GROWTH AND ENTEROTOXIN PRODUCTION OF A SELECTED STRAIN OF *Staphylococcus aureus* (isolated from heat treated milk in a ten-month survey) IN THE MANUFACTURE OF CHEDDAR CHEESE MADE FROM HEAT TREATED MILK

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GROWTH AND ENTEROTOXIN PRODUCTION OF A SELECTED STRAIN OF STAPHYLOCOCCUS AUREUS (ISOLATED FROM HEAT TREATED MILK IN A TEN-MONTH SURVEY) IN THE MANUFACTURE OF CHEDDAR CHEESE MADE FROM HEAT TREATED MILK

BY

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A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

A heat resistant enterotoxin-A-producing strain of Staphylococcus aureus was isolated from heat treating (at 61°C for 18 seconds) raw milk samples during a ten-month survey on raw milk obtained from the University of Manitoba Commercial Dairy. Baird-Parker medium proved to be the most suitable medium for the isolation and enumeration of thermally stressed cells of S. aureus. Employing Baird-Parker medium for enumeration, the percentage of S. aureus survivals in heat treated milk from the survey was 0-37.5%.

Growth and enterotoxin production of S. aureus in raw and heat treated milk were studied by inoculating test milks with various levels of the above mentioned test organism. Enterotoxin was detected in raw milk after 6-8 hours with an initial inoculum of 10^3-10^5 S. aureus per ml of milk. The minimal population associated with detectable enterotoxin was 1.30×10^7 S. aureus per ml of milk. Better growth and enterotoxin production resulted in heat treated milk (at 61°C for 18 seconds). Enterotoxin was detected after 4-6 hours. A minimal S. aureus population of 2.80×10^6 cells per ml resulted in enterotoxin production in heat treated milk.

Enterotoxin production was also demonstrated by enterotoxigenic S. aureus indigenous to raw milk (representing pre-heat treatment contamination) that survive the subpas-

teurization heat treatment, although the process was very much delayed due to a lag phase of 4-5 hours prior to the initiation of growth and multiplication. It was detected only after 12 hours with a comparatively high S. aureus population of 8.25×10^7 cells per ml of milk.

A comparison of cheddar cheese samples inoculated with three different levels of S. aureus using normal, slow, and partial slow starter cultures indicated that growth of S. aureus was most extensive in cheeses made with slow starter culture. Enterotoxin production in cheeses made with slow starter required a smaller S. aureus population than did cheeses made with normal starter. As few as 3.4×10^6 S. aureus per g of cheese resulted in enterotoxin production in slow cheese as compared to over 29 million in normal cheese. Similar responses were observed in comparing normal starter with partial starter failure, but to a much lesser extent. Enterotoxin was detected in partial slow cheeses after 5 hours with 1.20×10^7 S. aureus per g of cheese.

All three types of cheese achieved normal titratable acidity at various stages during the cheese manufacturing process. Cheeses made with slow and partial slow starter required a prolonged milk ripening period of 4-4.5 and 2.75-3 hours, respectively. Slow starter cheeses had pH values considerably greater than 5.4. Partial slow starter cheeses had pH values of 5.25-5.60. Cheeses made with normal starter had pH values of 5.4 or less after pressing. The moisture and salt content were normal for all cheese batches.

However, at any one staphylococcal inoculum level used, the fat content was higher in cheeses made with normal starter than in those cheeses made with slow starter.

Results obtained in this investigation approved the feasibility of the solid-phase radioimmunoassay for qualitative as well as quantitative detection of enterotoxin A production in cheddar cheese. This method has a sensitivity of detecting 0.31-1.0 ng of staphylococcal enterotoxin per g of cheese and a quantitative recovery of 94-95%.

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LIST OF ABBREVIATIONS

BP - Baird-Parker

BSA - bovine serum albumin

cpm - counts per minute

DNase - deoxyribonuclease

EDTA - ethylene diamine tetraacetic acid

HTST - high temperature short time

ng - nanogram

pg - picogram

RIA - radioimmunoassay

S110 - Staphylococcus 110

SEA - staphylococcal enterotoxin A

SPC - standard plate count

TEA - tomato yeast extract agar

TSA - trypticase soy agar

TSB - trypticase soy broth

thermonuclease - thermostable deoxyribonuclease

GENERAL INTRODUCTION

For many years, staphylococcal food-poisoning has been among one of the major types of food-borne diseases in North America. Within the past decade, there have been several outbreaks of staphylococcal food intoxication attributed to cheese (Allen and Stovall, 1960; Hendricks et al., 1959; Hausler et al., 1960; Zehren and Zehren, 1968a, 1968b). Thatcher et al. (1956) reported that Canadian cheeses sometimes contained large numbers of toxigenic staphylococci. In Ontario in 1971, a large quantity of cheddar cheese made from milk which received subpasteurization heat treatment was found to contain enterotoxin and was refused for consumer distribution (Duitschaever and Irvine, 1971).

Due to the ubiquitous nature of S. aureus, it is extremely difficult to obtain a raw milk supply that is entirely free of this organism. It has been demonstrated (Mattick et al., 1959; McLeod et al., 1962; Takahashi and Johns, 1959; Rougley and McLeod, 1961; Thatcher and Ross, 1960) that conditions can exist in the cheese manufacturing process such that large populations of staphylococci can develop within a relatively short period of time. This is especially critical when the milk being used for cheesemaking initially contains S. aureus.

Various heat treatments have been used to eliminate or substantially reduce S. aureus from milk for cheese manufacture. Subpasteurization at 60-61°C for 16-18 seconds is a common commercial practice in the Canadian cheese industry. Although subpasteurization has the advantage of achieving a better flavored cheese (Franklin and Sharp, 1962; Takahashi and Johns, 1959), strains of S. aureus surviving such heat treatment may constitute a potential health hazard. Conditions

may arise during cheesemaking which permit rapid growth of these organisms to numbers such that enterotoxin can be produced. If such a condition should occur, the enterotoxin formed will not be destroyed by the heat treatment that would destroy the organism. Therefore, the absence of S. aureus does not infer that enterotoxin is absent, but the presence of intensive growth of S. aureus signals the potential presence of enterotoxin.

Cheese made from heat treated milk is, by law, withheld from the consumer market for a period of 60 days, during which time any pathogenic organisms that might be present would be destroyed (AOAC, 1975). However, such a holding period does not have any effect on staphylococcal enterotoxins that may be present (Connell, 1959). Cheddar cheese made from raw milk (Hausler et al., 1960; Hendricks et al., 1959) or pasteurized milk (Zehren and Zehren, 1968a) has been implicated in outbreaks of staphylococcal food-poisoning. Therefore, the growth of S. aureus during the cheese manufacturing process must be restricted so that a population large enough to produce enterotoxin does not develop.

Takahashi and Johns (1959), Thatcher and Ross (1960), Tuckey et al. (1964), and Reiter et al. (1964) reported growth of S. aureus in cheeses made with normal and slow starter cultures. A much more rapid growth rate of S. aureus, however, was noted in cheeses made with slow starter (Reiter et al., 1964). Starter cultures, and their ability to produce acid, are largely responsible for insuring the safety of cheeses (Jezeski et al., 1967; Zehren and Zehren, 1968b). Furthermore, all cheeses containing enterotoxin had histories of poor acid development during cheese manufacture (Zehren and Zehren, 1968b). Apparently, other conditions, in addition to poor acidity, may also influence enterotoxin

production. However, owing to the inadequate procedures for the detection of staphylococcal enterotoxins and the insensitivity of the tests, much of the earlier research was directed toward factors that influence growth rather than enterotoxin production by S. aureus. There is little information available on the effects of natural environmental factors (such as moisture, salt, and fat content) on enterotoxin production by S. aureus in cheese.

Little is known about the extent of staphylococcal growth in cheese manufacture associated with the presence or absence of enterotoxin. In the only report of enterotoxin A production in cheddar cheese manufactured from pasteurized (65.6 °C) milk, Tatini et al. (1971b) detected enterotoxin when an S. aureus population of 28 million per g of cheese was reached. These workers detected enterotoxin by means of the microslide immunodiffusion technique with an estimated recovery of only 40-50%.

Conventional sensitive techniques for the detection of enterotoxins are not available in most food processing plants. Where such facilities are available, enterotoxin detection is too expensive to perform routinely. For these reasons, standards relating the levels of S. aureus to the production of enterotoxin become critical. Such standards are not available in Canada. Dr. H. Pivnick, Director of the Bureau of Microbial Hazards, reported in the Journal of Food Technology, January 1978, that "the Health Protection Branch expects to soon promulgate standards for cheese, from both unpasteurized and pasteurized milk." Understanding of the different chemical factors in the product as they relate to the presence or absence of enterotoxins is also important. The methods currently used by the U. S. Food and Drug Administration can detect 0.1-0.2 ug enterotoxin added to 100 g of food material. The

minimal practical limit of the microslide immunodiffusion method is approximately 0.1 ug per ml. Many laboratories have difficulty in achieving this sensitivity (Bergdoll et al., 1976). Therefore, there is demand for a more sensitive and suitable test for the detection of enterotoxin. The purpose of this study is to explore the relationships between the growth of S. aureus and the enterotoxin production, and between the chemical properties of the cheese and the presence of enterotoxin, in order to establish the necessary standards for cheddar cheese. In establishing such standards, one must always maintain high criteria of safety. Data observed in the general studies on staphylococcal enterotoxin production in different types of milk (Donnelly et al., 1968; Tatini et al., 1971a) and cheese (Tatini et al., 1971b), therefore, served as reference in setting up specific experimental controls and limits for this investigation. This study also investigates the feasibility of the solid-phase radioimmunoassay technique (which is still in the developmental stage) for the detection of enterotoxin in cheddar cheese.

REVIEW OF LITERATURE

Staphylococcal Food-poisoning

Staphylococcal food-poisoning is an intoxication due to the ingestion of preformed enterotoxin produced by *Staphylococcus aureus* in the food, rather than an infection due to the organism itself, as is the case with *Salmonella* food-poisoning (Dack, 1956; Elek, 1959). Thus, in order for the disease to occur, conditions must be favorable for an extensive growth of the organism with a concurrent production of enterotoxin in the food. Once the enterotoxin is present, the danger of food intoxication exists regardless of the destruction of the organisms thereafter. As little as 1 μg of enterotoxin per 100 g of food has been shown to cause illness (Casman and Bennett, 1965).

Following ingestion of food contaminated with sufficient quantities of enterotoxin, the onset of symptoms is quite rapid and characteristic. The symptoms usually appear within two to four hours, but the onset time may vary from half an hour to eight hours depending on individual susceptibility and the dosage consumed. Primary symptoms are nausea, vomiting, retching, abdominal cramps, and diarrhea. They are often accompanied by complications such as sweating, prostration, dehydration, shock, blood and mucus in the stools and vomitus. Fever is common, but subnormal body temperatures are not uncommon. The disease is acute and self-limiting, with complete recovery within twenty-four to seventy-two hours. In severe cases, hospitalization is necessary and supportive treatment is directed towards relieving shock, preventing dehydration, and controlling vomiting and diarrhea (Angelotti, 1969). Therefore, even though the disease is characterized by low mortality and relative short duration, the frequency of outbreaks

and severity of symptoms mark staphylococcal food-poisoning as an important food-borne hazard.

Steps leading to staphylococcal intoxication are as follows (Bryan, 1968):

(a) A reservoir for an enterotoxigenic strain of S. aureus.

(b) A mode of dissemination of the organism.

(c) Contamination of a food capable of supporting its growth.

(d) A temperature level for a time sufficient to permit adequate multiplication of the organism and enterotoxin production.

(e) The comsumption of a sufficient amount of enterotoxin by a susceptible person.

The largest reservoir for staphylococci is man, and the main foci of multiplication are the nose, skin, throat, and lesions (Williams, 1946; Williams, 1963). Staphylococci are widely distributed in air and dust, as well as in water, milk, food, feces (Minor and Marth, 1972a), and sewage (Angelotti, 1969). These pathogens can also infect animals and insects (Bryan, 1969; Moorehead and Weiser, 1946) which are then able to transfer the organisms through infections such as sepsis and mastitis. Hence, this ubiquity of staphylococci in man and his environment ensures widespread colonization in food regardless of care in food handling (Merson, 1973). It is not uncommon for raw milk, cheese (Minor and Marth, 1972a),and market meat samples (Joy, 1962) to contain detectable numbers of S. aureus. For this reason, staphylococcal food-poisoning continues to be one of the prevalent food-borne diseases.

Methods of controlling outbreaks of staphylococcal food-poisoning must be aimed at one or more of the above steps in the propagation of the intoxications. The only practical area for immediate control of the problem is the prevention or retardation of staphylococcal growth

in food. However, considerable efforts should also be directed towards minimizing opportunities for transmission of the organism which can be achieved by good personal hygiene and sanitary food processing. Some factors for control of this problem were reported by Troller (1976).

The Occurrence of Staphylococcal Food-poisoning in Milk and Dairy Products

Food-poisoning by S. aureus predates the scientific discovery of the organism (Dack, 1956). The first recorded outbreak appeared in 1884, when 300 cases of severe illness were reported in Michigan which were attributed to comsumption of cheddar cheese. Vaughn (1884) ingested filtrates extracted from the cheese and developed the illness. Microscopic examination of the cheese revealed spherical bacteria. He thus concluded that the bacteria produced a chemical poison responsible for the intoxication, but he was unable to identify the toxic material.

The first well-documented report which clearly identified staphylococci as the causative agents did not appear until 1914 when Barber (1914) studied a milk-poisoning outbreak in the Philippines and was able to isolate staphylococci from the milk of a cow with mastitis. The milk when consumed without prior refrigeration caused considerable gastroenteritis in human beings. The significance of this work went unrecognized for some years, and food-poisoning in general was ascribed to other bacterial agents (Connell, 1959).

Dack et al. (1930) isolated S. aureus from the cream filling of a Christmas cake responsible for a food-poisoning incident. Culture filtrates of the isolated bacteria, when swallowed by some human volunteers, produced gastroenteritis. Shortly after this report, comparing this information to several other cases in his files,

Jordan (1931) concluded that staphylococci were responsible for producing a toxic substance in food. Since then, the role of staphylococci as a cause of food-poisoning has been duly recognized. The long delay in this recognition is due largely to the focused attention on Salmonella or paratyphoid organisms, a group detectable with much less effort.

In a published review, Stone (1943) tabulated 82 reported outbreaks of staphylococcal food-poisoning for the period 1907-1939; 63 of these involved at least 4,123 individuals. These include seven outreaks caused by the consumption of raw milk with 506 persons involved, and six outbreaks in which butter was implicated. Other raw milk-poisoning outbreaks include a 57-case outbreak in a World War II defense plant (Perriello, 1944), one affecting 75 people in Africa (Drysdale, 1950), two cases in England and Wales (Smith, 1955), a family outbreak traced to mastitic cows (Wallace et al., 1960) and one affecting 26 campers also traced to mastitic cows (Bothwell, 1963).

Dauer (1960) reported five outbreaks attributed to the consumption of milk and milk products. These include one outbreak due to consumption of unpasteurized milk, one traced to ice-cream made with unrefrigerated raw milk, and five cases of gastroenteritis in a family group who ate cottage cheese. Two outbreaks due to the comsumption of pasteurized milk have been reported, one affecting 29 people (Hackler, 1939) and the other affecting 32 persons (Caudill and Meyer, 1943). Several outbreaks of staphylococcal food-poisoning associated with the consumption of spray-dried milk were also recorded. These include eight outbreaks that occurred in school canteens in England during 1953 involving 1,190 children (Anderson and Stone, 1955; Hawley and Benjamin, 1955; Hobbs, 1955), and a series of nineteen outbreaks of gastroenteritis which occurred among school children participating

in a school lunch program during a one month period in Puerto Rico (Armijo et al., 1957)

The occurrence of several outbreaks of staphylococcal food poisoning traced to contaminated cheese gave additional emphasis to the problem (Agenjo, 1952; Hendricks et al., 1959; Hausler et al., 1960). The U.S. Public Health Service has recorded several outbreaks during the period 1944-1952 (Hendricks et al., 1959). Tanner et al., (1953) reported 21 cases in eight families in Germany, based on symptoms and epidemiological evidence as being due to staphylococcal enterotoxin in cheese. Dauer and Sylvester (1954, 1955, 1956, 1957) recorded one outbreak from homemade cheese in 1953; nine cases from food containing cream cheese, and one family outbreak from cheese in 1954; nine cases due to cheddar cheese, from which staphylococci were isolated from the center of unopened samples in 1955; and one outbreak with 80 cases from cheese sauce in 1956.

In 1958, 200 persons became ill at a state institution in Iowa. Raw milk cheddar cheese, four to eight months old was the cause (Hendricks et al., 1959). About 87% of 85 cheeses were contaminated with β-hemolytic, coagulase-positive S. aureus, one of which was shown to be an enterotoxin producer. In the same year, a series of staphylococcal outbreaks in Wisconsin, Michigan and Indiana were reported (Allen and Stovall, 1960). Seventy-eight cultures of S. aureus were isolated from these cheeses and from the milk used in cheesemaking, as well as from swabs taken in different areas in the factory. Two of these were shown to be enterotoxin producers.

In 1961, an outbreak of cheese-poisoning occurred in two London hospitals. The cheese was found to contain approximately 200 million coagulase-positive S. aureus of phage type 42D per gram (Epsom, 1964).

Zehren and Zehren (1968a ; 1968b) reported another outbreak of staphylococcal food-poisoning which occurred in the fall of 1965 over a widespread area of the United States of America. Cheddar cheese and a small quantity of Kuminost and Monterey cheese produced from a Missouri dairy plant were found responsible. When the outbreak occurred, there were 2,112 batches of cheese still in storage, 59 of which were found to be toxic. One of the more toxic cheese batches was found to contain approximately 12 μg of enterotoxin per 100 g of cheese using the microslide immunodiffusion method.

Some recent recorded staphylococcal intoxications include one involving 22 cheese samples, each of which caused sickness in two to twenty-six persons (Miller, 1974); one involving 100 people in France caused by pastry cream; and incidents caused by pastries and Robbiole cheese (Caserio et al., 1975). For more detailed records, readers should consult the Morbidity and Mortality Weekly Reports issued by the U. S. Public Health Service.

Staphylococcus aureus - the Organism

1. History

Staphylococci were observed in pus and cultivated by Koch and Pasteur in 1878 and 1880, respectively. Sir Alexander Ogston is credited with applying the name "Staphylococcus" in 1881 because of the typical grape-like clusters of cocci he observed in cultures. The name, derived from the Greek nouns 'staphyle' ("bunch of grapes") and 'coccus' ("a grain or berry"), is very descriptive and appropriate. It was Rosenbach, however, who, after careful and systematic study of the organism, obtained in 1884, pure cultures of the organism on solid media.

Using the generic name "Staphylococcus", he classified the group into the albus and aureus forms. By the turn of the century, other varieties of Staphylococcus were studied and named.

2. Classification

According to the 8th edition of Bergey's Manual of Determinative Bacteriology (1974), the family Micrococcaceae can be delineated into three genera: Micrococcus, Staphylococcus, and Planococcus on the basis of motility, glucose fermentation, DNA content, and sensitivity to lysostaphin as follows:

FAMILY:	Micrococcaceae*		
GENUS:	Micrococcus	Staphylococcus	Planococcus
Gram (+) spherical cells	+	+	+
irregular clusters	+	+	-
tetrads	v	-	+
motility	-	-	+
G + C DNA content (moles%)	66-75	30-40	39-52
lysostaphin sensitivity	-	+	-
glucose fermentation (anaerobically)	-	+	-

*: + = most strains positive (90% or more); - = most strains negative (90% or more); d = some strains positive, some negative; v = characters inconsistent and in one strain may sometimes be positive, sometimes negative; NT = not tested.

Staphylococcus aureus is by far the best defined species in the

genus Staphylococcus, and is differentiated from the other two species, S. epidermis and S. saprophyticus, by its production of coagulases, heat-resistant nucleases, α - toxin, acid from mannitol anaerobically, and the presence of protein A in its cell wall. A delineation outline can be drawn as follows:

GENUS:	Staphylococcus*		
SPECIES:	S. aureus	S. epidermis	S. saprophyticus
coagulase production	+	-	-
mannitol:acid aerobically	+	d	d
anaerobically	+	-	-
thermostable nuclease	+	-	-
α -toxin production	+	-	-
enterotoxin production	+	-	-
cell wall: protein A	+	-	-
biotin for growth	-	+	NT
novobiocin sensitivity	+	+	-

*: + = most strains positive (90% or more); - = most strains negative (90% or more); d = some strains positive, some negative; v = characters inconsistent and in one strain may sometimes be positive, sometimes negative; NT = not tested.

S. aureus can be sub-divided into a number of varieties. Evidence has shown that it is possible to distinguish between strains from human and those from other animals by differences in phage susceptibility, antigenic components of the cell wall, sensitivity to anti-microbial agents, nutritional requirements, and biochemical characteristics

such as coagulation of different animal plasmas, hemolysin types, fibrinolysin production, and serological differences in nucleases (Baird-Parker, 1974). Baird-Parker suggested that the most acceptable of the proposed divisions of S. aureus is that dividing this organism into biotypes, and the most comprehensive of these is that proposed by Hájek and Marsalek (Baird-Parker, 1974) since such a separation has practical value, particularly to the food microbiologist, who not infrequently wishes to know the origin of a particular type of S. aureus found in a food. Hájek and Marsalek subdivided S. aureus into six biotypes: A, B, C, D, E, and F representing an origin from human, pig/poultry, cattle/sheep, hares, dogs, and pigeons respectively. More details can be found in the Recent Advances in Staphylococcal Research (Yotis, 1974). It is also possible to divide the biotypes further by phage typing (Meyer, 1967).

3. General Characteristics

Bergey's Manual of Determinative Bacteriology (1974) defines S. aureus as non-motile, Gram positive spherical cells, occurring singly, in pairs, in tetrads, and dividing in more than one plane to form irregular clusters. They are generally 0.8 to 1.0 μ in diameter. In addition to being facultatively anaerobic, they are catalase positive, non-photosynthetic and do not form spores. Many strains produce an orange or yellow pigment, particularly on media containing high levels of sodium chloride. Most strains form acetone from glucose, and ammonia from arginine, reduce nitrates to nitrites, and ferment a variety of carbohydrates. They can acidify litmus milk, and may cause coagulation.

The optimum temperature for growth is 37°C. They are also able to grow at 10° and 45°C, but these temperatures are very near the minimum and maximum growth temperatures, respectively.

4. Characteristics Suggested as Indices of Staphylococcal Enterotoxigenicity.

Based on research studies, several cultural and physiological characteristics have been suggested as indices of staphylococcal enterotoxigenicity. These characteristics include chromogenesis (Feldman, 1946; Evan and Niven, 1950; Muth, 1971), coagulase production, mannitol fermentation (Gwatkin, 1937; Joshi and Dale, 1963; Abd-El-Malek and Gibson, 1948), hemolysin production (Zemelman and Longeri, 1965), protein A production (Forsgren, 1970; Jay, 1971), gelatin liquefaction (Stone, 1935), and deoxyribonuclease production (Chesbro and Auborn, 1967). However, other workers found no correlation between enterotoxin production and characteristics such as pigmentation, hemolysin production, mannitol fermentation, gelatin liquefaction, lipolytic and proteolytic action, bacteriophage, and antibiotic sensitivity (Hussemann and Tanner, 1949; North, 1943; Clark et al. 1961; Otenhajmer et al., 1975).

Coagulase production is generally accepted to be the best indicator of potential pathogenicity. The coagulase test, suggested by Chapman (1944), is an indicator for the presence or absence of an enzyme capable of clotting citrated rabbit or human plasma. Coagulase, apparently is produced only during the exponential phase of growth (Pariza and Iandolo, 1969); however, being a protein, active protein synthesis, rather than growth per se is necessary for its production. However, some coagulase-negative Staphylococcus strains were found to

produce enterotoxin (Bergdoll et al., 1967; Thatch and Simon, 1956). It is questionable, therefore, whether coagulase production is a single prime determinant in enterotoxin production.

Recently, Lachica et al. (1969), suggested that the presence or absence of a heat-stable deoxyribonuclease (thermonuclease) could serve as an index of enterotoxigenicity. S. aureus, including enterotoxigenic strains, produces an extracellular deoxyribonuclease that is heat-stable (Weckman and Catlin, 1957; Lachica et al., 1969) and that can be rapidly assayed (4-6 hours) by an inexpensive and simple procedure (Lachica et al., 1971b). Close correlations between S. aureus growth and thermonuclease production, and between thermonuclease and enterotoxin production, were demonstrated in various types of cheese (Cords and Tatini, 1973), ham (Chesbro and Auborn, 1967), beef and pork (Lachica et al., 1972). Working with several different S. aureus strains, and a variety of foods, Tatini et al. (1975) showed that thermonuclease was detectable in every substrate tested, whenever S. aureus grew, but prior to the accumulation of detectable amounts of enterotoxins. Also, Lotter and Genigeorgis (1975) studied eight coagulase-negative enterotoxigenic strains and found that they all produced thermostable nuclease. On the basis of their data, and data reported elsewhere, they suggested that these organisms should be considered as variants or mutants of S. aureus.

In spite of all the extensive research studies, no single physiological characteristic, or combination of characteristics, possessed by S. aureus has yet been found to be an absolutely reliable indicator of enterotoxigenicity.

Isolation and Identification of S. aureus from Milk and Milk Products

I. Isolation

The selectivity of a medium used for the isolation of S. aureus is based on its high tolerance to inorganic and organic salts such as sodium chloride, lithium chloride, potassium tellurite, potassium thiocynate, and sodium azide; amino acids such as glycine (required to form cell-wall mucopeptide); and antibiotics such as polymyxin. However, such media will not suppress the growth of all micro-organisms other than S. aureus in a mixed flora. Therefore, various diagnostic tests such as the production of acid from mannitol, the hemolysis of erythrocytes, the coagulation of animal blood plasma, the hydrolysis of DNA, and the clearing of egg-yolk are incorporated in order to recognize the presence of this species on these media. For example, the tellurite glycine medium (Zebovitz et al., 1955) and the Vogel and Johnson agar (Vogel and Johnson, 1960) are based on the ability of S. aureus to aerobically reduce a tellurite salt to elemental tellurium, thus forming black colonies on agar media. The egg-yolk-sodium-azide agar (Lundbeck and Tirunarayanan, 1966), the tellurite-polymyxin-egg-yolk agar (Crisley et al., 1964), and the Baird-Parker medium are based on the ability of S. aureus to clear or precipitate egg-yolk by the production of lipoprotein lipases which splits the lipid moiety from the lipovitellenin present in egg-yolk (Tirunarayanan and Lundbeck, 1967), thus forming a clear zone around each colony in the agar (Hopton, 1961). Mannitol employed as the major source of carbon, along with a pH indicator, has been employed as a selective agent in media such as in Staphylococcus 110 medium (Chapman, 1946) and the mannitol salt agar. Fibrinogen has also been used as

a differential agent (McDivitt and Topp, 1964). It is digested by coagulase-positive staphylococci, thus forming halos around colonies on agar containing this agent. Some of the other differential media are milk salt agar, Polymyxin-B-sulfate medium, Bovine Blood agar, and Phenolphthalein phosphate agar with polymyxin (Gilbert et al., 1969). Opinions are divided as to the best media for the detection and isolation of S. aureus, and no single medium is suitable for all situations. For the isolation of these organisms from foods, the egg-yolk containing selective media, relying on the clearing of egg yolk as the diagnostic reaction, are most reliable (Baird-Parker, 1962; Crisley et al.,1964; Sinell and Baumgart, 1966; Holbrook et al., 1969). These media are very useful for detecting small numbers of S. aureus cells in the presence of the mixed flora found in many foods, while Baird-Parker medium is particularly effective for detecting organisms damaged by heating, drying, irradiation (Baird-Parker and Davenport, 1965), and freezing (Ostovar and Bremier, 1975). Organisms undergoing some physical stress normally are unable to grow on many of the media usually used for isolation. Most of the selective media used for isolating staphylococci are significantly inhibitory to the growth of one or more strains of S. aureus. Media with egg-yolk are generally less inhibitory to staphylococci (Baer et al., 1971) because egg-yolk was found to act as a protective agent for staphylococci. The protective action was more effective in tellurite-containing selective media than in salt-containing selective media (Stiles and Clark, 1974).

The isolation of S. aureus from a mixed flora such as that found in milk and milk products is complex, since many of the growth requirements and characteristics of S. aureus are the same or similar

to those of the other micro-organisms. Joshi and Dale (1963) found Tellurite-glycine agar to be unsatisfactory for the selection of coagulase-positive staphylococci from milk because most coagulase-negative staphylococci grew on the medium in the presence of milk (Joshi et al., 1963). Marshall et al., (1965) also found tellurite-glycine and Vogel-Johnson agars too inhibitory to be used for the enumeration of staphylococci in dry milk. Many coagulase-negative cocci and some rods were observed by McDivitt and Jerome (1965) to grow on Fibrinogen-polymyxin agar and Staphylococcus 110 medium used to isolate organisms from raw milk. Fibrinogen-tellurite-glycine agar was found (Jasper and Jain, 1965) very useful for isolating staphylococci from milk in the presence of other micro-organisms. Also, media containing 10% bovine blood were found suitable for detecting S. aureus in dairy products (Rammell and Howick, 1967; Munch-Peterson, 1970). Baird-Parker medium was found to be most effective in suppressing coagulase negative staphylococci (Gilbert et al., 1969). Moreover, it has been found superior in recovering sublethally heated cells of S. aureus (Gray et al., 1974; Collins-Thompson, et al., 1974; Stiles and Clark, 1974).

Sampling and sample preparation procedures are given by Walter (1967). Considerable evidence has been published (Busta and Jezeski, 1963 ; Stiles and Clark, 1974; Collins-Thompson et al., 1974) regarding the adverse effects of salt on physiologically impaired cells of S. aureus, such as are to be expected in processed food. Therefore, a method for the enumeration of S. aureus eliminating the use of salt was devised by the U. S. Food and Drug Administration. On the basis of the results from a collaborative study, the Associate Referee

recommends that direct plating with Baird-Parker medium be adopted as official first action for isolation of S. aureus from food. The method involves surface plating dilutions of a food sample on Baird-Parker medium, and incubating for 45-48 hours at 35-37°C. Typical black colonies are inoculated into brain heart infusion broth and the cultures are tested for coagulase activity after 18 hours of incubation at 35-37°C (Baer et al., 1975).

2. Identification

The most convenient and reliable current diagnostic test for S. aureus so far is the production of coagulases. However, it is important to realize the limitations of this test. Coagulases should be tested under carefully standardized conditions, using rabbit or human plasma in a tube test (Recommendations, 1965). According to Tager and Drummond (1965), the slide test in reality detects the "clumping factor" which is regarded as distinct from "coagulase", although recent observations by Blackstock et al. (1968) suggest that they may be closely related. The use of the slide test is also limited by the frequent necessity to subculture from a selective agar onto a non-selective agar to obtain reliable results.

Coagulases, although produced by almost all strains of S. aureus, may not,however, be detected for a variety of reasons (Baird-Parker, 1965b): - (i) growth under unsuitable conditions; (ii) use of plasmas deficient in fibrinogen; (iii) production of fibrinolysin, staphylokinase and/or coagulase-destroying factors. Most plasmas contain citrate as the anti-coagulation agent; citrate-utilizing bacteria (e.g. fecal streptococci and some members of the Enterobacteriaceae) may cause false

clotting of such plasmas (Evans et al., 1952). However, false-positive reactions can be reduced by adding 0.1% ethylenediaminetetraacetic acid to a citrate-containing plasma (Baer, 1968). Also, bacteria other than S. aureus, for example, Yersina (Pasturella) pestis (Eisler, 1961) may produce coagulases. Therefore, complete reliance on a single test, such as the coagulase test, for recognizing S. aureus, may lead to an erroneous diagnosis. Whenever possible, further tests should be done to confirm the identification.

Another important diagnostic characteristic of S. aureus is the production of heat-resistant nucleases (Chesbro and Auborn, 1967). It is important to note that, whereas S. aureus nucleases will resist boiling, similar nucleases produced by other organisms, including S. epidermis, are destroyed by heating (Lachica et al., 1971a). Thomas and Nambudripad (1974) reported that 30 out of 125 strains of Streptococcus fecalis produced thermonuclease. However, it was found to be optimally active in the acid pH range of 6.7 (Nambudripad, 1975). In this regard, staphylococcal thermonuclease is different because it is optimally active at a pH of about 9.0 (Tatini et al., 1976a). Recently, confusion and difficulty were reported from several workers (Sperber and Tatini, 1975; Rayman et al., 1975) in the interpretation of the coagulase test. Rayman et al. (1975) suggested that the thermonuclease test should be performed on cultures with doubtful coagulase reactions before classifying them as S. aureus.

Until lately, the coagulase test has been the most adopted diagnostic test in food processing plants. The need for the test, however, requires an additional day or two in most diagnostic schemes (AOAC , 1975; Thatcher and Clark, 1968). In efforts to develop a more

rapid test, the incorporation of plasma into plating media has been proposed. Orth and Anderson (1970) described a polymyxin-coagulase-mannitol agar medium which has overcome many problems encountered by previous workers with plate coagulase tests. The recent description of a CoDNase spot plate method by Mol and Vincentie (1975) appears to represent an important advance in media for detecting S. aureus. The method involved plating with CoDNA agar (DNase agar containing coagulase plasma) and incubating for 18-24 hours at 37°C. Coagulase positive colonies appear black, and are surrounded by a zone of precipitation, after the plate is flooded with 1 N HCl. DNase activity is demonstrated by a clear zone which is surrounded by a precipitation zone on a hazy clouded plate. The workers reported good agreement between the spot plate method and conventional tests for coagulase and thermonuclease activity indicating it promises to be a useful medium.

Very recently, Lachica (1976) reported a simplified thermonuclease test for the rapid identification of S. aureus. This involved heating of agar plates with grown colonies for 2 hours in a 60°C oven followed by an overlay with 10 ml of molten toluidine blue O-deoxyribonucleic acid agar. An S. aureus colony is identified by a bright pink zone after incubating for 3 hours at 37°C. Working with food sample analysis for a year, the author also reported that all suspected colonies confirmed as S. aureus were positive with the simplified thermonuclease test. Hence, such a rapid and simple test may prove to be an improvement over those in current use.

Other diagnostic tests such as phosphatase production (Tirunarayanan, 1968; Malveaux and San Clemente, 1967); alpha, beta and delta hemolysin production (Elek and Levy, 1950); and clearing of egg yolk

are considered to be less valuable since they are not specific for S. aureus (Baird-Parker, 1963; Kleck and Donahue, 1968; Kocur et al., 1966).

Staphylococcal Toxins with Emphasis on Staphylococcal Enterotoxin

1. Staphylococcal Toxins in General

The notion that pathogenic bacteria might produce their harmful effects by means of poisons is almost as old as the notion of pathogenic bacteria itself. Early in 1872, Klebs suggested that chemical substances ("sepsins") were responsible for the lesions caused by staphylococci, but produced no evidence for the existence of such substances. In 1888, working with diphtheria Bacillus, Roux and Yersin discovered that this "poisonous substance" was a "kind of enzyme", which they named, for the first time, a "toxin". Today, we understand toxins are distinct from simple chemical poisons by virtue of their microbial origin, high molecular weight, and their antigenicity. In general, there are two types of bacterial toxins, namely, "exotoxins" and "endotoxins". The prefixes exo- and endo- are now retained by convention mainly for historical reasons, and the fundamental difference between these toxins is concerned not so much with whether they are found outside or inside the bacterial cell, but with their structure. The exotoxins are proteins, probably without any nonprotein residues, and are antigenic; the endotoxins are antigenic complexes of protein, polysaccharide, and lipid. From a microbiological standpoint, exotoxins are produced generally by Gram positive organisms (except Shigella dysenteriae and Vibrio cholerae exotoxins) into the culture medium during the logarithmic, or declining phases of growth, but may be released on autolysis. Endotoxins are all produced

by Gram negative organisms from the bacterial cell wall, and are released only on autolysis.

S. aureus is Gram positive. It produces toxins which are protein in nature and are found outside the bacterial cells at all phases of growth. Hence, staphylococcal toxins are "exotoxins". Some of the exotoxins and their mode of action are listed below:

1. α - toxin (α hemolysin) - dermonecrosis, hemolytic
2. β - toxin (β hemolysin) - hemolytic to sheep, emesis in cats
3. γ - toxin (γ hemolysin) - hemolytic to rabbit leukocytes
4. δ - toxin - hemolytic
5. ε - toxin - hemolytic
6. hyaluronidase - spreading factor
7. coagulase - coagulates plasma
8. staphylokinase - fibrinolytic
9. enterotoxin - emetic
10. leukocidin - kills rabbit and human leukocytes

Additional information is furnished by Montie et al.(1970) and Cohen (1972).

2. Staphylococcal Enterotoxins

The heat stable toxins causing staphylococcal food-poisoning are referred to as enterotoxins. This term implies that their primary site of action is in the enteric tract of man and other animals (Lamanna and Carr, 1967). As yet, the enterotoxin has not been proven to act directly on the enteric tract, although there is some suggestive evidence of gastric and intestinal localization of iodinated toxin (Arghittu et al., 1962) and histopathological changes in the intestinal tract following oral toxin administration (Wang and Borison, 1951).

The gastrointestinal tract is extremely sensitive to stimuli from the nervous system and evidence has accumulated which suggests a direct nervous stimulus for the emetic action of this toxin (Sugiyama and Hayama, 1965; Sugiyama, 1966). However, the temptation to classify the staphylococcal toxin causing food-poisoning as an enterotoxin is great, since the gastrointestinal signs are the most prominent.

The purification of a protein that caused emesis in monkeys led to the discovery that staphylococci produce more than one enterotoxin (Bergdoll et al., 1959a) and the basis for differentiation being their reaction with specific antibodies. This basis was used in establishing a nomenclature, designating them as enterotoxins A, B, C, etc. (Casman et al., 1963). To date, enterotoxins A (Casman, 1960), B (Bergdoll et al., 1959a), C_1 and C_2 (Bergdoll et al., 1965), D (Casman et al., 1967), and E (Bergdoll et al., 1971) have been identified with the seventh enterotoxin F still in the process of isolation. Also, refined procedures for purification of enterotoxin A (Chu et al., 1966; Tan et al., 1969), B (Bergdoll et al., 1959b, 1961; Frea et al, 1963; Schantz et al., 1965), C_1 (Borja and Bergdoll, 1967), and E (Borja et al., 1972) have subsequently been published.

A. General Properties. The purified enterotoxins are fluffy, snow-white materials that are hygroscopic, and readily soluble in water and salt solutions (Chu et al., 1966). It does appear that the molecular weights of all enterotoxins are within 26,000 - 30,000, which is what one would expect in the case of a common group of proteins. Some of the chemical and physical properties of these enterotoxins have been tabulated by Bergdoll et al. (1974).

	ENTEROTOXIN				
	A	B	C_1	C_2	E
Nitrogen content, %	16.2	16.1	16.2	16.0	
Sedimentation coefficient ($S_{20,w}°$), S	3.03	2.89	3.00	2.90	2.60
Molecular weight	27,800 (34,700)	28,366	26,000 (34,100)	34,100	29,600
Isoelectric point, pH	7.26	8.60	8.60	7.00	7.00
Extinction, $E_{1\ cm}^{1\%}$	14.6	14.0	12.1	12.1	12.5

B. Stability of Enterotoxin. The enterotoxins in the active state are resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin, and papain. Pepsin destroys their activity at a pH of about 2 (Bergdoll, 1970), but is ineffective at higher pH values (Bergdoll, 1966).

The enterotoxins are considered to be quite heat resistant since boiling of crude enterotoxin solutions for 30 minutes does not destroy all their activity. The heat inactivation of enterotoxin A was found to be dependent upon the concentration of the enterotoxin (Hilker et al., 1968; Denny et al., 1971). Enterotoxin A was inactivated by less heat in a pH 7.2 phosphate buffer than in a pH 6.2 beef bouillon. In beef bouillon, heat inactivation was faster at pH 5.3 than at pH 6.2 . The Z values (the degrees Fahrenheit required to reduce the thermal death time tenfold) for the inactivation curves at pH 6.2 and 5.3 were 49.5° and 55°F (about 27°C and 30°C), respectively (Humber et al., 1975). The enterotoxin can also be inactivated by processes used in canning (Denny et al., 1966), although pasteurization or spray drying of milk as currently practiced does not inactivate enterotoxin B (Read and Bradshaw, 1966). Thermal inactivation of enterotoxin B was noted by Satterlee and Kraft (1969) to be quite rapid in phosphate buffer at

pH 7.4 and in 0.85% saline at 60-110°C. This rapid inactivation of toxin, however, accounted for only 65-75% of the initial toxin. The remaining toxin was inactivated more slowly. A more rapid loss of activity was observed at 70-80°C than at 90-100°C (Jamlang et al., 1971).

Irradiation of enterotoxin B was studied by Read and Bradshaw (1967) who determined that a dose of 5 Mrad of γ-irradiation was required to reduce the concentration of 31 µg of toxin to <0.7 µg per ml in Veronal buffer.

C. Antigenicity. As early as 1938, Davison et al., (1938) found that enterotoxin was antigenic by using subcutaneous injections of toxic filtrates from staphylococcal cultures in monkeys and kittens. It was not until 1952, however, that the property of antigenicity possessed by enterotoxin was demonstrated by an in vitro serological procedure. At the time, Surgalla et al. (1952) observed antigen-antibody reactions in agar and recognized the presence of a number of antigens in the enterotoxin preparation. Bergdoll et al. (1959b) indicated that their partially purified toxin preparation contained at least six antigens. They also provided evidence that immunologically distinct enterotoxins existed (Bergdoll et al., 1959b). Later, with the availability of highly purified enterotoxin, highly potent and specific antisera were produced (Casman and Bennett, 1964).

Initially, the only observable difference between enterotoxins C_1 and C_2 was thought to be in their isoelectric points, but on close examination it was apparent, in Ouchterlony plates, that the reaction of the enterotoxins with the heterologous antibodies

was not complete. The major antigenic sites are the same for both enterotoxins, but each apparently has a specific minor antigenic site. Bergdoll et al. (1971) showed that cross reactions of enterotoxin A with E antiserum, and E with A antiserum did occur. These reactions showed that enterotoxin A and E not only had major heterologous antigenic sites, but also had a common minor antigenic site. Gruber and Wright (1969) showed that enterotoxins B and C contained similar antigenic determinant groups. Information available concerning the enterotoxins shows that they are a closely related group of proteins with a common toxic action. Bergdoll et al. (1974) believed that the site responsible for the toxic action is a common one in all of the enterotoxins and is not involved in the antigenicity of the toxins.

Recently, Yamada et al. (1977) has demonstrated the heterogeneity of enterotoxin A by isoelectric focusing and disc electrophoresis. The toxin was found to be composed of three immunologically identical fractions with isoelectric points of 6.5, 7.0, and 8.0, two of which were considered to be charged isomers.

D. Composition. The enterotoxins are single polypeptide chains which contain relatively large amounts of lysine, aspartic acid, glutamic acid, and tyrosine (Bergdoll and Robbins, 1973). Each polypeptide chain appears to contain only two residues of half-cystine and one or two residues of tryptophan. The two half-cystine residues are cross-linked in the native enterotoxin to form a "cystine" loop. Several amino acid residues involving part of this loop appear to be the same for the different enterotoxins and may represent the toxic site. The other part

of the cystine loop may be involved in the antigenicity of the enterotoxin which is the basis for identifying them as enterotoxins A - E. The enterotoxin polypeptide chain contains one disulfide bridge which can be reduced without affecting the toxicity or antigenicity. At least a part of the tyrosyl and methionine residues, and the carboxyl and amino groups can be substituted without adversely affecting the toxicity of the molecule. The amino acid compositions of enterotoxins A and E are quite similar, while the compositions of B and C are similar. The terminal amino acids for the enterotoxins have been reported by Bergdoll et al. (1974) as follows:

	N-terminal amino acid	C-terminal amino acid
A	alanine	serine
B	glutamic acid	lysine
C_1	glutamic acid	glycine
C_2	glutamic acid	glycine
E	serine	threonine

Details concerning the composition, amino acid sequence, and conformation of the enterotoxins are furnished by Montie et al. (1970). In addition, the chemistry and characterization of staphylococcal enterotoxins have been adequately reported by Bergdoll et al. (1974).

E. Production. The production of high yields of enterotoxins from staphylococcal cultures and factors affecting their synthesis have been adequately reviewed by Minor and Marth (1972a) and Bergdoll (1970), respectively.

F. Effect of Enterotoxins. Staphylococcal enterotoxins cause emesis, diarrhea, enteritis, changes affecting the circulatory system (eg. leukocytosis), and death. More information is furnished by Montie et al., (1970).

The Presence, Growth, and Enterotoxin Production of S. aureus in Milk

Raw milk may become contaminated with staphylococci from several sources, but the principal one is probably the mastitic bovine udder. Williams (1941) studied raw milk samples from ten herds and found that more than 50% of the cows were shedding staphylococci in their milk. Staphylococcal counts in excess of 1,000 cells per ml were common, and most of the isolates were coagulase positive. This work was done before antibiotics were used to treat mastitis, and the problem of antibiotic resistance was not yet encountered. Clark and Nelson (1961) tested twenty Grade A raw milk samples and found they contained 25 to 3,300 coagulase positive staphylococci per ml of milk. Some workers (Warren and Arends, 1967; Geiges, 1972) have demonstrated that staphylococci could be transmitted between cows via milking machines.

Bell and Veliz (1952) found that enterotoxin was produced by

25 of 35 cultures of staphylococci isolated from the udder. Casman (1965) found 4.2% of 190 staphylococcal strains isolated from raw milk were enterotoxigenic, of which 76% produced enterotoxin A, and 24% produced B. More recently, Olson et al. (1970) isolated 157 strains from mastitic udders, eleven of which produced enterotoxin C, eleven others produced D, one produced both C and D, and none produced A and B.

Murray (1960) studied the influence of the holding temperature on the growth of staphylococci in naturally and artificially infected raw milk. He found that at 22°C or lower, there was very little growth and no enterotoxin production within 24 hours. However, if storage time at 22°C was increased to 48 hours of storage at above 22°C, there was a substantial increase in the number of staphylococci present. Holding raw milk samples at 4°C and 10°C (approximated by bulk tank and can holding conditions, respectively), Clark and Nelson (1961) demonstrated significant growth of staphylococci at 10°C for seven days, but not at 4°C.

In 1951, Smith (1951) reported that the normal microflora of milk tend to suppress the growth of staphylococci in accordance with the proportion of S. aureus and the natural microflora. He suggested that a product with a low microfloral count with a high percentage of staphylococci is more likely to cause food-poisoning than one with a higher microfloral count.

Donnelly et al. (1968) inoculated low and high count raw milk samples with S. aureus, then held the milks at different temperatures to determine both growth and enterotoxin production. Samples were done in duplicate. No enterotoxin was produced at 10°C. Enterotoxin A was detected after 5-9 hours at 35°C, 9-12 hours at 30°C, 18 hours at 25°C,

and 36 hours at 20°C. Although the indigenous microflora grew in both duplicate samples of low count raw milk (i.e. milks with similar initial populations), staphylococcal growth was found to be more pronounced in one than in the other. This more pronounced growth led to enterotoxin production in one milk sample but not in the other. Enterotoxin production in low count milk was more rapid at all temperatures tested when the highest inoculum of staphylococci was added. When high count raw milk was used, a larger S. aureus inoculum was required for enterotoxin production, and then the toxin appeared only at 35°C. Generally, a level of 5×10^7 S. aureus per ml was reached before enterotoxin was detected.

S. aureus population of two to three million per ml was noted by Tatini et al. (1971a) to produce detectable amounts of enterotoxin A in milk. This milk was relatively free of other competing micro-organisms. The toxin was detected after 4-6 hours in low count raw milk inoculated initially with $10^3 - 10^5$ S. aureus per ml. The literature reviewed indicates that:

(i) staphylococci are likely to be present in raw milk;

(ii) some of the staphylococci in raw milk can produce enterotoxin;

(iii) growth of staphylococci in raw milk can occur, and enterotoxin can develop as a result of such growth;

(iv) raw milk with a low initial microflora is more suitable for growth and enterotoxin production by staphylococci than is high count milk;

(v) there are differences among low count raw milks in their suitability for growth of staphylococci; and

(vi) holding temperatures above 20°C for more than 24 hours

are conducive for S. aureus proliferation and enterotoxin production.

Using phage-typing, it was shown that S. aureus isolates obtained from dairy products were predominantly 42D or Group IV of bovine origin. These isolates were more consistent in nature than any other phage group implicated in the production of enterotoxin (Thatcher and Simon, 1956; St. George et al., 1962).

Raw milk is normally pasteurized or subpasteurized before it is used for manufacturing purposes. Reiter et al. (1964) showed that the growth of S. aureus increased 70-fold in steamed milk, but only two-fold or less in raw or pasteurized milk. Donnelly et al.(1968) tested pasteurized milk as a substrate for staphylococci and found it was suitable for growth and enterotoxin production. From their data, it is evident that pasteurized milk was even more suitable for enterotoxin production than was low count raw milk, particularly when the smaller inoculum of staphylococci was used. Tatini et al. (1971a) in a similar study subjected the milk to several different heat treatments. Results obtained confirm that pasteurized milk (65.6°C for 16 sec) was more suitable for enterotoxin production than was the same milk in the raw state. The degree of heating, ranging from pasteurization to autoclaving, had little apparent effect on the capacity of milk to support enterotoxin production.

Raw and heated milk contain many volatile and non-volatile compounds (Kulshrestha and Marth, 1970) which were found to affect growth of S. aureus. Kodicek and Worden (1944) reported that 8 μg of fatty acids per ml of milk , particularly of linolenic and linoleic acid acid, were inhibitory to S. aureus. A 99% reduction in the growth of staphylococci was observed when media were adjusted

to a final pH of 5.0, 4.6, 4.5, 4.1, and 4.0 using acetic, lactic,citric, phosphoric and hydrochloric acids, respectively (Minor and Marth, 1970). Other fatty acids (Kabra et al., 1972 ; Podesta and Bertoldini, 1967 ; Vadhera and Harmon, 1964, 1965) have also been reported to retard growth of this bacterium. Kulshrestha and Marth (1970) tested 27 volatile compounds and found that fatty acids, aldehydes, amines, and diacetyl were inhibitory to S. aureus. Recently (Kulshrestha and Marth,1974), butyric, octanoic, and decanoic acids were found to be more effective against S. aureus than other fatty acids at 10 ppm. Also, formaldehyde, diacetyl, and chloroform inactivated S. aureus . Acetonitrile, ether, ethylene-dichloride and methylsulfone were significantly inhibitory to S. aureus only at high concentrations. Amines were found to be, in general, more inhibitory to S. aureus than the alcohols.

The Presence, Growth, and Enterotoxin Production of S. aureus in Manufactured Dairy Products

The origin of several well known outbreaks of staphylococcal food-poisoning have been attributed to cheese, non-fat dry milk and butter, with cheese as the chief vehicle to the problem. Here, cheese will be dealt with in some detail. The other dairy products have been reviewed by Minor and Marth (1972b).

Studies by Takahashi and Johns (1959) revealed that 36% of 333 phosphatase-positive samples of Canadian cheddar cheese contained staphylococcal populations in excess of 10,000 per g , and 6% of the samples had levels greater than 500,000 per g . The phosphatase test reveals inadequate pasteurization techniques. Sharpe et al.(1965) tested samples of cheese made from raw milk and found that 9% of these tested samples contained greater than 500,000 S. aureus per g. Mickelson

et al. (1961) collected samples from 20 varieties of cheese and found that 76% contained staphylococci, of which 70% were S. aureus, and 26% were S. epidermis. Coagulase-positive S. aureus were isolated from cheddar, bondost, blue and brick cheeses.

Thatcher et al. (1959) examined 49 cheese samples from a factory and found enterotoxin in eight samples. Donnelly et al. (1964) studied cheddar cheese samples incriminated in food-poisoning outbreaks, and found that 11 of 13 samples contained coagulase-positive staphylococci ranging from fifty to several million per g. They also examined 343 retail cheddar cheese samples, 20% of which contained coagulase-positive staphylococci ranging from 50 to greater than 200,000 per g. Later, 7 of 77 strains isolated from the outbreaks, and 9 of 155 isolated from market cheese samples were found to produce enterotoxin A (Donnelly et al., 1967). Zehren and Zehren (1968a) examined 2,112 vats of cheese suspected of harboring enterotoxin. Enterotoxin A was found in 56 vats of cheddar cheese, 2 vats of Monterey cheese and one vat of Kuminost cheese under conditions of subnormal acid development. As much as 12 µg of enterotoxin A per 100 g of cheese was found distributed throughout each cheese vat.

The problem of enterotoxin-containing cheese involves three distinct phases:

(i) Effect of milk quality on growth of staphylococci in cheesemaking

Thatcher and Ross (1960) showed that the following factors in relation to milk quality can contribute to the development of massive staphylococcal populations in cheese: (a) substantial contamination of the milk with staphylococci from the bovine udder; (b) inadequate overnight cooling; (c) the presence of antibiotic residues in milk

allowing the selective multiplication of the staphylococci and inhibition of the starter culture; (d) contamination with antibiotic resistant strains. However, Jezeski et al. (1961) showed that S. aureus counts observed in cheese made with normal starter were similar to those made from milk containing penicillin. They also found that heat treatment of milk prior to inoculation had no effect on growth of S. aureus during manufacture of cheddar and colby cheeses. The grade of milk used also had no effect on the growth of S. aureus.

Growth of S. aureus in raw milk held at 22°C and 32°C was found to be inversely correlated with the initial standard plate count of the milk (Takahashi and Johns, 1959). The rate of staphylococcal proliferation during the manufacture of cheddar cheese, and the ratio of whey bacterial population to curd bacterial population, were noted (McLeod et al., 1962) to be independent of the initial number of S. aureus in the milk. However, survival of staphylococci during curing was prolonged by increasing the number of S. aureus in milk.

(ii) Effect of starter activity on growth of staphylococci

Reiter et al. (1964) showed that the growth of S. aureus was inhibited in the presence of a lactic starter in raw, steamed, and pasteurized milk. When lactic acid, developed by the starter culture, was neutralized as it was formed, inhibition was found to be more than a function of pH. S. aureus was shown to grow more rapidly in cheese made with an inactive starter culture than with a normal active culture; the staphylococci also persisted longer in the former type of cheese than the latter type of cheese.

Jezeski et al. (1967) observed inhibition of growth of S. aureus in the presence of actively growing Streptococcus lactis in sterile or

steamed skim milk. Enterotoxin was produced by S. aureus when it was grown alone or when it was grown in the presence of the starter culture with the homologous bacteriophage. Normal lactic starter culture was able to prevent enterotoxin production by S. aureus. Gilliland and Speck (1972) tested six different lactic Streptococcus cultures for their ability to inhibit S. aureus. Inhibition was almost complete regardless of the starter culture type used or the amount of time, within limits, required for acid formation. The authors suggested that repression of staphylococci by lactic streptococci may involve production of antibiotics, hydrogen peroxide, and volatile fatty acids in addition to other organic acids.

(iii) Growth of staphylococci during cheesemaking

It is clearly evident that the growth of staphylococci is possible even when cheese is made using a normal process (Mattick et al., 1959; Reiter et al., 1964; Tuckey et al., 1964; Walker et al., 1961). Starter culture failure, however, during cheese manufacture can lead to more extensive growth of S. aureus (Reiter et al., 1964; Tatini et al., 1971b). Several investigators have studied the behavior of S. aureus during the manufacture of cheese. Results obtained from their studies are summarized: -

(i) S. aureus grew rapidly during the period of heat treatment following caesin coagulation (Thatcher and Ross, 1960).

(ii) The initial increase (after 2 hours) is largely attributed to the concentration effect when curd is formed (Tuckey et al., 1964).

(iii) Slight increases in growth continued throughout the cheese-making process until the curd was salted (Tuckey et al., 1964).

(iv) The number of staphylococci declines rapidly during curing and

aging (Mattick et al., 1959; Roughley and McLeod, 1961; Stiles et al., 1962).

Others investigators (Takashashi and Johns, 1959; Walker et al., 1961) made similar observations when they studied cheddar and Colby cheese, respectively. Cheddar and Colby cheeses were produced by Tatini et al. (1971b) from milk inoculated with different S. aureus populations. The data indicated the importance of both the starter culture activity and the initial concentration of staphylococci in determining whether or not a cheese becomes toxic. They concluded that cheese made normally could become toxic if it contained at least 15 million staphylococci per g for Colby, and 28 million per g for cheddar cheese. With starter culture failure, toxic cheese could result if 3 - 5 million staphylococci per g were presence.

Conditions Necessary for Growth and Enterotoxin Production

1. Nutritional Completeness of the Medium

Since enterotoxin production is related to the growth of S. aureus, it is, therefore, necessary that suitable growth conditions must be determined for the maximum amounts of enterotoxin production. Growth of S. aureus and production of enterotoxins in meat infusion broths such as veal, beef, beef heart, and brain heart have been reported (Surgalla et al., 1951; D'Arca, 1965; Hallander, 1965; Casman, 1958; McLean et al., 1968). Brain heart infusion has been reported (Casman and Bennett, 1963) as the best medium for producing high yields of enterotoxin A , whereas beef heart infusion with one percent of maltose resulted in better enterotoxin B production than any other medium (D'Arca, 1965). Simplified media using Protein Hydrolysate

Powder has been found to produce low yields of enterotoxin (Surgalla et al., 1951). Niacin and thiamine were required as supplements to the medium in order to achieve higher yields of enterotoxin (Kato et al., 1966). Supplementation of media with casein hydrolysate was found necessary for optimum growth and enterotoxin production (Jarvis and Lawrence, 1970).

According to studies by Surgalla (1947), enterotoxin was detected in the supernatants of cultures grown in media containing from 2 to 16 amino acids as the source of nitrogen. The simplest medium resulting in the production of enterotoxin contained arginine and cystine, plus nicotinic acid, thiamine, glucose, magnesium sulfate, ferrous ammonium sulfate, and potassium dihydrogen phosphate. The same experiment also showed that no amino acids were required for enterotoxin production other than those required for growth. Mah et al. (1967) found that the optimal growth of S. aureus S-6 occurred with glucose as the carbon source only when the medium contained 11 amino acids (glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine, and histidine) and three vitamins (thiamine, nicotinic acid, and biotin).

Wu and Bergdoll (1971a; 1971b) showed that a relatively large quantity of enterotoxin B was produced in a synthetic medium composed of amino acids, inorganic salts, and vitamins. The medium was based on the utilization of amino acid by S. aureus S-6 throughout its growth cycle. Aspartic acid, threonine, serine, glutamic acid, proline, glycine, and alanine were utilized rapidly; whereas only small amounts of tryptophan , cystine, tyrosine, phenylalanine, and methionine were used. Arginine appeared to be essential for enterotoxin production. Certain

peptide fractions have been shown to have a stimulatory effect on the production of enterotoxin. These peptides were found to contain large amounts of proline.

2. pH of the Medium

The response of S. aureus to acidity varies with strain, and is influenced by the size of the inoculum, type of medium, salt concentration, temperature, type of acid, and atmosphere (Genigeorgis et al., 1971a, 1971b; Genigeorgis et al., 1969; Genigeorgis and Sadler, 1966a; Hucker and Haynes, 1937; Thatcher et al., 1962). Genigeorgis et al. (1971c) reported a minimal pH of 4.0 for staphylococcal growth initiation, and an upper pH limit of 9.8 for growth. This initiation of growth was due either to a selection of acid-tolerant cells which initiated growth or to a pH shift to a higher value, caused by the release of metabolic products from non-reproducing living cells or by the decomposition products of dead cells. Enterotoxins B and C producing strains are influenced to a greater extent by pH than type A producing strains in the range 5.0 to 9.0 (Reiser and Weiss, 1969; Kato et al., 1966)

Minimal initial pH levels of 4.5, 4.7, and 4.7 were reported for the production of enterotoxins A, B, and C, respectively (Tatini et al., 1971a; Genigeorgis et al., 1971b). The optimal pH for enterotoxins B and C production is 6.8; whereas enterotoxin A synthesis occurs optimally over a range from pH 5.3 to 6.8 (Reiser and Weiss, 1969). Markus and Silverman (1969; 1970) reported an optimal pH of 8-8.5 in nitrogen free medium, and 7-7.5 in the presence of protein hydrolysates, for enterotoxin B production; and an optimal pH of 6.5-7.0 for enterotoxin

A production. Jarvis et al. (1973) reported that significantly more enterotoxin A was produced at a controlled pH of 6.5 in the fermentor than in shake-flask cultures. Since many foods are buffered at pH 6 to 6.5, the same authors suggested that some strains may, therefore, produce sufficient enterotoxin to cause food poisoning, although little or none may be produced when grown under normal testing procedures.

Enterotoxin production normally will not occur above pH 9.0, and is reduced by 50% at pH 8.0. At pH levels of 5.0 and below, little or no toxin is produced (Troller, 1976). Hence, enterotoxin is relatively resistant to pH ranges normally encountered in foods.

3. Temperature of the Product

Temperature ranges at which growth has been found to occur are quite broad varying from 6.7°C (Angelotti et al., 1961b) to a maximal temperature of 47.8°C (George et al., 1959; Reimann et al., 1972). Enterotoxin production, however, occurs within somewhat narrower temperature limits. The minimum and maximum temperature reported to permit enterotoxin production by S. aureus is 10°C (Genigeorgis et al., 1969) and 45-46°C (Scheusner and Harmon, 1971; Tatini et al., 1971a ; Scheusner et al., 1973),respectively. The optimal temperature for S. aureus growth is 37°C, whereas Tatini et al. (1971c) reported that production of enterotoxins A, B, C and D was stimulated in brain heart infusion broth at temperatures (40°C and 45°C) above the optimum growth temperature. Also, prolonged incubation (48-72 hr) at 37°C was observed by Reiser and Weiss (1969) to only occasionally result in production of additional quantities of enterotoxins A, B and C. Temperatures greater than 45°C and less than 5°C will control the normal growth and enterotoxin

production by this organism.

4. Water Activity of the Product

One of the more unique aspects of the growth of S. aureus is its ability to grow at relatively low water activity (a_w) levels. Using 14 strains of food-poisoning staphylococci, Scott (1953a) found that growth rates were reduced significantly at a_w levels below 0.94. Minimal a_w for aerobic growth in most laboratory media is approximately 0.86. Recently, Hill et al. (1975) obtained a minimal a_w of 0.83 for growth in a pork infusion medium, although no toxin was detected below an a_w of 0.86. Growth was obtained in nutrient broth at an a_w of 0.84 only when 1% yeast extract was added to the medium. The limiting a_w in the medium without yeast extract was 0.87. The stimulatory effect of yeast extract was believed to be due to the B complex vitamins, primarily thiamine, which has been shown to be essential for growth (Mah et al., 1967; Miller, 1972; Miller and Fung, 1972). The fact that pork is high in thiamine may also explain the difference in the limiting a_w noted between pork and laboratory media. Enterotoxin production is suppressed markedly by reduced a_w (Troller, 1971, 1973; Troller and Stinson, 1975). Enterotoxin production relative to cell yield virtually ceases at a_w levels ≤ 0.9; however, growth occurs at much lower a_w levels (Troller, 1976).

5. Associative Growth of Micro-organisms in Competition

S. aureus is not a good growth competitor, and under most circumstances, it tends to be suppressed by the growth of other organisms. The source of these competitive effects may be the results

of production of inhibitory substances, nutritional competition, or the alteration of various environmental factors to levels that are unfavorable. Several workers (Oberhofer and Frazier, 1961; Peterson et al., 1962a, 1962b; Peterson et al., 1964a, 1964b; Troller and Frazier, 1963a, 1963b) have investigated the repression of staphylococcal growth by other commonly occurring microflora in milk and other food products. In some situations, associative growth by competing bacteria did not affect the growth rates or maximal total staphylococcal counts, but did inhibit the production of enterotoxin (McCoy and Faber, 1966).

Peterson et al. (1962b) noted definite repressive effects on the growth of S. aureus by a mixture of saprophytic and psychrotrophic bacterial species. This repression became more pronounced as the staphylococcal population decreased and as the temperature approached room temperature or below. Saprophytic growth, however, was more inhibited as the salt concentration was increased (Peterson et al., 1964a). Incorporation of sucrose, lactose, dextrose, or a 4% concentration of corn oil also repressed saprophytic growth, allowing the staphylococci to dominate the population (Peterson et al., 1964b).

Some of the competitors inhibited S. aureus by elaborating low molecular weight or heat stable substances; others competed more effectively for amino acids than S. aureus. Dahia and Speck (1968)reported that culture filtrates of Lactobacillus lactis and L. bulgaricus contained hydrogen peroxide which was bacteriostatic to S. aureus. Inhibition of S. aureus by a micrococcal species was shown to be due to the utilization of certain amino acids by the Micrococcus necessary for

staphylococcal growth. The amino acids involved were glutamic acid, aspartic acid, arginine, and threonine (Dubos and Ducluzeau, 1969). Likewise, inhibition of an enterotoxigenic strain of S. aureus by Serratia marcescens was attributed to competition for amino acids (Troller and Frazier, 1963a, 1963b). Iandolo et al. (1965) reported that S. aureus MF31 was suppressed by Streptococcus diacetilactis which caused nicotinamide depletion.

6. Availability of oxygen

The nature of the gaseous atmosphere may influence growth and enterotoxin production. Staphylococci grow well under both aerobic and anaerobic conditions in laboratory media, although toxin production is normally somewhat reduced under anaerobic conditions. Aerated cultures produce toxin more rapidly and in larger amounts than do static cultures (Casman and Bennett, 1963; McLean et al., 1968). Staphylococci can grow and produce enterotoxins A and B, but not type C in meats under anaerobic conditions (Genigeorgis et al., 1969; Thatcher et al., 1962; Reimann et al., 1972). When meat is vacuum packed, oxygen is consumed without being replenished, and carbon dioxide is produced as a result of growth. This change favors facultative anaerobic lactobacilli which lowers the pH and competes with the staphylococci (Ingram, 1960).

Barber and Deibel (1972) found that in fermented sausages, staphylococcal growth had localized itself at the outer periphery of the sausage where the oxidation-reduction potential was greatest. Enterotoxin synthesis in inoculated sausages was suppressed when the oxygen content of the incubation atmosphere dropped below 10%, although S. aureus counts should have been sufficiently high (10^7 - 10^8 cells/g)

to support toxin production.

7. Salt Concentrations and Chemical Additives

Staphylococcal multiplication is possible aerobically in media with 16-18% salt (Genigeorgis and Sadler, 1966a; Mah et al., 1967; Scott, 1953b) and anaerobically in media with 14-16% salt (Peterson et al., 1964c; Scott, 1953b), provided the pH is near neutrality and the growth temperature is optimal. Ten percent salt in the brine is about the highest concentration which permits production of enterotoxins A, B, and C both in laboratory media and in semi-preserved meat products (Genigeorgis et al. 1971c; Markus and Silverman, 1969, 1970; McLean et al., 1968; Troller, 1971, 1972).

Genigeorgis et al. (1971c) studied the combination effect of salt and pH on the premise of initiating aerobic growth of five staphylococcal strains, producing enterotoxins A, B, C and D. The two factors interacted to produce inhibitory effects which neither produced alone. At pH values remote from neutrality, lower salt concentrations were required to prevent or delay growth. When combined with a moderate salt concentration of 7.4-7.7%, a titratable acidity of 2.2-2.7% was found to inhibit staphylococcal growth effectively (Helmy et al.,1975b). Recently, Helmy et al. (1975a) showed that the adaptation of some strains of S. aureus to high salinity resulted in more growth occurring in the presence of 15% salt. The organisms, however, became more sensitive to increases in acidity.

Neither sodium nitrate in concentrations up to 1000 ppm, nor sodium nitrite at levels up to 200 ppm, appears to affect staphylococcal growth or its production of enterotoxins A (Markus and Silverman, 1970)

and B (McLean et al., 1968). Later, Buchanan and Solberg (1972) found that nitrite had no effect even at 2,000 ppm when S. aureus was cultivated aerobically at pH 7.3. However, under anaerobiosis, sodium nitrate was inhibitory to S. aureus at 200 ppm (levels permitted in food products) at pH 6.3. By studying the metabolic activity, S. aureus cells were shown to metabolize nitrite when cultured aerobically. It was also found that nitrite inhibition of this bacterium involved extension of the adjustment phase, decrease in growth rate, and damage or destruction of some cells. The magnitude of inhibition was dependent upon the interaction of the nitrite concentration, initial pH, and partial pressure of oxygen.

The antibiotic nisin in concentrations of 500 IU (international units) was found to inhibit staphylococcal growth (Jarchovska, 1974). Addition of brewer's yeast (2% W/V) to whole milk was also shown to enhance production of enterotoxins A and D (Tatini et al., 1976b).

Heat Resistance and Recovery of S. aureus from Thermal Injury

Some information on thermal destruction of staphylococci is necessary, since a heat process of some sort is used in the manufacture of virtually all dairy products. The D_{60} value (time at 60°C to reduce population of cells by 90%) for S. aureus heated in a buffer solution or in a weak salt solution at pH 6.5 to 7.0 varies from about 0.5 to 2.5 min. (Walker and Harmon, 1966; Stiles and Witter, 1965; Thomas et al., 1966). Heat survival curves for S. aureus are sigmoidal rather than exponential, as would be expected from the generally accepted log order of death resulting from heated cells (Walker and Harmon, 1966; Singh, 1964). The reason for the nonlinearity of these curves may relate to the cluster-

forming tendencies of S. aureus, or to the differences in the heat resistance of S. aureus or to differences in the heat resistance of individual cells (Baird-Parker, 1972).

Three factors may alter the heat resistance of S. aureus:

1. Age of the Culture

As the age of the tested strain of S. aureus increased from 12 to 228 hours, Walker and Harmon (1966) found that the D_{55} value increased from 0.95 min to 3.0 min. Dabbach and Moats (1969) also found that actively growing cells in the logarithmic phase were less heat resistant than resting cells in stationary phase.

2. Composition of heating menstruum

Stiles and Witter (1965) found that the D_{60} value for S. aureus, heated in phosphate buffer, was higher at pH 6.5 than at 4.5, while the Z value was higher at pH 4.5 than 6.5. Walker and Harmon (1966) found that staphylococci were more resistant to heat in skim milk and cheddar cheese whey than in phosphate buffer and whole milk. A D_{60} value of 1.2-1.33 min for the former and 0.43-0.75 min for the latter were reported. Other D_{60} values quoted for S. aureus in skim milk range from 3.1 to 5.3 min (Kadan et al., 1963; Thomas et al., 1966). Working with strain 196E in raw skim milk, Heinemann (1957) found complete inactivation after 80 min at 57.2°C, 24 min at 60°C, 6.8 min at 62.8°C, 1.9 min at 65.6°C, and 0.14 min at 71.7°C. The Z value was found to be 9.2.

The heat resistance of S. aureus does not appear to change significantly in the presence of meat protein (Gross and Vinton, 1947; Thomas et al., 1966). However, the addition of sucrose in concentrations of 41-57% (Kaden et al., 1963) or sodium alginate (Scott and Strong, 1964),

to the heating menstruum, increased heat resistance of S. aureus; whereas the addition of hydrogen peroxide (Amin et al., 1966) or glucose (Calhoun and Frazier, 1966) reduced heat resistance. Serum solids also offered some protection, but milk fat, stabilizer, and emulsifier offered no protective action at the concentrations tested (Kadan et al., 1963). Staphylococci suspended in fat are extremely heat resistant ; the resistancy is greater in dry fat than in moist fat (Yesair et al., 1946).

3. Recovery Conditions

Heating causes a number of cellular changes in S. aureus. These changes include : (i) damage to the cytoplasmic membrane with resulting loss of permeability control allowing leakage of cytoplasmic constituents such as potassium, amino acids, proteins, and a 260-nm absorbing material (Iandolo and Ordal, 1966); (ii) decrease in metabolic activities such as catabolic capabilities and selected enzyme activities of glucose metabolism (Bluhm and Ordal, 1968); (iii) degradation of ribosomal ribonucleic acid (Rosenthal and Ordal, 1970); and (iv) partial denaturation of cell proteins (Iandolo and Ordal, 1966; Sogin and Ordal, 1967).

Busta and Jezeski (1961; 1963) found that heat damaged S. aureus grew poorly on Staphylococcus 110 medium, and this was confirmed by Mickelsen et al. (1963) who studied the recovery of S. aureus from cottage cheese. Lowering the salt concentration improved recovery with the selective medium. Salt tolerance returned if heat-treated cells were incubated in skim milk for 48 hours (Busta and Jezeski, 1964) . Also, when thermally stressed cells were incubated in trypticase soy broth for 4 hours, tolerance to salt and to other selective agents was

gradually regained (Gray et al., 1974). The liquid portion of Robertson's meat medium has been reported to diminish the inhibitory effect of 10% salt on staphylococcal growth (Maitland and Martyn, 1948); however, the effect of this medium on the recovery of thermally stressed cells has not been studied.

It is well established that heat damaged cells have a much longer lag period than unheated cells (Jackson and Woodbine, 1963) . However, when the injured cells reached the logarithmic phase of growth, they showed the same generation time as unheated cells. During this extended lag period, damaged cells undergo a complex repair mechanism. This repair phenomenon occurs in the absence of cell growth, cell wall synthesis, protein synthesis (Stiles and Witter, 1965), and without cell multiplication (Iandolo and Ordal, 1966; Hash, 1972). For repair, an energy source such as glucose (5%) or galactose (Stiles and Witter, 1965) is required together with a mixture of amino acids and phosphate (Iandolo and Ordal, 1966). However, Hurst et al. (1973) obtained good recovery of growth in the absence of sugar.

Lipid material in Gram positive bacteria is located only in the cell membrane (Kates, 1964). During injury, one-third of the lipid material was found to be lost, and there appeared to be an oversynthesis of C_{16} and C_{18} unsaturated fatty acids during cellular recovery (Hurst et al., 1973). S. aureus regained its salt tolerance during recovery, whereas a number of membrane functions were still impaired. One of the main steps in the recovery was the resynthesis of ribosomal ribonucleic acid (Sogin and Ordal, 1967; Haight and Ordal, 1969). This was substantiated by radiotracer experiments. The rate at which label was incorporated in the nucleic acid fraction paralleled that of recovery

and the return of salt tolerance.

A greater oxygen sensitivity was also observed in some of the heat damaged cells which indicated the possible loss of an enzyme such as catalase (Baird-Parker, 1965a). Thus, if through heating, the catalase of some of the surviving cells was destroyed aerobically, metabolizing cells lacking catalase could produce peroxide, which would then poison the cells, unless some external means of destroying the peroxide were present. This can well explain why heat damaged cells of S. aureus have been shown to recover more effectively in the presence of blood and pyruvate containing media (Sharpe et al., 1962; Baird-Parker and Davenport, 1965) since catalase is present in blood and pyruvate is also a strong oxidizer of peroxide. The recovery period required to improve the recoveries on media not containing pyruvate or catalase might be assumed to permit the damaged cells to resynthesize catalase and thus re-establish their ability to grow aerobically. Today, Baird-Parker medium is known to be the best selective and diagnostic medium for the recovery of heat damaged cells of S. aureus (Baird-Parker, 1962; Gray et al., 1974).

Heat shocked cultures were known to survive further heating in far greater numbers than unheated cells (Singh, 1964). The optimum temperature and pH for the recovery of heat damaged cells of S. aureus are 32°C and 6.0 , respectively (Allwood and Russell, 1966).

Detection of Staphylococcal Enterotoxins

Since staphylococcal food-borne illness is of world-wide occurrence, the need for a practical and sensitive method for the detection and estimation of enterotoxin in food has long posed a major

problem in this field.

1. Bioassays

Successful initial bioassays were carried out using various animal species such as kittens (Dolman et al., 1936; Dolman and Wilson, 1938, 1940; Fulton, 1943; Matheson and Thatcher, 1955) and rhesus monkeys (Surgalla et al., 1953).

2. Immunoassay

Reliable method for the detection and measurement of enterotoxin was not found until the property of antigenicity was applied to the problem. Since enterotoxins are antigenic, serological procedures based on antigen-antibody precipitin reaction have been developed. Among the methods that have been proposed are the single gel diffusion tube test (Surgalla et al., 1952), the double gel diffusion tube test (Bergdoll et al., 1959a, 1959b, 1961; Hall et al., 1965), the Ouchterlony plates (Casman, 1958; Bergdoll et al., 1965), the Wadsworth microslide (Wadsworth, 1957; Casman and Bennett, 1965), quantitative precipitin test (Silverman, 1963), hemagglutination inhibition (Morse and Mah, 1967; Johnson, 1967), reversed passive hemagglutination (Silverman et al., 1968), and immunofluorescence (Friedman and White, 1965; Genigerogis and Sadler, 1966b, 1966c; Smith et al., 1962; Stark, 1969, 1970). Among these tests, the single diffusion tube method and the quantitative precipitin test have been adapted for quantitative determination of the enterotoxins.

3. Radioimmunoassay

Radioimmunoassay is based on a reaction between radioactive ^{125}Iodine antigen and specific antibody, which results in the formation

of a labelled antigen-antibody complex. Iodine is preferentially attached on the tyrosyl residues of the proteins. The addition of unlabelled antigen to this system reduces the amount of labelled antigen-antibody complexes by competitive inhibition. Radioactivity contained in the complexes can be measured by collecting the complexes on membrane filters or by selective precipitation of the complex with ammonium sulfate. Using this method, the successful assay of enterotoxin B has been reported by Gruber and Wright (1967).

4. Solid-phase Radioimmunoassay

A new method of solid-phase radioimmunoassay has been devised (Catt and Tregear, 1967) based on the binding capacity of antibody onto polymers. The ability of antibody coated polymers to bind with radioactive tracer antigens provides the basis of this analysis. Two systems are presently utilized in the solid-phase radioimmunoassay ; (i) anti-enterotoxins are coupled to bromoacetyl cellulose (Collins et al., 1972; Collins et al., 1973) and (ii) polystyrene tubes are coated with anti-enterotoxins specific for the enterotoxins (Dickie et al., 1973; Johnson et al., 1971; Johnson et al., 1973; Jarvis, 1974) . Such binding is competitively and quantitatively inhibited by unlabelled antigen. The amount of labelled enterotoxin adsorbed onto anti-enterotoxin can be measured by radioactive counting. The amount of inhibition is equivalent to a given amount of unlabelled enterotoxin which can be determined from a standard curve. Both systems were reported to be satisfactory for enterotoxins A and B determination, but the immunological reactivity of anti-enterotoxin C_2 was significantly lower by the solid-phase method.

Recently, a double-antibody radioimmunoassay was described by Robern et al. (1975) for enterotoxin C_2. This method is based on the finding that gamma globulins have separate binding sites for their antigens and antibodies (Hunter, 1973). An antibody molecule may, therefore, form a complex with its antigen and then be complexed to a second antibody. A primary antigen-antibody complex is too small to be precipitated. A double anti-rabbit gamma globulin, prepared in goats, was used to precipitate the antigen-antibody complex of enterotoxin C_2 and anti-enterotoxin C_2. Employing this assay procedure, Robern et al. (1975) have reported a sensitivity of 100 picograms of enterotoxin per ml for the test. Because of this increased sensitivity, the same workers suggested that the solid-phase radioimmunoassay method be superseded by the double-antibody radioimmunoassay method for the detection of other staphylococcal enterotoxins. This would be beneficial especially when the enterotoxins are present in extremely small quantities or when the anti-enterotoxins adsorb poorly to plastic surfaces. Hence, this method may prove to be an improvement over those radioimmunoassays in current use.

SCOPE OF INVESTIGATION

Little information is available relative to the occurrence and survival of enterotoxigenic strains of Staphylococcus aureus in raw milk undergoing a subpasteurization heat treatment, and their subsequent fate during the manufacture of milk into cheese under conditions similar to those used in commercial cheese plants. With the advent of the immunodiffusion technique, much of the recent research was performed to investigate the growth and enterotoxin production of S. aureus in raw milk and in heated milk. However, the growth and enterotoxin production of enterotoxigenic S. aureus, that survive the subpasteurization heat treatment of raw milk, has not been studied. There is also little information available relating the levels of S. aureus to enterotoxin production during the cheese manufacturing process and the factors in the finished product responsible for the presence or absence of the enterotoxin produced. In the only report of enterotoxin production in cheese processing, Tatini et al.(1971b) inoculated S. aureus strains 196E and F265 into pasteurized milk used to make cheddar cheese. Until to day, pure commercial strains (or strains obtained from a similar source) have been employed in research studies on S. aureus (including isolation methods, thermal death studies, thermal recovery studies, growth studies, and enterotoxin production in food). However, model studies very often fail to work in actual food systems. Therefore, this research investigation was designed to simulate the situation in the dairy industry.

This study was initiated after it was established, through preliminary investigation, that pathogenic strains of S. aureus can be

isolated from heat treated milk. The scope of this investigation, therefore, consists of the following :

(1) To investigate the presence of indigenous S. aureus in raw milk, the percentage of their survivals in heat treated milk, and the possible occurrence of enterotoxigenic strains thereof.

(2) To investigate the growth and enterotoxin production of S. aureus in : (i) raw milk and (ii) heat treated milk. This experiment includes enterotoxigenic S. aureus that survive the subpasteurization heat treatment as well as those from post-heat treatment contamination.

(3) To investigate the growth and enterotoxin production of S. aureus during cheddar cheese manufacture using normal, partial slow, and slow starter culture.

(4) To determine the minimum and maximum levels of S. aureus associated with the presence and/or absence of enterotoxin production.

(5) To determine whether other conditions, such as moiture, salt, and fat content, in addition to acid development, may induce or enhance the production of enterotoxin in cheddar cheese manufacture.

It is also the intent of this study to investigate the feasibility of the solid-phase radioimmunoassay technique for the detection of staphylococcal enterotoxin in cheddar cheese.

MATERIALS

1. Microbiological Media

A. Baird-Parker medium :

a. Baird-Parker basal medium (Oxoid)
b. Egg-yolk tellurite emulsion (Oxoid)

B. Staphylococcus 110 medium (BBL)

C. Standard plate count agar (BBL)

D. Trypticase soy agar (Difco)

E. Trypticase soy broth (Difco)

F. Robertson's meat medium (Oxoid)

G. Brain heart infusion (Difco)

H. Peptone (Difco)

I. Tomato yeast extract medium :

a. Tomato juice agar (Difco)
b. Yeast extract (Difco)

This medium was made up by adding 3 grams of yeast extract to 51 grams of tomato juice agar contained in one litre of distilled water.

J. Skim milk (Difco)

K. Medium for anaerobic mannitol fermentation :

a. Tryptone (Difco)
b. Yeast extract (Difco)
c. Mannitol (BDH)
d. Bromocresol purple (Fisher Scientific Co.)
e. Noble Special agar (Difco)

L. DNase test agar (BBL)

M. Toluidine blue O - deoxyribonucleic acid agar :

a. Tris buffer
b. DNA (Difco)
c. NaCl
d. $CaCl_2$
e. Toluidine blue (Fisher Scientific Co.)
f. Noble Special agar (Difco)

N. 0.2 percent agar for microslide

This medium was made with 2 grams of Noble special agar (Difco in one litre of distilled water.

O. Gel diffusion agar :

a. sodium chloride
b. sodium barbitol
c. merthiolate (1:10,000)
d. Noble special agar (Difco)

P. Rabbit coagulase plasma with EDTA (Difco)

2. Chemicals

All chemicals used throughout this investigation were of analytical grade, unless stated otherwise.

3. Materials for cheesemaking

Raw milk samples, "Hansen" brand cheese starter culture , cheese color, commercial rennet, cheese clothes, and "cryovac" bags of 5-lb capacity were obtained from the Commercial Dairy, University of Manitoba.

4. Reagents and Materials for the detection of enterotoxins

A. Anti-enterotoxin A (Health Protection Branch, Ottawa)

B. Anti-enterotoxin B (Makor Chemicals, POB 6570, Jerusalem, Israel)

C. Anti-enterotoxin C (Health Protection Branch, Ottawa)

D. Enterotoxin A (Health Protection Branch, Ottawa ; and also from Serva Feinbiochemica, distributed in Canada by Terochem Laboratory Ltd., P. O. Box 8188, Edmonton, Alberta.)

E. Enterotoxin B (Makor Chemicals, POB 6570, Jerusalem, Israel)

F. Enterotoxin C (Health Protection Branch, Ottawa)

G. Labelled enterotoxins (Health Protection Branch, Ottawa)

H. Enterotoxin special solvent (Serva Feinbiochemica)

All the above stocks were kept frozen upon arrival until used. Before usage, stock preparations were diluted in accordance with the specific directions of the supplier of each shipment.

I. 0.1 M carbonate-bicarbonate buffer at pH 9.6 (Catt and Tregear, 1967).

J. Ceska's incubation buffer at pH 7.4 (Ceska et al., 1970) containing 1% bovine serum albumin.

K. Ion-exchange elution buffers (Reiser et al., 1974) :

a. 0.015 M sodium phosphate buffer containing 0.09% sodium chloride at pH 5.9 .

b. 0.15 M dibasic sodium phosphate buffer containing 0.9% sodium chloride at pH 6.8 .

L. Ion-exchange resin CG-50 (Serva Feinbiochemica)

M. Bovine serum albumin - COHN fraction 5, Sigma company.

N. Microslide templates (Serva Feinbiochemica)

O. 3M electrician tape (3M Canada Limited)

P. Logit-log graph paper was obtained from Robel Research Laboratories Limited, 2720 Howe Street, Ottawa.

5. The Anderson-Jacobson terminal (a typewriter controlled by computer) was employed for printing out the computer programs.

GENERAL METHODS

1. Serial Dilution

Appropriate serial dilutions were made by aseptically transferring a 1 ml sample into 9 ml of sterile 0.1% peptone water or 0.85% saline at each dilution.

2. Surface Plating

Surface plating was performed by spreading an appropriate dilution of the inoculum (or homogenate) over the surface of pre-poured and pre-dried agar medium according to Standard Methods (1960). Plating was done by spreading 0.1 ml of an inoculum onto a 9 cm petri-dish. Whenever a low number of bacteria is expected, plating was performed with a 1 ml aliquot onto a 24 cm petri-dish.

3. Pour-plating

Pour-plating was performed by aseptically transferring 1 ml of an inoculum into a sterile petri-dish and then the medium was poured as outlined in Standard Methods (1960).

All plating methods were performed as described above, unless stated otherwise.

CHAPTER I

Preliminary Studies : The Selection of a Suitable Medium for the Enumeration of Thermally Stressed Staphylococcus aureus in Heat Treated Milk.

INTRODUCTION

Recognition of sublethally impaired micro-organisms is essential to practical interpretations of data in such areas of food microbiology as food safety, food manufacture, food preservation and spoilage. Various treatments in food processing such as heat, cold, thawing, freeze-drying, dehydration, irradiation, and exposure to sanitizers or preservatives may induce sublethal damage in bacterial cells or spores. Cells are classified as injured rather than dead when they are damaged, but have the capability to function in an unrestrictive environment and restore a normal physiological state concomitant with initiation of growth and cell division. Therefore, contamination of foods with sublethally damaged cells that are capable of repairing the injury should be anticipated. From the literature reviewed in the previous section, several points on thermally stressed cells of S. aureus can be established:

(i) Relatively low temperatures, such as found in the pasteurization and subpasteurization of milk, can injure S. aureus.

(ii) Injured cells often are identified by their inability to proliferate under specific previously productive conditions. This inability can be demonstrated by lack of colony formation on solid minimal media.

(iii) During injury, numerous cellular changes have been implicated and related to an extended lag phase, resulting in delayed growth and inability to multiply. This prolonged lag phase may be minimized by medium adjuncts that compensate for modifications in nutritional requirements of injured cells.

Historically, several investigators (Stiles and Witter, 1965; Baird-Parker, 1972; Gray et al., 1974; Stiles and Clark, 1974) have identified certain media and procedures as superior for growth and recovery of thermally stressed cells of S. aureus. Yet, in all these experiments, pure bacterial cultures were studied, and cells were heated in a standard buffer system. Growth inhibition of thermally stressed S. aureus by salt-containing media has also been reported (Busta and Jezeski, 1963; Stiles and Clark, 1974; Collins-Thompson et al., 1974). This inhibition affected various S. aureus strains to different extents (Niskanen and Aalto, 1978). Therefore, comparative studies on the selectivity of media employing pure cultures of S. aureus are of limited value for the identification of thermally stressed S. aureus in mixed populations, such as are to be expected in food. Furthermore, many procedures may be adequate for enumerating normal S. aureus cells in food, but fail to quantitate thermally stressed cells in processed food. Procedures and media that lead to improper detection, enumeration, and identification of thermally stressed cells in mixed populations should, therefore, be adjusted so that accurate assessments of actual situations in food processing can be made. Hence, the purpose of this phase of the investigation was to select a medium and procedures suitable for the isolation and enumeration of S. aureus cells recovered from the subpasteurization heat treatment of milk.

EXPERIMENTAL METHODS

Five hundred to 700 ml of raw milk sample, from the University dairy herd, were collected in a sterile 1000 ml Erlenmeyer flask. Milk

samples were thoroughly mixed using a sterile magnetic stirrer. Milk samples were pour-plated either directly, or after serial dilution in sterile 0.1% peptone water, with standard plate count (SPC) agar, Baird-Parker (BP) medium, and Staphylococcus 110 (S110) medium. The bacterial counts on these media served as an index of the indigenous microflora and S. aureus in raw milk.

1. Procedures for Heat Treatment

The heating procedures for enumerating thermoduric bacteria were adopted from the Standard Methods for the Examination of Dairy Products (1960). Seven ml of a raw, whole milk sample were transferred aseptically into a 10 x 128 mm sterilized stoppered stainless steel test tube (test tubes used in high temperature short time studies). An assembly of ten test tubes was lowered into a water bath (Magni-Whirl, Blue M Electric, Illinois), thermostatically controlled at 61°C (142°F). A control test tube containing 7 ml of milk with an inserted thermometer (G. H. Zeal, England) was also included. The holding period was measured when the temperature of the milk in the control test tube reached 61°C. After holding for 18 seconds at this temperature, the test tubes were immediately immersed in an ice-water bath and cooled to 10°C.

2. Determination of the Number of Survivals

After cooling, tubes were thoroughly mixed with a vortex mixer. The tubes were then handled in the following manner to determine the number of survivals:

A. In order to determine whether BP and/or S110 medium was suitable for recovering sublethally heat stressed cells of

S. aureus, 1 ml from each of any five tubes was pour-plated, either directly or after serial dilutions in sterile 0.1% peptone water, with each of the following media: (i) trypticase soy agar (TSA), (ii) BP medium, (iii) S110 medium, and (iv) SPC agar. Colonies on SPC agar and TSA served as an index for the total bacterial count before and after the heat treatment, respectively.

B. Also, in order to determine whether enrichment broths were necessary for the recovery of thermally stressed cells of S. aureus, 1 ml of each of the remaining five tubes was added to 9 ml of various sterile enrichment broths, and subsequently incubated at 37°C for 24 hours. After incubation, the tube contents were mixed using a vortex mixer. A 1 ml sample from each tube, or its appropriate dilution, was pour-plated with each of the following media: TSA, BP medium, and S110 medium. The enrichment broths employed in this study were: trypticase soy broth (TSB), 5% glucose solution (GLU), Robertson's meat medium (RMM), and Robertson's meat medium with 7.5% salt (RMMS).

3. Plating Procedures

Pour-plating technique was employed using the various media. Plates were incubated at 37°C for 24-48 hours. Plates with S110 medium were further incubated for another 24 hours at room temperature to promote pigmentation of the S. aureus colonies. All platings were done in duplicate.

4. Identification of S. aureus and Other Micro-organisms

S. aureus colonies appeared round, opaque, flat, smooth, golden yellow to orange with an entire margin and raised edge on S110 medium. On BP medium, S. aureus appeared as circular, convex, smooth, moist, shiny black colonies, 1.0-1.5 mm in diameter, frequently with narrow off-white margins, and (i) with or without a clear zone extending into the opaque medium or (ii) surrounded by a clear zone with an inner opaque zone of precipitation. One to five colonies with the above characteristics were picked and examined for coagulase activity according to the procedures given by Thatcher and Clark (1968). Any degree of plasma coagulation was taken as evidence of coagulase activity (Section 40.036 AOAC, 1975). Coagulase positive colonies were considered as S. aureus.

Bacterial species, other than S. aureus, which survived the heat treatment, were partially identified by means of their morphological, biochemical, and physiological characteristics as described by Thatcher and Clark (1968), and Burrows (1965), as well as in the Bergey's Manual of Determinative Bacteriology (1974).

RESULTS

In the early phase of this investigation it was found that indigenous levels of S. aureus in the raw milk samples were very low. Since heat treatment of raw milk would further reduce the S. aureus population, it was necessary to choose a medium capable of detecting extremely small numbers of S. aureus survivals.

1. Heat Treatment of Raw Milk

Early investigations indicated that a coming-up time of two minutes was required to reach the water bath temperature (61°C) from room temperature. It was found that if the water bath was thermostatically controlled at 62°C or higher, the coming-up time of the heat treatment was reduced. However, the holding period of the heat treatment was in some cases reduced to less than 18 seconds. Therefore, in order to maintain a constant temperature of 61°C throughout the holding period, the water bath temperature was set at 61° $\pm$ 0.5°C. In order to achieve a shorter coming-up time, test tubes were tempered to 45°C in water before inserting them into the water bath. Using this approach, the coming-up time was decreased from 2 minutes to 55-60 seconds.

2. Microbial Examinations of Heat Treated Milk

Preliminary investigations comparing various direct plating media for the detection and isolation of S. aureus following heat treatment are given in Table 1. The results are summarized as follows:

(i) Prior to subpasteurization heat treatment, total bacterial counts per ml were relatively the same on SPC agar and TSA.

(ii) The total bacterial counts per ml observed on BP medium and S110 medium were relatively the same; however, both these media showed smaller viable counts as compared to SPC agar and TSA.

(iii) After subpasteurization heat treatment, similar viable counts were observed on SPC agar, TSA, and BP medium. Staphylococcus 110 medium showed minimal growth.

(iv) Microscopic examination, Gram stain, and coagulase activity indicated that the total viable counts observed on BP medium after

TABLE 1. Comparison of Various Plating Media for the Isolation of *Staphylococcus aureus*

Plating Media	Milk Sample Number	#TOTAL BACTERIAL COUNT / ml Prior Heat Treatment*	After Heat Treatment*
SPC	1	1.20×10^3	0.7×10^1
	2	1.35×10^3	1.5×10^1
	3	3.70×10^2	1.2×10^1
	4	1.80×10^5	4.5×10^1
BP	1	9.60×10^1	0.5×10^1
	2	1.02×10^2	1.5×10^1
	3	9.80×10^1	1.0×10^1
	4	4.80×10^2	2.0×10^1
S110	1	8.0×10^1	0
	2	8.4×10^1	0
	3	8.2×10^1	0
	4	4.0×10^2	1
TSA	1	1.20×10^3	1.1×10^1
	2	1.40×10^3	1.5×10^1
	3	4.80×10^2	1.4×10^1
	4	1.82×10^5	4.5×10^1

: average plate counts from 5 tubes with duplicate plating
* : sub-pasteurization heat-treatment was carried out at 61°C for 18 seconds
SPC : Standard Plate Count agar
BP : Baird-Parker medium
S110: Staphylococcus 110 medium
TSA : Trypticase Soy agar

subpasteurization were Gram positive cocci, exhibiting a coagulase activity greater than or equal to 3+.

(v) Although minimal viable counts were observed on S110 medium after subpasteurization, they nevertheless proved to be Gram positive cocci with a coagulase activity of 3+ or more.

(vi) Microscopic examination and biochemical tests (including coagulase test) for micro-organisms recovered on SPC agar and TSA after sub-pasteurization, indicated that Gram positive rods, Gram positive spore-formers, and Gram positive cocci were present. These organisms were partially identified as E. coli, Bacillus, and Micrococcus, respectively.

Preliminary studies performed on the various enrichment broths and plating media ascertained the following observations (Table 2):

(i) All the four enrichment broths were effective in the recovery of heat stressed (61 C°for 18 seconds) bacteria. Trypticase soy broth, however, showed the greatest recovery.

(ii) All the four enrichment broths serially plated on TSA, BP and S110 medium proved to be non-selective for the isolation of S. aureus.

(iii) Bacterial colonies isolated from BP medium serially diluted from RMM, RMMS, GLU, and TSB were shown to be Gram positive cocci. Many of these colonies, however, were shown to be coagulase negative. Gram positive and negative rods were not observed among the colonies tested.

(iv) Bacterial growth on TSA and S110 medium indicated profuse growth of Gram positive and Gram negative rods. Some of these bacteria were partially identified as Bacillus and E. coli.

TABLE 2. Comparison of Various Enrichment Broths and Plating Media for the Isolation of S. aureus

Plating media	Milk Sample Number	*BACTERIAL COUNT PER ml (X 10^2) Enrichment Broths RMM	RMMS	GLU	TSB
Baird-Parker medium	1	2	22	9	TNTC
	2	72	TNTC	83	TNTC
	3	52	77	70	TNTC
	4	90	TNTC	TNTC	TNTC
Trypticase Soy agar	1	TNTC	TNTC	TNTC	TNTC
	2	TNTC	TNTC	TNTC	TNTC
	3	154	TNTC	TNTC	TNTC
	4	TNTC	TNTC	TNTC	TNTC
Staphylococcus 110 medium	1	TNTC	40	78	TNTC
	2	TNTC	TNTC	TNTC	TNTC
	3	120	128	298	TNTC
	4	TNTC	TNTC	TNTC	TNTC

* : average plate counts from 5 tubes with duplicate plating

RMM : Robertson's Meat Medium

RMMS : Robertson's Meat Medium with 7.5% salt

GLU : 5% glucose solution

TSB : Trypticase Soy Broth

DISCUSSION

Results in this phase of the investigation indicated that thermally stressed cells of S. aureus were unable to recover in S110 medium. Baird-Parker medium, however, showed good recovery of thermally stressed S. aureus from a mixed microbial flora. These results agree with earlier reports in which: (i) S110 medium, due to its high sodium chloride concentration, was found to hinder the growth of sublethally stressed staphylococci (Busta and Jezeski, 1961, 1963; De Warrt et al., 1968; Collins-Thompson et al., 1974); and (ii) BP medium proved to be useful for the recovery of heat damaged cells of S. aureus (Baird-Parker, 1972; Stiles and Clark, 1974; Gray et al., 1974). Although the total bacterial counts observed on TSA were higher than those observed on BP medium, the growth of S. aureus per se on BP medium was higher than that observed on the TSA. This result was expected since TSA is a non-selective medium and most bacteria surviving the heat treatment were able to grow on it. Gray et al. (1974) also reported that growth of S. aureus on BP medium was consistently higher than that observed on TSA.

The second part of the preliminary study was to determine the effect of various enrichment broths, in combination with different plating media, on the recovery of the heat treated cells of S. aureus. As has been cited in the literature review, RMM has been reported by Maitland and Martyn (1948) to diminish the inhibitory effect of salt on staphylococcal growth. However, the effect of this medium on the recovery of thermally stressed cells of S. aureus has not been studied. Heat injured cells of S. aureus MF31 recovered in the presence of 5% glucose or galactose added to the heating menstruum (Stiles and Witter,

1965). Working with the same bacterial strain, Gray et al. (1974) demonstrated full recovery of growth after a four-hour incubation period in TSB. However, in both cases, pure bacterial cultures were studied, in contrast to natural milk microflora encountered in this investigation. All four enrichment broths were found to be effective in recovering bacterial cells surviving heat treatment of 61°C for 18 seconds. However, these media were not selective for S. aureus. Subpasteurization heat treatment at 61°C for 18 seconds is not severe enough to eliminate all the pathogenic bacteria in milk. Hence, pathogens other than S. aureus surviving such a heat treatment may have a repressive effect on the competitive growth of staphylococci. This is, perhaps, the reason for extensive growth of other bacteria, such as E. coli, Bacillus and Micrococcus species (these species were partially identified as outlined in the methodology) observed on the various plating media after incubation in enrichment broths. Several investigators (Peterson et al., 1962b; Donnelly et al., 1968; Jones et al., 1957) have reported growth inhibition of S. aureus due to high numbers of competing organisms.

With BP medium, plates were overcrowded with coagulase negative staphylococci even at the 10^{-2} dilution. This effect would be inconvenient for the isolation of coagulase positive S. aureus colonies when working with numerous milk aliquots. Moreover, to serve the purpose of enumerating heat survivals, the incubation period for recovery in growth before the initiation of cell multiplication has yet to be studied, and such a period might be different for different strains of S. aureus.

From the experimental results obtained, it appears that direct plating with BP medium is most suitable for the isolation and enumeration of S. aureus cells in heat treated milk. Results obtained in this

investigation should apply to a subpasteurization heat treatment of 61°C for 18 seconds. Whether similar responses would be observed with other heat treatment temperatures needs to be verified. Since enrichment broths offered no great advantage in the isolation of S. aureus from heat treated milk, the use of enrichment broths in future studies was omitted. Direct plating with BP medium was adopted for further studies. This part of the investigation was performed in the early summer of 1974. In the following year, 1975, direct plating with BP medium was adopted as the AOAC Official First Action Method for the enumeration of S. aureus in thermally processed food (Baer et al., 1975).

CHAPTER II

TITLE : A Ten-month Survey on *Staphylococcus aureus* Survivals in Heat Treated Milk.

INTRODUCTION

Within the past three decades, there have been many outbreaks of staphylococcal food-poisoning attributed to milk and milk products, as reviewed by Minor and Marth (1973). Several investigators have isolated enterotoxigenic strains of S. aureus from the bovine udder (Bell and Veliz, 1952; Olson et al., 1970), as well as from raw milk supplies (Casman, 1965). Other workers (Warren and Arends, 1967; Geiges, 1972) demonstrated that staphylococci could be transmitted between cows via milking machines. Therefore, the presence of S. aureus in milk and their ability to produce enterotoxins in manufactured products have been duly recognized.

Raw milk is usually pasteurized or subpasteurized before it is used for manufacturing purposes. A subpasteurization heat treatment of 60-61°C (140-142°F) for 16-18 seconds is currently employed by the Canadian dairy industry to obtain a better flavored cheddar cheese. However, subpasteurization temperatures are not sufficient to eliminate all pathogenic bacteria in milk. Cheddar cheese made from subpasteurized milk was found to contain staphylococcal enterotoxin (Duitschaever and Irvine, 1971). In outbreaks of staphylococcal food-poisoning attributed to milk products, enterotoxin is believed to have been formed in the milk prior to conversion to the manufactured product. Therefore, knowledge of the incidence of enterotoxigenic strains in raw milk and their chance of surviving subpasteurization heat treatment becomes critical.

Studies on the thermal death time of S. aureus have been performed by many workers (Angelotti et al., 1961; Busta and Jezeski, 1961; Singh, 1964; Walker and Harmon, 1965, 1966; Bhatt and Bennett, 1964), yet

little information is available on the percentage of S. aureus survivals in subpasteurized milk. Working with samples from one of the Minnesota cheese plants, Zottola (1964) found the percentage of S. aureus survivals in heat treated (63.8°C-66.5°C for 14-23 seconds) milk to be 0-17%. The percentage of survivals was calculated as the percent of samples showing S. aureus out of a total number of heated milk samples. No information is available from Canadian sources.

The purpose of this phase of the investigation was to obtain necessary information on:

(i) the indigenous counts of S. aureus in raw milk;

(ii) the percentage of S. aureus survivals in heat treated milk;

(iii) the correlation between (i) and (ii); and

(iv) the possible occurrence of enterotoxigenic strains of S. aureus in milk subsequent to a subpasteurization heat treatment of 61°C for 18 seconds.

EXPERIMENTAL METHODS

1. Source and Handling of Milk

Raw, whole milk samples were obtained from the Commercial Dairy, University of Manitoba, over a period of ten months so as to include cold and warm months. Twenty experimental trials were included in this survey. In every trial, milk samples were collected from the receiving line in two different batches at different times. Milk samples were pooled, mixed aseptically, and then heat treated at 61°C for 18 seconds. The method of heat treatment employed in this phase of the

investigation was essentially the same as that used in the previous chapter. An assembly of ten test tubes, plus one control test tube, was employed.

2. Plating Procedures

Milk samples in test tubes were thoroughly mixed with a vortex mixer and were then enumerated for S. aureus before and after the heat treatment using a pour-plate technique with BP medium. All platings were done in duplicate. Plates were incubated at 37°C for 24-48 hours before examination.

3. Isolation and Identification of S. aureus

A. Coagulase Test. The coagulase test was performed by adding approximately 0.1 ml of a brain heart infusion broth culture (Baird-Parker, 1969) to 0.3 ml of Difco rabbit plasma with EDTA. Tubes were incubated at 35-37°C and examined at intervals of 2, 4, 6, and 24 hours. Clot formation was rated 1+ through 4+ according to Thatcher and Clark (1968), and a 5+ score was assigned to a 4+ reaction when the clot was not displaced when the tube is inverted. Cultures yielding 2+ (or less) reactions were retested twice for coagulase production.

B. Identification Procedures. After incubation for 24 hours, typical S. aureus colonies were picked from BP medium and tested for catalase (Burrows, 1965) and coagulase production. Any degree of clot formation was taken as evidence of coagulase activity. The same petri-plates were further incubated for another 24 hours, and every additional typical colony was also tested for catalase and coagulase

production. With these additional colonies, clots of 3+ (or more) were required for the coagulase test to be conclusive.

Coagulase positive colonies were further examined microscopically by the Gram stain method (Burrows, 1965). Isolates, which were Gram positive cocci, catalase positive, and coagulase positive, were identified as S. aureus. S. aureus isolates were then maintained on TSA slants.

4. Calculation for the Percentage of Survivals

The total number of S. aureus isolated from heat treated milk is, therefore, the number of typical colonies on BP medium at 24 hour plus those positive colonies at 48 hour. The percentage of survivals was calculated as follows:

$$\% \text{ of survivals} = \frac{\text{number of S. aureus after heat treatment}}{\text{number of S. aureus before heat treatment}} \times 100$$

5. Statistical Analysis of the Survey

Data obtained on the indigenous levels of S. aureus in raw milk samples (X_i) and the percentage of survivals in heat treated milk (Y_i) were statistically analyzed for their correlation relationship. Since the bivariate population is not normally distributed, the Spearman's rank correlation method for non-parametric studies was adopted for the calculation of the correlation coefficient between the two variables (X_i, Y_i). The Spearman's rank correlation coefficient (r_s) was calculated as follows (Kendall, 1955; Snedecor and Cochran, 1972):

$$r_s = \frac{1 - 6 \sum (X_i - Y_i)^2}{n (n^2 - 1)}$$

where n = number of pairs of X_i, Y_i.

Data obtained in the cold months (below 0°C) and those obtained in the warmer months (above 0°C) were analyzed individually for their correlation coefficient. An overall correlation coefficient was also calculated. In each case, the correlation coefficient was tested for its significance by comparing the calculated "r_s" values with the "r_{n-2}" values given in statistical tables (Snedecor and Cochran, 1972) at the 5% and 1% significance levels.

RESULTS

Twenty samplings were performed over a period of ten months, from October 30, 1974 to August 27, 1975. Among these samplings, nine were drawn at temperatures below 0°C and eleven were drawn at temperatures above 0°C. Throughout the entire survey, total bacterial population (i.e. SPC) in raw and heat treated milk ranged from 3.7×10^2 to 1.8×10^5, and 2.3×10^1 to 4.5×10^2 cells per ml, respectively.

1. Percentage of S. aureus Survivals

The data of the survey on S. aureus in milk are presented in Table 3. During this ten-month period, the population levels of S. aureus in raw milk samples were found to be relatively low as compared to those found in commercial dairies. Approximately 50% of the raw milk counts were below 100 cells per ml, and all samples were below 1000 cells per ml.

TABLE 3. The Percentage of Survivals in Survey Heat treated Milk[#]

Date of Sampling	Mean Outdoor Temperature (°C)	*S. aureus Count per ml		% Survival
		Raw milk	Heated Milk	
October 30, 1974	2.75	150	20	13.33
November13, 1974	-3.30	49	15	30.61
November27, 1974	-7.80	60	12	20.00
December 4, 1974	-5.00	30	3	10.00
January 6, 1975	-7.70	75	0	0
January 20, 1975	-12.00	19	2	10.53
February 5, 1975	-22.00	12	2	16.66
February19, 1975	-7.90	17	0	0
March 5, 1975	-7.00	48	4	8.34
March 19, 1975	-5.60	17	3	17.65
April 9, 1975	2.60	160	15	9.38
April 30, 1975	2.20	260	10	3.85
May 14, 1975	5.30	270	20	7.41
May 28, 1975	13.40	365	25	6.85
June 4, 1975	12.20	900	140	15.55
June 18, 1975	16.60	40	15	37.50
July 2, 1975	20.00	525	50	9.52
July 16, 1975	27.10	190	40	21.05
August 13, 1975	17.90	390	27	6.92
August 27, 1975	11.30	85	10	11.76

* : average plate counts from 10 HTST test tubes

: heat treated at 61°C for 18 seconds

The highest count found was 900 per ml of raw milk. None of the raw milk samples was totally free of S. aureus. Indigenous levels of S. aureus in raw milk samples were usually found to be higher in the warmer months with temperatures above 0°C than those in the colder months with temperatures below 0°C.

A total of 2870 S. aureus survivals were isolated from heat treated milk during the ten-month survey, 124 of which were found to coagulate rabbit plasma with a definite clot of 3+ or more. Results of the coagulase reaction on the 124 S. aureus isolates are presented in Appendix Table 1. The remaining isolates produced a 2+/3+ clot, and were identified as S. aureus because they were isolated at 24 hour of incubation on BP medium. It is known (Baird-Parker, 1969) that certain strains of S. epidermis may also grow on the medium forming black colonies and causing clearing of egg yolk. At 24 hour, however, colonies of such strains are usually distinguishable from S. aureus by a very wide opaque zone around them which in turn is surrounded by a very narrow clear zone. At 48 hour, S. aureus may give a similar appearance. For this reason, all late egg-yolk clearers should be classified as S. aureus by a conclusive coagulase reaction with a clot at the level of 3+ or more.

S. aureus survivals in heat treated milk ranged from 0 to 140 cells per ml. Therefore, a subpasteurization heat treatment of 61°C for 18 seconds was not sufficient to eliminate all the S. aureus cells indigenous to raw milk. The percentage of survivals was calculated to be 0% to 37.5%.

2. Statistical Analysis of the Survey Data

Statistical analysis on the numbers of S. aureus in raw milk

and the percentage of survivals in heat treated milk is presented in Appendix Tables 2 and 3, and is summarized as follows:

Temperatures	n	$\Sigma(X - Y)^2$	r_s	r_{n-2} 5%	r_{n-2} 1%
below 0°C	9	117.5	0.0208	0.666	0.798
above 0°C	11	302.0	-0.3727	0.602	0.735
overall	20	1667.5	-0.2537	0.444	0.575

In the rankings of the survey data, a Spearman's rank correlation coefficient of 0.0208 with 9 pairs of observations was obtained for the colder months (below 0°C); whereas a coefficient of -0.3727 with 11 pairs of observations was obtained for the warmer months (above 0°C). An overall correlation coefficient of -0.2537 was obtained for the ten-month survey. Therefore, in all cases studied, there is no significant correlation between the two variables.

DISCUSSION

According to the microbiological standards in Canadian food and drug regulation, the limit set for aerobic colony count in milk for manufacturing purposes is $1 \times 10^6 - 2 \times 10^6$ cells per ml (Pivnick, 1978). Therefore, the SPC of raw milk in this ten-month survey are within the normal range encountered in commercial dairies. Standard plate counts of heat treated milk are much lower than those observed in raw milk, demonstrating that subpasteurization at 61°C for 18 seconds did substantially reduce the number of competing micro-organisms in the milk flora.

According to literature reviewed (Donnelly et al., 1968; Tatini et al., 1971), heat treated milk with a low bacterial count, such as observed in this experiment, is more susceptible to enterotoxin production by S. aureus than raw milk.

Regarding the initial S. aureus counts in raw milk, a marked seasonal effect is evident, higher counts reflecting warmer weather conditions. Indigenous levels of S. aureus in raw milk samples ranged from 17 to 900 cells per ml. The subsequent percentage of survivals ranged from 0 to 37.5%. One might expect that a low indigenous S. aureus level would eventually give a low percentage of survivals, and vice versa. Yet this is not the case according to statistical analysis on the data obtained. A Spearman's rank correlation coefficient of 0.0208, -0.3727, and -0.2537 was obtained for the colder months, the warmer months, and the overall ten months, respectively. Statistical analysis, therefore, revealed:

(i) a lack of direct correlation between the indigenous level of S. aureus and the percentage of survivals throughout the entire survey period; and

(ii) regardless of the weather conditions (i.e. above or below 0°C), this lack of correlation remains.

Results obtained from statistical analysis suggested that a high indigenous population of S. aureus was not an essential requirement for a subsequent high percentage of survivals. Hence, the danger of a potential health hazard may occur even with a fairly small initial population, if the subsequent percentage of S. aureus survivals is high.

Furthermore, the following factors have yet to be considered:

(i) The indigenous levels of S. aureus in the raw milk samples collected

from the University dairy herd (Commercial Dairy of the University of Manitoba) are extremely low in comparison with those of commercial dairies where milk is collected from various farms. Zottola (1964) has found a staphylococcal count of 1000-10,000 cells per ml of milk or more in the raw milk samples collected in four Minnesota cheese plants.

(ii) Employing laboratory techniques, the coming-up time for the test tubes to reach the holding temperature of 61°C was found to be 55-60 seconds, as presented in the previous chapter. In commercial practice, plate pasteurizers are utilized for heat treating raw milk used for cheese manufacture. A coming-up time of approximately ten seconds is required to reach the holding temperature. In general, the entire subpasteurization procedure (i.e. including the coming-up period, the holding period, and the cooling-down period) requires less than a minute (Hall and Trout, 1968). This means the heat treatment employed in this part of the investigation is far more severe than that employed in the commercial processing plants.

(iii) It should be emphasized that these experiments were conducted with 70-ml samples (ten test tubes with 7 ml of milk per tube). In commercial processing involving large volumes, the probability of survival and recovery is increased in proportion to the larger total numbers of S. aureus present.

Despite all the above limitations in this investigation, however, a total of 2870 coagulase positive S. aureus cultures were isolated from the heat treated milk during the ten-month survey. Some of these isolates were found to produce enterotoxins. Therefore, since entero-

toxigenic strains did occur in the heat treated milk during a small laboratory scale study such as this, they are more likely, to a greater extent, to occur in commercial heat treated milk used for cheese manufacture in the dairy industry.

Discussion of coagulase activity and enterotoxin production is presented in the next chapter.

CHAPTER III

TITLE : Characterization Tests on *Staphylococcus aureus* Isolates from the Ten-month Survey.

INTRODUCTION

All current procedures for the detection of S. aureus in foods regard a positive tube coagulase test as the definite identification of the organism (Section 46.040 AOAC, 1975). Additional tests such as enterotoxin production and phage typing are not routinely performed unless the sample is involved in food-poisoning outbreak. Therefore, the interpretation of the coagulase test is important to laboratories monitoring the microbiological quality of foods. The coagulase reactions are rated progressively 1+ through 4+ depending on the extent of clotting of the plasma. Due to the subjective nature of the coagulase test, confusion and uncertainty were reported regarding interpretation of the test (Rayman et al., 1975; Sperber and Tatini, 1975). This controversy was attributed to a disagreement as to the degree of clotting that should be considered as positive evidence of coagulase production. Sperber and Tatini (1975) suggested that cultures yielding 1+ through 3+ reactions warrant additional testing for characters such as anaerobic mannitol fermentation, thermonuclease production, and/or lysostaphin sensitivity before being confirmed as S. aureus. In addition, several factors have been reported which may affect the results of the coagulase reaction, including the type of plasma, the nature of the anticoagulant used, and variation from one lot to another (Orth et al., 1971; Sperber and Tatini, 1975).

The deoxyribonuclease (DNase) test is a routine diagnostic procedure used by some laboratories to distinguish between S. aureus and other Micrococcaceae (Morton and Cohn, 1972). The heat stable deoxyribonuclease (thermonuclease) test was reported recently as a useful confirmatory test for the identification of S. aureus (Sperber and Tatini, 1975),

including nearly all enterotoxigenic strains (Tatini et al., 1976).

The purpose of this phase of the investigation, therefore, was:

(i) to study the extent of clot formation in the coagulase test for the identification of S. aureus;

(ii) to evaluate the validity of the coagulase, DNase, and thermonuclease tests used for the identification of S. aureus;

(iii) to evaluate the validity of other characterization tests such as mannitol fermentation and gelatin hydrolysis for the identification of S. aureus;

(iv) to study the relation of these tests to the production of enterotoxin by S. aureus; and

(v) to choose an enterotoxigenic strain of S. aureus from the survey isolates for the studies on the enterotoxin production of S. aureus in milk and cheddar cheese presented in the later parts of this investigation.

EXPERIMENTAL METHODS

1. Selection of S. aureus Strains

S. aureus isolates, with a coagulase reaction of 3+ or more, were grown on S110 medium. Separate colonies of each isolate were obtained using dilution by streaking on S110 agar medium. Plates were incubated at 37°C for 24-48 hours followed by an additional 24 hours at room temperature for the development of pigmentation.

The differentiation of strains was achieved by determination of the following characteristics: (i) pigmentation, (ii) ability to ferment

mannitol, (iii) capacity for gelatin hydrolysis (Difco Manual, 1966), and (iv) morphology. Selected isolates were streaked onto TSA slants, and incubated for 18-24 hours at 37°C followed by storage at 4°C for further studies.

2. Characterization Tests on S. aureus Strains

S. aureus strains, which were Gram positive, yellow to orange in colour, catalase positive, coagulase positive (having a clot of 3+ or more), showing mannitol fermentation and gelatin hydrolysis, were chosen as test organisms for further studies. From each strain (type), only one representative S. aureus isolate was submitted for further studies.

These 20 representative strains were purified by cloning on BP medium through several successive transfers. Pure cultures were then grown in sterile reconstituted skim milk, heat treated at 61°C for 18 seconds, and recovered on BP medium by surface streaking. They were subsequently stored at 4°C on TSA slants as library samples until submitted for the following characterization tests:

A. Carbohydrate Fermentations. The ability of the strains to ferment glucose and mannitol anaerobically was determined by using the media and procedures outlined in the Recommendations (1965). Anaerobiosis was achieved in GasPak jars (BBL). Results were read after 5 days of incubation at 35°C.

B. DNase Production. The 20 strains were also examined for DNase production using DNase test agar as outlined in the BBL Manual of Products and Laboratory Procedures (1973). Plates were incubated at 35°C for 18-24 hours. DNase activity was indicated by a clear zone around the streak on plates which were flooded with hydrochloric acid.

C. Thermonuclease Production. Thermonuclease activity was determined by the method of Lachica et al. (1971b) using toluidine blue O-deoxyribonucleic acid agar. Approximately 20 ml of the agar were poured into a petri-dish. Ten small wells (3 mm in diameter) were cut into the agar. Overnight brain heart infusion broth cultures were steamed in a water bath for 15 minutes, and one drop was dispensed into an agar well. The detection of thermonuclease activity was also performed by heating the BP plates with grown colonies for 2 hours in a 60-80°C conventional oven and then overlaid with 10 ml molten toluidine blue O-deoxyribonucleic acid agar (Lachica et al., 1971b). Plates were incubated at 35-37°C, and examined after 4 and 24 hours. A thermonuclease producer was identified by a bright pink zone surrounding the colony.

3. Examination for Enterotoxin Production

A. Procedures for Enterotoxin Extraction. The sac culture assembly method of Donnelly et al. (1967) was used to obtain maximal yields of enterotoxins. A 0.85% saline solution was used in place of the phosphate buffer in this method. Several sac culture assemblies after inoculation were incubated on a gyratory shaker (Model G25, New Brunswick) at 37°C for 48 hours. After incubation, the liquid culture surrounding the sac was removed from the flask, and centrifuged at 23,500 x g for 20 minutes in a Sorvall centrifuge (Superspeed RC2-B). The resulting supernatant was frozen with 0.05% merthiolate until examined for enterotoxin.

B. Detection of Enterotoxins. Supernatant samples were tested for the production of enterotoxins A, B, and C by the solid-phase RIA technique in co-operation with the Health Protection Branch in Winnipeg (Appendix 4).

Enterotoxins D, E, and F were not available for similar examination during this research. Positive results were also confirmed by the conventional microslide immunodiffusion method performed in the Health Protection Branch.

C. Interpretation of Results. The logit-log plot devised by Rodbard et al. (1968) was employed. A standard curve of logit y (% trace binding) versus log x (concentration of enterotoxin) was plotted using the method of linear regression. Details of the methodology are presented in Chapter VII.

RESULTS

1. Characterization Tests on S. aureus Isolates

During the ten-month survey, 2870 S. aureus survivals were isolated from heat treated milk and 124 of these were found to coagulate rabbit plasma with a clot of 3+ or more. Results of the tests on coagulase reaction, aerobic mannitol fermentation, and gelatin hydrolysis are presented in Appendix Table 1. The coagulase reactions of 120 strains were completed within 2-4 hours. Four strains required further incubation overnight. These included one strain which produced a 3+ clot, and three strains which produced a 4+ clot. All 124 strains fermented mannitol aerobically and hydrolysed gelatin to a certain extent. Colony characteristics including chromogenesis on S110 medium and TSA slants as well as biochemical reactions to mannitol and gelatin, indicated at least 20 different strains of S. aureus were obtained. S. aureus isolates with similar strain characteristics were thus grouped together as shown in Table 4. From each group of isolates, one S. aureus culture was picked

TABLE 4. Characteristics of the 20 Different Strains of S. aureus#

Isolate Number	Colony Color S110	Color TSA	Mannitol fermentation	Gelatin hydrolysis	Coagulase reaction
2,4,32,65,113,123	O	O	S	+	5+
3,20,33,73,96	O	O	S	K	4+
11,48,75	O	O	+	+	5+
88,100	O	O	K	K	4+
10,22,35,56,66,84	O	O	+	K	3+
29,36,81,97,111,119	O	O	K	+	3+
13*,15,47,74,85,101, 114	O	O	S	S	5+
49,60,87,99,108,112, 116	O	W	K	S	3+
19,45,89,92,94,103, 104,117	O	W	S	S	4+
9,18,55,83	O	Y	S	+	5+
7,44,46,70,98	O	Y	S	K	3+
12,63,82,93,115	O	Y	K	+	4+
23,51	O	Y	K	K	3+
8,24,39,40,45,58,64, 67,76,79	O	Y	S	S	4+
42,50,52,118	O	Y	+	K	3+
6,16,34,57,90	Y	Y	S	+	4+
14,27,43	Y	Y	K	+	3+
25,37,38,80,95,124	Y	Y	K	K	3+
31,54,110,120	Y	Y	+	K	4+
5,26,30,69,107	Y	W	+	K	3+

* : This isolated strain was chosen as the test organism for further studies on the enterotoxin production of S. aureus in the manufacture of cheddar cheese.

Colony color : W = white
Y = pale yellow
O = orange to golden

Mannitol fermentation and gelatin hydrolysis : S = strong reaction
+ = positive reaction
K = very weak reaction

: All strains ferment glucose anaerobically.

(at random) to represent that particular strain. The 20 representatives (representing 20 different strains) were further tested for anaerobic carbohydrate fermentation, DNase activity, and thermonuclease production.

Table 5 shows the isolation number of the strains, the degree of clot formed in the coagulase test, and the relationship among coagulase, DNase, and thermonuclease production. All 20 strains fermented glucose anaerobically. Six strains did not ferment mannitol anaerobically, but were capable of fermenting mannitol aerobically. All 20 strains yielded DNase. One strain failed to produce thermonuclease.

2. Enterotoxin Production by S. aureus Isolates

All 20 strains (representatives) were grown for enterotoxin production, three of which were found to be enterotoxigenic. Two of the three enterotoxigenic strains were found to produce enterotoxin A, and one was found to produce both enterotoxin A and enterotoxin C. Data of the RIA performed on these isolates are presented in Appendices 28 and 29. Results are summarized as follows:

Isolate No. 13 - produces enterotoxin A

Isolate No. 4 - produces enterotoxin A

Isolate No. 7 - produces enterotoxins A and C.

Of the three enterotoxigenic strains tested, two were positive for both DNase and thermonuclease production, and the clotting of the plasma was sufficiently complete to be assigned a 3+ and 5+ rating. The single enterotoxin-A-producing S. aureus strain, Isolate No. 4, which was negative for thermonuclease production, was retested twice for enterotoxin, thermonuclease, and coagulase production with no change in the original

TABLE 5. Characteristic Tests Performed on the 20 Different Strains of *S. aureus*

ISOLATE NUMBER	COLONY COLOR S110	COLONY COLOR TSA	MANNITOL FERMENTATION aerobic	MANNITOL FERMENTATION anaerobic	GELATIN HYDROLYSIS	COAGULASE REACTION	DNase	THERMO-NUCLEASE	ENTERO-TOXINS
4	O	O	S	+	+	5+	+	-	A
20	O	O	S	+	K	4+	+	+	-
75	O	O	+	-	+	5+	+	+	-
100	O	O	K	+	K	4+	+	+	-
22	O	O	+	-	K	3+	+	+	-
36	O	O	K	+	+	3+	+	+	-
13*	O	O	S	+	S	5+	+	+	A
60	O	W	K	-	S	3+	+	+	-
45	O	W	S	+	S	4+	+	+	-
9	O	Y	S	+	+	5+	+	+	-
7	O	Y	S	+	K	3+	+	+	A,C
12	O	Y	K	+	+	4+	+	+	-
51	O	Y	K	-	K	3+	+	+	-
8	O	Y	S	+	S	4+	+	+	-
42	O	Y	+	+	K	3+	+	+	-
6	Y	Y	S	-	+	4+	+	+	-
43	Y	Y	K	+	+	3+	+	+	-
25	Y	Y	K	-	K	3+	+	+	-
31	Y	Y	+	+	K	4+	+	+	-
5	Y	W	+	+	K	3+	+	+	-

Colony color : W = white
Y = yellow
O = orange to golden

Mannitol fermentation : S = strong reaction
Gelatin hydrolysis : + = positive reaction
K = weak reaction

* : this strain was chosen as the test organism for further studies

results. Another S. aureus culture isolate from the same group (i.e. a similar strain), Isolate No. 113, was also tested and found negative for the thermonuclease production.

DISCUSSION

One of the main objectives of this phase of the investigation is to isolate a suitable enterotoxigenic strain of S. aureus for studies on growth and enterotoxin production in milk and cheddar cheese. Coagulase-negative enterotoxin-producing strains of S. aureus have been reported (Thatcher and Simon, 1956; Bergdoll et al., 1967); however, these are rare. For this reason, only those survey isolates yielding coagulase reactions at a level of 3+ or more were employed for this phase of the investigation. Of 2870 S. aureus isolated from the survey, 124 were found to coagulate rabbit plasma with a clot of 3+ or more. Of the 124 S. aureus isolates tested, some had very weak reactions on aerobic mannitol fermentation and/or gelatin hydrolysis. Some of the identified S. aureus isolates (yielding coagulase reactions of 2+) were capable neither of fermenting mannitol nor hydrolyzing gelatin. Therefore, aerobic mannitol fermentation as well as gelatin hydrolysis can only serve as characteristic tests for the differentiation of strains, but not as prime indices in the identification of S. aureus.

Based on pigmentation, coagulase reactions, aerobic mannitol fermentation, and gelatin hydrolysis, twenty different strains of S. aureus were picked for further characterization tests such as anaerobic carbohydrate fermentations, DNase, and thermonuclease production. Six of these strains were repeatedly negative for anaerobic utilization of

mannitol, but were capable of fermenting glucose anaerobically. These strains were identified as S. aureus since they produced thermonuclease and coagulated rabbit plasma with a clot of 3+ or more. Therefore, it does not appear that anaerobic mannitol fermentation is a reliable characteristic for the identification of S. aureus. This result is in agreement with the published data (Sperber and Tatini, 1975).

The coagulase, DNase, and thermonuclease test procedures rely on the ability of S. aureus to produce the respective enzymes. Currently, the tube coagulase test is the most widely used test for identifying S. aureus. With 97% of the 124 isolates, the coagulase reaction was complete within 2-4 hours. Therefore, 4 hours would be an ample incubation time for a coagulase test performed as described in the previous chapter. Occasionally, it might be necessary to continue the incubation overnight so that weak coagulase producers may become more pronounced. Since staphylococci can produce proteases that may dissolve the fibrin clot, the test should be read periodically so that false-negative reactions are avoided. Results obtained in this investigation indicated that S. aureus can be positively identified by the coagulase test alone if the extent of clotting of plasma is at the level of 3+ or more. This is in agreement with the findings of Rayman et al. (1975), but is at variance with those of Sperber and Tatini (1975), who consider only a 4+ clot as positive evidence in the identification of S. aureus. However, owing to the subjective nature of the test, this disagreement probably lies in the actual scoring of the clot formation rather than in the interpretation of the results.

In this study, all of the twenty different strains tested were DNase positive. Yet, DNase activity cannot be used as a reliable index

in identifying S. aureus because it could be produced by some saprophytic strains of S. epidermis (Zarzour and Belle, 1978). Recently, many investigators (Lachica et al., 1971a, 1972; Cords and Tatini, 1973; Chesbro and Auborn, 1967) have demonstrated close correlations between thermonuclease and enterotoxin production. However, in this investigation, one (Isolate No. 4) of the three enterotoxigenic strains failed to produce thermonuclease. Yet, Isolate No. 4 was identified as S. aureus by using other criteria such as coagulase activity, anaerobic mannitol fermentation, and enterotoxin production. Also, Rayman et al. (1975) have reported that one out of 63 enterotoxigenic strains was negative for thermonuclease production. Lachica et al. (1969) have also found that 4 of their 232 coagulase positive enterotoxigenic strains failed to produce thermonuclease. It is questionable, therefore, whether thermonuclease test can be employed as a single prime determinant for identifying S. aureus and enterotoxin production. Consequently, the suggestion of a replacement of the coagulase test by the thermonuclease test in the routine examination of foods for S. aureus would be premature at the present time, especially since enterotoxin-producing strains had been shown to be negative for thermonuclease production in this investigation as well as in others. As cited in the literature, thermonuclease (optimally active at pH 9) was shown to be produced exclusively by S. aureus (Tatini et al., 1976a). Hence, before relegating the coagulase test to a lesser position in the identification of S. aureus, a larger number of S. aureus strains from various sources and a wider range of bacterial strains must be tested for thermonuclease production to determine whether the test is indeed specific for S. aureus.

The thermonuclease test has the following advantages:

(i) It is relatively inexpensive.

(ii) It is rapid and easy to perform.

(iii) It is not influenced by as many factors and variations as the coagulase test. With all the strains tested, the color change occurred within 3-4 hours. There was no color reversion even after 24 hours of incubation.

(iv) It is not subjective in nature because it is operated on a purely qualitative basis.

For S. aureus strains yielding a 2+ clot (or less), thermonuclease test could be used to confirm the identity because it may reduce the possibility of error in the identification of S. aureus and will speed up reporting of the results. Moreover, Tatini et al. (1976a) have demonstrated that thermonuclease was detectable, in every substrate tested, prior to the accumulation of detectable amounts of enterotoxins. Therefore, if detectable amounts of thermonuclease are always produced under the processing conditions of a given system, and prior to the accumulation of detectable amounts of enterotoxin, thermonuclease assay could be a useful tool for screening batches that are likely to contain enterotoxin since testing every batch for the presence of enterotoxins is impractical.

Isolate No. 13 was chosen as the test organism for further study of growth and enterotoxin production in milk and cheddar cheese. This isolate was chosen for two reasons: (i) It grows well within 18-24 hours; and (ii) it produces enterotoxin more readily than any other cultures being examined. The logit-log plot of S. aureus isolate No. 13 is presented in Figure 1. Here, the standard curve (i.e. the linear regression line of the standard enterotoxin) shows that plots of logit y versus

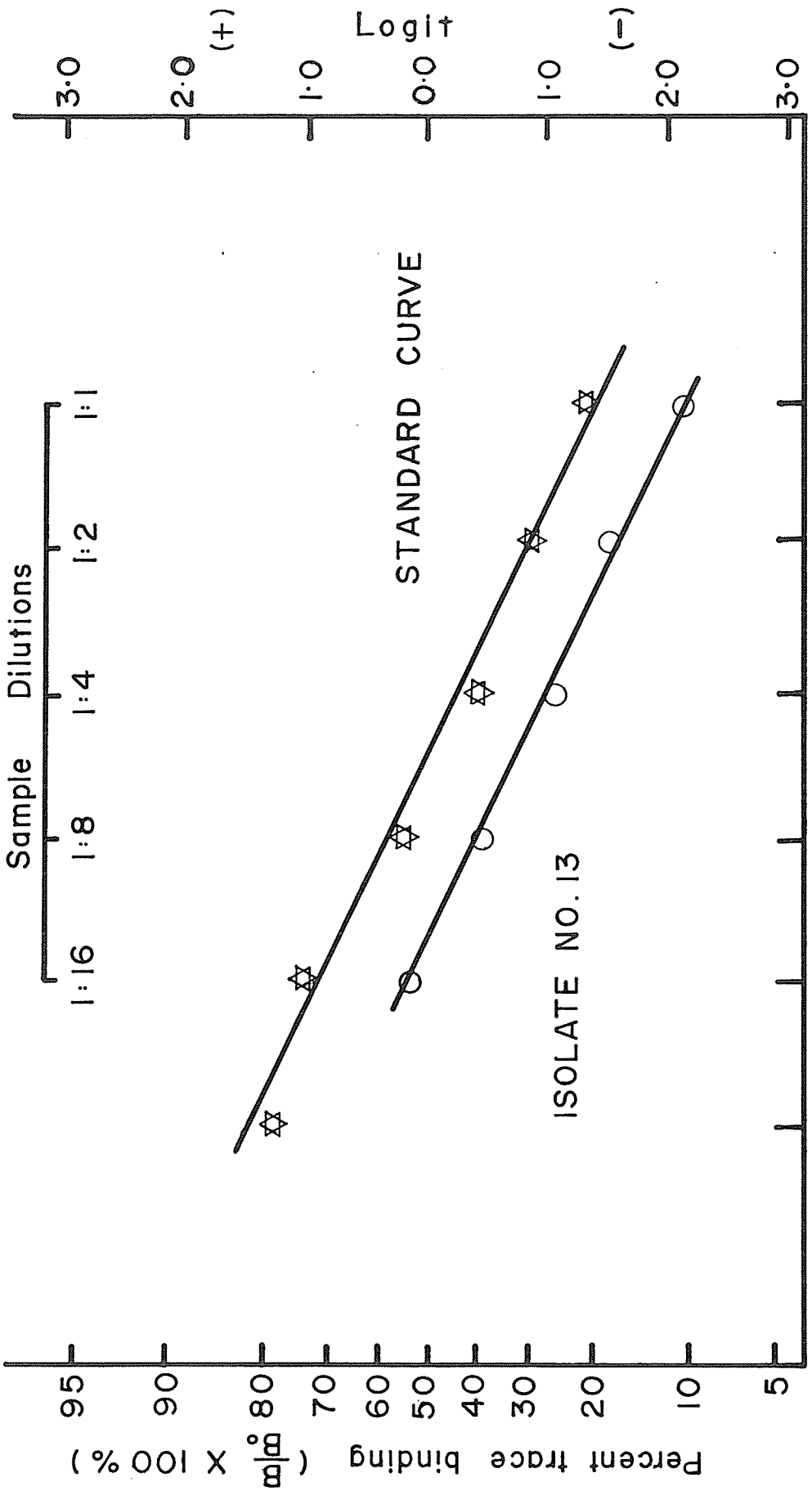


FIG. 1 LOGIT-LOG PLOT OF *S. aureus* ISOLATE NO. 13

log x fitted straight lines over the range of 0.31 to 10 ng for staphylococcal enterotoxin A (SEA). The linear regression line of Isolate No. 13 and that of the standard enterotoxin were parallel, indicating the presence of a protein with immunological identity.

CHAPTER IV

TITLE : Cultivation of *S. aureus* Isolate No. 13 for the Studies of Growth and Enterotoxin Production in Milk and Cheddar Cheese.

EXPERIMENTAL METHODS

1. Selection of S. aureus

Isolate No. 13, which was coagulase positive, DNase positive, thermonuclease positive, fermented mannitol anaerobically, and produced enterotoxin A, was chosen as the test organism for the studies on growth and enterotoxin production of S. aureus in milk and cheddar cheese.

2. Propagation of S. aureus Isolate No. 13

Cells were grown in 200 ml TSB, and incubated at a speed of 200-250 rpm on a gyratory shaker (Model G-25, New Brunswick) at 37°C for 24 hours. They were then centrifuged at 15,000 x g for ten minutes in a Sorvall (Superspeed RC2-B) centrifuge. Harvested cells were washed by two successive re-suspensions of a sterile 0.85% saline solution. The cells were then finally washed with 10 ml of sterile 0.85% saline into a sterilized flask, and gently mixed to provide a homogeneous suspension. A half-milliliter of this suspension was evenly spread over a TSA slant surface aided by sterile glass beads, and was then incubated for 24 hours at 37°C. After incubation, the resulting growth was washed with 10 ml of sterile 0.85% saline, and poured into a sterilized flask. This was kept as stock culture at 4°C until further transfer. Thrice weekly, cells were re-grown on an agar slant containing TSA.

3. Determination of Standard Curve for S. aureus Isolate No. 13

A. Experimental Procedures. Quantitative inoculation of heat treated milk with specific amounts of S. aureus for cheesemaking requires a methodology for obtaining different cell densities. Turbidity measure-

ments of bacterial suspensions were used as an index of cell densities. Turbidity was determined by measuring the transmittance of appropriately diluted suspensions of S. aureus using a Unicam SP 600 Series 2 spectrophotometer. Aliquots from a freshly prepared suspension of stock culture containing S. aureus were suitably diluted in 0.85% saline, and transmittance readings were then recorded for each dilution with saline as the blank. A wavelength of 340 nm was used for transmittance reading (Appendix Table 6). Transmittance measurements were performed in duplicate on 5-6 samples, containing 0.1-1.6 ml of S. aureus stock culture in 50 ml of 0.85% saline. Standard plate counts were performed for each transmittance reading using a serial dilution technique followed by surface plating each dilution on BP medium. All plating was done in duplicate. Plates were incubated for 24 hours at 37°C, and then read.

B. Standard Curve. A standard curve relating the percentage of transmittance versus logarithmic viable cell count was set up. To eliminate the considerable error as a result of subjective positioning of the line through the data points, construction of the linear function directly from the experimental data using the least-squares method was therefore adopted (Snedecor and Cochran, 1972).

C. Statistical Analysis. Results of the standard curve were examined by means of analysis of variance. Reliability of prediction of the Y_i values (logarithmic cell counts) on the X_i values (percentage of transmittance) was tested for significance by (i) comparison of the calculated 'F' value with the critical 'F' value in statistical tables, and (ii) the percentage of coefficient of determination (Snedecor and Cochran, 1972).

RESULTS

1. Standard Curve for S. aureus Isolate No. 13

A standard curve relating viable S. aureus growth and percent of transmittance is presented in Figure 2. Data of the standard curve are presented in Appendix Table 7. The standard curve presents the least-squared, best-fit regression line of the logarithmic cell count (Y-axis) versus the percent of transmittance (X-axis). Transmittance measurements varied within a 0-2.3% range from their average values. According to the standard curve, a 46% transmittance reading corresponds to a concentration of 10^8 cells per ml of the S. aureus stock suspension.

2. Statistical Analysis of the Standard Curve

Statistical analysis (Appendix Table 8) performed on the data used for the regression line revealed that:

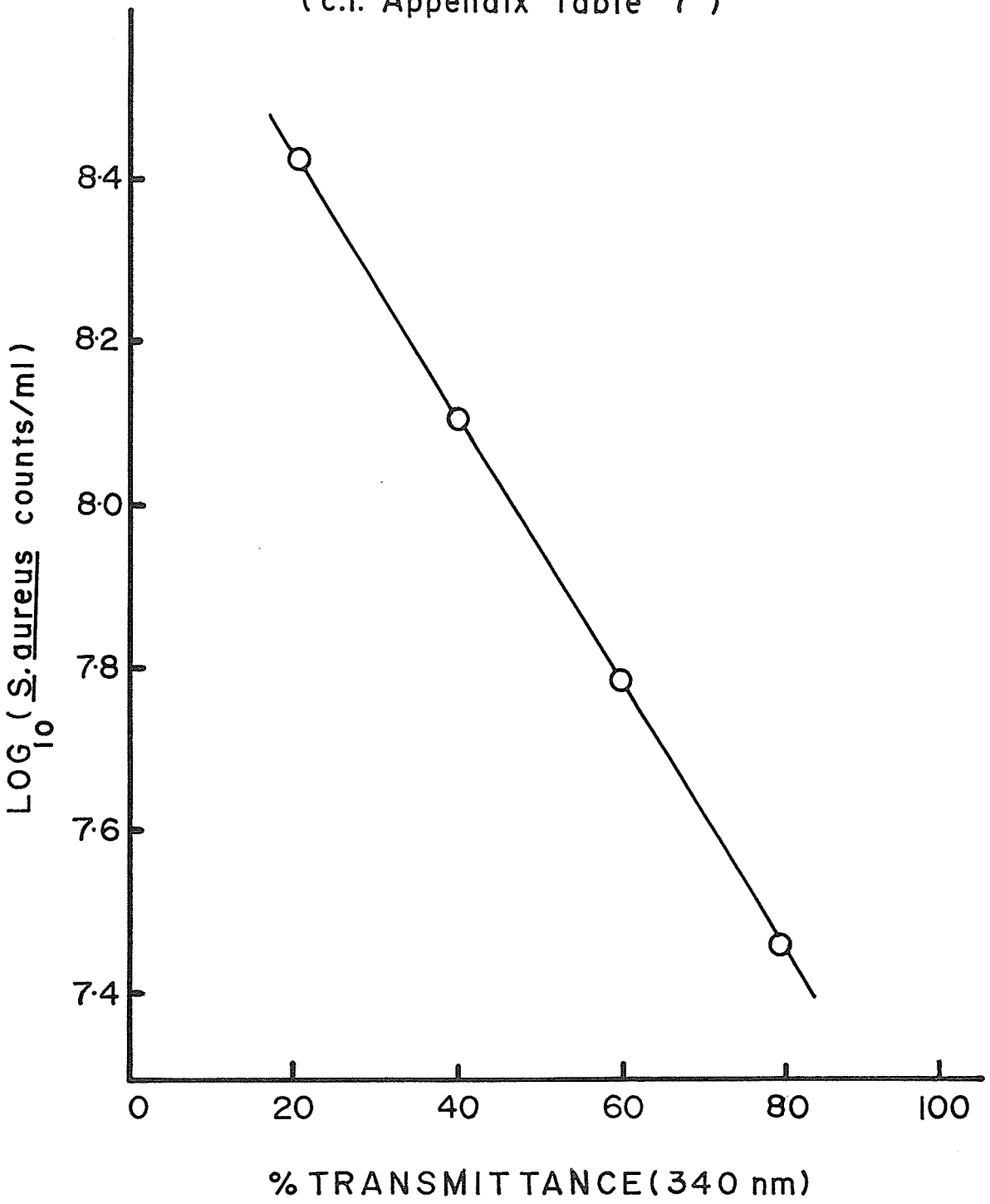
(i) The regression of Y_i on X_i was significant at the 1% level as well as the 0.5% level.

(ii) A 92% of the total sum of squares of variation was explained by the regression of Y_i on the X_i variable, leaving only a very small amount of deviation unexplained.

(iii) Such a high level of significance as presented in this case, therefore, indicated that transmittance measurements offered reliable measurements of cell numbers under the test conditions specified.

FIG. 2 STANDARD CURVE OF *S. aureus*

(c.f. Appendix Table 7)



CHAPTER V

TITLE : Growth and Enterotoxin Production of *S. aureus* Isolate No. 13 in Milk Used for the Cheddar Cheese Manufacture.

INTRODUCTION

Though fluid milk had been implicated in outbreaks of staphylococcal food-poisoning (Dauer, 1961), most large-scale outbreaks ascribed to dairy products were attributed to cheese (Hendricks et al., 1959; Zehren and Zehren, 1968; Miller, 1974; Caserio et al., 1975) and dried milk (Anderson and Stone, 1955; Armijo et al., 1957). Poor growth of S. aureus among other competing micro-organisms in the mixed flora (Casman et al., 1963; Reiter et al., 1964) and the low incidence of enterotoxigenic S. aureus in the dairy environment (Casman et al., 1967; Donnelly et al., 1967) may have contributed to the rare implication of fluid milk in staphylococcal intoxication. However, the incidence of enterotoxigenic strains of S. aureus in milk, as well as their ability to survive subpasteurization heat treatment, has been demonstrated in Chapter II of this investigation. Furthermore, during the processing of fluid milk into milk products, there is ample opportunity for this organism to grow and produce enterotoxins under favorable temperatures. Due to lack of a reliable and sensitive test for the detection of staphylococcal enterotoxins, much of the early research was directed toward factors that influence growth of S. aureus, including enterotoxigenic strains. The recent microslide and the new radioimmunoassay technology, together with modified methods for the concentration of enterotoxins, have therefore made it possible to study factors conducive to enterotoxin production.

Heat treatment has been used to eliminate or substantially reduce S. aureus in milk for cheese manufacture. Subpasteurization at 61°C for 16-18 seconds is a common commercial practice in the Canadian cheese industry. But, S. aureus including enteroxigenic strains were

demonstrated to survive this subpasteurization heat treatment at a rate of 0-37.5% as presented in Chapter II. Therefore, the surviving organisms may eventually produce enterotoxins in the finished product. In the dairy, milk is usually pumped immediately into the cheese vat for cheesemaking following the necessary heat treatment, while raw milk prior to heat treatment is usually held for a period of time. If milk is held long enough, multiplication of bacteria occurs at all the usual temperatures above the freezing point. Excessive numbers of enterotoxigenic S. aureus subsequent to multiplication, could later initiate growth and produce enterotoxin. Enterotoxin, if formed, would appear in the finished product. Therefore, pre-heat treatment contamination could be equally as important as post-heat treatment contamination. Growth and enterotoxin production by S. aureus in milk due to post-heat treatment contamination have been studied (Donnelly et al., 1968; Tatini et al., 1971). However, whether similar responses would be observed in the case of enterotoxigenic S. aureus that survive the heat treatment of milk needs to be verified.

Growth and enterotoxin production of S. aureus in milk were investigated by Donnelly et al. (1968). They inoculated low and high count raw/pasteurized milk with S. aureus, and then held the milks at 20, 25, 30, and 35°C to determine growth and enterotoxin production. Their data indicate that:

(i) Low count raw milk (10^4 SPC per ml) was more suitable for growth and enterotoxin production of S. aureus than high count raw milk (10^6 SPC per ml).

(ii) Good growth of S. aureus and enterotoxin production was found in low count raw milk as well as in high count raw milk after pasteurization.

Tatini et al. (1971a) did a similar experiment, but subjected the milk to several different heat treatments. Their data indicate additional information as follows:

(i) Heat treated milk was more suitable for enterotoxin production than the same milk in the raw state.

(ii) The degree of heating, which ranged from pasteurization to autoclaving, had little apparent effect on the capacity of milk to support enterotoxin production.

(iii) Minimal S. aureus populations of about two to three million per ml and a minimal incubation of four to six hours at 37°C were required to produce enterotoxin in milk.

It is necessary that these results be realized as we strive to produce low count raw milk, to use subpasteurization instead of pasteurization for the cheese manufacture, and as the milk is held for longer and longer periods before it is processed. With reference to these results, a research to establish standards relating S. aureus growth to enterotoxin production in milk used for cheese manufacture could be conducted to emphasize high criteria of safety.

The purpose of this investigation, therefore, was to obtain specific information on:

(i) the initial S. aureus population and its resulting population in milks associated with enterotoxin production in the finished product; and

(ii) the time required for enterotoxin production

in the case of post-heat treatment contamination as well as in the case of S. aureus which survive the heat treatment of milk.

EXPERIMENTAL METHODS

1. Selection and Propagation of S. aureus Culture

Isolate No. 13, an enterotoxin-A-producing strain isolated from the ten-month survey, was adopted for this phase of the investigation. It was propagated routinely by transfers as mentioned in the last chapter. At least two consecutive transfers were made prior to inoculating the culture into test milks.

2. Source and Handling of Test Milks

Raw, whole milk was obtained from the Commercial Dairy, University of Manitoba. Milk samples were collected from two different batches. After being pooled and mixed aseptically, this batch of milk mixture was stored at 4°C until used. Nine 800-ml portions were drawn aseptically from this batch of milk mixture as test milks, one of which acted as the control. Each 800-ml portion of the test milks was placed in a sterile, 1-litre, cotton-plugged Erlenmeyer flask containing a sterile magnet to break up bacterial clumps during the enumerating procedures. Unless stated otherwise, all test milks were tempered to 37°C before inoculating with S. aureus cultures. Test milks, with or without subpasteurization heat treatment, were incubated at 35°C and examined at 0, 4, 6, 8, and 12 hours to study the growth and enterotoxin production of S. aureus in milk. Samples of test milks, taken at plating intervals, were cooled immediately in an ice-water bath, and then stored at -20°C until tested for enterotoxin.

3. Procedures of Heat Treatment

Three 800-ml test milks were equilibrated to 61°C in a thermostatically controlled water bath (Magni Whirl, Blue M electric). Specified amounts of the culture suspension were added to the heating menstruum at 61°C. A 10 seconds coming-up time was incorporated at the beginning of the heating period. The mixture was agitated and maintained at this temperature for 18 seconds (holding period). After being heated, the test milk sample was immediately cooled to room temperature in an ice-water bath.

The heating chamber consisted of a 1-litre Erlenmeyer flask fitted with a rubber stopper with 3 sampling vents. A stirring shaft of an electric stirrer (Dyna-mix, Fisher) was inserted into one of the sampling vents. The whole chamber was then sterilized with the remaining sampling vents being sealed with stoppers. A thermometer was sterilized in boiling water for 15 minutes, and was then fitted into the sampling vent in place of the stopper. The whole heating chamber was immersed in a water bath.

4. Inoculation of S. aureus into Test Milks

Freshly prepared S. aureus culture suspension was added at different levels into three types of test milks. It was inoculated at:

(i) 10^3, 10^4, and 10^5 cells per ml into raw milk

(ii) 10^3, and 10^4 cells per ml into heat treated milk "A" (inoculum being added after the heat treatment)

(iii) 10^4, 10^5, and 10^6 cells per ml into heat treated milk "B" (inoculum being added before the heat treatment).

Culture suspensions were made with 24-hour S. aureus cultures which were

not refrigerated after incubation. Prior to inoculation, culture suspensions were shaken vigorously with sterile glass beads to reduce cellular clumping.

5. Plating Procedures

Standard plate counts were performed on raw and heat-treated test milks by pour-plating with SPC agar. Indigenous populations of S. aureus in raw and heat treated milks were determined using pour-plating and BP medium.

S. aureus counts at different incubating intervals were obtained by surface plating, in quadruplicate, 0.1 ml of the appropriate dilutions of milk samples onto pre-poured and pre-dried plates of BP medium. All plates were incubated at 37°C for 24 hours.

6. Extraction of Enterotoxin from Test Milks

The extraction procedure of Reiser et al. (1974) was used to separate enterotoxin A from 100-ml volumes of test milk samples. The extraction method consists essentially of a series of purification procedures such as acid precipitation, centrifugation, chloroform extraction, filtration, and chromatography with CG-50 ion exchange resins followed by concentration with 30% (w/w) polyethylene glycol 20,000 (Fisher Scientific).

7. Detection of Enterotoxin in Test Milks

The microslide immunodiffusion analysis was performed on all types of test milk samples as described by Reiser et al. (1974). Microslides were incubated for 24 hours at 37°C. Quantitative recovery of enterotoxin from the spiked milk samples was estimated to be 30-35%.

For an enterotoxin concentration of 6.25 µg/ml, an anti-enterotoxin dilution between 1:8 and 1:16 was required to give a reference line of precipitation midway between the two reactant wells.

RESULTS

1. Milk Quality and Heat Treatment

A. Milk Quality. Raw, whole milk obtained from the Commercial Dairy, University of Manitoba, is of Grade A raw milk according to the federal government regulation. S. aureus and the total number of bacteria indigenous to raw milk were enumerated before and after the subpasteurization heat treatment (61°C for 18 seconds). S. aureus count and total bacterial count (i.e. SPC) per ml of milk were observed as follows:

	Bacterial counts per ml of milk	
	before heat treatment	after heat treatment
S. aureus:	1.45×10^2	4.5×10^1
SPC:	1.20×10^3	9.5×10^1

B. Heat Treatment. Since S. aureus inoculum was injected when the internal temperature of the milk had equilibrated to 61°C, the coming-up time was, therefore, considered to be negligible. A ten seconds coming-up time was incorporated at the beginning of the heating period to account for the coming-up time of the plate pasteurizer employed in the dairy industry. The time for heat treatment, calculated from the time of injection of the inoculum to the time when the milk was cooled to room temperature, was found to be approximately one minute.

2. Microbiological Analysis of the Test Milks

The growth of S. aureus in the test milks is presented in Table 6 and in Figure 3. To determine the minimal population of S. aureus associated with detectable enterotoxin A in raw whole milk, three initial inoculum levels of S. aureus, 10^3 cells/ml, 10^4 cells/ml, and 10^5 cells/ml milk were employed. With an initial inoculum of 10^3 cells per ml of milk, a resulting population of 9.85×10^4, 1.02×10^6, 2.2×10^7, and 6.65×10^7 cells per ml of milk was reached after incubating at 35°C for 4, 6, 8, and 12 hours, respectively. With the higher inoculum (10^4 cells/ml), a population of 9.2×10^5, 1.3×10^7, 7.85×10^7, and 1.06×10^8 cells per ml of milk was reached after 4, 6, 8, and 12 hours of incubation. With an initial inoculum of 10^5 S. aureus per ml of milk, a population of 4.25×10^6, 8.0×10^7, 1.42×10^8, and 3.4×10^8 cells per ml of milk was attained in 4, 6, 8, and 12 hours.

To determine the minimal population associated with detectable enterotoxin in subpasteurized milk produced commercially for cheddar cheese manufacture, S. aureus was inoculated at levels of 10^3 and 10^4 per ml into heat treated milk (i.e. post-heat treatment contamination). With an initial inoculum of 10^3 and 10^4 S. aureus per ml of heat treated milk "A", a resulting population of 2.45×10^5, 2.8×10^6, 6.2×10^7, and 1.35×10^8 cells per ml of milk; and a population of 3.2×10^6, 7.25×10^7, 1.05×10^8, and 2.3×10^8 cells per ml of milk was reached in 4, 6, 8, and 12 hours, respectively.

It is the main interest of this part of the investigation to determine whether similar responses would be observed in the case of enterotoxigenic S. aureus indigenous to raw milk that survive a subpasteurization heat treatment. The effect of S. aureus indigenous to

TABLE 6. Growth of Staphylococcus aureus (Isolate No. 13) in Raw and Heat Treated Milk

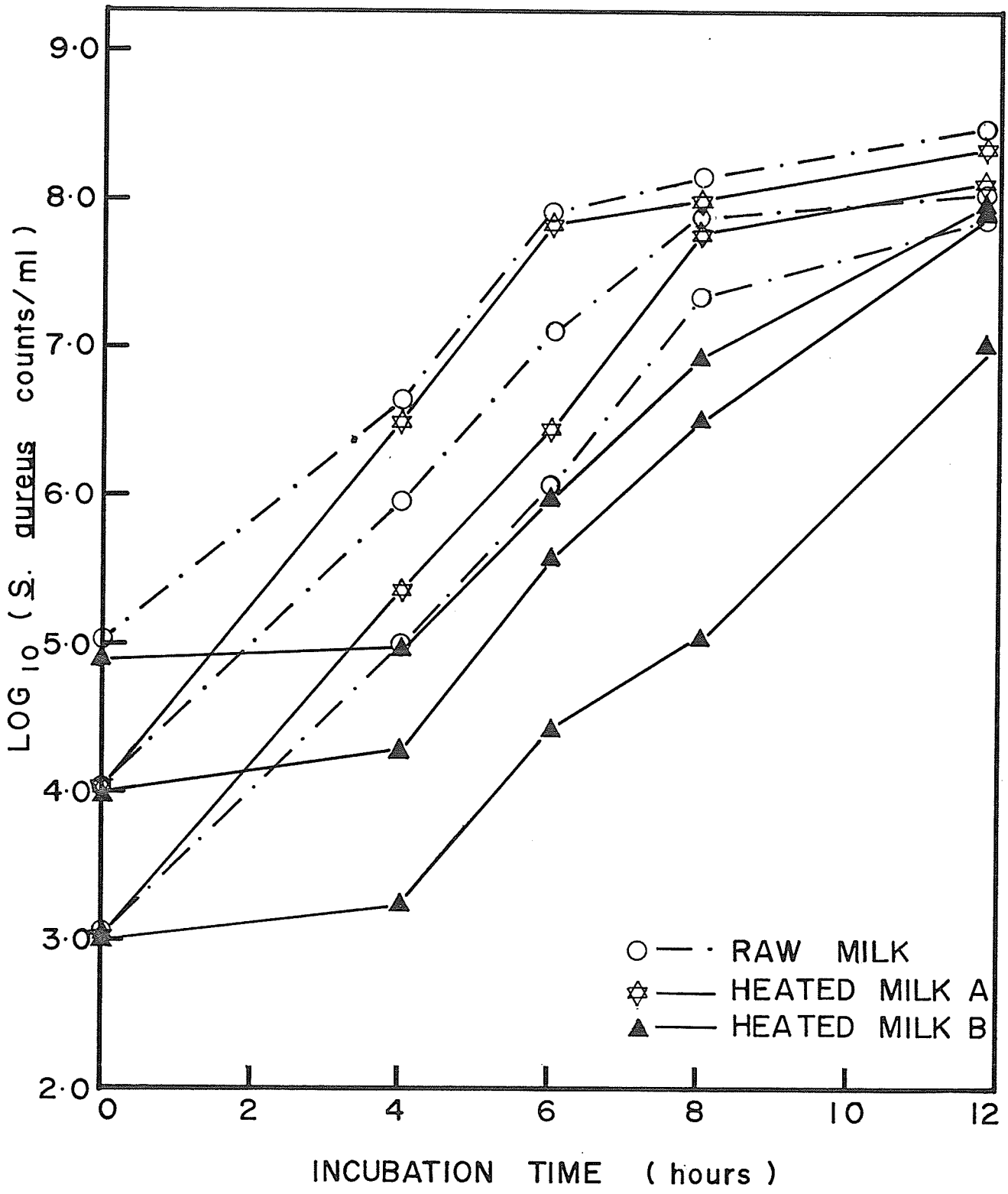
IDENTIFICATION OF MILK SAMPLE	S. aureus INOCULUM	S. aureus COUNT PER MILLILITER OF MILK				
		TIME OF INCUBATION AT 35°C (hours)				
		0	4	6	8	12
RAW MILK						
R1	10^3	1.20×10^3	9.85×10^4	1.02×10^6	2.20×10^7	6.65×10^7
R2	10^4	1.125×10^4	9.20×10^5	1.30×10^7	7.85×10^7	1.06×10^8
R3	10^5	9.75×10^4	4.25×10^6	8.00×10^7	1.42×10^8	3.40×10^8
HEAT TREATED MILK "A"						
A1	10^3	1.12×10^3	2.45×10^5	2.80×10^6	6.20×10^7	1.35×10^8
A2	10^4	1.125×10^4	3.20×10^6	7.25×10^7	1.05×10^8	2.30×10^8
HEAT TREATED MILK "B"						
B1	10^4*	1.05×10^3	1.85×10^3	2.65×10^4	1.125×10^5	1.02×10^7
B2	10^5*	1.02×10^4	2.15×10^4	3.75×10^5	3.20×10^6	8.25×10^7
B3	10^6*	8.65×10^4	9.60×10^4	9.85×10^5	8.90×10^6	1.04×10^8

* S. aureus counts in milk before heat treatment: B1 1.20×10^4
B2 1.12×10^5
B3 9.85×10^6

Heat treated milk "A" : S. aureus inoculum was added after the heat treatment of 61°C for 18 seconds

Heat treated milk "B" : S. aureus inoculum was added before the heat treatment

FIG. 3 GROWTH OF S. aureus IN TEST MILKS



raw milk (i.e. including pre-heat treatment contamination) on the growth and enterotoxin production in milk was studied by inoculating S. aureus at 10^4, 10^5, and 10^6 cells per ml into raw milk, heat treated at 61°C for 18 seconds, and then incubated at 35°C. In the first 4 hours of incubation, there was no apparent increase in growth as compared to the growth detected at 0 hour. After this lag phase, heated cells of S. aureus appeared to grow and multiply at a rate comparable to that of the unheated cells in heat treated milk "A". With an initial inoculum of 10^4, 10^5, and 10^6 S. aureus per ml of milk, a resulting population of 2.65×10^4, 1.125×10^5, and 1.02×10^7 cells per ml of milk was reached in heat treated milk "B" with the low initial inoculum; a population of 3.75×10^5, 3.2×10^6, and 8.25×10^7 cells per ml of milk was reached in the same type of milk with a higher inoculum; and a population of 9.85×10^5, 8.9×10^6, and 1.04×10^8 cells per ml of milk was reached with the highest level of inoculum after incubating for 6, 8, and 12 hours, respectively.

3. Enterotoxin Production in Test Milks

Results of enterotoxin production by S. aureus in the test milks are presented in Table 7. With an initial inoculum of 10^5 cells per ml, enterotoxin was detected in raw milk in 6 hours. With an initial inoculum of 10^3 and 10^4 S. aureus per ml of milk, enterotoxin was detected after 8 hours with 2.2×10^7 cells per ml of milk, and 6 hours with 1.3×10^7 cells per ml of milk, respectively.

With an initial inoculum of 10^3 S. aureus per ml of milk, enterotoxin was detected in heat treated milk "A" (i.e. post-heat treatment contamination) after 6 hours with 2.8×10^6 cells per ml of milk.

TABLE 7. Enterotoxin Production of S. aureus Isolate No. 13 in Raw and Heat Treated Milk

IDENTIFICATION OF TEST MILK	S. aureus INOCULUM	DETECTABLE ENTEROTOXIN TIME OF INCUBATION AT 35°C (hr) 0	4	6	8	12
RAW MILK						
R1	10^3	-	-	(-)	(+)	(+)
R2	10^4	-	(-)	(+)	-	(+)
R3	10^5	-	(-)	(+)	-	(+)
HEAT TREATED MILK "A"						
A1	10^3	-	-	(+)	-	(+)*
A2	10^4	-	(+)	(+)	-	(+)
HEAT TREATED MILK "B"						
B1	10^4	-	-	-	-	(-)**
B2	10^5	-	-	-	(-)	(+)*
B3	10^6	-	-	-	(-)	(+)*

(+) : enterotoxin detected

(-) : enterotoxin not detected

\- : not tested for enterotoxin

* : also submitted for RIA

** : no detectable enterotoxin even after 24 hours

With an initial inoculum of 10^4 cells per ml of milk, enterotoxin was detected after 4 hours with a population of 3.2×10^6 S. aureus per ml of heat treated milk "A".

Enterotoxin was not detected, however, after 12 hours in heat treated milk "B" (including pre-heat treatment contamination) which was obtained from the same batch of raw milk heat treated and incubated under identical conditions with an equivalent level of inoculum (10^4 cells/ml), although a staphylococcal population of 1.02×10^7 was attained. But, with higher initial inocula, 10^5 and 10^6 S. aureus per ml, enterotoxin was detected in heat treated milk "B" after 12 hours, reaching a maximum of 8.25×10^7 and 1.04×10^8 cells per ml of milk, respectively.

DISCUSSION

Depending upon the production and sanitation practices on farms, as well as the storage temperatures, the total bacterial count (i.e. SPC) of raw milk may vary from a few thousand up to 1×10^5 cells per ml or more for Grade A raw milk. Growth inhibition of S. aureus in raw milks of high bacterial count has been reported to be attributed to the high numbers of competing organisms in the milk (Donnelly et al., 1968; Tatini et al., 1971b). In establishing standards, the safety limit on the numbers of S. aureus related to enterotoxin production, therefore, should be set with low count Grade A raw milk. Raw milk with an SPC of 1.2×10^3 cells per ml, indeed, was most suitable for this part of the investigation. Grade A raw milks may contain few S. aureus cells, 10 to 3300 cells per ml (Clark and Nelson, 1961); whereas manufacturing grade

raw milks may contain over 1 x 10^4 cells per ml. Therefore, the inoculum levels chosen for this experiment, 10^3-10^5 cells per ml, were representative of the range of staphylococci found in some manufacturing grade raw milk. In both raw and pasteurized milks having a low SPC, enterotoxin was detected in minimal incubation times of 6 to 9 hours at 35°C, 9 to 12 hours at 30°C, 18 hours at 25°C, and 36 hours at 20°C after inoculation with 10^6 S. aureus per ml (Donnelly et al., 1968). In high count raw milk, enterotoxin was detected only in samples incubated at 35°C (Donnelly et al., 1968). Therefore, an incubation temperature of 35°C was chosen for this part of the investigation to ensure safety limit and to represent the temperature at which cheddar cheese is made.

Enterotoxin was detected in both raw and heat treated milk. However, it was produced with a much lower staphylococcal population in heat treated milk than in raw milk at any one inoculum level used. This result is in agreement with those of Tatini et al. (1971a), who reported better growth and enterotoxin production in heat treated milk than in raw milk. With an initial inoculum of 10^3 cells per ml, enterotoxin was detected in raw milk after 8 hours and in heat treated milk after 6 hours, reaching 2.2 x 10^7 and 2.8 x 10^6 S. aureus per ml of milk, respectively. With a higher inoculum, 10^4 cells per ml, enterotoxin was detected after 6 hours in raw milk and 4 hours in heat treated milk, reaching 1.3 x 10^7 and 3.2 x 10^6 S. aureus per ml of milk, respectively. Hence, the minimal population of S. aureus associated with detectable enterotoxin A in raw milk appears to be 1.3 x 10^7 cells per ml, while a minimal population of 2.8 x 10^6 cells per ml resulted in enterotoxin production in heat treated milk (SPC = 95 cells/ml). Donnelly et al. (1968) found a minimal population of 5 x 10^7 S. aureus per ml and a minimal incubation of 6 hours at

35°C were required for enterotoxin production in raw milk. The lower minimal population required for enterotoxin production observed in this investigation could be attributed to the better concentration and enterotoxin detection method employed in this study. Tatini et al. (1971a) reported that enterotoxin was detected after 4-6 hours at 37°C in low count raw milk (SPC = 80 cells/ml) with a minimal population of 2-3 million S. aureus per ml. Their results are very much similar to those obtained in the heat treated milk "A" of this investigation, in which a similar SPC level (95 cells/ml versus 80 cells/ml) was observed. With an initial inoculum of 10^4 S. aureus per ml, the same authors reported a minimal staphylococcal population of 12 million was required for enterotoxin production in heat treated milk (65.6°C for 16 seconds) after 6 hours at 37°C. The different minimal population required for enterotoxin production observed in this study, as compared to that reported by Tatini et al., could be attributed to the better recovery of S. aureus on the BP medium (versus their S110 medium), and possibly to the difference in the strains of S. aureus employed.

Growth and enterotoxin production of S. aureus indigenous to raw milk, that survive the subpasteurization heat treatment, has never been studied. An attempt to study this was made in this part of the investigation. Such an experiment also applies to the pre-heat treatment contamination of milk. Tatini et al. (1971b) speculated that "it is likely that heat injured S. aureus may not be able to recover and grow, or may grow but be unable to produce enterotoxin." Yet, the optimum temperature and pH for the recovery of heat injured cells of S. aureus are 32°C and 6.0, respectively (Allwood and Russell, 1966). It is of interest to note that the temperature at the early stages (including the

milk ripening stage) of the cheddar cheese manufacturing process is 30-32°C and the pH of milk is around 6.0. Therefore, contamination of cheese milk with sublethally heat damaged cells of S. aureus that are capable of repairing the injury would be expected. After the sub-pasteurization heat treatment of 61°C for 18 seconds, S. aureus showed no increase in growth during the first 4 hours of incubation. S. aureus survivors, however, multiplied ultimately at a rate similar to that of the unheated organisms in heat treated milk "A". Growth was initiated after a lag period of 4-5 hours. This lag phase could be attributed to a recovery period displayed by the S. aureus cells under a repair mechanism. As cited in the literature review, several workers (Iandolo and Ordal, 1966; Bluhm and Ordal, 1968; Rosenthal and Ordal, 1970) have reported that a host of cellular changes have been implicated and related to depressed growth capabilities of heat injured cells of S. aureus.

Enterotoxin was detected in heat treated milk "B", although it was produced at a much higher staphylococcal population than in either raw or heat treated milk "A". With an initial inoculum of 10^4 cells per ml, a resulting population of 1.02×10^7 S. aureus per ml was attained. Enterotoxin was not detected in this test milk even after 12 hours of incubation. As few as 3.2×10^6 S. aureus per ml resulted in enterotoxin production in heat treated milk "A" with an identical milk quality and an equivalent inoculum after only 4 hours. With an initial inoculum of 10^5 and 10^6 cells per ml, enterotoxin was detected in heat treated milk "B" after 12 hours, accompanied by a resulting population of 8.25×10^7 and 1.04×10^8 S. aureus per ml, respectively. Therefore, if S. aureus were subjected to a sublethal heat treatment and then given favorable conditions for growth, they could ultimately multiply and produce

enterotoxin although the process was delayed by a lag phase. The much higher staphylococcal population required for producing enterotoxin in heat treated milk "B" could be attributed to the loss of the ability to produce enterotoxin, partially or completely, by some of the thermally stressed cells of S. aureus recovering from the lag period. There were no published data with which to compare these results. However, whether similar responses would be observed in the cases of other sublethal heat treatment temperatures needs to be verified.

Ease of enterotoxin production in both types of heat treated milks ("A" and "B"), therefore, emphasizes the danger of: (i) pre-heat treatment contamination, (ii) post-heat treatment contamination, and (iii) subsequent exposure to growth temperatures adequate for S. aureus, such as those employed in the cheddar cheese manufacturing process. From the results obtained in this phase of the investigation, information on the possible occurrence of staphylococcal enterotoxin in cheese milk can be summarized as follows:

(1) The minimal indigenous S. aureus population in raw milk (i.e. before subpasteurization heat treatment) associated with detectable enterotoxin was 10^3 cells per ml.

(2) The minimal resulting S. aureus population in raw milk associated with detectable enterotoxin was 1.3×10^7 cells per ml.

(3) The minimal S. aureus population in heat treated milk (i.e. after subpasteurization heat treatment) associated with detectable enterotoxin was:

    (i) 10^3 cells per ml for post-heat treatment contamination.

    (ii) 10^5 cells per ml for pre-heat treatment contamination.

(4) The minimal resulting S. aureus population in heat treated milk associated with detectable enterotoxin was:

(i) 2.8×10^6 cells per ml for post-heat treatment contamination.

(ii) 8.25×10^7 cells per ml for pre-heat treatment contamination.

CHAPTER VI

TITLE : Growth and Enterotoxin Production of S. aureus Isolate No. 13 in Cheddar Cheese Manufacture : I. Manufacture and Analysis of Cheese.

INTRODUCTION

In the past two decades, cheese has been the dairy product most commonly involved in outbreaks of staphylococcal food-poisoning (Hendricks et al., 1959; Allen and Stovall, 1960; Hausler et al., 1960; Zehren and Zehren, 1968a; Miller, 1974; Caserio et al., 1975), and outbreaks were limited to cheddar and related types. The presence of S. aureus in milk as well as their ability to produce enterotoxin in manufactured cheese have therefore been duly recognized for quite some time.

To achieve a better flavored cheese, the Canadian cheese industry strives to produce cheese from subpasteurized (61°C for 18 seconds) milk rather than pasteurized milk. But Thatcher et al. (1956) reported that Canadian cheeses sometimes contained large numbers of toxigenic staphylococci. In Ontario in 1971, a large quantity of cheddar cheese, made from subpasteurized milk, was found to contain enterotoxin (Duitschaever and Irvine, 1971). Therefore, the growth of S. aureus during the cheese manufacturing process must be restricted so that a population large enough to produce enterotoxin does not develop.

Growth of S. aureus in cheese made with normal and slow starter was reported (Takahashi and Johns, 1959; Thatcher and Ross, 1960; Tuckey et al., 1964). However, a much more rapid growth was observed in cheese made with slow starter (Reiter et al., 1964). Due to inadequate procedures for the detection of staphylococcal enterotoxins, much of the early research was directed toward factors that influence growth rather than enterotoxin production by S. aureus. Recent development of serological procedures for the detection of enterotoxins, together with modified

methods for the concentration of enterotoxins in foods, has therefore made it possible to study the conditions conducive to enterotoxin production. It was found that all toxic cheese had histories of poor acid development during cheese manufacture (Zehren and Zehren, 1968b). Tatini et al. (1971b) also demonstrated that enterotoxin could be produced in cheese of normal acid development. Little information is available on staphylococcal growth in cheese manufacture associated with the presence or absence of enterotoxin. In the only report of enterotoxin production in cheddar cheese manufacture, Tatini et al. (1971b) detected the presence of enterotoxin with a minimal population of 28 million S. aureus per g of curd.

In the cheese manufacturing process, S. aureus may grow and reach a population size capable of producing enterotoxin prior to being inactivated by heat or low pH. The enterotoxins, being more resistant to heat and low pH, remain active. Consequently, examination of the finished product alone for the presence of viable S. aureus is of limited value in ascertaining whether or not S. aureus grew in the product to the extent that enterotoxins might be present. Yet, conventional sensitive techniques for the detection of enterotoxins are not available in most food processing plants. Where such facilities are available, enterotoxin detection is too expensive to perform routinely. Therefore, testing every batch of cheese for the presence of enterotoxins is almost impossible. Furthermore, rapid assay procedures are not available at present, nor are preparations of purified antigen-antisera available for all enterotoxins. For the above reasons, standards relating the levels of S. aureus in the cheese manufacturing process (before, during, and after) to the production of enterotoxin become critical. To date, such standards are not

available in Canada (Pivnick, 1978). Also, the conditions relating the chemical factors (pH, moisture content, salt content, and fat content) to the production of enterotoxin are important as well.

The purpose of this part of the investigation is, therefore, to explore:

(i) the relationship between the growth of S. aureus throughout the cheese manufacturing process and the enterotoxin production;

(ii) the relationship between the biochemical factors of cheese and the presence of enterotoxin; and

(iii) the time required for the production of enterotoxin in the cheese manufacturing process

with a view to establishing the necessary microbiological and biochemical standards for cheddar cheese. In establishing standards, one must maintain high criteria of safety. Results obtained by Tatini et al. (1971b), therefore, served as a reference to set up specific controls and limits for this investigation.

EXPERIMENTAL METHODS

1. Selection and Propagation of Bacterial Cultures

A. S. aureus. Isolate No. 13, an enterotoxin-A-producing strain isolated from the ten-month survey, was adopted for this phase of the investigation. It was propagated routinely by thrice weekly transfers as mentioned in Chapter IV.

B. Starter Cultures. Multiple strains of starter culture from Hansen's

(Redi-Set, Hansen's) were propagated routinely in sterilized reconstituted skim milk with daily fresh transfer using 1% inoculum. After each transfer, the cultures were incubated at 22°C (72°F) for 16-18 hours, and refrigerated until the next transfer. Starter activity was tested twice monthly according to the procedures outlined in Appendix 9.

2. Source and Handling of Milk

Raw, whole milk obtained from the University Commercial Dairy was collected in a stainless steel container. It was then heat treated in a steam chest with free flowing steam at 61°C (142°F) for 18 seconds. Temperature readings were routinely monitored. After heating at 61°C for 18 seconds, the container was immediately cooled in ice-cooled water to 30°C (86°F), and its content was immediately poured into the cheese vat for cheesemaking. Approximately 45-50 pounds of milk were used for each cheese batch.

3. Determination of Optimum Rate of Starter Failure for Cheesemaking

Addition of starter culture at a rate of 2% of milk is the normal commercial practice in the Canadian dairy industry. In this experiment, cheese batches were made with 2%, 1%, 0.5%, 0.3%, and 0.2% starter. Cheddar cheese Lot C2 and Lot C3 were made with 2% starter, and acted as control batches. Titratable acidity of milk and whey was measured at 30-minute intervals until the milk had ripened.

4. Inoculation of Cultures into Heat Treated Milk for Cheesemaking

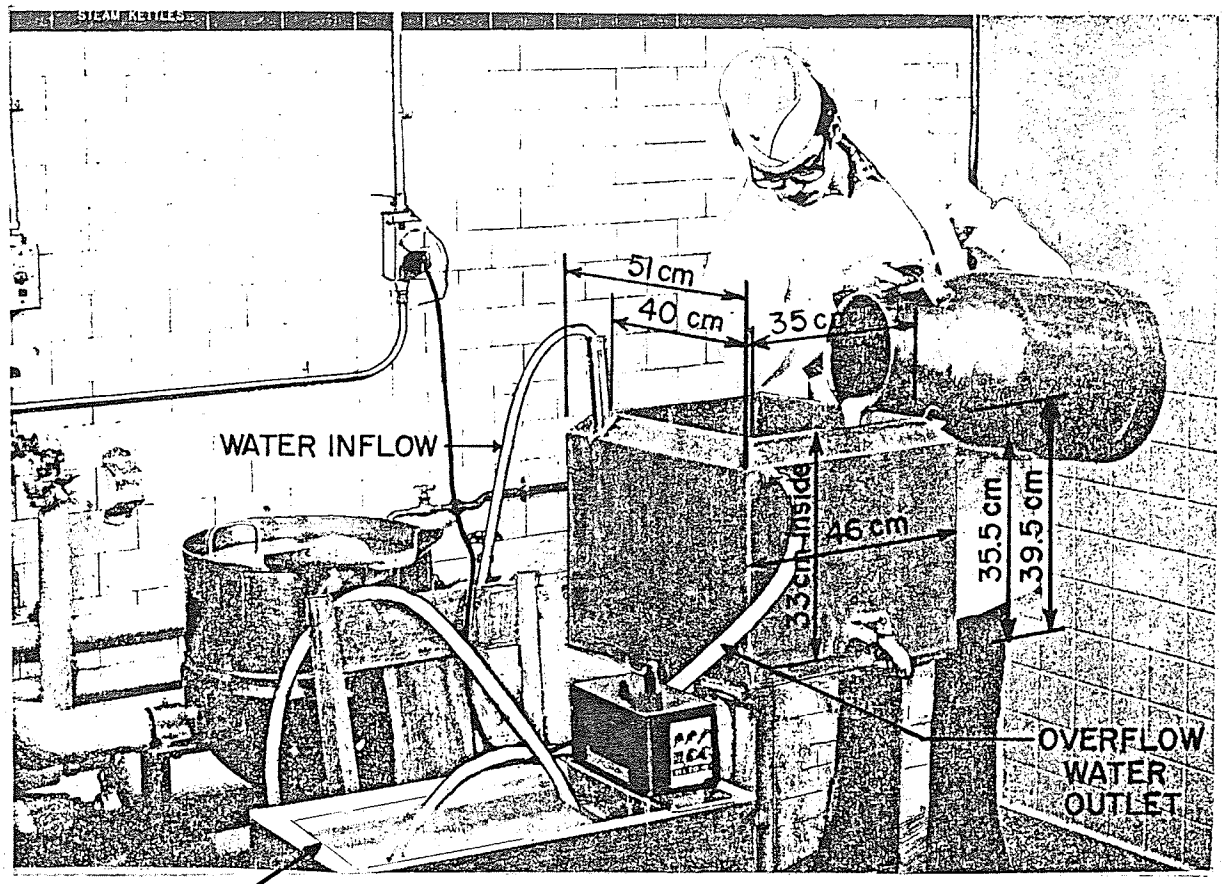
Specified amounts of freshly prepared, 24 hour S. aureus culture, and 16-18 hour starter culture, were added to the heat treated

milk in the cheese vat at 30°C (86°F). S. aureus culture was inoculated at three different levels: 60 cells per ml, 5 x 10^3 cells per ml, and 10^6 cells per ml of milk. An inoculum of 60 cells per ml was eliminated in "partial slow" cheese manufacture. Starter culture was added at a rate of 2%, 0.5%, and 0.3% of milk (v/v) for making "normal", "partial slow", and "slow" cheese, respectively. Freshly prepared cultures, which were not refrigerated after incubation, were shaken vigorously with sterile glass beads to reduce cellular clumping immediately before inoculating into heat treated milk used for the cheese manufacturing process.

5. Manufacture of Cheddar Cheese

Cheddar cheese was manufactured on a laboratory scale using procedures employed in the Canadian dairy industry. Details of the cheese manufacturing procedures were provided by the University of Manitoba Commercial Dairy as described in Appendix 10. Diagrams of the laboratory designed stainless steel cheese vat are presented in Figures 4 and 5.

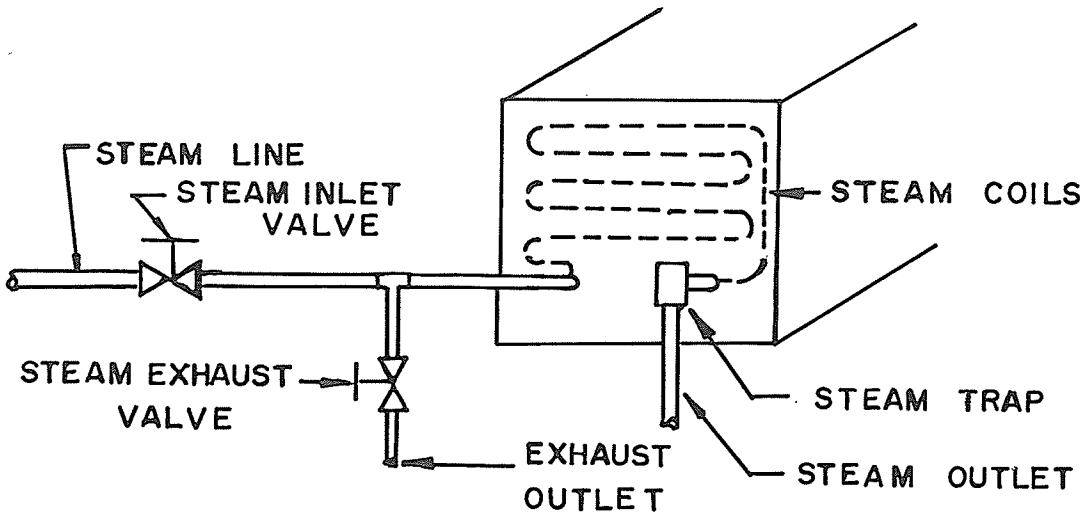
Cheeses, after pressing, were packaged in cryovac bags which were then evacuated to provide an air-free atmosphere. The packaged cheeses were then cured at 15°C (59°F) for 21 days, and subsequently stored at 4-5°C (39.2-41°F). The titratable acidity of the milk, and whey, and the pH of the curd were measured at various stages of the cheese manufacturing process. S. aureus counts, and total bacterial counts, including lactic acid bacteria, were determined throughout the cheesemaking and curing process. Cheese slurries were collected at various stages of the cheese manufacturing process, and were frozen at -20°C (-4°F) until tested for enterotoxin. After 21 days of curing,



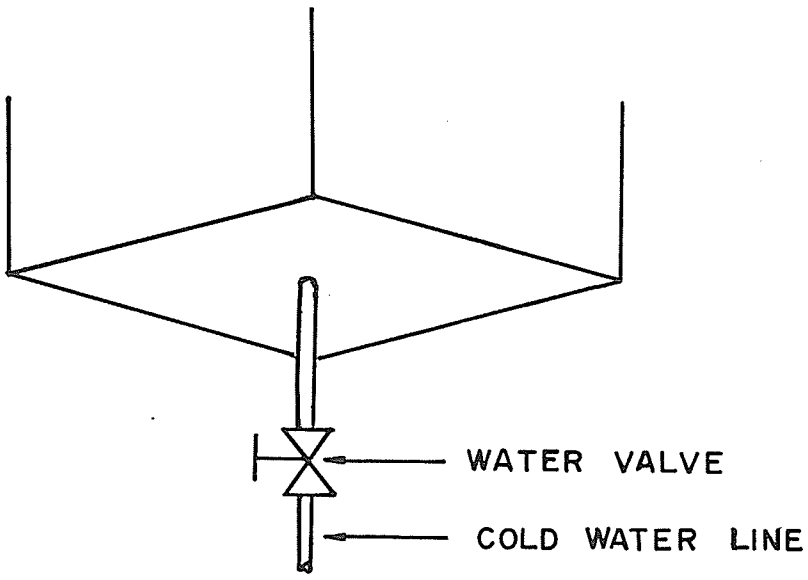
INNER WIDTH OF HOLLOW JACKET = 5 cm

THICKNESS OF STAINLESS STEEL SHEET = 0·25 cm

FIG. 4 Diagram of the Laboratory Scale Stainless Steel Cheese Vat for Cheese Manufacture



BACK PANEL OF THE VAT



BOTTOM OF THE VAT

FIG. 5 Details of Steam and Water Connections of the Laboratory Cheese Vat

cheeses were analyzed for their moisture, salt, and fat content. After 60 days of aging, cheese samples were collected, and frozen until analyzed for enterotoxin production.

6. Microbiological Analysis of Cheddar Cheese

Twenty-five g cheese samples were aseptically blended with 225 ml of 2% sodium citrate solution in an Osterizer at high speed for 3 minutes. The blended cheese samples were immediately cooled in an ice-water bath before plating. Appropriate serial dilutions, from the homogenate, were made for bacteriological analysis. S. aureus was analyzed on pre-poured and pre-dried BP medium using a spread-plate method. Plates were incubated at 37°C for 24 hours. Total bacterial counts were analyzed on tomato yeast extract medium using a pour-plate method. Plates were incubated at 32°C for 48 hours. All plating was done in duplicate.

7. Sampling of Packaged Cheese

Test samples were obtained with a sterile cheese trier (sterilized by dipping in alcohol followed by flaming). From each packaged cheese, at least three plugs were drawn perpendicularly to surface of cheese, one from the center, one near the outer edge, and a third half way between the other two. Each extracted plug was cut with a sterile knife one inch from the rind. The outer inch of the plug was inserted back into the bored hole, and immediately sealed with hot wax. The test samples were either used for immediate bacteriological analysis or ground into fine shreds with a cheese grinder for moisture, fat, and salt content determination. Samples drawn for enterotoxin detection were kept at 4°C until used.

8. Chemical Analysis of Milk, Whey, and Cheese

A. Titratable Acidity. The titratable acidity of milk and whey was measured by titrating 9 ml of the sample against 0.1N NaOH using phenolphthalein as the indicator as outlined by the Ontario Department of Agriculture for Testing Dairy Products (1961). The duplicate determinations conducted on milk and whey samples varied from their average values by 0-2%.

B. pH. pH of the cheese samples was measured either on an undiluted and slurried sample or in a 1:10 diluted (distilled water) and blended sample. A Radiometer Model 26 pH meter was used to measure the pH of the cheese samples.

C. Moisture Content. Two g samples of grated cheese were used for each moisture content determination. The moisture content of the cheese was performed on duplicate samples by the method outlined by the Department of Agriculture, Canada (1954). The moisture content of the cheese samples varied within $\pm$ 0.7% limit from their average values. The percentage of moisture was calculated as follows:

$$\text{percentage of moisture} = \frac{\text{Wt. OF SAMPLE} - \text{Wt. OF SOLID}}{\text{Wt. of SAMPLE}} \times 100$$

D. Salt Content. The salt content of the cheese samples was determined on 3 g samples using the titration method. In this method, 10 ml of 0.17N $AgNo_3$ were allowed to react with the salt in the cheese sample, and the excess $AgNO_3$ was then titrated with 0.17N potassium thiocyanate using saturated ferric ammonium sulfate as the indicator. Details of

the method are outlined by the Ontario Department of Agriculture for Testing Dairy Products (1961). The percentage of salt in the sample was calculated as follows:

$$\text{volume of } AgNo_3 \text{ combined with salt in cheese} = 10 \text{ ml } AgNo_3 - \text{volume of K thiocyanate titrated}$$

$$\text{percentage of salt} = \frac{\text{volume of } AgNO_3 \text{ combined with salt}}{\text{Wt. of cheese sample}} \times 100$$

E. Fat Content. The fat content of cheese samples was determined in duplicate by the Babcock test, as recommended by the Ontario Department of Agriculture for Testing Dairy Products (1961). The fat content of cheese samples varied from their average values by 0-1%.

9. Statistical Analysis

Results of S. aureus growth, total bacterial count, and cheese composition were analyzed by means of analysis of variance. A two way classification with interaction was applied. Differences among the three levels of inoculum as well as differences between the two types of starter were tested for significance by comparison of the calculated 'F' values with the critical 'F' values given in statistical tables (Snedecor and Cochran, 1972) at the 5%, 1%, and 0.5% significance levels.

10. Enterotoxin Production

Cheese samples were tested for the production of enterotoxin A by the solid-phase RIA technique. Detection of enterotoxin production is described in detail in the next chapter.

RESULTS

1. Optimum Rate of Starter Failure for Cheddar Cheese Manufacture

Cheddar cheese batches made with 1, 0.5, 0.3, and 0.2% starter (cheese batches E1, E2, E3, and E4) were compared to cheddar cheese batches using a 2% starter (cheese batches C2 and C3). The data obtained are presented in Table 8.

The ripening period of milk was found to be 1, 2, 2.75, and 4.5 hours for cheese batches made with 2, 1, 0.5, and 0.3% starter, respectively. Addition of starter culture at a rate of 0.2% of milk did not increase the acidity of milk during 8 hours of cheesemaking. Based on the results observed, the addition of starter, at a rate of 0.3% and/or 0.5% of milk, prolonged the milk ripening period so as to offer a considerable length of time for S. aureus cells to multiply. A starter rate of 0.3% of milk was, henceforth, adopted for "slow" cheddar cheese manufacture, whereas a starter of 0.5% of milk was employed to act as partial starter failure in the manufacture of "partial slow" cheddar cheese.

2. Manufacture of Cheddar Cheese

To determine growth and enterotoxin production of S. aureus in cheddar cheese manufacture, heat treated milk was inoculated with varying levels of S. aureus and then made into cheese using normal, slow, and partial slow starter. During the early stages of cooking, the curd particles in the cheese batches of slow and partial slow starter tended to be slightly more fragile than those in the batches of normal starter, and thus required gentle stirring during the first 20-30 minutes of cooking. Consequently, the final texture of the curd was softer and weaker in the "slow" and "partial slow" cheeses than that in the "normal" cheeses.

TABLE 8. Titratable Acidity of Milk and Whey in Cheddar Cheese Batches Made With Various Starter Rates.

Time of Sampling (hrs)	TITRATABLE ACIDITY (%)					
	Rate of Starter					
	2% C2	2% C3	1% E1	0.5 E2	0.3 E3	0.2 E4
0	0.150	0.155	0.150	0.155	0.165	0.160
1	0.180*	0.188*	0.160	0.165	0.163	0.160
1:30	0.195	-	0.170	0.170	0.168	0.160
2:00	0.150	0.150	0.180*	0.175	0.170	0.160
2:45	0.160	0.160	0.140	0.185*	0.172	0.160
3:00	0.185	0.185	0.140	0.140	0.175	0.160
3:30	0.260	-	0.150	0.140	0.178	0.160
4:00	0.500	0.480	0.170	0.165	0.182	0.160
4:30	0.580	0.570	0.300	0.170	0.195*	0.160

* time at which milk was ripened for rennet addition.

Starter cultures with titratable acidity of 0.84-0.90% were used for the manufacture of cheddar cheese. The time required for cheesemaking from starter inoculation to pressing was found to be 7, 8.5, and 10 hours for cheeses made with normal, partial slow, and slow starter, respectively. The increased time required for cheesemaking using a partial slow or slow starter was largely due to the prolonged milk ripening period. All three types of cheese achieved normal titratable acidity at various stages throughout the cheese manufacturing process. However, "partial slow" and "slow" cheeses took, respectively, 1.75-2 and 3-3.5 hours longer than the "normal" cheese did to raise the original titratable acidity of milk by 0.02% at the milk ripening stage of the cheese manufacturing process.

The titratable acidity of milk, whey, and pH of cheese are presented in Tables 9, 10, and 11 for "normal", "slow", and "partial slow" cheeses, respectively. Milling acidity of whey varied from 0.52% to 0.61% in "normal" cheeses, 0.56% to 0.63% in "slow" cheeses, and 0.55% to 0.62% in "partial slow" cheeses. Cheeses made with normal starter had pH values of 4.95-5.35 after pressing, while those made with slow starter had pH values of 5.45-5.8. "Partial slow" cheeses had pH values of 5.25-5.6. This phenomenon remained somewhat the same during curing and aging.

Statistical analysis of pH at curing is presented in Appendix Tables 11 and 12. Statistical analysis on the pH values revealed significant differences in: (i) cheeses made with normal starter and those made with slow starter; (ii) cheeses made with normal starter and those made with partial slow starter. There was also a significant difference existing among cheeses made with different levels of S. aureus inoculum.

TABLE 9. Titratable Acidity of Milk, Whey and pH of Cheese Curd in Cheddar Cheese Manufacture Using Normal Starter

	TITRATABLE ACIDITY OR pH					
	LEVEL OF *S. aureus*# INOCULUM + IDENTIFICATION OF CHEESE					
	60 cells/ml		5×10^3 cells/ml		10^6 cells/ml	
TIME OF SAMPLING	J23	M6	J19	F6	J30	F4
TA AT INOCULATION (0 hr)	0.160	0.165	0.160	0.160	0.160	0.160
TA AT SETTING (1 hr)	0.182	0.190	0.185	0.185	0.182	0.185
TA AT CUTTING (1:30 hr)	0.125	0.140	0.140	0.130	0.125	0.150
TA AT DRAINING (3:20 hr)	0.150	0.160	0.160	0.150	0.160	0.170
TA AT MILLING (5:30 hr)	0.610	0.580	0.580	0.560	0.520	0.610
pH OUT OF PRESS * (24 hr)	5.3	5.1	4.95**	5.02**	5.28	5.35
pH AT CURING * (1 month)	5.3	5.2	5.20	5.25	5.35	5.40

TA : Titratable acidity expressed as per cent lactic acid

* : was measured on a 1:10 diluted and blended sample in dilution water

** : pH was measured on an undiluted sample

\# : *S. aureus* inoculum was Isolate No. 13

TABLE 10. Titratable Acidity of Milk, Whey and pH of Cheese Curd in Cheddar Cheese Manufacture Using Slow Starter

	TITRATABLE ACIDITY OR pH					
	LEVEL OF *S. aureus*# INOCULUM + IDENTIFICATION OF CHEESE					
	60 cells/ml		5×10^3 cells/ml		10^6 cells/ml	
TIME OF SAMPLING	J26	F11	J21	M4	J28	M8
TA AT INOCULATION (0 hr)	0.160	0.160	0.160	0.155	0.150	0.160
TA AT SETTING (4 hr)	0.185	0.185	0.185	0.185	0.178	0.190
TA AT CUTTING (4:30 hr)	0.140	0.145	0.125	0.145	0.140	0.145
TA AT DRAINING (6:20 hr)	0.165	0.165	0.160	0.170	0.168	0.160
TA AT MILLING (8:30 hr)	0.600	0.56	0.63	0.58	0.61	0.63
pH OUT OF PRESS* (24 hr)	5.6	5.45	5.8	5.6	5.7	5.8
pH AT CURING* (1 month)	5.6	5.45	5.8	5.6	5.8	5.8

TA : Titratable acidity expressed as per cent lactic acid

* : was measured on a 1:10 diluted and blended sample in dilution water

: *S. aureus* inoculum was Isolate No. 13

TABLE 11. Titratable Acidity of Milk, Whey and pH of Cheese Curd in Cheddar Cheese Manufacture Using Partial Slow Starter

		TITRATABLE ACIDITY OR pH			
		LEVEL OF *S. aureus*# INOCULUM + IDENTIFICATION OF CHEESE			
		5×10^3 cells/ml		10^6 cells/ml	
TIME OF SAMPLING		M13	M20	A4	A13
TA AT INOCULATION	(0 hr)	0.165	0.155	0.160	0.160
TA AT SETTING	(2:30 hr)	0.190	0.190	0.185	0.185
TA AT CUTTING	(3:00 hr)	0.145	0.145	0.150	0.145
TA AT DRAINING	(4:50 hr)	0.170	0.165	0.175	0.175
TA AT MILLING	(7:00 hr)	0.550	0.560	0.610	0.620
pH OUT OF PRESS*	(24 hr)	5.25	5.35	5.45	5.60
pH AT CURING*	(1 month)	5.25	5.35	5.50	5.60

TA : Titratable acidity expressed as per cent lactic acid

* : was measured on a 1:10 diluted and blended sample in dilution water

\# : *S. aureus* inoculum was Isolate No. 13

3. Microbiological Analysis of Cheddar Cheese

Results on total bacterial counts in raw and heat treated milk are presented in Appendix Table 13. Total bacterial population in raw and heat treated milk ranged from 3.2×10^2 to 1.3×10^5 and 2.3×10^1 to 5.1×10^3 cells per ml, respectively. Raw milk employed in this study is, therefore, Grade A raw milk according to government standards.

Growth of S. aureus and total bacteria, including lactic acid bacteria, in cheddar cheese manufacture are presented in Tables 12, 13, and 14. Indigenous levels of S. aureus in heat treated milk for cheese manufacture ranged from 1.0×10^1 to 4.4×10^2 cells per ml of milk. With an initial inoculum of 60, 5×10^3, and 10^6 S. aureus per ml of milk, a resulting population of 2.9×10^3 (Lot J23) or 1.2×10^3 (Lot M6), 7.1×10^4 (Lot J19) or 7.85×10^4 (Lot F6), and 1.8×10^7 (Lot J30) or 2.25×10^7 (Lot F4) cells per g of cheese was reached in "normal" cheeses; a population of 3.05×10^5 (Lot J26) or 2.0×10^4 (Lot F11), 3.7×10^6 (Lot J21) or 1.55×10^6 (Lot M4), and 1.68×10^8 (Lot J28) or 1.11×10^8 (Lot M8) cells per g was reached in "slow" cheeses even after the cheeses were pressed. With an initial inoculum of 5×10^3 and 10^6 cells per ml of milk, a resulting population of 1.65×10^5 (Lot M13) or 1.55×10^5 (Lot M20), and 3.45×10^7 (Lot A4) or 4.8×10^7 (Lot A13) S. aureus per g was reached in "partial slow" cheeses. Therefore, there was a 15-28 fold increase in the staphylococcal count in cheeses made with normal starter, a 117-698 fold increase in cheeses made with slow starter, and a 31-52 fold increase in cheeses made with partial slow starter.

Initial populations of lactic acid bateria in the cheese milks ranged from 3.2×10^7 to 8.0×10^7 cells per ml of milk in the "normal"

TABLE 12. Growth of S. aureus Isolate No. 13 and Lactic Acid Bacteria in Cheddar Cheese Manufacture Using Normal Starter

MEDIUM AND TIME OF SAMPLING	BACTERIAL COUNT per ml OR g					
	LEVEL OF S. aureus INOCULUM and IDENTIFICATION OF CHEESE					
	60 cells/ml		5×10^3 cells/ml		10^6 cells/ml	
	J23	M6	J19	F6	J30	F4
HEATED MILK IN VAT*						
BP	4.5×10^1	1.0×10^1	1.0×10^1	1.5×10^1	2.0×10^1	1.0×10^2
ZERO HOUR (0 hr)						
BP	1.05×10^2	7.5×10^1	4.8×10^3	5.2×10^3	1.125×10^6	9.6×10^5
TEA	7.10×10^7	3.2×10^7	5.6×10^7	4.1×10^7	8.0×10^7	4.1×10^7
CUTTING (1:30 hr)						
BP	2.0×10^2	1.0×10^2	9.6×10^3	9.50×10^3	2.365×10^6	1.855×10^6
TEA	5.95×10^8	3.6×10^8	6.5×10^8	1.39×10^8	6.25×10^8	3.30×10^8
DRAINING (3:20 hr)						
BP	1.6×10^3	1.50×10^3	2.575×10^4	2.90×10^4	1.40×10^7	8.70×10^6
TEA	4.4×10^9	1.475×10^9	1.40×10^9	2.49×10^9	1.125×10^{10}	1.01×10^9
MILLING (5:30 hr)						
BP	2.45×10^3	2.80×10^3	4.4×10^4	6.9×10^4	1.525×10^7	2.9×10^7
TEA	1.72×10^{10}	7.15×10^9	6.3×10^9	3.6×10^9	7.0×10^9	3.4×10^9
DRESSING (7:00 hr)						
BP	5.55×10^3	3.10×10^3	7.9×10^4	9.8×10^4	1.675×10^7	3.80×10^7
TEA	6.80×10^9	4.25×10^9	7.2×10^9	4.6×10^9	6.05×10^9	2.85×10^9
OUT OF PRESS(24 hr)						
BP	2.9×10^3	1.2×10^3	7.1×10^4	7.85×10^4	1.8×10^7	2.25×10^7
TEA	4.2×10^9	1.3×10^9	2.0×10^9	2.55×10^9	4.5×10^9	1.90×10^9
21 DAYS CURING						
BP	6.0×10^2	3.0×10^2	3.0×10^4	4.0×10^4	4.5×10^6	2.20×10^6
TEA	4.9×10^7	1.205×10^8	8.85×10^8	6.85×10^7	3.3×10^8	6.25×10^7
AFTER 60 DAYS						
BP	-	-	1.85×10^3	3.55×10^3	4.25×10^5	2.0×10^5
TEA	7.25×10^5	7.7×10^6	1.325×10^6	6.70×10^6	5.55×10^7	3.0×10^6

TABLE 12 (CONTINUED)

* : to determine the level of indigenous S. aureus in heat-treated milk prior inoculation for cheesemaking

BP : Baird-Parker medium for the enumeration of S. aureus

TEA : Tomato yeast extract agar for the enumeration of lactic acid bacteria

- : Growth not detected in 1:10 dilution

TABLE 13. Growth of S. aureus Isolate No. 13 and Lactic Acid Bacteria in Cheddar Cheese Manufacture Using Slow Starter

MEDIUM AND TIME OF SAMPLING	BACTERIAL COUNT per ml OR g					
	LEVEL OF S. aureus INOCULUM and IDENTIFICATION OF CHEESE					
	60 cells/ml		5×10^3 cells/ml		10^6 cells/ml	
	J26	F11	J21	M4	J28	M8
HEATED MILK IN VAT*						
BP	4.4×10^2	2.0×10^1	2.95×10^2	2.80×10^2	1.2×10^1	2.0×10^1
ZERO HOUR (0 hr)						
BP	5.0×10^2	8.0×10^1	5.3×10^3	5.5×10^3	9.6×10^5	9.50×10^5
TEA	3.3×10^6	3.8×10^6	2.4×10^6	2.8×10^6	3.7×10^6	4.05×10^6
CUTTING (4:30 hr)						
BP	5.80×10^4_8	2.0×10^2_8	2.215×10^4_7	1.22×10^5_7	4.20×10^6_8	3.95×10^7_7
TEA	1.12×10^8	3.4×10^8	2.70×10^7	3.30×10^7	3.05×10^8	6.55×10^7
DRAINING (6:20 hr)						
BP	2.80×10^5_9	1.20×10^3_9	2.4×10^5_8	9.20×10^5_8	3.8×10^7_9	1.61×10^8_8
TEA	2.48×10^9	1.715×10^9	1.3×10^8	1.96×10^8	2.5×10^9	6.65×10^8
MILLING (8:30 hr)						
BP	7.85×10^5_9	3.0×10^3_9	4.90×10^6_8	1.28×10^6_8	1.68×10^8_9	3.95×10^8_8
TEA	2.80×10^9	3.35×10^9	6.75×10^8	8.60×10^8	3.0×10^9	9.75×10^8
DRESSING (10:00 hr)						
BP	3.75×10^5_9	4.0×10^4_9	6.30×10^6_9	3.4×10^6_9	2.0×10^8_9	2.21×10^8_8
TEA	3.35×10^9	4.35×10^9	1.04×10^9	4.3×10^9	4.3×10^9	5.25×10^8
OUT OF PRESS (24 hr)						
BP	3.05×10^5_9	2.0×10^4_9	3.7×10^6_8	1.55×10^6_8	1.68×10^8_9	1.11×10^8_8
TEA	1.03×10^9	1.88×10^9	3.2×10^8	3.70×10^8	1.04×10^9	4.85×10^8
21 DAYS CURING						
BP	6.65×10^4_7	3.1×10^3_6	8.6×10^5_7	6.70×10^5_7	3.10×10^7_8	3.10×10^7_8
TEA	5.0×10^7	7.3×10^6	4.9×10^7	5.15×10^7	1.45×10^8	1.56×10^8
AFTER 60 DAYS						
BP	5.05×10^4_5	1.50×10^3_6	7.75×10^5_6	3.35×10^5_6	4.15×10^6_6	1.98×10^6_6
TEA	9.75×10^5	3.85×10^6	2.21×10^6	2.23×10^6	4.45×10^6	7.95×10^6

TABLE 13. (CONTINUED)

* : to determine the level of indigenous S. aureus in heat-treated milk prior inoculation for cheesemaking

BP : Baird-Parker medium for the enumeration of S. aureus

TEA: Tomato yeast extract agar for the enumeration of lactic acid bacteria

TABLE 14. Growth of S. aureus Isolate No. 13 and Lactic Acid Bacteria in Cheddar Cheese Manufacture Using Partial Slow Starter

	BACTERIAL COUNT per ml OR per g			
	LEVEL OF S. aureus INOCULUM + IDENTIFICATION OF CHEESE			
	5×10^3 cells/ml		10^6 cells/ml	
	M13	M20	A4	A13
HEATED MILK IN VAT*				
BP	2.8×10^1	2.0×10^1	2.0×10^1	1.8×10^1
ZERO HOUR (0 hr)				
BP	5.1×10^3	5.05×10^3	1.04×10^6	9.25×10^5
TEA	8.8×10^6	8.25×10^6	8.25×10^6	9.65×10^6
CUTTING (3:00 hr)				
BP	1.1×10^4	1.235×10^4	6.25×10^6	5.95×10^6
TEA	1.2×10^8	1.015×10^8	8.40×10^7	8.65×10^7
DRAINING (4:50 hr)				
BP	2.0×10^5	1.28×10^5	1.51×10^7	1.20×10^7
TEA	6.25×10^8	6.55×10^8	4.70×10^8	3.50×10^8
MILLING (7:00 hr)				
BP	5.25×10^5	5.15×10^5	3.80×10^7	4.95×10^7
TEA	3.25×10^9	2.85×10^9	1.05×10^9	7.55×10^8
DRESSING (8:30 hr)				
BP	6.30×10^5	4.60×10^5	4.10×10^7	6.0×10^7
TEA	3.35×10^9	4.20×10^9	2.15×10^9	1.65×10^9
OUT OF PRESS(24 hr)				
BP	1.65×10^5	1.55×10^5	3.45×10^7	4.8×10^7
TEA	4.15×10^9	3.70×10^9	5.90×10^8	4.1×10^8
21 DAYS CURING				
BP	1.15×10^5	9.80×10^4	3.20×10^6	6.85×10^6
TEA	6.80×10^8	4.35×10^8	6.85×10^7	3.20×10^7
AFTER 60 DAYS				
BP	7.65×10^4	3.25×10^4	8.60×10^5	9.5×10^5
TEA	2.85×10^6	2.25×10^6	3.23×10^6	2.0×10^6

* : to determine the level of indigenous S. aureus in heat-treated milk prior inoculation for cheesemaking

BP :Baird-Parker medium for the enumeration of S. aureus

TEA:Tomato yeast extract agar for the enumeration of lactic acid bacteria

cheeses, 2.4×10^6 to 4.05×10^6 cells per ml of milk in the "slow" cheeses, and 8.0×10^6 to 9.65×10^6 cells per ml of milk in the "partial slow" cheeses. Therefore, total bacterial counts on tomato yeast extract agar at the early stage of cheesemaking indicated an apparent natural starter failure in the "slow" and "partial slow" cheese batches. Total bacterial counts at the dressing stage in "partial slow" and "slow" cheese batches, compared to "normal" cheese batches, however, indicated similar growth. But, "partial slow" cheeses took 1.75-2 hours and "slow" cheeses took 3-3.5 hours longer than the "normal" cheeses did to reach a similar bacterial population at the milk ripening stage of the cheese manufacture. Otherwise, the growth pattern of lactic acid bacteria appeared to be the same for all cheese batches.

Results on growth of S. aureus presented in Tables 12, 13, and 14 agreed with earlier published reports in that:

(1) Regardless of inoculum level used, rapid growth of S. aureus was found during the early stages of cheesemaking up to the milling stage, followed by little growth till the dressing time although the temperature in the curd during this period was favorable for staphylococcal growth (35°- 38°C), and declined gradually thereafter.

(2) Maximal growth of S. aureus was reached at the dressing stage of the cheese manufacture with the exception of Lot J30, in which maximal growth was achieved at the out-of-press stage. With an initial inoculum of 60 cells, 5×10^3 cells, and 10^6 cells per ml of milk, a population as large as 5.55×10^3 (Lot J23) or 3.1×10^3 (Lot M6), 7.9×10^4 (Lot J19) or 9.8×10^4 (Lot F6), and 1.675×10^7 (Lot J30) or 3.8×10^7 (Lot F4) S. aureus per g of curd was reached in "normal" cheeses; whereas an even

larger population of 3.75×10^5 (Lot J26) or 4.0×10^4 (Lot F11), 6.3×10^6 (Lot J21) or 3.4×10^6 (Lot M4), and 2.0×10^8 (Lot J28) or 2.21×10^8 (Lot M8) S. aureus per g was reached in slow starter cheeses. With an initial inoculum of 5×10^3 and 10^6 cells per ml of milk, a resulting population of 6.3×10^5 (Lot M13) or 4.6×10^5 (Lot M20), and 4.1×10^7 (Lot A4) or 6.0×10^7 (Lot A13) S. aureus per g of cheese was reached in cheeses made with partial slow starter.

(3) S. aureus counts decreased gradually during curing and aging.

(4) S. aureus multiplied considerably more rapidly in cheeses made with a slow starter than in cheeses made with a normal starter. They also multiplied slightly more rapidly in cheeses made with a partial slow starter than in those made with a normal starter. With an initial inoculum of 60 cells, 5×10^3 cells, and 10^6 cells per ml of milk, a resulting population of 2.9×10^3 (Lot J23) or 1.2×10^3 (Lot M6), 7.1×10^4 (Lot J19) or 7.85×10^4 (Lot F6), and 1.8×10^7 (Lot J30) or 2.25×10^7 (Lot F4) S. aureus per g of cheese was reached in normal starter cheeses; while a population of 3.05×10^5 (Lot J26) or 2.0×10^4 (Lot F11), 3.7×10^6 (Lot J21) or 1.55×10^6 (Lot M4), and 1.68×10^8 (Lot J28) or 1.11×10^8 (Lot M8) S. aureus per g of cheese was reached in "slow" cheeses even after the cheeses were pressed. With an initial inoculum of 5×10^3 cells and 10^6 cells per ml of milk, a resulting population of 1.65×10^5 (Lot M13) or 1.55×10^5 (Lot M20), and 3.45×10^7 (Lot A4) or 4.8×10^7 (Lot A13) S. aureus per g of cheese was reached in "partial slow" cheeses.

(5) A direct relationship between the counts of S. aureus in milk and those in the resulting cheeses was obtained.

(6) The extent of the staphylococcal multiplication during cheesemaking was independent of the initial numbers of S. aureus in the inoculated milk. At any one particular level of inoculum used in this investigation, the extent of S. aureus growth was different between the two individual batches made under identical conditions as presented in Figures 6-8.

(7) Survival of S. aureus during curing seemed to be prolonged by increasing the initial inoculum in milk. In this experiment, S. aureus growth was not detected in normal starter cheeses made with an initial inoculum of 60 cells per ml of milk. Yet, growth was found in cheeses made with higher inocula.

Statistical analysis performed on the bacterial counts in "normal" and "slow" cheeses is presented in Appendix Tables 14 to 17. Statistical analysis on these data revealed that:

(i) S. aureus counts obtained at the dressing and curing stages of cheese manufacture were significantly different in cheese batches made with normal and slow starter.

(ii) S. aureus counts obtained at similar stages of cheese manufacture were significantly different in cheese batches made with different levels of S. aureus inoculum.

(iii) There was no significant difference observed in the total bacterial count between the normal and slow starter cheeses made with any one particular S. aureus inoculum level. Similarly, no significant difference was observed within the "normal" or "slow" cheese batches made with varying S. aureus inoculum levels.

(iv) However, total bacterial counts observed in the dressing stage were significantly different in cheese batches made with normal starter at any one particular S. aureus inoculum level and those made with

FIG. 6 GROWTH OF S.aureus IN CHEDDAR CHEESE MANUFACTURE(S.aureus Inoculum : 60/ml)

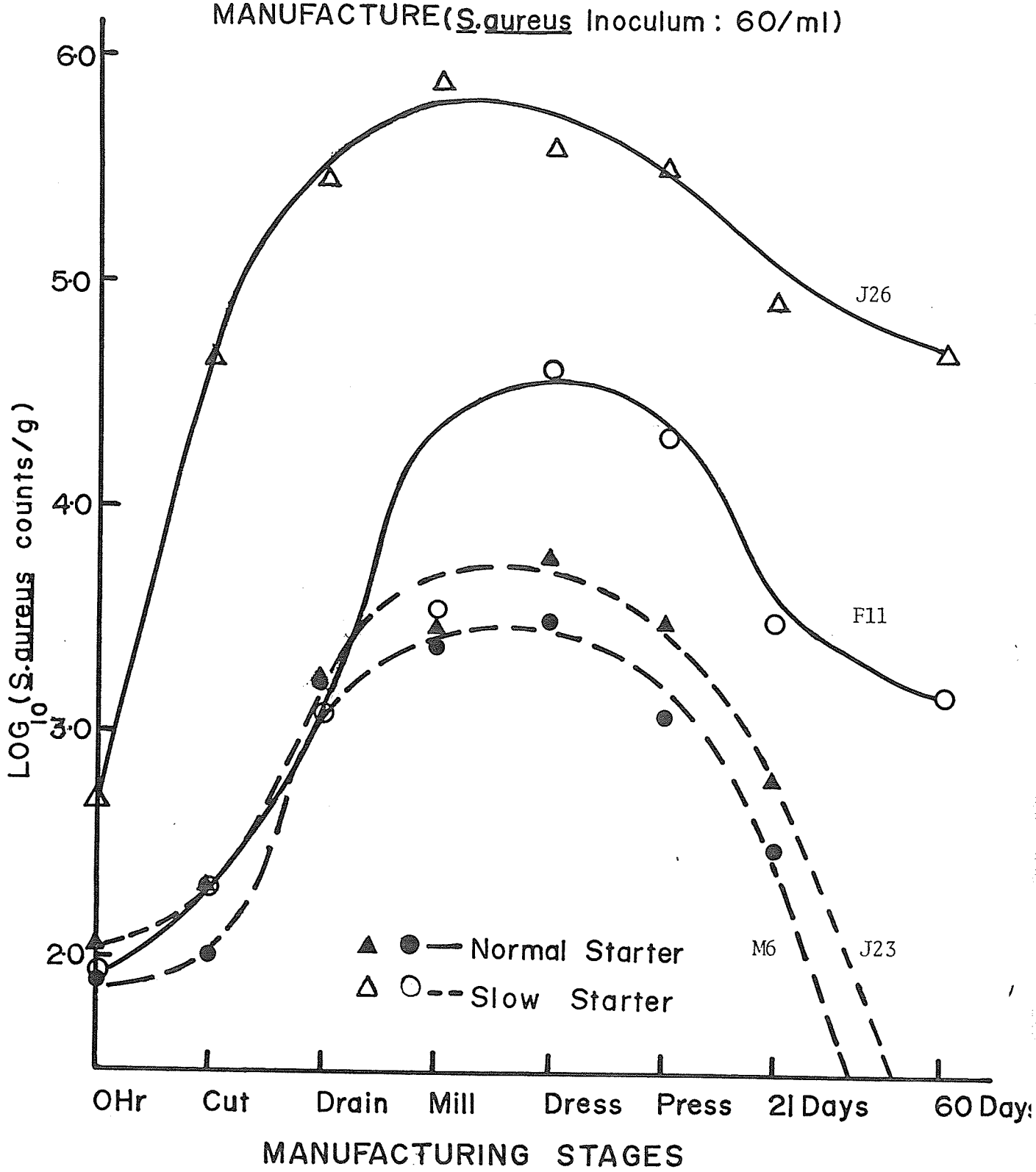


FIG. 7 GROWTH OF S. aureus IN CHEDDAR CHEESE MANUFACTURE (S. aureus Inoculum : 5×10^3/ml)

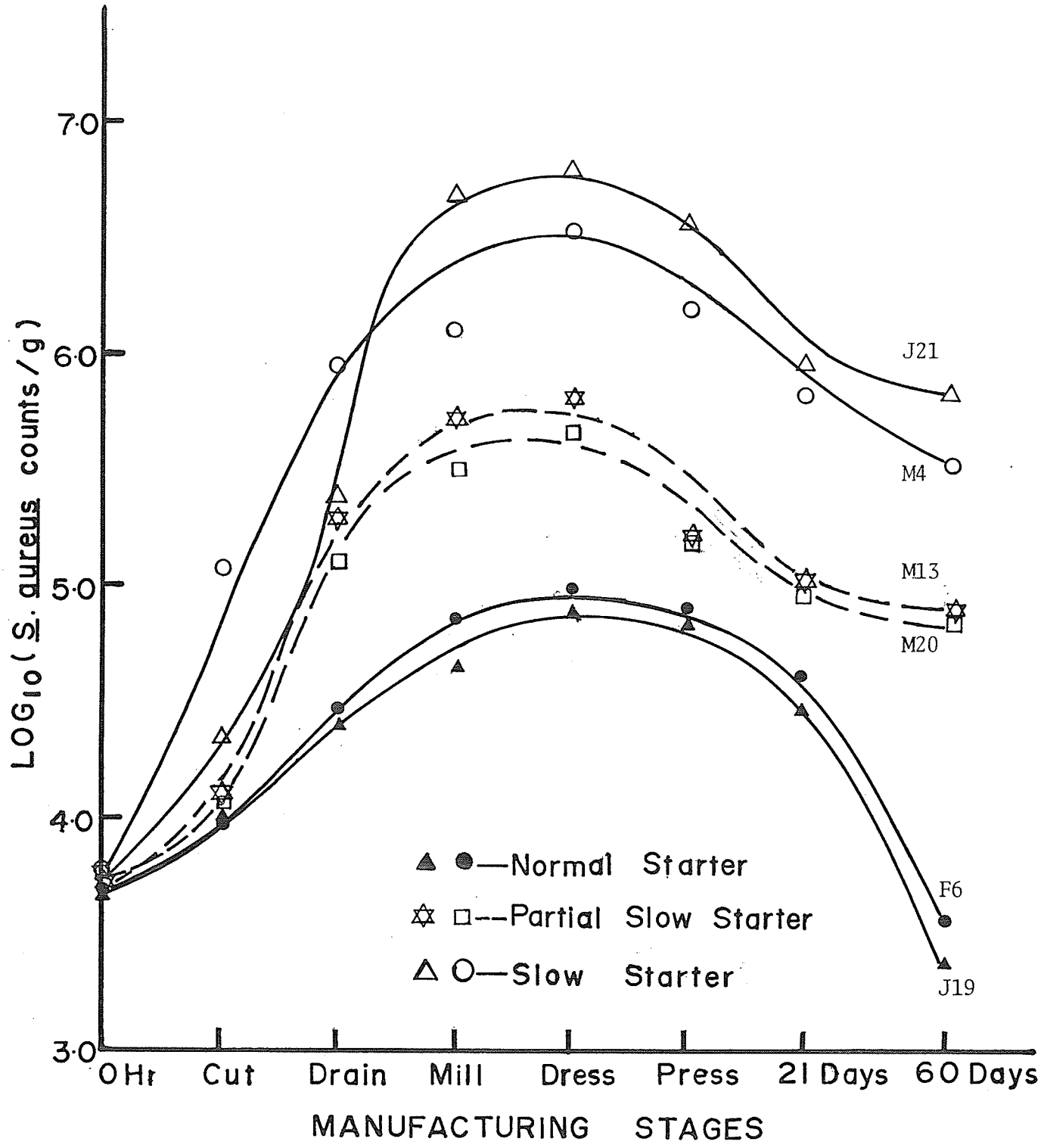
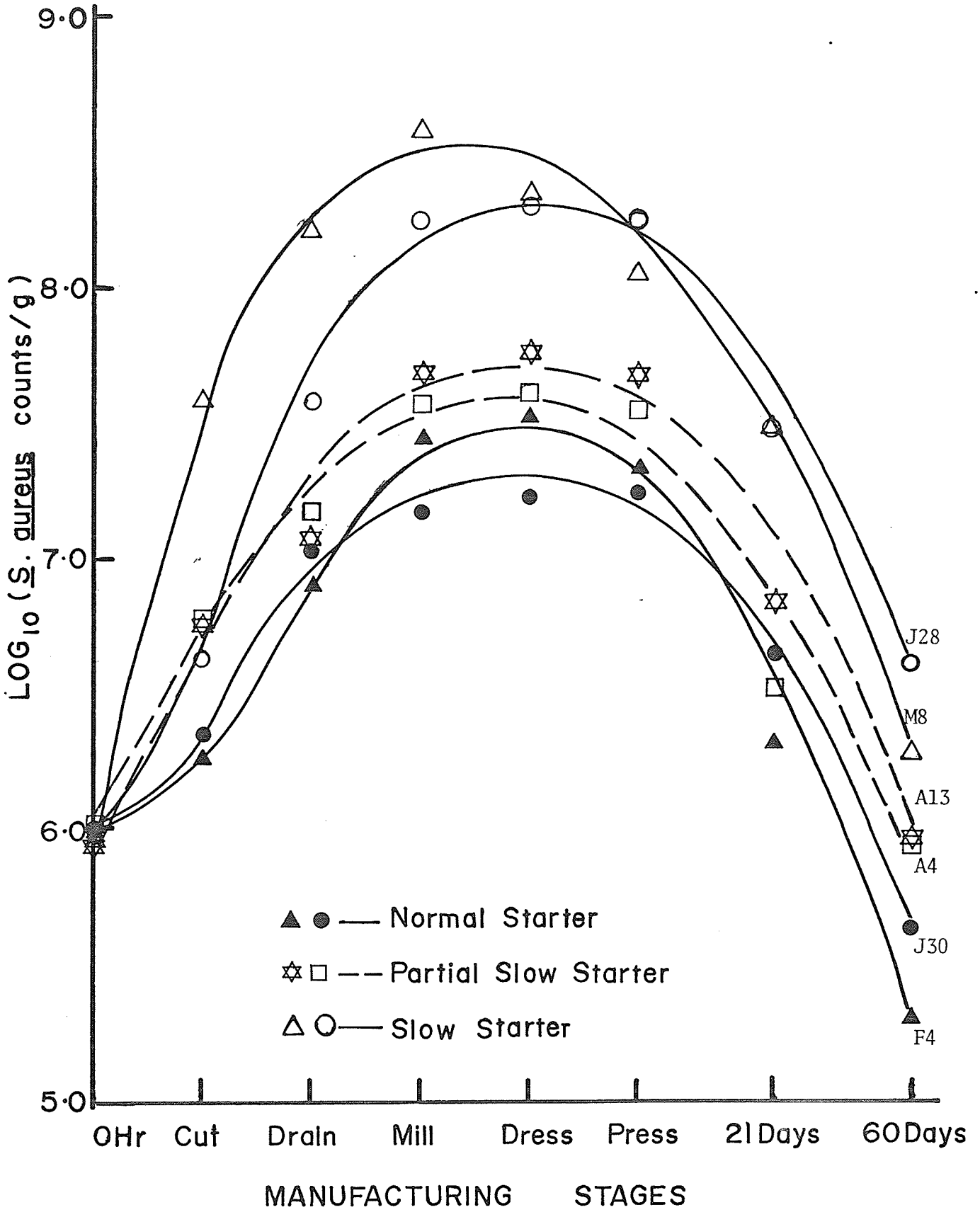


FIG. 8 GROWTH OF *S. aureus* IN CHEDDAR CHEESE MANUFACTURE (*S. aureus* Inoculum : 10^6/ml)



slow starter at another S. aureus inoculum level. Hence, the type of starter and the level of S. aureus inoculum created a combined interacting effect to produce significant differences in the total bacterial count among individual cheese batches. This interacting effect was not observed in the curing stage when the total bacterial population was dying out.

Statistical analysis performed on the bacterial counts in "normal" and "partial slow" cheeses is presented in Appendix Tables 18 to 21. Results similar to those of the "normal" and "slow" cheeses [(i) and (ii)] were obtained for the S. aureus counts in "normal" and "partial slow" cheeses. Similarly [as in (iii)], no significant difference was observed in the total bacterial counts between the "normal" and the "partial slow" cheeses made with any one particular S. aureus inoculum. Similarly, no significant difference was observed within the "normal" and "partial slow" cheeses made with varying S. aureus inoculum levels. In contrast, statistical analysis on the total bacterial counts revealed that the type of starter and the initial inoculum of S. aureus had no significant interacting effect on the counts observed in both stages of the cheese manufacture.

4. Chemical Analysis of Cheddar Cheese

Chemical analysis on the moisture, salt, and fat content of cheddar cheese made with normal, slow, and partial slow starter is presented in Tables 15, 16, and 17, respectively. All the cheese batches made were within the maximum limit of 40% moisture content, and within the minimum limit of 50% fat content (dry basis) as required by the federal government regulation.

TABLE 15. Moisture, Salt, and Fat Content of Normal Starter Cheese

	PERCENTAGE (%)					
	LEVEL of *S. aureus*# INOCULUM + IDENTIFICATION OF CHEESE					
	60/ml		5×10^3/ml		10^6/ml	
	M6	J23	J19	F6	J30	F4
MOISTURE CONTENT	34.495	38.988	37.750	36.123	39.175	38.050
SALT CONTENT	1.34	1.32	1.47	1.40	1.48	1.23
FAT CONTENT	34.0	33.5	35.5	33.5	31.5	32.5
FAT (DRY BASIS)*	51.904	54.906	57.028	52.444	51.787	52.461

* : fat on dry basis = $\frac{\text{fat content}}{100 - \text{moisture}} \times 100\%$

: *S. aureus* inoculum was Isolate No. 13

TABLE 16. Moisture, Salt, and Fat Content of Slow Starter Cheese

	PERCENTAGE (%)					
	LEVEL OF *S.aureus*[#] INOCULUM + IDENTIFICATION OF CHEESE					
	60/ml		5×10^3/ml		10^6/ml	
	J26	F11	J21	M4	M8	J28
MOISTURE CONTENT	37.490	36.658	36.520	39.858	38.793	40.070
SALT CONTENT	1.42	1.41	1.48	1.55	1.48	1.51
FAT CONTENT	32.0	33.0	33.0	31.0	31.0	31.0
FAT (DRY BASIS)*	51.191	52.097	51.984	51.544	50.647	51.727

* : fat on dry basis = $\frac{\text{fat content}}{100 - \text{moisture}} \times 100\%$

: *S. aureus* inoculum was Isolate No. 13

TABLE 17. Moisture, Salt, and Fat Content of Partial Slow Starter Cheese

	PERCENTAGE (%)			
	LEVEL OF *S. aureus*# INOCULUM + IDENTIFICATION OF CHEESE			
	5×10^3/ml		10^6/ml	
	M13	M20	A4	A13
MOISTURE CONTENT	38.150	38.125	38.658	39.340
SALT CONTENT	1.48	1.51	1.54	1.55
FAT CONTENT	33.0	33.0	31.5	31.0
FAT (DRY BASIS)*	53.355	53.333	51.351	51.105

* : fat on dry basis = $\frac{\text{fat content}}{(100 - \text{moisture})} \times 100\%$

: *S. aureus* inoculum was Isolate No. 13

Moisture contents of 36-39% were obtained in all cheese batches with the exception of Lot M6 and Lot J28, which had a moisture content of 34.5% and 40%, respectively. The salt content of the cheese batches ranged from 1.23-1.55%. A fat content of 31-35.5% was observed in the cheese batches. At any one particular S. aureus inoculum used, it was found that the fat content was higher in the cheeses made with normal starter than in those made with slow or partial slow starter.

Statistical analysis on the chemical composition of cheddar cheese batches is presented in Appendix Tables 22 to 27. Statistical analysis of these data revealed that:

(i) There were no significant differences between the moisture content of the cheeses made with normal starter and those made with slow starter. Also, the moisture content of the cheeses using different inoculum levels did not reveal any significant differences. Similar results were obtained from the statistical analysis on "normal" and "partial slow" cheeses.

(ii) No significant differences were found between the salt content of normal and slow starter cheese batches. Also, no differences were detected in those cheeses made with varying S. aureus inoculum levels. Similar results were obtained from the statistical analysis on "normal" and "partial slow" cheeses.

(iii) There were no significant differences in the fat content of the "normal" and "slow" cheeses made with different levels of S. aureus inoculum. The fat content found in "normal" cheese batches, however, was significantly different from that found in "slow" cheese batches.

(iv) Statistical analysis on the fat content of cheeses made with

normal and partial slow starter revealed significant differences in the fat content of cheeses made with different S. aureus inoculum levels. The fat content in cheese batches made with normal starter was not found to be different significantly from those batches made with partial slow starter.

DISCUSSION

Cheeses made with slow or partial slow starter were slightly softer in texture than those made with normal starter. Otherwise, there was no difference in texture and appearance. All three types of cheeses achieved normal milling and titratable acidity (Wilster, 1964) at various stages of the cheese manufacturing process. However, as demonstrated in Table 8, slow and partial slow starter took 4.50 and 2.75 hours to raise the original titratable acidity of milk by 0.02% at the milk ripening stage, thus producing an early starter failure. After pressing, the pH values for the "normal" cheeses were less than 5.4, whereas those for the "slow" cheeses were considerably higher than 5.4. Otherwise, there was no indication of starter failure. With a low S. aureus inoculum of 5×10^3 cells per ml, cheeses made with partial starter failure had pH values of 5.25 and 5.35. With a higher S. aureus inoculum, pH values for "partial slow" cheeses were 5.5 and 5.6. Therefore, the difference in pH values between "normal" and "partial slow" cheeses was less prominent. However, at any one S. aureus inoculum used, it was found that the pH values were higher in the "partial slow" cheeses than in the "normal" cheeses. Based on the above observations, cheese batches made

with a slow or partial slow starter (with high S. aureus inoculum) had a rather poor acid development in the early hours of cheesemaking, as well as in the finished product. Therefore, early starter failure could be indicated by abnormal pH observed at the out-of-press stage of the cheese manufacture. When this indication fails, as in the case of partial starter failure with a low S. aureus inoculum, early starter failure could always be detected by the total (lactic) bacterial count. Tatini et al. (1971b) reported that early starter failure may be indicated by abnormal milling acidity of whey or pH of cheese out of press, while partial starter failure was indicated only by low milling acidity. This disagreement on milling acidity as an indication of starter failure probably lies in the difference in the nature of starter failure employed. Tatini et al. (1971b) used an induced starter failure by lactic bacteriophage, whereas natural starter failures were employed in this investigation.

According to statistical analysis, the difference in pH values between the "normal" and "slow" cheese batches was significant. Also, differences among the pH values of cheese batches made with different levels of S. aureus inoculum were statistically significant. Similar results were obtained in comparing the "normal" cheeses with the "partial slow" cheeses. Thus, statistical evaluation of the data on pH values further indicated that poor acid development in the finished product resulted from early starter failure as well as extensive growth of S. aureus. The 7 toxic batches of cheddar cheese all had a pH value of 5.4 or higher. Tatini et al. (1971a) demonstrated enterotoxin production in sterile reconstituted nonfat dry milk at a pH as low as 4.5. However, differences in the pH values alone may not account for the production of

enterotoxin, since no enterotoxin was detected in Lot J26 and Lot F11, both representing relatively high pH values of 5.6 and 5.45, respectively. Although both Lot J26 and Lot F11 were slow starter cheeses, they were nevertheless made with a low initial S. aureus inoculum of 60 cells per ml of milk.

The numbers of lactic acid bacteria in a 2% starter were approximately 20-fold greater than those in a 0.3% starter, and 7-fold greater than those in a 0.5% starter. Lactic bacterial growth at the early hours of cheesemaking, therefore, indicated an apparent natural starter failure in the "slow" and "partial slow" cheese batches. Similar population levels were achieved in all the cheese batches at the draining stage of the cheese manufacturing process. However, "partial slow" cheeses took 1.75-2 hours and "slow" cheeses took 3-3.5 hours longer than the "normal" cheeses did to attain a similar population size. Statistical analysis revealed no significant differences in the lactic bacterial counts (i) between cheese batches made with normal and slow starter and (ii) among cheese batches made with different S. aureus inocula. The interacting effect created by the type of starter failure and the levels of S. aureus inoculum on the lactic bacterial counts in the individual cheese batches at the dressing stage could not be explained.

Results on growth of S. aureus in cheddar cheese of normal acidity were in agreement with earlier reports from other investigators (Takahashi and Johns, 1959; Roughley and McLeod, 1961; McLeod et al., 1962; Reiter et al., 1964; Tatini et al., 1971b). There was a 15-28 fold increase in S. aureus count in cheeses made with a normal starter. On the other hand, there was a 117-698 fold and 31-52 fold increase in cheeses made with a slow and partial slow starter, respectively. This

big contrast is expected, since S. aureus grow most rapidly during the early stages of cheesemaking, and are therefore most susceptible to changes in growth conditions caused by actively growing starter cultures. Hence, a prolonged milk ripening period, with a reduced population of lactic acid bateria, would give rise to extensive growth of S. aureus in cheeses made with starter failure. This also explains the lesser extensive growth of S. aureus in cheeses of partial starter failure as compared to that in cheeses of the slow starter. According to the findings of Takahashi and Johns (1959), there was a 10-fold increase in the staphylococcal count of cheese over that of the milk resulting from physical entrapment of organisms in the curd and was not due to growth. Studies on growth of S. aureus in cheesemaking have been reported. A 14-fold increase up to hooping was observed by Roughley and McLeod (1961). Takahashi and Johns (1959) reported obtaining a 15-45 fold increase at the end of cooking, and a 25-fold increase was observed by Tuckey et al. (1964) after pressing. Tatini et al. (1971b) reported a 16-24 fold increase in the curd during manufacture. The same authors showed that different strains of S. aureus and different types (single or mixed strains) of starter contribute to the variation in S. aureus increase. Tuckey et al. (1964) reported a further increase in S. aureus count in the first three weeks of ripening. However, results in this investigation agree with reports from other workers (McLeod et al., 1962; Roughley and McLeod, 1961; Takahashi and Johns, 1959) in that S. aureus count decreased gradually throughout the entire curing period.

At any one particular level of inoculum used in this investigation, the extent of S. aureus growth was different between the two individual batches (e.g. J30 and F4, J21 and M4 etc.) which were made

under identical conditions with an equivalent level of the same type of starter. This suggested that factors such as milk quality and milk microflora may alter the conditions for staphylococcal growth, and may eventually change the rate of growth of the test organism.

Results regarding the moisture content and fat content of the experimental cheeses were found to meet the standards set by the federal government (maximum moisture content 40%; minimum fat content 50%). The chemical composition of the cheese samples obtained from the experimental batches made with normal, partial slow, and slow starter revealed the following ranges:

MOISTURE CONTENT	34.5% - 40.0%
FAT CONTENT	31.0% - 35.5%
SALT CONTENT	1.23% - 1.55%

The salt content in the cheese batches was rather low, but this was not uncommon in a small laboratory scale production. Tatini et al. (1971b) reported a final salt content of 1.2-2.1%, and a final moisture content of 36.34-43.09%. The same workers found that the moisture content of the inhibited starter cheeses was considerably higher than that of the normal starter cheeses. They further suggested that this higher moisture content may have contributed to the production of enterotoxin in the inhibited starter cheeses, and the lack of enterotoxin in the "normal" cheeses. However, according to statistical analysis of the data obtained in this investigation, there are no significant differences in the moisture content between (i) cheeses made with normal and slow starter and (ii) cheeses made with normal and partial slow starter. Therefore, it is questionable whether moisture content can be a prime factor that will

enhance the production of enterotoxin.

At any one particular level of S. aureus inoculum used, the difference between the fat content of the "normal" cheeses and that of the "slow" cheeses was statistically significant. There were no published data with which to compare these results. Recently, several workers (Saggers and Stewart, 1968; Shah and Wilson, 1965; Vadehra and Harmon, 1969) have found that most of the S. aureus strains possess the enzyme lipase which is capable of hydrolyzing fat into free fatty acids. Other investigators (Ozdzynska and Kafel, 1968; Pazdiora and Serbus, 1970) have also reported that all the lipase-positive strains were coagulase positive, whereas lipase-negative strains did not produce this enzyme. By analyzing milk fat, several workers (Vedehra and Harmon, 1965; Luksdova and Vavrova, 1974) have demonstrated an increase in the total free fatty acids caused by the effect of staphylococcal lipase. This perhaps explains why the fat content of the "slow" cheeses is lower than that of the "normal" cheeses. However, whether this is the reason for producing low fat content in the "slow" cheeses needs to be verified. In the case of partial starter failure, statistical analysis revealed significant differences in the fat content of cheeses made with different S. aureus levels. The "partial slow" cheeses with a higher S. aureus inoculum had a fat content lower than those made with a lower inoculum.

A low fat content of 31-32.5% was obtained in 7 cheese batches, 6 of which were found to be toxic. Although cheese batch J21 was found to be toxic with a fat content of 33%, it nevertheless had a high pH value of 5.8 accompanied by an extensive growth of 6.3×10^6 S. aureus per g of cheese. Moreover, this batch of cheese was made with a slow starter. Therefore, low fat content alone could not be the prime

determinant for the presence of enterotoxin. It seems more likely that low fat content accompanied by starter failure and relatively high pH value with a high resulting staphylococcal population would then possibly signal a potential health hazard.

From the results obtained in this phase of the investigation, information on the chemical factors of cheddar cheese in relation to the possible absence of enterotoxin can be summarized as follows:

(1) The pH value of cheese out of press should be less than 5.4.

(2) The moisture content of the finished product should be less than 40%.

(3) The fat content of the finished product should be less than 33%.

CHAPTER VII

TITLE : Growth and Enterotoxin Production of S. aureus Isolate No. 13 in Cheddar Cheese Manufacture : II. Detection of Enterotoxin in Cheese

INTRODUCTION

Established conventional methods for the detection of enterotoxins in foods involve long and laborious purification and concentration procedures followed by assay using the microslide immunodiffusion technique (Casman and Bennett, 1965; Zehren and Zehren, 1968; Reiser et al., 1974). The yield of enterotoxin for these methods is rather low. In the only report of enterotoxin production in cheddar cheese manufacture, Tatini et al. (1971b) detected enterotoxin by the microslide technique with an estimated recovery of only 40-50%. The microslide immunodiffusion method is barely sufficient in sensitivity to detect the amount of enterotoxin which must be detected (i.e. 0.1-0.2 μg per 100 g of food, U.S. Food and Drug Administration). Furthermore, many workers have difficulty in achieving this sensitivity (Bergdoll et al., 1976).

Recently, research has been conducted on the development of the solid-phase RIA methods for the determination of staphylococcal enterotoxins (Collins et al., 1973; Johnson et al., 1973; Park et al., 1973). These methods are based on the competition between unlabeled and ^{125}I-labeled enterotoxins for antigen-binding sites in the anti-enterotoxin molecule. Unlabeled enterotoxin inhibits the attachment of labeled enterotoxin to an extent that is proportional to the amount of unlabeled enterotoxin present. The amount of labeled enterotoxin thus adsorbed can be measured by radioactive counting. Using the RIA technique, the requirement for purification and concentration is considerably reduced. Two systems are presently utilized in the solid-phase RIA. Employing bromacetyl-cellulose particles as the solid-phase, Collins et al. (1973) were capable of quantitating 10 ng of enterotoxins

per g of food. Johnson et al. (1973) adapted polystyrene tubes as the solid-phase with a potential recovery of 2-3 ng of enterotoxins per g of food. However, these workers failed to provide statistical evaluation to determine the actual sensitivity and precision of their assay procedures.

In attempting to assay enterotoxins in high protein foods, non-specific binding caused by the food components has been reported by several workers (Johnson et al., 1973; Bergdoll et al., 1976: Genigeogis and Kuo, 1976). This interference from food components was sufficiently large to seriously reduce the sensitivity and precision of the assay (Pober and Silverman, 1977). The purpose of this phase of the investigation, therefore, is:

(i) to develop extraction and assay procedures for the RIA with a view to minimizing the non-specific binding caused by food components; and

(ii) to determine the feasibility of the solid-phase RIA technique for the detection of enterotoxin in cheddar cheese.

EXPERIMENTAL METHODS

1. Extraction of Enterotoxin from Cheese Samples

A representative sample of cheese, consisting of 25-50 g, was blended with 2.5 volumes of Ceska's extraction/incubation buffer (Ceska et al., 1970). Extraction was performed as outlined by the Health Protection Branch, Department of Health and Welfare, Canada (Appendix 5).

Owing to the discovery of non-specific interference caused by

protein components in the cheese extract, the extraction method was modified with chloroform extraction and concentration by dialysis. Modifications are outlined as follows:

(i) Supernatant was obtained from a cheese sample using the original extraction procedures as listed in Appendix 5 (steps 1-9). The supernatant was then extracted with 20-30% chloroform (approximately 4:1).

(ii) The extraction mixture was centrifuged at 17,500 rpm for 20 minutes at 4°C (Sorvall centrifuge, Superspeed RC2-B), and the aqueous phase was separated from the chloroform layer by using a separatory flask.

(iii) The aqueous layer was then dialysed overnight in 30% (w/w) polyethylene glycol 20,000 (Fisher Scientific Co.) at 4°C.

(iv) After dialysis, the sac content was rinsed out with Ceska's incubation buffer.

Extracts were either assayed for enterotoxin immediately or frozen for assay at a later time. Details of the modifications are presented in Appendix 5.

2. Detection of Staphylococcal Enterotoxin A by the Solid-phase Radioimmunoassay

A. Iodination of Antigen. Staphylococcal enterotoxin A was labeled with ^{125}I, by the Health Protection Branch in Ottawa, using the Chloramine T method (Greenwood and Hunter, 1963). The specific activity of the ^{125}I-SEA was approximately 40 μCi per μg of protein.

B. Determination of Antibody Titer. Determination of antibody titer was performed by the Health Protection Branch in Winnipeg. Optimum sensitivity (50% uptake of ^{125}I-SEA) was obtained by using an anti-enterotoxin dilution of 1:2000 which was thus adopted for the RIA in this investigation.

C. Procedures of Radioimmunoassay. Procedures of the RIA method were provided by the Health Protection Branch in Winnipeg as outlined in Appendix 4. The following modifications were made:

(i) Aqueous cheese extract from the control cheese batches (those cheese batches made without S. aureus inoculation, namely, Lot C2 and Lot C3) was used as the diluent solution instead of Ceska's extraction buffer.

(ii) After the application of unlabeled enterotoxin, tubes were incubated at 4°C instead of 35°C. Tubes were shaken during incubation.

(iii) Unlabeled enterotoxin dilutions were allowed to equilibrate with anti-enterotoxin (several hours to overnight) prior to the administration of labeled enterotoxin.

(iv) After the administration of labeled enterotoxin, tubes were further incubated for several hours before counting.

(v) Due to the limited amount of cheese sampled, the original concentration of the cheese extract, in most cases, rather than a serial dilution, was used for the cheese sample assay.

Radioimmunoassay was performed using a single channel Beckman Biogamma analyser. This system has a counting efficiency of 65% and a background count rate of approximately 50 cpm.

D. Recovery Tests. Cheese samples obtained from the control batches (C2 and C3) were spiked with a specified dose of enterotoxin. Two ng per g of cheese were used in this investigation. Extraction procedures and RIA were performed on the spiked samples concurrent with the cheese samples tested for enterotoxin production. The ratio of the calculated dose to the spiked dose was used to determine the recovery of enterotoxin. The percentage of recovery was calculated as follows:

$$\text{percentage of recovery} = \frac{\text{calculated dose from RIA}}{\text{spiked dose in cheese homogenate}} \times 100$$

E. Interpretation of Results. The logit transformation (Rodbard et al., 1968), the simplest method to linearize conventionally obtained sigmoid dose-response curves of competitive binding assays, was applied for SEA-RIA. Calculation of the following results recorded in the RIA was performed as outlined:

(i) The average bound radioactivity expressed in cpm for each unlabeled enterotoxin dilution was calculated.

(ii) The average total radioactivity (in cpm) was also calculated for each unlabeled enterotoxin dilution.

(iii) The percentage of binding for each unlabeled enterotoxin dilution (B) was calculated as follows:

$$B = \frac{\text{average bound count}}{\text{average total count}} \times 100$$

(iv) The percentage of binding (B_o) for the negative control (tubes

without unlabeled enterotoxin), representing the maximum binding capacity of the labeled enterotoxin to the anti-enterotoxin molecules in a particular RIA, was determined as follows:

$$B_o = \frac{\text{average bound count of control}}{\text{average total count of control}} \times 100$$

(v) A standard curve was linearized by plotting logit y (where $y = \frac{B}{B_o} \times 100$) versus the logarithmic amount of unlabeled enterotoxin (i.e. log x). The regression equation of the standard curve was set up as follows:

$$\text{logit } y = I + S (\log_{10} x)$$

where I = intercept of regression line
S = slope of regression line

(vi) The logit y value for each dilution of an unknown sample (logit y_s) was also calculated in the same way as the unlabeled enterotoxin dilutions of the standard curve.

(vii) The estimated dose of an unknown sample, expressed in ng/ml, was calculated as follows:

$$\text{dose} = \text{antilog} \left(\frac{\text{logit } y_s - I}{S} \right)$$

Statistical analysis was performed on the values of the standard curve. The 95% confidence limits on the slope of the standard curve were also determined. Values of the curve were further analyzed by analysis of

variance. The 'F' test (Snedecor and Cochran, 1972) was used to examine the significance of the prediction of an unknown dose basing to the intercept and slope of the standard curve and the calculated logit y value of the unknown sample.

Sensitivity of the RIA was determined as the highest dilution of the unlabelled enterotoxin in the standard curve that gives less than 90% bound count of the negative control.

RESULTS

1. Extraction of Enterotoxin from Cheese Samples

Employing aqueous cheese extract (obtained from the control cheese batches), in place of Ceska's buffer, to obtain the standard curve values, difficulties were encountered in the RIA due to non-specific interference caused by some protein components in the cheese extracts. To determine the percentage of non-specific binding caused by the cheese components, standard curves, employing Ceska's buffer and aqueous cheese extract, were determined simultaneously using the solid-phase RIA. The percentage of non-specific binding (NSB %) caused by the protein components in the cheese extract, therefore, was calculated as follows:

$$\text{NSB \%} = (1 - R) \times 100$$

$$\text{where } R = \frac{B_o \text{ of RIA with cheese}}{B_o \text{ of RIA with buffer}}$$

providing both B_o values were obtained in the same assay. Employing the original extraction method, currently used by the Health Protection

Branch, the two standard curves obtained were found to cross each other as demonstrated in Figure 9. The percentage of 'R' was found to be 66%, which is not satisfactory. The 'R' value of 66% indicated that 34% reduction in the uptake of ^{125}I-SEA was attributed to the non-specific binding of the protein components in the cheese (Table 18). In addition, difficulties were encountered in recovering small amounts of enterotoxin present in the cheese samples. This could be attributed to the dilution of cheese sample to 2.5 times the original volume during the extraction procedures.

Results obtained so far suggested that partial purification and concentration of the cheese extracts are necessary. The extraction procedures were, therefore, modified. Most of the interfering contaminants in the cheese were removed by using chloroform extraction, centrifugation, and concentration by dialysis. These modifications resulted in obtaining two parallel standard curves as demonstrated in Figure 10, indicating the presence of a protein (enterotoxin) with immunological identity. An 'R' value of 92% obtained, however, indicated that 8% reduction in the uptake of ^{125}I-SEA was due to the non-specific binding of the cheese extract (Table 19). Further modifications were, therefore, initiated. Variations, as cited in the methodology, were made in the RIA procedures in an attempt to obtain a higher 'R' value. With this new approach, the two standard curves obtained were close, and they were parallel. Figure 11 and Table 20 demonstrate the results of one of the experimental trials. 'R' values of 95.7-96.3% were achieved in these experimental trials, indicating that only 3.7-4.3% reductions in the uptake of ^{125}I-SEA were attributed to the non-specific binding. The slight reductions were considered to be satisfactory,

Table 18. Inhibition of ^{125}I-SEA Uptake by Unlabelled Enterotoxin (with the original extraction and RIA method)

Unlabelled Enterotoxin (ng/ml)	Total count (cpm)	Bound count (cpm)	B(%)	$Y=\frac{B}{B_o}$ x100%	Logit Y
STANDARD CURVE (CESKA'S BUFFER)					
control*	16211#	1669	10.3		
0.63		1248	7.7	74.8	1.0880
1.25		1151	7.1	68.9	0.7955
2.50		1038	6.4	62.1	0.4938
5.00		745	4.6	44.7	-0.2128
10.0		486	3.0	29.1	-0.8905
20.0		283	1.8	17.0	-1.5892
40.0		178	1.1	10.7	-2.1218
	Logit Y = 0.9635 - 1.8752(log X)				
STANDARD CURVE (CHEESE EXTRACT)					
control*	16207#	1102	6.8		
0.63		940	5.8	85.3	1.7583
1.25		827	5.1	75.0	1.0986
2.50		794	4.9	72.1	0.9494
5.00		649	4.0	58.8	0.3557
10.0		519	3.2	47.1	-0.1161
20.0		357	2.2	32.4	-0.7355
40.0		211	1.3	19.1	-1.4435
	Logit Y = 1.4581 - 1.7033(log X)				

* control : tubes containing no unlabelled enterotoxin

Background count = 56 cpm (counts per minute)

: pooled total count

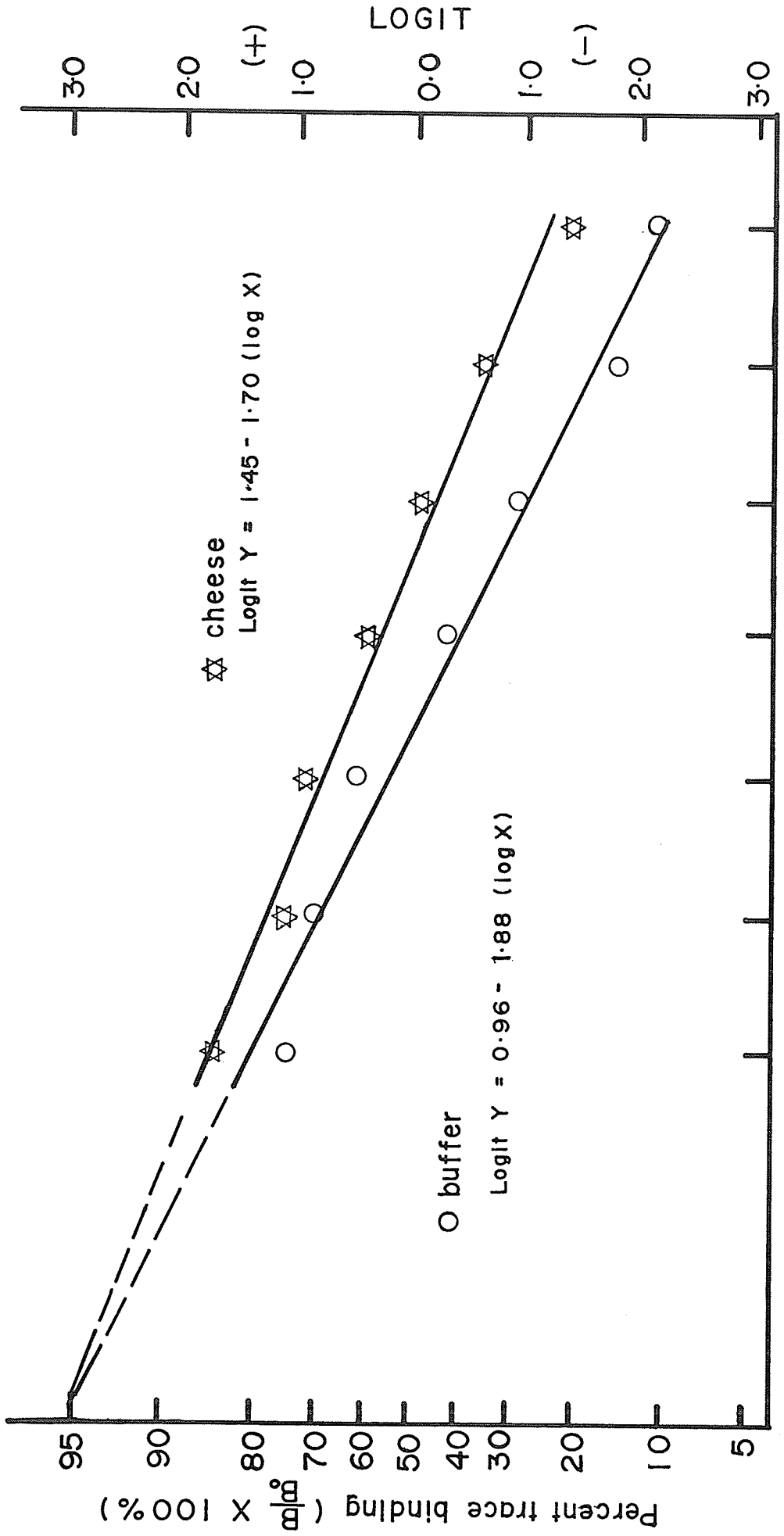


FIG. 9 LOGIT-LOG PLOT FOR STANDARD CURVES: Ceska's buffer vs. cheese extract (with the original extraction and RIA methods)

TABLE 19. Inhibition of ^{125}I-SEA Uptake by Unlabelled Enterotoxin (With the Modified Extraction Method)

Unlabelled Enterotoxin (ng/ml)	Total Count (cpm)	Bound count (cpm)	B(%)	$Y=\frac{B}{B_o}$ x100%	Logit Y
STANDARD CURVE (CESKA'S BUFFER)					
control*	20942#	1331	6.3		
0.63		1093	5.2	82.5	1.5506
1.25		1063	5.1	81.0	1.4500
2.50		846	4.0	63.5	0.5537
5.0		606	2.8	44.4	-0.2249
10.0		378	2.1	33.3	-0.6947
20.0		258	1.2	19.1	-1.4435
40.0		174	0.8	12.7	-1.9278
	Logit Y = 1.3465 - 2.0755(log X)				
STANDARD CURVE (CHEESE)					
control*	20947#	1226	5.8		
0.63		1091	5.2	89.7	2.1643
1.25		1066	5.1	87.9	1.9830
2.50		931	4.4	75.9	1.1472
5.0		779	3.7	73.8	0.5667
10.0		564	2.7	46.5	-0.1402
20.0		415	2.0	34.5	-0.6411
40.0		270	1.3	22.4	-1.2425
	Logit Y = 1.9406 - 1.9906(log X)				

* control : tubes containing no unlabelled enterotoxin

Background count = 63 cpm (counts per minute)

Date of RIA = November 4, 1976.

: pooled total count

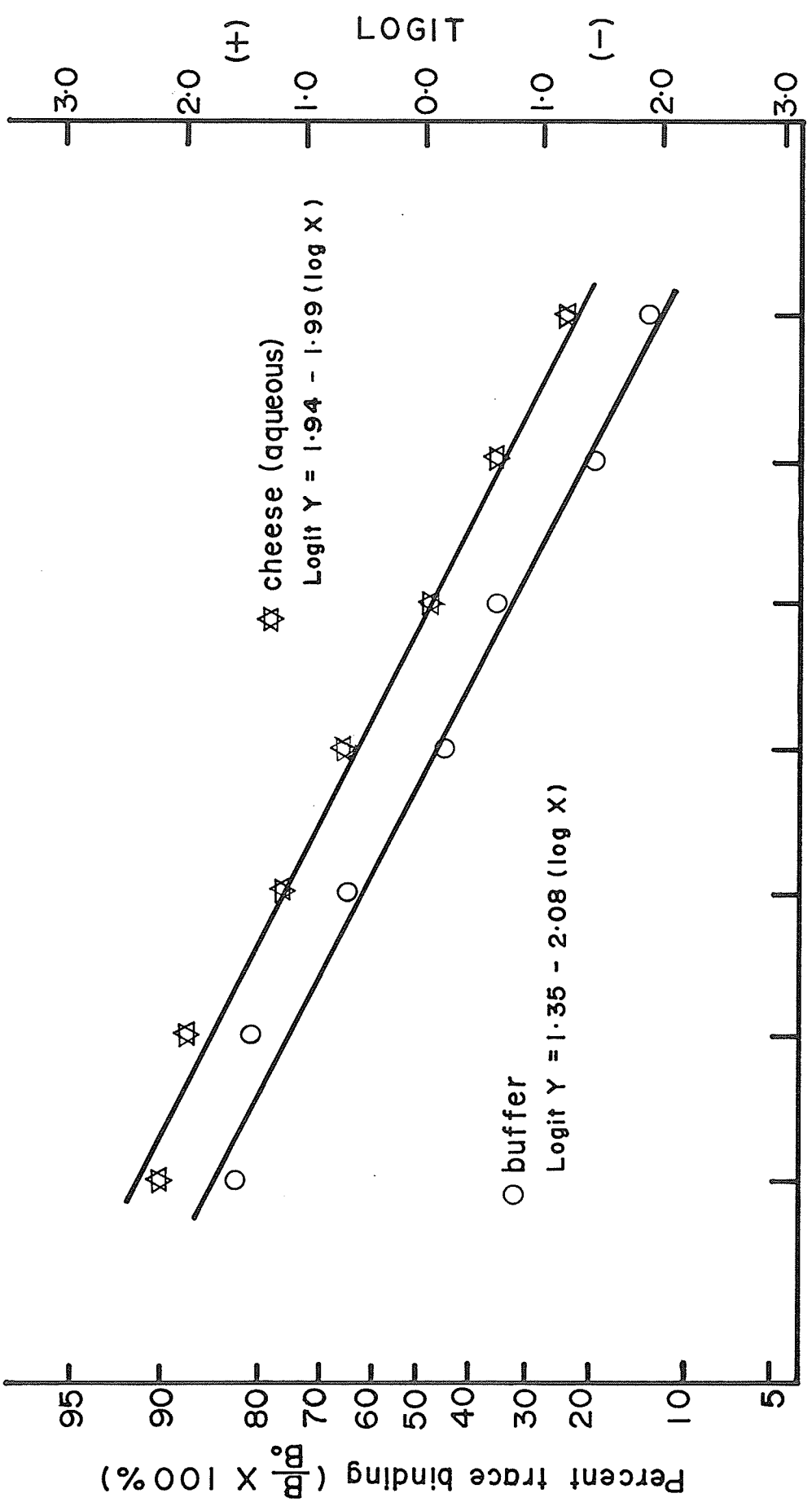


FIG. 10 LOGIT-LOG PLOT FOR STANDARD CURVE: Ceska's buffer vs. cheese extract (with the modified extraction method)

TABLE 20. Inhibition of ^{125}I-SEA Uptake by Unlabelled Enterotoxin (With the Modified Extraction and RIA Methods)

Unlabelled Enterotoxin (ng/ml)	Total count (cpm)	Bound count (cpm)	B(%)	$Y=\frac{B}{B_o}$ x100%	Logit Y
STANDARD CURVE (POOLED CESKA'S BUFFER)					
control*	15143#	1228	8.1		
0.63		1016	6.7	82.7	1.5645
1.25		979	6.5	79.7	1.3677
2.50		850	5.6	69.2	0.8095
5.00		778	5.1	63.4	0.5494
10.0		511	3.4	41.7	-0.3351
20.0		307	2.0	24.9	-1.1040
40.0		205	1.4	16.7	-1.6070
	Logit Y = 1.4527 - 1.8545(log X)				
STANDARD CURVE (POOLED CHEESE EXTRACT)					
control*	15195#	1185	7.8		
0.63		1012	6.7	85.4	1.7663
1.25		963	6.3	81.3	1.4696
2.50		865	5.7	72.9	1.2118
5.00		691	4.6	58.3	0.3351
10.0		542	3.6	45.8	-0.1684
20.0		346	2.3	29.2	-0.8857
40.0		222	1.7	18.8	-1.4631
	Logit Y = 1.6349 - 1.8746(log X)				

* control : tubes containing no unlabelled enterotoxin

Background count = 67 cpm (counts per minute)

Date of RIA = November 16, 1976.

: pooled total count

Note : anti-enterotoxin dilution used was 1:600

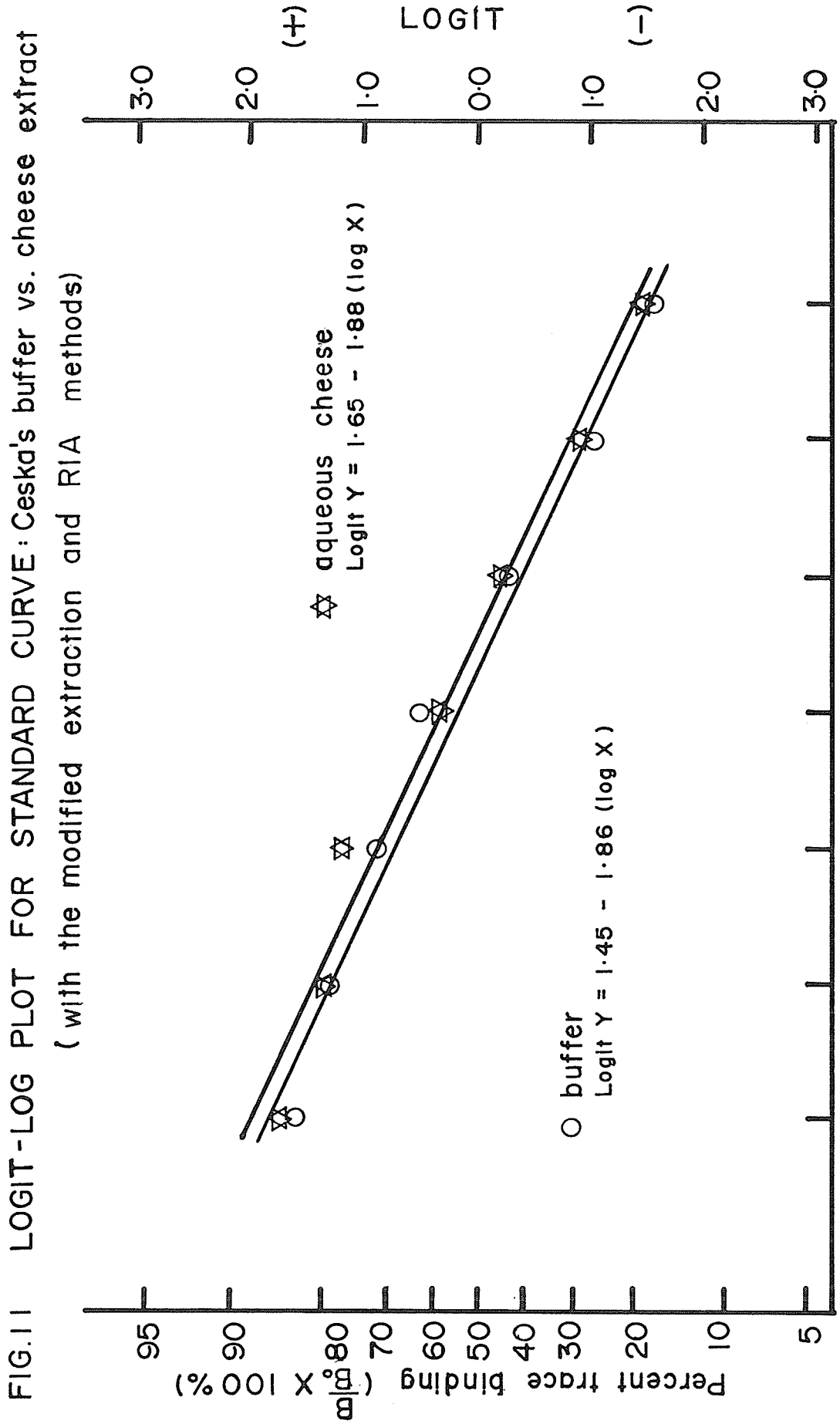


FIG. 11 LOGIT-LOG PLOT FOR STANDARD CURVE: Ceska's buffer vs. cheese extract (with the modified extraction and RIA methods)

since they might be due to random error. These modifications were, therefore, adopted for the assay of enterotoxins produced in the cheese samples.

2. Radioimmunoassays

The RIA procedure employed in this investigation was found to be sensitive to 0.31-1.0 ng of enterotoxin. Non-specific binding of the ^{125}I-SEA to the solid-phase (tubes without anti-enterotoxin and unlabeled enterotoxin) was either negative or insignificant. Radioimmunoassays on cheese samples revealed that anti-enterotoxin exhibited a maximum binding of 8.7-10.2% of the total ^{125}I-SEA. Data on the recovery of enterotoxin are presented in Appendix 30. Quantitative recovery of enterotoxin from the spiked cheese samples was estimated to be 94-95%.

3. Detection of Enterotoxin Production by S. aureus in Cheddar Cheese

A. Enterotoxin Production in Cheddar Cheese Made with Normal Starter

Enterotoxin was produced in cheddar cheese with normal acid development. Results of enterotoxin production by S. aureus in cheddar cheese made with normal starter are presented in Appendices 30-32, and summarized in Table 21.

No enterotoxin A was detected in Lot J23 and Lot M6, as well as in Lot J19 and Lot F6, which were made with an initial S. aureus inoculum of 60 and 5 x 10^3 cells per ml of milk, respectively. Enterotoxin was detected in Lot F4 after 5.5 hours with 2.9 x 10^7 S. aureus per g of cheese slurries. It was not detected at 24 hours in Lot J30, which was made under identical conditions with an equivalent level of

TABLE 21. Enterotoxin Production by S. aureus During Cheddar Cheese Manufacture with Normal Starter (c.f. Appendix 30-32).

	DETECTABLE ENTEROTOXIN					
	Level of S. aureus* + Identification of Cheese					
	60/ml		5×10^3/ml		10^6/ml	
TIME OF SAMPLING	J23	M6	J19	F6	J30	F4
CUTTING (1:30 hr)	-	-	-	-	-	(-)
DRAINING (3:20 hr)	-	-	-	-	-	(-)
MILLING (5:30 hr)	-	-	-	-	(-)	(+)
DRESSING (7:00 hr)	-	-	-	-	(-)	(+)
OUT OF PRESS(24hr)	-	-	-	-	(-)	(+)
60 DAYS	(-)	(-)	(-)	(-)	(-)	(+)
1 Year CURING	-	-	-	-	(-)	(+)

(+) : Enterotoxin detected

(-) : Enterotoxin not detected

\- : Not tested for enterotoxin

* : S. aureus inoculum in heat treated milk was Isolate No. 13.

inoculum. The resulting population in Lot J30, however, did not exceed 1.8×10^7 cells per g of cheese.

B. Enterotoxin Production in Cheddar Cheese Made with Partial Slow Starter

Results of enterotoxin production by S. aureus, presented in Appendices 30-32, are summarized in Table 22. No enterotoxin was detected in Lot M13 and Lot M20, which were made with an initial inoculum of 5×10^3 S. aureus per ml of milk. With an initial inoculum of 10^6 cells per ml of milk, enterotoxin was detected in Lot A4 and Lot A13 after 5 hours with 1.51×10^7 and 1.20×10^7 cells per g of cheese, respectively.

C. Enterotoxin Production in Cheddar Cheese Made with Slow Starter

Results of enterotoxin production by S. aureus in "slow" cheese, presented in Appendices 30-32, are summarized in Table 23. With an initial inoculum of 60 cells per ml of milk, S. aureus count increased to 7.85×10^5 cells per g in Lot J26 and 4.0×10^4 cells per g in Lot F11. No enterotoxin was detected in either cheese batch. Enterotoxin was detected in Lot J21 after 8.5 hours with 4.9×10^6 S. aureus per g of cheese, and in Lot M4 after 10 hours with 3.4×10^6 S. aureus per g. With an initial inoculum of 10^6 cells per ml of milk, enterotoxin was detected in Lot J28 and Lot M8 after 4.5 hours.

Enterotoxin persisted in all toxic cheese batches for over one year at 4°C. Non-toxic cheese batches remained negative for enterotoxin after curing for 60 days at 15°C and 4°C.

TABLE 22. Enterotoxin Production by S. aureus during Cheddar Cheese Manufacture with Partial Slow Starter (c.f. Appendix 30-32).

	DETECTABLE ENTEROTOXIN			
	Level of S. aureus*+ Identification of Cheese			
	5×10^3/ml		10^6/ml	
TIME OF SAMPLING	M13	M20	A4	A13
CUTTING (3:30 hr)	-	-	(-)	(-)
DRAINING (4:50 hr)	-	-	(+)	(+)
MILLING (7:00 hr)	-	-	(+)	(+)
DRESSING (8:30 hr)	-	-	-	-
OUT OF PRESS (24 hr)	(-)	(-)	(+)	(+)
60 DAYS	(-)	(-)	(+)	(+)
1 YEAR CURING	-	-	(+)	(+)

(+) : Enterotoxin detected

(-) : Enterotoxin not detected

\- : Not tested for enterotoxin

* : S. aureus inoculum in heat treated milk was Isolate No. 13.

TABLE 23. Enterotoxin Production by S. aureus during Cheddar Cheese Manufacture with Slow Starter (c.f. Appendix 30-32).

	DETECTABLE ENTEROTOXIN					
	Level of S. aureus*+ Identification of Cheese					
	60/ml		5×10^3/ml		10^6/ml	
TIME OF SAMPLING	J26	F11	J21	M4	J28	M8
CUTTING (4:30 hr)	-	-	-	-	(+)	(+)
DRAINING (6:20 hr)	-	-	(-)	(-)	(+)	(+)
MILLING (8:30 hr)	-	-	(+)	(-)	(+)	(+)
DRESSING(10:00 hr)	-	-	(+)	(+)	(+)	(+)
OUT OF PRESS(24hr)	-	-	(+)	(+)	(+)	(+)
60 DAYS	(-)	(-)	(+)	(+)	(+)	(+)
1 YEAR CURING	-	-	(+)	(+)	(+)	(+)

(+) : Enterotoxin detected

(-) : Enterotoxin not detected

- : Not tested for enterotoxin

* : S. aureus inoculum in heat treated milk was Isolate No. 13.

DISCUSSION

1. Extraction and Detection of Enterotoxin

Enterotoxin assay from foods by the microslide immunodiffusion technique is slow and laborious, requiring 3-4 days for completion. This is because the insensitivity of the method necessitates extensive concentration and purification. Tatini et al. (1971b) have claimed to achieve a sensitivity of 0.25 ug per 100 g of cheese. This sensitivity has not been achieved in this investigation. Bergdoll et al. (1976) reported that the minimal practical limit of this method is approximately 0.1 ug per g of food, and many workers have difficulty in achieving this sensitivity. Using the RIA method, it is possible to measure the toxin concentration in a food sample in one day, mainly because of the considerably reduced requirements for concentration and purification. In this investigation, an additional day was required for the concentration step in the extraction procedure when dealing with cheddar cheese samples in order to detect minute amount (i.e. less than 2 ng) of enterotoxin that might be present.

Currently, all the RIA were run with buffer as the medium for the determination of the standard curve. Food extracts were assayed and compared to the standard curve for positive results. However, most food systems somehow deviate from the standard buffer system. Therefore, it would be only proper to compare a given food extract to a similar system. Experimenting with aqueous cheese extract from control batches, in place of Ceska's buffer, as the medium for the standard curve, difficulty in obtaining a regression line identical to that of the buffer was encountered. This difficulty was imposed by the non-specific binding of the

cheese components to ^{125}I-SEA or anti-enterotoxin molecules. Owing to this inherent difficulty, the former extraction and RIA procedures (which were good for the bacterial dialysates and milk samples) were slightly modified.

Assaying SEA from extracts of cheese and boiled ham, no apparent interference by food material was reported by Dickie et al. (1973). In contrast, food components causing non-specific reactions in the assay procedure have been noted by several workers. It has been indicated that these food components are protein in nature (Johnson et al., 1973; Bergdoll et al., 1976; Genigeogis and Kuo, 1976). Non-specific binding caused by protein fractions in cheese may be due to the presence of a structural homologue which offers steric hindrance to the enterotoxin molecules, and may even preferentially bind to the anti-enterotoxin molecules. For future studies, gel isoelectric focusing and other electrophoretic techniques should be employed as analytical tools in elucidating some of the non-specific fractions found in the cheese extracts.

Bergdoll et al. (1976) reported that purification is necessary with certain food extracts in order to remove interfering compounds. Pober and Silverman (1977) found that it was necessary to extract cheese and chicken salad samples with purification procedures to make them more comparable to the buffer standards. In this investigation, partial purification, employing precipitation procedures and chloroform extraction, was found necessary to remove most of the interfering cheese components. Concentration by dialysis with polyethylene glycol 20,000 was also applied to extract enterotoxin from the cheese homogenates. However, this concentration step could be eliminated if the amount of

toxin in the sample is high. To detect levels of toxin below 2 ng, a twofold concentration of the sample would be sufficient. Using the modified extraction method, the non-specific binding of the cheese components was reduced to about 4%, indicating an insignificant inhibition of ^{125}I-SEA uptake by the cheese extracts. Recently, Pober and Silverman (1977) minimized the food component interference in milk samples by conditioning the anti-enterotoxin coated assay tubes overnight at 4°C with two ml of an aqueous food extract. However, their cheese slurry still contained some of the interfering food components. This procedure is very similar to the one used in this investigation, in which aqueous cheese extract was employed as the buffering medium which naturally conditioned the tubes during the assay. These authors also modified the current extraction method (for RIA usage) by applying chloroform extraction.

The solid-phase RIA technique employed in this investigation is sensitive to 0.31-1.00 ng of enterotoxin. Working with cheese extract, the maximum capacity of the labeled enterotoxin bound to the anti-enterotoxin molecules in the assays was found to be 8.7-10.2%. This maximum binding is rather low. Yet, negative control tubes (tubes containing no unlabeled enterotoxin) were included in every assay, and therefore the inherent low binding would be of little practical importance because all positive results were relatively compared to the results of the control tubes and those of the standard curve. The purpose of this investigation was to detect the presence or absence of enterotoxin in cheese samples (i.e. qualitative assays). However, quantitative recovery by the modified extraction procedure for separating enterotoxin from

spiked cheese samples and the assay by the solid-phase RIA was estimated to be 94-95% (versus 30-35% by the microslide assay). This high percentage of recovery indicated that SEA could be assayed from cheddar cheese extracts quantitatively and accurately.

Low binding capacity, nevertheless, could be attributed to: (i) the sloughing off of the anti-enterotoxin molecules from the solid-phase, and (ii) the deterioration of the labeled enterotoxin. Recently, Robern et al. (1978) purified the radioiodinated enterotoxin (^{125}I-SEA) peptides on Sephadex G-100. Two protein fractions were obtained. The aggregate fractions were removed and the monomeric fractions were used in the RIA. Removal of the aggregate fractions resulted in an increase in the initial binding of ^{125}I-SEA to anti-sera by about 30% and extended the stability of the enterotoxins. The formation of aggregate fractions after radioiodination of staphylococcal enterotoxin has been reported by several workers (Kauffman and Johnson, 1975; Niskanen and Lindroth, 1976). These radioiodinated aggregate fractions reduce binding capacity in the RIA test because: (i) they are bound by anti-sera in preference to the radioiodinated monomeric fractions; and (ii) they are not displaced as readily by non-radioactive staphylococcal enterotoxins as the monomeric fractions. Kauffman and Johnson (1975) showed that release of ^{125}I and aggregate fractions depends on specific activity of the labeled enterotoxin preparations and also on the storage time and temperature. They also found that the antigenicity of the labeled enterotoxin decreased during cold storage and has been estimated to correlate with specific activity of the preparations. In variance, Hiskanen and Lindroth (1976) have demonstrated that the iodination damage does not depend on the specific activity of the label, but rather it depends on the purity (99%)

of the label.

Lately, a double antibody RIA method was devised by Robern et al. (1975; 1978). In this method, a second antibody (anti-rabbit gamma globulin from goats) was employed to precipitate the primary antigen-antibody complex of the enterotoxin. Anti-enterotoxin molecules adsorbed to the solid-phase carrier could conceivably be displaced by other proteins in food. In the double antibody system, however, the primary antigen-antibody complex was in solution rather than adsorbed to a solid-phase, thus eliminating the displacement of the anti-enterotoxin from the solid-phase. The major problem with the double-antibody RIA method lies in the variability of the anti-rabbit gamma globulin (IgG) from preparation to preparation, thus giving inconsistent results. Recently, Bergdoll et al. (personal communication, 1978) have been working with protein A, in place of the second antibody, to precipitate the primary antigen-antibody complex. The protein A molecules, attached to the cell wall of S. aureus, bind specifically and strongly to IgG. Therefore, these methods, which are still in the experimental stage, may prove to be an advance step in the RIA technology. One limitation of the RIA technology at the present time is the requirement for purified enterotoxins (at least 90% pure) essential in the RIA method. Enterotoxins A, B, and C pose no particular problem; however, enterotoxin E is difficult to purify and D is even more so.

2. Enterotoxin Production in Cheddar Cheese

In reference to enterotoxin production, data obtained in this investigation revealed that enterotoxin could be produced in cheddar cheeses made with normal starter as well as in those cheeses made with

subnormal starter. However, an extremely high initial inoculum of S. aureus must be present to result in enterotoxin production in the normal starter cheeses. Extensive growth of the inoculum is also necessary. An initial inoculum of 10^6 cells of S. aureus per ml of milk was required to reach a population of 2.9×10^7 cells per g of cheese, in Lot F4, to be associated with enterotoxin in normal starter cheese; whereas an initial inoculum of only 3×10^5 cells per ml resulted in enterotoxin production in slow starter cheese. With partial starter failure, detectable enterotoxin was associated with an initial inoculum of 10^6 S. aureus per ml of milk and a minimal resulting population of 1.2×10^7 S. aureus per g of cheese slurries. Enterotoxin was not detected, however, in "normal" cheese batch J30, which was made under identical conditions with an equivalent level of initial inoculum, even with a resulting population of 1.8×10^7 cells per g of cheese. Therefore, actively growing starter in some way inhibited enterotoxin production by S. aureus. Other workers (Donnelly et al., 1968; Tatini et al., 1971a) have also reported a similar influence on enterotoxin by the microflora of raw milk, as well as by starter cultures in cheese (Tatini et al., 1971b). It is of interest to note that enterotoxin production was associated with 2.8×10^6 S. aureus per ml in low count heat treated milk (as presented in Chapter V) and 3.4×10^6 S. aureus per ml in slow starter cheese (as presented in Chapter VI). In contrast, no enterotoxin was detected when this population of 3 million was attained in raw milk with the presence of actively growing competing micro-organisms, as well as in cheese made with normal starter cultures. It is possible that the enterotoxin produced may have been destroyed somehow, or more likely, the conditions for enterotoxin production by S. aureus may have been altered by the growth of other

competing micro-organisms.

With an initial inoculum of 10^6 cells per ml of milk, enterotoxin was detected in normal starter cheese Lot F4 after 5.5 hours with 2.9×10^7 S. aureus per g of cheese slurries. Enterotoxin was not detected after 24 hours in Lot J30, which was made under identical conditions with an equivalent level of inoculum. The resulting population, however, did not exceed 1.8×10^7 S. aureus per g. Hence, a certain population has to be attained before enterotoxin would be produced in normal starter cheese. With an initial inoculum of 2.8×10^5 and 3.5×10^5 S. aureus per ml, Tatini et al. (1971b) obtained resulting populations of 2.8×10^7 and 1.7×10^7 cells per g, respectively. Enterotoxin was detected in the former cheese batch which was made with a single-strain starter, but not in the latter batch which was made with a mixed-type multiple-strain starter. These workers thus suggested that the type and population of starter may account for the difference in enterotoxin production. In this investigation, cheese batches Lot F4 and Lot J30 were made with the same type of starter with a similar starter population. Therefore, it is questionable whether the type of starter is the prime factor for causing enterotoxin production in one cheese but not in the other. It is more likely that the critical S. aureus population needed for enterotoxin production depends on factors such as milk quality and inherent microflora, which in turn determine the conditions for staphylococcal growth.

Enterotoxin was produced at a much lower staphylococcal population in cheese made with starter failure than in normal starter cheese. As few as 3.4×10^6 S. aureus per g of cheese (in Lot M4) resulted in enterotoxin in slow starter cheese as compared to over 29 million (in Lot F4) in normal starter cheese. The difference is less prominent with

partial slow starter cheese where a staphylococcal population of 1.2×10^7 cells per g of cheese was required for the presence of enterotoxin. With an initial inoculum of 10^6 cells per ml of milk, enterotoxin was detected in cheese Lot J28 and Lot M8 after 4.5 hours (both were made with slow starter) with 4.2×10^6 and 3.95×10^7 S. aureus per g of cheese, respectively. With an initial S. aureus inoculum of 10^6 cells per ml of milk, enterotoxin was detected in cheese batches A4 and A13 after 5 hours (both were made with partial slow starter) with 1.51×10^7 and 1.2×10^7 S. aureus per g of cheese slurries, respectively. Results obtained thus indicated that the time and growth of S. aureus required for enterotoxin production were dependent upon the degree and completeness of the starter failure. With an initial inoculum of 5×10^3 cells of S. aureus per ml of milk, enterotoxin was detected in cheddar cheese Lots J21 and M4. Both these batches were made with a slow starter. However, enterotoxin was detected in cheese Lot J21 after 8.5 hours of cheesemaking, and not in Lot M4, in which S. aureus did not reach as high a population as in Lot J21, 4.9 million versus 1.28 million. Enterotoxin was not detected in Lot M4 until after 10 hours when a S. aureus population of 3.4×10^6 cells per g of cheese was attained. Therefore, it appeared that again a certain staphylococcal population size has to be attained before enterotoxin could be produced in cheeses made with starter failure.

Results showed persistence of enterotoxin in all toxic cheese batches for over a year. In this study, the minimal population required to result in detectable enterotoxin was 29 million, 12 million, and 3.4 million for normal, partial slow, and slow starter cheeses, respectively. Tatini et al. (1971b) reported a population of 28 million and 3-5 million (S. aureus 196E and F265) for the enterotoxin production in cheddar

cheese made with normal and slow starter (starter failure induced by bacteriophage), respectively. The difference in growth reported in this study could be attributed to the higher reproducibility of the BP medium used in this investigation (versus the S110 medium used by Tatini et al.), and possibly to the difference in the strains of starter cultures and S. aureus employed. Besides, Tatini et al. reported a quantitative recovery of 50% for separating enterotoxin A from their cheese samples and the assay by the microslide technique. Therefore, different results would probably be obtained if the RIA technique was employed in place of the microslide technique for the detection of enterotoxin, since the RIA is much more sensitive.

The oxidation-reduction potential was not measured in this investigation. Yet, it has been reported (Chesbro and Aubourn, 1967; Kato et al., 1966; Thatcher et al., 1962) as a factor that has a direct influence on enterotoxin production. As has been cited in the literature review, enterotoxin production is substantially reduced under anaerobic conditions (McLean et al., 1968; Barber and Deibel, 1972; Jarvis and Lawrence, 1973). In future studies, if possible, the oxidation-reduction potential in the cheese samples should be measured. This is because the oxidation-reduction potential may account for the consistent presence of enterotoxin in cheeses of the slow starter and not in normal starter cheeses despite the attainment of similar populations. Since actively growing starter and other micro-organisms in cheese can alter the oxidation-reduction potential towards reducing conditions, and the magnitude of this change would be greater in cheese of normal starter than in slow starter cheese.

Data in this investigation apply to heat treated milk (at 61°C

for 18 seconds) used for cheddar cheese manufacture. Whether similar responses would be observed in the case of cheese milk of other heat treatment temperatures needs to be verified. In future studies, additional S. aureus strains should be tested relative to the variability of minimal population associated with enterotoxin.

From the results obtained in this phase of the investigation, the following information on the possible occurrence of SEA in cheddar cheese can be summarized:

1. The minimal initial S. aureus population in the cheese milk associated with detectable enterotoxin in the finished product was:
   (A) 10^6 cells per ml for cheese made with normal starter.
   (B) 10^6 cells per ml for cheese made with partial starter failure.
   (C) 5×10^3 cells per ml for cheese made with complete (slow) starter failure.

2. The minimal resulting S. aureus population in the cheese curd associated with detectable enterotoxin was:
   (A) 2.9×10^7 cells per ml for cheese made with normal starter.
   (B) 1.2×10^7 cells per ml for cheese made with partial starter failure.
   (C) 3.4×10^6 cells per ml for cheese made with complete starter failure.

3. The minimal time required to result in detectable enterotoxin was:
   (A) 5.5 hours for cheese made with normal starter.

(B) 4.8 hours for cheese made with partial starter failure.

(C) 4.5 hours for cheese made with complete starter failure.

GENERAL DISCUSSION AND CONCLUSIONS

A ten-month survey conducted on heat treated raw milk samples (obtained from the University of Manitoba Commercial Dairy) revealed a percentage of S. aureus survivals of 0-37.5%. Statistical analysis on survey data further suggested the possible danger of food-poisoning may result from even a fairly small indigenous staphylococcal population if the subsequent percentage of survivals is high. The survey also demonstrated the occurrence of enterotoxigenic strains of S. aureus in heat treated milk. Results obtained in this investigation suggest that S. aureus could cause a potential health problem in the milk and cheese industry. Since it is practically impossible to eliminate S. aureus from milk and cheese, criteria to ensure the safety of cheddar cheese should therefore be established.

Enterotoxin was detected in both raw and heat treated milk in this study. The minimal population of S. aureus associated with detectable enterotoxin in raw and heat treated milk was 1.3×10^7 cells and 2.8×10^6 cells per ml, respectively. Enterotoxin production was also demonstrated by S. aureus indigenous to raw milk. Those that survive the subpasteurization heat treatment of 61°C for 18 seconds attain a population of 8.25×10^7 cells per ml. Hence, ease of enterotoxin production in heat treated milks emphasizes the danger resulting from subsequent exposure to growth temperatures adequate for S. aureus such as those employed in the cheese manufacturing process.

Enterotoxin production was demonstrated in cheeses made with normal, partial slow, and slow starter. The minimal population required for enterotoxin production was 2.9×10^7, 1.2×10^7, and 3.4×10^6 cells

per ml for normal, partial slow, and slow starter cheeses, respectively.

Factors controlling growth and enterotoxin production are the nutritional completeness of the medium (i.e. chemical factors and milk quality), pH, temperature, moisture, inoculum size and type, and the effect of competing micro-organisms (i.e. starter cultures and milk microflora). The potential for staphylococcal enterotoxin production under various environmental factors must be considered in order to establish the necessary standards for cheddar cheese. Such standards must be realistic and yet maintain high criteria of safety. Ultimately, it is the sum of these factors which must concern the food hygienist and which determine the acceptance or rejection of a particular cheese lot. From the results obtained in this investigation, the following guidelines should be established to help in reducing the incidence of SEA food-poisoning in cheddar cheese:

1. The indigenous S. aureus population in raw milk before subpasteurization heat treatment should be less than 10^6 cells per ml of milk (which is much less than the staphylococcal population associated with enterotoxin, namely, 1.3×10^7 cells per ml).

2. The initial S. aureus population in heat treated milk used for cheesemaking should be less than 10^3 cells per ml.

3. The maximal resulting S. aureus population in the cheese curd should be less than 1×10^6 per g of curd (which is much less than the staphylococcal population associated with enterotoxin, namely 3.4×10^6 cells per g).

4. The starter population should be that of a 2% starter.

5. The titratable acidity at various stages of the manufacturing process should be normal.

6. The titratable acidity of whey at milling should be normal (i.e. greater than 0.5%).

7. The time required to raise the original acidity of milk by 0.02% should not be more than one hour.

8. The pH value of the cheese batch out-of-press should be less than 5.4.

9. The moisture content of the finished product should be less than 40%.

10. The percentage of fat on dry basis of the finished product should be more than 50%.

11. The fat content of the finished product should be less than 33%.

When these standards are met, the cheese product should probably not contain enterotoxin.

Results of this investigation demonstrated the feasibility of the solid-phase RIA for qualitative and quantitative detection of SEA production. The major current problem with this method is the availability of highly purified (> 90% pure) enterotoxins. The biggest problem is with the unidentified enterotoxins. As cited in Chapter III, thermonuclease test might be a possible replacement for the detection of staphylococcal enterotoxins in food. In future studies, efforts should be continued to determine (i) whether or not thermonuclease production is unique to S. aureus and (ii) whether or not thermonuclease is always

present along with the production of enterotoxin. Until this is achieved, enumeration of viable S. aureus at various stages of processing and examination of various environmental factors relative to enterotoxin production should remain as the analytical tool for routine quality control program in dairy processing plants, especially when the facilities for the detection of enterotoxin are not available.

SUMMARY

Tests of various plating media demonstrated that direct plating with Baird-Parker medium was most suitable for the isolation and enumeration of thermally stressed cells of Staphylococcus aureus from milk, especially when the initial staphylococcal count in the raw milk was low. Employing Baird-Parker medium for enumeration, the percentage of S. aureus survivals from heat treated milk over a ten-month period was found to be between 0% and 37.5%.

A heat resistant enterotoxin-A-producing strain of S. aureus, Isolate No. 13, was isolated from heat treating raw milk during a ten-month survey on raw milk samples obtained from the University of Manitoba Commercial Dairy. Isolate No. 13 was then used as the test organism for enterotoxin production studies in milk and in cheddar cheese manufacture. Growth and enterotoxin production of S. aureus in raw and heat treated milk were studied by inoculating test milks with varying concentrations of S. aureus. Enterotoxin was produced at a much lower S. aureus population in heat treated milk than in raw milk. With an initial inoculum of 10^3 - 10^4 S. aureus per ml of milk, enterotoxin was detected in raw milk after 6-8 hours, and in heat treated milk after 4-6 hours. The minimal population associated with detectable enterotoxin was 1.30×10^7 S. aureus per ml in raw milk, and 2.8×10^6 S. aureus per ml in heat treated milk.

Enterotoxin production was also demonstrated by enterotoxigenic S. aureus indigenous to raw milk that survive the subpasteurization heat treatment (61°C for 18 seconds), although the process was very much delayed due to a lag period of 4-5 hours prior to the initiation of

growth and multiplication. Enterotoxin was detected after 12 hours with a comparatively high S. aureus population of 8.25×10^7 cells per ml of milk.

The growth and enterotoxin production of S. aureus in cheddar cheese was determined by inoculating heat treated milk with three different levels of S. aureus. The inoculated milk was subsequently used to manufacture cheddar cheese using a normal (2%), or a partial slow (0.5%), or a slow (0.3%) starter. During cheesemaking, cheeses made from slow and partial slow starter required a prolonged milk ripening period of 4-4.5 hours and 2.75-3 hours, respectively. All cheese batches made with slow starter had pH values considerably higher than 5.4. All types of cheeses achieved normal titratable acidity at various stages in the cheese manufacturing process. The moisture and salt content were normal in all batches of cheese made. The fat content, however, was higher in cheeses made with a normal starter culture than in cheeses made with a slow starter culture at any particular level of S. aureus inoculum used. This phenomenon, however, was not found in comparing cheese batches made with a normal and a partial slow starter.

Cheddar cheese made with slow starter resulted in extensive growth of S. aureus. With an initial inoculum of 60 cells, 5×10^3 cells, and 10^6 cells per ml of milk, a corresponding population of 5.55×10^3 (J23) or 3.1×10^3 (M6), 7.9×10^4 (J19) or 9.8×10^4 (F6), and 1.8×10^7 (J30) or 3.8×10^7 (F4) cells per g of cheese was reached in normal starter cheeses; while a population of 3.75×10^5 (J26) or 4.0×10^4 (F11), 6.3×10^6 (J21) or 3.4×10^6 (M4), and 2.0×10^8 (J28) or 2.21×10^8 (M8) cells per g of cheese was reached in slow starter cheeses. With an initial inoculum of 5×10^3 cells and 10^6 cells per

ml of milk, a population of 6.3×10^5 (M13) or 4.6×10^5 (M20), and 4.1×10^7 (A4) or 6.0×10^7 (A13) cells per g of cheese was reached in partial slow starter cheeses.

Enterotoxin was produced at a much lower S. aureus population in slow starter cheeses than in normal starter cheeses. An initial inoculum of 10^6 cells per ml of milk was required to reach a population of 2.9×10^7 S. aureus per g of cheese, to be associated with enterotoxin in normal starter cheese. An initial inoculum of 5×10^3 cells per ml of milk resulted in enterotoxin production in slow starter cheese with a resulting population of as few as 3.4×10^6 cells per g of cheese. With an initial inoculum of 10^6 cells per ml of milk, enterotoxin was detected in partial slow starter cheese batches after 5 hours of cheese-making with 1.51×10^7 and 1.2×10^7 S. aureus per g of cheese. The minimal population required to result in detectable enterotoxin was 29 million, 12 million, and 3.4 million for normal, partial slow, and slow starter cheeses, respectively.

Enterotoxin persisted in all toxic cheese batches for over a year. Non-toxic cheese batches remained negative for enterotoxin after curing for 60 days.

A solid-phase radioimmunoassay (RIA) technique employing ^{125}I-labeled enterotoxin and polystyrene test tubes coated with specific anti-enterotoxin was used for the detection and quantitation of staphylococcal enterotoxin A (SEA) in samples collected at various stages of the cheddar cheese manufacturing process. The sensitivity of RIA was greatly reduced by protein components in cheese reacting with anti-enterotoxin or ^{125}I-SEA. This reactivity was minimized by the use of conditioned tubes and by the removal of non-specific interfering

compounds in cheese with an extraction procedure.

Extraction of SEA from cheese involved extraction of cheese at pH 4.6, centrifugation, and extraction of the supernatant fluid with chloroform at pH 7.4. The extract was then concentrated by dialysis with polyethylene glycol 20,000. The assay was a sequential RIA technique in which tubes were initially incubated with anti-enterotoxin and cheese extract (in incubation buffer containing 1% bovine serum albumin), and then with ^{125}I-SEA. The tubes were conditioned with aqueous cheese extract (acting as the incubation buffer) during the RIA analysis.

The entire RIA takes less than 24 hours, and has a sensitivity of 0.31-1.0 ng of enterotoxin per g of cheese, with a quantitative recovery of 94-95%. Non-specific inhibitions were 3.7-4.3%. Whenever the concentration procedure was applied, an additional 16-18 hours were required. The solid-phase RIA technique is sensitive, rapid, and easy to perform compared to the microslide immunodiffusion method. It is an attractive substitute for the conventional microslide immunodiffusion method.

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Appendix Table 1

Characteristics of 124 S. aureus Isolates From Heat treated Milk

Date of Isolation	Isolate Number	Colony Color on S110	Colony Color on TSA slants	Mannitol Fermentation	Gelatin Hydrolysis	Coagulase Reaction
Oct 30/74	1	W	Y	+	+	3+
"	2	O	O	S	+	5+
Nov 13/74	3	O	O	S	K	4+
Dec 4/74	4	O	O	S	+	5+
"	5	Y	W	+	K	3+
"	6	Y	Y	S	+	4+
"	7	O	Y	S	K	3+
"	8	O	Y	S	S	4+
"	9	O	Y	S	+	5+
"	10	O	O	+	K	3+
"	11	O	O	+	+	5+
"	12	O	Y	K	+	4+
"	13	O	O	S	S	5+
"	14	Y	Y	K	+	3+
"	15	O	O	S	S	5+
"	16	Y	Y	S	+	4+
"	17	O	O	K	S	3+
Jan 20/75	18	O	Y	S	+	5+
"	19	O	W	S	S	4+
"	20	O	O	S	K	4+
"	21	W	Y	+	+	3+
"	22	O	O	+	K	3+
"	23	O	Y	K	K	3+
Feb 5/75	24	O	Y	S	S	4+
"	25	Y	Y	K	K	3+
"	26	Y	W	+	K	3+
"	27	Y	Y	K	+	3+
"	28	W	Y	+	S	3+
"	29	O	O	K	+	3+
Mar 5/75	30	Y	W	+	K	3+
"	31	Y	Y	+	K	4+
"	32	O	O	S	+	5+
"	33	O	O	S	K	4+
"	34	Y	Y	S	+	4+
"	35	O	O	+	K	3+
"	36	O	O	K	+	3+
Mar 19/75	37	Y	Y	K	K	3+
"	38	Y	Y	K	K	3+
"	39	O	Y	S	S	4+
"	40	O	Y	S	S	4+
Apr 9/75	41	W	W	+	+	3+
"	42	O	Y	+	K	3+
"	43	Y	Y	K	+	3+
"	44	O	Y	S	K	3+
"	45	O	Y	S	S	4+
"	46	O	Y	S	K	3+

Appendix Table 1 (continued)

Date of Isolation	Isolate Number	Colony Color on S110	Colony Color on TSA slants	Mannitol Fermentation	Gelatin Hydrolysis	Coagulase Reaction
Apr 9/75	47	0	0	S	S	5+
"	48	0	0	+	+	5+
"	49	0	W	K	S	3+
"	50	0	Y	+	K	3+
"	51	0	Y	K	K	3+
"	52	0	Y	+	K	3+
Apr 30/75	53	W	Y	+	+	3+
"	54	Y	Y	+	K	4+
"	55	0	Y	S	+	5+
"	56	0	0	+	K	3+
"	57	Y	Y	S	+	4+
"	58	0	Y	S	S	4+
"	59	W	W	+	+	3+
"	60	0	W	K	S	3+
"	61	W	Y	+	+	3+
"	62	0	0	K	K	3+
May 14/75	63	0	Y	K	+	4+
"	64	0	Y	S	S	4+
"	65	0	0	S	+	5+
"	66	0	0	+	K	3+
"	67	0	Y	S	S	4+
"	68	0	Y	K	+	3+
"	69	Y	W	+	K	3+
"	70	0	Y	S	K	3+
May 28/75	71	W	Y	+	+	3+
"	72	W	W	K	K	3+
"	73	0	0	S	K	4+
"	74	0	0	S	S	5+
"	75	0	0	+	+	5+
"	76	0	Y	S	S	4+
"	77	W	Y	+	+	3+
"	78	W	Y	K	+	3+
"	79	0	Y	S	S	4+
"	80	Y	Y	K	K	3+
"	81	0	0	K	+	3+
"	82	0	Y	K	+	4+
June 4/75	83	0	Y	S	+	5+
"	84	0	0	+	K	3+
"	85	0	0	S	S	5+
"	86	W	Y	+	K	3+
"	87	0	W	K	S	3+
"	88	0	0	K	K	4+
"	89	0	W	S	S	4+
"	90	Y	Y	S	+	4+
"	91	W	W	K	+	3+
"	92	0	W	S	S	4+

Appendix Table 1 (continued)

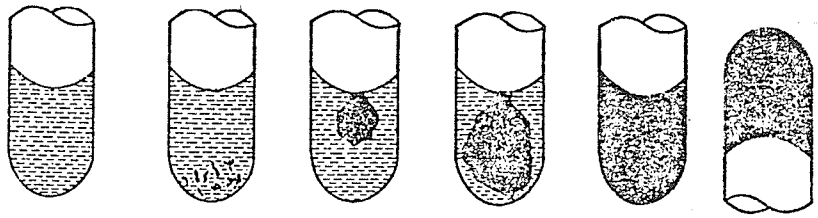
Date of Isolation	Isolate Number	Colony Color on S110	Colony Color on TSA slants	Mannitol Fermentation	Gelatin Hydrolysis	Coagulase Reaction
June 4/75	93	O	Y	K	+	4+
"	94	O	W	S	S	4+
"	95	Y	Y	K	K	3+
June 18/75	96	O	O	S	K	4+
"	97	O	O	K	+	3+
"	98	O	Y	S	K	3+
July 2/75	99	O	W	K	S	3+
"	100	O	O	K	K	4+
"	101	O	O	S	S	5+
"	102	W	Y	K	+	3+
July 2/75	103	O	W	S	S	4+
"	104	O	W	S	S	4+
"	105	W	W	K	+	3+
"	106	W	Y	+	+	3+
"	107	Y	W	+	K	3+
July 16/75	108	O	W	K	S	3+
"	109	W	Y	K	+	3+
"	110	Y	Y	+	K	4+
"	111	O	O	K	+	3+
"	112	O	W	K	S	3+
"	113	O	O	S	+	5+
"	114	O	O	S	S	5+
"	115	O	Y	K	+	4+
"	116	O	W	K	S	3+
"	117	O	W	S	S	4+
"	118	O	Y	+	K	4+
Aug 13/75	119	O	O	K	+	3+
"	120	Y	Y	+	K	4+
"	121	W	Y	K	K	3+
"	122	W	W	K	K	3+
Aug 27/75	123	O	O	S	+	5+
"	124	Y	Y	K	K	3+

Colony Color: W = white
Y = yellow
O = orange to golden

Mannitol fermentation and gelatin hydrolysis: S = strong reaction
+ = positive reaction
K = weak reaction

Appendix Table 1 (continued)

coagulase reaction:



negative 1+ 2+ 3+ 4+ 5+

negative - no evidence of fibrin formation
1+ - small unorganized clots
2+ - small organized clots
2+ - small organized clots
3+ - large organized clots
4+ - entire contents of tube coagulates but can be displaced when tube in inverted.
5+ - entire contents of tube coagulates and is not displaced when tube is inverted.

Appendix Table 2

Spearman's Rank Correlation Test

on

1. Cold months with temperatures below 0°C.

	Ranking Sample Number									
	2	3	4	5	6	7	8	9	10	
Raw milk count (X)	7	8	5	9	4	1	2½	6	2½	
% survivals (Y)	9	8	4	1	5	6	1	3	7	
Difference, d	-2	0	1	7½	-1	-5	1	3	-4½	$\Sigma d = 0$
d^2	4	0	1	56.25	-1	25	1	9	20.25	$\Sigma d^2 = 117.5$

Spearman's Rank Correlation Coefficient, $r_s = 0.0208^a$ (insignificant).

2. Warmer months with temperatures above 0°C.

	Ranking Sample Number											
	11	12	13	14	15	16	17	18	19	20	21	
Raw milk count (X)	4	6	7	8	11	1	10	5	9	2	3	
% survivals (Y)	5	1	4	2	9	11	6	10	3	7	8	
Difference, d :	-1	5	3	6	2	-10	4	-5	6	-5	-5	$\Sigma d = 0$
d^2 :	1	25	9	36	4	100	16	25	36	25	25	$\Sigma d^2 = 302$

$r_s = -0.3727^b$ (insignificant)

a - compared to "r" at the 5% level with 7 degrees of freedom.

b - compared to "r" at the 5% level with 9 degrees of freedom.

Appendix Table 3

Spearman's Rank Correlation Test

on

Overall Data of the Survey

Sample Number	Ranking (X) Raw Milk Count	(Y) % Survivals	Difference d	d^2
1	12	13	-1	1
2	8	19	-11	121
3	9	17	-8	64
4	5	10	-5	25
5	10	1½	8½	72.25
6	4	11	-7	49
7	1	15	-14	196
8	2½	1½	1	1
9	7	7	0	0
10	2½	16	-13½	182.25
11	13	8	5	25
12	15	3	12	144
13	16	6	10	100
14	17	4	13	169
15	20	14	6	36
16	6	20	-14	196
17	19	9	10	100
18	14	18	-4	16
19	18	5	13	169
20	11	12	-1	1

r_s* = -0.2737 (insignificant) $\Sigma d = 0$ $\Sigma d^2 = 1667.5$

*compared to "r" at 5% level of significance with 18 degrees of freedom.

Appendix 4

Solid-phase Radioimmunoassay of Staphylococcal Enterotoxins

I. Coating of tubes with anti-toxin solution

1. Dilute anti-toxin solutions with 0.1 M carbonate-bicarbonate buffer as follows: type A 1 : 2000
   B 1 : 5000
   C 1 : 1000

2. Pipette 2 ml of coating solution into each polystyrene tube, 16 mm X 150 mm (Beckman biovial).

3. Incubate tubes overnight at 35°C.

4. Rinse each tube twice using successive 3 ml rinse of 0.9 % saline per tube.

5. Invert tubes in cracks to let them drain until use. Tubes should be used within 2 or 3 hours.

II. Preparation of labelled enterotoxin for assay

Thaw and dilute stock solution of labelled enterotoxin to 20,000 - 30,000 cpm/0.5 ml in Ceska's incubating buffer containing 0.5% bovine serum albumin (COHN fraction 5, Sigma Company).

Type of Enterotoxin	cpm	µl/50 ml buffer
A	114,000	250
B	50,000	10
C	140,000	20

III. Preparation of standard curve

1. Dilute stock solution of unlabelled toxin (1,000 ng/ml) in Ceska's incubation buffer.

2. Prepare doubling dilutions 1:2, 1:4, 1:8, 1:16, 1:32 to 0.31 ng/ml in Ceska's incubation buffer.

3. Dilute stock solution of labelled toxin to 30,000-50,000 cpm/0.5 ml in incubation buffer, depending on the instructions accompanying each batch of labelled toxin.

4. Add 1.0 ml of each dilution of toxin + 0.5 ml of diluted labelled toxin to each of 3 tubes for a total volume of 1.5 ml/tube. Also, add 1.0 ml of incubation buffer + 0.5 ml of diluted labelled toxin to each of three tubes as a negative control for maximum binding

Appendix 4 (continued)

capacity.

5. Cap tubes, mix contents and count each tube for 0.5 minutes in γ-counter, to determine input count (Beckmann Biogamma analyser, single channel, efficiency 65%).

6. Incubate overnight at 35°C.

7. Pour out contents of tubes and rinse each tube twice with 0.9% saline (2 X 3 ml/per tube).

8. Count each tube for one minute in γ-counter to determine bound count.

IV. Radioimmunoassay of sample extract

This is set up in the same manner as the standard curve except for the following points, and is always run in conjunction with a standard curve.

1. No negative control is used, other than the one already set up with the standard curve.

2. Routinely, a dilution series of $2^0 - 2^{-4}$ is used. A higher dilution of extract should only be used if a very high level of toxin is expected in the sample.

Appendix 5

Enterotoxin Extraction from Cheese Samples

1. Where possible, weigh a 50 g representative sample and add to this (100 ml) Ceska's incubation buffer. If a smaller sample must be used, extract with 2½ volumes of buffer.

2. Blend 3 minutes at high speed.

3. Incubate at room temperature for about 30 minutes.

4. Centrifuge in large bottles for 30 minutes at 10,000 rpm (maximum RPM obtainable with large Sorvall rotor) at 4°C.

5. Filter supernatant through several layers of Kimwipes to remove solid and semi-solid fat (In some cases, the supernatant will be too thick to filter. This happens with some sausage extracts).

6. Precipitation:
   (a) Use 30-40 ml of the extract.

   (b) Adjust the pH of the extract to 4.6 using 1N HCl. Keep track of amount of HCl added for calculation of dilution factor.

7. Centrifuge extract 20 minutes at 17,500 rpm (maximum rpm obtainable with small Sorvall rotar) at 4°C.

8. Filter supernatant through grade 202 filter paper, making note of volume.

9. Adjust pH of supernatant to 7.4 $\pm$ 0.05 using 1N NaOH keeping a record of the amount added for calculation of dilution factor.

10. Centrifuge for 20 minutes at 17,500 rpm at 4°C.

11. Filter supernatant through grade 202 filter paper.

12. This extract can be either assayed for toxin immediately or frozen for assay at a later time.

Modification of Extraction

Steps 1-9 are essentially the same as those listed in the former method.

10. Add 20-30% chloroform (8 g/30 ml extract) to the supernatant, shake for 1 minute.

11. Centrifuge for 20 minutes at 17,500 rpm at 4°C.

Appendix 5 (continued)

12. Pour contents into a separatory flask, allow 5 minutes for chloroform to settle to the bottom, and draw off the choroform layer.

13. Pour top layer into a dialysis sac, (Canlab #D1615, pore size 24 angstroms), 8 inches for each original 30 ml sample, and dialyse overnight in 30% polyethylene glycol 20,000 at 4°C .

14. Rinse out the sac-contents thrice with a total volume of ¼ original sample volume (i.e. 7.5 ml buffer for a 30 ml original sample volume) of incubation buffer.

15. This extract can be either assayed for toxin immediately or frozen for assay at a later time.

Note: For extracts extracted by the original method, they should be thawed, and then chloroform should be added.

Using the same precedures as listed above, aqueous cheese extracts were obtained from the control cheese batches (C2 and C3, which are batches with no S. aureus inoculation). Aqueous cheese extracts were then served as a buffering medium for the radioimmunoassays.

Appendix Table 6

Selection of Wavelength for the Determination of Cell Turbidity of *Staphylococcus aureus* using a One ml Stock Culture in 50 ml Saline

WAVELENGTH	% TRANSMITTANCE
335	29.5
340	27.5
400	31.5
450	35.0
500	39.0
600	45.0

Appendix Table 7

S. aureus Count Versus % Transmittance at 340 nm

Assay Number	Transmittance (%) X	S. Count per ml* Y'	Log $_{10}$ Y' Y
	32.00	1.73×10^8	8.2380
	43.75	8.7×10^7	7.9395
1	51.50	5.3×10^7	7.7243
	62.50	4.7×10^7	7.6721
	82.50	2.20×10^7	7.3424
	22.0	2.07×10^8	8.3160
	30.0	1.41×10^8	8.1492
2	39.8	1.07×10^8	8.0294
	48.5	7.80×10^7	7.8921
	66.8	4.50×10^7	7.6532
	82.0	2.21×10^7	7.3444
	25.5	2.526×10^8	8.4024
	35.5	1.67×10^8	8.2227
3	42.5	1.363×10^8	8.1345
	52.5	1.016×10^8	8.0068
	60.0	8.25×10^7	7.9165
	82.0	3.20×10^7	7.5051
	24.5	2.585×10^8	8.4125
	34.0	1.85×10^8	8.2672
4	43.0	1.346×10^8	8.1290
	50.0	1.055×10^8	8.0233
	60.5	7.25×10^7	7.8603
	79.0	3.70×10^7	7.2682

* average count of duplicate platings

Appendix Table 7 (continued)

Calculated Points:

When X = 20 Y = 8.42484
X = 40 Y = 8.10544
x = 60 Y = 7.78604
X = 80 Y = 7.46664

Linear regression:

slope = -0.01597 intercept = 8.74424

regression equation : Y = 8.74424 - 0.01597(X)

Appendix Table 8

Statistical Analysis on the Standard Curve of *S. aureus*

I. Analysis of Variance Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F value[a]
regression	1	2.0602	2.0602	242.376***
deviations	21	0.1790	0.0085	
Total	22	2.2392		

a - compared to "F" with 1, 21 degrees of freedom.

* - significant at 5% level.
** - significant at 1% level.
*** - significant at 0.5% level.

II. Coefficient of determination, $r^2 = 0.92$

92% of the total variation is explained by the regression of Y on the X variable. (c.f. Appendix 10)

III. Standard error of the estimate slope, $S_b = 0.001025$.

IV. 95% confidence intervals for slope: -0.01597 ± 0.0025.

Appendix 9

The Horrall-Elliker Starter Activity Test

1. Mix 10 grams of pretested, spray-dried nonfat milk with 90 ml of distilled water and place it in an Erlenmeyer flask stoppered with cotton.

2. Sterilize the reconstituted milk in a flask at 15 lb pressure for 10 minutes.

3. Measure with a sterile pipette 10 ml of the sterile, reconstituted milk and place it in a sterile, screw-top test tube.

4. Adjust the temperature of the tube and milk to 100°F (37.5°C).

5. Measure 0.3 ml of the starter to be tested with a sterile pipette graduated to 0.1 ml and place it in the 10 ml of sterile milk. Mix it thoroughly by twirling or shaking.

6. Incubate the inoculated tube at 100°F for 3.5 hours.

7. Empty the 10.3 ml of incubated mixture into a clean flask for titration; rinse the tube with 5 ml of distilled water; add 1 ml of a 1% solution of phenolphthalein indicator; and titrate with N/10 sodium hydroxide to a faint pink color.

Interpretation:

1. Use of 4 ml or more of alkali indicates the starter is active.

2. Use of 3 to 3.5 ml of alkali indicates a "slow" culture.

3. Use of less than 3 ml of alkali indicates the starter is not active enough to use in a process where prompt acid development is required, like Cheddar.

Appendix 10

Procedure for Manufacturing Cheddar Cheese from Heat-Treated Milk

1. Preparation:
   (1) Wash vat with warm water and soda.
   (2) Rinse vat with hot water, and sanitize with 200 ppm chlorine solution.
   (3) Drain off vat and pump heat-treated milk into vat.

2. Inoculation:
   (1) Cool milk to 86°F (30°C).
   *(2) Add starter at the rate of 2%.

3. Ripening Period:
   Allow milk to ripen until the acidity of milk has increased from 0.01-0.02%.

4. Addition of coloring:
   Add cheese color at the rate of 75 ml per 1000 pounds of milk. Shake well before using and dilute about 20 times with cold water, (or add color at a rate recommended by local practices).

5. Addition of Rennet:
   (1) Add commercial calf rennet at the rate of 70 ml (or at the rate recommended) per 1000 pounds of milk.
   (2) Dilute rennet 40 times with pure cold water.
   (3) Stir milk thoroughly while rennet is being added and continue stirring for 3-5 minutes.
   (4) All motion should stop when bubbles appear unbroken on the surface.
   (5) Milk should set up in 25-30 minutes.

6. Cutting Curd:
   (a) When to cut:
      1. Curd breaks clean -- whey is clear.
      2. Can press curd away from side of vat.
      3. Two and one-half times the period from the time the rennet is added till the first sign of thickening appears gives the time for cutting.
   (b) How to cut:
      1. Cut lengthwise with a horizontal knife.
      2. Cut crosswise then lengthwise with a vertical knife.

7. Heating the curd:
   (a) Allow the curd to rest for a short time (i.e. for 5 to 10 minutes after cutting) until a film forms around the curd particles.

*Batches with slow starter were added at a rate of 0.3%.

Appendix 10 (continued)

(b) Regulation of heat
Raise the temperature of curd from 31°C to the required temperature by gradual increase at a rate to be determined on the basis of the following guidelines, relating to the acidity of whey, and the fat content of milk:

1. Length of heating time :-

Acidity of whey at cutting	Heating time
0.12%	60 minutes
0.13%	40 minutes
0.14%	30 minutes
0.145%	25 minutes
0.15%	20 minutes

2. Temperature level:-

Fat content	Temperature required
3 to 3.6%	34 to 36°C
4 to 5%	37 to 39°C

8. Draining of whey:
The time lapse between rennet addition (referred to as time of setting) and draining of whey, should be two and one quarter hours. Accordingly, after the curd is heated to the above desired temperature, hold it at this temperature for the remainder of the time period. The following points indicate the characteristic features at the time to draining:

(a) Curd is rubbery with no hollow centers.
(b) Acidity of whey ranges between 0.15 to 0.175% (when more than 0.155% acidity develops in the whey in less than two and one quarter hours from the time of setting, less starter should be added).

9. Cheddaring the curd:
(a) Piling curd
1. Pile curd along side of vat to drain.

(b) Cut curd in strips 3 to 4 inches wide and turn several times before piling.
1. Turn slabs every 10 to 15 minutes
2. In about one hour after draining, pile high and turn often to keep temperature uniform and to prevent a greasy curd.
3. Just before milling, pile 3 or 4 slabs high.

(c) As soon as the curd is piled, the whey should titrate 0.18 to 0.22% acid.

(d) The curd should be allowed to mat until the acidity of the whey reaches 0.51 to 0.55%.

Appendix 10 (continued)

(e) If the curd is firmer than usual, cut in wider slabs, pile sooner and higher.
1. If the curd is softer than usual, cut curd in smaller slabs and do not pile as soon or as high.

(f) Temperature of curd should not go below 32°C (32-34°C is the desirable range).

(g) Completion of cheddaring process:-
1. Acidity from 0.51 to 0.60%
2. Silky in appearance: velvety in feeling and tears like white chicken meat.

(h) Object of cheddaring:-
1. Regulate removal of whey.
2. Formation of characteristic body and texture

10. Milling curd:
(a) Stir pieces well (a new film is formed over the newly cut pieces):-
1. Should be impossible to squeeze whey from curd.
2. Stir 30 to 60 minutes: pieces should not mat together.

11. Salting curd:
(a) Add from one and a half to two and a half pounds of salt to one thousand pounds of milk.
(b) Stir well
(c) Allow to remain in vat until all of the salt is dissolved.

12. Hooping cheeses and pressing:
(a) Salt should be completely dissolved.
(b) Curd should be mellow and silky.
(c) Temperature between 30-32°C.
1. Have temperature high enough so the temperature will not have to be raised.

13. Pressing:
(a) Use light even pressure at first.
(b) Cheese is dressed in 30 minutes.

14. Dressing:
(a) Remove hoops from press.
(b) Invert hoop and remove cheese.
(c) Turn cheese upside down.
(d) Take press cloths off.
(e) Wet cheese with hot water.
(f) Pull out wrinkles in cheese cloth.

15. Pressing:
(a) Replace hoops in press and apply pressure as before
(b) Leave hoops in press overnight.

Appendix 10 (continued)

Note: In laboratory trials of Cheddar cheese manufacture, specially constructed stainless steel hoops (cheese capacity of about 5 pounds) were employed. After removal of the cheese from the press, it was trimmed to form even edges. It was transferred into a Cryovac bag, which was then evacuated and sealed.

Appendix Table 11

Statistical Analysis on pH Values at Curing Stage

(Normal and Slow Starter)

2 Way Classifications with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal Starter	5.30 5.20	5.20 5.25	5.35 5.40	31.70
Slow Starter	5.60 5.45	5.80 5.60	5.80 5.80	34.05
Sum	21.55	21.85	22.35	65.75

ANOVA TABLE

Source of Variance	Degree of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	0.46020	0.46020	71.260***
Level	2	0.08170	0.04085	6.325*
S X L	2	0.02165	0.00646	1.685
Error	6	0.03875	0.00646	
Total	11	0.60230		

*: significant at 5% level
**: significant at 1% level
***: significant at 0.5% level

Appendix Table 12

Statistical Analysis on pH Values at Curing
(Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	5.20 5.25	5.35 5.40	21.20
PARTIAL STARTER	5.25 5.35	5.50 5.60	21.70
Sum	21.05	21.85	42.90

ANOVA Table

Source of Variance	Degree of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	0.03125	0.03125	10.000*
Level	1	0.08000	0.08000	25.397**
S X L	1	0.00500	0.00500	1.600
Error	4	0.01250	0.00313	
Total	7	0.12875		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 13

Qualities of Milk Used for Cheddar Cheese Manufacture

A. For cheese batches made with normal starter :

	BACTERIAL COUNT per ml OR PERCENTAGE					
	Identification of Cheese Milk					
	J23	M6	J19	F6	J30	F4
SPC (raw milk)	7.6×10^4	4.9×10^2	1.8×10^3	3.7×10^2	8.4×10^2	1.1×10^5
SPC (heated milk)	2.1×10^2	3.8×10^1	2.3×10^1	4.1×10^1	7.7×10^1	7.3×10^2
fat* (%)	3.55	3.60	3.60	3.50	3.55	3.55

B. For cheese batches made with slow starter :

	BACTERIAL COUNT per ml OR PERCENTAGE					
	Identification of Cheese Milk					
	J26	F11	J21	M4	J28	M8
SPC (raw milk)	1.3×10^5	5.7×10^2	8.1×10^4	7.3×10^4	3.2×10^2	4.3×10^2
SPC (heated milk)	5.1×10^3	7.1×10^1	1.9×10^3	2.7×10^3	6.5×10^1	7.6×10^1
fat* (%)	3.45	3.50	3.60	3.50	3.55	3.55

Appendix Table 13 (continued)

C. For cheese batches made with partial slow starter :

	BACTERIAL COUNT per ml OR PERCENTAGE			
	Identification of Cheese Milk			
	M13	M20	A4	A13
SPC (raw milk)	1.4×10^3	5.9×10^2	9.6×10^2	3.7×10^3
SPC (heated milk)	6.7×10^1	7.5×10^1	6.8×10^1	4.95×10^1
fat* (%)	3.55	3.60	3.60	3.55

* : fat content was determined on duplicate samples of heat treated milk. Fat content of the raw milk samples was not determined.

Appendix Table 14

Statistical Analysis on S. aureus Count (logarithmic) at Dressing Stage of Cheddar Cheese Manufacture (Normal and Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM			
	60/ml	5 x 10^3/ml	10^6/ml	Sum
NORMAL STARTER	3.7443	4.8976	7.2240	21.9283
	3.4914	4.9912	7.5798	
SLOW STARTER	5.5740	6.7993	8.3010	40.1522
	4.6021	6.5315	8.3443	
Sum	17.4118	23.2196	31.4491	72.0805

ANOVA TABLE

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	5.6360	5.6360	55.5251***
Levels	2	24.8750	12.4375	122.5369***
S X L	2	0.3351	0.1676	1.6507
Error	6	0.6088	0.1015	
Total	11	31.4549		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 15

Statistical Analysis on Total Bacterial Count (logarithmic) at Dressing Stage of Cheddar Cheese Manufacture
(Normal and Slow Starter)

2 Way Classification with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal Starter	9.8325 9.6284	9.8573 9.6628	9.7818 9.4548	57.2176
Slow Starter	9.5250 9.6385	9.0170 9.6335	9.6335 8.7202	56.1677
Sum	37.6244	38.1706	37.5903	113.3853

ANOVA TABLE

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	0.09186	0.09186	0.7798
Levels	2	0.05302	0.02651	0.4501
S X L	2	19.37642	9.6882	82.2428*
Error	6	0.70670	0.1178	
Total	11	20.22800		

* : significant at 5% level
** : significant at 1% level
*** : significant at 0.5% level

Appendix Table 16

Statistical Analysis on *S. aureus* Count (logarithmic) After Curing for 60 Days (Normal and Slow Starter)

2 Way Classification with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal	2.0[a]	3.2672	5.6284	21.7468
Starter	2.0[a]	3.5502	5.3010	
Slow	4.7033	5.8893	6.6180	32.2084
Starter	3.1761	5.5250	6.2967	
Sum	11.8794	18.2317	23.8441	53.9552

a - assumed to be $\leq$ 100/ml.

ANOVA Table

Source of Variation	Degree of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	9.1204	9.1204	39.7230***
Levels	2	17.9171	8.9586	39.0181***
S X L	2	0.9102	0.4551	1.9821
Error	6	1.3778	0.2296	
Total	11	29.3255		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 17

Statistical Analysis on Total Bacterial Count (logarithmic) After Curing for 60 Days (Normal and Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
NORMAL STARTER	5.8603 6.8865	6.1222 6.8261	7.7443 6.4771	39.9165
SLOW STARTER	5.9890 6.5855	6.3443 6.3483	6.6484 6.9004	38.8159
Sum	25.3213	25.6409	27.7704	78.7324

ANOVA TABLE

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	0.1009	0.1009	0.3388(insig.)
Levels	2	0.8861	0.4431	1.4877(insig.)
S X L	2	0.0360	0.0180	0.0604(insig.)
Error	6	1.7868	0.2978	
Total	11	2.8098		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 18

Statistical Analysis on *S. aureus* Count (logarithmic) at Dressing Stage of Cheddar Cheese Manufacture (Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	4.8976 4.9912	7.2240 7.5798	24.6926
PARTIAL STARTER	5.7993 5.6628	7.6128 7.7782	26.8531
Sum	21.3509	30.1948	51.5457

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	0.5835	0.5835	25.7275**
Level	1	9.7768	9.7768	431.0758***
S X L	1	0.1215	0.1215	5.3571
Error	4	0.0907	0.0227	
Total	7	10.5725		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 19

Statistical Analysis on Total Bacterial Count (logarithmic) at Dressing Stage of Cheddar Cheese Manufacture
(Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	9.8573 9.6628	9.7818 9.4548	38.7567
PARTIAL STARTER	9.5250 9.6232	9.3324 9.2175	37.6972
Sum	38.6683	37.7865	76.4548

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	0.1403	0.1403	6.6969
Level	1	0.0972	0.0972	4.6396
S X L	1	0.0121	0.0121	0.5775
Error	4	0.0838	0.0209	
Total	7	0.3334		

* significant at 5% level
** significant at 1% level
*** significant at 1.5% level

Appendix Table 20

Statistical Analysis on S. aureus Count (logarithmic) after Curing for 60 Days (Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	3.2672 3.5502	5.6284 5.3010	17.7468
PARTIAL STARTER	4.8837 4.5119	5.9345 5.9777	21.3078
Sum	16.2130	22.8416	39.0546

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	1.5831	1.5831	38.7066**
Level	1	5.4923	5.4923	134.2861***
S X L	1	0.3182	0.3182	7.7790
Error	4	0.1637	0.0409	
Total	7	7.5593		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 21

Statistical Analysis on Total Bacterial Count (logarithmic) After Curing for 60 Days (Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	6.1222 6.8261	7.7443 6.4771	27.1697
PARTIAL STARTER	6.4548 6.3522	6.5092 6.3010	25.6172
Sum	25.7553	27.0316	52.7869

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	0.3013	0.3013	1.1184 (insig.)
Level	1	0.2036	0.2036	0.7558 (insig.)
S X L	1	0.2016	0.2016	0.7483 (insig.)
Error	4	1.0776	0.2694	
Total	7	1.7841		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 22

Statistical Analysis on Fat Contents of Cheddar Cheese
(Normal and Slow Starter)

2 Way Classification with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal Starter	34.0 33.5	35.5 33.5	31.5 32.5	200.5
Slow	32.0 33.0	33.0 31.0	31.0 31.0	191.0
Sum	132.5	133.0	132.5	391.5

ANOVA Table

Source of Variance	Degree of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	7.5208	7.5208	8.8050*
Levels	2	7.6250	3.8125	4.4632
S X L	2	1.2917	0.6459	0.7560
Error	6	5.1250	0.8542	
Total	11	21.5625		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 23

Statistical Analysis on Moisture Contents of Cheddar Cheese
(Normal and Slow Starter)

2 Way Classification with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal Starter	34.495 38.988	37.750 36.123	39.175 38.050	224.581
Slow Starter	37.490 36.658	36.520 39.858	38.793 40.070	229.389
Sum	147.631	150.251	156.088	453.97

ANOVA Table

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	1.9264	1.9264	0.6154 (insig.)
Levels	2	9.3713	4.6857	1.4960 (insig.)
S X L	2	0.4237	0.2119	0.0677
Error	6	18.7825	3.1304	
Total	11	30.5039		

Appendix Table 24

Statistical Analysis on Salt Content of Cheddar Cheese
(Normal and Slow Starter)
2 Way Classification with Interaction

	Levels of Inoculum			
	60/ml	5 X 10^3/ml	10^6/ml	Sum
Normal Starter	1.34 1.32	1.47 1.40	1.48 1.23	8.24
Slow Starter	1.42 1.41	1.48 1.55	1.48 1.51	8.85
Sum	5.49	5.90	5.70	17.09

ANOVA Table

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Values
Starter	1	0.031000	0.0310	5.0472 (insig.)
Levels	2	0.021025	0.0105	1.7095 (insig.)
S X L	2	0.002225	0.0011	0.1811
Error	6	0.036850	0.0061	
Total	11	0.091100		

Appendix Table 25

Statistical Analysis on Fat Contents of Cheddar Cheese
(Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	35.5 33.5	31.5 32.5	133.0
PARTIAL STARTER	33.0 33.0	31.5 31.0	128.5
Sum	135.0	126.5	261.5

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	2.5313	2.5313	3.8571
Level	1	9.0313	9.0313	13.7619*
S X L	1	0.2813	0.2813	0.4286
Error	4	2.6250	0.6563	
Total	7	14.4689		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 26

Statistical Analysis on Moisture Contents of Cheddar Cheese (Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5 X 10^3/ml	10^6/ml	Sum
NORMAL STARTER	37.750 36.123	39.175 38.050	151.098
PARTIAL STARTER	38.150 38.125	38.658 39.340	154.273
Sum	150.148	155.223	305.371

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	1.2601	1.2601	2.3024 (insig.)
Level	1	3.2195	3.2195	5.8825 (insig.)
S X L	1	0.3316	0.3316	0.6059 (insig.)
Error	4	2.1893	0.5473	
Total	7	7.0005		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix Table 27

Statistical Analysis on Salt Contents of Cheddar Cheese
(Normal and Partial Slow Starter)

2 Way Classification with Interaction

	LEVELS OF INOCULUM		
	5×10^3/ml	10^6/ml	Sum
NORMAL STARTER	1.47 1.40	1.48 1.23	5.58
PARTIAL STARTER	1.48 1.51	1.48 1.55	6.02
Sum	5.86	5.74	11.60

ANOVA Table

Source of Variance	Degrees of Freedom	Sum of Squares	Mean of Squares	F Values
Starter	1	0.0242	0.0242	2.6448 (insig.)
Level	1	0.0018	0.0018	0.1967 (insig.)
S X L	1	0.0050	0.0050	0.5465 (insig.)
Error	4	0.0366	0.0092	
Total	7	0.0676		

* significant at 5% level
** significant at 1% level
*** significant at 0.5% level

Appendix 28

Unless stated otherwise, computer programming on the data obtained in the solid-phase radioimmunoassays was written as follows:

```
//TOXINS JOB '1127,,,,CO=1,T=2,L=2,C=0','SUNNY IVY LAM',NOTIFY=ILAM
/*TSO
/*ROUTE PRINT LOCAL
// EXEC WATFIV
//GO.FT11F001 DD DSN=DATA.SET.NAME,DISP=SHR
//GO.SYSIN DD *

$JOB  WATFIV    SUNNY IVY,NOEXT,LINES=66,NOCHECK
C
C     VARIABLE TABLE
C
C     A       -  SUM OF LOG**2 (X)
C     B       -  SUM OF THE PRODUCT  LOGIT Y AND LOG X
C     C       -  SUM OF LOGIT Y
C     D       -  SUM OF LOG X
C     F       -  F STATISTICS
C     J,L,M   -  INDEXES FOR THE VECTORS
C     K       -  DEGREE OF FREEDOM OF REGRESSION
C     N       -  PARAMETER ON THE NUMBER OF DOSE SAMPLE TO SUBROUTINE
C     R       -  CORRELATION COEFFICIENT
C     S       -  SLOPE OF THE STANDARD CURVE
C     X       -  DOSE VALUES
C     Y       -  (%B / B0) X 100%
C     $B      -  %B
C     %B      -  (BOUND COUNT / TOTAL COUNT) X 100%
C     B0      -  %B OF THE NEGATIVE CONTROL
C     CV      -  COEFFICIENT OF VARIANCE
C     NO      -  A COUNT ON EACH STANDARD DOSE VALUE
C     SD      -  STANDARD DEVIATION
C     XX      -  LOGARITHMIC OF DOSE VALUE, THAT IS, LOG (X)
C     YY      -  LOGIT VALUE OF Y
C     AVG     -  THE AVERAGE OF BACKGROUND COUNTS
C     BLK     -  BLANK OF ONE BYTE
C     INT     -  INTERCEPT OF THE STANDARD CURVE
C     IPT     -  POSITION OF THE POINTS IN PLOTTING THE STANDARD CURVE
C     MSE     -  MEAN SQUARE OF ERROR
C     MSR     -  MEAN SQUARE OF REGRESSION
C     OUT     -  SUBSCRIPT OF THE DOSE VALUE TO BE ELIMINATED
C     RSQ     -  COEFFICIENT OF DETERMINATION
C     SSE     -  SUM OF SQUARE OF ERROR
```

```
C     SSR    -  SUM OF SQUARE OF REGRESSION
C     TOP    -  THE TUBE COUNT WITH THE GREATEST DIFFERENCE FROM THE
C               REST OF THE TUBE COUNTS WITHIN THE SAME TOXIN DILUTION
C     TSS    -  TOTAL SUM OF SQUARE OF REGRESSION
C     VEC    -  CHARACTER STRING OF 80 BYTES FOR EACH LINE OF THE CURVE
C               (GRAPH PLOTTING)
C     DATA   -  RADIOACTIVE COUNTS ON EACH TUBE(USED IN SUBROUTINE STAT)
C     DATE   -  DATE OF THE RADIOIMMUNOASSAY RUN
C     DIFF   -  DIFFERENCE OF A TUBE COUNT FROM THE AVERAGE OF TUBES'
C               COUNTS WITHIN THE SAME SAMPLE DILUTION
C     DOSE   -  DOSE VALUE, WHICH IS AN UNLABELLED TOXIN DILUTION
C     F001   -  F STATISTICS WITH 99% CONFIDENCE LEVEL
C     F005   -  F STATISTICS WITH 95% CONFIDENCE LEVEL
C     INUM   -  UNKNOWN SAMPLE NUMBER
C     SEES   -  STANDARD ERROR ESTIMATE OF THE SLOPE
C     SEEY   -  STANDARD ERROR ESTIMATE OF  YY
C     SUMX   -  SUM OF  X  VALUES, WHERE X IS A TUBE COUNT(SUBROUTINE STAT)
C     TIME   -  TIME IN MINUTE BEING COUNTED IN EACH TUBE
C     TYPE   -  TYPE OF UNKNOWN SAMPLES BEING ASSAYED
C     BKNUM  -  NUMBER OF BACKGROUND COUNTS
C     BLANK  -  A BLANK LINE WITH 80 BYTES
C     COUNT  -  RADIOACTIVE COUNTS IN EACH TUBE BEING ASSAYED
C     LABEL  -  VALUES ON THE VERTICAL AXIS OF THE CURVE
C     LOGIT  -  LOGIT FUNCTION
C     RDATA  -  SAME AS DATA BUT CONVERTED INTO REAL ATTRIBUTE
C     SPACE  -  SPACE ON EACH INTERVAL OF THE AXIS
C     SUMXY  -  SUM OF ( XX(J) - AVG OF XX ) ( YY(J) - AVG OF YY )
C     TIMES  -  THE NUMBER OF TUBES (PER DOSE VALUE) NOT BEING REJECTED
C     TLAVG  -  AVERAGE OF TOTAL COUNTS PER DOSE VALUE
C     TLNUM  -  NUMBER OF TUBES USED FOR ESTIMATING PIPETTING ERROR
C     TOXIN  -  THE TYPE OF STAPHYLOCOCCAL ENTEROTOXIN BEING ASSAYED
C     UBAVG  -  AVERAGE OF BOUND COUNTS PER UNKNOWN SAMPLE DILUTION
C     XXAVG  -  MEAN OF LOG (X)
C     YYAVG  -  MEAN OF LOGIT Y OF ALL X VALUES
C     AVGMXB -  AVERAGE OF MAXIMUM BINDING TUBES' BOUND COUNTS
C     AVGMXT -  AVERAGE OF MAXIMUM BINDING TUBES' TOTAL COUNTS
C     BKGAVG -  AVERAGE OF BACKGROUND COUNTS (IN SUBROUTINE STATISTICS)
C     CBOUND -  A CHARACTER STRING : "BOUND"
C     CTOTAL -  A CHARACTER STRING : "TOTAL"
C     DOSAVB -  AVERAGE OF BOUND COUNTS PER STANDARD DOSE VALUE
C     DOSAVT -  AVERAGE OF TOTAL COUNTS PER STANDARD DOSE VALUE
C     DOSECV -  COEFFICIENT OF VARIANCE OF DOSAVB OR DOSAVT
C     DOSESD -  STANDARD DEVIATION OF DOSAVB OR DOSAVT
C     DOSNUM -  THE NUMBER OF STANDARD DOSE VALUES
C     DOSPOS -  VALUE OF DOSE ON EACH LINE OF THE CURVE
C     INTVAL -  THE LENGTH OF INTERVAL BETWEEN TWO DOSE VALUES
C               ( IN LOGARITHMIC SCALE )
C     ISPACE -  NUMBER OF LINES BETWEEN TWO LABELS ON PRINTER
C     LOGITY -  POSITION OF SYMBOLS ON THE GRAPH
C     MAXBND -  NUMBER OF MAXIMUM BINDING TUBES (BOUND COUNT)
C     MAXTOT -  NUMBER OF MAXIMUM BINDING TUBES (TOTAL COUNT)
```

```
C     NUMDOS -  NUMBER OF DOSE (IN ELIMINATING DATA SECTION)
C     PIPERR -  PIPETTING ERROR
C     RSQ100 -  R**2  X  100%
C     SAMPTS -  SAMPTM, TIME OF SAMPLING IN CHEESEMAKING
C     STUBES -  NUMBER OF TUBES PER STANDARD DOSE VALUE
C     SUMSQX -  SUM OF X SQUARE, WHERE X IS THE RADIOACTIVE COUNT OF A
                TUBE (USED IN SUBROUTINE STATISTICS)
C     TUBENO -  THE TUBE NUMBER
C     UNKNOW -  TYPE OF UNKNOWN
C     UTUBES -  NUMBER OF TUBES PER UNKNOWN SAMPLE DILUTION
C     VECTOR -  THE WHOLE LINE OF THE PLOTTING BEING PRINTED
```

```
1040.        INTEGER  UNKNOW,UTUBES,STUBES,BKNUM,TUBENO,COUNT(10),
1050.       *         DOSNUM,TIMES,SPACE(7),TOTPRD(20),BNDPRD(20),
1060.       *         TLNUM,TOTDIL(20),BNDDIL(20),NOTHIN(10)/10*0/
1070.        REAL     TIME(10),DOSE(10),DOSAVT(10),DOSAVB(10),LOGIT,
1080.       *         LOGITY,INTVAL,$B(10),X(10),Y(10),XX(10),YY(10),
1090.       *         INT,LABEL(7),MSE,MSR,UTAVG(50),UBAVG(50),LOGX
1100.        CHARACTER DATE*8,TOXIN*40,TYPE*1,CTOTAL*5,CBOUND*5,
1110.       *          VEC*1(80),VECTOR*80,BLANK*80,BLK*1,LOTS*6(20)
1120.        LOGICAL ABSENS/.FALSE./
1130.        DATA     LABEL/0.31,0.63,1.25,2.5,5.0,10.,20./,
1140.       *         SPACE/7,8,7,7,8,7,1/,
1150.       *         CTOTAL/'TOTAL'/,CBOUND/'BOUND'/,
1160.       *         BLANK/'
1170.       *                              '/,
1180.       *         BLK/' '/
1190.        EQUIVALENCE (VEC,VECTOR)
1200.  C
1210.  C
1220.  C                       **********************
1230.  C                       *                    *
1240.  C                       *   MAIN   PROGRAM   *
1250.  C                       *                    *
1260.  C                       **********************
1270.  C
1280.  C
1290.  C
1300.  C     RUN INFORMATION
1310.  C     ===============
1320.  C
1330.        WRITE(6,143)
1340.        WRITE(6,100)
1350.  100   FORMAT('1',//,21X,'RUN INFORMATION',/,
1360.       *           21X,15('='),///  )
1370.        READ(11,101)DATE,UNKNOW,UTUBES,TYPE,TOXIN
1380.  101   FORMAT(A8,I4,I2,A1,A40)
1390.        WRITE(6,102)DATE
1400.  102   FORMAT('-',20X,'DATE OF RADIOIMMUNOASSAY    -',A8)
1410.        WRITE(6,103)TYPE,TOXIN,UNKNOW,UTUBES
1420.  103   FORMAT('0',20X,'STAPHYLOCOCCAL ENTEROTOXIN  -',4X,A1,//,
1430.       *           21X,'TYPE OF UNKNOWN             -',3X,A40,//,
1440.       *           21X,'NO. OF UNKNOWNS             -',I5,
1450.       *               '(POSITIVE RESULTS ONLY)',//,
1460.       *           21X,'NO. OF TUBES/UNKNOWN DIL''N  -',I5,//// )
1470.  C
1480.  C
1490.  C     STANDARD INFORMATION
1500.  C     ====================
1510.  C
1520.        READ(11,104)BKNUM,TLNUM,MAXTOT,MAXBND,DOSNUM,STUBES
1530.  104   FORMAT(6I3)
1540.        WRITE(6,105)BKNUM,TLNUM,MAXTOT,MAXBND,DOSNUM,STUBES
```

```
1550.  105   FORMAT('-',20X,'STANDARD INFORMATION',/,
1560.       *           21X,20('='),///,
1570.       *           21X,'NO. OF BACKGROUND TUBES          -',I5,//,
1580.       *           21X,'NO. OF TUBES (PIPETTING ERROR)   -',I5,//,
1590.       *           21X,'NO. OF MAXIMUM TOTAL TUBES       -',I5,//,
1600.       *           21X,'NO. OF MAXIMUM BOUND TUBES       -',I5,//,
1610.       *           21X,'NO. OF STANDARD DOSE VALUES      -',I5,//,
1620.       *           21X,'NO. OF TUBES/STANDARD DOSE VALUE -',I5   )
1630.        WRITE(6,143)
1640.  C
1650.  C
1660.  C     BASELINE STANDARDS
1670.  C     ==================
1680.  C
1690.        WRITE(6,106)
1700.  106   FORMAT('-',20X,'BASELINE STANDARDS',/,
1710.       *           21X,18('='),//,
1720.       *           24X,'TYPE',5X,'TUBE',6X,'COUNT',7X,'STD.DEV.',
1730.       *                5X,'C.V.',/,
1740.       *           21X,10('='),2X,4('='),2X,10('='),6X,8('='),
1750.       *                 4X,7('=')  )
1760.  C
1770.  C     BASELINE STANDARD : BACKGROUND
1780.  C                         ==========
1790.  C
1800.        WRITE(6,107)
1810.  107   FORMAT('0',20X,'BACKGROUND')
1820.        DO 1 J=1,BKNUM
1830.        READ(11,108)TUBENO,COUNT(J),TIME(J)
1840.  108   FORMAT(I2,1X,I6,1X,F3.2)
1850.  1     WRITE(6,109)TUBENO,COUNT(J)
1860.  109   FORMAT(' ',33X,I2,4X,I8)
1870.        IF(BKNUM.EQ.1)GOTO 2
1880.        CALL STAT(BKNUM,COUNT,TIME(1),0.,AVG,SD,CV)
1890.        WRITE(6,110)AVG,SD,CV
1900.  110   FORMAT('0',24X,'AVG ( CPM )',5X,F10.2,4X,F7.2,5X,F5.2)
1910.  C
1920.  C     BASELINE STANDARD : TOTAL (FOR ESTIMATING PIPETTING ERROR)
1930.  C                         ====================================
1940.  C
1950.  2     WRITE(6,111)
1960.  111   FORMAT('-',20X,'TOTAL (PIPETTING ERROR ESTIMATION)'/)
1970.        DO 3 J=1,TLNUM
1980.        READ(11,108)TUBENO,COUNT(J),TIME(J)
1990.  3     WRITE(6,109)TUBENO,COUNT(J)
2000.        IF(TLNUM.EQ.1)GOTO 4
2010.        CALL STAT(TLNUM,COUNT,TIME(1),0.,TLAVG,SD,CV)
2020.        WRITE(6,110)TLAVG,SD,CV
2030.  4     PIPERR=SQRT(ABS(SD**2-TLAVG))/TLAVG*100
2040.        WRITE(6,112)PIPERR
2050.  112   FORMAT('0',20X,'PIPETTING ERROR = SQRT|(S.D.**2 - AVG)',
```

```
2060.       *                '|/AVG  X 100% ','=',F5.2,'%' )
2070.  C
2080.  C     BASELINE STANDARD : MAXIMUM TOTAL
2090.  C                        =============
2100.  C
2110.        WRITE(6,113)
2120.  113   FORMAT('-',20X,'MAXIMUM TOTAL')
2130.        DO 5 J=1,MAXTOT
2140.        READ(11,108)TUBENO,COUNT(J),TIME(J)
2150.  5     WRITE(6,109)TUBENO,COUNT(J)
2160.        IF(MAXTOT.EQ.1)GOTO 6
2170.        CALL STAT(MAXTOT,COUNT,TIME(1),AVG,AVGMXT,SD,CV)
2180.        WRITE(6,110)AVGMXT,SD,CV
2190.  C
2200.  C     BASELINE STANDARD : MAXIMUM BOUND
2210.  C                        =============
2220.  C
2230.  6     WRITE(6,114)
2240.  114   FORMAT('-',20X,'MAXIMUM BOUND')
2250.        DO 7 J=1,MAXBND
2260.        READ(11,108)TUBENO,COUNT(J),TIME(J)
2270.  7     WRITE(6,109)TUBENO,COUNT(J)
2280.        IF(MAXBND.EQ.1)GOTO 8
2290.        CALL STAT(MAXBND,COUNT,TIME(1),AVG,AVGMXB,SD,CV)
2300.        WRITE(6,110)AVGMXB,SD,CV
2310.  8     BO=AVGMXB/AVGMXT*100
2320.        WRITE(6,115)BO
2330.  115   FORMAT('-',20X,'BO = (AVG MAX BOUND / AVG MAX TOTAL)',
2340.       *               '  X  100%  ','= ',F8.4,'%'   )
2350.  C
2360.  C
2370.  C     CONSTRUCT THE TABLE FOR TOTAL COUNT
2380.  C     ==================================
2390.  C
2400.        WRITE(6,116)CTOTAL,CTOTAL
2410.  116   FORMAT('1',//,21X,'STANDARD CURVE ',A5,' COUNT DATA ',/
2420.       *           21X,31('='),///
2430.       *           21X,'DOSE    TUBE     ',A5,' COUNT     ',
2440.       *               'S.D.    C.V.',/,
2450.       *           21X,47('=') )
2460.        NO = 0
2470.        DO 12 J = 1,DOSNUM
2480.        TIMES=STUBES
2490.        READ(11,*)DOSE(J),TIME(J),(COUNT(K),K=1,STUBES)
2500.        WRITE(6,120)
2510.  9     DO 11 K=1,TIMES
2520.        IF(TIMES.LT.STUBES)GOTO 10
2530.        WRITE(6,117)DOSE(J),NO+K,COUNT(K)
2540.        GOTO 11
2550.  10    WRITE(6,121)DOSE(J),COUNT(K)
2560.  11    CONTINUE
```

```
2570.  117   FORMAT(' ',20X,F5.2,4X,I2,8X,I8)
2580.        CALL STAT(TIMES,COUNT,TIME(J),AVG,DOSAVT(J),DOSESD,DOSECV)
2590.        WRITE(6,118)DOSAVT(J),DOSESD,DOSECV
2600.  118   FORMAT('0',22X,' AVG ( CPM ) = ',F10.2,2X,F8.2,F8.2)
2610.        IF(DOSECV.LT.5.)GOTO 12
2620.        WRITE(6,119)
2630.  119   FORMAT('+',65X,'** REJECT **',/)
2640.        CALL ERROR(TIMES,COUNT,DOSE(J))
2650.        IF(TIMES.GT.1)GOTO 9
2660.  12    NO=NO+3
2670.        WRITE(6,164)
2680.  164   FORMAT('-',20X,47('=') )
2690.  120   FORMAT('0')
2700.  C
2710.  C
2720.  C     CONSTRUCT THE TABLE FOR BOUND COUNT
2730.  C     ==================================
2740.  C
2750.        WRITE(6,116)CBOUND,CBOUND
2760.        NO = 0
2770.        DO 17 J=1,DOSNUM
2780.        TIMES=STUBES
2790.        READ(11,*)TIME(J),(COUNT(K),K=1,STUBES)
2800.        WRITE(6,120)
2810.  13    DO 15 K=1,TIMES
2820.        IF(TIMES.LT.STUBES)GOTO 14
2830.        WRITE(6,117)DOSE(J),NO+K,COUNT(K)
2840.        GOTO 15
2850.  14    WRITE(6,121)DOSE(J),COUNT(K)
2860.  121   FORMAT(' ',20X,F5.2,14X,I8)
2870.  15    CONTINUE
2880.        CALL STAT(TIMES,COUNT,TIME(J),AVG,DOSAVB(J),DOSESD,DOSECV)
2890.        WRITE(6,118)DOSAVB(J),DOSESD,DOSECV
2900.        IF(DOSECV.LT.5.)GOTO 17
2910.        WRITE(6,119)
2920.        CALL ERROR(TIMES,COUNT,DOSE(J))
2930.        IF(TIMES.GT.1)GOTO 13
2940.  17    NO=NO+3
2950.        WRITE(6,164)
2960.  C
2970.  C     TABLE FOR BOUND COUNT : ELIMINATING INCONSISTENT DATA
2980.  C                             ============================
2990.  C
3000.        NUMDOS=DOSNUM
3010.        DO 19 J=1,NUMDOS
3020.        IF(DOSE(J).NE.0.)GOTO 19
3030.        DOSNUM=DOSNUM-1
3040.        DO 18 K=J,DOSNUM
3050.        DOSAVT(K)=DOSAVT(K+1)
3060.        DOSAVB(K)=DOSAVB(K+1)
3070.  18    DOSE(K)=DOSE(K+1)
```

```
3080.  19    CONTINUE
3090.  C
3100.  C
3110.  C     STANDARD CURVE INFORMATION
3120.  C     ==========================
3130.  C
3140.        A=B=C=D=0.
3150.        WRITE(6,122)
3160.  122   FORMAT('1',//,21X,'STANDARD CURVE INFORMATION',/,
3170.       *           21X,26('='),//,
3180.       *           21X,'DOSE   = UNLABELLED TOXIN DILUTIONS ',
3190.       *                         '(IN NG/ML)',//,
3200.       *           21X,'B0     = %B OF NEGATIVE CONTROL',//,
3210.       *           21X,'X (J)  = DOSE CONCENTRATION OF JTH ',
3220.       *               'OBSERVATION (IN NG/ML)',//,
3230.       *           21X,'Y (J)  = (%B (J) / B0) X 100%',16X,
3240.       *             '<  ALSO CALLED B/B0 IN THE TABLE  >',//,
3250.       *           21X,'%B (J) = (BOUND COUNT(J) / TOTAL COUNT',
3260.       *             '(J)) X 100%',9X,'< FOR: J = 1,.....,N >',
3270.       *         //,21X,'XX (J) = LOG X OF JTH OBSERVATION',//,
3280.       *           21X,'YY (J) = LOGIT Y OF JTH OBSERVATION',
3290.       *           10X,'<  LOGIT Y  =  LN( Y / (100-Y) )  >',//)
3300.  C
3310.  C
3320.  C     STANDARD CURVE DATA
3330.  C     ===================
3340.  C
3350.        WRITE(6,123)
3360.  123   FORMAT('-',20X,'STANDARD CURVE DATA',/,
3370.       *           21X,19('='),////,
3380.       *           21X,90('*'),/,
3390.       *           21X,('*'),88X,('*'),/,
3400.       *           21X,'*','  DOSE   |  TOTAL COUNT  |  BOUND ',
3410.       *                   'COUNT  |   % B    |   B / B0  |',
3420.       *                   '  LOGIT Y  |   LOG X   ','*',/,
3430.       *           21X,('*'),88('='),('*')  )
3440.        DO 20 J=1,DOSNUM
3450.        $B(J)=DOSAVB(J)/DOSAVT(J)*100.
3460.        X(J)=DOSE(J)
3470.        Y(J)=$B(J)/B0*100.
3480.        XX(J)=ALOG10(X(J))
3490.        YY(J)=LOGIT(Y(J))
3500.        WRITE(6,124)X(J),DOSAVT(J),DOSAVB(J),$B(J),Y(J),
3510.       *            YY(J),XX(J)
3520.  124   FORMAT(' ',20X,'*',9X,'|',2(15X,'|'),10X,'|',2(11X,'|'),
3530.       *                  11X,'*',/,
3540.       *           21X,'*',F6.2,3X,'|',1X,F10.2,4X,'|',1X,F10.2,
3550.       *           4X,'|',1X,F6.2,3X,'|',1X,F7.2,3X,'|',2X,F7.4,
3560.       *           2X,'|',2X,F7.4,2X,'*' )
3570.        A=A+XX(J)**2
3580.        B=B+YY(J)*XX(J)
```

```
3590.          C=C+YY(J)
3600.    20    D=D+XX(J)
3610.          WRITE(6,162)
3620.    162   FORMAT(' ',20X,'*',88X,'*',/ ,21X,90('*')  )
3630.    C
3640.    C     UNWEIGHTED REGRESSION : SLOPE AND INTERCEPT
3650.    C     ==========================================
3660.    C
3670.          S=(C*D-B*DOSNUM)/(D**2-A*DOSNUM)
3680.          INT=(C-D*S)/FLOAT(DOSNUM)
3690.          WRITE(6,125)S,INT,INT,ABS(S)
3700.    125   FORMAT('-', 21X,'UNWEIGHTED REGRESSION',/,
3710.         *           21X,21('='),//,
3720.         *           21X,'S   =  SLOPE       = ',F6.2,//,
3730.         *           21X,'INT =  Y-INTERCEPT = ',F6.2,///,
3740.         *           21X,'REGRESSION EQUATION : LOGIT Y =',F6.2,
3750.         *                3X,F6.2,' X LOG (X)')
3760.          WRITE(6,142)ISIGN(1,IFIX(S)),BLK
3770.    C
3780.    C
3790.    C     PLOTTING THE STANDARD CURVE
3800.    C     ===========================
3810.    C
3820.          WRITE(6,126)
3830.    126   FORMAT('1',//,21X,'STANDARD CURVE',/,
3840.         *           21X,14('='),/,
3850.         *           21X,'VERTICAL   = DOSE (LOG SCALE) = NG/ML',/,
3860.         *           21X,'HORIZONTAL = RESPONSE         = LOGIT Y')
3870.          WRITE(6,127)
3880.    127   FORMAT('-',25X,'-3.2',6X,'-2.4',6X,'-1.6',6X,'-0.8',
3890.         *           7X,'0.0',7X,'0.8',7X,'1.6',7X,'2.4' )
3900.          WRITE(6,128)
3910.    128   FORMAT(' ',27X,'|',7('....''....|') )
3920.    C
3930.    C
3940.    C     PLOTTING OF THE VERTICAL AXIS :REFER TO DECLARATION
3950.    C     OF THE PROGRAM (UNDER THE COMMAND 'DATA')
3960.    C
3970.          M=1
3980.          WHILE(DOSE(M).LT.LABEL(1))DO
3990.               M=M+1
4000.          END WHILE
4010.          DO 28 K=1,7
4020.          IF(K.EQ.7)GOTO 21
4030.          INTVAL=(LABEL(K+1)-LABEL(K))/FLOAT(SPACE(K))
4040.          ISPACE=SPACE(K)
4050.    21    IF(K.EQ.7)INTVAL=0.
4060.          DO 27 L=1,ISPACE
4070.          IF(K.EQ.7.AND.L.GT.1)GOTO 29
4080.          DOSPOS=LABEL(K)+INTVAL*FLOAT(L-1)
4090.          VECTOR=BLANK
```

```
4100.  C
4110.  C      PLOTTING THE STANDARD CURVE : CALCULATED POINT
4120.  C                                   ================
4130.  C
4140.         LOGITY=S*ALOG10(DOSPOS)+INT
4150.         IPT=IFIX(41.5+LOGITY/0.08)
4160.         IF(IPT.LE.0)GOTO 25
4170.         VEC(IPT)='*'
4180.  C
4190.  C      PLOTTING THE STANDARD CURVE : ACTUAL AND MULTIPLE POINTS
4200.  C                                   ==========================
4210.  C
4220.         IF(M.GT.DOSNUM)GOTO 25
4230.         IF(DOSE(M).GE.DOSPOS.AND.DOSE(M).LE.DOSPOS+INTVAL)GOTO 22
4240.         GOTO 25
4250.  22     LOGITY=YY(M)
4260.         IPT=IFIX(41.5+LOGITY/0.08)
4270.         IF(IPT.LE.0)GOTO 25
4280.         IF(VEC(IPT).NE.' ')GOTO 23
4290.         VEC(IPT)='+'
4300.         GOTO 24
4310.  23     VEC(IPT)='@'
4320.  24     M=M+1
4330.  25     IF(L.EQ.1)GOTO 26
4340.         WRITE(6,129)VECTOR
4350.  129    FORMAT(' ',25X,' -',A70,'-')
4360.         GOTO 27
4370.  26     WRITE(6,130)LABEL(K),VECTOR
4380.  130    FORMAT(' ',20X,F5.2,' -',A70,'-')
4390.  27     CONTINUE
4400.  28     CONTINUE
4410.  29     WRITE(6,128)
4420.         WRITE(6,131)
4430.  131    FORMAT('0',20X,'SYMBOLS :  + = ACTUAL POINT',/,
4440.       *           32X,'* = CALCULATED POINT',/,
4450.       *           32X,'@ = MULTIPLE PT. <WHERE ACTUAL PT. ',
4460.       *               'COINCIDES WITH CALCULATED PT.> '  )
4470.  C
4480.  C
4490.  C      QUALITY CONTROL STATISTICS INFORMATION
4500.  C      ======================================
4510.  C
4520.         WRITE(6,132)
4530.  132    FORMAT('1',//,21X,'QUALITY CONTROL STATISTICS ',
4540.       *                  'INFORMATION',/,
4550.       *           21X,38('='),//,
4560.       *           21X,'N    = NUMBER OF DOSE VALUES IN THE ',
4570.       *               'STANDARD CURVE',///,
4580.       *           21X,'K    = DEGREES OF FREEDOM OF REGRESSION',
4590.       *       ///,21X,'N-1  = DEGREES OF FREEDOM OF TOTAL ',
4600.       *                       'VARIATIONS',///,
```

```
4610.       *           21X,'N-K-1= DEGREES OF FREEDOM OF ',
4620.       *               'UNEXPLAINED VARIATION'///,
4630.       *           21X,'J    = 1,....,N ' ///,
4640.       *           21X,'TSS  = TOTAL SUM OF SQUARES OF VARIATION',
4650.       *         //,25X,' = SUMMATION ( (YY(J) - ( SUMMATION ',
4660.       *               'YY(J) / N ) )**2 ) '///,
4670.       *           21X,'SSR  = SUM OF SQUARES OF VARIATION ',
4680.       *               'EXPLAINED BY  REGRESSION',//,
4690.       *           25X,' = S**2 X SUMMATION ( (XX(J) - ( SUM',
4700.       *               'MATION XX(J) / N ) )**2 )  '///,
4710.       *           21X,'SSE  = SUM OF SQUARES OF UNEXPLAINED ',
4720.       *               'VARIATION',//,
4730.       *           25X,' = TSS - SSR')
4740.        WRITE(6,156)
4750.  156   FORMAT('-',20X,'MSR  = SSR / K',///,
4760.       *           21X,'MSE  = SSE / ( N-K-1 )',///,
4770.       *           21X,'F    = MSR / MSE',///,
4780.       *           21X,'R**2        = COEFFICIENT OF DETER',
4790.       *               'MINATION = SSR / TSS',///,
4800.       *           21X,'R**2 X 100% = PERCENTAGE OF TSS ',
4810.       *               'EXPLAINED BY THE REGRESSION OF',//,
4820.       *           35X,'YY(J) ON X(J) ', ///,
4830.       *           21X,'SEE (Y)     = STANDARD ERROR ESTIMATE',
4840.       *                              ' OF YY(J)',///,
4850.       *           21X,'SEE (S)     = STANDARD ERROR ESTIMATE',
4860.       *                              ' OF THE SLOPE'  )
4870.  C
4880.  C
4890.  C     QUALITY CONTROL STATISTICS
4900.  C     =========================
4910.  C
4920.        TSS=SEES=SUMXY=0.
4930.        YYAVG=C/FLOAT(DOSNUM)
4940.        XXAVG=D/FLOAT(DOSNUM)
4950.        DO 30 J=1,DOSNUM
4960.        SEES=SEES+(XX(J)-XXAVG)**2
4970.        TSS=TSS+(YY(J)-YYAVG)**2
4980.  30    SUMXY=SUMXY+(XX(J)-XXAVG)*(YY(J)-YYAVG)
4990.        SSR=SEES*S**2
5000.        SSE=TSS-SSR
5010.        K=1
5020.        MSR=SSR/FLOAT(K)
5030.        MSE=SSE/FLOAT(DOSNUM-K-1)
5040.        F=MSR/MSE
5050.        R=SUMXY/SQRT(TSS*SEES)
5060.        RSQ=SSR/TSS
5070.        RSQ100=RSQ*100.
5080.        SEEY=SQRT(MSE)
5090.        SEES=SEEY/SQRT(SEES)
5100.        WRITE(6,133)
5110.  133   FORMAT('1',//,21X,'QUALITY CONTROL STATISTICS',/,
```

```
5120.       *           21X,26('='),////,
5130.       *           21X,'AOV  TABLE',/,
5140.       *           21X,10('='),////,
5150.       *           21X,60('*'),/,
5160.       *           21X,('*'),58X,('*'),/,
5170.       *           21X,'*',' SOURCE OF    DEGREES OF    SUM  ',
5180.       *                 'OF     MEAN',14X,'*',/,
5190.       *           21X,'*',' VARIATION     FREEDOM      ',
5200.       *               'SQUARES    SQUARE      F',6X,'*',/,
5210.       *           21X,('*'),1X,56('='),1X,('*'),/,
5220.       *           21X,('*'),58X,('*')  )
5230.        WRITE(6,134)K,SSR,MSR,F
5240.  134   FORMAT(' ',20X,'*',' REGRESSION',6X,I2,9X,F6.2,4X,F6.2,
5250.       *                   5X,F6.2,3X,'*'    )
5260.  C
5270.  C     QUALITY CONTROL STATISTICS : CHECKING SIGNIFICANCE OF F
5280.  C                                 ========================
5290.  C
5300.        READ(11,*)F001,F005,STUDET
5310.        IF(F.LT.F001)GOTO 31
5320.        WRITE(6,135)
5330.  135   FORMAT('+',76X,'**',2(/,21X,('*'),58X,('*') )  )
5340.        GOTO 32
5350.  31    IF(F.GT.F005)WRITE(6,136)
5360.  136   FORMAT('+',76X,'*',2(/,21X,('*'),58X,('*') )  )
5370.  32    WRITE(6,137)DOSNUM-K-1,SSE,MSE
5380.  137   FORMAT(' ',20X,'*',' DEVIATION',7X,I2,9X,F6.2,4X,F6.2,
5390.       *                   14X,'*',/,
5400.       *           21X,('*'),58X,('*'),/,
5410.       *           21X,('*'),1X,56('='),1X,('*'),/,
5420.       *           21X,('*'),58X,('*')  )
5430.        WRITE(6,138)DOSNUM-1,TSS
5440.  138   FORMAT(' ',20X,'*',' TOTAL',11X,I2,9X,F6.2,24X,'*',/,
5450.       *           21X,('*'),58X,('*'),/,
5460.       *           21X,60('*')  )
5470.        WRITE(6,139)K,DOSNUM-K-1,F005,K,DOSNUM-K-1,F001
5480.  139   FORMAT('-',20X,'F(',I1,',',I1,',.05) = ',F6.2,//,
5490.       *           21X,'F(',I1,',',I1,',.01) = ',F6.2,///,
5500.       *           21X,'*   : SIGNIFICANT AT 5% LEVEL',/,
5510.       *           21X,'**  : SIGNIFICANT AT 1% LEVEL',//// )
5520.        WRITE(6,140)
5530.  140   FORMAT('-',20X,'CORR. COEFF.     R**2     100% X R**2',
5540.       *                '     SEE(Y)     SEE(S)',/,
5550.       *           21X,59('='))
5560.        WRITE(6,141)R,RSQ,RSQ100,SEEY,SEES
5570.  141   FORMAT('0',20X,F8.4,7X,F6.2,5X,F8.2,6X,F8.4,3X,F8.4,// )
5580.  142   FORMAT('+',59X,I2,T62,A1)
5590.  143   FORMAT('1')
5600.        WRITE(6,155)S-STUDET*SEES,S+STUDET*SEES
5610.  155   FORMAT('-',20X,'95% CONFIDENCE INTERVAL FOR S = ',
5620.       *              F7.4,'< S < ',F7.4   )
```

```
5630.  C
5640.  C
5650.  C     UNKNOWN SECTION FOR TOTAL COUNT
5660.  C     ==============================
5670.  C
5680.        WRITE(6,144)CTOTAL
5690.  144   FORMAT('1',//,21X,'UNKNOWN SECTION FOR ',A5,' COUNT',/,
5700.       *           21X,31('='),//,
5710.       *           21X,'UNKNOWN  DILUTION  SAMPLE NO.   TIME OF ',
5720.       *               'SAMPLING   COUNT   S.D.    C.V.',/,
5730.       *           21X,71('=') )
5740.        CALL TABLES(UNKNOW,UTAVG,TOTPRD,TOTDIL,AVG,NOTHIN,LOTS)
5750.  C
5760.  C
5770.  C     UNKNOWN SECTION FOR BOUND COUNT
5780.  C     ==============================
5790.  C
5800.        WRITE(6,144)CBOUND
5810.        CALL TABLES(UNKNOW,UBAVG,BNDPRD,BNDDIL,AVG,NOTHIN,LOTS)
5820.  C
5830.  C
5840.  C     TABLE FOR UNKNOWN SECTION
5850.  C     =========================
5860.  C
5870.        WRITE(6,145)
5880.  145   FORMAT('1',//,17X,'UNKNOWN SECTION',/,
5890.       *           17X,15('='),//,
5900.       *           17X,'UNKNOWN | LOT | DILUTION |  TOTAL  |  ',
5910.       *               'BOUND  |   %B   |  B/BO  | LOGIT Y',/,
5920.       *           17X,72('=') )
5930.        I=MM=1
5940.        DO 35 J=1,UNKNOW
5950.        IPERID=BNDPRD(J)
5960.        ISTAR=0
5970.        DO 34 K=1,IPERID
5980.        IDILUT=BNDDIL(J)
5990.        A=B=C=D=VDOSEX=0.
6000.        MISS=0
6010.        DO 33 L=1,IDILUT
6020.        IF(NOTHIN(MM).EQ.0)GOTO 36
6030.        IF(NOTHIN(MM).EQ.I)GOTO 37
6040.  36    $BB=UBAVG(I)/UTAVG(I)*100.
6050.        UY=$BB/BO*100.
6060.        IF(UY.GE.100.)GOTO 39
6070.        YLOGIT=LOGIT(UY)
6080.        LOGX=(YLOGIT-INT)/S
6090.        WRITE(6,146)J,LOTS(J),2**(L-1),UTAVG(I),UBAVG(I),
6100.       *            $BB,UY,YLOGIT
6110.  146   FORMAT('0',18X,'000',T22,I2,3X,A6,T35,'1:',T37,I2,T42,
6120.       *           F10.2,2X,F8.2,F8.2,1X,F8.2,1X,F8.2,2X,F8.2)
6130.        GOTO 38
```

```
6140.  39    WRITE(6,148)J,LOTS(J),2**(L-1),UTAVG(I),UBAVG(I),$BB,UY
6150.  148   FORMAT('0',18X,'000',T22,I2,3X,A6,T35,'1:',T37,I2,T42,
6160.       *           F10.2,2X,F8.2,F8.2,1X,F8.2,5X,'**')
6170.        ABSENS=.TRUE.
6180.        ISTAR=ISTAR+1
6190.        I=I+1
6200.        GOTO 33
6210.  37    MM=MM+1
6220.        MISS=MISS+1
6230.        I=I+1
6240.        GOTO 33
6250.  38    I=I+1
6260.        XLOG10=ALOG10(32./2**(L-1))
6270.        A=A+XLOG10**2
6280.        B=B+XLOG10*YLOGIT
6290.        C=C+YLOGIT
6300.        D=D+XLOG10
6310.        IF(L.EQ.1)DUMMY=XLOG10
6320.  33    CONTINUE
6330.        IF(A.EQ.0..AND.B.EQ.0..AND.C.EQ.0..AND.D.EQ.0.)GOTO 40
6340.        L=L-MISS-1
6350.        VS=(C*D-B*(L-ISTAR))/(D**2-A*(L-ISTAR))
6360.        VINT=(C-D*VS)/FLOAT(L-ISTAR)
6370.        AJUSTY=VINT+VS*DUMMY
6380.        VLOGX=-(INT-AJUSTY)/S
6390.        VDOSEX=10**VLOGX
6400.        WRITE(6,150)VS,VINT,AJUSTY
6410.  150   FORMAT('0',16X,'SAMPLE REGRESSION SLOPE =',F8.4,/,
6420.       *          17X,'SAMPLE REGRESSION INTERCEPT =',F8.4,
6430.       *         /,17X,'SAMPLE ADJUSTED Y FOR 1:1 DILUTION =',
6440.       *               F8.4   )
6450.  40    WRITE(6,147)VDOSEX
6460.  147   FORMAT('0',16X,'CALCULATED DOSE = ',F7.4)
6470.  34    CONTINUE
6480.  35    CONTINUE
6490.        WRITE(6,158)
6500.  158   FORMAT(' ',16X,72('='))
6510.        IF(ABSENS)WRITE(6,149)
6520.  149   FORMAT(' ',16X,'** : --  Y > 100 ----> Y / (100-Y) ',
6530.       *              'IS NEGATIVE',/, 21X,'LOGIT Y CANNOT',
6540.       *              ' BE CALCULATED ----> NO TOXINS '  )
6550.        WRITE(6,160)
6560.  160   FORMAT('1')
6570.       .STOP ; END
6580.  C
6590.  C
6600.  C
6610.  C     ********************************************************
6620.  C     * FUNCTION LOGIT :   LOGIT Y  =  LN(  Y / (100-Y)  ) *
6630.  C     ********************************************************
6640.  C
```

```
6650.  C
6660.        REAL FUNCTION LOGIT(Y)
6670.        LOGIT=ALOG(Y/(100.-Y))
6680.        RETURN ; END
6690.  C
6700.  C
6710.  C                       **********************
6720.  C                       *                      *
6730.  C                       *     SUBROUTINES      *
6740.  C                       *                      *
6750.  C                       **********************
6760.  C
6770.  C
6780.  C
6790.  C     STATISTICS SUBROUTINE --  RETURNS THE FOLLOWINGS :
6800.  C     ================================================
6810.  C
6820.  C     MEAN      STANDARD DEVIATION      COEFFICIENT OF VARIANCE
6830.  C
6840.        SUBROUTINE STAT(N,DATA,TIME,BKGAVG,AVG,SD,CV)
6850.        INTEGER DATA(10)
6860.        SUMX=SUMSQX=0.
6870.        DO 1 J=1,N
6880.        SUMX=SUMX+FLOAT(DATA(J))/TIME-BKGAVG
6890.  1     SUMSQX=SUMSQX+(FLOAT(DATA(J))/TIME-BKGAVG)**2
6900.        AVG=SUMX/FLOAT(N)
6910.        IF(N.LE.1)GOTO 2
6920.        SD=SQRT((N*SUMSQX-SUMX**2)/FLOAT(N*N-N))
6930.        CV=SD/AVG*100.
6940.        RETURN
6950.  2     SD=CV=0.
6960.        RETURN ; END
6970.  C
6980.  C
6990.  C     ********************************************************
7000.  C     *WHEN CV > 5%, CONTROL WILL TRANSFER HERE TO ELIMINATE*
7010.  C     *THE DATA WITH THE LARGEST DIFFERENCE FROM THE REST.  *
7020.  C     *IF MORE THAN ONE DATA REMAINS,  THE REMAINING DATA   *
7030.  C     *WILL THEN BE TAKEN BACK FOR FURTHER RE-TESTING.      *
7040.  C     ********************************************************
7050.  C
7060.        SUBROUTINE ERROR(N,DATA,DOSE)
7070.        INTEGER OUT,DATA(10)
7080.        REAL DIFF(10),RDATA(10)
7090.        TOP=-999999.
7100.        SUMX=0.
7110.        DO 1 J=1,N
7120.        RDATA(J)=FLOAT(DATA(J))
7130.  1     SUMX=SUMX+RDATA(J)
7140.        AVG=SUMX/FLOAT(N)
7150.        DO 2 J=1,N
```

```
7160.          DIFF(J)=ABS(RDATA(J)-AVG)
7170.          IF(DIFF(J).LE.TOP)GOTO 2
7180.          TOP=DIFF(J)
7190.          OUT=J
7200.    2     CONTINUE
7210.          DATA(OUT)=DATA(N)
7220.          N=N-1
7230.          IF(N.EQ.1)DOSE=0.
7240.          RETURN ; END
7250.    C
7260.    C
7270.    C     SUBROUTINE TABLES -- CONSTRUCT A TABLE FOR THE TOTAL AND
7280.    C     ================     BOUND COUNTS IN THE UNKNOWN SECTION
7290.    C
7300.          SUBROUTINE TABLES(N,MEANS,PERIOD,DILUTE,AVG,NOTHIN,LOTS)
7310.    C
7320.          INTEGER TUBES,PERIOD(20),COUNT(10),DILUTE(20),NOTHIN(10)
7330.          CHARACTER LOT*6,SAMPTM*16,LOTS*6(20)
7340.          REAL TIME(10),MEANS(50)
7350.          I=MM=1
7360.          DO 4 J=1,N
7370.          READ(11,101)DILUTE(J),PERIOD(J),LOT
7380.    101   FORMAT(I2,1X,I2,1X,A6)
7390.          LOTS(J)=LOT
7400.          IPERID=PERIOD(J)
7410.          DO 3 K=1,IPERID
7420.          READ(11,102)SAMPTM
7430.    102   FORMAT(A16)
7440.          IDILUT=DILUTE(J)
7450.          DO 2 L=1,IDILUT
7460.          READ(11,*)TUBES,TIME(L),(COUNT(M),M=1,TUBES)
7470.          IF(TUBES.EQ.0)GOTO 5
7480.          WRITE(6,103)J,2**(L-1),LOT,SAMPTM
7490.    103   FORMAT('0',22X,'000',T26,I2,T33,'1:',T35,I2,T45,A6,
7500.         *                T60,A16 )
7510.          DO 1 M=1,TUBES
7520.    1     WRITE(6,104)COUNT(M)
7530.    104   FORMAT(' ',70X,I6)
7540.          CALL STAT(TUBES,COUNT,TIME(L),AVG,MEANS(I),SD,CV)
7550.          WRITE(6,105)MEANS(I),SD,CV
7560.          GOTO 6
7570.    5     NOTHIN(MM)=I
7580.          MM=MM+1
7590.    6     I=I+1
7600.    2     CONTINUE
7610.    105   FORMAT('0',55X,'AVG (CPM) =',F10.2,1X,F7.2,2X,F5.2)
7620.    3     CONTINUE
7630.          WRITE(6,106)
7640.    106   FORMAT('0',20X,71('-'),/)
7650.    4     CONTINUE
7660.          RETURN ; END

7670.    $ENTRY
7680.    /*
```

Appendix 28 (Cont'd)

The following sections in the computer outputs, presented in Appendix 29 - 32 (inclusive), were excluded since they were essentially the same as those presented in the output in this Appendix (Appendix 28).

- STANDARD CURVE INFORMATION
- QUALITY CONTROL STATISTICS INFORMATION

Computer output on the radioimmunoassay of staphylococcal enterotoxin "A" in the bacterial dialysates is presented as follows :

RUN INFORMATION

DATE OF RADIOIMMUNOASSAY - 22/9/1975.

STAPHYLOCOCCAL ENTEROTOXIN - A

TYPE OF UNKNOWN - BACTERIAL DIALYSATES

NO. OF UNKNOWNS - 3(POSITIVE RESULTS ONLY)

NO. OF TUBES/UNKNOWN DIL'N - 2

STANDARD INFORMATION

NO. OF BACKGROUND TUBES - 10

NO. OF TUBES (PIPETTING ERROR) - 10

NO. OF MAXIMUM TOTAL TUBES - 3

NO. OF MAXIMUM BOUND TUBES - 2

NO. OF STANDARD DOSE VALUES - 6

NO. OF TUBES/STANDARD DOSE VALUE- 3

BASELINE STANDARDS

TYPE	TUBE	COUNT	STD.DEV.	C.V.
BACKGROUND				
	1	3		
	2	3		
	3	5		
	4	3		
	5	7		
	6	7		
	7	6		
	8	9		
	9	2		
	10	2		
AVG (CPM)		47.00	24.52	52.17

TOTAL (PIPETTING ERROR ESTIMATION)

1	51640		
2	51107		
3	49980		
4	51116		
5	50959		
6	51280		
7	47755		
8	52029		
9	51736		
10	52402		
AVG (CPM)	102000.70	2633.88	2.58

PIPETTING ERROR = SQRT|(S.D.**2 - AVG)|/AVG X 100% = 2.56%

MAXIMUM TOTAL

1	50471		
2	51107		
3	51536		
AVG (CPM)	102029.00	1070.92	1.05

MAXIMUM BOUND

1	20954		
2	20978		
AVG (CPM)	20919.00	11.31	0.05

BO = (AVG MAX BOUND / AVG MAX TOTAL) X 100% = 20.5030%

STANDARD CURVE TOTAL COUNT DATA

DOSE	TUBE	TOTAL COUNT	S.D.	C.V.
0.31	1	51640		
0.31	2	51017		
0.31	3	51795		
AVG (CPM) =		102921.00	822.92	0.80
0.63	4	47966		
0.63	5	46218		
0.63	6	53004		
AVG (CPM) =		98078.31	7046.59	7.18**REJECT**
0.63		47966		
0.63		46218		
AVG (CPM) =		94137.00	2471.26	2.63
1.25	7	52402		
1.25	8	50876		
1.25	9	51736		
AVG (CPM) =		103295.60	1528.87	1.48
2.50	10	49980		
2.50	11	51116		
2.50	12	50959		
AVG (CPM) =		101323.00	1232.17	1.22
5.00	13	51280		
5.00	14	51209		
5.00	15	46903		
AVG (CPM) =		99547.63	5013.29	5.04**REJECT**
5.00		51280		
5.00		51209		
AVG (CPM) =		102442.00	101.19	0.10
10.00	16	47755		
10.00	17	51527		
10.00	18	52029		
AVG (CPM) =		100827.00	4671.56	4.63

STANDARD CURVE BOUND COUNT DATA

DOSE	TUBE	BOUND COUNT	S.D.	C.V.	
0.31	1	16862			
0.31	2	16270			
0.31	3	17456			
AVG (CPM) =		16815.66	592.94	3.53	
0.63	4	14498			
0.63	5	14465			
0.63	6	14503			
AVG (CPM) =		14441.66	17.28	0.12	
1.25	7	11842			
1.25	8	11336			
1.25	9	9936			
AVG (CPM) =		10991.00	987.32	8.98**	REJECT **
1.25		11842			
1.25		11336			
AVG (CPM) =		11542.00	357.77	3.10	
2.50	10	7684			
2.50	11	7964			
2.50	12	8156			
AVG (CPM) =		7887.66	237.32	3.01	
5.00	13	6002			
5.00	14	6168			
5.00	15	5648			
AVG (CPM) =		5892.33	265.57	4.51	
10.00	16	4370			
10.00	17	4508			
10.00	18	4472			
AVG (CPM) =		4403.00	71.52	1.62	

STANDARD CURVE INFORMATION
==========

DOSE = UNLABELLED TOXIN DILUTIONS (IN NG/ML)

B0 = %B OF NEGATIVE CONTROL

X (J) = DOSE CONCENTRATION OF JTH OBSERVATION (IN NG/ML)

Y (J) = (%B (J) / B0) X 100% < ALSO CALLED B/B0 IN THE TABLE >

%B (J) = (BOUND COUNT(J) / TOTAL COUNT(J)) X 100% < FOR: J = 1,.....,N >

XX (J) = LOG X OF JTH OBSERVATION

YY (J) = LOGIT Y OF JTH OBSERVATION < LOGIT Y = LN(Y / (100-Y)) >

STANDARD CURVE DATA

DOSE	TOTAL COUNT	BOUND COUNT	% B	B / B0	LOGIT Y	LOG X
0.31	102921.00	16815.66	16.34	79.69	1.3669	-0.5086
0.63	94137.00	14441.66	15.34	74.82	1.0892	-0.2007
1.25	103295.60	11542.00	11.17	54.50	0.1804	0.0969
2.50	101323.00	7887.66	7.78	37.97	-0.4909	0.3979
5.00	102442.00	5892.33	5.75	28.05	-0.9418	0.6990
10.00	100827.00	4403.00	4.37	21.30	-1.3070	1.0000

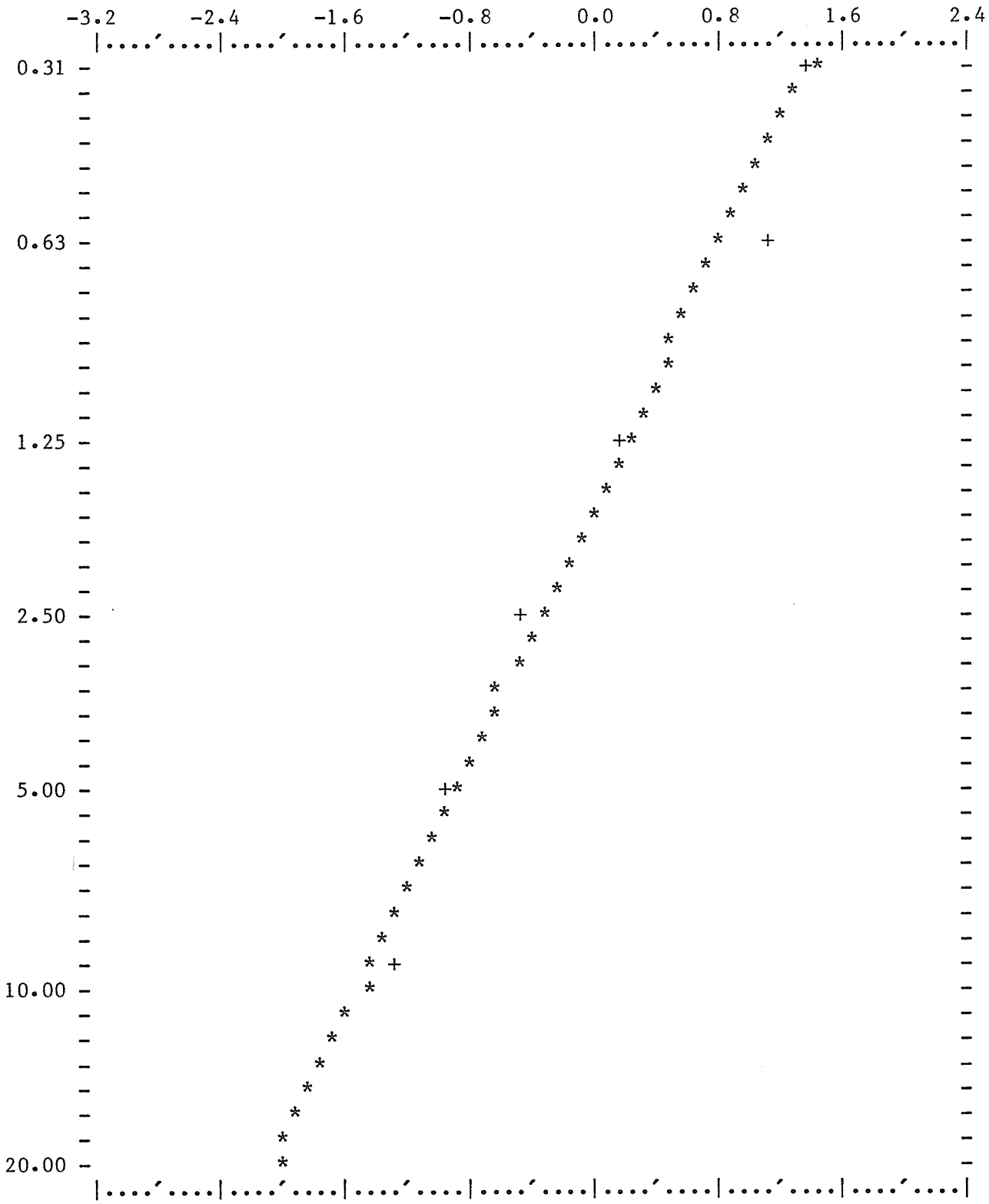
UNWEIGHTED REGRESSION

S = SLOPE = -1.91

INT = Y-INTERCEPT = 0.46

REGRESSION EQUATION : LOGIT Y = 0.46 - 1.91 X LOG (X)

STANDARD CURVE
==========
VERTICAL = DOSE (LOG SCALE) = NG/ML
HORIZONTAL = RESPONSE = LOGIT Y



SYMBOLS : + = ACTUAL POINT
* = CALCULATED POINT
@ = MULTIPLE PT. <WHERE ACTUAL PT. COINCIDES WITH CALCULATED PT.>

QUALITY CONTROL STATISTICS
==========

AOV TABLE
==========

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
REGRESSION	1	5.79	5.79	171.73**
DEVIATION	4	0.13	0.03	
TOTAL	5	5.92		

F(1,4,.05) = 7.71

F(1,4,.01) = 21.20

* : SIGNIFICANT AT 5% LEVEL
** : SIGNIFICANT AT 1% LEVEL

CORR. COEFF.	R**2	100% X R**2	SEE(Y)	SEE(S)
-0.9886	0.98	97.72	0.1836	0.1457

95% CONFIDENCE INTERVAL FOR S = -2.4181< S < -1.3999

QUALITY CONTROL STATISTICS INFORMATION
==========

N = NUMBER OF DOSE VALUES IN THE STANDARD CURVE

K = DEGREES OF FREEDOM OF REGRESSION

N-1 = DEGREES OF FREEDOM OF TOTAL VARIATIONS

N-K-1= DEGREES OF FREEDOM OF UNEXPLAINED VARIATION

J = 1,....,N

TSS = TOTAL SUM OF SQUARES OF VARIATION

= SUMMATION ((YY(J) - (SUMMATION YY(J) / N))**2)

SSR = SUM OF SQUARES OF VARIATION EXPLAINED BY REGRESSION

= S**2 X SUMMATION ((XX(J) - (SUMMATION XX(J) / N))**2)

SSE = SUM OF SQUARES OF UNEXPLAINED VARIATION

= TSS - SSR

MSR = SSR / K

MSE = SSE / (N-K-1)

F = MSR / MSE

R**2 = COEFFICIENT OF DETERMINATION = SSR / TSS

R**2 X 100% = PERCENTAGE OF TSS EXPLAINED BY THE REGRESSION OF YY(J) ON X(J)

SEE (Y) = STANDARD ERROR ESTIMATE OF YY(J)

SEE (S) = STANDARD ERROR ESTIMATE OF THE SLOPE

UNKNOWN SECTION FOR TOTAL COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	NO. 7		51002		
				51578		
			AVG (CPM) =	102533.00	810.81	0.79
0001	1: 2	NO. 7		51060		
				50947		
			AVG (CPM) =	101960.00	150.09	0.15
0001	1: 4	NO. 7		51085		
				50367		
			AVG (CPM) =	101405.00	1013.95	1.00
0001	1: 8	NO. 7		51843		
				50079		
			AVG (CPM) =	101875.00	2493.95	2.45
0001	1:16	NO. 7		51165		
				50809		
			AVG (CPM) =	101927.00	499.86	0.49
0002	1: 1	NO.13		50593		
				50611		
			AVG (CPM) =	101157.00	0.00	0.00
0002	1: 2	NO.13		50897		
				52750		
			AVG (CPM) =	103600.00	2620.09	2.53
0002	1: 4	NO.13		47631		
				47523		
			AVG (CPM) =	95107.00	135.76	0.14
0002	1: 8	NO.13		51822		
				51231		
			AVG (CPM) =	103006.00	834.46	0.81
0002	1:16	NO.13		50624		
				50521		
				52310		
			AVG (CPM) =	102256.30	2007.60	1.96

UNKNOWN SECTION FOR TOTAL COUNT (cont'd)

0003	1: 1	NO. 4	50217		
			51342		
			AVG (CPM) = 101512.00	1589.73	1.57
0003	1: 2	NO. 4	49501		
			51978		
			AVG (CPM) = 101432.00	3502.21	3.45
0003	1: 4	NO. 4	50268		
			47646		
			AVG (CPM) = 97867.00	3707.58	3.79
0003	1: 8	NO. 4	50688		
			50615		
			AVG (CPM) = 101256.00	90.51	0.09
0003	1:16	NO. 4	50410		
			50484		
			AVG (CPM) = 100847.00	101.19	0.10
0003	1:32	NO. 4	49970		
			52744		
			AVG (CPM) = 102667.00	3922.58	3.82

UNKNOWN SECTION FOR BOUND COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	NO. 7		2540		
				2288		
			AVG (CPM) =	2367.00	178.19	7.53
0001	1: 2	NO. 7		2686		
			AVG (CPM) =	2639.00	0.00	0.00
0001	1: 4	NO. 7		3298		
			AVG (CPM) =	3251.00	0.00	0.00
0001	1: 8	NO. 7		4420		
				4364		
			AVG (CPM) =	4345.00	39.40	0.91
0001	1:16	NO. 7		5656		
				6056		
				5990		
			AVG (CPM) =	5853.66	214.46	3.66

UNKNOWN SECTION FOR BOUND COUNT (cont'd)

0002	1: 1	NO.13		2212		
				2233		
			AVG (CPM) =	2175.50	14.70	0.68
0002	1: 2	NO.13		3820		
				3899		
			AVG (CPM) =	3812.50	55.79	1.46
0002	1: 4	NO.13		5148		
				5236		
			AVG (CPM) =	5145.00	62.10	1.21
0002	1: 8	NO.13		8332		
				8326		
			AVG (CPM) =	8282.00	0.00	0.00
0002	1:16	NO.13		11203		
				10937		
			AVG (CPM) =	11023.00	187.96	1.71
0003	1: 1	NO. 4		7873		
				7684		
				7642		
			AVG (CPM) =	7686.00	122.90	1.60
0003	1: 2	NO. 4		8289		
				8332		
			AVG (CPM) =	8263.50	32.00	0.39
0003	1: 4	NO. 4		11350		
				11213		
				10083		
			AVG (CPM) =	10835.00	695.34	6.42
0003	1: 8	NO. 4		15075		
				15318		
				16157		
			AVG (CPM) =	15469.66	567.64	3.67
0003	1:16	NO. 4		17730		
				17123		
				17693		
			AVG (CPM) =	17468.33	340.10	1.95
0003	1:32	NO. 4		19974		
				19764		
				19890		
			AVG (CPM) =	19829.00	105.32	0.53

UNKNOWN SECTION
==========

UNKNOWN	LOT	DILUTION	TOTAL	BOUND	%B	B/BO	LOGIT Y
0001	NO. 7	1: 1	102533.00	2367.00	2.31	11.26	-2.06
0001	NO. 7	1: 2	101960.00	2639.00	2.59	12.62	-1.93
0001	NO. 7	1: 4	101405.00	3251.00	3.21	15.64	-1.69
0001	NO. 7	1: 8	101875.00	4345.00	4.27	20.80	-1.34
0001	NO. 7	1:16	101927.00	5853.66	5.74	28.01	-0.94

SAMPLE REGRESSION SLOPE = -0.9431
SAMPLE REGRESSION INTERCEPT = -0.7414
SAMPLE ADJUSTED Y FOR 1:1 DILUTION = -2.1609
CALCULATED DOSE = 23.4615

0002	NO.13	1: 1	101157.00	2175.50	2.15	10.49	-2.14
0002	NO.13	1: 2	103600.00	3812.50	3.68	17.95	-1.52
0002	NO.13	1: 4	95107.00	5145.00	5.41	26.38	-1.03
0002	NO.13	1: 8	103006.00	8282.00	8.04	39.22	-0.44
0002	NO.13	1:16	102256.30	11023.00	10.78	52.58	0.10

SAMPLE REGRESSION SLOPE = -1.8523
SAMPLE REGRESSION INTERCEPT = 0.6678
SAMPLE ADJUSTED Y FOR 1:1 DILUTION = -2.1202
CALCULATED DOSE = 22.3376

0003	NO. 4	1: 1	101512.00	7686.00	7.57	36.93	-0.54
0003	NO. 4	1: 2	101432.00	8263.50	8.15	39.73	-0.42
0003	NO. 4	1: 4	97867.00	10835.00	11.07	54.00	0.16
0003	NO. 4	1: 8	101256.00	15469.66	15.28	74.51	1.07
0003	NO. 4	1:16	100847.00	17468.33	17.32	84.48	1.69
0003	NO. 4	1:32	102667.00	19829.00	19.31	94.20	2.79

SAMPLE REGRESSION SLOPE = -2.2647
SAMPLE REGRESSION INTERCEPT = 2.4983
SAMPLE ADJUSTED Y FOR 1:1 DILUTION = -0.9104
CALCULATED DOSE = 5.1917

==========

Appendix 29 (c.f. Appendix 28)

Computer programming on the data obtained in the solid-phase radioimmunoassay of staphylococcal enterotoxin C in bacterial dialysates was essentially the same as that presented in the previous Appendix (Appendix 28).

Computer output on the radioimmunoassay of staphylococcal enterotoxin C in the bacterial dialysates is presented as follows :

RUN INFORMATION
==========

DATE OF RADIOIMMUNOASSAY - 22/9/1975.

STAPHYLOCOCCAL ENTEROTOXIN - C

TYPE OF UNKNOWN - BACTERIAL DIALYSATES

NO. OF UNKNOWNS - 3(POSITIVE RESULTS ONLY)

NO. OF TUBES/UNKNOWN DIL'N - 2

STANDARD INFORMATION
==========

NO. OF BACKGROUND TUBES - 10

NO. OF TUBES (PIPETTING ERROR) - 10

NO. OF MAXIMUM TOTAL TUBES - 3

NO. OF MAXIMUM BOUND TUBES - 3

NO. OF STANDARD DOSE VALUES - 6

NO. OF TUBES/STANDARD DOSE VALUE - 3

BASELINE STANDARDS

TYPE	TUBE	COUNT	STD.DEV.	C.V.
BACKGROUND				
	1	7		
	2	4		
	3	6		
	4	6		
	5	4		
	6	3		
	7	5		
	8	5		
	9	3		
	10	5		
AVG (CPM)		48.00	13.17	27.43

TOTAL (PIPETTING ERROR ESTIMATION)

1	62672		
2	62754		
3	63920		
4	64475		
5	66112		
6	62990		
7	62530		
8	64979		
9	65006		
10	66414		
AVG (CPM)	128370.30	2874.10	2.24

PIPETTING ERROR = SQRT|(S.D.**2 - AVG)|/AVG X 100% = 2.22%

MAXIMUM TOTAL

1	63405		
2	63402		
3	62899		
AVG (CPM)	126422.60	581.90	0.46

MAXIMUM BOUND

1	21592		
2	21347		
3	22087		
AVG (CPM)	21627.33	376.93	1.74

BO = (AVG MAX BOUND / AVG MAX TOTAL) X 100% = 17.1072%

STANDARD CURVE TOTAL COUNT DATA

DOSE	TUBE	TOTAL COUNT	S.D.	C.V.
0.31	1	62672		
0.31	2	62754		
0.31	3	66414		
AVG (CPM) =		127845.30	4274.76	3.34
0.61	4	64761		
0.61	5	65456		
0.61	6	64340		
AVG (CPM) =		129656.60	1125.62	0.87
1.25	7	63920		
1.25	8	67210		
1.25	9	66308		
AVG (CPM) =		131577.30	3399.44	2.58
2.50	10	64475		
2.50	11	64557		
2.50	12	64979		
AVG (CPM) =		129292.60	543.06	0.42
5.00	13	66112		
5.00	14	62990		
5.00	15	65006		
AVG (CPM) =		129357.30	3166.54	2.45
10.00	16	62530		
10.00	17	59998		
10.00	18	59883		
AVG (CPM) =		121559.30	2990.93	2.46

STANDARD CURVE BOUND COUNT DATA

DOSE	TUBE	BOUND COUNT	S.D.	C.V.	
0.31	1	20081			
0.31	2	19737			
0.31	3	19290			
AVG (CPM) =		19654.66	396.41	2.02	
0.61	4	15781			
0.61	5	15816			
0.61	6	15580			
AVG (CPM) =		15677.66	126.83	0.81	
1.25	7	11809			
1.25	8	11884			
1.25	9	11182			
AVG (CPM) =		11577.00	385.33	3.33	
2.50	10	9381			
2.50	11	8799			
2.50	12	8778			
AVG (CPM) =		8938.00	342.23	3.83	
5.00	13	5961			
5.00	14	5769			
5.00	15	6145			
AVG (CPM) =		5910.33	188.07	3.18	
10.00	16	3031			
10.00	17	3084			
10.00	18	3426			
AVG (CPM) =		3132.33	214.40	6.84**	REJECT **
10.00		3031			
10.00		3084			
AVG (CPM) =		3009.50	37.52	1.25	

STANDARD CURVE DATA

DOSE	TOTAL COUNT	BOUND COUNT	% B	B / B0	LOGIT Y	LOG X
0.31	127845.30	19654.66	15.37	89.87	2.1826	-0.5086
0.61	129656.60	15677.66	12.09	70.68	0.8800	-0.2147
1.25	131577.30	11577.00	8.80	51.43	0.0573	0.0969
2.50	129292.60	8938.00	6.91	40.41	-0.3884	0.3979
5.00	129357.30	5910.33	4.57	26.71	-1.0095	0.6990
10.00	121559.30	3009.50	2.48	14.47	-1.7766	1.0000

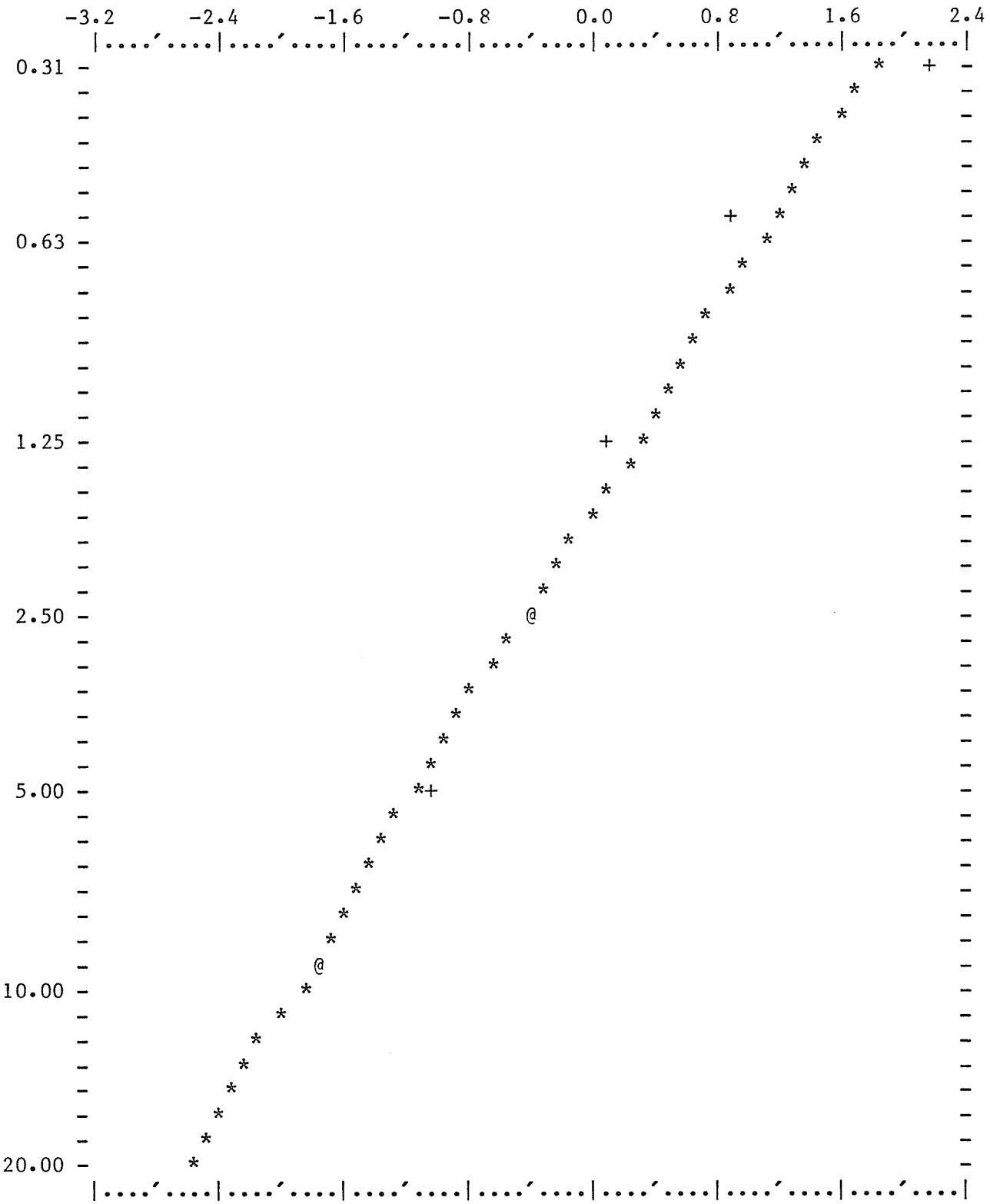
UNWEIGHTED REGRESSION

S = SLOPE = -2.45

INT = Y-INTERCEPT = 0.59

REGRESSION EQUATION : LOGIT Y = 0.59 - 2.45 X LOG (X)

STANDARD CURVE
==========
VERTICAL = DOSE (LOG SCALE) = NG/ML
HORIZONTAL = RESPONSE = LOGIT Y



SYMBOLS : + = ACTUAL POINT
* = CALCULATED POINT
@ = MULTIPLE PT. <WHERE ACTUAL PT. COINCIDES WITH CALCULATED PT.>

QUALITY CONTROL STATISTICS
==========

AOV TABLE
==========

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
REGRESSION	1	9.58	9.58	135.66**
DEVIATION	4	0.28	0.07	
TOTAL	5	9.87		

F(1,4,.05) = 7.71

F(1,4,.01) = 21.20

* : SIGNIFICANT AT 5% LEVEL
** : SIGNIFICANT AT 1% LEVEL

CORR. COEFF.	R**2	100% X R**2	SEE(Y)	SEE(S)
-0.9856	0.97	97.14	0.2658	0.2101

95% CONFIDENCE INTERVAL FOR S = -3.1812< S < -1.7127

UNKNOWN SECTION FOR TOTAL COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	NO. 4		67183		
				65838		
			AVG (CPM) =	132973.00	1898.55	1.43
0001	1: 2	NO. 4		64267		
				62620		
			AVG (CPM) =	126839.00	2328.76	1.84
0001	1: 4	NO. 4		66396		
				65820		
			AVG (CPM) =	132168.00	809.54	0.61
0001	1: 8	NO. 4		67642		
				63894		
			AVG (CPM) =	131488.00	5299.26	4.03
0001	1:16	NO. 4		66395		
				65873		
			AVG (CPM) =	132220.00	724.08	0.55
0001	1:32	NO. 4		65291		
				64720		
			AVG (CPM) =	129963.00	805.74	0.62
0002	1: 1	NO.13		61717		
				61039		
			AVG (CPM) =	122708.00	957.86	0.78
0002	1: 2	NO.13		62887		
				62037		
			AVG (CPM) =	124876.00	1200.75	0.96
0002	1: 4	NO.13		61629		
				60898		
			AVG (CPM) =	122479.00	1030.98	0.84
0002	1: 8	NO.13		67034		
				66710		
			AVG (CPM) =	133696.00	443.40	0.33
0002	1:16	NO.13		64500		
				63094		
			AVG (CPM) =	127546.00	1987.61	1.56
0002	1:32	NO.13		62684		
				62646		
			AVG (CPM) =	125282.00	0.00	0.00

0003	1: 1	NO. 7	64138		
			62456		
			AVG (CPM) = 126546.00	2378.36	1.88
0003	1: 2	NO. 7	69251		
			69273		
			AVG (CPM) = 138476.00	0.00	0.00
0003	1: 4	NO. 7	67141		
			67610		
			AVG (CPM) = 134703.00	677.31	0.50
0003	1: 8	NO. 7	63925		
			63964		
			AVG (CPM) = 127841.00	0.00	0.00
0003	1:16	NO. 7	65740		
			66904		
			AVG (CPM) = 132596.00	1649.16	1.24
0003	1:32	NO. 7	67316		
			65219		
			AVG (CPM) = 132487.00	2963.41	2.24

UNKNOWN SECTION FOR BOUND COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	NO. 4		22786		
				23461		
			AVG (CPM) =	23075.50	477.19	2.07
0001	1: 2	NO. 4		22320		
				21367		
			AVG (CPM) =	21795.50	673.81	3.09
0001	1: 4	NO. 4		23261		
				22718		
			AVG (CPM) =	22941.50	383.50	1.67
0001	1: 8	NO. 4		23895		
				24701		
			AVG (CPM) =	24250.00	569.74	2.35
0001	1:16	NO. 4		23158		
				23067		
			AVG (CPM) =	23064.50	62.99	0.27
0001	1:32	NO. 4		22407		
				22557		
			AVG (CPM) =	22434.00	104.92	0.47

0002	1: 1	NO.13		24803		
				24270		
			AVG (CPM) =	24488.50	376.77	1.54
0002	1: 2	NO.13		21976		
				22360		
			AVG (CPM) =	22120.00	271.29	1.23
0002	1: 4	NO.13		23071		
				23463		
			AVG (CPM) =	23219.00	277.13	1.19
0002	1: 8	NO.13		23557		
				23209		
			AVG (CPM) =	23335.00	246.06	1.05
0002	1:16	NO.13		24022		
				23938		
			AVG (CPM) =	23932.00	57.69	0.24
0002	1:32	NO.13		24109		
				22234		
			AVG (CPM) =	23123.50	1325.78	5.73
0003	1: 1	NO. 7		5986		
				6107		
			AVG (CPM) =	5998.50	85.51	1.43
0003	1: 2	NO. 7		9714		
				9785		
			AVG (CPM) =	9701.50	50.60	0.52
0003	1: 4	NO. 7		11084		
				11812		
			AVG (CPM) =	11400.00	514.74	4.52
0003	1: 8	NO. 7		15811		
				15086		
			AVG (CPM) =	15400.50	512.62	3.33
0003	1:16	NO. 7		20412		
				20072		
			AVG (CPM) =	20194.00	240.00	1.19
0003	1:32	NO. 7		23981		
				23279		
			AVG (CPM) =	23582.00	496.26	2.10

UNKNOWN SECTION
==========

UNKNOWN	LOT	DILUTION	TOTAL	BOUND	%B	B/BO	LOGIT Y
0001	NO. 4	1: 1	132973.00	23075.50	17.35	101.44	**
0001	NO. 4	1: 2	126839.00	21795.50	17.18	100.45	**
0001	NO. 4	1: 4	132168.00	22941.50	17.36	101.47	**
0001	NO. 4	1: 8	131488.00	24250.00	18.44	107.81	**
0001	NO. 4	1:16	132220.00	23064.50	17.44	101.97	**
0001	NO. 4	1:32	129963.00	22434.00	17.26	100.90	**

CALCULATED DOSE = 0.0000

0002	NO.13	1: 1	122708.00	24488.50	19.96	116.66	**
0002	NO.13	1: 2	124876.00	22120.00	17.71	103.54	**
0002	NO.13	1: 4	122479.00	23219.00	18.96	110.82	**
0002	NO.13	1: 8	133696.00	23335.00	17.45	102.03	**
0002	NO.13	1:16	127546.00	23932.00	18.76	109.68	**
0002	NO.13	1:32	125282.00	23123.50	18.46	107.89	**

CALCULATED DOSE = 0.0000

0003	NO. 7	1: 1	126546.00	5998.50	4.74	27.71	-0.96
0003	NO. 7	1: 2	138476.00	9701.50	7.01	40.95	-0.37
0003	NO. 7	1: 4	134703.00	11400.00	8.46	49.47	-0.02
0003	NO. 7	1: 8	127841.00	15400.50	12.05	70.42	0.87
0003	NO. 7	1:16	132596.00	20194.00	15.23	89.03	2.09
0003	NO. 7	1:32	132487.00	23582.00	17.80	104.05	**

SAMPLE REGRESSION SLOPE = -2.4376
SAMPLE REGRESSION INTERCEPT = 2.5243
SAMPLE ADJUSTED Y FOR 1:1 DILUTION = -1.1446
CALCULATED DOSE = 5.1185

==========

** : -- Y > 100 ----> Y / (100-Y) IS NEGATIVE
LOGIT Y CANNOT BE CALCULATED ----> NO TOXINS

Appendix 30 (c.f. Appendix 28)

Computer programming on the data obtained in the radioimmunoassays of staphylococcal enterotoxin A in the cheese samples was essentially the same as that presented in Appendix 28. Changes, however, were made in three sections as listed below :

1. In the " Declaration of the Program " :

```
5          DATA LABEL/0.63,1.25,2.5,5.0,10.0,20.0,40.0/,
          *     SPACE/7,8,7,7,8,7,1/,
          *     CTOTAL/'TOTAL'/,CBOUND/'BOUND'/,
          *     BLANK/'
          *                          '/,
          *     BLK/' '/,
          *     IONE/1/
```

2. In the section of setting up a table for the unknown samples :

```
      C     TABLE FOR UNKNOWN SECTION
      C     ========================
      C
241         WRITE(6.145)
242   145   FORMAT('1',//,8X,'UNKNOWN SECTION',/,
           *              8X,15('='),///,
           *              8X,'UNKNOWN  |     SAMPLE    | DILUTION |  ',
           *                 'TOTAL  |  BOUND  |   %B   | B/B0  | ',
           *                 'LOGIT Y | DOSE(X) ',/,
           *              8X,94('=')  )
243         I=MM=INUM=1
244         DO 35 J=1,UNKNOW
245         IPERID=BNDPRD(J)
246         DO 34 K=1,IPERID
247         IDILUT=BNDDIL(J)
248         DO 33 L=1,IDILUT
249         $BB=UBAVG(I)/UTAVG(I)*100
250         UY=$BB/B0*100
251         IF(UY.GE.100.)GOTO 39
252         YLOGIT=LOGIT(UY)
253         LOGX=(YLOGIT-INT)/S
254         DOSEX=10**LOGX
255         WRITE(6,146)INUM,LOTS(J),SAMPTS(J,K),L,UTAVG(I),UBAVG(I),
                        $BB,UY,YLOGIT,DOSEX
```

Appendix 30 (continued)

```
256   146   FORMAT('0',9X,'000',T12,I2,4X,A5,A16,T39,'1:',T41,I2,T46,
     *             F10.2,1X,F8.2,F8.2,2X,F8.2,1X,F8.2,1X,F8.2)
257         GOTO  38
258   39    WRITE(6,148)INUM,LOTS(J),SAMPTS(J,K),L,UTAVG(I),UBAVG(I),
     *           $BB,UY
259   148   FORMAT('0',9X,'000',T12,I2,4X,A5,A16,T39,'1:',T41,I2,T46,
     *             F10.2,1X,F8.2,F8.2,2X,F8.2,7X,'**' )
260         ABSENS=.TRUE.
261   38    I=I+1
262         INUM=INUM+1
263   33    CONTINUE
264   34    CONTINUE
265   35    CONTINUE
266         WRITE(6,158)
267   158   FORMAT('0',7X,94('=')  )
268         IF(ABSENS)WRITE(6,149)
269   149   FORMAT('0',7X,'** :  -- Y   100 --->  Y / (100-Y) IS',
     *             ' NEGATIVE',/,
     *          13X,'LOGIT Y CANNOT BE CALCULATED ---->  NO ',
     *             'TOXINS'  )
270         WRITE(6,160)
271   160   FORMAT('1')
272         STOP ; END
```

3. In the section of subroutine tables :

```
      C
      C     SUBROUTINE TABLES : CONSTRUCT A TABLE FOR THE TOTAL AND
      C     ================    BOUND COUNTS IN THE UNKNOWN SECTION
      C
      C
312         SUBROUTINE TABLES(N,MEANS,PERIOD,DILUTE,AVG,NOTHIN,LOTS,
     *                    SAMPTS)
313         INTEGER   TUBES,PERIOD(20),COUNT(10),DILUTE(20),NOTHIN(10)
      C
314         CHARACTER LOT*6,SAMPTM*16,LOTS*6(20),SAMPTS*16(20,7)
315         REAL      TIME(10),MEANS(50)
      C
316         I=MM=INUM=1
317         DO 4 J=1,N
318         READ(11,101)DILUTE(J),PERIOD(J),LOT
319   101   FORMAT(I2,1X,I2,1X,A6)
320         LOTS(J)=LOT
321         IPERID=PERIOD(J)
322         DO 3 K=1,IPERID
323         READ(11,102)SAMPTM
```

Appendix 30 (continued)

```
324   102   FORMAT(A16)
325         SAMPTS(J,K)=SAMPTM
326         IDILUT=DILUTE(J)
327         DO 2 L=1,IDILUT
328         READ(11,*)TUBES,TIME(L),(COUNT(M),M=1,TUBES)
329         IF(TUBES.EQ.0)GOTO 5
330         WRITE(6,103)INUM,L,LOT,SAMPTM
331   103   FORMAT('0',22X,'000',T26,I2,T33,'1:',T35,I2,T45,A6,T60,A16)
332         INUM=INUM+1
333         DO 1 M=1,TUBES
334   1     WRITE(6,104)COUNT(M)
335   104   FORMAT(' ',70X,I6)
336         CALL STAT(TUBES,COUNT,TIME(L),AVG,MEANS(I),SD,CV)
337         WRITE(6,105)MEANS(I),SD,CV
338         GOTO 6
339   5     NOTHIN(MM)=I
340         MM=MM+1
341   6     I=I+1
342   2     CONTINUE
343   105   FORMAT('0',55X,'AVG (CPM) =',F10.2,1X,F7.2,2X,F5.2)
344   3     CONTINUE
345         WRITE(6,106)
346   106   FORMAT('0',20X,71('-'),/)
347   4     CONTINUE
348         RETURN ; END
349   $ENTRY
350   /*
```

Additional changes, however, were made in each of the following three programs :

For cheese samples at the 'various manufacturing stages' :

DECLARATION OF THE PROGRAM
==========

```
1           INTEGER   UNKNOW,UTUBES,STUBES,BKNUM,TUBENO,NSAMPL,TLNUM
                      COUNT(10),DOSNUM,TIMES,SPACE(7),TOTPRD(20),
                      BNDPRD(20),TOTDIL(20),BNDDIL(20),NOTHIN(10)/10*0/


3           CHARACTER DATE*8,TOXIN*40,TYPE*1,CTOTAL*5,CBOUND*5,
                      VEC*1(80),VECTOR*80,BLANK*80,BLK*1,LOTS*6(20),
                      SAMPTS*16(20,7)
```

Appendix 30 (continued)

```
RUN INFORMATION
===============

10          READ(11,101)DATE,UNKNOW,UTUBES,TUBE,NSAMPL,TOXIN
11    101   FORMAT(A8,I4,I2,A1,I4,A40)

14          WRITE(6,103)TYPE,TOXIN,UNKNOW,NSAMPL,UTUBES
15    103   FORMAT('0',20X,'STAPHYLOCOCCAL ENTEROTOXIN  -',4X,A1,//,
           *           21X,'TYPE OF UNKNOWN            -',A40,//,
           *           21X,'NO. OF UNKNOWNS (BATCHES)  -',I5,//,
           *           21X,'NO. OF UNKNOWNS (SAMPLES)  -',I5,//,
           *           21X,'NO. OF TUBES/UNKNOWN DIL''N  -',I5,////  )
```

For cheese samples at '60 days of curing' :

Under "TABLE FOR THE UNKNOWN SECTION", changes were made at the follow-

ing lines :

```
242   145   FORMAT('1',//,16X,'UNKNOWN SECTION',/,
           *              16X,15('='),///,
           *              16X,'UNKNOWN |  LOT   | DILUTION |  TOTAL  |',
           *                  '  BOUND  |  % B  |  B / B0  |  LOGIT Y',
           *                  '  | DOSE(X)',/,
           *              16X,87('=')  )

256   146   FORMAT('0',18X,'000',T21,I2,5X,A6,T37,'1:',T39,I2,T42,
           *               F10.2,2X,F8.2,1X,F8.2,2X,F8.2,4X,F8.2,2X,
           *               F8.2)

259   148   FORMAT('0',18X,'000',T21,I2,5X,A6,T37,'1:',T39,I2,T42,
           *              F10.2,2X,F8.2,1X,F8.2,2X,F8.2,10X,'**')

262   33     CONTINUE
263   34     CONTINUE
264          INUM=INUM+1

267   158    FORMAT('0',15X,87('=')  )

269   149    FORMAT('0',16X,'** : --  Y > 100 ----> Y / (100-Y) IS',
           *               ' NEGATIVE',/,
           *           21X,'LOGIT Y CANNOT BE CALCULATED ----> NO ',
           *               'TOXINS'  )
```

Under the section "SUBROUTINE TABLES", changes were made at the follow-

Appendix 30 (continued)

ing lines :

```
314         CHARACTER  LOT*6,SAMPTM*16,LOTS*6(20)
```

Statement 325 was omitted.

Statement 332 was inserted between statement 346 and statement 347.

For cheese samples at 'out of press' :

Changes made were essentially the same as those described in the program for cheese samples at '60 days of curing'.

Additional changes, however, were made in the section of " Plotting the Standard Curve " at the following lines (c.f. Appendix 28) :

(i) at line 3880 :

```
      127  FORMAT('-',25X,'-2.4',6X,'-1.6',6X,'-0.8',7X,'0.0',7X,'0.8',
          *              7X,'1.6',7X,'2.4',7X,'3.2' )
```

(ii) at line 4150 :

```
           IPT=IFIX(31.5+LOGITY/0.08)
```

(iii)at line 4260 :

```
           IPT=IFIX(31.5+LOGITY/0.08)
```

Appendix 30 (Cont'd)

Computer output on the radioimmunoassay of staphylococcal enterotoxin A in cheese samples (collected in various cheese manufacturing stages) is presented as follows :

RUN INFORMATION
==========

DATE OF RADIOIMMUNOASSAY - 23/4/77

STAPHYLOCOCCAL ENTEROTOXIN - A

TYPE OF UNKNOWN - CHEDDAR CHEESE

NO. OF UNKNOWNS (BATCHES) - 10

NO. OF UNKNOWNS (SAMPLES) - 37

NO. OF TUBES/UNKNOWN DIL'N - 4

STANDARD INFORMATION
==========

NO. OF BACKGROUND TUBES - 10

NO. OF TUBES (PIPETTING ERROR) - 10

NO. OF MAXIMUM TOTAL TUBES - 3

NO. OF MAXIMUM BOUND TUBES - 3

NO. OF STANDARD DOSE VALUES - 7

NO. OF TUBES/STANDARD DOSE VALUE - 3

BASELINE STANDARDS
==========

TYPE	TUBE	COUNT	STD.DEV.	C.V.
BACKGROUND				
	1	7		
	2	5		
	3	9		
	4	7		
	5	4		
	6	4		
	7	6		
	8	2		
	9	6		
	10	8		
AVG (CPM)		58.00	20.98	36.17

TOTAL (PIPETTING ERROR ESTIMATION)

1	8808		
2	9040		
3	8543		
4	8788		
5	8901		
6	8597		
7	8615		
8	8639		
9	8896		
10	8827		
AVG (CPM)	17530.80	321.91	1.84

PIPETTING ERROR = SQRT|(S.D.**2 - AVG)|/AVG X 100% = 1.67%

MAXIMUM TOTAL

1	8691		
2	8672		
3	8796		
AVG (CPM)	17381.33	133.23	0.77

MAXIMUM BOUND

1	3122		
2	3137		
3	3129		
AVG (CPM)	1506.67	3.65	0.24

BO = (AVG MAX BOUND / AVG MAX TOTAL) X 100% = 8.6683%

STANDARD CURVE TOTAL COUNT DATA

DOSE	TUBE	TOTAL COUNT	S.D.	C.V.
0.67	1	8545		
0.67	2	8761		
0.67	3	8808		
AVG (CPM) =		17351.33	280.27	1.62
1.00	4	8896		
1.00	5	8682		
1.00	6	8820		
AVG (CPM) =		17540.66	216.74	1.24
2.00	7	8827		
2.00	8	8640		
2.00	9	9040		
AVG (CPM) =		17613.33	400.16	2.27
2.50	10	8878		
2.50	11	8543		
2.50	12	8639		
AVG (CPM) =		17315.33	344.96	1.99
3.30	13	8615		
3.30	14	8849		
3.30	15	8788		
AVG (CPM) =		17443.33	242.56	1.39
5.00	16	8901		
5.00	17	8761		
5.00	18	8597		
AVG (CPM) =		17448.00	304.28	1.74
10.00	19	8663		
10.00	20	8592		
10.00	21	8628		
AVG (CPM) =		17197.33	69.74	0.41

STANDARD CURVE BOUND COUNT DATA

DOSE	TUBE	BOUND COUNT	S.D.	C.V.	
0.67	1	2280			
0.67	2	1993			
0.67	3	2343			
AVG (CPM) =		1044.67	93.28	8.93**	REJECT **
0.67		2280			
0.67		2343			
AVG (CPM) =		1097.75	22.27	2.03	
1.00	4	1663			
1.00	5	1739			
1.00	6	1686			
AVG (CPM) =		790.00	19.48	2.47	
2.00	7	1386			
2.00	8	1331			
2.00	9	1296			
AVG (CPM) =		610.83	22.68	3.71	
2.50	10	1426			
2.50	11	1296			
2.50	12	1075			
AVG (CPM) =		574.83	88.73	15.44**	REJECT **
2.50		1426			
2.50		1296			
AVG (CPM) =		622.50	45.96	7.38**	REJECT **
3.30	13	952			
3.30	14	897			
3.30	15	923			
AVG (CPM) =		404.00	13.75	3.40	
5.00	16	773			
5.00	17	765			
5.00	18	757			
AVG (CPM) =		324.50	4.00	1.23	
10.00	19	502			
10.00	20	498			
10.00	21	516			
AVG (CPM) =		194.67	4.74	2.43	

STANDARD CURVE DATA

DOSE	TOTAL COUNT	BOUND COUNT	% B	B / B0	LOGIT Y	LOG X
0.67	17351.33	1097.75	6.33	72.99	0.9939	-0.1739
1.00	17540.66	790.00	4.50	51.96	0.0783	0.0000
2.00	17613.33	610.83	3.47	40.01	-0.4051	0.3010
3.30	17443.33	404.00	2.32	26.72	-1.0089	0.5185
5.00	17448.00	324.50	1.86	21.46	-1.2977	0.6990
10.00	17197.33	194.67	1.13	13.06	-1.8958	1.0000

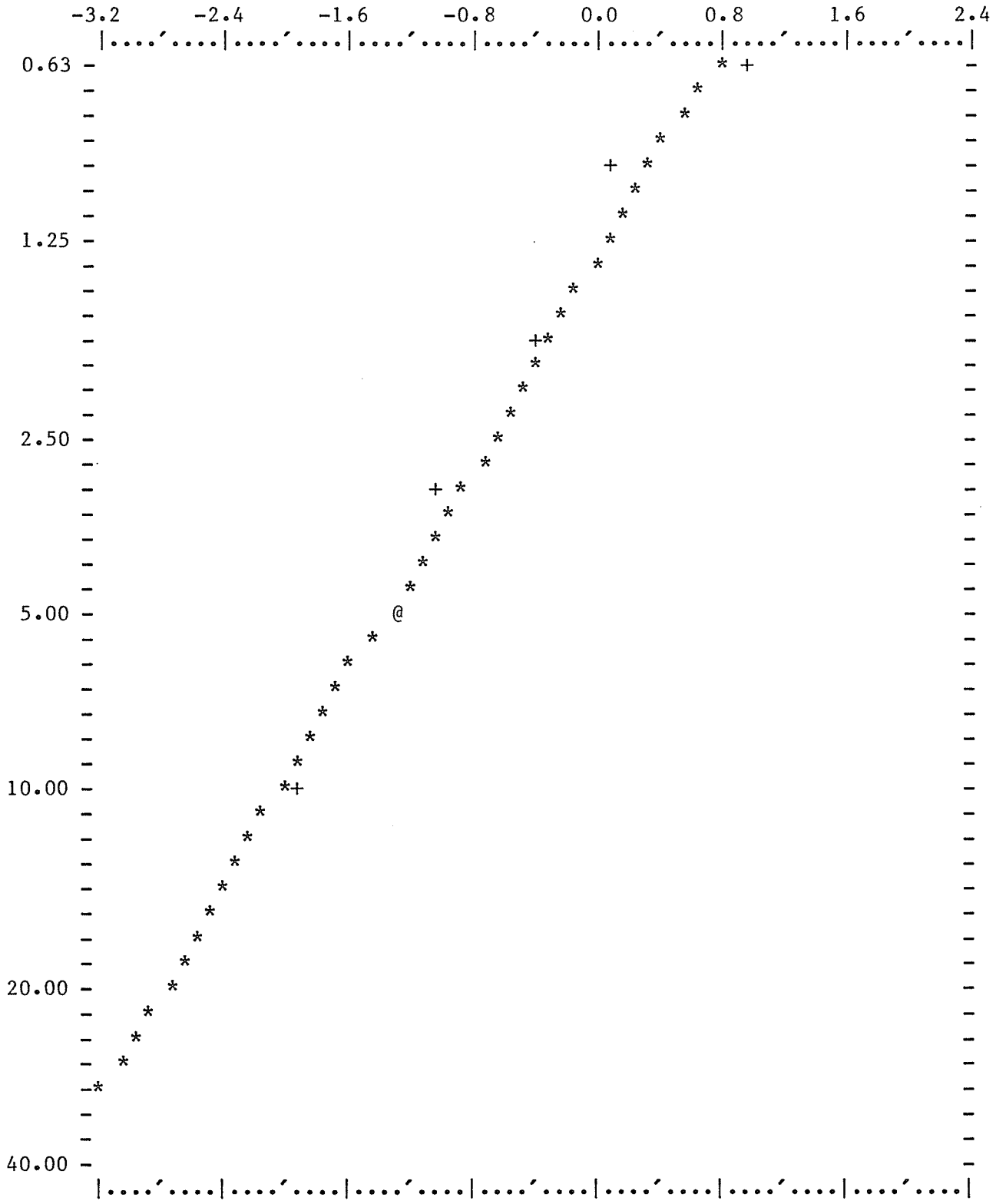
UNWEIGHTED REGRESSION

S = SLOPE = -2.33

INT = Y-INTERCEPT = 0.32

REGRESSION EQUATION : LOGIT Y = 0.32 - 2.33 X LOG (X)

STANDARD CURVE
==========
VERTICAL = DOSE (LOG SCALE) = NG/ML
HORIZONTAL = RESPONSE = LOGIT Y



SYMBOLS : + = ACTUAL POINT
* = CALCULATED POINT
@ = MULTIPLE PT. <WHERE ACTUAL PT. COINCIDES WITH CALCULATED PT.>

QUALITY CONTROL STATISTICS
==========

AOV TABLE
==========

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
REGRESSION	1	5.21	5.21	131.22**
DEVIATION	4	0.16	0.04	
TOTAL	5	5.37		

F(1,4,.05) = 7.71

F(1,4,.01) = 21.20

* : SIGNIFICANT AT 5% LEVEL
** : SIGNIFICANT AT 1% LEVEL

CORR. COEFF.	R**2	100% X R**2	SEE(Y)	SEE(S)
-0.9851	0.97	97.04	0.1993	0.2032

95% CONFIDENCE INTERVAL FOR S = -3.0377< S < -1.6174

UNKNOWN	SAMPLE	DILUTION	TOTAL	BOUND	%B	B/BO	LOGIT Y	DOSE(X)
001	M8 CUTTING	1: 1	17646.66	137.67	0.78	9.00	-2.31	13.54
002	M8 DRAINING	1: 1	17474.00	127.75	0.73	8.43	-2.38	14.53
003	M8 MILLING	1: 1	17490.00	106.33	0.61	7.01	-2.58	17.70
004	M8 DRESSING	1: 1	17678.00	106.00	0.60	6.92	-2.60	17.97
005	M8 1 YEAR CURING	1: 1	17667.50	95.17	0.54	6.21	-2.71	20.13
006	F4 CUTTING	1: 1	17310.00	2073.63	11.98	138.20	**	
007	F4 DRAINING	1: 1	17387.33	2018.17	11.61	133.90	**	
008	F4 MILLING	1: 1	17593.00	191.50	1.09	12.56	-1.94	9.36
009	F4 DRESSING	1: 1	17659.50	183.00	1.04	11.95	-2.00	9.90
010	F4 1 YEAR CURING	1: 1	17531.00	170.00	0.97	11.19	-2.07	10.66
011	J30 MILLING	1: 1	17708.00	2468.50	13.94	160.82	**	
012	J30 DRESSING	1: 1	17649.00	1901.25	10.77	124.28	**	
013	J30 1 YEAR CURING	1: 1	17440.50	1576.50	9.04	104.28	**	
014	J28 CUTTING	1: 1	17379.50	165.88	0.95	11.01	-2.09	10.85
015	J28 DRAINING	1: 1	17316.00	159.00	0.92	10.59	-2.13	11.33
016	J28 MILLING	1: 1	17564.66	138.33	0.79	9.09	-2.30	13.40
017	J28 DRESSING	1: 1	17508.00	129.25	0.74	8.52	-2.37	14.38
018	J28 1 YEAR CURING	1: 1	17510.00	114.75	0.66	7.56	-2.50	16.34
019	J21 DRAINING	1: 1	17427.00	1541.88	8.85	102.07	**	
020	J21 MILLING	1: 1	17428.50	261.25	1.50	17.29	-1.57	6.46
021	J21 DRESSING	1: 1	17603.00	220.88	1.25	14.48	-1.78	7.96
022	J21 1 YEAR CURING	1: 1	17447.00	202.00	1.16	13.36	-1.87	8.73
023	M4 DRAINING	1: 1	17434.00	1934.67	11.10	128.02	**	
024	M4 MILLING	1: 1	17448.00	1904.17	10.91	125.90	**	
025	M4 DRESSING	1: 1	17636.50	268.83	1.52	17.58	-1.54	6.33
026	M4 1 YEAR CURING	1: 1	17357.00	219.88	1.27	14.61	-1.77	7.87
027	A4 CUTTING	1: 1	17503.33	1939.67	11.08	127.84	**	
028	A4 DRAINING	1: 1	17667.50	205.88	1.17	13.44	-1.86	8.66
029	A4 MILLING	1: 1	17610.50	186.00	1.06	12.18	-1.98	9.69
030	A4 1 YEAR CURING	1: 1	17562.00	168.83	0.96	11.09	-2.08	10.76
031	A13 CUTTING	1: 1	17670.00	1845.00	10.44	120.46	**	
032	A13 DRAINING	1: 1	17488.00	194.17	1.11	12.81	-1.92	9.16
033	A13 MILLING	1: 1	17517.50	170.00	0.97	11.20	-2.07	10.65
034	A13 1 YEAR CURING	1: 1	17477.50	153.38	0.88	10.12	-2.18	11.91
035	C2 RECOVERY	1: 1	17508.50	638.88	3.65	42.10	-0.32	1.88
036	C2 RECOVERY	1: 1	17626.50	615.63	3.49	40.29	-0.39	2.03
037	C3 RECOVERY	1: 1	17517.00	635.38	3.63	41.84	-0.33	1.90

UNKNOWN
=======

SECTION
=======

** : -- Y > 100 ----> Y / (100-Y) IS NEGATIVE
LOGIT Y CANNOT BE CALCULATED ----> NO TOXINS

Appendix 31

Computer output on the radioimmunoassay of staphylococcal enterotoxin A in cheese samples (collected at the 'out-of-press' stage of the cheese manufacturing process) is presented as follows :

RUN INFORMATION
==========

DATE OF RADIOIMMUNOASSAY* - 16/11/1976.

STAPHYLOCOCCAL ENTEROTOXIN - A

TYPE OF UNKNOWN - CHEDDAR CHEESE(OUT-OF-PRESS)

NO. OF UNKNOWNS - 16

NO. OF TUBES/UNKNOWN DIL'N - 3

STANDARD INFORMATION
==========

NO. OF BACKGROUND TUBES - 10

NO. OF TUBES (PIPETTING ERROR) - 10

NO. OF MAXIMUM TOTAL TUBES - 3

NO. OF MAXIMUM BOUND TUBES - 3

NO. OF STANDARD DOSE VALUES - 7

NO. OF TUBES/STANDARD DOSE VALUE - 3

* anti-enterotoxin dilution used in this assay was 1:2500.

BASELINE STANDARDS
==========

TYPE	TUBE	COUNT	STD.DEV.	C.V.
BACKGROUND				
	1	33		
	2	37		
	3	31		
	4	39		
	5	31		
	6	33		
	7	28		
	8	35		
	9	31		
	10	36		
AVG (CPM)		66.80	6.68	10.00

TOTAL(PIPETTING ERROR ESTIMATION)

	1	7578		
	2	7561		
	3	7680		
	4	7669		
	5	7689		
	6	7707		
	7	7684		
	8	7523		
	9	7673		
	10	7595		
AVG (CPM)		15271.80	129.59	0.85

PIPETTING ERROR = SQRT|(S.D.**2 - AVG)|/AVG X 100% = 0.26%

MAXIMUM TOTAL

	1	7523		
	2	7612		
	3	7578		
AVG (CPM)		15075.20	89.08	0.59

MAXIMUM BOUND

	1	2764		
	2	2841		
	3	2836		
AVG (CPM)		1340.03	21.53	1.61

BO = (AVG MAX BOUND / AVG MAX TOTAL) X 100% = 8.8890%

STANDARD CURVE TOTAL COUNT DATA

DOSE	TUBE	TOTAL COUNT	S.D.	C.V.
0.63	1	7630		
0.63	2	7502		
0.63	3	7621		
AVG (CPM) =		15101.86	142.51	0.94
1.25	4	7671		
1.25	5	7707		
1.25	6	7561		
AVG (CPM) =		15225.86	151.65	1.00
2.50	7	7690		
2.50	8	7684		
2.50	9	7700		
AVG (CPM) =		15315.86	9.24	0.06
5.00	10	7680		
5.00	11	7547		
5.00	12	7607		
AVG (CPM) =		15155.86	132.42	0.87
10.00	13	7596		
10.00	14	7669		
10.00	15	7543		
AVG (CPM) =		15138.53	125.81	0.83
20.00	16	7704		
20.00	17	7689		
20.00	18	7605		
AVG (CPM) =		15265.20	106.13	0.70
40.00	19	7707		
40.00	20	7523		
40.00	21	7675		
AVG (CPM) =		15203.20	196.07	1.29

STANDARD CURVE BOUND COUNT DATA

DOSE	TUBE	BOUND COUNT	S.D.	C.V.
0.63	1	2574		
0.63	2	2726		
0.63	3	2750		
AVG (CPM) =		1274.87	47.71	3.74
1.25	4	2458		
1.25	5	2504		
1.25	6	2402		
AVG (CPM) =		1160.53	25.52	2.20
2.50	7	2358		
2.50	8	2210		
2.50	9	2278		
AVG (CPM) =		1074.20	37.03	3.45
5.00	10	1932		
5.00	11	2005		
5.00	12	1926		
AVG (CPM) =		910.37	21.98	2.41
10.00	13	1638		
10.00	14	1631		
10.00	15	1656		
AVG (CPM) =		754.03	6.43	0.85
20.00	16	1174		
20.00	17	1131		
20.00	18	1113		
AVG (CPM) =		502.87	15.67	3.12
40.00	19	904		
40.00	20	882		
40.00	21	898		
AVG (CPM) =		380.53	5.67	1.49

STANDARD CURVE DATA

DOSE	TOTAL COUNT	BOUND COUNT	% B	B / B0	LOGIT Y	LOG X
0.63	15101.86	1274.87	8.44	94.97	2.9379	-0.2007
1.25	15225.86	1160.53	7.62	85.75	1.7945	0.0969
2.50	15315.86	1074.20	7.01	78.90	1.3191	0.3979
5.00	15155.86	910.37	6.01	67.57	0.7343	0.6990
10.00	15138.53	754.03	4.98	56.03	0.2426	1.0000
20.00	15265.20	502.87	3.29	37.06	-0.5297	1.3010
40.00	15203.20	380.53	2.50	28.16	-0.9366	1.6021

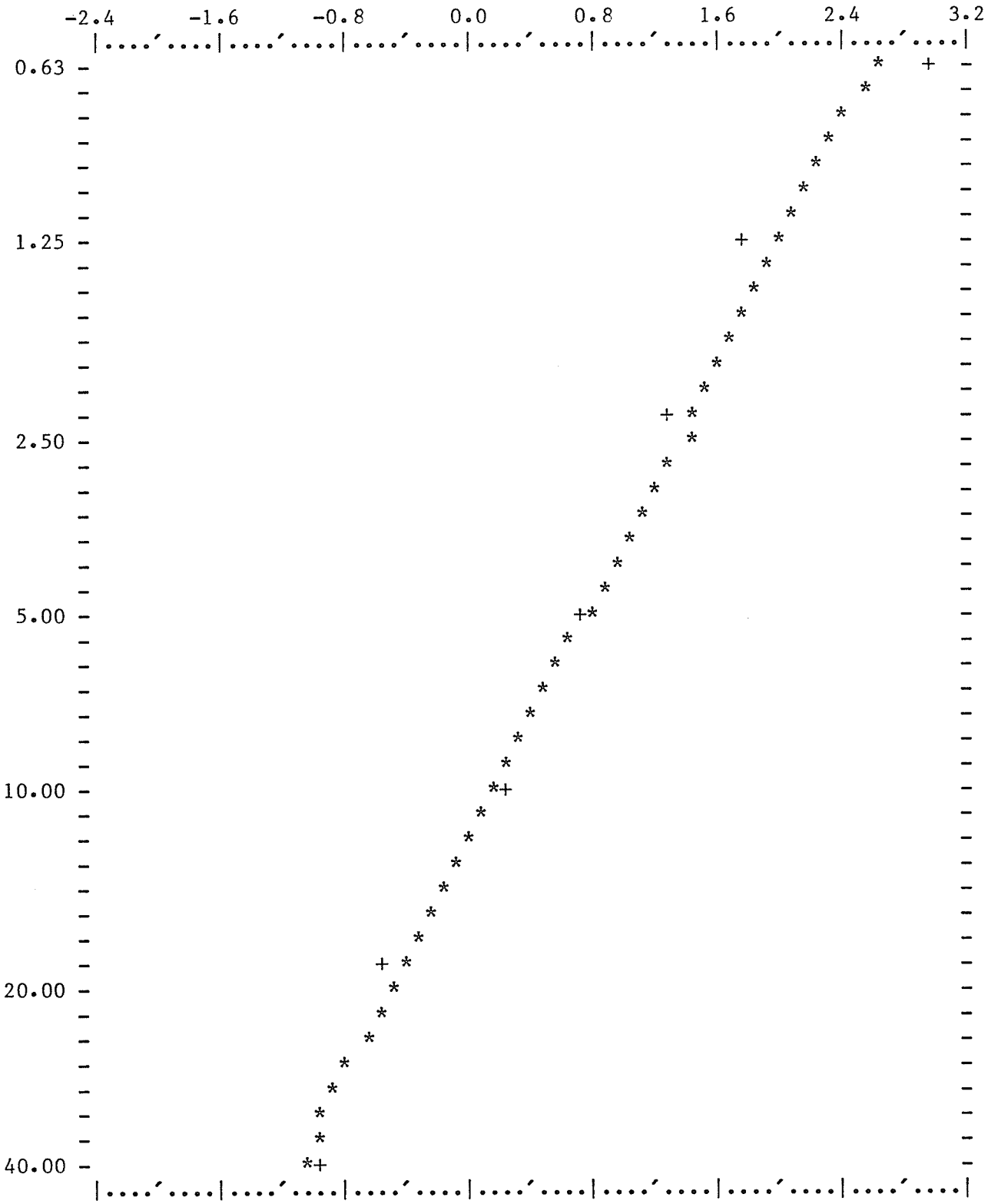
UNWEIGHTED REGRESSION

S = SLOPE = -2.06

INT = Y-INTERCEPT = 2.24

REGRESSION EQUATION : LOGIT Y = 2.24 - 2.06 X LOG (X)

STANDARD CURVE
==========
VERTICAL = DOSE (LOG SCALE) = NG/ML
HORIZONTAL = RESPONSE = LOGIT Y



SYMBOLS : + = ACTUAL POINT
* = CALCULATED POINT
@ = MULTIPLE PTS. <WHERE ACTUAL PT. COINCIDES WITH CALCULATED PT.>

QUALITY CONTROL STATISTICS
==========

AOV TABLE
==========

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
REGRESSION	1	10.74	10.74	293.43**
DEVIATION	5	0.18	0.04	
TOTAL	6	10.93		

F(1,5,.05) = 6.61

F(1,5,.01) = 16.26

* : SIGNIFICANT AT 5% LEVEL
** : SIGNIFICANT AT 1% LEVEL

CORR. COEFF.	R**2	100% X R**2	SEE(Y)	SEE(S)
-0.9916	0.98	98.32	0.1914	0.1203

95% CONFIDENCE INTERVAL FOR S = -2.4408< S < -1.6799

UNKNOWN SECTION FOR TOTAL COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	J23	OUT OF PRESS	7699		
				7562		
				7735		
			AVG (CPM) =	15263.86	182.43	1.20
0002	1: 1	M6	OUT OF PRESS	7605		
				7792		
				7671		
			AVG (CPM) =	15311.86	189.09	1.23
0003	1: 1	J19	OUT OF PRESS	7636		
				7519		
				7507		
			AVG (CPM) =	15041.20	141.91	0.94
0004	1: 1	F6	OUT OF PRESS	7675		
				7723		
				7663		
			AVG (CPM) =	15307.20	62.65	0.41
0005	1: 1	J30	OUT OF PRESS	7673		
				7595		
				7726		
			AVG (CPM) =	15262.53	131.45	0.86
0006	1: 1	F4	OUT OF PRESS	7787		
				7712		
				7716		
			AVG (CPM) =	15409.86	84.16	0.55
0007	1: 1	J26	OUT OF PRESS	7666		
				7650		
				7719		
			AVG (CPM) =	15289.86	71.26	0.47
0008	1: 1	F11	OUT OF PRESS	7574		
				7675		
				7691		
			AVG (CPM) =	15226.53	126.32	0.83

UNKNOWN SECTION FOR TOTAL COUNT (cont'd)

0009	1: 1	J21	OUT OF PRESS	7644 7689 7725		
			AVG (CPM) =	15305.20	81.06	0.53
0010	1: 1	M4	OUT OF PRESS	7624 7769 7676		
			AVG (CPM) =	15312.53	146.21	0.95
0011	1: 1	J28	OUT OF PRESS	7699 7692 7724		
			AVG (CPM) =	15343.20	32.00	0.21
0012	1: 1	M8	OUT OF PRESS	7660 7665 7614		
			AVG (CPM) =	15225.86	55.04	0.36
0013	1: 1	M13	OUT OF PRESS	7677 7637 7748		
			AVG (CPM) =	15307.86	111.81	0.73
0014	1: 1	M20	OUT OF PRESS	7786 7651 7741		
			AVG (CPM) =	15385.20	136.70	0.89
0015	1: 1	A4	OUT OF PRESS	7799 7740 7703		
			AVG (CPM) =	15427.86	96.22	0.62
0016	1: 1	A13	OUT OF PRESS	7655 7645 7678		
			AVG (CPM) =	15251.86	32.66	0.21

UNKNOWN SECTION FOR BOUND COUNT

UNKNOWN	DILUTION	SAMPLE NO.	TIME OF SAMPLING	COUNT	S.D.	C.V.
0001	1: 1	J23	OUT OF PRESS	3347		
				3309		
				3316		
			AVG (CPM) =	1595.20	10.33	0.65
0002	1: 1	M6	OUT OF PRESS	3430		
				3390		
				3326		
			AVG (CPM) =	1624.20	26.33	1.62
0003	1: 1	J19	OUT OF PRESS	3250		
				3297		
				3312		
			AVG (CPM) =	1576.37	16.25	1.03
0004	1: 1	F6	OUT OF PRESS	3474		
				3490		
				3561		
			AVG (CPM) =	1687.37	23.21	1.38
0005	1: 1	J30	OUT OF PRESS	3487		
				3513		
				3552		
			AVG (CPM) =	1691.87	16.49	0.97
0006	1: 1	F4	OUT OF PRESS	1507		
				1604		
				1638		
			AVG (CPM) =	724.70	33.99	4.69
0007	1: 1	J26	OUT OF PRESS	3268		
				3304		
				3293		
			AVG (CPM) =	1577.37	9.38	0.59
0008	1: 1	F11	OUT OF PRESS	3068		
				3176		
				3157		
			AVG (CPM) =	1500.03	28.89	1.93

UNKNOWN SECTION FOR BOUND COUNT (cont'd)

0009	1: 1	J21	OUT OF PRESS	1737		
				1684		
				1745		
			AVG (CPM) =	794.20	16.56	2.08
0010	1: 1	M4	OUT OF PRESS	1752		
				1826		
				1779		
			AVG (CPM) =	826.03	18.71	2.27
0011	1: 1	J28	OUT OF PRESS	1336		
				1298		
				1302		
			AVG (CPM) =	589.20	10.44	1.77
0012	1: 1	M8	OUT OF PRESS	1240		
				1181		
				1205		
			AVG (CPM) =	537.53	14.83	2.76
0013	1: 1	M13	OUT OF PRESS	3413		
				3506		
				3504		
			AVG (CPM) =	1670.37	26.63	1.59
0014	1: 1	M20	OUT OF PRESS	3178		
				3105		
				3199		
			AVG (CPM) =	1513.53	24.71	1.63
0015	1: 1	A4	OUT OF PRESS	1625		
				1626		
				1579		
			AVG (CPM) =	738.20	13.41	1.82
0016	1: 1	A13	OUT OF PRESS	1579		
				1514		
				1537		
			AVG (CPM) =	704.87	16.47	2.34

UNKNOWN SECTION
==========

UNKNOWN	LOT	DILUTION	TOTAL	BOUND	% B	B / B0	LOGIT Y	DOSE(X)
001	J23	1: 1	15263.86	1595.20	10.45	117.57	**	
002	M6	1: 1	15311.86	1624.20	10.61	119.33	**	
003	J19	1: 1	15041.20	1576.37	10.48	117.90	**	
004	F6	1: 1	15307.20	1687.37	11.02	124.01	**	
005	J30	1: 1	15262.53	1691.87	11.09	124.71	**	
006	F4	1: 1	15409.86	724.70	4.70	52.91	0.12	10.68
007	J26	1: 1	15289.86	1577.37	10.32	116.06	**	
008	F11	1: 1	15226.53	1500.03	9.85	110.83	**	
009	J21	1: 1	15305.20	794.20	5.19	58.38	0.34	8.34
010	M4	1: 1	15312.53	826.03	5.39	60.69	0.43	7.49
011	J28	1: 1	15343.20	589.20	3.84	43.20	-0.27	16.52
012	M8	1: 1	15225.86	537.53	3.53	39.72	-0.42	19.39
013	M13	1: 1	15307.86	1670.37	10.91	122.76	**	
014	M20	1: 1	15385.20	1513.53	9.84	110.67	**	
015	A4	1: 1	15427.86	738.20	4.78	53.83	0.15	10.25
016	A13	1: 1	15251.86	704.87	4.62	51.99	0.08	11.13

** : -- Y > 100 ----> Y / (100-Y) IS NEGATIVE
LOGIT Y CANNOT BE CALCULATED ----> NO TOXINS

Appendix 32

Computer output on the radioimmunoassay of staphylococcal enterotoxin A in cheese samples , collected at the '60 days of curing' stage of the cheese manufacturing process, is presented as follows :

RUN INFORMATION
==========

DATE OF RADIOIMMUNOASSAY - 15/12/1976.

STAPHYLOCOCCAL ENTEROTOXIN - A

TYPE OF UNKNOWN - CHEDDAR CHEESE(60 DAYS CURING)

NO. OF UNKNOWNS - 16

NO. OF TUBES/UNKNOWN DIL'N - 3

STANDARD INFORMATION
==========

NO. OF BACKGROUND TUBES - 10

NO. OF TUBES (PIPETTING ERROR) - 10

NO. OF MAXIMUM TOTAL TUBES - 3

NO. OF MAXIMUM BOUND TUBES - 3

NO. OF STANDARD DOSE VALUES - 7

NO. OF TUBES/STANDARD DOSE VALUE - 3

BASELINE STANDARDS
==========

TYPE	TUBE	COUNT	STD.DEV.	C.V.
BACKGROUND				
	1	8		
	2	6		
	3	4		
	4	4		
	5	5		
	6	6		
	7	4		
	8	4		
	9	6		
	10	8		
AVG (CPM)		55.00	15.81	28.75

TOTAL(PIPETTING ERROR ESTIMATION)

	1	4868		
	2	4818		
	3	4725		
	4	5080		
	5	4964		
	6	4886		
	7	5159		
	8	4789		
	9	4843		
	10	4811		
AVG (CPM)		9788.60	270.86	2.77

PIPETTING ERROR = SQRT|(S.D.**2 - AVG)|/AVG X 100% = 2.58%

MAXIMUM TOTAL

	1	4851		
	2	4782		
	3	4999		
AVG (CPM)		9699.66	221.61	2.28

MAXIMUM BOUND

	1	2076		
	2	2081		
	3	2098		
AVG (CPM)		987.50	5.76	0.58

BO = (AVG MAX BOUND / AVG MAX TOTAL) X 100% = 10.1808%

STANDARD CURVE TOTAL COUNT DATA

DOSE	TUBE	TOTAL COUNT	S.D.	C.V.
0.63	1	4724		
0.63	2	4868		
0.63	3	4950		
AVG (CPM) =		9639.66	228.71	2.37
1.25	4	4818		
1.25	5	4916		
1.25	6	4868		
AVG (CPM) =		9679.66	97.76	1.01
2.50	7	4811		
2.50	8	4921		
2.50	9	4759		
AVG (CPM) =		9605.66	165.38	1.72
5.00	10	4843		
5.00	11	4789		
5.00	12	4928		
AVG (CPM) =		9651.66	139.94	1.45
10.00	13	4745		
10.00	14	4826		
10.00	15	4964		
AVG (CPM) =		9635.00	221.32	2.30
20.00	16	5080		
20.00	17	5159		
20.00	18	4739		
AVG (CPM) =		9930.33	446.33	4.49
40.00	19	4886		
40.00	20	4868		
40.00	21	5237		
AVG (CPM) =		9939.00	416.05	4.19

STANDARD CURVE BOUND COUNT DATA

DOSE	TUBE	BOUND COUNT	S.D.	C.V.	
0.63	1	8985			
0.63	2	9400			
0.63	3	8754			
AVG (CPM) =		849.63	32.73	3.85	
1.25	4	8212			
1.25	5	8353			
1.25	6	9213			
AVG (CPM) =		804.27	54.18	6.74**	REJECT **
1.25		8212			
1.25		8353			
AVG (CPM) =		773.25	9.97	1.29	
2.50	7	7869			
2.50	8	7730			
2.50	9	8845			
AVG (CPM) =		759.80	60.76	8.00**	REJECT **
2.50		7869			
2.50		7730			
AVG (CPM) =		724.95	9.82	1.36	
5.00	10	6581			
5.00	11	6371			
5.00	12	8948			
AVG (CPM) =		675.00	143.11	21.20**	REJECT **
5.00		6581			
5.00		6371			
AVG (CPM) =		592.60	14.85	2.51	
10.00	13	1157			
10.00	14	1039			
10.00	15	1182			
AVG (CPM) =		508.00	38.19	7.52**	REJECT **
10.00		1157			
10.00		1182			
AVG (CPM) =		529.75	8.83	1.67	
20.00	16	950			
20.00	17	977			
20.00	18	923			
AVG (CPM) =		420.00	13.50	3.21	
40.00	19	817			
40.00	20	841			
40.00	21	829			
AVG (CPM) =		359.50	6.00	1.67	

STANDARD CURVE DATA
==========

DOSE	TOTAL COUNT	BOUND COUNT	% B	B / B0	LOGIT Y	LOG X
0.63	9639.66	849.63	8.81	86.57	1.8638	-0.2007
1.25	9679.66	773.25	7.99	78.47	1.2930	0.0969
2.50	9605.66	724.95	7.55	74.13	1.0528	0.3979
5.00	9651.66	592.60	6.14	60.31	0.4183	0.6990
10.00	9635.00	529.75	5.50	54.01	0.1606	1.0000
20.00	9930.33	420.00	4.23	41.54	-0.3415	1.3010
40.00	9939.00	359.50	3.62	35.53	-0.5959	1.6021

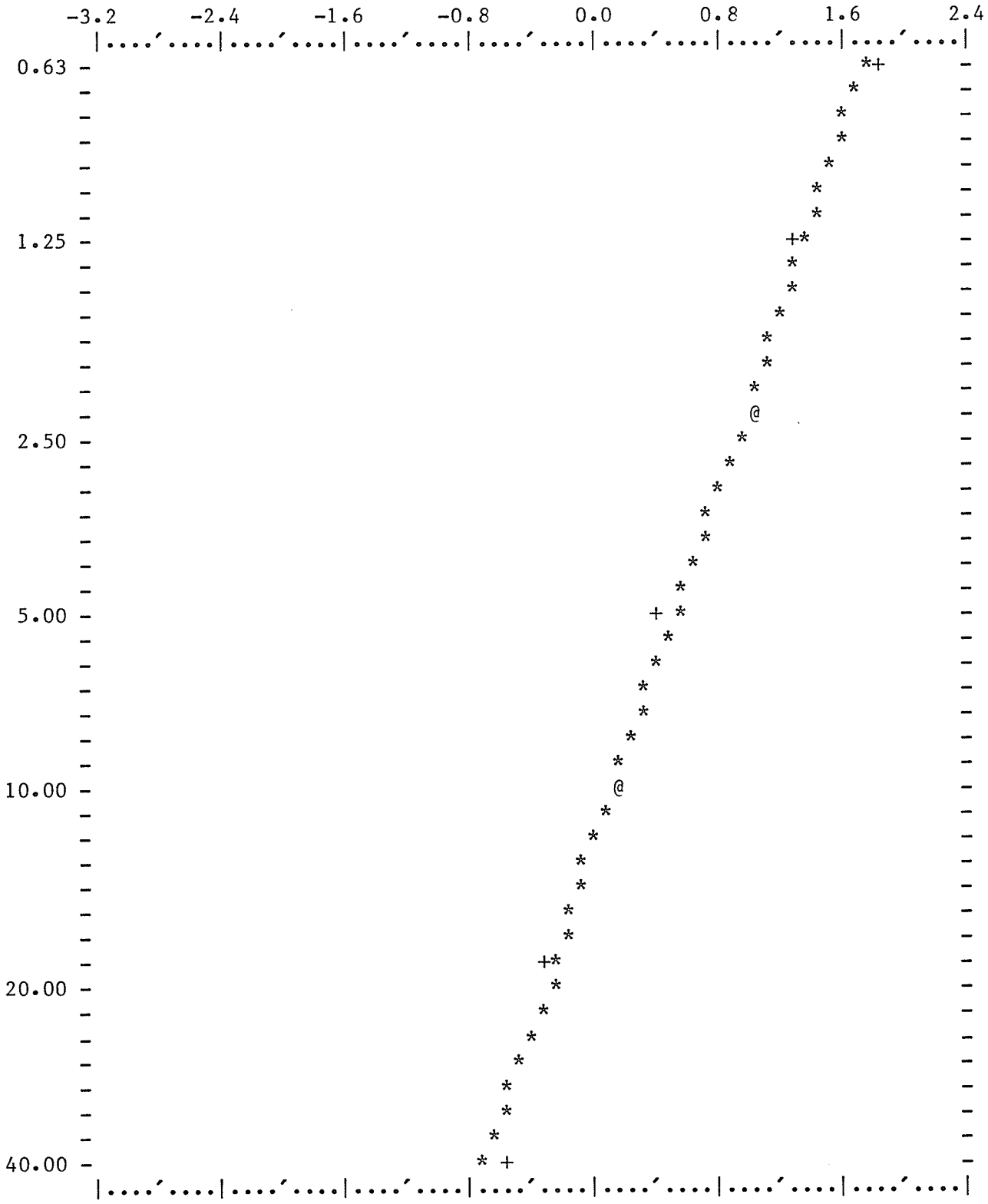
UNWEIGHTED REGRESSION
==========

S = SLOPE = -1.37

INT = Y-INTERCEPT = 1.51

REGRESSION EQUATION : LOGIT Y = 1.51 - 1.37 X LOG (X)

STANDARD CURVE
==========
VERTICAL = DOSE (LOG SCALE) = NG/ML
HORIZONTAL = RESPONSE = LOGIT Y



SYMBOLS : + = ACTUAL POINT
* = CALCULATED POINT
@ = MULTIPLE PTS. <WHERE ACTUAL PT. COINCIDES WITH CALCULATED PT.>

QUALITY CONTROL STATISTICS
==========

AOV TABLE
==========

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
REGRESSION	1	4.76	4.76	456.12**
DEVIATION	5	0.05	0.01	
TOTAL	6	4.81		

F(1,5,.05) = 6.61

F(1,5,.01) = 16.26

* : SIGNIFICANT AT 5% LEVEL
** : SIGNIFICANT AT 1% LEVEL

CORR. COEFF.	R**2	100% X R**2	SEE(Y)	SEE(S)
-0.9946	0.99	98.92	0.1021	0.0642

95% CONFIDENCE INTERVAL FOR S = -1.5738< S < -1.1677

UNKNOWN SECTION
==========

UNKNOWN	LOT	DILUTION	TOTAL	BOUND	% B	B / B0	LOGIT Y	DOSE(X)
001	J23	1: 1	9589.00	1012.67	10.56	103.73	**	
001	J23	1: 2	10360.33	1057.17	10.20	100.23	**	
002	M6	1: 1	9507.66	973.50	10.24	100.57	**	
003	J19	1: 1	9785.66	1073.00	10.97	107.70	**	
004	F6	1: 1	9670.33	991.67	10.25	100.73	**	
005	J30	1: 1	9521.00	1044.83	10.97	107.79	**	
005	J30	1: 2	9276.33	956.83	10.31	101.32	**	
006	F4	1: 1	9631.00	517.83	5.38	52.81	0.11	10.44
006	F4	1: 2	9944.33	631.50	6.35	62.38	0.51	5.40
007	J26	1: 1	9665.00	992.83	10.27	100.90	**	
007	J26	1: 2	9607.66	1047.33	10.90	107.07	**	
008	F11	1: 1	9600.33	1007.33	10.49	103.06	**	
008	F11	1: 2	9480.33	1012.67	10.68	104.92	**	
009	J21	1: 1	9634.33	552.33	5.73	56.31	0.25	8.23
009	J21	1: 2	9639.00	644.33	6.68	65.66	0.65	4.25
010	M4	1: 1	9848.33	577.33	5.86	57.58	0.31	7.55
010	M4	1: 2	9710.33	665.00	6.85	67.27	0.72	3.76
011	J28	1: 1	9427.00	450.33	4.78	46.92	-0.12	15.51
011	J28	1: 2	9439.00	548.33	5.81	57.06	0.28	7.82
012	M8	1: 1	9571.66	419.33	4.38	43.03	-0.28	20.21
012	M8	1: 2	9605.00	523.83	5.45	53.57	0.14	9.92
013	M13	1: 1	9696.33	1092.50	11.27	110.67	**	
013	M13	1: 2	9397.66	978.00	10.41	102.22	**	
014	M20	1: 1	9477.66	1008.17	10.64	104.48	**	
014	M20	1: 2	9536.33	974.00	10.21	100.32	**	
015	A4	1: 1	9689.66	525.67	5.43	53.29	0.13	10.11
015	A4	1: 2	9888.33	628.00	6.35	62.38	0.51	5.39
016	A13	1: 1	9451.00	492.33	5.21	51.17	0.05	11.66
016	A13	1: 2	9576.33	581.67	6.07	59.66	0.39	6.54

** : -- Y > 100 ----> Y / (100-Y) IS NEGATIVE
LOGIT Y CANNOT BE CALCULATED ----> NO TOXINS

RECOMMENDATIONS FOR FUTURE INVESTIGATIONS

Possible Research Aspects

1. In future studies, thermonuclease production in the toxic milk and cheese samples should be detected, so as to determine the correlation between thermonuclease production and enterotoxin production.
2. Survey studies can be conducted on (i) the thermonuclease content of various milk and cheese samples from different commercial dairies and (ii) the relationship between the S. aureus content and thermonuclease content of these samples.
3. Different S. aureus strains isolated from heat treated milk require to be tested relative to the variability of the minimum population associated with the presence of enterotoxins.
4. Relative large scale study on the maximum and minimum levels of S. aureus associated with the presence and absence of enterotoxin in other types of cheese.
5. Determination of the total free fatty acids in heat treated milk used for cheesemaking and subsequently in cheeses made with normal and subnormal starter.
6. Employing isoelectric focusing and electrophoretic techniques as analytical tools in elucidating some of the non-specific protein fractions found in the cheese extracts.
7. The applicaiton of tracer methodology and ion-exchange chromatography may provide a better understanding of the role of the proteolytic enzymes present in the cheese extracts (if there are any) in the cleavage or degradation of the active binding sites of the enterotoxin and/or anti-enterotoxin molecules.