PRODUCTION AND THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXINS IN MEAT SYSTEMS

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ABSTRACT

PRODUCTION AND THERMAL INACTIVATION
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By

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Staphylococcus aureus 265 and 243 which produce enterotoxins A and B, respectively, were inoculated into Genoa salami in the amount of 10^3, 10^5, and 10^7 cells/g of meat for the purpose of detecting the rate of growth and enterotoxin production. The spices and curing agents were added to the coarsely ground pork before inoculation, but no lactic starter culture was used. The stuffed salami was then cured, tempered, and heated at 38 C for 20 hr, 43 C for 2 hr, 49 C for 4 hr, and 54 C for 3 hr at relative humidity (RH) of 90%. After heating, they were moved to a drying room at 12 C with RH 67-72% for approximately 60 days.

At different stages of processing (after curing, tempering, heating, and during drying) samples were taken to determine the microbial populations, percentage of moisture, total acidity (expressed as lactic acid), pH and enterotoxin production. Samples composed of the outer 1 cm of surface and samples of the core were taken from salami inoculated with S. aureus 265. The staphylococcal and

total counts were higher in the surface samples than in the core samples. After tempering, 1.5×10^7, 2.8×10^8, and 4.9×10^8 staphylococcal cells/g were obtained from the surface of the salami inoculated with 10^3, 10^5, and 10^7 cells/g, respectively. In the core samples increases of 2.47 and 1.18 log cycles(s) occurred in the salami inoculated with 10^3 and 10^5 cells/g, respectively, and only a slight increase occurred in the salami inoculated with 10^7 cells/g. Heating caused a reduction of 1 to 2 log cycles in both surface and core samples. During the drying period, the populations gradually decreased. Populations greater than 10^6 cells/g remained in the surface portion of each salami throughout the drying process. In the samples taken after 8 or more days, approximately 0.2 ug of enterotoxin A was detected in 100 g of the surface samples of salami inoculated with 10^5 and 10^7 cells/g, but no enterotoxin was detected in the salami inoculated with 10^3 cells/g. The pH changes during processing were minute, whereas titratable lactic acid increased gradually.

Samples were taken from cross sections of the salami inoculated with S. aureus 243, and after tempering the staphylococcal populations were 1.0×10^6, 9.0×10^6, and 1.3×10^8 cells/g in the salami inoculated with 10^3, 10^5, and 10^7 cells/g, respectively. Enterotoxin B was not detected in any of the samples taken from salami inoculated with S. aureus 243.

Another portion of this investigation concerned the thermal inactivation of enterotoxins which are known to be heat stable proteins. The thermal stability varied tremendously in different heating menstrua and the specific effect of protein from beef broth was studied intensively. Crude enterotoxins were obtained by centrifuging a 24-hr staphylococcal culture and concentrating the supernatant against 40% polyethylene glycol. Samples of this crude enterotoxin at an initial concentration of 16 to 32 ug/ml were heated in TDT cans using a small retort. Inactivation of enterotoxin was then followed by plotting the log of titer vs heating time, and the D and Z values were determined from data obtained at 110.0, 115.6, 121.1, and 126.7 C. At all temperatures tested, D values were higher when crude enterotoxin A (SEA) was heated in beef broth than when heated in Brain Heat Infusion broth. Also crude enterotoxin B (SEB) had greater thermal stability in beef broth than in Brain Heart Infusion broth or veronal buffer; however, there was little difference among the Z values obtained in veronal buffer, Brain Heart Infusion or beef broth.

In order to study the reasons for the variation of D values in different heating menstrua, crude enterotoxins were partially purified by acid and ammonium sulfate precipitation, followed by gel filtration using a Sephadex G-100 column. The influence of various fractions of beef broth prepared by ultrafiltration through a PM 10 membrane, on the thermal stability of enterotoxin B was studied at

110 C. The fractions retained by the membrane had a significant protective effect, and the filtrate also showed slight protection. When the protein precipitate obtained by adding ammonium sulfate to the beef broth was added at 3.8 and 7.7 mg/ml to the veronal buffer, the D_{110} values of enterotoxin B in these heating menstrua were 51 and 70 min, respectively. But when the beef broth protein was dialyzed against veronal buffer prior to use, the D_{110} values of enterotoxin B were only 39 and 41 min, respectively, at protein concentrations comparable to those used with the non-dialyzed protein. Finally, when the dialysate was added back to the dialyzed protein, the protective effect was essentially restored to that of the non-dialyzed protein.

Therefore, in addition to the nonspecific protein effect, this investigation revealed the existence of a dialyzable factor in the beef broth which appeared to influence the thermal stability of the partially purified enterotoxin B. Preliminary studies suggest the factor has a molecular weight of less than 12,000 and possibly a protein fraction was involved in protecting the enterotoxins during heating. Data from additional experiments indicated this protective effect extends to enterotoxins A, C, and D. Isolation and characterization of the factor will be required for further understanding of its nature and reaction with enterotoxin during heating.

PRODUCTION AND THERMAL INACTIVATION OF STAPHYLOCOCCAL ENTEROTOXINS IN MEAT SYSTEMS

By

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INTRODUCTION

The manufacture of fermented sausages has long been considered a skilled art. Research on starter cultures of lactic acid bacteria has made it possible to use these microorganisms to overcome the problems involved during processing. However, "chance inoculation" and "back slopping" are still used to some extent in industry. In fact, recent cases of staphylococcal gastroenteritis were traced to Genoa salami containing up to 10^6 type A coagulase positive staphylococci/g produced by two commercial companies. Enterotoxin A was also detected in some of the samples (84, 85, 86). The widespread geographical locations of these outbreaks indicate that the cause was manufacturing procedures rather than mishandling by customers. One purpose of the investigation reported herein was to evaluate the processing procedures used for Genoa salami, determine the changes in microbial populations, measure enterotoxin production, and perform other chemical analyses of the salami.

The second area of investigation was the thermal inactivation of enterotoxins. Denny et al. (31) indicated

that enterotoxin A was inactivated faster in phosphate buffer than in beef bouillon at all concentrations tested, whereas, Satterlee and Kraft (63) demonstrated that during heating there was a more rapid loss of enterotoxin B in the presence of either myosin or metmyoglobin than when heated in phosphate buffer. The enterotoxins are known as thermally resistant proteins and heating menstrua have a great influence on the thermal stability of enterotoxins. However, the possible factors which cause the difference in stability have not been studied previously. Thus, it was the intention of this investigation to examine the effect of meat proteins on enterotoxins during heating at various temperatures.

LITERATURE REVIEW

Staphylococci were first named in 1881 because of the grapelike clusters of cocci, and their ability to produce toxin was demonstrated as early as 1884 (52). Since then, several reports have referred to food poisoning caused by these organisms. However, it was not until 1930 that Dack et al. (29) demonstrated in human volunteers that culture filtrates of staphylococci caused gastroenteritis.

There have been numerous publications about staphylococcal enterotoxin including some excellent review articles (5, 11, 12, 28, 52). The literature reviewed in this dissertation will be limited to publications pertinent to the various aspects of this study. The different types of enterotoxin were first studied by Bergdoll et al. (17) and Casman (22). To date, five staphylococcal enterotoxins designated as A, B, C, D, and E, have been classified according to their reactions with specific antibodies. Staphylococcal enterotoxin A (SEA) has been associated more frequently with food poisoning outbreaks than the other identified enterotoxins (23). Staphylococcal enterotoxin B (SEB), although occasionally involved in food poisoning, has been mostly associated with staphylococci isolated from

other human ailments (73), while staphylococcal enterotoxin C (SEC) has been produced by strains isolated from foods which were implicated in food poisoning outbreaks (13). Casman et al. (25) designated yet a fourth staphylococcal enterotoxin as strain D (SED), and indicated that the role of SED in food poisoning was second in frequency only to that of SEA. Then, in 1971, Bergdoll et al. (14) obtained a staphylococcal strain from chicken tetrazzini which was implicated in a food poisoning outbreak and found that the enterotoxin produced by this strain was different from enterotoxins A, B, C, or D. They thus presented evidence for identification of the fifth enterotoxin as E (SEE).

Purification and Selected Properties of Enterotoxins

Purified enterotoxins are fluffy, white materials that are highly hygroscopic and soluble in water and salt solutions. They are simple proteins which contain amino acids only. Enterotoxins have been purified by different groups of researchers. A freeze-dried preparation of SEA has been described by Chu et al. (27) in which SEA was purified by ion-exchange chromatography on carboxy-methyl-cellulose (CMC) and filtration through Sephadex G-100 and G-75 gels. The resultant purified SEA is a protein with 34,500 molecular weight determined by sedimentation and diffusion methods. Moreover, other properties, including the sedimentation diffusion coefficient and intrinsic viscosity were also determined. The term ED_{50} is defined

as the amount of enterotoxin which causes emesis in 50% of the animals challenged. The ED_{50} of SEA *per os* (ingestion) was 5 ug per monkey (2-3 Kg) and approximately 0.17 ug per monkey by intravenous injection.

Bergdoll *et al.* (15, 16) reported a significant purification of SEB by a combination of acid precipitation, adsorption on Amberlite IRC-50, ethanol precipitation, and starch-bed electrophoresis. A partial purification by a combination of ethanol precipitation, filtration on Sephadex and electrophoresis on Sephadex was then reported by Frea *et al.* (33), while Schantz *et al.* (64) described a method of purification of SEB on a large scale. The procedures in the latter method involved removal of the toxin from the culture and from the bulk of impurities with Amberlite CG-50 resin and purification to a high degree by chromatography on CMC. The purified SEB was a simple protein with a molecular weight of 35,300. The dose required to produce emesis or diarrhea in monkeys was 0.1 ug by intravenous injection and 0.9 ug by oral feeding per Kg of animal weight. Other properties of the purified toxin were also studied.

Purification of SEC from *S. aureus* strain 137 was reported by Borja and Bergdoll (19) who purified it by column chromatography on CMC and filtration through Sephadex G-75 and G-50 gels. The molecular weight was 34,100 determined by sedimentation and diffusion measurement. The isoelectric point of SEC was determined as

8.6 in veronal buffer of 0.1 ionic strength, while the toxicity required to produce emesis in rhesus monkeys (2-3 Kg) within 2-5 hours after intragastric administration was 5 ug. SEC produced from another strain, S. aureus 361, was purified similarly by Avena and Bergdoll (7), and when administered to monkeys, the ED_{50} was 5 to 10 ug intragastrically and 0.5 ug by intravenous injection. Bergdoll et al. (14) reported that SEE was also purified on a CMC column and gel filtration on Sephadex.

The isoelectric value of SEA is 6.8 which is in contrast to 8.6 for SEB (13). Differences in isoelectric pH may result from a difference in the number of basic groups as shown in SEC_1, and SEC_2 (7, 13). SEC_1 produced by strain 137 has an isoelectric point of 8.6 and SEC_2 produced by strain 361 has an isoelectric point of 7.0. The cross reactions of enterotoxins A and B, B and E, B and C, and A and E were studied (14) and these reactions indicated that an antigenic relationship might exist among all the enterotoxins. Such relationship among the enterotoxins could be revealed by further studies in this area.

Thermal Inactivation of Enterotoxins

In 1930 Dack et al. (29) reported that enterotoxins were relatively stable to heat. Since then, many workers have studied a variety of parameters in relation to the heat inactivation of enterotoxins.

Enterotoxin A. Purified SEA was reported to be relatively heat labile as compared with purified SEB (27). When 200 ug/ml of SEA were dissolved in 0.05 M sodium phosphate at pH 6.85 and heated at 60 C for 20 min a 50% loss in the serological reaction was observed, while heating a similar SEA solution at 70 C for 3 min resulted in a 60% decrease in the serological reaction. No antigen-antibody reaction was obtained after heating the SEA at 80 and 100 C for 3 and 1 min, respectively. However, other reports on heating of crude SEA have shown a much higher heat resistance. Heat inactivation of SEA in 0.04 M veronal buffer at pH 7.2, using Pyrex thermal death time tubes, was studied by Hilker et al. (40). They reported that total inactivation of 90 ug/ml was not accomplished by heating for 18 min at 121.1 C, 29 min at 115.5 C, 47 min at 110 C, 67 min at 104.4 C, or 106 min at 100 C. At least 3.25 ug/ml remained after each of these time-temperature treatments, while barely detectable amounts (1 ug/ml) were found after heating broth containing 21 ug/ml at the same time-temperature combinations. The slope of the inactivation curve (Z value) was about 27.8 C (50 F) (31, 40). Soo et al. (70) reported thermal inactivation of partially purified enterotoxin A and D in sodium acetate and phosphate buffer at different pH's, temperatures and toxin concentrations. SEA showed a more rapid loss of activity at 70 C than at 80 or 90 C and a more rapid inactivation at pH 4 to 5.5 than at pH 6.0 to 7.5.

Thermal inactivation curves for concentrations of 60, 20, and 5 ug/ml of SEA in beef bouillon were determined by Denny et al. (31). The initial concentration of 60 ug/ml had the highest end points, and the initial concentrations of 5 ug/ml had the lowest end points. However, the thermal inactivation of enterotoxin A was not directly proportional to concentration, since the difference in the end point at each of the heating temperatures was greater between 5 and 20 ug/ml than between 20 and 60 ug/ml. Such an increase in heat resistance associated with the increase in SEA concentration could be explained in part by a protective effect afforded one protein molecule by other protein molecules (31). It was also found that at all toxin concentrations tested SEA was inactivated by less heat in a pH 7.2 phosphate buffer than in beef bouillon (31). The detection of SEA in the above experiments was done serologically, while some earlier work on detection of heat-inactivated SEA was performed by observing emesis in cats and monkeys to which enterotoxin had been administered. In this previous work a Z value of 48 F was obtained from the heat-inactivation curve of crude SEA based on the cat emetic reaction to intraperitoneal injection (32).

It is obvious from the above findings of different investigators that the slopes of the thermal inactivation curve (Z values) are all about 27.8 C (50 F) regardless

of the toxin concentrations, media in which the toxins were heated, or the methods of detecting the toxin.

Enterotoxin B. Schantz et al. (64) reported the biological activity of purified SEB was retained after heating a solution at 60 C and pH 7.3 for as long as 16 hours. Also at 100 C for 5 min, less than 50% of the biological activity was destroyed. The times and temperatures required to inactivate SEB in veronal buffer were studied by Read and Bradshaw (60). Pure (99+%) and crude SEB were diluted in 0.04 M veronal buffer to 30 ug/ml and heated in an oil bath. The respective D values of crude and purified SEB were 64.5 and 52.3 min at 99 C, 40.5 and 34.4 min at 104.4 C, 29.7 and 23.5 min at 110 C, 18.8 and 16.6 min at 115.6 C, and 11.4 and 9.9 min at 121 C. It is noteworthy that the crude preparation was slightly more thermostable at all temperatures. The Z value for purified enterotoxin B was 32.4 C (58.5 F). Read and Bradshaw (59) also determined the thermal inactivation of purified SEB in raw milk at temperatures of 210 to 260 F, using an initial enterotoxin concentration of 30 ug/ml. D values were 68.5, 46.2, 26.1, 16.6, 9.4, and 6.2 min at 210, 220, 230, 240, 250, and 260 F, respectively. The time required to inactivate 30 ug/ml of enterotoxin B to levels believed to be lower than those emetic to humans was calculated, using the relationship F=4D.

Satterlee and Kraft (63) demonstrated the effect of meat slurry, myosin, and metmyoglobin (MetMb) on the thermal inactivation of SEB. They found the initial thermal inactivation of SEB was faster at 80 C than at 100 or 110 C with or without the presence of meat protein. Heating SEB at 60, 80, and 100 C in the presence of either myosin or MetMb resulted in a more rapid loss of the toxin than when heated in phosphate-saline buffer. Similar results were obtained for SEB in ground meat slurry. It was suggested that the rapid loss of SEB in the slurry might be due to two factors: (a) some of the enterotoxin may bind to meat proteins and thus become undetectable by the gel diffusion technique; and (b) the toxin that is not bound may be inactivated rapidly by the heat.

Stinson and Troller (71) reported that SEB underwent a rapid, initial decomposition during the first 20 seconds of heating at 149 C. A slower rate of decomposition then followed on further heating. They also demonstrated SEB was more stable when heated in menstruum of 0.90 a_w than 0.99 a_w. However, this protective effect diminished after 200 seconds of heating.

The effect of pH, protein concentration and ionic strength on heat inactivation of highly purified SEB was later studied by Jamlang et al. (41). They confirmed the findings of other researchers (63) who had noted a more rapid loss of immunological activity at 70 to 80 C than at 90 to 100 C. Such loss of immunological activity at 70 to

80 C did not follow first order kinetics, which is contrary to the usual pattern for the denaturation of protein. There was a rapid loss of activity and accompanying visible aggregation when the enterotoxin was heated at 70 and 80 C, but heating at 100 C redissolved the aggregate (41). Jamlang et al. (41) also studied the heat inactivation of SEB at a concentration of 100 ug/ml in 0.08 M sodium phosphate buffer. When the pH was changed from 6.4 to 4.5 or to 7.5, no large change occurred in the inactivation time at 70 C as long as the ionic strength was maintained at 0.1. At 100 C, SEB was more stable at pH 6.4 than at pH 4.5 or 7.5. It was also observed that a high initial concentration of SEB (up to 350 ug/ml) was associated with higher destruction at both pH 4.5 and 7.5 when heated for 10 min at 70 C.

Enterotoxin C. Avena and Bergdoll (7) reported that solutions of purified SEC from S. aureus strain 361 heated to 52 C developed turbidity which increased with an increase in temperature. The serological activity of SEC was reduced to about 20% of normal when the protein was heated at 100 C for 1 min. The purified SEC produced by S. aureus strain 137 was studied by Borja and Bergdoll (19). They indicated no loss of antigenicity when enterotoxin solutions were incubated for 0.5 hr at 60 C. A turbid solution resulted from incubation of SEC at 60 C for 1 hr. A study of the crude enterotoxins showed that

after 5 hr of heating at 80 C, both crude SEB and SEC retained 5-10% residual serological activity. Heating SEB at 100 C for 3 hr and heating SEC at 121 C for 30 min totally inactivated these toxins (34). Both SEB and SEC were inactivated slightly more rapidly at 80 C than at 100 C during the initial heating period of about 10 to 30 min. These phenomena have been described before by other workers (41, 63). After 24 hr of reactivation at 25 C, toxin treated at 100 C lost more acitivity than toxin treated at 80 C, at the initial and prolonged heating times. Other conclusions were: (a) Crude SEC was more heat stable than crude SEB under similar experimental conditions; (b) toxins subjected to "sublethal" time-temperature treatment had the ability to reactivate, but totally inactivated toxins did not have the ability to reactivate; (c) the reactivation was temperature dependent; and (d) mechanical stress or oxidation induced by agitation during heat treatment caused permanent damage to the toxin.

Reactivation is an interesting phenomenon in heating experiments. It has been proposed that proteins unfold during heating. If there is no permanent damage, reassociation of the toxin molecules may occur during cooling (34). The reversibility of this reaction provides reasonable assurance that chemical modifications, which effect disulfide bonds, have not taken place. The proteinaceous products of reversible thermal denaturation, though highly disordered, are not randomly coiled, but

retain regions of ordered structure. Native proteins also may undergo aggregation without prior unfolding (74).

Enterotoxin D. Little research has been conducted on the thermal inactivation of SED. Soo et al. (70) studied the thermal stability of partially purified SED in sodium acetate and phosphate buffer at different pH's, temperatures and toxin concentrations. They reported greater inactivation at 80 C than at 70 or 90 C, and at pH 6.5 to 7.5 than at pH 4.0 to 6.5.

Effect of Environmental Factors on Staphylococcal Growth and Enterotoxin Production

The effects of pH, curing agents, oxygen tension and temperature on staphylococcal growth and enterotoxin production are reviewed with an emphasis on meat products. The growth and production of enterotoxin A by S. aureus strain 234 in inoculated raw beef, raw pork, cooked pork and canned ham were studied by Casman et al. (26). Enterotoxin A production and good growth of cells occurred in all of the meat samples. Although growth was better in cooked meat, there was no significant increase in enterotoxin production. Poor growth in ground raw beef was observed and this may have been due to the inability of the staphylococci to compete with the other organisms present in foods (26). In 1963 studies of the detection of enterotoxin were carried out in the Microbiology Division of the Food and Drug Administration by use of a serological method.

Casman and Bennett (24) described a method for extraction and serological detection of trace amounts of enterotoxin A and B in foods incriminated in outbreaks of staphylococcal food poisoning. The procedures for examining foods for the presence of the enterotoxin were divided into 3 steps: (a) separation of the enterotoxin from insoluble constituents, (b) separation of enterotoxin from soluble extractives, and (c) concentration of the extract and examination by a gel diffusion test performed on appropriately prepared slides. The method of extraction of enterotoxin from sausage used by Barber and Deibel (9) was different, however, from that developed by Casman and Bennett (24) in that the Barber and Deibel method involved (a) acid precipitation of the extract, (b) heat precipitation of the extract, and (c) stirring of the extract in wet carboxymethyl cellulose CM 22 resin for the adsorption of the enterotoxin. The resin was then poured into a column and the adsorbed enterotoxin could be eluted by a salt solution.

Scott (67) grew fourteen food-poisoning strains of Staphylococcus aureus at 30 C in various media of known water activity. Aerobic growth was observed at water activities (a_w) between 0.999 and 0.86. The rate of growth and the yield of cells were both reduced substantially when the water activity was less than 0.94. Aerobic growth processed at slightly lower water activities than anaerobic growth. Typical intermediate moisture foods have a_w from 0.6 to 0.85 and moisture contents from 20 to 50% on a dry

solid basis (58). In order to study the effect of a_w on the staphylococci in intermediate moisture foods, desorption samples were prepared by direct mixing of a food sample with the humectant, while absorption samples were made by freeze-drying a food and rehumidifying in desiccators equilibrated at various a_w with salt solutions (58). By studying an intermediate moisture product (strained pork), Labuza et al. (46) reported that S. aureus cells were able to grow in desorption samples at 0.84 a_w, but not at 0.75 a_w. Whereas, in the adsorption samples the visable population decreased at all a_w investigated (0.68 to 0.90 a_w). Plitman et al. (58) studied the effect of 2 different isotherms (adsorption and desorption) of strained chicken and lean pork loin on the viability of staphylococci. They showed that the a_w of the medium and the moisture content were both important factors in regulating the biological response of S. aureus. Growth was observed in the adsorption samples at 0.92 a_w. When glycerol was used as the humectant, growth of S. aureus was inhibited at 0.88 a_w in adsorption samples and at 0.865 a_w in desorption samples (58). However, a_w of 0.83 in pork has been reported to support growth of S. aureus 265-1 and enterotoxin A was detected at 0.86 a_w (76).

Troller (81, 82) studied the effect of water activity on the production of SEA and SEB as well as cell growth. Total numbers and rate of growth of S. aureus C-243, (an enterotoxin B producing strain) were diminished

at low a_w levels, and enterotoxin synthesis was extremely sensitive to reduction in a_w. Addition of glycerol to reduce the a_w from 0.99 to 0.97 caused a decrease in the SEB production from 55 ug/ml to less than 5 ug/ml after 60 hrs incubation. A 99% decrease in SEB production occurred when Protein Hydrolysate Powder (PHP) and NZ amine NAK were added to a medium to lower the a_w from 0.99 to 0.98. Results of similar studies, employing strain 196 E which produces SEA, indicated that this organism was capable of producing enterotoxin at a much lower a_w than that required for the production of SEB. A reduction of the a_w from 0.99 to 0.945 reduced SEA by 55% to 60%, depending on the solute employed for a_w adjustment.

The effects of different environmental conditions and curing ingredients on the growth and enterotoxin production of staphylococci have been studied intensively. The presence of nitrate was reported to have little effect on the pH tolerance, either aerobically or anaerobically (47). A pH range of 4.7 to 9.4 was reported for the growth of S. aureus 243, and 5.1 to 9.0 for the production of SEB (65). However, Peterson et al. (56) found that staphylococci when in the presence of a saprophytic bacterial population could only multiply appreciable in the pH range of 6 to 8 and at temperatures above 20 C. Reiser and Weiss (61) found an initial pH of 6.8 resulted in higher yields of SEB and SEC than did either pH 6.0 or 5.3, while

SEA was produced at the same rate within the range of pH 5.3 to 6.8. Kato et al. (43) obtained growth and production of SEA in the pH range of 5.0 to 8.0 without observing a marked difference within this range. Production of SEB was not influenced by adding 2% salt to the medium; whereas, an additional increase in salt greatly decreased the toxin production. Neither $NaNO_3$ in concentrations up to 1000 ppm nor $NaNO_2$ at concentrations of 200 ppm affected cell growth or enterotoxin B production in broth, while the combination of nitrate at 200 ppm or 600 ppm and nitrate at 120 ppm reduced the toxin titer to 37.5% (51). Yet another report (21) showed that there was a bactericidal effect of $NaNO_2$ at a concentration of 500 ppm at pH 6.3 compared to a previously reported concentration of 200 PPM at pH 5.6 or less being required for inhibition (75).

Genigeorgis et al. (37) inoculated a variety of hams cured in the laboratory with 10^3 to 10^6 cells of S. aureus strain S-6 and incubated them anaerobically at 10, 22, and 30 C for up to 16 weeks. SEB was detected in hams with an original pH above 5.3, and containing 0.54 ppm undissociated nitrous acid and up to 9.2% NaCl. Genigeorgis et al. (36) also studied, with a factorial design, the effects of pH and NaCl concentration on the probability of 5 staphylococcal strains initiating aerobic growth in BHI broth at 30 C. The probability of one cell initiating growth could be calculated from equations which relate the

NaCl concentration and the pH of the medium to log reductions of a staphylococcal population in a given condition. The same research group (38) then studied the probability of staphylococcal growth initiation in pasteurized, cured meat and compared it with initiation growth in BHI broths. The findings indicated that the meat environments were more conducive to growth of staphylococci than were the BHI broths. Fewer cells were required to initiate growth in meats than in BHI broths at the same NaCl concentration and pH. All meats supporting enterotoxin production had good staphylococcal growth (> 4×10^7 cells per g), but some samples with 10^8 cells per g did not contain SEB or SEC.

Barber and Deibel (9) studied the effect of pH and oxygen tension on staphylococcal growth and enterotoxin formation in fermented sausage. When commercial fermented sausage contained large numbers of viable staphylococci, these organisms were always located in the outermost areas where the oxygen tension was highest. The pH values varied and no correlation could be made between the lactic acid bacterial count and pH of the sausage. In addition to the analysis of the commercial sausage, portions of 100 g of the laboratory formulated sausage mix were prepared with various concentrations of glucono delta lactone (GDL). Significant growth of staphylococci was observed aerobically at GDL concentrations up to 1.3% in the presence of oxygen.

A lower concentration of 1.1% GDL was necessary to decrease the anaerobic counts. When Pediococcus cerevisiae culture was added, it failed to suppress the staphyloccal growth completely in the surface areas. An inoculum ratio of 10^6 pediococci to 10^1 staphylococci allowed the staphylococci to attain a population of 10^3 to 10^4 cells/g on the surface of the sausage. In nonacidulated sausage S. aureus strain 9 did not produce SEA at 0% oxygen, even after 120 hr incubation. The cell growth and enterotoxin production in buffered BHI after 72 hr at 37 C were studied under aerobic conditions. The lowest pH which supported SEA, SEB, SEC, and SEE was 4.9, 5.0, 4.9, and 4.8 respectively.

The combined effects of pH, NaCl, and sodium nitrate on SEA production were studied by using a dialysis sac technique in BHI broth (79). In the presence of sodium nitrite, growth and enterotoxin production decreased as the pH decreased below 7.0. At pH 7.0, nitrite concentration up to 300 ug/ml had no effect on toxin production, while cell growth occurred at pH 7.0 with 12% NaCl regardless of nitrite level (0 to 300 ug/ml). Growth and production of SEC by S. aureus strain 137, in 3% PHP + 3% NAK with 0 to 12% NaCl, and an initial pH of 4.00 to 9.83 were studied during an 8 day incubation period at 37 C (35). When the inoculum contained at least 10^8 cells/ml, growth and toxin production were initiated at pH values as low as 4.00 and

as high as 9.83 at 0% salt. Markus and Silverman (50) demonstrated that nonreplicating staphylococci in a nitrogen free medium were able to release SEB following incubation at 37 C for 10 hr. Therefore, it has not been determined whether the growth and toxin production at pH 4.0 were caused by selection of some acid tolerant cells, or by the shifting of pH of the medium to a higher pH by metabolic products of nonreplicating cells and decomposition products of dead cells.

The minimum reported temperature permitting staphylococcal growth in custard, chicken a la king, and ham salad was 6.7 C (6). Genigeorgis et al. (37) detected enterotoxin B in inoculated cured meat at 10 C after at least 2 weeks incubation. Better toxin production was obtained at 30 C than at 22 C or 10 C. McLean et al. (51) found that after 112 hr incubation in BHI broth at 16, 20, or 37 C, the same optical density of cell growth was obtained, but the amounts of enterotoxin B in the culture supernatant were 8, 20, and 340 ug/ml, respectively. Scheusner (65) reported the temperature range for the growth and enterotoxin production of S. aureus strains 265, 243, 493, and 315 which produced enterotoxins A, B, C, and D, respectively. All strains grew in the temperature range of 13 to 45 C, with no growth at 7 or 50 C. Enterotoxins were produced from 19 to 45 C, with the exception of SEB which was produced at 13 C but not at 45 C. Scheusner and Harmon (66) also studied the growth and toxin production

of the various strains in foods at 19 to 45 C. Enterotoxins were detected in vanilla pudding incubated at the previously mentioned temperature range, and enterotoxins B, C, and D were detected at much lower populations at 45 C than at 19, 26, or 37 C. At 19, 26, and 37 C S. aureus strain 265 tended to produce measurable SEA at lower populations than the other strains. Yet another report (77) indicated that the production of enterotoxins A, B, C, and D was stimulated at 40 to 45 C. These temperatures were above the optimum for cell production, which is 37 C.

Staphylococcal Foodborne Outbreaks in Genoa Salami

Staphylococcal food poisoning has always been responsible for a large proportion of the foodborne outbreaks reported to the Center for Disease Control. In 1972, there were 34 outbreaks and 1,948 cases caused by staphylococci, which constituted 25% of the total outbreaks and 32.5% of the total cases. Among these cases 59% involved meat (beef and pork) as the vehicle (83). Mildly processed cured-meat products together with manufactured meat products such as ham and bacon are the food items most commonly incriminated in food poisoning, with the causative organism often being S. aureus (20).

Fermented sausage, on the other hand, has not been a significant item in the incidence of this type of food poisoning, and several reports of gastroenteritis associated with Genoa salami are described in detail here. On

May 10, 1971, a man from Denver, Colorado, experienced nausea, vomiting, abdominal cramps, and diarrhea 3 hrs after he consumed one package of Genoa salami (84). A second package purchased at the same time was cultured and yielded a heavy growth of coagulase positive staphylococci, Salmonella bredeney, S. derby, and S. manhattan. Other samples of salami obtained in Colorado by the USDA were found to contain 1,000 to 1,000,000 coagulase positive staphylococci/g. SEA was detected in two of three samples but no salmonella isolations were obtained. In mid-May 1971, a man in Kenosha, Wisconsin, became ill twice with nausea, vomiting, and diarrhea approximately 4 hrs after having eaten Genoa salami. Laboratory studies on the samples brought in by the patient revealed 30,000 coagulase positive staphylococci/g; no salmonella were isolated. Other samples obtained by the Kenosha Health Department were cultured and yielded 210,000, 270,000, and 230,000 coagulase positive staphylococci/g. Another case reported in Washington involved 5 persons who experienced nausea and vomiting approximately 4 hours after eating salami purchased from the same store. Genoa salami purchased at that store by officials of the health department was found to contain 1.2 million coagulase positive staphylococci/g. No analysis for enterotoxin was performed. All the Genoa salami involved in the above outbreaks were produced by the Armour Company at a single plant

in Minnesota. Samples obtained by the USDA directly from the plant contained 1,000 to 100,000 coagulase positive staphylococci/g, and two out of three samples were positive for SEA. As a result of these findings, Armour Company halted production at the plant and initiated a recall of the product under supervision of the USDA (84).

Genoa salami manufactured by George A. Hormel and Co. was also reported to be incriminated in the outbreaks (85). Three children from Chicago, Illinois, experienced vomiting and diarrhea 3 hrs after eating sliced Genoa salami. Samples collected from the same store were cultured and yielded 150,000, 292,000, and 790,000 coagulase positive staphylococci/g. In July 1971 five members of a family in Allegheny county, Pennsylvania suffered nausea, vomiting, cramps and diarrhea 2 1/2 to 4 1/2 hours after eating Sam Remo Genoa stick salami also made by Hormel. Again coagulase positive staphylococci were obtained. Other outbreaks incriminating Hormel's Dilusso Genoa salami were reported in Pennsylvania and Minnesota. Two other separate incidents of staphylococcal food poisoning involving Genoa salami, reported from Florida, warrant mention, since the counts of coagulese positive staphylococci were in excess of 1 million/g in one case, and 500,000/g in another. Based on these discoveries, the Florida State Department of Health issued a "Stop Sale" order prohibiting the sale of all Hormel and Armour Genoa salami within the state of Florida,

and the Hormel company issued a voluntary recall of Genoa salami monitored by the USDA (85). On September 14, 1971, gastroenteritis attributed to San Remo Genoa stick salami was again reported in Baltimore, Maryland, where laboratory studies of the salami demonstrated the presence of 400,000 coagulase positive staphylococci/g, and SEA was detected (86).

From the information provided by these outbreaks, it is obvious that the cause of food poisoning was not due to mishandling by the customer or to contamination after processing. A contaminated ingredient or faulty processing procedures used by both companies was probably responsible for organism growth and enterotoxin production.

MATERIALS AND METHODS

Cultures

Staphylococcus aureus strains 265, 243, 493, and 494 supplied by the late Dr. E. P. Casman, U. S. Food and Drug Administration Microbiology Laboratory in Washington, D.C., were used for the production of enterotoxins A, B, C, and D, respectively. The cultures were maintained on Brain Heart Infusion (BHI) agar (Difco) at 4 C and subcultured bimonthly.

Genoa Salami

Processing of Genoa Salami. Several batches of Genoa salami were made from pork inoculated with washed cells of *S. aureus* strains 265 and 243. Boston butts were purchased through the MSU Food Store, wrapped in Cryovac bags and placed in a freezer at -30 C for a month prior to use. Each batch of sausage was made of twenty to 25 lb of meat which was thawed in a refrigerator at 4 C for two days. The ratio of lean meat to fat was approximately 4 to 1. After thawing, the meat was cut into strips and ground through a 1/2-inch plate which was attached to a

model 5010 meat grinder (Toledo Scales Company). Spices and curing agents (Table 1) were mixed in by hand. The meat was then ready for inoculation.

Table 1.--Spices and curing agents used in Genoa salami formulation.

Ingredient	Grams/20 lbs Meat	Concentration (g/kg meat)	Source
salt	306.2	33.7	MSU store
white pepper	11.34	1.25	McCormick
whole pepper	2.84	0.313	McCormick
sodium nitrite	0.71	0.0782	Mallinckrodt
sodium nitrate	5.68	0.626	Baker
garlic	1.89	0.208	Meisel
dextrose	68.1	7.50	Difco

S. aureus strains 265 and 243 were each inoculated into 50 ml BHI broth and incubated at 37 C in a rotary shaker (New Brunkswick Scientific Company) at 150 rpm for 24 hr. The cultures were transferred daily for two days prior to use. Cells were collected by centrifugation at 12,000 x g for 10 min, washed twice, and resuspended in 100 ml phosphate buffered dilution blanks (4). After several preliminary experiments, a 24-hr staphylococcal culture in BHI broth was determined to have a plate count of approximately 5×10^9 to 1×10^{10} cells/ml. Four batches of meat (5 lb each) were then each inoculated as follows:

(1) Forty-six ml of the 1/10 dilution of the suspended cells inoculated into 5 lb of meat was equivalent to an inoculum of 10^7 cells/g of meat.

(2) Inoculation of 46 ml of the 1/1,000 dilution of the suspended cells into 5 lb of meat resulted in 10^5 cells/g of meat.

(3) Inoculation of 46 ml of the 1/100,000 dilution of the suspended cells into 5 lb of meat resulted in 10^3 cells/g of meat.

(4) A non-inoculated control batch was made by adding 46 ml of phosphate buffer dilution blank to 5 lb of meat.

After inoculation, the meat was spread in layers about 6 in. thick and covered with parchment paper. Following incubation for two days at 4 C, it was re-ground and stuffed into pre-soaked tied collagen casings (9 x 56 cm; Brechteen Company) with a 4-liter hand stuffer (F. Dick). The casings of salami were hung on sticks and kept in the cooler at 4 C for an additional 4 days and then put in a tempering room at 20 to 25 C with relative humidity at 80% for 2 days. Following tempering, they were moved to a smoking chamber and heated in air (without smoke) at 38 C for 20 hr, 43 C for 2 hr, 49 C for 4 hr and 54 C for 3 hr at 80 to 90% relative humidity. Finally, after heating, the salami were showered with cool water for 3 min, dried at room temperature for 3 hr, then moved to a

drying room at 12 C with a relative humidity of 72% for approximately 60 days.

All the contaminated equipment was autoclaved at 121°C for 45 min after use. The non-autoclavable parts were soaked in Lysol overnight before cleaning.

Sampling Methods. Two sampling methods were used. Samples composed of the outer 1 cm of surface and samples of the core were taken from salami inoculated with S. aureus 265. When S. aureus 243 was inoculated, however, samples were taken from cross sections of the salami.

Samplings were examined at different stages of processing as follows: (a) after inoculation, (b) before tempering, (c) after tempering, (d) after heating, and (e) at intervals during drying.

Enumeration of Microbial Populations. Fifty grams of the sample were blended for 2 min in 200 ml of phosphate buffered dilution water in a Waring blender.

Staphylococcal counts were made in pre-poured plates of Mannitol Salt Agar (MSA; Difco) and Vogel Johnson Agar (VJA; Difco). Duplicate samples of 0.1-ml aliquots of the appropriate dilutions were deposited on MSA and VJA plates and then spread evenly with sterile bent glass rods. The plates were incubated at 37 C for 2 days. Following incubation, the typical S. aureus colonies were picked and the coagulase test performed. Only coagulase positive colonies were counted. The aerobic total count was made

by the pour-plate technique in Plate Count Agar (PCA; Difco) with the plates being incubated at 32 C for 3 days. Lactic acid bacteria were enumerated on pour plates of Lactobacillus Selective Agar (LBS; BBL) with the plates being incubated at 32 C for 2 days.

Lactic Acid Determination. A 20-g sample of salami was blended for 2 min in 180 ml deionized water in a Waring blender. The homogenate was filtered through a Whatman #1 filter paper (W & R Balston, Ltd.), and portions of filtrate corresponding to 5 g of sample were titrated with 0.1 N NaOH to pH 8.3. The percentage of lactic acid was calculated by the following formula:

$$\frac{\text{ml of NaOH x normality of NaOH x 9}}{\text{weight of sample in grams}} = \% \text{ lactic acid}$$

pH Determination. The pH of the filtrate obtained as described previously was measured by a Corning single electrode on a Beckman Research pH Meter.

Moisture Determination. Approximately 5 g of sample was spread evenly in an aluminum moisture dish of 5.5 cm in diameter (Sargent & Co.). The sample was dried in an air oven at 100 C for 16 to 18 hr and cooled in a desiccator. The weight loss was expressed as % moisture loss.

Determination of Water Activity. A hygrosensor elements (No. 547535, Hygrodynamics, Inc.) was mounted

in a rubber stopper which fit a 6-oz baby food jar and attached to a hygrometer indicator. Approximately 20 g of the sample was placed in the jar. Water activity measurements were carried out after the samples were equilibrated for 24 hr.

Extraction of Enterotoxin from Salami. Enterotoxin was extracted from the samples according to the method described by Casman and Bennett (24) with some modifications as described by Barber and Deibel (9).

A 100-g sample was blended, adjusted to pH 7.5, centrifuged, extracted with chloroform and dialyzed. The extract was then heated to 55 C in a water bath and immediately chilled in ice. After repeated chloroform extractions the extract was acidified to pH 4.5 and centrifuged. The supernatant was adjusted to pH 6.0 and added to 1.5 g of wet carboxymethyl cellulose (Whatman) suspended in 0.01 M sodium phosphate buffer. Enterotoxin, when present, was adsorbed onto the resin by an ion exchange reaction. The slurry was poured into a column (2 x 40 cm) and then eluted by 150 ml of 0.2 M sodium phosphate buffer containing 0.2 M NaCl at pH 7.5. After elution, the sample was concentrated, extracted with chloroform, and lyophilized in a Virtis Freeze Mobile (The Virtis Company, Inc.). A small amount (0.2 ml) of saline was used to re-dissolve the lyophilized extract. The presence of the enterotoxin in the extract was determined by the microslide gel diffusion method (24).

Production and Preparation of Enterotoxins

Crude Enterotoxins. Each S. aureus strain was inoculated into 50 ml of BHI broth and incubated at 37 C on a rotary shaker (New Brunswick Scientific Company) at approximately 150 rpm for 24 hr. The culture was transferred daily for 2 days prior to use. Four-ml aliquots of the 24-hr culture were inoculated into 30 1-liter flasks, each containing 400 ml of BHI broth, and the flasks were incubated at 37 C for 24 hr while being agitated. After incubation, the broth culture was centrifuged in a Sorvall RC-2 refrigerated (4 C) centrifuge at 12,000 x g for 10 min. The supernatant was then concentrated for 48 hr at 4 C in cellulose Dialyzer Tubing (diameter 1 7/8 in.; Arthur H. Thomas Company), covered with 40% (20 M) polyethylene glycol (PEG); (Union Carbide). After concentrating, the outside of the tubing was washed thoroughly with tap water and was dialyzed for another 4 hr in deionized water. Following dialysis, the material remaining inside the tubing was removed by washing with sterile deionized water and was designated as crude enterotoxin.

The final concentration of enterotoxin and protein in the crude enterotoxin was measured. Protein concentration was determined by a method described by Lowry (49), and bovine serum albumin (Sigma Chemical Company) at concentrations of 0 to 100 ug/ml was used as a standard.

Partially Purified Enterotoxins. Sephadex G-100 gels (Pharmacia Fine Chemicals) were treated by soaking in 0.04 M veronal buffer (VB) at pH 7.4 for 3 days at room temperature. After a few washings, the slurry was poured carefully into a 3.5 x 60 cm column. The packed column was then washed by VB which contained 0.02% sodium azide as an antimicrobial agent. A solution of 0.04% Dextran 2000 (Pharmacia Fine Chemicals) was passed through the bed to check for homogeneity. The column was then ready to use. Approximately 50 mg of crude SEB prepared as described before was concentrated to 20 ml in 40% PEG. After centrifugation of the concentrate at 12,000 x g for 10 min, the supernatant was carefully applied to the Sephadex column. The operating pressure was about 20 cm water and the flow rate was maintained at approximately 20 ml/hr. The total elution took about 40 hr. Absorbance at 280 nm, a non-specific measure of protein concentration, was measured by a UV monitor (Gilson) and registered on a recorder (model SR, Sargent). The eluant of 4 ml/tube was collected on a fraction collector (LKB 7000A Ultrorac, LKB-Produkter AB, Sweden). The concentration of SEB and the amount of protein in each pooled fraction of 40 ml were determined. The fractions corresponding to the toxin peak were pooled and designated as partially purified SEB, which was stored in aliquots of 20 ml in a freezer at -30 C. After elution, the column was washed extensively with eluant before the

next application. Crude SEC was also partially purified by the same procedures.

Both crude SEA and SED had lower specific activities (ug enterotoxin/mg protein) compared to those of the crude SEB and SEC. Therefore, more purification steps were necessary for SEA and SED. Concentrated crude SEA was first acidified to pH 4.5 by adding 3 N HCl. After centrifugation at 12,000 x g for 10 min, the pH of the supernatant was adjusted to 7.4 and the precipitate discarded. Solid ammonium sulfate (Fisher Scientific Company) was added slowly to bring the supernatant to 35% saturation. After stirring for 30 min, the precipitate was removed by centrifugation for 10 min at 12,000 x g, and ammonium sulfate was again added to bring the supernatant to 65% saturation. After 30 min the precipitate was collected by centrifugation and taken up in 40 ml VB. Approximately 20 ml of the enterotoxin preparation containing 44 mg of SEA was loaded on a Sephadex G-100 column. The same elution procedures were followed as described in the purification of SEB and SEC. Twelve mg of SED in 20 ml of VB, prepared as described for SEA, were also purified by passing through the Sephadex G-100 column.

Heating Menstrua Used to Inactivate Enterotoxins

Veronal Buffer. A 0.04 M veronal buffer was prepared by dissolving 8.25 g of sodium barbital (Mallinckrodt)

in deionized water. The pH was titrated to 7.4 by adding acetic acid (Mallinckrodt), and the volume was then brought up to 1 liter.

BHI Broth. Thirty-seven g of BHI were dissolved in 1 liter of deionized water and autoclaved at 121 C for 15 min. The final pH was 7.4.

Beef Broth. The constituents in 100 g of the beef broth (BB) (Campbell Soup Company) were:

Protein	3.6 g	iron	0.2 mg
fat	0.0 g	sodium	670 mg
carbohydrate	1.9 g	potassium	90 mg
crude fiber	0.1 g	vit. A	trace
ash (salt free)	0.3 g	thiamine	trace
solids	7.4 g	riboflavin	0.02 mg
calcium	trace	niacin	1.0 mg
phosphorus	24 g		

Each can of BB (300 ml) was diluted to 600 ml with deionized water and pH was adjusted to 7.4 with 1 N NaOH.

Ultrafiltration Fractions of Beef Broth (5x, 2.5x, and filtrate). After the pH was adjusted to 7.4, BB was ultrafiltered through a PM 10 membrane in an Amicon cell (Amicon Corporation) under nitrogen pressure of 55 psi. Each can of BB was concentrated to 60 ml and subsequently diluted to 120 ml with deionized water. This was a 5-fold (5x) concentration, considering that one can of BB contained 600 ml after dilution. The filtrate was also diluted to half of its concentration. A portion of the 5x concentration was diluted to 2.5x by adding an equivalent volume of deionized water. The protein concentrations

of the 5x concentrate, 2.5x concentrate, and the diluted filtrate were determined.

Non-dialyzed Beef Broth Protein. Sufficient ammonium sulfate was added to cans of BB at pH 7.4 to achieve 80, 90, or 95% saturation. The precipitate was collected by centrifugation at 16,000 x g for 10 min. It was then dissolved in 30 ml VB and designated as non-dialyzed beef broth protein. The effect of this non-dialyzed BB protein on the heat inactivation of enterotoxin was investigated by determining the D values of enterotoxins obtained by heating the enterotoxins in menstrua containing different concentrations (3.8, 5.8, 7.7, and 11.5 mg/ml) of non-dialyzed BB protein.

Dialyzed Beef Broth Protein. Thirty ml of the non-dialyzed BB protein previously described was dialyzed against 270 ml VB, and the volume was increased to 60 ml in the tubing after dialyzing for 16 hr. Assuming that equilibrium of dialysis was approached, 80% of the dialyzable substances would be present in the dialysate which was saved for future studies. Further dialysis against 2000 ml VB was performed immediately to reduce the amount of dialyzable substance inside the dialysis tubing to a negligible amount. After dialysis, the content of the tubing was designated as the dialyzed BB protein.

Thermal Inactivation of Enterotoxins

There are several methods commonly used in the study of thermal resistance of microorganisms or thermal inactivation of biochemical compounds. In this study, the thermal death time (TDT) can method was used (3). Enterotoxin (16 to 32 ug/ml) added to a heating menstruum was placed in a small can (2 8/16" x 6/16"; American Can Company) and the volume was brought up to 13 ml by adding VB. The cans were then sealed, and subjected to heat at 110.0, 115.6, 121.1, 126.7±0.1 C in a small steam retort (80). The time lag to reach the steam temperature in the center of the can was determined by thermocouple measurements to be 20 sec and the holding times used were corrected for this thermal lag. The heating times were 0 to 60 min at intervals of 10 min, or 0 to 30 min at intervals of 5 min, depending on the rate of the inactivation of enterotoxin. Immediately after heating, the cans were cooled by water at 11.7 C in the retort. Inactivation of SEB was performed in duplicate, whereas, only single determinations for inactivation of SEA, SEC, and SED were performed due to the limited supply of antitoxin available.

Assay for Enterotoxin

Reference enterotoxins A, C, and D and their corresponding antitoxins were obtained from the late Dr. Casman. Enterotoxin B and antitoxin B were purchased from Makor Chemical Ltd. (Jerusalem, Israel). The lyophilized

preparations were rehydrated in a sterile diluent consisting of a mixture of one part of BHI broth and 9 parts of fluid base which contained 0.02 M phosphate buffer, 0.85% NaCl, and 0.01% merthiolate (Eli Lilly). The pH of the diluent was 7.4. Reference enterotoxins were diluted to give concentrations of 1 to 2 ug/ml. Antitoxins were diluted as follows: Anti-A, 1:40; Anti-B, 1:20, Anti-C, 1:10, and Anti-D, 1:24. The concentrations of the various enterotoxins were determined serologically by the microslide gel diffusion method of Casman and Bennett (24). The slides were incubated in a moist chamber for 3 days at approximately 21 C prior to examination. A precipitation line which formed between the antitoxin well and the sample well indicated the presence of enterotoxin in the sample. This method gives a semi-quantitative determination of enterotoxin with a sensitivity of approximately 1 ug/ml.

Assay for Heat Inactivated Enterotoxins

After the heat treatment, the TDT cans were opened and two-fold serial dilutions (1/2, 1/4, 1/8, 1/16, and 1/32) of the samples were made in the previously described diluent. Twenty-five µl of the non-diluted, 1/2 diluted, and 1/4 diluted samples were each placed in a well on one slide. A fourth well on the same slide was filled with reference enterotoxin. The other 3 dilutions, 1/8, 1/16, and 1/32 of the heated enterotoxin, were placed on another slide and the corresponding antitoxin of the enterotoxin

was deposited in the center well. After incubation, the slides were examined, and the reciprocal of the highest dilution of the sample which showed a positive test was determined as the titer of the enterotoxin.

Analysis of Data on Heat Inactivated Enterotoxin

The end point method of analysis was performed (18). At constant temperature, the presence or absence of enterotoxin in each dilution of the heated sample was indicated by a + or - sign on semi-log paper with log of titer on the ordinate axis and heating time on the abscissa. A straight line was drawn above every survival point (+) and below or through every inactivation point (-) (80). The D value, time (in min) required for 90% inactivation of enterotoxin at a given temperature, was taken directly from the straight line. The Z value, temperature required to reduce D value by 90%, could be obtained from a straight line by plotting the log of D values against various heating temperatures.

Recombination of Dialysate and Dialyzed Beef Broth Protein

The dialysate of BB protein and dialyzed BB protein were prepared as described previously. Dialyzed BB protein at concentrations of 3.8 and 7.7 mg/ml were added to 5 and 10 ml of dialysate, respectively. Five and 10 ml of the dialysate were the volumes calculated to contain concentrations close to the amount of dialyzable substances in 3.8 and 7.7 mg/ml of non-dialyzed BB protein.

Analysis of Dialysate of Beef Broth Protein

Chelation with Disodium Ethylenediamine Tetraacetate. A 50 mM solution of disodium EDTA (Baker) was prepared in deionized water and the pH was adjusted to 7.4. A 1.3 ml aliquot of the solution was added to 10 ml of the dialysate of BB protein. After incubation for 30 min at room temperature, 7.7 mg/ml of the dialyzed protein was mixed with the dialysate treated with EDTA. This mixture of dialyzed BB protein and treated dialysate was used as a heating menstruum for SEB. A control experiment was performed by heating SEB in 5 mM EDTA to determine if disodium EDTA had any adverse effect on the immunological reaction of SEB.

Digestion of Dialysate by Proteolytic Enzymes. Stock solutions of trypsin and type II α-chymotrypsin (Sigma Chemical Company) were dissolved in VB to concentrations of 1 mg/ml. Seventy ml of BB dialysate were digested by 0.35 ml of trypsin and 0.35 ml of α-chymotrypsin at 25 C for 3 hr. The protein concentration of the dialysate was 0.8 mg/ml; therefore, the amount of each enzyme was 0.63% of the protein in the dialysate. The proteolytic enzymes were then inhibited by Phenylmethyl Sulfonyl-Fluoride (PMSF: Sigma Chemical Company). A stock solution of PMSF (1 mg/ml) was prepared in 2-propanol and 0.49 ml was calculated to be necessary to inhibit the enzymes added in 70 ml dialysate. Phenylmethyl sulfonyl fluoride

was then added to the enzyme-treated dialysate and incubated at 25 C for 1 hr. Ten ml aliquots of this treated dialysate were placed in 6 TDT cans and dialyzed BB protein was added to a final concentration of 7.7 mg/ml. Partially purified SEB was heated in this menstruum at 110 C for periods of time ranging from 0 to 60 min at intervals of 10 min. Three other heating menstrua described below were prepared as controls and differed in the treatment of the dialysate:

(a) Seventy ml of BB dialysate were incubated at 25 C for 3 hr. The dialysate was then treated with 0.49 ml of PMSF at 25 C for 1 hr, after which 7.7 mg/ml of dialyzed BB protein was added.

(b) Trypsin (0.35 ml) and α-chymotrpysin (0.35 ml) were inhibited with 0.49 ml of PMSF prior to the addition of 70 ml of BB dialysate and 7.7 mg/ml of dialyzed BB protein.

(c) A solution containing 70 ml dialysate and dialyzed BB protein (7.7 mg/ml) was prepared.

Effect of Non-dialyzed Beef Broth Protein on the D_{110} of Partially Purified SEA, SEC, and SED

Partially purified SEA (16 to 32 ug/ml) was heated at 110 C in VB containing non-dialyzed BB protein with final concentrations of 1.9, 3.8, 5.8, 7.7, and 11.5 mg/ml. The D_{110} values were determined at each concentration of

added protein. The same procedures were followed for partially purified SEC and SED.

RESULTS

Genoa Salami Inoculated with S. aureus 265

Microbial Populations. Samples of salami were obtained for analyses at day 0 (after the meat was inoculated), 6 days (after curing in the cooler), 8 days (after tempering), 9 days (after heating), 29 and 63 days (during drying). Figures 1 to 4 illustrate the data obtained on the aerobic plate counts, staphylococcal counts, and the lactic acid bacterial counts. Figure 1 illustrates the growth patterns of S. aureus 265 in the inoculated salami. The staphylococcal population remained the same or decreased slightly after six days of curing in the cooler. After tempering, counts of 1.5×10^7, 2.8×10^8, and 4.9×10^8 cells/g were obtained from the surfaces of the salami inoculated with 10^3, 10^5, and 10^7 staphylococci/g, respectively. In the core samples, however, increases of 2.47 and 1.18 log cycles occurred in the salami inoculated with 10^3 and 10^5 cells/g, respectively, while only a slight increase occurred in the salami inoculated with 10^7 cells/g. Heating caused a reduction of 1 to 2 log cycle(s) in populations in both surface and core samples. During the

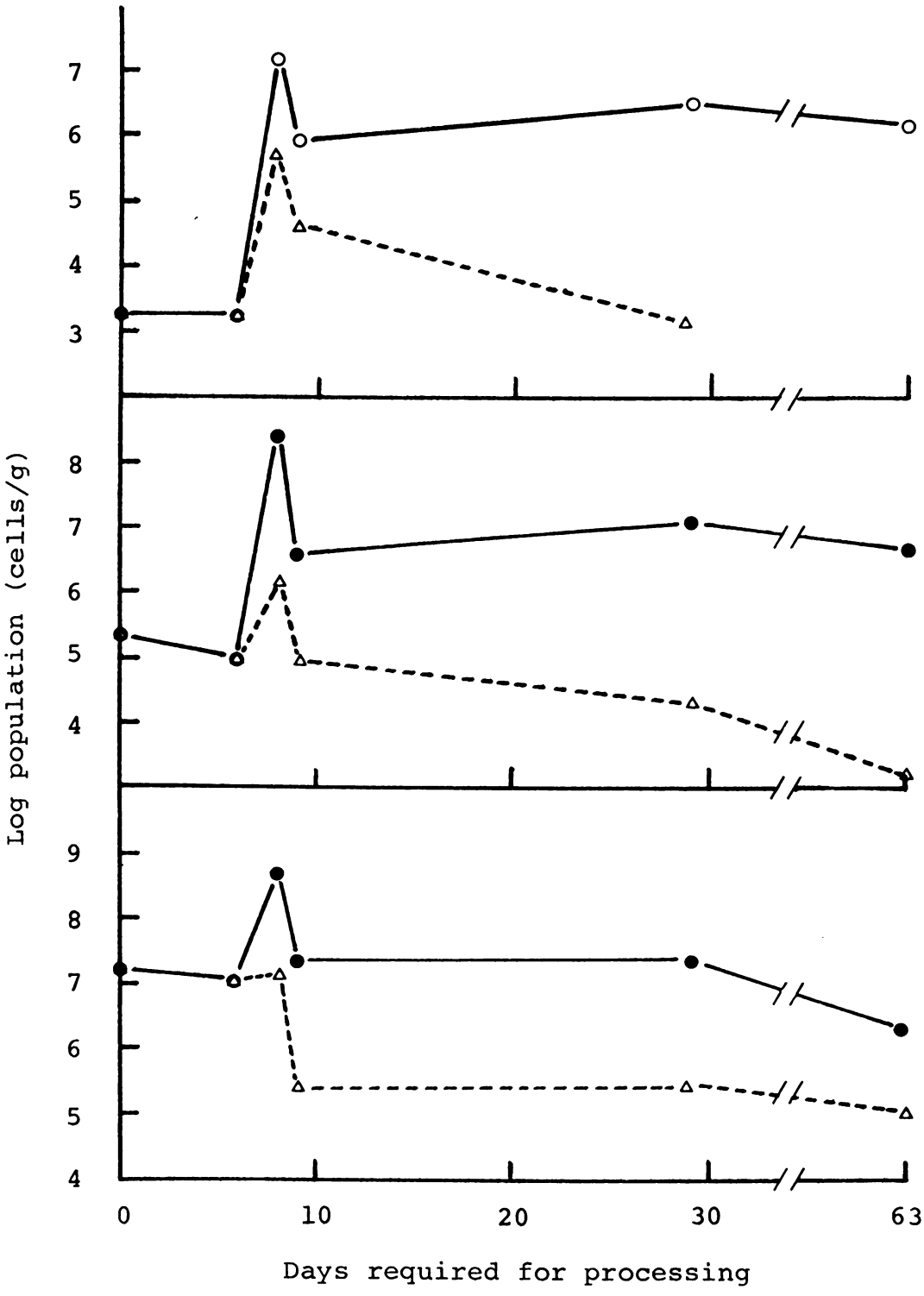

Figure 1 -- Populations of S. aureus 265 as determined on MSA plates, and enterotoxin A produced in salami inoculated with 10^3(top), 10^5(middle) and 10^7(bottom) cells/g. Legend: -o- surface sample; -Δ- core sample. Shaded symbols indicate enterotoxin A was detected.

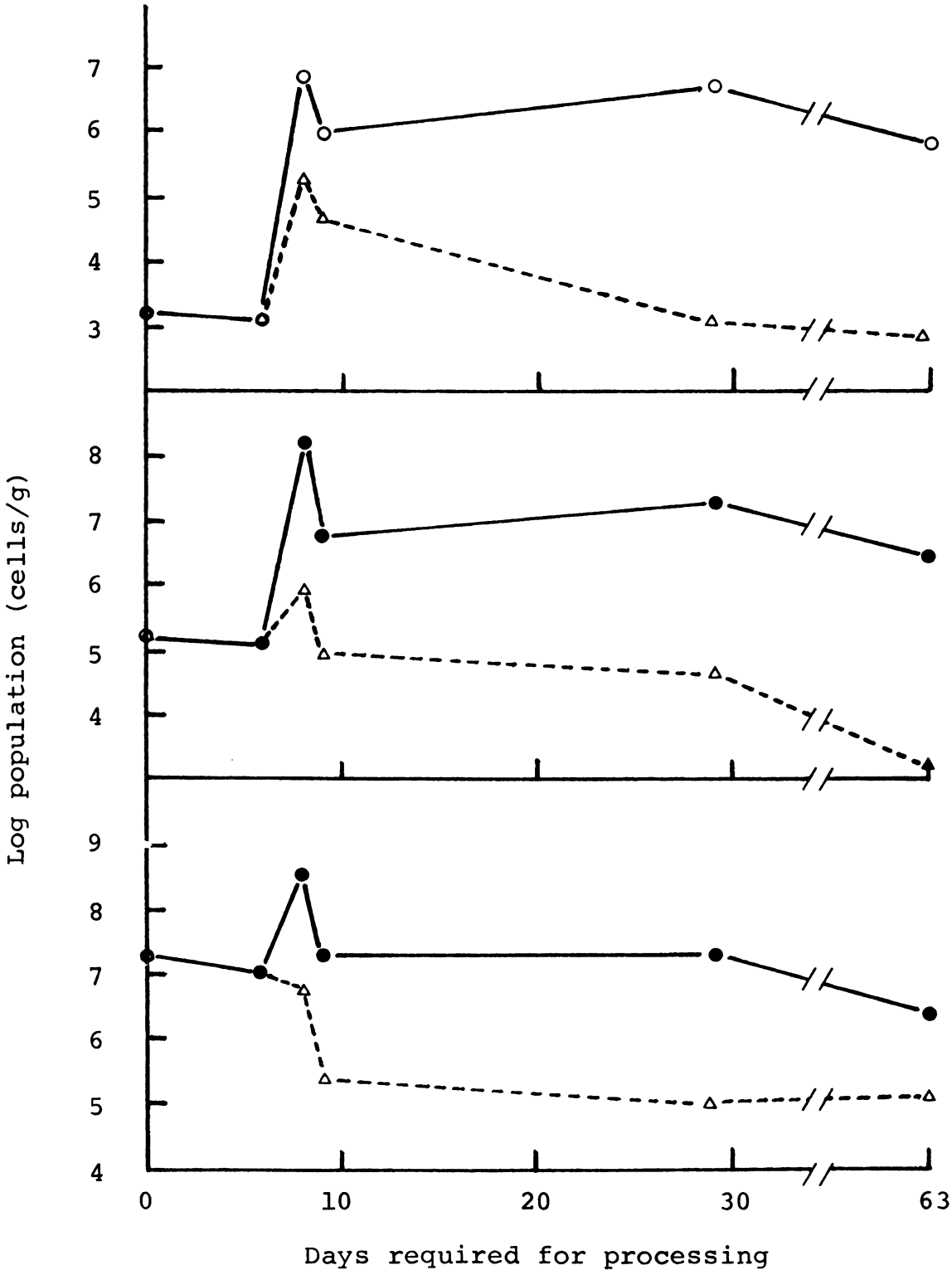

Figure 2 -- Populations of S. aureus 265 as determined on VJA plates, and enterotoxin A produced in salami inoculated with 10^3(top), 10^5(middle) and 10^7(bottom) cells/g. Legend: -o- surface sample; -Δ- core sample. Shaded symbols indicate enterotoxin A was detected.

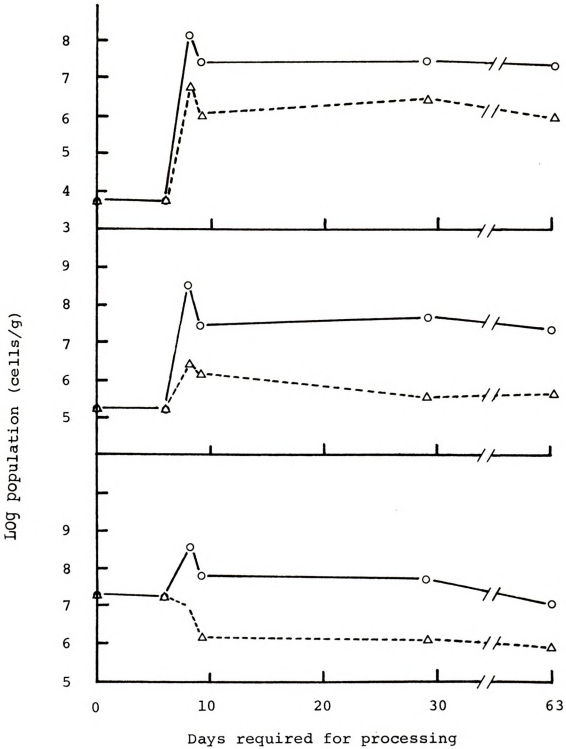

Figure 3 -- Aerobic plate counts of salami inoculated with 10^3(top), 10^5(middle) and 10^7(bottom) S. aureus cells/g. Legend: -o- surface sample; -Δ- core sample.

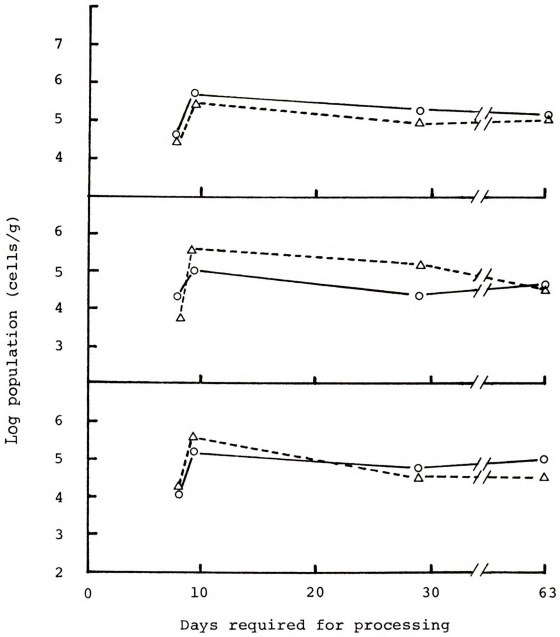

Figure 4 -- Populations of lactic acid bacteria in salami inoculated with 10^3(top), 10^5(middle) and 10^7(bottom) S. aureus 265 cells/g. Legend: -o- surface sample; -Δ- core sample.

drying period, the populations gradually decreased and after drying, less than 10^7 cells/g were recovered in the surface of each salami and less than 1.0×10^3, 1.7×10^3, and 1.2×10^5 cells/g were recovered in the cores of salami inoculated with 10^3, 10^5, and 10^7 cells/g, respectively. The data obtained from samples plated on VJA corresponded with those obtained on MSA plates (Figure 2).

Growth patterns for the aerobic plate counts were similar to those of the staphylococci, except that during the drying period the total populations decreased less than the staphylococcal populations (Figure 3). Figure 4 represents the data obtained on the populations of the lactic acid bacteria. The original population of these organisms in the pork was less than 150 cells/g of meat, but the count increased to more than 10^5 cells/g in the samples taken after the salami was heated. When samples were taken either from the surface or from the core portions of the salami, minor differences between the populations of lactic acid bacteria were observed. During drying there was no significant decrease in the population of these bacteria.

Chemical Analyses. The pH changes during processing were minute in both surface and core samples (Table 2). In the samples taken after 9 or more days, the surface portions had slightly lower pH than the core portions. Total acidity, expressed as lactic acid, increased in both

Table 2.--Determination of pH and lactic acid produced in salami inoculated with 10^3, 10^5, and 10^7 S. aureus 265 cells/g.

Days	10^3				10^5				10^7			
	Surface	Core	Surface	Core	Surface	Core	Surface	Core	Surface	Core	Surface	Core
	pH		% acid		pH		% acid		pH		% acid	
0*	6.17		0.38		6.18		0.39		6.12		0.40	
6*	6.20		0.39		6.21		0.38		6.22		0.39	
8	6.18	6.17	0.48	0.43	6.14	6.17	0.50	0.44	6.19	6.16	0.43	0.44
9	6.06	6.22	0.56	0.48	6.09	6.25	0.57	0.47	6.10	6.30	0.53	0.46
29	6.15	6.25	0.63	0.56	6.19	6.31	0.63	0.58	6.22	6.31	0.55	0.55
63	6.17	6.22	0.79	0.72	6.27	6.32	0.65	0.68	6.20	6.33	0.64	0.70

*At 0 and 6 days sausage mixture had not been placed in casings.

surface and core samples during processing. Data in Table 2 illustrate that at the end of processing more lactic acid was present in the surface samples from salami inoculated with 10^3 cells/g than those from salami with higher inocula (10^5 and 10^7 cells/g). However, in the core samples the amounts of lactic acid produced were similar at different rates of inoculation.

Enterotoxin Production. In the samples taken after eight or more days, approximately 0.2 µg of enterotoxin A was detected in 100-g portions of surface samples from salami inoculated with 10^5 and 10^7 S. aureus 265 cells/g meat, but no enterotoxin was detected in the salami inoculated with 10^3 cells/g.

Genoa Salami Inoculated with S. aureus 243

Microbial Populations. Samples were taken from cross sections of this salami instead of from the surface and core as was done with the previous group of samples. Figure 5 illustrates the data obtained on the staphylococcal population of the inoculated salami. The counts were slightly lower after curing for six days in the cooler, while determinations after tempering indicated that the populations increased to 1.0×10^6, 9.0×10^6, and 1.3×10^8 cells/g in the salami inoculated with 10^3, 10^5, and 10^7 cells/g, respectively. Heating on the 9th day reduced the populations by 3.70 to 4.77 log cycles in

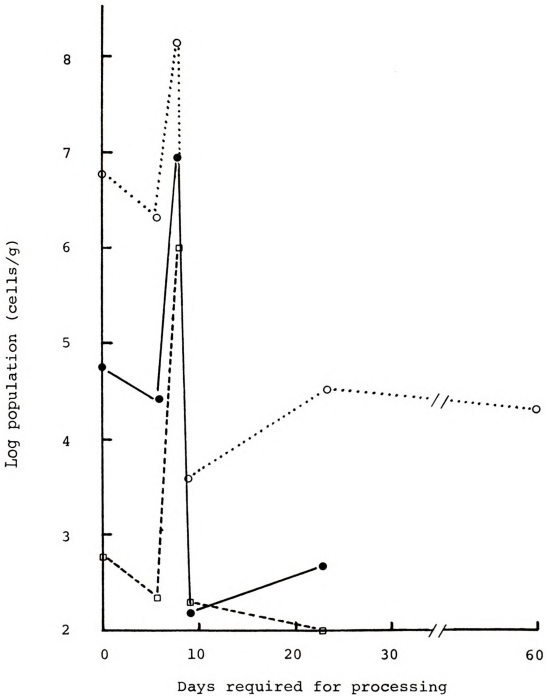

Figure 5 -- Populations of S. aureus 243 as determined on MSA plates from salami inoculated with 10^3 (□), 10^5 (●) and 10^7 (○) cells/g.

different samples of salami, but a slight increase in population at the next sampling time on the 24th day was observed. On the 59th day, a large population (2.3×10^4 cells/g) remained in the salami inoculated with 10^7 cells/g. The data in Figure 6 show the aerobic plate counts of the salami. Again, the populations increased during tempering and decreased during heating, whereas, little change in the populations occurred during drying. Data on the population of lactic acid bacteria are summarized in Table 3. No signficant competitive effect was observed between the lactic acid bacteria and the staphylococcal inocula at the various concentrations.

Table 3.--Population of lactic acid bacteria in non-inoculated salami and in salami inoculated with S. aureus 243.

Days	Non-inoculated Control	S. aureus Inocula (cells/g) 10^3	10^5	10^7
	Lactic Acid Bacteria Counts (cells/g)			
0	<150	<150	<150	<150
6	<150	<150	<150	<150
8	2.5×10^4	1.7×10^5	1.3×10^4	2.5×10^4
9	9.5×10^3	1.6×10^4	4.0×10^4	3.2×10^4
23	6.0×10^4	5.4×10^4	4.4×10^4	4.4×10^4
59	4.8×10^3	5.5×10^3	3.6×10^3	2.6×10^4

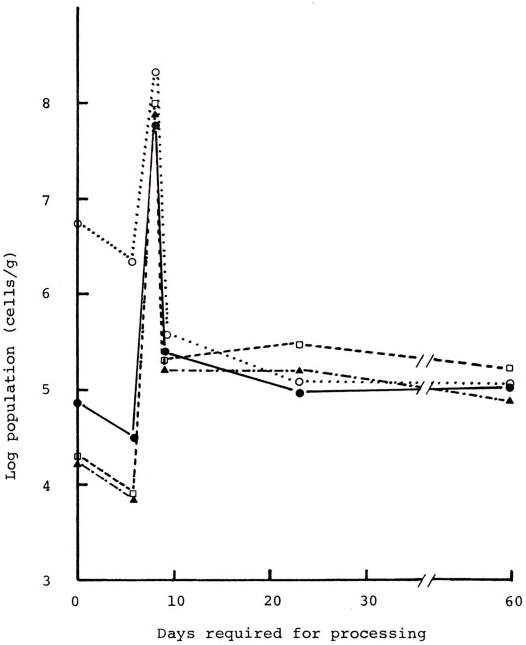

Figure 6 -- Aerobic plate counts of noninoculated salami (▲) and of salami inoculated with S. aureus 243 at 10^3 (□), 10^5 (●) and 10^7 (○) cells/g.

Chemical Analyses. The changes in pH between sampling intervals were insignificant, and the total acidity expressed as lactic acid increased gradually during processing. The non-inoculated salami contained more lactic acid when sampled at 23 and 59 days than the salami inoculated with 10^3, 10^5, and 10^7 cells/g. However, the amount of inoculum had little influence on the lactic acid content of the inoculated salami (Table 4).

Table 4.--Determination of pH and lactic acid produced in non-inoculated salami and in salami inoculated with S. aureus 243.

	Inocula (cells/g)							
	Control		10^3		10^5		10^7	
Days	pH	% acid	pH	% acid	pH	% acid	pH	% acid
0	6.11	0.43	6.11	0.43	6.11	0.43	6.11	0.43
6	6.14	0.44	6.14	0.44	6.12	0.46	6.16	0.45
8	6.12	0.51	6.19	0.49	6.19	0.48	6.14	0.49
9	6.07	0.55	6.02	0.53	6.14	0.54	6.15	0.53
23	6.10	0.76	6.20	0.61	6.23	0.62	6.24	0.61
59	6.16	0.88	6.21	0.79	6.15	0.83	6.18	0.78

Data showing the % moisture content and a_w of the salami are recorded in Table 5. The salami gained 1.5% moisture during heating and lost 16.5% during the drying period. The salami sampled at the end of the drying period had a moisture content of 43.4% and a_w of 0.84.

Table 5.--Determination of moisture content and a_w of a representative blend of salami.

Days	Moisture (%)	a_w
0	N.D.*	N.D.
6	58.2	~1.00
8	58.4	0.98
9	59.9	N.D.
23	55.3	0.97
59	43.4	0.84

*no determination

Enterotoxin Production. Enterotoxin B was not detected in any of the samples taken from salami inoculated with 10^3, 10^5, and 10^7 S. aureus 243 cells/g meat.

Thermal Inactivation of Enterotoxins

Thermal Inactivation of Crude Enterotoxins A and B. The thermal stability of crude SEA and SEB was determined at 110, 115.6, 121.1, and 126.7 C. In the range of temperatures and toxin concentration studied, the inactivation of toxin increased logarithmically with the increase in heating time. D values were obtained by the end point method of analysis. An example is shown in Figure 7. The presence or absence of enterotoxin in the heated sample

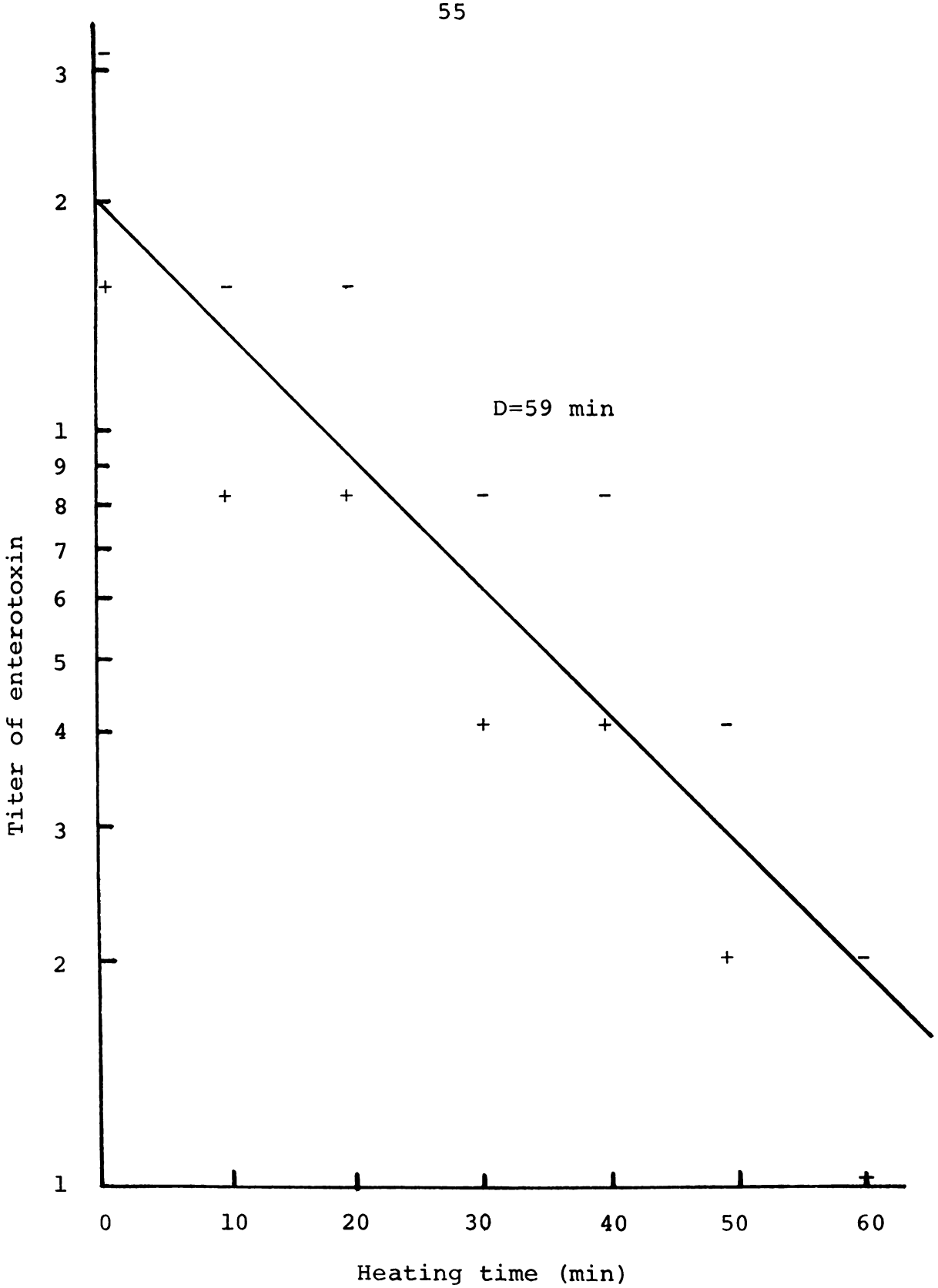

Figure 7 -- The logarithmic thermal inactivation time plot of SEA in BHI broth at 110 C.

is indicated by positive (+) or negative (-) signs. In this example the D_{110} value of SEA was calculated to be 59 min.

Tables 6 and 7 show the effects of heating crude SEA and SEB in different menstrua at 110.0, 115.6, 121.1, and 126.7 C. At all temperatures tested, D values were higher when crude SEA was heated in BB than when heated in BHI broth. Also crude SEB had greater thermal stability in BB as shown by the higher D values in BB than in BHI broth or VB.

The D values of SEA and SEB are not compared here, since crude preparations of both enterotoxins contain substantial protein and other substances which have definite effects on the thermal inactivation of the enterotoxins. Thermal inactivation curves were constructed by fitting a straight line to the D values at various temperatures on semi-log paper (Figures 8 and 9). The Z value, which is the reciprocal of the slope of the thermal inactivation curve, was determined and little difference between (or among) the Z values was found from heating the enterotoxin in different menstrua (Tables 6 and 7).

Partial Purification of Entertoxins. Broth cultures containing SEA, SEB, SEC, and SED were concentrated in 40% polyethylene glycol to 72, 62, 59, and 24-fold, respectively. SEA and SED were produced at lower concentrations (titers 4 and 1.5, respectively). Therefore, two

Table 6.--Effect of heating menstrua on the thermal stability of crude SEA.

Heating Menstruum	D value at				Z	
	110.0 C	115.6 C	121.1 C	126.7 C	C	F
Brain Heart Infusion (BHI)	59	38	24	14	26.7	48
Beef broth (BB)	89	55	34	22	27.2	49

Table 7.--Effect of heating menstrua on the thermal stability of crude SEB.

Heating menstruum	D value at				Z	
	110.0 C	115.6 C	121.1 C	126.7 C	C	F
Veronal buffer (VB)	34	25	16	10	31.1	56
Brain Heart Infusion (BHI)	43	29	20	14	32.8	59
Beef broth	69	44	34	21	33.3	60

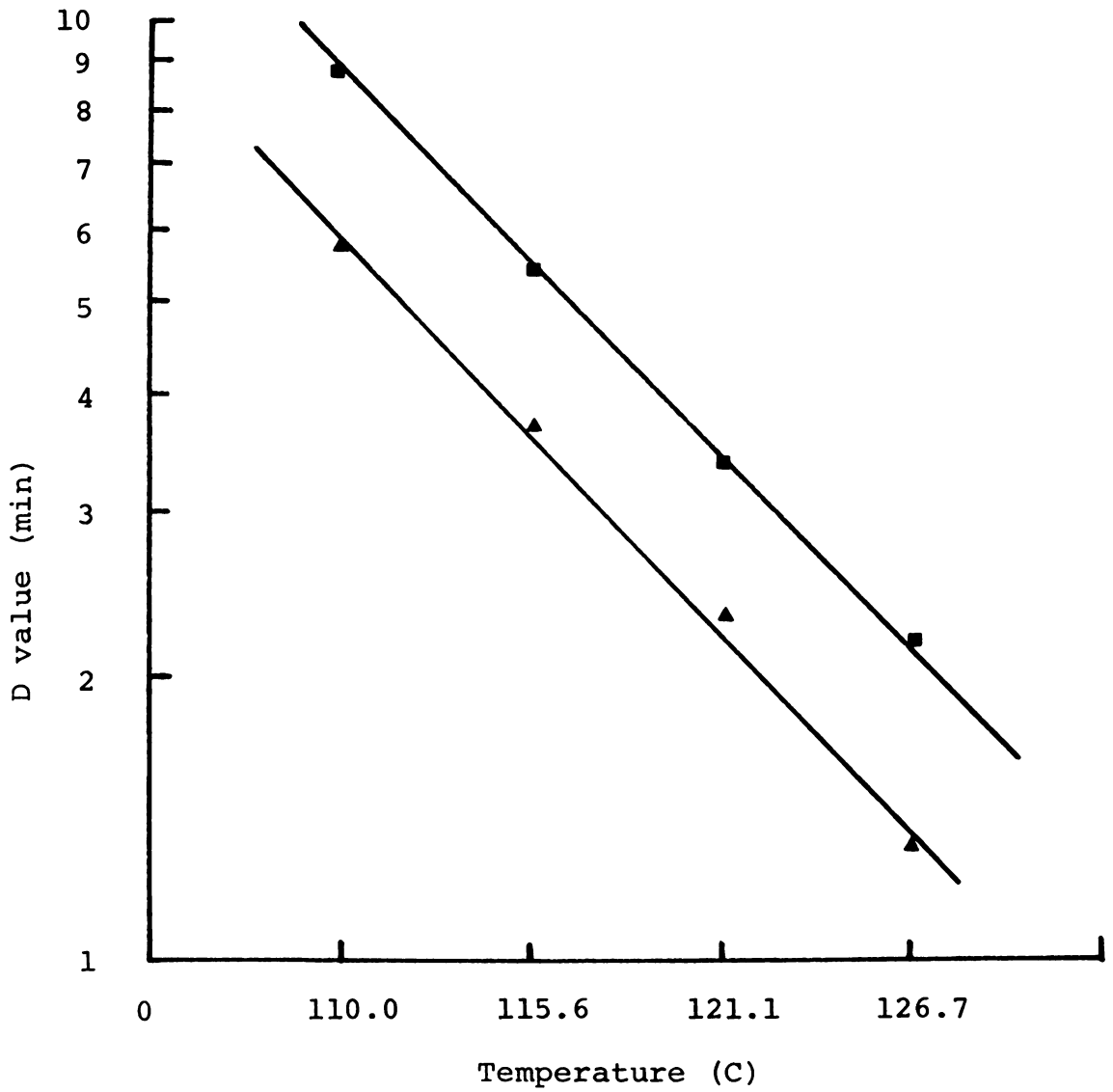

Figure 8 -- Thermal inactivation curves of SEA in BB (■) and in BHI(▲).

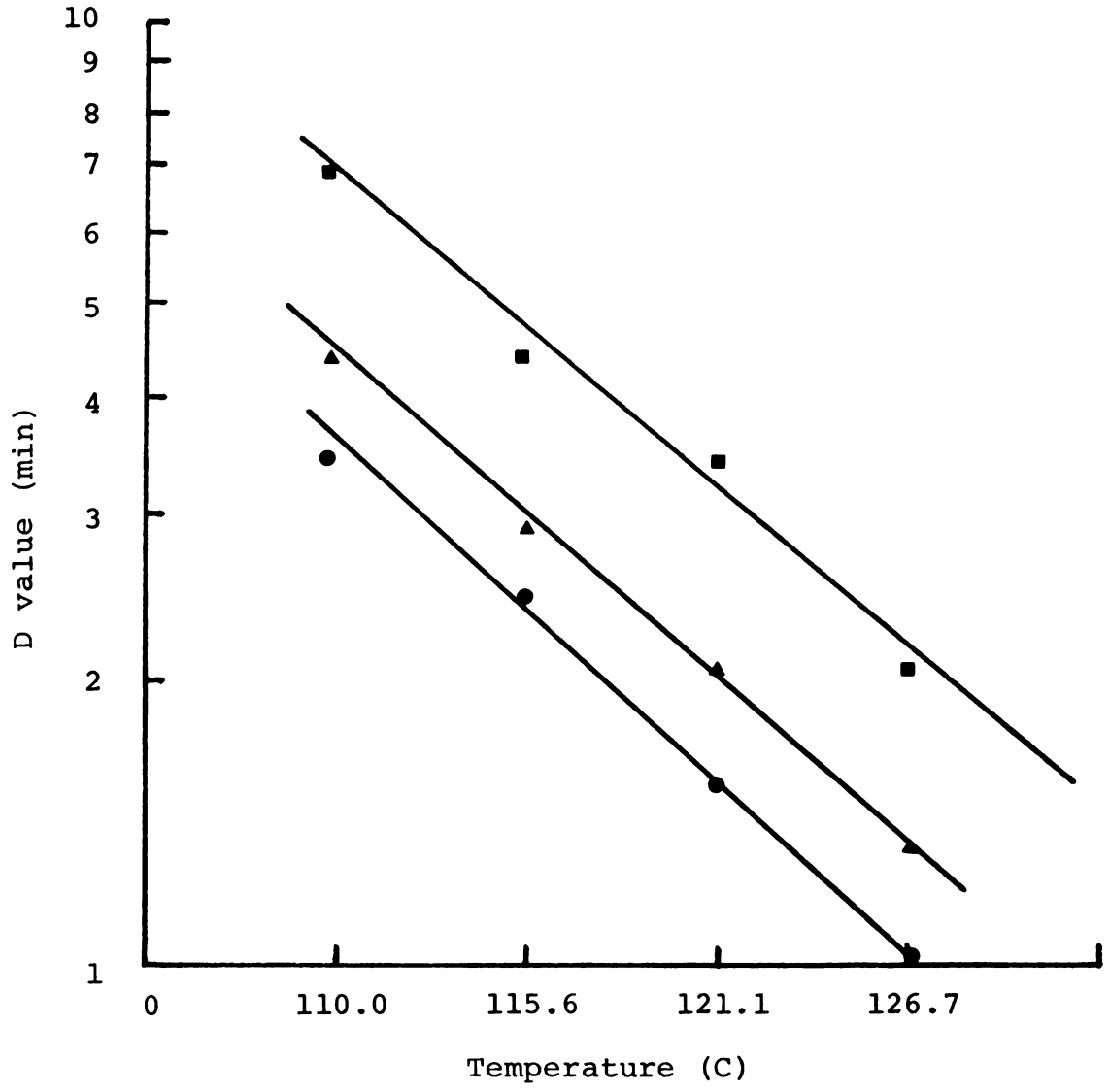

Figure 9 -- Thermal inactivation curves of SEB in BB (■), BHI (▲) and VB (●).

purification steps, namely acid and ammonium sulfate precipitation, were performed on the crude SEA and SED. The procedures resulted in 130, 53, 74, and 112-fold purification of the crude SEA, SEB, SEC, and SED, respectively, as indicated in Table 8. In order to use the same initial concentration of enterotoxins in the heat inactivation study, different volumes of these partially purified enterotoxins were added to a TDT can to make the final concentration of 16 to 32 μg/ml. Total protein concentrations in the volume of the partially purified enterotoxins used in each TDT can were 5.1 mg in 0.5 ml of SEA, 1.5 mg in 0.3 ml of SEB, 0.7 mg in 0.2 ml of SEC and 36.6 mg in 3 ml of SED.

Table 8.--Partial purification of enterotoxins.

Procedure	Specific activity of enterotoxins (ug enterotoxin/mg total protein)			
	SEA	SEB	SEC	SED
BHI culture	0.24	3.77	7.53	0.09
Concentrations in 40% PEG	0.90	21.8	68.6	0.55
Acid and $(NH_4)SO_4$ precipitation	5.20	--	--	1.22
G-100 Sephadex column	31.3	200.8	555.6	9.84
Total purification (fold)	130	53	74	112

Effect of Ultrafiltered Fractions of Beef Broth on the Heat Inactivation of Partially Purified SEB. Data in Table 9 illustrate the effect of ultrafiltered fractions (5x, 2.5x, and the filtrate) on the D_{110} of partially purified SEB. The protein concentration in the heating menstrua ranged from no protein in VB to 32.2 mg/ml in 5x BB. An increase in protein concentration up to 16.2 mg/ml in the heating menstruum resulted in an increase in the D_{110} value; however, further increase of protein concentration from 16.2 mg/ml to 32.2 mg/ml (in 5x BB) did not cause a corresponding increase in D_{110}.

Table 9.--Effect of heating menstrua on D_{110} of SEB.

Menstruum	Protein (mg/ml)	D_{110} (min)
Veronal buffer	0	18
Beef broth--1x	10.5	60
Beef broth--2.5x*	16.2	74
Beef broth--5x*	32.2	78
Filtrate	3.2	47

*concentrated by ultrafiltration using an Amicon PM 10 membrane filter

Effect of Beef Broth Protein on the Heat Inactivation of Partially Purified SEB. Dialyzed or non-dialyzed BB protein (the ammonium sulfate precipitate of beef broth)

added at concentrations of 3.8 and 7.7 mg/ml were used as the heating menstrua for partially purified SEB (Table 10). When SEB was heated in non-dialyzed BB protein, the D_{110} values obtained in the presence of 7.7 mg/ml of protein were higher than those obtained in 3.8 mg/ml. D values were greater when BB protein precipitated with 95% ammonium sulfate was added to the heating menstruum than when BB protein precipitated with 80% ammonium sulfate was added to the heating menstruum. On the other hand, the D_{110} values were similar when SEB was heated in the presence of either 3.8 or 7.7 mg/ml of dialyzed BB protein. It was also found that higher D_{110} of SEB was obtained in the presence of non-dialyzed BB protein than in dialyzed BB protein at comparable concentrations (Table 10). The data imply that the original non-dialyzed BB protein contained a dialyzable factor which significantly affected the thermal stability of the partially purified SEB.

Recombination of Dialysate and Dialyzed Beef Broth Protein. The D_{110} values of 31 and 32 min were obtained when SEB was heated in the dialyzed BB protein at concentrations of 3.8 and 7.7 mg/ml (Figure 10). When SEB was heated in 5 and 10 ml of the dialysate, the D_{110} values were 33 and 34 min, respectively (data not shown). This indicates that the dialysate or dialyzed BB protein alone, at the concentrations described, had a limited effect on the thermal stability of SEB. The recombination of the BB

Table 10.--Effect of concentration and treatment of beef broth protein on the thermal stability of SEB.

$(NH_4)SO_4$ Used for Precipitation	Treatment of BB Protein	Concentration of Protein	D_{110}
(%)		(mg/ml)	(min)
80	non-dialyzed	3.8	44
80	non-dialyzed	7.7	68
95	non-dialyzed	3.8	51
95	non-dialyzed	7.7	70
80*	dialyzed	3.8	34
80*	dialyzed	7.7	37
95	dialyzed	3.8	39
95	dialyzed	7.7	41

*80% $(NH_4)SO_4$ precipitate of 5 x BB obtained from the ultrafiltration of BB.

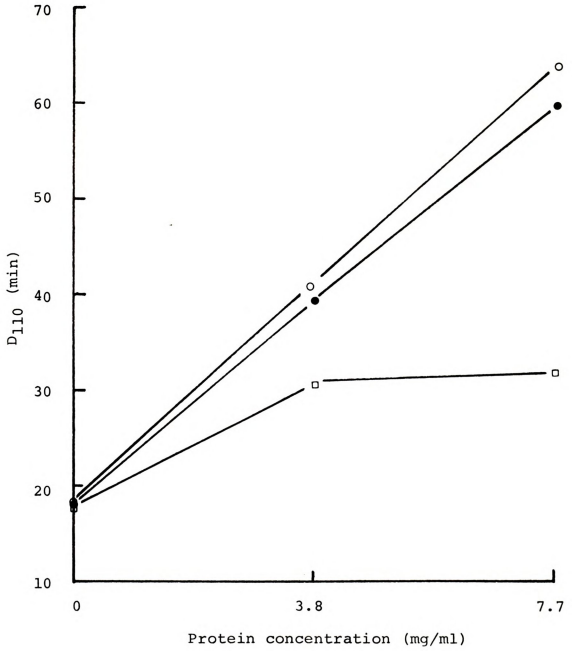

Figure 10 -- Effect of recombination of dialysate and dialyzed beef broth protein on D_{110} of SEB. Legend: -□- dialyzed protein; -●- nondialyzed protein; -○- recombination of dialyzed protein and dialysate.

protein was prepared by adding 5 ml and 10 ml of the dialysate, respectively, to 3.8 and 7.7 mg/ml of the dialyzed BB protein. By heating the SEB in these recombinations, the protective effect toward SEB was essentially restored to that of the non-dialyzed BB protein (Figure 10).

Analysis of the Dialysate. An experiment was performed to examine the nature of the dialyzable factor present in the dialysate. EDTA was added to chelate metal ions which may have been in the dialysate (Table 11). A D_{110} of 75 min was obtained when EDTA was added to a final concentration of 5 mM in the dialysate before adding the dialyzed protein. However, treatment of the dialysate with trypsin and chymotrypsin at 25 C for 3 hr decreased the protective effect which was attributed to the dialysate, and a lower D_{110} value of 51 min was obtained (treatment 3). In treatment 4 the dialysate was first incubated with PMSF, followed by the addition of dialyzed protein. The D_{110} of 73 min showed that PMSF has no adverse effect on the immunological reaction of SEB and antitoxin. In treatment 5 the proteolytic enzymes were inhibited by the PMSF prior to the addition of dialysate and dialyzed protein, and a D_{110} of 70 min indicated a complete inhibition of the enzymes by PMSF.

Effect of Non-dialyzed Beef Broth Protein on the Partially Purified Enterotoxins. Data presented in Figure 11 indicate the effect of different concentrations

Table 11.--Effect of treatment of the beef broth dialysate on the thermal inactivation of SEB at 110 C.

Treatment of dialysate*	D_{110} (min)
1. None	65
2. EDTA[a] at 25 C for 1 hr	75
3. Trypsin-chymotryspin[b] at 25 C for 3 hr, then inhibited with PMSF[c] at 25 C for 1 hr	51
4. PMSF at 25 C for 1 hr	73
5. Trypsin-chymotrypsin, previously treated with PMSF, at 25 C for 3 hr, then added dialysate	70

*The heating menstruum also contained VB and 7.7 mg/ml of dialyzed BB protein.

a. 5mM

b. 0.63% (enzyme/protein)

c. 6.97 µg/ml dialysate

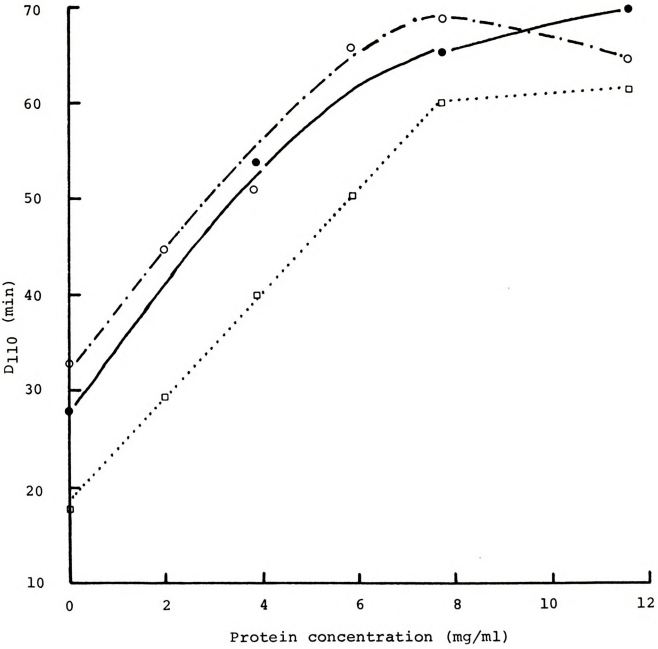

Figure 11 -- Effect of different concentrations of nondialyzed beef broth protein on the thermal inactivation of SEA(o), SEB (□) and SEC(●).

of BB protein on the D_{110} values of partially purified SEA, SEB, and SEC. Because of the different preparations and different purities of the enterotoxins, it is not intended here to compare the heat stability of the 3 enterotoxins. There was a linear increase in the D_{110} values for the enterotoxins when non-dialyzed BB protein increased from 0 to approximately 6 mg/ml (Figure 11). Partially purified SED was the least purified enterotoxin in comparison with SEA, SEB, and SEC. A gradual but linear increase in the D_{110} value of SED was observed when the non-dialyzed BB protein was increased from 0 to 7.7 mg/ml in the heating menstruum. However, the increase in concentration of dialyzed BB protein in the heating menstruum showed no effect on the D_{110} of SED (Figure 12), an occurrence which can be explained by the presence of relatively large amounts of protein other than enterotoxin in the partially purified SED. Three ml of SED, containing 36.6 mg of total protein, was used in each TDT can. A D_{110} of 45 min was obtained by heating SED in a menstruum containing 2.8 mg/ml of protein (36.6 mg/13 ml). In other words, 2.8 mg/ml of protein was already present along with the SED before any other protein was added to the heating menstruum. The effect of dialyzed protein may have already reached the maximum at a concentration around 2.8 mg/ml, thus an additional increase in the amount of dialyzed protein did not cause a corresponding increase in D_{110} values.

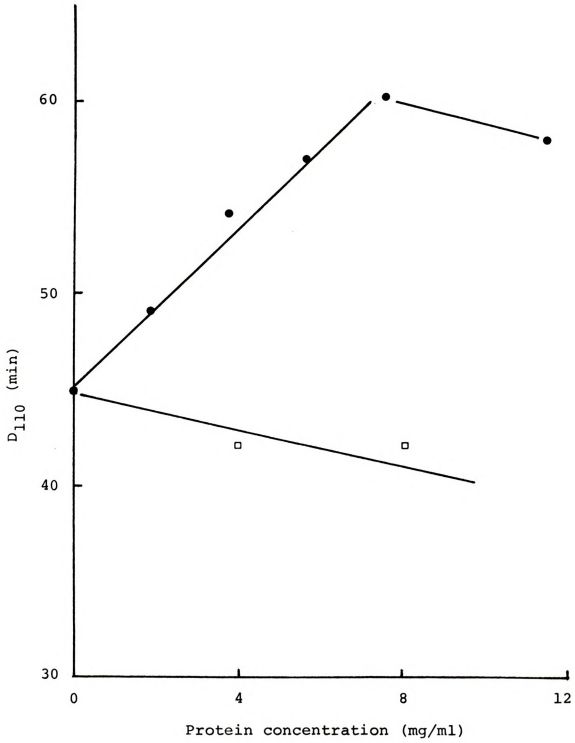

Figure 12 -- Effect of different concentrations of beef broth protein on the thermal inactivation of SED. Legend: -●- nondialyzed BB protein; -□- dialyzed BB protein.

DISCUSSION

Genoa Salami

Genoa salami is a variety of dry, Italian-type fermented sausage which is a very popular item in the United States. The fermentation is carried out by lactic acid bacteria which are either added or naturally present. Failure to produce an acceptable product in the processing of Genoa salami is highly possible when the fermentation is dependent on the indigenous bacterial flora of the meat, or introduction from the equipment. Therefore, starter cultures of lactic acid bacteria may be introduced in the manufacturing of dry and semi-dry sausages (54).

This investigation, however, did not utilize starter cultures for the following reasons:

(a) The use of starter cultures in producing dry, Italian-type sausage is not universally accepted in industry, and dependence upon "chance inoculation" and "back slopping" is still common practice (30).

(b) The intention was to study a simulated condition in which a failure of the added starter culture occurred.

(c) The inoculated S. aureus cells were given a chance to compete with the indigenous microbial flora in the meats.

The method of processing used in this investigation is an invention of Armour & Company (68), and is particularly suitable for the production of dry, Italian-type sausage products using artificial casings.

In studying core samples and surface samples, which consisted of the outer 1 cm of the salami, the surface samples always had higher staphylococcal and total counts. These results verify the findings of Barber and Deibel (9) who reported that the uneven distribution of the microbial populations was mainly due to difference in the oxygen tension. They also indicated that the anaerobic condition in the core samples decreased the potential for enterotoxin production. Therefore, in the examination of sausage for staphylococcal population and the presence of enterotoxin, the analyses should be performed on the surface samples.

There are many factors which influence the toxin-producing ability of an enterotoxigenic strain. The minimum population of staphylococci which supports the production of enterotoxin has often been discussed. By using a cellophane sac technique (23), Tompkin et al. (79) illustrated the relationship between the number of staphylococci attained in the sac after 96 hr at 37°C and the presence of SEA. Enterotoxin A was present in two out of four BHI broth samples containing 10^7 staphylococci/ml,

but when the staphylococci increased to 10^8 cells/ml, SEA was detected in 82% of the samples. A staphylococcal count ranging from 1.0×10^7 to 4.0×10^7 cells/g was proposed by Barber and Deibel (9) as the minimum number that produced detectable SEA within 24 hr in laboratory formulated sausages. Scheusner and Harmon (66) investigated enterotoxin production in commercial foods such as banana, chocolate, and coconut cream pies which were inoculated with 10^5 cells/g of S. aureus 265. SEA was detected after 68-hr incubation at 21°C, and the terminal counts of staphylococci were 5.0×10^7 to 2.0×10^8 cells/g. In this investigation, SEA was detected in samples after tempering which contained 2.8×10^8 and 4.9×10^9 staphylococci/g. On the other hand, SEA was not detected in a similar sample containing 1.5×10^7 cells/g which corresponded to the lower limit of the population reported to support the production of SEA in some other products.

The highest population of S. aureus 243 obtained in the inoculated salami was 1.3×10^8 cells/g and no SEB was detected. However, since the sampling method consisted of taking cross sections of the salami, the SEB, if present, may have been diluted. Previous reports indicated that a population of 8.3×10^8 S. aureus S-6 cells/g in laboratory-formulated sausage (9) and 3.3×10^7 S. aureus 243 cells/g in canned sweet potato (66) were able to produce measurable quantities of SEB. It should be noted that the variations in staphylococcal strains and environmental conditions have

a great influence on the production of enterotoxin. Thus, although the production of enterotoxin is always accompanied by the attainment of high cell population, good growth of staphylococci is not necessarily an indication of the presence of enterotoxin (9, 44, 62, 66).

Samples taken on the 23rd day showed a slight increase in the staphylococcal populations in the surface samples from salami inoculated with S. aureus 265 (Figure 1 and 2) and there was also an increase in the samples from salami inoculated with S. aureus 243 when sampled on the 29th day (Figure 5). Ordal (55) reported that thermally induced lesions in sublethally heat-treated staphylococci must be repaired before the cells can multiply and divide. Therefore, staphylococcal populations measured in samples taken immediately after heating may not include heat-injured cells. The increase in populations at the next sampling time after heating may be due to recovery of heat-injured cells as well as multiplication of staphylococci.

The effect of a_w on enterotoxin production and growth of S. aureus has been studied by Troller (81, 82). By adjusting media containing NZ Amine NAK and partially hydrolyzed protein (PHP) to various a_w levels, he demonstrated that the production of SEB by S. aureus C-243 was strongly inhibited by a reduction in a_w from 0.99 to 0.98 despite the attainment of populations above 10^9 cells/mg. Under similar experimental conditions, studies on SEA indicated that S. aureus 196E was capable of

producing SEA at an a_w of 0.90 and final cell counts remained above 10^8 cells/ml (82). In this investigation, the a_w dropped from 0.99 to 0.98 during tempering of the salami (Table 5). Therefore, the reduction of a_w in the salami presumably is a limiting factor in the production of SEB, and SEB was not detected in salami inoculated with S. aureus 243 even though final populations were as high as 1.3×10^8 cells/g.

In contrast to the growth of staphylococci, the anaerobic condition in the core sample does not retard the growth of lactic acid bacteria, since they are microaerophilic organisms. As a result, the samples taken from different locations of the salami do not show any significant difference in the population of lactic acid bacteria. Fifteen species of lactic acid bacteria were examined by Haines and Harmon (39) for their ability to influence the growth of S. aureus and the production of enterotoxin in associative culture. They demonstrated that the streptococci were most inhibitory, followed by Pediococcus cerevisiae, while the lactobacilli and Leuconostoc citrovorum were not inhibitory to staphylococcal growth but did slightly suppress the production of enterotoxin. On the other hand, the presence of S. aureus had no inhibitory effect on the population of the lactic acid bacteria tested when the initial population of both S. aureus and the effector organisms were 10^5 cells/ml. In this investigation, data in Figure 4 further illustrates that variation

in number of S. aureus 265, within the population range studied, did not have a significant effect on the population of lactic acid bacteria in salami. Similar results were also obtained on the population of lactic acid bacteria when S. aureus 243 was inoculated into the salami at various rates (Table 3).

Data in Table 2 show that more lactic acid was produced in the surface samples from salami with low staphylococcal inoculum (10^3 cells/g) than those with high inocula (10^5 and 10^7 cells/g). This would indicate that high populations of staphylococci in the surface samples might be competing with the lactic acid bacteria, resulting in reductions in the amount of lactic acid production.

The desired pH of a fermented sausage ranges from 4.8 to 5.4 at the end of processing. Therefore, the salami made in this study were not ideal products because the pH values were >5.4. The population of lactic acid bacteria in the salami increased from less than 150 in the non-inoculated pork to approximately 10^5 cells/g at the end of the heating period, whereas, Acton et al. (1) reported more than 10^8 cells/g were found after 48 hr in a controlled fermentation when a starter culture of lactic acid bacteria was inoculated at 2×10^6 cells/g. Therefore, the fermentation in the non-inoculated salami was a slow and incomplete process in comparison to that in the inoculated salami with lactic starter culture. The temperature of

20 to 25C during tempering may also have contributed to the slow rate of fermentation (1). The lactic acid produced over a period of 2 months caused no significant reduction in the pH value of the salami, presumably due to the buffering ability of the meat and the slow rate of acid production. However, low pH values may not inhibit the growth and enterotoxin production by staphylococci. Enterotoxin B can be produced in cured meat at an initial pH of 5.0 (62), while an initial pH of 4.5 in reconstituted nonfat milk solids has been reported to support SEA production (78), and the lowest pH value reported to support the production of SEC was 4.0 in a PHP-NAK broth inoculated with 1×10^8 cells/ml (35). Furthermore, Baran (8) reported that during the production of a dry turkey sausage, an increase in staphylococcal population from 5.6×10^3 to 1.8×10^6 S. aureus 243 cells/g was obtained even when a starter culture of Pediococcus cervevisiae was used. Although SEB was not detected, the high population of staphylococci in the product could be a potential health hazard. In conclusion, a more direct approach for the control of staphylococci in Genoa salami is to reduce or eliminate contamination by the staphylococci in the raw ingredients or during processing.

Thermal Inactivation of Enterotoxins

The changes that take place in protein molecules during heat denaturation constitute a complex class of reactions. Some of the physico-chemical changes related to

denaturation are: (a) molecular parameters including molecular size, shape, and optical properties, (b) interactions between protein molecules such as aggegation and phase transition, and/or (c) interactions of proteins with other compounds, for example, solubility, binding ability, enzymatic and immunological behavior (42). A detailed discussion of these topics, however, is outside the scope of this dissertation.

In this investigation, the thermal inactivation of enterotoxins was detected by the loss of immunological activity. Some exotoxins can be denaturated without influencing the antigenic specificity; however, other investigators have found that the loss of toxic activity of enterotoxins assayed by injection into cats, paralleled the loss of immunological activity detected by a double gel diffusion assay (41, 60).

Heat treated protein may aggregate or coagulate when the aggregate reaches macroscopic size. In this investigation, some of the experiments involved the use of beef broth protein in the heating menstrua. Whether submicroscopic protein aggregates were formed was not determined; however, the beef broth protein was not coagulated after heating which facilitated the serological assay of the enterotoxins. The data on thermal inactivation of enterotoxins were analyzed by the end point method of analysis which was originally applied for the analysis of

thermal death time (TDT) data. Difficulties may arise in drawing a TDT curve to fit the data. Townsend et al. (80) stated that the curve should be determined as follows:

(a) "A survival point is considered as positive data and the curve must be above (higher in temperature or longer in time than) every survival point."

(b) "Destruction points are indicative but not positive owing to the phenomenon of 'skips' (survival of organisms at a time beyond that at which sterility is indicated). In general, a thermal death curve should lie beneath as many destruction points as possible and still be above all survival points."

(c) "The slope of the thermal death time curve should be parallel to the general trend of the survival and destruction points."

In this investigation, the heated enterotoxin was diluted two-fold and solutions with titers of 2, 4, 8, 16, and 32 were assayed to quantitate the amount of enterotoxin. The wide intervals between titers may have caused some variation in the D values, and the time intervals of heating (10 min in most of the cases) may have also contributed to variations. However, due to the voluminous number of samples and the dilutions involved, it was impractical to run the experiment at smaller time intervals.

D and Z Values of Crude Enterotoxins A and B. At all temperatures tested the D values were higher when crude

SEA was heated in BB than when heated in BHI (Table 6). Similar data shown in Table 7 indicate that SEB was most stable in BB, followed by BHI broth, and least stable in VB. These results agree with the findings of Denny et al. (31) who reported that the heat stability of SEA in beef bouillon was 3 to 5 times greater than in 0.15 M phosphate buffer. However, Satterlee and Kraft (63) indicated more rapid loss of SEB in ground meat slurry than in phosphate buffer. This might be due to low recovery of enterotoxin during extraction from the meat slurry.

The Z value obtained from the TDT curve is an important heat parameter. By knowing Z and a D value at one temperature (T_1), the D value at any other temperature (T_2) can be calculated by the following formula (72):

$$\log D_2 - \log D_1 = \frac{1}{Z}(T_1 - T_2)$$

The Z values determined for SEA (26.7 and 27.2 C) and SEB (31.1, 32.8, and 33.3 C) were very close to those reported previously by other authors (31, 32, 40, 60). Both crude SEA and SEB are extremely stable during heating as shown by the high D and Z values in Tables 6 and 7. In order to give an indication of their degree of thermal stability, a few examples of thermal inactivation studies on proteins other than enterotoxins are mentioned below. Licciardello et al. (48) reported the Z value of type E botulinum toxin to be 7.5 C (13.5 F). Calculating from their data, the D_{71} value was approximately 0.64 min when

the toxin was heated in a haddock substrate. Another study by Amelunxen and Lins (2) on the comparative thermostability of 11 enzymes from Bacillus stearothermophilus and a mesophilic B. cereus indicated that with two exceptions, the enzymes from the thermophile demonstrated greater thermostability than the enzymes from the mesophile. For example, when alkaline phosphatase was heated at 70 C for 10 min, the D value of the enzymes was calculated to be 188 min for the thermophile compared to 34.7 min for the mesophile. When the thermal exposure was increased to 80 C for 10 min, the alkaline phosphatase from the thermophile had a D value calculated to be 10 min, and no activity was detected in the enzyme from the mesophile.

Denny et al. (31) stated that the increase in heat stability of SEA corresponded to the increase in toxin concentration, and they further explained that other protein molecules had a protective effect on the toxin molecule. Therefore, D values cannot be compared when initial concentrations are different nor when enterotoxin is heated in different menstrua. It is interesting that higher D values were obtained when crude SEA and SEB were heated in BB than when heated in BHI broth. According to the Lowry determination (49), the protein concentrations of BB and BHI were 12 and 17 mg/ml, respectively. Therefore, the protection of enterotoxins afforded by the heating menstrua is not in direct proportion to the protein concentrations when the proteins originate from different sources.

Studies of Partially Purified SEB in Beef Broth. The protective effect of protein in the thermal inactivation of enterotoxins has been suspected (31). However, little research has been conducted to study the effect of protein in relation to enterotoxin during heating. In order to find the role of protein during thermal inactivation of enterotoxin, it was necessary to reduce the total protein content in the crude enterotoxin to a minimum amount. A simple purification scheme (Table 8) was followed. Such purification was very successful with SEB and SEC and fairly successful with SEA. In this research project, a series of experiments was designed to study this protein-enterotoxin relationship during thermal inactivation. Beef broth protein was chosen because of its protective effect of crude SEA and SEB during heating and because it is readily available commercially. Also no extraction of enterotoxin from BB is required before assaying the toxin by the microslide method.

Ultrafiltration of BB through a PM 10 membrane, which permits passage of particles with a maximum molecular weight of ca. 10,000 was used to separate the large molecules, such as proteins and polysaccharides, from the small molecules, such as peptides, sugars, and salts. Since SEB showed more heat stability in concentrated BB than in the filtrate (Table 9), it was assumed that the increase in the amount of protein in the concentrated BB may have caused this increase in heat stability in SEB. In order to prove

this assumption, BB protein collected by ammonium sulfate precipitation was added either dialyzed or non-dialyzed to the heating menstrua at different concentrations. The D values obtained are given in Table 10. The data demonstrate that the thermal stability of SEB is significantly influenced by the concentration of BB protein in the heating menstruum. In addition to the non-specific protective effect(s) of protein, there is a specific protective effect associated with a dialyzable fraction of the BB protein. During dialysis, cellulose tubing with an average pore diameter of 480 mm was used permitting the passage of low molecular weight compounds while retaining materials with a molecular weight of ca. 12,000 and higher. Therefore, the dialyzable factor presumably has a molecular weight of less than 12,000.

The dialyzable factor was initially suspected to be a metal ion, since metal ions are believed to increase the thermostability of macromolecules (57, 69). Therefore, EDTA was added to the dialysate to chelate the available metal ions, but EDTA did not interfere with the thermal protection of SEB attributed to the dialysate (Table 11). In order to determine whether the factor was a protein, the dialysate was treated with proteolytic enzymes trypsin and chymotrypsin and the resulting dialysate possessed less thermal protection for SEB. These data suggest that the dialyzable factor contained a protein fraction which is necessary for conveying increased thermal stability to SEB.

The D value of 51 min (Table 11, treatment 3) may indicate an incomplete digestion of the factor by the trypsin and chymotrypsin under described experimental conditions, or it may suggest that the factor has a more complicated structure than that of a simple protein. Another possible explanation is that after digestion the remnants of the factor still protect the enterotoxins during heating but to a lesser extent. The dialyzable factor demonstrated a specific thermostable influence on SEB because 0.2 mg/ml of the dialysate protein in the heating menstruum showed approximately the same degree of thermal protection to SEB as 3.8 mg/ml of the dialyzed BB protein. Furthermore, this protective effect does not appear to be specific for SEB since evidence from heating experiments conducted with partially purified SEA, SEC, and SED indicate these enterotoxins possessed greater thermal stability in the presence of the non-dialyzed BB protein.

Some proteins extracted from thermophilic microorganisms have been highly purified and characterized. Homologous proteins from mesophilic organisms were compared and similarities were found which included molecular weight, subunit composition, amino acid composition, and primary amino acid sequences (10, 45). It is generally agreed that a thermally stable protein molecule is usually a flexible molecule, containing a high level of hydrophobic and charged amino acids (69). Further studies on the three dimensional structure of the proteins may help elucidate

the mechanism(s) of thermophily (69). At present, the existence of an intrinsic thermostability in the proteins from thermophilic organisms is the popular hypothesis used to explain the thermophile in molecular terms. However, a contrary example was provided by the study of a catalase from an unspecified thermophile by Nakamura (53). The crude enzyme isolated from bacterial cells contained an S factor which suppressed the activity of the catalase. The S factor could be removed from the crude enzyme by charcoal treatment which would result in a shift of the optimum temperature for enzymatic activity from 65 to 60 C. The physical properties and the nature of the S factor were not reported.

In this investigation, an exogenous factor from BB was found which stabilized enterotoxins during heating. Due to its specific thermostabilizing effect as described previously, the effect shown by the small amount of protein in the dialysate leads to the assumption that a specific protein-to-protein interaction between the factor and the enterotoxin molecule was responsible for a major portion of stability of enterotoxin during heating. Further studies are required for a detailed understanding of the nature of the factor, its reaction toward enterotoxins during heating, and whether the protection is due to one particular protein or a specific group of proteins.

CONCLUSIONS

1. The inoculation of Staphylococcus aureus 265 at the rate of 10^3, 10^5, and 10^7 cells/g into Genoa salami resulted in 1.5×10^7 to 4.9×10^8 cells/g in the surface samples of the salami after tempering. Enterotoxin A was detected in the surface samples from salami inoculated with 10^5 and 10^7 cells/g, but not in salami inoculated with 10^3 cells/g. Although high populations were obtained from samples of cross sections of salami inoculated with S. aureus 243, no enterotoxin B was detected. The low water activity in the salami may have caused a suppression of the enterotoxin producing ability of S. aureus 243.
2. The salami produced in this investigation was not a desirable product, since the pH was higher than 5.4. The low population of lactic acid bacteria suggests inadequate fermentation by the lactic organisms naturally present in meat. The use of lactic starter cultures is recommended for better control of the fermentation process.

3. The D values for thermal inactivation of crude enterotoxins A and B varied significantly in different heating menstrua. At all temperatures tested higher D values for crude SEA were obtained in beef broth than in Brain Heart Infusion broth. Crude enterotoxin B had higher thermal stability in beef broth than in Brain Heart Infusion broth or veronal buffer. Whereas, little difference existed between (or among) the Z values obtained from heating the enterotoxin in different menstrua.
4. The variation of D values in heating menstrua was caused by a variety of factors. In this investigation, the high thermal stability of enterotoxins in beef broth was shown to be associated with a nonspecific effect provided by the dialyzed beef broth protein and a specific effect attributed to a dialyzable factor in the beef broth. Preliminary studies suggest that the dialyzable factor which confers thermal stability to enterotoxins has a molecular weight of less than 12,000. A portion of the factor, composed of protein, is believed to be involved in the protection of enterotoxins during heating.

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APPENDIX

APPENDIX

GLOSSARY

Brain Heart Infusion	BHI
Beef Broth	BB
carboxymethyl cellulose	CMC
enterotoxin A	SEA
enterotoxin B	SEB
enterotoxin C	SEC
enterotoxin D	SED
ethylenediamine tetraacetate	EDTA
glucono delta lactone	GDL
Lactobacillus Selective Agar	LBS
Mannitol Salt Agar	MSA
phenylmethyl sulfonyl fluoride	PMSF
polyethylene glycol	PEG
thermal death time	TDT
veronal buffer	VB
Vogel Johnson Agar	VJA