

1978

# Combined enrichment procedure for the detection of salmonellae in foods

William Henry Sveum  
*Iowa State University*

Follow this and additional works at: <https://lib.dr.iastate.edu/rtd>

 Part of the [Agriculture Commons](#), and the [Food Science Commons](#)

## Recommended Citation

Sveum, William Henry, "Combined enrichment procedure for the detection of salmonellae in foods " (1978). *Retrospective Theses and Dissertations*. 6422.  
<https://lib.dr.iastate.edu/rtd/6422>

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact [digirep@iastate.edu](mailto:digirep@iastate.edu).

## INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure you of complete continuity.
2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.
3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.
4. For any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.
5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.

University  
Microfilms  
International

300 N. ZEEB ROAD, ANN ARBOR, MI 48106  
18 BEDFORD ROW, LONDON WC1R 4EJ, ENGLAND

7907285

SVEUM, WILLIAM HENRY  
COMBINED ENRICHMENT PROCEDURE FOR THE  
DETECTION OF SALMONELLAE IN FOODS.

IOWA STATE UNIVERSITY, PH.D., 1978

University  
Microfilms  
International 300 N. ZEEB ROAD, ANN ARBOR, MI 48106

**Combined enrichment procedure for the detection  
of salmonellae in foods**

by

**William Henry Sveum**

**A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of  
The Requirements for the Degree of  
DOCTOR OF PHILOSOPHY**

**Major: Food Technology**

**Approved:**

Signature was redacted for privacy.

**In Charge of Major Work**

Signature was redacted for privacy.

**For the Major Department**

Signature was redacted for privacy.

**For the Graduate College**

**Iowa State University  
Ames, Iowa**

**1978**

**TABLE OF CONTENTS**

	<b>Page</b>
<b>INTRODUCTION</b>	<b>1</b>
<b>LITERATURE REVIEW</b>	<b>4</b>
<b>MATERIALS AND METHODS</b>	<b>28</b>
<b>RESULTS AND DISCUSSION</b>	<b>50</b>
<b>SUMMARY</b>	<b>103</b>
<b>CONCLUSIONS</b>	<b>107</b>
<b>LITERATURE CITED</b>	<b>109</b>
<b>ACKNOWLEDGEMENTS</b>	<b>123</b>

## LIST OF TABLES

	Page
Table 1. Composition of conventional tetrathionate and combined tetrathionate broths per liter	30
Table 2. Composition of SC broth and the selenite combined basal media per liter	32
Table 3. Comparison of heat treatments used to produce a population of thermally stressed <u>S. typhimurium</u>	51
Table 4. Effect of heating <u>E. coli</u> suspensions at 52 C for 30 min	51
Table 5. Effect of adding selenite to SEL 1 broth 0, 4, and 6 h after inoculation with heat injured salmonellae and grown for 24 h	58
Table 6. Number of thermally injured salmonellae recovered from lactose and combined selenite broths after 24 h incubation and following an additional 24 h incubation in SC broth	59
Table 7. Number of thermally injured salmonellae recovered from lactose and CTET broths after 24 h incubation and following an additional 24 h incubation in TET broth	59
Table 8. Percent of freeze injured salmonellae after 4 h incubation at 35 C in nonselective recovery media	60
Table 9. pH values of salmonellae enrichment media after 24 h incubation	72
Table 10. Number of salmonellae present in Ringer's solution suspensions stored for 0, 24 and 48 h at 5 C	75
Table 11. Comparison of conventional and combined methods for the recovery of salmonellae from frozen ground beef	79
Table 12. Comparison of conventional and combined methods for the recovery of <u>Salmonella</u> serotypes from frozen ground beef	83
Table 13. Competitive flora present in frozen turkey roasts	85

	Page
Table 14. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts	87
Table 15. <u>Salmonella</u> species isolated from samples of frozen control turkey roasts	88
Table 16. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts	89
Table 17. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts incubated at 35 C and 43 C	91
Table 18. Comparison of conventional and combined methods for the recovery of salmonellae from dried beef gravy, vegetable beef soup, and spray dried egg albumen inoculated with salmonellae	94
Table 19. Comparison of FA and conventional identification methods for the detection of salmonellae in various foods inoculated with known salmonellae	97
Table 20. Comparison of conventional and combined methods for the recovery of salmonellae from various foods inoculated with salmonellae	100
Table 21. Comparison of conventional and combined methods for the recovery of salmonellae from various foods inoculated with salmonellae and enriched for 24 and 48 h	101

## LIST OF FIGURES

	Page
Figure 1. Schematic outline of the MPN presumptive and confirmed tests used to determine the number of <u>E. coli</u> and salmonellae in <u>Salmonella</u> isolation media	39
Figure 2. Schematic outline of <u>Salmonella</u> isolation methods	46
Figure 3. Growth of heat injured <u>S. typhimurium</u> in lactose broth and CTET broth	55
Figure 4. Growth of heat injured <u>S. anatum</u> in lactose broth and CTET broth	56
Figure 5. Growth of uninjured <u>S. typhimurium</u> and <u>E. coli</u> in various salmonellae enrichment media	63
Figure 6. Growth of heat injured <u>S. typhimurium</u> and <u>E. coli</u> in various salmonellae enrichment media	66
Figure 7. Growth of heat injured <u>S. anatum</u> and <u>E. coli</u> in various salmonellae enrichment media	68
Figure 8. Growth of heat injured <u>S. heidelberg</u> and <u>E. coli</u> in various salmonellae enrichment media	70
Figure 9. Bacterial counts of beef patties during frozen storage of 60 days	78
Figure 10. Direct FA staining effects when the combined and conventional enrichment media were used to enrich samples of frozen ground beef containing <u>S. typhimurium</u>	98



## INTRODUCTION

Foods that contain Salmonella present a significant health hazard to the consumer (National Academy of Sciences, 1969). There are more than 1700 known Salmonella serotypes and variants; every serotype is potentially capable of producing an infection in man (Edwards and Galton, 1967). Consumption of food products containing these organisms may result in salmonellosis, a foodborne or waterborne infection characterized by gastroenteritis, usually 12 to 36 h following consumption of the contaminated source. Salmonellosis has become the most important animal-borne bacterial infection of man (Weiner, 1974). The presence of salmonellae in animal feeds also poses problems to the poultry and livestock industries. Because of salmonellae infections there are losses due to the death of young livestock and poultry as well as decreased milk and egg production and reduced weight gain. Also, the need to produce salmonellae free products requires that the food industry maintain costly testing and control programs and absorb losses due to the disposal of contaminated products.

The Center for Disease Control (CDC) recorded 23,285 isolations from humans in 1976 (Center for Disease Control, 1977). This was a decrease of 160 cases from the previous year but doesn't reflect a decrease in the incidence of salmonellosis. Fewer cases were reported because reporting centers have reduced their activities primarily due to budgetary reductions.

Beef or beef products were the most probable vehicle of transmission involved in the 42 outbreaks reported to CDC in 1976. Cohen and Blake (1977) reported that between the years of 1963 and 1975 the number of meat-associated outbreaks, particularly beef, increased while poultry-associated outbreaks showed no significant trend, and egg-associated outbreaks declined significantly. However, the number of cases reported to CDC is only a small percentage of the true incidence of salmonellosis in the United States. Aserkoff et al. (1970) made an estimate of the number of actual cases. They stated, that, "Data on the number of unreported cases obtained from investigations of several large epidemics suggest that the actual incidence may be as great as 100 times that reported."

A great deal of research has been done in the area of salmonellae detection methodology because improvements would be beneficial to both the consumer and food processor. Much of this work has involved the development of more effective enrichment media used to enhance the isolation of salmonellae from foods.

The present study was undertaken to determine the feasibility of combining the separate steps of preenrichment and selective enrichment used in salmonellae assays into a single enrichment step. This was attempted by adding the appropriate selective agents by pipette to a nonselective basal medium after a sufficient period of preenrichment to allow injured salmonellae to undergo

cellular repair in the absence of inhibitory agents. Following the addition of selective agents, an environment would be created in which the resuscitated salmonellae would proliferate but the growth of competitors would be inhibited. The purpose of this modified enrichment procedure was to provide a reliable method which would shorten incubation time, reduce the materials and labor required, and enhance the detection of injured salmonellae in comparison to conventional salmonellae detection procedures.

## LITERATURE REVIEW

### Introduction

Before literature reports are presented, an introductory discussion is given here on the basis for the methodology involved.

The methods used to examine various foods for Salmonella often differ from those procedures utilized by clinical and public health laboratories. Samples must undergo some type of liquid enrichment step since food or feed samples cannot be plated directly onto selective agars, particularly when the products are processed by heat, cold, or drying. Commonly used procedures for analyzing foods for salmonellae utilize 5 basic steps: (i) preenrichment and/or selective enrichment; (ii) plating the enrichment broth on selective or differential agars; (iii) biochemical screening; (iv) serological screening; and (v) final identification of the isolates.

Liquid enrichment media are used to favor the resuscitation of injured salmonellae and enhance the growth of those salmonellae present. Since foods undergo processing steps such as heating, freezing, and drying, any salmonellae not destroyed may exist in a sublethally injured state. Highly selective media may not allow these injured organisms to regain viability. Such organisms may be undetected, and then the result might be the release of products containing Salmonella into consumer channels. Similarly, competing gram negative bacteria, such as pseudomonads, Proteus, and coliforms, unless inhibited by a selective enrichment step, may inhibit or mask the growth of salmonellae on selective agars.

These problems are overcome by using both nonselective and selective enrichment media which favor the resuscitation and resulting multiplication of those stressed salmonellae present. A nonselective enrichment step enables injured cells to undergo repair in the absence of inhibitory selective agents. Following this nonselective preenrichment step, portions of the enrichment or products that are highly contaminated and haven't undergone sublethal processing are inoculated into selective enrichment media. The multiplication of competitors is limited by the selective enrichment step while those salmonellae present are able to multiply readily. Thus, higher levels of Salmonella result from selective enrichment and increase the number of salmonellae plated on selective agars.

#### Preenrichment

Processed foods may often contain a population of stressed or sublethally injured microorganisms (Busta, 1976). Unless analytical procedures are used that take this phenomenon under consideration, injured salmonellae in such products may not be detected. The preenrichment step was designed to alleviate the problem by providing a nonselective environment in which stressed salmonellae could recover. Greater levels of salmonellae would then be available for inoculation into selective media. This would enhance the detection of low numbers of Salmonella in the presence of large populations of competing microorganisms. Preenrichment is not recommended for heavily

contaminated products such as raw meats. Use of a nonselective enrichment would favor the growth of other organisms in the food or feed which would interfere with the isolation of those salmonellae present. Inactive cells readily regain metabolic activities in a suitable nonselective preenrichment medium.

The usefulness of preenrichment was suggested by Slocum (cited in Dack, 1955). One of the first investigators to demonstrate the effectiveness of preenrichment to enhance the detection of salmonellae was Thomson (1955). He was able to recover Salmonella paratyphi B from flour stored for a period of one year by utilizing nutrient broth as an enrichment medium. No salmonellae were recovered from flour samples inoculated directly into selenite F broth after only 5 months storage. Byrne et al. (1955) enhanced the isolation of Salmonella from active dried yeast by incubating the yeast in distilled water for 24 h prior to selenite F enrichment.

The most commonly used preenrichment medium is lactose broth which has demonstrated the best recovery rates (Troller, 1976, and Litchfield, 1973). The use of lactose broth as a preenrichment medium was first described by North (1961). The detection of low levels of salmonellae in dried egg albumen was greatly improved when lactose broth was used as a recovery medium prior to selective enrichment. Several workers confirmed the effectiveness of North's procedure of preenrichment in lactose broth. Taylor and Silliker (1961) observed that preenrichment in lactose broth was superior to direct inoculation of

tetrathionate and selenite-cystine broths. They also found lactose preenrichment to be more efficient than preenrichment in buffered water. Taylor et al. (1964) found the direct enrichment method of the Food Hygiene Laboratory of England and the procedure of using lactose broth as a preenrichment medium in the United States to be comparable. The effectiveness of preenrichment in lactose broth before selective enrichment was also demonstrated in studies by Hall et al. (1964). Preenrichment in lactose broth was adopted for use in salmonellae assays by the Food and Drug Administration (1969), the Association of Official Analytical Chemists (Poelma, 1967) and the International Commission on Microbial Specifications for Foods (Thatcher and Clark, 1968). The usefulness of preenrichment in lactose broth was more recently demonstrated by Gabis and Silliker (1974). Preenrichment in lactose broth prior to selective enrichment increased the recovery of salmonellae from high moisture foods.

Because most Salmonella are unable to ferment lactose, many investigators have advocated the use of preenrichment media containing carbohydrates that salmonellae are able to ferment. Salmonella, though, can readily multiply to large populations in lactose broth. Mannitol purple sugar broth was used for preenrichment by Taylor (1961). Preenrichment in this medium was superior to direct enrichment or centrifugation of samples. Taylor and Silliker (1961), in confirming the lactose preenrichment method of North, observed no difference in recovery among dulcitol, lactose, mannitol, or purple sugar broths. Geritcher and Sechter (1966) compared recovery in different carbohydrate broths to direct

selective enrichment. They found preenrichment to be superior but found no advantage of dulcitol or mannitol broths over lactose broth. More recently, van Schothorst and van Leusden (1972) also observed no advantage of dulcitol broth over lactose broth for preenrichment of nonfat dry milk containing injured salmonellae. Kaper et al. (1977) isolated more salmonellae from estuarine water samples that were preenriched in dulcitol and inositol broths in comparison to preenrichment in lactose broth. However, water samples interact quite differently with salmonellae enrichment media than do food or feed samples.

Numerous methods of preenrichment which do not utilize lactose broth have been presented in the literature. Salmonellae in egg albumen were concentrated by Sugiyama et al. (1960) by preenriching samples in lauryl tryptose or nutrient broths containing polyvalent antiserum. Improved recoveries were obtained by centrifuging the flocculated organisms prior to selective enrichment. Taylor and Silliker (1961) also enhanced detection of salmonellae using centrifugation of samples. The centrifugation step increased recoveries by separating the bacteria from soluble foods which had caused reductions in the selectivity of enrichment broths. Preenrichment of whole dried egg in nutrient broth was more efficient than direct enrichment in selenite broth (Hobbs, 1963) or tetrathionate broth (Galton et al. 1964).

Sterile water or reconstituted nonfat dry milk containing low concentrations of crystal violet or brilliant green dyes was suggested by North (1960) for use as a preenrichment medium for dried foods. Poelma et al. (1969) also



confirmed the usefulness of reconstituting dried foods in sterile water containing brilliant green dye. The Food and Drug Administration (1976) currently recommends this preenrichment procedure for various desiccated foods.

Suspending food samples for several hours in phosphate buffer followed by overnight incubation in lactose broth was suggested by Montford and Thatcher (1961). Mossel and Ratto (1970) detected more injured Enterobacteriaceae in dried foods incubated for 1 to 6 h at room temperature in tryptone soya peptone broth than in samples incubated at 30 C overnight in lactose broth. Thermally injured S. typhimurium multiplied more readily in trypticase soy broth (TSB) than in lactose, nutrient, or lauryl tryptose broth (Clark and Ordal, 1969). Resuscitation of stressed salmonellae subjected to desiccation was more complete in TSB than lactose broth (van Schothorst and van Leusden, 1972). No differences in recovery were observed by Wilson et al. (1975) when either TSB or sterile distilled water were used as preenrichment media for salmonellae-contaminated samples of pressed yeast and dried inactive yeast.

Recently, the use of buffered peptone water has been suggested by many researchers. Edel and Kampelmacher (1973, 1974) used buffered peptone water as a preenrichment medium for comparative studies of Salmonella isolations from feeds and naturally or artificially contaminated minced meat. Their preenrichment method produced more salmonellae isolations than methods in which direct selective enrichment was utilized. Buffered peptone water was equal to lactose broth as a preenrichment medium for recovery of salmonellae from egg

products (van Schothorst and van Leusden, 1975a). Vassiliadis et al. (1976) recovered more salmonellae from minced meat preenriched in buffered peptone water than those samples placed directly in selective enrichment broths. In another comparative study (Smith, 1977), low numbers of salmonellae were detected using buffered peptone water prior to selective enrichment of animal feeds. Harvey and Price (1977) enhanced the recovery of Salmonella from sewage samples by using buffered peptone water as a preenrichment medium prior to selective enrichment in tetrathionate broth. More salmonellae were isolated by Thomason et al. (1977) from environmental samples enriched with buffered peptone water than lactose broth or directly enriched in selective media. They believed their results were due to the ability of the competitors to reach much higher levels in lactose broth than in buffered peptone water, and thus not be suppressed sufficiently in the selective media. Thomason and Dodd (1978) isolated more salmonellae from ground beef, pork sausage, and chicken livers which were either preenriched in buffered peptone water or directly enriched in tetrathionate than from those samples preenriched in lactose broth.

### Selective Enrichment

Selective enrichment broths are used in Salmonella isolation procedures to favor and enhance the growth of salmonellae while inhibiting other organisms present in the food sample. The various selective media utilized have been

extensively reviewed by Galton et al. (1968), Litchfield (1973), and Fagerberg and Avens (1976).

There are numerous types of Salmonella enrichment media but the most commonly utilized for food samples are selenite-cystine broth (North and Bartram, 1953) a modification of Liefson's (1936) selenite broth and Mueller's (1923) tetrathionate broth as modified by Kauffmann (1930, 1935) with the addition of brilliant green and bile salts. Kauffmann (1935) demonstrated the need for selective enrichment when he used tetrathionate brilliant green broth to examine fecal specimens from patients with acute gastroenteritis. The number of isolations of Salmonella on brilliant green phenol red agar (Kristensen et al. 1925) increased by 500 per cent when enrichment was used rather than no selective enrichment prior to plating.

Since many modifications of the existing salmonellae enrichment media have been made, it is apparent that no one medium is capable of providing consistent isolations of salmonellae. Part of this variability is due to diversity among serotypes and the detrimental effects that various foods have on the selectivity of the enrichment broths. Silliker and Taylor (1958) demonstrated a decrease in selectivity of selenite and tetrathionate broths due to the addition of different foods. Modifications are made to enhance the suppression of competing gram negative organisms such as Proteus, Pseudomonas, and coliforms, and various gram positive bacteria.

The modification of selenite F broth by North and Bartram (1953) included the addition of cystine and a decreased phosphate concentration. Cystine enhanced the growth of salmonellae in the presence of large amounts of organic material. Use of selenite-cystine broth resulted in enhanced isolations of salmonellae from egg products compared to recoveries with selenite F broth. Another modification of selenite F broth included the addition of brilliant green alone (Stokes and Osborne, 1955) or with sulfapyridine (Osborne and Stokes, 1955). These modifications were designed to inhibit Escherichia and Proteus while allowing very low numbers of salmonellae to reach detectable levels. Silliker et al. (1964) reported the selectivity of selenite broth was improved by the addition of a sterile filtrate of feces which aided in the inhibition of competitors. The substitution of dulcitol for lactose in selenite broth (Raj, 1966) was claimed to improve the isolation of salmonellae from seafoods. Grau and Smith (1972) described mannitol-selenite-cystine broth which gave improved recoveries of salmonellae from sheep feces in comparison to nutrient broth, tetrathionate, and mannitol-selenite broths.

Numerous modifications of tetrathionate broth have been made to aid in the isolation of salmonellae from foods. Kauffmann's addition of brilliant green to tetrathionate broth (1930, 1935) has been widely accepted by many regulatory agencies (Fagerberg and Avens, 1976). In order to reduce the growth of Proteus in tetrathionate broth, Galton et al. (1950) added sodium sulfathiazole, Jeffries (1959) used novobiocin, and Jameson (1961) added

1% sodium lauryl sulfate and bismuth sulfite to suppress the growth of Proteus in sewer samples. A problem exists with these modifications though, the possibility exists of increasing the inhibitory effects of the medium against Salmonella (Litchfield, 1973).

The most commonly utilized modification of tetrathionate broth with or without brilliant green is the addition of a wetting agent, sodium heptadecyl sulfate (Tergitol 7). This compound aids in dispersing and emulsifying the fat layer in the enrichment broth when fatty foods are analyzed (Galton et al. 1954). Morris and Dunn (1970) added Tergitol 7 to tetrathionate brilliant green broth and reported the improved detection of salmonellae in pork sausage. Another modification of tetrathionate broth by Hajna and Damon (1956) has not been evaluated extensively. These workers enriched tetrathionate brilliant green broth by including yeast extract, dextrose and mannitol, and replaced the bile salts with sodium deoxycholate.

Many investigators have reported the inhibition of various serotypes in commonly used enrichment media. Literature reports indicate that no one medium allows maximum recovery for all serotypes (Fagerberg and Avens, 1976, and Kafel and Bryan, 1977). Gundstrup et al. (1969) suggested using both tetrathionate and selenite-cystine broths to insure detection of all salmonellae since inhibitory compounds which effected certain Salmonella were detected in selenite broth. The use of two different enrichment media in combination has

been demonstrated to increase recovery of salmonellae by many investigators (Huhtanen and Naghski, 1972; Tompkin and Kueper, 1973; Gabis and Silliker, 1974; and Silliker and Gabis, 1974).

Because certain Salmonella serotypes are unable to multiply in selenite and tetrathionate broths, several media that utilize different inhibitory compounds have been introduced. Brilliant green when added to MacConkey broth (Smith, 1959) increased isolations of S. choleraesuis. Gram negative broth (Hajna, 1955) is less inhibitory to Salmonella than other selective enrichment media but its usefulness as an enrichment medium for foods has not been determined. Cox et al. (1972) examined poultry products and chicken feces and obtained somewhat lower recoveries with gram negative broth than with selenite-cystine and Rappaport broths.

Rappaport broth as described by Rappaport et al. (1956) is an enrichment broth containing magnesium chloride and malachite green, which permits unrestricted growth of salmonellae but inhibits coliforms. Even though the medium was originally developed for use with clinical specimens, several groups have obtained variable results when using Rappaport broth as an enrichment medium for foods (Litchfield, 1973). Recently, Vassiliadis et al. (1976) demonstrated a slight superiority of enrichment of minced meat in Rappaport broth in comparison to enrichment in tetrathionate broth.

Banic (1964) described a magnesium selenite medium which was superior to tetrathionate broth for isolating S. typhi but unsuccessful in

comparison to other enrichment broths for detecting other salmonellae. Two enrichment media, strontium chloride and strontium selenite broths, which also incorporated specific ions to achieve a selective environment were introduced by Iveson and MacKay-Scollay (1969). These media are claimed to suppress the growth of Proteus more readily than selenite or tetrathionate broths. The analysis of salmonellae contaminated samples from man, animals, meat products, and abattoir effluents showed greater isolations with strontium selenite and strontium chloride broths than selenite F broth (Iveson and MacKay-Scollay, 1972). Strontium selenite was also superior to selenite F broth in detecting S. typhi (Chau and Forrest, 1972).

Hargrove et al. (1971) introduced neutral red-lysine-iron-cystine broth for the isolation of Salmonella from dairy products. This medium was modified by Hoben et al. (1973) for use in determining the presence of salmonellae in foods. Their modification, lysine-iron-cystine-neutral red broth was useful as a rapid technique in screening Salmonella negative samples and presumptively identifying salmonellae positive products. Lysine-iron-cystine-neutral red broth was used as an alternative to conventional plating media for the presumptive identification of salmonellae (D'Aoust, 1977a). Satisfactory results were obtained with samples first enriched in tetrathionate brilliant green broth but unreliable results were obtained from samples enriched first in selenite-cystine broth.

Although many claims have been made advocating the effectiveness of the aforementioned media, the literature indicates that of all the media reviewed, selenite-cystine and tetrathionate broths or minor modifications of them will give the highest recovery of salmonellae from foods. Since various serotypes vary in their response to selenite-cystine and tetrathionate broths, the use of both in parallel is recommended for maximum detection of salmonellae. Selenite-cystine and tetrathionate broths are recommended for use as selective enrichment media by the Food and Drug Administration (1976), the Association of Official Analytical Chemists (1975a) and the National Academy of Sciences (1971).

### Injury

When subjected to environmental stresses, microorganisms may become sublethally injured. A characteristic of this injured state is the inability to tolerate conditions in which normal cells would readily multiply. When a suitable recovery medium is provided, a damaged cell may undergo resuscitation and regain its normal metabolic functions. Processes utilized in the manufacture of foods such as freezing, freeze drying, heating, desiccation, acidification, and irradiation will induce an injured condition. Busta (1976) indicated the need to recognize the importance of injured microorganisms in foods so that adequate procedures would be used to determine the presence of spoilage types and pathogenic organisms after being stressed.

The detection and enumeration of injured microorganisms in foods has in the last few years received much attention in the literature. This subject has been



reviewed by Ray and Speck (1973a); Kueck (1974); Lanz (1975); van Schothorst (1976); Busta (1976); and Hurst (1977). Injury of foodborne microorganisms with an emphasis on the Enterobacteriaceae has been associated with cells exposed to heating (Clark and Ordal, 1969; Tomlins and Ordal, 1971; Wilson and Davies, 1976; Tomlins and Ordal, 1976; and D'Aoust, 1978), freezing (Ingram and Mackey, 1976; and Speck and Ray, 1977), freeze drying (Sinskey and Silverman, 1970; Ray et al. 1971a and 1971b), drying (van Schothorst and van Leusden, 1972 and 1975b), irradiation (Licciardello et al. 1970; and Maxcy, 1977), sanitizers (Scheusner, et al. 1971a) and acidification (Roth and Keenan, 1971). Two manifestations of environmental stresses will be reviewed: the sensitivity of injured cells to selective agents and the time required for repair of stressed cells.

Many investigators have demonstrated the increased susceptibility of injured cells to secondary stresses. For purposes of this review concerning selective agents, injury can be defined as an increased or totally new sensitivity to selective agents, antimicrobial compounds or similar substances present in the growth medium being utilized (Busta, 1976). The sublethally impaired microorganisms are inhibited by conditions which would not affect the growth of normal cells. Because of this effect, microbiological procedures that utilize selective media may fail to detect injured organisms that are unable to replicate in an inhibitory environment. A major characteristic of injured cells is an extended lag phase, commonly referred to as the time required to

repair cellular damage or undergo resuscitation.

As indicated in the introductory statement to this section, injured microorganisms, if provided with a suitable, noninhibitory recovery medium, are able to repair cellular damage and return to a normal physiological state. Various repair times have been reported with many researchers demonstrating the ability of impaired cells to repair damage within 1 to 6 h following inoculation into the appropriate medium.

Using heat injured S. typhimurium, Clark and Ordal (1969) observed that the injured cells required 5 h to repair in TSB. Tomlins and Ordal (1971), using the same organism, determined that resuscitation occurred after 4 h in citrate-glucose-minimal salts broth. The injured cells were defined as those members of a population that had lost the ability to replicate in the presence of 2% NaCl incorporated into eosin methylene blue agar (EMBS). In comparison to TSB, repair was slightly slower in lactose, nutrient, and lauryl sulfate tryptose broths. An 8 h lag period was observed when selective media, tetrathionate and selenite F broths were used as recovery menstrooms. Thermally injured S. seftenberg 4969 cells were able to repair within 3 h in a minimal medium containing salts and glucose (Wilson and Davies, 1976). Repair in lactose broth was as rapid but resulted in slightly lower cell numbers. Resuscitation was slower and produced lower yields in nutrient, trypticase soy yeast extract (TSYE), and tetrathionate broths. Selenite broth, however, was extremely toxic to injured cells and inhibited recovery.

Sinskey and Silverman (1970) found that Escherichia coli ML30 cells when injured by freeze drying were unable to multiply on a minimal salts plating medium. Metabolic damage could be repaired, however, after a 5 h resuscitation period in citrate-minimal salts broth. S. anatum NF3 cells were also stressed by freeze drying (Ray et al. 1971a). The injured cells were unable to replicate in the presence of 0.25% sodium deoxycholate. Tolerance to this bile salt was regained following 2 to 3 h incubation in sterile water or lactose broth. This was demonstrated by the increased number of colonies formed on xylose-lysine-peptone (XLP) agar containing 0.25% sodium deoxycholate.

Several investigators have studied the lag period caused by sublethal freezing treatments. Ray and Speck (1973b) and Warseck et al. (1973) found that substantial repair of stressed coliforms would occur in TSB within 1 to 2 hours. Injured coliforms, when surface plated on trypticase soy agar (TSA), were also able to undergo repair within 1 to 2 h (Speck et al. 1975). Cells of S. anatum NF3, after being frozen by Ray et al. (1972), were unable to form colonies when surface plated on XLP agar plus 0.2% sodium deoxycholate. Most of the injured salmonellae were repaired in TSYE broth or citrate-glucose-minimal salts broth after 1 to 2 h incubation prior to surface plating on XLP agar plus 0.2% sodium deoxycholate.

Resuscitation of sublethally impaired cells in dried foods has also been investigated. Mossel and Ratto (1970) determined that 1 to 6 h incubation in

tryptone soya peptone broth was a sufficient restoration period for injured Enterobacteriaceae. However, optimum results were obtained when foods were incubated only 1 to 2 h in the nonselective medium prior to selective enrichment. Similar results were observed by van Schothorst and van Leusden (1975b) who determined the recovery time required for injured salmonellae in dried milk powder. These investigators determined that most injured salmonellae were insensitive to bile salt components after 3 h incubation in buffered peptone water. A number of cells still remained sensitive after the 3 h recovery period. They believed that some salmonellae may be more severely injured than others, thus requiring a much longer resuscitation period. Martin et al. (1976) used catalase to enhance the recovery of sublethally injured S. typhimurium which had been subjected to reduced water activity and storage. The addition of catalase to the surface of EMBS agar plates increased the number of stressed cells by preventing the accumulation of hydrogen peroxide in or around the injured cells.

As previously discussed, injured cells are sensitive to secondary stresses such as sodium deoxycholate, salt, components of Salmonella selective enrichment media, and exposure to minimal media which require the injured microorganisms to synthesize essential metabolites. Exposure to additional secondary stresses have also been reported to inhibit the repair of sublethally injured microorganisms. A susceptibility to chloramphenicol, streptomycin, and actinomycin D, which does not normally occur in uninjured E. coli cells was demonstrated by freeze drying E. coli (Sinskey and Silverman, 1970). Exposure

of E. coli to a quaternary ammonium compound caused the cells to become sensitive to bile salts with sodium deoxycholate being most inhibitory (Scheusner et al. 1971b). Hartman et al. (1975) and Speck et al. (1975) demonstrated the increased recovery of stressed coliforms in the absence of bile salts and dyes, the selective agents of violet red bile agar. Starvation of a microbial population in a low nutrient aquatic environment will also induce cellular stress. Klein and Wu (1974) observed increased susceptibility of starved microorganisms to the transient stress of warm agar used in pour plate procedures. Recoveries of stressed coliforms from water samples was improved by using a 2 h enrichment in TSYE broth, a rich nonselective medium, prior to exposure to selective agents (Bissonnette et al. 1977).

#### Rapid Methods

The literature contains many reports of improved techniques or alternative methods to the existing procedures recommended for the isolation of salmonellae from foods. These standard methods may require from 5 to 7 days for complete identification of salmonellae positive samples. Because so much time is ordinarily required, many rapid techniques have been developed to detect Salmonella as well as to improve existing methods. In order for any modifications of existing procedures or rapid methods to be adopted for use, they must be demonstrated to be as efficient as the conventional 5 to 7 day procedures. These methods are outlined in the Bacteriological Analytical Manual (BAM) of the

Food and Drug Administration (1976), and in the Association of Official Analytical Chemists reference manual (1975a). There are five basic steps utilized with these recommended procedures, including: (i) preenrichment and/or selective enrichment; (ii) plating the enrichment broth on selective agars; (iii) biochemical screening; (iv) serological screening; and (v) final identification, based on more biochemical and serological tests. Since reports on rapid methods for salmonellae are quite profuse, this portion of the literature review will be limited to those rapid methods or procedure modifications which could be utilized in conjunction with the combined preenrichment-selective enrichment technique.

Many investigators have demonstrated the feasibility of using a single composite sample from a lot rather than multiple smaller samples. The pooling of samples decreases the number of analyses needed since only the composite sample that is Salmonella-positive need be further tested to determine which subsample is contaminated. This technique is especially useful when the frequency of salmonellae isolations is low because savings in time, materials, and labor are made by pooling samples. The Committee on Salmonella of the National Academy of Sciences (National Academy of Sciences, 1969) discussed "wet compositing" as an approach to control procedures for salmonellae detection. The technique involved the pooling of preenrichment cultures by adding 1 ml from each of 10 enrichment broths to 10 ml of double strength selective broth. Results from this compositing

procedure agreed favorably with conventional sampling procedures. Price et al. (1972) pooled 10 25-g samples and observed no apparent loss in sensitivity as compared to Food and Drug Administration (1969) sampling methods using direct selective enrichment. No significant difference was observed by Huhtanen et al. (1972) when meat- and bone-meal samples were examined as a single 300-g sample or 10 30-g samples. Several types of dried foods were tested for Salmonella by using composites, 60 25-g samples, 15 100-g samples, and 3 500-g samples (Silliker and Gabis, 1973). High-moisture foods contaminated with salmonellae were also pooled in 13 25-g, 3 108.3-g, 1 325-g, and 1 500-g subsamples by Gabis and Silliker (1974). Any of the compositing techniques was able to detect the salmonellae present in any one subsample. The U. S. Department of Agriculture (1974) also recommends the use of 3 500-g samples, rather than 60 25-g samples. Maximum size of a composite unit acceptable to the Food and Drug Administration is a 375-g sample consisting of a series of 25-g subsamples (Olson, 1975).

Salmonellae may be detected in foods by modifications of the fluorescent-antibody technique (FA) introduced by Coons et al. (1942). There are several distinct advantages that the direct FA method has over conventional isolation procedures; the capability to detect salmonellae in mixed culture, the sensitivity of the assay, and the ability to determine the presence of salmonellae in samples within 50 to 55 h. Arkhangel'skii and Kartoshova (1962) were the first investigators to use the FA procedure to detect salmonellae in foods. Following

this initial study, many investigators have developed modifications of the FA method for the rapid detection of Salmonella in foods. Cherry et al. (1975) have adequately reviewed many of these applications of the FA technique. Because many research groups have found the direct FA technique equal in efficiency to conventional cultural methods as a screening procedure, a FA method for the detection of salmonellae in foods was adopted by the Association of Official Analytical Chemists (1975b) and the Food and Drug Administration (1976). The FA technique is particularly suited for the examination of foods in which the incidence of salmonellae is low; such as chocolate and chocolate products (D'Aoust, 1977b).

Another rapid method which also utilizes conventional enrichment procedures is the enrichment serology (ES) method, introduced by Sperber and Deibel (1969). Detection of Salmonella can be completed within 50 h through the serological identification of salmonellae by using pooled poly "H" antisera rather than FA staining. Boothroyd and Baird-Parker (1973) found that the ES procedure detected 98% of the samples that were positive by conventional means. Use of the ES technique as a screening method was preferred by Mohr et al. (1974) over FA and conventional methods because they obtained fewer false positives with ES. The effectiveness of ES and FA procedures were reviewed by Hilker (1975). Almost all Salmonella-positive samples will be detected by FA methods but many false positive samples will not be screened out due to nonspecific staining, a major problem associated with the FA technique



(Swaminathan et al. 1978a). Fewer false positives are produced by the ES method but 1 to 3% of the positive samples will not be detected.

Another rapid technique capable of detecting salmonellae in foods within 50 h is the enzyme-labeled antibody technique (ELAT) developed by Krynski and Heimsch (1977). Samples are enriched using the cultural techniques of Sperber and Deibel (1969) and analyzed for salmonellae by placing portions of the enrichment broth on membrane filters. The membranes are first immersed in rabbit antflagella antibody, washed, and then immersed in peroxidase-labeled goat anti-rabbit antibody. In the presence of the appropriate substrate, peroxidase causes color reactions which will occur on spots containing salmonellae. The authors stated that the ELAT method was sensitive to fewer salmonellae cells,  $10^5$  Salmonella per ml than the  $5 \times 10^7$  per ml needed for detection by FA or ES techniques. False positive results were obtained though, when "H" antisera contained nonspecific "O" antibodies which caused nonspecific staining.

The identification of presumptive Salmonella isolates from selective agar plates requires the use of numerous biochemical tests. Currently, several commercially available miniaturized multitest kits are available which can be used to identify Enterobacteriaceae isolates from foods. There is a high degree of correlation between the results of comparison studies determining the efficiency of the multitest techniques to conventional biochemical procedures. Most evaluations of these multitest systems have been designed to compare their efficiency to conventional tube methodology for the identification of clinical

specimens. Recently, several reports have dealt with the usefulness of these systems for identification of isolates from foods. Cox et al. (1977) reviewed the commercially available multitest systems for the identification of Enterobacteriaceae. Five multitest kits were evaluated for their ability to identify salmonellae isolates from naturally contaminated foods by Poelma et al. (1977). The API-20 Enteric (Analytab Products, Inc.) and Minitek (BioQuest, BBL) systems were found to be adequate to differentiate Salmonella from other Enterobacteriaceae without additional biochemical tests which are required with the Pathotec Rapid I-D System (General Diagnostics Division), Enterotube (Roche Diagnostics), and the Modified Expanded R/B Enteric Differential System (Diagnostic Research, Inc.). Cox and Mercuri (1978) also determined that the API minikit was superior to the R/B system for identifying Enterobacteriaceae isolated from poultry and meat products. These results were also confirmed by Guthertz and Okoluk (1978) who determined the identity of organisms isolated from comminuted beef, pork, and turkey using the API, Pathotec, Minitek, and Inolex Enteric 1 (Inolex Biomedical Division) multitest systems. Both the API and Minitek methods provided more accurate and rapid identification of Enterobacteriaceae isolates than the other two systems.

This literature review indicates the lack of universally accepted methods for the isolation of salmonellae from processed foods. Some uniformity has been achieved by virtue of the Food and Drug Administration's specified methods

(Food and Drug Administration, 1976). Although many enrichment alternatives exist to the generally accepted preenrichment and selective enrichment techniques, it is apparent that both these procedures are generally needed to detect the presence of sublethally injured salmonellae in foods. Use of lactose broth as a resuscitation medium and SC and TET broths as isolation media has been demonstrated to be a reliable method. Several rapid techniques have also been described which greatly shorten the time needed to detect salmonellae in foods with the FA procedure being most widely accepted. The recent introduction of miniaturized multitest kits has also reduced the problem of time required in biochemically identifying presumptive Salmonella isolates by making it possible to rapidly test many more cultures at one time. Application of the combined enrichment procedure would utilize the benefits of both nonselective and selective enrichment in a shorter time period. Used in combination with techniques such as the FA procedure, rapid detection of Salmonella in foods could be achieved.

## MATERIALS AND METHODS

### Organisms

Cultures of S. typhimurium, S. heidelberg, S. anatum, and S. infantis, originally isolated from poultry meat and E. coli isolated from raw milk were obtained from the collection in the Department of Food Technology, Iowa State University, Ames, Iowa. The identity of each Salmonella serotype was confirmed serologically by the State Hygienic Laboratory, Iowa City, Iowa.

The cultures were maintained on TSA slants. Each culture was transferred monthly to a fresh slant, incubated at 35 C ( $\pm$  1 C) for 20 h and stored at about 5 C.

### Conventional Media

The following media were utilized: lactose (Lac) broth, selenite-cystine (SC) broth, tetrathionate (TET) broth, brilliant green bile 2% (BGB 2%) broth, brilliant green (BG) agar, Salmonella-Shigella (SS) agar, bismuth sulfite (BS) agar, Levine eosin methylene blue (EMB) agar, triple sugar iron (TSI) agar, and lysine iron (LIA) agar. The media were obtained from Difco Laboratories, Detroit, Michigan, except for TSB and TSA obtained from BBL, Cockeysville, Maryland. The manufacturers directions for preparation were followed for each medium.

### Combined Enrichment Media

Four combined enrichment media were utilized, selenite one (SEL 1) broth, selenite two (SEL 2) broth, selenite three (SEL 3) broth, and combined tetrathionate (CTET) broth. The enrichment broths combined preenrichment in a nonselective basal medium similar to lactose broth and selective enrichment in SC broth and TET broth.

CTET broth as previously described by Sveum and Hartman (1977) consisted of (per liter): lactose broth (Difco), 13 g; sodium thiosulfate, 30 g; calcium carbonate, 10 g; and bile salts (Difco), 1 g. The composition of CTET and TET broth is presented in Table 1. The combined basal medium differed from TET basal broth due to the presence of lactose and beef extract, components of lactose broth. The CTET basal medium was adjusted to pH 6.8, heated to boiling, and 225 ml aliquots were aseptically dispensed into sterile 500 ml flasks.

Tetrathionate, the selective agent of TET and CTET broths is not formed until iodine is added to the basal media. Sodium thiosulfate reacts with iodine to form tetrathionate according to the reaction:  $2 \text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O} + \text{I}_2 + [\text{KI}] \rightarrow \text{Na}_2\text{S}_4\text{O}_6 \cdot 2 \text{H}_2\text{O} + 2 \text{NaI} + [\text{KI}]$ . Iodine is added by pipette from a stock solution containing 6 g  $\text{I}_2$  and 5 g KI per 20 ml of sterile distilled water. An inhibitory concentration of tetrathionate is obtained by adding 2 ml of iodine per 100 mls of TET broth, therefore 225 ml of CTET basal required 4.5 ml of the iodine solution.

Table 1. Composition of conventional tetrathionate and combined tetrathionate broths per liter

Tetrathionate		Combined Tetrathionate	
Sodium thiosulfate	30 g	Sodium thiosulfate	30 g
Calcium carbonate	10 g	Calcium carbonate	10 g
Bile salts	1 g	Bile salts	1 g
Proteose peptone	5 g	Peptone	5 g
Iodine solution <sup>a</sup>	20 ml	Lactose	5 g
		Beef extract	3 g
		Iodine solution <sup>a</sup>	20 ml

<sup>a</sup>Dissolve 60 g of iodine crystals and 50 g of potassium iodide in 200 ml of sterile distilled water.

Three different selenite combined enrichment media were used in this study. SEL 1, introduced by Sveum and Hartman (1977) consisted of (per liter): lactose broth, 13 g; disodium phosphate, 10 g; and L-cystine, 0.01 g. The components of SEL 2 were (per liter): lactose, 5 g; peptone, 5 g; disodium phosphate, 10 g; and L-cystine, 0.01 g. SEL 3 ingredients were (per liter): lactose, 4 g; tryptone, 5 g; disodium phosphate, 10 g; and L-cystine, 0.01 g. Table 2 lists the constituents of the combined selenite broths and conventional SC broth. The selenite basals were adjusted to pH 6.8, and 225 ml quantities were added to 500 ml flasks and sterilized (121 C, 15 min).

The selective agent of SC broth, sodium acid selenite, is not present in the combined selenite basals. A selective environment was created by aseptically adding 10 ml of a filter sterilized solution of 9 g sodium acid selenite (BBL, Cockeysville, MD) per 100 ml of water to 225 ml of each selenite basal medium. SEL 1 and SEL 2 differed in composition from SC broth because they contained the components of lactose broth, peptone and beef extract, or peptone without beef extract, respectively. SEL 3 and SC broths were equal in composition.

#### Production of Heat Injured Salmonellae

Cultures of heat injured salmonellae were prepared, using a modification of the method of Clark and Ordal (1969). Frozen stock cultures were prepared for use as inocula by placing 0.1 ml of an actively growing culture into 10 ml of TSB and freezing the cultures at about -29 C. Tubes were thawed when needed

Table 2. Composition of SC broth and the selenite combined basal media per liter

SC		SEL 1		SEL 2		SEL 3	
Disodium phosphate	10.00 g	Disodium phosphate	10.00 g	Disodium phosphate	10.00 g	Disodium phosphate	10.00 g
Tryptone	5.00 g	Peptone	5.00 g	Peptone	5.00 g	Tryptone	5.00 g
Lactose	4.00 g	Lactose	5.00 g	Lactose	5.00 g	Lactose	4.00 g
L-cystine	0.01 g	Beef extract	3.00 g	L-cystine	0.01 g	L-cystine	0.01 g
Sodium acid selenite	4.00 g	L-cystine	0.01 g	Selenite solution <sup>a</sup>	40.00 ml	Selenite solution <sup>a</sup>	40.00 ml
		Selenite solution <sup>a</sup>	40.00 ml				

32

<sup>a</sup>Dissolve 40 g of sodium acid selenite in 400 ml of distilled water and filter sterilize the solution.



and the entire 10 ml was inoculated into a 500 ml flask containing 200 ml TSB. The culture was incubated for 16 h at 37 C on a rotary shaker. Cells were harvested by centrifuging a 40 ml sample at 2000 x g for 10 min at room temperature. The supernatant was discarded, and the cells were washed once in 100 mM sodium phosphate buffer (pH 6.0), centrifuged, and resuspended in 5 ml of phosphate buffer. The salmonellae were thermally stressed by adding the 5 ml suspension to 195 ml of preheated phosphate buffer and heating the cells at 52 C for 30 min under constant agitation in a water bath.

The extent of injury in the heat stressed population was determined by removing samples at various intervals and diluting them in 0.1% peptone distilled water blanks. This dilution menstroom was used for all enumeration procedures in this project unless otherwise noted. Appropriate dilutions were surface-plated in 0.1 ml amounts on predried TSA and EMBS plates which were incubated for 48 hr at 35 C. The number of colonies on TSA represented all viable cells, both injured and uninjured. Counts on EMBS represented only the number of uninjured cells in the population. The number of injured cells present in the heated buffer was equal to the difference between the TSA and EMBS counts.

#### Production of Heat Injured E. coli

The same technique used to heat injure salmonellae was utilized to produce a population of thermally stressed E. coli cells.

### Production of Freeze Injured Salmonellae

Modifications of the techniques of Ray et al. (1971a) and Ray et al. (1972) were utilized to produce populations of freeze injured salmonellae. Frozen stock cultures were thawed when needed and the entire contents added to 200 ml of TSB in a 500 ml flask. The culture was incubated for 16 h at 37 C on a rotary shaker. Cells were harvested by centrifuging a 30 ml sample at 2000 x g for 10 min at room temperature. The supernatant was decanted, and the cells were washed once in either 100 mM sodium phosphate buffer (pH 6.0), sterile H<sub>2</sub>O, or Ringer's solution consisting of (per liter): 960 ml of 0.154 M NaCl, 20 ml of 0.154 M KCl, and 20 ml of 0.11 M CaCl<sub>2</sub>. The suspensions were centrifuged again and the salmonellae were resuspended in 30 ml of the appropriate freezing menstruum. The cells were freeze injured by adding 1 ml of the suspension to 9 ml of the appropriate medium and uniformly suspending the cells in a 18 x 150 mm test tube with a vortex mixer. Freeze injury inducing treatments were: (i) immersion in liquid nitrogen (LN<sub>2</sub>) for 2 min; (ii) placement in carbon dioxide snow (CO<sub>2</sub> snow) for 10 min; or (iii) exposure to mechanical freezing in a blast freezer at -29 C for 24 h. Following the initial freezing treatment the cultures were stored at -29 C until needed.

The extent of injury in a frozen population was determined by thawing a frozen suspension in a beaker of water at 5 C for about 20 min. Samples were diluted in peptone water and surface plated on predried plates of TSA and BG agar or TSA plus 0.25% sodium deoxycholate (TSAD), and XLP prepared as

described by Ray et al. (1971a) and XLP plus 0.25% sodium deoxycholate (XLPD). The number of colonies on TSA and XLP represented all viable cells, both injured and uninjured in the population. Counts on BG, TSA, and XLPD represented the number of uninjured salmonellae in the frozen suspension. The number of injured cells in the frozen culture was equal to the difference between the TSA and BG or TSAD counts or the XLP and XLPD counts.

### Resuscitation Studies With Heat and Freeze Injured Salmonellae

#### Repair of heat injured salmonellae

The time required for heat injured S. typhimurium or S. anatum to undergo resuscitation was determined by inoculating lactose and CTET broths with thermally stressed cells. Immediately following production of injured salmonellae, 1 ml of the heated suspension containing either  $10^2$ ,  $10^4$ , or  $10^6$  organisms was added to the recovery media which were incubated at 35 C. Samples were removed at 0, 4, 8, 12, 16, and 24 h after inoculation and surface plated on TSA and EMBS agar plates. The plates were enumerated after incubation for 48 h at 35 C to determine the time required for the stressed salmonellae to repair in the recovery media. Repair was demonstrated by the ability to replicate on EMBS agar. The pH of the enrichment media was measured after 24 h incubation.

### Addition of selective agents

Selective agents were added at various times to combined selenite basals and CTET broth which contained heat injured salmonellae in order to determine the most effective preenrichment period. Sodium acid selenite and iodine were added at 0, 4, and 6 h after inoculation with different concentrations of salmonellae. Combined basal broths that contained no selective agents were used as controls. The recovery of the stressed salmonellae in lactose, SC, and TET broths was also analyzed. Following preenrichment in lactose broth, 1 ml aliquots were transferred to SC and TET broths. The number of Salmonella present in the conventional media was compared to the level obtained in the combined media. Salmonellae growing in the recovery media were enumerated by surface plating on TSA and EMBS. All culture broths were incubated at 35 C for 24 h except in some experiments when incubation was extended to 48 h. The pH of each medium was recorded after the required incubation period.

### Repair of Freeze Injured Salmonellae

The ability of freeze injured cells to repair in lactose broth and the combined basal media when incubated at 35 C was studied. Cultures of freeze injured salmonellae were thawed in a beaker of water at 5 C until thawed (approximately 20 min). The suspension was diluted to obtain a concentration of  $10^5$  cells per ml in each recovery medium. Samples were removed at the time of

inoculation and 4 h later and surface-plated on XLP and XLPD or TSA and TSAD in order to determine the rate of repair in the enrichment broths.

### Competition Studies

#### Growth of *S. typhimurium* and *E. coli* in conventional and combined enrichment media

Cultures of *S. typhimurium* and *E. coli* were prepared by inoculating 200 ml of TSB in 500 ml flasks with 10 ml of a frozen stock culture. The suspensions were incubated at 37 C for 16 h on a rotary shaker. Following inoculation, the cultures were diluted appropriately to obtain an inoculum containing approximately 100 cells per ml. Five ml of the *E. coli* suspension and 1 ml of the *S. typhimurium* culture were added to each of nine salmonellae recovery media to obtain a desired ratio of approximately five *E. coli* to one *S. typhimurium*. The total number of viable cells in each suspension was determined following inoculation by surface plating aliquots of the inoculum on TSA plates which were incubated at 35 C for 24 h.

Recovery media and methods used were: direct enrichment in SC and TET broths for 24 h; preenrichment in lactose broth for 24 h followed by selective enrichment in SC and TET broths for 24 h; or enrichment in the combined enrichment media, CTET, SEL 1, SEL 2, and SEL 3 for 24 h. All enrichments were incubated at 35 C and selective agents were added to the combined enrichment broths after 4 h incubation. *E. coli* and *S. typhimurium* concentrations were

estimated using techniques described in the next section.

#### Recovery of heat injured salmonellae and *E. coli* in conventional and combined enrichment media

Cultures of *S. typhimurium*, *S. anatum*, *S. heidelberg*, and *E. coli* were heat injured at 52 C and the extent of injury was determined as previously described. An inoculum was prepared immediately by diluting the heated cells to obtain a concentration of 100 organisms per ml. Again, 5 ml of the *E. coli* suspension and 1 ml of the salmonellae cultures were used as inocula for each of the nine recovery media. The ratio obtained with this inoculum was approximately ten *E. coli* per one *Salmonella* serotype. The number of organisms in each enrichment broth was determined using the procedure discussed in the next section.

#### Enumeration of *E. coli* and salmonellae in mixed cultures

A three-tube Most Probable Number (MPN) technique was used for estimating numbers of *E. coli* (Mayou, 1976) and salmonellae (Galton et al. 1968) in the recovery media. Serial 10-fold dilutions were prepared from each medium. Depending on the enrichment broth being sampled, 10 ml, 1 ml, and 0.1 ml from the appropriate dilution and 0.1 ml of the succeeding 10-fold dilution were used as inocula.

*E. coli* was enumerated by first inoculating a series of lactose broth fermentation tubes (Figure 1). Double strength lactose broth was used for the

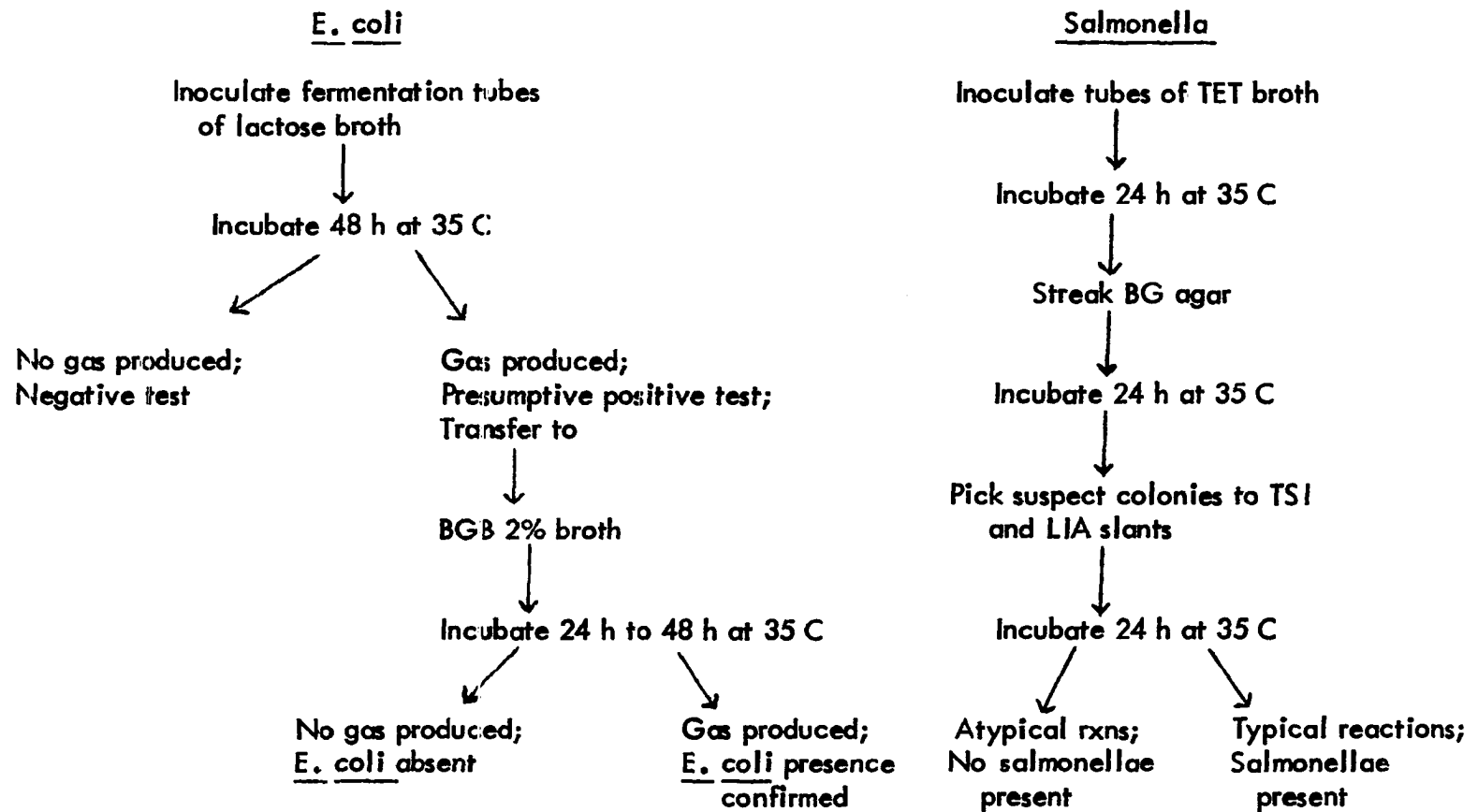


Figure 1. Schematic outline of the MPN presumptive and confirmed tests used to determine the number of E. coli and salmonellae in Salmonella isolation media.

10 ml sample and single strength for the remaining samples. Lactose broth containing acid and gas after 48 h at 35 C was a presumptive positive test for E. coli. E. coli was confirmed by gas formation after subculturing all positive tubes in BGB 2% broth.

The number of salmonellae was determined by inoculating a series of tubes containing TET broth. Double strength TET broth was utilized for the 10 ml sample and single strength for the remaining inocula. Each TET broth tube was incubated at 35 C for 24 h and then streaked for isolation on BG agar (Figure 1). Salmonellae were confirmed by picking characteristic colonies to TSI and LIA. Cultures demonstrating typical salmonellae reactions were confirmed using Salmonella O Polyvalent A-I antisera (Difco). The pH of each recovery medium was measured after sampling.

#### Determination of a Recoverable Level of Salmonellae From Frozen Inoculated Ground Beef

Suspensions of S. typhimurium and S. heidelberg were prepared as previously described from frozen stock cultures. Each Salmonella culture was diluted through a series of 10-fold dilutions to obtain an approximate level of  $10^5$  cells per ml and  $10^2$  cells per ml. A ml of each serotype was "spot" inoculated in several locations on the surface of 50 g ground beef patties prepared from lean ground beef obtained from a local supermarket. Each patty was wrapped in aluminum foil and frozen by three different methods: (i) immersion in LN<sub>2</sub> for 2 min; (ii) packed in CO<sub>2</sub> snow for 10 min; and (iii) placed in a



mechanical air blast freezer at -29 C for 24 h. Following initial freezing, the patties were stored at -29 C in styrofoam containers.

The frozen patties were analyzed for salmonellae using a 3 tube MPN technique after one week of storage. Patties were rapidly thawed at room temperature. One patty was added to 450 ml of lactose broth in a sterile blender jar and homogenized for 2 min using an Osterizer (John Oster Co., Milwaukee, Wisconsin) at low speed. One hundred ml of the homogenate was added to 3 separate sterile quart jars. Ten ml of the same suspension were inoculated into sterile test tubes while 1 ml and 0.1 ml samples were added to 10 ml of lactose broth. One ml of each lactose broth culture was transferred to 10 ml of TET broth after incubation for 24 h at 35 C (Figure 1). Salmonellae in the TET enrichments were confirmed using the previously described procedures. Control beef patties were also examined for Salmonella using methods outlined in BAM.

#### Preparation of a Standardized Inoculum

A modification of the technique of Kafel and Bryan (1977) was used to prepare cultures of salmonellae containing a known level of viable cells. Frozen stock cultures were thawed when needed and the contents incubated at 35 C for 20 h. Ten-fold serial dilutions of the broth culture were prepared in Ringer's solution.

The number of salmonellae in each dilution was determined by pour plating

samples with TSA. The plates were counted after 24 h incubation at 35 C. Ringer's suspensions were stored for 24 and 48 h at 5 C. Following storage in the Ringer's solution, the Salmonella cultures were replated to observe any changes in the number of living cells. This procedure in which cell suspensions were stored for 24 h was used for studies in which various foods were inoculated with a known level of salmonellae ranging from 1 to 10 per gram of sample.

### Food Product Studies

#### Preparation and analysis of ground beef frozen by LN<sub>2</sub>, CO<sub>2</sub> snow, and mechanical freezing

Frozen lean ground beef was obtained from the Department of Animal Science, Iowa State University, Ames, Iowa. The ground beef was thawed overnight at 5 C, and 25-g patties were prepared the following morning. Each patty, except for control samples was inoculated with salmonellae by "spot" inoculating different areas of the surface to obtain approximately 10 salmonellae per gram. The inoculum, containing known levels of S. typhimurium, S. anatum, and S. heidelberg was prepared in Ringer's solution. Each patty was wrapped in aluminum foil and frozen by one of the previously described freezing techniques. The internal temperature at the center of the patties at the end of the LN<sub>2</sub> and CO<sub>2</sub> snow treatments was measured by using copper-constantan thermocouples attached to a model 8691 millivolt potentiometer (Leeds and Northrup Co., Philadelphia, PA). After initial

freezing, the patties were stored in styrofoam boxes at  $-29\text{ C}$ .

The patties were examined for levels of mesophiles and psychrotrophs and the presence of salmonellae after 0 and 60 days frozen storage. Mesophilic and psychrotrophic counts were determined after duplicate control samples were thawed at room temperature ( $26\text{--}28\text{ C}$ ). Each patty was homogenized in 225 ml of peptone water for 2 min. Samples were pour plated with TSA and mesophiles counted after 48 h incubation at  $30\text{ C}$ . The number of psychrotrophs was determined after incubation at  $7\text{ C}$  for 7 days.

The inoculated samples were analyzed for salmonellae 24 h after initial freezing by means of the combined enrichment methods and conventional BAM procedures for raw, unprocessed meats. Duplicate patties thawed at room temperature were placed in blender jars containing 225 ml of SEL 2, SEL 3, or CTET basals or TET plus brilliant green (TETBG) or SC broth and homogenized for 2 min. Selective agents were added to the basal broths after 4 h incubation. Each enrichment broth was incubated for 24 h at  $35\text{ C}$  prior to selective plating.

Patties examined following two months storage were divided into two lots. The same procedures and enrichment media were used but half the samples were incubated at  $35\text{ C}$  and the remaining patties at  $43 \pm 1\text{ C}$  (Kafel and Bryan, 1977). Also, the enrichment media were plated on selective agars after 24 and 48 h incubation. Each recovery medium was tested for salmonellae using the methods described in "Comparison Procedure."

### Preparation and analysis of frozen turkey roasts

Three different brands of frozen turkey roasts consisting of white and dark meat or all white meat were purchased from various supermarkets in Ames, Iowa. Twenty-five-g samples prepared from roasts thawed overnight at 5 C were added to 225 ml of the combined enrichment media and lactose broth in blender jars. Half of the combination of turkey meat and enrichment broths were inoculated with a known concentration of S. typhimurium, S. anatum, and S. heidelberg prepared in Ringer's solution. The remaining samples served as controls. The inoculated and control samples were homogenized for 2 min and assayed for salmonellae utilizing procedures described in "Comparison Procedure." After 4 h incubation, selective agents were added to each combined enrichment basal broth.

The comparison study was repeated 3 times using different inoculum levels, incubation times and temperatures. In the first experiment, about 10 salmonellae per gram were added to the enrichment broths which were analyzed for salmonellae after 24 h incubation at 35 C. Approximately 1 to 2 salmonellae per gram were added in study two and each enrichment broth was assayed for salmonellae after 24 and 48 h incubation at 35 C. Enrichment media in experiment three were seeded with 1 to 2 salmonellae per gram and examined after 24 and 48 h incubation at either 35 C and 43 C.

The number of mesophiles and psychrotrophs present in each roast was determined by counting colonies from TSA pour plates prepared from homogenized

control samples in 0.1% peptone water. Mesophiles were counted on plates incubated at 30 C for 48 h and psychrotrophs on plates held at 7 C for 7 days.

#### Preparation and analysis of dried beef gravy, vegetable beef soup, and spray dried egg albumen

Samples of dried gravy and soup were purchased from supermarkets in Ames, Iowa. Two different samples of spray dried egg were obtained from Dr. P. A. Hartman, Department of Bacteriology, Iowa State University, Ames, Iowa. The total number of mesophiles present in each type of dried food was determined by colony counts from TSA pour plates prepared from homogenized samples and incubated for 48 h at 30 C. Each dried product was assayed for the presence of natural Salmonella contaminants using BAM methods. Inocula containing a known level of S. typhimurium and S. heidelberg were prepared in Ringer's solution. Twenty-five g of the dried food product were added to the combined enrichment broths and lactose broth, which were then inoculated with 1 to 2 salmonellae per gram. A selective environment was created in each combined broth by adding selective agents after 4 h enrichment. The enrichment broths were incubated at 35 C for 24 and 48 h and assayed for salmonellae utilizing the comparison methods described in "Comparison Procedure."

#### Comparison procedure

The combined enrichment technique was used to examine each inoculated and control sample of the various products analyzed. Procedures

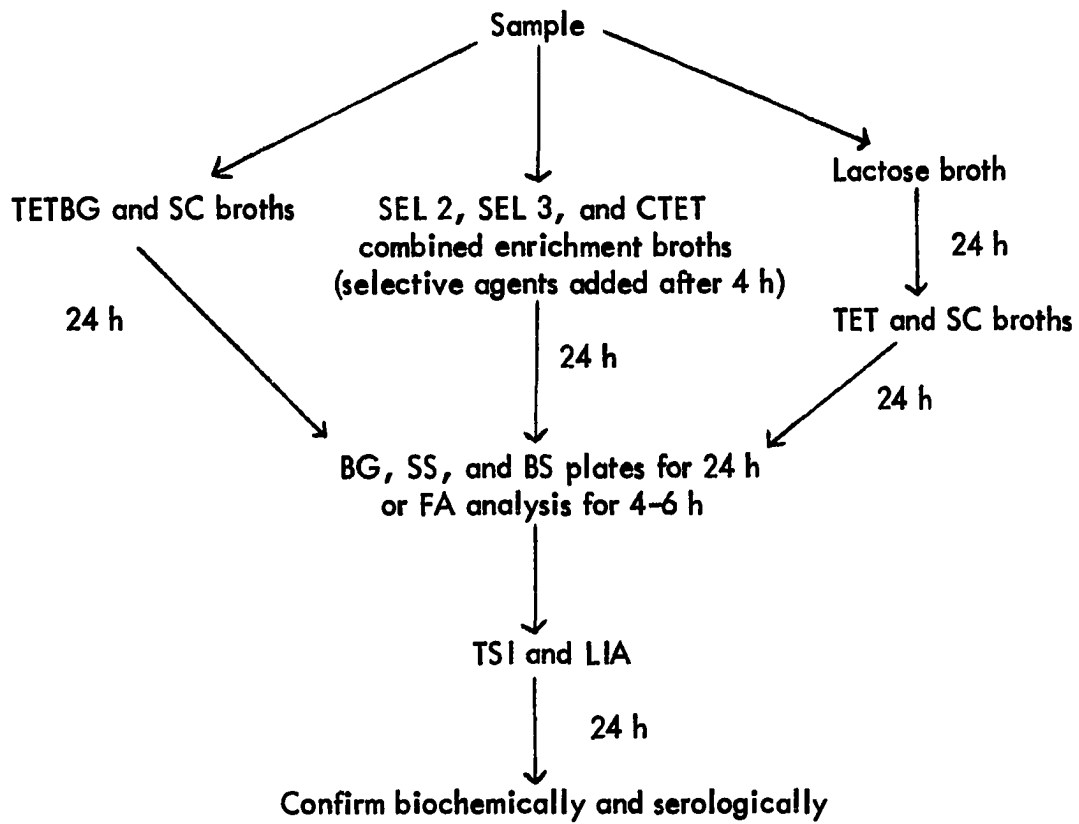


Figure 2. Schematic outline of Salmonella isolation methods.

used are shown in the flow chart (Figure 2). The methods outlined in BAM for each type of food examined were used as control procedures. Each culture broth was adjusted to pH  $6.8 \pm 0.2$  following addition of the sample. One ml of each conventional lactose pre-enrichment broth was transferred to 10 ml of SC and TET broths. All conventional and combined enrichment broths were incubated for the appropriate time and temperature described in the preceding section for the products assayed. Each culture broth was then streaked on predried plates of BG, SS, and BS agars. Any characteristic Salmonella-like colonies were transferred to TSI and LIA and confirmed biochemically and serologically. The presence of salmonellae in the food products was also determined using the direct FA technique.

#### Fluorescent Antibody Procedure

Modifications of the techniques of Haglund et al. (1964) and the Food and Drug Administration (1976) were utilized to detect salmonellae in food products with the direct FA technique. Ten ml of SC broth was inoculated with 1 ml of each conventional and combined enrichment broth from the food product analysis after the described incubation conditions. The SC cultures were grown for 4 h at 35 C. A loopful of the suspension was placed on a dry clean slide and allowed to air dry. The organisms were fixed to the slide by immersion in Kirkpatrick's reagent (60 ml absolute alcohol, 30 ml chloroform, and 10 ml formalin) for 3 min at room temperature. The fixative was removed by rinsing

with 95% ethyl alcohol for 1 min and then allowing the slides to air dry. The antiserum used in this study was a pooled polyvalent "OH" antiserum which had been conjugated with fluorescein isothiocyanate (Clinical Sciences, Inc., Whippany, NJ). A drop of this conjugate was placed on a smear and incubated in a moist chamber for 20 min at 37 C. The slides were removed from the chamber and washed for 30-60 min in two changes of phosphate buffered saline which consisted of (per liter):  $\text{Na}_2\text{HPO}_4$ , 1.18 g;  $\text{NaH}_2\text{PO}_4$ , 0.22 g, and NaCl, 8.5 g. Each slide was rinsed with distilled water, drained, and air dried prior to examination. Slides were mounted with pH 8.5 phosphate buffered glycerol (1 part pH 8.5 buffer with 9 parts glycerol) and covered with a glass coverslip.

Slides were examined on a Reichert Zetopan fluorescent microscope equipped with incident light fluorescence. The light source was an 8 Osram HBO 200 mercury vapor lamp. The microscope was equipped with a 3BG12 exciter filter, OG515 barrier filter, KG2 heat absorbing filter and a 490 nm dichroic interference beam splitter.

Photomicrographs were made using a Zeiss Photomicroscope III and Ektachrome Film, asa 200 (Eastman Kodak, Co., Rochester, NY). The microscope was equipped with incident light fluorescence.

#### Biochemical and Serological Identification of Presumptive

#### Salmonella Isolates

Those isolates producing typical Salmonella-positive reactions in TSI and LIA were confirmed as Salmonella species by biochemical and



serological tests. Presumptive salmonellae isolates were inoculated into urea agar slants and reacted with Salmonella O polyvalent A-1 antiserum. Organisms that lacked urease and agglutinated the antiserum were confirmed biochemically by tests described by Edwards and Ewing (1972). Cultures from control samples of frozen turkey roasts biochemically confirmed as Salmonella species were forwarded to the State Hygienic Laboratory, Iowa City, Iowa, for serological confirmation.

### Statistical Analysis

Differences between enrichment methods used to enumerate populations of both injured and uninjured salmonellae and E. coli were determined by an analysis of variance followed by calculation of least significant differences (Steele and Torrie, 1960). This same analysis was used to determine the effect of freezing methods on the bacterial population of frozen ground beef.

The Chi-square test ( $X^2$ ) was used to determine whether significant differences existed between the combined and conventional enrichment media used to isolate salmonellae from the foods analyzed (Steele and Torrie, 1960).

## RESULTS AND DISCUSSION

### Production of Heat Injured Salmonellae Cultures

Table 3 demonstrates the effect of different heating temperatures used to sublethally injure a population of S. typhimurium cells. Injury was determined by comparing the number of salmonellae on TSA plates to the number on EMBS agar, the stress medium. Only 50% of the organisms heated at 48 C for 30 min were injured in this study. Heating for 45 min provided little advantage over 30 min heating. Eighty-five percent were injured at 50 C but a desired level of 99% stressed cells was achieved by heating S. typhimurium suspensions at 52 C for either 30 or 45 min. The population was more than 99 % injured at 54 C but this treatment was very lethal since greater numbers of cells were killed or more severely injured. Salmonellae in subsequent studies, were heat shocked at 52 C since this technique consistently produced suspensions of 99% or greater injured salmonellae. S. heidelberg, S. anatum, and S. infantis, when heated at 52 C were also injured to the same extent as S. typhimurium.

### Production of Heat Injured E. coli

Cultures of E. coli cells, when heated at 52 C, were more resistant to the sublethal heat treatment than the salmonellae. When heated at 52 C, only 18% of the population was injured (Table 4). Injury was no greater at 55 C, and all the cells were destroyed when heat shocked at 60 C.

Table 3. Comparison of heat treatments used to produce a population of thermally stressed S. typhimurium

Time of Exposure	Percentage of Population Sublethally Injured			
	48 C	50 C	52 C	54 C
30 min	50%	85%	99%	99.9%
45 min	70%	93%	99%	99.9%

Table 4. Effect of heating E. coli suspensions at 52 C for 30 min

Replication <sup>a</sup>	Percentage of Population Sublethally Injured
1	19%
2	21%
3	13%
mean	18%

<sup>a</sup>Each replication represents the average of three different heated cultures .

### Production of Freeze Injured Salmonellae

The extent of injury in the frozen salmonellae cultures was determined by comparing the counts on nonselective media, TSA and XLP with counts on selective media, BG or TSAD and XLPD, respectively. The use of BG agar as a stress medium was found to be an unreliable method because uninjured cells were also inhibited when surface plated on it. Kroniger and Banwart (1978) observed fewer salmonellae on surface plates of BG agar in comparison to plate count agar surface inoculated with the same concentration of cells. The use of TSAD and XLPD agars as stress media was more reliable than BG agar and these two injury determination methods were used in succeeding studies. The freezing treatments caused a reduction of approximately one log cycle in total numbers and effectively produced populations generally containing about 90% freeze injured salmonellae.

### Repair of Heat Injured Salmonellae

The ability of heat injured salmonellae to undergo resuscitation in the combined and conventional enrichment media was ascertained. Information obtained from these experiments was used to determine the appropriate time at which to add selective agents to the nonselective combined basal broths. Adding selective agents before the injured salmonellae had undergone repair might inhibit their resuscitation, which would detract from the possible value of the combined enrichment procedure.

Repair in the combined basal broths is feasible because of their general nonselective nature. The combined media are similar to lactose broth, a nonselective peptone medium. CTET basal broth contains bile salts which makes it more selective than the combined selenite media. The bile salts suppress the multiplication of gram positive microorganisms but have no effect on the growth of gram negative bacteria. Suppression of enterics other than salmonellae is achieved by the toxic effects of tetrathionate and thiosulfate (Palumbo and Alford, 1970). Individually these salts are not toxic but in combination a synergistic lethal action occurs. The mechanism of tetrathionate inhibition is not known but these authors found that only growing cells are killed, since nongrowing organisms were unaffected. Most salmonellae and Proteus are not inhibited in TET broth because they possess tetrathionate reductase whereas E. coli lacks this enzyme. The enzyme reduces tetrathionate to thiosulfate. Knox (1945) and Knox et al. (1943) suggested that tetrathionate served as an alternative hydrogen acceptor to oxygen which extends the log phase for salmonellae as oxygen is depleted. Palumbo and Alford (1970) also stated it is known that tetrathionate inactivates sulfur-containing enzymes and interferes with the synthesis and activity of cell wall and membrane components. Tetrathionate is not formed in CTET broth until a solution of iodine is added.

The inhibitory action of SC broth, a peptone based medium is also not known. The medium contains selenium, a very toxic element to many

microorganisms, in the form of sodium selenite (Shrift and Boulette, 1974). Salmonellae are one of the few groups of organisms in which so many species display a natural tolerance to selenite. Weiss et al. (1965) investigated the mechanism of selenite toxicity. Selenite's inhibitory action is similar to that of tetrathionate because its toxic action is a growth-related phenomenon. These workers observed the rapid uptake of selenium during early incubation of cultures and proposed two mechanisms to explain its toxicity. The selenite may react with sulfhydryl groups of enzymes or be incorporated into analogues of sulfur-containing compounds since selenium closely resembles the sulfur present in amino acids. Susceptibility to selenium coincided with the rate of selenite uptake by E. coli, Proteus vulgaris, and S. thompson. E. coli incorporated up to twice as much selenium as the other organisms during the early stages of incubation and was inhibited by lower concentrations of selenite. Each selenite basal broth is nonselective until sodium selenite is added which creates a selective environment.

The repair of S. typhimurium and S. anatum in lactose and CTET basal broth containing no tetrathionate is illustrated in Figures 3 and 4, respectively. These typical growth curves indicate the time required for resuscitation of the injured population. S. anatum cells were not as severely injured as the S. typhimurium population, which is indicated by the large difference in EMBS counts at 0 time. Almost the entire S. anatum population repaired within 4 h in each medium. S. typhimurium cells also repaired rapidly with the majority

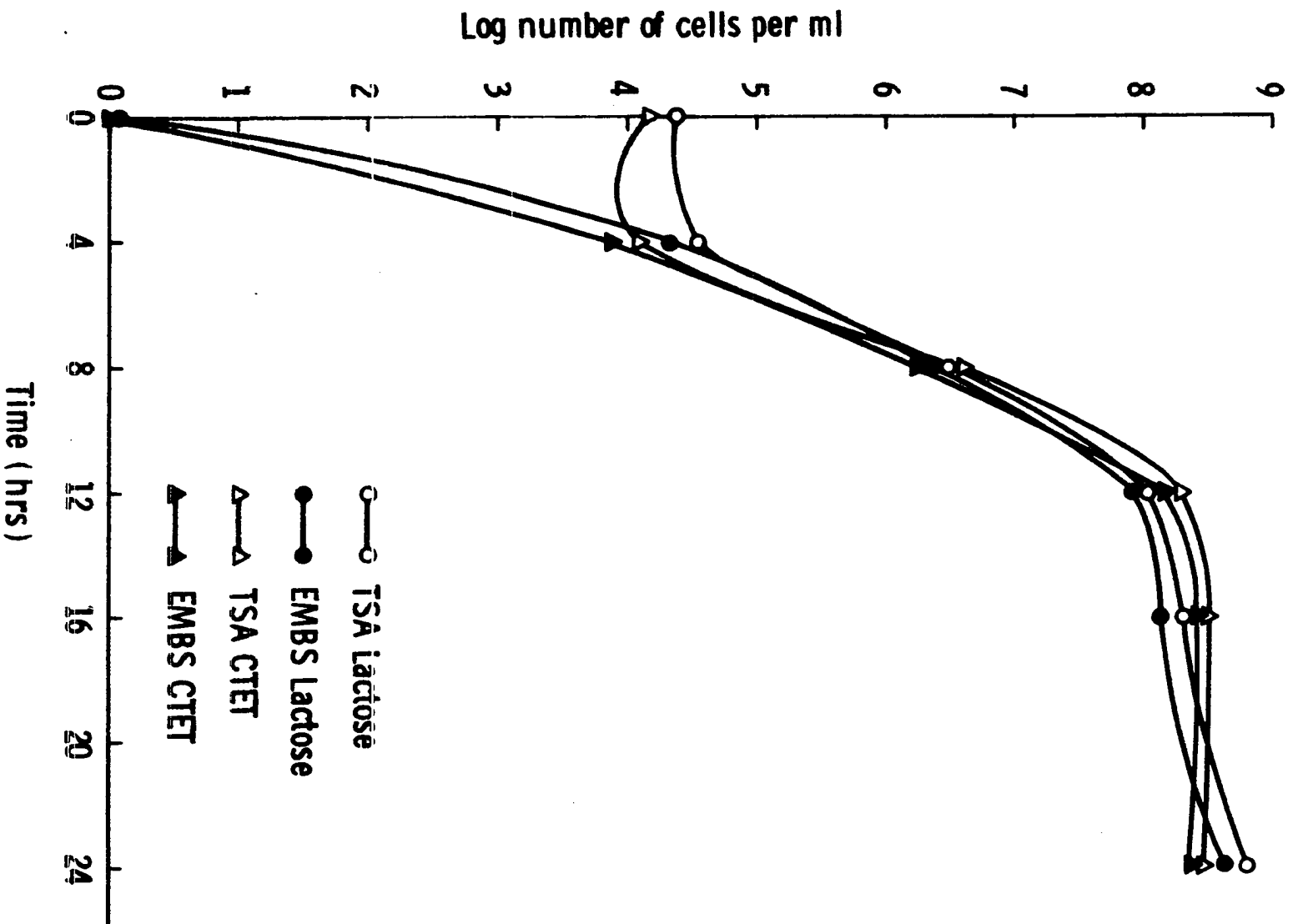


Figure 3. Growth of heat injured *S. typhimurium* in lactose broth and CTET broth.

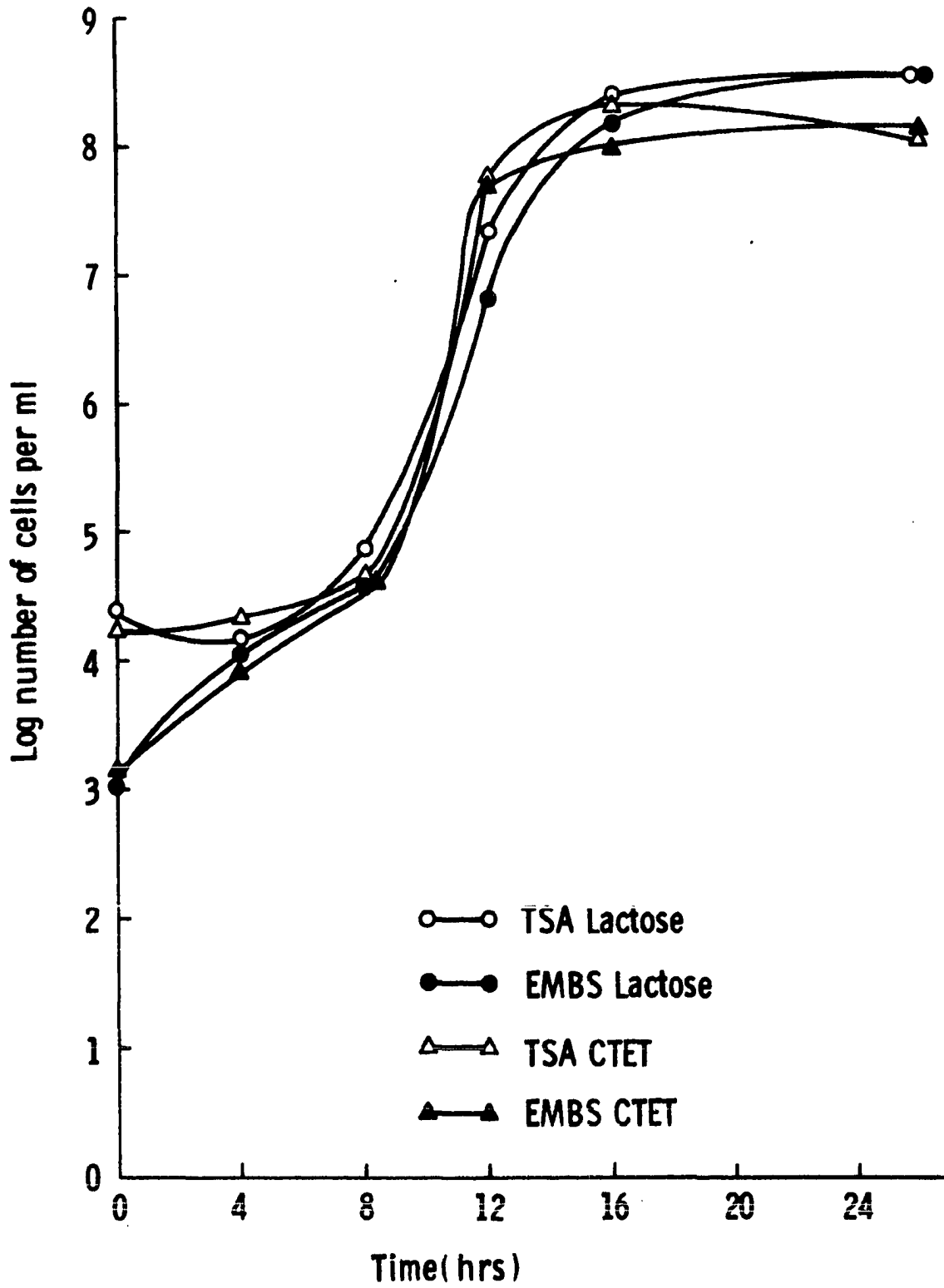


Figure 4. Growth of heat injured *S. anatum* in lactose broth and CTET broth.



of the population undergoing resuscitation within 4 h. Recovery from heat injury occurred in CTET broth just as readily as in lactose broth even though bile salts were present in the combined medium. Slightly higher levels of salmonellae were observed in lactose broth than in CTET broth after 24 h but the difference was not significant. Observations from similar growth curve studies also indicated that the majority of the heat injured salmonellae had undergone repair after 4 to 6 h incubation in lactose and CTET broths (Figs. 3 and 4).

Using this information, additional experiments were done to determine the appropriate time at which to add sodium selenite to SEL 1 broth. Table 5 illustrates the effect of adding selenite to SEL 1 broth containing heat injured salmonellae after 0, 4, and 6 h incubation at 35 C. Repair and subsequent multiplication was greatest in the basal broth containing no selenite. The repair of the stressed cells was not inhibited by the presence of selenite in the medium at the time of inoculation. injured S. typhimurium and S. anatum may not be sensitive to the inhibitory effects of selenite. Since little difference was observed when adding selenite either 4 or 6 h after inoculation, a 4 h preenrichment was thought to be sufficient. Recovery of salmonellae in CTET broth also indicated that a 4 h nonselective enrichment phase was long enough to allow a majority of the stressed cells to undergo repair. Selective agents were added after a 4 h preenrichment period in subsequent experiments.

Table 5. Effect of adding selenite to SEL 1 broth 0, 4, and 6 h after inoculation with heat injured salmonellae and grown for 24 h

Time of addition	<u>S. anatum</u>	<u>S. typhimurium</u>
	No. bacteria per ml x 10 <sup>8</sup>	
0	2.80	1.40
4	1.70	1.10
6	1.90	.59
No selenite added	5.50	4.60

Tables 6 and 7 illustrate the growth of injured salmonellae in the combined and conventional enrichment media. Multiplication was greater in lactose broth than in CTET or the combined selenite media but the difference was very small. Even following the addition of selective agents after 4 h incubation, the salmonellae grew to levels comparable to those reached in lactose broth which contains neither tetrathionate or selenite. Aliquots of the lactose broth preenrichment cultures were transferred to SC and TET broths. SC broth contained substantially more salmonellae than the combined selenite broths but the populations attained in TET and CTET broths were quite similar. These results indicate that a 4 h preenrichment phase is long enough to allow the resuscitation of injured salmonellae. The addition of selective agents should not be delayed longer than 4 h in order to prevent the extensive growth of competitors which would occur in the absence of tetrathionate and selenite.

Table 6. Number of thermally injured salmonellae recovered from lactose and combined selenite broths after 24 h incubation and following an additional 24 h incubation in SC broth

Organism	No. bacteria per ml $\times 10^8$				
	Lac	SEL 1	SEL 2	SEL 3	LacSC
<u>S. anatum</u>	2.7	1.6	2.1	—	11.0
<u>S. typhimurium</u>	2.8	1.1	1.6	2.9	6.0

Table 7. Number of thermally injured salmonellae recovered from lactose and CTET broths after 24 h incubation and following an additional 24 h incubation in TET broth.

Organism	No. bacteria per ml $\times 10^8$		
	Lac	CTET	LacTET
<u>S. anatum</u>	2.8	1.3	3.90
<u>S. typhimurium</u>	3.3	1.4	.94

#### Repair of Freeze Injured Salmonellae

The repair of freeze injured salmonellae after 4 h incubation in various recovery media is presented in Table 8. Except for the S. heidelberg suspensions, each culture contained more than 90% stressed cells. Resuscitation occurred in each repair medium; however, complete repair of the entire population did

not occur in any of the enrichment broths during the 4 h period of repair. Repair was greatest in SEL 3 broth and similar in CTET broth, both of which allowed the recovery of more than 50% of the injured cells. Less than 50% of the cells repaired in either lactose or SEL 2 broths which were comparable in efficiency. The least repair was demonstrated by S. anatum cells which probably were more susceptible to the freezing treatment than S. typhimurium or S. heidelberg.

Table 8. Percent of freeze injured salmonellae after 4 h incubation at 35 C in nonselective recovery media

Organism <sup>a</sup>	Recovery Medium <sup>b</sup>			
	Lac	SEL 2	SEL 3	CTET
<u>S. heidelberg</u>	50	65	22	33
<u>S. typhimurium</u>	67	29	23	24
<u>S. anatum</u>	65	93	61	80
Mean	61	62	35	45

<sup>a</sup>Injury was induced by freezing cultures suspended in phosphate buffer for 2 min in LN<sub>2</sub>.

<sup>b</sup>Results are expressed as that percentage of the population that had not undergone repair after 4 h at 35 C.

Although repair was not complete in the recovery media after 4 h, resuscitation was more rapid in SEL 3 and CTET broths than lactose or SEL 2 broths. This demonstrates the nonselective nature of the combined enrichment broths, similar to the conventional preenrichment medium, lactose broth. A 4 h nonselective enrichment phase was sufficiently long to allow the repair of many injured cells since half the salmonellae had undergone resuscitation in this time period.

Freeze injured salmonellae in this study required a longer repair period than shown by results in other reports on the repair of freeze injured salmonellae and the experiments with heat injured S. typhimurium and S. heidelberg. Extension of the nonselective enrichment step, while permitting more salmonellae to resuscitate prior to the addition of selective agents, might also allow competitors to reach higher numbers. Even though more viable salmonellae may be present, the chances of detecting them could be greatly reduced due to the growth of more competing microorganisms.

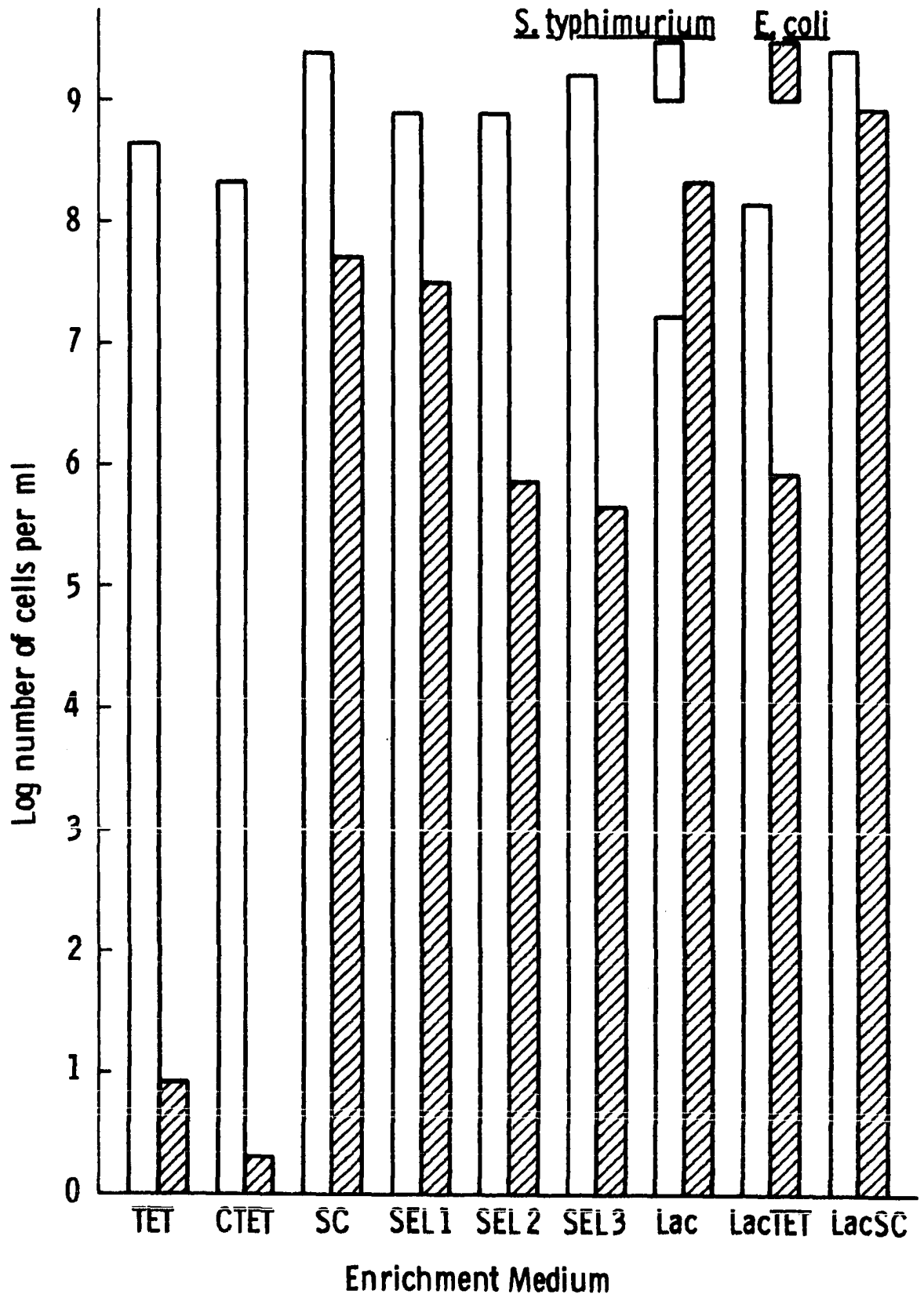
A compromise similar to that reached when observing the repair of heat injured salmonellae in the combined enrichment media can also be applied to the repair of freeze injured salmonellae. A preenrichment phase less than 4 h based on these studies would only allow the recovery of less than 50% of the stressed salmonellae. Any preenrichment step delayed longer than 4 h could allow competing organisms to reach a level that may mask or inhibit the growth of the new viable Salmonella present.

Enumeration of Uninjured S. typhimurium and E. coli  
In Salmonellae Enrichment Media

Figure 5 illustrates the ability of S. typhimurium to multiply to measurable levels in the combined enrichment broths when grown in a mixed culture with E. coli. Conventional enrichment media were used as control procedures. The initial population in each recovery medium was approximately 4 to 5 E. coli cells per S. typhimurium cell per ml. The results represent the mean counts obtained from three replications of this competition study.

The growth of E. coli was significantly suppressed only in TET and CTET broths. The cells multiplied to  $10^5$  or more per ml in the other enrichment media used. Fewer E. coli cells were present in combined selenite broths than SC broth but only SEL 3 broth was significantly ( $P < 0.05$ ) more inhibitory than SC broth. S. typhimurium reached levels greater than  $10^8$  organisms per ml in all enrichments except lactose broth. Due to its nonselective properties, more E. coli cells grew in lactose broth and may have interfered with the multiplication of the salmonellae. Lactose broth was significantly ( $P < 0.05$ ) less effective than SC broth but comparable to the other media used to enumerate S. typhimurium. Since S. typhimurium was able to multiply in the presence of E. coli, the feasibility of the combined enrichment technique was further tested using heat injured salmonellae and E. coli cells. The ratio of E. coli to salmonellae in the culture broths was also increased to approximately 10 to 1.

**Figure 5. Growth of uninjured S. typhimurium and E. coli. in various salmonellae enrichment media.**



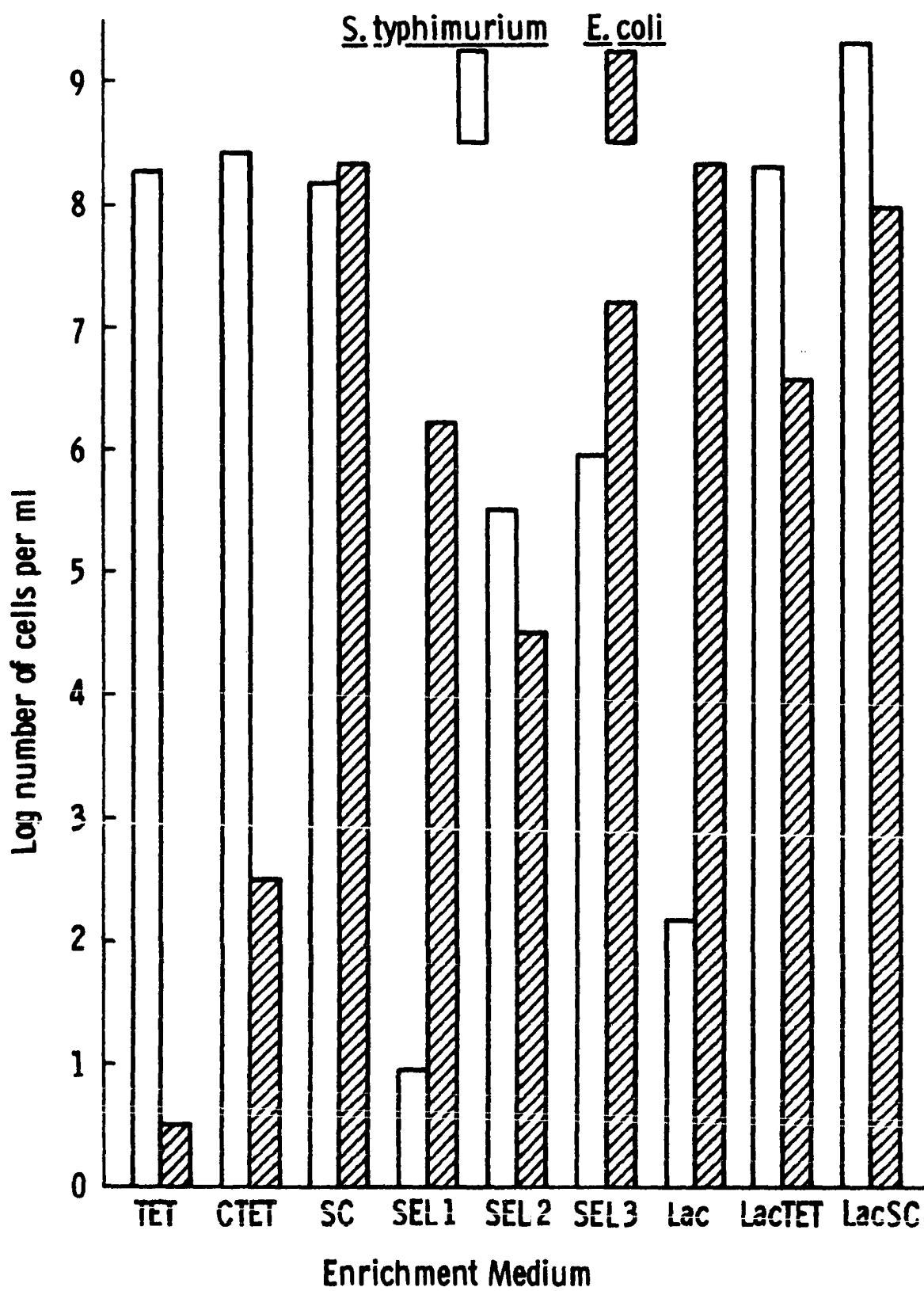


Enumeration of Heat Injured Salmonellae and E. coli  
In Salmonellae Enrichment Media

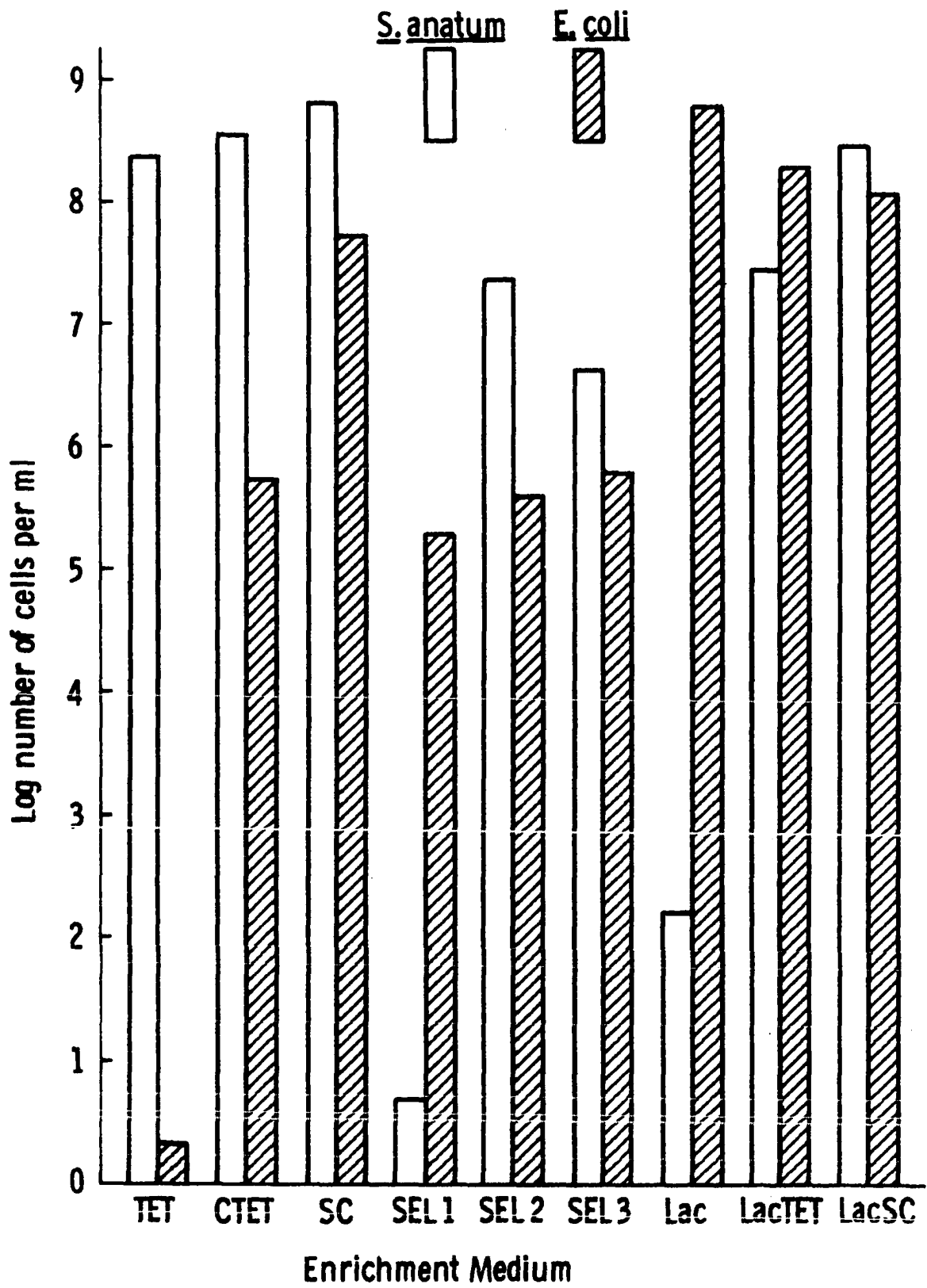
Figures 6, 7, and 8 indicate the ability of heat injured salmonellae to undergo repair in various salmonellae enrichment media and multiply to measurable levels when grown with stressed E. coli cells. The initial concentration of the mixed population per ml was about 11 E. coli per S. typhimurium cell, 13 E. coli per S. anatum cell, and 9 E. coli per S. heidelberg cell. The experiment was repeated three times with each serotype and the results represent the mean counts of these replications.

Multiplication of E. coli was again significantly inhibited in TET and CTET broths although CTET did not suppress the cells as greatly as direct enrichment in TET broth. The data from this experiment confirms the need for a selective enrichment step in salmonellae assays. Salmonellae were greatly overgrown in lactose broth by the E. coli cells. E. coli reached levels of  $10^8$  per ml while salmonellae numbers averaged only  $10^2$  per ml. When aliquots of the lactose preenrichments were transferred to SC and TET broths the salmonellae grew to greater concentrations but E. coli cells also multiplied to quite high levels. These organisms could have interfered with the isolation of salmonellae on selective agars prepared from SC and TET broths. The combined technique alleviates this problem of competitor overgrowth by utilizing only a 4 h preenrichment step as opposed to the 24 h period used in conventional assays. The selenite combined broths also inhibited the growth of E. coli more

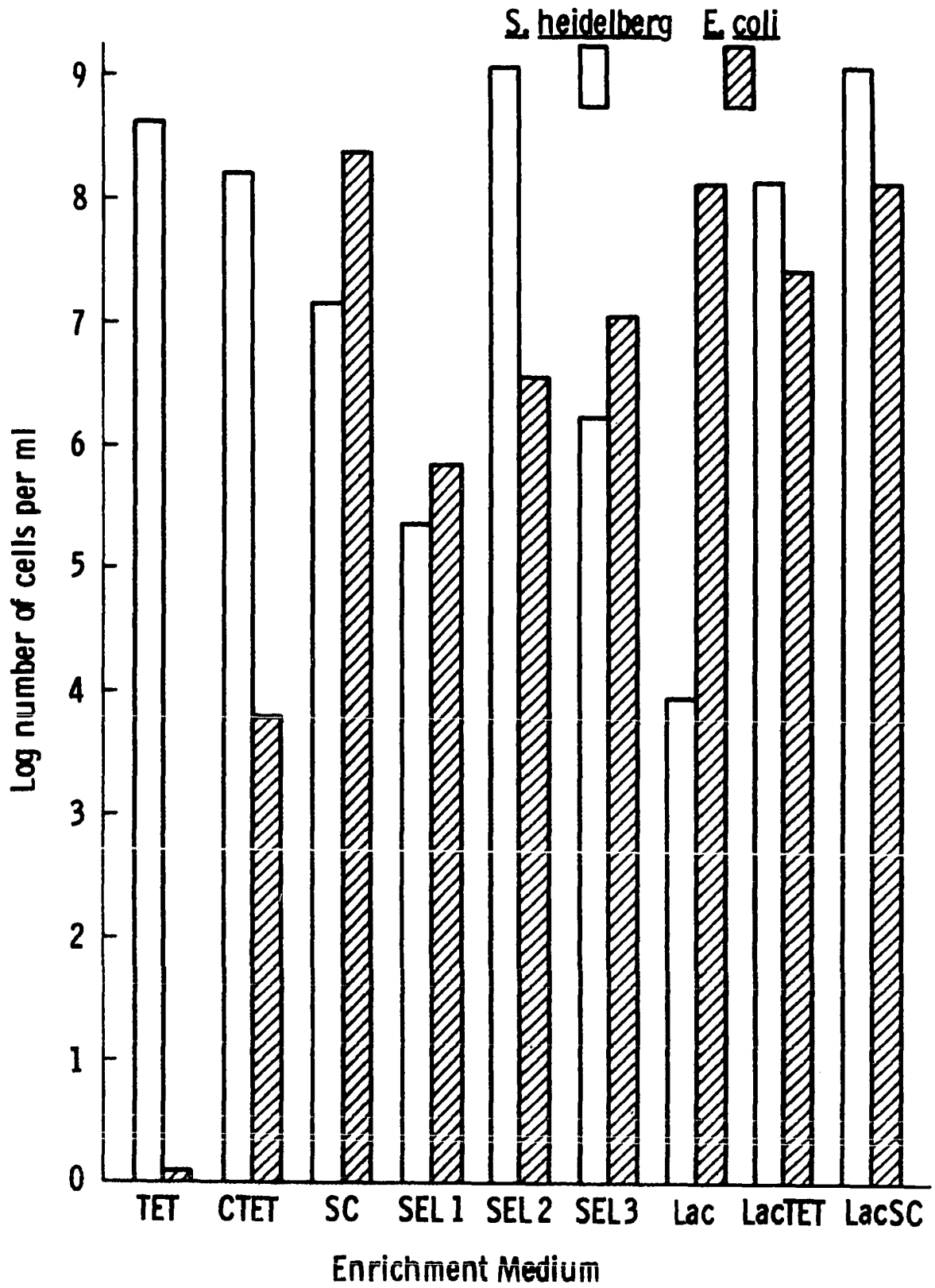
**Figure 6. Growth of heat injured S. typhimurium and E. coli in various salmonellae enrichment media.**



**Figure 7. Growth of heat injured S. anatum and E. coli in various salmonellae enrichment media.**



**Figure 8. Growth of heat injured S. heidelberg and E. coli in various salmonellae enrichment media.**



effectively than methods using SC broth. SEL 2 broth suppressed the multiplication of E. coli significantly ( $P \leq 0.01$ ) better than SEL 3 broth.

There was no significant difference ( $P \leq 0.01$ ) between methods when comparing the recovery of injured salmonellae in CTET broth to growth in TET broth with or without a lactose preenrichment step. Growth of salmonellae in SEL 2 broth was not significantly different ( $P \leq 0.05$ ) from that obtained with direct selective enrichment in SC broth. Preenrichment in lactose broth followed by selective enrichment in SC broth produced a significantly greater population of salmonellae than enrichment in the combined selenite media.

The pH of each enrichment medium recorded after 24 h incubation is indicated in Table 9. Salmonellae will grow under optimal conditions in

Table 9. pH values of salmonellae enrichment media after 24 h incubation

Inoculum	TET	CTET	SC	SEL 1	SEL 2	SEL 3	Lac
Injured cells	6.5	5.2	6.0	6.8	6.8	6.8	4.8
Uninjured cells	6.4	5.2	6.1	6.2	6.4	6.4	5.0

artificial media over a pH range of 4.0 to 9.0 and are selectively favored between pH 5.8 and 6.3 (Troller, 1976). pH values recorded during the competition studies all fall within this growth range. Lower recoveries of salmonellae in the combined selenite media may have been caused by the higher



pH values which do not favor salmonellae. Although the pH of CTET broth was quite low after 24 h incubation, salmonellae were still able to multiply to the same concentrations as those observed at pH 6.4 to 6.5 in TET broth. Low pH did not appear to inhibit the growth of salmonellae but pH values near 6.4 to 6.8 favored E. coli in media containing selenite.

Of the three serotypes used in this study, S. anatum multiplied significantly better in the recovery media than S. typhimurium or S. heidelberg. Whether the organisms used as an inoculum were heat injured or not was also significant. The effectiveness of the combined selenite media was quite different when heat injured cells were used. Much lower concentrations of salmonellae, two or more log cycles, were observed in the combined selenite broths as opposed to the levels observed when the multiplication of normal cells was studied. CTET broth allowed injured cells to multiply as readily as uninjured organisms.

These studies demonstrate the feasibility of the combined enrichment procedures. CTET broth was as effective as methods using TET broth. SEL 2 and SEL 3 broths also allowed salmonellae to reach levels comparable to those attained in SC broth. Because injured salmonellae did not grow as well in SEL 1 broth, it was eliminated from future comparison studies. The lower selectivity of SEL 1 broth may be explained by the presence of beef extract which is not a component of SEL 2 or SEL 3 broths. Leifson (1936) reported that beef extract and especially meat infusion reduced the toxicity of selenite.

The E. coli cells were probably less inhibited in SEL 1 broth because of this reduced selectivity and overgrew the salmonellae.

Following these preliminary studies, additional experiments were designed to evaluate the ability of the combined methods to detect the presence of salmonellae in naturally and artificially contaminated foods. The effectiveness of the combined techniques were compared directly to BAM procedures recommended for each type of food analyzed in the comparison study.

#### Detection of Salmonellae From Frozen Ground Beef By the 3 Tube MPN Technique

The MPN method of Galton et al. (1968) was capable of detecting 10 salmonellae per gram present in inoculated frozen ground beef patties. This observation indicated that enough salmonellae survived the freezing treatments to be enumerated. Future experiments could then be designed using ground beef seeded with a small number of Salmonella. Following the freezing and storage of these samples, detectable levels of salmonellae would still be present to provide a reliable comparative test of the efficiency of the various liquid enrichment media used. Samples would not be Salmonella negative due to the death of the organisms but would be because of apparent differences in the effectiveness of the various enrichment media used for recovery.

#### Effect of Storing Salmonellae in Ringer's Solution

Table 10 demonstrates the reproducibility of the technique of preparing a standardized inoculum in Ringer's solution. The number of salmonellae

present in the refrigerated suspensions of S. typhimurium or S. heidelberg remained constant over a period of 48 h storage. This procedure was a reliable method that made it possible to inoculate foods with a known concentration of salmonellae ranging from 1 to 10 per gram. Inocula of S. anatum cells prepared in Ringer's and used with the various foods assayed, also showed no decrease in numbers during refrigerated storage.

Table 10. Number of salmonellae present in Ringer's solution suspensions stored for 0, 24 and 48 h at 5 C

Time of Storage (h)	<u>S. typhimurium</u> <u>S. heidelberg</u>			
	No. bacteria per ml x 10 <sup>9</sup>			
0	1.8	1.9	2.1	1.8
24	1.6	2.0	1.8	1.9
48	1.6	1.6	1.9	1.7

#### Isolation of Salmonellae From Frozen Ground Beef

Ground beef was selected as a type of food to be analyzed in the comparison study for several reasons. The flora of frozen ground beef consists of several competitors, such as coliforms with pseudomonads and Moraxella-Acinetobacter being most predominant (Reddy, 1978). Unless these organisms

are inhibited by the selective enrichment step they could interfere with the detection of salmonellae in the samples of beef. The popular use of commercially prepared frozen beef patties by food service establishments then warrants examination in this study. Although few cases of foodborne disease have been attributed to ground beef (Goepfert, 1976), the potential for Salmonella contamination still exists during production and distribution of the product. Surkiewicz et al. (1975) and Swaminathan et al. (1978b) have reported the presence of salmonellae in samples of retail ground beef. The possibility remains that ground beef could be a source of salmonellae in foodborne disease outbreaks. Freezing the inoculated patties might also injure some of the salmonellae present. Temperatures recorded inside the beef patties during cryogenic freezing,  $-78\text{ C}$  for  $\text{CO}_2$  snow and  $-196\text{ C}$  for  $\text{LN}_2$ , may have been sublethal freezing treatments. An injured population of salmonellae in the samples would serve to test the ability of the combined enrichment broths to detect injured cells.

Figure 9 indicates the number of mesophiles and psychrotrophs enumerated in the control samples of beef patties frozen by three methods. The competitive flora was enumerated after 0 and 60 days storage to determine whether any decrease in the total number of microorganisms had occurred. Slight reductions in the level of mesophiles and psychrotrophs were observed after 60 days storage, but the decrease was not statistically significant.

The effect of freezing methods on the survival of microorganisms on the

patties was also determined. No statistical difference between mechanical freezing, CO<sub>2</sub> snow, and LN<sub>2</sub> freezing techniques was observed. Reddy (1978) found a more lethal effect using cryogenic freezing with CO<sub>2</sub> snow and LN<sub>2</sub> than mechanical freezing. He also observed a decrease in surviving micro-organisms during storage of frozen beef patties. Neither of these phenomena were as apparent in this study. This may have been due to the low initial flora present in the ground beef used. The beef may have contained a majority of organisms capable of surviving the freezing treatments. Because of handling requirements during processing, each patty had been prepared from frozen beef which had been quick frozen with CO<sub>2</sub> snow. This initial freezing treatment may have effectively selected organisms resistant to later freezing treatments. However, the initial freezing treatment did not affect comparisons in the study on recovery of salmonellae from the ground beef.

Isolation of salmonellae from patties before and after frozen storage is indicated in Table 11. Before frozen storage, all samples enriched in SEL 2, CTET, TETBG, and SC broths were salmonellae positive. Four negative samples were observed when SEL 3 broth was used as the recovery medium. Since salmonellae were present in each patty, negative samples occurred in SEL 3 broth due to its inability to allow the salmonellae to reach detectable levels while suppressing the growth of competing organisms.

When beef patties were analyzed after 60 days storage the method of analysis was modified. The same enrichment media were used but the culture

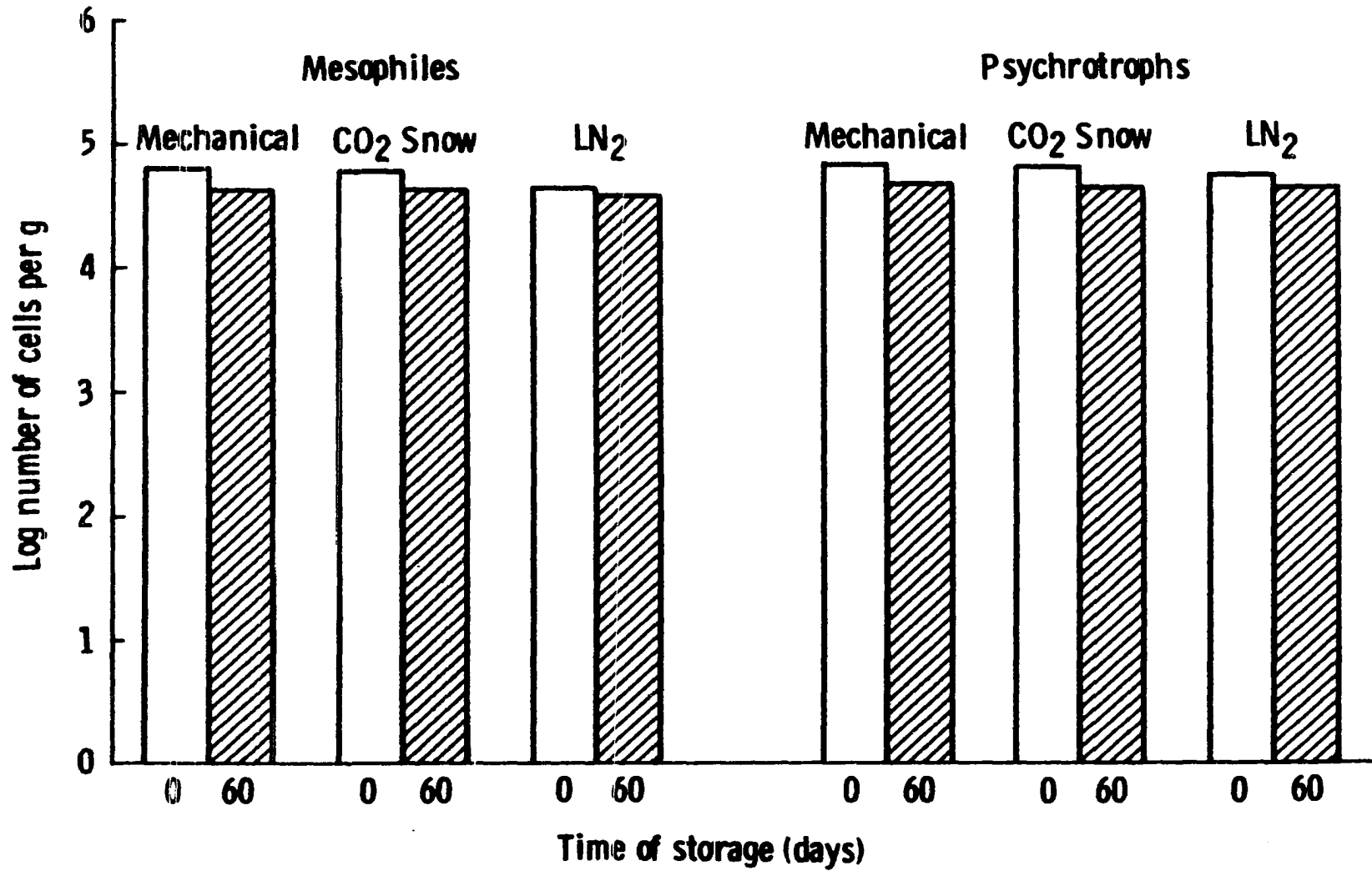


Figure 9. Bacterial counts of beef patties during frozen storage of 60 days.

Table 11. Comparison of conventional and combined methods for the recovery of salmonellae from frozen ground beef

Method of Freezing	Storage Time (days at -29 C)	No. of Samples <sup>b</sup>	Number of salmonellae positive replications <sup>a</sup>									
			Combined <sup>c</sup>				Conventional <sup>c</sup>					
			SEL 2		SEL 3		CTET		SC		TETBG	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Mechanical	0	6	6		5		6		6		6	
	35 C	3	1	2	1	2	3	3	3	3	3	3
	43 C	3	2	3	1	2	3	3	3	3	3	3
CO <sub>2</sub> Snow	0	6	6		4		6		6		6	
	35 C	3	1	3	1	2	3	3	3	3	3	3
	43 C	3	2	3	1	2	3	3	3	3	3	3

		0	6	6		5		6		6		6	
LN <sub>2</sub>	60	35 C	3	2	2	1	1	3	3	3	3	3	3
		43 C	3	2	3	1	2	3	3	3	3	3	3
Total	60	0	18	18		14		18		18		18	
		35 C	9	4	7	3	5	9	9	9	9	9	9
		43 C	9	6	9	3	6	9	9	9	9	9	9

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>All the enriched samples examined after 0 days storage were incubated at 35 C while the patties analyzed after 60 days frozen storage were incubated at either 35 C or 43 C.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24 h incubation when the patties were stored 0 days and after 24 and 48 h incubation following 60 days storage.



broths were streaked onto selective agars after 24 and 48 h incubation at either 35 C or 43 C. Increasing the incubation period and elevating the incubation temperature were done to increase the possibility of detecting salmonellae with the various enrichment broths.

Incubation at 43 C has been demonstrated by Morris and Dunn (1970), Silliker and Gabis (1974), Kafel and Bryan (1977), and Gabis and Silliker (1977) to enhance the detection of salmonellae in meat products. The higher incubation temperature suppresses the multiplication of competitors which interfere with the isolation of Salmonella from foods. Sveum and Hartman (1977) increased the number of isolations of salmonellae from spray dried egg white by extending incubation from 24 to 48 h. Increased isolations of salmonellae have been obtained by lengthening the incubation period of selective enrichment broths from 24 to 48 h (Galton et al. 1968 and Edel and Kampelmacher 1973, 1974).

Table 11 also includes the results of the analysis of frozen patties after 60 days storage. Every sample enriched in CTET, TETBG, and SC broths was salmonellae positive using the modified incubation procedures. Negative samples were observed when patties were enriched in SEL 2 and SEL 3 broths. More salmonellae isolations occurred in SEL 2 broth at both incubation times and temperatures but the difference was not significant. The number of positive samples increased by approximately 50% when the combined selenite broths

were incubated an additional 24 h at either 43 C or 35 C. Although more salmonellae were isolated in SEL 2 and SEL 3 broths incubated at 43 C than at 35 C, the difference was not statistically significant.

The analysis of frozen beef patties demonstrates the practical use and reliability of CTET broth since it was as efficient as the conventional TETBG broth. SEL 2 and SEL 3 broths were not as effective as SC broth. The observation that fewer patties were positive in the combined selenite media after 60 days storage indicates that a reduction of the salmonellae population may have occurred. The combined selenite media appear to be less sensitive to low numbers of Salmonella. Methods of freezing did not significantly affect the detection of salmonellae in the ground beef but the serotypes used as inocula did cause variations in recovery. Table 12 illustrates differences in detection due to serotype. When the patties were initially sampled, only those inoculated with S. anatum produced any negative results (Table 12). After 60 days storage, there were also more negative samples observed for meat inoculated with S. anatum than with S. heidelberg and S. typhimurium (Table 12). This might be explained by the slow rate of repair of S. anatum cells as demonstrated in resuscitation studies using the combined enrichment media. Because S. anatum repaired more slowly, the addition of selenite after just 4 h incubation may have inhibited its recovery in SEL 2 and SEL 3 broths.

Table 12. Comparison of conventional and combined methods for the recovery of Salmonella serotypes from frozen ground beef

Serotype	Storage Time (days at -29 C)	No. of Samples <sup>b</sup>	Number of salmonellae positive replications <sup>a</sup>									
			Combined <sup>c</sup>						Conventional <sup>c</sup>			
			SEL 2		SEL 3		CTET		SC		TETBG	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
<u>S. typhimurium</u>	0	6	6		6		6		6		6	
	35 C	3	1	3	1	2	3	3	3	3	3	3
	43 C	3	2	3	1	2	3	3	3	3	3	3
<u>S. anatum</u>	0	6	6		2		6		6		6	
	35 C	3	0	1	0	0	3	3	3	3	3	3
	43 C	3	3	3	0	0	3	3	3	3	3	3

	0	6	6	6	6	6	6	6	6	6	6	6
<u>S. heidelberg</u>	60	35 C	3	1	3	0	2	3	3	3	3	3
		43 C	3	0	3	0	3	3	3	3	3	3

---

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>All the enriched samples examined after 0 days storage were incubated at 35 C while the patties analyzed after 60 days frozen storage were incubated at either 35 C or 43 C.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24 h incubation when the patties were stored 0 days and after 24 and 48 h incubation following 60 days storage.

### Isolation of Salmonellae From Frozen Turkey Roasts

Frozen turkey roasts were included in the comparison study because they are often naturally contaminated with Salmonella. Guthertz et al. (1976 and 1977) have reported the presence of salmonellae in comminuted turkey meat obtained from retail sources. Frozen roasts are also examples of a processed meat product which would require a preenrichment step in order to enhance the detection of stressed salmonellae. The preenrichment of processed meats has been recommended by Gabis and Silliker (1974), Edel and Kampelmacher (1973), and Vassiliadis et al. (1976). Competitive flora, including pseudomonads and Proteus present on the roasts, unless suppressed by a selective enrichment step, would interfere with the isolation of salmonellae.

The average number of mesophiles and psychrotrophs on the roasts is indicated in Table 13. These levels are similar to the number of competitors

Table 13. Competitive flora present in frozen turkey roasts

Samples <sup>a</sup>	Mesophiles	Psychrotrophs
	No. bacteria per g x 10 <sup>4</sup>	
A, B, C	6.0	4.3
D, E, F	15.0	17.0
G, H, I	7.2	7.0

<sup>a</sup>Counts were determined by averaging the number of organisms enumerated in duplicate control samples of three roasts at each sampling time.

observed on the ground beef patties. This presented the combined enrichment media with a similar concentration of organisms to suppress. The roast flora was of a different type than that observed on ground beef. More Proteus organisms grew on selective agars prepared from all the Salmonella enrichment broths. However, they were effectively suppressed on BG agar by incorporating 5 mg of sulfadiazine per liter into the agar base.

Samples of the roasts were inoculated with salmonellae to compare the ability of each enrichment medium to detect a known concentration of cells. Also, control samples were analyzed by each comparison method to determine the effectiveness of the media for isolating natural Salmonella contaminants.

Table 14 contains the results from the analysis of turkey roasts inoculated with 8 to 10 salmonellae per gram. Control samples A and B were contaminated with S. san diego and S. st paul, respectively (Table 15). CTET, TET and SC broths isolated these contaminants and also detected all the samples inoculated with salmonellae. The natural contaminants were not found with either SEL 2 or SEL3 enrichment broths. Roast samples inoculated with S. typhimurium were not positive in one of two cases in both combined selenite broths, and the SEL 2 medium did not detect S. heidelberg in one seeded sample. Because fewer salmonellae were isolated from roasts enriched in SEL 2 and SEL 3 broths, the incubation period was extended by 24 h for the same reasons previously discussed.

Data in Table 16 indicates the effect of incubation time when samples were incubated for 24 and 48 h in the conventional and combined enrichment media.

Table 14. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts

Sample <sup>b</sup>	Number of salmonellae positive samples <sup>a</sup>				
	Combined <sup>c</sup>			Conventional <sup>c</sup>	
	SEL 2	SEL 3	CTET	LacSC	LacTET
	24 h	24 h	24 h	24 h	24 h
A Control	-	-	1+	1+	1+
<u>S. typhimurium</u>	1+	1+	2+	2+	2+
B Control	-	-	1+	1+	1+
<u>S. heidelberg</u>	1+	2+	2+	2+	2+
C Control	-	-	-	-	-
<u>S. anatum</u>	2+	2+	2+	2+	2+
Tot- Control	-	-	2+	2+	2+
al Salmonellae	4+	5+	6+	6+	6+

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>Duplicate enriched control and inoculated (8 to 10 Salmonella per g) turkey roast samples were incubated at 35 C.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, BS agar after 24 h incubation at 35 C.

Table 15. Salmonella species isolated from samples of frozen control turkey roasts

Sample	<u>Salmonella</u> spp. isolated <sup>a</sup>
A	<u>S. san diego</u>
B	<u>S. st paul</u>
D	<u>S. heidelberg</u>
G	<u>S. anatum</u>
	<u>Arizona hinshawii</u>
H	<u>S. bredeney</u>

<sup>a</sup>Identified by the State Hygienic Laboratory, Iowa City, Iowa.

Only sample D was naturally contaminated with S. heidelberg which was isolated from CTET, TET, and SC broths (Table 15). One control sample was salmonellae negative after 48 h incubation in SC broth. Neither SEL 2 broth or SEL 3 broth gave positive results from roasts inoculated with S. heidelberg.

Turkey samples D, E, and F were inoculated with about 1 to 2 salmonellae per gram. This provided a more rigid test to determine the ability of the combined enrichment procedure to isolate low numbers of salmonellae. Only S. typhimurium was detected in the combined selenite broths. The 48 h incubation period also increased the isolations of this serotype. All inoculated samples were salmonellae positive at each sampling time in SC and TET broths. One roast inoculated with S. anatum was falsely negative in CTET broth after 48 h incubation.



Table 16. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts

Sample <sup>b</sup>	Number of salmonellae positive samples <sup>a</sup>										
	Combined <sup>c</sup>				Conventional <sup>c</sup>						
	SEL 2		SEL 3		CTET		LacSC		LacTET		
	24	48	24	48	24	48	24	48	24	48	
D	Control	-	-	-	-	2+	2+	2+	1+	2+	2+
	<u>S. typhimurium</u>	1+	2+	1+	2+	2+	2+	2+	2+	2+	2+
E	Control	-	-	-	-	-	-	-	-	-	-
	<u>S. anatum</u>	-	-	-	-	2+	1+	2+	2+	2+	2+
F	Control	-	-	-	-	-	-	-	-	-	-
	<u>S. heidelberg</u>	-	-	-	-	2+	2+	2+	2+	2+	2+
Total	Control	-	-	-	-	2+	2+	2+	1+	2+	2+
	Salmonellae	1+	2+	1+	2+	6+	5+	6+	6+	6+	6+

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>Duplicate enriched control and inoculated (1 to 2 Salmonella per g) turkey roast samples were incubated at 35 C.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, BS agar after 24 and 48 h incubation.

Because salmonellae were not isolated from known positive samples when using the combined selenite broths, an additional experiment was designed to possibly increase their selectivity. All culture broths were analyzed after 24 and 48 h incubation at either 35 C or 43 C. The elevated incubation temperature was used to possibly increase the sensitivity of the combined selenite media. Approximately 1 to 2 salmonellae per gram were added to the inoculated roast samples. Data from this experiment are presented in Table 17.

All control and inoculated roasts enriched in SEL 2 and SEL 3 broths were salmonellae negative except for a single sample containing S. heidelberg which was positive in SEL 2 broth after 24 h at 43 C. All inoculated samples were salmonellae positive when enriched in CTET and TET broths. One sample containing S. anatum incubated at 35 C in SC broth was negative after both 24 and 48 h incubation. S. anatum and Arizona hinshawii were detected in control sample G and S. bredeney was isolated from sample H (Table 15). CTET, TET, and SC broths all detected the presence of these natural contaminants when incubated at 43 C. Only SC broth isolated S. bredeney from roast H when enriched at 35 C for 48 h. Incubation of control sample G at 35 C resulted in salmonellae isolations only when CTET broth was used.

These studies with frozen turkey roasts demonstrated the reliability of CTET broth. Only one more inoculated sample was detected using the conventional tetrathionate medium than CTET broth. However, one additional

Table 17. Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts incubated at 35 C and 43 C

Sample <sup>b</sup>			Number of salmonellae positive samples <sup>a</sup>									
			Combined <sup>c</sup>					Conventional <sup>c</sup>				
			SEL 2		SEL 3		CTET		LacSC		LacTET	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
G	Control	35 C	-	-	-	-	+	+	-	-	-	-
		43 C	-	-	-	-	+	+	+	+	+	+
	<u>S. anatum</u>	35 C	-	-	-	-	+	+	-	-	+	+
		43 C	-	-	-	-	+	+	+	+	+	+
H	Control	35 C	-	-	-	-	-	-	-	+	-	-
		43 C	-	-	-	-	+	+	+	+	+	+
	<u>S. typhimurium</u>	35 C	-	-	-	-	+	+	+	+	+	+
		43 C	-	-	-	-	+	+	+	+	+	+
I	Control	35 C	-	-	-	-	-	-	-	-	-	-
		43 C	-	-	-	-	-	-	-	-	-	-
	<u>S. heidelberg</u>	35 C	-	-	-	-	+	+	+	+	+	+
		43 C	+	-	-	-	+	+	+	+	+	+

Total	Control	35 C	-	-	--	-	+	+	-	+	-	-
		43 C	-	-	--	-	2+	2+	2+	2+	2+	2+
	Salmonellae	35 C	-	-	--	-	3+	3+	2+	2+	3+	3+
		43 C	1+	-	--	-	3+	3+	3+	3+	3+	3+

---

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>Single enriched control and inoculated (1 to 2 Salmonella per g) turkey roast samples were incubated at 35 C and 43 C.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, BS agar after 24 and 48 h incubation.

control sample was salmonellae positive in CTET broth but negative in TET broth. All control samples positive in SC broth were salmonellae negative when enriched in SEL 2 and SEL 3 broths. The combined selenite media also failed to isolate salmonellae from many inoculated samples. The inefficiency of the combined selenite media is probably due to their inability to suppress the multiplication of competitors as effectively as the other enrichment media. Streak plates prepared from SEL 2 and SEL 3 broths contained more non-salmonellae-like colonies than on agars streaked from CTET, TET, and SC broths. This greater concentration of competitors may have inhibited the growth of the salmonellae in the enrichment media or masked their presence on the selective agars.

#### Isolation of Salmonellae From Dried Foods

Dried food, including dried soup and gravy mixes, and spray dried egg albumen, was the third type of food analyzed in the comparison study. These products were chosen because they must undergo a preenrichment phase in order to determine the presence of salmonellae sublethally injured by the drying processes. The presence or absence of natural salmonellae contaminants in each dried product was determined utilizing the comparison procedures. No salmonellae were isolated from dried gravy or soup but a Salmonella species was detected in dried egg sample C using CTET broth (Table 18). The serotype of the isolate could not be determined by the State Hygienic Laboratory, Iowa City,

Table 18. Comparison of conventional and combined methods for the recovery of salmonellae from dried beef gravy, vegetable beef soup, and spray dried egg albumen inoculated with salmonellae

Type of Food	Sample	No. of inoculated samples per recovery method <sup>a</sup>	Number of salmonellae positive replications <sup>b</sup>									
			Combined <sup>c</sup>				Conventional <sup>c</sup>					
			SEL 2		SEL 3		CTET		LacSC		LacTET	
			24	48	24	48	24	48	24	48	24	48
Gravy	A	2	2	2	2	2	2	2	1	2	2	
Gravy	B	1	1	1	1	1	1	1	1	1	1	
Soup	A	2	2	1	2	2	2	2	2	2	2	
Soup	B	1	1	1	1	1	1	1	1	1	1	
Egg	A	2	2	2	2	2	2	2	2	2	2	
Egg	B	2	2	2	2	2	2	2	2	2	2	
Egg	C	1	0	0	0	0	1	0	0	0	0	
Total	7	11	10	10	9	10	10	11	10	9	10	10

<sup>a</sup>All the samples were inoculated with 1 to 2 salmonellae per gram except spray dried egg white sample C, which was naturally contaminated with salmonellae.

<sup>b</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>c</sup>Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24 and 48 h incubation at 35 C.

Iowa. The number of mesophiles enumerated in dried gravy mix per gram was  $4.0 \times 10^4$ . The soup mix contained  $6.0 \times 10^3$  mesophiles per gram and the salmonellae negative egg samples had  $8.2 \times 10^2$  mesophiles per gram.

Because no salmonellae contaminants were found, each sample was inoculated with 1 to 2 Salmonella per gram. Every enrichment medium was analyzed for salmonellae after 24 and 48 h incubation at 35 C. CTET broth was again as efficient as TET broth, which is indicated in Table 18. SEL 3 broth was equal to SC broth in efficiency, but SEL 2 broth detected one additional sample negative by the other selenite enrichment methods.

These results for dried foods again demonstrate the reliability of CTET broth as was found for beef and turkey, but SEL 2 and SEL 3 broths were also as efficient as the conventional SC medium. However, the increased sensitivity of the combined selenite broths must be interpreted with caution. Fewer competitors were present in the dried foods than in the meat products previously examined. The nature of the flora in the desiccated foods was also different. Less gram negative organisms were observed and more gram positive sporeforming rods were encountered. The combined selenite media were probably more reliable in this work because the salmonellae might have been able to reach greater numbers due to the absence of large populations of competing gram negative microorganisms.

### Detection of Salmonellae in Foods With the FA Technique

Detection of salmonellae in the various foods analyzed in the comparison study by cultural methods and the FA technique is presented in Table 19. There were no false positive samples determined by the FA procedure but one turkey roast sample which was culturally positive in TET broth was a false negative sample by the FA test. Only 30 h were required to detect the presence of salmonellae in the samples with the FA technique while cultural methods required 5 days.

The pooled "OH" antiserum absorbed to both whole cells and flagella present in the enrichment broths. Identification of Salmonella positive samples using the pooled antiserum was readily made because cells were relatively easy to identify as salmonellae because of the fluorescing flagella. Flagella were not observed in all samples positive by FA analysis. Background fluorescence was more of a problem with the ground beef and turkey samples than the dried products which did not contain as much fat as the meat samples.

Figure 10 illustrates typical fields containing fluorescing cells. The samples were ground beef inoculated with S. typhimurium and enriched in both the conventional and combined enrichment media. Fewer cells were consistently observed on FA slides prepared from the combined selenite broths, with SEL 3 broth containing the fewest cells per positive field. Slides made from SC, TET, and CTET broths generally contained similar numbers of fluorescing cells per field. Results from this study indicate that salmonellae will reach levels detectable



Table 19. Comparison of FA and conventional identification methods for the detection of salmonellae in various foods inoculated with known salmonellae

Sample	Confirmation Method	Enrichment Medium				
		SEL 2	SEL 3	CTET	SC	TET
Ground Beef A	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Ground Beef B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Ground Beef C	FA	+	-	+	+	+
	Conventional	+	-	+	+	+
Turkey Roast I	FA	+	-	+	+	-
	Conventional	+	-	+	+	+
Turkey Roast G	FA	-	-	+	+	+
	Conventional	-	-	+	+	+
Dried Gravy B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Dried Soup B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Dried Egg A	FA	+	+	+	+	+
	Conventional	+	+	+	+	+

by the direct FA technique within 24 h in the combined enrichment broths.

#### Statistical Analysis of the Comparison Study

Results of the study made to determine the effectiveness of the combined enrichment broths in comparison to conventional media used for the isolation of Salmonella are listed in Table 20. The data represent only those samples inoculated with salmonellae and incubated at 35 C for 24 h. Table 21 includes the same samples as in Table 20 but only those which were tested for salmonellae after both 24 and 48 h incubation.

**Figure 10. Direct FA staining effects when the combined and conventional enrichment media were used to enrich samples of frozen ground beef containing S. typhimurium.**

Upper left - TET broth

Upper right - CTET broth

Lower left - SC broth

Lower right - SEL 2 broth

Lower center - SEL 3 broth

PLEASE NOTE:

Dissertation contains color  
photographs which will not  
reproduce well in xerography.  
Filmed in the best possible  
way.

UNIVERSITY MICROFILMS.

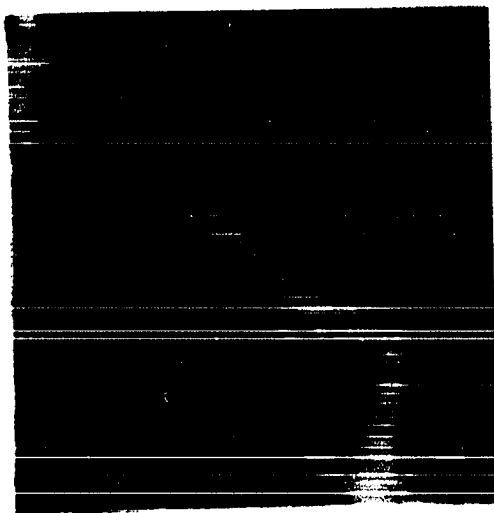
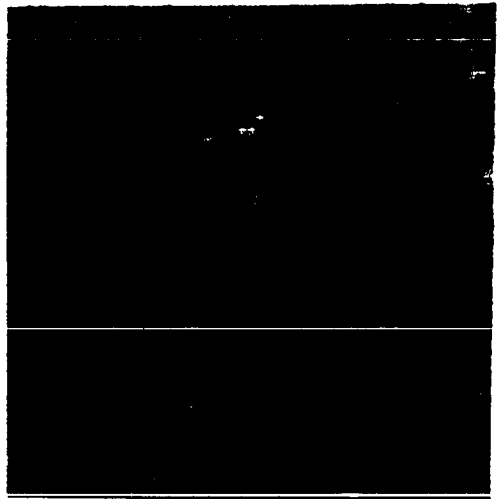
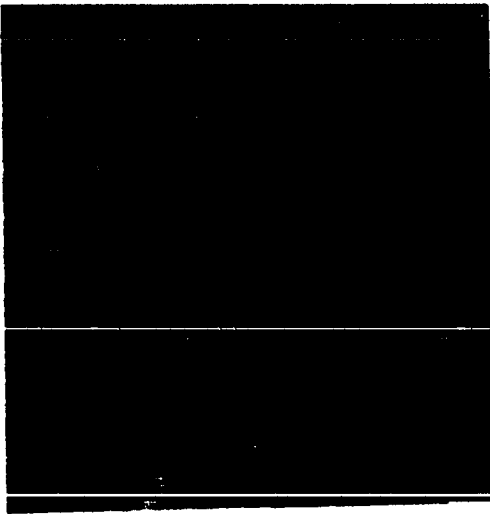
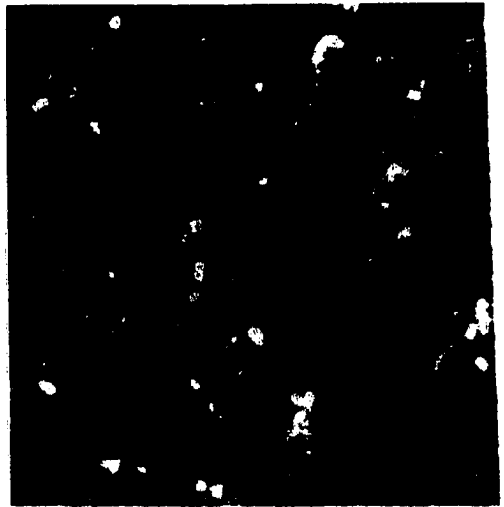
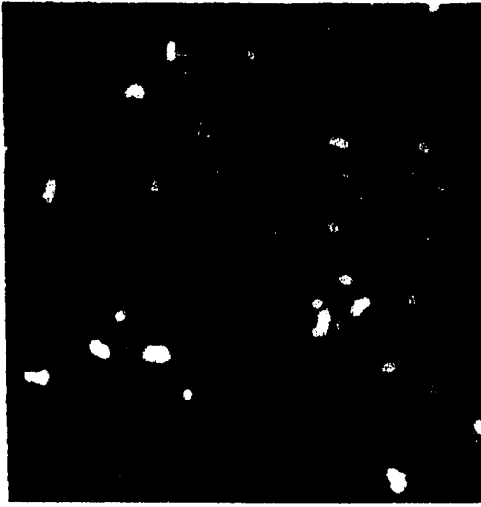


Table 20. Comparison of conventional and combined methods for the recovery of salmonellae from various foods inoculated with salmonellae

Type of Food	No. of Lots	No. of inoculated samples per recovery method	Number of salmonellae positive replications <sup>a</sup>				
			Combined <sup>b</sup>			Conventional <sup>b</sup>	
			SEL 2	SEL 3	CTET	SC	TET
			24 h	24 h	24 h	24 h	24 h
Ground Beef	9	27	22	17	27	27	27
Turkey Roast	9	15	5	6	15	14	15
Dried Soup	2	3	3	2	3	3	3
Dried Gravy	2	3	3	3	3	3	3
Dried Eggs	1	4	4	4	4	4	4
Total	23	52	37	32	52	51	52

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24 h incubation at 35 C.

Table 21. Comparison of conventional and combined methods for the recovery of salmonellae from various foods inoculated with salmonellae and enriched for 24 and 48 h

Type of Food	No. of Lots	No. of inoculated samples per recovery method	Number of salmonellae positive replications <sup>a</sup>									
			Combined <sup>b</sup>						Conventional <sup>b</sup>			
			SEL 2		SEL 3		CTET		SC		TET	
			24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Ground Beef	9	9	4	7	3	5	9	9	9	9	9	9
Turkey Roast	6	9	1	2	1	2	9	8	8	8	9	9
Dried Soup	2	3	3	3	2	3	3	3	3	3	3	3
Dried Gravy	2	3	3	3	3	3	3	3	3	2	3	3
Dried Egg	1	4	4	4	4	4	4	4	4	4	4	4
Total	23	28	15	19	13	17	28	27	27	26	28	28

<sup>a</sup>A replication was recorded as positive if a single Salmonella sp. was isolated from it.

<sup>b</sup>Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24 and 48 h incubation at 35 C.

A total of 52 samples was inoculated with salmonellae and assayed after 24 h incubation. CTET broth detected every positive sample, as did TET broth. SC broth failed to isolate salmonellae from only one turkey roast sample. Use of SEL 2 broth resulted in 15 negative samples and SEL 3 broth did not detect the salmonellae in 20 samples. A Chi-square analysis of these data indicated no significant difference existed between CTET, TET, and SC broths for use as salmonellae enrichment media. SC broth was significantly more effective than SEL 2 and SEL 3 broths. Although more salmonellae isolations occurred in the SEL 2 medium than the SEL 3 combined broth, the difference was not significant.

The additional 24 h incubation period enhanced the detection of salmonellae in foods enriched in SEL 2 and SEL 3 broths. This increase in efficiency was not statistically significant at the 5% level of probability. The improvement was probably caused by the multiplication of the salmonellae to detectable levels after 48 h incubation. Since competitors do not seem to be as readily inhibited in the combined selenite broths as in CTET broth, a 48 h incubation period needs to be used to improve the effectiveness of the selenite media.

## SUMMARY

Studies were made to determine the feasibility of combining preenrichment in lactose broth and selective enrichment in tetrathionate (TET) or selenite-cystine (SC) broths, media used for salmonellae assays, into a single combined enrichment procedure. This was achieved by adding selective agents to nonselective basal broths following an appropriate preenrichment period. Injured salmonellae were able to repair in the absence of selective agents and competitors were inhibited following the addition of the agents. Tetrathionate and selenite are the selective components of TET and SC broths respectively. Tetrathionate was formed in combined tetrathionate (CTET) basal broth when a solution of iodine was added by pipette. Selenite was also added by pipette from a stock solution to nonselective combined selenite (SEL 1, SEL 2, and SEL 3) basal broths. The combined technique was used to detect the presence of both injured and uninjured salmonellae in foods.

The principle of combining preenrichment and selective enrichment into a single enrichment technique has been previously demonstrated. Alford and Knight (1969) obtained favorable results by delaying the addition of selenite to SC broth which contained no selective agent at the time of inoculation with Salmonella contaminated foods. Lanz and Hartman (1976) also demonstrated the feasibility of combined enrichment procedures by detecting injured and uninjured coliforms using timed-release capsules containing brilliant green and brilliant



green plus oxgall. The capsules were added to nonselective media at the time of inoculation and the media gradually became selective as the agents were released. This principle was also adopted for use in the isolation of salmonellae from foods by Sveum and Hartman (1977). Iodine or selenite was gradually released from timed-release capsules added at the time of inoculation and the media progressively became selective. This method was not as sensitive as the conventional techniques used because a selective environment was gradually formed which failed to suppress the growth of competitors. This problem was alleviated in the present study because selective conditions were formed immediately following the addition of the selective agents.

Heat injured and freeze injured salmonellae were able to undergo resuscitation in the combined enrichment media. The rates of repair were similar to those observed in lactose broth but freeze injured cells recovered from stress more slowly than heat injured salmonellae. Preenrichment for 4 h was found to be sufficiently long to allow for the recovery of injured salmonellae. Determining the presence of sublethally stressed salmonellae in foods is essential because injured salmonellae have been demonstrated to still be pathogenic (Sorrells et al. 1970). The isolation methods utilized must allow for the resuscitation of injured cells but must also suppress the growth of competitors. Growth of E. coli cells was inhibited in CTET broth as effectively as in TET broth. The multiplication of E. coli was not suppressed substantially in the combined selenite media.

The efficiency of the combined procedures was compared to conventional techniques used to isolate salmonellae from foods. Three types of food, frozen ground beef, frozen turkey roasts, and dried foods were analyzed for salmonellae. Each category of food was also inoculated with low levels of salmonellae in order to determine the efficiency and to gain some idea of the sensitivity of the combined methods.

In comparison to conventional methods used to examine the foods for Salmonella, CTET broth was as reliable as TET and SC broths. SEL 2 and SEL 3 broths detected salmonellae in dried foods more readily than in the meats examined. The combined selenite technique possibly was not as effective as CTET broth when low numbers of salmonellae were present in food containing a high concentration of competing microorganisms. The effectiveness of the selenite combined procedure can be enhanced by incubating the media an additional 24 h. In studies comparing the ability of each medium to isolate salmonellae from foods, SEL 2 broth was a more reliable enrichment medium than SEL 3 broth but neither was as dependable as SC broth.

The presence of salmonellae in the foods assayed could be determined in 30 h using the direct FA technique and the combined enrichment procedure. Fluorescent stains prepared from the combined selenite broths contained fewer fluorescing cells per field than slides prepared from the other enrichment broths; CTET was therefore more adaptable for FA staining than the combined selenite media.

The results of this study demonstrate the feasibility of combining the separate steps of preenrichment and selective enrichment used in salmonellae assays. A reduction in incubation time was obtained utilizing the combined method because only 24 h were needed for incubation of samples containing stressed salmonellae. Forty-eight hours are required when using the conventional procedures which utilize separate 24 h nonselective and selective enrichment periods.

Reductions in labor and manhours required for Salmonella assays were also achieved utilizing the combined enrichment technique. Transfers did not have to be made from preenrichment media to selective enrichment broths. Also, only one set of media and glassware had to be prepared and cleaned using the combined technique while conventional methods required two sets of media and glassware.

## CONCLUSIONS

1. Successful heat injury of salmonellae for experimental purposes can be achieved by heat shocking the cells at 52 C for 30 min.
2. Heat injured S. typhimurium and S. anatum will undergo repair in lactose broth and the combined tetrathionate broth within 4 h, so that selective enrichment of salmonellae is possible after that time.
3. In general, heat injured salmonellae undergo cellular repair more rapidly than freeze injured salmonellae in various enrichment media.
4. Combined tetrathionate broth is more effective than combined selenite media for inhibiting E. coli in salmonellae detection. Combined tetrathionate broth also produces more reliable results than the combined selenite media do in comparison with conventional methods for isolating salmonellae from foods.
5. Combined tetrathionate broth is as effective as conventional tetrathionate broth in determining salmonellae in foods, and the determination can be completed within 30 h when the direct fluorescent antibody technique is used for confirmation.
6. Detection of salmonellae in ground beef is improved by lengthening the incubation period to 48 h and by incubating at 43 C instead of 35 C when combined selenite media are used.
7. Combined selenite broths are more effective in detecting salmonellae in dried foods than in other foods because fewer competing gram

negative bacteria are present in the dried products.

8. Combined selenite media are not as efficient as selenite-cystine broth in salmonellae enrichment.

9. It is feasible to combine the separate steps of preenrichment and selective enrichment into a single procedure so that both phases of salmonellae isolation can be done in 24 h instead of 48 h.

10. Use of the combined enrichment procedure substantially reduces the materials and manhours required for salmonellae assays.

## LITERATURE CITED

- Alford, J. A., and N. Knight. 1969. Applicability of aeration and delayed addition of selenite to the isolation of salmonellae. *Appl. Microbiol.* 18:1060-1064.
- Arkhangel'skii, I. I., and V. M. Kartoshova. 1962. Accelerated methods of detecting Salmonella in milk. *Veterinariya* 9:74-78.
- Aserkoff, B., S. A. Schroeder, and P. S. Brachman. 1970. Salmonellosis in the United States--A five year review. *Amer. J. Epid.* 92:13-24.
- Association of Official Analytical Chemists. 1975a. Official methods of analysis of the association of official analytical chemists. 12th ed. Washington, D. C.
- Association of Official Analytical Chemists. 1975b. Fluorescent antibody (FA) method. Official first action. Section 46. AO3-46. AO6. *J. A.O. A.C.* 58:417-419.
- Banic, S. 1964. A new enrichment medium for salmonellae. *J. Hyg.* 62: 25-28.
- Bissonnette, G. K., J. J. Jezeski, G. A. McFeters, and D. G. Stuart. 1977. Evaluation of recovery methods to detect coliforms in water. *Appl. Environ. Microbiol.* 33:590-595.
- Boothroyd, M., and A. C. Baird-Parker. 1973. The use of enrichment serology for Salmonella detection in human foods and animal food. *J. Appl. Bacteriol.* 36:165-172.
- Busta, F. F. 1976. Practical implications of injured microorganisms in food. *J. Milk Food Technol.* 39:138-145.
- Byrne, A. F., M. M. Rayman, and M. D. Schneider. 1955. Methods for the detection and estimation of numbers of Salmonella in dried eggs and other food products. *Appl. Microbiol.* 3:368-372.
- Center for Disease Control. November, 1977. Salmonella surveillance; annual summary 1976. Center for Disease Control, Atlanta, Georgia.

- Chau, P. Y., and C. R. Forrest. 1972. Further study of strontium selenite and selenite F broths for the isolation of Salmonella typhi. *J. Clin. Pathol.* 24:966.
- Cherry, W. B., B. M. Thomason, J. B. Gladden, N. Halsing, and A. M. Murlin. 1975. Detection of salmonellae in foodstuffs, water, and feces by immunofluorescence. *Ann. N. Y. Acad. Sci.* 254:350-368.
- Clark, C. W., and Z. J. Ordal. 1969. Thermal injury and recovery of Salmonella typhimurium and its effect on enumeration procedures. *Appl. Microbiol.* 18:332-336.
- Cohen, M. L., and P. A. Blake. 1977. Trends in foodborne salmonellosis outbreaks: 1963-1975. *J. Food Prot.* 40:798-800.
- Coons, A. H., H. J. Creech, R. M. Jones, and E. Berliner. 1942. The demonstration of pneumococcal antigen in tissue by the use of fluorescent antibodies. *J. Immunol.* 45:159-170.
- Cox, N. A., and A. J. Mercuri. 1978. Comparison of two minikits (API and R-B) for identification of Enterobacteriaceae isolated from poultry and meat products. *J. Food Prot.* 41:107-110.
- Cox, N. A., B. H. Davis, J. H. Kendall, A. B. Watts, and A. R. Colmer. 1972. Salmonella in the laying hen. 3. A comparison of various enrichment broths and plating media for the isolation of Salmonella from poultry feces and poultry food products. *Poultry Sci.* 51:1312-1316.
- Cox, N. A., F. McHan, and D. Y. C. Fung. 1977. Commercially available minikits for identification of Enterobacteriaceae. *J. Food Prot.* 40:866-872.
- Dack, G. M. 1955. Methodology for Salmonella in food. *Bacteriol. Rev.* 4:275-276.
- D'Aoust, J. Y. 1977a. Limitations of lysine-iron-cystine-neutral red broth in the presumptive identification of Salmonella. *Appl. Environ. Microbiol.* 34:595-596.
- D'Aoust, J. Y. 1977b. Salmonella and the chocolate industry. A review. *J. Food Prot.* 40:718-727.

- D'Aoust, J. Y. 1978. Recovery of sublethally heat-injured Salmonella typhimurium on supplemented plating media. *Appl. Environ. Microbiol.* 35:483-486.
- Edel, W., and E. H. Kampelmacher. 1973. Comparative studies on the isolation of sublethally injured salmonellae in nine European laboratories. *Bull. Wld. Hlth. Org.* 48:167-174.
- Edel, W., and E. H. Kampelmacher. 1974. Comparative studies on Salmonella isolation from feeds in ten laboratories. *Bull. Wld. Hlth. Org.* 50:421-426.
- Edwards, P. R., and M. M. Galton. 1967. Salmonellosis. *Adv. Vet. Sci.* 11:1-63.
- Edwards, P. R., and W. H. Ewing. 1972. Identification of Enterobacteriaceae. 3rd ed. Burgess Publishing Co., Minneapolis, Minnesota.
- Fagerberg, D. J., and J. S. Avens. 1976. Enrichment and plating methodology for Salmonella detection in food. A review. *J. Milk Food Technol.* 39:628-646.
- Food and Drug Administration. 1969. Bacteriological analytical manual for foods. 2nd ed. United States Food and Drug Administration, Washington, D. C.
- Food and Drug Administration. 1976. Bacteriological Analytical Manual for Foods. 4th ed. United States Food and Drug Administration, Washington, D. C.
- Gabis, D. A., and J. H. Silliker. 1974. ICMSF methods studies. II. Comparison of analytical schemes for detection of Salmonella in high moisture foods. *Can. J. Microbiol.* 20:663-669.
- Gabis, D. A., and J. H. Silliker. 1977. ICMSF methods studies IX. The influence of selective enrichment broths, differential plating media, and incubation temperatures on the detection of Salmonella in dried foods and feed ingredients. *Can. J. Microbiol.* 23:1225-1231.
- Galton, M. M., C. L. Stucker, H. B. McElrath, and A. V. Hardy. 1950. A preliminary study of tetrathionate brilliant green bile broth with added sulfathiazole for isolation of Salmonella from dogs. *Bacteriol. Proc.* 1950:115.



- Galton, M. M., W. D. Lowery, and A. V. Hardy. 1954. Salmonella in fresh and smoked pork sausage. *J. Infect. Dis.* 95:232-235.
- Galton, M. M., J. R. Boring, and W. T. Martin. 1964. Salmonellae in foods. A review of methods for isolation and a suggested procedure. National Communicable Disease Center, Atlanta, Georgia.
- Galton, M. M., G. K. Morris, and W. T. Martin. 1968. Salmonellae in foods and feeds. U.S. Department of Health, Education, and Welfare. National Communicable Disease Center, Atlanta, Georgia.
- Gerichter, C. B., and I. Sechter. 1966. Comparison of methods for the isolation of Salmonella from bone meal. *Appl. Microbiol.* 14:711-715.
- Goepfert, J. M. 1976. The aerobic plate count, coliform and Escherichia coli content of raw ground beef at the retail level. *J. Milk Food Technol.* 39:175-178.
- Grau, F. H., and M. G. Smith. 1972. A comparison of methods for the enrichment of salmonellae from sheep faeces. *J. Appl. Bacteriol.* 35:531-536.
- Gundstrup, A. S. P., K. Grunnet, and G. J. Bonde. 1969. Salmonellae antagonists and salmonella enrichment media. *Hlth. Lab. Sci.* 6:221-226.
- Guthertz, L. S., and R. L. Okoluk. 1978. Comparison of miniaturized multitest systems with conventional methodology for identification of Enterobacteriaceae from foods. *Appl. Environ. Microbiol.* 35:109-112.
- Guthertz, L. S., J. T. Fruin, D. Spicer, and J. L. Fowler. 1976. Microbiology of fresh comminuted turkey meat. *J. Milk Food Technol.* 39: 823-829.
- Guthertz, L. S., J. T. Fruin, R. L. Okuluk, and J. L. Fowler. 1977. Microbial quality of frozen comminuted turkey meat. *J. Food Sci.* 42:1344-1346.
- Haglund, J. R., J. D. Ayres, A. M. Paton, A. A. Kraft, and L. Y. Quinn. 1964. Detection of Salmonella in eggs and egg products with fluorescent antibody. *Appl. Microbiol.* 12:447-450.

- Hajna, A. A. 1955. A new enrichment broth medium for gram-negative organisms of the intestinal group. *Public Health Lab.* 13:83-89.
- Hajna, A. A., and S. R. Damon. 1956. New enrichment and plating media for the isolation of Salmonella and Shigella organisms. *Appl. Microbiol.* 4:341-345.
- Hall, H. E., D. F. Brown, and R. Angelotti. 1964. The quantification of salmonellae in foods by using the lactose preenrichment method of North. *J. Milk Food Technol.* 27:235.
- Hargrove, R. E., F. E. McDonough, and R. J. Reamer. 1971. A selective medium and presumptive procedure for detection of Salmonella in dairy products. *J. Milk Food Technol.* 34:6-11.
- Hartman, P. A., P. S. Hartman, and W. W. Lanz. 1975. Violet red bile 2 agar for stressed coliforms. *Appl. Microbiol.* 29:537-539.
- Harvey, R. W. S., and T. H. Price. 1977. Observations on pre-enrichment for isolating Salmonellas from sewage polluted natural water using Muller-Kauffmann tetrathionate broth prepared with fresh and desiccated ox bile. *J. Appl. Bacteriol.* 43:145-148.
- Hilker, J. S. 1975. Enrichment serology and fluorescent antibody procedures to detect salmonellae in foods. *J. Milk Food Technol.* 38:227-231.
- Hobbs, B. C. 1963. Techniques for the isolation of Salmonella from eggs and egg products. *Ann. Inst. Pasteur.* 104:621-637.
- Hoben, D. A., D. H. Ashton, and A. C. Peterson. 1973. A rapid, presumptive procedure for the detection of Salmonella in foods and food ingredients. *Appl. Microbiol.* 25:123-129.
- Huhtanen, C. N., and J. Naghski. 1972. Effect of type of enrichment and duration of incubation on Salmonella recovery from meat and bone meal. *Appl. Microbiol.* 23:578-585.
- Huhtanen, C. N., J. Naghski, and E. S. Dellamonica. 1972. Efficiency of Salmonella isolation from meat-and-bone meal of one 300-g sample versus ten 30-g samples. *Appl. Microbiol.* 23:688-692.
- Hurst, A. 1977. Bacterial injury: A review. *Can. J. Microbiol.* 23:935-944.

- Ingram, M., and B. M. Mackey. 1976. Inactivation by cold. Pages 111-151 in F. C. Skinner and W. B. Hugo, ed. Inhibition and inactivation of vegetative microbes. Academic Press, New York 1976.
- Iveson, J. B., and E. M. MacKay-Scollay. 1969. Strontium chloride and strontium selenite enrichment broth media in the isolation of Salmonella. J. Hyg. 67:457-464.
- Iveson, J. B., and E. M. MacKay-Scollay. 1972. An evaluation of strontium chloride, Rappaport, and strontium selenite enrichment for the isolation of salmonellae from man, animals, meat products and abattoir effluents. J. Hyg. 70:367-384.
- Jameson, J. E. 1961. A study of tetrathionate enrichment techniques, with particular reference in two new tetrathionate modifications used in isolating salmonellae from sewer swabs. J. Hyg. 59:1-13.
- Jeffries, L. 1959. Novobiocin-tetrathionate broth: A medium of improved selectivity for the isolation of salmonellae in faeces. J. Clin. Pathol. 12:568-571.
- Kafel, S., and F. L. Bryan. 1977. Effects of enrichment media and incubation conditions on isolating salmonellae from ground-meat filtrate. Appl. Environ. Microbiol. 34:285-291.
- Kaper, J. B., G. S. Saylor, M. M. Baldini, and R. R. Colwell. 1977. Ambient-temperature primary nonselective enrichment for isolation of *Salmonella* spp. from an estuarine environment. Appl. Environ. Microbiol. 33:829-835.
- Kauffmann, F. 1930. Die Technik der Typhenbestimmung in der Typhusparatyphus-Gruppe. Zentralbl. Bakt. Orig. 119:152-160.
- Kauffmann, F. 1935. Weitere erfahrungen mit dem kombinierten Anreicherungsverfahren für Salmonellabacillen. J. Hyg. Infektionskr. 117:26-32.
- Klein, D. A., and S. Wu. 1974. Stress: A factor to be considered in heterotrophic microorganism enumeration from aquatic environments. Appl. Microbiol. 27:429-431.
- Knox, R. 1945. The effect of tetrathionate on bacterial growth. Brit. J. Exp. Pathol. 26:146-150.

- Knox, R., P. G. H. Gell, and M. R. Pollock. 1943. The selective action of tetrathionate in bacteriological media. *J. Hyg.* 43:147-158.
- Kristensen, M., U. Lester, and A. Jurgens. 1925. Use of trypsinized casein, brom-thymol-blue, brom-cresol-purple, phenol-red and brilliant green for bacteriological nutrient media. *Brit. J. Exp. Pathol.* 6:291-299.
- Kroniger, D. L., and G. J. Banwart. 1978. Effect of various selective agars on the growth of rare and common salmonellae. *J. Food Sci.* 43:1328-1329.
- Krynski, E. P., and R. C. Heimsch. 1977. Use of enzyme-labeled antibodies to detect Salmonella in foods. *Appl. Environ. Microbiol.* 33:947-954.
- Kueck, L. D. 1974. Effects of freezing and heating on production of enterotoxin type A Staphylococcus aureus. M. S. Thesis. Iowa State University, Ames, Iowa.
- Lanz, W. W. 1975. A new MPN method for coliform enumeration. Ph.D. Thesis. Iowa State University.
- Lanz, W. W., and P. A. Hartman. 1976. Timed-release capsule method for coliform enumeration. *Appl. Environ. Microbiol.* 32:716-722.
- Leifson, E. 1936. New selenite enrichment media for the isolation of typhoid and paratyphoid (Salmonella) bacilli. *Amer. J. Hyg.* 24:423-432.
- Licciardello, J. J., J. T. R. Nickerson, and S. A. Goldblith. 1970. Recovery of salmonellae from irradiated and unirradiated foods. *J. Food Sci.* 35:620-624.
- Litchfield, J. H. 1973. Salmonella and the food industry - Methods for isolation, identification and enumeration. *Crit. Rev. Food Technol.* 3:415-456.
- Martin, S. E., R. S. Flowers, and Z. J. Ordal. 1976. Catalase: Its effect on microbial enumeration. *Appl. Environ. Microbiol.* 32:731-734.
- Maxcy, R. B. 1977. Comparative viability of unirradiated and gamma irradiated bacterial cells. *J. Food Sci.* 42:1056-1059.

- Mayou, J. 1976. MPN — Most probable number. Pages 152-162 in M. L. Speck, ed. Compendium of methods for the microbiological examination of foods. American Public Health Association, Washington, D. C. 1976.
- Mohr, H. K., H. L. Trenk, and M. Yeterian. 1974. Comparison of FA methods and enrichment serology for the detection of Salmonella. Appl. Microbiol. 27:324-328.
- Montford, J., and F. S. Thatcher. 1961. Comparison of four methods of isolating salmonellae from foods, and elaboration of a preferred procedure. J. Food Sci. 26:510-517.
- Morris, G. K., and C. G. Dunn. 1970. Influence of incubation temperature and sodium heptadecyl sulfate (tergitol no. 7) on the isolation salmonellae from pork sausage. Appl. Microbiol. 20:192-195.
- Mossel, D. A. A., and M. A. Ratto. 1970. Rapid detection of sublethally impaired cells of Enterobacteriaceae in dried foods. Appl. Microbiol. 20:273-275.
- Mueller, L. 1923. Un nouveau milieu d'enrichissement pour la recherche du bacille typhique et des paratyphiques. Compt. Rend. Soc. Biol. 89:434-437.
- National Academy of Sciences. 1969. An evaluation of the Salmonella problem. Committee on Salmonella, Division of biology and agriculture, national research council. National Academy of Sciences, Washington, D. C.
- National Academy of Sciences. 1971. Reference methods for the microbiological examination of foods. Food Protection Committee, Food and Nutrition Board, Division of Biology and Agriculture, National Research Council, National Academy of Sciences, Washington, D. C.
- North, W. R. 1960. Use of crystal violet or brilliant green dyes for the determination of salmonellae in dried food products. J. Bacteriol. 80:861.
- North, W. H. 1961. Lactose pre-enrichment method for isolation of Salmonella from dried egg albumen. Appl. Microbiol. 9:188-195.
- North, W. R., and M. T. Bartram. 1953. The efficiency of selenite broth of different compositions in the isolation of Salmonella. Appl. Microbiol. 1:130-134.

- Olson, J. C., Jr. 1975. Development and present status of FDA Salmonella sampling and testing plans. *J. Milk Food Technol.* 38: 369-371.
- Osborne, W. W., and J. H. Stokes. 1955. A modified selenite brilliant-green medium for the isolation of Salmonella from egg products. *Appl. Microbiol.* 3:295-299.
- Palumbo, S. A., and J. A. Alford. 1970. Inhibitory action of tetrathionate enrichment broth. *Appl. Microbiol.* 20:970-976.
- Poelma, P. L. 1967. Detection and identification of Salmonella from dried whole egg, dried egg yolk, and dried egg white: report of a collaborative study. *J. A.O.A.C.* 50:753-763.
- Poelma, P. L., C. R. Wilson, A. Romero, and A. Padron. 1969. Detection and identification of Salmonella from dry milk. *J. A.O.A.C.* 52: 455-458.
- Poelma, P. L., A. Romero, and W. H. Andrews. 1977. Rapid identification of Salmonella and related foodborne bacteria by five biochemical multitest systems. *J. Food Sci.* 42:677-680.
- Price, W. R., R. A. Olsen, and J. E. Hunter. 1972. Salmonella testing of pooled pre-enrichment broth cultures for screening multiple food samples. *Appl. Microbiol.* 23:679-682.
- Raj, H. 1966. Enrichment medium for selection of Salmonella from fish homogenate. *Appl. Microbiol.* 14:12-20.
- Rappaport, F., N. Konforti, and B. Navon. 1956. A new enrichment medium for certain Salmonella. *J. Clin. Pathol.* 9:261-266.
- Ray, B., and M. L. Speck. 1973a. Freeze-injury in bacteria. *CRC Critical Rev. Clinical Lab. Sci.* 4:161-213.
- Ray, B., and M. L. Speck. 1973b. Discrepancies in the enumeration of Escherichia coli. *Appl. Microbiol.* 25:494-498.
- Ray, B., J. J. Jezeski, and F. F. Busta. 1971a. Effect of rehydration on recovery, repair, and growth of injured freeze-dried Salmonella anatum. *Appl. Microbiol.* 22:184-189.

- Ray, B., J. J. Jezeski, and F. F. Busta. 1971b. Repair of injury in freeze-dried Salmonella anatum. *Appl. Microbiol.* 22:401-407.
- Ray, B., D. W. Janssen, and F. F. Busta. 1972. Characterization of the repair of injury induced by freezing Salmonella anatum. *Appl. Microbiol.* 23:803-809.
- Reddy, K. V. 1978. Effect of cryogenic and conventional freezing on microorganisms on poultry and beef. Unpublished M. S. Thesis. Iowa State University, Ames, Iowa.
- Roth, L. A., and D. Keenan. 1971. Acid injury of Escherichia coli. *Can. J. Microbiol.* 17:1005-1008.
- Scheusner, D. L., F. F. Busta, and M. L. Speck. 1971a. Injury of bacteria by sanitizers. *Appl. Microbiol.* 21:41-45.
- Scheusner, D. L., F. F. Busta, and M. L. Speck. 1971b. Inhibition of injured Escherichia coli by several selective agents. *Appl. Microbiol.* 21:46-49.
- Shrift, A., and R. F. Boulette. 1974. Form of selenium in selenite enrichment media for isolation of salmonellae. *Appl. Microbiol.* 27:814-816.
- Silliker, J. H., and W. I. Taylor. 1958. Isolation of salmonellae from food samples. II. The effect of added food samples upon the performance of enrichment broths. *Appl. Microbiol.* 6:228-232.
- Silliker, J. H., and D. A. Gabis. 1973. ICMSF methods studies. I. Comparison of analytical schemes for detection of Salmonella in dried foods. *Can. J. Microbiol.* 19:475-479.
- Silliker, J. H., and D. A. Gabis. 1974. ICMSF methods studies. V. The influence of selective enrichment media and incubation temperatures on the detection of salmonellae in raw frozen meats. *Can. J. Microbiol.* 20:813-816.
- Silliker, J. H., R. H. Deibel, and P. T. Fagan. 1964. Enhancing effect of feces on isolation of salmonellae from selenite broth. *Appl. Microbiol.* 12:100-105.

- Sinskey, T. J., and G. J. Silverman. 1970. Characterization of injury incurred by Escherichia coli upon freeze-drying. *J. Bacteriol.* 101: 429-437.
- Smith, H. W. 1959. The isolation of salmonellae from mesenteric lymph nodes and faeces of pigs, cattle, sheep, dogs, and cats and from other organs of poultry. *J. Hyg.* 57:266-273.
- Smith, P. J. 1977. A standard technique for the isolation of Salmonella from animal feeds. *J. Hyg., Camb.* 79:449-461.
- Sorrells, K. M., M. L. Speck, and J. A. Warren. 1970. Pathogenicity of Salmonella gallinarum after metabolic injury by freezing. *Appl. Microbiol.* 19:39-43.
- Speck, M. L., and B. Ray. 1977. Effects of freezing and storage on microorganisms in frozen foods: A review. *J. Food Prot.* 40:333-336.
- Speck, M. L., B. Ray, and R. B. Read, Jr. 1975. Repair and enumeration of injured coliforms by a plating procedure. *Appl. Microbiol.* 29:549-550.
- Sperber, W. H., and R. H. Deibel. 1969. Accelerated procedure for Salmonella detection in dried foods and feeds involving only broth cultures and serological reactions. *Appl. Microbiol.* 17:533-539.
- Steele, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Company, Inc., New York.
- Stokes, S. L., and W. W. Osborne. 1955. A selenite brilliant green medium for the isolation of Salmonella. *Appl. Microbiol.* 3:217-220.
- Sugiyama, H. G., G. M. Dack, and G. Lippitz. 1960. Agglutinating antiserum for the isolation of Salmonella with special reference to isolation from egg albumen. *Appl. Microbiol.* 8:205-209.
- Surkiewicz, B. F., M. E. Harris, R. P. Elliot, J. F. Macaluso, and M. M. Strand. 1975. Bacteriological survey of raw beef patties produced at establishments under federal inspection. *Appl. Microbiol.* 29: 331-334.
- Sveum, W. H., and P. A. Hartman. 1977. Timed-release capsule method for the detection of salmonellae in foods and feeds. *Appl. Environ. Microbiol.* 33:630-634.



- Swaminathan, B., J. C. Ayres, and J. E. Williams. 1978a. Control of nonspecific staining in the fluorescent antibody technique for the detection of salmonellae in foods. *Appl. Environ. Microbiol.* 35:911-919.
- Swaminathan, B., M. A. B. Link, and J. C. Ayres. 1978b. Incidence of salmonellae in raw meat and poultry samples in retail stores. *J. Food Prot.* 41:518-520.
- Taylor, W. I. 1961. Isolation of salmonellae from food samples. V. Determination of the method of choice for enumeration. *Appl. Microbiol.* 9:487-490.
- Taylor, W. I., and J. H. Silliker. 1961. Isolation of salmonellae from food samples. IV. Comparison of methods of enrichment. *Appl. Microbiol.* 9:484-486.
- Taylor, W. I., B. C. Hobbs, and M. E. Smith. 1964. Comparison of two methods for the isolation of salmonellae from imported foods. *Appl. Microbiol.* 12:53-56.
- Thatcher, F. S., and D. S. Clark. 1968. *Microorganisms in foods, their significance and methods of enumeration.* University of Toronto Press, Toronto, Canada 1968.
- Thomason, B. M., and D. J. Dodd. 1978. Further studies on enrichment procedures for isolating salmonellae from raw meat and poultry. *Amer. Soc. Microbiol. Ann. Meeting* 1978:193. (Abstr.)
- Thomason, B. M., D. J. Dodd, and W. B. Cherry. 1977. Increased recovery of salmonellae from environmental samples enriched with buffered peptone water. *Appl. Environ. Microbiol.* 34:270-273.
- Thomson, S. 1955. The numbers of pathogenic bacilli in faeces in intestinal diseases. *J. Hyg.* 53:217-224.
- Tomlins, R. I., and Z. J. Ordal. 1971. Requirements of Salmonella typhimurium for recovery from thermal injury. *J. Bacteriol.* 105:512-518.
- Tomlins, R. I. and Z. J. Ordal. 1976. Thermal injury and inactivation in vegetative bacteria. Pages 153-190 in F. C. Skinner and W. B. Hugo, ed. *Inhibition and inactivation of vegetative microbes.* Academic Press, New York 1976.

- Tompkin, R. B., and T. V. Keuper. 1973. Factors influencing detection of salmonellae in rendered animal by-products. *Appl. Microbiol.* 25:485-487.
- Troller, J. A. 1976. Salmonella and Shigella. Pages 129-155 in M. P. Defiguereido and D. F. Splittstoesser, ed. *Food Microbiology: Public Health and Spoilage Aspects*. The AVI Publishing Company, INC Westport, Connecticut. 1976.
- United States Department of Agriculture. 1974. Toward global salmonellae test. *Agric. Res.* 22:15.
- van Schothorst, M. 1976. Resuscitation of injured bacteria in foods. Pages 317-328 in F. A. Skinner and W. B. Hugo, ed. *Inhibition and inactivation of vegetative microbes*. Academic Press, New York 1976.
- van Schothorst, M., and F. M. van Leusden. 1972. Studies on the isolation of injured salmonellae from foods. *Zbl. Bakt. Hyg., I. Abt. Orig. A* 221:19-29.
- van Schothorst, M., and F. M. van Leusden. 1975a. Comparison of several methods for the isolation of salmonellae from egg products. *Can. J. Microbiol.* 21:1041-1045.
- van Schothorst, M., and F. M. van Leusden. 1975b. Further studies on the isolation of injured salmonellae from foods. *Zbl. Bakt. Hyg., I. Abt. Orig. A* 230:186-191.
- Vassiliadis, P., J. A. Papadakis, D. Karais, and K. Trichopoulos. 1976. Enrichment in Mueller-Kauffmann's broth and Rappoport's broth from buffered peptone water in the isolation of salmonellae from minced meat. *J. Appl. Bacteriol.* 40:349-354.
- Warseck, M., B. Ray, and M. L. Speck. 1973. Repair and enumeration of injured coliforms in frozen foods. *Appl. Microbiol.* 26:919-924.
- Weiner, H. 1974. The origins of salmonellosis. (Libr. Congr. Card No.: 74-79229). Animal Health institute, Washington, D. C.
- Weiss, K. F., J. C. Ayres, and A. A. Kraft. 1965. Inhibitory action of selenite on Escherichia coli, Proteus vulgaris, and Salmonella thompson. *J. Bacteriol.* 90:857-862.

- Wilson, C. R., W. H. Andrews, and P. L. Poelma. 1975. Evaluation of cultural methods to isolate Salmonella from pressed yeast and dried inactive yeast. *J. Milk Food Technol.* 38:383-385.
- Wilson, J. M., and R. Davies. 1976. Minimal medium recovery of thermally injured Salmonella seftenberg 4969. *J. Appl. Bacteriol.* 40:365-374.

## ACKNOWLEDGEMENTS

I am deeply grateful to Dr. Allen A. Kraft, my major professor, for his interest, guidance, useful suggestions, encouragement, and friendship which he provided during the course of this investigation and in the preparation of this dissertation. The valuable suggestions of Dr. Homer W. Walker were sincerely appreciated. I am indebted to Dr. Paul A. Hartman who served as my major professor during my master's program for helping to develop my interest in food microbiology and encouraging me to continue my graduate studies. The help and advice of the other members of my graduate committee, Dr. John G. Holt and Dr. Harry E. Snyder, was greatly appreciated. Also, thanks are extended to Robert H. Champion and Mary Hampton for their able technical assistance. The statistical advice and assistance of Dr. Donald K. Hotchkiss was appreciated. Special thanks to my wife, Jeanette, for the encouragement and assistance she provided during the course of this project and throughout my graduate education.

This investigation was supported by the Iowa Agriculture and Home Economics Experimental Station, Ames, Iowa; project nos. 2028 and 2252, and by Airco Industrial Gases, Inc.