phospholipases, lipoprotein lipases, esterases, lyases, coagulases, nitrases and heat-stable nucleases.

Enterotoxin

Staphylococcus aureus causes a food-poisoning syndrome in man upon the ingestion of preformed enterotoxins present in the food product. Enterotoxins are single, unbranched

water-soluble peptides (M.W. 30,000-35,000) that cause gastroenteritis by acting on the intestines (Baird-Parker, 1971; Minor and Marth, 1972). To date, there are 8 serologically different enterotoxins identified. The 8 are given the letter-numerical designations of A, B, C_1, C_2, C_3, D, E, and F (Payne and Wood, 1974). The letter designations for enterotoxin types were established on May 9, 1962 at the American Society for Microbiology Meeting in Kansas City, Mo. (Casman *et al.*, 1963). Staphylococcal enterotoxins A and D (SEA and SED) have been implicated in the majority of the food poisoning outbreaks caused by *S. aureus* (Jay, 1978).

Enterotoxins are produced by cells when they grow in a laboratory medium or a food product. Because the toxin causing the syndrome is produced outside the body, food poisoning caused by *S. aureus* is classified as a food intoxication (Minor and Marth, 1972). The minimum number of cells needed to produce a sufficient quantity of enterotoxins to cause food poisoning is generally agreed to be $>10^6$ cells/g (Baird-Parker, 1971; Barber and Deibel, 1972; Genigeorgis *et al.*, 1971a). Optimum conditions for the production of all enterotoxin types include: 1) a pH in the range of 6.0 to 8.0, 2) a temperature of 37 to 40°C, 3) an Aw greater than 0.94, and 4) an environment of high O_2 tension

(Jay, 1978). The point should be made that enterotoxin production takes place in a more narrow range of environmental conditions than the multiplication of _S. aureus_. Conditions favorable for the production of specific enterotoxins differ depending on the type of enterotoxin being produced (Minor and Marth, 1972). Not all strains of _S. aureus_ are capable of producing enterotoxins or are enterotoxigenic (Baird-Parker, 1971). On the other hand, many enterotoxigenic staphylococci are able to produce more than one type of enterotoxin (Gilbert and Wieneke, 1973; Payne and Wood, 1974).

Another important physical characteristic of these enterotoxins is the remarkable heat stability of these proteins. As a general rule, enterotoxins can be boiled for 15 minutes without any or only minimal loss in activity. SEB is the most heat stable of the enterotoxins followed by SEC and SEA (Bergdoll, 1973).

Enterotoxin A

Enterotoxin type A is the toxin that is most often implicated in staphylococcal food poisoning outbreaks. Freeze-dried enterotoxin type A is described as a fluffy, snow-white material that is highly hygroscopic and very soluble in water and salt solutions (Chu _et al_., 1966; Minor and Marth, 1972). The molecular weight has been estimated to be

about 34,400. The isoelectric point is pH 6.8. The active toxin is trypsin resistant to trypsin and has a maximum absorption at 277 mμ (Chu *et al.*, 1966).

Staphylococcal enterotoxin type A is produced during the log phase of growth (Bergdoll *et al.*, 1974). Optimum conditions for the production of SEA are pH 7.0 (Carpenter and Silverman, 1976), aeration, Aw > 0.90 (Lotter and Leistner, 1978) and a temperature of 37°C. Enterotoxin A is normally produced in smaller amounts than the other serological types (Jarvis *et al.*, 1973; Kato *et al.*, 1966). For example, SEA can be produced under ideal conditions in amounts of 5 to 6 μg/ml; quantities of SEB can be produced at levels of 350 μg/ml (Reiser and Weiss, 1969). SEA, however, appears to be produced in more uniform amounts from strain to strain (Kato *et al.*, 1966).

There are several explanations why many researchers believe that SEA is implicated in more food poisoning outbreaks than the other serological types. The first is that most enterotoxigenic *S. aureus* isolated from foods produce SEA (Jay, 1978). In one survey of staphylococci isolated from foods, Payne and Wood (1974) found that 49% produced enterotoxin A. In another survey, Hall (1968) found 62.4% of the enterotoxigenic organisms produced SEA. The second explanation is that the

optimal pH range of 6.0 to 7.0 for production of SEA is commonly encountered in foods (Markus and Silverman, 1970).

Discovery of Staphylococcal Food Poisoning

Elek (1959) chronicled the history of the discovery of staphylococcal food poisoning. He lists J. Denys as the first researcher to correlate a food poisoning case to staphylococci in 1894. The case involved meat consumed from a sick cow infected with staphylococci. All the persons who ate the meat became ill while one person in the family who did not eat the meat did not become ill. Elek notes R. Owen as the next significant researcher; Owen corroborated Denys' findings in 1907. His work involved an acute gastroenteritis in 19 persons who had consumed dried beef contaminated with staphylococci. The next investigator of note was Barber who in 1914 investigated acute gastro-intestinal upsets. He traced these upsets to milk from cows infected with mastitis. He reproduced this disease in himself by consuming cream from the milk of these cows.

The final important investigators in the elucidation of staphylococcal food poisoning were Dack *et al.* (1930). These investigators are credited with reporting the first

thorough examination of food poisoning by staphylococci (Elek, 1959) and showed conclusively that some strains of *S. aureus* are able to produce food poisoning (Jay, 1978). The research involved an outbreak of intestinal upsets from eating a Christmas cake. After eliminating metallic poisons or *Salmonella* as possible causes, they grew strains of cocci found in the food. The filtrates of these organisms were toxic to a rabbit and human volunteers. These cocci were *Staphylococcus aureus*.

Pathology of Staphyloccal Food Poisoning

After ingestion of food containing sufficient amounts of enterotoxin, the onset of symptoms occurs in approximately 4 hours with a range of 1 to 6 hours (Jay, 1978; Minor and Marth, 1972). The time of onset is consistent with the syndrome for a food intoxication; most symptoms of an intoxication occur within 1 to 10 hrs. The symptoms start with salivation followed by nausea, vomiting, abdominal cramps (which are usually quite severe), diarrhea, sweating, headache, prostration and sometimes a fall in body temperature. Complications can occur, including dehydration, shock and blood and mucus in the vomitus and stools. The symptoms last for 24 to 48 hours; the illness is rarely

fatal (Jay, 1978; Minor and Marth, 1972). Treatment involves maintaining fluid balance and bed rest.

The mode of action of enterotoxin on the intestines is unclear. The target areas for binding and inducing vomiting and diarrhea in humans appear to be the abdominal viscera and gastrointestinal tract (Jay, 1978; Sugiyama and Hayama, 1964). The amount of enterotoxin needed to produce the symptoms in humans is believed to be <1 μg (Bergdoll, 1972).

Food Poisoning Outbreaks

A wide variety of foods are involved in staphylococcal food poisoning outbreaks (Banwart, 1981; Jay, 1978). In a summary of the foods involved in staphylococcal food poisoning outbreaks compiled by the CDC for the years 1971-1976 (Banwart, 1981), the top 3 implicated foods were meat, poultry and salads in decreasing order. Jay (1978) listed the usual two "common denominators" of foods implicated in these food poisoning outbreaks as convenience foods made by hand and improper refrigeration after preparation. Despite the wide variety of foods implicated, research shows that red meats are involved in over 40% of cases of staphylococcal food poisoning.

Food Poisoning Outbreaks in Meats

Fresh meats are less involved in food poisoning outbreaks than cured or fermented meats. Banwart (1981) lists the primary reason for this as being that fresh meats are held under refrigeration because they are perishable. Also, fresh meats contain more competitive organisms that can inhibit the growth of *S. aureus* (Casman *et al.*, 1963; Troller and Frazier, 1963). Banwart (1981) reported that cured meats often are mishandled by allowing them to remain at room temperature and therefore may be implicated more often. Peterson *et al.* (1964) noted that NaCl (an important ingredient in cured products) acts as a selective agent for staphylococci by being antagonistic to saprophytic organisms. Data summarized by Bryan (1976) support this theory by showing that of 251 meat products implicated, 76 were ham products.

Cooked meats should be safe if produced so as to contain low numbers of organisms and then are held at temperatures above 140°F or below 40°F (Jay, 1978).

Food poisoning outbreaks from fermented meats are the result of bad manufacturing practices (Lee *et al.*, 1977). Lee *et al.* (1977) noted that lactic acid bacteria inhibit growth of *S. aureus*.

Detection of Enterotoxins

Use of animals

The best method to demonstrate the presence of enterotoxin in foods is to feed the suspected food to humans (Jay, 1978; Minor and Marth, 1972). Due to the lack of volunteers (Jay, 1978) and the lack of practicality of the method (Minor and Marth, 1972), researchers have turned to animals. Animals that have been used in these analyses include dogs, pigs, pigeons, frogs, and rabbits. The two animal subjects that have been used most frequently are kittens and monkeys (Jay, 1978; Minor and Marth, 1972).

The kitten test, developed by Dolman *et al.* (1936) consists of an intraperitoneal dose of filtrate from an enterotoxin-producing culture of *S. aureus* into a kitten. If the enterotoxin is present, vomiting is produced in the animal. The problems with the kitten test are: 1) the lack of consistent sensitivity of kittens to the toxin, and 2) other toxic substances that can also induce vomiting necessitating the need for heat treatment of the filtrate (Minor and Marth, 1972).

The detection of enterotoxins using monkeys involves the preparation of a filtrate from the suspected food and administering the filtrate via a catheter (Jay, 1978). The advantages of the monkey test are: 1) the filtrate can be administered orally, 2) the monkeys give the emetic

response only in the presence of enterotoxin (Bergdoll, 1972), and 3) young Rhesus monkeys are thought to be the most susceptible animals to enterotoxins other than man, although some believe cats are more susceptible (Denny et al., 1966). Some disadvantages are: 1) the monkeys may exhibit some variability in their response to oral doses of enterotoxin (Surgalla et al., 1953), and 2) monkeys become less sensitive to the emetic response of enterotoxin for a short time after the continued administration of the toxin (Sugiyama et al., 1962; Sugiyama and Hayama, 1964).

Serological Detection of Enterotoxin

Serological detection of staphylococcal enterotoxin is based on the theory of antigen-antibody reactions. If the antigen reacts with the proper antibody at the right titer, binding will take place. The use of serology for the detection of enterotoxins presents the investigator with two specific problems. The first is the need for a separate reagent (antibody) to detect each enterotoxin (Bergdoll et al., 1976). Secondly, enterotoxin is produced or may be present (especially in foods) in small amounts. This requires either concentration to get detectable levels of enterotoxin or an extremely sensitive test.

In the hope of making detection more sensitive and easier to do, several serological methods have been devised. These tests include: 1) the Microslide method (Casman and Bennett, 1965), 2) an Immunofluorescence method (Stark and Middaugh, 1969), 3) Radioimmunoassay (Bergdoll and Reiser, 1980), 4) a Reversed Passive Hemagglutination Assay (Silverman *et al.*, 1968), and the 5) Enzyme-linked Immunosorbent Assay (Freed *et al.*, 1982; Notermans *et al.*, 1978).

Microslide method

The antigen-antibody precipitation reaction will be seen as a white band if it takes place in an agar medium (Minor and Marth, 1971). This concept was developed by Ouchterlony (1958) into a double-diffusion technique in Petri plates with the agar containing the antigen and antibody; Ouchterlony cites Oudin as having applied the same concept to tubes in 1946. Wadsworth (1957) modified the method of Ouchterlony by using a microscope slide coated with a thin layer of agar.

Although some enterotoxin detection analyses are performed with Ouchterlony plates and tubes (Fung and Wagner, 1970), the most frequently used method is the Wadsworth method. The microslide method, although considered to be unreliable and archaic by some, is still widely used in many laboratories (Freed *et al.*, 1982) and recognized by the FDA

Bacteriological Analytical Manual for Foods (Olson, 1976) and the Compendium of Methods for the Microbiological Examination of Foods (Bergdoll and Bennett, 1976).

The basic method involves the application of a thin layer of agar to a microscope slide. The filtrate and antibody are added to wells on the slide. Formation of a white band of precipitate between wells containing filtrate and antibody constitutes a positive reaction (Bennett and McClure, 1976; Bergdoll et al., 1976).

Two major problems with the test have caused many laboratories to abandon this test in favor of other methods. The first is that the test is not very sensitive. The lower limit of sensitivity of this test for foods is approximately 0.1 μg enterotoxin/ml of filtrate; many researchers have difficulty detecting enterotoxin at this level (Bergdoll et al., 1976). The second major problem is the length of time required to obtain results from this test. Because of the lack of sensitivity of the method, concentration becomes necessary and takes several days. This need, along with the incubation time, can require a total time of 3 to 6 days (Bergdoll et al., 1976). Although some modifications have been made in the method (Reiser et al., 1974), the test still takes up to 3 days.

Fluorescent Antibody Technique

One method that has been proposed as a rapid detection method for the presence of enterotoxin in foods is the Fluorescent Antibody Technique (FAT). The theory behind the method involves the development of antibodies to the enterotoxin and conjugating them to fluorescein isothiocynate (Minor and Marth, 1972). The conjugated antiserum (conjugate) is used to stain smears of broth cultures or food slurries. These preparations are incubated for a period of time and then the unreacted conjugate is washed off (Stark and Middaugh, 1969). Detection of fluorescence on the slide when observed under a U.V. microscope indicates the presence of enterotoxin. The method can be quantitated by measuring the degree of cell fluorescence (Stark and Middaugh, 1969).

The major advantage of this test is the lack of the necessity for concentrating the food extract. This decreases the amount of time needed to run the test (Stark and Middaugh, 1969).

Two problems exist with this method that bear mentioning. The first and most important problem with the test is sensitivity. Genigeorgis and Sadler (1966b) detected as little as 0.05 μg enterotoxin/ml while Bergdoll (1970) lists the sensitivity at only 1 μg/ml. Another problem with the method is the need for expensive equipment; namely, the U.V. microscope.

Reversed Passive Hemagglutination Assay and the Radio-immunoassay

Two methods have been proposed that have proven to increase the sensitivity of detecting enterotoxin. The methods are the Reversed Passive Hemagglutination Assay (RPHA) and the Radioimmunoassay (RIA) (Bergdoll *et al.*, 1976; Bergdoll and Reiser, 1980).

The RPHA is based on the adsorption of specific antibody to tannic acid-treated sheep red blood cells. When these cells come into contact with enterotoxin, a hemagglutination reaction occurs (Silverman *et al.*, 1968).

There are a number of advantages to using the RPHA. The test is very sensitive and can detect levels of 1.5 ng enterotoxin/ml of food without concentration of the food sample (Bergdoll *et al.*, 1976). Since no concentration of food extracts is necessary, the method is fast and can be done in only a few hours (Silverman *et al.*, 1968). Bergdoll *et al.* (1976) list two problems encountered with the test that have caused many researchers to become disillusioned with the RPHA. The first is the nonadsorption of the antibody to the red blood cells which results in the absence of agglutination of the cells in the presence of enterotoxin; this yields false-positive results. This problem has been related to low titer antisera. The second problem is the nonspecific agglutination of proteins in the food extracts.

Although the problem can be resolved by the use of trypsin or by partial purification of the food extract (Reiser *et al*., 1974), it also increases the time needed to perform the analysis. Bennett *et al*. (1973) along with Bergdoll *et al*. (1976) concluded that the method was unreliable.

The radioimmunoassay (RIA) is a method that is extremely sensitive for the detection of enterotoxin. Bergdoll and Reiser (1980) credit Dr. Robert A. Monroe for initiating the use of the RIA technique for detection of staphylococcal enterotoxins. Bergdoll and Reiser (1980) describe the basic RIA technique as a given antigen (or unknown sample) competing with a known amount of the same antigen labeled with a radioactive nucleide. The amount of antigen present is calculated from a standard curve prepared from results obtained when known amounts of unlabeled antigen are used. This technique is the basis for many different tests (Miller *et al*., 1978).

Conflicting reports have been published on the sensitivity of this technique. Bennett *et al*. (1973) detected a minimum of 10 ng of SEA/g of food while Johnson *et al*. (1973) were able to detect 1 ng SEA/g of food. Bergdoll and Reiser (1980) list the sensitivity of the method of 1 ng toxin/g of food and the ability to perform the analysis in one day as advantages of this technique.

Bergdoll and Reiser (1980) list two problems which de-

ters the RIA test from becoming a widely used method. The first is the need for expensive equipment and radioactive materials that many laboratories may have a problem providing. The second is the need for purified enterotoxins which are rare and very expensive. Kuo and Silverman (1980) also list nonspecific adsorption as a problem.

Enzyme-Linked Immunosorbent Assay

The method that presently is showing promise for the detection of enterotoxins is the Enzyme-Linked Immunosorbent Assay (ELISA). The key selling point of this assay is that it is as sensitive and as rapid as the RIA, <1 ng/g food, without the use of radioactive materials (Freed *et al.*, 1982; Kuo and Silverman, 1980).

Kuo and Silverman (1980) stated that the principle of their ELISA method is identical with the RIA. The method, like the RIA (Bergdoll and Reiser, 1980), basically involves the antigen (or unknown sample) competing with a known antigen-enzyme conjugate for antibody sites. The amount of antigen present is calculated from a standard curve established with known amounts of antigen. The major difference between the RIA and the ELISA is that the RIA uses a radioactive nucleide that is measured; in the ELISA method, the measurement is related to color formation by the reaction of the enzyme and the substrate with both relating to the amount of toxin present (Freed *et al.*, 1982; Kuo and Silver-

man, 1980).

Kuo and Silverman (1980) list the advantage of the ELISA as: 1) no radioactive compounds are needed, 2) common equipment can be used, and 3) a positive reaction can be visually evaluated. They also state that the major disadvantage of the assay is the problem of nonspecific adsorption of proteins.

Screening of Foods for the Presence of Enterotoxin

A major trend in research on *S. aureus* in foods is the use of assays that would serve as an indicator for the presence of enterotoxin. Tatini *et al.* (1976) state that direct detection of enterotoxins in foods is expensive and is based on methods presently not feasible. They list five qualities that a substance should meet to qualify as an indicator for enterotoxin; it should: 1) be rapidly and inexpensively assayed for, 2) be produced by all strains of *S. aureus*, including all enterotoxigenic strains, 3) be produced only by *S. aureus* or can be differentiated by some other test (pH optimum, etc.), 4) be produced in detectable amounts under conditions that permit the growth of *S. aureus* or the production of detectable amounts of enterotoxins, and 5) be able to survive processing conditions to the same extent that enterotoxins do.

Many substances produced by *S. aureus* have been studied in an attempt to associate their presence with the presence of enterotoxins; they include gelatinase, phosphatase, lysozyme, lecithinase, lipase, thermonuclease, fermentation of carbohydrates, and coagulase (Jay, 1978; Lachica *et al.*, 1969; Minor and Marth, 1971; Niskanen and Nurmi, 1977). The only substances that have been employed with any success have been coagulase and thermonuclease. Two additional properties, production of protein A and fibrinogen affinity, are presently of interest to some researchers (Carret *et al.*, 1982).

Coagulase Assay

Coagulase is an extracellular enzyme produced by *S. aureus*; it clots citrated or oxylated rabbit or human blood plasma (Minor and Marth, 1971). The enzyme has thrombokinase-like activity in that it initiates the conversion of fibrinogen to fibrin. The bacterial cells become coated with fibrin and then clump together forming a clot-like appearance in the test procedure (Clancy, 1974; Willett, 1980).

The coagulase test is carried out by adding citrated rabbit or human blood plasma to a broth culture (in a tube or on a slide) and incubating for a suitable period of time. If the enzyme is present, a fibrin clot is formed

(Clancy, 1974).

Early studies on coagulase and enterotoxin production (Evans and Niven, 1950) established the belief that almost all food-poisoning strains of S. aureus were coagulase-positive. Although much evidence has been accumulated to show that this was a false assumption (Willett, 1980), many researchers still agree that a high percentage of the strains of S. aureus that cause food poisoning produce coagulase (Baird-Parker, 1974; Gramoli and Wilkinson, 1978; Jay, 1978). The production of coagulase is not necessarily a stable characteristic in all cultures. Some cultures have been reported to lose this trait during laboratory storage (Lachica et al., 1969).

Two major problems are encountered with the coagulase test. The first problem relates to false-positive results. Some bacteria, particularly S. epidermidis, produce pseudocoagulases that coagulate blood plasma (Wegrzynowicz et al., 1980). Baird-Parker (1965) reported that citrate-utilizing bacteria such as Pseudomonas aeruginosa, Serratia marcescens and Streptococcus faecalis can cause false positives in this test. Rayman et al. (1975) cited the second major problem as the difficulty in interpreting the results of the test. The tube coagulase test is semiquantitative with the degree of clotting measured by +1 (lowest degree) to +4 (highest degree). The test is very subjective and there is a question

as to what degree of clotting exhibits a positive test. The subjective nature of this test could lead to an inaccurate interpretation of the results (Barry *et al.*, 1973; Rayman *et al.*, 1975).

A significant problem concerning the coagulase assay for foods is the need for viable organisms. The assay can be performed in raw, unprocessed products but if the organisms have been killed by processing, the assay cannot be used. Enterotoxin, which can withstand processing conditions, can still be present. Another analysis is then necessary for the screening of processed foods for enterotoxins.

Thermonuclease Test

Enzyme

In microbial literature, reference to thermonuclease generally means the extracellular enzyme produced by pathogenic strains of *S. aureus* (Anfinsen et al., 1971). The enzyme is a small, globular protein with a molecular weight of 17,000 and consists of 149 amino acids. The enzyme, described as a 3'-nucleotidohydrolase that can hydrolyze RNA or DNA, cleaves the 5'-phosphoryl diester with exo- and endo-activity. The result of this reaction produces 3'-phosphomononucleotides and dinucleotides (Cuatrecasas *et al.*, 1967). The enzyme is most reactive at the sugars attached to

adenine-thymine but can cause some breakdown of those sugars attached to cytosine and guanine (Anfinsen et al., 1971). The greater activity of the enzyme on DNA than RNA is believed to be based on the base preference (Cuatrecasas et al., 1967). The enzyme has even greater activity on denatured DNA (Anfinsen et al., 1971; Cuatrecasas et al., 1967).

One important physical property of the enzyme is its dependence on Ca^{+2} (Anfinsen et al., 1971; Cuatrecasas et al., 1967; Heins et al., 1967). In fact, the pH optimum of the enzyme, which is between 9 and 10, depends on the concentration of Ca^{+2} (Cuatrecasas et al., 1967). The optimum temperature is reported to be 50°C (Kamman and Tatini, 1977).

Thermostability or the maintenance of activity at high temperatures is the most remarkable physical property of this enzyme. In one of the earliest studies on this enzyme, Cunningham et al. (1956) reported that the enzyme withstands boiling for 15 minutes without any appreciable loss in activity. Cuatrecasas et al. (1967) reported that the enzyme had no loss of activity after boiling for 20 minutes and Chesbro and Auborn (1967) reported that the enzyme was stable for 15 minutes at 121°C. Enzyme stability is enhanced by the presence of Ca^{+2} ion, serum albumin, and nucleotide breakdown products (Cuatrecasas et al., 1967;

Sulkowski and Laskowski, 1968).

The enzyme is produced by S. aureus early during the log phase of growth and continues into the stationary phase (Carpenter and Silverman, 1976; Erickson and Deibel, 1973a). Most researchers agree that at least 10^6 cells/ml are needed to produce nuclease in detectable amounts (Koupal and Deibel, 1978; Tatini et al., 1975, 1976).

Arvidson et al. (1971) showed that nuclease is produced under more restrictive conditions than those for growth. Optimum conditions for the production of the enzyme are difficult to determine. These optimum conditions will differ based on the growth conditions such as the type of medium used, amount of aeration, strain used, and the concentration of Ca^{+2} ion (Arvidson et al., 1971; Fox and Holtman, 1968; Tatini et al., 1976; Weckman and Catlin, 1957). Most researchers agree, however, that conditions that stimulate the production of nucleases are conditions that stimulate large numbers of cells of S. aureus ($>10^6$ cells/ml). As conditions become less favorable for the growth of the organisms, less, if any, nuclease is produced (Arvidson et al., 1971).

Nuclease vs. enterotoxin

The primary significance of thermonuclease (DNAse) in food microbiology research is screening for enterotoxin in foods (Tatini et al., 1976). The reason for assaying for

nuclease as an indicator for the presence of enterotoxin is the high correlation between the presence of enterotoxin and nuclease (Cunningham *et al.*, 1956; Niskanen and Nurmi, 1977). Lachica *et al.* (1969) reported that 95% of the enterotoxin-producers elaborated nuclease. Tatini *et al.* (1976) reported data showing that 98.3% of the enterotoxigenic strains produced nuclease. In addition to the high nuclease-enterotoxin correlation, Cords and Tatini (1973) and Ibrahim and Baldock (1981) reported that nuclease can be detected in cheese before enterotoxin and in the presence of lower numbers of cells. Lachica *et al.* (1972c) and Ibrahim and Baldock (1981) reported that nuclease, like enterotoxin, is stable under food storage conditions. Some studies have demonstrated that nuclease production is commensurate with enterotoxin production and factors that affect toxin production affect nuclease production (Niskanen and Nurmi, 1976; Tatini *et al.*, 1975). In fact, Niskanen and Nurmi (1977) and Weckman and Catlin (1957) reported that strong DNAse activity was correlated with highly toxigenic strains.

There are several drawbacks in using the thermonuclease assay for screening for the presence of enterotoxin. One limitation is that not all enterotoxin-producers elaborate DNAse (Rayman *et al.*, 1975). Jarvis and Lawrence (1971) suggested that nuclease production may be under a different

metabolic control than enterotoxin. Todd et al. (1981) reported finding enterotoxin in a food product when no thermonuclease was detected. Park et al. (1978) have suggested that all foods may not be adaptable to the thermonuclease assay.

Other nuclease-producing organisms

One major limitation to the thermonuclease test is the presence of other nucleases produced by organisms other than S. aureus. Microorganisms other than S. aureus that produce nucleases include species from the genera Bacillus, Serratia, Streptococcus, Micrococcus, Lactobacillus, Staphylococcus and Pseudomonas (Jeffries et al., 1957; Lachica and Deibel, 1969; Miller et al., 1971). Fortunately, most of the nucleases these other organisms produce are not thermostable (Lachica and Deibel, 1969) and do not cause a problem in the thermonuclease assay.

Other microorganisms that produce thermostable nucleases are Streptococcus faecalis var. faecalis, S. faecalis var. zymogenes (Batish et al., 1982), Staphylococcus epidermidis (Gramoli and Wilkinson, 1978) and "minor" species of Staphylococcus (intermedius and hyicus) (Devriese and Van De Kerckhove, 1979). The nucleases that these organisms produce can be distinguished from S. aureus. Batish et al. (1982) reported that the optimum pH for the enterococcal nuclease is 6.7 as

opposed to the alkaline pH (9.0-10.0) of the thermonuclease from *S. aureus* and can be used as a distinguishing characteristic. The nucleases produced by the other species of *Staphylococcus* are produced in smaller amounts and give weak reactions in the thermonuclease tests (Devriese and Van De Kerckhove, 1979). This difference helps to distinguish the nucleases of *S. aureus* from those of other species of *Staphylococcus*.

Inactivation of thermonuclease

Another factor concerning the use of the thermonuclease assay as an indicator of enterotoxin in foods is the possibility of proteolytic activity breaking down the enzyme while in the food product. Such activity would cause negative results in the assay although enterotoxin would be present. Potential sources of proteolytic enzymes that could degrade the thermonuclease are microorganisms present in the food products.

Some organisms are added to the food product, for example in some types of cheeses, to produce proteolysis for organoleptic purposes and are a potential source of proteolytic enzymes that could degrade the thermonuclease (Tatini *et al.*, 1976). The food product may also have organisms in the normal flora that could produce proteolytic enzymes capable of degrading the thermonuclease. Organisms of this latter group that have been shown to

produce such enzymes are Streptococcus faecalis subsp. liquefaciens and Bacillus subtilis (Lachica et al., 1972c; Medwid and Grant, 1980). Medwid and Grant (1980) characterized the factor from S. faecalis subsp. liquefaciens as heat labile, nondialyzable and optimally active at 55°C and pH 7.0.

Two investigators believe that the potential problem of thermonuclease degradation is not an insurmountable one. Lachica et al. (1972c) stated that the enzyme is stabilized against proteolytic cleavage near the binding site by the presence of Ca^{+2} and by nucleotides which may be present in many foods, particularly those of animal origin. Furthermore, Cords and Tatini (1973) explain that the inactivation of the thermonuclease could be detected by adding thermonuclease to the product and reassaying after incubation at an appropriate temperature.

Coagulase test vs. thermonuclease test

Many researchers believe that assaying for thermonuclease is superior to the coagulase assay as an indicator of the presence of enterotoxin or the pathogenicity of S. aureus. Barry et al. (1973) and Menzies (1977) considered the thermonuclease assay to be simple, less time-consuming and less expensive when compared to the coagulase test. Jasper (1973) states that the thermo-

nuclease assay was just as reliable as the coagulase assay in his study. Furthermore, Lachica *et al.* (1969) reported that a greater percentage of the enterotoxin-producers he studied were thermonuclease producers (93%) than produced coagulase. He suggested this as a good reason for using the thermonuclease assay instead of the coagulase assay. Other advantages in using the thermonuclease assay is that the coagulase property can be lost during storage (Lachica *et al.*, 1969) while the thermonuclease is stable during storage (Lachica *et al.*, 1972c). Also, the thermonuclease assay can be used on pre- and post-processed foods (Tatini *et al.*, 1976); the coagulase test can only be used on preprocessed foods.

Because the thermonuclease assay, like the coagulase test, is not 100% accurate, Barry *et al.* (1973) suggested that the two tests be used to complement each other. Rayman *et al.* (1975) also suggested that assaying for thermonuclease would be very useful for confirming unsure coagulase results. They reported that there was a correlation between coagulase and thermonuclease production; Weckman and Catlin (1957) reinforced this theory by reporting that more coagulase-positive strains produced thermonuclease than do coagulase-negative strains.

Thermonuclease assays

Assays for thermonuclease activity all contain three basic components. The first is a source of DNA in a liquid or an agar-based medium. The second is a heat treatment of the suspected material. When assaying for unpurified nuclease produced by S. aureus, the sample is usually a supernatant of a broth culture or of a blended mixture of the food product. The material is heated for 10 or 15 minutes at 100°C to remove any heat labile nucleases that may interfere with the assay.

The third and final component is a means of detecting the activity of the enzyme. This has been accomplished by three basic techniques. The first is based on the spectrophotometric measurement of absorption of DNA (Alexander et al., 1961; Kunitz, 1950) or a turbid colloid (Houck, 1959). In measuring the absorption of DNA, the biological compound is known to absorb maximally at 260 nm due to the presence of nucleotides (Clark and Switzer, 1977). When measuring the absorbance of DNA in a system, the absorption at 260 nm increases as the nucleic acid is degraded and nucleotides are released. The increase in absorption of 260 nm is proportional to the activity of the thermonuclease (Clark and Switzer, 1977). The method of measuring the turbid colloid is based on the observation that

polymerized nucleic acid will form a stable, turbid colloid in acidified serum albumin (Houck, 1958). When the colloid is exposed to the nuclease, it will be broken down and the absence of the colloid can be measured by a difference in absorption. The difference in absorption is proportional to the activity of the enzyme.

Spectrophotometric assay: DNA absorption

The spectrophotometric assay for thermonuclease is usually based on light absorption at 260 nm of nucleotides produced from DNA. Another method, however, assays thermonuclease activity by measuring the deoxyribose component (as opposed to the nucleotide component) at a wavelength of 600 nm (Burton, 1956). As the DNA is broken down, this component accumulates causing an increase in absorption that is proportional to enzyme activity. This procedure has been described as tedious, time-consuming (Wadström, 1967) and not as reproducible as measuring nucleotide absorption (Arvidson and Holme, 1971).

The spectrophotometric method for assaying thermonuclease in foods was used by Chesbro and Auborn (1967). The supernatant of a suspected sample was added to a DNA-containing glycine buffer ($CaCl_2$, glycine) adjusted to pH 8.6 and allowed to react for 20 to 30 minutes at 37°C. The absorption of the mixture was then measured at 260 nm. An in-

crease in adsorption at 260 nm represented enzyme activity.

The advantages of the spectrophotometric method are: 1) accuracy (Kamman and Tatini, 1977), 2) sensitivity, and 3) ability to quantitate. The disadvantages of the method are: 1) laborious and time-consuming (Koupal and Deibel, 1978; Lachica *et al.*, 1972a; Sharpe and Woodrow, 1971), 2) need for expensive equipment (Kamman and Tatini, 1977), and 3) ineffective with weak nuclease activity or in the presence of excessive impurities (Kamman and Tatini, 1977; Lachica *et al.*, 1972a).

Spectrophotometric assay: Turbidimetric assay

Another spectrophotometric assay is based on the light absorption of a turbid colloidal suspension formed by polymerized nucleic acid in acidified serum albumin (Houck, 1959).

The method of Houck (1959) modified by Erickson and Deibel (1973b) involves the addition of the enzyme to a DNA-containing borate buffer (boric acid, borax, Ca^{+2}) adjusted to pH 9.0. After a reaction period of 15 minutes at 37°C, the reaction is stopped by adding 4N HCl which also develops the turbid colloid. The absorbance is read at 600 nm.

The advantages of this assay as noted by Erickson and Deibel (1973b) are: 1) it is sensitive, 2) it

requires no special apparatus or technical skill, and 3) it is unaffected by the purity of the enzyme. The disadvantages of the assay are essentially the same as for the other spectrophotometric assay; namely, 1) the need for expensive equipment and 2) it is laborious and time-consuming.

The second method is visual and consists of three types: colorimetric, fluorescent, and acid precipitation. The colorimetric method involves the incorporation of a dye into a medium containing DNA. Toluidine blue and methyl green are two dyes that are used in this method. The DNA associates with the dye yielding a color based on the wavelength of light absorbed. When the enzyme breaks down the DNA, the dye is disassociated from the DNA causing a color change and indicating nuclease activity. The fluorescent method is basically the same as the colorimetric method, except that a decrease in fluorescence, indicates nuclease activity. The acid precipitation method involves the activity of nuclease on DNA in the system followed by the addition of strong HCl ($\geq$1 N) which precipitates the nucleate salts (Jeffries *et al*., 1957). Nuclease activity is shown by clear areas in the agar where the nuclease has broken down the DNA.

Colorimetric method: Toluidine blue

This assay is based on the unique reaction of toluidine blue O (TB) with DNA. Toluidine blue has the ability of absorbing maximally at different wavelengths under different conditions (Lachica *et al.*, 1971; Michaelis and Granick, 1945). Toluidine blue alone has a maximum absorbance at 625 nm. It associates with DNA by intercalating between bases and the color is blue because light is absorbed at a longer wavelength. When the DNA is degraded, the dye associates with the agar and is pink because light is absorbed at a shorter wavelength. The color change is used as an indication of nuclease activity (Lachica *et al.*, 1971).

A method developed by Lachica *et al.* (1971, 1972a) involves the addition of the enzyme or unknown supernatant of sample to a well in toluidine blue-DNA agar (DNA, TB, agar, $CaCl_2$ and NaCl) at pH 9.0. The plate is incubated for 3 hours at 37°C. The presence of pink halos indicates nuclease activity. The sensitivity of the assay is 5 ng/ml.

The advantages of the assay are: 1) low detection limit, 2) speed, and 3) no special techniques or equipment are required (Lachica *et al.*, 1971, 1972a). The major disadvantage of the assay is the problem of improper color development (Emswiler-Rose *et al.*, 1980; Koupal and Deibel,

1978) which may be due to protein interference (Lachica *et al.*, 1972b).

Colorimetric method: Methyl green

This nuclease assay is based on the interaction of a dye, methyl green, with DNA. Methyl green is capable of staining polymerized DNA (Kurnick, 1950). When the DNA is depolymerized or degraded, the color disappears; the degree of color disappearance can be related to nuclease activity (Horney and Webster, 1971). This method has been applied to spectrophotometric assays of nuclease activity (Horney and Webster, 1971) but the bulk of the research involves using media containing an incorporated methyl green-DNA complex (Horney and Webster, 1971; Smith *et al.*, 1969).

A method developed by Horney and Webster (1971) for nucleases other than *S. aureus* involves the addition of the enzyme or suspected sample to a well in DNA-methyl green agar (methyl green, DNA, sodium acetate, EDTA, sodium phosphate, $MgCl_2$, Tris-HCl) at pH 6.0 (Kunitz, 1950) followed by incubation for 6 hours at 37°C. The detection limit of the assay was <1 ng/ml.

The advantages of the assay are: 1) low detection limit, 2) simple equipment and no special techniques required, 3) impure systems can be used (Lachica *et al.*, 1972a), and 4) the test can be quantitated (Horney and Webster, 1971). The

major disadvantages of the assay are the need for highly polymerized DNA (Lachica *et al.*, 1972a) and the pH of 6.5 which is out of the optimum range for thermonuclease from *S. aureus* which has an optimal activity in the range pH 9 to 10. The methyl green complex has greatest stability at pH 7.5.

Fluorescent method: Acridine orange

In this assay, acridine orange dye intercalates with DNA or slips in between adjacent base pairs (Stryer, 1981). Since the molecule fluoresces under ultraviolet light, fluorescence indicates the location of DNA. The activity of the enzyme is measured by a decrease in fluorescence as a result of breakdown of DNA (Lachica and Deibel, 1969; Lanyi and Lederberg, 1966).

A method developed by Lachica and Deibel (1969) involves the addition of supernatant from a sample (boiled for 15 minutes) to a paper filter disc and overlaying it with acridine orange-deoxyribonucleate agar (DNA salt, $KHPO_4$, agar, and acridine orange), adjusting to pH 9.0 and incubating for 2 to 5 hours at 37°C. Halos of clearing when viewed with a U.V. light are indicative of nuclease activity.

The advantages of the assay are that it is rapid and could be used in MPN procedures for food products (Lachica

and Deibel, 1969). The obvious disadvantage is the use of the mutagenic dye and ultraviolet light (Lachica et al., 1971).

Acid precipitation   This nuclease assay is based on the acid precipitation of the salts of DNA. In media containing DNA, the nucleate salts react with acid to produce a precipitate (Jeffries et al., 1957). When DNA has been degraded, no reaction takes place; the amount of precipitate can be related to enzymatic activity.

A method developed by Koupal and Deibel (1978) involves the addition of the enzyme or unknown supernatant to a well in Koupal-Deibel DNA agar (DNA, agar, NaCl, trizma base, and $CaCl_2$) at pH 9.0. The preparation is incubated at 50°C for 1 hour; the plate is then flooded with 4N HCl to precipitate any unhydrolyzed DNA. Clear areas represent nuclease activity with the minimum detection level consistently at 10 ng/ml or g of food product.

The advantages of the assay are: 1) simplicity and rapidity, 2) easy reproducibility of results, 3) simple equipment needed, and 4) pure and impure systems can be used (Emswiler-Rose et al., 1980; Jarvis and Lawrence, 1969; Koupal and Deibel, 1978). The disadvantages of the test are: 1) the need for strong acid, 2) lack of quantitation (Koupal and Deibel, 1978), and 3) protein interference (Lachica et al., 1972a).

A method that has not been used extensively is the radioactive-labeled DNA complex (Sharpe and Woodrow, 1971). To determine nuclease activity, the complex is added to a liquid buffer system containing the enzyme. After a suitable reaction time, the complex is removed and the released ^{3}H is proportional to the nuclease activity.

Thermonuclease test in meats

The thermonuclease test for meat and meat products is suggested particularly for processed meats when no viable *S. aureus* are present (Koupal and Deibel, 1978; Tatini *et al.*, 1976). Even when viable organisms are present, the thermonuclease test provides a very rapid assay to determine if *S. aureus* has grown to numbers $>10^{6}$ cells/g (Tatini *et al.*, 1976).

There are two basic methods to determine presence of thermonuclease in meats: 1) by the direct application of the meat product to the detection medium, or 2) by the extraction of the nuclease from the meat product. The thermonuclease assays that are used by most investigators today are the metachromatic agar-diffusion (MAD) method of Lachica *et al.* (1971, 1972b) and the acid precipitation method developed by Koupal and Deibel (KD) (1978).

Lachica *et al.* (1972b) reported a direct food application in their MAD assay. Naturally and artificially con-

taminated meat products were cut into small 5-mg portions, steamed for 15 min and placed on the toluidine blue-O-DNA (TB) medium. The medium was incubated for 3 hours at 37°C; the appearance of bright pink halos indicated nuclease activity. Problems with the assay included the development of greenish halos with some assays and improper pink color development (Emswiler-Rose *et al.*, 1980; Lachica *et al.*, 1972b). Another direct application method used by Emswiler-Rose *et al.* (1980) involved the use of the casings of contaminated sausages on TB and KD agars. This method was based on research by Barber and Deibel (1972) that showed that most of the growth and toxin production of *S. aureus* exists in the outermost part of the sausage where the oxygen tension is highest. They reported that the KD agar gave more consistent results than the TB agar.

Two basic procedures are used today to extract thermonuclease from foods. The method of Cords and Tatini (1973) starts with the blending of 20 g of food sample followed by pH adjustment to 4.5. The mixture is centrifuged at 23,500 x g for 15 minutes at 5°C. Cold 3M trichloroacetic acid is added to the supernatant. The preparation is centrifuged, adjusted to pH 8.5, boiled and cooled. The resultant solution is then used in the thermonuclease assay. This extraction procedure has been used predominantly in the MAD assay (Niskanen and Nurmi, 1976;

Park *et al*., 1978, 1979; Tatini *et al*., 1976).

The Koupal-Deibel extraction procedure is shorter and consists of blending 15 to 20 g of sample with 30 to 40 ml of water followed by the adjustment of the pH to 5.5. The mixture is boiled for 20 min, cooled and centrifuged for 45 min at 7500 rpm. The supernatant is used in the thermonuclease assay.

Park *et al*. (1978, 1979) found the extraction procedure of Cords and Tatini (1973) to be particularly applicable to meats. They state this is not true for all foods, particularly egg-containing products. A modified extraction method has been developed to facilitate the extraction of thermonuclease from these other foods.

Staphylococcus aureus in meat

The presence of *S. aureus* and the potential for food poisoning from this organism have always been a problem in meats. The organism is part of the normal flora of meats. In fact, the major source of *S. aureus* in the environment is from warm-blooded animals (Baird-Parker, 1974); Jay (1978) notes that many domestic animals harbor *S. aureus*. The factors that control the numbers of *S. aureus* in meat and the presence of enterotoxin are: 1) pH, 2) the oxidation/reduction potential, 3) temperature, 4) Aw, and 5) the numbers and types of competing

organisms present (Jay, 1978). Other factors, not inherent in the meat product, that also affect the number of *S. aureus* in the meat product are: 1) the way the product is handled, packaged, and stored and 2) the cut of meat (Jay, 1978).

Staphylococcus aureus in fresh meats

When an animal is killed and the muscle is converted into meat, several important chemical changes take place to influence the growth of *S. aureus* and other bacteria. The major changes include: 1) the loss of oxygen in the muscle, 2) the pH drop that takes place due to the breakdown of glycogen to lactic acid, and 3) the absence of the immune response due to the absence of the lymphatic system and circulating white blood cells (Jay, 1978; Forrest, 1975).

The depletion of O_2 in the muscle causes the oxygen tension to be the highest at the surface and reduced in the interior of the meat. Although *S. aureus* is a facultative anaerobe able to grow in the absence of air (Baird-Parker, 1974), the organism is known to proliferate and produce toxins to a greater extent in the presence of O_2 (Arvidson *et al.*, 1971; Barber and Deibel, 1972).

Perhaps the most important change in the muscle that could potentially affect *S. aureus* is the drop in pH

(Forrest, 1975). The change in pH has been described as a gradual drop from 7.0 to 5.6 in 6 to 8 hours (Forrest, 1975). This drop in pH alone does not affect _S. aureus_ because it is able to survive and grow at a pH as low as 4.2 (Baird-Parker, 1974; Barber and Deibel, 1972; Lechowich _et al._, 1956).

An inherent factor of the meat that controls growth of _S. aureus_ is the normal flora (McCoy and Faber, 1966; Peterson _et al._, 1962, 1964). The major sources of organisms in fresh meats are: 1) animal lymph nodes, 2) knife used for exsanguination, 3) hide of the animal, 4) intestinal tract, 5) hands of the handlers, 6) cutting knives, and 7) storage bins (Ayres, 1955). Some of the common bacteria found on meats include the genera _Pseudomonas_, _Moraxella_, _Escherichia_, _Serratia_, _Streptococcus_, and _Proteus_ (Ayres, 1960; Jay, 1978). Casman _et al._ (1963) demonstrated the ability of saprophytic organisms to inhibit growth of _S. aureus_ by showing how well the pathogen grew after the removal of these organisms by heating.

Many of the saprophytic organisms found on meats successfully outcompete _S. aureus_ for nutrients (Troller and Frazier, 1963). Troller and Frazier (1963) list the genera of these competing organisms as _Serratia_ and _Pseudomonas_. The inhibition of _S. aureus_ by these organisms is

dependent on environmental conditions such as pH, temperature, etc., *S. aureus*, of course, competes most effectively at its optimum growth conditions (McCoy and Faber, 1966; Troller and Frazier, 1963).

Some organisms found on meats control and inhibit the growth of *S. aureus* by producing inhibitory substances (Troller and Frazier, 1963). Troller and Frazier (1963) list these organisms as *Bacillus cereus*, *Proteus vulgaris*, *E. coli* and *Aerobacter aerogenes*. These organisms can produce antibiotic substances that are Seitz-filterable, dialyzable and stable at 90°C for 10 min (Troller and Frazier, 1963).

Staphylococcus aureus in processed meats

Processed meats are made by the use of procedures that alter the characteristics of fresh meat. These procedures include salting, smoking, precooking and fermentation (Forrest, 1975). Processing of meats has evolved as a means of preserving meat; today, however, it is primarily used for the flavor. Many of these processing procedures have a direct effect on the survival and growth of *S. aureus* in meats. Two important processes are: curing and fermentation.

Curing

Meat curing is defined as the application of salt, color-fixing ingredients, and seasonings to meat in order to impart unique properties to the end product (Forrest, 1975).

1. Nitrite. Desrosier and Desrosier (1977) list the functions of nitrite in meat curing as stabilization of color, development of flavor, inhibition of growth of a number of food poisoning and spoilage microorganisms and the retardation of development of rancidity. Nitrite may be directly added to foods or nitrate may be added. The latter compound is then reduced to nitrite by microbial action.

The common amount of nitrite allowed in meats by the USDA is 156 ppm (Forrest, 1975).

The degree of the effect of nitrite on *S. aureus* in cured meats is controlled by environmental conditions, namely pH and the presence of O_2 (Buchanan and Solberg, 1972; Lechowich *et al.*, 1956). Low pH is more effective in preventing growth (Buchanan and Solberg, 1972; Lechowich *et al.*, 1956; Tarr, 1941). Castellani and Niven (1955) reported that as the pH of a medium is lowered by one unit, the bacteriostatic effect of nitrite is increased 10-fold.

The increase in the bacteriostatic activity of nitrite

by a drop in pH is attributed primarily to the formation of nitrous acid (Castellani and Niven, 1955; Shank et al., 1962). The nitrous acid formed from the nitrite is an effective bactericidal agent. In meat, at a pH of 5.5 to 6.0, some nitrite is converted to nitrous acid (Forrest, 1975). Nitrous acid is maintained at the highest concentrations in the pH range of 4.5 to 5.5 and bactericidal activity is at a maximum under these conditions (Shank et al., 1962). Theories concerning the action of nitrous acid on the bacterial cell are damage to the bacterial cell and making constituents in the medium unavailable to the cell (Castellani and Niven, 1955; Shank et al., 1962).

The other major environmental condition that affects the bacteriostatic action of nitrite on S. aureus is the absence of O_2. Several researchers have reported that nitrite inhibits growth of S. aureus to the greatest extent under anaerobic conditions with almost no effect under some aerobic conditions (Buchanan and Solberg, 1972; Castellani and Niven, 1955; Lechowich et al., 1956). Buchanan and Solberg (1972) reported this inhibition at a pH as high as 7.2. Castellani and Niven (1955) suggested the bacteriostatic action under anaerobic conditions lies in the ability of nitrite to interfere with a system involving an essential sulfhydryl compound. Buchanan and Solberg (1972) theorized that nitrite may inhibit the growth of S. aureus by blocking

sulfhydryl sites of either coenzyme A or alpha-lipoic acid which interferes with the normal metabolism of pyruvate.

2. NaCl: Salt (NaCl) and nitrite are the two main ingredients used in meat curing (Forrest, 1975). Forrest (1975) lists the two purposes of salt in meat as being a preservative agent and a flavoring agent. Flavoring is probably the most important function because the low level of salt used in the product today has little, if any, preservative action. Processed meats contain 1 to 5% salt with the vast majority of sausages containing 2 to 3% salt (Kramlich, 1978).

The preservative effect of salt is due to its dehydrating ability which alters the osmotic pressure so bacteria cannot grow (Desrosier and Desrosier, 1977; Marsh, 1983). *S. aureus* is one of the organisms most resistant to salt with growth and/or survival at levels of salt up to 15 to 20% (Baird-Parker, 1974; Jay, 1978). Therefore, at the percent concentrations of salt added to meats, salt in the product will not inhibit the growth of this bacterium. In fact, Peterson *et al*. (1964) found that concentrations of salt above 3.5% decreased the numbers of antagonistic saprophytic organisms and enabled growth of *S. aureus*. As the concentration of salt is increased, however, enterotoxin production decreases and stops before growth ceases (Hojvat and

Jackson, 1969). Like all other factors affecting growth of *S. aureus*, the bacteriostatic ability of salt is affected by other environmental factors such as pH (Genigeorgis and Sadler, 1966a; Genigeorgis *et al.*, 1971a; Hojvat and Jackson, 1969). As these environmental factors become more adverse for the organism, the bacteriostatic activity of the salt increases.

Public health aspects of NaCl usage

There is general concern in the medical community today concerning the role of salt in hypertension. Although the relationship between high sodium intake and hypertension is not a simple cause and effect (Sebranek *et al.*, 1983), most researchers agree that sodium intake is a contributing factor (Page, 1981). Manufacturers of processed foods have been encouraged to reduce the levels of sodium in their products. Compounds used in the processing of meats that contribute sodium are sodium nitrite, sodium phosphates, sodium ascorbate as well as nonfat dry milk and soy derived proteins (Marsh, 1983). Sebranek *et al.* (1983) state, however, that sodium chloride is the compound that is responsible for the greatest portion of sodium in processed meat products.

In an effort to comply with the demand to reduce sodium in their products, manufacturers have been searching for

substitutes for NaCl. Some of these compounds have included potassium chloride (KCl), magnesium chloride ($MgCl_2$), calcium chloride ($CaCl_2$) and lithium chloride (LiCl) (Marsh, 1983). Research is still being carried out on the safety and consumer acceptance of sodium-reduced meat products containing certain of these compounds. In addition to these concerns, Sebranek et al. (1983) list other aspects that must be considered, including textural changes, decreased moisture retention and product appearance.

Phosphates

The use of phosphates, previously prohibited in sausage (Forrest, 1975), is now allowed in these products (Desrosier and Desrosier, 1977). There are several benefits from the use of alkaline phosphates in meat processing: increased water holding capacity, increased tenderness and juiciness of the product, improvement in cured meat color, and protection against browning (Forrest, 1975).

The use of phosphates (sodium tripolyphosphate, heaxametaphosphate, acid pyrophosphate, pyrophosphate and disodium phosphates) alone or in combination is restricted to not more than 0.5% in the finished product (Bard and Townsend, 1978). Their effect on bacteria, particularly

S. aureus, is not clear.

Fermented meats

A fermented food is a food product that is preserved by the action of microorganisms inoculated into or inherent to the product. Fermented meats are normally sausages prepared by adding curing and seasoning ingredients to ground meat, stuffing the mixture into casings and incubating for a period of time at 80 to 95°F (Jay, 1978). The organisms normally used as starter cultures are from the group of bacteria generally known as lactic acid bacteria. These organisms produce lactic acid and include the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* (Groups D and N) (Jay, 1978).

The inhibitory actions these microorganisms provide against *S. aureus* (Graves and Frazier, 1963) are the low pH due to the production of lactic acid, the production of inhibitory substances antagonistic to *S. aureus* and by outcompeting the staphylococci for vital nutrients in the meat system.

Lactic acid causes a drop in pH and has bacteriostatic action against *S. aureus* (Haines and Harmon, 1973). Bacteriostatic action is dependent on the presence of O_2 in the system. Barber and Deibel (1972) reported that *S. aureus* was more acid tolerant aerobically than anaerobically. The drop in pH can affect toxin production

without affecting growth (Barber and Deibel, 1972). Haines and Harmon (1973) reported that lactic acid inhibited growth of *S. aureus* in early but not late stages of incubation.

Another theory on the effect of lactic acid bacteria on *S. aureus* is the production of inhibitory substances. Inhibitory substances such as diplococcin and nisin are produced by certain species of *Streptococcus* (Hirsch and Wheater, 1951; Oberhofer and Frazier, 1961). Lactic acid bacteria may also inhibit *S. aureus* by the production of hydrogen peroxide (Dahiya and Speck, 1968).

Finally, there is evidence to show that lactic acid bacteria can out compete *S. aureus* for nutrients in the medium (McCoy and Faber, 1966). Haines and Harmon (1973) reported that competition for vital nutrients, particularly niacin and biotin, inhibited growth of *S. aureus*. Iandolo *et al.* (1965) reported that the depletion of nicotinamide by lactic acid bacteria inhibited growth of *S. aureus*. The extent of inhibition is dependent on environmental conditions such as temperature, pH, etc. (Graves and Frazier, 1963).

MATERIALS AND METHODS

The experiments in this research project consisted of several phases: 1) development of a quantitative method for the determination of thermonuclease, 2) evaluation of environmental conditions on the production of thermonuclease, and 3) the examination of extraction procedures used for the detection of nuclease in a meat product.

Measurement of Thermonuclease Activity

Koupal-Deibel DNA medium

Koupal-Deibel (KD) medium used in this work was developed and described by Koupal and Deibel (1978). The medium was prepared by adding to 100 ml of distilled water 0.6 g Trizma Base (Sigma Chemical Co., St. Louis, Mo., Cat. No. T 1503), 0.045 g DNA (Sigma Chemical Co., St. Louis, Mo., Cat. No. D 1632), 1.25 g Agar (Difco Laboratories, Detroit, Michigan, Cat. No. 0140-01-0), 1.00 g NaCl (Fisher Scientific Co., Pittsburgh, Pa., Cat. No. S-271), and 0.2 ml of 0.01M $CaCl_2$. The pH was adjusted to 9.0 with 1N HCl. This mixture was boiled for 20 minutes and stored in rubber-stoppered 125 ml flasks at room temperature.

When needed, the medium was steamed to melt the agar and 15 ml was pipetted into 100 x 15 mm plastic Petri dishes (Fisher Scientific Co., Pittsburgh, Pa., Cat. No. 8-757-13)

allowed to cool for at least five hours at room temperature. Wells were cut in the agar with a No. 2 cork borer. Agar plugs were removed by the borer under vacuum suction.

Samples to be tested for the presence of thermonuclease were added to the wells by two methods: Pasteur pipettes (Fisher Scientific Co., Pittsburgh, Pa., Cat. No. 13-678-6B) when not quantitated and by microliter pipette (Rainin Instrument Co., Woburn, Mass., Cat. No. P-200) with pipette tips (Brinkman Instruments, Inc., Westbury, N.Y., Cat. No. 2235130-3) when quantitation was desired. The Pasteur pipette was attached to a bulb and the sample was added until the well was filled. When the microliter pipette was used, 30 μl and 20 μl of sample were examined.

Purified thermonuclease

The nuclease used in this research project was micrococcal nuclease purchased from Worthington Farms (Millipore Corp., Freehold, NJ). Two samples of the nuclease were needed to provide enough nuclease for all experiments. Each lot contained 15,000 units (unit = μmoles of substrate used or product formed/ minute). In terms of purity or specific activity, the samples contained 14,925 units/mg (used primarily in quantitative assay development experiments) and 12,319 units/ mg, respectively.

Dilution of the purified nuclease

The enzyme was diluted using either 0.05 M Trizma base, pH 8.8 $\pm$ 0.1 or Brain Heart Infusion (BHI) pH 7.0 $\pm$ 0.1 except in specified experiments (Difco Laboratories, Detroit, Michigan, Cat. No. OD 37-01-6). The nuclease solution was diluted to obtain nanogram or unit quantities/ml.

To obtain ng/ml quantities of thermonuclease for the development of the quantitative assay, the following procedure was used. One milligram or 10^6 ng of nuclease was added to 10 ml of 0.05 Trizma base giving 10^5 ng of nuclease per ml. Then 0.1 ml of the solution was added to screw cap tubes giving 10^4 ng of nuclease/tube. These tubes were frozen until needed. At that time, the solution was further diluted with 9.9 ml of BHI or 0.05 M Trizma base or BHI broth to obtain 1000 ng nuclease/ml. The nuclease was serially diluted giving nuclease concentrations of 640 ng/ml, 500 ng/ml, 320 ng/ml, 100 ng/ml, 80 ng/ml, 75 ng/ml, 50 ng/ml, 25 ng/ml, 20 ng/ml, 10 ng/ml and 5 ng/ml.

The dilution of the nuclease, in terms of units, was accomplished by adding 15,000 units of the purified nuclease to 10 ml of 0.05M Trizma base, giving 1,500 units of nuclease per ml. Then, 0.1 ml of the solution containing

1,500 units/ml was added to screw cap tubes giving 150 units of nuclease/tube. These tubes were kept frozen until needed. At that time, the solution was further diluted with 9.9 ml of BHI to obtain 15.0 units of nuclease/ml. The nuclease was serially diluted giving nuclease activities of 7.50 units/ml, 1.50 units/ml, 0.75 units/ml, 0.15 units/ml and 0.08 units/ml.

These solutions were then added to wells cut in the agar in Petri dishes and incubated for 1-2 hours at 50°C. After the incubation period, the plates were flooded with 4N HCl. The acid precipitated unreacted DNA and circular clear zones were present when enzymatic activity occurred. The zones in the white precipitate were easier to detect if the plate was placed on a black background.

Measuring thermonuclease activity

Thermonuclease activity was measured by placing the Petri dish in an antibiotic-zone reader (Accu-Tech Corp., New York, NY) which had a magnifying glass and a black background. The magnification of the zones and the black background improved visibility and measurement.

The zones were measured with vernier calipers (Fisher Scientific, Pittsburgh, Pa., Cat. No. 12-122) along the horizontal and vertical axes. A measurement was taken

also of the well along the horizontal and vertical axis. The measurement called "clearing zone" was calculated by using the following formula;

$$\left[\frac{\text{Diameter horizontal clearing zone (mm)} + \text{diameter vertical clearing zone (mm)}}{2}\right] - \left[\frac{\text{Diameter horizontal well (mm)} + \text{Diameter vertical well (mm)}}{2}\right] = \text{Clearing zone}$$

Organism

The organism used was *Staphylococcus aureus* Z88 and was obtained from Dr. William Sveum, Armour and Company, Scottsdale, Arizona. The organism was isolated originally by Dr. Sita R. Tatini, University of Minnesota and was involved in a food poisoning outbreak. The organism, which produces staphylococcal enterotoxin A (SEA), is noted for production of high levels of thermonuclease.

An active culture of the organism was maintained on trypticase soy agar slants; maintenance transfers were made at least once a month. Short-term storage of the slants was at 4°C and for long-term storage the organism was kept in a frozen state at -80°C in a sterile 10% glycerol-BHI mixture (Gherna, 1981).

The inoculum for the BHI broth cultures was taken from stock cultures on trypticase soy agar. BHI broth was incubated for 12 to 18 hours at 35 to 37°C. The cells

were centrifuged and washed once with sterile BHI broth. BHI broth was sterilized in 150-ml quantities in 250-ml flasks and stored at room temperature until used.

Evaluation of Environmental Factors

1. pH. The initial pHs of the broth used in these experiments were 5.3, 6.3, 7.0 (the pH of the control flask) and 8.3 $\pm$ 0.1. Adjustment of the pH was made before autoclaving by using either 1N HCl or 1N NaOH. The pH varied at times from 0.1-0.3 of a unit after autoclaving.

2. NaCl. The concentrations of NaCl were calculated on a weight percent basis (Fritz and Schenk, 1974) to obtain 5%, 10% and 15%.

$$\text{Wt. \%} = \frac{100 \times \text{grams of solute}}{\text{grams of solvent} + \text{grams of solute}}$$

Based on the above equation, 5 grams of NaCl were added per 95 ml of BHI, 10 grams of NaCl were added per 90 ml of BHI and 15 grams of NaCl were added to 85 ml of BHI to obtain 5%, 10% and 15% concentrations, respectively. The 15% solution had to be heated to completely dissolve the salt. The solutions (media) were distributed into flasks, autoclaved, cooled and stored at room temperature.

3. KCl. The concentrations of KCl (Fisher Scientific, Pittsburgh, Pa., Cat. No. P-217) used in these experiments were the same as those used for NaCl, 5%, 10% and 15%.

The calculations used to determine the amount of KCl added to the BHI to obtain the respective percentage concentrations were made on a weight percent basis.

The weights added were the same as those described previously for NaCl. The solutions were prepared the same, distributed into flasks, sterilized, cooled and stored.

4. Phosphate. The phosphate used in this experiment was sodium acid pyrophosphate (SAPP) (Stauffer Chemical Co., Westport, Conn.). The concentrations used, calculated on a weight percent basis, were 0.1%, 0.25% and 0.5%.

Incubation of flasks

The flasks were incubated at three different temperatures: 15°, 22° and 37°C. A different level of inoculum was used at each temperature. A level of 10^2 to 10^4 cells/150 ml of broth was inoculated into flasks incubated at 37°C, 10^4 to 10^6 cells/150 ml of broth were inoculated into flasks incubated at 22°C and 10^6 to 10^8 cells/150 ml of broth were inoculated into flasks incubated at 15°C.

During the incubation period, the flasks were aerated by shaking at constant speed on one of three shakers. They were a Fisher Shaking Water Bath, Model 127 (Fisher Scientific Co., Pittsburgh, Pa.), a Gyrotory Shaker, Model

G-76 (New Brunswick Scientific Co., New Brunswick, N.J.), and a Laboratory Rotator, Model G2 (New Brunswick Scientific Co., New Brunswick, N.J.).

Measuring bacterial growth

Growth of *S. aureus* in the broth experiments was monitored by light absorbance measurements. A standard curve was produced for *S. aureus* growth in control BHI flasks incubated for 48 hours at 15°, 22°, or 37°C, respectively. Growth was measured every two hours by removing a sample for an aerobic plate count on Trypticase Soy Agar. Absorbance readings of broth cultures were made at 660 nm at the same time (Koch, 1981) with a spectrophotometer (Bausch and Lomb, Rochester, N.Y.). Plate counts were taken every 2 to 4 hours along with absorbance readings until the absorbance readings did not change by more than 0.5 units. Absorbance readings were taken on undiluted culture until the readings were greater than 0.2. The sample was then diluted with BHI to obtain readings less than 0.2. When the absorbance readings had stabilized, readings were made every 4-8 hours Numbers of organisms in the flasks with added materials were estimated by comparing absorbance readings with the standard curve. When carrying out the absorbance readings on cultures, the blank used to standardize the spectrophotometer had to be the same solution as used

for the culture.

Measurement of thermonuclease activity

During incubation of the flasks, thermonuclease production was monitored by taking a 4-ml sample every 8 hours for the 48-hour period. The sample was boiled for 10 minutes prior to sampling. The solution was centrifuged for five minutes and the supernatant was assayed for thermonuclease.

The Koupal-Deibel DNA medium was used to determine thermonuclease activity. The protocol for the test used included the use of a 20 μl nuclease sample, 15 ml of medium/plate, and incubation of the plate for 2 hours at 50°C.

The first step for obtaining a quantitative estimate of nuclease was to establish a standard curve using purified nuclease. Five replicate wells were measured for each known concentration; the "cleared zone" values were plotted against log of concentration. Five replicates of each unknown well were tested also. "Cleared zone" measurements were compared to the standard curve to determine the nuclease concentration.

Plate counts

Plate counts were performed following the aerobic plate count method as outlined by the Compendium of Methods for the Microbiological Examination of Foods (Gilliland *et al*., 1976).

Samples were taken every 2-4 hours from the control flasks and absorbance measured until the absorbance readings had stabilized. When the absorbance readings had stabilized, counts were taken every 4-8 hours.

Counts were taken every 24 and 48 hours in flasks containing BHI plus added materials. The numbers of organisms present were primarily monitored by the absorbance readings. The gram stain procedure was used to periodically check for contamination.

Nuclease production vs. numbers of *S. aureus*

S. aureus was inoculated into flasks and incubated for a period of time until nuclease was produced. The time for nuclease production was estimated from previous results. Thermonuclease was generally assayed for each hour for 5 to 7 hours. The thermonuclease assay was qualitative only. Plate counts were taken every other hour during the 5 to 7 hour time period.

Extraction of Thermonuclease from Meat

Two procedures for the extraction of thermonuclease from meat were compared using sausage samples obtained from Dr. William Sveum (Armour and Company, Scottsdale, Arizona). Sausage samples contained *S. aureus* at levels of 10^5 to 10^6 cells/g while others were uninoculated and used as controls.

1. Method based on assay of Koupal and Deibel (1978):

The method of Koupal and Deibel involved using 20 grams of the outer 3 to 5 mm of the sausage sample and blending with 40 g of distilled water. The sample was blended for 3 to 5 minutes with a homogenizer (Sorvall Omni Mixer, Ivan Sorvall Inc., Norwalk, Conn., Cat. No. OM-1150), and poured into a centrifuge tube. The pH was adjusted to 5.5 with 1N HCl and placed in a boiling water bath for 20 minutes. The tubes were then centrifuged for 45 minutes at 7500 rpm at 5°C in a refrigerated centrifuge. The supernatant of the sample was used for the thermonuclease qualitative assay.

2. Method based on assay of Tatini et al. (1976):

This method involved blending 20 g of the outer 3 to 5 mm of the sausage sample with 40 ml of distilled water. The sample was adjusted to pH 4.5 in a 100- or 150-ml beaker. The material was centrifuged at 14,000 rpm

(23,500 x G) for 15 minutes at 5°C in a refrigerated centrifuge. Cold 3M trichloroacetic acid (TCA) was added in the proportion of 1.5 ml TCA/30 ml supernatant (Dr. William Sveum, Personal Communication, Armour and Company, Scottsdale, Arizona). The solution was centrifuged as previously stated. The supernatant was decanted and the precipitate containing the nuclease was resuspended in 2 ml of 0.05M Trizma buffer pH 9.1. The solution then was adjusted to a final pH 8.2-8.6. This preparation was heated for 15 minutes in a boiling water bath after which it was cooled rapidly in ice. The supernatant of the sample was used in the thermonuclease qualitative assay.

RESULTS AND DISCUSSION

Development of Quantitative Assay for Staphylococcal Thermonuclease

Preliminary research involved examination of the Koupal-Deibel method as a potential quantitative as well as qualitative assay for thermonuclease. Initially, at levels of 5 ng of thermonuclease/ml of sample, clearing in the medium was inconsistent; after repeated trials, however, activity as manifested by clearing zones became consistent. The explanation for the inconsistent then consistent clearing reactions is not understood but is attributed, in part, to generally improved techniques. Due to the initial inconsistent readings and the very small clearings observed in further trials, 5 ng was taken to be the limit of sensitivity. The concentration of 5 ng/ml of thermonuclease is equivalent to the 5 ng amount noted by Koupal and Deibel (1978) as the limit of sensitivity; however, they could not consistently detect this concentration when extracting from food products. Lachica *et al.* (1972b) found this concentration to be the limit of their assay using the metachromatic agar diffusion technique.

Figure 1 shows results from preliminary experiments; diameters of clearing zones are plotted against the log thermonuclease concentration, ng/ml. Utilizing the statistical model of predicting a known value from an unknown value

Figure 1. The relationship of the diameter of clearing versus the log of the thermonuclease concentration, ng/ml

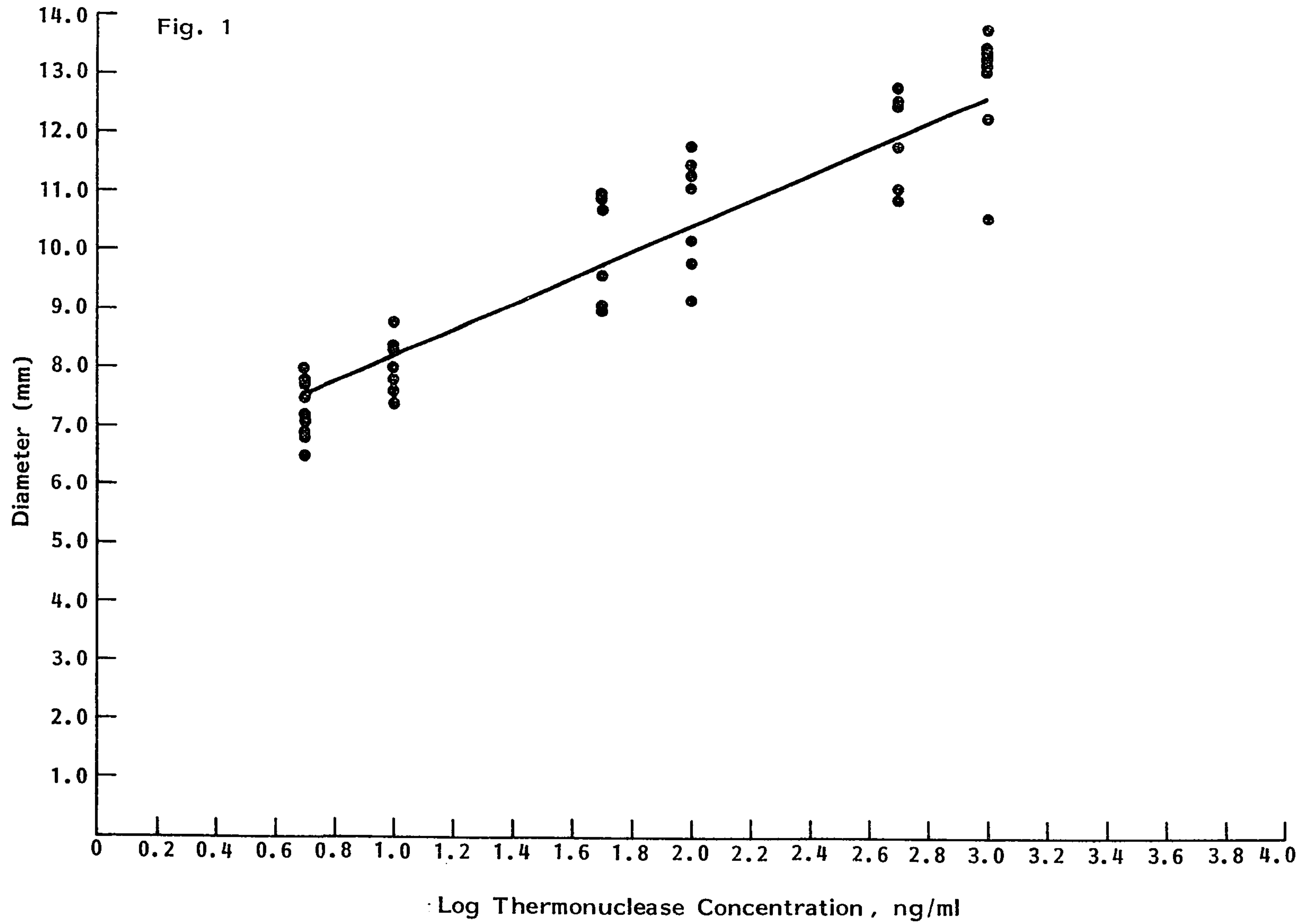
Fig. 1
Diameter (mm)
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
Log Thermonuclease Concentration, ng/ml

(Cox, 1971; Snedecor and Cochran, 1980), the thermonuclease concentration was placed on the x axis and the diameter of clearing was placed on the y axis. The analysis of variance (Table 1) showed that 88% of the variability in the measured diameters could be explained by the log of the thermonuclease concentration (LGTN) and another 7% of variability was attributable to differences among plates. The variability among plates was small compared to the variability due to concentration.

Table 1. Regression and analysis of variance on the data from diameter measure vs. log thermonuclease concentration

Source	DF	Type I SS	F Value	PR>F	R^2
Plate	8	20.73	13.12	0.01	0.07
LTTN	1	251.27	1271.72	0.01	0.88
			Variance 0.20		

y intercept - 5.99 Slope - 2.21

Several reasons may be given to explain why the plate on which the diameter was measured contributed to the size of the clearing zones. In agar diffusion assays with round Petri dishes, there have been reports that wells have to be equally spaced from the center to get reproducible results (Bartlett and Mazens, 1971;

Hallynck and Pijck, 1978). Since the wells were cut at random in this assay, this could have influenced the measurements between plates. The second factor that may have influenced plate-to-plate variation is the convexity of the Petri dish. Convexity of the Petri dish influences the thickness of the agar and can influence the diffusion of the enzyme through the agar. Thickness of the agar can vary from one plate to another (Hallynck and Pijck, 1978). Finally, day-to-day variations in technique may have contributed to plate-to-plate variation. Because the levels of nuclease used was in very small increments, a small error in technique could have also contributed to the variations in measurements.

The analysis of variance (Table 1) gives the slope (m) of the line $y = mx+b$ in Figure 1 as 2.21 and the intercept (b) as 5.99. This linear relationship between the log of the thermonuclease concentration and diameter measurement was reported by Cords and Tatini (1973) and Lachica _et al_. (1972b) using toluidine blue (TB) DNA medium. A strong linear relationship occurred throughout the range of concentrations from 5 to 1000 ng/ml. The linear relationship formed the basis for the development of the quantitative assay. With this relationship, the concentration of thermonuclease could be predicted, within

limits, by the size of the diameter of the cleared zone (Cox, 1971; Snedecor and Cochran, 1980).

At each concentration of thermonuclease, there is assumed to be a population of diameters that are normally distributed with equal variance. The variance of the diameters in this experiment was 0.20 (Table 1). In the prediction model for enzyme concentrations from given diameters, we wanted an experimental procedure for which the variance of the diameter populations is as small as possible. In the procedure used, the measurement of the clearing was always horizontal in relation to the Petri plate orientation. The problem with only one measurement was that the clearing was not always perfectly circular.

One method for reducing this bias in measurement was to measure the zones horizontally are vertically and then average the two values. An F-test was used to determine if this change in experimental procedure led to diameter populations of smaller variance. The estimated variances in Table 2 suggest that the average clearing (AC) procedure results in less variation than that of the populations from the horizontal (HOR) measuring procedure ($F_{60,60}$ = 1.98, p<.01), and equal to that of the populations from the vertical (VER) measuring procedure ($F_{60,60}$ = 0.93,

Table 2. Variance of horizontal, vertical and average clearing measuring procedures on data from measuring procedures vs. log thermonuclease concentration

Measuring procedure	Variance
Horizontal $_{60,60}$	.10
Vertical $_{60,60}$	.05
Average clearing $_{60,60}$	.05

$p > .25$). Since the average clearing (AC) measure also has the added stability of being the average of two lengths, it is preferred to either the horizontal or vertical measurements alone.

During the development of the quantitative assay, results showed the 20 µl and 30 µl filled the well (15 ml agar/plate) without overfilling or underfilling. Using the 30 µl sample might be expected to give significantly larger clearing measurements. To determine if there was a significant difference between measured clearings, the analysis of variance (ANOVA) procedure was used.

The results in Table 3 show a low insignificant F value (0.73) and a low contribution to the variability in measured diameters. No significant difference was observed in the measurement whether a 20 µl or 30 µl

Table 3. Analysis of variance on data from average clearing vs. log thermonuclease concentration using 20 μl or 30 μl per well

Source	DF	ANOVA SS	F value	Pr > F
Plate	5	14.20	17.67	0.01
Amt	1	0.12	0.73	0.40
LGTN	4	77.47	120.50	0.01
Amt*TN	4	0.63	0.99	0.42

sample was used. The 20 μl sample was chosen for future use because this would hypothetically prevent overfilling any wells that may not have had the proper agar thickness or prevent spilling the nuclease out of the wells when handling the plate.

As discussed earlier, the AC measurement of clearing size was preferred over VER and HOR measurements. However, AC itself was thought to have had a deficiency. Since wells make up part of the measurement, AC would best estimate clearings due to enzyme activity if all wells were of equal size. The well sizes differed when utilizing the cutting in this experiment as outlined by Koupal and Deibel (1978). The sizes of the wells varied from 5.00 mm to 5.50 mm depending on the experiment. Hallynck and Pijck (1978) noted that cutting wells in agar

with a cork borer can lead to wells with unevenly cut edges. To remove this potential source of inaccuracy, the measurement referred to as cleared zone (CZ) was developed. This basic computation attempts to account for differences in well sizes when measuring zone diameters. This computation subtracts the average of the horizontal well measure (HORW) and the vertical well measure (VERW) from the AC. The formula used was:

$$CZ = AC - \frac{HORW-VERW}{2} .$$

The results of the quantitative assay were used for establishing a standard curve showing the concentration of nuclease based on clearing size. A major concern when producing the standard curve was whether the curve could be reproduced daily or whether the difference between measurements made on different days were significant. Lachica _et al_. (1972b) found that in their metachromatic agar diffusion assay that the standard curve needed to be produced daily due to variance in measurements. This factor was considered previously when significant differences were observed in measurements from plate-to-plate.

Using the reduction in sums of squares principle, the slope of the fitted regression line (in the regression of CZ on LGTN) differed significantly from Day 1 to Day 2.

From Figure 2, the estimated slopes from Day 1 and Day 2 are 1.39 and 2.30, respectively. The mean of readings from Day 2 (3.98) was also larger than Day 1 (3.45). This result shows that the standard curve used to determine the activity of nuclease in an unknown sample has to be run at the same time that the unknown sample is measured. This result verifies the hypothesis made in the preliminary experiment and is not unusual in biological systems. Many biological systems have daily variation within them (Hewitt, 1977). As previously mentioned, day-to-day variation in technique could also account for some of the variation.

When carrying out preliminary research on the growth of *S. aureus* Z88 in Brain Heart Infusion broth (BHI), the decision was made to use BHI to dilute the enzyme rather than Trizma because the organisms were grown in BHI broth.

Preliminary research showed that nuclease diluted with BHI gave larger clearings than nuclease diluted with Trizma. Also, it indicated that extension of the time that the nuclease remained in the wells produced larger clearing zones.

To test the significance of these two observations, an experiment was designed. The experiment involved four dilution media and four times of incubation. The four

Figure 2. The relationship of the clearing in agar (CZ readings) versus the log of the thermonuclease concentration, ng/ml on two different days

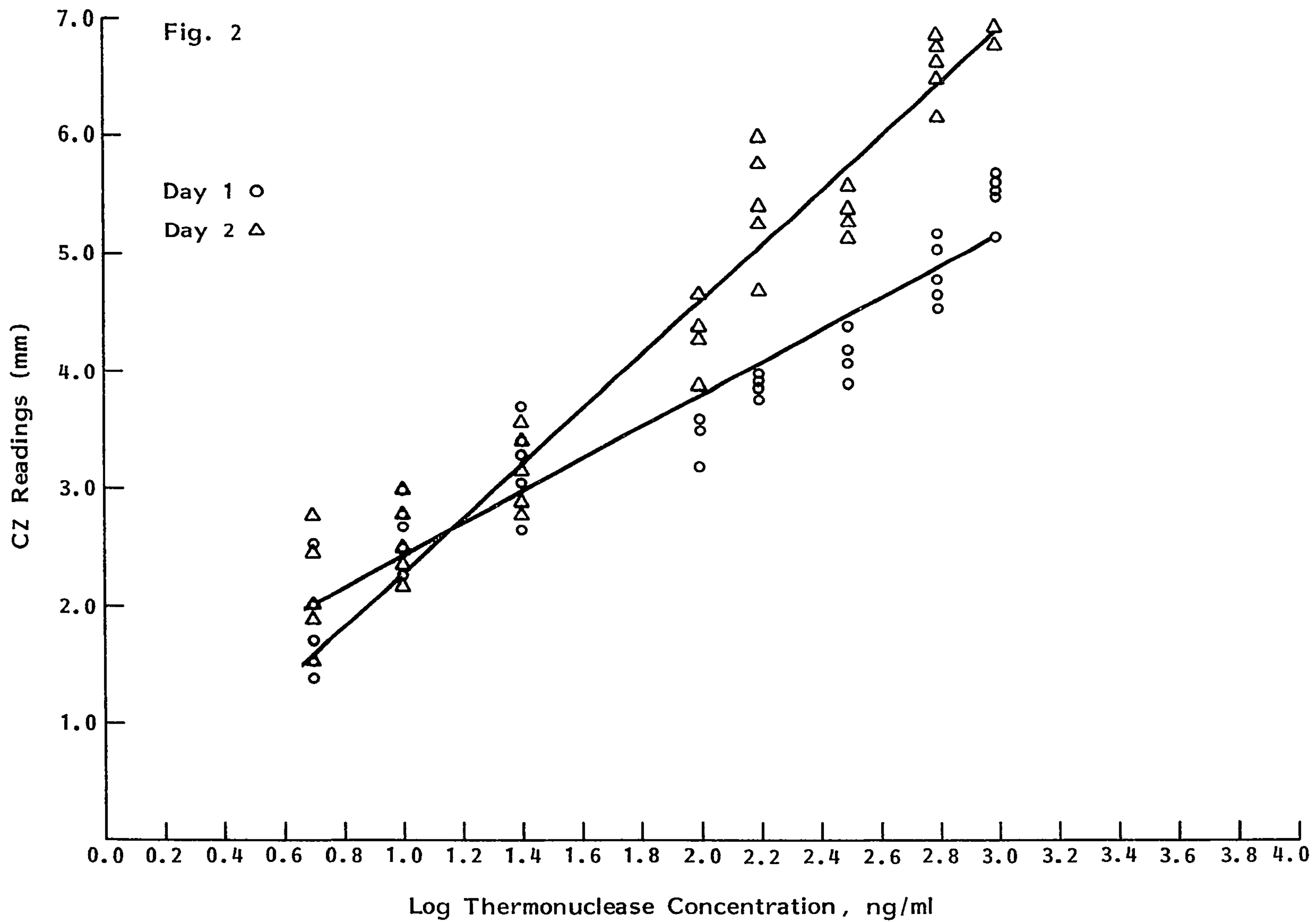
Fig. 2
Day 1
Day 2
CZ Readings (mm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
Log Thermonuclease Concentration, ng/ml

dilution media used were BHI at pHs of 6.99 (B) and 8.88 (H) and Trizma at pHs of 6.99 (R) and 8.88 (T). By using the same media at different pHs, it was hoped to determine the influence of pH on nuclease activity insofar as clearing was concerned. Times of incubation were staggered every 20 minutes giving incubation times of 1 hour (hr), 1 hr and 20 minutes (min), 1 hr and 40 min and 2 hrs.

An analysis of variance showed that there was a significant difference in the measured clearings based on F values at the different time periods and dilution media. The results appear in Table 4.

The means in Table 5 show that as the incubation times increased, CZ readings increased. The least significant difference for these comparisons is approximately 0.17 at the 5% level. The differences between these means is greater than 0.17, therefore, incubating at the 4 different times gave significantly different CZ readings. The means in Table 5 and the absence of significant interactions in the data in Table 4 also indicate that these time differences are consistent for all nuclease concentrations (activities) and diluting media.

The increase in size of the clearings with time was not totally unexpected. Lachica _et al_. (1972b) saw an

Table 4. Analysis of variance on data from cleared zone readings vs. log thermonuclease concentration using various dilution media and incubation times

Source	DF	Type I SS	F value	PR >F
Time	3	24.64	32.06	0.01
Medium	3	63.84	83.03	0.01
LGTN	5	385.43	300.79	0.01
Tm*Med	9	4.27	1.85	0.06
Tm*LGTN	15	2.95	0.77	0.71
Med*LGTN	15	1.61	0.42	0.97
Tm*Med*LGTN	45	4.92	0.43	0.99

Table 5. Means of cleared zone readings at different incubation times on data from cleared zone readings vs. log thermonuclease concentration

Incubation Time	CZ means (mm)
1 hour	3.71
1 hour, 20 min	3.95
1 hour, 40 min	4.24
2 hours	4.48

increase in zone diameter with time in their metachormatic agar diffusion assay. Enzyme activity is measured in units (mmoles of substrate used/min) (Clark and Switzer, 1977). Therefore, the longer the incubation period, the more substrate is broken down and the larger should be the CZ measurements within limits. Other assays for thermonuclease are carried out at longer incubation times (>1 hr) but most of these assays are run at temperatures lower than 50°C (Jarvis and Lawrence, 1969; Lachica *et al.*, 1972b). The increase in size in relation to enzyme concentration is of particular interest at lower concentrations or activities. By increasing the size of the CZ readings, enzyme samples of lower activities are easier to detect and measure; incubating for only 1 hour may not result in a detectable reaction.

Two hours incubation time was chosen for future assay work. The longer incubation time was thought to increase the detection of low activities of nuclease. Any longer incubation time would be counter productive to the speed of the assay.

The means in Table 6 show that purified nuclease diluted with BHI, irrespective of pH, gave higher overall CZ means than purified nuclease diluted in Trizma. The means in Table 6 and the absence of significant inter-

Table 6. Means of cleared zone readings with different dilution media on data from cleared zone readings vs. log thermonuclease concentration

Medium	CZ reading means (mm)
BHI - pH 6.99	4.56
BHI - pH 8.88	4.57
Trizma - pH 6.99	3.56
Trizma - pH 8.88	3.69

actions in the data in Table 4 indicate that the media differences are consistent for all nuclease concentrations and times. At the least significant difference level of 0.17, the two media at different pHs were not significantly different while the two different media, regardless of pH, were significantly different. Therefore, the differences in activity of the diluting media are attributable to the constituents of the media and not due to pH.

This observation may be explained by increased nuclease stability in the BHI broth. Research has shown that the enzyme is more stable when the following are present: nucleotide components, Ca^{+2} ion and bovine serum albumin (Lachica *et al.*, 1972c; Sulkowski and Laskowski, 1968). The ingredients for BHI are basically an extract

of beef brain and heart with salts added. There would be bovine serum albumin, degraded nucleotides present as well as traces of Ca^{+2} from the added salts. If the enzyme were more stable, this stability would enable the enzyme to retain much of its activity over a longer period of time. Trizma does not contain all these components and the enzyme would not necessarily be as stable.

Based on these results, BHI was chosen as the dilution medium for future assays. The greater stability of the enzyme in BHI should reduce variation in CZ measurements.

Applying the changes discussed previously (BHI dilution medium, 2 hour incubation time), a standard curve was developed with results illustrated in Figure 3. The results of regression analysis gave a relatively low estimated CZ population variance (.05) and an R^2 of 91%. The low variance estimate lends support to the hypothesis that the CZ populations associated with BHI medium have as small a variance as those associated with a Trizma medium. This "low" variance estimate is expected to be more stable from one experiment to another due to the stability of the enzyme in BHI. The equation of the fitted regression line is CZ = 2.39 + (0.89) LGTN. This equation can be used to predict the concentration of nuclease produced by *S. aureus* in growth medium.

Figure 3. The relationship of the clearing in agar (CZ readings) versus the log of the thermonuclease concentration, ng/ml when BHI was used as the diluting medium

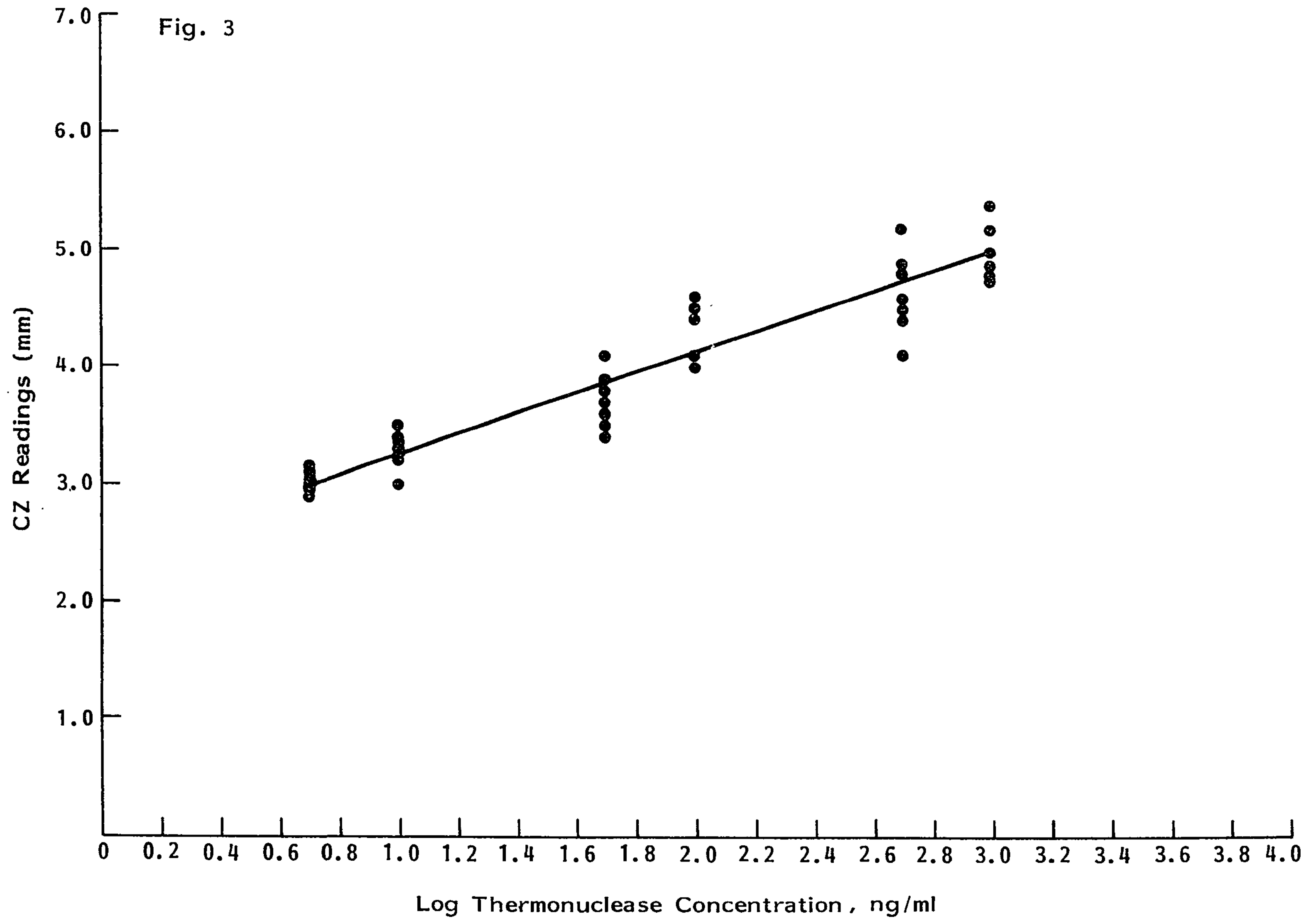
Fig. 3
7.0
6.0
5.0
4.0
3.0
2.0
1.0
CZ Readings (mm)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
Log Thermonuclease Concentration, ng/ml

Confidence limits were placed on predictions based on the regression line of the standard curve. In the prediction of x from y (i.e., nuclease concentration from the diameter) Equation 1 was used. The term of particular note in the equation is the m term. This term is equal to the number of measurements made on the unknown sample. If the prediction of the activity of the unknown sample gives a confidence interval larger than desired, making more measurements on the unknown sample theoretically reduces the interval due to the m term is in the denominator of a fraction in the following equation.

$$P\{\overline{X} \pm [\frac{\frac{y^*-\overline{Y}}{b_1} - t_{.05/2,N-2}\frac{S}{b_1}\sqrt{(\frac{1}{m}+\frac{1}{N})(1-C^2)+(\frac{1}{\Sigma(X_i-\overline{X})^2})(\frac{y^*-\overline{y}}{b_1})^2}}{1-C^2}]$$

$$\leq X^* \leq \overline{X} + [\frac{\frac{y^*-\overline{y}}{b_1} + t_{.05/2,N-2}\frac{S}{b_1}\sqrt{(\frac{1}{m}+\frac{1}{N})(1-C^2)+(\frac{1}{\Sigma(X_i-\overline{X}^2)})(\frac{y^*-\overline{y}}{b_1})^2}}{1-C^2}]\}$$

$$= 1-\alpha$$

where

$$C^2 = \frac{t^2{\alpha/2, N-2}\ S^2}{b_1{}^2\varepsilon(X_i-\overline{X})^2} \qquad \text{Equation 1}$$

where

t = t value at .05 level with 58 df

b_1= estimated slope

$\bar{y}$ = mean of cleared zone readings

$\bar{X}$ = mean of log of thermonuclease concentration

S^2=variance

S = standard deviation

α = level of error in confidence interval

y^*=average clearing size of nuclease

x^*=estimate of nuclease concentration

m = number of readings for the average clearing size

N = number of measures to produce the standard curve

P = probability

Table 7 shows the results of the confidence interval calculations made on a hypothetical reading from the standard curve in Figure 3. Table 7 shows that at the 95% (α = .05) confidence level, the more measurements taken, the smaller the interval. For our purposes, taking one reading gave an interval that was too large. Taking more than 10 readings would be excessive in relation to the time needed to run the experiment. The difference in the confidence interval between 5 and 10 readings was not large enough, 83.41 versus 59.80, respectively, to merit 10 readings in relation to time. Therefore, five readings per unknown sample were used for prediction

Table 7. Confidence intervals of a sample problem using data from cleared zone readings vs. log thermonuclease concentration with BHI medium at 2 hour incubation

Suppose Y* = 4.01 is the average of m clearing sizes for an enzyme of unknown concentration

Using Equation 1:

Confidence intervals of nuclease concentration (α=.05)

	Log confidence interval ng/ml (.05)	Confidence interval ng/ml (.05)
m=1	1.33 - 2.38	21.52 - 237.64
m=5	1.61 - 2.10	41.08 - 124.50
m=10	1.68 - 2.03	47.61 - 107.42
m=-∞	1.71 - 1.92	61.32 - 83.40

purposes and five readings per known nuclease concentration were used to produce the standard curve.

Up to this point, the assay for the enzyme has been based on clearing zone (mm) versus ng of thermonuclease/ml. Working with purified nuclease, this assay is valid. However, when measuring the unpurified enzyme from S. aureus cultures, activity in terms of enzyme units would be the proper measure. An enzyme is measured primarily in units and is expressed in terms of amount of substrate broken down or product formed per minute (Clark

and Switzer, 1977). If the enzymes being measured are not purified to the same degree, a ng of one enzyme would not give the same reaction as a ng of the other. Therefore, when using the assay developed here for unpurified enzyme produced by cultures of *S. aureus* Z88, clearing zone versus units of enzyme is a more accurate measure.

Figure 4 shows a plot of clearing zones versus units in a standard curve using the same data as shown in Figure 3. The same slope is obtained as when clearing zones versus thermonuclease activity is plotted. The variance (.05) is the same as well. Therefore, changing to activity did not affect the assay adversely.

Effect of Environmental Conditions on Thermonuclease Production

Temperature

Many researchers agree that the presence of nuclease is an indication of $>10^6$ cells/ml of *S. aureus* is or have have been present (Tatini *et al.*, 1975, 1976). The growth of *S. aureus* Z88 at 15°, 22° and 37°C in numbers $>10^6$ cells/ml always resulted in the production of nuclease within 48 hours. A major problem when using the three temperatures was the number of cells needed in the inoculum for the production of $>10^6$ cells/ml within 48 hours. Also, the extent of the lag and log phases and

Figure 4. The relationship of the measure of agar clearing (CZ readings) vs. the log of the activity units/ml

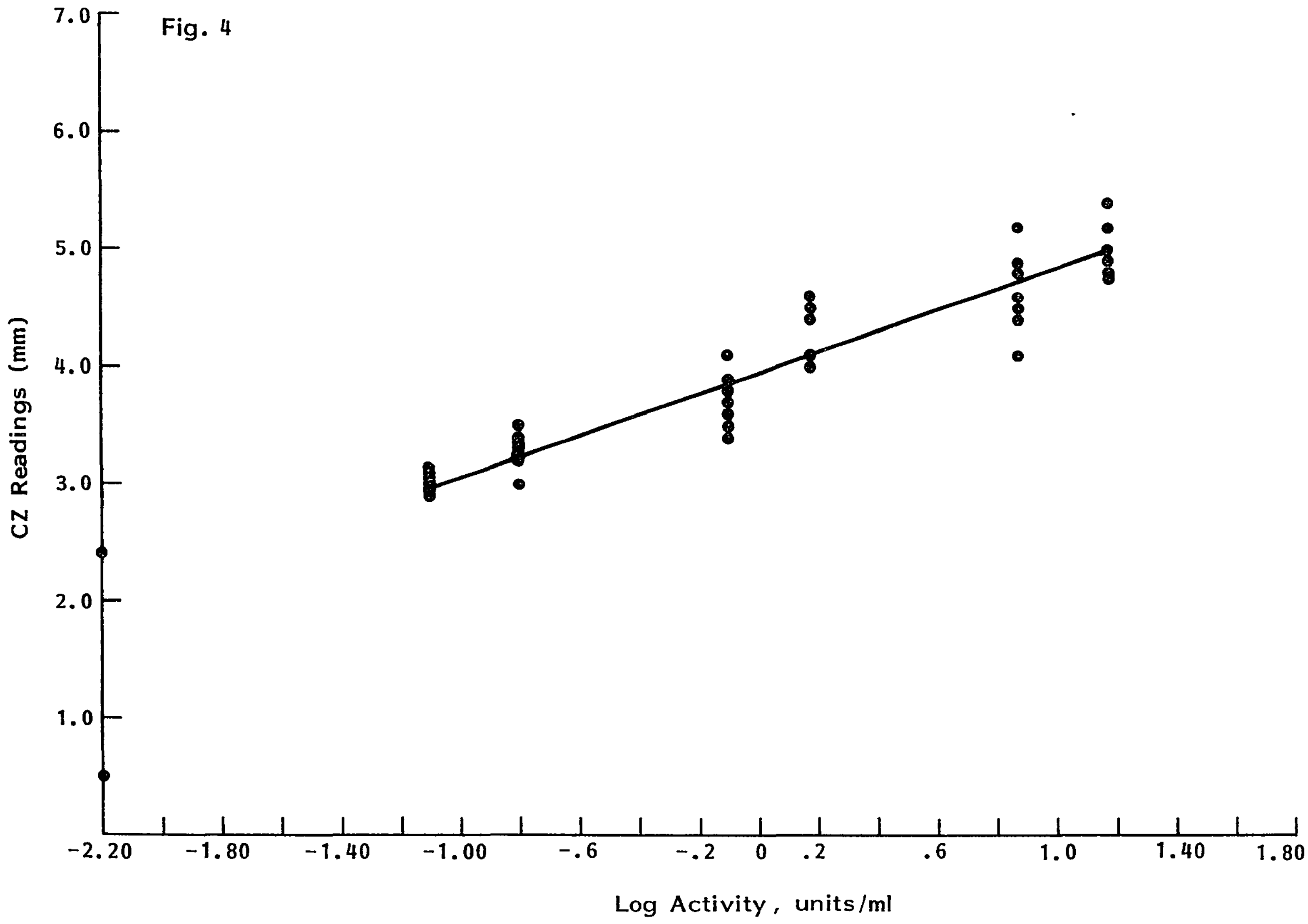
Fig. 4
CZ Readings (mm)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
-2.20
-1.80
-1.40
-1.00
-.6
-.2
0
.2
.6
1.0
1.40
1.80
Log Activity, units/ml

the onset of the stationary phase.

As the temperature of incubation decreased, a larger inoculum of cells was required to produce 10^6 cells/ml within the 48 hour incubation period. At 37°C, 10^2 to 10^4 cells were added to the flasks; at 22°C, 10^4 to 10^6 cells were added; and at 15°C, 10^6 to 10^8 cells were added to the flasks. This gave an initial concentration of cells at <30 - $6.7x10^1$ cells/ml in flasks at 37°C, $6.7x10^1$ - $6.7x10^3$ cells/ml in flasks at 22°C, and $6.7x10^3$ - $6.7x10^5$ cells/ml in flasks at 15°C.

Research has shown that upon transferring bacteria from one medium to another, a lag phase takes place (Dawes, 1976; Postgate, 1967). The extent of the lag phase or time for the development of growth in the new medium is dependent on how different the change in environment (medium) is from the original medium, how well the organism grows in the new environment and the number of organisms inoculated (Dawes, 1976; Postgate, 1967). The medium was the same in this experiment (BHI broth) with the major difference being in the temperature. At 37°C, the temperature did not vary much from the temperature at which the cells for the inoculum (35°C) were grown. S. aureus grows optimally at 37°C and, therefore, the lower inoculum of cells sufficed at this temperature. Temperatures of 22°C and 15°C varied to a greater

extent from the optimum temperature of 35° to 40°C. The temperature of 22°C is less favorable than 37°C but more favorable than 15°C. Other workers have also shown that growth of S. aureus at lower than optimum temperatures requires a large initial number of cells (Dawes, 1976; Postgate, 1967). Therefore, 15°C required a larger inoculum than did 22°C to give $>10^6$ cells/ml within 48 hours.

Figure 5 shows that at 37°C the log phase begins within 2 to 3 hours and the stationary phase was reached in 11 to 12 hours. Under these conditions, $\geq 10^6$ cells/ml was reached in 8 to 10 hours. At 22°C (Figure 6), the log phase does not start until after 4 to 6 hours and the stationary phase was not reached until 34 to 36 hours. The log phase was longer at 22°C (28 to 30 hours) than at 37°C (8 to 9 hours). The level of 10^6 cells/ml was reached in 20 to 22 hours, 12 to 14 hours later than at 37°C. At 15°C (Figure 7), the log phase starts even later (6 to 8 hours) and the stationary phase was not reached within the 48 hour period; in fact, the numbers of cells were still increasing after 48 hours. A concentration of 10^6 cells/ml was attained in 24 to 28 hours.

The measure of absorbance on the turbidity of the medium was used to monitor growth of S. aureus Z88 during the 48 hour incubation period. Figure 8 shows the typical result of absorbance readings versus log cells/ml obtained

Figure 5. Log number of *S. aureus* Z88 cells/ml present in BHI broth in 0-48 hours at 37°C

Figure 6. Log number of *S. aureus* Z88 cells/ml present in BHI broth in 0-48 hours at 22°C

Figure 7. Log number of *S. aureus* Z88 cells/ml present in BHI broth in 0-48 hours at 15°C

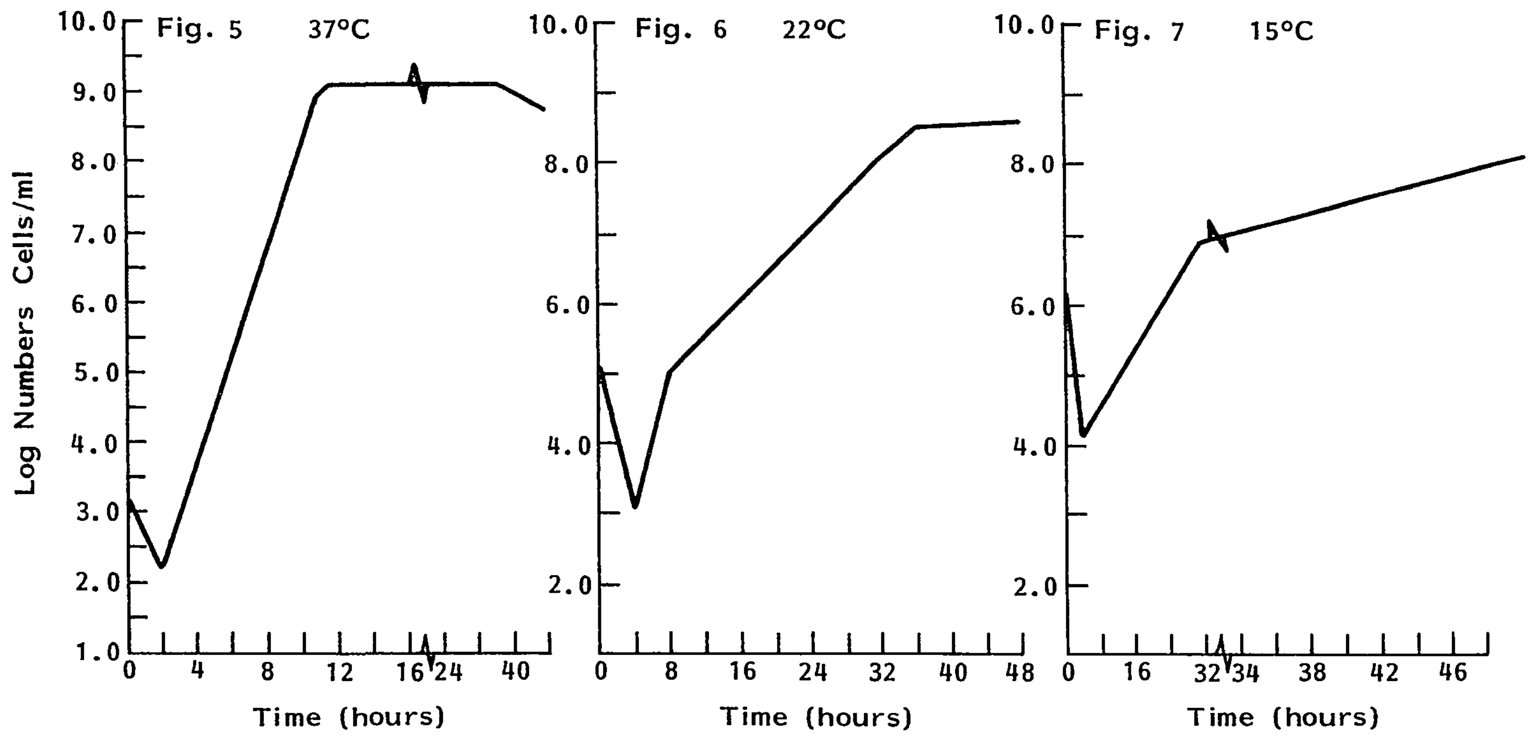
Fig. 5
37°C
Fig. 6
22°C
Fig. 7
15°C
Log Numbers Cells/ml
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
4
8
12
16
24
40
16
24
32
40
48
32
34
38
42
46
Time (hours)
Time (hours)
Time (hours)

from plate counts. The readings in this figure are for cultures incubated at 37°C and the absorbance was not measurable until at least 10^6 cells/ml were present. At this point, the growth was in log phase. At 10^8 cells/ml, however, the curve shows a large increase in absorbance without a parallel increase in numbers of organisms. At this point the organism was in stationary phase. Therefore, when absorbance was read, at least 10^6 cells/ml were present and when the absorbance readings stabilized, at least 10^8 cells/ml were or had been present.

Figure 9 shows variation of absorbance with time at the three temperatures. Generally, when absorbance could be recorded at all three temperatures, 10^6 cells/ml were present. Both at 37°C and 22°C, the stationary phase began when absorbance readings exceeded .700. At 15°C, no stationary phase occurred within 48 hours; and in this instance, the absorbance readings exceeded .200.

Tables 8 through 10 show the size of the clearing zones from the assay plates and the corresponding nuclease activity for S. aureus Z88 grown in BHI broth at 37°, 22° and 15°C. Table 8 shows that nuclease activity could be detected after 8 hours of incubation; the units of nuclease present were too low to predict with confidence the

Figure 8. Log numbers of *S. aureus* Z88 cells/ml present in BHI broth at measured absorbance (660 nm) at 37°C

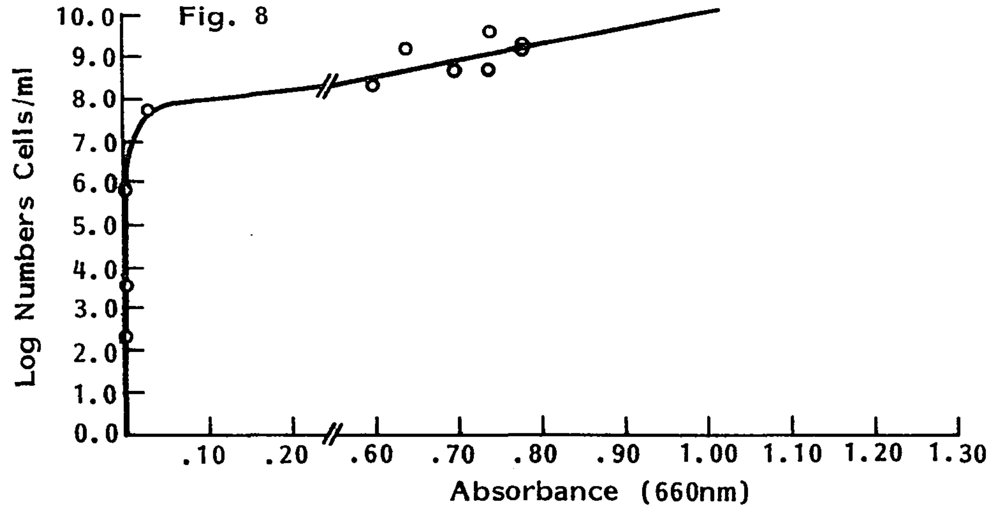
Fig. 8
Log Numbers Cells/ml
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
.10
.20
.60
.70
.80
.90
1.00
1.10
1.20
1.30
Absorbance (660nm)

Figure 9. The average absorbance readings (660 nm) of *S. aureus* Z88 in BHI broth incubated at 37°, 22° and 15°C during 0-48 hours

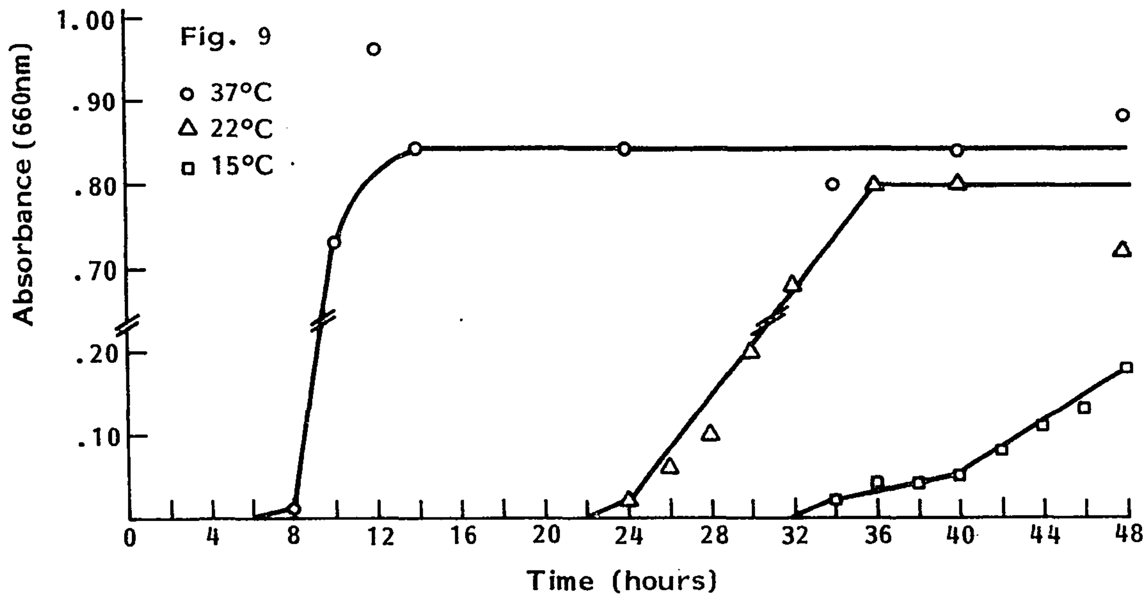
Fig. 9
o 37°C
Δ 22°C
□ 15°C
Absorbance (660nm)
1.00
.90
.80
.70
.20
.10
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (hours)

activity of nuclease present. Nuclease activities were much higher after 16 hours. Many of the readings exceeded the limit of the standard curve.

Nuclease production at 22°C (Table 9) was not evident until after 24 hours. Activity readings after 24 hours were lower than those taken at 32 through 48 hours in which many readings exceeded the standard curve limit. Activity at 15°C (Table 10) was not detected until after 32 hours. Nuclease activity increased after 40 and 48 hours. The level of activity produced was not as high at 15°C as was produced at 37°C and 22°C.

S. aureus Z88 produced nuclease at all three temperatures. Table 11 shows the number of cells/ml when nuclease was first detected. The table show $>10^6$ cells/ml present when nuclease was present. From the research presented here, it cannot be determined whether *S. aureus* Z88 can produce as high an activity of nuclease at 15°C as was produced at 22°C and 37°C since the stationary phase was not reached in 48 hours.

Some of the CZ measurements exceeded that were out of the range of the standard curve. The very high production of thermonuclease by *S. aureus* Z88 produced very high CZ values. Readings out of range at the lower end of the curve (<0.08 units) are due to low activities. Thermonuclease

Table 8. Levels of thermonuclease activity in cultures of *S. aureus* Z88 grown in BHI broth for 48 hours at 37°C

Replicate	CZ readings (mm)	Units of activity/ml	Time (h)	Confidence interval (95%)
1	1.89	<0.08	8	-
2	2.26	<0.08	8	-
3	2.29	<0.08	8	-
1	6.14	11.6	16	6.47-21.2
2	5.52	4.57	16	2.63- 8.02
3	6.49	>15.0	16	-
1	5.99	9.27	24	5.22-16.7
2	5.85	7.51	24	4.26-13.4
3	5.86	7.62	24	4.32-13.6
1	6.22	13.1	32	7.27-24.0
2	7.13	>15.0	32	-
3	6.96	>15.0	32	-
1	6.49	>15.0	40	-
2	6.46	>15.0	40	-
3	6.54	>15.0	40	-
1	6.22	13.1	48	7.27-24.0
2	7.11	>15.0	48	-
3	7.16	>15.0	48	-

Table 9. Levels of thermonuclease activity in cultures of *S. aureus* Z88 grown in BHI broth for 48 hours at 22°C

Replicate	CZ Readings (mm)	Units of activity/ml	Time (h)	Confidence interval (95%)
1	2.31	<0.08	24	-
2	3.72	0.12	24	0.06-0.24
3	3.74	0.13	24	0.06-0.25
1	6.54	>15.0	32	-
2	6.61	>15.0	32	-
3	6.62	>15.0	32	-
1	6.62	>15.0	40	-
2	6.60	>15.0	40	-
3	6.73	>15.0	40	-
1	6.78	>15.0	48	-
2	7.01	>15.0	48	-
3	6.94	>15.0	48	-

Table 10. Levels of thermonuclease activity in cultures of *S. aureus* Z88 grown in BHI broth for 48 hours at 15°C

Replicate	CZ Reading (mm)	Units of activity/ml	Time (h)	Confidence interval (95%)
1	3.04	0.10	32	0.07-0.15
2	3.40	0.17	32	0.12-0.25
3	4.02	0.45	32	0.32-0.64
1	4.18	0.58	40	0.41-0.82
2	4.33	0.72	40	0.51-1.02
3	4.47	0.90	40	0.64-1.27
1	4.79	1.46	48	1.04-2.08
2	4.77	1.42	48	1.01-2.01
3	4.84	1.58	48	1.12-2.24

Table 11. Nuclease activity compared with numbers of *S. aureus* Z88 cells/ml grown in BHI broth. Time indicates the first observation of nuclease activity

Replicate	CZ Reading (mm)	Units of activity/ml	Cells/ml	Time (h)	Temp (°C)
1	1.89	<0.08	$2.3x10^{7}$	8	37
2	2.26	<0.08	$2.5x10^{7}$	8	37
3	2.29	<0.08	$6.2x10^{7}$	8	37
1	2.31	<0.08	$3.2x10^{6}$	24	22
2	3.72	0.12	$4.9x10^{6}$	24	22
3	3.74	0.13	$1.0x10^{7}$	24	22
1	3.04	0.10	$3.8x10^{7}$	32	15
2	3.40	0.17	$3.6x10^{7}$	32	15
3	4.02	0.45	$8.0x10^{7}$	32	15

could not be detected at the low levels consistently during the development of the quantitative assay and in addition difficulty in measuring very small clearing zones was encountered in the assay.

These measurements served as a reference or control in subsequent observations for the comparison of S. aureus Z88 grown in BHI broth in which the pH was varied and to which NaCl, KCl and sodium acid pyrophosphate (SAPP) were added.

pH

Figures 10 through 12 show the results of absorbance of cultures of S. aureus Z88 in BHI broth at initial pHs of 5.3, 6.3, 7.0 and 8.3 incubated for 48 hours at temperatures of 15°, 22° and 37°C. These figures show that at pH 6.3 the results were similar to those of the control flasks (pH 7.0). In other words, pH 6.3 had no significant influence on the growth of S. aureus Z88 in comparison to the control. Various researchers have reported that pH has no effect on the growth of S. aureus until it drops below 6.0 (Arvidson and Holme, 1971; Jarvis et al., 1973). At pH 5.3 and 8.3, absorbance could not be observed with the spectrophotometer as soon as at pH 6.3. Based on these absorbance readings,

Figure 10. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth at initial pHs of 5.3, 6.3, 7.0 and 8.3 incubated for 48 hours at 15°C

Figure 11. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth at initial pHs of 5.3, 6.3, 7.0 and 8.3 incubated for 48 hours at 37°C

Figure 12. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth at initial pHs of 5.3, 6.3, 7.0 and 8.3 incubated for 48 hours at 22°C

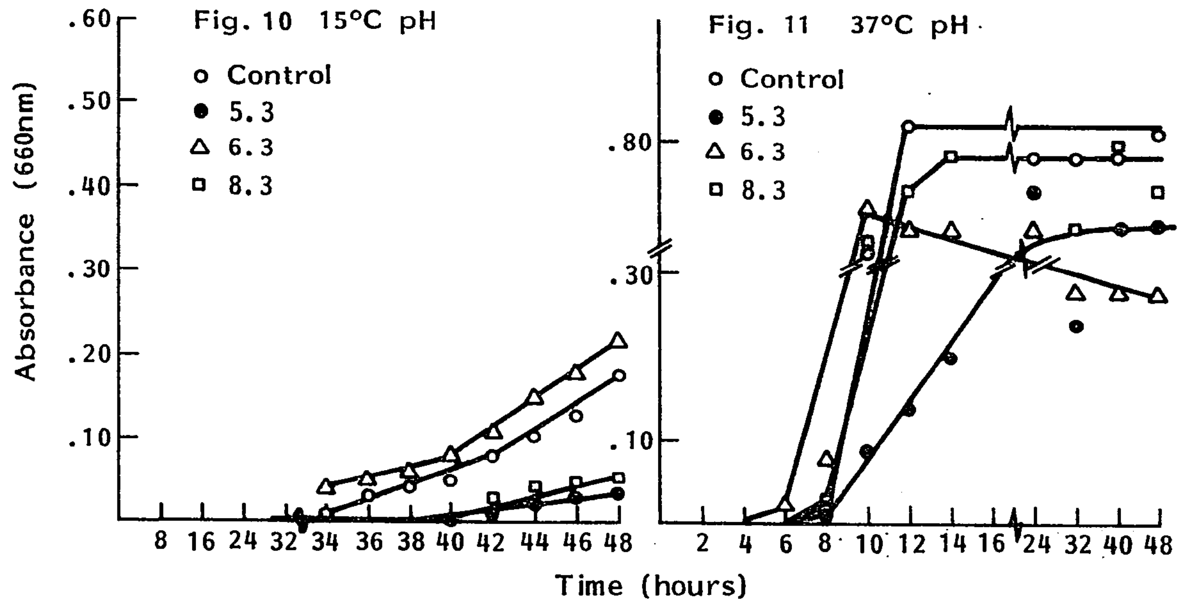
Fig. 10 15°C pH
Control
5.3
6.3
8.3
.60
.50
.40
.30
.20
.10
Absorbance (660nm)
8 16 24 32 34 36 38 40 42 44 46 48
Fig. 11 37°C pH
Control
5.3
6.3
8.3
.80
.30
.10
2 4 6 8 10 12 14 16 24 32 40 48
Time (hours)

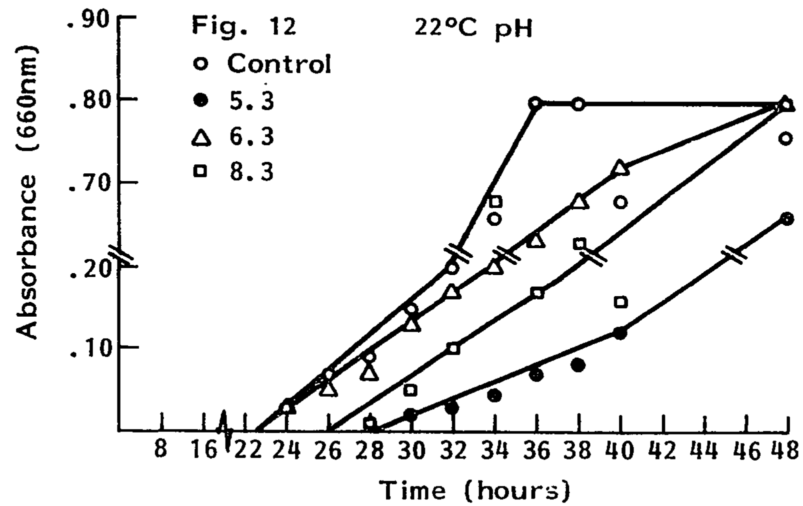
Fig. 12 22°C pH
Control
5.3
6.3
8.3
.90
.80
.70
.20
.10
Absorbance (660nm)
8 16 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (hours)

pH 5.3 appeared more adverse to the growth of *S. aureus* Z88 than was pH 8.3 over all three temperatures. This finding supports other observations that the growth of *S. aureus* is more adversely affected by acid pHs than by alkaline pHs (Arvidson and Holme, 1971; Jarvis *et al.*, 1973).

Tables 12 through 14 show the production of nuclease by *S. aureus* Z88 at the three pHs. Nuclease was detected at pH 6.3 at the same time as for pH 7.0 and at about the same level of activity. This observation was consistent over all three temperatures and lends credence to the assumption that at 6.3, growth and the production of nuclease are the same as in the control (pH 7.0).

Table 12 shows that at pH 8.3, nuclease is produced at higher activities than at 5.3, and growth at both pHs reached stationary phase.

At 22°C (Table 13) the enzyme was produced at higher levels of activity at 5.3 than was observed at 37°C. This could be due to the slower growth rate allowing the cells to survive longer and produce more nuclease. The high levels of nuclease still were not produced as soon as at 8.3. At 15°C (Table 14) nuclease activity was detected after 40 and 48 hours at pH 8.3 and after 48 hours at pH 5.3. Perhaps a combination of factors including the low pH and the cold temperature (15°C) that delayed growth to a greater extent at 5.3 contributed to activity being

Table 12. Nuclease activity of cultures of *S. aureus* Z88 grown in BHI broth at pHs 5.3, 6.3, 7.0 and 8.3 at 37°C

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
5.3	8	-	-	-
5.3	16	1.59	<0.08	-
5.3	24	5.27	3.14	1.82-5.44
5.3	32	2.04	<0.08	-
5.3	40	5.42	3.93	2.27-6.87
5.3	48	1.93	<0.08	-
6.3	8	2.71	<0.08	-
6.3	16	6.03	9.85	5.53-17.8
6.3	24	5.95	8.73	4.92-15.7
6.3	32	6.18	12.3	6.86-22.6
6.3	40	6.18	12.3	6.86-22.6
6.3	48	6.09	12.5	6.96-22.9
7.0	8	1.89	<0.08	-
7.0	16	6.14	11.6	6.48-21.2
7.0	24	5.99	9.28	5.22-16.7
7.0	32	6.22	13.1	7.27-24.0
7.0	40	6.49	19.7	10.7 -36.9
7.0	48	6.22	13.1	7.27-24.0
8.3	8	-	-	-
8.3	16	5.39	3.76	2.17- 6.56
8.3	24	6.00	9.42	5.29-17.0
8.3	32	5.71	6.08	3.47-10.8
8.3	40	6.57	>15.0	-
8.3	48	5.71	6.08	3.47-10.8
Experiment II				
5.3	8	-	-	-
5.3	16	3.81	0.12	0.06-0.23
5.3	24	5.09	1.36	0.73-2.47
5.3	32	5.37	2.31	1.24-4.24
5.3	40	5.15	1.52	0.82-2.77
5.3	48	5.36	2.27	1.22-4.16
6.3	8	3.26	<0.08	-
6.3	16	6.09	9.09	4.68-17.5
6.3	24	6.17	10.6	5.41-20.5
6.3	32	6.41	>15.0	-
6.3	40	6.29	13.3	6.72-26.1
6.3	48	6.46	>15.0	-

Table 12 (Continued)

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
7.0	8	3.06	<0.08	-
7.0	16	6.14	10.0	5.12-19.4
7.0	24	6.14	10.0	5.12-19.4
7.0	32	6.37	>15.0	-
7.0	40	6.77	>15.0	-
7.0	48	6.35	14.9	7.49-29.5
8.3	8	-	-	-
8.3	16	3.81	0.12	5.71-21.8
8.3	24	6.20	11.2	-
8.3	32	6.77	>15.0	-
8.3	40	6.76	>15.0	-
8.3	48	7.00	>15.0	-

Table 13. Nuclease activity of cultures of *S. aureus* Z88 grown in BHI broth at pHs 5.3, 6.3, 7.0, and 8.3 at 22°C

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
5.3	8	-	-	-
5.3	16	-	-	-
5.3	24	-	-	-
5.3	32	4.56	0.58	0.30- 1.10
5.3	40	5.97	7.73	3.91-15.6
5.3	48	5.69	4.61	2.38- 9.11
6.3	8	-	-	-
6.3	16	-	-	-
6.3	24	2.84	<0.08	-
6.3	32	6.59	>15.0	-
6.3	40	6.75	>15.0	-
6.3	48	7.03	>15.0	-
7.0	8	-	-	-
7.0	16	-	-	-
7.0	24	2.31	<0.08	-
7.0	32	6.54	>15.0	-
7.0	40	6.62	>15.0	-
7.0	48	6.78	>15.0	-

Table 13 (Continued)

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
8.3	8	-	-	-
8.3	16	-	-	-
8.3	24	-	-	-
8.3	32	6.57	>15.0	-
8.3	40	6.11	10.0	5.00-20.5
8.3	48	6.61	>15.0	-

<u>Experiment II</u>

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
5.3	8	-	-	-
5.3	16	-	-	-
5.3	24	-	-	-
5.3	32	4.67	0.61	0.33-1.11
5.3	40	6.17	10.6	5.41-20.5
5.3	48	6.52	>15.0	-
6.3	8	-	-	-
6.3	16	-	-	-
6.3	24	4.66	0.60	0.32-1.09
6.3	32	6.56	>15.0	-
6.3	40	6.75	>15.0	-
6.3	48	6.86	>15.0	-
7.0	8	-	-	-
7.0	16	-	-	-
7.0	24	4.83	0.83	0.44-1.50
7.0	32	6.74	>15.0	-
7.0	40	6.88	>15.0	-
7.0	48	6.87	>15.0	-
8.3	8	-	-	-
8.3	16	-	-	-
8.3	24	-	-	-
8.3	32	5.90	6.33	3.31-12.0
8.3	40	6.65	>15.0	-
8.3	48	6.70	>15.0	-

Table 14. Nuclease activity of cultures of S. aureus Z88 grown in BHI broth at pHs 5.3, 6.3, 7.0 and 8.3 at 15°C

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
5.3	8	-	-	-
5.3	16	-	-	-
5.3	24	-	-	-
5.3	32	-	-	-
5.3	40	-	-	-
5.3	48	2.65	<0.08	-
6.3	8	-	-	-
6.3	16	-	-	-
6.3	24	-	-	-
6.3	32	4.52	0.97	0.69-1.37
6.3	40	4.38	0.78	0.55-1.11
6.3	48	4.94	1.84	1.31-2.62
7.0	8	-	-	-
7.0	16	-	-	-
7.0	24	-	-	-
7.0	32	3.04	0.10	0.07-0.15
7.0	40	4.18	0.58	0.41-0.82
7.0	48	4.79	1.46	1.04-2.08
8.3	8	-	-	-
8.3	16	-	-	-
8.3	24	-	-	-
8.3	32	-	-	-
8.3	40	2.72	<0.08	-
8.3	48	3.68	0.27	0.19-0.38
Experiment II				
5.3	8	-	-	-
5.3	16	-	-	-
5.3	24	-	-	-
5.3	32	-	-	-
5.3	40	-	-	-
5.3	48	2.81	<0.08	-

Table 14 (Continued)

pH	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
6.3	8	-	-	-
6.3	16	-	-	-
6.3	24	-	-	-
6.3	32	2.60	<0.08	-
6.3	40	4.76	1.08	0.62-1.85
6.3	48	4.92	1.48	0.85-2.54
7.0	8	-	-	-
7.0	16	-	-	-
7.0	24	-	-	-
7.0	32	2.80	<0.08	-
7.0	40	4.64	0.86	0.49-1.46
7.0	48	5.01	1.76	1.02-3.04
8.3	8	-	-	-
8.3	16	-	-	-
8.3	24	-	-	-
8.3	32	-	-	-
8.3	40	3.25	<0.08	-
8.3	48	3.71	0.14	0.18-0.24

detected at pH 5.3 later than at pH 8.3. The data on nuclease production at different pHs show that both pH 5.3 and 8.3 delay growth of the *S. aureus* Z88 and that at pH 5.3 production of nuclease can be lower and even delayed to a greater extent than at pH 8.3. These results may be due to lower levels of nuclease produced at pH 5.3. Research by Arvidson and Holme (1971) and Jarvis *et al.* (1973) reported that at pHs below 6.0, nuclease is produced at lower levels than at pHs greater than 6.0.

Sodium acid pyrophosphate (SAPP)

Figures 13 through 15 show the effects of SAPP on the absorbance of cultures of *S. aureus* Z88 at the three temperatures. SAPP had no major effect on the growth of the organism; absorbance readings of *S. aureus* Z88 in the presence of the phosphate closely paralleled the readings of the control flasks regardless of incubation temperature and phosphate concentration.

Although there was no major effect of the phosphate on the growth of *S. aureus* Z88, there was a general trend; that is, in most readings, the higher the concentration of SAPP, the lower were the initial absorbance readings and the corresponding plate counts (Table 15). An explanation for this difference could be due to the pH of

Figure 13. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth with 0%, 0.1%, 0.25% and 0.5% of sodium acid pyrophosphate (SAPP) for 48 hours

Figure 14. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth with 0%, 0.1%, 0.25% and 0.5% of sodium acid pyrophosphate (SAPP) for 48 hours.

Figure 15. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth with 0%, 0.1%, 0.25% and 0.5% of sodium acid pyrophosphate (SAPP) for 48 hours at 22°C

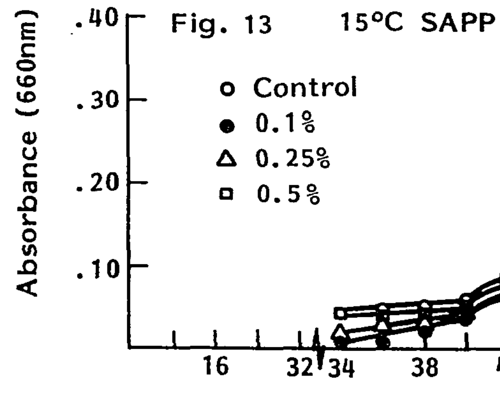

Fig. 13
15°C SAPP
Control
0.1%
0.25%
0.5%
Absorbance (660nm)
.40
.30
.20
.10
16
32
34
38
42
46

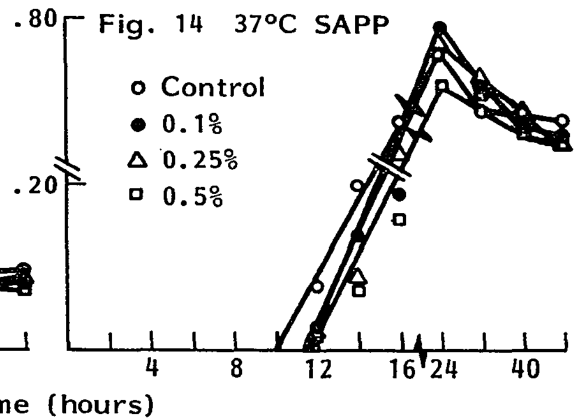

Fig. 14
37°C SAPP
Control
0.1%
0.25%
0.5%
.80
.20
4
8
12
16
24
40
Time (hours)

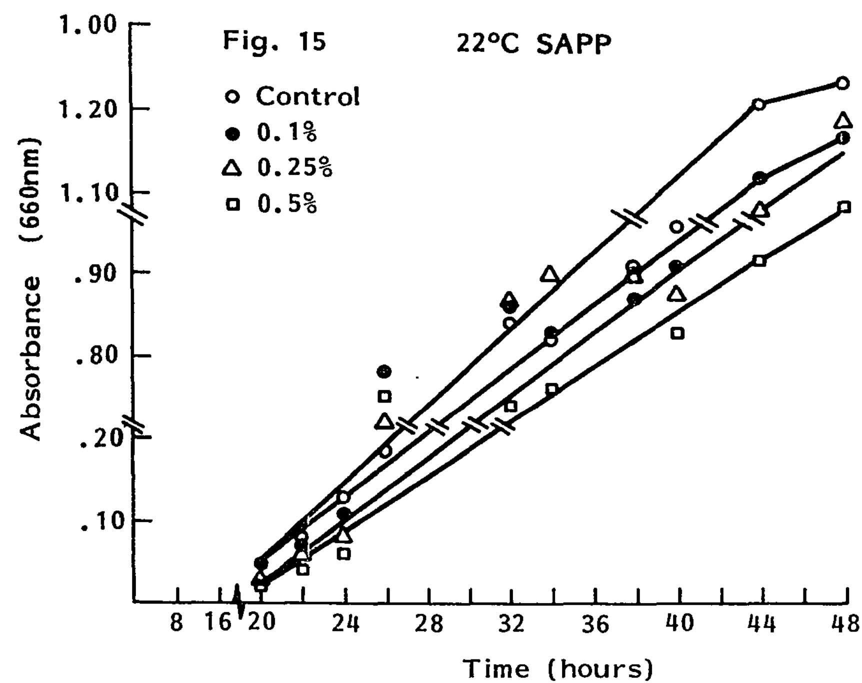

Fig. 15
22°C SAPP
Control
0.1%
0.25%
0.5%
Absorbance (660nm)
1.00
1.20
1.10
.90
.80
.20
.10
8
16
20
24
28
32
36
40
44
48
Time (hours)

Table 15. Average peak counts and absorbance readings of *S. aureus* Z88 grown in BHI broth at SAPP concentrations of 0%, 0.1%, 0.25%, and 0.5% at 15°C, 22°C, and 37°C

SAPP %	Temp C°	Time (h)	Absorbance	Plate counts (cells/ml)
0	15	24	-	$2.6x10^5$
0.1	15	24	-	$3.6x10^5$
0.25	15	24	-	$2.6x10^5$
0.5	15	24	-	$1.4x10^5$
0	15	48	.144	$1.1x10^8$
0.1	15	48	.130	$4.6x10^7$
0.25	15	48	.102	$6.0x10^7$
0.5	15	48	.092	$5.1x10^7$
0	22	24	.130	$1.1x10^8$
0.1	22	24	.106	$9.5x10^7$
0.25	22	24	.082	$6.7x10^7$
0.5	22	24	.055	$3.6x10^7$
0	22	48	1.270	$1.4x10^9$
0.1	22	48	1.140	$1.5x10^9$
0.25	22	48	1.170	$1.4x10^9$
0.5	22	48	.970	$3.5x10^8$
0	37	24	.724	$5.1x10^8$
0.1	37	24	.782	$5.8x10^8$
0.25	37	24	.746	$4.2x10^8$
0.5	37	24	.642	$3.3x10^8$
0	37	48	.570	$4.4x10^8$
0.1	37	48	.516	$3.5x10^8$
0.25	37	48	.538	$4.7x10^8$
0.5	37	48	.524	$3.5x10^8$

the medium. Table 16 shows the initial pHs of three samples of media. As the concentration of the SAPP is increased, the pH of the medium decreased. This is due to the ionization of the sodium acid pyrophosphate (Knipe, 1982). The number of organisms being lower initially at 24 hrs may be due to the lowering of the pH. The measured pH samples were never lower than 6.0. As previously discussed, a pH no lower than 6.0 did not greatly influence the growth of *S. aureus* when compared with the optimum pH 7.0 (Arvidson and Holme, 1971; Jarvis *et al.*, 1973). Therefore, pH may not fully account for these results.

Tables 17 through 19 show nuclease activity in the presence of SAPP. The level of nuclease produced in the presence of SAPP in the initial readings when nuclease was detected was lower than flasks as the concentration of SAPP was increased. The explanation for this result requires further study.

NaCl

Figure 16 shows the effect of NaCl on the absorbance of cultures of *S. aureus* Z88 at the temperatures 22° and 37°C. No growth was detected by absorbance at 15°C at all NaCl concentrations during the 48 hour incubation period. At 22°C, absorbance readings were observed only in 5% NaCl within 48 hours. At 37°C, absorbance was observed in 5% and 10% NaCl within 48 hours.

Table 16. pHs of BHI broth with added SAPP in percent concentrations of 0%, 0.1%, 0.25% and 0.5% of 3 autoclaved samples

SAPP (%)	pH sample 1	pH sample 2	pH sample 3
0	7.17	7.27	6.91
0.10	6.90	7.08	6.58
0.25	6.58	6.80	6.43
0.50	6.32	6.53	6.05

Table 17. Nuclease activity of cultures of S. aureus Z88 grown in BHI broth containing various concentrations of SAPP at 37°C

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	6.61	>15.0	-
0	24	6.28	>15.0	-
0	32	6.05	>15.0	-
0	40	6.45	>15.0	-
0	48	6.25	>15.0	-
0.1	8	-	-	-
0.1	16	6.33	>15.0	-
0.1	24	6.17	>15.0	-
0.1	32	6.56	>15.0	-
0.1	40	6.38	>15.0	-
0.1	48	6.57	>15.0	-
0.25	8	-	-	-
0.25	16	5.74	>15.0	-
0.25	24	6.24	>15.0	-
0.25	32	6.69	>15.0	-
0.25	40	6.42	>15.0	-
0.25	48	6.41	>15.0	-
0.5	8	-	-	-
0.5	16	4.62	2.79	1.77-4.86
0.5	24	6.02	>15.0	-
0.5	32	6.42	>15.0	-
0.5	40	6.01	>15.0	-
0.5	48	6.51	>15.0	-

Table 17 (Continued)

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment II				
0	8	-	-	-
0	16	6.11	>15.0	-
0	24	6.30	>15.0	-
0	32	5.81	14.1	9.89-19.8
0	40	6.08	>15.0	-
0	48	6.57	>15.0	-
0.1	8	-	-	-
0.1	16	5.68	11.3	7.97-15.8
0.1	24	6.19	>15.0	-
0.1	32	6.21	>15.0	-
0.1	40	6.33	>15.0	-
0.1	48	6.41	>15.0	
0.25	8	-	-	-
0.25	16	5.31	5.99	4.30-8.28
0.25	24	6.35	>15.0	-
0.25	32	6.04	>15.0	-
0.25	40	6.53	>15.0	-
0.25	48	6.05	>15.0	-
0.5	8	-	-	-
0.5	16	4.69	2.08	1.52-2.83
0.5	24	6.11	>15.0	-
0.5	32	6.28	>15.0	-
0.5	40	6.00	>15.0	-
0.5	48	5.85	>15.0	-

Table 18. Nuclease activity of cultures of *S. aureus* Z88 grown in BHI broth containing various concentrations of SAPP at 22°C

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	-	-	-
0	24	6.37	5.82	3.50- 9.73
0	32	6.40	6.04	3.63-10.1
0	40	7.08	14.2	8.29-24.6
0	48	7.38	>15.0	-

Table 18 (Continued)

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
0.1	8	-	-	-
0.1	16	-	-	-
0.1	24	6.12	4.25	2.58- 7.04
0.1	32	6.87	10.9	6.43-18.7
0.1	40	7.18	>15.0	-
0.1	48	7.37	>15.0	-
0.25	8	-	-	-
0.25	16	-	-	-
0.25	24	6.09	4.09	2.48- 6.77
0.25	32	7.02	13.2	7.71-22.8
0.25	40	7.00	12.9	7.52 22.2
0.25	48	7.55	>15.0	-
0.5	8	-	-	-
0.5	16	-	-	-
0.5	24	4.91	0.92	0.57- 1.50
0.5	32	6.73	9.16	5.43-22.8
0.5	40	6.34	5.60	3.38-9.36
0.5	48	7.33	>15.0	-
Experiment II				
0	8	-	-	-
0	16	-	-	-
0	24	2.62	<0.08	-
0	32	5.34	2.73	1.78- 4.21
0	40	5.57	3.83	2.49- 5.95
0	48	6.46	14.2	8.90-23.0
0.1	8	-	-	-
0.1	16	-	-	-
0.1	24	3.41	.16	0.10- 0.25
0.1	32	5.69	4.57	2.96- 7.13
0.1	40	5.78	5.22	3.37- 8.17
0.1	48	6.42	13.4	8.41-21.6
0.25	8	-	-	-
0.25	16	-	-	-
0.25	24	2.55	<0.08	-
0.25	32	5.41	3.02	1.98- 4.68
0.25	40	5.76	5.07	3.28- 7.92
0.25	48	5.94	6.61	4.24-10.4

Table 18 (Continued)

SAPP (%)	Time (h)	CZ Readings (mm)	Unit activity/ml	Confidence interval (95%)
0.5	8	-	-	-
0.5	16	-	-	-
0.5	24	2.34	<0.08	-
0.5	32	5.28	2.50	1.64- 3.85
0.5	40	5.66	4.38	2.84- 6.81
0.5	48	6.27	10.76	6.80-17.2

Table 19. Nuclease activity of cultures of S. aureus Z88 grown in BHI broth containing various concentrations of SAPP at 15°C

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	-	-	-
0	24	-	-	-
0	32	4.13	0.35	0.21-0.57
0	40	3.24	0.11	0.07-0.19
0	48	5.93	3.35	2.04-5.51
0.1	8	-	-	-
0.1	16	-	-	-
0.1	24	-	-	-
0.1	32	3.23	0.11	0.06-0.20
0.1	40	4.06	0.32	0.19-0.52
0.1	48	5.49	1.92	0.18-3.13
0.25	8	-	-	-
0.25	16	-	-	-
0.25	24	-	-	-
0.25	32	2.88	<0.08	-
0.25	40	5.18	1.30	0.80-2.11
0.25	48	4.82	0.83	0.51-1.34

Table 19 (Continued)

SAPP (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
0.5	8	-	-	-
0.5	16	-	-	-
0.5	24	-	-	-
0.5	32	2.72	<0.08	-
0.5	40	4.15	0.36	0.22-0.58
0.5	48	3.75	0.21	0.13-0.36
Experiment II				
0	8	-	-	-
0	16	-	-	-
0	24	0	0	-
0	32	3.22	0.12	0.08-0.19
0	40	3.73	0.25	0.16-0.40
0	48	4.54	0.84	0.55-1.28
0.1	8	-	-	-
0.1	16	-	-	-
0.1	24	-	-	-
0.1	32	2.64	<0.08	-
0.1	40	3.19	0.11	0.07-0.18
0.1	48	3.86	0.31	0.20-0.48
0.25	8	-	-	-
0.25	16	-	-	-
0.25	24	-	-	-
0.25	32	3.69	0.23	0.15-0.37
0.25	40	4.04	0.40	0.26-0.62
0.25	48	4.52	0.81	0.54-1.25
0.5	8	-	-	-
0.5	16	-	-	-
0.5	24	-	-	-
0.5	32	2.72	<0.08	-
0.5	40	3.31	0.13	0.09-0.22
0.5	48	4.16	0.48	0.31-0.74

Figure 16. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI broth with 0%, 5% and 10% NaCl for 48 hours at 22° and 37°C

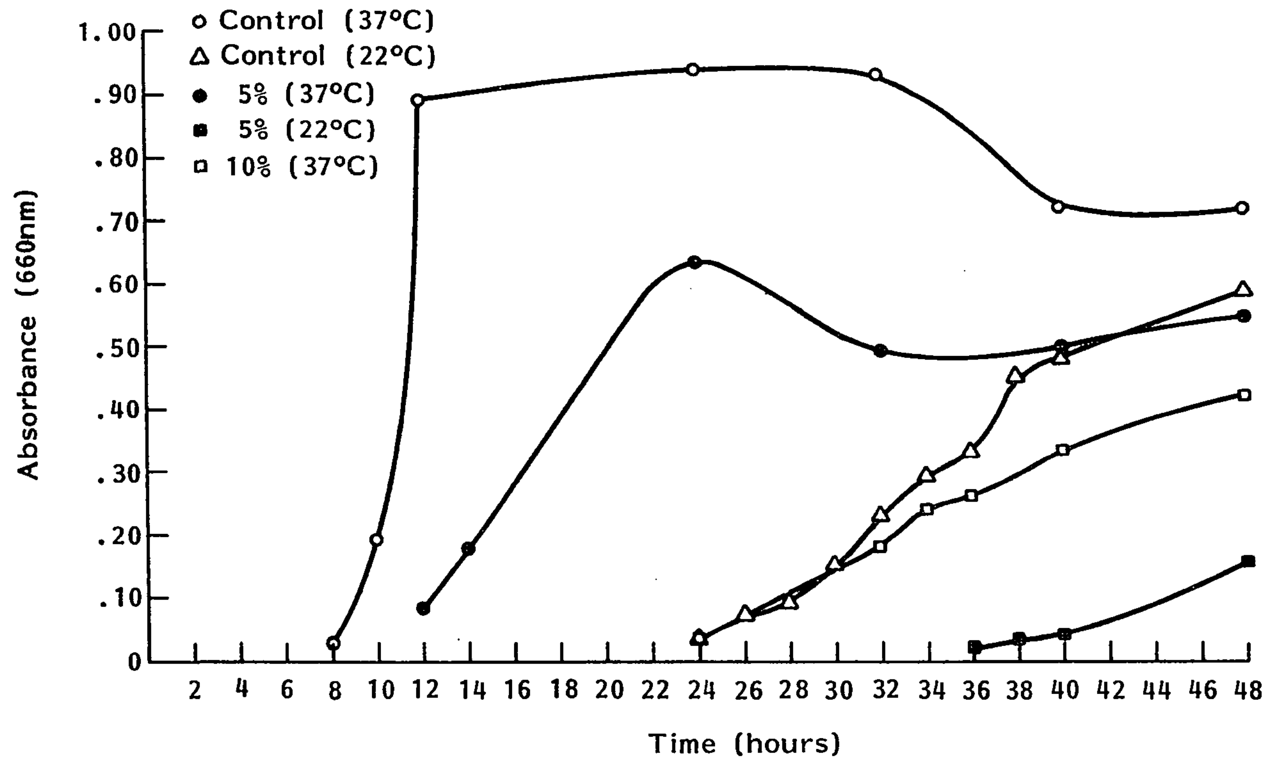
Fig. 16
NaCl
o Control (37°C)
Δ Control (22°C)
● 5% (37°C)
■ 5% (22°C)
□ 10% (37°C)
Absorbance (660nm)
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48
Time (hours)

S. aureus is noted for its ability to survive and grow in higher salt concentrations than are many other bacteria (Jay, 1978; Minor and Marth, 1971). Although *S. aureus* has been reported to grow in 15 to 20% NaCl (Baird-Parker, 1974; Genigeorgis and Sadler, 1966a; Jay, 1978) no absorbance readings were observed at 15% NaCl even at the optimum temperature of 37°C within 48 hours. Plate counts showed that numbers of cells did increase in 48 hours but not in numbers sufficient to produce an absorbance reading.

Growth in the medium containing $\geq$5% NaCl is dependent on other environmental conditions such as temperature (Genigeorgis and Sadler, 1966a; Genigeorgis *et al.*, 1971a; Hojvat and Jackson, 1969; Lotter and Leistner, 1978). As these conditions become more adverse to the growth of *S. aureus*, the organism becomes more sensitive to NaCl (Genigeorgis and Sadler, 1966a; Genigeorgis *et al.*, 1971a; Hojvat and Jackson, 1969). This would explain the decrease in growth in the presence of NaCl as the temperature decreased.

Tables 20 and 21 show nuclease activity of *S. aureus* Z88 in the presence of NaCl. The primary observation is that at 37°C, activity in 10% NaCl was lower than that produced in 5% NaCl and the control flasks. The absorbance readings for 10% NaCl at 48 hours were lower

Table 20. Nuclease activity of cultures of _S. aureus_ Z88 grown in BHI broth containing various concentrations of NaCl at 37°C

NaCl (%)	Time (h)	CZ Reading (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	3.06	<0.08	-
0	16	6.14	10.0	6.48-21.2
0	24	6.14	10.0	6.48-21.2
0	32	6.37	<15.0	-
0	40	6.77	<15.0	-
0	48	6.35	14.9	7.49-29.5
5	8	-	-	-
5	16	4.08	0.20	0.10- 0.37
5	24	6.35	14.9	7.49-29.5
5	32	6.64	<15.0	-
5	40	6.59	<15.0	-
5	48	6.76	<15.0	-
10	8	-	-	-
10	16	-	-	-
10	24	-	-	-
10	32	-	-	-
10	40	2.37	<0.08	-
10	48	5.32	2.10	1.13-3.85
Experiment II				
0	8	3.41	0.15	0.87-0.24
0	16	6.51	>15.0	-
0	24	6.56	>15.0	-
0	32	6.72	>15.0	-
0	40	6.62	>15.0	-
0	48	6.89	>15.0	-
5	8	-	-	-
5	16	-	-	-
5	24	6.51	>15.0	-
5	32	5.93	6.56	3.93-10.9
5	40	6.68	>15.0	-
5	48	5.76	5.08	3.06- 8.25
10	8	-	-	-
10	16	-	-	-
10	24	3.42	0.15	0.09-0.25
10	32	5.85	5.82	3.49-9.60
10	40	5.55	3.70	2.25-6.03
10	48	5.67	4.44	2.68-7.26

Table 21. Nuclease activity of cultures of S. aureus Z88 grown in BHI broth containing various concentrations of NaCl at 22°C

NaCl (%)	Time (h)	CZ Reading (mm)	Unit activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	-	-	-
0	24	2.31	<0.08	-
0	32	6.54	>15.0	-
0	40	6.62	>15.0	-
0	48	6.78	>15.0	-
5	8	-	-	-
5	16	-	-	-
5	24	-	-	-
5	32	-	-	-
5	40	4.84	0.96	0.50- 1.84
5	48	5.91	6.92	3.52-13.9
Experiment II				
0	8	-	-	-
0	16	-	-	-
0	24	4.83	0.83	0.44-1.50
0	32	4.75	>15.0	-
0	40	6.88	>15.0	-
0	48	6.88	>15.0	-
5	8	-	-	-
5	16	-	-	-
5	24	-	-	-
5	32	-	-	-
5	40	4.88	0.91	0.49-1.65
5	48	5.30	2.02	1.09-3.70

than for 5% NaCl and the control (Figure 16). In Table 22, the numbers of cells produced at 48 hours in 10% NaCl were not as high as that produced in the control or in the presence of 5% NaCl at the stationary phase (24 and 48 hours). The lower number of organisms could be responsible for the lower activity readings.

At 22°C, nuclease activities in 5% NaCl did not reach those in the control. Since growth did not reach the stationary phase in 5% NaCl, the nuclease activity difference in this experiment can be attributed only to the lack of maximum growth in the presence of 5% NaCl. Greater variation in nuclease activity was observed in the presence of NaCl than in its absence and reproducible results were difficult to obtain. Additional work needs to be done to obtain a satisfactory explanation.

Table 22. Average plate counts, absorbance and CZ readings of S. aureus Z88 at 24 and 48 hours in BHI at NaCl concentrations of 0%, 5% and 10% at 37°C

NaCl (%)	CZ Readings (mm)	Time (h)	Absorbance	Numbers cell/ml
0	6.35	24	0.983	1.4×10^9
5	6.43	24	0.636	2.6×10^8
10	1.71	24	0.028	1.3×10^6
0	6.62	48	0.820	7.9×10^8
5	6.26	48	0.546	2.4×10^8
10	5.50	48	0.424	8.4×10^7

KCl

Figures 17 and 18 show the effects of KCl on the absorbance of cultures of *S. aureus* Z88 grown at the temperatures of 22° and 37°C. No growth occurred at 15°C at any of the KCl concentrations to produce absorbance readings or nuclease activity. At 22°C, absorbance readings were observed in 5% and 10% KCl in 48 hours. At 37°C, absornbnce readings were observed in all three KCl concentrations.

Greater numbers of cells of *S. aureus* Z88 were observed in the presence of high concentrations of KCl than of NaCl. The primary effect NaCl has on the growth of an organism, among other theories, is the reduction in the water activity (Aw) of the environment (Marsh, 1983; Terrell, 1983). KCl, like NaCl, is a salt and has this same capacity to produce ions and to control A_w. NaCl, however, has a lower molecular weight (58.46) than does KCl (78.56). On a percentage basis, a higher number of moles of NaCl would be present than KCl; therefore, more ions theoretically are produced giving a lower Aw. This theory is beneficial in helping to explain growth at higher percentage concentrations of KCl than NaCl.

At 37°C (Figure 17), absorbance readings indicative of growth could be taken at about the same time for 5%

Figure 17. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI with 0%, 5%, 10% and 15% KCl for 48 hours at 37°C

Figure 18. The average absorbance (660 nm) of *S. aureus* Z88 grown in BHI with 0%, 5%, 10% and 15% KCl for 48 hours at 22°C

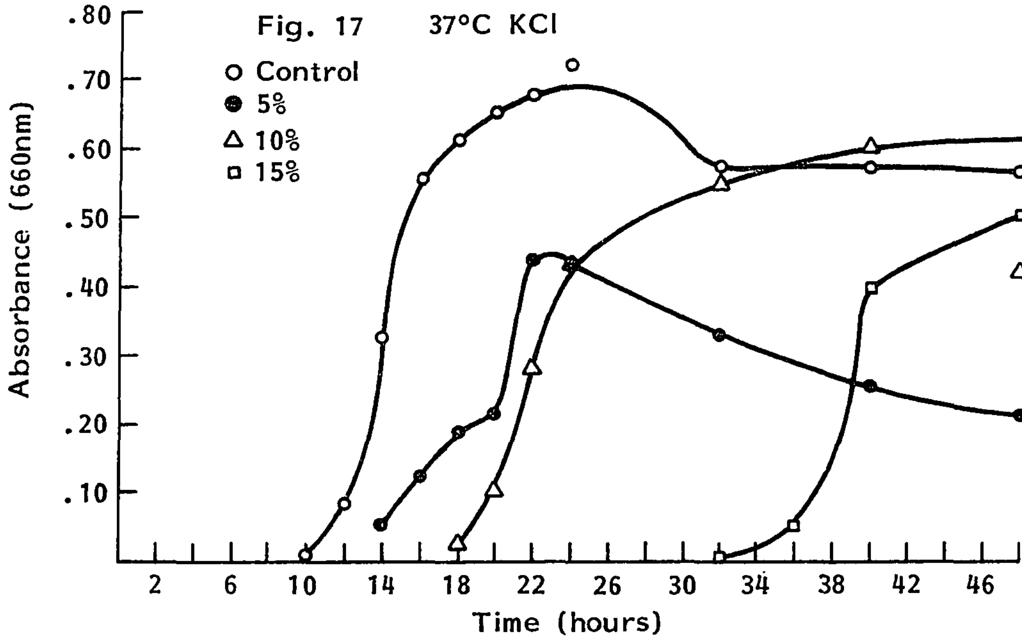
Fig. 17 37°C KCl
Control
5%
10%
15%
Absorbance (660nm)
.80
.70
.60
.50
.40
.30
.20
.10
2 6 10 14 18 22 26 30 34 38 42 46
Time (hours)

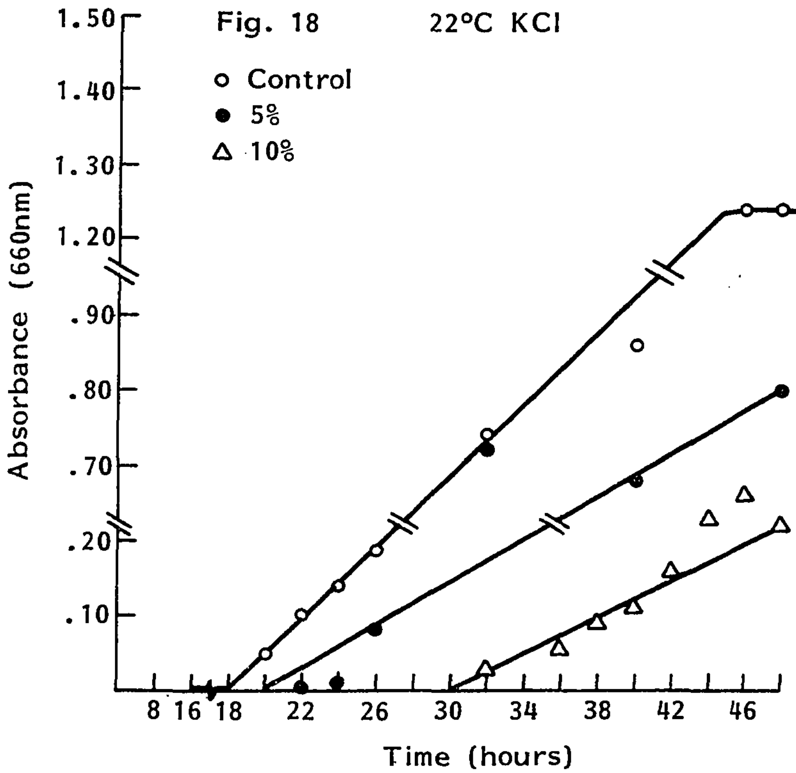
Fig. 18 22°C KCl
Control
5%
10%
Absorbance (660nm)
1.50
1.40
1.30
1.20
.90
.80
.70
.20
.10
8 16 18 22 26 30 34 38 42 46
Time (hours)

KCl and 5% NaCl (Figure 16). Readings at 10% KCl occurred sooner than for 10% NaCl; and the stationary phase was observed earlier. At 22°C (Figure 18) growth was observed earlier in the presence of 5% KCl than in the presence of 5% NaCl (Figure 16).

Tables 23 and 24 show the measurements of the clearing zones and thermonuclease activities in the presence of various levels of KCl. The data in Table 23 show that the final (48 hours) nuclease activity levels at 10% KCl were as high as those produced in 5% KCl and the control. This result differs from that for NaCl which had lower nuclease readings at 48 hours in the presence of higher concentrations of NaCl. The data in Table 25 explain this difference. Unlike the results in Table 22, the numbers of organisms present at 48 hours in 10% KCl were not lower than those at 5% KCl and the control. Therefore, nuclease activity would be expected to be as high at 10% KCl as in the control and 5% KCl.

Tables 24 and 26 on the other hand, showed that the activity measured nor the number of cells with KCl present did not reach the same level as in the control. With the numbers of cells being lower than the control at 10% KCl, nuclease activity was not expected to be as high.

Table 23. Nuclease activity of cultures of *S. aureus* Z88 grown in BHI broth containing various concentrations of KCl at 37°C

KCl (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	6.61	>15.0	-
0	24	6.28	>15.0	-
0	32	6.05	>15.0	-
0	40	6.45	>15.0	-
0	48	6.25	>15.0	-
5	8	-	-	-
5	16	5.38	12.1	6.57-19.8
5	24	6.15	>15.0	-
5	32	6.58	>15.0	-
5	40	6.05	>15.0	-
5	48	6.43	>15.0	-
10	8	-	-	-
10	16	-	-	-
10	24	6.62	>15.0	-
10	32	6.68	>15.0	-
10	40	6.00	>15.0	-
10	48	6.46	>15.0	-
15	8	-	-	-
15	16	-	-	-
15	24	-	-	-
15	32	-	-	-
15	40	2.42	<0.08	-
15	48	5.33	11.02	6.03-18.0
Experiment II				
0	8	-	-	-
0	16	6.11	>15.0	-
0	24	6.30	>15.0	-
0	32	5.81	14.6	9.90-19.8
0	40	6.08	>15.0	-
0	48	6.57	>15.0	-
5	8	-	-	-
5	16	5.09	4.12	2.98-5.65
5	24	6.29	>15.0	-
5	32	6.53	>15.0	-
5	40	6.40	>15.0	-
5	48	6.32	>15.0	-

Table 23 (Continued)

KCl (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
15	8	-	-	-
15	16	-	-	-
15	24	-	-	-
15	32	-	-	-
15	40	2.60	<0.08	-
15	48	4.46	1.41	1.03-1.91

Table 24. Nuclease activity of cultures of *S. aureus* Z88 grown in BHI broth containing various concentrations of KCl at 22°C

KCl (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment I				
0	8	-	-	-
0	16	-	-	-
0	24	6.37	5.82	3.50- 9.73
0	32	6.40	6.04	3.63-10.1
0	40	7.08	14.2	8.29-24.6
0	48	7.38	>15.0	-
5	8	-	-	-
5	16	-	-	-
5	24	3.83	0.24	0.14-0.39
5	32	6.30	5.33	3.21-8.88
5	40	5.81	2.88	1.76-4.72
5	48	6.27	5.13	3.10-8.55
10	8	-	-	-
10	16	-	-	-
10	24	-	-	-
10	32	4.33	0.45	0.27-0.73
10	40	4.87	0.88	0.54-1.43
10	48	5.82	2.91	1.78-4.78

Table 24 (Continued)

KCl (%)	Time (h)	CZ Readings (mm)	Units activity/ml	Confidence interval (95%)
Experiment II				
0	8	-	-	-
0	16	-	-	-
0	24	2.62	<0.08	-
0	32	5.34	2.73	1.78- 4.21
0	40	5.57	3.83	2.49- 5.95
0	48	6.46	14.2	8.90-23.0
5	8	-	-	-
5	16	-	-	-
5	24	-	-	-
5	32	4.97	1.58	1.04-2.42
5	40	5.32	2.65	1.73-4.09
5	48	5.21	2.25	1.48-3.47
10	8	-	-	-
10	16	-	-	-
10	24	-	-	-
10	32	2.46	<0.08	-
10	40	3.08	0.10	0.06-0.16
10	48	4.32	0.60	0.40-0.93

Table 25. Average plate counts, absorbance and nuclease readings of *S. aureus* Z88 at 24 and 48 hours in BHI broth at 0%, 5%, and 10% KCl concentrations at 37°C

KCl (%)	Time (h)	CZ Readings (mm)	Absorbance	Number cells/ml
0	24	6.29	0.724	$5.1x10^8$
5	24	6.22	0.438	$1.8x10^8$
10	24	6.15	0.436	$1.7x10^8$
0	48	6.41	0.566	$4.9x10^8$
5	48	6.38	0.212	$1.2x10^8$
10	48	6.22	0.420	$7.2x10^8$

Table 26. Average plate counts, absorbance and nuclease readings of *S. aureus* Z88 at 24 and 48 hours in BHI broth at 0%, 5% and 10% KCl concentrations at 22°C

KCl (%)	Time (h)	CZ Readings (mm)	Absorbance	Number cells/ml
0	24	6.37	.130	$1.1x10^8$
5	24	3.83	.040	$5.6x10^5$
10	24	-	-	$2.7x10^4$
0	48	7.38	1.270	$1.4x10^9$
5	48	6.27	.816	$5.6x10^8$
10	48	5.82	.352	$1.4x10^8$

Number of Cells versus Nuclease Production

Temperature

The data in Table 27 compare the production of nuclease and the numbers of cells/ml of growth medium. Nuclease was encountered in some instances when the count was $\geq 1.0x10^5$ cells/ml, regardless of the temperature; nuclease, however, was always present when the numbers exceeded 10^6 cells/ml.

Although most research has shown that the presence of nuclease is an indication of the presence of $\geq 10^6$ cells/ml of *S. aureus* (Koupal and Deibel, 1978; Tatini *et al.*, 1975, 1976), there have been reports of nuclease

Table 27. Comparison of numbers of cells of *S. aureus* Z88 per ml and time of detection of nuclease in BHI broth incubated at 15°, 22° and 37°C

Temp (°C)	Time (h)	Presence of nuclease	Number cells/ml
15	28	-	$4.6x10^6$
15	29	+	-
15	30	+	$6.8x10^6$
15	30	-	$1.4x10^5$
15	31	-	-
15	32	+	$1.2x10^5$
22	20	-	$4.6x10^5$
22	21	-	-
22	22	+	$3.9x10^6$
22	20	-	$1.5x10^6$
22	21	-	-
22	22	+	$2.8x10^6$
37	8	-	$1.0x10^6$
37	9	+	-
37	10	+	$4.4x10^7$
37	8	-	$1.5x10^6$
37	9	+	-
37	10	+	$5.3x10^7$

being present at counts of 10^5 cells/g (Niskanen and Nurmi, 1976), 10^4 cells/g (Tatini *et al.*, 1975) and 10^3 cells/g (Chesbro and Auborn, 1967). Cords and Tatini (1973) attribute the variability in results to several factors including the nature of the growth medium, growth conditions, variability in the recovery of *S. aureus* and the variability in nuclease production between strains.

SAPP

Comparisons of numbers of cells and times of nuclease detection in the presence of SAPP are shown in Tables 28 through 30. As observed previously, nuclease was sometimes detected when about 10^5 cells/ml were present but nuclease was always detected when cell numbers exceeded 10^6/ml. SAPP at any level did not consistently delay production of thermonuclease.

pH

Tables 31 through 33 show the effect of initial pH on the production of nuclease. Again, the nuclease was detectable in some instances when the number of cells were in the 10^5/ml range and was always detected when numbers $>10^6$ cells/ml, regardless of pH and temperature. At an initial pH of 6.3, nuclease was produced at times closely paralleling that in the control flasks. As explained previously, at this pH, *S. aureus* grows as well as at the optimum pH of 7.0 (Arvidson and Holem, 1971; Jarvis *et al.*, 1973).

The time for nuclease production at the initial pH values of 8.3 and 5.3 closely paralleled one another. Results in the environmental studies might not have led to this conclusion because the level of nuclease present was higher at 8.3 than at 5.3. The conclusion from the data in Tables 31 through 33 and the environmental studies is

Table 28. Comparison of numbers of cells of *S. aureus* Z88 per ml and time of detection of nuclease in BHI broth containing various concentrations of SAPP at 15°C

SAPP (%)	Time (h)	Presence of nuclease	Number cells/ml
0.1	28	-	$1.1x10^6$
0.1	29	-	-
0.1	30	+	$2.0x10^6$
0.1	30	-	$3.6x10^5$
0.1	31	-	-
0.1	32	+	$2.4x10^5$
0.25	30	-	$1.2x10^6$
0.25	31	-	-
0.25	32	+	$3.1x10^6$
0.25	30	-	$1.2x10^5$
0.25	31	-	-
0.25	32	+	$2.5x10^5$
0.5	30	-	$1.9x10^6$
0.5	31	+	-
0.5	32	+	$3.2x10^6$
0.5	30	-	$2.0x10^5$
0.5	31	-	-
0.5	32	+	$2.1x10^5$

Table 29. Comparison of numbers of cells of *S. aureus* Z88 per ml and time of detection of nuclease in BHI broth containing various concentrations of SAPP at 22°C

SAPP (%)	Time (h)	Presence of nuclease	Number cells/ml
0.1	22	-	3.1×10^6
0.1	23	+	-
0.1	24	+	8.2×10^6
0.1	22	-	2.4×10^6
0.1	23	+	-
0.1	24	+	9.0×10^6
0.25	22	-	1.5×10^6
0.25	23	-	-
0.25	24	+	3.6×10^6
0.25	22	-	1.4×10^6
0.25	23	+	-
0.25	24	+	2.9×10^6
0.5	22	-	7.6×10^5
0.5	23	-	-
0.5	24	+	1.4×10^6
0.5	22	-	3.5×10^5
0.5	23	-	-
0.5	24	+	1.3×10^6

Table 30. Comparison of numbers of cells of *S. aureus* Z88 per ml and time of detection of nuclease in BHI broth containing various concentrations of SAPP at 37°C

SAPP (%)	Time (h)	Presence of nuclease	Number cells/ml
0.1	8	-	$4.3x10^5$
0.1	9	+	-
0.1	10	+	$7.9x10^6$
0.1	8	-	$5.4x10^5$
0.1	9	+	-
0.1	10	+	$9.8x10^6$
0.25	8	-	$4.5x10^5$
0.25	9	+	-
0.25	10	+	$1.3x10^6$
0.25	8	-	$4.3x10^5$
0.25	9	+	-
0.25	10	+	$1.4x10^6$
0.5	8	-	$7.5x10^5$
0.5	9	+	-
0.5	10	+	$1.0x10^7$
0.5	8	-	$2.4x10^5$
0.5	9	+	-
0.5	10	+	$5.8x10^6$

Table 31. Comparison of numbers of cells of *S. aureus* Z88 per ml and time detection of nuclease in BHI broth at pHs 5.3, 6.3 and 8.3 at 15°C

pH	Time (h)	Presence of nuclease	Number cells/ml
5.3	46	-	$1.3x10^7$
5.3	47	+	-
5.3	48	+	$8.2x10^6$
5.3	44	-	$2.8x10^5$
5.3	45	+	-
5.3	46	+	$1.2x10^7$
6.3	28	-	$5.4x10^6$
6.3	29	+	-
6.3	30	+	$1.4x10^7$
6.3	28	-	$6.6x10^5$
6.3	29	+	-
6.3	30	+	$1.1x10^6$
8.3	46	-	$4.0x10^5$
8.3	47	+	-
8.3	48	+	$9.6x10^5$
8.3	44	-	$4.3x10^5$
8.3	45	-	-
8.3	46	+	$1.3x10^6$

Table 32. Comparison of numbers of cells of *S. aureus* Z88 per ml and time detection of nuclease in BHI broth at pH 5.3, 6.3 and 8.3 at 22°C

pH	Time (h)	Presence of nuclease	Number cells/ml
5.3	28	-	$2.2x10^6$
5.3	29	-	-
5.3	30	+	$5.2x10^6$
5.3	26	-	$1.8x10^6$
5.3	27	-	-
5.3	28	+	$8.4x10^6$
6.3	22	-	$4.4x10^6$
6.3	23	-	-
6.3	24	+	$6.2x10^6$
6.3	20	-	$1.2x10^6$
6.3	21	-	-
6.3	22	+	$2.8x10^6$
8.3	28	-	$3.9x10^5$
8.3	29	-	-
8.3	30	+	$1.6x10^6$
8.3	28	-	$1.6x10^6$
8.3	29	+	-
8.3	30	+	$4.6x10^6$

Table 33. Comparison of numbers of cells of *S. aureus* Z88 and time detection of nuclease in BHI broth at pH 5.3, 6.3 and 8.3 at 37°C

pH	Time (h)	Presence of nuclease	Number cells/ml
5.3	8	-	$6.4x10^{4}$
5.3	9	-	-
5.3	10	+	$6.8x10^{5}$
5.3	10	-	$1.3x10^{5}$
5.3	11	+	-
5.3	12	+	$1.2x10^{6}$
6.3	8	-	$1.1x10^{6}$
6.3	9	-	-
6.3	10	+	$2.6x10^{7}$
6.3	8	-	$7.0x10^{5}$
6.3	9	+	-
6.3	10	+	$3.6x10^{7}$
8.3	8	-	$6.7x10^{4}$
8.3	9	-	-
8.3	10	+	$6.5x10^{5}$
8.3	8	-	$1.3x10^{5}$
8.3	9	-	-
8.3	10	+	$1.8x10^{6}$

the level of nuclease produced at the initial 8.3 pH was much higher than that at the initial pH of 5.3. This result is the same as found by Jarvis *et al.* (1973, 1975) and Arvidson and Holme (1971) that a pH below 6.0 reduces the level of thermonuclease and other extracellular proteins produced by *S. aureus*. The detection of the presence of thermonuclease later at pH 8.3 (46 and 47 hours) in the experiments at 15°C than in the environmental assays (40 hours) was conceivably due to the combination of extreme pH and temperature at which the organism was grown.

NaCl and KCl

The data in Tables 34 through 36 show the same consistent result; nuclease was routinely detected when the count was in the 10^5 to 10^6 range with a time lapse occurring before nuclease was detected regardless of NaCl or KCl concentration or temperature.

The tables provide a comparison of the effects of NaCl and KCl on nuclease production. Actually, 5% KCl and 5% NaCl did not differ greatly in the time that nuclease was produced at 22° and 37°C. Available information would support the contention that NaCl would have a greater effect on *S. aureus* growth than KCl because of the difference in the amount of ions produced that could lower

Table 34. Comparison of numbers of cells of S. aureus Z88 and time detection of nuclease in BHI broth containing various concentrations of NaCl at 22° and 37°C

NaCl (%)	Temp (°C)	Time (h)	Presence of nuclease	Number cells/ml
5	22	28	-	$1.3x10^4$
5	22	29	-	-
5	22	30	+	$3.8x10^6$
5	22	28	-	$1.4x10^4$
5	22	29	-	-
5	22	30	+	$4.8x10^6$
5	37	12	-	$5.0x10^5$
5	37	13	+	-
5	37	14	+	$4.0x10^5$
5	37	12	-	$6.2x10^5$
5	37	13	+	-
5	37	14	+	$8.0x10^5$
10	37	34	-	$2.4x10^5$
10	37	35	-	-
10	37	36	+	$3.6x10^5$
10	37	34	-	$2.1x10^6$
10	37	35	-	-
10	37	36	+	$4.6x10^6$

Table 35. Comparison of numbers of cells of <u>S. aureus</u> Z88 and time detection of nuclease in <u>BHI broth</u> containing various concentrations of KCl at 22°C

KCl (%)	Time (h)	Presence of nuclease	Number cells/ml
5	30	-	$5.0x10^6$
5	31	+	-
5	32	+	$5.0x10^6$
5	28	-	$5.4x10^5$
5	29	-	-
5	30	+	$3.8x10^6$
10	38	-	$1.6x10^6$
10	39	-	-
10	40	+	$7.6x10^6$
10	38	-	$1.4x10^6$
10	39	-	-
10	40	+	$2.4x10^6$

Table 36. Comparison of numbers of cells of <u>S. aureus</u> Z88 and time detection of nuclease in <u>BHI broth</u> containing various concentrations of KCl at 37°C

KCl (%)	Time (h)	Presence of nuclease	Number cells/ml
5	8	-	$1.8x10^4$
5	9	-	-
5	10	+	$1.0x10^5$
5	10	-	$6.0x10^5$
5	11	+	-
5	12	+	$2.7x10^6$
10	14	-	$2.2x10^5$
10	15	+	-
10	16	+	$4.8x10^6$
10	14	-	$2.5x10^5$
10	15	-	-
10	16	+	$1.5x10^6$

Table 36 (Continued)

KCl (%)	Time (h)	Presence of nuclease	Number cells/ml
15	46	-	$4.7x10^5$
15	47	-	-
15	48	+	$2.5x10^6$
15	46	-	$1.1x10^5$
15	47	-	-
15	48	+	$7.8x10^5$

the Aw. Most researchers suggest using the same ionic strength of KCl when replacing NaCl in processed products (Dr. William Sveum, Personal Communication, Armour and Company, Scottsdale, Arizona; Terrell, 1983). At a level of 5%, NaCl does not severely hinder nuclease production based on the results. A time difference in nuclease production was observed when 10% NaCl and 10% KCl were present at 37°C, showing that 10% NaCl has a much greater effect on delaying nuclease production than 10% KCl. In fact, 10% KCl did not hinder nuclease production at 37°C much more than 5% NaCl did.

Extraction of Nuclease From Sausage Samples

The sausage samples supplied by Dr. William Sveum (Armour and Company, Scottsdale, Arizona) had been inoculated with 10^6 cells of *S. aureus* Z88 and were known to contain thermonuclease and enterotoxin A. The samples were examined for the presence of thermonuclease by using the extraction methods of Koupal and Deibel (1978) and Tatini *et al*. (1976) with minor modifications.

Both assays were equally capable of detecting thermonuclease in the sausage. The Tatini method, however, gave clearing zones that were easier to see than those in the Koupal-Deibel assay. The clearing zones in the Koupal-Deibel medium were cloudier and not as distinct. These differences in reactions in the medium may be due to the more extensive extraction procedure used in the method of Tatini *et al*. (1976). This method involved an adjustment to an acidic pH (4.5), addition of 3M trichloroacetic acid (TCA), addition of Trizma buffer, boiling and several centrifugations. The Koupal-Deibel method involved only an adjustment to an acid pH, boiling and centrifugation. Fewer steps are desirable for a rapid test, but in this instance, less of the interfering substances may have been removed, therefore, giving a less clear reaction.

The Koupal-Deibel extraction method, on the other

hand, is simpler and involves the use of fewer materials and equipment.

Finally, the extraction procedure of Koupal and Deibel left a fatty top layer when the sausage was used. This lipid layer can interfere with the assay. To remove this potentially hindering layer, cooling the tube for 15 to 30 minutes at 4°C or using a refrigerated centrifuge (4°C) solidified the layer and it was removed with a spatula to gain access to the aqueous layer containing the enzyme.

In conclusion, the extraction method of choice depends on the needs of the individual running the test. The Koupal-Deibel extraction method is a good assay if one is looking for a simple procedure that is easy to run and gives a clearing reaction in a qualitative assay. The method of Tatini *et al*. (1976) is a better assay if one is looking for a reaction that is clearer and would be the method of choice if the method were quantitative as well as qualitative. This would also be the method of choice to detect low amounts of thermonuclease because of the clearer reaction.

SUMMARY AND CONCLUSIONS

A quantitative assay was developed for the measurement of the concentration and activity of thermonuclease present in a sample. The basis of the method is that of Koupal and Deibel (1978) in which the thermonuclease activity is detected by the development of clearing zones in agar containing DNA. The quantitative assay was developed in an effort to determine the amount of nuclease produced by S. aureus Z88 under a variety of conditions.

The assay detected the presence of thermonuclease down to a level of 5 ng/ml (0.08 units/ml). The relationship between clearing size and the log of the concentration of activity of nuclease was shown to be linear from 5 ng/ml (0.08 units/ml) through 1,000 ng/ml (15.0 units/ml). In attempting to quantitate the method, however, disadvantages that became evident were the spread in the confidence interval (α=.05) and the necessity of taking extra measurements to reduce the confidence intervals (α=.05). Although the method could not provide exact nuclease activities due to the variability in the measurements, the assay was a useful tool in providing a rough estimate of the activity of thermonuclease produced under various environmental conditions.

In the development of the assay, information was gathered that might prove useful in the qualitative use of the test. First of all, incubation for longer periods of time increases the size of the clearing. This information would prove highly useful if the investigator suspected low amounts of thermonuclease present and did not see a clearing reaction after one hour. The enzyme is more stable in BHI broth, probably due to the presence of certain constituents. If all these constituents are not present, the enzyme may not be as stable and easy to detect. Finally, using the lowest level of nuclease the assay can detect, it could be used as a standard when running samples and limit the chance of any false positives occurring.

The growth studies showed that, with the appropriate number of cells/ml in the initial inoculum, *S. aureus* can multiply and produce nuclease in BHI broth at the initial pHs of 5.3, 6.3, 7.0 and 8.3, in the presence of SAPP at levels of 0.1, 0.25 and 0.5% and at temperatures of 15°, 22° and 37°C. Growth and nuclease production varied in 5%, 10% and 15% KCl and 5%, 10% and 15% NaCl, depending on the temperature. No nuclease production at 15°C was observed at the concentrations of KCl and NaCl and incubation times used.

Growth and nuclease production by *S. aureus* Z88

at pH 6.3 parallel growth and nuclease production at the optimum pH (7.0). At pH values of 8.3 and 5.3, growth and nuclease production were delayed but appeared when numbers of cells exceeded 10^5/ml in some experiments but always when numbers were greater than 10^6 cells/ml.

SAPP had no definitive affect on growth and on nuclease production of *S. aureus* Z88 at all three temperatures. There was a noticeable trend, however, that as the concentration of the SAPP increased, plate counts and activities also decreased.

NaCl had a more pronounced effect on nuclease production and on growth of *S. aureus* Z88 than did KCl. The organism grew more rapidly and produced relatively higher activitives of nuclease in the presence of higher percentages of KCl than NaCl.

The numbers of cells present when nuclease was first detected was greater than 10^5 cells/ml in some experiments; thermonuclease was always present when counts were greater than 10^6 cells/ml.

The results of studies involving the extraction of nuclease from sausage showed that the method of Tatini *et al.* (1976) yielded clearer zones than the method of Koupal and Deibel (1978). The Tatini method, however, requires more time and equipment than the Koupal and Deibel method; thus, a compromise must be made in deciding if clearer zones or

shorter time is of paramount importance. For a qualitative test as might be used in quality control, time may be more important. In the Koupal-Deibel method, lipid was found to be a potential hindrance in the assay. Refrigeration soon after or during centrifugation and removing the solidifed lipid solved this problem.

Based on the results of the experiments the conclusions of this research are:

1. The relationship between the measured clearing zone and the log of the nuclease concentration or activity is linear from 5 ng (0.08 units) to 1000 ng (15.0 units).

2. The limit of sensitivity of the assay was 5 ng/ml (0.08 units/ml).

3. The longer the incubation time up to two hours, the larger clearing zone.

4. The thermonuclease enzyme gave larger clearing zones when diluted with BHI broth than with Trizma buffer, regardless of pH.

5. Day-to-day variation influenced the size of the clearing zones.

6. A larger inoculum of *S. aureus* Z88 was needed to produce nuclease in 48 hours in BHI broth when low temperatures were used.

7. When incubation of *S. aureus* was lower than 37°C (22° and 15°C) growth and nuclease production in *S. aureus*

Z88 were delayed.

8. Growth and nuclease production were essentially the same at the initial pH values of 7.0 and 6.3, but at pHs 8.3 and 5.3, growth and nuclease production are delayed.

9. SAPP appeared to delay the growth and nuclease production by *S. aureus* Z88.

10. NaCl was more detrimental than KCl to growth and nuclease production.

11. In some experiments, nuclease was present when the plate count was greater than 10^5 cells/ml but nuclease was always present when the count was greater than 10^6 cells/ml.

12. Extracts prepared by the method of Tatini *et al.* (1976) yielded clearer reactions in the K-D medium than did the method of Koupal and Deibel (1978).

RECOMMENDATIONS

The quantitative assay used in this research provides a measure of thermonuclease production. Due to the amount of variability between the measurements, the assay needs some refinement. Suggestions would include the investment in equipment to cut wells in a more uniform manner and a zone reader that would make the zones of clearing in the agar easier to measure. Sensitivity of the assay could be increased by reducing the percent of agar present within limits. Refinement of the quantitative assay would give more confidence in the qualitative assay particularly as to its sensitivity.

Studies should be continued on how various compounds might affect nuclease production in foods and thus affect the usefulness of thermonuclease as an indicator of the growth of S. aureus and the presence of enterotoxin. Comparisons of NaCl and KCl could be done on a molar basis to give equivalent ionic strengths. Research with phosphates, including sodium acid pyrophosphate should be continued to determine what effects these compounds may have on growth and nuclease production. These experiments should be done in broth and in meat products as well. Analysis for the presence of enterotoxin should be done in conjunction with thermonuclease analyses.

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