Suggested short title :

DESTRUCTION OF SALMONELLA IN CHOCOLATE

STUDIES ON THE DESTRUCTION OF SALMONELLA IN CHOCOLATE BY HEAT, ULTRASONICS AND ULTRAVIOLET

by

Byong H. Lee

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

Agricultural Chemistry & Physics
Macdonald College of McGill University
Montreal, Quebec

September, 1976

Suggested short title :

DESTRUCTION OF SALMONELLA IN CHOCOLATE

M.Sc. Agricultural Chemistry

Byong H. Lee

ABSTRACT

STUDIES ON THE DESTRUCTION OF SALMONELLA IN CHOCOLATE BY HEAT, ULTRASONICS AND ULTRAVIOLET

Detailed studies were made on the destruction of Salmonella by heat, ultrasonics and UV irradiation. Ninety-six percent of Salmonella eastbourne was destroyed by a six day storage period at 5C. An additional storage period of one year led to the destruction of 99% of the cells. S. eastbourne was the most heat sensitive in sucrose solution and S. senftenberg was by far the least heat sensitive of any of the other five strains that were checked. The heat resistance of S. eastbourne (D = 4.5 hr) in chocolate was about the same as that of S. senftenberg (D = 4.6 hr). S. typhimurium (D = 6.6 hr) was more resistant than S. senftenberg in milk chocolate. Ultrasonic treatment (10 min; 250 watt) destroyed over 99.999% of S. eastbourne in peptone solution (0.1%). The same treatment had little effect on the destruction of Salmonella in milk chocolate.

Ultraviolet irradiation (12×10^3 erg/cm^3/sec; 2 min) destroyed over 99.999% of the S. eastbourne cells in thin layers (0.5, 1.0 and 2.0 mm) of peptone solution. Similarly, S. eastbourne cells under a thin layer of chocolate (0.1 mm), were completely destroyed as indicated by direct plate count. Different serotypes of Salmonella were equally sensitive to UV and there was no observable photoreactivation and excision repair on agar and under a thin layer of chocolate. The efficiency of UV irradiation on the destruction of S. eastbourne was affected by cell density and cell age.

RESUME

M.Sc. Byong H. Lee Chimie Agricole

ETUDE SUR LA DESTRUCTION PAR LA CHALEUR, LES RAYONS ULTRASONIQUES ET ULTRA VIOLETS DE LA SALMONELLE

Des études détaillées furent poursuivies sur la destruction de la Salmonelle par la chaleur, les rayons ultrasoniques et les irradiations U.V. Quatre vingt seize pour cent de la Salmonelle eastbourne furent détruites en les entreposant durant six jours à 5C. Une période additionnelle de un an a résulté en la destruction de 99% de ses cellules. S. eastbourne fut la plus sensible à la chaleur dans une solution de sucrose et S. senftenberg fut de loin la moins sensible à la chaleur de toutes les cinq autres variétés qui furent vérifiées. La résistance à la chaleur de la S. eastbourne (D=4.5 hr) dans le chocolat est à peu près la même que celle de la S. senftenberg (D=4.6 hr). S. typhimurium (D=6.6 hr) fut plus résistante que la S. senftenberg dans le lait au chocolat. Le traitement ultrasonique a détruit (10 min; 250 watt) plus de 99.999% de la S. eastbourne dans une solution de peptone (0.1%). Le même traitement eut peu d'effet sur la destruction de la Salmonelle dans le lait au chocolat.

L'irradiation à l'ultraviolet (12×10^3 erg/cm^2/sec; 2 min) a détruit plus de 99.999% des cellules de la S. eastbourne en couches minces (0.5, 1.0 et 2.0 mm) de solution de peptone. De même, les cellules S. eastbourne sous une mince couche de chocolat furent complètement détruites, tel qu'indiqué par un comptage sur plaque. Différents sérotypes de Salmonelles furent également sensibles au UV et il n'y eut pas de photoréactivation observée et de cicatrisation d'excision sur agar ainsi que sous une minche couche de chocolat. L'efficacité de l'irradiation UV sur la destruction de la S. eastbourne était affectée par la densité des cellules et par leur âge.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Dr. B.E. Baker, for his encouragement, advice and criticism throughout the course of this work. Thanks are also extended to the other faculty members for their help: Drs. R.H. Common, P.A. Anastassiadis, H.J. Hueckel, T. Beveridge, and Professor G.O. Henneberry.

The technical advice of Dr. E.S. Idziak, Department of Microbiology, Macdonald College and Dr. T. Matula, Bio-research Laboratory, Pointe Claire, Quebec, is greatly appreciated.

Special thanks are expressed to Mr. K. Maurer and Mr. Slaby who helped in the construction and operation of ultrasonic and UV sterilizer apparatus.

I also wish to thank Mrs. D.L. Hendren for the typing of the thesis and my wife for her assistance with the typing.

Finally, the author wishes to thank Comet Confectionary, Limited, St. Hyacinthe, Quebec and the Department of Industry, Trade and Commerce, Canada for financial support accorded to this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL INTRODUCTION

Salmonella, a group of diarrhea causing pathogenic bacteria has emerged as one of the major causes of food poisoning.

Although protein foodstuffs of animal origin, such as meat, poultry, egg and dairy products are usually recognized as vehicles of infection, salmonellosis has seldom been associated with chocolate products. Most recently, the sudden and increased incidents of Salmonella infection, caused by S. eastbourne in chocolate, received widespread publicity because of the commercial distribution of the contaminated products. Because of this, some attention has been given to the detection and control of Salmonella in milk chocolate. Most of the difficulties encountered in the chocolate industry have arisen from the fact that Salmonella are extremely difficult to destroy in contaminated chocolate. The high sugar and fat content, coupled with the low moisture content of chocolate, appear to be the most important factors, contributing to the heat resistance of Salmonella as previously shown in dried egg and milk. The sterilization of chocolate has also posed a considerably greater problem because of its great instability to heat in the range of effective pasteurization.

In view of the fact that milk chocolate cannot be heated sufficiently to destroy Salmonella without adverse effects on chocolate quality, it would be highly desirable to have a method which would be used to sterilize milk chocolate just

before the moulding operation. There is no existing methodology for sterilization of chocolate and also for the precise detection of Salmonella, available to the chocolate industry. This investigation was undertaken to collect information which will be helpful in the development of a workable method for the detection of Salmonella and for the sterilization of chocolate on a continuous basis. The present study deals with the use of UV irradiation, heat and ultrasonic for the sterilization of chocolate.

LITERATURE REVIEW

I. The Role of Salmonella in Food Poisoning

1. Historical

The term Salmonella, refers to a group of diarrhea-causing pathogenic bacteria which was formerly called "Paratyphoid Bacteria". It was derived from the name Salmon, who first described a member of the group (Salmon et al., 1886). Three species of Salmonella are generally recognized namely, S. cholearaesuis, S. typhi and S. enteriditis: the first two do not contain serotypes but S. enteriditis contains about 1500 serotypes.

Since the first report of botulism food poisoning in 1735, there has been a marked increase in the incidence of the disease although some of this increase may be attributed to an improvement in reporting. Gärtner (1888) reported the first fatal case of Salmonella poisoning which occurred in Germany. It was the result of the consumption of raw meat. Since this incident Salmonella poisoning has been a major concern to public authorities (Bryan, 1974; D'Aoust et al. 1975; Todd, 1976). It is estimated that only 1-10% of the actual number of human infections due to Salmonella is presently being reported. According to Davis et al., (1973), Salmonella cause a variety of illnesses, ranging from typhoid and paratyphoid fevers to meningitis, septicemia, osteomyelitis, pneumonia, and endocarditis, in addition to their most frequent manifestation, gastroenteritis.

The symptoms of Salmonella gastrointestinal disorders (salmonellosis) are characterized by nausea, vomiting, abdominal pain, and diarrhea, followed by headache and chills.

It has been estimated that about two million people have Salmonella infections per year and these infections caused approximately 500 deaths in the U.S.A. alone and approximately 120 deaths including young infants in England yearly. The indirect losses to the American economy has been estimated to be 100 million dollars annually (Eickhoff, 1966; Prost and Riemann, 1967). Following active salmonellosis, the organisms occasionally become established in the host and give rise to a carrier state which may be present for a long period of time (Prost and Riemann, 1967).

The mechanism of food poisoning by members of the Salmonella groups is not fully understood but Dack and Davison (1938) and Prost and Riemann (1967) postulated that it is most likely caused by joint action of the bacterial cell and the endotoxin which is liberated during lysis of the bacterial cells. Epidemiological evidence appeared to corroborate the experimental evidence that living organisms are essential in outbreaks of food-borne infection caused by Salmonella. It is a generally accepted fact that a large number of Salmonella organisms are necessary to produce food poisoning but McCullough and Eisele (1951) stated that the infectious dose may vary with species of Salmonella and with the individual concerned. Edwards and Galton (1967) and Thatcher and Clark (1968) also stated that any

serotypes of Salmonella in foods is potentially infectious to man, either directly upon consumption of food or indirectly through secondary contamination of utensils, processing equipment, or other foods.

2. Sources of Salmonella

Human: Salmonella are frequently present in the intestinal tract of some individuals and thus, it is very easy to understand the way in which the organisms gain entry to food, during its preparation, handling and serving. Edward and Bruner (1943) studied the 224 cultures isolated from the stools of patients with gastroenteritis and found that most prevalent species are S. typhimurium, S. montevideo, S. oranienburg, S. newport, and S. anatum in order of importance. In recent years, a number of epidemics have been related to S. choleraesuis, S. newport, S. anatum, S. enteriditis, S. blockley, S. oranienburg, S. derby, S. brandenrup, S. saint-paul, S. infantis, S. montevideo, S. ready, S. newington (Prost and Riemann, 1967; Rowe, 1973; Handzel, 1974).

Poultry and poultry products: Mallick and Rao (1964) studied salmonellosis produced by the poultry specific S. gallinarum-pullorum which can cause high mortality in hens. Numerous publications in recent years have reported a frequent occurrence of symptomless infection with various Salmonella types, particularly with S. gallinarum-pullorum and S. typhimurium in hens (Sharma and Singh, 1963; Sadler and Corstvet, 1965). Recent reports from various countries state that 2.6 to 7.0

percent of eggs are contaminated with S. typhimurium. Wilder and MacCready (1966), Bryan et al. (1968), and Morris and Wells (1970) reported that uncontaminated fresh and frozen poultry, such as chicken and turkey, are frequently contaminated during evisceration and chilling in the processing plant. Schneider (1946), Ager et al. (1967), and Board (1973) reported that unpasteurized egg salad, frozen, or dried eggs, yolks, or albumin have been a major source of salmonellosis since World War II. Their inclusion as supplements in other food products such as cake or cookie dry mixes or noodles have widened their impact on the consumer. This eventually resulted in FDA regulations requiring Salmonella-free egg products. Other species of birds, such as turkeys, ducks, geese and fowl, also suffer from similar diseases produced mainly by S. typhimurium. Most recently a S. reading outbreak was caused by failure to refrigerate turkeys (Report, 1974).

Meat and dairy products: Of the numerous foodstuffs used by man, meat and meat products are among the most frequent sources of Salmonella since the first report on swine plague. During recent years, a number of serious epidemics have been caused by ground meats, sausages, meat salads, and various other meat preparations (Wilson et al., 1961; Weissman and Carpenter, 1969; Morris and Dunn, 1970; Carpenter et al., 1973). Galton et al. (1954) observed that swine are readily infected during transportation and in the holding lots at the abattoir or subsequently on the pork assembly line. Hobbs (1961) also stated

that the major source of contamination of fresh beef seems to occur from plant equipment and/or at the retail butcher shop. Milk has also been the cause of Salmonella food poisoning, although it is rarely subject to primary contamination because of almost universal pasteurization of milk. Dumas et al. (1961) stated that the contamination originates from the feces of the cows, a diseased cow or from a human carrier. The incidence of salmonellosis from manufactured milk products remains low, although S. typhimurium or S. typhi infection in the past resulted from consumption of several different types of cheeses. Collins et al. (1968) reported the recent outbreak of 29 cases of S. newbrunswick associated with nonfat dry milk.

Other animal species: Household animals, chiefly dogs and cats, sometimes have salmonellosis but most often they are asymptomatic carriers (Day et al. 1963, Smith, 1959; Mackel et al., 1952). Animal offal or by-products used as protein supplements in their foods also may carry the infection. Other animal species such as rats and mice (Caldwell et al., 1966), turtles (Lowenstein et al., 1971), parrot family, rodents and insects (Welch et al., 1941), were found to be a serious cause of symptomless infections.

Processed foods and miscellaneous products: Some Salmonella outbreaks have been attributed to processed foods such as coconut (Wilson and Mackenzie, 1955), dried yeast (Elliot, 1966; Lennington, 1967), cotton seed protein (McCall et al., 1966), food coloring, carmine dye (Lang et al., 1967), and

chocolate (Lennington, 1967; Handzel, 1974). Other commodities in which Salmonella have been reported include thyroid, pancreatin, pepsin and liver powders (Guill, 1967), flour (Thompson, 1953), sugar and spices (Bartram, 1966), soya flour (Edwards, 1965), and bacterial enzyme drain cleaners (Lennington, 1968), sandwiches (McCroan et al., 1964), fish and fish products (Appelman et al., 1964). Finally, modern sewage treatment plants often discharge more salmonella-laden effluent into the waterways than can be attributed to industrial waste or sewage outlets (Harvey et al., 1969).

II. The Chemical and Microbiological Aspects of Chocolate Process

There is a great deal of published information on manufacturing process for chocolate products (Minifie, 1970; Lees and Jackson, 1973). The manufacture of chocolate involves the grinding of nib to produce chocolate liquor and the subsequent mixing of this with sugar, milk solid, etc. A flow sheet of the process is shown in Fig. 1.

1. Ingredients

Cocoa beans develop in the pods of the cacao tree (Theobroma cacao). The raw cocoa beans are fermented to facilitate development of flavor precursors and to remove the adhering pulp from the beans. Howat (1968) postulated that the chemical and biological changes that take place during fermentation are associated with enzymatic action, yeast fermentation, and oxidation and condensation resulting in the elimination of the

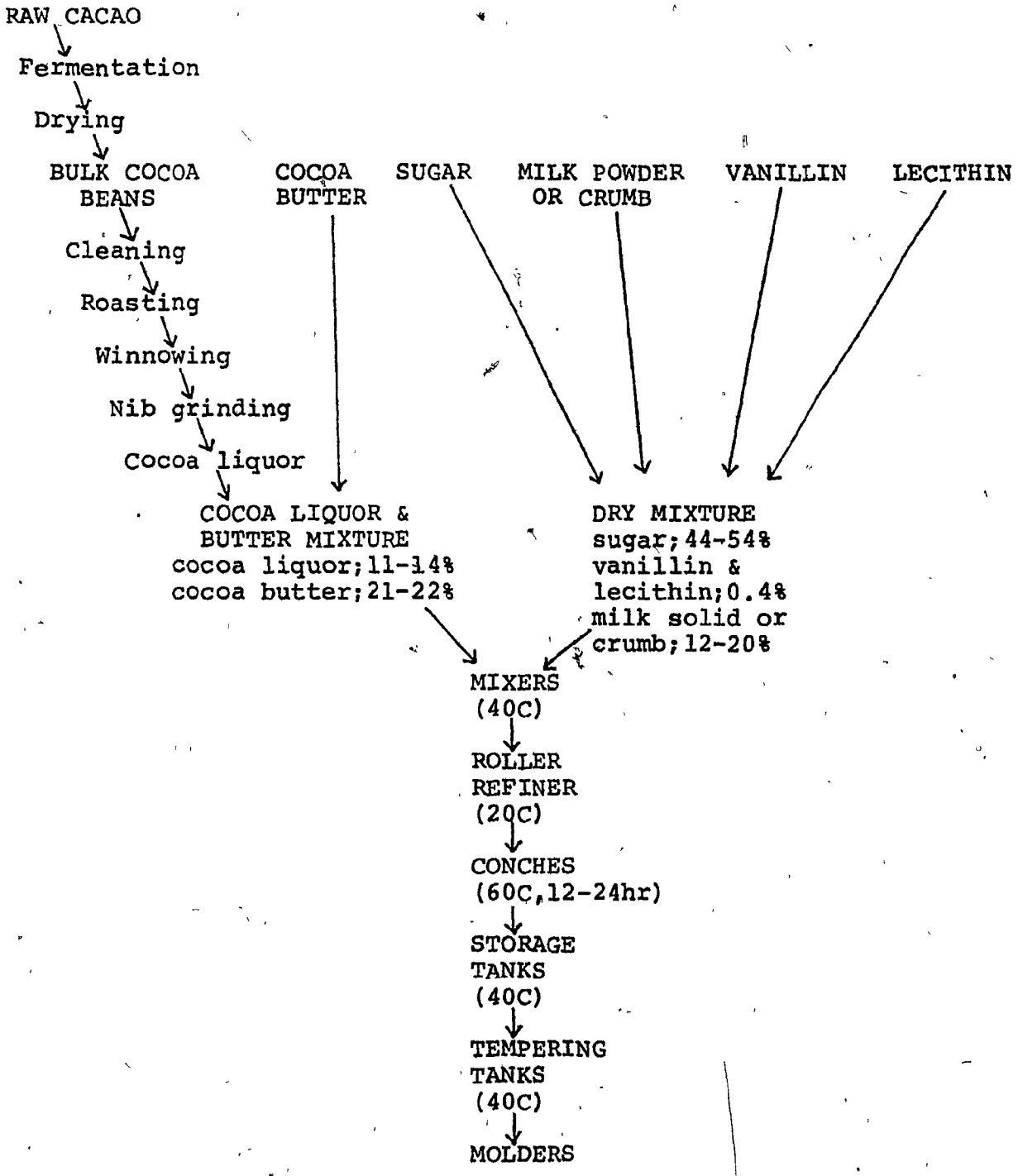


Fig. 1. Chocolate Processing Operations

bitter taste. Bracco et al. (1969) discussed the hydrolytic phenomena which could explain variations in the contents of nitrogen compounds, phenolic substances and carbohydrates of the cocoa bean. Forsyth and Quesnel (1963) showed that the predominant microflora at the start of fermentation are yeasts, which are then replaced by lactic acid bacteria, acetic acid bacteria, and finally spore-forming bacilli. Quesnel (1965) also observed that, with the breakdown of pigment cells, there is a destruction of the germinating power of the seed, partly by the high temperatures, and by the increased quantities of acetic acid formed in the pulp. The fermented beans are then dried approximately to 6% moisture. The dried beans are expected to carry a large number of bacteria of various types. Howat (1968) and Minifie (1970) stated that any possible health hazards arising from the proliferation of Salmonella and other bacteria are likely to be eliminated during roasting of the beans at 105 to 150°C. Barrile et al. (1971) observed, however, that the initial total counts per gram of bean ranged 3×10^5 to 4.7×10^7 and after roasting at 150°C for 40 min., the counts ranged between $10^3 - 10^5$ per gram of bean. Roasting of cocoa beans at 165°C and 180°C for 30 minutes led to an extreme bactericidal effect but these temperatures imparted scorched flavor to the beans. Some work (Rombouts, 1952; Gabis et al., 1970; Barille, 1971; Ostovar, 1972) has been done on the identification of the microorganism in raw cocoa beans. They

found that the majority of microorganisms in unroasted beans belonged to the genus Bacillus, Streptococcus, Micrococcus, E. coli, Flavobacterium and Microbacterium. Roasted beans contained organisms such as B. stearothermophilus and B. coagulans.

The winnowing process is necessary to separate the shell from the coteledon which breaks up into segments (nibs). It has been suggested that the winnowing operation could be a source of contamination if care is not taken to keep the machinery reasonably clean. The temperature achieved during grinding of the nibs does not eliminate contamination.

Ingredients such as sugar or sugar syrups do not present bacteriological problems. Nuts, desiccated coconut and, to a lesser extent, dried fruit such as raisins have been known to create special problems. Minifie (1970) and Doolin (1972) pointed out that desiccated coconut has been known to contain Salmonella, chiefly Salmonella senftenberg. This has been attributed to the very poor sanitary conditions in some of the factories in the tropics. The authors also noted the presence of aflatoxin in peanuts arising from molds growing on the kernels caused by poor harvesting, drying and storage conditions. Cross-contamination from unheated material has been known to have occurred in chocolate factories due to the use of equipment (bunkers, trays, conveyers) for the raw and processed beans. Dried fruits such as raisins and cherries

are used extensively in both moulded chocolate and assorted chocolate. It is known that the bacteriological contamination can arise due to the transfer of Salmonella or other pathogen from the hands or clothes of workers to the products.

Recently, milk powder was considered a greater potential source of Salmonella contamination than of the other basic ingredients of milk chocolate. Collins et al. (1968) described outbreaks of salmonellosis caused by dried milk and Armstrong et al. (1970) described similar Salmonella outbreaks which were attributed to an imitation ice cream. Although milk products present some bacteriological hazard to manufacturers, most pathogenic bacteria are likely to be eliminated by the various heat treatments that are applied to milk. Anon (1968) stated that the contamination of chocolate crumb can be more readily controlled if it is formulated in the factory than if the crumb is imported.

2. Processing operations

It is generally agreed (Foster, 1968; Goepfert and Biggie, 1968; Barrile and Cone, 1970; Rieschel and Schenkel, 1971) that the process involved in chocolate manufacture does not destroy Salmonella once the product has been contaminated; there is not sufficient heat involved in the process to sterilize chocolate since Salmonella is very resistant to heat under the dry conditions found in chocolate. The conching process is primarily designed to aid in the development of flavor and in the removal of undesirable volatile acid residues

that result from the fermentation and other processes. Conching is usually done at 40 - 52°C for milk chocolate and 60 - 70°C for dark chocolate. Minifie (1970) stated that higher temperature could be used to give caramelized flavours in milk chocolate and to give a slightly burnt flavour in dark chocolate. It was suggested that milk chocolate, which contains a lower proportion of nibs, should be conched at a lower temperature to prevent thickening of the chocolate. Minifie (1970) postulated that thickening of milk chocolate during conching is due to chemical changes of the milk protein. Tumermar and Webb (1965) reported that heat treatment could partially dephosphorylate casein, denature whey proteins, initiate protein-lactose interactions, increase acidity and alter the ionic equilibrium. Any of these alterations can conceivably influence the subsequent physical and chemical behavior of milk proteins. The precise effect of heat on milk and the chocolate ingredients as well as the ingredients in foods in general is difficult to predict with certainty. The traditional longitudinal conche is being replaced by rotary conches which handle larger quantities of chocolate and use higher temperatures. Lees and Jackson (1973) discussed recently variations in conching procedures, including a process involving circulation bed systems, where chocolate is continuously passing through a heated pipe and subjected to ultrasonic conching. Ultrasonics are used to develop a certain motion in the chocolate which leads to the exposure of

new surfaces of the chocolate to the air. Other processes, developed by the British Food Manufacturing Industry Research Association, achieve complete roasting and conching within a 30 minute period (140 - 160°C).

Vaeck (1960) suggested that there are four different forms, γ(17C, M.P), α(21-24C), β'(27-29C), β(34-35C), of crystalline cocoa butter in chocolate. The β crystal is the only stable form and the purpose of tempering is to crystallize chocolate in that stable form which is necessary to ensure a long shelf-life. Chocolate is usually heated to 48.8°C at which temperature all the cocoa butter becomes liquid; the melted chocolate is then cooled to 26.6°C where crystallizing takes place to produce both the stable and unstable forms of fat. On heating to 31 - 32°C, all the unstable crystals are remelted except the stable β- crystal. A defect known as bloom is known to be caused by using improperly tempered chocolate, improper cooling methods, contamination with incompatible fats, and fluctuating storage temperatures. Moisture conditions can also cause fat blooming.

III. The Specific Problem of Salmonella in Chocolate

1. The incidence of Salmonella infection in chocolate

Before 1966, there had not been reported a single case of Salmonella infection in confectionary products; it has been reported to be present in ingredients of chocolate such as dried milk, coconut and carmine dye. Foster (1968) and

Depew (1968) stated that at least seven major chocolate producers encountered Almonella contamination in finished products and recalled the products from the market during 1966 to 1967. According to WHO (1969) and the Laboratory Centre for Disease Control (1974), chocolate products have been found previously to contain Salmonella and to be the cause of an outbreak (WHO, 1973) of an infection. Most recently, over 200 cases of Salmonella eastbourne infections in Canada and the United States were reported in the period of July, 1973 to April, 1974. An extensive investigation both by the Health Protection Branch (HPB) in Canada and by Centre Disease Control in the U.S.A. (CDC, vol. 23 (4) and 23 (9), 23 (10), 1974) showed that Christmas-wrapped chocolate items manufactured by one major chocolate company in Canada were the vehicle of the epidemic. The major group affected were children between 1 and 4 years of age and the number of affected males and females were nearly equal. It was reported that the chocolate contained approximately 2.5 cells (S. eastbourne) per gram and that Salmonella infection was the result of the ingestion of at least 100 cells. These numbers were found to be much lower than doses previously reported to have given rise to outbreaks of Salmonella infection. A survey of the plant revealed the presence of S. eastbourne in roasted cocoa beans, in the bean processing room, and in a variety of end products (CDC, 1974; D'Aoust et al., 1975; Handzel, 1974; Craven et al., 1975). The inspection also indicated that cross-

contamination between raw and roasted beans could cause re-contamination, after the roasting step.

2. Survival of Salmonella in chocolate

The manufacture of chocolate by this specific company started with roasting the beans at 125°C for 30 minutes, refining and grinding the beans into cocoa liquor which is then mixed with cocoa butter. The dry ingredients such as sugar, salt, and imported crumbs are then blended into the mass. According to D'Aoust et al. (1975), the conching conditions at 55 - 60°C for less than 5 hours appeared to be ineffective in the destruction of Salmonella. The moisture content of the chocolate was also less than 1% which could act as a protective environment from heat.

It has been established that the thermal resistance of Salmonella is 600 to 700 times higher in dried egg white than in liquid egg white; a one week storage period at 50°C was required to destroy Salmonella in dried egg (Rasmussen et al., 1964). The authors noted that S. senftenberg 775 W inoculated into meat and bone meal was killed in 15 min. at 68°C; a heating period of 30 minutes at 82°C was required to eliminate Salmonella from naturally contaminated meal.

A similar situation seemed to exist with chocolate products. Previous studies by Goepfert and Biggie (1968), Foster (1968), Barrile and Cone (1970) and Rieschel and Schenkel (1971) have shown that several Salmonella serotypes, such as S. anatum, S. senftenberg 775 W, and S. typhimurium,

became heat resistant in chocolate; this suggests that chocolate acts as a protective environment against thermal damage to bacteria. Goepfert and Biggie (1968) found that heating milk chocolate at 70°C for seven hours and thirteen hours was required to kill 90% of S. senftenberg and S. typhimurium, respectively. Rieschel and Schenkel (1971) also reported that 40 hours of conching (72°C) was required to kill 90% of S. enteritidis and S. typhimurium in chocolate. Barrile and Cone (1970) studied the heat resistance of S. anatum in milk chocolate as a function of added moistured content and found that S. anatum was much more susceptible to heat in the presence of trace water. The D value, (a heating time to kill 90%) at 71°C was reduced from 20 hrs. to 8 hrs. by the addition of 1% water and to four hours by the addition of 2% to milk chocolate. Barrile et al. (1970) made further studies on high temperature (127°C) short time (HTST) sterilization, on a pilot plant scale. They observed that chemical and physical change took place in the milk chocolate before complete destruction of Salmonella even at temperature as low as 90°C. They suggested that the milk solids, especially the milk proteins, may be the sensitive component since chocolate liquor alone was not destabilized at the same temperature. It is also clear from the relevant work on the thermal destruction of Salmonella that the organism in chocolate cannot be eliminated by methods which are most often employed to destroy Salmonella in foods. On the other hand, Foster (1968) and Rieschel and

Schenkel (1971) stated that the possibility of Salmonella growth in milk chocolate and other confectionary products are negligible because of their low moisture content. Foster (1968), Barrile et al. (1970), Rieschel and Schenkel (1971), Dockstader & Gromes (1971) and Tamminga et al. (1976) studied the effect of long term storage on the survival of Salmonella in milk chocolate and found that Salmonella can survive in chocolate for quite long periods of time. Riechel and Schenkel (1971) detected Salmonella in milk chocolate even after 15 months storage at room temperature. Dockstader and Gromes (1971) also detected S. anatum and S. newington in contaminated milk chocolate after a four year storage period. Barrile et al. (1970) studied the effect of storage on the survival of S. anatum in milk chocolate at room temperature. The results of this study showed a gradual decrease (74%) in numbers of survivors but Salmonella was still discovered after 15 months storage, even with low initial numbers. Most recently, Tamminga et al. (1976) measured the rate of destruction of S. eastbourne and S. typhimurium in milk and bitter chocolate at room temperature. They showed that there was a rapid destruction of S. eastbourne and S. typhimurium but both types survived a nine months storage period. S. typhimurium was less resistant than S. eastbourne and both died off more rapidly in bitter chocolate than in milk chocolate.

IV. Detection and Identification of Salmonella in Foods

The International Committee on Microbiological Specifications for Foods (ICMSF) was formed in 1962 to establish internationally acceptable methods of analysis and to reach agreement on the essential supporting methods. Thatcher and Clark (1968) and ICMSF (1974) published books on methods of enumeration and methods of sampling. The methods outlined in the U.S. F.D.A.'s Bacteriological Analytical Manual (Olson , 1972) and the Association of Official Analytical Chemists (Horwitz, 1970).

In general, the development of a rapid technique for detection of Salmonella that would be fully satisfactory for all foods and serotypes has not yet been achieved but there have been significant contributions in this field during the last few years. These methods are always subject to change and there is currently a great deal of technical work on improved methods for the detection of Salmonella and other microorganisms. All methods are similar in principle and embody the procedural steps of pre-enrichment, enrichment, plating on selective agars, and taxonomic determination of colonies suspected of being Salmonella. In general, pre-enrichment favors the rapid recovery of Salmonella from the physiologically inactive state in foods that have been subjected to heat desiccation, preservation, freezing, high osmotic pressure and change in pH. The complexity of identification procedures is due to the fact that Salmonella in foods originate through faecal contamination which

is usually associated with other Enterobacteria such as E. coli, Proteus citobacter, Shigellae. The methods are designed to favour the multiplication of Salmonella and restrict the growth of other microorganisms.

Fantasia et al. (1969) and Sperber and Deibel (1969) described the Enrichment Serology method for detection of Salmonella in food, feeds and pharmaceutical products. This method is similar to cultural methods (BAM), except that each selective enrichment broths are inoculated into M broth and the pooled poly H antisera are used for agglutination. Non-motile Salmonella variants are not detected and thus the sensitivity and reliability of this method as a screening test has not been accepted. The fluorescent antibody method has been used to detect Salmonella; it is a rapid, economical screening test for large numbers of samples. The application of this technique was first used for examination of milk by Arkhomgel'skii and Kartoshova (1962). Georgala and Boothroyd (1964) also employed the technique to detect Salmonella in raw meat and indicated that the method had great promise. The fluorescent antibody technique was also found suitable for the detection of Salmonella in egg products (Silliker et al., 1966), animal feeds (Laramore and Moritz, 1969) and non-fat dry milk (Reamer and Hargrove, 1972). A simplified fluorescent antibody, employing the immunofluorescent microscope has also been reported by Markovits and Burboeck (1971). A modified radiometric technique has been used recently as a rapid method to detect

Salmonella in foods by Previte (1972), Waters (1972) and Wekell and Martinsen (1975). The radiometric technique is a measure of the quantity of $^{14}CO_2$ produced by the total microflora present. These studies suggested that radiometric techniques can be applied directly to detect bacteria in dairy products only when high numbers of bacteria are present. More recently, Dockstader and Gromes (1971) suggested a more rapid and sensitive method to detect low levels of contamination by Salmonella in chocolate which involved the use of synthetic fabrics. The technique was used to concentrate the Salmonella cells to a level that was detectable by fluorescent antibody examination.

V. Control of Salmonella in Foods

1. General control measures

The control of Salmonella in foods presents a difficult and serious problem to the food processor since Salmonella are widely distributed in nature and have a very wide range of hosts including domestic meats and pet animals. The problem is further complicated by the intrinsic difficulties associated with the detection of Salmonella when present at low concentrations in foods. Prost and Riemann (1967) and Hobbs and Christian (1973) have described the control of Salmonella and other food poisoning organisms. Food plants attempt to avoid Salmonella contamination by good sanitation, careful control of milk and water supplies, improved methods of processing,

elimination of human carrier from plants, control of cross-contamination and multiplication of Salmonella in the equipment and raw materials. The literature on the occurrence and distribution of Salmonella is overwhelming. Bryan (1974) noted that the types of food which can cause salmonellosis are usually nonsterile processed products. Control of contamination must depend on a kill step involving sterilization, radiation or acidification, etc. Anon (1968) suggested that greater emphasis must be placed on the control of the raw materials which can be heated since the destruction of Salmonella in chocolate is difficult. The authors showed the importance of separating raw materials from finished or in-line products, the classifying of employees who handle finished goods and those who handle raw materials, controlling air, moisture, equipment and pests. Krauss (1972) and D'Aoust et al. (1975) discussed in some detail the problems and means of quality control in chocolate processing. The authors point out that primary consideration should be given to (a) the isolation of cocoa bean storage, and roasting areas, (b) increasing roasting, conching and holding temperatures. They also pointed out that it can be dangerous and expensive to rely only on the testing of end products.

2. Destruction of Salmonella by chemicals

Acidulants serve numerous purposes in modern food processing including the preservation of the growth of micro-organism. Microorganisms are sensitive to acid in varying

degrees; the acid produced by one type of organism often will inhibit multiplication of another type. Much of the preservative effect from acid is due directly to the hydrogen ion concentration and its destabilizing influence on proteins; acids, however, which produce the same pH are not always equally effective since the anions of certain acids also exert some effect (Nunheimer and Fabian, 1940). It is well recognized that acids enhance the lethality of heat and the degree of acidity, tolerable in foods from the standpoint of palatability, is never sufficient to ensure food sterility. Ayres (1966) showed that complete destruction of Salmonella in egg yolk adjusted at pH 4.5 with acetic acid, takes place in two hours at 40°C or four days at 15°C. Foster (1968) studied the interaction of several environmental factors such as pH, sucrose, and heat, which can be used to control Salmonella in any confectionary product. The Salmonella were not able to grow at pH 4.1 and at 30, 37, 43°C but they did grow at all three temperatures when the pH was raised to 4.6. He also showed that Salmonella grew in 40% sucrose at pH 4.0 but it did not grow at pH 6.0 and thus he could prevent growth either by increasing sucrose concentration to 45% or by reducing the pH slightly. Goepfert (1969) also studied the destruction of Salmonella by various food acidulants such as volatile fatty acids and obtained results which were similar to those reported by Ayres (1966). In addition he also showed that glucose, sucrose, and peptone at the 20% levels protected Salmonella

from the lethal effect of 0.5% acetic acid.

Many chemicals have been in use to kill microorganisms but most of these are not permitted in foods. Sorbic acid, sodium and calcium propionates, and nitrite are permitted in certain foods to control Salmonella. Antibiotics have also been used as food preservatives, feed additives, and therapeutic agents to control Salmonella (Prost and Riemann, 1967; Weiser et al., 1971). Investigators have used various chemicals such as hydrogen peroxide (Ayres and Slosberg, 1949; Naguib and Hussein, 1972; Chu et al., 1975), beta-propiolactone (Bruch and Koesterer, 1962), and ethylene oxide (Mayr and Kaemmerer, 1959) to control Salmonella.

3. Destruction of Salmonella by heat

It is well recognized that heat is the most reliable and universally applicable method of sterilization. Most mesophilic nonspore-forming bacteria may be killed by moist heat at 60C for 30 min. Staphylococcus aureus and Streptococcus faecalis, however, require an exposure time of 60 min at 60C. A temperature of 80C for 5 to 10 min destroys the vegetative forms of all bacteria, yeast and molds. Spores of Clostridium botulinim, which are very heat-resistant are destroyed in 4 min at 120C or 30 min at 100C. To ensure sterilization, a temperature of about 121C (15 lbs. steam) and an exposure time of 15 min is used. Sterilization by dry heat requires higher temperatures and a longer period of heating than does sterilization with steam. The three theories

most generally accepted concerning bacteria destruction by heat are that (a) heat inactivates vital enzymes, (b) the cells become intoxicated with their metabolic waste products, or (c) certain destructive changes take place in the physical state of essential lipids (Lammann & Mallette, 1953). It has been well documented that nonspore-forming Salmonella are highly sensitive to moist heat and that the usual method for milk pasteurization is sufficient to kill even large numbers of Salmonella cells. Ng et al. (1969) reported that different serotypes of Salmonella do not vary much in their heat resistance except S. senftenberg 775 W. They found that D values of 0.9 to 1.3 min at 57°C for most of the Salmonella that were tested. Ng (1966) reported that D values for Salmonella are 1 to 2 sec at 65.6°C in whole milk, 0.3 min at 65.6°C in whole egg, and 0.5 min at 60°C in egg yolk. Bergquest (1961) showed that dry heat treatment of dried egg white at 54.4°C for seven days is effective in destroying Salmonella. Garibaldi (1966) and Riemann (1966) discussed the mechanism of thermal destruction of Salmonella in a defined system. They observed that amino acids, peptides, RNA and traces of calcium and magnesium leak out of the cells during heat treatment. In recent years, many workers observed that the heat resistance of Salmonella increases as the water activity (a_w) of the medium decreases. Goepfert (1968), Barrile and Cone (1970) and Goepfert et al. (1970) studied the relationship between water activity and heat resistance of

Salmonella; they found that the thermal resistance of Salmonella increased as the water activity of the heating medium was reduced.

It is a well known fact that freezing temperatures have germicidal properties; cold is not, however, an effective means of destroying bacteria in foods. Repeated freezing and thawing are much more destructive than prolonged storage at freezing temperature but these methods have not proved to be successful in destroying microorganisms. Weiser et al. (1971) showed that Salmonella typhi could be frozen in liquid air at -182 to -190°C for 20 hours without destroying the viability of these organisms. If bacteria are frozen rapidly to temperatures below -35°C, ice crystals which form within the cell, produce a lethal effect during defreezing. If cultures are dried in vacuo from the frozen state, by lyophilization, the initial viability is greatly enhanced.

4. Destruction of microorganisms by ultrasonics

There are many reports in the literature on the application of ultrasonic energy in the food industry for emulsification, bacterial destruction including sterilization of liquid foods and improvement of flavor or odour (Johnson, 1974). Considerable work has been done on the use of ultrasonic with dairy products, especially with milk. Beckwith and Weaver (1936) studied the effects of ultrasonic on the destruction of bacteria in milk under various conditions. A process for sterilizing milk by ultrasonics has been patented

recently in Britain (Brit. Pat. 1, 17982, 1970) and U.S.S.R. (USSR Pat. 438406, 1974). Haevecker (1970) described the application of ultrasonics in certain areas of the fruit processing industry. Khadzhiiski (1970) used ultrasonics in the wine industry to accelerate maturation and for sterilization. Avakyan (1972, 1974) studied the continuous cold sterilization of wine by ultrasonics and by UV irradiation. It is known that microorganisms differed markedly in their sensitivity to ultrasonics. The most susceptible are gram-negative rods such as Salmonella and E. coli and among the most resistant are the Staphylococci.

5. Destruction of Salmonella by radiation

a. Ionizing irradiation

X-rays, microwaves, infra-rays, UV light and ionizing radiations are electromagnetic. The ionizing radiation such as X-rays, gamma-rays, and cathode rays (electrons) are of great practical value for purpose of sterilization, because of their great penetrating power. Gamma-irradiation produced by Cobalt 60 has been applied to the destruction of Salmonella in foods and other products which are especially damaged by heat treatment (IAEA, Vienna, 1963-1973). Gamma irradiation has been used successfully in the processing of meat, fish products, vegetables and spices; milk and milk products, however, appeared to be extremely sensitive to ionizing irradiations as it is indicated by the changes in flavor which are associated with the use of this type of irradiation. Comer et al. (1963) and

Lineweaver (1966) used gamma irradiation for the pasteurization of egg products. Grundewaid (1973) studied the use of gamma-irradiation for the control of pests in chocolate products. Organoleptic tests showed that chocolate coating was much more sensitive to the irradiation than was the filling material. The application of cathode ray (electron beams or beta radiation) has also been used for the sterilization of dairy and other food products. Proctor et al. (1953) and Nickerson et al. (1957) indicated that the use of cathode irradiation is feasible to destroy Salmonella, especially S. typhimurium and S. senftenberg in liquid, frozen egg white and egg powder.

b. Ultraviolet irradiation

The effects of UV irradiation on microorganisms have been studied extensively since the lethal action of sunlight on certain bacteria was first demonstrated by Dowes and Blunt as early as 1877. Since the introduction of more efficient sources of germicidal UV energy at 2537 A (Rentschler et al., 1941), numerous quantitative studies have been performed to establish the relationship between wavelength, energy and type of organism that are affected by the radiation. Kelner (1949), Setlow et al. (1963), and Howard-Flanders (1968) have suggested that the main consequence of UV irradiation of microorganisms is the formation of a pyrimidine dimer between adjacent thymines on opposite strands of DNA; these changes are believed to be the cause of most of the lethal and mutagenic effects. The killed state did not necessary represent, however,

irreversible destruction of viability. The damaging effects by UV irradiation could be reversed in some strains of bacteria by (1) photoreactivating enzyme which splits pyrimidine dimers, thus restoring the normal DNA structure (Kelner, 1949; Novick and Szilard, 1949; Wulff and Rupert, 1962) by (2) excision repair enzymes which occur in the dark subsequent to UV irradiation (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) and by (3) post-replication (recombination) repair mechanism, which is the most recently described and the least understood of the repair mechanisms (Rupp & Howard-Flanders, 1968). Excision repair appeared to be the primary cause of an enhanced resistance to UV irradiation by some strains of bacteria. The detailed studies on enzymatic repair mechanisms by UV irradiation have been reviewed by Witkin (1969).

Although it has been well established that the energy of UV irradiation is low and its power of penetration is very poor, the possible applications of UV irradiation have been reported for controlling various pathogen and spoilage microorganisms in drinking water (Huff et al., 1965; Muller et al., 1972), milk (Supplee, 1930; Burton, 1951; Werner et al., 1972), eggs (Ijichi et al., 1964), meats and poultry (Reagan et al., 1973; Yndestad et al., 1972; Mudretsova-viss and Zavyalova, 1975) and wine (Avakyan, 1972, 1974). Among the many other applications of UV irradiation are the 1) treatment of cider to improve keeping quality, 2) treatment of maple sap,

3) treatment of brines in cheese making to control growth of microorganisms, 4) control of microorganisms in sugar refineries and cheese ripening rooms, and 5) control fungi and bacteria in cold storerooms and in laboratories, particularly in the fermentation industries (Johnson, 1974). The primary application of UV irradiation is in the control of airborne infection, where it is used for disinfecting enclosed areas, such as hospital wards and operating rooms. Burton (1951) demonstrated that 99% of the bacteria in milk could be destroyed by UV irradiation but no commercialization of the process has been made, due to the poor keeping quality of the treated milk. Stoutz (1969) proposed a method for the treatment of milk which involved successive exposures of that milk to IR radiation (2 - 10 sec). An Australian patent (Aquitron Corp., 1969) described a process for the sterilization of milk by UV irradiation using UV permeable pipelines in which the milk is exposed to UV continuously.

Benesi (1956) and Oppenheimer et al. (1959) developed a method in which thin films of fluids can be exposed to UV radiation; the process is now used for the sterilization of plasma and vaccines. Curran and Tamsma (1960) used the same technique for the sterilization of whole milk but without complete success. Werner et al. (1972) described a process for the sterilization of milk in which very thin films of milk are exposed to UV irradiation; the exposed milk had a vitamin D potency of 400 - 500 IU/l. Ijichi et al. (1964) studied the

effect of UV irradiation on the destruction of Salmonella in thin films of egg white (liquid). They found that UV irradiation (7.22×10^4 ergs/cm/sec) reduced Salmonella (typhimurium and senftenberg) by a factor of 10^6 to 10^7. It has been shown that UV irradiation having wavelengths which are shorter than 200 nm give rise to ozone formation; ozone has a pungent smell and a strong oxidizing action on the fats which are present in foodstuffs (Nilson, 1963). Weiser et al. (1971) noted that UV irradiation can lead to the destruction of thiamine, ascorbic acid, riboflavin, niacin, other vitamins, in addition to glutathione, many endogenous enzymes, and natural antioxidants in fats.

MATERIALS AND METHODS

I. Bacterial Strains

The culture of S. eastbourne was supplied by Comet Chocolate Ltd., St. Hyacinthe, Quebec, Canada. Other serotypes, e.g. S. alachua S-1041, S. anatum S-515, S. montevideo 75-1806, S. senftenberg 75-1450, S. typhimurium 75-1794, S. infantis MCC-605, and S. tennessee MCC-621, were obtained from the Health Protection Branch (Health and Welfare, Canada) and from Macdonald College (Culture Collection Centre).

II. Media

Unless otherwise stated, the media constituents and other chemicals that were used in these studies were of reagent grade or were Difco products.

III. Maintenance of Cultures

Stock cultures were stored at 4°C on tryptic soy agar slants and were subcultured monthly. These stock cultures were also maintained in sterile skim milk (freeze-dried). Working cultures were transferred daily in tryptic soy broth and incubated for 24 hours at 35°C. A loopful of the broth culture was streaked on tryptic soy agar and brilliant green agar plates which were then incubated at 35°C for 24 hours and again stored at 4°C.

IV. Source of Chocolate

A manufacturer of milk chocolate supplied the chocolate used in this study. It contained cocoa liquor (11-14%), milk solid or crumb (11-20%), sugar (44-54%), cocoa butter (21-22%), lecithin (0.4%). Milk crumb contained whole dry milk powder (80-85%), sugar (10-15%) and cocoa powder (3%).

V. Detection and Identification of Salmonella

Salmonella was detected in chocolate by the method outlined in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (Olson, 1972). Larger samples (50 g) were used, however, instead of the 25 g sample prescribed in the manual; in addition 10 ml of pre-enrichment in 90 ml of enrichment broths (selenite cystine) were used instead of 1 ml of preenrichment in 10 ml of enrichment broths. Presumptive Salmonella cultures, based on the biochemical reactions on Triple Sugar Iron (TSI) and Lysine Iron agar were next tested with Bacto-salmonella antisera.

VI. Inoculation of Chocolate with Salmonella

Salmonella cells resulting from 24 hr growth (35°C) in tryptic soy broth (500 ml) were harvested centrifugally and then resuspended in sterile skim milk (20 ml) and lyophilized. The lyophilized cultures were dispersed (VirTis 45 mixer, 5000 rpm, 5 min) in Salmonella free, molten milk chocolate (45°C) to

give viable counts of approximately 300 per plate.

VII. Enumeration of Salmonella in Chocolate

Three separate samples (10 g or 100 g) of chocolate were blended (VirTis 45 mixer; 2000 rpm) for two minutes with reconstituted skim milk (10%) to give a final concentration of 1 gm of chocolate/ml. One ml of the blended sample was added to 9 ml of selenite-cystine broth. For direct plate count, 0.1 ml samples of appropriate dilutions were surface plated on brilliant green agar (3 different plates) using a sterile bent glass-rod and a turntable spreader (Fisher). The plates were incubated at 35°C for 24 hours and the colonies were counted by use of a Bacterial Colony Counter (Fisher Scientific Co., N.Y.). When the number of Salmonella was too low to permit the use of the plating technique, viable Salmonella was enumerated by the three tube Most Probable Number (MPN) technique.

EXPERIMENTAL

I. Detection and Identification of Salmonella in Chocolate

1. Sensitivity of the methods (Bacteriological Analytical Manuals and Direct Plating Count) for detection of Salmonella in chocolate

It is generally accepted that the method for the detection of Salmonella in foods, described in the Standard Bacteriological Analytical Manual (BAM; Olson,1972) is slow, cumbersome and expensive (Thatcher and Clark, 1968). It has been customary to specify that pathogen such as Salmonella should be absent from foods but the International Commission on Microbiological Specification for Foods (ICMF, 1974) stated that no feasible sampling plan can ensure complete absence of a particular organism (i.e., zero tolerance) and also it is not yet commercially possible to market some food without the risk of some pathogens being present. Any improvement in the methods of detection of pathogens, which could help the manufacturer in assuring that his product and its ingredients are free of these organisms, would be highly desirable. The sensitivity of a method is rarely known precisely for a given food because changes in the nature of the food or in its microbial ecology can modify the sensitivity of the method (Thatcher and Clark, 1968).

Experiments were performed to determine the level of Salmonella which could be detected by the standard BAM methods

with certain changes (Figure 2). The predetermined num[illegible] of viable S. eastbourne in peptone solution (0.1%) was dispersed in chocolate (50 g; 45°C). The 50 g sample was then blended (VirTis 45; 2 min; 2000 rpm) in reconstituted skim milk (10 g skim milk powder/90 ml water/2 ml brilliant green solution). Table 1 summarized the results of three separate experiments on the detection (BAM and direct plating methods) of Salmonella in an artificially contaminated chocolate. Based on the results in Table 1, it would appear that it is possible to detect 20-30 Salmonella cells per 50 g of chocolate by the direct plate count technique. The lower level (2 - 3 cells) of Salmonella was not detected by this method before pre-enrichment growth for 24 hr, (Stage 1). The results in Table 1 also show that most S. eastbourne at the level of above 2-3 cells could be detected by the modified BAM method after pre-enrichment growth in reconstituted skim milk. The chocolate sample in experiment 3 was contaminated with Salmonella before S. eastbourne was incorporated into the chocolate; this was confirmed by an independent laboratory.

2. Identification of Salmonella in milk chocolate

Fig. 3a and 3b showed the results of presumptive Salmonella detection before serological testing and Table 2 summarized the results of identification in commercial milk chocolate by a method given in Figure 2. Various serotypes of Salmonella have been detected and identified in certain samples of chocolate obtained from a commercial laboratory during the

Place 50 g chocolate in 100 ml skim milk and brilliant green mixture* (solution A).

↓

Stage 1 Blend and inoculate into solution A and incubate for 24 hrs at 35°C.

↓

Stage 2 10 ml of the inoculated solution A was added to 90 ml of selenite cystine and tetrathionate broths; incubate for 24 hrs at 35°C.

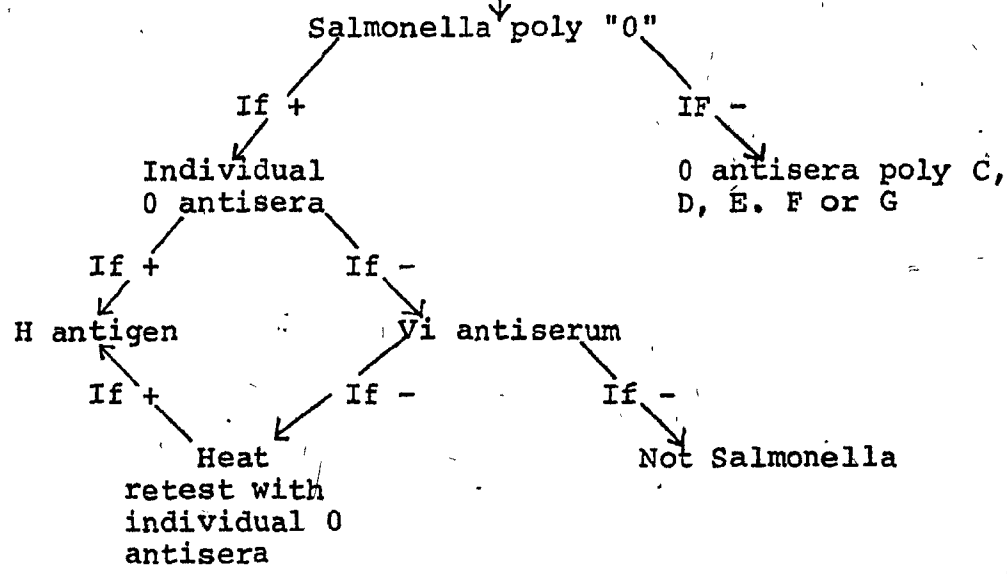
↓

Stage 3 Streak on selective differential plates (BG,SS,BS) and incubate for 24 hrs at 35°C.

↓

Stab and streak on TSI and LIA plates and incubate for 24 hrs at 35°C.

↓



*10 g skim milk powder/90 ml water; 2 ml brilliant green solution was added to the 98 ml of the reconstituted skim milk.

Figure 2. Schematic Representation of Analytical Method.

Table 1

Detection of *S. eastbourne* in Artificially Contaminated Chocolate

Expt.	Stage of Culture (see Fig.2)	No. of cells contaminated/50 g chocolate			
		0	2-3	20-30	200-300
1	0*	-	-	+	+
	Stage 1	-	+	+	+
	Stage 2	-	+	+	+
	Stage 3	-	+	+	+
2	0*	-	-	+	+
	Stage 1	-	+	+	+
	Stage 2	-	+	+	+
	Stage 3	-	+	+	+
3	0*	+	+	+	+
	Stage 1	+	+	+	+
	Stage 2	+	+	+	+
	Stage 3	+	+	+	+

* Immediately after homogenizing of the chocolate in reconstituted skim milk, 0.1 ml of blended mixture was plated directly on each of four agar (brilliant green) plates and incubated for 24 hrs at 35°C.

+, - indicates presence and absence of Salmonella.

Figure 3a. Detection of Presumptive Salmonella on Selective Differential Plates; Brilliant Green (1), Bismuth Sulfite (2) and Salmonella-Shigella (3)

Figure 3b. Detection of Presumptive Salmonella on Triple Sugar Iron (TSI) Agars; Control (1), Yellow(acid) Slant with H_2S (2), Yellow (acid) Slants without H_2S(3), and Red (alkaline) Slants without H_2S(4).

Figure 3a.

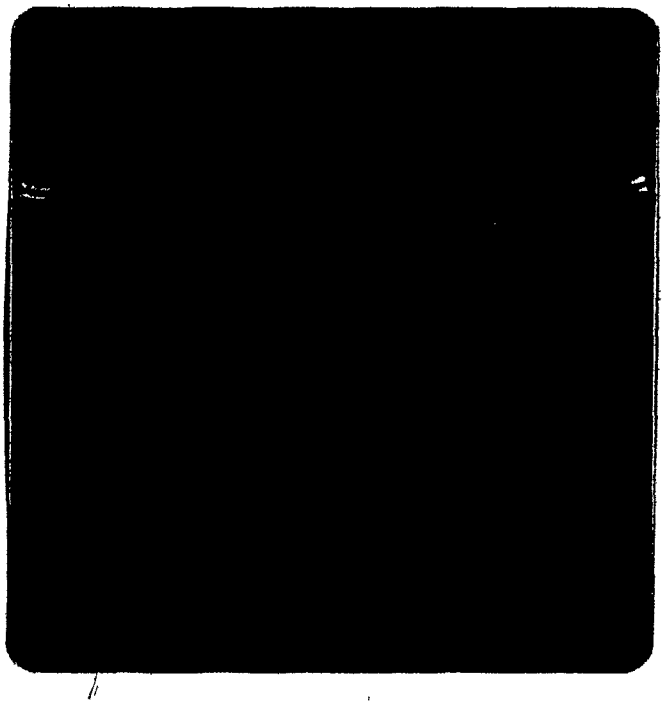


Figure 3b.

Table 2

Identification of Salmonella in Milk Chocolate by Cultural (BAM) and Serological Tests

Sample Number	Group	O antigen	H antigen*		Identification
			Specific	Nonspecific	
1	E_1	3,10	z_4, z_{23}	(1,7)	S.adabraka
2	C_1	6, 7	r	1,2	S.virchow
3	C_1	6, 7	r	1,6	S.nigeria
4	C_1	6, 7	r	1,5	S.infantis
5	C_1	6, 7	r	1,7	S.colindale
6	C_1	6, 7	r	1,5	S.infantis
7	C_1	6, 7	r	1,5	S.infantis
8	B	4, 5	r	1,5	S.bradford
9	E_1	3,10	e,h	1,6	S.anatum
10	E_1	3,10	e,h	1,2	S.vejle
11	E_1	3,10	e,h	1,5	S.muenster
12	E_1	3,10	e,h	1,7	S.nyborg

* Confirmed both by Spicer-Edward H and flagella H antisera tube test.

period of February, 1975-January, 1976. S. eastbourne, which caused a serious human infection in 1974, was not detected in the samples.

3. Variability of direct plate count method

Experiments were performed with peptone solution (0.1%) and chocolate containing S. eastbourne to determine the efficiency of the plating technique. A suspension (0.1 ml) of S. eastbourne culture was diluted with peptone solution (0.1%) and then was plated directly on brilliant green agar plates, prepared in advance and dried for 18 hr at room temperature. A sample of Salmonella contaminated chocolate was prepared by mixing freeze-drying cells (20 mg) with milk chocolate (3000 g) by means of a Waring blender. A sample (10 g or 25 g) contaminated chocolate was homogenized (VirTis 45) with reconstituted skim milk (90 ml or 75 ml) for 2 min at 2000 rpm. The blended mixture (0.1 ml) was plated on each of five brilliant green agar plates and the number of Salmonella cells were counted as described previously (page 34). Table 3 lists the number of the analysis (5 replicates) of three samples of the peptone suspension of Salmonella cells and three samples of the contaminated chocolate. The Coefficient of Variation* varied approximately 3.2 to 8.2% for the peptone solution and 3.8 to 6.3% for the contaminated chocolate. The selectivity of brilliant green agar decreased gradually as the prepared plates were stored

* $C.V. = \frac{S.D.}{\text{Amount Present}} \times 100$

Table 3

Variability of Direct Plate Count Method for the Determination of Viable Count of S. eastbourne in Peptone Solution and in Chocolate

Replicate	Count/Plate[a]			Count/Plate[b]		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
1	200	182	139	255	542	496
2	221	188	142	245	510	498
3	203	190	120	289	492	445
4	194	199	120	256	532	449
5	203	190	126	263	510	463
Mean	204	190	129	262	517	470
S.D	10.1	6.1	10.5	16.6	19.8	25.3

[a] Plates prepared by using 0.1 ml of peptone solution containing S. eastbourne.

[b] Plates prepared by using 0.1 ml of reconstituted skim milk containing S. eastbourne contaminated chocolate.

at room temperature. The plates were, therefore, used within 24 hrs when they were stored at room temperature and within seven days when they were stored at 5°C as suggested by Thatcher and Clark (1968).

II. The Destruction of Salmonella by Heat

1. The destruction of S. eastbourne in milk chocolate stored at 5°C.

The effect of long term storage (room temperature) on the destruction of Salmonella in chocolate was studied by Barrile et al. (1970), Rieschel and Schenkel (1971), Dockstader and Gromes (1971) and Tamminga et al. (1976). In general, the authors agree that the initial population of Salmonella decreases gradually as the length of the storage period increases but that Salmonella is likely to survive for long periods of time in chocolate stored at room temperature, in spite of the low water activity (a_w) of the chocolate. Experiments were performed to investigate the fate of S. eastbourne in milk chocolate during refrigerated storage at 5°C. Tempered chocolate (10 g) was inoculated with S. eastbourne suspension (0.1 ml; 3.5×10^7 cells) in sterile petri-dish (35 x 10 mm) and were stored at 5°C. Number of survivors was determined (page 34) at 2-day intervals for the first 10 days after six months and one year storage periods. The results in Table 4 show that the loss of viable S. eastbourne was negligible during the first two days of storage but counts were reduced to approximately 1 log cycle during the six day storage period. The reduction in viable S. eastbourne during 12 months was approximately 2 logs.

2. The destruction of S. eastbourne in sucrose solution (54%) at 57°C

It is generally recognized that the heat resistance of Salmonella in aqueous suspensions can be enhanced greatly by the addition of sucrose which results in the reduction of water

Table 4

The Destruction of S. eastbourne in Milk Chocolate Stored at 5°C

Storage (days)	Number/g	% kill
0	5.8×10^5	0
2	5.2×10^5	10
4	1.6×10^5	73
6	2.6×10^4	96
8	2.6×10^4	96
10	2.8×10^4	95
180	9.8×10^3	98
360	7.8×10^3	99

activity (Goepfert et al., 1970; Gibson, 1973; Corry, 1974). As milk chocolate has a high content of sucrose (44-54%), it was deemed important to investigate the effect of this compound on the heat resistance of Salmonella. A cell suspension (0.5 ml; 0.1% peptone solution; O.D = 0.09, 660 nm) was added to a sucrose solution (4.5 ml; 60%) contained in a capped test tube (150 x 15 mm). The sucrose solution had been previously heated to 57°C. During the heating period, samples (0.5 ml) were withdrawn and were added to 4.5 ml of diluent. Suitable dilutions were made and the solutions (0.1 ml) were surface plated on duplicate plates of brilliant green

agar and tryptic soy agar. It may be seen from the results reported in Table 5 that a heat period of 35 min was required to kill completely S. eastbourne cells in a suspension containing 1.4×10^8 cells/ml. The Decimal Reduction Time (D Value), which is the heating time required to kill 90% (or to reduce the cells by 1 log cycle), was approximately 6.9 min. A comparison of the results of the present experiment with those obtained by other workers (Goepfert et al., 1970) indicate that S. eastbourne is one of the most sensitive groups that have been studied. To check this point, seven strains of Salmonella were tested for heat resistance using similar cell suspensions (54% sucrose solution). The log 10 of the number of survivors was plotted as a function of time. The

Table 5

The Destruction of S. eastbourne in Sucrose Solution (54%) at 57°C

Temp(min)	Number/ml	% kill	D Value(min)
0	1.4×10^8	0.00	
5	2.0×10^7	85.70	6.8
10	2.8×10^6	98.00	
15	3.3×10^5	99.80	6.5
20	5.5×10^4	99.96	
25	1.3×10^4	99.98	7.4
30	2.3 x 103	99.99	
35	0	100.00	Ave. 6.9

survivor curves were extrapolated to obtain the D value. The results in Fig. 4 show that S. eastbourne and S. infantis were the most heat sensitive group; they had D values of 6.2 and 7.3 min respectively. S. alachua, S. typhimurium, S. montevideo, and S. tennessee had D values which were intermediate between that of the sensitive group (S. eastbourne; S. infantis) and the resistant S. senftenberg. S. senftenberg was shown to be by far the most resistant and this is in agreement with the results reported in the literature. Although S. anatum is generally regarded as being heat resistant, in our laboratory, however, it was shown to be fairly heat sensitive (D value; 6.7). On the other hand, S. anatum was not completely killed after 60 min at 57°C and it seemed to multiply in 54% sucrose containing trace amounts of peptone broth after an incubation period of 70 min at 57°C (Table 6). The recoveries

Table 6

The Destruction of S. anatum by Heating (57°C) in Sucrose Solution (54%)

Time	B.G agar		Tryptic soy agar	
	Number/ml	% kill	Number/ml	% kill
0	1.7×10^6	0	5.0×10^7	0
10	2.3×10^5	98.7	9.0×10^5	98.2
20	1.3×10^4	99.7	3.5×10^4	99.4
30	3.8×10^4	99.98	5.1×10^3	99.99
40	4.6×10^2	99.999	9.9×10^2	99.998
50	1.6×10^2	99.9991	3.1×10^2	99.9995
60	9.0×10^1	99.9995	1.5×10^2	99.9997
70	1.9×10^3	99.89	8.8×10^2	99.998

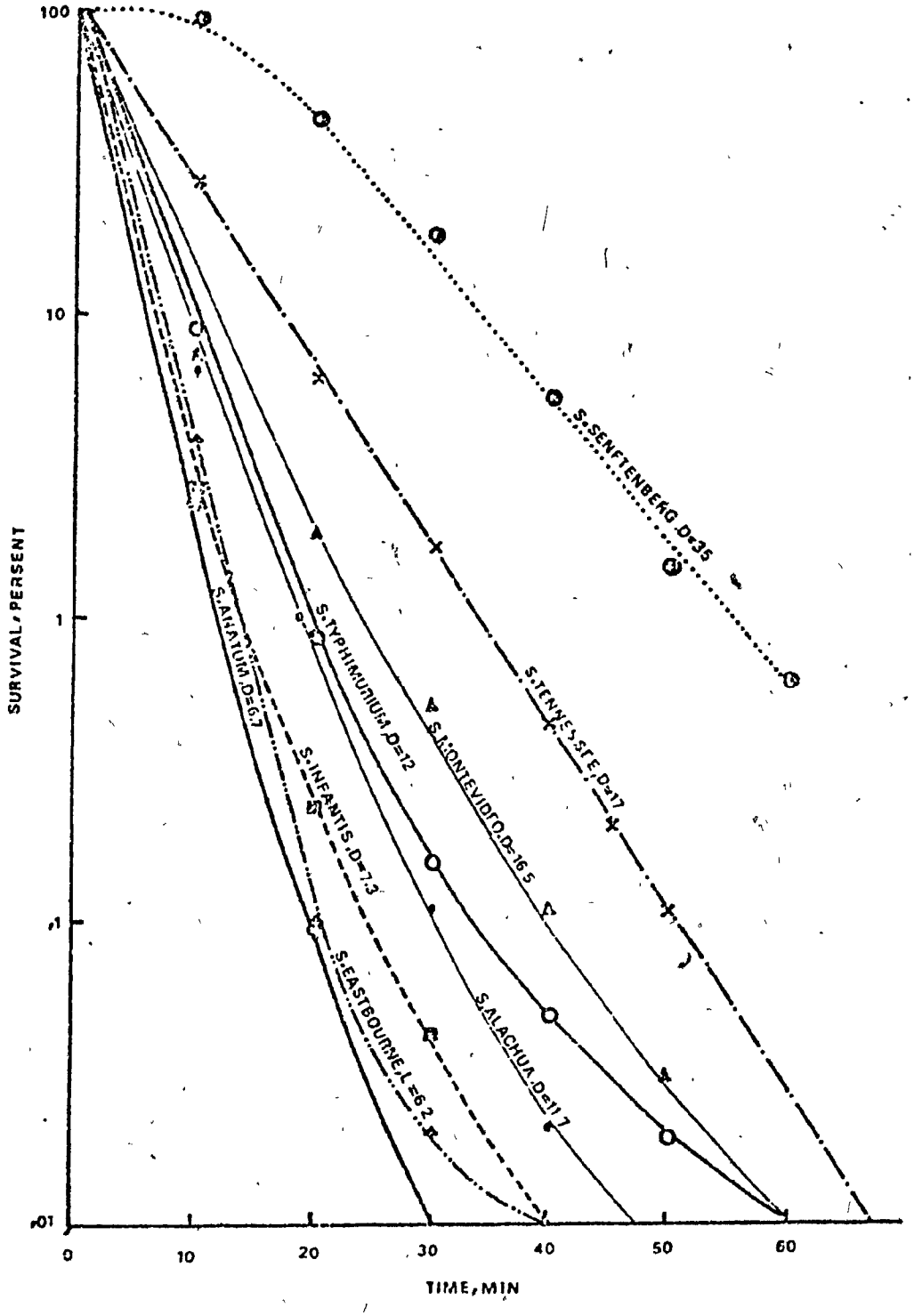


Figure 4. The Destruction of Different Serotypes of Salmonella in Sucrose Solution (54%) at 57°C.

of S. anatum were also different when the treated organism was incubated in two different media, e.g. brilliant green agar and tryptic soy agar. S. eastbourne did not exhibit this difference in recovering in the two media.

3. The destruction of S. eastbourne, S. senftenberg and S. typhimurium in milk chocolate heated at 71°C

Earlier investigations indicated that several Salmonella strains, such as S. anatum, S. senftenberg and S. typhimurium are extremely difficult to destroy in milk chocolate; this suggested that chocolate exerted a protective action against the destruction of the cells by dry heat (Goepfert and Biggie, 1968; Barrile and Cone, 1970; Barrile et al., 1970; Rieschel and Schenkel, 1971). Tamminga et al. (1976) assumed that S. eastbourne could have higher D value than most other serotypes of Salmonella, although they did not observe the heat resistance of S. eastbourne in milk chocolate. To check this point, 10 mg of lyophilized cells (S. eastbourne; S. senftenberg; S. typhimurium) were blended (VirTis 45 homogenizer; 5000 rpm; 10 min) into molten chocolate (200 g). Samples (10 g) of the contaminated chocolate were placed in flasks which were then stoppered (No. 9 rubber) and heated in a water bath (71°C). Samples were withdrawn at various time intervals and the number of cells that survived was determined both by direct plate count and most probable number technique as described previously (page 34).

The results in Table 7 showed that the D value of

Table 7

The Destruction of S. eastbourne, S. senftenberg and S. typhimurium in Milk Chocolate at 71°C.

Time (hrs)	S. eastbourne		S. senftenberg		S. typhimurium	
	No./g[a]	% kill	No./g	% kill	No./g	% kill
0	4.4×10^4	0	1.4×10^4	0	5.0×10^4	0
2	1.2×10^4	72.7	3.7×10^3	73.6	2.5×10^4	50.0
4	6.9×10^3	84.3	2.4×10^3	82.9	1.0×10^4	80.0
6	1.7×10^3	96.1	6.2×10^2	95.6	6.0×10^3	88.0
8	8.7×10^2	98.1	3.0×10^2	97.9	3.0×10^3	94.0
10	2.6×10^2	99.4	8.0×10^1	99.4	1.2×10^3	98.6
12	9.0×10^1	99.8	0	100.0	8.0×10^2	99.4
24	0	100.0	0	100.0	NT[b]	-
D value (hr)		4.5		4.6		6.6

[a] Viable counts based on direct plate count for 0 to 10 hrs and MPN value for 12 and 24 hrs.

[b] Not tested.

S. eastbourne was 4.5 hr, which is very close to the D value (4.6 hr) of S. senftenberg. It will be noted that a heat treatment of 12 hours at 71°C was required to kill completely S. senftenberg cells (1.4×10^4 cells/g) dispersed in milk chocolate. S. eastbourne cells in the chocolate (4.4×10^4 cells/g) were not completely destroyed after 12 hrs at 71°C. S. typhumurium

(D value; 6.6 hr) was more resistant than S. senftenberg.

III. The destruction of Salmonella by Ultrasonics

1. The destruction of S. eastbourne and S. anatum in peptone solution (0.1%) and in milk chocolate

There are many reports in the literature on the destruction of microorganisms including sterilization of liquid foods such as milk (Beckwith and Weaver, 1936; Brit. Pat. 1, 17982, 1970; U.S.S.R. Pat. 438406, 1974), and wine (Avakyan, 1972 and 1974) by ultrasonics. A new ultrasonic conching process has recently been developed where ultrasonics are used to develop a certain motion in the chocolate (Lees and Jackson, 1973). In a preliminary experiment designed to study the destruction of Salmonella in aqueous suspension by ultrasonics, a suspension of Salmonella cells (50 ml; OD = 0.09, 660 nm; 5°C) in peptone solution (0.1%) was exposed for various periods of time (2 - 30 min) to ultrasonic vibration (Braunsonic 1510; 100 watt; Braunsonic, N.J., U.S.A.). The result in Table 8 showed that 99.7 to 99.99% of the cells were destroyed after 10 min exposure. There were slight differences in resistance between S. eastbourne (D, 3.0 min) and S. anatum D, 2.1 min). S.anatum showed a tailing effect after 4 min treatment of ultrasonics. It was observed that a considerable amount of heat (43°C; 10 min) was generated in the suspension during the ultrasonic treatment. The experiment was repeated in a suspension of Salmonella (25 ml; 5°C) using a probe before and

Table 8

The Destruction of S. eastbourne and S. anatum in Peptone Solution (0.1%; 50 ml) by Ultrasonics[a]

Time (min)	S. eastbourne		S. anatum	
	Number/ml[b]	% kill	Number/ml	% kill
0	6.3×10^6	0	5.0×10^7	0
2	1.0×10^6	84.0	2.0×10^5	99.6
4	5.5×10^5	92.3	1.5×10^4	99.87
6	1.0×10^5	98.4	9.0×10^3	99.98
8	4.0×10^4	99.4	7.0×10^3	99.986
10	2.0×10^4	99.7	5.5×10^3	99.99
20	2.9×10^2	99.99	7.5×10^2	99.999
30	1.3×10^2	99.998	2.5×10^2	99.9995

[a] Braunsonic ultrasonic generator, model 1510; 100 watt; Braunsonic, N.J., U.S.A.

b Direct plate count method as described on page 34.

after the end of the probe had been covered with a layer (0.5 cm) of silicon polymer. The result in Table 9 showed that over 99.999% of the cells were destroyed after 10 min exposure before the end of the probe had been covered with silicon but only 90% of the cells were killed after the end of the probe had been covered with silicon. The experiment was repeated using a dispersion (VirTis 45; 5000 rpm; 10 min) of S. eastbourne cells in milk chocolate and the end of the probe which was not covered with silicon. Table 10 shows that only about 26% of S. eastbourne cells were destroyed by a 10 min exposure

Table 9

The Destruction of S. eastbourne in Peptone Solution (0.1%; 25 ml) by Ultrasonics[a]

Time (min)	Probe without silicon		Probe with silicon[b]	
	Number/ml	% kill	Number/ml	% kill
0	8.3×10^7	0	4.6×10^7	0
2	3.0×10^6	99.64	3.9×10^7	15
4	7.1×10^5	99.87	1.2×10^7	74
6	1.1×10^4	99.99	6.8×10^6	85
8	1.1×10^3	99.999	5.5×10^6	89
10	2.0×10^2	99.9999	4.7×10^6	90

[a] Braunsonic 1510 (250 watt).

[b] End of probe was covered with a layer (0.5 cm) of silicon polymer.

Table 10

The Destruction of S. eastbourne in Milk Chocolate by Ultrasonics*

Time (min)	Number/g of chocolate	% kill
0	1.9×10^5	0
10	1.4×10^5	26
20	9.0×10^4	53
30	5.0×10^4	74

* Braunsonic 1510 (100 watt; 42°C)

to ultrasonics. No change in the flavor of the chocolate was obtained by a panel of five after the chocolate had been exposed to the ultrasonic treatment.

IV. The Destruction of Salmonella by UV Irradiation

1. Preliminary experiments

Thin disks (ca. 1 mm) of milk chocolate were prepared by use of a hand microtome. The disks were placed over Salmonella deposited on tryptic soy agar plates. The plates were then subjected to UV irradiation for 5 to 10 min using Sylvania germicidal lamp with reflector (G15 T8, 15 watt, General Electric, U.S.A.). The lamp was placed at a distance of 18 cm from the agar plate; this gave an intensity of approximately 12×10^3 erg/cm^2/sec. The UV irradiated and nonirradiated plates were then incubated (35°C) for 24 hrs. An inspection of the plates (Fig. 5) indicated that S. eastbourne cells on the agar surface were completely destroyed by 5 min exposure to UV irradiation at room temperature. No detectable destruction of S. eastbourne was noted on the section of the plates that was covered by the thin layer of the chocolate.

Previous workers have shown that UV irradiation can destroy microorganisms in buffered cell suspension, water (Huff et al., 1965; Muller et al., 1972), milk (Werner et al., 1972), wine (Avakyan, 1972 and 1974), egg white and whole egg (Ijichi et al., 1964), and plasma (Oppenheimer et al., 1959). Prior to an investigation of the penetration of UV irradiation into a

Figure 5. The Destruction of S. eastbourne in Thin Disks of Chocolate by UV Irradiation.

1. Nonirradiated Control Plate
2. Irradiated Tryptic Soy Agar Plate

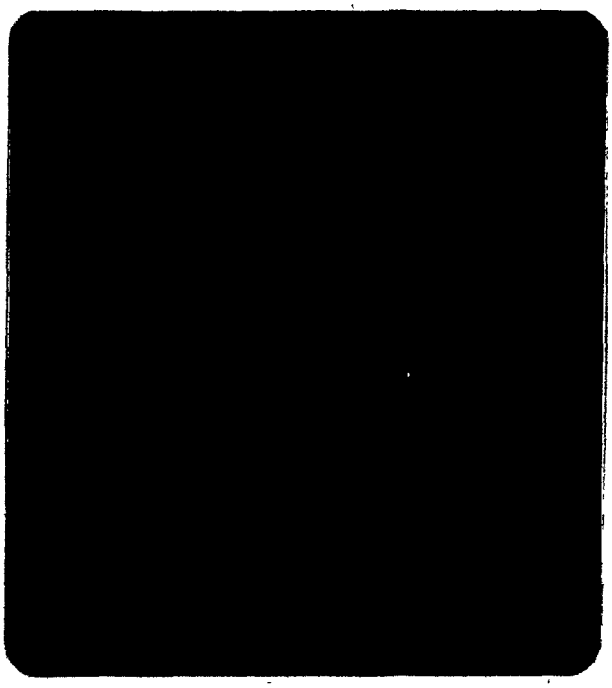


Figure 5

chocolate film, experiments were performed to find the efficiency of killing in peptone solution (0.1%). Different volumes (0.45, 0.9, 1.8 ml) of a suspension (0.1% peptone solution; OD = 0.09 at 660 nm) of S. eastbourne cells were placed on petri-dishes (35 x 10 mm); this gave layers of liquid of 0.5, 1.0 and 2.0 mm in thickness. These thin layers of cell suspension were exposed to UV irradiation (12 x 10^3 erg/cm^2/sec, measured by Black-Ray UV meter, UV Products, Inc., Model No. J225, San Gabriel, Calif.). Samples of the appropriate dilutions of the UV irradiated suspensions were plated on brilliant green agar and the number of colonies were counted after an incubation (35°C) period of 24 hrs.

Table 11 and Fig. 6 showed that the UV irradiation killed over 99.999% of S. eastbourne in 1 to 2 min. The efficiency of destruction was more pronounced in the 0.5 mm layer and survivors were virtually negligible after 3 - 4 min to the UV irradiation. No survivors were detected in any of the films (0.5, 1.0 and 2.0 mm) after a 5 min exposure to the UV irradiation by the direct plate count technique.

2. UV penetration of chocolate films

The apparatus, which was constructed for studying UV penetration of thin layers of chocolate, consisted of steel disks (3-½" diam. x 1" thickness) which had circular depressions (0.1 mm, 0.5 mm, 1.0 mm depth x 1.89" width) and a thermometer well that permits the precise measurement of the temperature of the chocolate layer. The steel block was heated to the

Table 11

Survival of S. eastbourne in Peptone Solution (0.1%) Exposed to UV Irradiation (12×10^3 erg/cm^2/sec) in Different Layers

Exposure Time (min)	Thickness	Number/ml	% Survivor
0	0.5	1.4×10^8	100
	1.0	1.4×10^8	100
	2.0	1.4×10^8	100
1	0.5	1.5×10^3	1.1×10^{-3}
	1.0	0.8×10^3	1.3×10^{-3}
	2.0	9.0×10^2	6.0×10^{-4}
2	0.5	1.7×10^2	1.7×10^{-4}
	1.0	7.2×10^2	5.0×10^{-4}
	2.0	8.1×10^2	6.0×10^{-4}
3	0.5	5.0×10^1	4.0×10^{-5}
	1.0	1.1×10^2	8.0×10^{-5}
	2.0	2.1×10^2	1.5×10^{-4}
4	0.5	2.0×10^1	1.4×10^{-5}
	1.0	1.2×10^2	8.0×10^{-5}
	2.0	1.5×10^2	1.0×10^{-4}
5	0.5	0	Negative
	1.0	0	"
	2.0	0	"

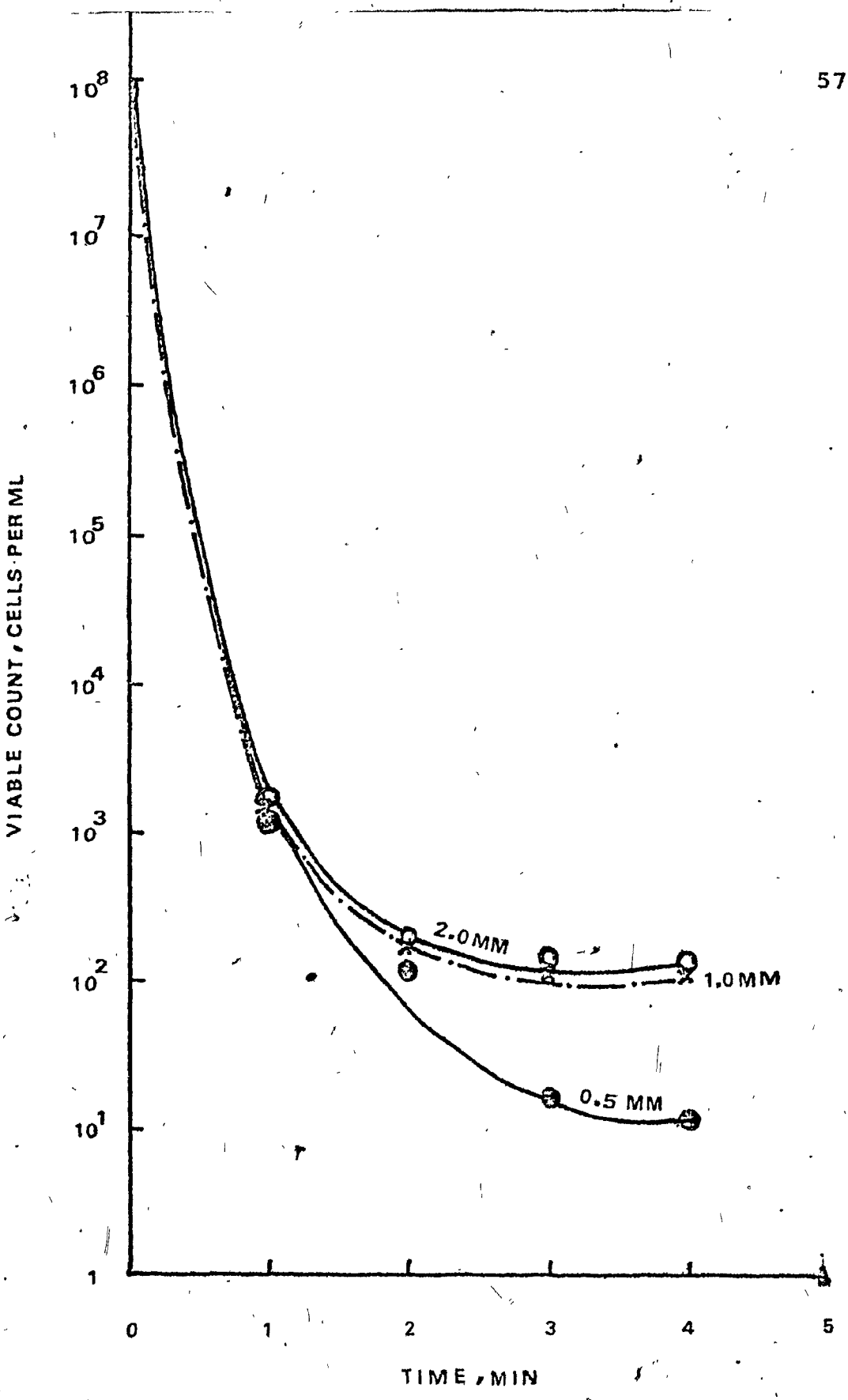


Figure 6. The Destruction of S. eastbourne in Peptone Solution (0.1%) by UV Irradiation (Film thickness; 0.5 mm, 1.0 mm, 2.0 mm).

desired temperature (45° C) by means of an electric heater (Corning, U.S.A.) and then portions (0.1 ml) of S. eastbourne suspensions (24 hr culture, 0.09 OD at 660 nm) in peptone solution (0.1%) were dried at the bottom of the depression of the steel disk (Fig. 7a).

A chocolate layer was placed over the thin layer of cells and the excess was removed by means of a microtome knife (No. 7120-E Blade Handle, Scientific Apparatus, A.H. Thomas Co., Pa., U.S.A.). The chocolate layer was then exposed to UV irradiation. At the end of the exposure period, the chocolate and the cells at the bottom of the depression were removed by use of 50 ml brilliant green skim milk (10% w/v) and a Pasteur transfer pipette (dispo. P5211, Canlab). The volume of the rinsings was adjusted to 100 ml with brilliant green skim milk and the resultant mixture was then blended in a homogenizer (VirTis 45, Gardiner, N.Y.) for 2 min at 2000 rpm (Fig. 7b). Portions (0.1 ml) of the blended sample were surface plated immediately on brilliant green agar plates which were then incubated at 35°C for 24 hrs. Survivors of Salmonella were counted by means of Bacterial Colony Counter (Fisher Scientific Co.). Both the irradiated and nonirradiated control samples were subjected to the same procedure. The experiment was performed in a room which was illuminated with yellow light (G.E. Gold fluorescent bulbs). Table 12 summarized the results of this study. It will be noted that S. eastbourne cells, which were covered with an 0.1 mm layer of

Figure 7a. Apparatus for Studies on Penetration of UV into Chocolate; a Chocolate Layer on a Circular Depression (0.1 mm depth x 1.89" width) of the Steel Disk (3.5" diam. x 1" thickness).

Figure 7b. A Blended Mixture of Chocolate and Reconstituted Skim Milk (B.G.) in VirTis Homogenizer before Salmonella Analysis.

Figure 7a.

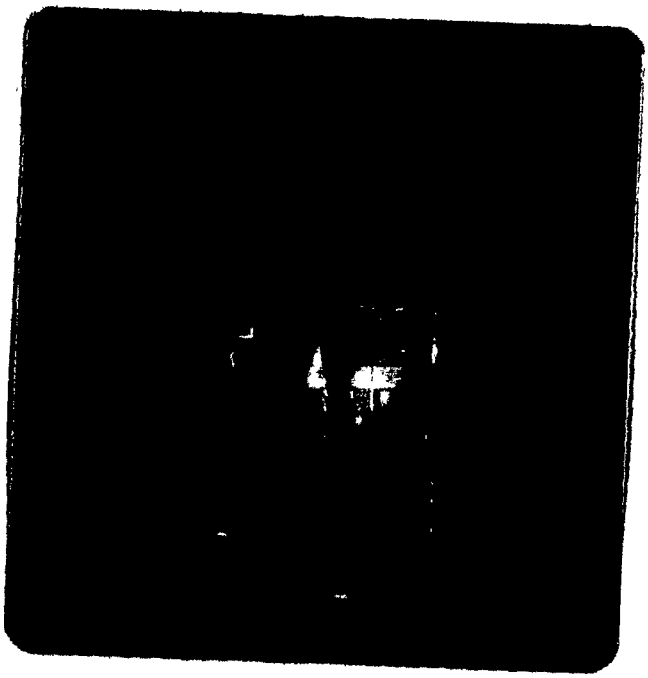


Figure 7b.

chocolate, were completely destroyed after 1.5 min exposure to UV irradiation ($10x10^3 erg/cm^2/sec$) at 45C (Fig. 8). Ten min were required to kill 75% of the cells when the chocolate film was 0.5 mm and no detectable destruction was noted when the film thickness was greater than 0.5 mm. As the data reported in Table 12 which were obtained by the direct plate count method indicated, it was reasonable to assume that survivors fewer than 20 per 100 ml may not be detected by direct plating technique because of inherent error in the relatively small size of plating. Physiologically inactive cells may also not be directly recovered on selective brilliant green agar. Therefore, more detail studies were made on the effect of preenrichment and enrichment growth on the survival of S. eastbourne both by direct plate count and most probable number techniques.

The experiment with the 0.1 mm film of chocolate as reported in Table 12 was repeated. In this experiment, however, the mixture of exposed chocolate and cells, which was removed from the steel disk by means of brilliant green skim milk, was subjected to a preenrichment and enrichment growth; portions (0.1 ml) of blended mixture were surface plated on triplicate brilliant green agar plates and each of blended mixture (10 ml) in test tubes (150 x 15 mm) were then incubated at 35C for 24 hrs. The number of colonies on brilliant green agar was counted after 24 hrs at 35C and portions (1 ml) of a preenrichment culture, which had not shown growth at 35 C for 24 hrs,

Figure 8. The Destruction of S. eastbourne in an 0.1 mm Layer of Chocolate by UV Irradiation.

1. Colonies of S. eastbourne on Brilliant Green Agar after Plating 0.1 ml Portion of Original Non-irradiated Sample. Actual Counting was done after 1/10 Dilution of the Original Sample.
2. Absence of Salmonella Showing Destruction on Brilliant Green Agar in the UV Treated Mixture.

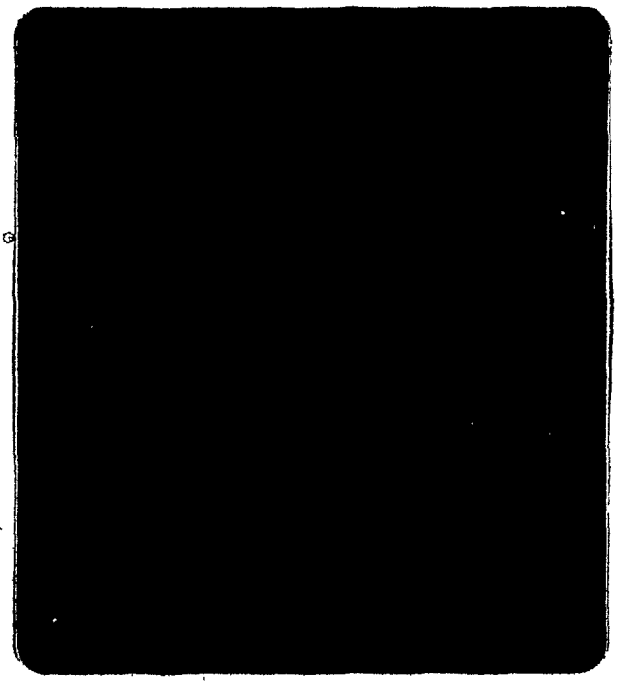


Figure 8.

Table 12

The Effect of Chocolate Thickness on Destruction of S. eastbourne by UV Irradiation.

Exposure time (min)	0.1 mm		0.5 mm		1.0 mm	
	No/ml*	% kill	No/ml	% kill	No/ml	% kill
0	1.9×10^5	0	9.2×10^4	0	1.5×10^5	0
0.5	4.6×10^4	76	9.1×10^4	0	1.7×10^5	0
1.0	7.0×10^3	97	9.3×10^4	0	1.5×10^5	0
1.5	0	100	9.6×10^4	0	1.6×10^5	0
2	0	100	1.1×10^5	0	1.7×10^5	0
4	0	100	7.4×10^4	20	1.7×10^5	0
6	0	100	6.8×10^4	26	1.6×10^5	0
8	0	100	2.0×10^4	78	-	-
10	0	100	2.3×10^4	75	1.5×10^5	0

* Mean number of survivors recovered on duplicate B.G. agar by direct plate count method as described on page 34.

Table 13

The Effect of Enrichment Growth on the Survival of S. eastbourne after UV Irradiation

Exposure time (min)	Count/ml[a]	MPN/ml[b]	% kill	
0	6.5×10^5		0	
2	1.4×10^4		97.8	
4	1.0×10^4		98.5	
6	Negative	0.23	>99.999	c
8	"	0.15	>99.999	
10	"	0.07	>99.999	

[a] Viable numbers of survivors on triplicate brilliant green agar by direct plate count.

[b] Survivors estimated by MPN technique using selenite cystine broth and plating B.G agar.
3 positives(3,0,0) reported as 23 per 100 ml.
2 positives(2,1,0) reported as 15 per 100 ml., etc.

[c] Based on the original cell number (6.5×10^5 cells/ml) by direct plate count method.

were reincubated in selenite cystine broth for MPN determination. The results in Table 13 show that S. eastbourne cells (6.5×10^5 cells/ml) under an 0.1 mm film of chocolate were not detected by direct plating technique after 6 min exposure to UV irradiation but approximately 0.001% fraction was recovered after the treated sample was subjected to a pre-enrichment and enrichment cultivation.

3. The effect of cell population on the destruction of Salmonella by UV irradiation

A series of experiments were performed to investigate some of the factors which might affect the efficiency of destruction of Salmonella by UV irradiation. In preliminary experiments, the effects of age and humidity of the cells on the sensitivity of Salmonella to UV irradiation were investigated. UV irradiation of S. eastbourne cells which were at different stage of growth (generation time: 30 min) in tryptic soy broth, indicated that the stationary phase of S. eastbourne is more resistant than that of the log phase. This is in agreement with the reports of other workers (Rentschler et al., 1941). Preliminary experiment also showed that dry cells are extremely

resistant to UV irradiation. The resistance of S. eastbourne cells to the irradiation was found, however, to be affected greatly by the cell density. A detailed study was therefore made on the effects of cell density and cell age on the efficiency of Salmonella destruction by UV irradiation.

Samples (0.1 ml) of S. eastbourne culture which had been incubated for 18 hrs (OD = 0.9) and 24 hrs (OD = 1.3) were plated on brilliant green agar plates after serial dilutions and were then exposed (10 to 120 sec; 12 x 10^3 erg/cm^2/sec) to UV irradiation at room temperature. The exposed plates were then incubated (35°C) for 24 hrs and the number of survivors was ascertained by direct plate count method as described on page 34. Table 14 illustrates that S. eastbourne cells on the agar plates are in general destroyed rapidly by UV irradiation when the concentration of cells was not too high. The results also show that the resistance of cell to UV irradiation depends on the age of the cells. For example, when the cell density was 2.0×10^6 cells/ml (18 hrs growth; OD = 0.9), the destruction was completed. On the other hand, when the cell density was 1.4×10^6 cells/ml in a somewhat older culture (24hrs growth; OD = 1.3), complete destruction was not achieved under the same condition of irradiation.

Further experiments were carried out in which Salmonella cells, which were beneath an 0.1 mm layer of chocolate, were exposed to UV irradiation. Number of survivors was determined by direct plate count method as described previously. The

Table 14

The Effect of Cell Population on the Destruction of S. eastbourne by UV Irradiation

Bacterial Dilution	Number/ml after UV treatment[a]					
	0	10 sec[b]	30 sec[b]	60 sec[b]	90 sec[b]	120 sec[b]
Expt. I						
0[c]	1.4×10^{9}	***	1.0×10^{4}	5.0×10^{3}	3.0×10^{2}	1.5×10^{2}
10^{-1}	1.4×10^{8}	***	1.0×10^{3}	8.0×10^{1}	0	0
10^{-2}	1.4×10^{7}	***	3.0×10^{1}	2.0×10^{1}	0	0
10^{-3}	1.4×10^{6}	**	0	0	0	0
10^{-4}	1.4×10^{5}	**	0	0	0	0
10^{-5}	1.4×10^{4}	1.2×10^{4}	0	0	0	0
10^{-6}	1.4×10^{3}*	1.4×10^{3}	0	0	0	0
Expt. II						
0[d]	2.0×10^{8}	1.1×10^{3}	3.5×10^{2}	1.3×10^{2}	7.0×10^{1}	5.0×10^{1}
10^{-1}	2.0×10^{7}	3.5×10^{2}	1.0×10^{2}	0	0	0
10^{-2}	2.0×10^{6}	0	0	0	0	0
10^{-3}	2.0×10^{5}	0	0	0	0	0
10^{-4}	2.0×10^{4}	0	0	0	0	0
10^{-5}	2.0×10^{3}	0	0	0	0	0
10^{-6}	2.0×10^{2}*	0	0	0	0	0

[a] Intensity of 12×10^{3} erg/cm^{2}/sec at room temperature.

[b] Period of UV treatment.

[c] 0.1 ml portions of S. eastbourne culture in tryptic soy broth (24 hrs incubation; OD = 1.3 at 660 nm) plated on B.G agar.

[d] 0.1 ml portions of S. eastbourne in tryptic soy broth (18 hrs incubation; OD = 0.9 at 660 nm) plated on B.G agar.

**** Massive growth.

** Slightly reduced growth

* Actual growth and other numbers at control estimated.

results in Table 15 indicate that S. eastbourne cells at a concentration of 2.2×10^5 cells/ml or less in both experiments were not detected after the chocolate layer and underlying cells had been irradiated for 5 min. When the cell density, however, was 4.1×10^5 cells/ml, the destruction under the given set of conditions was 99.3%.

4. The effect of UV irradiation on photoreactivation or excision repair of S. eastbourne and S. typhimurium

It has been established that the damaging effects by UV irradiation can be reversed in some strains of bacteria by enzymatic repair mechanisms such as photoreactivation (Kelner, 1949; Wulff and Rupert, 1962), excision repair (Setlow and Carrier, 1964) and Post replication (Rupp and Howard-Flanders, 1968).

Experiments were performed to investigate the photoreactivation and excision repair of two serotypes of Salmonella after UV irradiation. The experiment was carried out under white light, yellow light and in the dark; these conditions were the same as Singh (1975) in similar experiments. Samples (0.5 ml) of cell suspensions (0.1% peptone solution; OD = 0.09 at 660 nm) of S. eastbourne and S. typhimurium were exposed to UV

Table 15

The Effect of the Cells (S. eastbourne) under an 0.1 mm Layer of Chocolate on the Efficiency of Destruction by UV

Absorbance of Cell Culture[a]	Number/ml[b]		
	Nonirradiated	Irradiated	% kill
Expt. I			
0.13	1.7×10^5	0	100
0.09	8.5×10^4	0	100
0.05	1.5×10^4	0	100
0.01	0	0	0
Expt. II			
0.13	4.1×10^5	3.0×10^3	99.3
0.09	2.2×10^5	0	100
0.05	2.0×10^4	0	100
0.01	0	0	0

[a] Absorbance measured at 660 nm

[b] Based on direct plate count technique.

irradiation (19×10^3 erg/cm^2/sec) for 30 to 120 sec in sterile petri-dishes (35x10 mm). After the UV treatment, the samples (0.1 ml) were removed and diluted in peptone solution (0.1%). Samples (0.1 ml) of appropriate dilutions were spread on the surface of brilliant green agar plates. The plates were incubated at 35°C for 24 hrs and the colonies were counted; the mean of three replicates was used to calculate the number of

cells that survived. The results in Table 16 showed that no striking differences were observed in the recovery of S. eastbourne and S. typhimurium cells which were exposed to UV and then incubated in yellow and white light. S. eastbourne cells, however, showed some photorecovery in darkness after 120 sec exposure of UV; this was not observed with S. typhimurium. A pronounced tailing effect was observed in both types of cells under all conditions after 30 sec exposure to UV irradiation.

Another experiment was performed in given conditions on excision repair and tailing effect exhibited by S. eastbourne cell suspensions. Samples of appropriate dilution of S. eastbourne cell suspensions were surface plated on brilliant green agar and the plates were subjected to UV irradiation (12×10^3 erg/cm^2/sec) for 2 to 10 sec.

Table 17 showed that the tailing effect or repair mechanism, which was observed with the cell suspensions (0.1% peptone solution), was not observed with the irradiated agar plates. UV survival data of S. eastbourne on the surface of the agar was exponential, showing one hit mechanism. No definite conclusions can be drawn from the results of these experiments.

An additional experiment was carried out to investigate possible photoreactivation and excision repair. Portions (0.1 ml) of S. eastbourne suspensions (24 hrs culture, OD = 0.09 at 660 nm) in peptone solution (0.1%) were dried (45°C) at the bottom of the depression of the steel disk. A chocolate layer

Table 16

The effect of UV Irradiation on Photoreactivation or Excision Repair of S. eastbourne and S. typhimurium in Peptone Solution

Exposure time(sec)	Yellow[a] (No/ml)		White[b] (No/ml)		Darkness[c] (No/ml)	
	S.east.	S.typhi.	S.east.	S.typhi.	S.east.	S. typhi.
0	$1.1x10^7$	$9.7x10^7$	$1.1x10^8$	$9.7x10^7$	$1.1x10^8$	$9.7x10^7$
30	$9.0x10^3$	$1.1x10^4$	$1.7x10^4$	$1.5x10^4$	$1.2x10^4$	$1.1x10^4$
60	$3.6x10^3$	$6.1x10^3$	$2.0x10^3$	$9.5x10^3$	$4.6x10^3$	$8.9x10^3$
90	$2.1x10^3$	$2.5x10^3$	$2.9x10^3$	$4.7x10^3$	$5.3x10^3$	$5.2x10^3$
120	$4.8x10^3$	$1.1x10^2$	$2.3x10^3$	$5.4x10^2$	$1.2x10^4$	$2.8x10^3$

[a] G.E. gold fluorescent bulbs and 25 watt yellow light bulbs which do not emit at wavelength below 540 nm.

[b] 50 watt ordinary white light bulbs.

[c] 50 watt light bulbs wrapped in black clotch.

Table 17

The Effect of UV Irradiation on Photoreactivation or Excision Repair of S. eastbourne on the Surface of Brilliant Green Agar

Exposure time[a] (sec)	Yellow		White		Dark	
	Count[b]	% Survivor	Count	% Survivor	Count	% Survivor
0	580	100	580	100	580	100
2	47	7.2	56	9.7	40	6.9
4	2	0.3	3	0.5	1	0.2
6	0	0	0	0	0	0
8	0	0	0	0	0	0
10	0	0	0	0	0	0

[a] Intensity of $12x10^3 erg/cm^2 sec$.

[b] Mean number of colonies recovered on three replicate brilliant green agar plates.

was placed over the thin layer of cells and the excess was removed by means of a microtome knife. The chocolate layer was then exposed to UV irradiation under different illumination conditions. Number of survivors was recovered on three replicate B.G agar as described in previous method (page 34) under the condition of yellow, white and dark. The results in Table 18 showed that there were no observable differences in the recovery of UV exposed cells under the layer (0.1 mm) of chocolate which were incubated under the conditions of yellow, white and darkness.

In order to ensure that the destruction of Salmonella on the agar surface is due solely to UV irradiation or the production of inhibitor, brilliant green agar plates were irradiated before S. eastbourne cells were placed on the plates. Portions (0.1 ml) of S. eastbourne suspensions (24 hrs culture; OD = 0.09 at 660 nm) in peptone solution (0.1%) after serial dilutions were surface plated on brilliant green agar. Number of colonies were counted after 24 hrs at 35°C. The results in Table 19 showed that there was no difference in the assay of S. eastbourne cells on pre-irradiated and nonirradiated plates, which indicate that S. eastbourne destruction on the agar plate was solely the effect of UV irradiation rather than the effect of inhibitors which are formed during UV irradiation.

5. The effect of UV intensity on the destruction of S.eastbourne

Samples (0.5 ml) of a S. eastbourne suspension (0.1% peptone solution; 24 hrs culture) were placed on sterile petri-

Table 18

The Effect of UV Irradiation on Photoreactivation or Excision Repair of S. eastbourne under a Chocolate Layer (0.1 mm)

Exposure* time(sec)	Yellow		White		Dark	
	No/ml	% Survivor	No/ml	% Survivor	No/ml	% Survivor
0	$4.1x10^5$	100	$4.8x10^5$	100	$3.7x10^5$	100
30	$3.5x10^5$	85.4	$3.7x10^5$	77.0	$3.4x10^5$	91.8
69	$1.7x10^5$	41.5	$1.5x10^5$	31.3	$1.1x10^5$	29.7
90	$1.5x10^4$	3.7	-	-	-	-
120	$1.2x10^4$	2.9	$4.5x10^4$	9.4	$1.7x10^4$	4.6

* Intensity of $19x10^3 erg/cm^2/sec$.

Table 19

Number of Colonies on Irradiated and Nonirradiated ~~D.~~G Agar Plates

Exposure time*(min)	Count/Plate	
	1	2
0	200	205
2	180	200
4	180	185
6	210	190
8	240	230
10	245	200

* Intensity of $19x10^3 erg/cm^2/sec$.

Table 20

The Effect of UV Intensity on the Destruction of S. eastbourne

Distance* (cm)	Intensity (measured)	Number/ml	% Survivor
	Control	2.3×10^{7}	
36	4.8×10^{5}	1.6×10^{3}	7.0×10^{-3}
24	7.2×10^{5}	1.0×10^{3}	4.0×10^{-3}
12	1.3×10^{6}	5.5×10^{2}	2.4×10^{-3}
6.2	2.3×10^{6}	3.1×10^{2}	1.3×10^{-3}
0.5	3.6×10^{6}	1.5×10^{2}	6.5×10^{-4}

* Distance between source and sample.

Table 21

The Effect of Shortwave and Longwave UV Irradiation on Destruction of S. eastbourne

Exposure time (min)	Rayteck shortwave		Rayteck longwave	
	Number/ml	% kill	Number/ml	% kill
0	5.8×10^{8}	0	5.5×10^{8}	0
1	2.2×10^{7}	96	5.6×10^{8}	0
2	1.0×10^{7}	98	6.0×10^{8}	0
3	0	100	6.5×10^{8}	0
4	0	100	6.7×10^{8}	0

dishes and then exposed (2 min) to different intensity of UV irradiation. Portions (0.1 ml) of the treated sample were surface plated on B.G agar plates and number of survivors was counted after 24 hrs at 35°C. The different intensities were observed by varying the distance between the source and the sample. The intensity of the irradiation was measured by means of UV meter (Model No. J225; Ultraviolet Products Inc., San Gabriel, Calif., U.S.A.). Table 20 showed that percentage survival ranged from 0.0007 to 0.007% when the UV source to sample distance ranged between 0.5, to 36 cm, respectively.

6. The effect of shortwave and longwave UV on the destruction of S. eastbourne

Experiments were conducted to observe the killing effect of shortwave UV and longwave UV on the destruction of Salmonella. Portions (0.5 ml) of S. eastbourne suspensions (0.1% peptone solution; 0.09 absorbance at 660 nm) were placed in sterile petri-dishes (35x10 mm) and exposed for 1 to 4 min using laboratory scale UV lamp (Rayteck, Conn., U.S.A.). Data in Table 21 showed that S. eastbourne cells were completely killed by a treatment with shortwave UV(8.3×10^3 erg/cm^3/sec) but the destruction of S. eastbourne was not observed at all by longwave (4.6×10^3 erg/cm^2/sec) of UV irradiation.

7. The effect of UV irradiation on the destruction of different Salmonella serotypes

Experiments have been conducted to ascertain the difference in resistance of several serotypes of Salmonella to

UV irradiation. Portions (0.1 ml) of S. eastbourne and other seven serotype suspensions (24 hrs culture, 0.09 OD at 660 nm) in peptone solution (0.1%) were plated on three replicate brilliant green agar and were subjected to UV irradiation ($19 \times 10^3 erg/cm^2/sec$). Number of survivors was counted after 24 hrs incubation at 35°C.

The results of this study are illustrated in Fig. 9. It will be noted that the five serotypes (S. anatum, S. typhimurium, S. senftenberg, S. montevideo, S. alachua), which are considered to be heat resistant and the three serotypes (S. infantis, S. eastbourne, S. tennessee), which are considered to be heat sensitive in sucrose (Goepfert et al., 1970), did not differ markedly in their resistance to UV irradiation. Six second exposure to an intensity of $12 \times 10^3 erg/cm^2/sec$ destroyed all serotypes completely. It seems therefore that the serotypes which differed in their heat resistance did not display the same difference in sensitivity to UV irradiation.

8. The destruction of S. eastbourne in a thin film of milk chocolate by use of a continuous UV sterilizer

Figures 10,11 and 12 show a diagram of the apparatus which was constructed by Metal and Electrical Products, Corp. Ltd., Montreal. The apparatus was designed for the continuous UV irradiation of chocolate and comprised four components: 1) UV lamp units (H) which consist of 40 lamps (15 watt, G15 T8, Sylvania germicidal lamps, General Electric, U.S.A.); the lamps were 4.5 cm apart and produced a total intensity of approximately

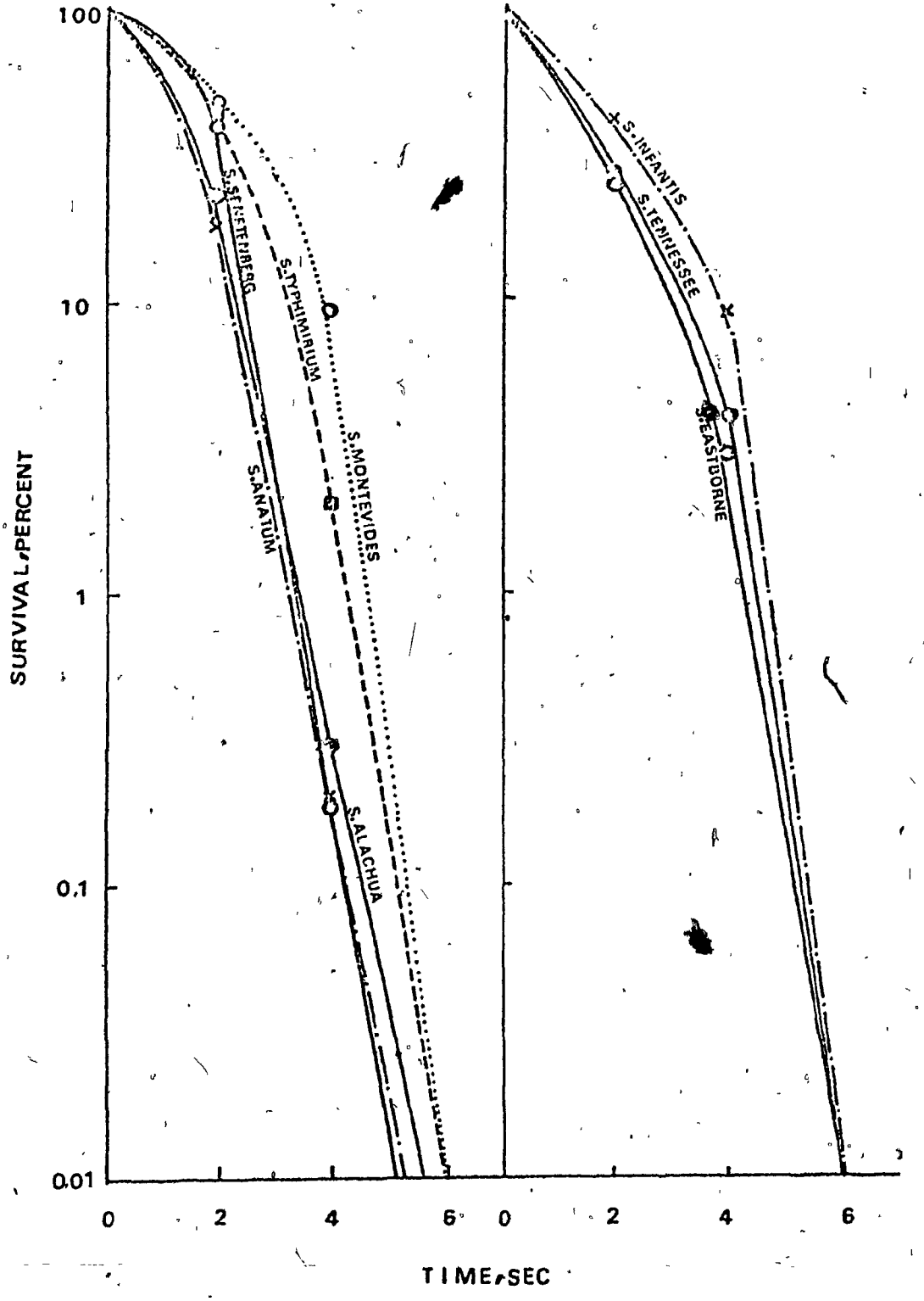


Figure 9. The Effect of UV Irradiation on the Destruction of Different Salmonella Serotypes.

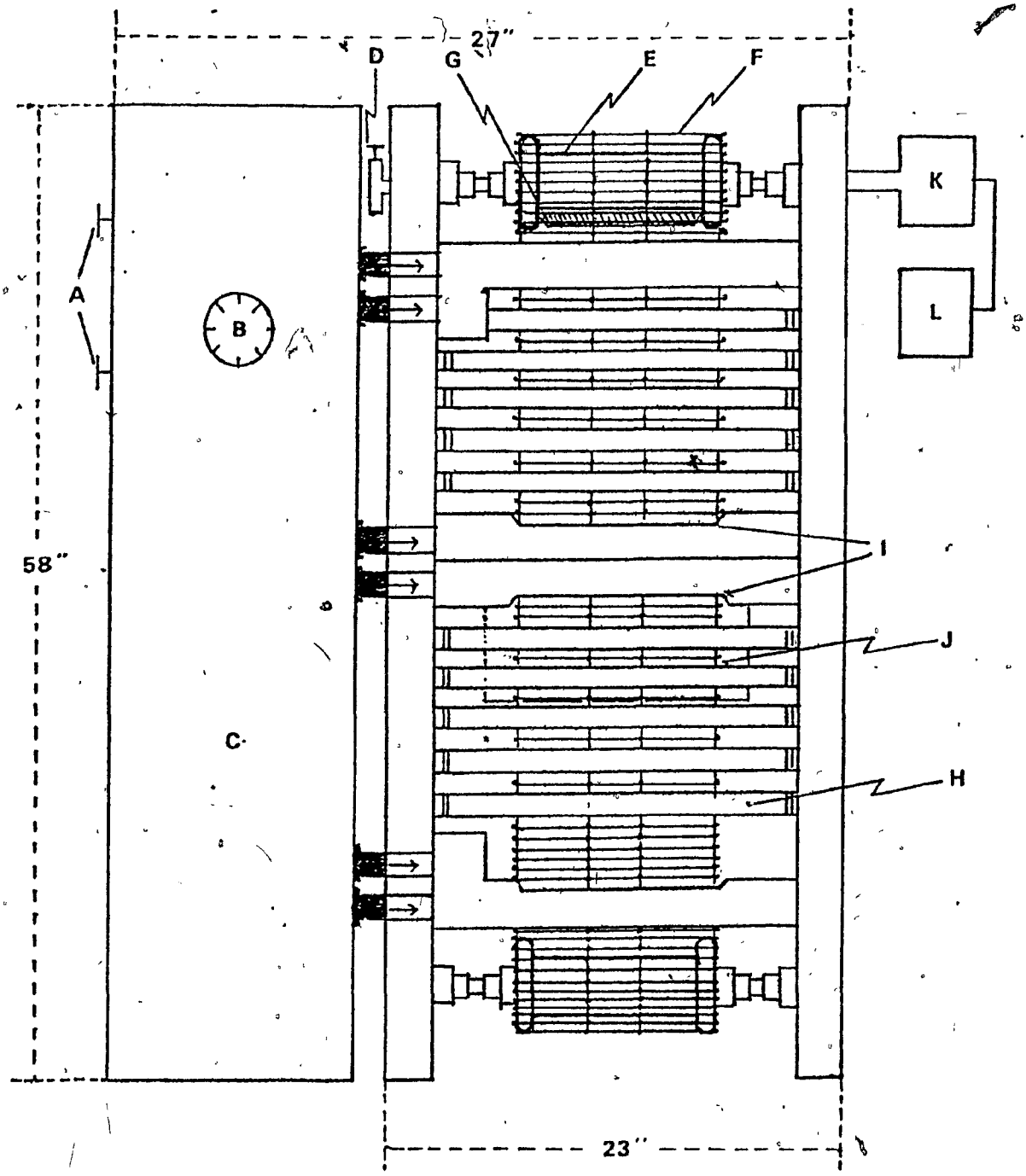


Figure 10. Specification of a Front Section of the Continuous UV Sterilizer; A. Hot Air Controller (10 knobs), B. Thermometer for A, C. Hot Air Blower (10), D. Temperature Controller, E. Roller (7.4x2.5"), F. Wire Mesh Belt (7.7" width), G. Film Scrapers(20x1.5") H. UV Lamps (10 for 1 set), I. Hot Air Outlet, J. Ultrasonic Vibrators, K. Roller Motor, L. Speed Controller

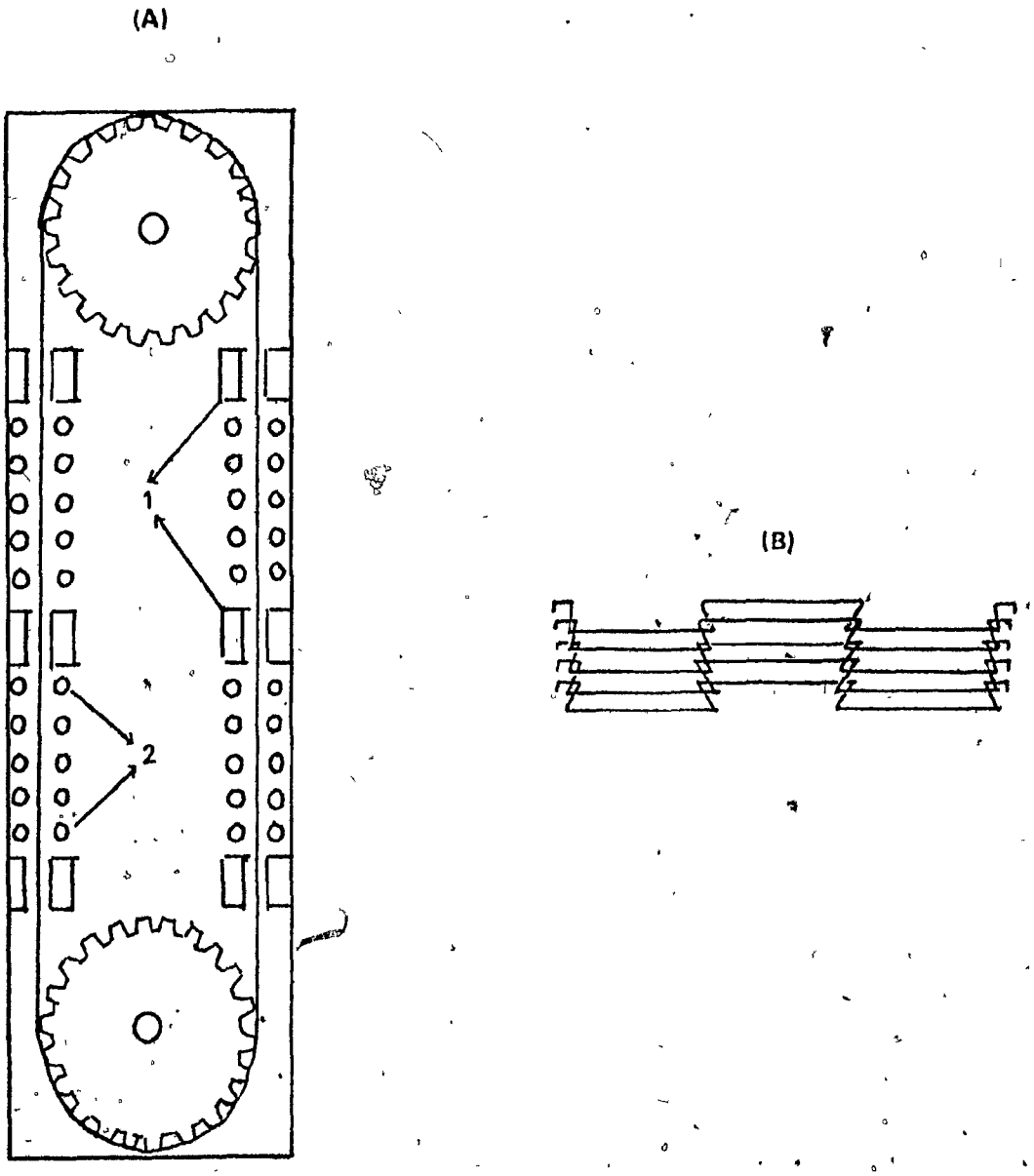


Figure 11. A) A Cross Section of Diagram of the Continuous UV Sterilizer:
1. Hot Air Outlet 2. UV Lamps.
B) A Net of Wire Mesh Belt.

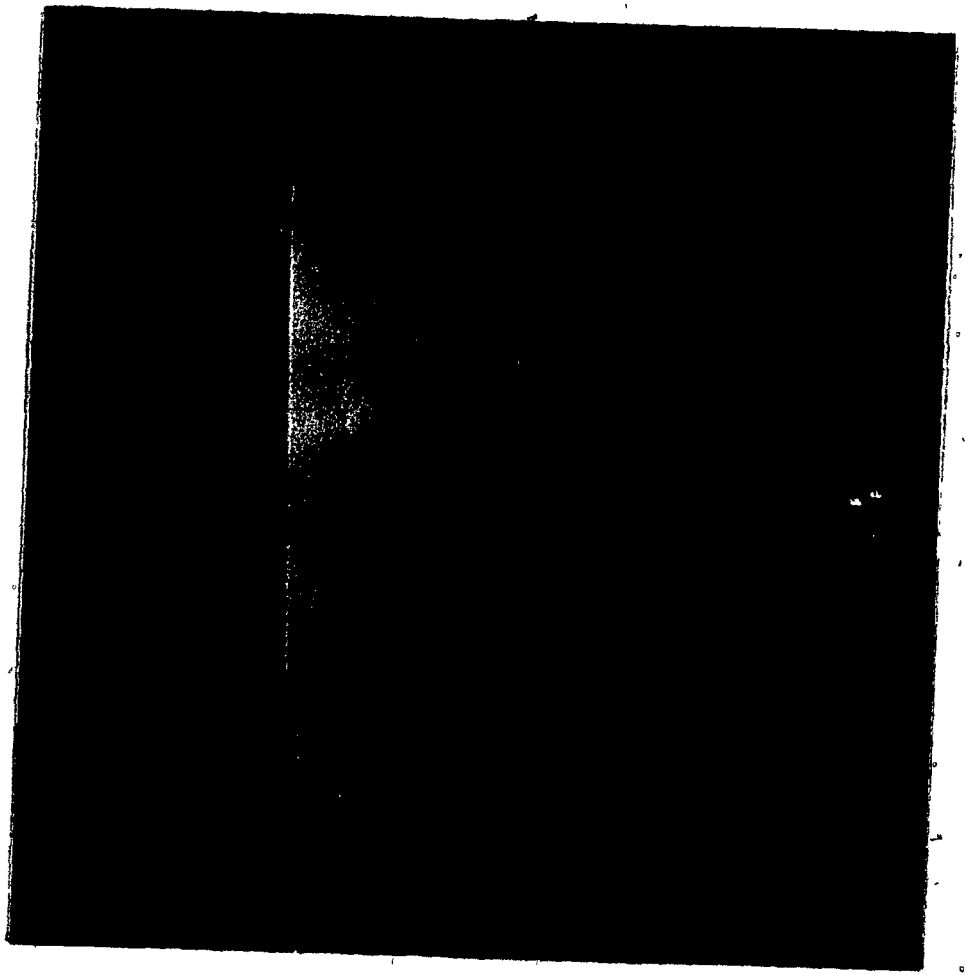


Figure 12. Photograph of Machine Designed to Expose a Continuous Film of Chocolate to UV Irradiation.

40×10^3 erg/cm^2/sec on side of the film of chocolate, 2) Stainless-steel wire belt(F) which was designed to carry a 1 mm film of chocolate; the wire belt was driven by roller (E) which was controlled by roller motor (K) and speed controller (L). 3) Hot air curtains (I) for the purpose of which was to maintain the film of chocolate at 60°C; hot air curtain valves (A) were opened at maximum speed and temperature of the roller (E) was controlled at 60°C by using thermocontroller (D), and 4) ultrasonic vibrators (J) which were to agitate the film and expose new surfaces of chocolate film to the UV irradiation.

S. eastbourne contaminated chocolate was prepared by dispersing the lyophilized cells (20 mg) into the molten chocolate (3000 g) at 45°C to give approximately 10^3 - 10^4 cells/g of chocolate as described in Methods (page 33). The molten chocolate (60°C) contained in a 6 liter beaker was fed slowly on to the surface of the thermoroller (D). Two rubber slips (G) were attached to the thermoroller (D) to feed a thin film of chocolate on the wire mesh belt (F). When the wire mesh belt was completely covered with chocolate, the speed of the belt was adjusted so as to give different exposure times. After the chocolate on the belt had been exposed to the UV irradiation, the lights were turned off and the chocolate was removed from the belt by use of a microtome knife placed across the moving belt. Samples (10 or 20 g) of UV treated and nontreated chocolate were placed in homogenizing flasks (VirTis 45 model)

and thoroughly mixed (5000 rpm; 5 min). The % destruction of S. eastbourne cells was estimated by direct plate count and by most probable number techniques as described on page 34.

The results of a preliminary experiment are shown in Table 22. It will be noted that the average (3 sample) Salmonella kill obtained with the five min exposure time was 91%. Numerous subsequent runs were made with the continuous UV sterilizer in which different methods were used to apply the molten chocolate to the moving belt and to maintain the uniform film when the belt passed over the rollers. The results of the analysis of UV exposed chocolate were extremely erratic and no conclusive results were obtained.

Table 22

The Destruction of S. eastbourne in a Chocolate Film by Use of a Continuous UV Sterilizer[a]

Exposure time(min)[b]	Number/g of Chocolate				
	Sample 1	Sample 2	Sample 3	Ave.	% kill
0	-	3.9×10^4	7.1×10^4	5.5×10^4[c]	0
2	1.4×10^4	5.4×10^3	2.7×10^4	1.4×10^4	75
5	4.0×10^3	3.0×10^3	5.6×10^3	4.9×10^3	91

[a] Room temperature and humidity, 36°C, 51%; air curtain controls at maximum; temperature of chocolate film (60°C); temperature of roller (E, 60°C).

[b] This was regulated by ranging the speed of the moving belt.

[c] Analysis of untreated chocolate (60°C).

DISCUSSION

In experiments designed to investigate a level of Salmonella which could be detected by the standard microbiological methods, it appeared that Salmonella may be detected directly in the range of 20 - 30 cells per 50 g of chocolate by direct plate count technique without preenrichment and enrichment growth. The coefficient of variation of the direct plating technique varied by 3.2 to 8.2% for the peptone solution and 3.8 to 6.3% for the chocolate. The standard deviation associated with the value observed in the chocolate analyses was much higher than that observed in the analysis of peptone solution, possibly because of uneven distribution of Salmonella cells in the chocolate. The Bacteriological Analytical Manual (BAM) variation method was able to detect as few as 2 - 3 cells in 50 g chocolate after preenrichment and enrichment procedure. The media and antiserum (Bacto) employed in serotyping tests was also suitable for the final identification of the presumptive Salmonella in milk chocolate, although it was occasionally necessary to increase the motility of the test organism. The results of the sensitivity of the standard method indicated that the BAM methods are quite suitable for the purpose of detection and identification of Salmonella in chocolate products. The possibility also existed that direct plate count method for quantitating Salmonella in chocolate may not recover cells that were injured by heat or freeze-drying. Control samples were

analyzed, however, in the same way as were the treated samples and recovery was not considered to be a problem with the brilliant green agar. It was considered, therefore, that the techniques which were being used were adequate for the quantitation of Salmonella in chocolate; it was necessary to use the MPN method when the number of Salmonella was too low to permit the use of plating techniques. It should be pointed out, however, that even with the highly sensitive method, the validity of the conclusion to be deduced from analytical data will be dependent largely on the adequacy of the sampling plan used in conjunction with the test.

The cold storage of chocolate contaminated with S. eastbourne appeared to have some destructive effect on Salmonella population; this could be the result of temperature shock. Experiments showed that approximately 96% of the S. eastbourne in milk chocolate was destroyed during a 6 day storage period at 5°C; during the next 12 months approximately 99% were destroyed. Barrile et al. (1970) also observed the survival of Salmonella that was stored at room temperature for 15 months. The rapid initial (10 days) reduction of Salmonella cells observed by the authors might be the result of osmotic shock (Mossel and Koopman, 1965). The destruction of Salmonella in chocolate during storage could be due to the low moisture content of the chocolate or it might be associated with the anticyanin compounds that are present in cocoa beans. The antimicrobial effect of cocoa on Salmonella and other bacteria was reported

previously by Gabis and Langlóis (1967) and Busta and Speck (1968). More recently, Ostovar (1973) observed that the destruction of Staphycococcus aureus was much greater in dark chocolate which contains more cocoa than does milk chocolate. Tamminga et al. (1976) also observed that S. eastbourne and S. typhimurium died off more rapidly in bitter chocolate than in milk chocolate when it was stored at room temperature. Although cold storage is not an effective means of destroying Salmonella in chocolate, Salmonella may be eliminated from chocolate by a combination of temperature shock, antimicrobial low water activity and cocoa constituents, if the level of contamination is low.

Milk chocolate contains a high content of sucrose (44-54%) and this compound provides a good protective environment for the heat destruction of Salmonella. Experiments with eight serotypes of Salmonella showed that S. eastbourne is more heat sensitive (D = 6.2 min) in sucrose solution (54%) than any of the other serotypes that were tested; S. senftenberg (D = 35 min) was by far the most heat resistant under the same conditions. These results are in agreement with those reported by Goepfert et al. (1970) and Corry (1974) in connection with the heat resistance of S. senftenberg. They noted, however, that the difference in heat resistance between S. senftenberg and the other serotypes decreased as the concentration of sucrose was increased. This suggested that the response of the individual strains to the change in water activity (a_w) is not uniform.

Other workers (Barrile and Cone, 1970; Goepfert et al.,1970) considered that S. anatum is one of the heat resistant Salmonella in milk chocolate and in sucrose. It was demonstrated in our laboratory, however, that S. anatum (S-515, HPB, Canada) is not destroyed in 54% sucrose heated at 57°C for 70 min. Our result suggested that S. anatum which survived with the heat treatment could be a variant and, with time, the resistant cells survived and may multiply under the experimental condition. Although the conching is usually done at 45 - 60°C for milk chocolate, Barrile and Cone (1970) observed that physical properties of milk chocolate are not seriously altered at 71°C. The results of the experiment on its heat resistance of S. eastbourne and S. senftenberg in milk chocolate at 71°C showed that S. eastbourne displayed an enormous increase in heat resistance (D = 4.5 hr) when the cells were dispersed in chocolate where there were conditions of low moisture. The D value for S. eastbourne (D = 4.5 hr) was about the same as that for S. senftenberg (D = 4.6 hr). The results also indicated that a dry heating of 12 hrs (71°C) was required to kill completely S. senftenberg (1.4×10^4 cells/g) dispersed in milk chocolate, whereas, S. eastbourne (4.4×10^4 cells/g) was not completely destroyed after 12 hrs. S. typhimurium (D = 6.6 hr) was more resistant than S. senftenberg, which is in agreement with those reported by Goepfert and Biggie (1968). Our previous results in 54% sucrose showed that S. senftenberg was approximately six times as resistant to moist heat as S. typhimurium.

Ng (1966) reported that S. senftenberg 775W was approximately 25 times more resistant to moist heat than was S. typhimurium: other workers reported that S. typhimurium became more resistant to dry heating than did S. senftenberg 775W in chocolate (Goepfert and Biggie, 1968) and in animal feed (Riemann, 1968). Goepfert and Biggie (1968) found that a heating period (70°C) of 7.3 hrs (D value) and 13.6 hrs (D value) were required to kill 90% of S. senftenberg 775W and S. typhimurium respectively in milk chocolate. Rieschel and Schenkel (1971) reported that a heating (72°C) period of 24 hrs was required to kill 90% of S. enteriditis and S. typhimurium in milk chocolate. These collective data suggest that the conching procedure (60°C; 12 - 24 hrs) used in the processing of chocolate is not sufficient in destroying Salmonella. Barrile and Cone (1970) studied the heat resistance of S. anatum in milk chocolate as a function of added moisture content; they found that S. anatum was very susceptible to heat in the presence of traces of water. It is assumed that carbohydrates increase the heat stability of the cell by reducing the water activity (a_w) of the protoplasm (Gibson, 1973).

In experiments on the bactericidal effect of ultrasonics it was observed that over 99.999% of S. eastbourne was destroyed in peptone solution (0.1%) after a treatment period of 10 min. There was a slight difference in resistance between S. eastbourne (D = 3.0 min) and S. anatum (D = 2.1 min). S. anatum showed a tailing effect after 4 min treatment. Weiser et al. (1971)

pointed out that the lethal effect of ultrasonics depends on the number of organisms per unit of volume as well as the shape, size and age of the microorganisms. The destruction of S. eastbourne in milk chocolate was negligible and considerable heat (73°C) was generated during ultrasonic treatment (2 min). The ultrasonic probe which was sealed with silicon to prevent heat generation was inefficient for the destruction of Salmonella in peptone solution (0.1%). Ultrasonic vibration is known to produce hydrogen peroxide in liquids containing oxygen and to cause depolymeration of macromolecules and intramolecular regroupings (Weiser et al., 1971; Joklik and Smith, 1972). Beckwith and Weaver (1936) showed that Saccharomyces ellipsoideus suspended in grape juice was destroyed rapidly by ultrasonic treatment. The present work has demonstrated that ultrasonics are not effective for the destruction of Salmonella in chocolate under the conditions that were employed. Other workers (Joklik and Smith, 1972) have also stated that the use of ultrasonics is not practical for the purpose of sterilization Lees and Jackson(1973), however, made use of ultrasonics in a process developed for the conching of chocolate.

A detailed study was made on the effect of film thickness on the efficiency of Salmonella destruction by UV irradiation. UV irradiation for 1 - 2 min at an intensity of 12×10^3 erg/cm^2/sec killed over 99.999% of S. eastbourne in 0.5, 1.0 and 2.0 mm layers of peptone solution (0.1%). The destruction of S. eastbourne was more effective in 0.5 mm layer than in the 1.0

and 2.0 mm layers after 3 - 4 min exposure. A distinct tailing effect was noted in the 1.0 and 2.0 mm layer of peptone solution (0.1%). No survivors were detected (direct plate count), however, in the 0.5, 1.0 and 2.0 mm layer of the solution after an UV exposure time of 5 min. The preliminary studies, on the destruction of S. eastbourne cells under the thin disks of solid chocolate prepared by use of a hand microtome indicated that the penetration of UV energy was very low in the crystallized solid chocolate. It seemed likely that the destruction of Salmonella cells would be more effective in melted chocolate where the cocoa butter crystals (M.P. 43.3°C) could not interfere with the penetration. It is well known that UV does not penetrate into solid and penetrates into liquid very slightly (Joklik and Smith, 1972). To obtain some information on the penetration of UV irradiation into chocolate, S. eastbourne cells were deposited on the bottom of a depression precisely cut in steel disk and the liquid chocolate (45°C) was placed over the film of cells. It was assumed that the radiation must pass through the layer of chocolate before reaching the cells. The results of this study showed that S. eastbourne cells (1.9×10^4 cells/ml) deposited on the bottom of a steel disk that was covered with a 0.1 mm layer of liquid chocolate (45°C) were completely destroyed (direct plate count method) after an UV irradiation period of 1.5 min; the intensity of the irradiation was 19×10^3 erg/cm^2/sec. Ten minutes was required to kill 75% of the cells under an 0.5 mm layer of

chocolate and no detectable destruction was observed when the film thickness was greater than 0.5 mm. S. eastbourne cells which were completely destroyed by UV irradiation were according to the direct plate count method; the cells (approximately 0.001% fraction), however, recovered when the sample was pre-enriched and then enriched in selenite cystine broth for MPN determination. It appeared that few survivors were not detected by direct plating technique because of inherent error in the relatively small size of plating. Physiologically inactive cells may also not be detected directly on selective brilliant green agar. It was assumed that a negative test by direct plating method indicates that survivors are fewer than 20 cells per 100 ml. The results of the effect of cell density and cell age on the efficiency of Salmonella destruction by UV irradiation indicates that the destruction of Salmonella by UV irradiation was affected by cell density and cell age. When the cell density was 2.0×10^6 cells/ml in 18 hr grown cells (0.9 absorbance), the destruction was completed but when the cell density was 1.4×10^6 cells/ml in 24 hrs culture (1.3 absorbance), the destruction was not completely achieved under the same set of conditions. S. eastbourne cells (2.2×10^5 cells/ml or less) under an 0.1 mm layer of chocolate were destroyed after 5 min exposure of UV but when the cell density was 4.1×10^5 cells/ml, the destruction was 99.3%. The effect of cell density and cell age on UV irradiation was previously reported by Rentschler et al. (1941), Witkin (1946) and Mudretsova-Viss and Zavyalova

(1975) who showed that high cell density and stationary phase cells markedly reduced the efficiency of bacterial destruction by UV irradiation. Consequently, it is generally accepted that UV irradiation has no effect on microorganisms that are shielded or protected from the incident beams. The minimal dose for complete destruction of S. eastbourne was 7.2×10^4 erg/cm^2/sec on the agar surface. The destruction of Salmonella was solely the effect of UV irradiation since experiments showed that no inhibitors were formed during the UV irradiation. When the UV source to sample distance ranged between 0.5 to 36 cm, the destruction of S. eastbourne cells ranged from 99.9993 to 99.993%. Longwave UV energy appeared to be ineffective for the destruction of S. eastbourne.

The UV survival curve of S. eastbourne and S. typhimurium which were obtained when cell suspensions (0.1% peptone) were incubated in yellow, white light and darkness, showed a distinct tailing effect in solution after 30 sec exposure to UV irradiation (19×10^3 erg/cm^2/sec). There was no further increase in UV survival above the tail level, when S. eastbourne and S. typhimurium were exposed to yellow and white light. S. eastbourne cells showed an upward inflection in the darkness after 120 sec exposure to UV irradiation (19×10^3 erg/cm^2/sec). The tail obtained after 120 seconds irradiation of S. eastbourne cells comprised a significant fraction (approximately 0.01%) of the original cell population. Further studies on photorecovery of S. eastbourne showed that the tailing effect and/or dark

repair (excision) mechanism which were observed in the liquid medium was not observed on the surface of the agar or under the 0.1 mm layer of chocolate. The inhibition of colony formation by UV irradiation on the surface of agar was exponential and this suggests a single hit mechanism; it is therefore reasonable to assume that the distinct tailing effect in the liquid may be due to a multicomponent curve produced by a population composed of organisms with different target size (genome). Setlow et al. (1963) indicated that UV resistant cells of E. coli have mechanisms for repairing a pyrimidine dimer, whereas sensitive cells do not. Lewis and Kumpta (1972) also observed a similar tailing effect in UV survival curve of Micrococcus sp. and the possibility of genetic heterogeneity in the cell population was ruled out since clones picked up from the tail showed a similar UV response as the parental population. A similar effect was observed by Hayes (1966) in experiments which E. coli B clones, taken from the tail after exposing to low doses of UV irradiation, showed a similar tailing effect, indicating absence of genetic heterogeneity. According to Hayes (1966), the inactivation of cells prior to the tail is due to faulty coordination between various steps in excision (dark) repair and the tail component may be due to an UV-induced increase in the efficiency of the repair complex. More recently, Moss and Davies (1974) studied the effect of photoreactivation, excision and recombination repair on the survival of UV irradiation E. coli K-12 mutants and found that

when excision repair or both excision and recombination repair are operative, dose modification is no longer observed and a large tail is exhibited on the survival curve. Harm (1968) considered that the limit on the amount of damage that can be repaired by the excision repair mechanism is related to the proximity of UV-induced lesions to one another along the DNA helix. More detailed studies using Salmonella mutants and radioactive assay of DNA by the methods of Moss and Davies (1974) and Doudney (1974) will be necessary to clarify the occurrence of repair mechanisms of S. eastbourne and other serotypes. Singh (1975) also observed the occurrence of photorepair of UV-induced killing in a wide range of light, in blue-green algae and algal virus. Different serotypes of Salmonella are equally sensitive to UV irradiation. The results are in agreement with those who report that Salmonella are very susceptible to UV irradiation (Proctor et al., 1953; Nickerson et al., 1957; Ijichi et al., 1964; Lineweaver, 1966). . . . They found that one of the most heat resistant strain of S. senftenberg was similar to S. typhimurium and other serotypes, in sensitivity to UV and ionizing radiation.

Experiments in which S. eastbourne cells were dispersed in milk chocolate and then exposed to UV irradiation using a continuous UV sterilizer showed that complete destruction was not achieved after 8 min exposure period at an intensity of $40 \times 10^3 erg/cm^2/sec$. In a preliminary experiment, 91% of S. eastbourne was destroyed after 5 min exposure by the UV sterilizer

at 60°C. It was not possible to reproduce this result in several experiments. In the preliminary experiment (91% kill), freeze-drying cells were blended into the milk chocolate by use of a Waring blender; this might have been responsible for the 91% kill which was obtained. It was evident, however, from the several months of experimental work on the continuous UV sterilizer that the design of the equipment did not permit the production of a uniform film of chocolate over the total area of the belt. The film of chocolate between the wires of the belt was less than 0.1 mm and this permitted a high degree of destruction of Salmonella cells in this film of chocolate. The film of chocolate around the wires of the belt and especially at the side of the belt was much thicker and was not uniform. Furthermore, the film of chocolate carried by the belt was destroyed when the belt moved over the rollers. Attempts to reform the film and to maintain a uniform film, by use of flexible rubber scrapers installed on either side of the belt beneath the rollers, were unsuccessful. Valuable experience has been gained in this experiment with the continuous UV sterilizer. This is especially true in connection with the physical properties of liquid chocolate. The design of a new apparatus for the continuous irradiation of chocolate is now under way. The design is based on the use of a flexible steel belt on which a film of chocolate can be formed and removed much in the same way as it is done in the roller-drum drier. The work on the UV sterilizer of chocolate by a continuous process

will proceed as soon as the new equipment is built and installed in our laboratories.

Other workers (Ijichi et al., 1966) have attempted to destroy S. typhimurium in a thin film of egg white and whole egg, by UV irradiation. They observed the development of off-flavor and odor when an incident UV energy of 7.22×10^4 erg/cm^2/sec were used. These workers reported, that this film (50 μ) of egg white protein at a feed rate of 100 ml per min permitted the passage of 57% of an incident UV energy (2537Å). Benesi (1956) and Oppenheimer (1959) originally developed a method in which thin films of fluids can be exposed to UV irradiation; the process is now used for the sterilization of plasma and vaccines. Curran and Tamsma (1960) used the same technique for the sterilization of whole milk but without complete success. Werner et al. (1972) also described a process for the sterilization of milk in which a very thin film of milk is exposed to UV irradiation; the exposed milk had a vitamin D potency of 400 - 500 ID/L.

SUMMARY

1. The levels of detection of Salmonella ranged from 20 - 30 cells per 50 g of chocolate by direct plate count technique without preenrichment and enrichment procedure. Low levels of Salmonella in the range of 2 - 3 cells per 50 g of chocolate were detected only by a modification of the method described in the Bacteriological Analytical Manual. The media and antiserum employed in serotyping tests was suitable for the final identification of the presumptive Salmonella in chocolate. The coefficient of variation of the direct plating technique varied between 3.2 and 8.2% for the peptone solution and between 3.8 and 6.3% for the chocolate.

2. Experiments showed that storage (5°C) of milk chocolate contaminated with S. eastbourne, for a period of six days, destroyed about 96% of the cells. A further storage period of one year led to the destruction of 99% of S. eastbourne cells.

3. A comparison of the heat resistance of S. eastbourne and seven serotypes of Salmonella in 54% sucrose at 57°C indicated that S. eastbourne (D = 6.2 min) was more heat sensitive than any of the other seven serotypes. S. senftenberg (D = 35 min) was by far the most heat resistant. The D value for S. eastbourne (D = 4.5 hr) was about the same as that for S. senftenberg (D = 4.6 hr) in milk

chocolate. S. typhimurium (D = 6.6 hr) was more resistant than S. senftenberg in milk chocolate.

4. The efficiency of ultrasonic destruction of Salmonella differed markedly in peptone solution (0.1%) and in milk chocolate. Over 99.999% of S. eastbourne was destroyed in peptone solution (0.1%) after a period of treatment of 10 min. There was a slight difference in resistance between S. eastbourne (D = 3.0 min) and S. anatum (D = 2.1 min). S. anatum showed a tailing effect after 4 min treatment in peptone solution. In a similar experiment in which milk chocolate was used, the amount of the destruction of S. eastbourne was negligible.

5. Ultraviolet irradiation (12×10^3 erg/cm^2/sec) for a time of 2 min destroyed over 99.999% of S. eastbourne in films (0.5, 1.0, and 2.0 mm) of peptone solution (0.1%). A distinct tailing effect was noted in the 1.0 and 2.0 mm layer of peptone solution. No survivors of S. eastbourne were detected (direct plate count) in the 0.5, 1.0 and 2.0 mm layer of the solution after an UV exposure time of 5 min at an intensity of 12×10^3 erg/cm^2/sec.

6. S. eastbourne cells (1.9×10^4 cells/ml) which were covered with an 0.1 mm layer of molten chocolate were completely destroyed (direct plate count) after an UV irradiation period of 1.5 min at an intensity of 19×10^3 erg/cm^2/sec; the cells (approximately 0.001%), however, recovered when

they were further checked with most probable number technique after preenrichment and enrichment procedure.

7. The efficiency of the destruction of S. eastbourne by UV irradiation was governed by the density and the age of the cells.

8. The minimal dose of UV irradiation that was required for complete destruction of Salmonella was 6 sec at an intensity of $12 \times 10^3 erg/cm^2/sec$. It was observed that no inhibitors were formed during the UV irradiation.

9. Experiments showed that the destruction of S. eastbourne by longwave UV irradiation was negligible.

10. Different serotypes of Salmonella were equally sensitive to UV irradiation. There was no observable photo-reactivation and excision repair of Salmonella cells on agar or under a layer (0.1 mm) of chocolate. There was, however, excision repair (dark) of S. eastbourne cells in peptone solution.

11. A machine was designed and constructed for the destruction (UV) of Salmonella in a thin continuous film of chocolate. Unsuccessful attempts were made to use the machine for the sterilization of chocolate on a continuous basis. The failure to achieve the same degree of destruction of Salmonella as was achieved with the static film was because of difficulties involved in the prodution of an uniformly thin (0.1 mm) film of chocolate.

REFERENCES

Ager, E.A., Nelson, K.E., Galton, M.M., Boring, J.R., and J.R. Jernigan. 1967. Two outbreaks of egg-borne salmonellosis and implications for their prevention. J. Am. Med. Assoc. 199, 122.

Anon. 1968. Precautions will reduce Salmonella threat for chocolate producer. Manufacturing Confectioner. 48, 39-41.

Appelman, M.D., Brain, N., and J.M. Shewan. 1964. A study of some organisms of public health significance from fish and fish products. J. App. Bact. 27, 69.

Aquitron Corp. 1969. Sterilizing. Australian Pat. 292, 563. Cited in Encyclopedia of Food Technol. (1974).

Arkhomgel'skii, I.I., and V.M. Kartoshova. 1962. Accelerated methods of detecting Salmonella in milk. Veterinariya 9, 74-78.

Armstrong, R.W., Fodor, T., Curlin, G.T., Cohen, A.B., Morris, G.K., Martin, W.T., and J. Feldman. 1970. Epidemic Salmonella gastroenteritis due to contaminated imitation ice-cream. J. Am. Apidem. 91, 300.

Avakyan, B.P. 1972. Cold sterilization of wine. Cited in Food Sci. Technol. Abstr. 5, 7L4.

Avakyan, B.P. 1974. Inhibition of secondary growth of micro-organisms in wine treated simultaneously with UV rays and ultrasound. Biologicheskii Zhunal Armenii. 25(3), 36-39. Cited in Food Sci. Technol. Abstr. 2, 3H365.

Ayres, J. 1966. In the destruction of Salmonellae. 22-25. A Report of the Western Exp. Sta. Collaborators Conf. Albany, Calif. March.

Ayres, J.C., and H.M. Slosberg. 1949. Destruction of Salmonella in egg albumen. Food Technol. 3, 180-183.

Barrile, J.C., and J.R. Cone. 1970. Effect of added moisture on the heat resistance of Salmonella anatum in milk chocolate. Appl. Microbiol. 19, 177-178.

Barrile, J.C., Cone, J.F., and P.G. Keeney. 1970. A study of Salmonella survival in milk chocolate. Manufacturing Confectioner. 50(9), 36-39.

Barrile, J.C., Ostovar, K., and P.G. Keeney. 1971. Microflora of cocoa beans before and after roasting at 150°C. J. Milk Food Technol. 34(7), 369-371.

Bartram, M.T. 1966. How to detect and control mycotoxins; occurrence of Salmonellae and mycotoxins. Candy Ind. 127, 37.

Beckwith, T.D., and C.E. Weaver. 1936. Sonic energy as a lethal agent for yeast and bacteria. J. Bacteriol. 32, 361-373.

Benesi, E. 1956. Design of a centrifugal filmer for the ultraviolet irradiation of liquids. General Motors Eng. J. 3, 2.

Bergquest, D.H. 1961. Eggs. In food dehydration, Vol. 2.W.B. Van Arsdel and M.J. Copley (Editors). Avi Publishing Co., Westport, Conn.

Board, R.G. 1973. The microbiology of eggs (a review). In egg science and technology. 46-60. Avi Publishing Co., Westport, Conn.

Boyce, R.P., and P. Howard-Flanders. 1964. Release of ultraviolet light induced thymine dimers from DNA in E. coli K-12. Proc. Natl. Acad. Sci., U.S.A., 51, 293-300.

Bracco, U., Grailhe, N., Rostagno, W., and R.H. Egli. 1969. Analytical evaluation of cocoa curing in the Ivory Coast. J. Sci. Food. Agr. 20, 713.

Bruch, C.W., and M.G. Koesterer. 1962. Destruction of enteric bacteria in liquid egg with beta-propiolactone. Appl. Microbiol. 10, 123-128.

Bryan, F.L., Ayres, J.C., and A.A. Kraft. 1968. Salmonella associated with further-processed turkey-products. Appl. Microbiol. 16, 1.

Bryan, H.C. 1974. Microbiological food hazards today - based on epidemiological information. Food Technol. (Sept.) 51-63.

Burton, H. 1951. Ultraviolet irradiation of milk. Dairy Sci. Abstr. 13, 229-244.

Busta, F.F., and M.L. Speck. 1968. Antimicrobial effect of cocoa on Salmonellae. Appl. Microbiol. 16, 424.

Caldwell, W.J., Stulberg, C.S., and W. D. Peterson. 1966. Somatic and flagellar immunofluorescence of Salmonella. J. Bacteriol. 92, 1177-1187.

Carpenter, J.A., Elliot, J.C., and A.E. Reynolds. 1973. Isolation of Salmonella from pork carcasses. Appl. Microbiol. 25, 731-734.

Center for Disease Control (CDC). 1974. Morbidity and Mortality Weekly Report. Vol. 23(4). January 26.

Center for Disease Control (CDC). 1974. Morbidity and Mortality Weekly Report. Vol. 23(9). March 2.

Center for Disease Control (CDC). 1974. Morbidity and Mortality Weekly Report. Vol. 23(10). March 9.

Chu, H.D., Leeder, J.G., and S.G. Gilbert. 1975. Immobilized catalase reactor for use in peroxide sterilization of dairy products. J. Food Sci. 40, 641-643.

Collins, R.N., Troger, M.D., Goldsby, J.B., Borling, J.R., Coohon, P.B., and R.N. Barr. 1968. Interstate outbreak of Salmonella newbrunswick infection traced to powdered milk. J. Am. Med. Assoc. 203(10), 838-844.

Comer, A.G., Anderson, G.W., and E.H. Garrard. 1963. Gamma irradiation of Salmonella species in frozen whole egg. J. Can. Microbiol. 9, 321-327.

Corry, J.E.L. 1974. The effect of sugars and polyols on the heat resistance of Salmonellae. J. Appl. Bacteriol. 37, 31-43.

Craven, P.C., Mackel, D.C., Baine, W.B., Barker, W.H., Gangarosa, E.J., Goldfield, M., Rosenfeld, H., Altman, R., Lachapelle, G., Davies, J.W., and R.C. Swanson. 1975. International outbreak of Salmonella eastbourne infection traced to contaminated chocolate. The Lancet (April 5). 788.

Curran, H.R., and A. Tamsma. 1960. Some observations in the ultraviolet irradiation of milk (centrifilmer process) with emphasis upon organoleptic effects and sporicidal efficiency. J. Dairy. Sci. 43, 410.

Dack, G.M., and E. Davison. 1938. Salmonella food poisoning-infection or intoxication ? Food Research. 3, 347-349.

D'Aoust, J.Y., Avis, B.J., Thisdele, P., Durante, A., Brisson, N., Dragon, D., Lachapelle, G., Johnston, M., and R. Laidley. 1975. Salmonella eastbourne outbreak associated with chocolate. J. Inst. Can. Sci. Technol. Aliment. 8(4), 181-184.

Davis, B.D., Duebecco, R., Eisen, H.N., Ginsberg, H.S., Wood, W.B., and M. McCusty. 1973. The enteric bacilli and similar gram-negative bacteria. Microbiology (second ed) Harper and Row Publ. Inc.

Day, W.H., Tames, E., and C.D. Healther. 1963. Salmonellosis in the dog. J. Am. Vet. Res. 24, 156-158.

Depew, F.M. 1968. The Salmonellae - a current challenge. Manufacturing Confectioner. 48(3), 31-32.

Dockstader, W.B., and R.J. Gromes. 1971. Detection and survival of Salmonella in milk chocolate. Bacteriol. Proceeding. 971, 7.

Doolin, G.S. 1972. Quality control in the confectionary industry. J. Milk Food Technol. 35(7), 424-431.

Doudney, C.O. 1974. Loss of photoreversibility of damage to deoxyribonucleic acid replication in ultraviolet-irradiated E. coli B/r thy trp. J. Bacteriol. 120, 394-398.

Dumas, A., Foley, A.R., and J.P. Breton. 1961. A milk borne epidemic of Typhoid fever in Montmagny, Quebec, 1959. J. Can. Public Health. 52, 68-71.

Edwards, P.R. 1965. Salmonellosis - an expanding problem. Proc. Nat. Conf. on Salmonellosis. 11-13th March, 1964. Communicable Disease Centre, Atlanta, Georgia.

Edwards, P.R., and D.W. Bruner. 1943. The occurrence and distribution of Salmonella. J. Infect. Dis. 72, 58-67.

Edwards, P.R., and M.M. Galton. 1967. Salmonellosis. Advan. Vet. Sci. 11, 1.

Eickhoff, J.C. 1966. In the destruction of Salmonellae, 17. A report of the Western Expt. Sta. Collaborators Conf. Albany, Calif. March.

Elliot, R.P. 1966. An evaluation of public health hazards from the microbiological contamination of foods. Quart. Bull. Fd. and Drug Off., U.S.A.. 30, 8.

Fantasia, L.D., Sperber, W.H., and R.H. Deibel. 1969. Comparison of two procedures for detection of Salmonella in food, feed, and pharmaceutical products. Appl. Microbiol. 17, 540-541.

Forsyth, W.G.C., and V.C. Quesnel. 1963. The mechanism of cocoa curing. Advances Enzymol. 25, 457-492.

Foster, E.M. 1968. The NCA research program at the Food Research Institute. Manufacturing Confectioner. 48, 33-37.

Gabis, D.A., and B.E. Langlois. 1967. Effect of cocoa powder used in chocolate milk on bacterial growth. J. Dairy Sci. 50, 945.

Gabis, D.A., Langlois, B.E., and A.W. Rudwik. 1970. Microbiological examination of cocoa powder. Appl. Microbiol. 20, 644-645.

Galton, M.M., Smith, W.V., McElrath, H.B., and A.V. Hardy. 1954. Salmonella in swine, cattle, and the environment of abattoirs. J. Infect. Dis. 95, 236-245.

Garibaldi, J.A. 1966. In the destruction of Salmonellae, 34-37. A report of the Western Expt. Sta. Collaborators Conf. Albany, Calif. March.

Gärtner, A. 1888. Korresp. d. allg. ärzll. Ver. Thüringen. 17, 233.

Georgala, D.L., and M. Boothroyd. 1964. A rapid immunofluorescence technique for detecting Salmonellae in raw meat. J. Hyg. (camb) 62, 319-327.

Gibson, B. 1973. The effect of high sugar concentrations on the heat resistance of vegetative microorganisms. J. Appl. Bact. 36, 365.

Goepfert, G.M. 1969. The interrelationship between pH, temperature, oxygen supply, and available water, on growth and survival of Salmonella organisms with special reference to confectioner. 49, 68-72.

Goepfert, J.M., and R.A. Biggie. 1968. Heat resistance of Salmonella typhimurium and Salmonella senftenberg 775W in milk chocolate. Appl. Microbiol. 16, 1939-1940.

Goepfert, J.M., Iskander, I.K., and C.H. Anurndson. 1970. Relation of the heat resistance of Salmonellae to the water activity of the environment. Appl. Microbiol. 19, 429-433.

Grundewaid, T. 1973. Irradiation of chocolate and chocolate products. Zeitschrift für Lebensmittel untersuchung und Forschung. 151(1), 13-17. Cited in Food Sci. and Technol. 6K 62.

Guill, J.H. 1967. Current regulatory status of the Salmonella problem. Manufacturing Confectioner. 47, 30.

Haevecker, E. 1970. Patented applications of ultrasonics in the fruit processing industry. Lebensmittel Industrie 17, No. 2, 63. Cited in Encyclopedia of Food Technol. Avi. 1974.

Handzel, S. 1974. Outbreak of food poisoning due to Salmonella eastbourne. Epidemiol. Bull. (Health and Welfare, Canada) 18(4), 54.

Harm, W. 1968. Effects of dose fractionation of ultraviolet survival of E. coli. photochem. Photobiol. 7, 73-86.

Harvey, R.W.S., Price, T.H., Foster, D.W., and W.C. Griffiths. 1969. Salmonellae in sewage; a study in latent human infection. J. Hyg. Camb. 67, 517.

Hayes, R.H. 1966. The interpretation of microbiol. inactivation and recovery phenomenon. Radiat. Res. Suppl. 6, 1-29.

Hobbs, B.C. 1961. Public health significance of Salmonella carriers in livestock and birds. J. Appl. Bacteriol. 24, 340-352.

Hobbs, B.C., and J.H.B. Christian. 1973. The microbiological safety of food. Proceed. of the eighth Int. Symp. on Food Microbiol. Reading, England, Sept. 1972. Acad. Press, London and N.Y.

Horwitz, W. ed. 1970. Official methods of analysis of the association of official analytical chemists, eleventh ed. with supplements. Assoc. of Official Analytical Chemists, Washington, D.C.

Howard-Flanders, P. 1968. DNA repair. Ann. Rev. Biochem. 37, 175-200.

Howat, G.R. 1968. Chocolate manufacture. Process Biochem. 3, 31-35.

Huff, C.B., Smith, H.F., Boring, W.D., and N.A. Clarke, 1965. Study of ultraviolet disinfection of water and factors in treatment efficiency. Publ. Hlth. Rep. 80, 695-705.

IAEA, Vienna. 1963. Radiation control of Salmonella in food and feed products. Technical Report Series No. 22, 148P.

IAEA, Vienna. 1966. Food irradiation proceedings of a symposium karlsruhe, 6-10 June. 956P.

IAEA, Vienna. 1967. Microbiological problems in food preservation by irradiation. Proceedings a panel, Vienna, 27 June-1 July. 145P.

IAEA, Vienna. 1972. Food irradiation information No. 1 and 2. International project in the field of food irradiation.

IAEA, Vienna. 1973. Factors influencing the economical application of food irradiation. Proceedings of a panel held in Vienna. 14-18 June. 197.

Ijichi, J. 1966. In the destruction of Salmonellae, 50-52. A report of the Western Expt. Sta. Collaborators Conf. Albany, Calif. March.

Ijichi, J., Hammerle, O.A., Lineweaver, H., and L. Kline. 1964. Effects of ultraviolet irradiation of egg liquids on Salmonella destruction and performance quality with emphasis on egg white. Food Technol. 18, 1628-1632.

International Commission on Microbiological Specifications for Foods (ICMSF). 1974. Microorganisms in foods, Vol. II. sampling for microbiological analysis; principles and specific applications. Univ. of Toronto Press.

Johnson, A.H. 1974. Ultrasonic energy. P912. In Encyclopedia of Food Technol. Avi Publishing Co.

Johnson, A.H. 1974. Ultraviolet radiation. P915. In Encyclopedia of Food Technol. Avi Publishing Co.

Joklik, W.K., and D.J. Smith. 1972. Chap. 11. Sterilization and disinfection. Zinsser Microbiol. 15th ed.

Kelner, A. 1949. Effect of visible light on the recovery of Streptomyces griseus conidia from ultraviolet irradiation injury. Proc. Natl. Acad. Sci. U.S.A. 35, 73-79.

Khadzhiiski, D. 1970. Use of ultrasonics in wine production. Logarstro Vinarstro 18, No. 5, 30. Cited in Food Sci. Technol. Abstr. 2, 5H545.

Krauss, P. 1972. How to control and ensure quality in chocolate processing. Manufacturing Confectioner. 52, 38-41.

Lammanna, C., and M.F. Mallette. 1953. Basic bacteriology. The Williams and Wilkins Co., Baltimore.

Lang, D.J., Kunz, L.S., Martin, A.R., Schroeder, S.A., and A. Thompson. 1967. Carmine as a source of nosocomial salmonellosis. New Engl. J. Med. 276, 829.

Laramore, C.R., and C.W. Moritz. 1969. Fluorescent-antibody techniques in detection of Salmonella in animal feed and feed ingredients. Appl. Microbiol. 17, 352-254.

Lees, R., and E.B. Jackson. 1973. Sugar confectionary and chocolate manufacture. Leonard Hill Books, Great Britain.

Lennington, K.R. 1967. FDA looks at the Salmonella problem-ever present threat to industry. Manufacturing Confectioner. 47, 31.

Lennington, K.R. 1968. Salmonella found in drain cleaners. Food Chem. News. 10, 9.

Lewis, N.F., and U.S. Kumta. 1972. Evidence for extreme UV resistance of Micrococcus Sp. NCTC 10875. Biochem. Biophys. Res. Com. 47, 1100-1105.

Lineweaver, H. 1966. In the destruction of Salmonella, 47-49. A report of the Western Expt. Sta. Collaborators. Conf. Albany, Calif.

Lowenstein, M.H., Lamm, S.H., Gangarosa, E.J., and H.W. Anderson. 1971. Salmonellosis associated with turtles. J. Infect. Dis. 124, 433.

Mackel, D.C., Galton, M.M., Gray, H., and A. Hardy. 1952. Salmonellosis in dogs. J. Infect. Dis. 91, 15-18.

Mallick, B.B., and S.B.V. Rao. 1964. Indian Vet. J. 41, 439-441.

Markovitz, A., and P. Burboeck. 1971. A complete economical system for rapid mass screening of Salmonella contamination. Presented at the 80th Annual Meeting of the A.O.A.C.

Mayr, G., and H. Kaemmerer. 1959. Fumigation with ethylene oxide. Food Manuf. 34, 169.

McCall, C.E., Collins, R.N., Jones D.B., Kaufmann, A.F., and P.S. Brachman. 1966. An interstate outbreak of salmonellosis traced to a contaminated food supplement. J. Am. Epidemiol. 84, 32.

McCroan, J.E., McKinley, T.W., Brim, A., and W.C. Henning. 1964. Staphylococci and Salmonellae in commercial wrapped sandwiches. Pub. Hlth. Rep., Wash. 79, 497.

McCullough, N.B., and C.W. Eisele. 1951. Experimental human salmonellosis. J. Infect. Dis. 88, 278.

Minifie, B.W. 1970. Chocolate, cocoa, and confectionary; science and technology. Avi Publishing Co., Westport, Conn.

Morris, G.K., and C.H. Dunn. 1970. Influence of incubation temperatures and sodium heptadecyl sulfate on the isolation of Salmonellae from pork sausage. Appl. Microbiol. 20, 192.

Morris, G.K., and J.G. Wells. 1970. Salmonella contamination in a poultry processing plant. Appl. Microbiol. 19, 795.

Moss, S.H., and D.J.G. Davies. 1974. Interrelationship of repair mechanisms in UV-irradiated E. coli. J. Bacteriol. 120, 15-23.

Mossel, D.A.A., and M.J. Koopman. 1965. Losses in viable cells of Salmonellae upon inoculation into dry animal feeds of various types. Poultry Sci. 44, 890-892.

Mudretsova-viss, K.A., and D.V. Zavyalova. 1975. Effect of ultraviolet radiation on the microflora of meat. Applied Biochem. and Microbiol. 9(4), 542-545.

Muller, G., Goethe, H., and R. Herrmann. 1972. Bacteriological examinations for disinfecting drinking water of ships by means of UV irradiation. Zbi. Bakt. I. Abt. Orig. 215, 361-372.

Naguib, Kh., and L. Hussein. 1972. The effect of hydrogen peroxide treatments on the bacteriological quality and nutritive value of milk. Milchwissenschaft 27, 758.

Ng, H. 1966. The destruction of Salmonellae, 39-41. A report of the Western Expt. Sta. Collaborators Conf. Albany, Calif. March.

Ng, H. Bayne, H.G., and J.A. Garibaldi. 1969. Heat resistance of Salmonella; the uniqueness of Salmonella senftenberg 775 W. Appl. Microbiol. 77, 78-82.

Nickerson, J.T.R., Charm, S.E., Beagle, R.C., Lockhart, E.E., Proctor, B.E., and H. Lineweaver. 1957. Use of high-voltage cathode rays to destroy salmonella in liquid and frozen egg white and egg white solids. Food Technol. 11, 159.

Nilson, T. 1963. The use of ultraviolet radiation in food industries. Proc. 2nd meeting of food preservation by ionizing radiation. Stockholm. 79.

Novick, A., and L. Szilard. 1949. Experiments on light-reactivation of ultraviolet-inactivated bacteria. Proc. Natl. Acad. Sci. U.S.A. 35, 591-600.

Nunheimer, T.D., and F.W. Fabian. 1940. Influence of organic acids, sugars and sodium chloride upon strains of food poisoning staphylococci. J. Am. Public Health. 30, 1040.

Olson, J.C., ed. 1972. Salmonella. Chapter VIII. Bacteriological analytical manual, U.S. Food and Drug Administration, Washington, D.C.

Oppenheimer, F., Benesi, E., and A.R. Taylor. 1959. The ultraviolet irradiation of biological fluids in thin flowing films. J. Am. Public Health 49, 903.

Ostovar, K. 1972. Isolation and characterization of micro-organisms involved in the fermentation on Trinidas cacao beans. Dis. Abstr. International, B33(1), 344, Order No. 72-19-360(En)(Penn. State Univ., Univ. Park, U.S.A.).

Ostovar, K. 1973. A study on survival of Staphylococcus aureus in dark and milk chocolate. J. Food Sci. 38, 663-664.

Previte, J.J. 1972. Radiometric detection of some food bacteria. Appl. Microbiol. 24, 535-539.

Proctor, B.E., Joslin, R.P., Nickerson, J.J.R., and E.F. Lockhart. 1953. Elimination of Salmonella in whole egg powder by cathode ray irradiation of egg magma prior to drying. Food Technol. 7, 291.

Prost, E., and H. Riemann. 1967. Food-borne salmonellosis. Ann. Rev. Microbiol. 495-528.

Quesnel, V.C. 1965. Agents inducing the death of cacao seeds during fermentation. J. Sci. Food Agr. 16, 441-447.

Rasmussen, O.G., Hansen, R., Jacobes, N.J., and O.H.M. Wilder. 1964. Dry heat resistance of Salmonellae in rendered animal by-products. Poultry Sci. 43, 1151-1157.

Reagan, J.O., Smith, G.C., and Z.C. Carpenter. 1973. Use of ultraviolet light for extending the retail caselife of beef. J. Food Sci. 38, 929-931.

Reamer, R.H., and R.E. Hargrove. 1972. Twenty-four hour immuno-fluorescence technique for the detection of Salmonellae in non-fat dry milk. Appl. Microbiol. 23, 78-81.

Rentschler, H.C., Nagy, R., and G. Manuromseff. 1941. Bactericidal effect of ultraviolet radiation. J. Bacteriol. 41, 745-774.

Report. 1974. Salmonella reading outbreak by turkey caused by failure to refrigerate. Morbidity Mortality Weekly Rept. (U.S.A.) 24(33), 284.

Riemann, H. 1966. In the destruction of Salmonellae, 26-34. A report of the Western Expt. Sta. Collaborators Conf. Albany, Calif. March.

Riemann, H. 1968. Effect of water activity on the heat resistance of Salmonella in "dry" materials. Appl. Microbiol. 16, 1621-1622.

Rieschel, H., and J. Schenkel. 1971. Das rerhalten von Microorganismen, speziell salmonellen, in schokoladenwaren. Alimenta 10, 57-66.

Rombouts, J.E. 1952. Observations on the microflora of fermenting cocoa benas in Trinidad. Appl. Bacteriol. 15(1), 103.

Rowe, B. 1973. Salmonellosis in England and Wales. In the microbiological safety of food, P165-180. Ed. B.C. Hobbs and J.H.B. Christian. Academic Press, London and N.Y.

Rupp, W.D., and P.Howard-Flanders. 1968. Discontinuities in the DNA synthesized in an excision-defective strain of E. coli following UV irradiation. J. Mol. Biol. 31, 291-304.

Sadler, W.W., and R.E. Corstvet. 1965. Second survey of market poultry for Salmonella infection. Appl. Microbiol.13,348-351.

Salmon, D.H., and S. Theobald. 1886. Rep. Com. Agr., Washington, D.C.; 1885, U.S. Bureau of Am. Indust. 2nd Ann. Rept. P184.

Schneider, M.D. 1946. Investigation of Salmonella content of powdered whole egg with not more than two percent moisture content. II. General survey on occurrence of species of Salmonella in high quality egg powder. Food Res. 11, 313-318.

Setlow, R.B., and W.L. Carrier. 1964. The disappearance of thymine dimers from DNA; an erro-correcting mechanism. Proc. Natl. Acad. Sci. U.S.A., 51, 226-231.

Setlow, R.B., Swenson, P.A., and W.L. Carrier. 1963. Thymine dimers and inhibition of DNA synthesis by ultraviolet irradiation of cells. Science, 142, 1464-1466.

Sharma, S.P., and C.M. Singh. 1963. Indian J. Microbiol.3, 13-16.

Silliker, H.H., Schmall, A., and J.Y. Chira. 1966. The fluorescent antibody techniques as a means of detecting Salmonellae in foods. J. Food Sci. 31, 240-244.

Singh, P.K. 1975. Photoreactivation of UV-irradiated blue-green algae and algal virus LPP-1. Arch. Microbiol. 103, 297-302.

Smith, H.W. 1959. The isolation of Salmonellae from the mesenteric lymph nodes and faces of pigs, cattle, sheep, dogs, and cats and from other organs of poultry. J. Hyg. 57, 266-273.

Sperber, W.H., and R.H. Deibel. 1969. Accelerated procedure for Salmonella detection in dried foods and feeds involving only broth cultures and serogical reactions. Appl. Microbiol. 17, 533-539.

Stoutz, W.P. de. 1969. Process for preserving milk. West Ger. Pat. 1, 300, 813(German). Cited in Encyclopedia Food Technol. (1974).

Supplee, A.R. 1930. The effect of ultraviolet radiation on market milk. J. Milk Technol. 16, 251.

Tamminga, S.K., Beumer, R.R., and E.H. Kampelmacher. 1976. Survival of Salmonella eastbourne and Salmonella typhimurium in chocolate. J. Hyg. Camb. 76, 41-47.

Thatcher, F.S., and D.S. Clark (eds). 1968. Microorganisms in foods. Their significance and methods of enumeration. (Toronto, Canada; Univ. of Toronto Press and Oxford, England; Oxford Univ. Press).

Thompson, S. 1953. Paratyphoid fever and bakers confectionery. Mon. Bull. Minist. Hlth. 12, 187.

Todd, E.C.D. 1976. The first annual summary of food-borne disease in Canada. J. Milk Food Technol. 39(6), 426-431.

Tumermar, L., and B.H. Webb. 1965. Coagulation of milk and protein denaturation. In fundamentals of dairy chemistry, B.H. Webb and A.H. Johnson (Editors). Avi Publishing Co. Westport, Conn.

Vaeck, S.V. 1960. The four polymorphic forms α, β, β', γ in cocoa butter. Manufacturing Confectioner 40(6), 35.

Waters, J.R. 1972. Sensitivity of the $^{14}CO_2$ radiometric method for bacterial detection. Appl. Microbiol. 23, 198-199.

Weiser, H.H., Mountney, G.J., and W.A. Gould. 1971. Chap. 17. Radiation in food preservation. P269-282. Practical Food Microbiol. and Technol. Second Ed. Avi Co.

Weissman, M.A., and J.A. Carpenter. 1969. Incidence of Salmonellae in meat and meat products. Appl. Microbiol. 17, 899.

Wekell, M.M., and C.S. Martinsen. 1975. Growth of Salmonella heidelberg at room temperature in irradiated and non-irradiated potato chip dip. J. Milk Technol. 38, 259-261.

Welch, H., Ostrolenk, M., and M.T. Bartram. 1941. Role of rats in the spread of food poisoning bacteria of the Salmonella group. J. Am. Public Health 31, 332-340.

Werner, M., Janecke, H., and R. Brendel. 1972. Vitamin D enrichment of milk by UV irradiation. Milchwissenschaft 27(9), 562-569.

Wilder, A.N., and R.A. MacCready. 1966. Isolation of Salmonella from poultry, poultry products and poultry processing plants in Massachusetts. New Eng. J. Med. 274, 1453-1460.

Wilson, E.R.S., Paffenbarger, M.J., Foter, M.J., and K.H.Lewis. 1961. Prevalence of Salmonella in meat and poultry products. J. Infect. Dis. 109, 166.

Wilson, M.M., and E.F. Mackenzie. 1955. Typhoid fever and salmonellosis due to the consumption of infected desiccated coconut. J. Appl. Bacteriol. 18, 510.

Witkin, E.M. 1946. Nuclear segregation and the delayed appearance of induced mutations in E. coli. Cold Spring Harbor Symposia Quant. Biol. 16, 357-371.

Witkin, E.M. 1969. Ultraviolet-induced mutation and DNA repair Ann. Rev. Microbiol. 23, 487-514.

World Health Organization (WHO). 1969. Salmonella surveillance 2nd quarter 1968. Weekly Epidemiological Record Vol. 44. No. 19, May 19.

World Health Organization (WHO). 1973. Salmonella surveillance other than S. typhi and S. paratyphi, 1971. Weekly Epidemiological Record Vol. 48. No. 39, September 28.

Wulff, D.C., and S.S. Rupert. 1962. Disappearance of thymine photodimer in ultraviolet irradiated DNA upon treatment with a photoreactivating enzyme from baker's yeast. Biochem. Biophys. Res. Commun. 7, 237-240.

Yndestad, M., Underdal, B., and J. Novdal. 1972. The effects of UV radiation on the bacterial counts and shelf-life of chicken carcasses. Acta Agriculture, Scandinavica, 22(3), 169-172.