CAPILLARY TUBE AGAR-DIFFUSION SYSTEM FOR

DETECTION OF STAPHYLOCOCCAL THERMONUCLEASE

by

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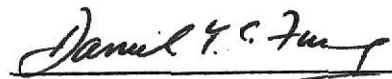
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Staphylococcal food poisoning remains one of the most common types of food poisonings. This is despite the extensive research that has been done on Staphylococcus aureus. During the period 1975-1979, 540 food poisoning outbreaks were reported to the Centers for Disease Control (CDC 1977, 1979, 1981a, b) with S. aureus responsible for 28% of the outbreaks. Therefore, early detection of Staphylococcal cells and enterotoxins produced by them in food or food ingredient is of crucial public importance. But determination of viable S. aureus population in finished food products as a means of ascertaining whether or not enterotoxin may be present is of limited value because the viable cells after attaining sizable populations may die subsequently or may be killed by various processing techniques employed. Thus, a staphylococcal count on a finished product may be misleading, and cannot be used as an index of safety.

Ever since Cunningham et al. (1956) discovered Staphylococcal heat-stable nuclease, considerable interest and study has been focused on this enzyme as an indicator of the possible previous presence of S. aureus, and therefore the possible presence of heat stable enterotoxins. Thermonuclease enzyme meets the criteria of being produced by nearly all S. aureus including all enterotoxigenic strains. It can be produced to detectable amounts under conditions that allow S. aureus growth and survives or withstands processing conditions, such as heat and low pH, which are similar to those of enterotoxins (Tatini et al., 1976). Because of the laborious and time-consuming procedures of enterotoxin

1

detection, this indirect detection of S. aureus and/or the enter-
toxins in foods is a tremendous advantage to researchers.

The remarkable heat-stability of nuclease has been confirmed by
other researchers (Elston and Fitch, 1964; Lachica et al., 1969;
Chesbro and Auborn, 1967; Tatini et al., 1976). In a laboratory,
Staphylococcal thermonuclease has been shown to withstand inactivation
for at least 5 hours in a solution if protected by calcium ions, bovine
serum albumin and one of the products of enzymatic reaction (Sulkowiski
and Laskowiski, 1968).

Currently, the most widely used method for detection of thermonuclease
is the metachromatic agar-diffusion (Lachica et al., 1971). Other
methods include HCl flooding technique (Jeffries et al., 1957), overlay
method (Lachica et al., 1969), serological specificity of thermonuclease
(Lachica et al., 1979), fluorescent acridine orange incorporated in the
DNA-agar and observed under ultraviolet light (Lanyi and Lederberg,
1966), and simplified thermonuclease test (STN) (Lachica, 1976).

The screening for thermonuclease in foods is intended to be a rapid
diagnostic procedure. In some cases, it may be employed as a
confirmatory test to coagulase test or as a principle test where
coagulase is negative. It is recognized that on occasions S. aureus
may lose the characteristic of producing coagulase but still retain
toxigenicity and pathogenicity.

The purpose of this study was to develop a capillary tube agar-
diffusion system to investigate and determine the quantitative activities
of Staphylococcal thermonuclease. Secondly, study any relationship
between thermonuclease and coagulase production and thirdly, to study

the possible use of thionin to detect thermonuclease activities. This system was intended to be simple to perform and use minimum amount of reagents to accurately detect thermonuclease.

LITERATURE REVIEW

Occurrence of Staphylococcus aureus

Staphylococcal food poisoning continues to be an important
problem for food processors, food service workers and consumers.
The ubiquity of staphylococci has long been recognized. They are
found on the body surfaces and respiratory passages of men and animals
(Gould, 1955). Some strains of these gram positive, non-motile, pigment
producing cocci, under suitable conditions, can cause food poisoning.
This syndrome is characterized by nausea, vomiting, diarrhea, headache,
sweating and general malaise and weakness beginning two to six hours after
ingestion of the food. The symptoms may last for 24-48 hours. Although
the illness is seldom fatal, complications including dehydration and
shock can accompany severe attacks. Mortality is low, but there are
some fatal cases on record by Elek (1959) and Meyer (1953).

Since staphylococci were recognized by Pasteur in 1880 and were
classified by Rosenbach in 1884, food poisoning cases of staphylococci
origin have continually been described (Elek, 1959; Bryan, 1976;
Casman, 1967; Smith et al., 1983). The importance of staphylococci as
food-poisoning organisms is best emphasized further by the data
released by the Centers for Disease Control (CDC 1977, 1979). From
1969 to 1972, staphylococci accounted for the largest proportion of
reported food-poisoning outbreaks of bacterial etiology ranging from
37 to 46%. During the period of 1976, 1977 and 1978, the syndrome
caused 19.8%, 15.9% and 14.9% of the total number of outbreaks in the
United States respectively (CDC, 1981a). These figures rank S. aureus

4

second to Salmonella in this same period with 19.8% of the total
outbreaks and 32.4% of the total 6,806 cases (CDC, 1981b).

The first recorded outbreak of staphylococcal food intoxication
appeared in 1884, the same year Rosenbach described the genus
Staphylococcus. Vaughin (1884) ingested material extracted from the
cheese suspected to have caused 300 cases of severe illness in
Michigan and he contracted the same symptoms observed in the victims.
Vaughin's conclusion was that a bacteria produced a chemical poison
responsible for the illness. However, the first well-documented report
which clearly identified staphylococci as food poisoning agents was in
1914 by Barber (1914). In this study, Barber reported a consistent
isolation of staphylococci from the milk of a cow suffering from
mastitis as the cause of a milk-borne outbreak illness in the Philippines.
The significance of this work remained unrecognized until 1930 when
Dack et al. (1930) isolated a yellow Staphylococcus present in
large numbers in a Christmas cake responsible for a food-poisoning
incident. Sterile filtrates, prepared from broth in which the
organism was grown, produced illness when swallowed by some human
volunteers.

Many foods have been implicated in staphylococcal food poisoning.
High protein foods primarily have been implicated in food-poisoning
outbreaks. Hodge (1960) reported that 99% of the staphylococcal
outbreaks surveyed were caused by cooked proteins. In such foods,
staphylococci grow without check from the normal competition and may
build up to extremely high numbers. Hodge (1960) reported left-over
foods as being responsible for 94% of the outbreaks, and foods containing

a mixture of two or more ingredients were responsible for 67% of staphylococcal outbreaks. Prominent in terms of frequency are cooked meat and poultry products, cheese, custard or cream filled pastries, milk, dried milk and salads containing potato, egg or shrimp (Merson, 1973). Recently $\underline{S}$. $\underline{aureus}$ have been detected in bakery products which are considered low risk foods because of their low moisture content, high baking temperature and unusually high sugar concentration (Sankaran and Leela, 1983). Among food poisoning incidents reported in the United Kingdom during 1969-72, 30% were traced to bakery products and in 9 out of 10 instances, the causative agent was $\underline{S}$. $\underline{aureus}$ (Seiler, 1978). The heat stable proteins called staphylococcal enterotoxins produced by enterotoxigenic $\underline{S}$. $\underline{aureus}$ strains are the ones responsible for staphylococcal illnesses. Angelloti et al. (1961) have well established that staphylococci can multiply between 6.7°C and 45.5°C. Vandenbosch et al. (1973) established that 40°C was the optimal temperature for toxin production. It has been reported that toxin is produced by the cells at the late log phase (Markus and Silverman, 1970; Morse et al., 1969). Little information is available on the numbers of cells of staphylococci present in foods implicated in food-poisoning outbreaks except for the reports by Hobbs (1955) and Casman and Bennett (1963) that there must be several millions of staphylococci present in the foodstuff before there is sufficient toxin formed to cause illness. Gilbert et al. (1972) reported that large numbers, usually greater than 1×10^6 cells/g, must be present, or must have been present at one time, to produce enough enterotoxin to cause symptoms. Tatini et al. (1976) reported that 5×10^5 to 1×10^6 cells/g must be present prior to

accumulation of detectable amounts of enterotoxins. Bryan (1968) has suggested that for staphylococcal intoxication to occur, a) there must be a reservoir for the enterotoxigenic strain of S. aureus; b) contamination of a food that is capable of supporting the growth of the organism; c) a mode of dissemination of the organism; d) a temperature level for a length of time sufficient to permit adequate multiplication of the organism and toxin production; and e) consumption of a sufficient amount of enterotoxin by a susceptible person. Successful control measures can eliminate one or more of these factors. The most practical control measures prevent growth of staphylococci by adequate chilling below 5°C or hot holding of foods above 45°C.

Other etiologic agents such as Salmonella, Clostridium perfringens and Vibrio parahaemolyticus are frequent causes of food poisoning in some countries but overall staphylococcal food poisoning is probably the leading cause of food-borne illness in the world. In some countries obtaining records about food-poisoning is impeded because, aside from the fact that other problems may be of more immediate concern, it is difficult to determine how important the staphylococci are in the digestive disturbances common to these countries. Although the type of foods consumed in any given country may affect the incidence of poisoning, the populations of most countries consume foods that can support the growth of staphylococci and the production of enterotoxin given time-temperature abuse conditions.

Isolation and enumeration of S. aureus in foods

Staphylococcal contamination in food may arise from any

sources and the presence of toxin in food may be a result of the growth of the organism, therefore contamination of food with S. aureus may be viewed in two ways. First, the number of staphylococci present may serve as an index of sanitation practices during processing. Second, when present in large numbers they signal the potential presence of enterotoxin and further that poor sanitary practices and inadequate time-temperature control prevailed during processing and handling.

In attempting to isolate S. aureus strains from food, the microbiologist is often faced with the presence of low numbers of detectable organisms per gram of sample and often these numbers are attenuated as a result of industrial processing conditions or prolonged storage which is often characteristic of mass-produced and distributed in foods. The choice of an isolation and an enumeration procedure may be based on an estimate as to the population per gram of sample. As always, the element of time is important and need for an early analytical conclusion must be a consideration in the choice of methods.

A number of media have been developed for isolation and enumeration of S. aureus. But no specific test may be useful in every case to isolate and recover the staphylococci from a wide variety of foods in which they are found. As a result, attempts have been made to find a combination of selective and enrichment media that will support the growth of the staphylococci and at the same time suppress the growth of other microflora present that tend to overgrow the staphylococci. Among the common media are Mannitol Salt agar (Chapman, 1945), Staphylococcus medium No. 110 fortified with egg yolk (Herman

. and Morelli, 1960), Tellurite glycine agar (Zebovitz et al., 1966) and egg yolk-sodium azide agar, EYAA (Hopton, 1961). Other media have also been developed such as Baird-Parker medium (Baird-Parker, 1962), Tellurite polymyxin egg-yolk agar (TPEY) (Crisely et al., 1964), KRANEP agar (Sinell and Baumgart, 1967) used extensively in Germany and Phenolphthalein diphosphate with added polymyxin (PPAP) developed by Barber and Kuper (1951) and modified by Hobbs et al., (1968).

Baird-Parker medium is widely used because of its lack of inhibition of injured staphylococci, its selectivity and ease of recognition of colonies of S. aureus. It has been approved for use in AOAC with or without pre-enrichment step (Baer et al., 1971a, Baer et al., 1971b). It's also recommended for enumeration of S. aureus in meat and meat products by the International Organization for Standardization. The enrichment broth described by AOAC is trypticase soy broth containing 10% sodium chloride (Baer et al., 1971b). However, Baird-Parker medium also has disadvantages (Baird-Parker, 1962). Some strains of Proteus vulgaris form colonies on this medium that are indistinguishable from colonies of S. aureus and some strains of S. aureus do not produce clearing of egg-yolk. Some group D Streptococci, micrococci, corynebacteria and some members of the family Enterobacteriaceae grow on Baird-Parker medium and form black colonies (De Waart et al., 1968), but none of these organisms are able to clear egg-yolk.

Because of conflicting reports as to the efficiency of different media for isolation and enumeration of staphylococci, the problem of choosing a medium has been simplified by an International Committee

on Microbiological Specifications for Foods established by the International Association of Microbiological Societies (IAMS). This committee has published and recommended techniques to detect and enumerate microorganisms in foods (Thatcher and Clark, 1968). Five media have been recommended to isolate staphylococci from foods: Vogel-Johnson agar, Baird-Parker medium, egg-yolk sodium azide agar, tellurite-polymyxin egg yolk agar, and milk salt agar. The confusion that exists with regard to the methods for quantitative recovery of S. aureus can also be solved by individual investigators execution of their own judgement in selecting a suitable medium for their specific studies. Quantitative estimations of S. aureus in foods are useful indicators of adherence or non-adherence to good manufacturing practices. For this reason, specified count levels are undoubtedly an important part of microbiological quality standards for certain foods. Therefore, the importance of development of data on which to base such standards needs no emphasis.

Enterotoxins

Synthesis and chemical composition of enterotoxins by S. aureus

Six types of enterotoxins (A, B, C, D, E, and F) have been detected, based on their distinct immunological differences. This sequential lettering system was adopted for systematic nomenclature of staphylococcal enterotoxins since a possibility exists that more enterotoxins may be discovered (Casman et al., 1963). A comparison of some properties of enterotoxins A, B, C_1, C_2, D and E are shown in Table 1 and amino acid composition of the enterotoxins in grams per

100 gram protein is presented in Table 2. Staphylococcal enterotoxin F associated with toxic-shock syndrome patients is the most recent to be discovered (Bergdoll et al., 1981; Nottermans and Dufrenne, 1982). Its properties have not been very well established. Bergdoll et al. (1981) reported that the enterotoxin has a molecular weight of approximately 20,000 daltons and isoelectric point of 6.8 while Nottermans and Dufrenne (1982) reported a molecular weight of 23,000 daltons and isoelectric point of 7.2.

In 1960, Casman (1960) reported two serologically different types of enterotoxins. One of these types, produced by S. aureus strain 196E was designated type "F" (food poisoning) and the other from strain 243, was designated as type "E" (enteritis). The nomenclature was changed in 1962 to conform with the rules for naming other bacterial toxins. Type F became known as enterotoxin A and type E as enterotoxin B. Enterotoxin C (Bergdoll et al., 1965), enterotoxin D (Casman et al., 1967) and enterotoxin E (Bergdoll et al., 1971) were later identified as enterotoxins.

The enterotoxins are simple, relatively low molecular weight proteins that are hydroscopic and easily soluble in water and salt solutions (Bergdoll et al., 1974). The compounds in the active state are resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin and papain. Bergdoll (1970) reported that pepsin destroys their activity at a pH of about 2 but it is ineffective at higher pH values. The molecular weights reported in the literature differ, and only the value for enterotoxin B has been decided definitely. Its molecular weight of 28,366 daltons (Dayhoff, 1972) was calculated from the amino

Table 1. Some properties of enterotoxins

Property	Enterotoxins					
	A[a]	B[b]	C_1[c]	C_2[d]	D	E[e]
Emetic dose ED_{50} UG/monkey	5	5	5	5-10	20	10
Nitrogen content (%)	16.2	16.1	16.2	16.0		
Sedimentation coeff $(S^o_{20}, W)S$	3.03	2.78	3.00	2.90		
Molecular weight	27,800	28,366	34,100	34,000	27,300	29,600
Isoelectric point	6.9	8.6	8.6	7.0	7.4	7.0
Max. absorption (mμ)	277	277	277	277	278	277
Extinction ($E^{1\%}$ 1cm)	14.6	14.4	12.1	12.1	10.1	12.5

[a]Schantz et al. (1972)
[b]Bergdoll et al. (1965)
[c]Borja and Bergdoll (1967)
[d]Avena and Bergdoll (1967)
[e]Borja et al. (1972)

Table 2. Amino acid composition of the enterotoxins in grams per 100
gram protein

| Amino acid | Enterotoxins | | | | | |
	A^a	B^b	$C_1{}^c$	$C_2{}^c$	D	E^d
Lysine	11.26	14.85	14.43	13.99	12.93	10.83
Histidine	3.16	2.34	2.91	2.87	2.66	3.04
Arginine	4.02	2.69	1.71	1.75	3.38	4.50
Aspartic acid	15.53	18.13	17.85	18.39	16.67	15.10
Threonine	5.96	4.50	5.31	5.80	4.51	6.36
Serine	2.99	4.05	4.58	4.81	5.09	4.72
Glutamic acid	12.36	9.45	8.95	8.93	13.15	12.15
Proline	1.35	2.11	2.16	2.23	1.41	1.93
Glycine	2.96	1.78	2.99	2.90	2.69	4.10
Alanine	1.94	1.32	1.85	1.60	2.00	2.38
Half-cystine	0.66	0.68	0.79	0.74	0.74	0.81
Valine	4.93	5.66	6.50	5.87	4.13	4.36
Methionine	0.96	3.52	3.20	3.60	1.07	0.45
Isoelucine	4.11	3.53	4.09	4.02	6.00	4.30
Leucine	9.78	6.86	6.54	5.13	9.25	10.08
Tyrosine	10.63	11.50	9.80	10.27	7.23	9.79
Phenylalanine	4.31	6.23	5.35	5.25	4.76	4.47
Tryptophan	1.46	0.95	0.99	0.84	0.62	1.51
Amide NH_3	1.80	1.66	1.71	1.62	1.71	1.66

[a]Schantz et al. (1972)
[b]Bergdoll et al. (1965)
[c]Huang et al. (1967)
[d]Borja et al. (1972)

acid sequence as reported by Huang and Bergdoll (1970). The values given in Table 1 are those considered to be closest to the actual values. From Table 2, it has been determined that enterotoxins contain relatively large amounts of lysine, aspartic acid, glutamic acid and tyrosine. The amino acid, compositions of Enterotoxins A and E are quite similar. For example the methionine content of staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin E (SEE) is much less than for staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin C (SEC). There are also differences in the lysine, aspartic acid, glutamic acid and leucine contents. Accumulated evidence as reported by Bergdoll et al. (1974) seem to indicate that synthesis of staphylococcal enterotoxins may take place in at least two different ways, with SEB and SEC synthesized one way and, SEA, SED and SEE the other way. Also the amount of SEB and SEC produced by different organisms within a given strain vary greatly from any given strain. Organisms that produce large amounts of SEB and SEC (200-500 µg/ml or more for SEB) may be isolated, but on continued transfer, enterotoxin production may recede. In the case of other enterotoxins, increasing the enterotoxin production is very difficult, even when mutating agents are used. However, Friedman and Howard (1971) were able to increase enterotoxin A production from approximately 3 µg/ml to 57 µg/ml by 13 mutations with N-methyl-N'-nitro-N-nitrosoguanidine. One theory is that enterotoxins B and C production are plasmid related while the others are chromosomally related.

Enterotoxin B has been studied in greater detail than other enterotoxins because of the larger amounts of toxin produced by the

enterotoxin B producing S. aureus strains. Some researchers (McLean et al., 1968; Morse et al., 1969) have reported that enterotoxin B is produced at the beginning of the stationary phase, whereas SEA is produced during the exponential phase of growth (Markus and Silverman, 1970). Miller and Fung (1973) determined the minimum requirements for enterotoxin B production revealing that the enterotoxin could be produced in a synthetic medium containing leucine, proline, valine, arginine, cystine, and phenylalanine, six inorganic salts, four vitamins and monosodium glutamate as the energy source. The amount of enterotoxin produced was 5-6 μg/ml by S. aureus strain S-6, with minimal growth. Increasing the concentration of the amino acids did not increase the growth or enterotoxin production. Wu and Bergdoll (1971) studied the synthesis of SEB (Strain S-6) in a medium containing 18 amino acids and found that the amount of each amino acid required is dependent on its utilization by the organisms. The medium was supplemented with ammonium sulfate, five inorganic salts, and two vitamins. Nothing was added as a specific energy source. Neither arginine nor cystine were required in large amounts, but arginine was essential for initiating growth and was a limiting factor in enterotoxin production. Not as much enterotoxin B was produced in an equivalent concentration of the amino acid medium as was produced in the PHP medium normally used for enterotoxin production, but the amount was only slightly less (125 μg/ml versus 150 μg/ml). It is expected that additional modifications in the concentrations of the various amino acids would result in even higher yields of the enterotoxin. Other physical and chemical factors such as aeration, incubation, temperature

and pH influence synthesis of enterotoxins (Casman and Bennett, 1963; Jarvis et al., 1973; Dietrich et al., 1972; Tatini et al., 1981; Reiser and Weiss, 1969).

Stability of staphylococcal enterotoxins

The staphylococcal enterotoxins are much more stable than most proteins as reported by Davison and Dack (1939) who observed that the potency of enterotoxins could only be gradually decreased by prolonged boiling or autoclaving. Since then, several other researchers have reported the heat-stability of enterotoxins. Denny et al. (1971) determined that boiling crude solutions of enterotoxins for 30 minutes does not destroy all of the activity and Schantz et al. (1965) reported that the activity of an enterotoxin B was retained even after heating at 60°C for 16 hrs.

A number of studies have been conducted to obtain more specific information about the heat stability of the enterotoxins. Most of them are reported in terms of loss of reaction with the specific antibodies, primarily with the single-gel-diffusion tube method (Oudin test), which has a minimal sensitivity of 1 μg/ml. This method is useful in determining the thermal inactivation curves, but it is not possible to determine the small amounts of enterotoxin that may remain and are adequate to cause staphylococcal food poisoning.

The thermal resistance of enterotoxins is primarily dependent on relative purity and the concentration of the toxin. Denny et al. (1971) and Hilker et al. (1968) reported that the time required to inactivate enterotoxin A depends on the amount of enterotoxin present in the sample.

The larger the amount of toxin, the longer the time required to reduce the content to less than 1 µg/ml as determined by the single gel-diffusion tube test. The heating medium may also affect the inactivation of the enterotoxin. When beef bouillon was used as the heating medium in the inactivation of enterotoxin A (20 µg/ml) at pH 6.2 Denny et al. (1971) found that much longer heating time at 121°C was required than when 0.15M phosphate buffer at pH 7.2 was used as the medium. The times found were 37 and 10 minutes respectively. More concentrated enterotoxin solutions also required longer heating times for inactivation of 27 and 37 minutes for 5 µg/ml and 20 µg/ml, respectively.

All the studies dealing with the heating of enterotoxin A at 100°C show that this enterotoxin is gradually inactivated which is consistent with the findings for enterotoxin B (Fung et al., 1973). An attempt to determine the effect of heating on enterotoxin A and D in milk, skim milk, cream, cheese, whey, and sausage by Soo et al. (1974) showed little loss of toxin in milk, skim milk, or cream under pasteurization conditions (72°C for 15 seconds). These investigators reported that it would be difficult to thermally inactivate enterotoxins A and D in milk without adversely affecting the milk. Enterotoxin B is also very little affected by pasteurization (Read and Bradshaw, 1966).

Loss of activity of enterotoxin B was reported to be greater at 80°C than at either 60°C or 100°C (Jamlang et al., 1971; Satterlee and Kraft, 1969; Fung et al., 1973) after a short inactivation time of about 30 minutes. Reichert and Fung (1976) determined that enterotoxins B and C lost immunological activity rapidly during the first 10 to 30 minutes when heated at 80 or 100°C. They attributed this phenomenon to

low-temperature aggregation of protein molecules. After standing for 24 hrs. some reactivation of the toxin was noted, the degree of reactivation depending on the heating time and temperature, with greater recovery after heating at 80°C.

All information available indicates that enterotoxin in food is not easily inactivated by heat and that the larger the amount present the more heat required to reduce the quantity to below detection levels. However in general, the higher the temperature the more rapidly the enterotoxin is denatured, with the times and temperatures used in normal processes of canning foods commercially.

Enterotoxins are resistant to proteolytic enzymes. Studies show that pepsin destroys the activity of enterotoxin B at a pH 2 but is ineffective at high pH values (Bergdoll, 1970; Schantz et al., 1965). This resistance to proteolytic enzymes explains why the toxins are active when ingested, even in the presence of pepsin, because the pH of the stomach is much higher than 2 after ingestion of food. Therefore the enterotoxins remain in active stage to cause food-poisoning illness.

Production of coagulase

The coagulase test is the most widely used method for predicting the presence of toxigenic and pathogenic staphylococci (S. aureus) from closely related Micrococcaceae (Baird-Parker et al., 1976; Davidson et al., 1982). The results of this test indicate the presence or absence of an enzyme able to clot citrated or oxalated rabbit or human blood plasma. The cells of the test organism are mixed with plasma and incubated at 37°C either on a microscope slide (Cadness-Graves

et al., 1974) or in a small test tube (Chapman et al., 1941). Coagulase
has also been detected by pour plate method (Parisi et al., 1973).

The relationship between coagulase and toxin production has been
studied. Lachica et al. (1969) found that from 275 coagulase-positive
strains of S. aureus, 93% were enterotoxigenic and 95% produced heat-
stable nuclease. Rayman et al. (1975) reported a 100% correlation between
coagulase and enterotoxin production in 63 isolates of S. aureus isolated
from foods and personnel involved in food poisoning outbreaks. However,
enterotoxin production may not be an exclusive characteristic of
coagulase-positive S. aureus as it was one time thought in the past
(Evans and Niven, 1950; Evans et al., 1950). Some coagulase-negative
staphylococci that are able to produce enterotoxin have been isolated
from foods (Thatcher and Simon, 1956) and have been incriminated in
outbreaks of staphylococcal food poisoning (Breckinridge and Bergdoll;
1971).

Staphylococcus epidermidis, Staphylococcus hyicus and Staphylococcus
intermedias have been reported that are coagulase-positive (Devriese
and Oeding, 1975; Hajek, 1976; Phillips and Kloos, 1981). These
coagulase-positive strains can be distinguished from S. aureus by DNA-
DNA hybridizations but this technique is not available to many laboratories.
Coagulase may also be produced by bacteria other than staphylococci;
for example, Yersinia pestis (Eisler, 1961). Therefore it is important
that isolates be gram-stained and tested for catalase before performing
a coagulase test.

Under some conditions, coagulase may be present but not detected
by the coagulase test. Plasma may fail to react because of appreciable

concentrations of specific antibodies directed against coagulase or because they are fibrinogen-deficient (Minor and Marth, 1976). Production of fibrinolysin, staphylokinase, and coagulase-destroying factors (Lominski et al., 1953; Munch-Petersen, 1961) by staphylococci may inhibit the action of coagulase on plasma. The other staphylococci produced proteases may dissolve the fibrin clot, resulting in a false-negative reaction (Sperber and Tatini, 1975). Therefore, it is important to read the test periodically to avoid false-negative reactions.

Occurrence and chemical composition of thermonuclease

Enzymes which catalyze the breakdown of nucleic acids by hydrolysis of phosphodiester bonds have been found in almost all biological systems. Some ribonucleases are quite specific for Ribonucleic acids (RNA), others, the deoxyribonucleases act only on Deoxyribonucleic acids (DNA), while a third group of non-specific nucleases is active against either nucleic acid. Staphylococcal thermonuclease (or micrococcal nuclease as it is often known) belongs to the non-specific nucleases.

Cunningham et al. (1956) while studying deoxyribonucleases from various sources, observed that an enzyme in the culture medium of Micrococcus pyogenes var aureus catalyzes the degradation of DNA yielding 3'-deoxynucleotides. Later the enzyme was determined to have unusual properties of requiring Ca^{2+} as a cationic activator instead of Mg^{2+} and prior to separation from the original culture medium, it could be boiled with little or no loss of activity. This unusual stability of the enzyme was a promise that it might be obtainable in sufficiently free of interfering enzymes, so that a study of the mechanism of action

of DNA could be undertaken. Laskowiski (1971) reported that staphylococcal
thermonuclease can degrade DNA to a mixture of nucleoside 3'-mono-
phosphates and oligonucleotides with 3'-phosphate termini (Fig. 1).
It attacks RNA and, preferentially, heat-denatured DNA.

The enzyme primarily operates as an endonuclease and has a small
molecular weight (Dirksen and Dekker, 1960; Bernadi and Griffe, 1964).
This uniqueness of thermonuclease operating primarily as an endonuclease
coupled with other favorable properties of the enzyme, suggest that the
micrococcal nuclease would serve well as a conformation-sensitive probe
of DNA. The unusual heat stability of staphylococcal thermonuclease
and the considerable difference in the rate with which the enzyme attacks
native and denatured DNA are taken advantage of in this probe of DNA
conformation. This was also advantageous in differentiating S. aureus
from other microorganisms that produce heat-labile nucleases.

The primary structure of staphylococcal thermonuclease, V8 strain
was established by Taniuchi and Anfinsen (1966) and Taniuchi et al.
(1967, 1968). The amino acid sequence was determined by cleavage of
extracellular nuclease of S. aureus with cyanogen bromide that permitted
the production of linear arrangement of five fragments. The sequences
of the tryptic peptides prepared from these fragments together with
partial chymotryptic peptides isolated from digests of the intact
nuclease were used to determine the nuclease amino acid composition
shown in Figure 2. The calculated molecular weight of nuclease was
16,807 daltons as determined from the amino acid sequence (Taniuchi et al.,
1967). Schechter and Chen (1970) confirmed further that nuclease is a
single polypeptide chain of 149 amino acids with no cystine or cysteine

Figure 1. Schematic representation of (A) complex formation between staphylococcal nuclease and polynucleotide substrate; (B) inhibitory oligonucleotides bearing 5'-phosphoryl-termini; and (C) a slowly hydrolyzed 3'-phosphoryloliog-nucleotide. The major substrate bonding regions of the enzyme or "phosphate subsites" are indicated as P1, P2, and P3. The hydrolytic site (H) consists of a region related to P1 which recognizes the phosphodiester bond (A), and a region which recognizes the sugar-base moiety whose 5'-OH is linked to the phosphate group (B).

A

B

ACTIVE SITE

Hydrolytic site

C
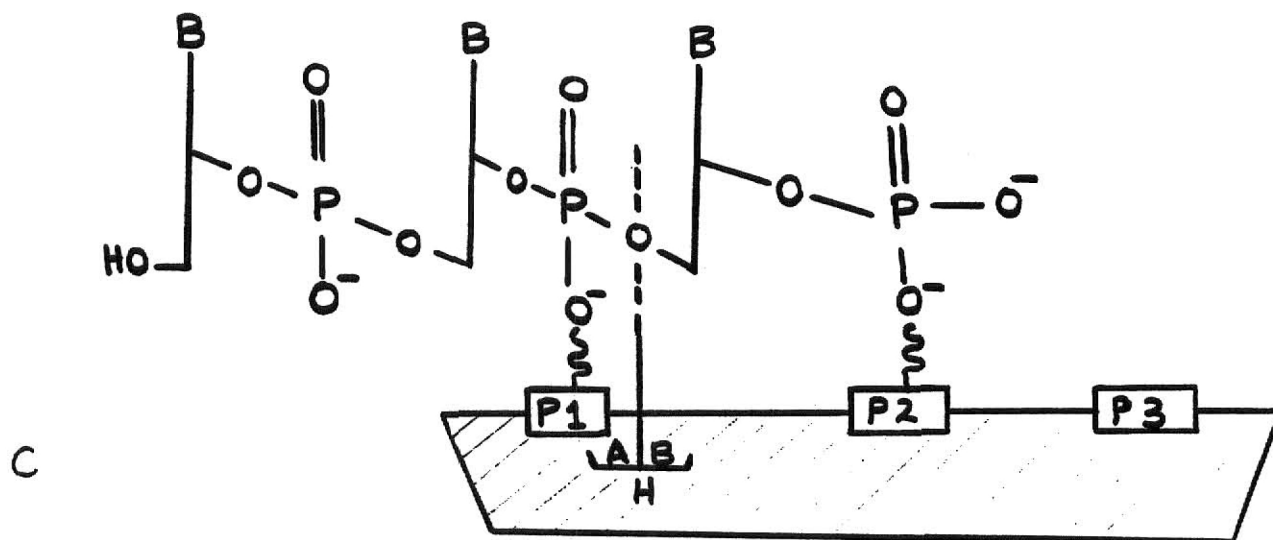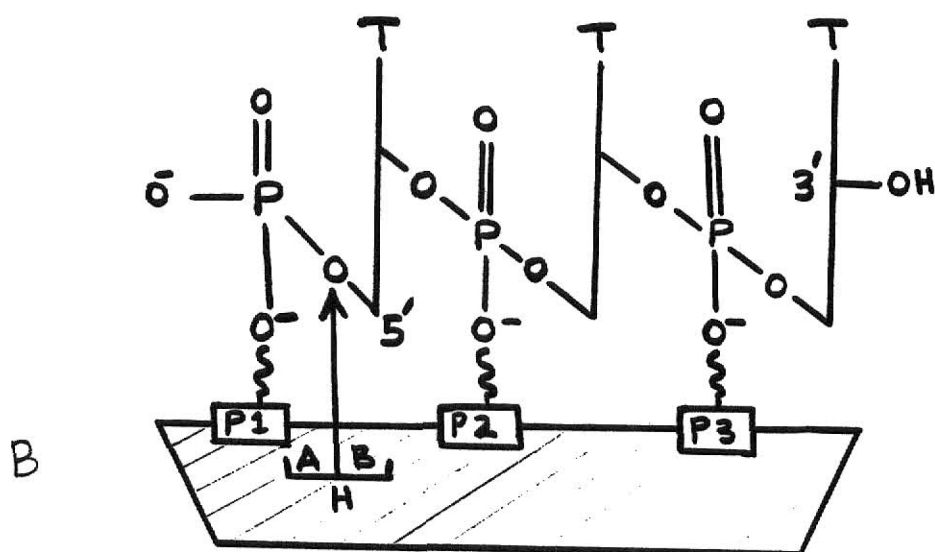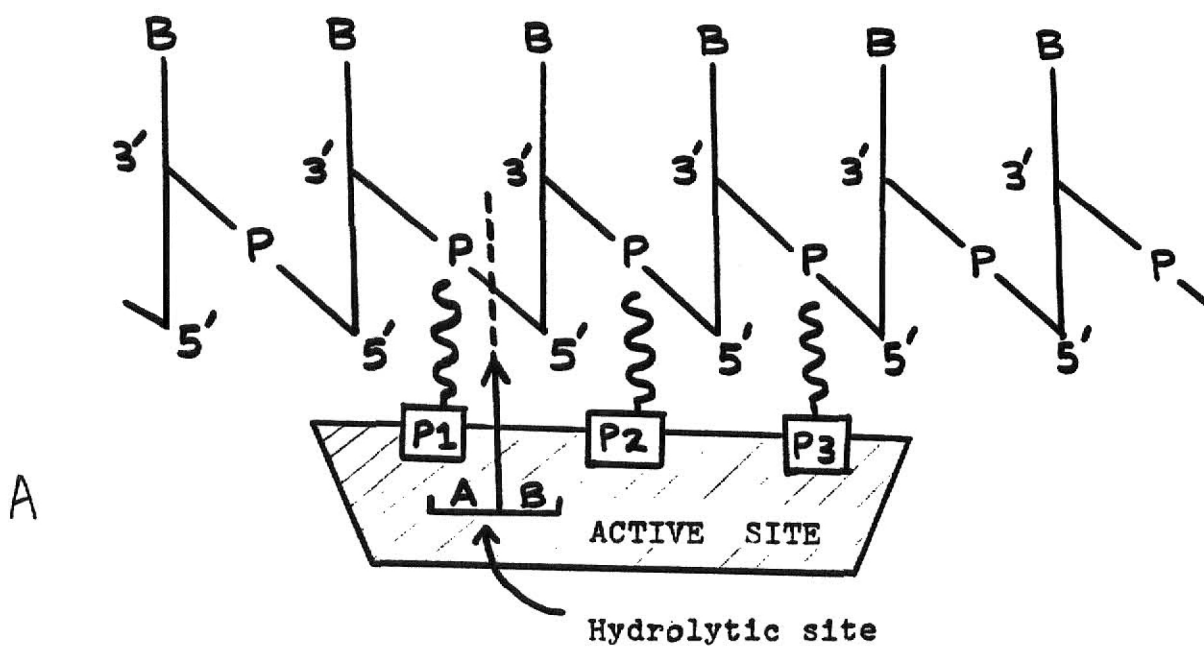
Figure 2. Proposed amino acid composition of staphylococcal
nuclease from S. aureus strain V8.

```
                                                  10
NH2-Ala-Thr-Ser-Thr-Lys-Lys-Leu-His-Lys-Glu-Pro-Ala-Thr-Leu-Ilu
    30                       .                        20            |
GLn-Pro-Gly-Lys-Tyr-Met-Leu-Lys-Val-Thr-Asp-Gly-Asp-Ilu-Ala-Lys
|                                        40
Met-Thr-Pre-Arg-Leu-Leu-Leu-Val-Asp-Thr-Pro-Gln-Thr-Lys-His-Pro
          60                                                    |
Lys-Thr-Phe-Ala-Ser-Ala-Glu-Pro-Gly-Tyr-Lys-Glu-Val-Gly-Lys-Lys
|                              70
Lys-Met-Val-Glu-Asn-Ala-Lys-Lys-Ilu-Glu-Val-Glu-Phe-Asn-Lys-Gly
                    90                                      |80
Asp-Ala-Tyr-Ilu-Tyr-Ala-Leu-Gly-Arg-Gln-Tyr-Lys-Asp-Thr-Arg-Gln
|                   100
Gly-Lys-Met-Val-Asn-Glu-Ala-Leu-Val-Arg-Gln-Gly-Leu-Ala-Lys-Val
                         120                                     |
Lys-Arg-Leu-Leu-Gln-Glu-His-Thr-Asn-Asn-Pro-Lys-Tyr-Val-Tyr-Ala
|        130                                             143
Glu-Lys-Lys-Ser-Glu-Ala-Gln-Ala-Lys-Leu-Asn-Ilu-Trp-Ser-Glu-Asn
                              149                           |
                          HOOC-Gln-Gly-Ser-Asp-Ala-Asp
```

residues and there is a single tryptophan residue in position 140.

Taniuchi and Anfinsen (1966) derived partial sequence of the S. aureus Foggi strain enzyme molecule for a comparative amino acid study of the two enzymes with strain V8. The sequence of a 13-residue peptide was presented, which is similar to the sequence in the corresponding cyanogen bromide fragments of the S. aureus V8 strain, except that leucine 124 in the later is replaced by histidine. Bohnert and Taniuchi (1972) also studied the complete amino acid sequence of the Foggi strain thermonuclease (Figure 3). The results are similar to those obtained by Taniuchi et al., (1967) except that residues 77, 143, and 144 were assigned as asparagine, asparagine and aspartic acid respectively in staphylococcal nuclease V8 strain. They suggested a re-examination of the residues in staphylococcal nuclease V8 strain before considering whether mutational changes in them had occurred. Thermonuclease from S. aureus V8 strain differs further from thermonuclease of Foggi strain at position 124 which contains leucine in V8 and histidine in the Foggi strain.

Heat stability and calcium requirement of thermonuclease

Thermonuclease is completely dependent on calcium ions for its activity (Cuatrecas et al., 1967; Cunningham et al., 1956; Weckman and Catlin, 1957). The calcium requirement depends on pH and vice versa, emphasizing the need for precise description of experimental conditions when describing the properties of thermonuclease. The optimum pH for the thermonuclease activity is at 10 for 1 mM of calcium or 9.5 for 10 mM of calcium (Blackburn, 1977; Ibrahim, 1981; Kamman and Tatini, 1977). At similar pH values of 10 and 9.5 when there is maximum

Figure 3. The amino acid sequence of staphylococcal nuclease
          Foggi strain.

```
         1                                    10
NH₂-Ala-Thr-Ser-Thr-Lys-Lys-Leu-His-Lys-Glu-Pro-Ala-Thr-Leu-Ile
                                            20                  |
 Pro-Gln-Gly-Lys-Try-Met-Leu-Lys-Val-Thr-Asp-Gly-Asp-Ile-Ala-Lys
 |                                 40
 Met-Thr-Phe-Arg-Leu-Leu-Leu-Val-Asp-Thr-Pro-Glu-Thr-Lys-His-Pro
                                                      50   |
 Lys-Thr-Phe-Ala-Ser-Ala-Glu-Pro-Gly-Tyr-Lys-Glu-Val-Gly-Lys-Lys
 |                               70
 Lys-Met-Val-Glu-Asn-Ala-Lys-Lys-Ile-Glu-Val-Glu-Phe-Asp-Lys-Gly
                   60                                    | 80
 Asp-Ala-Tyr-Ile-Tyr-Ala-Len-Gly-Arg-Gly-Tyr-Lys-Asp-Thr-Arg-Gln
 |        100                                         110
 Gly-Lys-MetpVal-Asn-Glu-Ala-Leu-Val-Arg-Gln-Gly-Leu-Ala-Lys-Val
                                120                            |
 Lys-Arg-Leu-His-Gln-Glu-His-Thr-Asn-Asn-Pro-Lys-Tyr-Val-Tyr-Ala
 |        130                             140
 Ser-Glu-Ala-Gln-Ala-Lys-Lys-Glu-Lys-Leu-Asn-Ile-Trp-Ser-Glu-Asp
                                149                           |
                            COOH-Gln-Gly-Ser-Asp-Ala-Asn
```

thermonuclease activity on DNA, the activity of RNA is lower and increase with a slight decrease in pH value. Sulkowiski and Laskowiski, Sr. (1962) have suggested that the slower rate of hydrolysis of RNA compared with that of DNA may be due to the replacement of deoxythymidylic acid by deoxyuridylic acid, since the thermonuclease has considerably less affinity toward the later.

Dirksen and Dekker (1960) have shown that the rate at which the thermonuclease attacks its substrate depends markedly on the later's conformation. However, Ca^{2+} may not be primarily involved in producing conformational changes in the substrate since there is no significant changes in Ca^{2+} requirements when the amounts of DNA or RNA are altered (Blackburn, 1977). Therefore, the precise role of the Ca^{2+} in the mechanism of the action of the enzyme is obscure.

Staphylococcal thermonuclease is stable at pH values as low as 0.1 and at a concentration of 0.015 mg/ml it still shows no loss of activity on boiling for 20 minutes at 100°C (Sulkowiski and Laskowiski, Sr., 1968). Von Hippel and Felsenfeld (1964) reported that thermonuclease was protected during initial stages of the reaction when Ca^{2+}, bovine serum albumin and native DNA were present. Sulkowiski and Laskowiski, Sr. (1968) substituted the reaction products for the native DNA. The simultaneous presence of 0.01 M Ca^{2+}, 0.05% bovine serum albumin and one of the products of enzymatic reaction was found to protect the enzyme from thermal inactivation in solution for at least 5 hours. The protective action probably involves interaction between all the three components, albumin, Ca^{2+} and products, since when any of them was omitted, the remaining two were ineffective.

Several researchers (Cunningham et al., 1956; Chesbro and Auborn, 1967; Privat de Garilhe, 1967) have observed the remarkable resistance of thermonuclease enzyme to heat. Erickson and Deibel (1973) attempted to gain more precision in estimating the heat resistance of the enzyme by using the D-value (time at a given temperature to effect a 1-log decrease in enzyme activity) concept. The supernatant portions of the enzyme in sealed pyrex tubing was heated in an oil bath equilibrated at 100°C, 120°C and 130°C. The D-value at 100°C was 180 minutes, at 120°C was 34 minutes and 130°C was 16.6 minutes. These data reinforce the observation regarding the remarkable heat stability of the staphylococcal thermonuclease as well as afford quantitation of the enzyme's thermostability. The Z-value (increase in degrees of fahrenheit required to cause a 1-log decrease in D-value) was calculated from the D-values obtained by Erickson and Deibel (1973). A Z-value of 51°F (28.3°C) was obtained for the enzyme. This is indicative of unusually high heat stability, because many of the more heat-resistant bacterial spores have Z-values of approximately 18°F (Frazier, 1967).

Factors affecting thermonuclease production

Various experimental conditions have been observed to affect the production of staphylococcal thermonuclease. The effect of agitation-aeration was studied on five isolated coagulase positive S. aureus strains cultivated at room temperature in brain heart infusion (100 ml in 300-ml flasks) by Weckman and Catlin (1957). After cultivation for 40 hrs., viscometric deoxyribonuclease (DNase) tests were carried out on the cultures. DNase activity was one hundred to one thousand times

greater with shaken than with unshaken cultures. Erickson and Deibel (1973) also noted an increase ranging from 10- to 105-fold in thermonuclease production for S. aureus incubated at 37°C, for 24 hrs. under aerated conditions. To determine if small changes in oxygen tension would influence thermonuclease production, atmospheres of 0, 5, 10, and 20% oxygen were examined for their effect by using S. aureus strains 100 and S-6. After incubation at 37°C for 48 hrs at which the supernatant fluid was assayed for thermonuclease activity, a marked increase at 5% oxygen tension, followed by modest but detectable increases at the successively higher oxygen tensions was observed. This was an indication that anaerobic incubation may radically diminish the production of staphylococcal thermonuclease.

Glucose inhibition of the production of staphylococcal thermonuclease has been reported (Privat de Garilhe, 1967; Erickson and Deibel, 1973). These researchers have shown that glucose concentration not only influences the final pH value of the culture, but also thermonuclease production is dependent on pH. Final pH values of the media decrease with increase in glucose concentration, and concomitant decrease in thermonuclease production are also observed. The inhibitory effect can be associated with a decrease in pH value because of fermentation of the sugar. The optimum pH for thermonuclease production was determined by Erickson and Deibel (1973) to be 8.3 and use of a tris (hydroxymethyl) aminomethane buffer system results in an increased production of the enzyme as compared to that obtained with a phosphate buffer.

Sodium chloride may enhance staphylococcal growth in raw whey because of its inhibitory effect on the competing microorganisms present

in the whey. However, because of the decrease in pH, eventually sodium chloride appears to be also inhibitory to S. aureus (Ahmed et al., 1983). This inhibition of S. aureus growth may be transformed into less thermo-nuclease production. A substantial delay of several days in the production of thermonuclease has also been reported in pasteurized minced cod with potassium sorbate inoculated with S. aureus (Lynch and Potter, 1982). This delay in thermonuclease production is indicative of a similar delay in enterotoxin production.

Production of thermonuclease as a tool to identify S. aureus

The most widely used criteria for distinguishing S. aureus from other toxigenic and pathogenic saprophytic organisms is the test for the production of coagulase enzyme. Among other enzymes produced by S. aureus are: phosphatase, proteases, penicillinase, lipase, catalase, lysozyme, lactate dehydrogenase and hyaluronidase (Abramson, 1972). Of these enzymes, staphylococcal thermonuclease is unique because of its heat-stability. However, some investigators have reported heat-stable nuclease production by other microorganisms. Thomas and Nambudripad (1974) reported that 3 out of 12 strains of Streptococcus fecalis produced thermostable nuclease. The thermonuclease of S. fecalis was found to be optimally active in the acid pH range, i.e. pH 6.7 (Thomas and Nambudripad, 1974). In this regard staphylococcal thermo-nuclease produced by S. aureus strains is different because it is optimally active at a pH of about 9.5. Positive heat-stable nuclease have also been reported for S. intermedias, S. hyicus (Devriese and Van de Kerchkove, 1979), S. epidermidis (Devriese and Oeding, 1975; Jones et al.,

1963; Zarzour and Belle, 1978). Zarzour and Belle studied 450 S. aureus strains and 70 isolates of S. epidermidis and found that 447 (99.3%) and 3 of S. aureus strains and S. epidermidis isolates respectively were positive for staphylococcal thermo-stable nuclease. Morton and Cohn (1972) demonstrated that 98% of the 304 coagulase-positive cultures and 13.5% of 200 coagulase-negative cultures produced thermo-stable nuclease. It appears from these results that a small percentage of S. epidermidis strains may be thermo-stable nuclease, whereas some S. aureus strains may not produce thermonuclease.

All 450 clinical isolates identified as S. aureus yielded thermo-stable nuclease (Zarzour and Belle, 1978). These results support the findings of other investigators. Rayman et al. (1975) reported that all 103 clinical isolates of S. aureus tested were positive for thermo-nuclease. Sperber and Tatini (1975) also found that all 439 strains identified as S. aureus yielded thermonuclease. Lachica et al. (1971) reported that many S. epidermidis strains produce heat-labile nucleases. Therefore from these results of various investigators, it appears that thermostable nuclease production is a consistant property by which S. aureus can be identified or confirmed.

Production of thermonuclease as an index of pathogenicity

Since cells of S. aureus may be killed by heat or low pH during processing or by cold temperature during storage, viable counts of S. aureus as an indicator of food-borne hazards from enterotoxins may be misleading. Analysis for the presence of thermonuclease has been proposed for screening of foods (Chesbro and Auborn, 1967; Cords and

Tatini, 1973; Lachica et al., 1972; Tatini et al., 1975, 1976). Thus the test for thermonuclease production, which is simple to perform compared to analysis of enterotoxins, has a useful place in the diagnostic laboratory for the recognition of potential pathogens. Assaying for a heat-stable nuclease, produced specifically and characteristically by S. aureus is a more accurate measure of significant growth in the product. The thermonuclease as well as enterotoxins can survive processing procedures that kill the organisms; therefore, each is a better indicator of staphylococcal growth than are viable counts.

Studies on the relationship between thermonuclease and enterotoxins has been reported. Chesbro and Auborn (1967) extracting thermonuclease from ham and assaying by spectrophotometric means demonstrated that in ham experimentally inoculated with S. aureus, a good correlation existed between S. aureus population and thermonuclease content. Lachica et al. (1969) studied the relationship between thermonuclease and enterotoxin. These investigators found that from 275 coagulase-positive strains of S. aureus, 93% were enterotoxigenic and 95% produced heat stable nuclease. They concluded that these three characteristics appear to have a high correlation among them. Niskanen and Koiranen (1977) examined a total of 276 S. aureus strains isolated from routine sampling, food poisoning outbreaks and mastitic milk for the production of enterotoxins A, B, C, D and E and thermonuclease production. They found that all of S. aureus strains examined produced thermonuclease and 142 (51%) produced one or more of enterotoxins A, B, C, D and E. Bugrova (1981) reported that enterotoxins and thermo-stable nuclease occurred concurrently in 94% of S. aureus strains isolated during staphylococcal

food intoxications. These results appear to confirm the fact that thermonuclease and enterotoxin production correlate equally well. This is also confirmed by a study done by Tatini et al. (1975). These investigators working with a variety of foods, noted a close relationship between thermo-stable nuclease production, growth of $\underline{S}$. $\underline{aureus}$ and production of the most commonly occurring enterotoxins (A and D) in food poisoning outbreaks.

However, some researchers have reported a lack of correlation between nuclease production and enterotoxins. Lee et al. (1975) did not detect any correlation between thermonuclease and enterotoxins production in experimentally inoculated batches of macaroni and noodle dough. The toxic dough samples were negative for thermonuclease which indicates that there may have been some inhibition of heat-stable nuclease production or production of low levels not sensitive to DNase assay procedure. Erickson and Deibel (1973) working on 13 strains of $\underline{S}$. $\underline{aureus}$ that produce enterotoxins A, B, C_1, C_2, D and E found no correlation between thermonuclease activity and enterotoxin type.

Although no definitive statement can be made about the production of thermonuclease as an indicator of pathogenicity, it is essential to regard foods showing positive thermonuclease as a health hazard.

Methods for the detection of thermonuclease

HCl flooding technique

The HCl flooding technique was first demonstrated by Jeffries et al. (1957). The bacteria are grown on agar plates containing large amounts of nucleic acids. The nuclease activity is then demonstrated

by flooding colonies of the test organism on agar with 1N HCl to precipitate unhydrolyzed DNA. The presence of DNase is indicated by a clear zone surrounding a colony of organisms amidst the precipitated nucleic acid. This method was modified by Jarvis and Lawrence (1969). These investigators spread 1 ml of a hot mixture of agar, calf thymus DNA mixed with equal volume of 0.1 M Tris-HCl buffer and 0.005 M $CaCl_2$ for the assay of staphylococcal nuclease. A hole 2.7 mm in diameter was bored in the agar and 0.004 ml of the supernatant containing DNase was added. The slides were then dipped in 1 N HCl for 15 seconds after incubation at 37°C for 20 hrs and washed with water. The quantity of the staphylococcal nuclease present is measured by the diameter of the clear zones. The method was further modified by Smith et al. (1969) by using 0.005% concentration of methyl green as an indicator. Methyl green dye combines with highly polymerized DNA and so the nuclease activity is indicated by clearing of the dye around the colony.

Overlay method

The overlay method (Lachica and Deibel, 1969) is based on the ability of a fluorescent acridine orange dye combination with DNA forming a complex in which the dye molecules intercalate between successive layers of base pairs (Lerman, 1963). This interaction enhances the green fluorescence of the dye when observed under ultra-violet light. Lachica and Deibel (1969) discovered an overlay method which avoided the direct addition of acridine orange and nucleic acid to the growth medium and a heat stability determination of the enzyme could be afforded. A 24-hour semi-solid agar culture of S. aureus

was overlaid with acridine orange-DNase agar mixture and incubated at
1-3 hours. When viewed under ultraviolet light clear halos around
colonies producing DNase were observed. A dehydrated DNase test agar
is available from Difco (Detroit, Michigan) to which acridine orange is
added during preparation.

The method has one shortcoming: the relatively complex manipulation
necessitated by the use of an ultraviolet light and the sensitivity
of the acridine orange fluorescence to quenching by proteins.

Metachromatic agar-diffusion

A metachromatic agar-diffusion (MAD) microslide technique has been
developed by Lachica et al. (1971, 1972) for quantitative assay of
staphylococcal thermonuclease. In this method the metachromatic
properties of toluidine blue 0 are applied to detect thermonuclease.
The aptness of this approach was indicated by the development of a
toluidine blue-flooding technique by Streitfeld et al. (1962) to
investigate the extracellular DNase activity of various species or
strains of Pseudomonas.

The best known metachromatic basic dyes are toluidine blue 0 and
thionin. They are useful in histochemical studies because of their
deviance from Beer's law when dissolved in water (Michaelis and Granick,
1945; Lachica et al., 1969). They stain some histological morphological
elements such as cellular nuclei or the cytoplasma of lymphocytes. When
a sample containing thermonuclease enzyme is placed in a well cut in
the toluidine blue 0-DNA-agar mixture, a bright pink halo is obtained
after 4 hrs of incubation at 37°C. Lachica et al. (1972) assayed the

thermonuclease in milk demonstrating that neither protein nor opacity of the experimental material interferes with the measurements.

Elimination of the need for purification of the enzyme has made this method the most used in determination of thermonuclease activity. The method is suitable for the rapid, routine screening of large numbers of samples by a single laboratory worker. The MAD technique is more specific than both the HCl-flooding technique and overlay method because heat-labile enzyme of S. epidermidis and Micrococcus spp can be inactivated before testing.

Other tests

The serological specificity of thermonuclease elaborated by S. aureus strains of human origin has been demonstrated using three antisera and 407 strains of staphylococci from diverse human and animal sources (Lachica et al., 1979). This is based on the observation that thermonucleases produced by S. aureus are serologically distinct from the thermonucleases produced by other coagulase-positive staphy- lococci. Lachica and coworkers demonstrated that strains of S. aureus of biotypes A to D including enterotoxigenic and methicillin-resistant strains produced thermonucleases that were inhibited by sera A, B and C. Strains of biotypes E and F (S. intermedius) and the four coagulase- positive S. epidermidis strains had thermonucleases that were not at all inhibited by sera A and B and only at diultions of 1:16 and 1:14 of serum C was there inhibition of thermonucleases of S. intermedius and S. epidermidis strains respectively. These results indicate that thermonuclease seroinhibition test is a convenient and reliable means

for distinguishing S. aureus from other coagulase-positive staphylococci.

Another method for thermonuclease detection involves the colony overlay procedure (Lachica, 1976) by using TDA medium. Preheated plates with grown colonies are overlaid with 10 ml of molten TDA mixture. S. aureus are identified by a bright pink zone in 3 hrs at 37°C. This method is referred to as simplified thermonuclease (STN). Boothy et al. (1979) modified the disc overlay procedure of Lachica et al. (1969) to subject known heat-labile and heat-stable cultures grown on PCA plates to various time-temperature treatments to accurately differentiate heat-stable from heat-labile nuclease producing strains. It was found that 65°C for 2.5 hours denatured all heat-labile nucleases produced by bacteria other than S. aureus, but left thermonuclease intact.

Stadhouders et al. (1981) described a rapid and simple method for detection of staphylococcal thermonuclease in cheese. Discs of cheese are placed in petri dishes after which TDA mixture is poured carefully. After solidification of the agar and incubation at 37°C for 24 hrs, pink zones form around the discs of cheese if thermonuclease is present. The disadvantage of this method is because of its less sensitivity and long incubation time although it is simple especially when many samples have to be investigated.

Enzymatic detection of S. aureus in foods

Thermonuclease enzyme as a possible means for detecting food contamination by S. aureus was suggested by Chesbro and Auborn (1967). This was because nearly all pathogenic staphylococci produced this unique and remarkable heat-stable nuclease (Jeffries et al., 1957;

Elston and Fitch, 1964). The importance of a heat-stable indicator for the possible contamination by S. aureus is because enterotoxins are heat-stable and can cause food poisoning even after food has been cooked or reheated (Read and Bradshaw, 1966; Fung et al., 1973).

Measurement of thermonuclease activity is a sensitive means for the detection of foods contaminated with metabolic products of S. aureus. The usefulness of the thermonuclease as an indicator of such contamination is increased by the relative rapidity with which it can be conducted. The test is also simpler and less expensive to perform than an analysis of enterotoxins.

Park et al. (1978) evaluated the thermonuclease assay method on a variety of foods which were naturally or artificially contaminated with S. aureus, or to which staphylococcal thermonuclease had been added. In meats, poultry, dairy products, and sandwich fillings, the method allowed detection of as little as 10 ng (0.002 units) thermonuclease per gram. In raw ground beef, egg noodles, spaghetti and boiled egg yolk, the lower limit of detection was 50 ng thermonuclease per gram. Ahmed et al. (1983) reported detection of thermonuclease in unsalted pasteurized whey after 24 hrs at 37°C incubation when the S. aureus count was 1.6 x 10^8 cells per milliliter. Analysis of these samples revealed the presence of enterotoxin A. These results differ from those of Miller and Ledford (1977) who detected thermonuclease but not enterotoxin at 1.7 x 10^7 S. aureus per milliliter after 24 hours at 37°C.

Thermonuclease has been detected in sausage (Emswiler-Rose et al., 1980; Koupal and Deibel, 1978), cheese (Cords and Tatini, 1973), milk (Koupal and Deibel, 1978), ham slices and potato salad (Chesbro

Auborn, 1967). Detection of thermonuclease immediately implies that high S. aureus counts were present at one time in the food and that the presence of enterotoxin is possible. Low levels or complete lack of detection of thermonuclease may not in any case be a confirmation of absence of enterotoxin in food because production and recovery of the enzyme is influenced by the type of food.

The purposes of this study were (1) to adapt the pasteur pipette diffusion-agar system for quantification of thermonuclease activities, (2) to find any relationship between thermonuclease and coagulase production and (3) to study the use of thionin dye as an alternative to toluidine blue O in the agar mixture using this quantitative system.

MATERIAL AND METHODS

Preparation of Toluidine Blue-DNA-Agar Mixture

Preliminary experiments were conducted to determine the best combination of Toluidine Blue-DNA-Agar (TDA) mixture which would exhibit both good color contrast and a distinct precipitation band. The TDA mixture was prepared as described by Lachica et al. (1971) and modified by Ibrahim (1981). To 0.015 gm DNA (Difco) in a 250-ml Erlenmeyer flask, 50 ml of 0.01 M Tris (Hydroxymethyl) Aminomethane buffer at pH 10, 0.5 g NaCl, 0.5 g noble agar (Difco) and 1.5 ml of 0.5M $CaCl_2 \cdot 2H_2O$ were added. Before adding 0.15 ml of 0.1M Toluidine blue-O the mixture was boiled for 20 minutes until DNA and agar were completely dissolved and then cooled to 45°C. Pasteur pipettes of the same diameter were introduced into the TDA solution. By suction action, the TDA solution was brought to about half the length of the thinner portion of a pasteur pipette. The tip of the pipette was sealed with plasticine. The pipettes were then stored in a covered moist chamber at 4°C until when required.

Assaying the samples

To each tube containing TDA mixture 0.1 ml of supernatant liquid sample was added, a quick jerk of the pipette caused the liquid sample to come in contact with the TDA agar in the capillary tube (Figure 4).

The tubes were then covered with parafilm and incubated in a chamber at 50°C for 4 hours. After incubation, the length of the precipitation band in the TDA mixture, indicating the presence of thermonuclease

Figure 4.   Capillary tube agar-diffusion system for thermonuclease
          detection.

Parafilm

Pasteur   Pipette

Liquid   sample

Precipitation   band

Thermonuclease   agar
column

Plasticine   seal
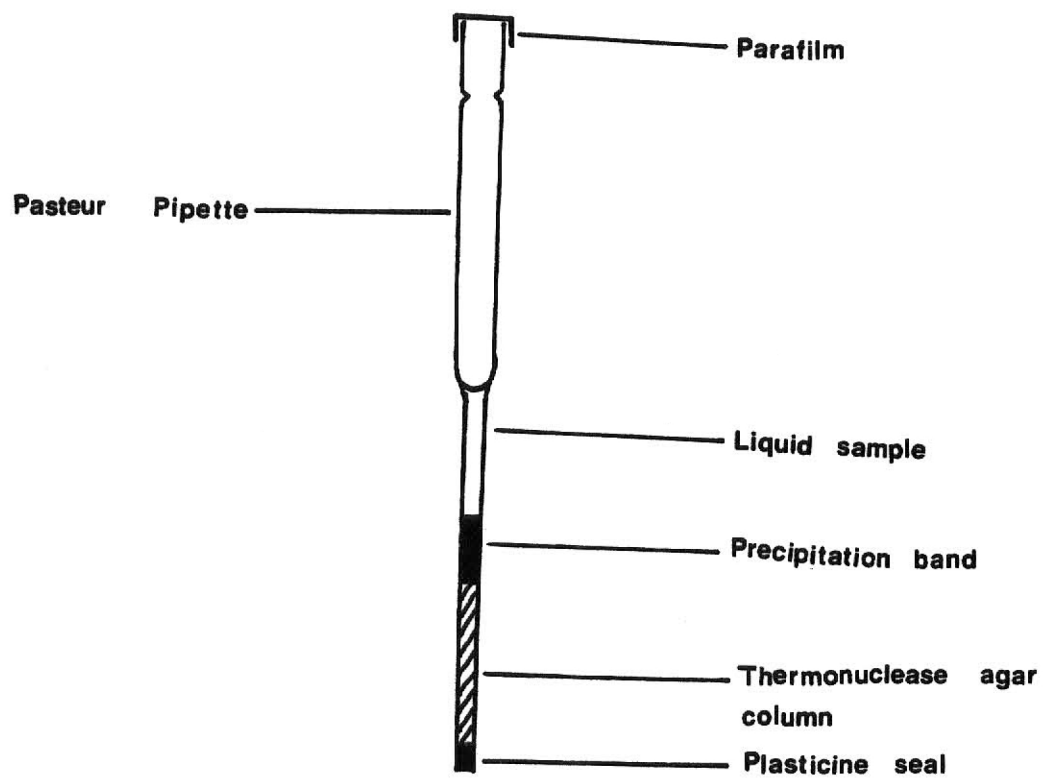
activity, was measured in millimeters by use of an optical micrometer (Bausch and Lamb, Inc., Rochester, N.Y.). All tests throughout this study were done in duplicate. All values reported are averages of duplicate samples.

Standard curve for thermonuclease

The standard curve was prepared using a purified Micrococcal nuclease (Sigma Chemical Co., St. Louis, MO) dissolved in 1 ml of distilled water containing 0.1% bovine serum albumin. One milligram of the standard enzyme (purified Micrococcal nuclease) contained 83 units of DNase activity or 0.083 units per 1.0 µg. A series of purified thermonuclease standard dilutions were prepared in Tris buffer solution at pH 10. The thermonuclease dilutions were as follows: 1:2, 1:4, 1:5, 1:8, 1:10, 1:16, 1:32, 1:50, 1:64, 1:100, 1:128, 1:256, 1:500 and 1:1000. The purified thermonuclease was heated at 100°C for 20 minutes before preparing serial dilutions.

The diluted samples were added to TDA mixture capillary tubes in duplicates and incubated at 50°C for 4 hours. After incubation, the precipitation band in the TDA was measured in millimeters and averaged for each dilution sample. Standard thermonuclease curve was plotted in concentration (Units/ml) against band length millimeters on a semi-log paper.

Organisms tested

The following strains of Staphylococcus aureus were tested with the capillary tube agar-diffusion and metachromatic agar-diffusion systems for thermonuclease and for coagulase capillary tube system determination:

1. MF 131
2. 5106 R
3. FAD 209 P
4. 137
5. 707-4 R
6. 100
7. 326
8. 472
9. 48 S pen R
10. 57-∅K
11. 5925
12. 639-451
13. 69129
14. 740-SEL
15. 8325-4∅11
16. 83 A
17. COLS
18. DU-4916
19. DUMAS
20. FRI 273
21. FRI 279
22. IS P2
23. PS 80
24. RN 1304(PI 169)
25. RN 2425
26. RN 450
27. S-6
28. S 6-262

All the cultures were obtained from the Food Products and Microbiology Laboratory, Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas. Identities of the S. aureus cultures were confirmed by using morphology and pigmentation, gram-reaction, thermonuclease and coagulase production. The characteristics of the organisms corresponded well with the descriptions in Bergey's Manual (Buchanan and Gibbon, 1974).

The cultures were kept in nutrient agar slants at 4°C and transferred every three months. Working cultures were prepared by aseptically transferring one loopful of culture from stock culture into 50 ml of brain heart infusion (Difco) and incubating in a waterbath shaker (125 rpm) at 37°C for 18 hours.

Thermonuclease and coagulase production by strains of S. aureus

Twenty-eight strains of S. aureus were transferred to 50 ml of brain heart infusion in 250 ml Erlenmeyer flasks and grown in a waterbath shaker (125 rpm) at 37°C for 18 hours. After incubation, the culture broths were boiled at 100°C for 20 minutes in an oilbath, cooled and then centrifuged at 10,000 x g (Beckman model J-21) at 4°C for 15 minutes. The cell-free supernatant portions of 0.1 ml were added into each of the duplicate TDA capillary tube agar-diffusion pipettes. The pipettes were then covered with parafilm and incubated at 50°C for 4 hours. After incubation, the length of the precipitation band was measured and averaged for each culture.

In order to ascertain the efficiency of capillary system versus a more conventional system of measuring thermonuclease activity, the metachromatic agar-diffusion (MAD) method of Lachica et al. (1971) was used as a comparison. Five milliliters of TDA mixture at 45°C was pipetted into 15 x 13 mm petri dishes and allowed to solidify on a levelled surface. The reagent was then cooled at 4°C before 6.3 mm diameter wells, about 2 cm apart, were covered with parafilm to prevent evaporation and incubated at 50°C for 4 hours. After incubation, the diameter of the pink zone developed around the wells were measured with calipers (two readings per well). A standard curve was constructed by adding varying known quantity of standards in the well then measure the resultant zone diameter.

Twenty-eight strains of S. aureus cultures, for the production of coagulase, were transferred into 250 ml Erlenmeyer flask containing 50 ml of brain heart infusion broth. The cultures were grown in a

waterbath shaker (125 rpm) at 37°C for 18 hours. The cells were removed by centrifugation at 10,000 x g (Beckman model J-21) for 15 minutes at 4°C.

The plasma-fibrinogen agar (PF) was prepared as described by Kohl and Johnson (1980) and modified for capillary tube agar assay by Ocasio and Fung (1983). In a 125 ml Erlenmeyer flask, 0.5 g of Noble agar (Difco) was dissolved in 25 ml of 0.01M sodium phosphate buffer with 0.6M NaCl at pH 7.2, heated until the solution became clear, and then tempered to 48°C. In another 125 ml Erlenmeyer flask, 0.15 g of fibrinogen was dissolved in 24 ml of the same buffer for 5 minutes; then 1.0 ml of plasma and 0.5 ml of a 1:100 solution of ethylmercurithioso-lecylic acid sodium salt were added. The temperature of the resulting PF solution was brought to 37°C in a waterbath. The PF solution was added to the agar solution, and the combined PF-agar solution was held at 40°C in a waterbath. Pasteur pipettes were introduced into the PF-agar solution and the suction brought to about half way into the thinner part of the pipettes. The tip of the pipettes was sealed with plasticine and stored in a chamber at 4°C for 12 hours.

The cell-free supernatants were added (0.1 ml of liquid sample) into the PF-agar. After a quick jerk of the pipette to make the liquid come in contact with the agar, the tubes were covered with parafilm and incubated at 37°C for 24 hours. At the end of incubation, the precipitation band length in the agar was measured in millimeters.

Comparison of the detection of thermonuclease by use of Toluidine Blue-and Thionin-DNA-Agar mixtures

Several organic dyes (pinacyanol chloride, methylene blue, thionin,

and acridine orange) were tested in the DNA-agar system to test whether another dye could be used for DNA-agar besides Toluidine blue. Preliminary data showed that thionin has good possibility for further testing. Thionin-DNA-Agar (ThDA) mixture was prepared by adding 0.015 g of 0.1M DNA (Difco), 0.5 g of NaCl, 0.5 g Noble agar (Difco), and 1.5 ml of 0.5M $CaCl_2:2H_2O$ to 50 ml of 0.01M Tris (hydroxymethyl) aminomethane buffer at pH 10 in a 125 ml Erlenmeyer flask. The mixture was boiled for 20 minutes until agar and DNA dissolved completely and then tempered to 45°C before adding 0.15 ml of 0.01M thionin dye. The mixture was sucked into the capillary tubes as described for TDA. A standard curve for ThDA was constructed using varying known concentration of purified Micrococcal nuclease into the ThDA then measure the resultant band lengths.

Cell-free supernatant prepared from twenty eight S. aureus strains was then added each to duplicates of TDA and ThDA mixtures in the pipettes. The pipettes were covered with parafilm and incubated at 50°C for 4 hours. At the end of incubation, the precipitation band lengths in the agar mixtures were measured.

TDA mixture shelf-life

A culture of S. aureus S 6-262 was grown in brain heart infusion at 37°C for 18 hours in a waterbath shaker at 125 rpm. The culture broth was centrifuged at 10,000 x g for 15 minutes at 4°C. The cell-free supernatant was boiled in an oil bath at 100°C for 20 minutes.

Then 20 ml of the supernatant was transferred with sacs made by tying off sections of cellulose dialysis tubing (molecular weight cutoff:

14,000, Fisher Scientific Co.) and then dialyzed against against 50% polyethylene glycol (M.W. 20,000, Fisher Scientific Co.) for 12 hours. The resultant aliquot was resuspended in 5 ml of Tris buffer and placed in 8 screw capped tubes (15 x 45 mm) in 1 ml portions. Four tubes were immediately frozen and the other four were stored at 4°C.

The samples (one from 4°C and one from the freezer) were assayed for thermonuclease activity after periods of 1 hour, 3, 6, and 9 weeks using the TDA mixture capillary tubes. The frozen samples were thawed before assaying for thermonuclease activity. The precipitation bands were measured after incubation at 50°C for 4 hours.

Toluidine Blue O- and Thionin-DNA-Agar mixtures sensitivity

A concentrated culture broth of S. aureus S 6-262 was diluted as follows: 1: 0, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256. The diluted samples were added to TDA and ThDA in duplicates and incubated at 50°C for 4 hours. After incubation, the precipitation bands were measured to determine the sensitivity of toluidine blue and thionin dyes to detect thermonuclease activity.

Inactivation of thermonuclease

To study heat inactivation of thermonuclease, 1.2 ml of the cell-free boiled (100°C for 20 min) supernatants prepared from S. aureus strains S 6-262, 740-SEL and 472, selected on the basis of large thermonuclease production, was pipetted into each of a number of ampoules (1.2 size, Wheaton Scientific Co.). The ampoules were heat sealed. Sets of sealed ampoules for each S. aureus strains were then placed into an oilbath maintained at 80, 100 and 121°C. Heat up times were as previously

determined by Fung et al. (1973) to be 70 S, 40 S and 40 S for 80,
100 and 121°C oil baths respectively. The ampoules were heated for time
intervals from 20 minutes to 5 hours. The ampoules were then removed,
cooled in an ice bath for 10 minutes, and then opened. A 0.1 ml amount
of heated sample was added to duplicate capillary tube agar-diffusion system
pipettes containing TDA mixture. The pipettes were covered with parafilm
and incubated at 50°C for 4 hours. Thermonuclease activity was estimated
by matching precipitation band lengths with those on a standard curve. The
results of heated samples were reported as percentage of activity compared
with unheated control.

Thermonuclease recovery from liquid food

Concentrated portions of 5 ml of cell-free supernatant S. aureus
strains of S-6 and S-6-262 thermonuclease were added to 25 ml of homemade
chicken broth. The inoculated broth was stored at 4°C for 12 hours for
complete uptake of the enzyme into the food.

A 10 ml inoculated broth sample was boiled at 100°C for 20 min and
then dialyzed against 50% polyethylene glycol for 12 hours at 4°C.
The resultant aliquot was resuspended in 2 ml of tris buffer. Con-
centrated sample portions of 0.1 ml were added to each of the duplicate
pipettes containing TDA mixture for thermonuclease activity determination.

In another determination of thermonuclease recovery, two loopfuls
of S. aureus strains S-6 and S-6-262 were aseptically inoculated in 100 ml
of chicken broth in 250 ml Erlenmeyer flask. After incubation at 37°C
for 18 hours in a water bath shaker, the broth was centrifuged at 10,000 x
g at 4°C for 15 minutes followed by boiling at 100°C for 20 minutes in an
oil bath. Then 10 ml cell-free supernatant was dialyzed against 50%

polyethylene glycol and resuspended in 2 ml of tris buffer. The
concentrate supernatant was then assayed for thermonuclease activity.

Detection of enterotoxin B from liquid food

Portions of 4 ml concentrated cell-free supernatants of S. aureus
S-6 and S 6-262 were added to 20 ml of chicken broth. The samples
were stored at 4°C for 12 hours for complete uptake of the cell-free
supernatant into the food. The broth samples were concentrated against
50% polyethylene glycol and resuspended in 0.5 ml of phosphate buffer at
pH 7.4. The samples were then assayed for enterotoxin activity.

The enterotoxin B activity was determined according to the method
of Fung and Wagner (1971). A 1:10 dilution of antiserum agar was prepared
by mixing 0.8 ml of antiserum for staphylococcal enterotoxin B (Sigma
Chemical Co.) with 3.2 ml of phosphate buffer saline (0.02M, pH 7.4)
and 4.0 ml of melted and tempered (48°C) 1% noble agar (Difco). By
suction a small volume of the agar-antiserum mixture was brought to
approximately half the length of the thinner portion of pasteur pipette.
The tip of the pipette was sealed with plasticine.

To each capillary tube containing agar-antiserum mixture, 0.1 ml
of concentrated sample of enterotoxin B prepared (as described above)
from S. aureus S-6 and S 6-262 were added to capillary tubes and given
a quick jerk to cause the liquid sample to come in contact with the
antiserum agar in the capillary tube. The pipettes were covered with
parafilm and incubated at 37°C for 24 hours. The presence of precipitation
band in the antiserum agar was examined after incubation. Quantitation
of enterotoxin B was achieved by comparing the average band with the

band length on the standard curve to obtain the concentration in μg/ml.

Detection of thermonuclease, coagulase, and enterotoxin B in separate agar and in one tube with combined agar

Cell-free concentrated supernatants from S. aureus strains S-6, S-100, S 6-262, COLS and DU-4916 were assayed for thermonuclease, coagulase and enterotoxin B using TDA, PF-agar and antiserum B agar respectively. Then, TDA was combined with antiserum B agar and TDA-antiserum B-PF agar at pH 7.4 in triplicate capillary tubes labelled "Super tubes". TDA tubes for thermonuclease activity were incubated at 50°C for 4 hours, PF-agar and antiserum B agar tubes were incubated at 37°C for 24 hours. The "Super tubes", containing combined agar, were in two sets, one set incubated at 50°C for 4 hours and the other at 37°C for 24 hours. After incubation, the precipitation band lengths in TDA, PF agar, antiserum and combined agar were measured in millimeters.

RESULTS

Development of capillary tube agar-diffusion system

The standard curve (Fig. 5) for the detection of thermonuclease activity was developed using TDA mixture and serially diluting a purified commercial micrococcal enzyme of known concentration. Portions of the diluted enzyme were added in duplicate to capillary tubes containing TDA mixture. After incubation at 50°C for 4 hours, the precipitation band in the agar was measured in millimeters by an optical micrometer.

The regression line fitted the equation $Y = 3.30 + 3.38X$ where Y equals precipitation band length in millimeters and X equals logarithm of the thermonuclease concentration in units per milliliter. A linear relationship was observed between the length of the precipitation band and the logarithm of the thermonuclease concentration. This relationship was in the domain 0.14 to 34.86 U/ml and over the range of 1 to 8.5 mm. The correlation coefficient of the line was high ($r = 0.98$).

The precipitation band was read immediately after 4 hours incubation because the clarity of the band was observed to diminish with longer incubation period.

Production of thermonuclease and coagulase by S. aureus strains

A total of 28 S. aureus strains were tested for thermonuclease (by TDA and MAD methods) and coagulase production (Table 3). The strains were cultured in brain heart infusion broth at 37°C under constant shaking for 18 hours. The culture broths were centrifuged,

Figure 5.   Standard curve for thermonuclease activity by capillary
tube agar-diffusion system using TDA.
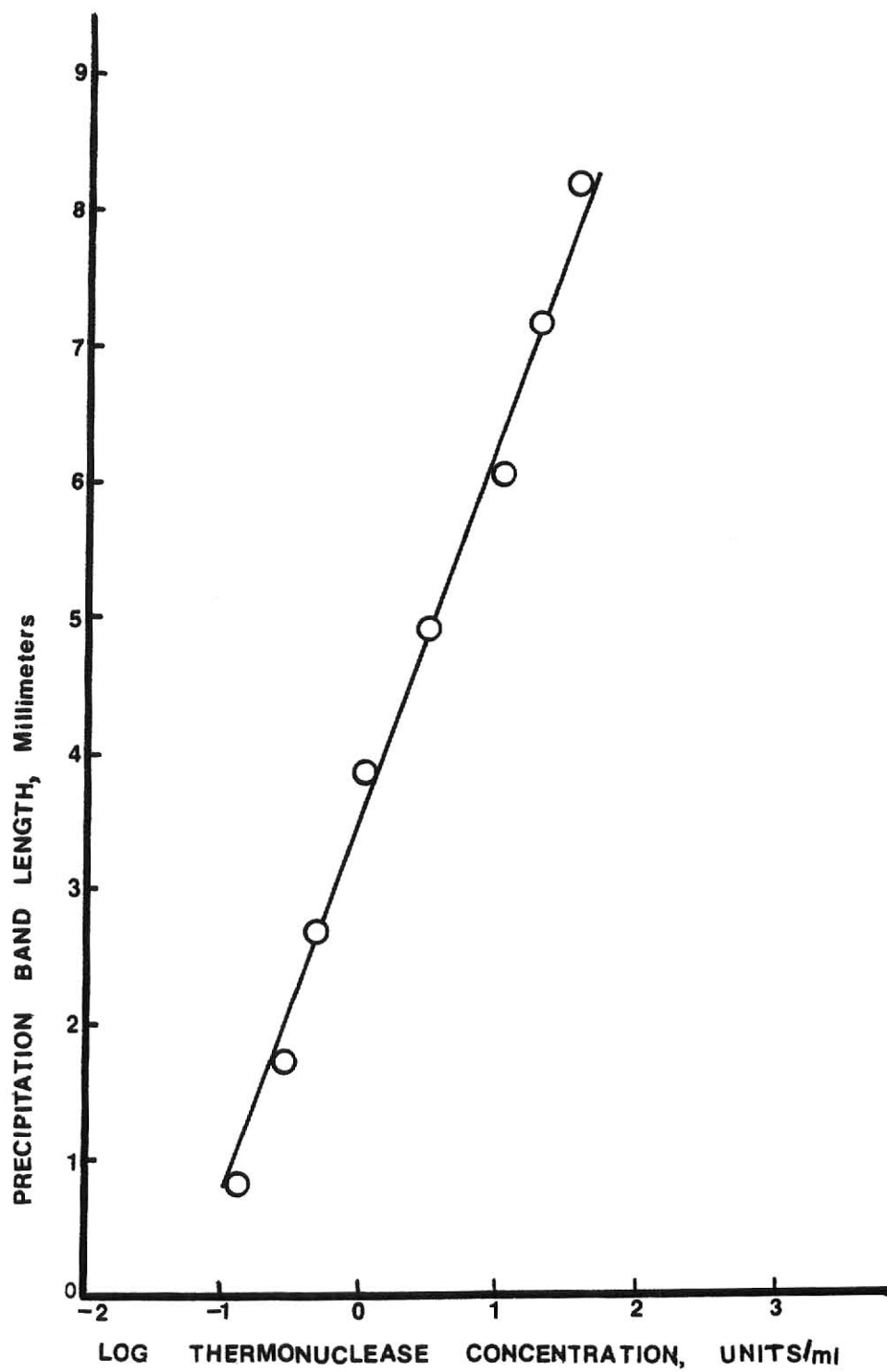
Table 3. Thermonuclease activities of S. aureus measured by capillary tube agar-diffusion method and metachromatic agar-diffusion technique and coagulase activities measured by capillary tube method.

S. aureus strain	Thermonuclease activity U/ml		Coagulase activity U/ml
	TDA Capillary Method	MAD Method	
100	1.78	2.34	12.02
137	0.58	1.02	4.65
326	2.69	4.89	7.41
472	10.12	15.85	1.44
48 S pen R	5.01	7.75	3.98
5106 R	0.25	0.12	2.63
57 - ø K	5.37	8.15	2.29
5925	2.88	4.16	1.74
639-451	3.31	5.00	13.18
69129	1.26	2.29	3.72
707-4R	0.38	0.27	2.75
740-SEL	7.41	10.56	2.00
8325 - 4 ø 11	2.00	2.50	1.12
83 A	0.72	2.00	12.59
COLS	0.53	1.14	3.98
DU-4916	6.03	6.40	5.89
DUMAS	1.78	1.50	1.00
FAD 209 P	2.24	1.86	1.78
FRI 273	12.02	15.39	3.55
FRI 279	7.41	10.34	3.02
IS P 2	1.17	1.38	1.17
MF 31	4.07	4.40	2.63
PS 80	5.37	9.85	2.19
RN 1304	6.03	10.01	5.25
RN 2425	4.68	4.46	15.85
RN 450	5.01	9.72	17.78
S-6	5.37	12.28	2.19
S 6-262	15.14	30.60	7.94

and the supernatants were collected and used as the enzyme preparation.
All strains were positive for thermonuclease and coagulase production.
The differences between individual strains in the thermonuclease and
coagulase production was greater for thermonuclease than coagulase,
factors in excess of 60 and 17 times respectively.

Strain 5106 R was the lowest thermonuclease producer with 0.25 U/ml
(TDA capillary method). Strain S 6-262 produced the most thermonuclease,
15.14 U/ml (TDA capillary method). Strains DUMAS and RN 450 had the
lowest (1.00 U/ml) and highest (17.78 U/ml) coagulase activities,
respectively. MAD method for the detection of thermonuclease showed a
higher coefficient of variation (CV = 104.3) compared to capillary
tube agar-diffusion method (CV = 85.4). A significant relationship
(p = 0.0001) between TDA capillary tube and MAD methods was determined.
TDA capillary tube readings differed from MAD results by a 1.6 ratio.
The reason for this difference may be due to a fitting error in the
standard curve.

Although all strains produced thermonuclease and coagulase, no
linear relationship was established in the S. aureus strains tested.

Toluidine Blue-DNA-Agar mixture shelf life

In order to ascertain the storage stability of TDA, two sets of
concentrated cell-free supernatants were stored, one set at 4°C and
the other in a freezer. Each set had six samples. A sample from each
set was assayed for thermonuclease activity in TDA mixture prepared and
stored at 4°C in a closed chamber to avoid dehydration for a period of up
to 9 weeks. The results summarized in Table 4 show that after 9

Table 4.   Shelf life of TDA mixture in capillary tubes

	Thermonuclease activity[*] (Units/ml)			
Test liquid	1 hr	3 weeks	6 weeks	9 weeks
4°C	52.48	53.70	53.70	52.60
Frozen	54.95	52.80	53.09	53.30

*TDA mixture stored at 4°C.

weeks TDA mixture was still stable to be used for the detection of thermonuclease without any loss in activity. The two sets of thermonuclease were stored at different temperatures to check if any change in thermonuclease activity was due to the loss of activity by the enzyme rather than the TDA mixture. Since all results were essentially identical, it is concluded that both enzyme and TDA mixture are stable in cold storage.

Quantitative measurement of thermonuclease activity using Thionin-DNA-agar and Toluidine blue-DNA-agar mixtures

All the $\underline{S}$. $\underline{aureus}$ strains examined showed thermonuclease activity in Thionin-DNA-Agar (ThDA) and TDA. Table 5 shows the results obtained by testing 28 strains grown in brain heart infusion broth, centrifuged and assayed for thermonuclease activity using both the (ThDA) and TDA capillary tube agar-diffusion method. Quantitation of thermo-nuclease activity in ThDA was by preparing a standard curve of known concentration of a purified micrococcal nuclease (Fig. 6). The highest and lowest thermonuclease activities for TDA and ThDA media were shown by strains S 6-262 and 5106 R respectively. The correlation of the two dyes was high ($r = 0.95$, $p = 0.0001$). The average ratio of TDA to ThDA results was 1.18. This ratio difference may be due to the fitting error of the standard curves.

Sensitivity tests of TDA and ThDA

A concentrated cell-free supernatant from $\underline{S}$. $\underline{aureus}$ strain S 6-262 was diluted serially and assayed for thermonuclease activity to determine the sensitivity of TDA and ThDA to detect the presence of thermonuclease. This was intended to ascertain the usefulness of each media in detecting low amounts of the enzyme in dilute samples. As is shown in Table 6,

Figure 6. Standard curve for thermonuclease activity by capillary

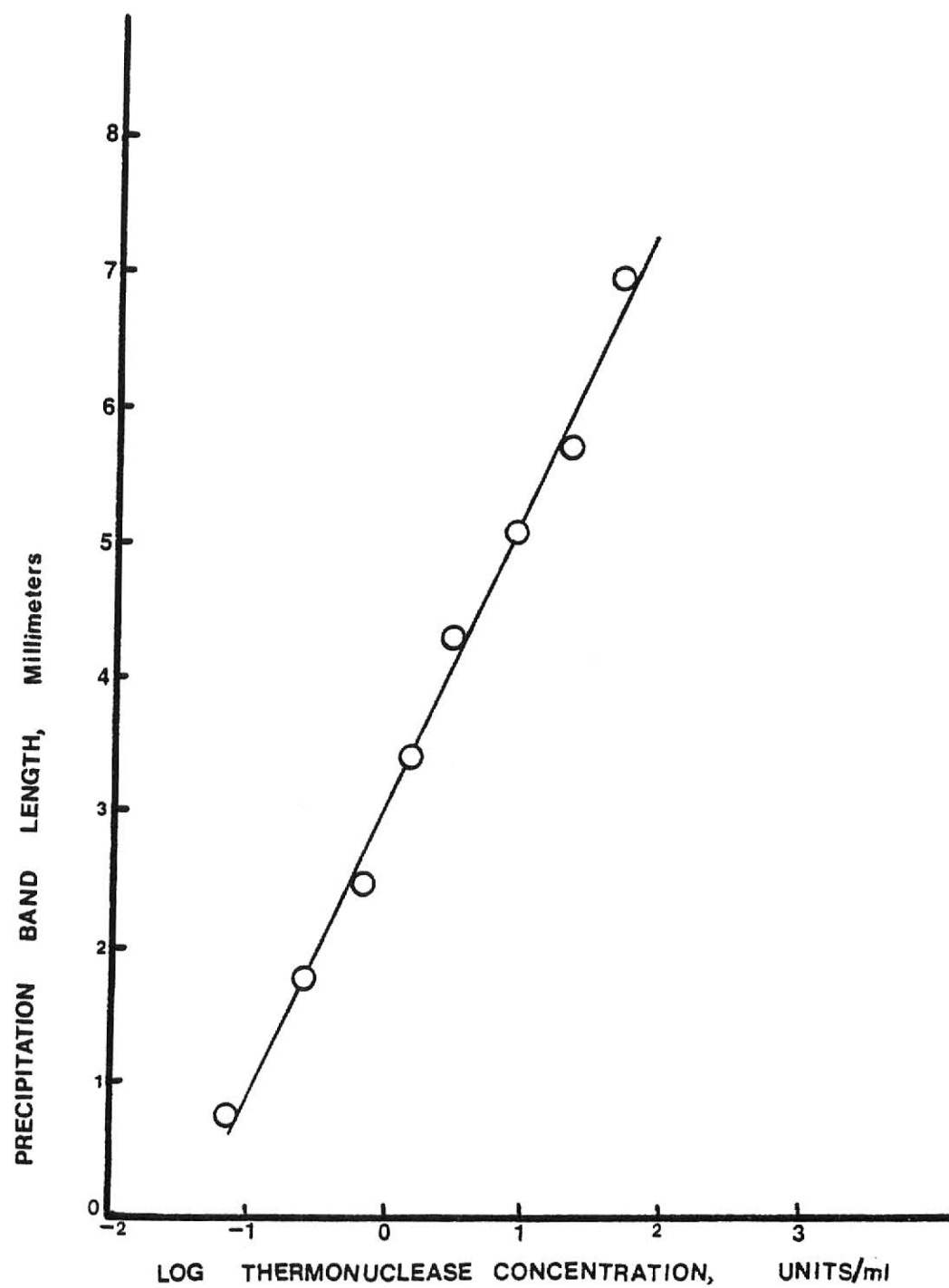tube agar-diffusion system using ThDA.

Table 5. Quantitative detection of thermonuclease activities by
two dyes.

S. aureus strain	Thermonuclease activity U/ml	
	TDA[a]	ThDA[b]
100	1.78	1.56
137	0.58	0.45
326	2.69	2.35
472	10.12	8.40
48 S Pen R	5.01	4.80
5106 R	0.25	0.15
57 - ø K	5.37	4.50
5925	2.88	2.41
639-451	3.31	3.00
69129	1.26	0.90
707-4 R	0.38	0.45
740-SEL	7.41	7.48
8325-4 ø 11	2.00	1.80
83 A	0.72	0.61
COLS	0.53	0.40
DU-4916	6.03	5.12
DUMAS	1.78	1.60
FAD 209 P	2.24	2.85
FRI 273	12.02	9.89
FRI 279	7.41	6.18
IS P2	1.17	1.00
MF 31	2.04	1.63
PS 80	5.37	4.60
RN 1304	6.03	5.15
RN 2425	4.68	3.89
RN 450	5.01	4.20
S-6	5.37	4.60
S 6-262	15.14	13.18

[a]TDA = Toluidine blue-DNA-agar

[b]ThDA = Thionin-DNA-agar

Table 6. Sensitivity of TDA and ThDA to detect thermonuclease
activities of $\underline{S}$. $\underline{aureus}$

	Thermonuclease activity (mm)	
Dilution	TDA	ThDA
1:0	8.45	7.00
1:2	7.30	5.9
1:4	7.00	5.0
1:8	6.60	4.3
1:16	5.90	3.4
1:32	4.80	2.6
1:64	3.70	1.7
1:128	2.50	1.0
1:256	1.50	0*

*No thermonuclease activity was evident.

the thermonuclease detection band length in ThDA was less than in TDA at 1:0 dilution. At 1:256 dilution no thermonuclease activity was observed in ThDA mixture while TDA showed 1.5 mm precipitation band indicating an activity of the enzyme.

Since the diffusion bands are longer in TDA compared with ThDA and lower levels of enzyme (higher titer) were detectable in TDA it is concluded that TDA is still the superior agar system for thermonuclease detection.

Inactivation of thermo-stable nuclease

Thermal-destruction curves of thermonuclease were prepared to determine the thermal stability of this enzyme. Concentrated cell-free supernatants from S. aureus strains 472, 740-SEL and S 6-262 that had shown earlier to produce large quantities of thermonuclease were heated at 80, 100 and 121°C for a time interval ranging from 20 minutes to 5 hours. Figures 7, 8 and 9 show the thermal inactivation curves of thermonuclease at 80, 100 and 121°C. After 5 hours of heating at 80°C thermonuclease retained 4.5%, 7.0% and 7.0% residual activity for samples prepared from S. aureus strains S 6-262, 740-SEL and 472, respectively. At 100°C, 1.5%, 2.0% and 2.0% residual thermonuclease activity still remained after heating the samples for 5 hours. Heating at 121°C inactivated thermonuclease activity completely after 3 hours for all samples.

Recovery rates of thermonuclease and enterotoxin from liquid food

Since thermonuclease is heat stable and can be detected relatively easily, recovery of the enzyme was performed to ascertain the usefulness

Figure 7. Inactivation of thermonuclease from <u>S</u>. <u>aureus</u> S 6-262.
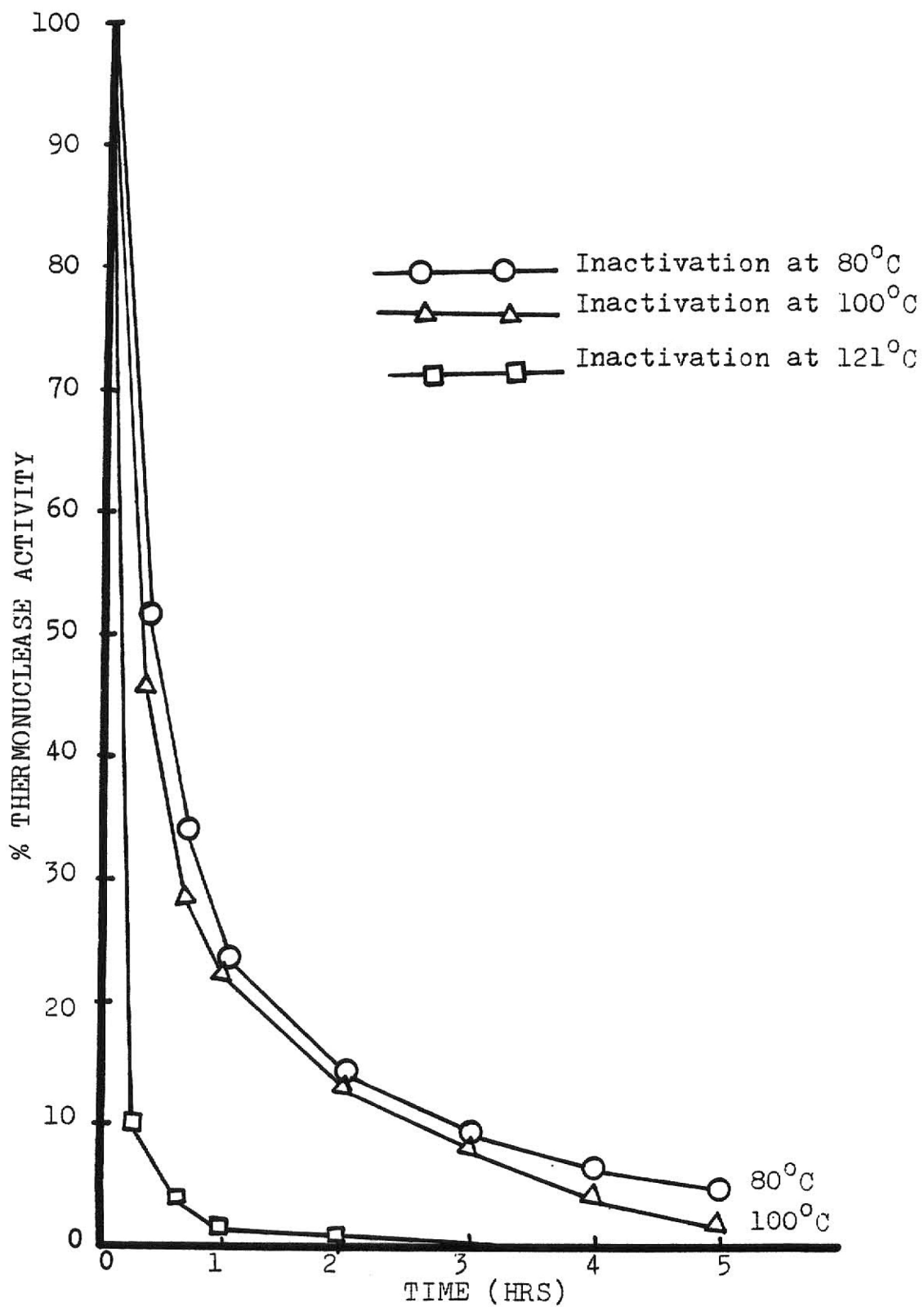
Figure 8. Inactivation of thermonuclease from <u>S</u>. <u>aureus</u> 740-SEL.
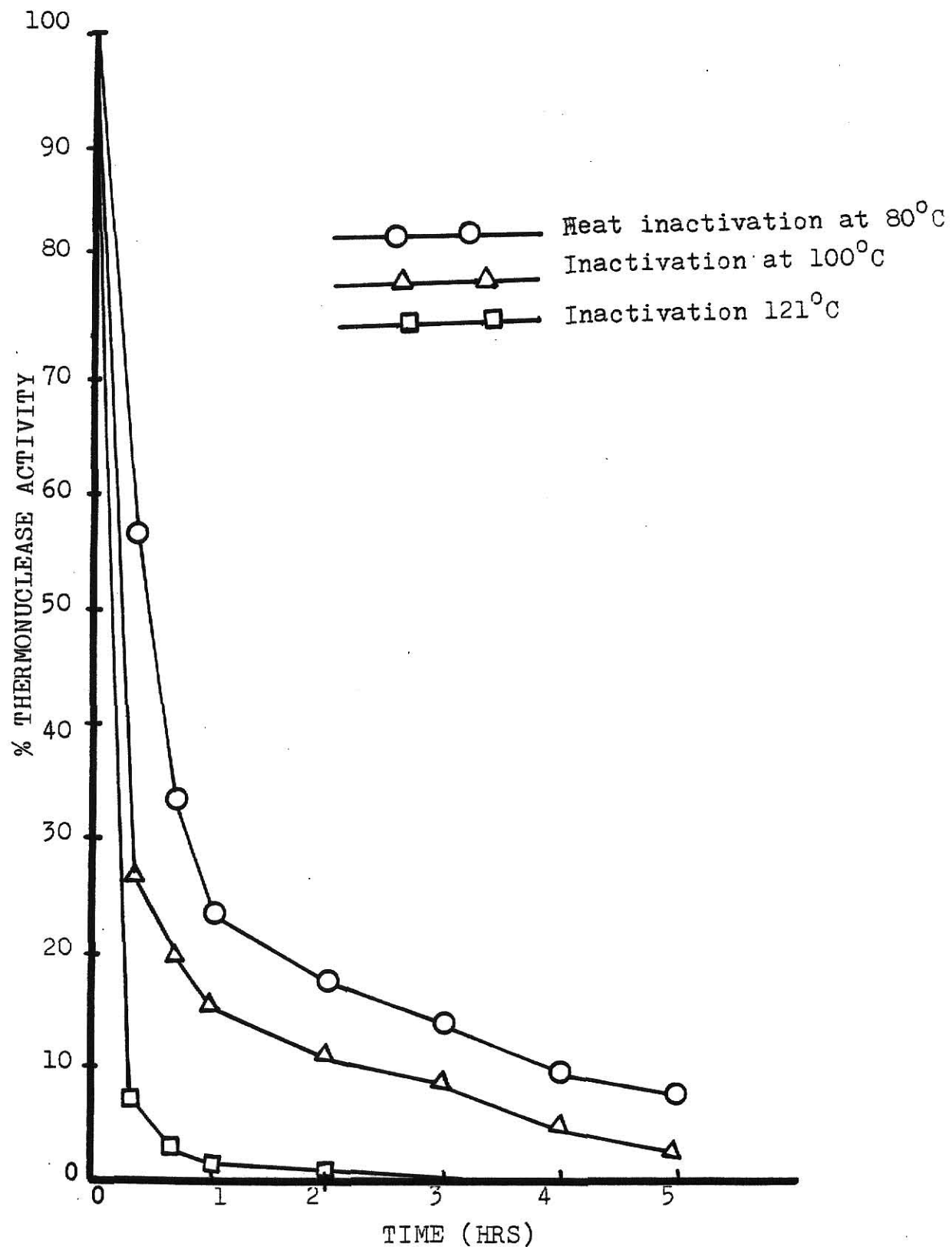
Figure 9.   Inactivation of thermonuclease from S. aureus 472.
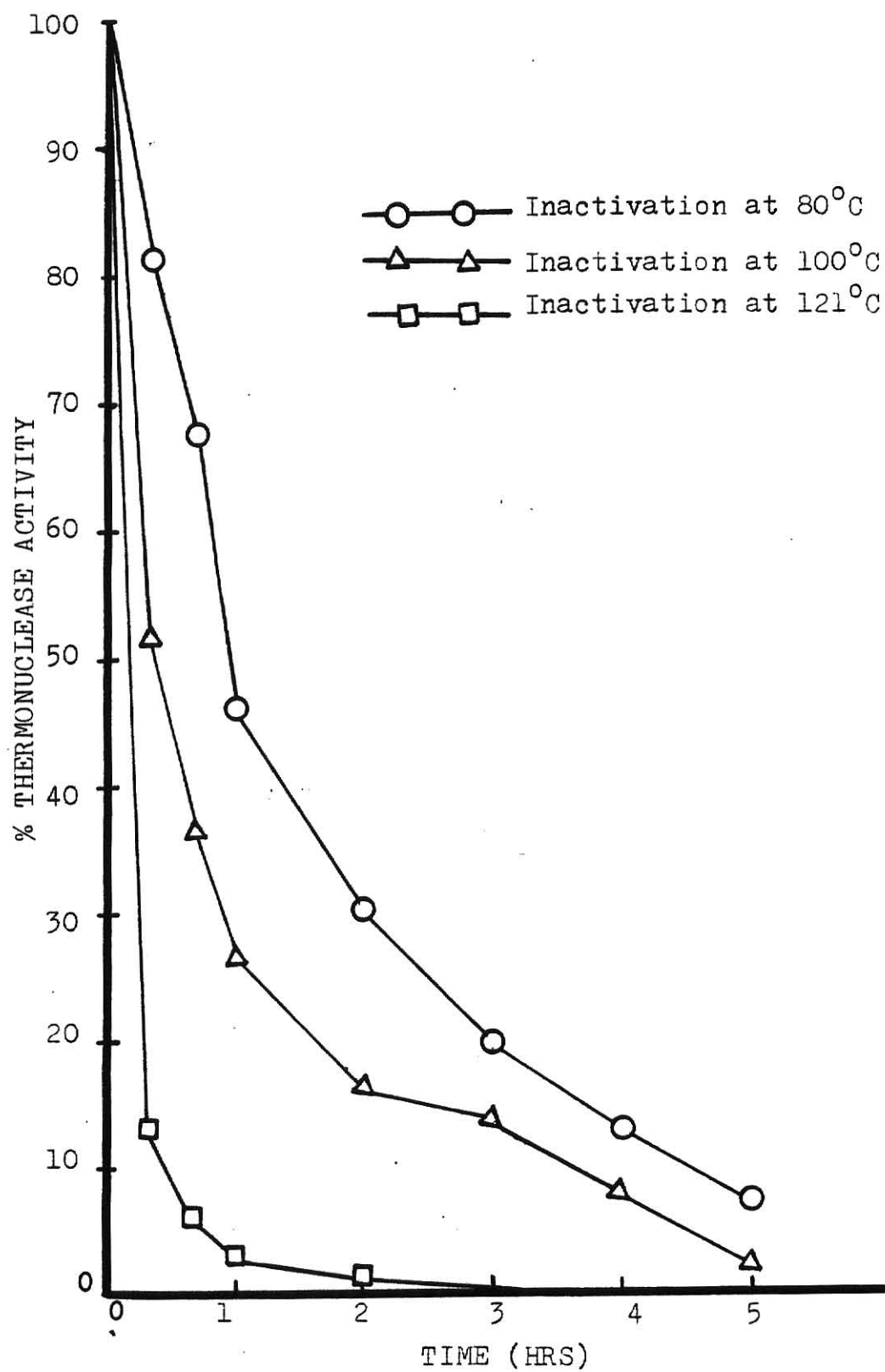
Table 7. Detection of thermonuclease and enterotoxin B from chicken broth.

Strain	Amount added		Amount recovered		% recovery		Cells grown	
	Thermo-nuclease U/ml	Entero-toxin μg/ml	Thermo-nuclease U/ml	Entero-toxin μg/ml	Thermo-nuclease	Entero-toxin	Thermo-nuclease U/ml	Entero-toxin μg/ml
S-6	12.10	10.00	7.08	3.56	58.51	35.6	1.12	0.56
S 6-262	25.70	0*	16.00	0*	62.26	0*	1.86	0*

*No enterotoxin B activity was evident.

of thermonuclease as a means of detecting food contamination by S. aureus at one time. Table 7 shows the recovery of both thermonuclease and enterotoxin B from chicken broth. Thermonuclease was detected in all samples of foods in which cell-free supernatant was added and those contaminated by inoculation of 2 loopfuls of viable S. aureus strains S-6 and S 6-262. The thermonuclease recovery rates were 58.51% and 62.26% from the original thermonuclease concentration of 12.10 and 25.70 U/ml. Enterotoxin B was detected in both samples of foods. The recovery rate was 35.6% for S-6 strain.

Detection of thermonuclease, coagulase and enterotoxin B separately and in one tube with combined agar

Table 8 shows the activities of five S. aureus strains in TDA, PF-agar, antiserum agar, antiserum agar-TDA and antiserum PF-TDA agar. This test was intended to serve as a diagnostic method whereby activity of any one property of S. aureus (thermonuclease, coagulase and/or enterotoxin production) can be used as an indication of possible presence of S. aureus. All strains were positive for thermonuclease, coagulase and enterotoxin B except for strain S 6-262 that did not show any activity in antiserum B agar. In this study the units/ml activity of thermonuclease and coagulase as well as μg/ml of enterotoxin B could be determined in the individual agar system. However, in the combined system such measurement cannot be determined, instead the presence of migration band indicates the occurrence of one or more metabolites of S. aureus in the supernatants.

Table 8. Determination of thermonuclease, coagulase and enterotoxin
B in TDA, PF-agar and Antiserum agar separately and in TDA-
antiserum agar and TDA-PF-antiserum agar combined media

	Staphylococcus aureus strains (mm)				
	S-6	S-100	S 6-262	COLS	DU-4916
Thermonuclease[a]	6.20	5.80	8.35	6.85	6.80
Coagulase[b]	4.80	4.55	3.80	4.15	4.40
Enterotoxin B[b]	6.0	4.70	0	4.25	3.40
50°C/4 hrs	2.50	2.95	4.60	3.95	2.75
TDA + Antiserum agar					
37°C/24 hrs	7.25	7.95	8.75	8.20	5.45
50°C/4 hrs	2.40	3.00	4.50	4.2	2.90
TDA + Antiserum + PF agar					
37°C/24 hrs	7.10	6.80	8.70	8.05	5.30

[a]Incubated at 50°C for 4 hrs

[b]Incubated at 37°C for 24 hrs

DISCUSSION

Development of capillary tube agar-diffusion system

The radial-diffusion test on agar plate was originally developed in order to assay the potency of antibiotics. The test was later applied to determine the activity of various enzymes such as amylases, proteases and lipases (Mottonen, 1970). The enzyme solution is dispensed in small volumes into holes cut in the agar. The diffusing enzyme acts on the substrate to produce a visible reaction around the spot. The radial-diffusion method was modified by Fung and Wagner (1971) for immunological determination of staphylococcal enterotoxins and Ocasio and Fung (1983) for the determination of staphylocoagulase activity.

In this study, the TDA and ThDA were adapted into a capillary tube agar-diffusion system to determine thermonuclease activity by single gel diffusion. Figures 5 and 6 show straight-lines linear relationship observed between the precipitation band length and the logarithm of the thermonuclease concentration. The working range in this system was between 0.14 U/ml and 34.86 U/ml.

Production of thermonuclease and coagulase by S. aureus

All strains of S. aureus examined in this study produced both thermonuclease and coagulase. The production of thermonuclease and coagulase are important properties that may be useful for identifying these strains. Different strains of S. aureus produce varying amounts of thermonuclease as is coagulase. No significant relationship (r = .005) exists between coagulase and thermonuclease quantitative production. This is in agreement with Rayman et al. (1975).

Numerous investigators have suggested the use of thermonuclease test for routine testing of foods for possible contamination by S. aureus. This possibility was applied in the recovery of thermonuclease from liquid food. Thermonuclease was easily detected from artificially contaminated chicken broth. The enzyme was also easily recovered from chicken broth inoculated with viable S. aureus. Enterotoxin B was detected from the food inoculated with S. aureus.

The results of this study show that capillary tube agar-diffusion system is a useful tool when determining the concentration of thermo-nuclease and coagulase produced by S. aureus strains. Although no definite statement can be made about the role of thermonuclease production in staphylococcal pathogenicity, it is of interest that all strains be regarded as potentially pathogenic if they produce this enzyme alone or in addition to coagulase. However, negative results for thermonuclease and/or coagulase is no guarantee of non-pathogenicity. The simplicity, rapidity, inexpensiveness, small sample volume required for analysis and lack of ambiquity of the reaction to result in misreading of the thermonuclease test make capillary tube agar-diffusion system an important research tool. Based on these advantages, and the results in this study, thermonuclease test is as important as coagulase test. The usefulness of the thermonuclease test as an indicator of contamination of S. aureus is increased by the relatively short incubation period (4 hrs.) as compared to coagulase test (4-24 hrs) and the laborious and time consuming enterotoxins analysis. The reagents and equipment (pasteur pipettes) are readily available cheaply to most laboratories.

Table 8 shows the activities of thermonuclease, coagulase and enterotoxin B in separate and combined agar. Any activity observed in the combined agar (TDA-PF-agar-antiserum agar) could be taken as a possible presence of thermonuclease, coagulase and/or enterotoxin. This may serve as a useful screening method for the contamination of S. aureus.

In this study, a number of advantages were noted for the capillary tube agar-diffusion method compared to radial-diffusion (MAD). Dehydration of the media in capillary tubes is less likely to occur compared to tests on petri dishes or microslides used for MAD method. Any evaporation is likely to affect the degree of enzyme diffusion because of more solidification of the agar media. The uniformity of the holes cut in the agar also depends on the length of time left to solidify and the temperature. Measurement of the precipitation band lengths in capillary tubes is much easier and more accurate with an optical micrometer than with calipers or magnification lenses attached to a scale that are commonly used in the MAD method.

Comparison of Toluidine Blue- and Thionin-DNA-agar mixtures to detect thermonuclease

Thionin and Toluidine Blue (TB) are some of the commonly used histochemical dyes. In the radial-diffusion agar, the metachromatic properties of these dyes are exploited to detect thermonuclease activity.

The aptness of this approach was first indicated by the development of a TB-flooding technique by Streitfeld et al. (1962). The nature of the normal coloring effect of a basic dye with nucleic acid may be inferred from the fact that the dye, when combined with nucleic acid, deviates from Beer's law, in contrast to its behavior in aqueous

The purpose of this experiment was to study if thionin dye incorporated in DNA-agar mixture could detect thermonuclease activity as well or better than toluidine blue. As is shown in Table 5, Thionin-DNA-Agar (ThDA) mixture showed a shorter precipitation band length and as the enzyme was diluted, the sensitivity to detect low levels of the enzyme was drastically reduced compared to TDA. The color contrast was not as distinct as in TDA mixture. This may be explained by the fact that different dyes have different straining abilities. Thionin as a dye to detect thermonuclease deserves further studies.

TDA mixture shelf life was determined to be remarkable stable (Table 4). After more than 2 months, the TDA mixture in capillary tubes was still stable and produced satisfactory results. This is in agreement with Lachica et al. (1969). The toluidine blue inhibitory properties towards gram-positive bacteria may be attributed partly to this TDA stability.

Heat stability of thermonuclease

Heat stability of thermonuclease has been reported by several investigators (Cunningham et al., 1956; Weckman and Catlin, 1957;

Lachica and Deibel, 1969; Erickson and Deibel, 1973a). The results
in this investigation are in agreement with heat stability of thermo-
nuclease. It was desirable in this study to know the levels of thermal
stability of thermonuclease to know whether it can withstand the high
temperatures and times, similar to those utilized during cooking.

The heat stability profiles of thermonuclease were compared to
those by Fung et al. (1973) for enterotoxin B and Ocasio and Fung (1983)
for staphylocoagulase. Thermonuclease showed high degree of stability
when heated at 80, 100 and 121°C. As is shown in figures 7, 8 and 9,
the degree of activity decreases with longer exposure time at each
temperature. This behavior is similar to that reported by Fung et al.
(1973) for enterotoxin B and Ocasio and Fung (1983) for staphylocoagulase.
At 121°C after 20 minutes, 9.5%, 7.5% and 12.0% residual activity was
found for strains S 6-262, 740-SEL and 472 respectively. This is
relatively similar to 10% residual activity after 34 minutes heating
at 120°C reported by Erickson and Deibel (1973a). No increased loss
of activity was observed at 80°C than at 100°C as has been reported for
enterotoxin B and C (Fung et al., 1973; and Jamlang et al., 1971).
Fung et al. (1973) attributed this anomaly phenomenon to the formation
of low temperature protein aggregates at 80°C. However, no such
observation was noted in this investigation for thermonuclease. This
agrees with a report by Ocasio and Fung (1983) on coagulase inactivation.
Thermonuclease and coagulase, although stable to different degrees,
do not exhibit formation of aggregates of protein molecules at low
temperatures such as enterotoxins B and C.

That thermonuclease can withstand heating at 80 and 100°C

for 5 hours and be inactivated after 3 hours at 121°C explains the uniqueness and remarkable stability of this enzyme. The survival of the enzyme in high temperatures for a long time (5 hrs.) makes it a useful property for screening possible staphylococci contamination of foods.

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CAPILLARY TUBE AGAR-DIFFUSION SYSTEM FOR

DETECTION OF STAPHYLOCOCCAL THERMONUCLEASE

by

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The detection of Staphylococcus aureus in food is essential for the prevention of food poisoning. The coagulase test is the most widely used method for screening the presence of staphylococci. The detection of heat-stable nuclease produced by strains of S. aureus has been suggested as a possible alternative to coagulase to detect food contamination. This enzyme (thermonuclease) has a unique property of withstanding the normal cooking temperatures so that the presence or possible presence of S. aureus at one time can be detected.

The metachromatic agar-diffusion technique described by Lachica et al. (1971) is based on radial diffusion of thermonuclease through agar containing Deoxyribonucleic acid (DNA) as a substrate and Toluidine Blue dye indicator. In this study, Toluidine blue-DNA-agar (TDA) mixture was adapted into a capillary tube agar-diffusion system based on a single gel diffusion of enzymes. In this system, a liquid sample containing thermonuclease is added into a pasteur pipette containing TDA mixture column, the enzyme diffuses through the agar and forms a band. The pink DNA hydrolysis band moves down the agar column in a capillary tube at a rate corresponding to the concentration of thermonuclease.

In this investigation, thermonuclease and coagulase production by S. aureus strains was studied. The capillary tube agar-diffusion showed a significant correlation with metachromatic agar-diffusion method. No linear relationship was observed between the amount of thermonuclease and coagulase produced although all strains were positive for both enzymes. Using the capillary tube agar-diffusion method, thermonuclease

was detected in liquid food. This system is easy to perform, saves materials and the band can be measured easily with an optical micrometer.

The possibility of thionin dye for use in DNA-Agar beside toluidine blue for the detection of thermonuclease using a capillary tube system was studied. Diffusion band lengths in Thionin-DNA-Agar were shorter compared to Toluidine blue-DNA-agar indicate that Toluidine blue is superior for thermonuclease detection. TDA was determined to be stable in cold storage (4°C) for over two months in capillary tube system which makes it a convenient system for routine use in diagnostic laboratory.

The heat inactivation profiles of thermonuclease were determined. Thermonuclease was found to be remarkably heat stable. The enzyme showed residual activities after heating for 5 hrs. at 80 and 100°C. The complete inactivation of the enzyme was achieved after heating for 3 hrs. at 121°C. This heat stability profiles are similar to those of staphylococcal enterotoxins B and C (Fung et al., 1973) and staphylo-coagulase (Ocasio and Fung, 1983).