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CAPILLARY AGAR TUBE SYSTEM FOR STAPHYLOCOAGULASE

by

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INTRODUCTION

Ever since Loeb (1903) first demonstrated the clotting of goose plasma by staphylocoagulase, an enzyme produced extracellularly by certain strains of staphylococci, considerable interest and study have been focused in this phenomenon. The production of staphylocoagulase has been accepted as an index of pathogenicity (Eksdet, 1960; Victor et al., 1969; Rayman et al., 1975) and as a key test for the identification of Staphylococcus aureus (AOAC, 1975; Baer et al., 1976; Kloos and Smith, 1980).

Current methods for the detection of staphylocoagulase are generally not quantitative, require relatively large amounts of materials and some are difficult to perform. The two most widely used methods are the tube method (Chapman et al., 1941) and the slide method (Cadness-Graves et al., 1943). Quantitative methods recently developed include a nephelometric assay (Stutzenberger et al., 1966), a dye-diffusion method (Fung and Kraft, 1968), a radial diffusion slide assay (Kohl and Johnson, 1980) a microfiltration procedure (Sahni et al., 1981) and a chromogenic substrate assay (Engles et al., 1981).

Staphylococcus aureus produces a class of heat stable toxins called staphylococcal enterotoxins which are responsible for staphylococcal food intoxication. The presence of the enterotoxin is independent of the viability of S. aureus in a food. Thus, the only real test for the presence of enterotoxin is to directly detect the enterotoxin in the food. Unfortunately, the procedure of enterotoxin detection is time consuming and laborious; thus researchers have been seeking easier

methods to indirectly detect the presence of S. aureus and/or the enterotoxins in food. One of the compounds tested as an indirect measurement of S. aureus contamination in food is the heat stable deoxyribonuclease (Chesbro and Kuborn, 1967; Lachica, 1980), an enzyme produced extracellularly by S. aureus. The utilization of this enzyme as an indicator of contamination is due to its remarkable heat stability (Elston and Fitch, 1964) which is similar to that of staphylococcal enterotoxins (Fung et al., 1973).

Another possibility is to use staphylocoagulase. However, heat stability of this compound has not been well established. Tager (1948) found that purified staphylocoagulase was relatively heat labile, losing 99% of its activity by boiling in water for 15 minutes. Zolli and San Clemente (1963) reported that crude staphylocoagulase remained active after heating for 5 min. at 80 C.

The present study was undertaken to develop a simple to perform quantitative capillary agar tube system for the detection of staphylocoagulase, based on the principle of single gel diffusion of enzymes. Our aim was to develop a sensitive, accurate and easy to perform system using minimum amount of reagents and sophisticated equipment to quantified staphylocoagulase. The second objective was to use the new system to study heat inactivation profiles of staphylocoagulase in laboratory media and food systems.

LITERATURE REVIEW

Public Health Significance of Staphylococcus aureus

Staphylococcus aureus, a Gram-positive coccus, is ubiquitous in the environment and produces a variety of extracellular enzymes and products. Among these products is a class of heat stable proteins called staphylococcal enterotoxins. The ability of certain strains of S. aureus to produce enterotoxins when these strains proliferate in a variety of foods is a major public health concern. The symptoms resulting upon the ingestion of staphylococcal enterotoxin usually develop within four hours. Although the symptoms of the illness are variable they usually include: diarrhea, nausea, vomiting, abdominal cramps, sweating, headache, prostration, and sometimes, drop in body temperature. Even though symptoms generally last 24-48 hours, and mortality rate is very low, the resultant discomfort, the frequency of occurrence, and the implications it makes of the sanitary conditions in general, make staphylococcal food poisoning an important subject of investigation for the food microbiologist.

First studies in the syndrome are attributed to Denys (1894), and later to Barber (1914) who produced in himself the symptoms of the disease by ingesting milk contaminated with a culture of S. aureus. The ability of some strains of S. aureus to cause food intoxication was conclusively proven by Dack et al. (1930) who showed that symptoms could be produced in human volunteers by feeding them culture filtrates of S. aureus.

Staphylococcal food poisoning has consistently been among the top three causative agents of food-borne disease during the last five decades in the U.S.A. along with Clostridium perfringens and Salmonella.

According to the United States Public Health Services (Feig, 1950), during the period between 1945 and 1947 staphylococcal food poisoning accounted for 82% of all food poisoning outbreaks. Even though more recent figures do not rank staphylococcal food poisoning nearly as high it still is among the top three food-poisoning agents. For instance, in 1968 (USDHEW, 1970) the syndrome still lead the list being responsible for 23.8% of the total outbreaks and 25.2% of the total 17,567 cases involved. During the years of 1972 and 1973, staphylococcal food poisoning accounted for 25.0% of the outbreaks and 32.5% of the total cases in 1972 and 15.7% of the outbreaks and 16.5% of the total cases in 1973 (USDHEW, 1974). In both years, S. aureus ranked second only to Salmonella in the number of outbreaks. In 1975 staphylococcal poisoning was again the first cause of food-borne illness with 23.6% of the total outbreaks. 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, 1981). These figures ranked the staphylococcal intoxication second only to Salmonella in each of the three years mentioned above. The most recent reports from the Centers of Disease Control for 1979 have indicated that S. aureus ranked second to Salmonella with 19.8% of the total outbreaks and 32.4% of the total 6,806 cases (CDC, 1981). Since staphylococcal intoxication is a nonreportable disease the numbers tabulated by health officials are just a fraction of the actual number of cases.

Production of Staphylocoagulase as as Index of Pathogenicity

A large number of enzymes and metabolic products are produced by cells of S. aureus. Among the enzymes reported to be produced by this organism are: staphylocoagulase, hyaluronidase, phosphatase, deoxyribonuclease, penicillinase, proteases (caseinase, gelatinase, staphylokinase, and Muller factor), lipase, catalase, lysozyme and lactate dehydrogenase (Abramson, 1972). The production of staphylocoagulase has been widely accepted as an index of pathogenicity. Although not all coagulase-positive strains of S. aureus are capable of causing food poisoning, and some coagulase-negative staphylocci are enterotoxigenic, a good relationship between coagulase production and pathogenicity is well documented.

Eksdet and Yotis (1960) demonstrated that from a group of 25 strains of S. aureus; (16 were coagulase-positive and 9 coagulase-negative), all coagulase-positive strains were rapidly fatal when injected intracerebrally into mice, while the coagulase-negative strains had little effect under the same conditions.

Pulverer and Entel (1967) examined over 1,000 healthy animals for the presence of coagulase-positive staphylococci. These workers isolated 453 coagulase-positive staphylococci, 15% of which were potentially pathogenic based on phage typing, antibiograms and physiological properties. The relationship between coagulase and toxin production was studied by Victor et al. (1969). These investigators found that from 275 coagulase-positive strains of S. aureus, 93% were also enterotoxigenic, and 95% produced heat stable deoxyribonuclease and concluded that these three characteristics appeared to have a high correlation among them.

Rayman et al. (1975) reported a 100% correlation between coagulase production and enterotoxin production in 63 isolates of S. aureus obtained from foods and personnel involved in food poisoning outbreaks. Of the 63 isolates, 62 were positive for both heat stable deoxyribonuclease and coagulase production, and in every instance clotting of the plasma was of a 4+ rating. These workers also found that from 53 isolates from routine microbiological analyses of foods, 28 were enterotoxigenic, and in all cases also produced coagulase. From the 25 non-enterotoxigenic cultures, 18 were coagulase-positive. Nine of the 18 coagulase-positive non-enterotoxigenic cultures yielded a coagulase reaction rated at 2+ or less. A total of 103 clinical isolates of staphylococci were positive for both thermonuclease and coagulase production.

On the other hand, many other workers have challenged the validity of coagulase as an indicator of pathogenicity. For instance, Noble (1966) investigated 221 isolates of staphylococci and concluded that pathogenicity or virulence, with regard to extracellular enzymes (including coagulase), had no direct relationship to virulence as demonstrated by mice experiments.

The early hypothesis that coagulase protects S. aureus from phagocytosis in human sera (Wlodarczak and Jeljaszewicz, 1959; Ekstedt and Yotis, 1960) has also been the subject of dispute among the scientific community. Borchardt and Pierce (1964) suggested that coagulase was not the factor responsible for staphylococcal survival within leukocytes. The same was observed by Cawdery et al. (1969) who showed that the process

of defibrination utilized by earlier workers resulted in significant loss of leukocytes from the blood; which in turn affected the results obtained in early investigations.

Regardless of the past and present studies, coagulase testing continues to be the single most important test in identifying S. aureus and in assuming pathogenicity in most clinical and food laboratories in the U.S.A.

Staphylocoagulase Production as a Tool for Staphylococci Characterization

Most clinical and food laboratories utilize the tube coagulase test as the most efficient and reliable method for distinguishing S. aureus from other Micrococcaceae (AOAC, 1975; STSM, 1965; Kloos and Smith, 1980; Baird-Parker et al., 1976; Davidson et al., 1982). Several investigators (Sperber and Tatini, 1975; and Rayman et al., 1975) have determined that the coagulase test is a valid means of identifying S. aureus, provided that only a firm clot that does not move when the tube is tipped, is considered a positive reaction. Sperber and Tatini (1975) investigated 508 coagulase positive presumptive S. aureus. Of these, 439 strains which gave a 4+ coagulase reaction were identified as S. aureus on the basis of other biochemical tests. The other 69 strains produced coagulase reactions of 2+ and 3+, and were found not to be S. aureus on the basis of other characteristics. Zarzour and Belle (1978) characterized 520 clinical and environmental isolates of the family Micrococcaceae that fermented glucose anaerobically. Of these, 450 isolates coagulated the rabbit plasma, produced thermostable nuclease

and were identified as S. aureus. This yielded a 100% correlation between both characteristics. The authors suggested thermostable nuclease as a good confirmatory procedure on clinical isolates yielding a 2+ or 1+ clot in the coagulase test.

More evidence of the validity of the coagulase test for characterizing S. aureus was provided by Yrios (1977). In his work, 627 clinical isolates of Micrococcaceae were characterized. Of these, 416 were classified as S. aureus. All 416 strains produced a 3+ to 4+ clot formation in heparinized pig plasma, whereas 415 isolates produced similar reactions in citrated rabbit plasma. Of the remaining 211 non-S. aureus isolates, 16 produced weak 1+ to 2+ reactions. He also reported that heparinized pig plasma appeared to be superior, because most of the isolates produced well formed 4+ clot in this plasma. It has been recently confirmed that a few species of staphylococci other than Staphylococcus aureus also exhibit the ability to produce coagulase. Schleifer et al. (1976) reported that 80 strains of coagulase-positive and heat-stable deoxyribonuclease producing staphylococci isolated from man and various animals were proven to be divided into two major groups based on cell wall composition. Based on biotypings, coagulase-positive staphylococci species have been subdivided into six biotypes (A to F) (Hajek, and Marsalke, 1971). The traditional species of S. aureus have been limited to biotypes A to D, whereas biotypes E and F have been included into a new species names S. intermidus (Hajek, 1976). S. hycus,

a species containing some coagulase-positive strains has been successfully distinguished from other coagulase-positive staphylococci by DNA-DNA hybridizations (Phillips and Kloos, 1981).

Even though the above mentioned investigations are indicative of the need of caution when using coagulase as a sole resource for the identification of S. aureus, most clinical laboratories still use this practice mainly due to the fact that S. intermedius and S. hycus are of rare occurrence and mainly their importance is solely of veterinary concern. These two species have not been isolated from human clinical material (Goldstein and Roberts, 1982).

Purification and Chemical Composition of Staphylocoagulase

Before any molecular properties, or the mode of action or the contribution toward pathogenicity can be definitely elucidated, purification of staphylocoagulase to homogenicity is essential. Through the years, many workers have approached this problem with increasing success, but to date, the complete purification of staphylocoagulase is yet to be achieved.

Tager (1948) purified staphylocoagulase 300-fold using cycles of alcohol precipitation and ammonium sulfate saturation. Duthie and Haughton (1958) obtained an electrophoretically homogeneous preparation showing an isoelectric point of pH 5.3 and a minimum mean molecular weight of approximately 44,000. They obtained only a 17-fold purification. A chemically and serologically homogeneous staphylocoagulase was obtained by Zolli and San Clemente (1963) who claimed 3,700-fold increase in activity for their homogeneous preparation.

Blobel, et al. (1960) purified coagulase 387-fold but their preparation was not homogeneous by immunological and electrophoretic criteria. Stutzenberger and San Clemente (1968) achieved a 45-fold increase in activity and a preparation highly homogeneous by immunological standards.

A significant improvement came from Siwecka and Jeljaszewicz (1968). These workers obtained a 5120-fold purification with a recovery of 35%. The highly active preparations were electrophoretically homogeneous. They reported an isoelectric point at pH 5.85, and suggested the presence of sugars as structural components.

Bas et. al. (1974) were able to purify the enzyme 35,700 fold. Contaminating proteins were present only in trace amounts. The molecular weight of the extremely active preparation was of 61,000 $\pm$ 2,300 daltons as determined by sodium dodecylsulphate-gel electrophoresis and gel filtration. The degree of purification found was seven times higher than the highest purification previously described. They investigated the chemical composition of their highly purified preparation and suggested that staphylocoagulase was a single chain protein with aspartic acid as N-terminal amino acid. The amino acid composition of the preparation is presented in Table 1. No carbohydrate was found in their highly purified preparations, as determined by the carbohydrate staining of the polyacrylamide gels. This implication challenges the earlier suggestion that sugar was a structural component of staphylocoagulase (Siwecka and Jeljaszewicz, 1968).

One of the most recent attempts to purify staphylocoagulase was conducted in Japan by Igarashi et al. (1979). They failed to improve

Table 1. Amino Acid Composition of Staphylocoagulase[a]

Amino Acid	g Amino acid/100g protein	Nearest integer to mole amino acid/mole protein
Lysine	9.10	43
Histidine	1.89	8
Arginine	4.01	16
Aspartic Acid	13.18	70
Threonine	4.74	29
Serine	3.71	26
Glutamic acid	17.58	83
Proline	2.82	18
Glycine	3.38	36
Alanine	5.09	44
Cystine (half)	1.64	10
Valine	6.96	43
Methionine	1.51	7
Isoleucine	6.42	35
Leucine	7.70	42
Tyrosine	3.99	15
Phenylalanine	4.53	19
Tryptophan	0.60	2

[a]From Bas et al. (1974)

the degree of purification achieved by Bas et al. (1974), their preparation showed a 7,600 fold increase in activity. The purified preparation gave a single precipitin band in immunodiffusion tests against anti-crude and anti-purified staphylocoagulase sera. However, the final product was shown to contain one major and two minor components by sodium dodecyl gel electrophoresis. The molecular weight of the purified staphylocoagulase was about 70,000 (major band), 58,000 and 52,000 for the two minor bands, respectively. Chemical analysis of the material indicated that its terminal NH_2 residue is a single residue of isoleucine and cysteine residues were not present. This observation is in disagreement with the report of Bas et al. (1975) who reported aspartic acid as the N-terminal amino acid. These two groups of investigators used different strains of S. aureus, which may have accounted for the differences.

A variety of molecular weights for staphylocoagulase have been proposed, ranging from 5,000 (Murray and Gohdes, 1960), 10,000 (Tager and Drummond, 1965), 18,000 (Zajdel et al., 1976), 44,000 (Duthie and Haughton, 1958) and to 90,000 daltons (Tirunarayan, 1966). Different molecular weights of staphycoagulase were reported even among studies dealing with the same strain. For instance, at least three different molecular weights have been reported for the clotting activity of S. aureus strain 104 (Bas et. al., 1974; Murray & Gohdes, 1960; and Tager and Drummond, 1965). In an effort to clarify the reasons for the lack of uniformity among reports on the chemical and physical properties of

staphylocoagulase from S. aureus strain 104, Reeves et al. (1981) purified the clotting activity 46,000-fold by cycles of acid and ammonium sulfate precipitation, DEAE-cellulose and hydroxyapatite chromatography, and sephadex G-200 electrophoresis. However, absolute purity was not achieved. Elution of clotting activity from denaturing and non-denaturing polyacrilamide gels revealed the presence of four distinct molecular forms. Molecular weights of the forms were approximately 31,500, 34,800, 44,800 and 56,800 as determined by at least four different techniques. They reported that the forms differ in shape from one another as determined by estimated frictional ratios. They proposed that the establishment of the multiple molecular forms helps explain the diversification of molecular weights previously reported. They also concluded that the clotting properties purified were not spherical and that such asymmetry can lead to problems in determining molecular weights by gel filtration when globular proteins were used as standards. Carbohydrate content of the purified material was not more than 5%. The presence of carbohydrates in the purified materials agrees with the findings of Siwecka and Jeljaszewicz (1968).

The results of the purification outline indicate a tremendous divergence in regard to homogenicity. This suggests that staphylocoagulase either may be a single protein which can exist in more than one molecular form or that there is more than one form of staphylocoagulase. In any case, the elucidation of this problem is essential before the chemical composition of staphylocoagulase is revealed.

Bound Coagulase or Clumping Factor

The term "Bound Coagulase" was introduced by Duthie (1954b) to describe a clotting factor associated with the cell wall of certain strains of S. aureus which may or may not produce free coagulase. He reported that the clumping factor was antigenically distinct from free coagulase; and that the clumping associated with the slide coagulase test was due to the clumping factor rather than free coagulase.

McNeil (1968) reported no correlation between clumping factor, coagulase and virulence. The clumping factor is believed to act directly on fibrinogen, whereas free coagulase requires an accessory factor (Abramson, 1972). Nevertheless, it is important to clarify that the clumping factor is considered a separate entity from free coagulase, and that all the efforts toward purification previously described here were conducted using coagulase and not the clumping factor.

Production and Biosynthesis of Staphylocoagulase

According to Duthie (1954a) and Martson and Fahlberg (1960) staphylocoagulase is produced by test cultures in the lag phase and continues throughout the logarithmic growth phase. Several organic compounds have been reported to stimulate the release of coagulase from staphylococcal cells. Martson and Fahlberg (1960) found that L-glutamic acid a L-lysine stimulated the production of coagulase on chemically defined medium. Tirunarayanan (1966) reported the stimulation of coagulase production by glucose and sodium bicarbonate. He reported optimum production of staphylocoagulase at pH values between 7.3 and 7.9.

Serum albumin has been responsible for enhancing staphylocoagulase elaboration by 10-fold or more (Davis, 1951; Altenbern, 1966). Increased coagulase production was also reported with culture shaking (Fahlberg and Martson, 1960; Haughton and Duthie, 1959; Stutzenberger and San Clemente, 1968; Tager, 1948).

Engels et al. (1978) studied the effect of different cultivation conditions on the production of staphylocoagulase. The study showed that a casein hydrolysate medium was superior for coagulase production when compared to four other media tested. They also reported that Mg^{2+} concentration was critical for coagulase production being optimal at 0.05 to 0.10 M while higher concentrations became inhibitory. Glucose was found to be inhibitory when used in concentrations higher than 0.2% (W/V) and coagulase production decreased as the ratio of the culture volume to the flask volume increased. Both of the last two results were in agreement with earlier findings (Stutzenberger and San Clemente, 1968).

Mechanism of Action of Staphylocoagulase

In the presence of a plasma cofactor or an accessory factor, staphylocoagulase transforms soluble fibrinogen into insoluble peptides (Smith and Hale, 1944). This accessory factor has been called "coagulase reacting factor" (CRF) by Tager (1948) who drew the same conclusions.

Staphylocoagulase probably reacts with the CRF present in plasma forming a complex which in turn can act upon the fibrinogen. Drummond and Tager (1962) along with others (Haughton and Duthie, 1959; Scheraga and Laskowski, 1957; Zajdel et. al., 1973), reported that the action of

the coagulase-CRF complex upon fibrinogen involved a preliminary stage of proteolysis similar to that caused by thrombin, an enzyme responsible for physiological blood clotting.

Haughton and Duthie (1959) found that like thrombin, and certain other proteins with coagulating capabilities, coagulase-CRF complex possesses esterease activity on a synthetic substrate, N-2 toluene-p-sulfonyl-L-arginine methyl ester. Drummond and Tager (1963) noted that when fibrinogen was converted to insoluble fibrin and fibrino peptides, the latter were released in a manner similar to the thrombin degradation of fibrinogen. They reported both types of fibrino peptides (from coagulase and thrombin) were physicochemically identical suggesting that coagulase-CRF complex resulted in thrombin formation. Fibrinogen clotting induced by the coagulase-CRF complex or by thrombin was accompanied by the release of about 3% of the total nitrogen; implicating proteolysis occurrence. Soybean trypsin inhibitor did not affect neither coagulase nor thrombin activities, but diisopropylfluorophosphate inhibited both thrombin and coagulase activities.

Even though there are many similarities between the coagulase-CRF and thrombin, differences among these two substances are also well documented. For instance, Tager and Drummond (1965) reported that fibrin resulting from the action of coagulase-CRF complex has different properties as compared with thrombin produced fibrin in that it does not aggregate blood platelets. Zajdel et al. (1973) warned that despite the many similarities between coagulase-CRF and thrombin; several differences among these substances were evident. This report mentioned

the resistance of coagulase to such thrombin inhibitors such as heparin, hirudin and plasma antithrombrins. They demonstrated that the coagulase-CRF complex and thrombin had different in vivo properties, as shown by intravenously injecting both or either substances into rabbits. Finally these investigators proved that coagulase reaction was only dependent on the prothrombin part of plasma and that therefore the CRF can now be identified as prothrombin. The mechanism of action of staphylocoagulase proposed by these workers is shown in Figure 1.

The earlier hypothesis of proteolysis being a key intermediate step in the degradation of fibrinogen by staphylocoagulase (Drummond & Tager, 1963) was challenged by Hemker et al. (1975). These investigators found: (a) that optimal amounts of the active reaction product (coagulase-CRF) are found when equimolar amounts of prothrombin and staphylocoagulase are added together; (b) that the molecular weight of coagulase-CRF equals the sum of the molecular weights of coagulase and prothrombin; (c) that the amino acid composition of coagulase-CRF complex cannot be distinguished from the sum of the amino acid composition of prothrombin and staphylocoagulase; (d) that the N-terminal amino acids of coagulase-CRF complex were those of prothrombin (alanine) and staphylocoagulase (aspartic acid); (e) that an antibody against coagulase-CRF precipitates prothrombin and staphylocoagulase but not thrombin. These results led the authors to conclude that coagulase-CRF complex is the result of a stoichiometric reaction in which one molecule of prothrombin interacts with one molecule of staphylocoagulase, and that the limited proteolysis does not play a role in the mechanism.

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Figure 1. Mechanism of Action of Staphylocoagulase[a]

[a]Modified from Zajdel et al. (1973)

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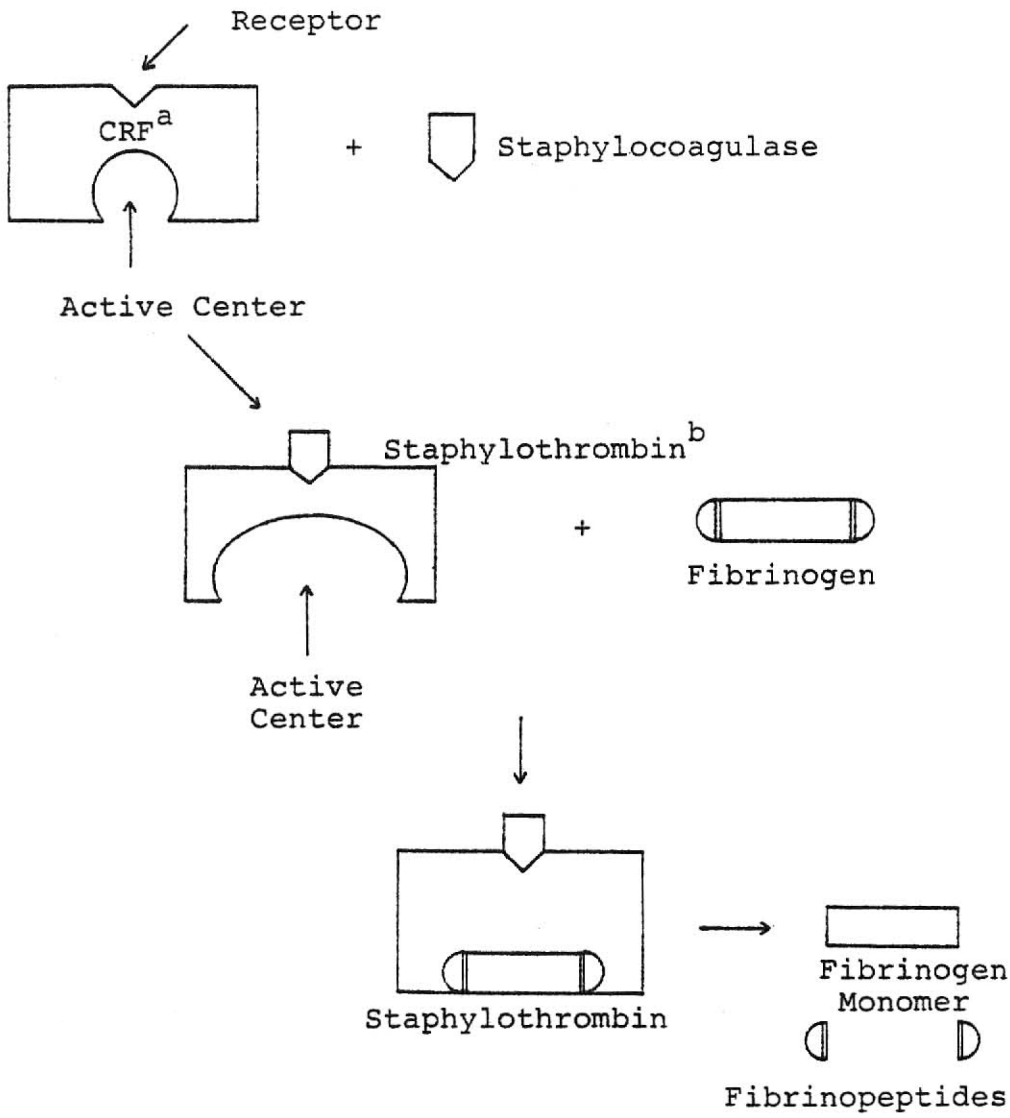


[a]Coagulase Reacting Factor

[b]Also called CRF-Coagulase Complex

Factors Influencing Staphylocoagulase Detection

Probably the single most important factor influencing the detection of staphylocoagulase is the source of the serum used to provide the substrates for the enzyme. Even though the need for standardization of reagents and procedures has been stressed (Hajek et al., 1968; Tager and Drummond, 1965; Jeljaszewicz, 1968), a variety of reagent compositions are currently being used. Meyer (1965) suggested using simultaneous testing with human and bovine plasmas, as well as plasmas from different animal species for differentiating strains of S. aureus. The use of diabetic plasma has been found superior to normal human plasma in yielding satisfactory results (Yearsley and Carter, 1966). Hajek et al. (1968) indicated that the type of plasma utilized for coagulase test should be determined by the clinical origin of the specimen tested. They proposed that human plasma should be used to test for diseases of human origin and animal plasma in those of animal origin. The United States Food and Drug Administraton (1969) has recommended the use of rabbit plasma for detecting S. aureus on the basis of rapidity of the reaction and the lack of inhibitors of coagulase production that are sometimes found in human serum.

Duthie and Lorenz (1952) reported that cow, sheep, dog, guinea pig and mouse plasmas exhibited a relative deficiency in coagulase reacting factor (CRF), whereas the plasmas of man, monkey, horse, cat, pig, fowl and rabbit contained substantially more CRF. In a similar work, Orth et al. (1971) found that the whole sera of different species

have the following relative concentrations of CRF arranged from the strongest to the weakest as follows: human > pig > rabbit > horse > bovine > chicken and lamb. They also reported the plasmin content of the sera; may lead to false negative tests due to the action of proteases. The plasmin activities of the different sera in descending order of activity was as follows: rabbit > human > lamb > horse > bovine > chicken and pig. These results led these authors to recommend the use of pig plasma. In contrast, Boothby, et al. (1979) classified the performance of pig plasma in the pour-plate method for coagulase detection as inadequate and undependable. These investigators selected agar with 5% rabbit plasma over pig plasma and combinations of rabbit and pig plasma on the basis of clarity of precipitation halos in the pour plate test. On the other hand, Sperber and Tatini (1975) concluded that the mixture of pig and rabbit plasma proved better than either plasma alone in characterizing 508 isolates by the tube test. Yrios (1977), in a comparison of rabbit and pig plasma in the tube coagulase test, found that out of 627 isolates of Micrococcaceae 211 were found to be coagulase negative by both types of plasmas. Of the 416 remaining isolates all of them were found to clot heparinized pig plasma; whereas 415 clot citrated rabbit plasma.

Another important factor influencing the reliability of the coagulase reaction is the nature of the anticoagulant utilized to prevent coagulation of plasma due to the natural clotting (Thrombin) activities present in plasma. Bayliss and Hall (1964) investigated the influence of

type of anticoagulant upon plasma coagulation by organisms other than S. aureus. They tested the performance of such anticoagulants as acid-citrate-dextrose, ethylenediamine tetraacetate (EDTA), balanced oxalate, potassium and sodium oxalates and heparin. These investigators found that out of 200 organisms tested, 12 were able to coagulate citrated plasma. Only 6 S. aureus strains used as controls coagulated plasma other than the one treated with citrate. It was determined by chromatographic testing and other means that the coagulation of citrated plasma by organisms other than S. aureus resulted from the capability of these organisms to metabolize citrate. The utilization of citrate resulted in the release of calcium which is then available for the conversion of prothrombin into thrombin, and the completion of the normal physiological clotting of plasma.

The utilization of heparin over other anticoagulants has been recommended by several workers (Orth et al., 1971; Yrios, 1977). However, the suitability of EDTA for this purpose is also well documented (Finegold et al., 1978; Kloos and Smith, 1980).

The action of staphylokinase and the staphylococcal Muller factor upon the activation of the plasminogen-plasmin system present in normal plasma also has a direct effect upon coagulase testing. The plasmin thus formed causes fibrinolysis (proteolytic degradation of the fibrinogen clot) which results in the disappearance of the clot observable during coagulase test and therefore can lead to false negative reactions (Orth et al., 1971). This activity can be abolished by soybean trypsin inhibitor (Hutchinson, 1962; Orth et al., 1969).

To avoid the occurrence of false negative results due to fibrinibinolysis during the tube coagulase testing, taking reading periodically for up to 6 h and then reexamination after 24 hrs. has been recommended (Baer, 1976; Finegold et al., 1978; MacFaddin, 1981).

Orth et. al. (1969) reported that the incorporation of trypsin inhibitor (egg white or soybean) into coagulase testing was useful not only for protecting against false negative reactions but also in reducing the reaction time of coagulase produced by organisms which produced plasminogen activators as well as organisms which did not activate plasminogen to plasma. These investigators also studied the influence of plasma concentration on the reaction mixture. They found the reaction time decreases with increases in plasma concentration until a concentration of about 40%.

Stutzenberger et al. (1966) concluded that the relative activity of the coagulase increases with the plasma concentration up to a concentration of 2% as determined nephelometrically. This result differs sharply from those of Orth et al. (1969), but it must be clarified that the source of plasma as well as the assay procedure were completely different. The activity of the reaction also increased with the fibrinogen concentration up to about 2.5 mg/ml. Sodium chloride was added to help reduce the electrostatic repulsion between fibrinogen molecules and therefore accelerate the polymerization process. It was found that a concentration of NaCl between 0.05 M to 0.06 M was the best for this purpose. The ideal pH for the reaction was between 7.1 and 7.5.

Methods for the Determination of Staphylocoagulase

The Tube Coagulase Test.

The tube coagulase test first described by Chapman et al. (1941) is the most utilized and reliable test for the detection of staphylocoagulase (Sperber and Tatini, 1975; Sperber, 1976). The test has been through many modifications ever since its first description. Many types of plasmas from different species, anticoagulants, incubation times and temperatures and other factors have been tested in efforts to improve the test.

To date the official (AOAC, 1975) procedure calls for transferring colonies of suspected S. aureus into brain heart infusion broth, and incubating at 35 to 37 C overnight. Then add about 0.1 ml of the culture into 0.5 ml of coagulase plasma. Incubate the mixture at 35 to 37 C, check periodically for up to about 6 h and recheck negative cultures after 24 hrs. Rank positive reactions from 1+ to 4+ according to the degree of coagulation observed (Baer, 1976; AOAC, 1975; Kloos and Smith, 1980). The subjective procedure of semiquantifying the tube coagulase reaction by rating the clotting reactions progressively from 1+ to 4+ has been the subject of disagreement and confusion. In an effort to clarify this problem, Sperber and Tatini (1975) presented the correct interpretation of the tube coagulase test as described earlier by Turner and Schwartz (1958). These authors described a negative test as one where no evidence of fibrin formation is observable; 1+ is one in which only small unorganized clots are formed; the 2+ and 3+ ratings are assigned when small and large organized clots respectively are observed. In the 4+ reaction the entire content of tube coagulates and is not displaced when tube is inverted.

The Slide Coagulase Test.

The slide test is conducted by mixing the growth of an isolated colony with a drop of plasma laying on a microscope slide. The formation of clumps is considered a positive test. Cadness-Graves et al. (1943) described this test and made a large scale comparison with the tube coagulase test. They found a high correlation between the two tests. Of 442 clinical isolates, 440 were positive for both tests. Further studies by the same workers found 48 strains which were positive by the tube test but negative by the slide test, while three were tube negative and a slide positive.

Duthie (1954a) conclusively proved that the slide method tested for bound coagulase (clumping factor) which is a separate identity from free coagulase. Van der Vijver et al. (1972) tested 400 staphylococci isolates from nasal carriers and clinical material found that 10% of 100 isolates which gave a positive tube reaction failed to clot plasma in the slide test when Difco's rabbit coagulase plasma was used and 4% when fresh human plasma was used.

Morton and Cohn (1972) reported that neither the clumping factor nor the production of phosphatase correlated well with coagulase production when 504 clinical isolates were tested. Flandoris and Carret (1981) proposed a passive hemagglutination test for the detection of the clumping factor or bound coagulase from S. aureus. The test was conducted by emulsifying one or two staphylococcal colonies in one drop of sheep erythrocytes coated with human fibrinogen on a slide. The agglutination of erythrocytes was considered a positive reaction. The clumping

activities of 580 staphylococci of different species were tested by both the hemagglutination and slide method and a good correlation between them was reported.

Currently the slide test is considered a screening test for S. aureus and negative cultures must be rechecked by the tube method (Goldstein and Roberts, 1982).

The Pour Plate Method.

The pour plate method along with tube and slide tests are the most common procedures for testing the production of coagulase by S. aureus (Boothby et al., 1979). Penfold (1944) and Reid and Jackson (1945) inoculated the surface of a medium containing plasma with culture of staphylococci. Colonies of coagulase-positive staphylococci were differentiated by the surrounding coagulation zone. Duthie and Lorenz (1952) incorporated human fibrinogen and plasma in a nutrient medium and poured it into plates; colonies of coagulase positive staphylococci were surrounded by an opaque ring in this medium. A common disadvantage of these early differential media was that they were not selective enough, and the growth of the staphylococci could be suppressed by high numbers of other organisms present in the sample (10^8 to 10^{10} per gram in feces for example) (Finegold and Sweeney, 1961).

Orth and Anderson (1970) were successful in improving the selectivity of a medium containing coagulase reacting factor (CRF) and fibrinogen by the addition of polymxin B; which is highly inhibitory of most gram negative intestinal bacteria but to which S. aureus is resistant. These investigators combine the plate method detection of coagulase

with the detection of mannitol fermentation by adding mannitol and the phenol red into the medium. The presence of coagulase was indicated by an opaque, gray zone, or halo, radiating outward from the colony and was easily observed by indirect illumination with a dark background. The fermentation of mannitol was evidenced by a yellow, acid reaction around the colony.

One of the disadvantages of the plate method for coagulase detection is that fresh agar must be prepared each time, due to unstability of plasma during storage. Van der Vijver et al. (1972) recommended the utilization of stable human prothrombin as a substitute for plasma. They concluded that diluting the prothrombin preparation 1/30 before adding it into the agar gave the optimal concentration of the former. These investigators compared their plate method to the tube test using 400 isolates from nasal carriers and clinical material and found a 100% correlation between the two procedures.

One of the major concerns when using the plate method is the possible disappearance of the halos surrounding the colony due to fibrinolysis or protelysis. To avoid this problem, Orth and Anderson (1970) used plasminogen-free coagulase reacting factor. Boothby et al. (1979) developed a plate method for coagulase containing soybean trypsin inhibitor to inactivate the plasmin present in serum (Sherry and Troll, 1954); and therefore prevent false negative reactions due to the action of staphylokinase (fibrinolysis). These authors reported a 100% correlation between their method and the tube coagulase when 261 isolates were tested.

Parisi et al. (1973) compared the suitability of the pour plate method with the surface inoculation procedure for coagulase detection by the plate method. In this study, 26 cultures of coagulase tube positive S. aureus show coagulase activity by the pour plate method; whereas seven (26.9%) of them produced zones of precipitated fibrin which were either faint or not detectable by the surface inoculation method.

Quantitative Tests.

Tager and Hales (1947) determined coagulase activity quantitatively by two-fold dilutions in 2% peptone. The serial dilutions were mixed with equal volumes of noninhibitory human plasma previously diluted 1:5. Titers of coagulase activity were recorded as the reciprocal of the highest dilution which caused an organized clots after 24 hrs. at 57 C. In the decades subsequent to this publication, this method, now the most widely used procedure for the quantification of coagulase, has been the subject of numerous modifications (Fahlbert and Martson, 1960; Altenbern, 1966; Pariza and Iandolo, 1969; Reeves et al., 1981).

Duthie (1954a) used clotting time as a means to quantify coagulase. This investigator added measured volumes (0.1 ml or 0.2 ml) of a coagulase standard preparation into 0.2 ml of 0.4% (W/V) solution of bovine fibrinogen containing also 0.5% (V/W) rabbit plasma in tubes 5.0 x 0.8 cm. Each tube was held against a black background in a warm illuminated box and tapped at intervals until a definite clot appeared; this time was measured accurately. The reciprocal of the coagulase concentration plotted against the clotting time gave a straight line which was used as

a standard curve for futher analyses. A similar procedure was described by Soulier et al. (1967) and followed by Engels et al. (1978) and Bas et al. (1974). The latter investigator conducted the procedure by adding 0.1 ml of sample containing staphylocoagulase into a reagent mixture of rabbit plasma and bovine fibrinogen in a glass tube. At the addition of the sample, a stopwatch was started. The moment of coagulation was assessed by hand with a 5 x 30 mm stainless steel hook, and the time recorded at that moment. Coagulation times obtained with dilutions from a standard preparation were used to plot a standard curve.

All these methods, depending on dilutions and clotting times, are too subjective due to the interpretation of clot formation, which in many cases can be confusing. Stutzenberger et al. (1966) developed a nephelometric assay based in the increase of light scattering which accompanies the clotting of fibrinogen. These investigators constructed a standard curve for the quantification of staphylocoagulase by plotting the coagulase concentration against the nephelometric units per minute. Wegrzynowicz et al. (1969) proposed a simple standardized procedure for the determination of staphylocoagulase. These workers quantified staphylocoagulase on the basis of the time necessary to clot a reaction mixture containing euglobulin as a source of both fibrinogen and coagulase reacting factor. They defined one unit of coagulase as the amount of enzyme that clots a standard euglobulin solution containing 0.25% of clottable protein, 0.01 M aminocaproic acid and 20 units of heparin per ml in 25 seconds. These investigators found that the use of euglobulin fraction of plasma has a substantial advantage over fibrinogen. Systems

with fibrinogen shown unproportionally long clotting times owing to a lack of sufficient amount of coagulase-reacting factor.

Kohl and Johnson (1980) described a quantitative, Radial Diffusion Slide Assay for staphylocoagulase. This system was based on the principle of radial gel diffusion of enzymes (Schill and Schumacher, 1972). An agar mixture containing fibrinogen, rabbit plasma, phosphate buffer and merthiolate was pipetted on standard microscope slides. Upon solidification wells were cut into the agar and filled with 0.007 ml of the sample. After incubation time of 12 to 16 h an opaque ring of fibrin precipitation surrounded the well. The diameter of the ring increased proportionally with the concentration of staphylocoagulase in the sample. Fung and Lahellec (1980), in a preliminary study, incorporated the agar mixture of Kohl and Johnson (1980) into the thinner portion of a Pasteur pipette and were able to detect staphyloccoagulase activity of S. aureus. The potential of this system to quantitate staphylocoagulase exists but have not been studied in detail.

Sahni et al. (1981) developed a microtitration procedure for quantitative determination of staphylocoagulase using fibrinogen-coated red blood cells. This assay was performed by conducting 1.5-fold dilutions of a coagulase sample in a microtiter plate containing a saline diluent, a suspension of red blood cells coated with firbinogen and pooled fresh, citrated pig or rabbit plasma as a source of coagulase reacting factor. One hemagglutination unit was defined as the reciprocal of the highest dilution showing hemagglutination, as determined by the presence of a complete carpet of cells covering the bottom of the well in the microtiter plate. Engel et al. (1981) utilized a synthetic chromogenic

substrate called chromozin TH (N-tosyl-glycyl-L-propyl-L-arginyl-p-nitroanalide hydrochloride) for coagulase quantification. The thrombin-like activity of the staphylothrombin complex cleaves the synthetic substrates by limited proteolysis, liberating the yellow p-nitroaniline. The reaction was performed in microtiter plates. After incubation for 1 hr. to 2 hrs. at 37 C, the change in light absorbance was measured. A standard curve was constructed by plotting the linear relationship observed between the concentration of coagulase and the correspondent reaction rate as determined by the increase in absorbance after different periods of incubation.

Other Tests.

Alami and Kelly (1959) presented a soft agar tube medium which utilized albumin, plasma and fibrinogen as reagents. Using this system these investigators were able to simultaneously detect the staphylococcal clumping factor and the free coagulase activity. A compact colony and a coagulation zone were considered positive reactions for the clumping factor and the free coagulase respectively. Fung and Kraft (1968) added crystal violet after a 2 hrs. incubation period of coagulation between plasma and sample culture. The dye diffused through the pre-existing clot at a rate inversely proportinal to the amount of coagulation. An eight-point semi-quantitative scale representing the different degrees of plasma coagulation, observed by different enzyme concentrations was developed on the basis of their observations.

Anadam (1971) impregnated paper strips with reagents for the simultaneous detection of coagulase production and mannitol fermentation. Of 244 staphylococci strains tested, 232 showed complete correlation between methods, the P value being 0.951 which exceeded the 95% confidence limits. Recently, Goldstein and Roberts (1982) investigated the sensitivity and specificity of a new microtube coagulase tests developed for the API system (Analytab Products, Plainview, N.Y.). It consisted of rabbit plasma with 2.5% EDTA iyophilized in API microtubes on API strips. The tests were observed for gelatination of plasma each hour for 7 hrs. and after 24 hrs. of incubation at 37 C.

Enzymatic Detection of Staphylococcus aureus in Foods

Most of the investigations in the possibilities of using enzymatic products as an indication of S. aureus contamination in foods have been directed toward the detection of heat stable deoxyribonuclease (Chesbro and Auborn, 1967; Lachica, 1980). The utilization of this enzyme as an indicator of staphylococci growth is due to its remarkable heat stability (Elston and Fitch, 1964; Chesbro and Auborn, 1967). The importance of an indicator which is heat stable is due to the fact that staphylococcal enterotoxins are very heat stable and can cause food poisoning even after the food has been cooked or reheated (Read and Bradshaw, 1966; Fung et al., 1973).

The alternative to using coagulase as an enzymatic detection of staphylococcal contamination has not been proposed. But before this possibility is studied, coagulase must be proven to be heat stable and to be produced in a large amount of food commodities with and without

competition from other microorganisms. So far only a small number of literature dealing with these conditions is available.

Walker et al. (1947) reported that a preparation of crude coagulase was found to remain active after autoclaving at 120 C. On the other hand, Tager (1948) found that purified coagulase was relatively labile, losing 99% of its activity by boiling in water for 15 minutes. Zolli and San Clemente (1963) reported that crude coagulase remained active after heating at 56 C for 48 hours. Unpublished data by Fung and Shahin (1980) showed that coagulase activities of 33 strains of S. aureus retained their ability to clot plasma after heating for 5 minutes at 80 C. Ocasio and Fung (1983) reported that coagulase preparations of 28 strains of S. aureus remained active after periods of heating at 80 C, 100 C and 121 C.

The purposes of the present investigation were (1) to perfect the Pasteur pipette system for quantification of staphylocoagulase activities, and (2) to study the heat stability of staphylocoagulase using this quantitative system.

MATERIALS AND METHODS

Development of Capillary Agar Tube System

Preparation of Assay Agar

The plasma-fibrinogen agar (PF-agar) described by Kohl and Johnson (1980) was prepared following the procedure described by these authors. The substrates for the enzyme consisted of ethylenediaminetetraacetic acid rabbit plasma (Difco) and Type I bovine fibrinogen (Sigma Chemical Co.) suspended in Noble agar (Difco). In a 125 ml Erlenmeyer flask, 0.5 g of this agar was dissolved in 25 ml of 0.01 M sodium phosphate buffer with 0.06 M 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 ethylmercurithiosolecylic acid sodium salt were added. The temperature of the resulting plasma-fibrinogen (PF) solution was brought to 37 C in a water bath. The PF solution was added to the agar solution and then the combined PF-agar solution was held at 40 C in a water bath. The resulting volume is enough for preparing about 500 capillary tubes.

Preparation of Capillary Tubes

A 9-inch size Pasteur pipette was introduced into PF-agar solution. By suction the mixture was brought to about half way into the thinner part of the Pasteur pipette. The tip of the pipette was sealed with plasticine. The tubes were then stored in a moist chamber at 4 C until needed (within a month time).

Addition of Liquid Samples

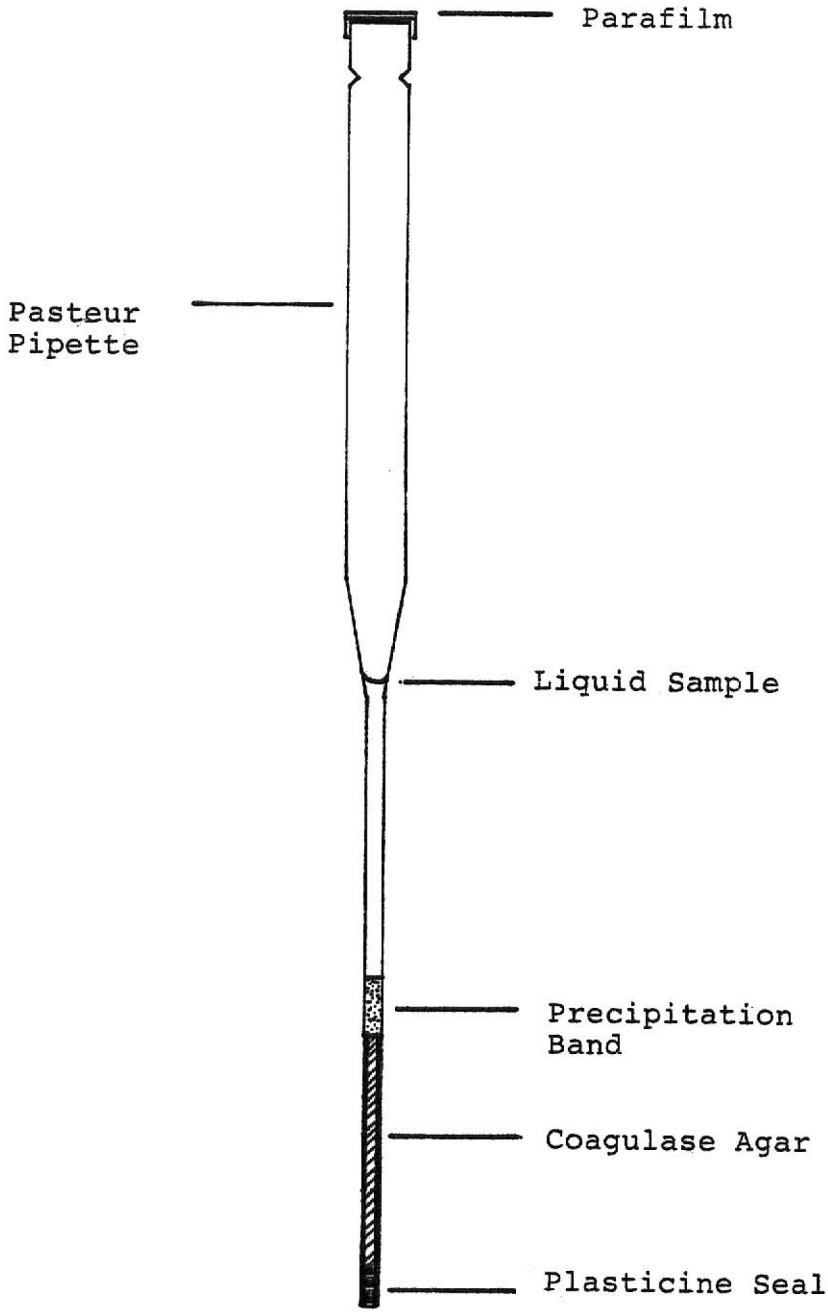
To the tubes containing PF-agar, 0.1 ml of liquid sample (liquid culture or cell free concentrate) was added; a quick jerk of the Pasteur pipette caused the liquid sample to come in contact with the agar in the capillary tube (Fig. 2).

The tubes were then covered with parafilm and incubated in a moist chamber at 37 C for various intervals up to 48 hours. (Generally 24 hours was used as a standard.) After incubation time, the precipitation band in the agar (indicating the presence of staphylocoagulase in the liquid) was measured in millimeters by an optical micrometer (Bausch and Lamb, Inc., Rochester, NY).

Crude Standard for Staphylocoagulase

Since no purified staphylocoagulase was commercially available crude staphylocoagulase was used as standards for comparative studies. A culture of S. aureus strain S-6 obtained from Kansas State University microbial collection was grown in nutrient agar overnight and then transferred into Brain Heart Infusion Broth (Difco) and incubated at 37 C in a water bath shaker at 200 rpm for 18 hrs. Cell-free supernatant from this culture was obtained by centrifugation at 10,000 x g at 4 C (Beckman model J-21B) for 10 minutes. One hundred milliliter portions of cell-free supernatant was then transferred into sacs made by tying off sections of cellulose dialysis tubing (m.w. cut off: 14,000; Fisher Scientific Co.) 10 cm wide and 30 cm long, and then dialyzed against 40% polyethylene glycol (m.w. 20,000; Fisher Scientific Co.) for 15 hr. The resultant aliquot was resuspended in 4 ml of phosphate buffer and placed

Figure 2. Capillary Agar Tube System for Staphylocoagulase Detection.


Parafilm
Pasteur
Pipette
Liquid Sample
Precipitation
Band
Coagulase Agar
Plasticine Seal

in small screw cap tubes (15 by 45 mm). Ethylmercurithiosolesylic acid sodium salt (merthiolate) was added to the culture fluid to make a final concentration of 1:10,000. The fluid was then frozen at -20 C. Before use a tube sample (containing 2 ml) was thawed in an ambient temperature water bath, serially diluted and disposed into test pipettes.

Staphylocoagulase concentration was determined by the inverse of titer method (Pariza and Iandolo, 1969) as modified by Kohl and Johnson (1980). A series of staphylocoagulase standard dilutions was prepared in the phosphate buffer previously described. These staphylocoagulase dilutions were as follows: 1:2; 1:5; 1:10; 1:14; 1:16; 1:18; 1:25; 1:30; 1:35; 1:40; 1:45; 1:50; 1:55; 1:60; 1:65; 1:70; 1:75; 1:80; 1:85; 1:90; 1:95; 1:100; 1:110; 1:20; 1:130; 1:140; 1:145; 1:150. To a series of test tubes (12 by 100 mm), each containing 0.3 ml of EDTA rabbit plasma (Difco) were added 0.3 ml of the diluted samples. After incubation for 3 hrs. at 37 C, the tubes were observed for fibrin formation. A positive test was defined as any visible adherence of fibrin material to a hooked stainless steel sterile loop. The units of staphylocoagulase were defined as the reciprocal of the highest dilution of a sample yielding a positive test.

Effect of Reagents Concentrations Upon the Capillary Agar Tube System

The influence of the soybean trypsin inhibitor (SBTI) as well as different concentrations of plasma were studied. Thirty-six different agar mixtures containing different levels of these two substances were tested. The compositions of these agar mixtures ranging in concentration from 0 mg/ml of SBTI and 2% plasma to 20 mg/ml of SBTI

and 20% plasma is shown in Table 2. The fibrinogen concentration was held constant at 0.5%. The agar mixtures were prepared as follows: 1.0 g of Noble agar (Difco) was dissolved in 50 ml of 0.01 M sodium phosphate buffer with 0.06 M NaCl at pH 7.2, heated until the solution became clear and then tempered at 48 C. In a small test tube, a 10% fibrinogen solution was prepared by mixing 0.625 g of Bovine Type I Fibrinogen with 6.25 ml of the phosphate buffer and mixed for 5 minutes. Three different solutions containing 100 mg/ml; 10 mg/ml and 0.1 mg/ml of SBTI were prepared by dissolving 200 mg, 0.1 mg and 0.1 mg of SBTI into 2.0 ml, 10 ml and 100 ml of buffer respectively.

The proper amount of each reagent solution (Table 2) was added to a test tube (12 x 100 mm) to make up a final volume of 1 ml. Then 0.02 ml of a 1:100 solution of ethylmercurithiosolecylic acid sodium salt was added into the mixture. The reagent mixture was then tempered at 37 C and 1 ml of the tempered agar was added to the preparation. Pasteur pipettes were then used for the preparation of the capillary agar tube system from each agar mixture.

From the above described mixtures it was expected to obtain an ideal plasma concentration which in turn would be used to determine the ideal fibrinogen concentration.

Twenty more agar mixtures (Table 3) with different levels of fibrinogen, ranging from 0.10% to 0.75%, were tested. The plasma concentration was held at 2% for all cases.

Table 2. Components of Agar Mixtures Containing Variable Concentrations of Plasma and Soybean Trypsin Inhibitor (SBTI)

SBTI mg/ml		% Plasma[d] 2	5	10	20
0	SBTI[a]	0 ml	0 ml	0 ml	0 ml
	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.86 ml	0.80 ml	0.70 ml	0.50 ml
0.001	SBTI[a]	0.01 ml	0.01 ml	0.01 ml	0.01 ml
	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.83 ml	0.77 ml	0.67 ml	0.47 ml
0.01	SBTI[a]	0.10 ml	0.10 ml	0.10 ml	0.10 ml
	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.74 ml	0.68 ml	0.58 ml	0.38 ml
0.1	SBTI[b]	0.01 ml	0.01 ml	0.01 ml	0.01 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.83 ml	0.77 ml	0.67 ml	0.47 ml
1.0	SBTI[b]	0.10 ml	0.10 ml	0.10 ml	0.10 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.74 ml	0.68 ml	0.58 ml	0.38 ml
5	SBTI[c]	0.10 ml	0.10 ml	0.10 ml	0.10 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.76 ml	0.70 ml	0.60 ml	0.40 ml
10	SBTI[c]	0.20 ml	0.20 ml	0.20 ml	0.20 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.66 ml	0.60 ml	0.50 ml	0.30 ml

Table 2. (cont.)

		% Plasma[d]			
		2	5	10	20
15	SBTI[c]	0.30 ml	0.30 ml	0.30 ml	0.30 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.56 ml	0.50 ml	0.40 ml	0.20 ml
20	SBTI[c]	0.40 ml	0.40 ml	0.40 ml	0.40 ml
	Plasma	0.04 ml	0.10 ml	0.02 ml	0.40 ml
	Buffer	0.46 ml	0.40 ml	0.30 ml	0.10 ml

a. 0.1 mg/ml SBTI solution

b. 10 mg/ml SBTI solution

c. 100 mg/ml SBTI solution

d. Difco rabbit plasma resuspended in phosphate buffer.

All solutions contained; 0.1 ml of a 10% solution of bovine fibrinogen, 0.02 ml of 1:100 solution of merthiolate and 1.0 ml of a 2% solution of molten noble agar.

Table 3. Components of Agar Mixtures Containing Variable Concentrations of Plasma and Bovine Fibrinogen.

% Bovine Fibrinogen		% Plasma			
		2	5	10	20
	Fibrinogen[a]	0.02 ml	0.02 ml	0.02 ml	0.02 ml
0.1	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.94 ml	0.88 ml	0.78 ml	0.58 ml
	Fibrinogen	0.04 ml	0.04 ml	0.04 ml	0.04 ml
0.2	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.92 ml	0.86 ml	0.76 ml	0.56 ml
	Fibrinogen	0.06 ml	0.06 ml	0.06 ml	0.06 ml
0.3	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.90 ml	0.84 ml	0.74 ml	0.54 ml
	Fibrinogen	0.10 ml	0.10 ml	0.10 ml	0.10 ml
0.5	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.86 ml	0.80 ml	0.70 ml	0.50 ml
	Fibrinogen	0.15 ml	0.15 ml	0.15 ml	0.15 ml
0.75	Plasma	0.04 ml	0.10 ml	0.20 ml	0.40 ml
	Buffer	0.81 ml	0.75 ml	0.65 ml	0.45 ml

a - 10% Bovine Fibrinogen solution was used in all mixtures

All solutions contained; 0.2 ml of merthiolate and 1.0 ml of a 2% solution of molten Noble agar.

The criteria for selecting the best agar mixture included the clarity of the precipitation band and sensitivity of the system. The clarity or intensity of the precipitation band was rated from 1 (lowest) to 4 (highest). In judging the sensitivity of the system, the basic assumption was that the longer the band the more sensitive would be the system.

Finally, three reagent mixtures were selected and each one was tested at two agar concentrations (0.5% and 1.0%). The resulting 6 different mixtures were used to construct 6 standard curves after each of several incubation times. These curves were constructed as follows: serial dilutions of concentrated cell free supernatant containing coagulase were made; a 0.1 ml volume of each dilution was transferred into duplicate pipettes of each type of reagent mixture. The pipettes were then incubated at 38 C and length of the precipitation band of the agar mixture was determined at 1,4,8,12,16, and 24 hrs. Standard curves were constructed for all reagent mixtures after each incubation period by plotting the logarithm of the coagulase concentration against the length of the precipitation band. The criteria for selecting the best agar mixture included the sensitivity of the system at a given incubation time, the correlation between the log of the concentration and length of the band, and the clarity of the band.

Organisms Tested

The following strains of Staphylococcus aureus were tested in the capillary tube and the conventional systems for coagulase determinations to compare the effectiveness of the capillary system for coagulase determination:

(1) 137	(15) DU-MAS
(2) ISP2	(16) RN 2425
(3) COLS	(17) DU 4916
(4) 472	(18) 5106 R
(5) 196E	(19) FRI 279
(6) 570K	(20) RN 1304
(7) 5925	(21) MF 31
(8) PS80	(22) FRI 273
(9) S-6 262	(23) 69129
(10) 8325	(24) 639-45I
(11) FAD 209P	(25) 83A
(12) 48S	(26) 740-SEL
(13) S-6	(27) 707-4R
(14) RN 450	(28) 13N 2909

Salmonella enterititidis, Staphylococcus albus, Serratia marcescens and Escherichia coli were used as negative controls. All the cultures were obtained from the Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas; except for S. aureus strains S-6 and 13N 2909 which were obtained from J.J. Iandolo, Department of Biology, Kansas State University, Manhattan, Kansas.

Identities of the staphylococcal cultures were confirmed by colony morphology and pigmentation, Gram-reaction, thermonuclease and coagulase production. A mannitol fermentation test was conducted for the strains yielding less than a +4 coagulase reaction. Non-S. aureus cultures were confirmed by using cell morphology, gram reaction and the Minitek system (BBL Microbiology system, Cockeysville). The characteristics of the named bacteria corresponded well with their descriptions in Bergey's Manual (Buchanan and Gibbon, 1974).

All 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 100 ml of Brain Heart infusion (Difco) and incubating in a water bath shaker (200 rpm) at 37 C for 24 hrs.

Staphylocoagulase Productions by Strains of S. aureus

Twenty-eight of S. aureus plus cultures of Escherichia coli, Serratia marcescens, Salmonella enteritidis and Staphylococcus albus (as negative controls) were given in nutrient agar slants overnight at 37 C, then transferred to Brain Heart Infusion Broth (Difco) and grown in a water bath shaker at 200 rpm for 18 hrs. After the incubation period the cells were removed by centrifugation at 10,000 x g (Beckman model J-21 B) for 10 minutes.

Portions (0.1 ml) of the cell-free supernatant were then added into each of triplicates capillary agar system pipettes. The pipettes were then covered with parafilm and incubated at 37 C for 24 hrs. After incubation time, the length of the precipitation band, if any was present

was measured. The conventional tube method was conducted according to manufacturer's directions (Difco). Two drops of each culture were pipetted using a 1 ml serological pipette into small test tubes (15 x 45 mm) containing 0.5 ml of EDTA rabbit plasma and covered with parafilm. The tubes were then checked for positive reactions after 4 hrs. at 37 C by observing the tubes for plasma coagulation without disturbing the clot, and rechecked at 24 hrs. The reaction was rated from 1+ to 4+, using the criteria of Turner and Schwartz (1958) (Fig. 3).

Staphylocoagulase Production by S. aureus S-6 in Laboratory Media

A culture of S. aureus S-6 was grown in Brain Heart Infusion (BHI) at 37 C for 18 hrs. in a water bath shaker. The cells were washed twice in the sterile medium before being used as inoculum for 100 ml of sterile BHI in a 300 ml Klett flask. The inoculum was diluted to a final reading of 5 Klett units. The flask was then placed in a water bath shaker (200 rpm) at 37 C. Samples consisting of 1.0 ml of culture were aseptically taken every 20 min. A 0.1 ml volume of this sample was utilized for making serial dilutions in phosphate buffer. For the bacterial enumeration, 1.0 or 0.1 ml of each dilution were plated in duplicate plates of staphylococcus medium 110 (Difco) and incubated at 32 C for 48 hrs before colony numbers were determined. The rest of the sample was placed in a small test tube and ethylmercurithiosolecylic acid sodium salt was added to make up a concentration of 1:10,000. This concentration was shown to inhibit growth inside the Pasteur pipettes in preliminary experiments. Such a growth could lead to misleading results during the determination of coagulase. At this point 0.1 ml portions of

Figure 3. Interpretation of Tube coagulase Test[a]

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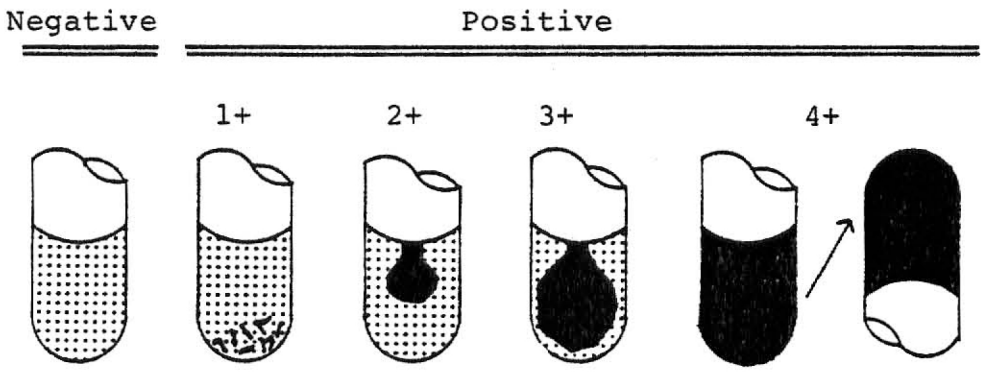
[a]From Turner and Schwartz (1958)



Negative	No Evidence of Fibrin Formation
1+ Positive	Small Unorganized Clots
2+ Positive	Small Organized Clot
3+ Positive	Large Organized Clot
4+ Positive	Entire Content of Tube Coagulates and is not Displaced when Tube is inverted

portions of the sample were added into the capillary agar tube system pipettes. The pipettes were covered with parafilm and incubated at 37 C for 24 hrs. before measuring the length of the precipitation band. The experiment was continued until the culture reached the stationary phase as determined by the unadjusted Klett reading.

Enterotoxin B Detection

Enterotoxin B was determined by 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 (0.02 M, pH 7.4) and 4.0 ml of melted and tempered (48 C) 1% agar (Noble agar, Difco). By suction, a small volume of warm antiserum agar was brought up to about half way the thinner portion of a Pasteur pipette (9 inch size). The tip of the pipette was then sealed with plasticine. After solidification of the agar all Pasteur pipettes were stored in a moist chamber at 4 C.

Samples were assayed by pipetting 0.1 ml into the top of one of the Pasteur pipettes. A quick jerk caused the liquid sample to come in contact with the antiserum agar. The pipettes were then covered with parafilm and incubated at 37 C for 24 hrs. After incubation the pipettes were examined for the presence of a precipitation band in the antiserum agar. The length of this band was proportional to the concentration of enterotoxin in the sample. Quantitation of enterotoxin B was achieved by preparing a standard curve of known enterotoxin concentration (μg/ml) vs. the band length (mm) plotted in semi-log paper. Sensitivity limit of this assay is about 1-10 μg/ml of toxin B.

Detection of Staphylocoagulase and Enterotoxin B from Liquid Foods

Portions of 3 ml cell-free supernatant of S. aureus S-6 containing staphylocoagulase in a concentration of 23 u/ml and enterotoxin B in a concentration of 73 µg/ml were added into 15 ml portions of chicken or beef broth. The theoretical concentration of staphylocoagulase inside the food was of 3.3 u/ml, and of enterotoxin B was 12.2 µg/ml. Half of the contaminated food portions were transferred into 100 ml beakers sealed with a rubber stopper equipped with a thermometer and heated in an oil bath at 80 C for 5 min.

Then 10 ml of each heated and unheated sample was dialyzed against 40% polyethylene glycol for 15 hrs. The resultant aliquot inside the dialysis tube was resuspended in 0.75 ml of the same sterile food. At this point 0.1 ml of the concentrated food was pipetted into each of the duplicate pipettes for the capillary agar tube systems for staphylocoagulase and the antiserum agar system. The pipettes were then covered with parafilm and incubated for 24 hrs. at 37 C. After the incubation period the concentrations of staphylocoagulase and enterotoxin B were determined by matching the lengths of the precipitation band against the previously constructed standard curves.

Heat Stability of Staphylocoagulase

To study heat inactivation of staphylocoagulase, 1.2 ml of the cell-free supernatant was pipetted into each of a number of ampoules (1.2 ml size; Wheathon Scientific Co.). The ampoules were heat sealed, and grouped in sets. Different sets of ampoules were then placed into 3 different oil baths maintained at 80,100 and 121 C respectively. Heat-up

times for an identical system were previously determined (Fung et al., 1973) to be 70S,40S and 40S respectively for the 80,100 and 121 C oil baths. The vials were then heated for time intervals from 3 min. to 5 hrs. The ampoules were removed from the oil bath and placed in an ice bath for 10 min. and then opened. A 0.1 ml portion of heated sample was pipetted into duplicate capillary agar tube system pipettes. The pipettes were covered with parafilm and incubated. After 24 hrs. at 37 C the staphylocoagulase concentration was determined.

In order to determine if the enzyme possessed reactivation properties similar to those of staphylococcal enterotoxins (Fung et al., 1973) the following procedure was conducted: 0.4 ml samples of heat treated supernatant were placed into duplicate petri dishes (10 cm by 1.5 cm height). The petri dishes were then sealed by tape. It was previously determined that evaporation of liquid in the petri dishes was negligible and that no viable organisms were found in the heat treated supernatants. One petri dish was incubated at 25 C and the other one at 4 C; after 24 hrs. staphylocoagulase activities were determined as previously described.

Fung et al. (1973) suggested that reactivation observed in heat treated staphylococcal toxin could be related to unfolding and refolding of toxin molecules, and that if this was the case, mechanical agitation of toxin solution during heating should effect the stability of toxins. To study this possibility in staphylocoagulase, portions (25 ml) of staphylocoagulase containing supernatant were placed in 100 ml beakers containing magnetic stirring bars. Each beaker was sealed with a stopper equipped with a thermometer and a syringe for removal of samples.

The solutions were heated at 80 C and 95 C with or without stirring. After 10 and 60 minutes of heating, 1.0 ml samples were withdrawn. Inactivation and reactivation profiles were then studied as described in the previous paragraph. As a control one of the samples was stirred for 5 hrs. at 25 C.

In order to compare the stability of concentrated staphylocoagulase to that of the unconcentrated preparations, portions (10 ml) of concentrated and unconcentrated supernatant were placed into 50-ml beakers. The beakers were sealed with a rubber stopper equipped with a thermometer. The solutions were heated at 80 and 95 C for 10 min., and the inactivation and reactivation profiles of both preparations studied. The concentrated staphylocoagulase was prepared as it has been described previously except that Brain Heart Infusion was used as the resuspension volume.

Methods of Statistical Analysis

The comparative data was computer analyzed by the following procedures (SAS User's Guide, 1982):

Analysis of variance was conducted using the SAS ANOVA procedure for balanced data sets (equal number of replications per treatment combination) and General Linear Model (GLM) for unbalanced data sets. Multiple comparisons between means were made using Fisher's (protected) Lease Significant Different (LSD) at alpha = 0.05. Pearson's correlations were computed with the CORR procedure.

RESULTS

Development of Capillary Agar Tube System

Figure 4 shows the standard curve developed for staphylocoagulase quantification by means of the capillary tube system. The curve was developed by serially diluting a concentrated, cell free preparation (containing staphylocoagulase) from 150 u/ml to 1 u/ml. Portions (0.1 ml) of the diluted sample were applied into triplicate test capillary tubes. After 24 hrs. incubation at 37 C the opaque cylindrical zones which appeared in the agar, were measured in millimeters by an optical micrometer. A linear relationship was observed between the length of the precipitation band and the logarithm of the staphylocagulase concentration, the length of the band being directly proportional to the staphylocoagulase concentration present in the sample. The length of the band ranged in size from 1.2 mm to 8.1 mm. The lowest coagulase activity detectable with the assay was about 1.7 u/ml. The correlation coefficient for the linear relationship was of 0.98. Two straight line slopes were obtained, one for concentrations higher than 2.34 u/ml, and another for smaller concentrations. Every point in the graph represents the mean of the three tests.

Effect of Reagent Concentrations Upon the Capillary Agar Tube System

Figure 5 shows the effect of plasma and soybean trypsin inhibitor (SBTI) upon the length of the precipitation band observed in the capillary tube system after incubation for 24 hrs. at 37 C. Both the SBTI and plasma concentrations significantly influenced the length of the band ($p = .0001$) as shown in the General Linear Model in Table 4.

Figure 4. Standard Curve for Staphylocoagulase Activity by the Capillary Agar Tube System.

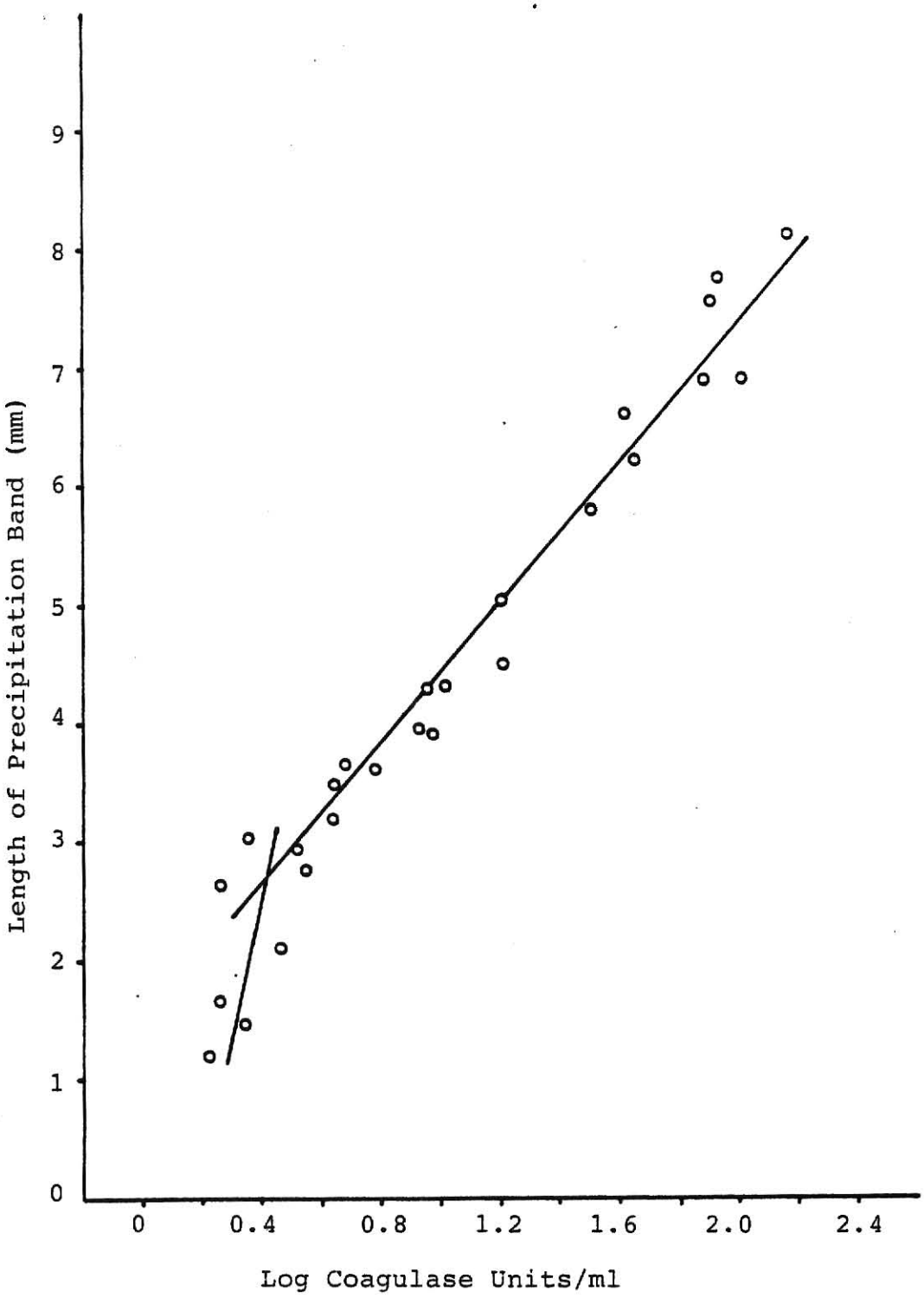

Length of Precipitation Band (mm)
0
1
2
3
4
5
6
7
8
9
0
0.4
0.8
1.2
1.6
2.0
2.4
Log Coagulase Units/ml

Figure 5. Effect of SBTI-Plasma Concentration on the Length of the Precipitation Band.

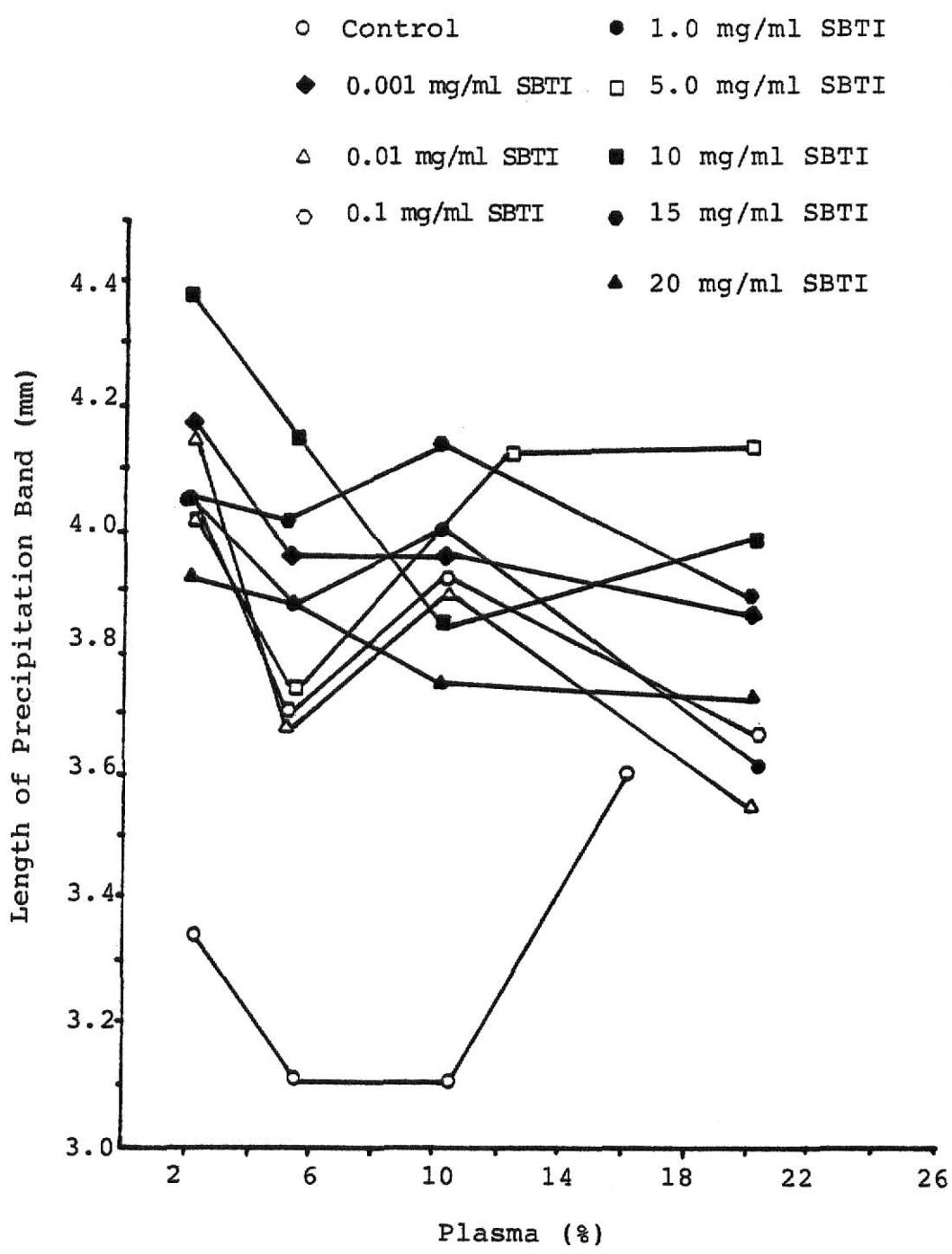

Control
0.001 mg/ml SBTI
0.01 mg/ml SBTI
0.1 mg/ml SBTI
1.0 mg/ml SBTI
5.0 mg/ml SBTI
10 mg/ml SBTI
15 mg/ml SBTI
20 mg/ml SBTI
Length of Precipitation Band (mm)
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2
6
10
14
18
22
26
Plasma (%)

Table 4. General Linear Model Procedures
Effect of Plasma and SBTI Upon the Length of the Precipitation Band

Source	DF	Type III SS	MS	F	P
Plasma	3	2.0151008	0.67170027	14.87	0.0001
SBTI	8	10.6453770	1.33067210	29.46	0.0001
Plasma*SBTI	24	3.7104070	0.15460030	3.42	0.0001
Error	176	7.9493333	0.04516670		

All the concentrations of SBTI used, significantly increased (alpha = 0.05) the length of the precipitation band over that of the control group, except at the 20% plasma concentration. As little as 0.001 mg/ml of SBTI significantly increased the length of the precipitation band over the control samples.

In all mixtures tested the length of the band decreased when the concentration of plasma was increased from 2% to 5%. The length of the band increased or remained the same when the plasma concentration increased from 5% to 10% except in agar mixtures containing 10 mg/ml and 20 mg/ml of SBTI. Going from 10% to 20% plasma again decreased the length of the band in all samples except in 5 mg/ml and 10 mg/ml SBTI containing samples and in the control group. Therefore, no clear relationship could be established between the length of the band and the plasma or the SBTI concentration (Fig. 5). It appears that the length of the band is dependent upon the relative proportions of these two components rather than the concentration of either one.

Table 6 shows the means of each of the agar mixtures mentioned above as well as rating for the clarity of the band observed. On the basis of the length of the band (the longer the band, the more sensitive the assay), the clarity of the band, and "good" performance while saving materials; five plasma - SBTI combinations were selected for further analysis, in order to find the most appropriate combinations for the capillary agar tube system. The selected mixtures were: (a) 2% plasma and 0.001 mg/ml SBTI, (b) 2% plasma and 0.01 mg/ml SBTI, (c) 2% plasma and 0.1 mg/ml SBTI, (d) 2% plasma and 1.0 mg/ml SBTI.

Table 5. General Linear Model Procedures
Effect of Plasma and Fibrinogen Upon the Length of the Precipitation Band

Source	DF	Type IV SS	MS	F	P
Plasma	3	0.12086301	0.04028767	1.0	0.4002
Fibrinogen	3	3.94562922	1.31520974	32.58	0.0001
Plasma*Fibr.	9	0.77254938	0.08583882	2.13	0.0382
Error	70	2.82550000	0.04036429		

Table 6. Comparison of Means of Precipitation Band Length Obtained with Different Plasma-SBTI Concentrations

Reagent Mixture	Length of Band	Clarity* of Band	No. of Observations
1. 0 mg/ml SBTI			
A	3.33^{k}	+2	6
B	3.06^{l}	+2	6
C	3.10^{jk}	+2	6
D	4.04^{k}	+3	6
2. 0.001 mg/ml SBTI			
A	4.17^{ab}	+2	6
B	3.96^{bcdef}	+2	6
C	3.96^{bcdef}	+2	6
D	3.86^{efghi}	+2	5
3. 0.01 mg/ml SBTI			
A	4.16^{ab}	+2	6
B	3.68^{ghi}	+3	6
C	3.90^{defgh}	+3	6
D	3.55^{jk}	+3	6
4. 0.10 mg/ml SBTI			
A	4.05^{bcde}	+3	6
B	3.70^{ghij}	+4	6
C	3.92^{cdefg}	+3	6
D	3.67^{hij}	+3	6
5. 1.0 mg/ml SBTI			
A	4.06^{bcde}	+3	6
B	3.87^{efgh}	+4	6
C	4.0^{bcde}	+4	6
D	3.62^{ij}	+4	6

Table 6. (cont.)

Reagent Mixture	Length of Band	Clarity* of Band	No. of Observations
6. 5 mg/ml SBTI			
A	4.02^{bcde}	+3	6
B	3.73^{fghij}	+3	6
C	4.10^{abcd}	+3	5
C	4.12^{abcd}	+3	5
7. 10 mg/ml SBTI			
A	4.35^{a}	+3	6
B	4.17^{ab}	+3	6
C	3.85^{efghi}	+3	6
D	3.98^{bcde}	+3	5
8. 15 mg/ml SBTI			
A	4.05^{bcde}	+4	6
B	4.20^{bcd}	+4	6
C	4.14^{abc}	+4	6
D	3.88^{efgh}	+4	6
9. 20 mg/ml SBTI			
A	3.90^{cdefg}	+4	6
B	3.88^{efgh}	+4	6
C	3.65^{ij}	+4	6
D	3.62^{j}	+4	6

Sample codes:

A - 2% rabbit plasma

B - 5% rabbit plasma

C - 10% rabbit plasma

D - 20% rabbit plasma

At least one subscript in common denotes the sample means are not significantly different at the alpha = 0.05 level.

*The clarity of the band was rated from the lowest capacity (+1) to the highest (+4)

Figure 6 shows the effect of plasma and fibrinogen combinations upon the length of the precipitation band. The level of fibrinogen significantly ($p = .0001$; Table 5) influenced the length of the precipitation band. The length of the band decreased with increased concentrations of fibrinogen. This relationship was particularly strong in the samples containing 2% plasma. The plasma concentration did not significantly influence ($p = 0.4$) the length of the band in this case.

Table 7 shows the means for each of the plasma-fibrinogen combinations studied as well as the rating for the clarity of the band. On the basis previously described, the combination of 0.2% fibrinogen and 2% plasma was selected as the best for the capillary tube method.

Based on the above data (Tables 6 and 7), the combination of 2% plasma, 0.2% fibrinogen and 0.001 mg/ml SBTI was selected as the most appropriate mixture. However, when serial dilutions of the concentrated cell free supernatant used as standard was added to this preparation a high degree of fibrinolysis (disappearance of the precipitation band) was observed in many of the dilutions tested. This phenomenon occurred only to a small degree when constructing the standard curve for the assay containing the agar designed by Kohl and Johnson (1980). The reason for the relative resistance of the former agar to fibrinolysis was that it contained 0.3% fibrinogen rather than 0.2%. Therefore, the precipitation band observed (as a result of fibrinogen degredation) was more concentrated, and in turn more resistant to fibrinolytic compounds. The fact that this was not observed when unconcentrated samples were used obviously suggests that the fibrinolytic compounds were concentrated

Figure 6. Effect of Plasma-Fibrinogen Concentration on the Length of the Precipitation Band.

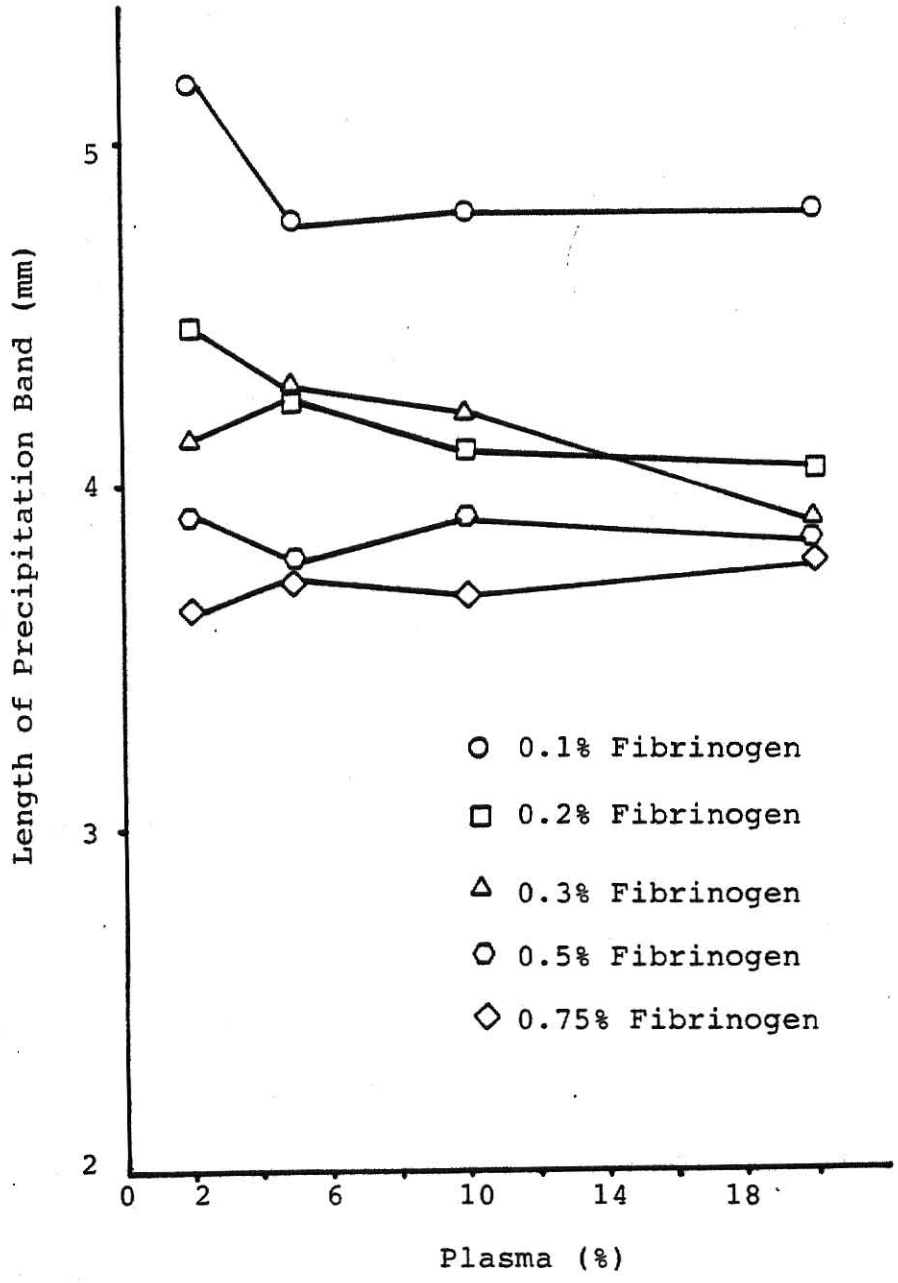

Length of Precipitation Band (mm)
5
4
3
2
0
2
6
10
14
18
Plasma (%)
0.1% Fibrinogen
0.2% Fibrinogen
0.3% Fibrinogen
0.5% Fibrinogen
0.75% Fibrinogen

Table 7. Comparison of Means of Precipitation Band Length Obtained with Different Plasma-Fibrinogen Concentrations.

Reagent Mixture	Length of Band	Clarity of Band	No. of Observations
1. 0.05% bovine Fibrinogen			
A	N*	---	6
B	N	---	6
C	N	---	6
D	N	---	6
2. 0.10% bovine Fibrinogen			
A	5.16^{a}	+1	6
B	4.78^{a}	+1	6
C	4.80^{a}	+2	6
D	4.80^{a}	+2	6
3. 0.20% bovine Fibrinogen			
A	4.46^{b}	+3	6
B	4.24^{bc}	+3	6
C	4.10^{d}	+3	6
D	4.02^{de}	+3	6
4. 0.30% bovine Fibrinogen			
A	4.14^{cd}	+4	6
B	4.27^{bc}	+4	5
C	4.22^{bc}	+4	5
D	3.90^{def}	+4	5
5. 0.50% bovine Fibrinogen			
A	3.92^{def}	+4	5
B	3.78^{efg}	+4	6
C	3.93^{de}	+4	5
D	3.84^{efg}	+4	6

Table 7. (cont.)

Reagent Mixture	Length of Band	Clarity of Band	No. of Observations
6. 0.75% bovine Fibrinogen			
A	3.64^{g}	+4	6
B	3.73^{efg}	+4	5
C	3.68^{efg}	+4	5
D	3.77^{efg}	+4	5

Sample codes:

A - 2% rabbit plasma

B - 5% rabbit plasma

C - 10% rabbit plasma

D - 20% rabbit plasma

*No visible reaction occurred. Means with at least one subscript in common are not significantly different at alpha = 0.05 level.

in a more efficient rate than the coagulase during the concentration procedure.

Adding 10 mg/ml of SBTI instead of 0.001 mg/ml, completely inhibited the occurrance of fibrinolysis. In order to decide the minimal SBTI concentration necessary to protect the assay against fibrinolysis, as well as providing optimum sensitivity, agar mixtures containing 0.1 mg/ml SBTI, 1.0 mg/ml SBTI and 10 mg/ml SBTI were designed. Yet another parameter was added; the effect of agar concentration was studied by making the three agar mixtures described above with 0.5% and 1% Noble agar (Difco). As a control, a mixture containing no SBTI, 2% plasma and 0.2% fibrinogen was also run, at both 0.5% and 1.0% agar.

Figures 7,8, and 9 show the standard curves for the 0.1 mg/ml SBTI, 1.0 mg/ml SBTI and 10 mg/ml SBTI respectively (at 1.0% agar concentration) after 1 hr., 4 hrs., 8 hrs., 12 hrs., 16 hrs and 24 hrs. Figure 7 shows the performance of the agar containing 0.1 mg/ml of SBTI. No fibrinolysis was observed in any of the dilutions. The assay was able to detect down to 2.3 u/ml within 6 hrs, incubation and 0.82 u/ml within 6 hrs, incubation and 0.82 u/ml within 16 hrs. The correlation coefficient was very high (>0.95) for all times tested.

Figure 10 shows the performance for the agar mixture containing the 0.1 mg/ml of SBTI but half the agar concentration (0.5%). This preparation was able to detect 2.3 u/ml within 4 hrs., and 0.82 u/ml within just 8 hrs. This was the fastest of any of the reaction mixtures tested. The correlation was high (>0.95) after all times tested.

Figure 7. Agar Mixture Containing 0.1 mg/ml of SBTI and 1% Agar.

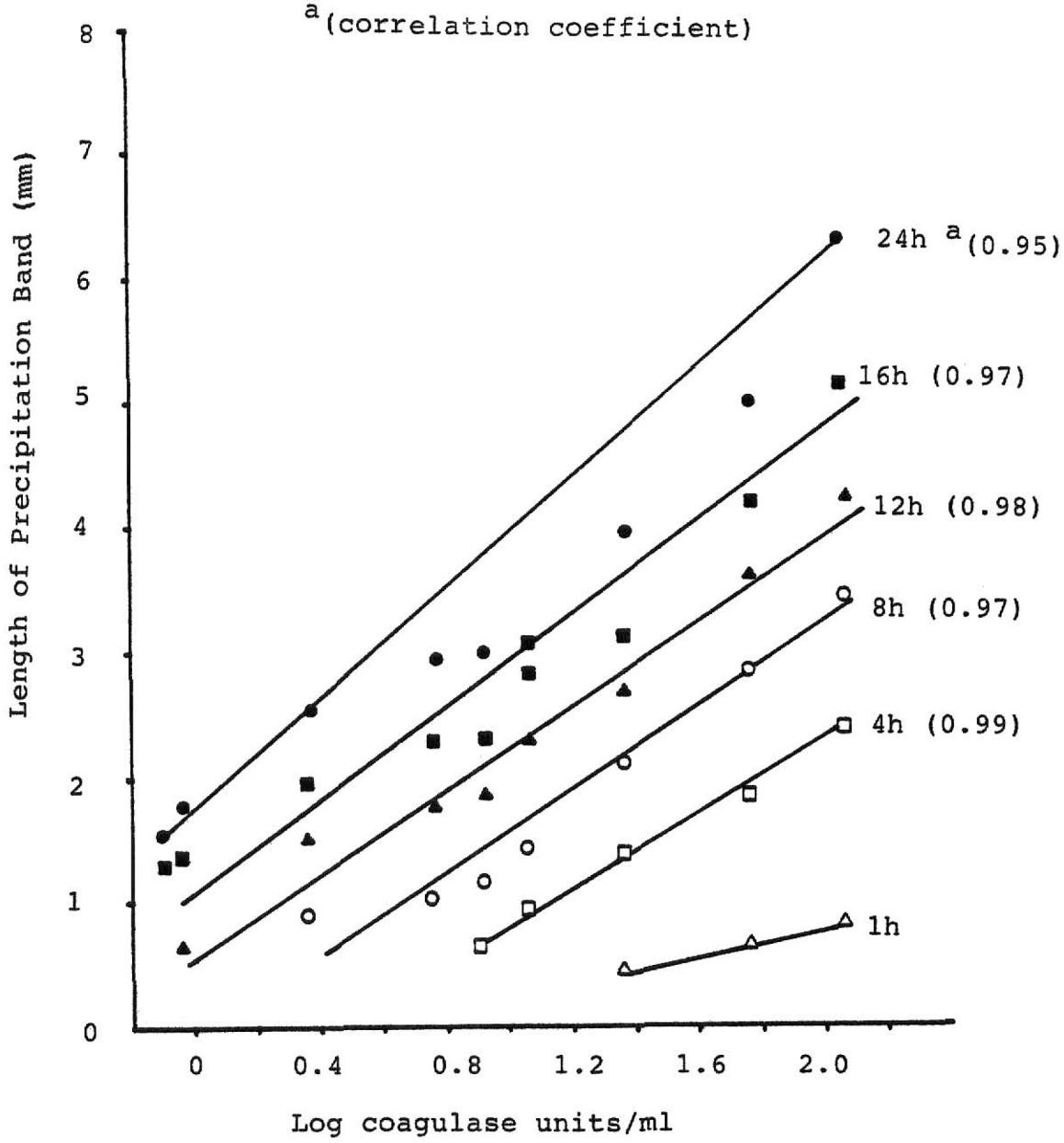

[a](correlation coefficient)
Length of Precipitation Band (mm)
0
1
2
3
4
5
6
7
8
0
0.4
0.8
1.2
1.6
2.0
Log coagulase units/ml
24h [a](0.95)
16h (0.97)
12h (0.98)
8h (0.97)
4h (0.99)
1h

Figure 8. Agar Mixture Containing 1.0 mg/ml of SBTI and 1% Agar

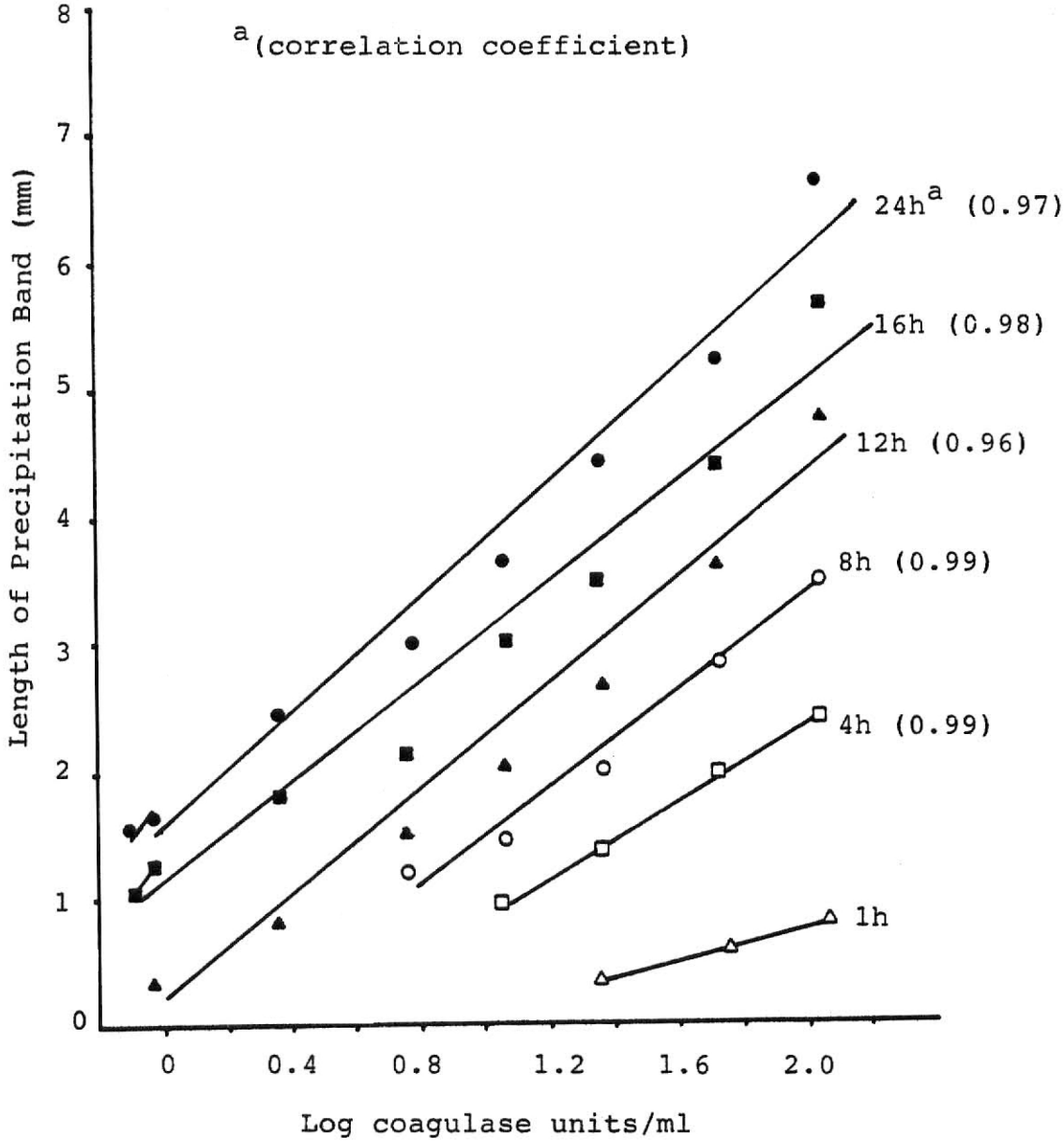

[a](correlation coefficient)
Length of Precipitation Band (mm)
Log coagulase units/ml
24h[a] (0.97)
16h (0.98)
12h (0.96)
8h (0.99)
4h (0.99)
1h
0
1
2
3
4
5
6
7
8
0
0.4
0.8
1.2
1.6
2.0

Figure 9. Agar Mixture Containing 10 mg/ml of SBTI and 1% Agar.

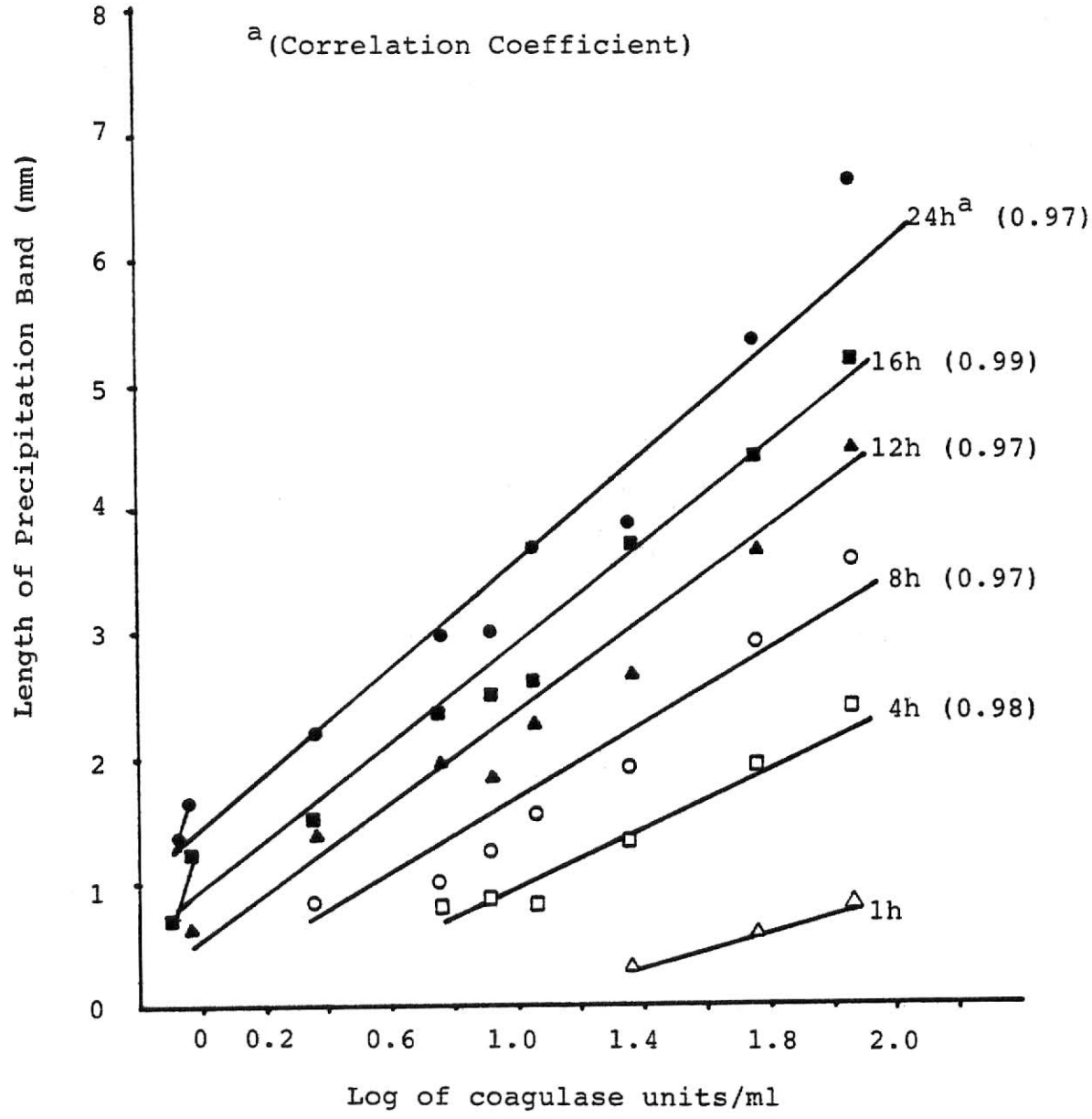

[a](Correlation Coefficient)
Length of Precipitation Band (mm)
Log of coagulase units/ml
24h[a] (0.97)
16h (0.99)
12h (0.97)
8h (0.97)
4h (0.98)
1h
0
1
2
3
4
5
6
7
8
0
0.2
0.6
1.0
1.4
1.8
2.0

Figure 11 shows the standard curves for the agar mixture containing 1.0 mg/ml of SBTI and an agar concentration of 0.5%. Figure 12 presents the standard curve for the agar mixtures containing 10 mg/ml of SBTI and agar concentration of 0.5%. All standard curves showed two straignt line slopes; one slope for enzyme concentration of 2.3 u/ml or higher and another for enzyme concentration smaller than 2.3 u/ml.

Table 8 summarizes the performance of all the agar mixtures tested. The agars without SBTI showed a high degree of fibrinolysis. Therefore, no standard curves could be constructed for those mixtures. As little as 0.1 mg/ml of SBTI provided protection from these phenomena.

Agar mixtures containing 0.5% agar provided better sensitivity in shorter time as compared to those containing 1% agar. The mixtures containing 0.5% agar also showed an improved band opacity. This is mainly due to the fact that the lower agar content caused the solution to be clearer, and therefore, creating a better contrast with the precipitation band. The best sensitivity was that of the mixture containing 0.1 mg/ml of SBTI and 0.5% agar. The correlation coefficients for all agars were very high, proving the suitability of this assay for measuring coagulase activity.

Based on the data in Table 8 the agar mixture containing 2% plasma, 0.2% fibrinogen, 0.1 mg/ml of SBTI and 0.5% agar was selected as the best mixture for the capillary tube assay. Table 9 shows the ingredients needed for preparing 50 ml of this mixture, which is enough for 500 units of the assay.

Figure 10. Agar Mixture Containing 0.1 mg/ml of SBTI and 0.5% Agar.

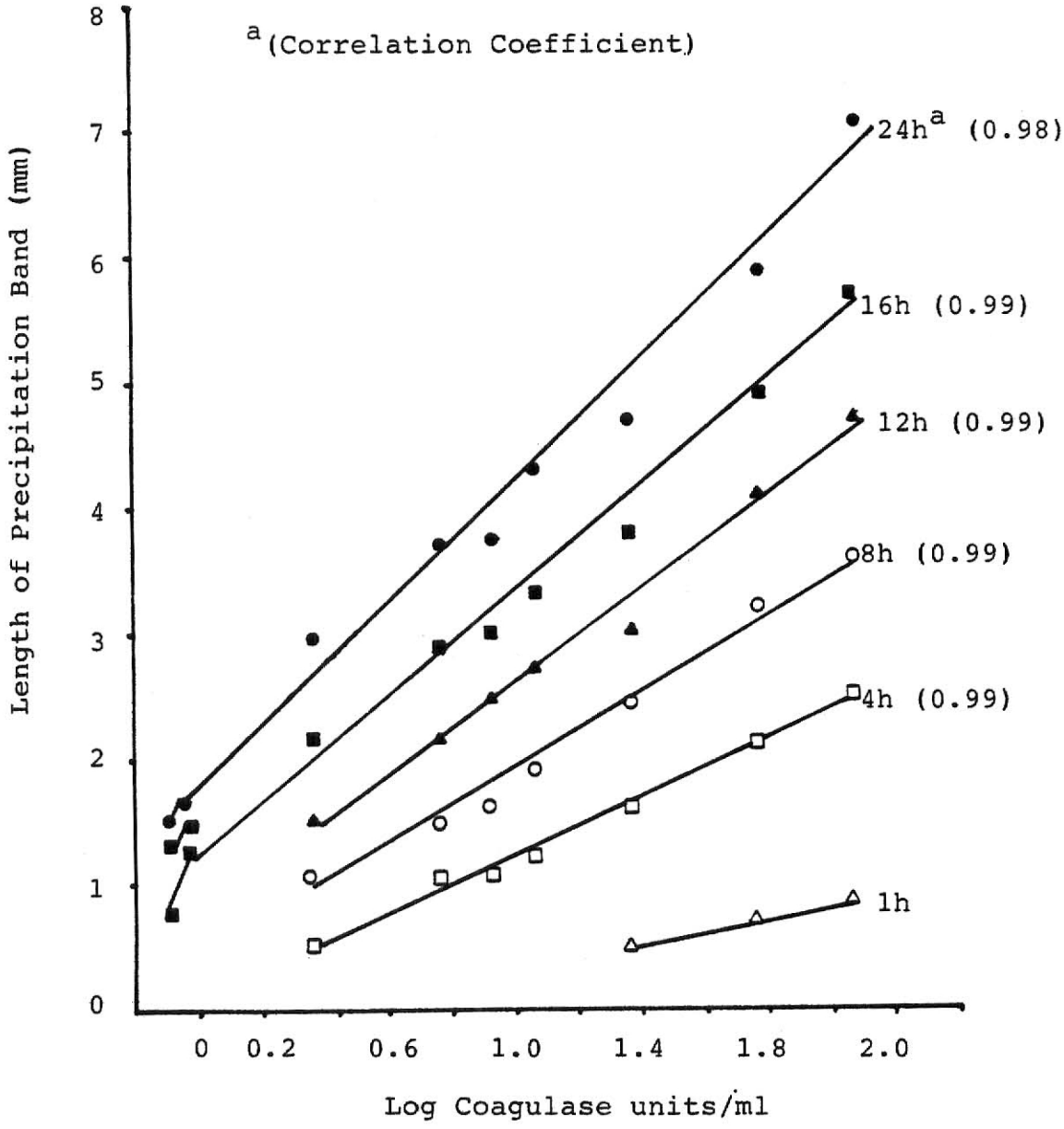

[a](Correlation Coefficient)
24h[a] (0.98)
16h (0.99)
12h (0.99)
8h (0.99)
4h (0.99)
1h
Length of Precipitation Band (mm)
Log Coagulase units/ml
0
1
2
3
4
5
6
7
8
0
0.2
0.6
1.0
1.4
1.8
2.0

Figure 11. Agar Mixture Containing 1.0 mg/ml of SBTI and 0.5% Agar.

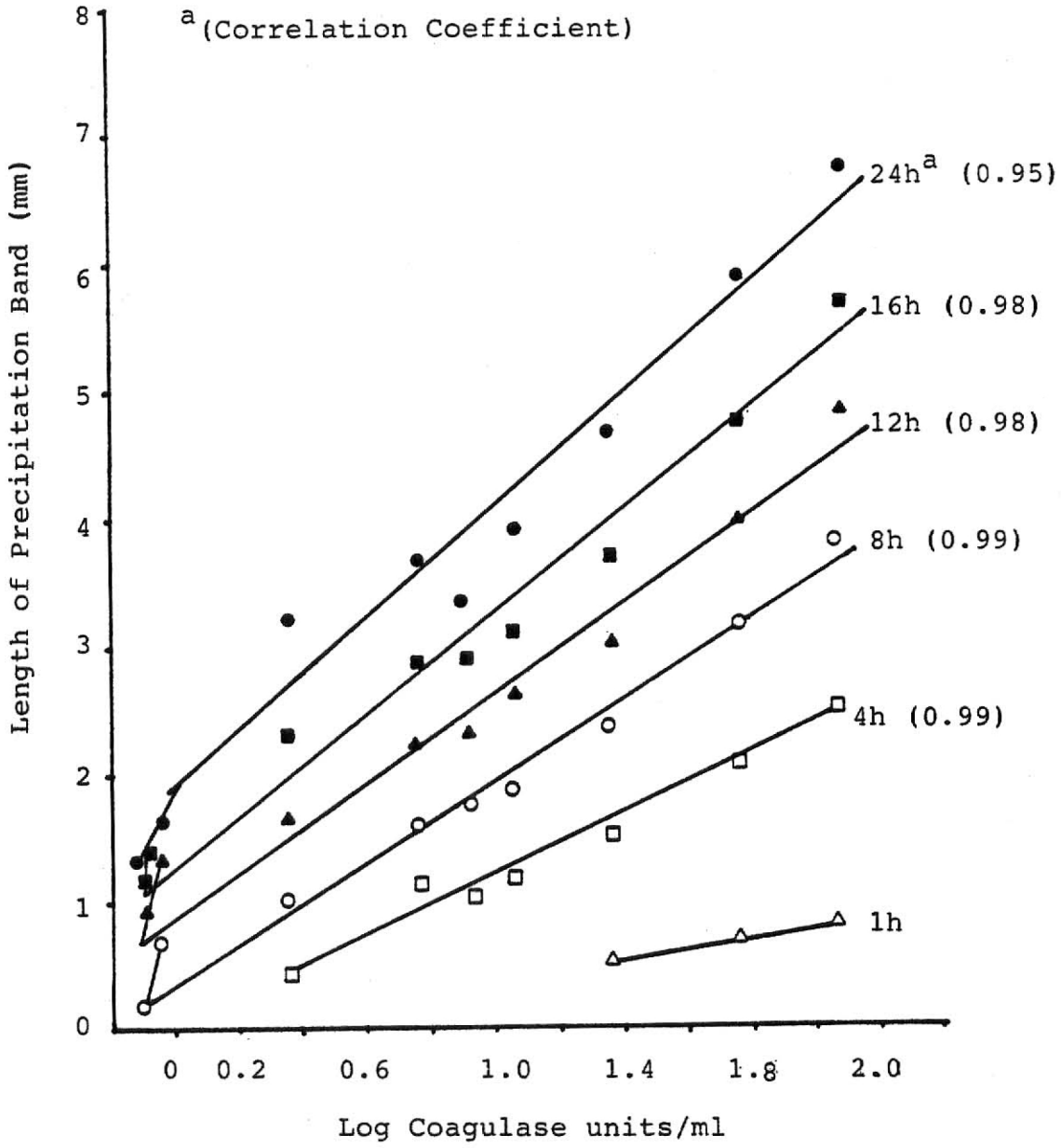

[a](Correlation Coefficient)
Length of Precipitation Band (mm)
Log Coagulase units/ml
24h[a] (0.95)
16h (0.98)
12h (0.98)
8h (0.99)
4h (0.99)
1h
0
1
2
3
4
5
6
7
8
0
0.2
0.6
1.0
1.4
1.8
2.0

Figure 12. Agar Mixture Containing 10 mg/ml of SBTI and 0.5% Agar.

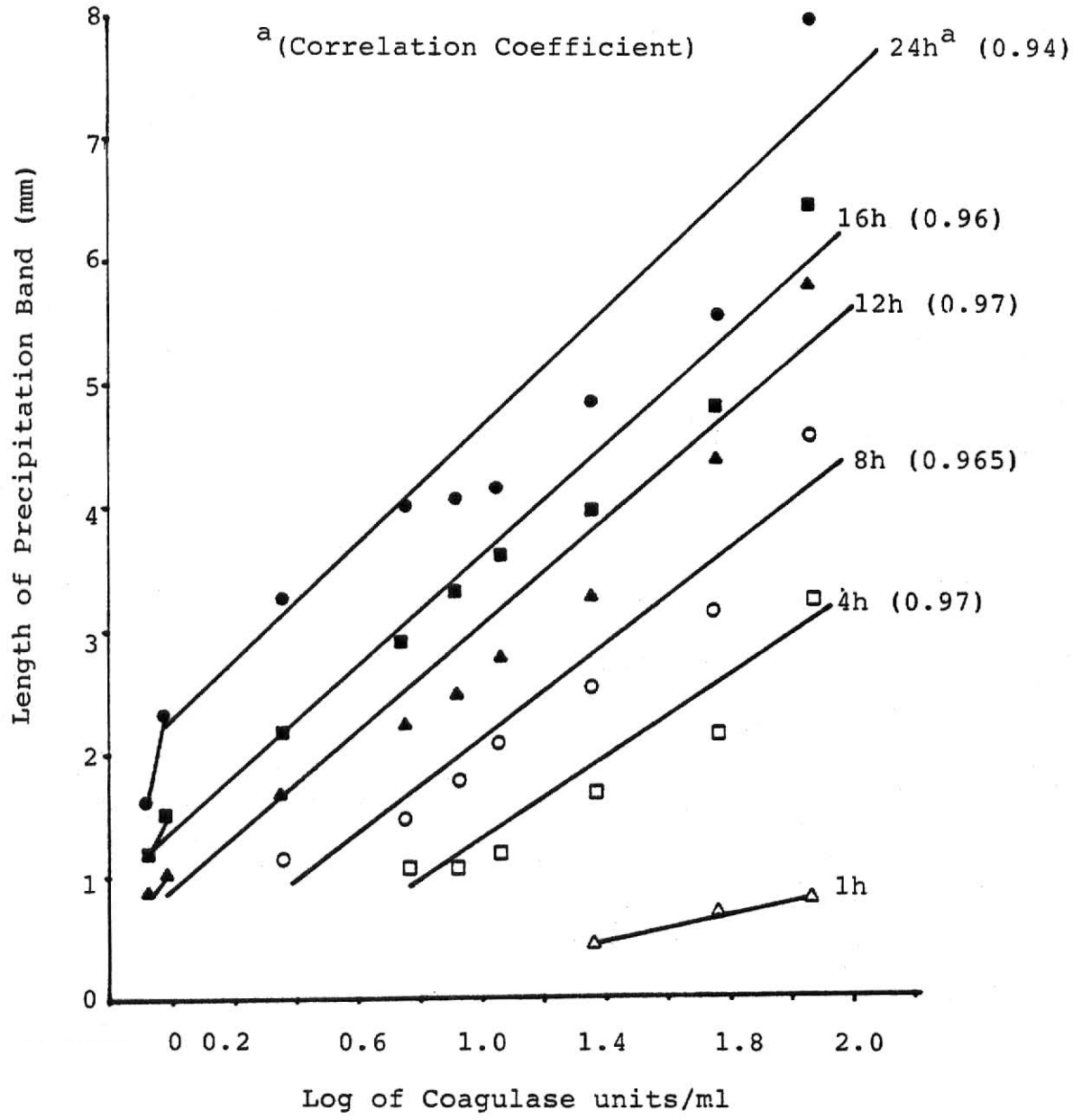

[a](Correlation Coefficient)
24h[a] (0.94)
16h (0.96)
12h (0.97)
8h (0.965)
4h (0.97)
1h
Length of Precipitation Band (mm)
Log of Coagulase units/ml
0
1
2
3
4
5
6
7
8
0 0.2
0.6
1.0
1.4
1.8
2.0

Table 8. Performance of Reagent Mixtures on the Capillary Tube System

Reagent* Mixture	Incubation time for max. sensitivity (hours)	Clarity of the band	Correlation coefficient	Degree of Fibrinolysis
1	---	---	---	complete
2	16	+3	0.967	None
3	16	+3	0.977	None
4	16	+3	0.987	None
5	---	---	---	complete
6	8	+4	0.986	None
7	12	+4	0.987	None
8	12	+4	0.965	None

Sample code:

SBTI

1 - 0 mg/ml
2 - 0.1 mg/ml
3 - 1.0 mg/ml
4 - 10.0 mg/ml
> 1.0% agar

5 - 0 mg/ml
6 - 0.1 mg/ml
7 - 1.0 mg/ml
8 - 10 mg/ml
> 0.5% agar

*All mixtures contained 2% plasma and 0.2% Fibrinogen

Table 9. Preparation and Ingredient for Improved Capillary Agar Tube System

Solution A:	-0.25 g Noble agar (0.5%) -25 ml of 0.01 M sodium phosphate buffer with 0.06 M NaCl at pH 7.2 Tempered at 48°C
Solution B:	-0.15 g Type I bovine fibrinogen (0.3%) -1.0 ml rabbit plasma (EDTA) (2.0%) -0.05 g Soybean Trypsin Inhibitor (0.1%) -0.5 ml of 1:100 Merthiolate (1:10,000) -24 ml same buffer Tempered at 37°C
Solution A + Solution B = Plasma - Fibrinogen agar	Tempered at 40°C

*Enough for about 500 units

Staphylocoagulase Production by S. aureus S-6 in Laboratory Media

Figure 13 shows the production of staphylocoagulase by S. aureus S-6 cultured under constant shaking (200 rpm) at 37 C in Brain Heart Infusion (Difco). The study was carried in a 300 ml Klett flask with 100 ml of medium. Coagulase was produced within 20 minutes of the log phase, and all throughout the log phase, increasing from 0 u/ml to 21 u/ml by the end of log phase. A small amount of coagulase (from 21 to 26 u/ml) was produced during early stationary phase. No additional coagulase production was observed during stationary phase.

Staphylocoagulase Production by Strains of S. aureus

Table 10 shows the production of staphylocoagulase by strains of S. aureus cultured in Brain Heart Infusion at 37 C, under constant shaking for 24 hrs. Coagulase was detected by both the capillary tube and conventional tube test. All the S. aureus strains tested were coagulase positive by both assays, with the exceptin of strain 13N 2909 which was negative in both tests. No coagulase production was observed in the controls containing cultures of E. coli, Serratia marcescens, Salmonella and Staphylococcus albus.

Strain 137 was the lowest coagulase producer with 4.9 u/ml which was equivalent to a 2+ reaction in the conventional test. The highest production was that of Strain 707-4R with 102.3 u/ml.

Figure 13. Growth and Staphylocoagulase Production by S. aureus S-6 at 37°C and 200 rpm in Brain Heart Infusion.

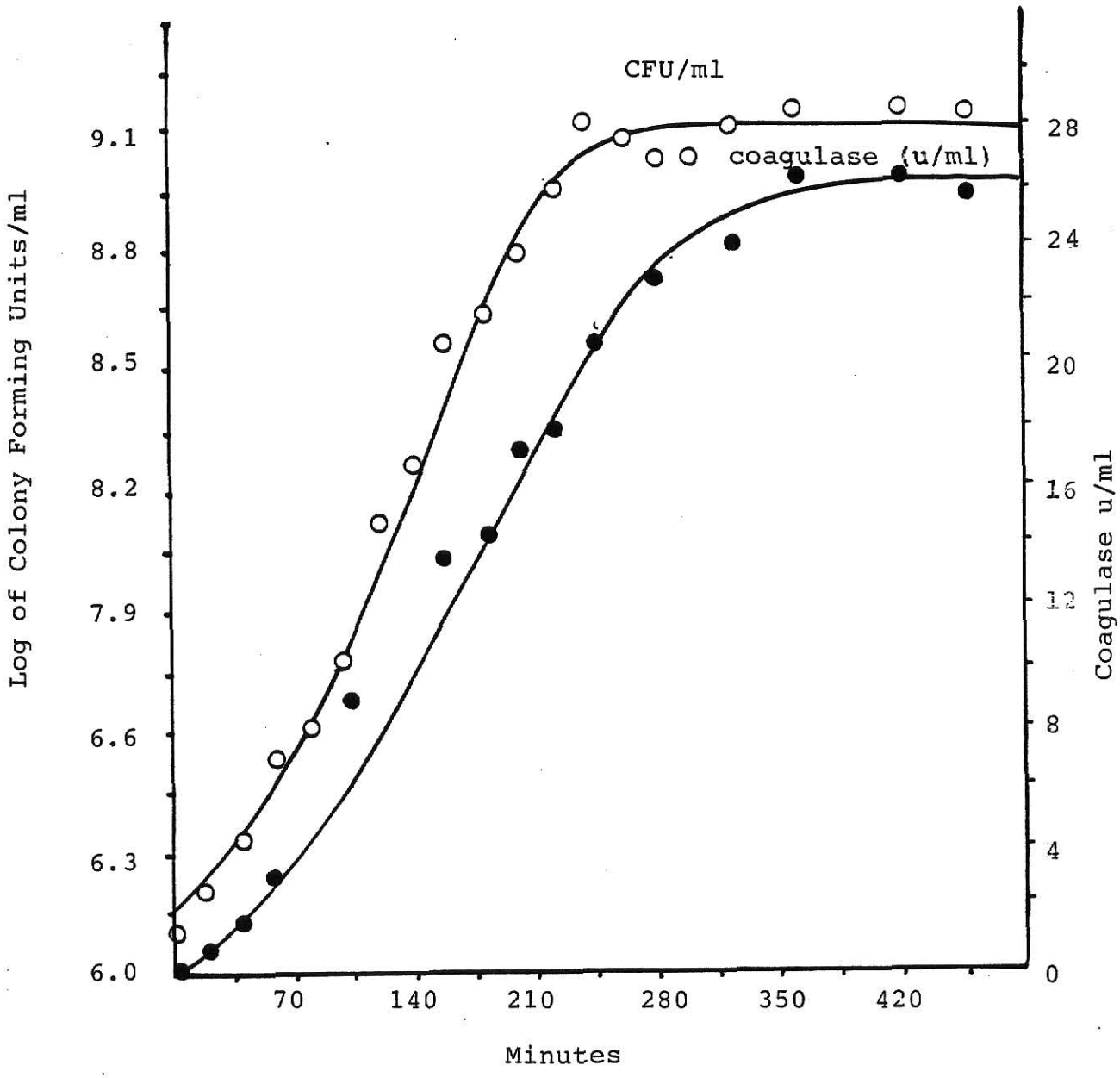

CFU/ml
coagulase (u/ml)
Log of Colony Forming Units/ml
Coagulase u/ml
Minutes
9.1
8.8
8.5
8.2
7.9
6.6
6.3
6.0
28
24
20
16
12
8
4
0
70
140
210
280
350
420

Table 10. Coagulase Activities of *Staphylococcus aureus* Measured by the Capillary Tube Method and the Conventional Tube Method.

Strain	Band length (mm)	Coagulase unit/ml	Conventional 4 hr test
137	3.45	4.9	2+
I SP 2	3.67	5.8	2+
COLS	4.40	9.5	2+
472	3.40	4.8	3+
196-E	4.40	9.5	3+
570 K	4.63	11.5	3+
5925	5.57	22.9	3+
PS80	6.00	31.6	3+
S-6 262	6.00	31.6	3+
8325	4.03	7.2	4+
FAD 209P	4.40	9.5	4+
48S	4.53	10.5	4+
S-6	5.43	20.9	4+
RN 450	5.43	20.9	4+
DU-MAS	5.43	20.9	4+
RN2425	5.60	22.9	4+
DU4916	5.60	22.9	4+
5106R	5.67	23.9	4+
FRI 279	5.77	25.1	4+
RN 1304	5.93	30.2	4+
MF 31	6.13	34.7	4+
FRI 273	6.25	36.3	4+
69129	6.30	38.0	4+
639-45I	6.37	39.8	4+
83A	6.57	45.7	4+
740-SEL	7.30	79.4	4+
707-4R	7.75	102.3	4+
13N2909	Neg.	0.0	--
E. coli	Neg.	0.0	--
Serratia marcescens	Neg.	0.0	--
Salmonella enteritidis	Neg.	0.0	--
Staphylococcus albus	Neg.	0.0	--

Detection of Staphylocoagulase and Enterotoxin B from Liquid Foods

In order to ascertain the potential usefulness of the capillary tube system for the detection of Staphylocoagulase in foods, two food systems were contaminated with culture supernatant containing staphylocoagulase and enterotoxin B. Table 11 shows the recovery of both enterotoxin B and staphylocoagulase from heated and unheated chicken and beef broth. Staphylocoagulase and enterotoxin B were detected in all samples of heated and unheated foods. Staphylocoagulase was recovered more efficiently than enterotoxin B in all cases. This proves the suitability of staphylocoagulase as a possible means of testing for enterotoxin presence in food. Controls containing uncontaminted foods did not show staphylocoagulase or enterotoxin activity.

Heat Stability of Crude Staphylocoagulase from S. aureus S-6

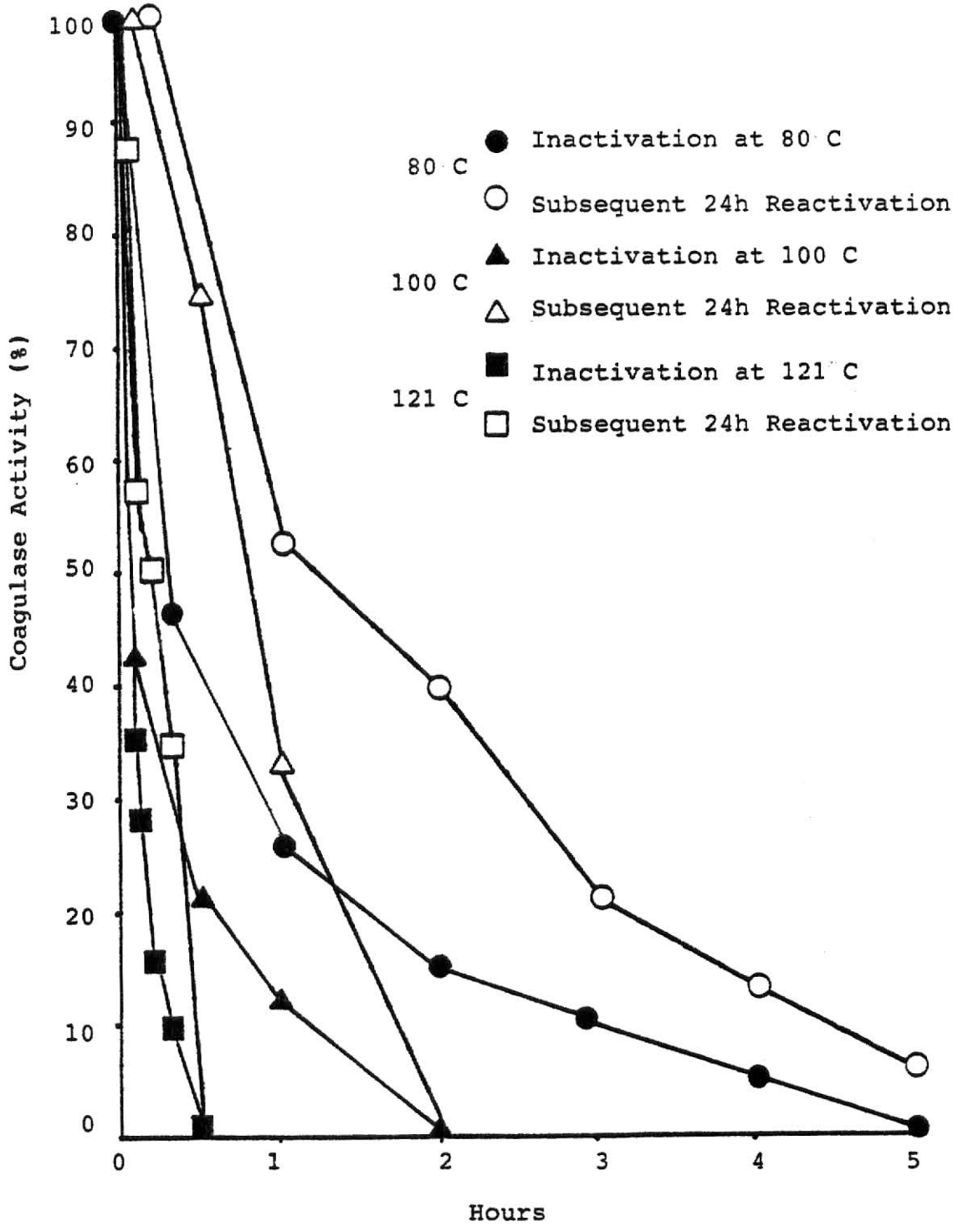
The cell free superantant of S. aureus S-6, containing a known staphylocoagulase concentration (26 u/ml) was used throughout this experiment. Figure 14 shows the thermal inactivation curves of crude staphylocoagulase at 80,100 and 121 C. After 4 hrs. of heating at 80 C, staphylocoagulase retained some (5.8%) residual enzymatic activity. No activity was retained after 5 hrs. at the same temperature. Heating at 100 C and 121 C inactivated staphylocoagulase activity after 2 hrs. and 30 min., respectively. After 24 hrs. reactivation at 25 C, staphylocoagulase treated at all three temperatures regained a certain amount of activity, with the exception of the preparations that were heated for 2 hrs. and 30 min. at 100 C and 121 C respectively. Complete reactivation (100%) was observed on the samples 30 min. and 5 min. at 80 C and 100 C respectively.

Table 11. Detection of Staphylocoagulase and Enterotoxin B from Treated Liquid Foods

Food	Amount added		Amount recovered		% Recovery		Concentration Factor
	coagulase (u/ml)	toxin (µg/ml)	coagulase (u/ml)	toxin (µg/ml)	coagulase	toxin	
Beef Broth unheated	3.8	12.2	19.9	32.8	39.1	20.7	13
Beef Broth* heated	3.8	12.2	17.4	10.0	34.0	6.3	13
Chicken Broth unheated	3.8	12.2	15.1	27.0	29.6	17.1	13
Chicken Broth heated*	3.8	12.2	14.5	16.3	28.4	10.3	13

*Heated at 80°C for 5 minutes.

Figure 14. Heat Inactivation and Subsequent Reactivation at 25 C of Staphylocoagulase.


100
90
80
70
60
50
40
30
20
10
0
Coagulase Activity (%)
0
1
2
3
4
5
Hours
80 C
Inactivation at 80 C
Subsequent 24h Reactivation
100 C
Inactivation at 100 C
Subsequent 24h Reactivation
121 C
Inactivation at 121 C
Subsequent 24h Reactivation

Reactivation to some degree also occurred when the heated samples were allowed to reactivate at 4 C for 24 hrs. as shown in Figure 15. The level of activity recovered at 4 C significantly smaller ($p = 0.0001$) than that observed at 25 C at all heating temperatures. Complete reactivation did not occur in any sample at 4 C. After 24 hrs. reactivation at 4 C samples heated for 100 min. and 30 min. at 100 C and 121 C, did not reactivate to any degree.

The degree or activity recovered after 24 hrs. reactivation periods at 25 C or 4 C, decreased as the enzyme achieved greater degrees of inactivation at all three temperatures studied.

Effect of Stirring on Heat Stability

Since heat inactivation and subsequent reactivation might be related to the unfolding and refolding of staphylocoagulase molecules, mechanical agitation during heating should affect the stability of the enzyme. Mechanical stress or oxidation (as a result of stirring) may cause the enzyme molecules to unfold or partially unfold, resulting in loss of activity. Table 12 shows the effect of stirring on curde staphylocoagulase. Heating and stirring of the crude enzyme at 80 C and 95 C for 10 min. and 60 min. resulted in significantly ($p = 0.0001$) more inactivation as compared to the unstirred preparation. Reactivation patterns of both stirred and unstirred samples were similar to those observed in the previous experiment. No reactivation occurred in the stirred samples heated at 95 C for 60 min. after 24 hrs. at 25 C or 4 C while the unstirred sample treated under the same condition retained

Figure 15. Heat Inactivation and Subsequent Reactivation at 4 C of Staphylocoagulase.

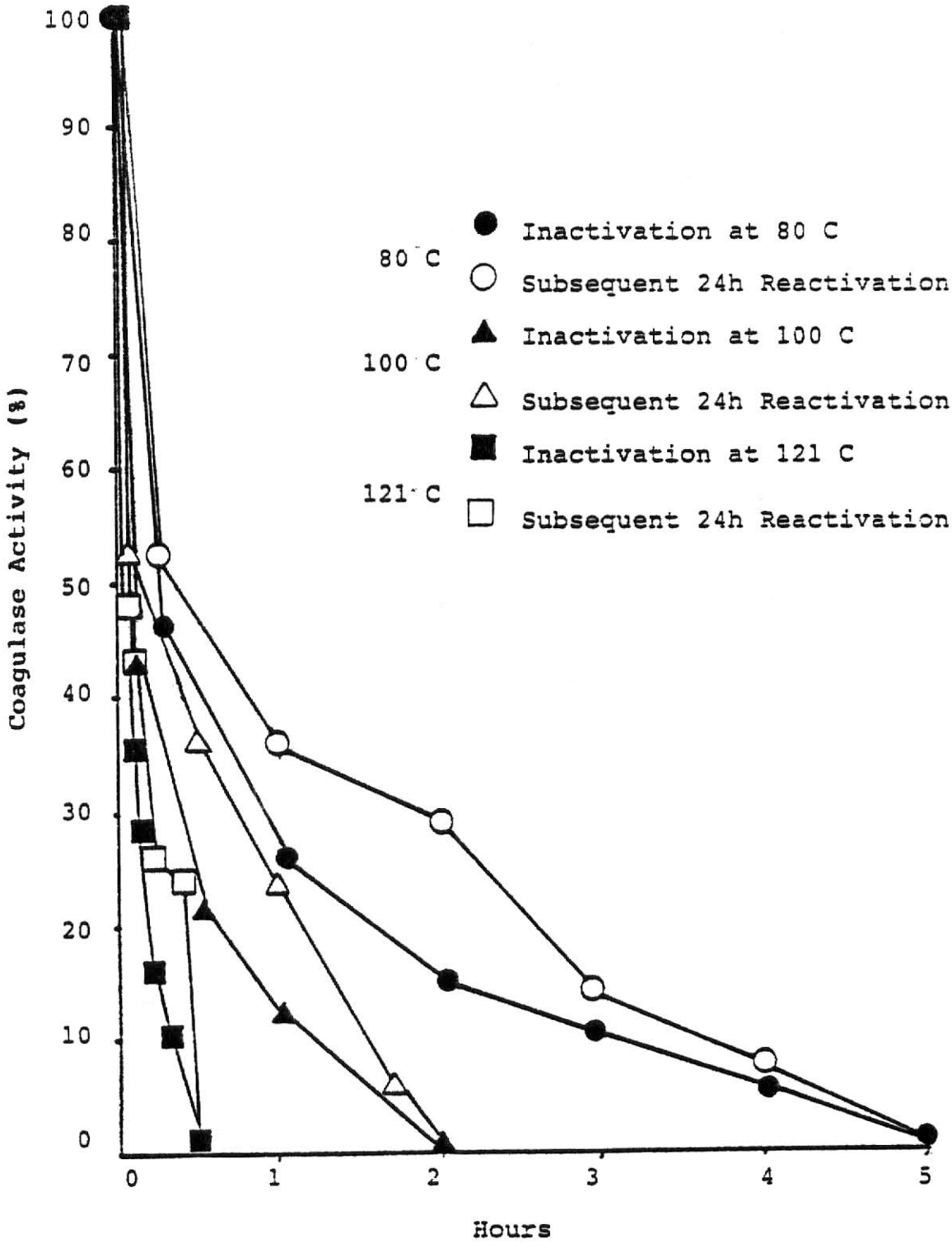

100
90
80
70
60
50
40
30
20
10
0
Coagulase Activity (%)
0
1
2
3
4
5
Hours
80 C
Inactivation at 80 C
Subsequent 24h Reactivation
100 C
Inactivation at 100 C
Subsequent 24h Reactivation
121 C
Inactivation at 121 C
Subsequent 24h Reactivation

Table 12. Effect of Stirring on Heat stability of Staphylocoagulase.

Heating temp. (c)	Activation of coagulase	Staphylocoagulase activity (%)			
		10 min. heating		60 min heating	
		stirred	unstirred	stirred	unstirred
80	Inactivation	13.1	37.9	0.0	27.5
	24h reactivation at 25°C	69.0	125.0	45.6	63.0
	24h reactivation at 4°C	43.6	79.3	0.0	41.6
95	Inactivation	9.9	20.8	0.0	0.0
	24h reactivation at 25°C	60.2	57.0	0.0	27.5
	24h reactivation at 4°C	13.8	23.9	0.0	0.0

27.5% of its original activity after 24 hrs. at 25 C. These results indicated that permanent damage (loss of ability to reactivate) to the enzyme molecules was caused by this combination process. The level of activity present in the unstirred samples after 24 hrs. at either 25 C or 4 C were higher than those of the stirred samples for all samples. Heating at 95 C causes more inactivation than heating at 80 C.

Effect of Concentration on Heat Stability

The characteristic heat inactivation and subsequent reactivation behavior of staphylococcal coagulase may be related to the presence of some "protecting" molecules in the culture medium. If these molecules are of smaller molecular weight than the coagulase molecule, then concentration by dialysis should have an effect on the stability of the enzyme. Table 13 shows the stability of a concentrated cell-free supernatant containing coagulase as compared to its unconcentrated portion, when heated for 10 min. at 80 C or 95 C. After heating for 10 min. at 95 C, the concentrated sample lost all its enzymatic activity while the unconcentrated retained 48.9% of the original activity. Heating at 80 C also caused more inactivation of the concentrated sample as compared to the unconcentrated sample indicating the loss of some "protecting" molecules or factor in the concentrated sample. Reactivation after 24 hrs. at 25 C was observed in all cases except in the concentrated sample heated at 95 C for 10 min., in which reactivation did not occur. Over 100% (115%) of the original activity was observed in the unconcentrated sample heated at 80 C for 10 min. after 24 hrs. reactivation period at 25 C. This phenomenon was also observed during

the effect of the sitrring study in the sample treated also at 80 C for 10 min. (see Table 12). This behavior might suggest the inactivation of some inhibitor of coagulase activity present in the culture medium. In other words a mild heating treatment of the culture medium may inactivate the action of an inhibitor while leaving the enzyme with near complete or complete reactivation capacity. Heating at 95 C caused more inactivation than heating at 80 C for both concentrated and unconcentrated samples.

Table 13. Effect of Concentration by Dialysis on the Heat Stability of Coagulase

Heating* temp. (c)	Activation of coagulase	Staphylocoagulase activity (%) concentrated supernatant	Staphylocoagulase activity (%) unconcentrated supernatant
	Inactivation	23.0	48.9
80	24h reactivation at 25°C	57.0	115.0
	Inactivation	0.0	21.0
95	24h reactivation at 25°C	0.0	60.0

*All samples were exposed to the heating temperature for 10 minutes.

DISCUSSION

Development of Capillary Agar Tube System

The principle of radial diffusion in gels containing substrates has been widely applied in the quantitative determination of enzymes (Schill and Schumacher, 1972). A modification of this principle (single gel diffusion) was utilized by Fung and Wagner (1971) for the immunological determination of staphylococcal enterotoxins, inside capillary tubes. Kohl and Johnson (1980) applied the principle of radial gel diffusion in developing a slide assay for staphylocoagulase. These investigators utilized an agar mixture containing bovine fibrinogen and rabbit plasma as substrates for the enzyme.

The present investigation has successfully proven the adaptability of the agar mixture developed by the above investigators into a capillary system. This system utilizes the principle of single gel diffusion in the determination of staphylocoagulase. Figure 4 shows the linear relationship between the length of the precipitation band observed after standard incubation time (24 hrs.) and the logarithm of the coagulase concentration. The lowest coagulase activity detectable was ca. 1.7 u/ml.

Effect of Reagent Concentrations Upon the Capillary Agar Tube System

In order to develop a most suitable agar mixture for quantification of staphylocoagulase various combinations of plasma, fibrinogen and trypsin inhibitors were investigated. Orth et al. (1969) reported that increasing the plasma concentration decreased the clotting time in the tube coagulase test up to a certain limit. Stutzenberger et al.

(1966) found that increasing the plasma concentration up to 2% of the total reaction mixture or the fibrinogen concentration up to 0.3% increased the light scattering activity of the staphylocoagulase reaction.

The use of trypsin inhibitor to prevent the occurrance of fibrinolysis during the coagulase test is well documented (Hutchison, 1962; Boothby et al., 1979). Orth et al. (1969) reported that the addition of trypsin inhibitor not only provided protection against fibrinolysis but also reduced the clotting time in the tube coagulase test. Increasing the concentration of trypsin inhibitor up to 15 mg/ml resulted in faster clotting time.

In this investigation it was difficult to predict the effect of the substrates (plasma and fibrinogen) or trypsin inhibitor concentration. Although other workers (Orth et al., 1969; Stutzenberger et al., 1966) suggested that increased amounts of substrates improved the degree of coagulation as well as reduced reaction time. In our system the detection of the reaction was not only dependent upon the reaction rate but also in the diffusion rate; we expect that increasing the substrate concentration in the gel will result in more compacted precipitation and therefore the diffusion of the enzyme will be retarded. This in turn would result in lower sensitivity for the assay. Mestecky et al. (1969) reported that decreasing the substrate concentration increased the sensitivity for the radial diffusion determination of alpha-amylase. Fung (1973) also reported that reducing the concentration of the antiserum in the agar gel increased the sensitivity of the single gel diffusion determination of staphylococcal enterotoxins. Since we

observed that increasing the sensitivity of the mixture resulted in longer precipitation bands at any given concentration of staphylocoagulase tested, we used the length of the band as an indirect indication of the sensitivity of the test.

Figures 5 and 6 show the effect of plasma-SBTI and plasma-fibrinogen combinations respectively upon the length of the precipitation band. Increasing the concentration of SBTI significantly ($p = 0.0001$) increased the length of the precipitation band when other components were held constant. However, the pattern in which the SBTI concentration influenced the length of the band was unpredictable as shown in Figure 5. Multiple comparison analysis (Table 6) showed that the means of the samples containing SBTI were significantly (alpha = 0.05 level) higher than those without SBTI. This observation agrees with those of Orth et al. (1969) in that the trypsin inhibitor improved coagulase activity. However, it can not be concluded whether the effect was due to the inactivation of fibrinolytic substances by trypsin inhibitor or the actual activation of staphylocoagulase molecules. In any case the addition of SBTI was proving useful in increasing the sensitivity of the capillary agar tube assay. But again, increasing the concentration of plasma did not follow any particular pattern in relation to band length. These results indicate that the length of the band was dependent upon the relative proportions of SBTI and plasma rather than in the concentration of either one.

The concentration of plasma did not significantly influence the length of the band in the absence of SBTI (Table 5). This data suggests that in the presence of SBTI, more coagulase molecules were activated

resulting in variable needs for coagulase reacting factor (present in plasma). Therefore, the plasma concentration in the assay was of significant influence. This result also suggests that the SBTI effect was at least partially due to the activation of coagulase.

The concentration of fibrinogen significantly ($p = 0.0001$) influenced the length of the band. The length of the band increased as the concentration of fibrinogen decreased. Since fibrinogen is the true substrate of staphylocoagulase (plasma only provides the coagulase reacting factor needed for transforming the enzyme into its active form; and SBTI increases the activity of the enzyme in a manner yet unknown) therefore, this observation agrees with that of Mestecky et al. (1969) in that reducing the amount of substrate increased the sensitivity of the gel diffusion systems.

Of the 56 combinations of SBTI, plasma and fibrinogen, (Tables 2 and 3) combinations containing 2% plasma, 0.2% fibrinogen and SBTI concentrations of 0.1 mg/ml, 1.0 mg/ml and 10 mg/ml were selected as the most appropriate for the capillary tube assay. The three combinations were tested at 0.5% and 1.0% concentration of agar, making a total of six combinations.

Table 8 summarizes the performance of the last six mixtures analyzed. Reducing the agar concentration increased the sensitivity and the clarity of the band in all cases. The complete criteria for selecting the best mixture included sensitivity, incubation time necessary for such sensitivity, clarity of the band, protection against fibrinolysis, saving of materials, and correlation between the length of the band and the

log 10 of the coagulase concentration. Based on the above criteria the agar mixture containing 2.0% plasma, 0.2% fibrinogen, 0.1 mg/ml of SBTI and 0.5% agar was recommended for the capillary tube system.

The system proved to be highly specific as shown by the 100% correlation with the tube coagulase test when testing 28 strains of S. aureus.

In conclusion the system presented here showed an excellent correlation with the conventional tube test, it is a valuable tool for quantifying staphylocoagulase, facilitates measurement of results, offers a tremendous saving of materials, protects against false negative results due to fibrinolysis and is easy to perform. The improved mixture increased the sensitivity of the system at least two-fold (from 1.7 u/ml to at least 0.82 u/ml; lower concentrations were not tested) as compared with the original sensitivity of the system, when the agar mixture developed by Kohl and Johnson (1980) was used. The incubation time recommended by these investigators was also improved from 12 hrs. or 16 hrs. to only 8 hrs. The Pasteur pipettes were prepared in advance and stored at 4 C in a humid chamber for as long as a month without loss in activity.

This test would be ideal for large screening tests since large amounts of assays can be run with minimal effort and materials.

Staphylocoagulase Production by S. aureus

Davies (1951) reported that staphylocoagulase was produced only during the latter part of the lag phase of growth. Other investigators (Duthie, 1954a; Martson and Fahlberg, 1960) have reported the production of staphylocoagulase to be evident in the lag phase and continue through

the logarithmic phase of growth. The present investigation agrees with the latter observations. Staphylocoagulase was produced all throughout the logarithmic phase of growth. Only small amounts of production were detected during the early stationary phase (5 u/ml). The amount of staphylocoagulase remained unaffected past the first 105 minutes of the stationary phase. Therefore, the production of staphylocoagulase accompanied the growth rate in a very close fashion in the laboratory medium studied. In this regard staphylocoagulase can be considered a primary metabolite of S. aureus.

Different strains of S. aureus are known to produce varying amounts of staphylocoagulase in vitro. Some researchers advocated that in order to characterize a staphylococcus as S. aureus the organism must produce staphylocoagulase to form a 4+ clot (Sperber and Tatini, 1975; Rayman et al., 1975; and Yrios, 1977). The results of this study have shown that a wide variety of concentrations of staphylocoagulase were detected even within the strains of S. aureus yeilding a 4+ tube test. This indicates that the semiquantitative scale of the conventional method is not sensitive enough to differentiate staphylocoagulase activities of strains of S. aurues. Therefore, the use of the capillary agar tube system would be most useful when trying to draw a demarcation line as to what concentration of staphylocoagulase should be considered an absolute proof of S. aureus.

Another potential practical application of the capillary agar tube system is its utilization for the detection of staphylocoagulase produc tion in liquid foods or food extracts. In this investigation it was

possible to detect staphylocoagulase from contaminated chicken and beef broth. Since few interfering substances were present in these foods a simple concentration by dialysis was sufficient to detect small concentrations of staphylocoagulase. More studies are needed to determine the production of staphylocoagulase in various more complicated food systems in the presence or absence of competitive organisms.

Heat Stability of Staphylocoagulase from S. auerus S-6

Heat stability of staphylocoagulase is not well documented. Zolli and San Clemente (1963) described the stability of staphylocoagulase after low temperature long time treatment (56 C for 48 hrs.).

Data on the heat stability of crude staphylocoagulase at temperatures and times similar to those utilized during cooking or reheating of foods would be of interest to the food microbiologist since S. aureus produces a variety of heat stable metabolites such as staphylococcal enterotoxins and heat stable deoxyribonuclease. Unpublished data by Fung and Shahin (1981) from this laboratory showed that crude staphylocoagulase from 33 S. aureus strains retained most of its activity after heating at 86 C for 5 min.

The purpose of this experiment was to study in detail the heat stability profiles of staphylocoagulase from a known enterotoxigenic S. aureus S-6, and to compare these profiles to those obtained in a similar study by Fung et al. (1973) for enterotoxin B produced by the same strain.

Staphylocoagulase showed a high degree of stability when heated for various intervals of times at 80 C, 100 C and 121 C. Figure 14 shows the heat inactivation profiles of staphylococcal coagulase from strain S-6; as expected, the degree of activity decreases with longer exposure time to each temperature. This behavior is similar to that reported for enterotoxin B by Fung et al. (1973). The decrease in activity in those preparations exposed to 121 C was higher than those exposed for the same time at 100 C. The same was observed when comparing the 100 C treatment to the 80 C treatment. This last observation was somehow in disagreement with the preliminary work of Fung and Shahin (unpublished data) who found a large loss of activity at 80 C than at 100 C. However, these workers utilized the semiquantitative rating of the tube coagulase test for reaching their conclusions; this might have led to misleading results. Also, the difference in strains used may have been responsible for the disagreement. Increased loss of activity at 80 C, as compared to 100 C, has also been reported for staphylococcal enterotoxins B and C (Statterlee and Kraft, 1969; Jamalang et al., 1971). Fung et al. (1973) found this phenomenon to be true for heating periods of 10 min. or less, and attributed it to the formation of low temperature protein aggregates. In any case, such phenomenon for staphylocoagulase was not observed in this investigation, suggesting that staphylocoagulase differs from enterotoxin B in that low temperature aggregation of protein molecules are not formed. Staphylocoagulase as well as enterotoxin B showed the ability to regain some activity after the original loss of activity due to heat exposure. Reactivation occurred to some degree after 24 hrs. at

25 C at all three temperatures except in the samples heated at 2 hrs. and 20 min. at 100 C and 121 C when the enzyme was totally inactivated. The reactivation properties of staphylocoagulase might be explained by the hypothesis proposed by Fung et al. (1973) for explaining the same behavior by staphylococcal enterotoxins B and C. Heat may cause enzyme molecules to unfold partially or completely. During heat treatments that do not result in complete inactivation (reactivation occurs), some enzymes molecules totally unfold, some partially unfold and some molecules remained intact. Our assay most likely detected only intact molecules, since even small changes in the molecular configuration of an enzyme may cause loss in its affinity toward its specific substrate. Therefore, partially or completely unfolded molecules were not detected. However, the partially unfolded enzyme molecules may reassociate throughout a period of time and proper temperature into enzymatically active molecules. And this is what we have called "reactivation." On the other hand, in cases where no reactivation was observed, the likelihood is that the heat exposure through a "lethal" time interval caused complete unfolding of all enzyme molecules. Such molecules were unable to reassociate as partially unfolded molecules would do, and therefore reactivation could not take place. The level of activity observed after 24 hrs. reactivation at 25 C was significantly ($p = 0.0001$) higher than that of 4 C.

The degree of reactivation decreased as the enzyme achieved greater degree of inactivation. This is in agreement with our hypothesis. Since longer exposure will cause more molecules to go from partially unfolded to completely unfolded state.

The higher the amount of completely unfolded molecules, the smaller will be the degree of reactivation achieved. Therefore, it may be concluded that the ability to regain activity by enzyme molecules would be influenced by time, temperature and the amount of inactivation incurred.

The inactivation and reactivation profiles for staphylocoagulase from S. aureus S-6 were surprisingly similar to those reported by Fung et al. (1973) for staphylococcal enterotoxin B produced by the same strain. At 80 C the enzyme did not achieve its lethal exposure (time required to completely inactivate the enzyme so that no reactivation can occur at 25 C for 24 hrs.) after 5 hrs.; the toxin showed the same behavior. The lethal exposure for the enzyme at 100 C and 121 C was of 120 min. and 30 min. respectively, while the toxin lasted for 180 min. and 30 min. for the same treatment.

If the proposed unfolding-refolding theory is in fact the phenomenon taking place during the heat inactivation and reactivation of staphylocoagulase, then mechanical agitation of enzyme solutions should effect the stability of the enzyme. In this study it was found that stirring indeed influences the heat stability of the enzyme. Stirring significantly increased ($p = 0.001$) the degree of inactivation as compared to no-stirring after heat exposures at 80 C and 95 C for 10 and 60 min. The degree of reactivation was as before influenced by the amount of inactivation incurred in any given sample. Therefore, the amount of reactivation observed for the stirred sample was less than that of the unstirred samples. Stirring alone did not affect the enzymatic activity of staphylocoagulase, indicating that stirring had no effect upon intact

enzyme molecules. Heating might cause partial unfolding of some or all of the enzyme molecules, depending upon time and exposure. Mechanical stress or oxidation (stirring) can cause the partially unfolded molecules to become totally unfolded, or they can prevent renaturation so less reactivation can occur.

Heating at 95 C caused more inactivation than heating at 80 C for both the stirred and unstirred samples, suggesting that low temperature protein aggregates were not formed in neither case.

That the culture medium exerts some kind of protection from heat inactivation to the coagulase molecules has been previously reported (Zolli and San Clemente, 1963). The same observation has been made for staphylococcal enterotoxins (Fung et al., 1973).

In this investigation the cell-free broth concentrated by dialysis showed higher sensitivity to heat as compared to the unconcentrated broth, indicating that some "protecting" factor present in the medium was lost during the concentration procedure. Since the dialysis tube used in this investigation retained molecules of molecular weight 14,000 or larger, it could be assumed that the "protecting" factor was of smaller molecular weight. The small aliquot obtained after concentration was resuspended in about 5 times its volume of the same sterile medium. The fact that the reconstituted medium (containing all its original components) did not provide the missing protection, indicates that the "protecting" factor was produced during the growth of the cells in the culture medium.

The inactivation-reactivation behavior of the concentrated and unconcentrated samples were similar to the one observed in the previous results described here. Heating at 95 C provided faster inactivation than heating at 80 C. And the degree of reactivation was directly related to the degree of inactivation incurred.

The confirmation of the heat stability of staphylocoagulase can open interesting possibilities of food safety implications. The possibility of using staphylocoagulase as an enzymatic indicator of S. aureus in heated food could be explored. Other similarities of the enzyme with other metabolic products such as toxin and heat stable nucleases may be studied. The question as to why S. aureus being a mesophilic bacteria, produces so many heat stable products could be raised and studied.

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CAPILLARY AGAR TUBE SYSTEM FOR STAPHYLOCOAGULASE

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ABSTRACT

The tube coagulase test is the most common technique for the detection of staphylocoagulase production by Staphylococcus aureus (Baer et al., 1976). As this method is not quantitative some workers (Sahni et al., 1981; Engels et al., 1981) recently developed quantitative procedures for staphylocoagulase. However, these methods are difficult to perform and required sophisticated instrumentation.

Kohl and Johnson (1980) described a quantitative method for staphylocoagulase based on the principle of radial diffusion of enzymes through a gel containing their substrates. In this investigation the agar mixture described by Kohl and Johnson has been improved by the manipulation of the substrates (plasma and fibrinogen) and the addition of soybean trypsin inhibitor. The mixture was then adapted into a capillary tube system for the detection of staphylocoagulase based on the principle of single gel diffusion of enzymes.

The capillary agar tube system is composed of a 9-inch Pasteur pipette with an agar mixture (containing plasma and fibrinogen as substrates for staphylocoagulase), partially filling the thinner portion of the pipette. A liquid sample containing staphylocoagulase is added through the wider portion of the Pasteur pipette. The enzyme diffuses through the agar at a rate proportional to the concentration of the enzyme in the sample. As the enzyme diffuses it reacts with its substrates causing the denaturation of fibrinogen into insoluble fibrin and fibrinopeptides. This results in the formation of a visible precipitation band in the agar. The length of the band being proportionally

related to the concentration of the enzyme.

The capillary agar tube system showed a good correlation with the conventional tube method, it is quantitative, requires a shorter incubation period than that of the Kohl and Johnson system (8 hrs.vs. 12-16), results in savings of materials and is easy to perform. This test would be ideal for large scale screening tests.

Using this capillary agar tube system the heat inactivation and reactivation profiles of staphylocoagulase were obtained. Staphylocoagulase was found to be very heat stable, and heat inactivation and reactivation profiles were similar to those of staphylococcal enterotoxins B and C (Fung et al., 1973).