EVALUATION, ASSAY, DISTRIBUTION, AND SURVEY OF STAPHYLOCOCCUS AUREUS THERMONUCLEASE IN MILK AND CHEESE PRODUCTS

by

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This thesis is dedicated to my mother and father. And finally to my wife, Marsha, for her patience and support in fulfilling this assignment, I extend a husband's love and gratitude.

Gene Leong Hong
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ABSTRACT

Evaluation, Assay, Distribution, and Survey of *Staphylococcus aureus* Thermonuclease in Milk and Cheese Products

by

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Utah State University, 1975

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The metachromatic agar-diffusion test of Lachica, Hoeprich, and Genigeorgis (1971) was modified by adding 17 ml of the agar medium to a 100 by 15 mm plastic petri dish, using 5 µl samples and 3 mm wells. The test detected 0.5 ng/ml of thermonuclease in 3 hr at 37 °C. This was equivalent to $10^6$ to $10^7$ cells/ml of *Staphylococcus aureus* strains 196 E, 14458, and 19095 grown in Brain Heart Infusion (BHI) broth. The test sensitivity was decreased ten fold in raw and pasteurized milk.

One-half gram of cheese was dissolved in 4.5 ml of 0.1 M sodium citrate for quantitative studies. When staphylococcal ($>8 \times 10^8$/ml) contaminated raw milk was pasteurized and made into cheese, more thermonuclease activity/ml was noted in the curd than whey. During prolonged storage at 4 °C, thermonuclease activity decreased in raw and pasteurized milk and laboratory made cheese.

*Bacillus subtilis*, *Streptococcus faecalis var liquefaciens*, *Streptococcus thermophilus*, and *Streptococcus cremoris* strain ML reduced thermonuclease activity during growth incubation in BHI broth.
Thermonuclease was detected directly in staphylococcal contaminated laboratory aged cheese (>10^7/gm) without prior purification, concentration, or extraction procedures.
INTRODUCTION

Vaughn in 1884, recorded the first case of enterotoxic staphylococcal food poisoning in cheese products. Since then, a number of similar staphylococcal food poisoning outbreaks involving cheese products have occurred (Dack, 1956; Donnelly, Black, and Lewis, 1964). Investigators, including Galton and Steele (1961), Mickelsen et al. (1961), Donnelly, Black, and Lewis (1964), Sharpe et al. (1965), and Zehren and Zehren (1968b), have demonstrated either the increase in staphylococcal mastitis or the prevalence of pathogenic and enterotoxic staphylococci in raw or pasteurized cheese products.

In the past, the two primary methods for detecting pathogenic and enterotoxin producing staphylococci in cheese products have been to test for a specific enterotoxin or the enzyme coagulase. Both methods are slow, bothersome, and expensive.

The detection in cheese products of another enzyme, unique to the species *Staphylococcus aureus* was evaluated in this study. Presence of thermonuclease could pinpoint areas of *S. aureus* contamination during food processing. The thermonuclease could also be used as a screening device for possible enterotoxic foods. Both the thermonuclease and the enterotoxin are heat stable and can be found in the absence of viable staphylococcal cells.

This study included attempts to develop and evaluate a more sensitive, rapid and simple test for the detection of thermonuclease in cheese products. Observations were conducted on the whey-curd
distribution of the enzyme during a laboratory cheese making process. Enzyme stability experiments were determined in raw and pasteurized milk and laboratory made cheese. Finally a survey was conducted on the prevalence of thermonuclease in various market Colby and Cheddar cheese products.
REVIEW OF LITERATURE

Although pathogenic and enterotoxic staphylococci produce a number of toxins that can cause toxicosis in man, this review and research are mainly concerned with the food poisoning or enterotoxic aspect. An excellent set of monographs in the book *The Staphylococci*, edited by Cohen (1972), is highly recommended if additional information on pathogenic staphylococci is desired.

Staphylococcal food poisoning

A summary of food-borne disease outbreaks in 1969 reported that 25 percent of all cases were attributed to staphylococcal food poisonings (Mortality and Morbidity Weekly Report No. 15, 1970). This was greater than any single agent of food-borne illness.

The symptoms, which include acute gastrointestinal upset followed by projectile vomiting and diarrhea, appear 2 to 3 hr after the toxin is ingested. The number of actual cases are probably higher than reported since the symptoms last a few hours and recovery is complete with a very low mortality rate (Burrows, 1968).

The enterotoxins are relatively heat stable and are formed by most coagulase-positive strains. Eighty-one percent of the *S. aureus* strains classified were of phage types 6/47 or 42D. Not all strains in these phage types were enterotoxic. The former type is one of the most common carried by nasal carriers, while the latter is a frequent cause of bovine mastitis (Burrows, 1968).
There are five basic staphylococcal enterotoxins labeled A through E, respectively. Enterotoxin B is produced in larger quantities than any other enterotoxins but enterotoxin A is most often implicated in food poisoning. The interrelationship of these enterotoxins with respect to food poisoning and pathogenicity has not been established (Bergdoll, 1972).

Chesbro and Auborn (1967) commented that perhaps 30 percent of all pathogenic staphylococci produce enterotoxin. However, the presence of a large number of *S. aureus* is still considered to be strong evidence of enterotoxic contamination (Thatcher and Clark, 1968). The author's view is the same as Donnelly, Black, and Lewis (1964), who feel that the presence of large or small numbers of pathogenic staphylococci in foods is, to say the least, undesirable.

Pathogenic and enterotoxic staphylococci in cheese

In cheese products, staphylococcal detection has become important with the increasing concern over staphylococcal mastitis (McCoy, 1959; Galton and Steele, 1961; Burrows, 1968). This concern has led to a number of surveys to show the presence of various staphylococcal enzymes from cheese products.

A survey by Mickelsen et al. (1961) on American and foreign cheeses isolated coagulase-positive staphylococci in 7.2 percent of 125 samples. Donnelly, Black, and Lewis (1964) found coagulase-positive staphylococci in 20 percent of 343 market cheese samples. Zehren and Zehren (1968b) detected enterotoxin A in 59 of 2,112
cheese lots which had been seized by the Federal Drug Administration for possible enterotoxin content. One lot contained 12 µg of enterotoxin A/100 g of cheese. The most dramatic survey was conducted by Sharpe et al. (1965) on a variety of cheeses from England and Wales. They detected $5 \times 10^5$ coagulase-positive staphylococci/g of cheese in 9 percent of 910 samples of cheese made with raw milk.

There is some evidence that the above counts of staphylococci were much higher than indicated. Jezeske et al. (1961) and Walker, Harmon, and Stine (1961) demonstrated that viable staphylococcal cells decreased in number during the normal cheese aging process.

Thatcher, Simon and Walters (1956), Walters (1959), and Galton and Steele (1961), have shown the prevalence of coagulase-positive staphylococci in raw milk. Therefore, their presence in cheese products made with raw milk is not surprising.

Partially because of these problems, most cheese products are made with pasteurized milk. However, some surveys have detected pathogenic or enterotoxic staphylococci in cheese made from pasteurized milk (Thatcher, Simon, and Walters, 1956; Mickelsen et al., 1961; Donnelly, Black, and Lewis, 1964; Zehren and Zehren, 1968b). They could be present because of improper pasteurization or post pasteurization contamination.

The two traditional methods for detection of pathogenic or enterotoxic staphylococci in cheese products have been to test for the enzyme coagulase or for a specific enterotoxin. Both methods are laborious, time consuming, and expensive.
Coagulase is generally detected in cheese by blending a curd sample with sterile distilled water, spreading a portion of the blend upon a selective medium and incubating for 18 hr at 37 C. A typical colony is transferred to a broth medium and incubated for an additional 18 hr to allow growth. Finally, the broth culture is tested with coagulase medium (Thatcher, Simon, and Walters, 1956; Mickelson et al., 1961; Donnelly, Black and Lewis, 1964; Sharpe et al., 1965).

Coagulase detection cannot be the sole determinant in identifying pathogenic staphylococci. Baird-Parker mentioned that such organisms as Pseudomonas aureginosa, Pasteurella pestis, actinomycetes and strains of Streptococcus faecalis clot plasma (Whipple, 1965).

Staphylococcal enterotoxin in foods is most effectively tested by reacting on enterotoxin with its specific antibody. Various immunological tests including a microslide test, double gel-diffusion tube tests, quantitative precipitin tests, immunoflourescence tests, and hemagglutination tests can detect less than 1 µg/ml quantities of enterotoxin (Silverman, 1963; Casman and Bennett, 1963; Morse and Mah, 1967; Bergdoll, 1972).

The testing time for enterotoxin determination has decreased. Gandhi and Richardson (1971) developed a capillary tube immunological assay which detects 1 µg/ml of staphylococcal enterotoxin A, B, or D in less than 1 hr. Immunological tests require purification and possible concentration of the enterotoxin from the food which could take days.

The reverse passive hemagglutination test of Silverman, Knott, and Howard (1968) detected 1 µg of enterotoxin B in unconcentrated
extract from 100 g of food. The Food Research Laboratories have reported false positive tests, possibly from interfering material (Bergdoll, 1972).

Radioimmunoassay techniques show promise in being sensitive to as low as 0.005 µg of purified and crude enterotoxin in foods. But, there has been insufficient data on their value. Interfering substances may prevent accurate results (Bergdoll, 1972).

Besides disadvantages of cost and time, all immunological tests are governed by the limited availability of the antiserum specific for its enterotoxin. Also, unidentified enterotoxins may exist which could cause illness in man.

Thermonuclease

Tests for staphyloccal thermonuclease in foods as an indicator of pathogenic or enterotoxic staphylococci may eliminate some of the above problems. To this date, no other microorganism besides S. aureus has been reported to produce a thermonuclease. Many workers have suggested that the production of thermonuclease and coagulase be measured as indicators of pathogenicity in staphylococci (Burns and Holtman, 1960; Brandish and Willis, 1970; Lachica, Genigeorgis, and Hoeprich, 1971; Maughan, 1972; Erickson and Deibel, 1973). Verification comes from surveys such as the one by Lachica, Weiss, and Deibel (1969). They showed that 95 percent of 250 enterotoxin producing strains of staphylococci produced a thermonuclease and 93 percent produced coagulase. In 1971, Lachica, Hoeprich, and Genigeorgis reported that all 305 coagulase-positive isolated from various sources produced thermonuclease.
The calcium activated thermonuclease discovered by Cunningham, Catlin and DeGarilhe in 1956 has a molecular weight of 16,807. It consists of 149 amino acids in a single polypeptide chain with no disulfide bridging. Heat (65°C), acid (pH 2), base (pH 11.5), urea (8 M), methanol (49 percent v/v), and dioxane (40 percent v/v) can cause structural disruptions, but these disruptions are rapidly and completely reversible (Abramson, 1972). The presence of calcium ions and nucleotides, especially deoxythymidine 3' 5' diphosphate, protect the more sensitive bonds in the vicinity of the binding site from cleavage by proteolytic enzymes (Lachica, Hoeprich, and Reiman, 1972).

The enzyme is a phosphodiesterase which cleaves DNA or RNA to produce 3' phosphomonomonucleoside. The enzyme acts primarily as an endonuclease but in the latter stages acts as an exonuclease (Abramson, 1972). The optimum pH is reported to be between 8 and 10 depending on the calcium ion concentration (Barman, 1969; Erickson and Deibel, 1973; Abramson, 1972).

Erickson and Deibel (1973) confirmed the heat stability of the enzyme. They reported a D value (time at a given temperature to effect a one-log decrease in enzyme activity) of 16.6 minutes at 130°C for thermonuclease.

It was believed that pathogenic staphylococci strains varied little in the amount of thermonuclease produced (Cunningham, Catlin, and DeGarilhe, 1956; Alexander, Heppel, and Hurwitz, 1961). Recent reports indicate that nuclease activity varies with the different strains of pathogenic staphylococci (Lachica, Genigeorgis, and Hoeprich, 1971; Maughan, 1972; Erickson and Deibel, 1973).
Thermonuclease production in pure broth culture was inhibited by anaerobic conditions and stimulated by aerobic conditions (Chesbro and Auborn, 1967; Erickson and Deibel, 1973). But the reverse was found when thermonuclease was tested in ham slices (Chesbro and Auborn, 1967). This could have been caused by other bacteria or by chemical stimulants.

Lachica, Hoeprich, and Reiman (1972) reported on the stability of thermonuclease to prolonged storage. The enzyme was stored in butter, banana cream filling, and sterile reconstituted dry milk at 5°C. Stability was also checked in Colby cheese and meat spreads of beef, turkey, and chicken at room temperature. Three different portions of nonfat dry milk with the enzyme added was stored at 37, 25, and 5°C. All the samples were sealed in airtight containers. After 10 months, all foods exhibited nuclease activity. Of the dried milk samples, only the milk stored at 37°C exhibited a decrease in activity.

Chesbro and Auborn (1967) reported that the amount of thermonuclease recovered, using a spectrophotometric assay, was related to the type of food analyzed. For a given type of food, the level of recovery of the enzyme was reproducible. Generally starchy foods gave the highest recovery; meats intermediate; and fat, rich foods the lowest recovery. Maughan (1972) employed ammonium sulfate extraction from cheese slurries and tested the enzyme with the metachromatic agar-diffusion test of Lachica, Genigeorgis, and Hoeprich (1971). He attributed the 41 percent loss in enzyme activity to trapping of the enzyme in fat.
Detection of thermonuclease in foods

Although there are a relatively large number of tests for nuclease, few have been successful in detecting thermonuclease in foods.

One assay method used a standard spectrophotometric procedure. The presence of nucleases was determined by a loss in absorbance when deoxyribonucleic acid was read at 260 nm. Chesbro and Auborn (1967) reported the sensitivity of the test to be approximately $9.5 \times 10^{-3} \mu g$ or .34 unit of enzyme activity. A laborious purification process is required to reduce the levels of naturally occurring oligonucleotides and nucleotides. Equipment and material cost are also detrimental factors (Lachica, Hoeprich, and Genigeorgis, 1972).

Chesbro and Dunlap (1972) eliminated the purification step with a radioassay technique. This method was reported to be 20 times more sensitive than the spectrophotometric method. This advantage is offset by the extra precautions necessary to handle the radioactive C$^{14}$ labeled substrate and the extra cost for materials and equipment.

Only the methods using methyl green and toluidine blue have proven applicable to impure systems (Lachica, Hoeprich, and Franti, 1972).

The methyl green method requires a highly polymerized deoxynucleic acid substrate. For opaque systems, disc-agar-diffusion and HCL-flood techniques are available. The latter is limited by protein interference (Lachica, Hoeprich, and Franti, 1972).

The metachromatic agar-diffusion (MAD) miroslide test of Lachica, Genigeorgis, and Hoeprich (1971), does not require a highly polymerized deoxyribonucleic acid substrate and quantitatively reproducible
results are obtained with impure, opaque systems containing high protein concentrations. The test utilizes the metachromatic effect of agar and DNA in toluidine blue. When an enzyme preparation is placed in a well cut in the agar, a clear or bright pink hydrolysis zone develops. The size of the zone depends upon the enzyme concentration, time, and temperature of incubation.

The substrate was remarkably stable. No sterilization was needed, even for storage at room temperature for up to four months. Satisfactory results were obtained even after the mixture had been subjected to several melting cycles. The stability characteristics of the substrate could be attributed to toluidine blue inhibition of gram positive bacteria (especially spore formers) and the stability of the toluidine-DNA complex to heat.

Thermonuclease was quantitatively measured and mixed in the foods listed on page 9. Liquid food samples were heated at 97°C for 15 minutes and transferred with a platinum loop to wells in the MAD medium. Small particles of solid food were steamed for 15 min and placed in wells in the MAD medium for testing. Concentrations as low as 0.005 µg/ml and as high as 2.0 µg/ml of thermonuclease were detected after incubation at 37°C for 3 hours. Chocolate pie filling was the only exception in that the lower limit was 2.0 µg/gm.

They were also able to detect thermonuclease in chicken and turkey food products which had been involved in food poisoning cases. Maughan (1972) used a modified procedure of the above test to indicate that chicken was the source of staphylococcal contamination in a food
poisoning case at Utah State University. He verified his results by finding $3 \times 10^8$ staphylococci/g in the chicken tissue.

Lachica, Hoeprich, and Genigeorgis (1972) determined the correlation of thermonuclease and enterotoxin production in beef and pork processed at different pH values (5.8 to 6.75) and salt concentrations (3.75 to 7.78 percent). The meats were inoculated with varying amounts ($10^6$ to $10^6$ cells/ml) of a *S. aureus* strain producing enterotoxin B. Four of the 16 samples failed to produce detectable enterotoxin B, while all exhibited thermonuclease activity.

Maughan (1972) utilized a laborious ammonium sulfate extraction process in conjunction with pressure dialysis to achieve a 59 percent thermonuclease recovery from cheese slurries. The procedure was a modified MAD test in that the incubation time was extended to 13 hr and a higher purified DNA substrate was used. The medium was incubated in a special humidity chamber to decrease evaporation loss. The sensitivity of the test was 0.1 to 0.01 µg/ml of thermonuclease which was less than Lachica, Hoeprich, and Genigeorgis (1971) reported in Colby cheese.

Tests utilizing methyl green and toluidine blue seemed the most practical for detecting thermonuclease in foods. Besides being applicable in impure and opaque systems, both have the advantage of being convenient, simple, rapid, and inexpensive. Also the MAD test has been shown to detect nanogram quantities of thermonuclease in foods without prior purification, concentration, or extraction.
MATERIALS AND METHODS

Bacterial cultures

Staphylococcus aureus strains, 196E, 14458, and 19095, producing predominately enterotoxins A, B, and C, respectively, were obtained from the Nutrition and Food Sciences Department, Utah State University, Logan, Utah. Stock cultures were maintained on Brain Heart Infusion (BHI) agar plates (Difco Laboratories, Detroit, Michigan) and stored at 4 °C.

When a culture was required for an experiment, a loopful of stock culture was inoculated into a flask containing 100 ml of BHI broth and incubated at either 22 or 37 °C. Purity of each culture was determined by colony characteristics after streaking on BHI agar plates and by using a Gram's stain.

Streptococcus cremoris M18 and P2, and Streptococcus lactis AM2, used for cheese curd preparation, were obtained from the Nutrition and Food Sciences Department, Utah State University, Logan, Utah. Stock cultures were maintained in sterile skim milk and stored at 4 °C. Before using, cultures were transferred into sterile skim milk and incubated at 22 °C for 18 hr.

Other bacteria were obtained from the Bacteriology and Public Health Department, Utah State University, Logan, Utah. Stock cultures were maintained and activated for experiments in the same manner as the staphylococcal cultures.
Nuclease test media preparation

The metachromatic agar-diffusion (MAD) microslide medium of Lachica, Genigeorgis, and Hoeprich (1971) was prepared as described in Table 1.

Table 1. Metachromatic agar-diffusion medium composition formula

<table>
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<th>Materials</th>
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<tr>
<td>Tris Buffer pH 9.0 0.05 M</td>
<td>1 liter</td>
</tr>
<tr>
<td>DNA (Difco)</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Bacto-Agar (Difco)</td>
<td>10.0 g</td>
</tr>
<tr>
<td>CaCl$_2$ 0.01 M</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Toluidine Blue 0.1 M</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

Before adding the toluidine blue solution, it is essential that the DNA and agar are completely dissolved by boiling. After mixing the ingredients together, the mixture was left in the same mixing flask and stored at room temperature. For use, the mixture was re-heated to boiling and 17 ml of the cooled solution was placed in a plastic petri dish (100 by 15 mm) and allowed to solidify. Ten to 15 wells, 3 mm in diameter, were cut in the agar and the cut agar aspirated with a mental cannula attached to a vacuum. The same batch of Bacto-Agar (control number 498426) was employed for all experiments requiring agar unless otherwise stated.
Liquid samples were boiled 15 min and 5 µl delivered into a well using a 10 µl Hamilton syringe. Approximately 50 mg of solid food samples were steamed for 15 min and embedded in the agar. Thermonuclease activity was detected by a clear zone of DNA hydrolysis around the well after incubation at 37 °C for 3 hr. The Hamilton syringe was rinsed between samples with 95 percent ethyl alcohol followed by several rinsings with distilled water.

This modification of the original MAD procedure incorporated delivery of samples with a Hamilton syringe instead of a platinum loop. The well size was increased from 2 mm to 3 mm and liquid samples volumes increased from 3 µl to 5 µl. The agar medium was placed in a plastic petri dish. This allowed room for more samples and minimized evaporation. Plates remained in good condition for 48 hr if the sides of the plates were sealed with masking tape.

The MAD medium was replaced with DNase Test Agar with Methyl Green (Difco) to compare sensitivities utilizing the above procedure. The original instructions required the medium to be sterilized by autoclaving. But since all tests were completed within 3 1/2 hr and the medium was freshly prepared before each test, no sterilization was performed. A positive nuclease test is indicated by a clear zone around the well.

**Substitutions and simplifications of the metachromatic agar-diffusion test**

Stock solutions of highly polymerized calf thymus DNA (Sigma Chemical Company, St. Louis, Mo.) were made to be used in place of
Difco DNA in an attempt to improve the sensitivity of the nuclease test. A 100 ml solution containing NaCl (0.1 M), ethylenediaminetetraacetic acid (EDTA) (0.001 M), and Tris buffer (0.01 M, pH 7) was used to dissolve 1 g of the dried, fibrous DNA by continuous gentle stirring for 4 days. The mixing was accomplished under aseptic refrigerated conditions.

A more refined agar called Ion Agar #2 (Colab Laboratories Inc., Chicago Heights, Illinois) was substituted in place of Bacto-Agar (Difco). At a concentration of 0.85 percent, Ion Agar gave a surface to a medium which was suitable for streaking microorganisms.

Filter paper strips (Whatman Filter Paper Numbers 1, 7, 40, 41, and 42, W. R. Balston, Ltd., England) were soaked in heated solutions of the MAD medium. After drying the paper strips at room temperature, drops of heated nuclease solutions were placed on the filter paper strips. Then the strips of filter paper were incubated at 23 and 37 C for up to 24 hr.

Heated MAD medium was placed in 3 by 115 mm Wintrobe tubes (Clay-Adams, Inc., New York, New York) and allowed to solidify to compare longitudinal and radial reactions. A 5 µl thermonuclease sample was layered on top of the medium with a Hamilton syringe and then the tubes were incubated at 37 C for up to 24 hr.

**Laboratory cheese processing**

Cheese was made in the laboratory according to the procedure of Read et al. (1965b). The lactic streptococcus strains used were ML_8, and AM_2 in the ratio of 1 to 2. Strains P_2 and AM_2 were also used.
in the same 1 to 2 ratio. Modifications to the procedure included pressing the curd overnight instead of 1 hr and placing the curd at 4 C after salting instead of 15.6 C.

To simulate staphylococcal contamination, 10 ml of an 18 hr BHI broth culture of a S. aureus strain was inoculated into 1000 ml of raw milk and incubated at 30 C for 4 hr prior to pasteurization and cheese processing.

Staphylococcal plate counts

Duplicate plate counts of staphylococcal cells, using 0.1 ml of the appropriate dilutions of the sample, were obtained by surface spreading onto prepoured and dried plates of Staphylococcus Medium 110 (Difco). Plates were incubated at 37 C for 2 to 3 days. Plates with 30 to 300 colonies were counted.
RESULTS AND DISCUSSION

MAD medium sensitivity and modification studies

The sensitivity of the following nuclease tests was determined with a 5.0 to 0.0005 µg/ml decimal dilutions series of partially purified *S. aureus* nuclease (Grade V, Sigma). Brain Heart Infusion (BHI) broth was chosen as the diluent because it had been the growth medium for strains of staphylococci in experiments to correlate number of cells with amount of nuclease produced.

The modified MAD test detected from 5 µg/ml down to 0.0005 µg/ml of thermonuclease in 3 hr at 37°C. This was 10-fold more sensitive than reported by Lachica, Genigeorgis, and Hoeprich (1971).

In an attempt to improve the sensitivity of the MAD test, a more refined agar and DNA was substituted in the formula. The refined DNA was a highly polymerized calf thymus DNA (Sigma) which was substituted for the Difco DNA at a concentration of 0.3 g/liter. In the heated form, both media had the same blue color, but upon solidification, the medium containing Sigma DNA turned a purple color while the original medium remained blue. The color difference did not interfere with the reading of positive zones. Results on both the original and the substituted medium were identical in 3 hr at 37°C at high and low thermonuclease concentrations.

Maughan (1972) utilized the Sigma DNA in the MAD medium throughout his experiments. However, he used a more concentrated solution of
DNA (3.0 g/l) than stated in the original formula (0.3 g/l). This and solubility was probably the main reason for his decrease in sensitivity (lower limit of 0.1 µg/ml) and the longer incubation period (13 hr) required for test results.

Difco DNA was chosen to be used in the following experiments because there was no pre solution required before addition to the medium. The Sigma DNA took approximately 4 days of aseptic agitation at 4 C to dissolve.

The modification of using a more refined Ion Agar was discarded because the medium turned a color similar to a positive test at concentrations of 0.85, 0.8, 0.7 and 0.6 percent. To simplify the test, filter paper strips soaked in heated MAD medium and dried at room temperature showed no visual changes after incubation at 37 C for up to 24 hr with thermonuclease concentrations up to 5 µg/ml.

In 3 mm by 115 mm tubes, 5 µg/ml of thermonuclease were detected in 3 hr at 37 C by a red zone occurring at the interface. By layering a small amount of heated MAD medium on top of the thermonuclease samples in the tube, the sensitivity of the test was increased 10 fold (0.5 µg/ml). No increase in sensitivity was detected by increasing the incubation period to 24 hr or by decreasing the agar concentration from 0.9 to 0.5 percent.

Sensitivity of the DNase Test Agar with Methyl Green (Difco)

The DNase Test with Methyl Green (Difco) showed no thermonuclease activity after 3 hr incubation at 37 C with 5 µg/ml of thermonuclease.
The following deletions and substitutions were made in the formula to increase the sensitivity of the test since the medium was formulated for the growth of microorganisms.

Tryptose was eliminated since it was basically an enrichment factor for growth. Also, the low dye concentration and neutral pH in the original formula was to allow for growth of microorganisms. Calcium chloride was added since calcium ions are essential for the reaction.

The optimum pH was found to be between 8 and 9 (Table 2). The optimum dye concentration at pH 8.5 was between 0.04 to 0.08 percent (Table 3). The results represent the means of 8 trials.

With the following modifications listed in Table 4, the medium was able to detect 0.05 µg/ml in 3 hr at 37°C. The results indicated the modified MAD test to be the most sensitive test for thermonuclease and was used in subsequent experiments.

Table 2. Maximum zone diameters produced by .05 µg/ml thermonuclease in 3 hr at 37°C on modified DNase Test Agar with Methyl Green at various pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of zone diameter (mm)</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>4.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 3. Maximum zone diameters produced by .05 μg/ml thermonuclease in 3 hr at 37°C on the modified DNase Test Agar with Methyl Green at various dye concentrations

<table>
<thead>
<tr>
<th>Dye concentration (%)</th>
<th>.02</th>
<th>.04</th>
<th>.06</th>
<th>.08</th>
<th>.10</th>
<th>.12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of zone diameter (mm)</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 4. Ingredients in the modified DNase Test Agar with Methyl Green

<table>
<thead>
<tr>
<th>Materials</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-Agar</td>
<td>1.5 g</td>
</tr>
<tr>
<td>DNA (Difco)</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>CaCl₂ (0.01 M)</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Methyl Green</td>
<td>0.06%</td>
</tr>
<tr>
<td>Tris Buffer pH 8.5 0.05 M</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Correlation of zone diameter with μg of thermonuclease/ml

To determine the amount of thermonuclease required to produce a certain size zone diameter, 0.5 to 0.0005 μg/ml decimal dilutions series of Sigma nuclease in BHI broth was tested using the modified MAD procedure. The data in Table 5 reflect zone diameters after 3 hr of incubation at 37 C.

Figure 1 contains a standard curve of data from Table 5.

Table 5. Zone diameter sizes produced by 0.5 to .0005 μg/ml decimal dilutions of Sigma S. aureus nuclease Grade V in Brain Heart Infusion (Difco) broth in 3 hr at 37 C

<table>
<thead>
<tr>
<th>μg of thermonuclease/ml</th>
<th>Zone size (mm)</th>
<th>Average of 4 readings (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>12 12 12 12</td>
<td>12</td>
</tr>
<tr>
<td>0.5</td>
<td>11 11 10 10</td>
<td>10.5</td>
</tr>
<tr>
<td>0.05</td>
<td>9 8 8 8</td>
<td>8.25</td>
</tr>
<tr>
<td>0.005</td>
<td>6 6 6 6</td>
<td>6</td>
</tr>
<tr>
<td>0.0005</td>
<td>5 5 5 5</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 1. Relationship between zone diameters produced in the modified metachromatic agar-diffusion medium and decimal dilutions of Sigma S. aureus nuclease Grade V in Brain Heart Infusion (Difco) broth and raw and pasteurized milk after 3 hr incubation at 37 C.
Pasteurized and Raw Milk  ●
Brain Heart Infusion Broth  ○
Thermonuclease studies in raw and pasteurized milk

The sensitivity of thermonuclease in raw and pasteurized milk was determined by adding known concentrations of Sigma nuclease. The thermonuclease was mixed in the milk for 30 min at room temperature prior to testing with the modified MAD medium. Table 6 data indicate very little difference in enzyme detection in the two types of milk. Figure 1 also portrays a plot of the data.

The lines in Figure 1 indicate that corresponding amounts of thermonuclease produce smaller zones in milk than in broth. The thermonuclease may be trapped in certain milk substances such as fats. The presence of naturally occurring proteases inactivating the nuclease from milk or from microorganisms may reduce activity. These possibilities are explored in subsequent tests.

Stability experiments were conducted by placing a known concentration of thermonuclease in 100 ml of raw and pasteurized milk. Both milk samples were from identical sources and only differed in that one sample was pasteurized. After mixing, both milk samples were stored at 4 C. At various time intervals, the samples were tested for thermonuclease activity. The results are summarized in Table 7.

Thermonuclease was more stable in pasteurized than in raw milk. Possible reasons could be the inactivation of naturally occurring proteolytic enzymes and/or the destruction of proteolytic microorganisms by heat treatment during pasteurization. Losses in pasteurized milk suggest that microorganisms which survive pasteurization might be involved.
Table 6. Zone diameter sizes produced by known concentrations of thermonuclease in raw and pasteurized milk

<table>
<thead>
<tr>
<th>µg of thermonuclease/ml</th>
<th>Zone size (mm)</th>
<th>Average of 4 readings (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11 10 10 10 10</td>
<td>10.25</td>
</tr>
<tr>
<td>0.5</td>
<td>8.5 8.5 8.0 8.0</td>
<td>8.25</td>
</tr>
<tr>
<td>0.05</td>
<td>7.0 7.0 7.0 7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>0.005</td>
<td>5.0 5.0 5.0 5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.0005</td>
<td>very slight zone</td>
<td>-</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11 10 10 10 10</td>
<td>10.25</td>
</tr>
<tr>
<td>0.5</td>
<td>8.0 8.0 8.0 8.5</td>
<td>8.13</td>
</tr>
<tr>
<td>0.05</td>
<td>7.0 7.0 7.0 7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>0.005</td>
<td>5.0 5.0 5.0 5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>0.0005</td>
<td>very slight zone</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7. Stability of thermonuclease in raw and pasteurized milk stored at 4 C

<table>
<thead>
<tr>
<th>Days of Incubation</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone size*</td>
<td>10</td>
<td>10</td>
<td>9.5</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pasteurized milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone size*</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8.5</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

*Average diameter of 4 readings in mm.
Quantitative studies of thermonuclease in cheese curd

Before thermonuclease could be studied quantitatively in cheese curd, a method was developed to dissolve the curd to liberate the enzyme.

Raw milk contaminated with 10 ml of BHI broth containing *S. aureus* strain 196 E was pasteurized, and then processed into cheese. Samples of cheese curd (0.5 g) were mixed with 4.5 ml of various concentrations of sodium citrate. The mixture was heated and mixed thoroughly for 20 min in a boiling water bath until dissolved in the test tube.

The cheese sample dissolved in 0.1 M sodium citrate gave the largest zone of thermonuclease activity.

The use of citrate to dissolve the curd was evaluated because citrate binds calcium ions and calcium ions are essential for the thermonuclease reaction. One ml of .01 to 0.5 M CaCl₂ was added to the curd-citrate solution. The results indicated no increase in size of the zone diameter over the control with no excess calcium ions added.

In higher concentrations of CaCl₂, the curd reforms and causes a corresponding decrease in the size of the zone diameter. The probable reason for this is that the thermonuclease once again becomes trapped in the curd.
Table 8. Zone diameter size of thermonuclease from staphylococcal contaminated cheese curd dissolved with various concentrations of sodium citrate

<table>
<thead>
<tr>
<th>Amount of sodium citrate</th>
<th>Size of zone (mm)</th>
<th>Average in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 M</td>
<td>4.0 4.0 4.0 4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>0.5 M</td>
<td>4.5 4.5 4.5 4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>0.1 M</td>
<td>6.0 6.5 6.0 6.0</td>
<td>6.13</td>
</tr>
<tr>
<td>0.05 M</td>
<td>5.0 5.5 5.0 5.0</td>
<td>5.13</td>
</tr>
</tbody>
</table>

The distribution of thermonuclease in whey and curd

To establish the distribution of thermonuclease in whey and curd during a laboratory cheese process, a known amount of thermonuclease was added to raw milk, mixed, and then the milk was pasteurized. One half milliliter of milk was added to 4.5 ml of 0.1 M sodium citrate and tested for thermonuclease.

During cheese processing, whey samples were diluted in the same manner as the milk and tested for thermonuclease. After processing, 0.5 g curd samples were dissolved in 4.5 ml of 0.1 M sodium citrate and tested for thermonuclease.

The above experiment was repeated with S. aureus strain 196 E, replacing the known thermonuclease in the milk. The results of the experiments are listed in Table 9.

Plate counts indicated between $10^6$ to $10^9$ staphylococci/ml were present in staphylococcal contaminated milk samples prior to
Table 9. Thermonuclease distribution in whey and curd in a laboratory cheese process

<table>
<thead>
<tr>
<th>Thermonuclease + milk</th>
<th>Size of zone in mm (average of 8 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurized milk sample</td>
<td>8.0</td>
</tr>
<tr>
<td>Whey sample after cooking</td>
<td>8.0</td>
</tr>
<tr>
<td>Whey sample after pressing</td>
<td>8.0</td>
</tr>
<tr>
<td>Cheese curd</td>
<td>8.0</td>
</tr>
<tr>
<td><em>Staphylococcus aureus (196 E) + milk</em></td>
<td></td>
</tr>
<tr>
<td>Pasteurized milk samples</td>
<td>8.0</td>
</tr>
<tr>
<td>Whey sample after cooking</td>
<td>7.5</td>
</tr>
<tr>
<td>Whey sample after pressing</td>
<td>7.5</td>
</tr>
<tr>
<td>Cheese curd</td>
<td>8.75</td>
</tr>
</tbody>
</table>

*Greater than $8 \times 10^8$ staphylococci/ml.
pasteurization and cheese making. No staphylococci were present on plate counts after pasteurization.

When just the enzyme was added to milk and staphylococcal counts were lower than \(8 \times 10^8\) staphylococci/ml, there was equal thermonuclease activity detected per ml in the curd and the whey. However, when staphylococcal counts were greater than \(8 \times 10^8\) cells/ml there was a greater increase in thermonuclease activity in the curd than in the whey. A possible reason for this is that with a higher amount of staphylococci being trapped in the curd, there may be a corresponding accumulation of staphylococcal enzyme in the curd.

**Stability of thermonuclease during aging of laboratory cheese curd**

Staphylococcal contaminated cheese milk was pasteurized and lactic streptococci strains ML₈ and AM₂ were used to make cheese. During various time intervals, thermonuclease activity was detected as the cheese aged at 4°C.

The results in Table 10 indicate an initial drop of thermonuclease activity, but later the enzyme activity stabilizes.

**Correlation of numbers of staphylococci with thermonuclease production**

Three flasks containing 100 ml of BHI broth were inoculated with one loopful of *S. aureus* strains 196 E, 14458, and 19095, respectively. The broth cultures were incubated at 37°C and samples were removed to
Table 10. Thermonuclease stability during aging of laboratory made cheese at 4 C

<table>
<thead>
<tr>
<th>Time in days</th>
<th>Size of zones (mm) Average of 4 readings</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td>8.0</td>
</tr>
<tr>
<td>10</td>
<td>7.0</td>
</tr>
<tr>
<td>15</td>
<td>6.5</td>
</tr>
<tr>
<td>20</td>
<td>6.0</td>
</tr>
<tr>
<td>25</td>
<td>6.0</td>
</tr>
<tr>
<td>30</td>
<td>6.0</td>
</tr>
</tbody>
</table>

test for thermonuclease and to run corresponding plate counts at selected time intervals.

The results (Figure 2) indicate that the three strains produced different amounts of thermonuclease. The first sign of thermonuclease activity was noted when there was between $10^6$ to $10^7$ staphylococci/ml. Thermonuclease activity continued to increase while the cells were in the stationary phase of growth which may aid in staphylococcal detection in foods.

Detection of thermonuclease in staphylococcal contaminated aged cheese

Ten 50-gram samples of staphylococcal contaminated Cheddar and Colby cheese were obtained through the generosity of Dr. Sita Tatini, Department of Food Science and Nutrition, University of Minnesota,
Figure 2. Correlation between number of cells of three *Staphylococcus aureus* strains/ml in Brain Heart Infusion (Difco) broth and zone diameter of thermonuclease activity using the modified metachromatic agar-diffusion medium.
Log S. aureus cells/ml vs Zone Diameter (mm)

- S. aureus strain 14458
- S. aureus strain 19095
- S. aureus strain 196 E
St. Paul, Minnesota. Information from Dr. Tatini on the cheese samples is listed in Table 11.

Nine of the experimental cheese samples were aged for 2 months at 40 °F and held in frozen storage (-4 °F) for over 3 years. The Schreiber cheese sample was aged for over 6 years at 40 °F and was never held in frozen storage.

Thermonuclease activity was detected only in the Schreiber cheese sample. None of the other samples exhibited thermonuclease activity even when the MAD medium was incubated an additional 21 hours at 37 °C. This would indicate that the thermonuclease test is not as sensitive as enterotoxin determination.

The maximum number of staphylococci/gm in the cheese samples was approximately the minimum amount of staphylococci/ml needed to produce the first detectable signs of thermonuclease in BHI broth. Also, our tests indicate that thermonuclease activity decreased during storage.

It may have been possible to detect thermonuclease in the cheese milk and cheese soon after manufacturing.

**Direct assay for thermonuclease in laboratory made cheese**

Laboratory cheese made with cheese milk containing between $10^7$ and $10^9$ staphylococci/ml and the Schreiber cheese sample exhibited thermonuclease activity without prior curd solution dissolving. The sample curd (about 50 mg) was steamed for 15 min and placed on the surface of the MAD medium. Incubation time of 3 hr at 37 °C was sufficient to give a positive test.
Table 11. Data on staphylococcal contaminated aged cheese obtained from the University of Minnesota

<table>
<thead>
<tr>
<th>Lot no.</th>
<th>Initial S. aureus strain inoculum in cheese milk (per ml)</th>
<th>Maximum S. aureus population in cheese (per gram)</th>
<th>Enterotoxin in 100 grams of cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ch</td>
<td>$9.5 \times 10^3$ (196 E)</td>
<td>1.2 million</td>
<td>-</td>
</tr>
<tr>
<td>8a Ch</td>
<td>$4.7 \times 10^3$ &quot;</td>
<td>1.7 &quot;</td>
<td>-</td>
</tr>
<tr>
<td>10a Ch</td>
<td>$4.9 \times 10^3$ &quot;</td>
<td>3.3 &quot;</td>
<td>+</td>
</tr>
<tr>
<td>14 Ch</td>
<td>$6.0 \times 10^3$ &quot;</td>
<td>1.1 &quot;</td>
<td>-</td>
</tr>
<tr>
<td>20 Co</td>
<td>$11.0 \times 10^3$ (F265)</td>
<td>1.0 &quot;</td>
<td>-</td>
</tr>
<tr>
<td>23 Co</td>
<td>$0.98 \times 10^3$ (196 E)</td>
<td>0.75 &quot;</td>
<td>-</td>
</tr>
<tr>
<td>24a Co</td>
<td>$1.2 \times 10^3$ &quot;</td>
<td>12.0 &quot;</td>
<td>+</td>
</tr>
<tr>
<td>29 Ch</td>
<td>$1.8 \times 10^3$ (F265)</td>
<td>0.12 &quot;</td>
<td>-</td>
</tr>
<tr>
<td>Hb Ch</td>
<td>$130.0 \times 10^3$ &quot;</td>
<td>8.5 &quot;</td>
<td>+</td>
</tr>
</tbody>
</table>

Schrieber Ch No information; involved in food poisoning ---- +

a Showed low milking acidity (0.37% T.A.) and probably had a partial failure of mixed type (WC) starter. Final pH of cheese was, however, normal (5.25).

b Bacteriophage added to induce lactic starter failure.

c Enumerated on S110 medium.

Ch = Cheddar cheese   Co = Colby cheese
Survey of a variety of market Colby and Cheddar cheese products for thermonuclease

Forty samples of market Colby and Cheddar cheese samples (Table 12) were obtained from retail outlets in the midwest and western area of the United States. All samples showed no presence of thermonuclease after a total of 18 hr at 37 C.

A turbid ring may appear around the well and may be mistaken for a positive test when extending the incubation period for cheese testing. The possible reason for this is the absorption of the milky cheese solution into the agar, which does not diffuse further than 5 mm. A positive test can be readily detected since the zone size will increase beyond 5 mm with increased incubation time.

Inactivation of thermonuclease by bacteria in broth culture

Eleven bacterial cultures associated with dairy fermentation products or bacteria normally present in raw milk were tested for the possibility of deactivating thermonuclease. Each bacterial culture was inoculated into 20 ml of BHI broth containing a known amount of thermonuclease. At the beginning and end of one week, samples were removed and tested for thermonuclease. Table 13 indicates the results obtained.

The results indicate that a number of bacteria will cause an inactivation of thermonuclease with time.

*Streptococcus faecalis* var. *liquefaciens* and *Bacillus subtilis* caused the greatest inactivation of thermonuclease of the organisms
Table 12. Commercial brand names of Colby and Cheddar cheese products tested for thermonuclease

<table>
<thead>
<tr>
<th>No.</th>
<th>Brand Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Safeway-Natural Cheddar Cheese (KCA)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Safeway-Colby Cheese</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Safeway-Mild Cheddar Cheese</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Safeway-Natural Cheddar Tillamook</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Kraft-Mild Longhorn Cheddar Cheese (48)</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Kraft-Mild Cheddar (44)</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Kraft-Cracker Barrel 60 (1E45263)</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Kraft-Mild Cheddar (44) (75)</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Kraft-Mild Cheddar (44) (45)</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Kraft-Mild Cheddar-Sliced Sandwich Size</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Kraft-Mild Longhorn (48) (35)</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Kraft-Cracker Barrel Cheddar (11 143 E 263)</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Kraft-Sandwich Slices</td>
<td></td>
</tr>
<tr>
<td>14.</td>
<td>Kraft-Mellow Cracker Barrel (12 17 EC 2)</td>
<td></td>
</tr>
<tr>
<td>15.</td>
<td>Kraft-Mild Colby</td>
<td></td>
</tr>
<tr>
<td>16.</td>
<td>Arden-Natural Cheddar (165W)</td>
<td></td>
</tr>
<tr>
<td>17.</td>
<td>Schreiber-Mellow Cheddar Cheese (319)</td>
<td></td>
</tr>
<tr>
<td>18.</td>
<td>Borden-Wisconsin Mellow Cheddar</td>
<td></td>
</tr>
<tr>
<td>19.</td>
<td>Lake to Lake Wisconsin-Cheddar Cheese</td>
<td></td>
</tr>
<tr>
<td>20.</td>
<td>Cache Valley-Cheddar Cheese</td>
<td></td>
</tr>
<tr>
<td>21.</td>
<td>Arden-Mild Cheddar</td>
<td></td>
</tr>
<tr>
<td>22.</td>
<td>Longhorn-Cheddar Cheese</td>
<td></td>
</tr>
<tr>
<td>23.</td>
<td>Challenge-Natural Cheddar Cheese</td>
<td></td>
</tr>
<tr>
<td>24.</td>
<td>Oregon Tillamook-Natural Cheddar Cheese</td>
<td></td>
</tr>
</tbody>
</table>
Table 12. Continued

<table>
<thead>
<tr>
<th></th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Albertson-Mild Cheddar</td>
</tr>
<tr>
<td>26</td>
<td>Cold Pack Cheese-Mild Club</td>
</tr>
<tr>
<td>27</td>
<td>Lady Lee-Mild Cheddar</td>
</tr>
<tr>
<td>28</td>
<td>Challenge-Natural Cheddar</td>
</tr>
<tr>
<td>29</td>
<td>Pauly's Wisconsin Sharp</td>
</tr>
<tr>
<td>30</td>
<td>Schreiber-Mellow Cheddar (145)</td>
</tr>
<tr>
<td>31</td>
<td>Orchard Park-Sharp Cheddar</td>
</tr>
<tr>
<td>32</td>
<td>Pauly's Colby Cheese</td>
</tr>
<tr>
<td>33</td>
<td>Woody's Natural Wisconsin Cheddar</td>
</tr>
<tr>
<td>34</td>
<td>Lake to Lake-Mild Cheddar Cheese</td>
</tr>
<tr>
<td>35</td>
<td>Lake to Lake-Mild Colby Cheese</td>
</tr>
<tr>
<td>36</td>
<td>Orchard Park-Mild Colby</td>
</tr>
<tr>
<td>37</td>
<td>Farndale-Extra Sharp Cheddar Cheese</td>
</tr>
<tr>
<td>38</td>
<td>Milwaukee-Sharp Natural Cheddar Cheese</td>
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<tr>
<td>39</td>
<td>Milwaukee-Mild Colby Cheese</td>
</tr>
<tr>
<td>40</td>
<td>Milwaukee-Mild Cheddar Cheese</td>
</tr>
<tr>
<td>41</td>
<td>Milwaukee-Medium Cheddar Cheese</td>
</tr>
</tbody>
</table>
Table 13. Inactivation of thermolysinase by bacterial cultures in BHI (Difco) broth at 37°C

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>0 days</th>
<th>7 days</th>
</tr>
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<tbody>
<tr>
<td><strong>Streptococcus faecalis var. liquefaciens</strong></td>
<td>6.0</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>6.0</td>
<td>4.0</td>
</tr>
<tr>
<td><strong>Streptococcus thermophilus</strong></td>
<td>6.0</td>
<td>4.5</td>
</tr>
<tr>
<td><strong>Lactobacillus bulgaricus</strong></td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Streptococcus lactis</strong></td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Staphylococcus epidermidis</strong></td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Lactic streptococcus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. cremoris ML8</td>
<td>6.0</td>
<td>5.0</td>
</tr>
<tr>
<td>S. cremoris P2</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>S. lactis AM2</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>S. cremoris MLb</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>S. cremoris P2b</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>S. lactis AMb</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

a Incubated at 45°C.
b Incubated at 23°C.
tested and both are commonly found in raw milk.

More interesting is that *Streptococcus thermophilus* and *Streptococcus cremoris* strain ML$_8$ (at 37 C) caused a slow inactivation of thermonuclease and both bacteria are used to produce a number of dairy products.

*Streptococcus cremoris* strain ML$_8$ was used to make the cheese used in determining the stability of thermonuclease during aging at 4 C.
CONCLUSIONS

1. The metachromatic agar-diffusion test of Lachica, Hoeprich, and Genigeorgis (1971) was modified by adding 17 ml of agar reagent to a 100 by 15 mm plastic petri dish and using 5 µl samples in 3 mm wells. The test proved to be a rapid, simple, sensitive, and inexpensive test for the detection of thermonuclease. The sensitivity of the test (0.0005 µg/ml) using BHI broth as the diluent, was greater than the modified DNase Test with Methyl Green (0.05 µg/ml).

2. Standard curves of known amounts of thermonuclease versus size of zone diameter can be used to determine unknown amounts of thermonuclease in milk, whey, or cheese curd.

3. When identical amounts of thermonuclease were placed in raw pasteurized milk and BHI broth, there was a tenfold decrease in the milk samples (0.005 µg/ml) than in the broth.

4. Thermonuclease activity will decrease in raw and pasteurized milk during storage at 4 C. The inactivation is more rapid in the raw milk samples.

5. Thermonuclease can be studied quantitatively in cheese by dissolving 0.5 g of cheese in 4.5 ml of 0.1 M sodium citrate. This procedure established that thermonuclease activity will decrease but stabilized during storage in cheese.

6. When staphylococcal (8 x 10^8/ml or more) contaminated cheese milk was pasteurized and made into laboratory cheese, more thermonuclease activity/ml was detected in the curd than in the whey. When the above procedure was repeated with less than the above staphylococci/
ml or with just the enzyme, there was equal distribution of thermo-
nuclease activity/ml in whey and curd.

7. Using three *S. aureus* strains, detectable thermonuclease was
evident when there was approximately $10^6$ to $10^7$ *S. aureus* cells/ml
of BHI broth at 37 C. The results indicated that staphylococcal
strains produce different amounts of thermonuclease. The staphylococci
continued to produce thermonuclease in the stationary phase of growth
which may aid in detecting staphylococcal contamination in foods.

8. Staphylococcal ($10^6$-$10^7$ cells/gm) contaminated aged cheese
obtained from the University of Minnesota showed no detectable thermo-
nuclease activity except for the sample involved in food poisoning.
This indicates enterotoxin was a more sensitive indicator of staphylo-
coccal contamination than thermonuclease.

9. Direct detection of thermonuclease can be made in staphylo-
coccal ($10^7$ to $10^9$ staphylococci/g) contaminated aged laboratory
made cheese without prior purification, concentration, or extraction
of thermonuclease.

10. A survey of 40 market Colby and Cheddar cheese products
showed no evidence of thermonuclease activity even after extending the
incubation period to 18 hours.

11. Various bacteria caused inactivation of thermonuclease in
BHI broth. These included organisms commonly found in raw milk
(*Streptococcus faecalis* var. *liquefaciens* and *Bacillus subtilis*) as
well as bacteria used to make dairy fermentation products (*Strepto-
coccus thermophilus* and *Streptococcus cremoris* MLg).
BIBLIOGRAPHY


Horwood, M. P., and V. A. Minch. 1951. The numbers and types of bacteria found on the hands of food handlers. Food Research 16:133.


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Master of Science


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