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A NEW MPN METHOD FOR COLIFORM ENUMERATION.  
Iowa State University, Ph.D., 1975  
Bacteriology

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A new MPN method for coliform enumeration

by

Wayne William Lanz

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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Major: Bacteriology

Approved:

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INTRODUCTION

Physical and chemical stresses impair the metabolic and reproductive functions of microorganisms. The cells become injured and fail to grow in selective media. Resuscitation of the injured cells occurs rapidly in a non-selective nutritional medium (Clark and Ordal, 1969; Tomlins and Ordal, 1971; and Ray and Speck, 1973b). The repaired cells are insensitive to the selective inhibitors and regain their ability to reproduce in the selective medium.

Methods employed to enumerate coliforms in foods and water by using selective media may fail to detect a substantial portion of the population of injured cells. False-negative results, or erroneously low counts, might be obtained; this can affect the true evaluation of the sanitary histories of foods and water. The primary purpose of this work, therefore, was to develop methods to enumerate both healthy and injured coliforms in foods and water. A further objective was to combine the presumptive and confirmed tests for the examination of coliforms. To achieve these goals, a simple and easy method using timed-release, wax-coated capsules was developed.
LITERATURE SURVEY

The use of an indicator organism to evaluate the safety of potable water was first suggested by Schardinger (1894). His proposal was based on the report of Escherich (1887) that *Bacterium coli* (*Escherichia coli*) was invariably isolated from human stools. Schardinger postulated that shedding of *Salmonella typhi*-infected feces into water would coincide with contamination by *E. coli*. He acknowledged that not all waters contaminated with *E. coli* would contain *S. typhi*, but if all contaminated waters were rejected the incidence of typhoid fever would be reduced.

During the 80 years after Schardinger's proposal, the use of *E. coli* as an indicator organism in food and water examination has been extensively developed. Mossel (1967) reviewed the current status of microbial indicators of pollution. The primary objective of the research reported herein was the development of improved or alternative media and methods for isolation of injured coliforms from food and water. The literature review will be confined to this specific area of the overall problem of coliform isolation and identification.

Development of Brilliant Green Bile 2% Broth

The incorporation of bile or bile salts into media for the isolation of *E. coli* was first described by MacConkey
Two media were reported: an agar medium containing 0.5% sodium taurocholate, 2% peptone, 1% lactose, and 1.5% agar, and a broth medium of 0.5% sodium taurocholate, 2% peptone, 0.5% glucose, and litmus. MacConkey (1905) improved the broth medium by replacing litmus with neutral red and modifying the carbon source, if desired, to a 1% carbohydrate or alcohol concentration.

The bile salt medium was almost immediately accepted in England but not in the United States; glucose broth was recommended for use in coliform tests in Standard Methods for Water Analysis (American Public Health Association, 1905). The acceptance in the United States of bile-containing media was altered when Jackson (1907) described a 1% lactose-10% fresh bile medium. Negative tests, caused by the overgrowth of non-coliform bacteria, and false positive tests, caused by synergistic gas production, were reduced in the lactose-bile medium; both false-negative and false-positive tests were encountered when glucose broth was used. Jackson (1908) improved the 1% lactose-10% bile medium by the addition of 1% peptone; gas was produced at a more rapid rate and in increased quantity than in the lactose-bile medium. Hale and Melia (1910) compared glucose broth with the 1% lactose-10% bile and 1% lactose-10% bile-1% peptone broths. Gas production occurred in more tubes when glucose broth was used than when either of the bile-containing media
were used. Bile inhibited the growth of *E. coli*; this inhibition increased with attenuation. Attenuated *E. coli* were defined as organisms that, after inoculation into unsterile surface water, survived extended storage at 8, 20, or 37 C. Hale and Melia surmised that attenuated forms were unimportant because they did not represent fresh contamination by "vigorous" *E. coli*.

Jackson and Muer (1911) developed a beef liver broth that was non-inhibitory to attenuated *E. coli*. The broth differed from the 1% lactose-10% bile-1% peptone medium; 50% beef liver infusion was substituted for fresh bile and glucose for lactose. In *Standard Methods for the Examination of Water and Sewage* (American Public Health Association, 1912), glucose, 1% lactose-10% bile-1% peptone, and the beef liver broths were all described. The beef liver broth was recommended "as it gives a larger number of attenuated forms, has better rejuvenating power, and gives fewer anomalies and greater and more rapid gas production".

Comparative studies of lactose and 1% lactose-10% bile-1% peptone broths (Jordan, 1913; Creel, 1914) confirmed reports that 10% bile was inhibitory to coliforms as well as non-coliforms. These investigations led to the recommendation of lactose broth for the primary isolation of coliforms in the 1917 edition of *Standard Methods for the Examination of Water and Sewage* (American Public Health Association,
1917). Confirmation was on Endo agar (Endo, 1904) followed by a completed test using lactose broth.

In the meantime, Melia (1915) replaced the 10% fresh bile in 1% lactose-10% bile-1% peptone broth with 5% oxgall, which was more uniform in quality than the fresh bile. The 5% oxgall broth was more sensitive than fresh bile, as indicated by higher percentages of E. coli detected in the 45 water samples tested by using the two media. A 5% oxgall concentration was preferred to lower levels (1 to 3%) because the 5% level prevented interference by growth of streptococci.

A separate line of study concerning the inhibition of bacteria by triphenylmethane dyes was simultaneously being made. Reports by Krumweide and Pratt (1914), Krumweide et al. (1916), and Kligler (1918) stimulated the use of triphenylmethane dyes in media formulations. Triphenylmethane dyes inhibited the growth of Gram-positive bacteria and had little effect upon the growth of Gram-negative bacteria. Different dyes possessed similar selective action and the most effective concentration of each dye varied. The application of these reports to water examination was made by Hall and Ellefson (1917, 1919). A 1:20,000 or 1:100,000 concentration of gentian violet in 1% lactose broth inhibited growth of anaerobic and aerobic, gas-producing sporeformers and enhanced the isolation of E. coli.
A 1:20,000 concentration was recommended below this concentration *E. coli* was inhibited and at a concentration of 1:100,000 some "spurious" positive tests were encountered.

Using the same approach, Muer and Harris (1920) altered the broth of Melia (1915) by adding brilliant green. The formulation for brilliant green bile 5% (BGB5%) was: 5% oxgall, 1% lactose, 1% peptone, and 1:10,000 brilliant green. This concentration of brilliant green prevented the growth of *Clostridium perfringens*, *Clostridium sporogenes*, and other anaerobes. A concentration of 1:100 brilliant green in BGB5% was needed to inhibit *E. coli*. Higher numbers of *E. coli* were enumerated with BGB5% than with lactose broth, 1% lactose-10% bile-1% peptone broth, or gentian violet (1:20,000) lactose broth. Winslow and Dolloff (1922) discovered that a portion of the brilliant green toxicity disappeared in the presence of bile salts. In lactose broth, brilliant green inhibited *E. coli* at a 1:1,000,000 concentration and *Enterobacter aerogenes* at 1:100,000. When 5% bile was added, the concentration required for inhibition increased to 1:1,000 for *E. coli* and 1:500 for *E. aerogenes*.

Levine (1922) investigated the effects of bile concentrations (0.5 to 10%) in 0.5% peptone upon the growth of *E. coli* and *E. aerogenes*. *E. coli* was stimulated by 0.5, 1.0, and 2.0% bile; maximum growth was obtained at 1 to 2% concentrations. Five per cent was slightly inhibitory and
10% was detrimental. A similar effect was noted with *E. aerogenes*; 1% bile produced maximum growth. Apparently this report was overlooked for several years.

In 1923, the American Water Works Association Committee No. 1 assigned the investigation of BGB5% to McCrady (Dunham *et al.*, 1925). In over 12,000 positive presumptive tests made during 1923 and 1924, lactose broth was more sensitive than BGB5% broth. Of the positive presumptive tubes subjected to the completed test, 42% of the lactose broth tubes were completed compared with 95% for BGB5%. This advantage of BGB5% over lactose broth was overshadowed by the fact that total completions obtained with the bile broth were only 77% of those obtained with lactose broth. The BGB5% medium was thought to selectively inhibit attenuated coliforms and, when used for confirmation, this inhibition was not a factor. Jordan (Dunham *et al.*, 1925) stated that "the fundamental concept should be the need of finding every organism of this group that has any vestige of metabolic activity left at the moment of sampling", indicating that attenuated forms had regained importance.

A further comparison of lactose and BGB5% broths was made using water samples from Lake Michigan (Ruchhoft, 1926). A total of 303 positive presumptive lactose broth tubes were confirmed on Endo agar by standard methods (American Public Health Association, 1917) compared to 161 for BGB5%. On the
recommendation of Jordan (Ruchhoft, 1926), BGB5% was used for confirmation. The BGB5% medium compared favorably with the standard methods confirmatory medium (Endo agar); 686 BGB5% confirmations were positive compared to 675 confirmations obtained by using Endo agar. This report, and the one by Dunham et al. (1925), emphasized that BGB5% broth should be used only for confirmation of lactose-positive presumptive tubes.

Investigations by Dunham and Schoenlein (1926) verified Levine's report (1922) that 2% oxgall was the optimum bile concentration for growth of E. coli. In addition, with a 1:75,000 brilliant green concentration in 2% oxgall medium, E. coli was not inhibited. The formulation for brilliant green bile 2% (BGB2%) was: 2% oxgall, 1% lactose, 1% peptone, and 1:75,000 brilliant green. Comparative studies indicated that BGB2% supported more rapid growth of E. coli than did BGB5%. Hale (1927) reported, however, that BGB2% permitted the growth of Clostridium welchii (Cl. perfringens) whereas BGB5% did not. He concluded, "... if we wish to inhibit spore-formers; and that is the purpose of this media, ... It is far preferable and indeed advantageous to inhibit some of the attenuated colon organisms".

Jordan (1927) reviewed the usage of lactose broth versus 2 and 5% bile broths for presumptive inoculation. Two objections to lactose broth were: "1. The complexity
of growth in lactose broth is often so great as to make the isolation of colon group organisms difficult. 2. Synergism is responsible for a very large number of cases in which gas is produced, but no plate growth follows. On water supplies where this occurs, the 'presumptive' test is recognized as having little significance'. In comparing BGB2% with BGB5% as presumptive or confirmatory media, Jordan noted that:
(a) 2% bile was slightly superior to the 5% bile medium for both presumptive and confirmatory tests, (b) a higher degree of completion occurred with BGB2% when either the 2 or 5% bile medium was used for the presumptive tests, and (c) no cultures were discarded due to the presence of sporeformers when either bile medium was used for presumptive inoculation. In only 3 cases out of 1,487 tests were spores found when either bile medium was used. This disproved the report of Hale (1927) that BGB2% permitted the growth of sporeformers. Jordan, in supporting the use of BGB2% broth for primary isolation, suggested that parallel inoculations of both lactose and BGB2% broths should be made. Later, Jordan (1932) continued his campaign for the 2% bile medium by outlining an "optional" procedure in which parallel tubes of both media were used. Three reviewers of this report (W. M. Frost, M. H. McCrady, and L. J. Reed), however, recommended lactose broth for presumptive tests followed by confirmation with BGB2%. 
Lactose broth was compared to BGB2% and BGB5% for primary isolation of coliforms from 83 water samples (Butterfield, 1933). Based on completions by procedures outlined in Standard Methods for the Examination of Water and Sewage (American Public Health Association, 1925), the per cent recoveries for the media were: lactose broth, 100%; BGB2% broth, 81%; and BGB5% broth, 72%. A comparison was also made between confirmation by a standard method (Endo agar) versus EGB2% and EGB5% broths. The per cent confirmations of positive presumptive lactose broth tubes in BGB2% and BGB5% were 95% compared to 100% when Endo agar was used.

Horwood and Heifetz (1934) examined 50 water samples for coliforms by using four presumptive media: lactose broth, crystal violet broth (Salle, 1930), BGB2% broth, and methylene blue brom cresol purple broth (Dominick and Lauter, 1929). Lactose and BGB2% broths were the most sensitive; gas was produced in 25% of the presumptive tubes. The other two media were less efficient. Based on completed tests, the per cent recovery was 85% for BGB2% compared to 70% for lactose broth.

Ruchhoft (1935) and Ruchhoft and Norton (1935) tested seven presumptive media with 73 isolated coliform strains. A standardized suspension of each test organism was inoculated into three decimal dilutions (15 tubes/dilution) of each medium. The productivity from the highest to the
lowest was lactose broth, buffered lactose broth, fuchsin lactose broth (Ritter, 1932), methylene blue brom cresol purple broth, BGB2% broth, crystal violet broth, and formate ricinoleate broth (Stark and England, 1935). Fuchsin lactose, BGB2%, and formate ricinoleate broths were most effective in inhibiting sporeforming lactose fermentors and were too inhibitive for primary isolation. A second investigation was made using several media for confirmation; BGB2% and formate ricinoleate broths compared well with eosin methylene blue (EMB) agar, which was recommended in standard methods (American Public Health Association, 1925). A combined confirmatory test was also employed; positive tubes of BGB2% and formate ricinoleate broths (confirmatory tests) were "completely" confirmed on EMB agar. More coliforms were recovered in BGB2% broth than in formate ricinoleate broth as indicated by 85% of the BGB2% tubes being "completely" confirmed on EMB agar compared to 62% for formate ricinoleate broth. Ruchhoft suggested that "the ricinoleate medium while not fermented by sporeforming lactose fermentors is fermented with gas production by non-lactose fermentors, not belonging to the coli-aerogenes group".

Further evidence supporting BGB2% as a confirmatory medium was reported by McGrady (1937, 1939). In a collaborative study involving 21 laboratories, 1,213 water samples
were examined by employing five confirmatory media: BGB2% broth, crystal violet broth, fuchsin lactose broth, formate ricinoleate broth, and EMB agar. The results indicated "the brilliant green bile confirmatory method to be the most generally satisfactory of the selective procedures. The results also indicated that the brilliant green bile method compared very favorably with the usual Standard Methods 'completed test' and, for the examination of most waters, might advantageously replace it".

Several other investigations were made comparing confirmation by BGB2% broth to the completed test (Howard et al., 1941; Kelly, 1940; Smith, 1941; Taylor, 1940; and Wattie, 1943). In each study, recoveries obtained when BGB2% broth were used were equal to or slightly higher than recoveries obtained by using the completed test outlined in standard methods (American Public Health Association, 1936). Howard et al. (1941) also suggested using BGB2% for the confirmatory test instead of the standard methods completed test because the BGB2% method involved less media and was less time consuming than the completed test.

In the ninth edition of Standard Methods for the Examination of Water and Sewage (American Public Health Association, 1946), BGB2% was adopted as an alternative confirmatory medium to Endo or EMB agars. Prior to this date, BGB2% was listed in standard methods as one of several
"optional" media that could be used for parallel presumptive inoculation with lactose broth. According to the present edition of standard methods (American Public Health Association, 1971), BGB2β broth, Endo agar, or EMB agar are all permitted for use as confirmatory media. The preferred method, however, was BGB2β for confirmation followed by Endo or EMB agar; subculturing on solid media was necessary to isolate coliform colonies which could then be subjected to the completed test.

The knowledge acquired in developing BGB2β broth for water analysis was readily adapted to the examination of food. McCrady and Langevin (1932) and McCrady and Archambault (1934) compared gentian violet broth (Kessler and Swenarton, 1927) and BGB2β broth for the enumeration of coliforms from 139 pasteurized milk samples. Similar results were obtained with both broth media; plate counts were considered inadequate because small numbers of coliforms could not be detected. BGB2β broth was recommended because "anomalies with positives in higher dilutions and negatives in lower dilutions were less frequently encountered" than with gentian violet broth. Further support for BGB2β was obtained when 86% of 153 positive BGB2β presumptives from 29 pasteurized milk samples and 99% of 112 positive BGB2β presumptives from 20 raw milk samples were confirmed.
The Standard Methods for Milk Analysis (American Public Health Association, 1935) included provisional methods for microbiological examinations. The only procedure for enumeration of coliforms was a "tentative" method in which BGB2% broth was used for presumptive tests; no confirmatory or completed tests were described.

Several media were investigated for possible use with dairy products. Crystal violet broth was compared with BGB2% in the examination of milk by Stark and Curtis (1935). Small numbers of cells initiated growth and produced gas in BGB2% broth but not in the crystal violet broth. Stark and Curtis (1936) also studied the inhibitory effects of five broths. Crystal violet, methylene blue brom cresol purple, gentian violet, BGB2%, and formate ricinoleate broths were tested for their inhibitory qualities against three *Bacillus* and seven *Clostridium* species. Formate ricinoleate, BGB2%, and crystal violet broths inhibited the growth of all ten cultures; methylene blue brom cresol purple and gentian violet broths did not. The inhibitory qualities of the media were also tested when 1.0 ml of milk was added to each medium. Formate ricinoleate still inhibited the growth of all ten organisms; BGB2% inhibited seven and crystal violet only inhibited four cultures. The authors concluded that the selective inhibition by BGB2% and crystal violet broths was partially destroyed by milk protein and, therefore,
recommended formate ricinoleate broth for the examination of milk.

Babel and Parfitt (1936) examined 133 ice cream samples using formate ricinoleate broth, BGB2% broth, deoxycholate agar (Leifson, 1935), and violet red bile agar. Using 0.1-ml samples, coliforms were detected in a greater percentage of the samples with the broth media than with the agar media. No difference was noted between either of the two broths. A further comparison of recovery media was made by Bartram and Black (1936). Four agar and six broth media were used to enumerate coliforms from 25 raw milk samples. The highest percentage of confirmed samples (32%) was obtained with neutral red bile agar, violet red bile agar, and BGB2% broth. Formate ricinoleate broth was considered unsatisfactory because only 8% of the samples were confirmed.

In 1939, violet red bile agar, deoxycholate agar, and formate ricinoleate broth were all adopted by Standard Methods for the Examination of Dairy Products (American Public Health Association, 1939) in addition to BGB2% broth. Fournelle and Macy (1950) demonstrated that BGB2% was superior to ricinoleate broth. As a result, ricinoleate broth was dropped from standard methods (American Public Health Association, 1960). The use of BGB2% broth for the presumptive coliform test was also adopted in Recommended Methods for the Microbiological Examination of Foods.
Metabolic Injury

Exposure of an organism to unfavorable stresses or sub-lethal conditions may result in physiological injury. Supplying a suitable environment may allow the injured cell to recover and proliferate.

Metabolic injury has been reviewed by Kueck (1974) and Allwood and Russell (1970). Injury has been demonstrated in cells exposed to heating (Iandolo and Ordal, 1966; Jackson and Woodbine, 1963; and Kaufman et al., 1959), freezing (Arpai, 1962; Moss and Speck, 1966; Postgate and Hunter, 1963; Straka and Stokes, 1959; and Warseck et al., 1973), and freeze-drying (Fry and Greaves, 1951; Ray et al., 1971b; and Sinskey and Silverman, 1970). In the present investigation, two characteristics of injured cells will be reviewed: the extended lag period and the increased sensitivity to selective media.

Eijkman (1908) introduced the injury concept when he reported that the longer *E. coli* was heated (52 °C for 0.5 to 35 min), the greater was the recovery period (3 to 15 days). The exposure to heat was directly responsible for the time required to resume growth and cell division. Hershey (1939) demonstrated that the lag period for *E. coli* was also progressively lengthened as temperatures were increased. When
E. coli was heated at 51 C for 15 min, the time required for repair was 1.6 hr; when heated at 56 C for 15 min, resuscitation took 7.9 hr. The extended lag seemed directly related to injury and not to the selection of resistant cells.

Changes in nutritional requirements caused by heat injury were first described by Curran and Evans (1937). E. coli survivors of lethal heat (98 C) were more exacting in their nutritional requirements than unheated cultures. Increased counts from the heated cultures were detected when blood, 10% glucose, 10% yeast extract, beef infusion, and beef extract were incorporated into the media. These constituents were considered essential for accurate enumeration of bacteria subjected to lethal stresses. Nelson (1943) used nine heat-injured bacteria to compare their recovery rates on several media. Two coliforms, E. coli 5 and E. coli 57, were heated in skim milk at 55 or 57 C for 5 and 10 min. More coliforms were recovered on beef infusion agar than on nutrient or glucose-minimal salts agars. Similar results were obtained with the other seven organisms. The inhibition of heat-injured cultures by selective media was reported by Labots (1959). E. coli was heated in raw milk at 72 C for 5 sec and enumerated with Endo agar, violet red bile agar, and BGB2% broth. The numbers of survivors were: Endo agar, 92,500/ml; BGB2% broth, 23,000/ml; and violet red
bile agar, 64/ml. In a second experiment, *E. coli* was heated in raw milk, pre-heated milk, or nutrient broth at 70 or 71°C for 5 sec. More injured cells were again recovered on Endo agar than by using either BGB2% broth or violet red bile agar.

Busta and Jezeski (1963) investigated the nutritional requirements of heat-injured Gram-positive bacteria. *Staphylococcus aureus* 196E was heated in milk at 60°C for 13 to 43 min. Injured staphylococci were detected on plate count agar and by milk enrichment but lost their ability to multiply and form colonies on a medium containing 7.5% NaCl. Stiles and Witter (1965) reported similar findings with heated *S. aureus* MF31.

Alterations in the nutritional requirements of frozen cells were first described by Gunderson and Rose (1948). *E. coli* and *E. aerogenes* were each inoculated into 250-g samples of chicken chow mein and frozen at -25°C. The survivors were recovered on tryptone-glucose-extract and violet red bile agars. Immediately after freezing, counts on violet red bile agar were only 49% (*E. coli*) and 59% (*E. aerogenes*) of the counts obtained on tryptone-glucose-extract agar. The percentages of coliforms able to grow on violet red bile agar decreased during cold storage; only 19% (*E. coli*) and 27% (*E. aerogenes*) grew after 5 days.

Hartsell (1951) studied the inhibitory effects of
selective media on freeze-injured salmonellae. Cultures were rapidly frozen in fresh beef at -25 C for 4 hr and stored at -9 C. Immediately after freezing, the *Salmonella typhi* counts on MacConkey (selective) agar were 86% of the counts obtained on yeast extract-veal infusion (non-selective) agar. This percentage decreased to 9% after 2 mo of storage to almost zero after 4 mo of storage. Similar results were observed on deoxycholate (selective) agar and yeast extract-veal infusion agars using *Salmonella oranienburg*. The counts on deoxycholate agar immediately after freezing were 84% of the counts obtained on yeast extract-veal infusion agar; the percentage decreased to 3% in 2 mo.

Nakamura and Dawson (1962) investigated freeze-injured *Shigella sonnei*. Cultures of *S. sonnei* were suspended in nutrient broth, saline, or skim milk and frozen at -20 C. The surviving populations were enumerated on nutrient agar, blood heart infusion agar, and a salt-vitamin synthetic medium. The synthetic medium was unsuitable for recovery of *S. sonnei* in all frozen suspensions; more cells were recovered on blood heart infusion and nutrient agars. When meat extract, peptone, and Casamino acids were added singly or in combination to the synthetic medium, however, recoveries were comparable to those obtained using nutrient and blood heart infusion agars.
Straka and Stokes (1959) continued the investigations concerning the metabolic injury of bacteria exposed to freezing. Frozen cultures of *E. coli* and a *Pseudomonas* species were enumerated on trypticase-soy agar and a citrate-glucose-salts minimal medium. Injured, dead, and unharmed cells were defined. Injured cells were those that, after exposure to stress, grew on a nutrient medium such as trypticase-soy agar but not on the minimal agar. Dead cells failed to grow on the nutrient or minimal media; unharmed cells were those that grew on both media. These definitions were expanded to include selective media (Busta and Jezeski, 1963; Stiles and Witter, 1965). Injured cells grew on non-selective medium but not on the selective medium; unharmed cells grew on both media.

In addition to the reports of Eijkman (1908) and Hershey (1939), numerous studies concerning an extended lag period due to sublethal heat treatment have been reported. Iandolo and Ordal (1966), and Sogin and Ordal (1967) heated *S. aureus* MF31 at 55°C for 15 min. The injured cells (99% of the survivors) exhibited an extended lag in which their ability to grow on a 7.5% NaCl medium was regained. Resuscitation in trypticase-soy broth and in a Casamino acid-carbohydrate-salt medium took approximately 4 hr. The nutritive requirements for the complete recovery consisted of an energy source, a mixture of amino acids, and phosphate.
Clark et al. (1968) exposed *Streptococcus faecalis* R57 to 60°C for 15 min. Greater than 99% of the survivors were injured, based on their inability to reproduce in trypticase-soy broth plus 6% NaCl compared to growth in trypticase-soy broth. The lag period for the heated cells was determined in several media. Resuscitation was complete within 5 hr in trypticase-soy and tryptone-phosphate broths. The lag period was extended to 8.5 hr in an azide dextrose broth; the extension was considered the result of azide in the medium.

Clark and Ordal (1969), and Tomlins and Ordal (1971) repeated the same sublethal heat treatment procedures with *Salmonella typhimurium*. Exposure at 48°C for 30 min caused 90 to 99% of the survivors to become injured, based on their inability to grow on EMB agar containing 2% NaCl. Repair of the heated cells took 4 hr in a citrate-minimal salts medium and 5 hr in trypticase-soy broth. Resuscitation in lactose, nutrient, and lauryl sulfate tryptose broths was only slightly slower than in trypticase-soy broth; growth rates in all media after recovery were approximately the same. Using selective media, tetrathionate and Selenite F broths, the lag period was extended to approximately 8 hr.

The effect of sublethal heat treatment on *E. coli* B was determined by Mukherjee and Bhattacharjee (1970). A phosphate buffer suspension of *E. coli* B was heated at 52°C,
divided into three portions, and stored at 0.5, 20, and 37 C. Recovery occurred within 1 hr at 20 and 37 C; no recovery was apparent within 4 hr at 0.5 C.

The extended lag period caused by lethal freezing treatments has been extensively studied by Ray and Speck (1972a). An aqueous suspension of *E. coli* NCSM was frozen at -78 C for 10 min followed by thawing at 8 C for 30 min. More than 90% of the survivors were injured, based on their failure to reproduce on trypticase soy-0.3% yeast extract agar containing 0.1% deoxycholate. Preliminary reports indicated that repair took 3 hr in trypticase-soy broth and 4 hr in minimal salts medium at 20 C. Further studies (Ray and Speck, 1972b) revealed, however, that resuscitation of 70 to 80% of the injured cells occurred within 1 hr in 0.5% K₂HPO₄ or K₂HPO₄ plus MgSO₄ solutions at 25 C. Subsequent investigations (Ray and Speck, 1973b) revealed that repair, as indicated by the initiation of cell multiplication, was complete in trypticase soy-yeast extract broth between 30 and 60 min at 35 or 45 C, between 60 and 90 min at 30 C, and between 90 and 120 min at 25 C.

The effect of freezing on salmonellae was also investigated by Ray et al. (1972). *Salmonella anatum* NF3 was frozen in an aqueous solution at -78 C for 10 min and thawed at 4 C for 75 min. Approximately 97% of the survivors were injured, based on their inability to form colonies on xylose-
lysine-peptone agar containing 0.2% deoxycholate. Repair in trypticase soy-yeast extract broth and in a citrate-glucose-minimal salts broth was complete in 1 hr.

The effect of freeze-drying upon *E. coli* was determined by Sinskey and Silverman (1970). *E. coli* ML30 was suspended in 2% gelatin, frozen at -40°C for 12 hr, and dried 8 hr at 49°C. Over 99% of the cells were killed, almost all during the drying process; 28% of the survivors were injured, based on their inability to grow on deoxycholate lactose agar. Recovery in a citrate-minimal salts medium required 5 hr at 37°C. With the addition of various nitrogen sources to the minimal medium, the lag period was shortened; 0.1% Casamino acids shortened the lag to approximately 1.5 hr and 0.1% peptone or 0.1% trypticase shortened the lag to 3 hr. Carbon sources, such as glycerol or glucose, when added to the minimal salts medium, had no effect on the lag period.

The extended lag period of freeze-dried salmonellae was investigated by Ray *et al.* (1971a). *S. anatum* NP3, suspended in reconstituted 10% non-fat dry milk, was frozen in dry ice-acetone and dried for 40 hr at room temperature. Approximately 60% of the cells were killed; 70% of the survivors were injured, based on their failure to grow on xylose-lysine agar containing 0.25% sodium deoxycholate. Resuscitation in water took 2 to 3 hr at 25°C. Brilliant green (0.002%) water, lactose broth, 0.3% peptone, 0.1% pyruvate,
and a complex medium (Casamino acids, tryptone, yeast extract, and salts) were compared to water for their ability to resuscitate injured cells. Repair in lactose broth was slightly faster than in the other menstrua, but no significant difference was detected between repair in all solutions, including water.
MATERIALS AND METHODS

Cultures

Two cultures of *E. coli* were included in this investigation. A culture of *E. coli* B was kindly supplied by Dr. Peter A. Pattee, Department of Bacteriology, Iowa State University, Ames, Iowa. A student in an introductory Bacteriology course at Iowa State University provided a culture of *E. coli* (strain S) that he isolated from river water. Both cultures were checked for purity by streaking on eosin methylene blue agar (Difco) followed by characterization with the IMViC reactions (Parr, 1936).

Each culture was maintained on trypticase-soy agar (Difco). After monthly transfers to fresh medium and incubation at 35°C for 18 hr, the cultures were stored at 4°C.

Media

All media, including brilliant green bile 2% (BGB2%) broth, EC medium, lactose broth, lauryl sulfate tryptose (LST) broth, M-Endo-MF broth, M-FC broth, trypticase-soy broth, deoxycholate lactose (DL) agar, eosin methylene blue (EMB) agar, tryptone-glucose-extract (TGE) agar, and violet red bile (VRB) agar, were obtained from Difco Laboratories, Detroit, Michigan. Each medium was prepared according to the manufacturer's directions except VRB and DL agars which were sterilized in an autoclave for 5 min at 121°C.
Phosphate buffered water (Butterfield, 1932), sterilized at 121 °C for 15 min, was used as the diluent for all dilutions.

Two basal media, lactose broth and a 1% lactose (Fisher Scientific)-1% peptone (Difco) broth, were utilized with capsule I. One basal medium, a 1% lactose-1% peptone-2% oxgall (Difco) broth, was used with capsule II. Ten milliliters of each medium were dispensed into 18 x 150 mm fermentation tubes and sterilized at 121 °C for 15 min.

Preparation of Capsule I

Bacto-agar (Difco), 3 g; brilliant green (Matheson, Coleman, and Bell), 20 mg; and oxgall, 30 g; were dispensed into a 150-ml beaker. Distilled water was added to bring the suspension to a volume of 100 ml. The suspension was heated to boiling with constant stirring and the volume again adjusted to 100 ml. After boiling for 3 to 5 min, the suspension was covered and cooled to 60 °C in a water bath. A 1.0-ml tuberculin syringe (Becton-Dickinson) was used to dispense 0.5-ml quantities of the 60-°C suspension into #0 gelatin capsules (Eli Lilly and Company). Each capsule was closed, immersed in distilled water for approximately 5 sec, and dried for 22 to 24 hr at room temperature under an air current. The last steps sealed the two halves of the capsule into one unit, and an air pocket was trapped in the upper third of the capsule.
The dehydrated capsule was then coated with melted and cooled (75 to 80°C) commercial beeswax. This method was suggested by Dr. Paul Levine, Drake University, Des Moines, Iowa (personal communication). The bottom half of the capsule was dipped into the melted wax and allowed to air dry forming a thin wax coat. The top half was coated in a similar manner. Care was taken so that a small "window", approximately 2 mm² (1.5 to 3.0 mm) on one side near the bottom, remained uncoated. The wax coat provided structural rigidity to the gelatin capsule.

Preparation of Capsule II

The same procedure that was used to make capsule I was also employed for capsule II. Capsule II, however, contained only Bacto-agar and brilliant green; oxgall was omitted.

Estimation of Brilliant Green Concentration

Concentrations of brilliant green were measured by the method of Conn (1961). A stock solution (200 μg/ml) was prepared by dissolving 50 mg of brilliant green in 250 ml of 50% ethyl alcohol. Three milliliters (600 μg) were diluted in distilled water to 200 ml (3 μg/ml) and the absorbance was measured at wavelengths between 740 and 340 nm; a Beckman Model DB Spectrophotometer and 1 cm² matched quartz cuvettes were used. A standard curve was made by preparing suitable
dilutions from the stock solution and measuring their absorbance at 630 nm.

Diffusion of Brilliant Green

Two procedures were developed to measure the diffusion of brilliant green from capsule I or II into a broth medium.

Beckman Model DB Spectrophotometer method

A wax-coated, gelatin capsule was added to each of 15 tubes containing 10 ml of tempered basal medium (35 C for 15 min), and each tube was incubated in a 35-C water bath. At specified intervals (1, 2, 4, 6, 8, and 24 hr), a 0.2-ml aliquot was removed from each tube and diluted 1:10 with 1.8 ml of the same basal medium. After mixing, the absorbance at 630 nm was recorded.

Technicon AutoAnalyzer method

Wax-coated capsules were dispensed into tubes containing 10 ml of tempered basal medium (35 C for 15 min); the tubes were incubated in a 35-C water bath. At specific times (1, 2, 4, 8, or 24 hr), a Pasteur pipette was inserted to the base of a tube of medium and the broth was pumped at a rate of 1 ml/min through the continuous flow cell of a colorimeter. The relative concentration of brilliant green in the basal medium was assayed by measuring the absorbance at 630 nm.
Production of Heat-Injured Coliforms

A modification of the method of Maxcy (1970) was used. A culture of *E. coli* B or *E. coli* S was inoculated into trypticase-soy broth; the broth was incubated at 35 C for 18 hr and stored at 5 C for 2 to 5 days. To obtain young cells, 0.1 ml of the stored culture was used to inoculate 10 ml of trypticase-soy broth and the tube was incubated with shaking for 6 hr in a 32-C water bath.

Reconstituted non-fat milk solids (Carnation), 10% w/v, autoclaved for 15 min at 121 C, was used as the menstruum for the heat-injury investigations. A 1.0-ml inoculum of the 6-hr culture was added to 99 ml of tempered (15 min at 57 or 60 C) reconstituted sterile non-fat milk solids in a milk dilution bottle. The sample was heated with shaking (15 sec every min) in a water bath at 57.0±0.2 C (*E. coli* B) or 60.0±0.2 C (*E. coli* S). At specified times (2, 4, 6, and 8 min), a 1.0-ml aliquot was removed and diluted 1:100 with 99 ml of cooled (room temperature) diluent. Serial dilutions and platings were made according to Standard Methods for the Examination of Dairy Products (American Public Health Association, 1972). Zero time readings were made by adding 1.0 ml of the 6-hr culture to 99 ml of room temperature reconstituted sterile non-fat milk solids. After mixing, 1.0 ml was serially diluted and plated by the same method. The elapsed time from sampling to plating was 20
min or less, so error due to holding times was insignificant (Hartman and Weber, 1972).

The total viable coliform population was enumerated by using TGE agar. VRB agar was used to detect uninjured cells. The difference between counts obtained on TGE and VRB agars represented the number of injured cells. Injured and uninjured cells were also enumerated with the 5-tube most probable number (MPN) method by utilizing BGB2% broth, lactose broth, LST broth, lactose broth plus capsule I, 1% lactose-1% peptone (LP) broth plus capsule I, and 1% lactose-1% peptone-2% oxgall (LPO) broth plus capsule II.

Examination of Water

Collection of water samples

Twelve surface water samples were collected from each of five sites on the Des Moines River and one site on the Raccoon River in central Iowa (Fig. 1). Each sample was refrigerated during transit and stored at 4 to 6 C until analyses could be made. The time between collection and examination did not exceed 24 hr.

Most probable number method

A 5-tube MPN method was used, as described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971), except for a few minor modifications. Dilutions of 1:10 were prepared by serially
Fig. 1. Location map for the six sampling sites on the Des Moines and Raccoon Rivers.
diluting 10 ml of sample with 90 ml of diluent. One milliliter of each sample or dilution was used per tube for the inoculum in each dilution series of five fermentation tubes. This procedure differed from that of the standard methods in which 10, 1.0, or 0.1 ml were recommended as the inocula for each dilution series. In the first investigation, BGB2% broth, lactose broth, LST broth, and lactose broth plus capsule I were used. In the second study, BGB2% broth, lactose broth, LST broth, LP broth plus capsule I, and LPO broth plus capsule II were compared. The capsules were dispensed into tubes containing the appropriate basal media immediately after inoculation.

The schedules for inoculation of the MPN presumptive and confirmed tests are shown in Fig. 2. To readily facilitate handling of all cultures, a second modification of standard methods was employed. In subculturing from one broth to another, one drop of medium from a Pasteur pipette was transferred instead of a loopful of broth from a 3-mm loop. As indicated in Fig. 2, all positive presumptive tubes, except BGB2% broth and lactose broth plus capsule I, were transferred to EC medium and BGB2% broth. Further confirmation was made by streaking confirmed BGB2% tubes on EMB agar. Positive presumptive tubes of BGB2% broth and lactose broth plus capsule I were subcultured in EC medium and on EMB agar.
Inoculate fermentation tubes of lactose broth, LST broth, LP broth plus capsule I, and LPO broth plus capsule II

- **Incubate 24 to 48 hr at 35.0±0.5°C**
  - **No gas produced; Negative Test**
  - **Gas produced; Positive Test; Transfer to BGB2% broth**
    - **Incubate 24 to 48 hr at 35.0±0.5°C**
      - **No gas produced; Coliform group absent**
      - **Gas produced; Coliform group confirmed; Transfer to EMB agar**
        - **Incubate 24 to 48 hr at 35.0±0.5°C**
          - **Negative colonies; Coliform group absent**
          - **Typical or atypical coliform colonies; Coliform group confirmed**

- **Gas produced; Negative Test**
  - **Transfer to EC medium**
    - **Incubate 24 hr at 44.5±0.2°C**
      - **No gas produced;**
      - **Fecal coliform group absent**
      - **Gas produced; Fecal coliform group present**
    - **Transfer to EMB agar**
      - **Incubate 24 to 48 hr at 35.0±0.5°C**
        - **Negative colonies; Coliform group absent**
        - **Typical or atypical coliform colonies; Coliform group confirmed**

Fig. 2. Schematic outline of the MPN presumptive and confirmed tests used in the examination of water.
Membrane filtration method

The method used for enumeration of coliforms and fecal coliforms by membrane filtration was reported in Application Manual AM302, Biological Analysis of Water and Wastewater, 1972, Millipore Corporation, Bedford, Massachusetts. Serial dilutions of 1:10 were prepared and 40 ml of each sample or dilution were filtered through a 0.45 μm pore size, pre-sterilized filter. Between filtration of samples, the Millipore filter apparatus (funnel and holder) was boiled for 4 to 6 min to prevent transfer of coliforms from one sample to another. After filtration, the inoculated filter was transferred to a sterile pad containing 1.8 to 2.0 ml of broth. M-Endo-MF broth was used to detect coliforms; fecal coliforms were enumerated with M-FC broth. Plates containing filters on pads moistened with M-Endo-MF broth were incubated at 35 C for 24 hr; plates containing filters on pads moistened with M-FC broth were immersed in a 44.5±0.2 C water bath for 24 hr. Coliforms growing on M-Endo-MF medium were dark red with a "typical" coliform sheen. Rough, blue colonies on M-FC medium were presumed to be fecal coliforms.

Plate count method

The pour plate procedure utilizing VRB agar was described in Recommended Methods for the Microbiological Examination of Foods (American Public Health Association, 1966). Serial dilutions of 1:10 were made and 1.0-ml aliquots of
each sample or dilution were used as the inocula. Tempered (44 to 46 °C) VRB agar was mixed with the inoculum and allowed to solidify; each plate was overlayed with approximately 5 ml of tempered VRB agar. Upon solidification, the plates were incubated at 35 °C for 48 hr and red colonies 0.5 mm or larger in diameter were counted. Five representative colonies from each plate were subjected to confirmation in BGB2% broth.

Examination of Food

Collection of food samples

Seven samples of hamburger, twelve of frozen vegetables, and thirteen of various dried foods were obtained from stores in Ames, Iowa. Eight fresh raw milk samples were supplied through the courtesy of Anderson-Erickson Dairy, Des Moines, Iowa. The hamburger and raw milk were stored at 4 °C and the frozen vegetables at -20 °C until examined.

Most probable number method

The 5-tube MPN procedure used in this study was reported in Recommended Methods for the Microbiological Examination of Foods (American Public Health Association, 1966). Initial dilutions of 1:100 were made by homogenizing 20 ml (g) of sample in 180 ml of diluent for 2 min. Serial dilutions of 1:100 (1 ml in 99 ml of diluent) were prepared and 1.0- or 0.1-ml quantities were used as the inocula. In all investigations, the following media were included: BGB2% broth,
lactose broth, LST broth, lactose broth plus capsule I, LP broth plus capsule I, and LPO broth plus capsule II. The schedules for the inoculation of the MPN presumptive and confirmed tests were identical to those used for the examination of water (Fig. 2) except EC medium was omitted and positive tubes of lactose broth plus capsule I were confirmed with BGB2%. A departure from recommended methods was also employed; a Pasteur pipette instead of a 3-mm diameter loop was used to subculture from one tube of broth to another.

**Plate count method**

The pour plate procedure, utilizing DL and VRB agars, was described in the section for the examination of water. Aliquots (1.0 or 0.1 ml) of the samples or dilutions prepared for the MPN tests were used for the inocula. The plates were incubated at 35°C for 48 hr. Five representative colonies (0.5 mm or larger in diameter) from each plate were transferred to BGB2% broth for confirmation.
RESULTS

Evolution of the Wax-Coated Gelatin Capsule

Preliminary investigations were undertaken to develop a timed-release tablet similar to one described by Matelova et al. (1972). Compressed sucrose tablets were coated with 30% ethanolic shellac and/or 5% polyvinyl acetate in acetone (Matelova et al., 1972). The number and type of coats utilized were directly proportional to the time required for the tablet to dissolve. After several unsuccessful studies on development of a timed-release tablet for coliform determinations (data not shown), two modifications were made. First, the tablet concept was abandoned and gelatin capsules were used. The second modification was to incorporate Alka-Seltzer (Miles Laboratories) into the capsule ingredients in addition to the selective inhibitor, brilliant green. Carbon dioxide would be released from the Alka-Seltzer upon contact with water, causing the capsule to move rapidly up and down in the medium and ensure uniform diffusion of the dye in the medium. This technique was unsuccessful because the brilliant green was not completely distributed throughout the medium. Numerous attempts at coating capsules with ethanolic shellac and/or polyvinyl acetate also met with failure. The time required to initiate release of the ingredients from coated capsules varied from 1 to 24 hr. Several
subsequent attempts to formulate tablets and pills were also unsuccessful.

One procedure that was partly satisfactory was to incorporate the ingredients into agar plugs. When dried, an agar plug was added to each tube of basal medium at the time of incubation. The agar plugs were fragile, therefore, the agar containing inhibitors was dispensed into capsules for structural support. The capsules floated at the top of the medium due to an air bubble trapped within each capsule during preparation. The ingredients diffused uniformly throughout the medium. Dehydration of the agar plugs within the capsules facilitated handling and provided a long storage life. During dehydration, however, the capsules frequently separated into two halves and the air bubbles were released. Immersion of the capsules in water prior to dehydration caused the gelatin to become sticky and each capsule was sealed into one unit, maintaining the air pocket.

Uncoated capsules were satisfactory when the incubation temperature was 25 C. At an incubation temperature of 35 C, the gelatin capsules melted and the agar plugs sank to the bottom of the tubes forming concentrated layers of brilliant green. As a result, coating of the capsules again became essential. Beeswax was recommended (Dr. Paul Levine, Drake University, Des Moines, Iowa), but was initially unsatisfactory; only minimal diffusion occurred from a beeswax-coated
capsule within 24 hr. To increase diffusion, a small "window" (1.5 to 3.0 mm) below the trapped air pocket was left uncoated. Water entered the capsule through this small "window", initiating rehydration of the agar plug; the air bubble remained intact. The beeswax provided structural rigidity to the capsule at 35 C.

Procedures recommended for the "sterilization" of M-FC and M-Endo-MF broths were modified for use in preparing the capsules. The agar suspension was heated to boiling followed by boiling for 3 to 5 min. Any contaminating coliforms within the ingredients were destroyed. The high concentration of brilliant green and oxgall, plus the dehydrated state of the agar plug, prevented growth of other bacteria. "Pasteurization" of the exterior of the capsules was accomplished by dipping the dehydrated capsules in melted (75 to 80 C) beeswax. Sterility tests were made by incubating 40 tubes of basal medium, each containing a capsule, at 35 C for 48 hr. Negative tests indicated that no further "sterilization" was required.

The amount of brilliant green used in the agar suspension was adjusted so that the final concentration per capsule was 133 ug. Upon liberation of the brilliant green from the capsule into 10 ml of basal medium, the concentration of brilliant green in the medium was 13.3 ug/ml, the same level that was proposed for BGB2% (Dunham and
Schoenlein, 1926).

A photograph of capsule I is shown in Fig. 3. The brilliant green agar plug is the dark area in the bottom two-thirds of the capsule. The slight indentation or deformation was the result of dehydration. The trapped air bubble is indicated by the light area at the top of the capsule. When added to a liquid medium, the capsule floated and rehydration of the submerged agar plug was initiated. Water was absorbed through the uncoated "window", causing the agar to swell to its original form. The rehydration process was complete within 15 to 20 min. The swelling opened a minor

Fig. 3. Capsule I, containing brilliant green, oxgall, and agar.
rupture in the bottom of the capsule and wax structure. Release of brilliant green and oxgall into the medium occurred through the uncoated "window" and rupture. The agar plug remained intact within the floating wax structure; the rupture was not sufficient to affect separation of the agar plug from the wax structure.

Estimation of Brilliant Green Concentration

An absorbance scan of brilliant green from 740 to 340 nm is shown in Fig. 4. Maximum absorbance at 632 to 628 nm confirmed the report of Conn (1961). A second peak at 420 nm was probably caused by an impurity in the brilliant green. Subsequent measurements of brilliant green concentrations were made at 630 nm. The standard curve, using concentrations from 1 to 10 ug/ml, indicated that absorbance was directly proportional to concentration up to 5 ug/ml.

Diffusion of Brilliant Green

The concentration of brilliant green released from capsule I into lactose broth was recorded on the Beckman Model DB Spectrophotometer. The data (Table 1) represent the average of two trials; each trial consisted of 15 capsules selected at random from a lot of 250. The medium to be assayed was diluted, usually 1:10, so that the absorbance values were within the linear portion of the standard curve. Only 13.3% (1.8 ug/ml) of brilliant green was released in 1
Fig. 4. The absorbance scan of a 3 ug/ml concentration of brilliant green measured from 740 to 340 nm.

A Beckman Model DB Spectrophotometer was used to record absorbance. The 3 ug/ml solution was prepared by diluting 3 ml of a stock solution (200 ug/ml) to 200 ml with distilled water.
Table 1. Diffusion of brilliant green from capsule I into lactose broth at 35 C.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Mean (ug/ml)</th>
<th>Range (ug/ml)</th>
<th>Per cent Diffused</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8</td>
<td>0.8-2.7</td>
<td>13.1</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>1.9-6.8</td>
<td>30.7</td>
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<tr>
<td>4</td>
<td>8.9</td>
<td>6.3-12.3</td>
<td>65.0</td>
</tr>
<tr>
<td>6</td>
<td>11.1</td>
<td>8.0-13.2</td>
<td>81.0</td>
</tr>
<tr>
<td>8</td>
<td>12.2</td>
<td>9.9-13.8</td>
<td>89.1</td>
</tr>
<tr>
<td>24</td>
<td>13.7</td>
<td>12.6-14.6</td>
<td>100.0</td>
</tr>
</tbody>
</table>

*Capsule I contained brilliant green, oxgall, and agar.*
hr, 30.7% (4.2 ug/ml) in 2 hr, and 65.0% (8.9 ug/ml) in 4 hr. The final mean concentration of 13.7 ug/ml compared well with the 13.3 ug/ml proposed for BGB2% (Dunham and Schoenlein, 1926). We estimated that a variation in dye release of ±10% would be acceptable for routine use. A 10% variation would permit final dye concentrations in the range of 13.3±1.3 ug/ml, or 12.0 to 14.6 ug/ml. The final range of brilliant green concentration experienced (Table 1) was 12.6 to 14.6 ug/ml, which indicated that adequate "quality control" had been achieved in capsule preparation; the capsules could be used for additional experiments.

The same procedure was used to measure brilliant green released from capsule II into LPO medium. The data (Table 2) were accumulated from 15 capsules selected at random from a lot of 250. Initial diffusion was more rapid (19.2% in 1 hr, 43.2% in 2 hr) than with capsule I. The mean, range, and per cent diffused from 4 to 24 hr were approximately the same for both capsules I and II. The final mean concentration was low (12.5 ug/ml) but within 6% of the desired mean of 13.3 ug/ml. The range of the dye-diffusion values at 24 hr (last column, Table 2) was 11.5 to 14.2 ug/ml; this exceeded slightly the 10% limit of variation for one capsule, however, the remaining dye-release values all fell within our theoretical allowable range.

The relative concentration of brilliant green released
Table 2. Diffusion of brilliant green from capsule II\textsuperscript{a} into LPO\textsuperscript{b} broth at 35 C.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>24</th>
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<tr>
<td>Mean (ug/ml)</td>
<td>2.4</td>
<td>5.4</td>
<td>8.4</td>
<td>10.7</td>
<td>11.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Range (ug/ml)</td>
<td>1.6-3.6</td>
<td>3.7-7.6</td>
<td>6.7-10.2</td>
<td>9.1-12.2</td>
<td>10.8-13.5</td>
<td>11.5-14.2</td>
</tr>
<tr>
<td>Per cent Diffused</td>
<td>19.2</td>
<td>43.2</td>
<td>67.2</td>
<td>85.2</td>
<td>93.1</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Capsule II contained brilliant green and agar.

\textsuperscript{b}LPO = 1% lactose-1% peptone-2% oxgall.
from capsule I into lactose broth was measured by the Technicon AutoAnalyzer (Fig. 5). The exact concentration could not be monitored because absorbancies larger than 0.360 (5 µg/ml on the standard curve) were encountered. A gradient formed during the first few hours, but was not apparent except at the very top of the medium when diffusion was complete. This minute area at the top represented the liquid portion above the "window" of the floating capsule.

The diffusion of brilliant green from capsule II into LPO broth was also measured by using the Technicon AutoAnalyzer (Fig. 6). An increase in the base line was caused by oxgall present in the medium; this increase, calculated as part of the total absorbance, was insignificant. The results were not as dramatic as with capsule I; a minor gradient formed initially but dissipated after several hours.

Heat-Injury Studies

Preliminary investigations on the heat injury of E. coli S were made at 57, 60, and 62 C (Maxcy, 1970). Optimum results, indicated by approximately a 90 to 99% kill and a high injury rate (41 to 47%), were obtained by heating at 60 C for 6 min (Table 3). Mean TGE counts from three trials at 60 C represented the total E. coli S population. Counts on VRB agar indicated the uninjured E. coli S. The difference between the TGE and VRB counts represented injured cells.
Fig. 5. Brilliant green concentrations (measured from the bottom to the top of the tubes) in tubes of lactose broth at various times after inoculation.

Brilliant green was released from capsule I (brilliant green, oxgall, and agar) into lactose broth at 35°C. A Technicon AutoAnalyzer was used to record absorbance at 630 nm. The medium was pumped from the bottom of the tube at a rate of 1 ml/min. The medium at the bottom was first to be removed; the medium at the top was the last to be removed.
Fig. 6. Brilliant green concentrations (measured from the bottom to the top of the tubes) in tubes of LPO broth at various times after inoculation.

Brilliant green was released from capsule II (brilliant green and agar) into LPO (1% lactose-1% peptone-2% oxgall) broth at 35°C. A Technicon AutoAnalyzer was used to record absorbance at 630 nm. The medium was pumped from the bottom of the tube at a rate of 1 ml/min. The medium at the bottom was first to be removed; the medium at the top was the last to be removed.
Table 3. Enumeration of heated (60 C) E. coli S on TGE\textsuperscript{a} and VRB\textsuperscript{b} agars by the method of Maxcy (1970).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TGE counts</th>
<th>VRB counts</th>
<th>Per cent killed\textsuperscript{c}</th>
<th>Per cent injured\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$7.7 \times 10^7$</td>
<td>$6.9 \times 10^7$</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>$1.4 \times 10^7$</td>
<td>$9.9 \times 10^6$</td>
<td>82</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>$1.3 \times 10^7$</td>
<td>$7.6 \times 10^6$</td>
<td>83</td>
<td>41</td>
</tr>
<tr>
<td>6</td>
<td>$1.0 \times 10^7$</td>
<td>$5.3 \times 10^6$</td>
<td>87</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>$5.8 \times 10^6$</td>
<td>$3.2 \times 10^6$</td>
<td>92</td>
<td>45</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Tryptone-glucose-extract agar.

\textsuperscript{b}Violet red bile agar.

\textsuperscript{c}TGE counts at zero time minus TGE counts at specified time/TGE counts at zero time.

\textsuperscript{d}TGE counts at specified time minus VRB counts for that time/TGE counts at the specified time.

At zero time, VRB agar detected only 90% of the TGE counts, indicating that VRB was inhibitory to some unheated E. coli S cells.

Optimum results of heating E. coli B at 55, 57, and 60 C were obtained at 57 C. Table 4 summarizes the data of two trials. At zero time only 74% of the unheated population grew on VRB agar; therefore, unheated E. coli B was more sensitive than unheated E. coli S to VRB. The per cent kill of E. coli B (96% at 6 min) was within the proposed 90 to 99% range. The per cent injured (99%) was approximately twice
Table 4. Enumeration of heated (57 C) E. coli B on TGE\(^a\) and VRB\(^b\) agars by the method of Maxcy (1970).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>TGE counts</th>
<th>VRB counts</th>
<th>Per cent killed(^c)</th>
<th>Per cent injured(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.1 x 10(^7)</td>
<td>8.1 x 10(^6)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>6.9 x 10(^6)</td>
<td>8.5 x 10(^5)</td>
<td>37</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>1.8 x 10(^6)</td>
<td>7.3 x 10(^4)</td>
<td>84</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>4.8 x 10(^5)</td>
<td>6.6 x 10(^3)</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>8</td>
<td>7.8 x 10(^4)</td>
<td>6.0 x 10(^2)</td>
<td>99</td>
<td>99</td>
</tr>
</tbody>
</table>

\(^a\)Tryptone-glucose-extract agar.

\(^b\)Violet red bile agar.

\(^c\)TGE counts at zero time minus TGE counts at specified time/TGE counts at zero time.

\(^d\)TGE counts at specified time minus VRB counts for that time/TGE counts at the specified time.

that of E. coli S. Because of the high injury obtained with E. coli B, it was used for the production of heat-injured cells in subsequent investigations.

The MPN capsule methods for enumerating heat-injured E. coli B were compared to selected procedures routinely used. Table 5 contains a summary of data obtained during four trials. The TGE counts were less than lactose broth counts, confirming the report of Ray and Speck (1973a) that tempered (44 to 46 C) agar may reduce counts. All broth cultures yielded higher counts than did VRB agar. The capsule media
detected two to three times the number of injured cells than did BGB2% broth.

Table 5. Comparison of MPN capsule methods with selected methods for enumerating heat-injured E. coli B.<sup>a</sup>

<table>
<thead>
<tr>
<th>Media</th>
<th>Counts (x 10&lt;sup&gt;6&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone-glucose-extract agar</td>
<td>11.0</td>
</tr>
<tr>
<td>Violet red bile agar</td>
<td>0.3</td>
</tr>
<tr>
<td>Lactose broth</td>
<td>15.0</td>
</tr>
<tr>
<td>Lauryl sulfate tryptose broth</td>
<td>12.0</td>
</tr>
<tr>
<td>Brilliant green bile 2% broth</td>
<td>3.5</td>
</tr>
<tr>
<td>Lactose broth plus capsule I</td>
<td>6.4</td>
</tr>
<tr>
<td>LP&lt;sup&gt;b&lt;/sup&gt; broth plus capsule I</td>
<td>9.3</td>
</tr>
<tr>
<td>LPO&lt;sup&gt;c&lt;/sup&gt; broth plus capsule II</td>
<td>8.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Heated at 57°C for 6 min.

<sup>b</sup> LP = 1% lactose-1% peptone.

<sup>c</sup> LPO = 1% lactose-1% peptone-2% oxgall.

Examination of Water

Six samples were collected from each site for six consecutive weeks. The 36 water samples (Group A) were examined for coliforms with the MPN method using lactose broth, LST broth, and lactose broth with capsule I (Table 6). Positive tubes of lactose broth with capsule I were assumed to be
Table 6. Comparison of three MPN media for enumeration of coliforms from 36 water samples (Group A).

<table>
<thead>
<tr>
<th>Media</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>580</td>
<td>432</td>
<td>74</td>
<td>411</td>
<td>95</td>
<td>70</td>
</tr>
<tr>
<td>LST</td>
<td>580</td>
<td>455</td>
<td>78</td>
<td>427</td>
<td>94</td>
<td>74</td>
</tr>
<tr>
<td>Lac-I</td>
<td>580</td>
<td>---</td>
<td>---</td>
<td>384</td>
<td>--</td>
<td>66</td>
</tr>
</tbody>
</table>

\(^{a}\) LST = lauryl sulfate tryptose; Lac-I = lactose plus capsule I.
confirmed because BGB2# was the confirmatory medium for lactose and LST broths. The percentage of positive presumptive tests was 74% for lactose broth and 78% for LST broth. The percentage of positive presumptive tubes confirmed with BGB2# broth was 94% for lactose broth and 95% for LST broth. Overall, LST broth was the most efficient; 74% of the inoculated presumptive tubes were confirmed. Lactose broth with capsule I was the least efficient with 66% of the inoculated presumptive tubes positive.

The enumeration of fecal coliforms from Group A water samples was made using EC medium incubated at 44.5±0.2 C as the confirmatory medium (Table 7). Lactose broth containing

Table 7. Comparison of three MPN media for detection of fecal coliforms from 36 water samples (Group A).

<table>
<thead>
<tr>
<th>Presumptive media</th>
<th>Number of EC medium inoculated</th>
<th>Number of positives</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>316</td>
<td>188</td>
<td>59</td>
</tr>
<tr>
<td>LST</td>
<td>343</td>
<td>174</td>
<td>51</td>
</tr>
<tr>
<td>Lac-I</td>
<td>299</td>
<td>194</td>
<td>65</td>
</tr>
</tbody>
</table>

\(^a\) LST = lauryl sulfate tryptose; Lac-I = lactose plus capsule I.

\(^b\) Confirmation of fecal coliforms.
capsule I showed the highest recovery (65%) of the three presumptive media tested.

Of the 36 samples (Group A) examined, 24 were also investigated using BGB2% broth as a presumptive MPN medium (Table 8). Positive tubes of BGB2% and lactose broth plus capsule I were interpreted as being confirmed. Both BGB2% and lactose broth with capsule I were slightly less efficient than the lactose and LST broth methods; 69% of the inoculated presumptive tubes were positive using BGB2% and lactose broth plus capsule I compared with 71% for lactose broth and 75% for LST broth. When EC medium was used for confirmation, BGB2% broth, lactose broth, and lactose broth plus capsule I recovered the most fecal coliforms; 61 to 63% of the tubes were positive, compared to only 48% for LST broth.

In one experiment, six samples (one from each site) were incubated in lactose broth plus capsule I at 44.5±0.2 C. Of the 90 tubes inoculated, 20 were positive, indicating a low recovery (22%) of fecal coliforms; 48, 61, and 63% recovery rates were obtained with the presumptive media at 35 C followed by confirmation with EC medium at 44.5±0.2 C (Table 8). The capsule method, therefore, was not applicable without further modification for initial incubation at high temperature to select for fecal coliforms.

Coliforms from the 36 water samples (Group A) were also
Table 8. Comparison of four MPN media for enumeration of coliforms and fecal coliforms from 24 water samples included in Group A.

<table>
<thead>
<tr>
<th>Media</th>
<th>Number of tubes inoculated</th>
<th>Number of inoculated confirmed</th>
<th>Per cent inoculated confirmed</th>
<th>Number of EC medium inoculated</th>
<th>Number of EC medium positives</th>
<th>Per cent EC medium positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>370</td>
<td>261</td>
<td>71</td>
<td>194</td>
<td>119</td>
<td>61</td>
</tr>
<tr>
<td>LST</td>
<td>370</td>
<td>277</td>
<td>75</td>
<td>203</td>
<td>98</td>
<td>48</td>
</tr>
<tr>
<td>BGB2%</td>
<td>370</td>
<td>255</td>
<td>69</td>
<td>180</td>
<td>114</td>
<td>63</td>
</tr>
<tr>
<td>Lac-I</td>
<td>370</td>
<td>255</td>
<td>69</td>
<td>180</td>
<td>109</td>
<td>61</td>
</tr>
</tbody>
</table>

\(^{a}\)LST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; Lac-I = lactose plus capsule I.

\(^{b}\)Confirmation of fecal coliforms.
enumerated using M-Endo-MF broth (membrane filtration) and VRB agar (pour plate). Fecal coliforms were detected with M-FC broth (membrane filtration) in addition to EC medium. The geometric means of the coliform and fecal coliform counts for the 36 samples are given in Table 9. Mean coliform counts with the MPN media were based on confirmed counts. Representative colonies on VRB agar were also

Table 9. Geometric means of coliform and fecal coliform counts from 36 water samples (Group A) as determined by MPN, membrane filtration, and pour plate procedures.

<table>
<thead>
<tr>
<th>Media</th>
<th>Coliforms per 100 ml</th>
<th>Fecal coliforms per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>4,700</td>
<td>1,000</td>
</tr>
<tr>
<td>LST</td>
<td>7,200</td>
<td>890</td>
</tr>
<tr>
<td>Lac-I</td>
<td>3,900</td>
<td>960</td>
</tr>
<tr>
<td>M-Endo-MF</td>
<td>7,100</td>
<td>950</td>
</tr>
<tr>
<td>M-FC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VRB</td>
<td>10,000</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) LST = lauryl sulfate tryptose; Lac-I = lactose plus capsule I; VRB = violet red bile.

\(^b\) Lactose, LST, and Lac-I were used with the MPN method, M-Endo-MF and M-FC with membrane filtration, and VRB with the pour plate procedure.
confirmed with BGB2%. VRB plate counts yielded higher numbers (10,000/100 ml) of coliforms than did the other four media tested. LST broth (7,200/100 ml) and M-Endo-MF broth (7,100/100 ml) were also efficient. Lactose broth (4,700/100 ml) and lactose broth with capsule I (3,900/100 ml) were the least efficient. An analysis of variance (Walker and Lev, 1969), calculated by using the log$_{10}$ value (geometric value) of each sample, showed that there was no significant difference at the 95% level between any two coliform isolation media. The geometric means for each medium used to detect fecal coliforms were similar (890 to 1,000/100 ml); again no statistical difference (95% level) was observed between any two media.

The MPN method using BGB2% was included in the investigation of 24 samples from Group A (Table 10). Differences between the numbers of coliforms detected with each medium were not as dramatic as those shown in Table 9. Lactose broth plus capsule I recovered more coliforms (4,500/100 ml) than did BGB2% (4,200/100 ml) but the mean count was lower with lactose broth plus capsule I than with lactose broth alone (4,700/100 ml). The fecal coliform values for each medium were again quite similar. An analysis of variance (Walker and Lev, 1969), using log$_{10}$ values, indicated that no significant difference (95% level) existed between counts from either the coliform or fecal coliform media.
Table 10. Geometric means of coliform and fecal coliform counts from 24 water samples (Group A) as determined by MPN, membrane filtration, and pour plate procedures.

<table>
<thead>
<tr>
<th>Media(^a,b)</th>
<th>Coliforms per 100 ml</th>
<th>Fecal coliforms per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>4,700</td>
<td>1,100</td>
</tr>
<tr>
<td>LST</td>
<td>6,300</td>
<td>960</td>
</tr>
<tr>
<td>BGB2%</td>
<td>4,200</td>
<td>1,000</td>
</tr>
<tr>
<td>Lac-I</td>
<td>4,500</td>
<td>1,100</td>
</tr>
<tr>
<td>M-Endo-MF</td>
<td>6,400</td>
<td></td>
</tr>
<tr>
<td>M-FC</td>
<td></td>
<td>870</td>
</tr>
<tr>
<td>VRB</td>
<td>7,400</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) LST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; Lac-I = lactose plus capsule I; VRB = violet red bile.

\(^b\) Lactose, LST, BGB2%, and Lac-I were used with the MPN method, M-Endo-MF and M-FC with membrane filtration, and VRB with the pour plate procedure.

Thirty-six water samples (Group B) were examined for coliforms by using the MPN method with lactose broth, LST broth, BGB2% broth, LP broth plus capsule I, and LPO broth plus capsule II (Table 11). Positive presumptive tubes of LP broth plus capsule I, LPO broth plus capsule II, LST broth, and lactose broth were confirmed in BGB2%. The per
Table 11. Comparison of five MPN media for enumeration of coliforms from 36 water samples (Group B).

<table>
<thead>
<tr>
<th>Media $^a$</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>565</td>
<td>404</td>
<td>72</td>
<td>376</td>
<td>93</td>
<td>67</td>
</tr>
<tr>
<td>LST</td>
<td>565</td>
<td>420</td>
<td>74</td>
<td>393</td>
<td>94</td>
<td>70</td>
</tr>
<tr>
<td>BGB2%</td>
<td>565</td>
<td>---</td>
<td>---</td>
<td>362</td>
<td>--</td>
<td>64</td>
</tr>
<tr>
<td>LP-I</td>
<td>565</td>
<td>368</td>
<td>65</td>
<td>357</td>
<td>97</td>
<td>63</td>
</tr>
<tr>
<td>LP0-II</td>
<td>565</td>
<td>401</td>
<td>71</td>
<td>387</td>
<td>97</td>
<td>69</td>
</tr>
</tbody>
</table>

$LST = $ lauryl sulfate tryptose; $BGB2% = $ brilliant green bile 2%; $LP-I = 1\%$ lactose-1\% peptone plus capsule I; $LP0-II = 1\%$ lactose-1\% peptone-2\% oxgall plus capsule II.
cent of positive presumptive tubes confirmed with BGB2% was higher (97%) with LP and LPO broths than with lactose broth (93%) or LST broth (94%). LST broth was again the most efficient overall; 70% of the inoculated presumptive tubes were confirmed. LPO broth plus capsule II was also highly efficient; 69% confirmation was obtained when LPO broth was used.

Fecal coliforms were detected in the 36 Group B samples with EC medium (Table 12). LP broth with capsule I and BGB2% showed the highest recovery; 72 and 70% of the tubes

Table 12. Comparison of five MPN media for enumeration of fecal coliforms from 36 water samples (Group B).

<table>
<thead>
<tr>
<th>Presumptive media</th>
<th>Number of EC medium inoculated</th>
<th>Number of positives</th>
<th>Per cent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>271</td>
<td>173</td>
<td>64</td>
</tr>
<tr>
<td>LST</td>
<td>273</td>
<td>162</td>
<td>59</td>
</tr>
<tr>
<td>BGB2%</td>
<td>257</td>
<td>180</td>
<td>70</td>
</tr>
<tr>
<td>LP-I</td>
<td>279</td>
<td>202</td>
<td>72</td>
</tr>
<tr>
<td>LPO-II</td>
<td>267</td>
<td>165</td>
<td>62</td>
</tr>
</tbody>
</table>

*LST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; LP-I = 1% lactose-1% peptone plus capsule I; LPO-II = 1% lactose-1% peptone-2% oxgall plus capsule II.

*Confirmation of fecal coliforms.
were positive, respectively. LST broth was again the least efficient for recovery of fecal coliforms; 59% of the EC medium tubes were positive.

Coliforms from the 36 Group B samples were also enumerated using M-Endo-MF broth (membrane filtration) and VRB agar (pour plate). Fecal coliforms were also counted with M-FC broth (membrane filtration). Table 13 shows the geometric means of the coliforms and fecal coliforms for the 36 samples. The coliform values for all MPN media, except BGB2%, were based on confirmed counts. Coliform-like colonies on VRB agar were also confirmed with BGB2%. VRB plate counts (6,200/100 ml) again detected the highest numbers of coliforms. LST broth (5,500/100 ml), M-Endo-MF broth (4,800/100 ml), and LPO broth (4,700/100 ml) were also quite efficient. An analysis of variance (Walker and Lev, 1969) showed that no significant difference (95% level) existed between any two coliform isolation media. M-FC broth recovered 1,600/100 ml fecal coliforms. The MPN media, used in conjunction with EC medium, detected approximately the same numbers of fecal coliforms (1,100 to 1,300/100 ml). Again, no statistical difference (95% level) was observed between any two fecal coliform isolation media.

A comparison was also made of the standard methods used for the enumeration of coliforms and fecal coliforms from surface waters. Table 14 shows the geometric means of the
Table 13. Geometric means of coliform and fecal coliform counts from 36 water samples (Group B) as determined by MPN, membrane filtration, and pour plate procedures.

<table>
<thead>
<tr>
<th>Media</th>
<th>Coliforms per 100 ml</th>
<th>Fecal coliforms per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>4,300</td>
<td>1,100</td>
</tr>
<tr>
<td>LST</td>
<td>5,500</td>
<td>1,100</td>
</tr>
<tr>
<td>BGB2%</td>
<td>3,700</td>
<td>1,200</td>
</tr>
<tr>
<td>LP-I</td>
<td>3,500</td>
<td>1,200</td>
</tr>
<tr>
<td>LPO-II</td>
<td>4,700</td>
<td>1,300</td>
</tr>
<tr>
<td>M-Endo-MF</td>
<td>4,800</td>
<td></td>
</tr>
<tr>
<td>M-FC</td>
<td></td>
<td>1,600</td>
</tr>
<tr>
<td>VRB</td>
<td>6,200</td>
<td></td>
</tr>
</tbody>
</table>

*LST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; LP-I = 1% lactose-1% peptone plus capsule I; LPO-II = 1% lactose-1% peptone-2% oxgall plus capsule II; VRB = violet red bile.

*Lactose, LST, BGB2%, LP-I, and LPO-II were used with the MPN method, M-Endo-MF and M-FC with membrane filtration, and VRB with the pour plate procedure.

coliforms and fecal coliforms for the 72 water samples of Groups A and B. Coliform counts with lactose and LST broths were confirmed in BGB2%. LST recovered higher numbers of coliforms (6,300/100 ml) compared to M-Endo-MF broth.
Table 14. Geometric means of coliform and fecal coliform counts from 72 water samples (Groups A and B) as determined by MPN and membrane filtration procedures.

<table>
<thead>
<tr>
<th>Media(^a,b)</th>
<th>Coliforms per 100 ml</th>
<th>Fecal coliforms per 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>4,500</td>
<td>1,100</td>
</tr>
<tr>
<td>LST</td>
<td>6,300</td>
<td>1,000</td>
</tr>
<tr>
<td>M-Endo-MF</td>
<td>5,800</td>
<td></td>
</tr>
<tr>
<td>M-FC</td>
<td></td>
<td>1,200</td>
</tr>
</tbody>
</table>

\(^a\) LST = lauryl sulfate tryptose.

\(^b\) Lactose and LST were used with the MPN method and M-Endo-MF and M-FC with membrane filtration.

(5,800/100 ml) and lactose broth (4,500/100 ml). An analysis of variance (Walker and Lev, 1969) showed that no significant difference (95% level) existed between the three media. The mean values (1,000 to 1,200/100 ml) for fecal coliforms were similar for all three media. Again, no statistical difference (95% level) was noted between mean counts from the fecal coliform media.

Examination of Food

Pour plate and MPN procedures were employed for coliform analyses of selected foods. Coliform colonies on DL
and VRB agars were confirmed in BGB2%; five representative colonies/duplicate plate were subjected to confirmation. The MPN methods included use of lactose broth, LST broth, BGB2% broth, lactose broth plus capsule I, LP broth plus capsule I, and LPO broth plus capsule II. All positive presumptive tubes were confirmed with BGB2% broth.

Each food sample was plated on VRB agar shortly after collection to determine the approximate coliform counts so that appropriate dilutions could be made for subsequent experiments. After preliminary counts were estimated, a complete study using the MPN and pour plate methods was made with six selected samples of each variety of food.

**Raw milk samples**

Six samples, with counts on VRB agar between 290 and 8,800 coliforms/ml, were further examined by the MPN method using six media (Table 15). Lactose broth yielded the highest percentage of positive presumptive tests (90%); LST broth had 85% positive, lactose broth plus capsule I had 79% positive, and LP broth plus capsule I and LPO broth plus capsule II had 71% positive. Confirmations of positive presumptive tubes were 93, 94, and 96% for LPO broth with capsule II, LP broth with capsule I, and lactose broth with capsule I, respectively, compared to 94% for lactose broth and 87% for LST broth. Lactose broth was the most efficient medium overall; 85% of the inoculated presumptive tubes were
<table>
<thead>
<tr>
<th>Media^a</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>120</td>
<td>108</td>
<td>90</td>
<td>102</td>
<td>94</td>
<td>85</td>
</tr>
<tr>
<td>LST</td>
<td>120</td>
<td>102</td>
<td>85</td>
<td>89</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>BGB2%</td>
<td>120</td>
<td>---</td>
<td>--</td>
<td>91</td>
<td>--</td>
<td>76</td>
</tr>
<tr>
<td>Lac-I</td>
<td>120</td>
<td>95</td>
<td>79</td>
<td>88</td>
<td>93</td>
<td>73</td>
</tr>
<tr>
<td>LP-I</td>
<td>120</td>
<td>85</td>
<td>71</td>
<td>80</td>
<td>94</td>
<td>67</td>
</tr>
<tr>
<td>LPO-II</td>
<td>120</td>
<td>85</td>
<td>71</td>
<td>83</td>
<td>98</td>
<td>69</td>
</tr>
</tbody>
</table>

^aLST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; Lac-I = lactose plus capsule I; LP-I = 1% lactose-1% peptone plus capsule I; LPO-II = 1% lactose-1% peptone-2% oxgall plus capsule II.
confirmed. The other five media were less efficient, with 67 to 76% of the inoculated presumptive tubes being confirmed.

In addition to the six broth media, coliforms were also detected using VRB and DL agar pour plates. Based on confirmed coliforms/ml, the geometric means of the six raw milk samples were: lactose broth, 150,000; DL agar, 140,000; VRB agar, 110,000; LST broth, 57,000; BGB2% broth, 54,000; lactose broth plus capsule I, 43,000; LPO broth plus capsule II, 29,000; and LP broth plus capsule I, 18,000. Of the 60 representative colonies picked from the VRB or DL agar plates, 45 (75%) of the VRB colonies and 47 (78%) of the DL colonies were confirmed.

**Hamburger samples**

Six samples, each a different batch of ground beef, yielded preliminary counts on VRB agar between 3,100 and 80,000 coliforms/g. A further investigation was made with the MPN method using six media (Table 16). The highest percentage of positive presumptive tests was obtained with LST broth (54%), followed by lactose broth (48%), LPO broth plus capsule II (40%), lactose broth plus capsule I (38%), and LP broth plus capsule I (34%). The percentage of confirmed positive presumptives was 100% for the three capsule media compared to 98% for LST broth and 84% for lactose broth. Overall, LST broth was the best medium; 53% of the
Table 16. Comparison of six MPN media for enumeration of coliforms in six hamburger samples.

<table>
<thead>
<tr>
<th>Media</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>90</td>
<td>43</td>
<td>48</td>
<td>36</td>
<td>84</td>
<td>40</td>
</tr>
<tr>
<td>LST</td>
<td>90</td>
<td>49</td>
<td>54</td>
<td>48</td>
<td>98</td>
<td>53</td>
</tr>
<tr>
<td>BGB2%</td>
<td>90</td>
<td>--</td>
<td>--</td>
<td>38</td>
<td>--</td>
<td>42</td>
</tr>
<tr>
<td>Lac-I</td>
<td>90</td>
<td>34</td>
<td>38</td>
<td>34</td>
<td>100</td>
<td>38</td>
</tr>
<tr>
<td>LP-I</td>
<td>90</td>
<td>31</td>
<td>34</td>
<td>31</td>
<td>100</td>
<td>34</td>
</tr>
<tr>
<td>LP0-II</td>
<td>90</td>
<td>36</td>
<td>40</td>
<td>36</td>
<td>100</td>
<td>40</td>
</tr>
</tbody>
</table>

*LST = lauryl sulfate tryptose; BGB2% = brilliant green bile 2%; Lac-I = lactose plus capsule I; LP-I = 1% lactose-1% peptone plus capsule I; LP0-II = 1% lactose-1% peptone-2% oxgall plus capsule II.*
inoculated presumptive tubes were confirmed in BGB2% broth. The other five media were less efficient, with 34 to 42% confirmation of the inoculated presumptive tubes.

Coliforms were enumerated using VRB and DL agar pour plates in addition to the six broth media. The geometric means of the six hamburger samples, based on confirmed coliforms/g, included: DL agar, 42,000; VRB agar, 24,000; LST broth, 22,000; lactose broth, 17,000; LPO broth plus capsule II, 14,000; BGB2% broth, 13,000; lactose broth plus capsule I, 6,800; and LP broth plus capsule I, 6,400. Of the 60 coliform-like colonies picked from the VRB and DL agar plates, 51 (85%) of both the VRB and DL colonies were confirmed.

Frozen vegetable samples

Twelve frozen vegetable samples were plated in VRB agar. Low numbers of coliforms (1 to 6/g) were present in six samples; no coliforms were present in the other samples. Birdseye tiny peas, Libby's mixed vegetables, Libby's peas and carrots, Libby's sweet corn, Rosedale peas and carrots, and Silverdale mixed vegetables were chosen for further examination. The recovery of coliforms by each MPN medium is shown in Table 17. Double strength media, to which had been added two capsules, was used for the lowest dilution. The inoculum for the double strength media was 10 ml of a 1:10 dilution of each sample. Lactose broth yielded the highest
**Table 17.** Comparison of six MPN media for enumeration of coliforms in six frozen vegetable samples\(^a\).

<table>
<thead>
<tr>
<th>Media</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>90</td>
<td>43</td>
<td>48</td>
<td>12</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td>LST</td>
<td>90</td>
<td>39</td>
<td>43</td>
<td>23</td>
<td>59</td>
<td>26</td>
</tr>
<tr>
<td>BGB2(%)</td>
<td>90</td>
<td>--</td>
<td>--</td>
<td>21</td>
<td>--</td>
<td>23</td>
</tr>
<tr>
<td>Lac-I</td>
<td>90</td>
<td>32</td>
<td>36</td>
<td>25</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td>LP-I</td>
<td>90</td>
<td>33</td>
<td>37</td>
<td>22</td>
<td>67</td>
<td>24</td>
</tr>
<tr>
<td>LPO-II</td>
<td>90</td>
<td>30</td>
<td>33</td>
<td>20</td>
<td>67</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^a\)Birdseye tiny peas, Libby's mixed vegetables, Libby's peas and carrots, Libby's sweet corn, Rosedale peas and carrots, and Silverdale mixed vegetables.

\(^b\)LST = lauryl sulfate tryptose; BGB2\(\%\) = brilliant green bile 2\%; Lac-I = lactose plus capsule I; LP-I = 1\% lactose-1\% peptone plus capsule I; LPO-II = 1\% lactose-1\% peptone-2\% oxgall plus capsule II.
percentage of positive presumptive tubes (48%) followed by LST broth (43%) and the three capsule media (33 to 37%). The percentage of positive presumptives confirmed with BGB2% was lowest with lactose broth (28%) and increased to 59% for LST broth, 67% for LP and LPO broths, and 78% for lactose broth plus capsule I. The percentage of inoculated presumptive tubes confirmed was approximately the same (22 to 28%) for all media except lactose broth (13%).

In addition to the six MPN media, coliforms were enumerated using VRB and DL agar pour plates. The geometric means (coliforms/g) of the confirmed counts made on the six samples were: lactose broth plus capsule I, 14; LST broth, 12; BGB2% broth, 9.9; LP broth plus capsule I, 9.9; LPO broth plus capsule II, 8.1; lactose broth, 3.0; DL agar, 1.7; and VRB agar, 1.7. Of 14 coliform-like colonies picked from VRB agar and 15 from DL agar, only one colony from each medium was confirmed.

**Dried food samples**

A preliminary examination by plating in VRB agar was made on 13 dried cake mixes, muffin mixes, bread mixes, corn meal, and corn starch. Only six samples, Betty Crocker white cake mix, Betty Crocker blueberry muffin mix, Betty Crocker peach muffin mix, Duncan Hines white cake mix deluxe II, Pillsbury white cake mix, and Pillsbury nut bread mix, yielded counts. No counts were obtained on the other seven
samples. Recovery of the coliforms in the six samples by each MPN medium is shown in Table 18. Double strength media, containing two capsules, was used for the lowest dilution. The inoculum for the double strength media was 10 ml of a 1:10 dilution of each sample. Only 33 to 36% of the presumptive tests were positive with all media. The percentage of positive presumptives confirmed with BGB2% was low; only 6% of the lactose and LST broths, and 7% of the LP and LPO broths were confirmed. The percentage of inoculated presumptive tubes confirmed was also low (0 to 3%) for all media.

Coliforms were detected using VRB and DL agar pour plates in addition to the six broth media. The geometric means of the six dried food samples, based on confirmed coliforms/g, were: LPO broth plus capsule II, 1.1; BGB2% broth, 0.7; lactose broth, 0.7; LP broth plus capsule I, 0.7; LST broth, 0.7; lactose broth plus capsule I, 0; DL agar, 0; and VRB agar, 0. Of the 60 colonies picked from the VRB and DL agar plates, none was confirmed in BGB2% broth.
Table 18. Comparison of six MPN media for enumeration of coliforms in six dried food samples\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Media \textsuperscript{b}</th>
<th>Number of tubes inoculated</th>
<th>Number of positive presumptives</th>
<th>Per cent positive presumptive</th>
<th>Number of positive confirmed</th>
<th>Per cent presumptives confirmed</th>
<th>Per cent inoculated confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>90</td>
<td>31</td>
<td>34</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>LST</td>
<td>90</td>
<td>32</td>
<td>36</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>BGB2%</td>
<td>90</td>
<td>--</td>
<td>--</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Lac-I</td>
<td>90</td>
<td>30</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LP-I</td>
<td>90</td>
<td>30</td>
<td>33</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>LPO-II</td>
<td>90</td>
<td>30</td>
<td>33</td>
<td>3</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Betty Crocker white cake mix, Betty Crocker blueberry muffin mix, Betty Crocker peach muffin mix, Duncan Hines white cake mix deluxe II, Pillsbury white cake mix, and Pillsbury nut bread mix.

\textsuperscript{b}LST = lauryl sulfate tryptose; BGB2\% = brilliant green bile 2\%; Lac-I = lactose plus capsule I; LP-I = 1\% lactose-1\% peptone plus capsule I; LPO-II = 1\% lactose-1\% peptone-2\% oxgall plus capsule II.
DISCUSSION

The initial goal of this research was to develop a capsule, pill, or tablet method that would enumerate injured coliforms in water and food. Two broths, BGB2% or LST, were initially proposed for use as the "parent" medium in the capsule, pill, or tablet methods. BGB2% was chosen because brilliant green, the selective inhibitor in BGB2%, could easily be assayed compared to sodium chloride or sodium lauryl sulfate found in LST broth. Furthermore, BGB2% was used for the confirmed test in the examination of water and, therefore, a combination presumptive-confirmed test could be developed. Consequently, the formulations for the basal media, LP and LPO broths, were based on the formulation for BGB2% with both inhibitors (brilliant green and oxgall) removed from LP broth or, only brilliant green removed as with LPO broth.

A great deal of time and effort was expended in attempts to develop the timed-release, wax-coated capsule. Initially we proposed to have the contents of the capsule released between 6 and 10 hr after inoculation because various periods of pre-enrichment from 5 to 24 hr had been used for salmonellae; pre-enrichment was usually overnight (18 hr) to conform to workday routines. According to Ray and Speck (1973b), and Sinskey and Silverman (1970), however, repair
of injured *E. coli* in a nutritional medium took only 1 to 3 hr at 35 and 37 C. The three basal media (lactose, LP, and LPO broths) were considered nutritional media because LP broth contained 1% lactose and 1% peptone, LPO broth contained 1% lactose, 1% peptone, and 2% oxgall, and lactose broth contained 0.3% beef extract, 0.5% peptone, and 0.5% lactose. Carbohydrates had no effect on the lag period, but 0.1% peptone shortened the lag of freeze-dried *E. coli* (Sinskey and Silverman, 1970). Resuscitation of the injured cells in each of the three basal media was, therefore, assumed to be complete within 1 to 3 hr. The concentration of brilliant green released from the capsules into the basal media reached 4.2 ug/ml and 5.4 ug/ml at 2 hr (Tables 1 and 2); this should have been sufficient time for resuscitation of injured cells (Ray and Speck, 1973b; Mukherjee and Bhattcharjee, 1970). The gradient which formed during the initial hours of diffusion (Fig. 5 and 6) was not a factor because at no time did the brilliant green concentration exceed 13.3 ug/ml and the gradient had disappeared by 4 hr incubation.

The results of the heat-injured *E. coli* B studies were similar to results obtained by Maxcy (1970). Counts of heat-injured *E. coli* B cells in lactose broth plus capsule I (Table 5) were only 43% of the counts obtained with lactose broth; recovery of the heat-injured cells was not complete
in lactose broth at 2 hr. If recovery had been complete in 1 hr, as reported for heat-injured *E. coli* (Mukherjee and Bhattacharjee, 1970), counts in both media would have been approximately the same. Lower counts obtained in LP and LPO broths compared to lactose broth indicated that recovery was also not complete in these two basal media before the brilliant green concentrations reached inhibitory proportions. A significant amount of repair, however, had taken place in each basal medium because counts of heat-injured cells using the capsule media were two to three times the numbers of counts obtained by using the "parent" BGB2% broth (Table 5). The 2% oxgall in LPO broth appeared to be only slightly inhibitory to the heat-injured cells, although a valid comparison could not be made between LP and LPO broths because a different capsule was used in each medium. Based on counts obtained in LST and lactose broths, and on TGE agar, the inhibitory effects of sodium lauryl sulfate in LST broth (Mallman and Darby, 1941) did not extend to heat-injured cells; LST broth was essentially a non-selective medium. The inhibitory effects of neutral red, crystal violet, and bile salts on the heat-injured cells were apparent by the very low counts obtained in VRB agar.

**Examination of Water**

Although the resuscitation of heat-injured cells by the
capsule methods was not complete, sufficient repair had occurred (43%) to warrant further study with water samples. Results with the MPN, membrane filtration, and pour plate methods in enumerating coliforms in water samples indicated that few injured coliforms were present in the 24 Group A or 36 Group B samples (Tables 9, 10, and 13). Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971) stated that only counts from plates with 30 to 300 colonies were statistically accurate. This accuracy was difficult to attain because the number of coliform colonies enumerated in VRB agar and M-Endo-MF broth were less than 30 in 16 water samples. During the collection of Group B samples, heavy run offs had occurred and water samples from all sites, except site five, contained large amounts of soil. Large numbers of non-coliform colonies in M-Endo-MF broth and non-fecal coliforms in M-FC broth, using membrane filtration, overwhelmed the selectivity of the broth and enumeration was difficult. Since statistically there was no difference detected between counts using any of the six broth media, the capsule method was comparable to standard methods for enumeration of coliforms in water samples.

The combination presumptive-confirmed tests initially proposed for the capsule method proved successful. The lower numbers of false-positives (3%) obtained using LP and LPO broths compared to 7% in lactose broth and 6% in LST broth
(Table 11) indicated that confirmation of the LP and LPO broths in BGB2% is unnecessary; only 3% of 769 positive presumptive tests were negative after confirmation. The smaller numbers of false-positives reported herein compared to earlier studies (Dunham, et al., 1925) were based on the interpretation of gas production. Early standard methods (American Public Health Association, 1925) stated that if gas production in any quantity was present in the presumptive media after 48 hr, confirmation was required. Presently, standard methods (American Public Health Association, 1971) recommends more stringent requirements for gas production, which tends to eliminate from consideration many of the false-positives earlier encountered.

A minor modification of the capsule method, the addition of phosphate buffer to the basal media, is suggested. Using the six broths with the MPN test, LST broth was observed to yield the highest quantity of gas/tube of all the broths tested. No quantitative measurements of gas were taken, but upon observations of several thousand tubes, this phenomenon was noted consistently. Discounting the inhibitors, sodium chloride and sodium lauryl sulfate, the only constituent found in LST broth and not the other MPN broths was phosphate buffer. In spite of earlier reports that lactose broth was superior to buffered lactose broth (Ruchhoft, 1935; Ruchhoft and Norton, 1935), the addition of a phosphate buffer to the
basal media might increase gas production facilitating its interpretation more readily.

The number of fecal coliforms confirmed by EC medium depended upon the recovery of fecal coliforms in the presumptive medium. The results of several investigations (Tables 7, 8, and 12) indicated that the selectivity for fecal coliforms by the "parent" broth, BGB2%, was retained using capsule I in lactose and LP broths. A portion of the inhibitory qualities of the EC test towards non-fecal coliforms was due to the elevated incubation temperature (Perry and Hajna, 1933). Results of an experiment using the lactose broth-capsule I medium incubated at 44.5±0.2 C indicated that the capsule methods in their present form could not be used as a combined presumptive-confirmed test for the examination of fecal coliforms in water samples. Modifications, such as reducing the "window" size or eliminating the "window" in the capsules, might decrease the diffusion rate of brilliant green and increase the usefulness of the capsule method at temperatures above 35 C. A second modification would be the addition of magnesium stearate, which is commonly used to slow diffusion rates of active ingredients from commercial capsules and pills; incorporation of magnesium stearate in a timed-release capsule might decrease the diffusion rate of brilliant green.

We feel that with minor modifications, the timed-release
capsule methods could possibly be used as a presumptive-confirmed test for routine use in water examination. Major modifications, such as the use of LST broth as the "parent" broth, could also be incorporated into the capsule method. What we wish to emphasize, however, is the principle of the method, and that is the transformation of a non-selective medium into a selective medium by diffusion of an inhibitor(s) from a capsule into a basal medium.

**Examination of Food**

The capsule methods could also be used as a combined pre-enrichment and presumptive test for the enumeration of coliforms in raw milk and hamburger; the fewest number of false-positives was obtained from raw milk (Table 15) and hamburger (Table 16) using the capsule methods, and this number was minimal (0 to 7%). The same conclusion could not be made when frozen vegetables and dried foods were analyzed with the capsule method (Tables 17 and 18), indicating that a confirmatory medium was needed for enumeration of coliforms in these foods. The low numbers of coliforms detected in the frozen vegetables and dried foods dictated the use of double strength basal medium containing two capsules because the lowest dilution, 10 ml of the sample homogenate (10^{-1} g/ml), was used for the inoculum. When the 10-ml sample was dispensed into the medium, it settled to the bottom of the
tube and prevented diffusion of the brilliant green throughout the lower one-fourth of the medium. This could have been avoided by placing the tubes on a shaker until diffusion was complete, therefore, providing mixing of the inhibitors in the basal media. The selectivity of the capsule media was reduced when the large amounts of organic materials (10 ml) were added to the media and non-coliform gas producers grew; as a result, high percentages of false-positives were obtained by the three capsule media. This phenomenon occurred in the double strength capsule media used for the analyses of both the frozen vegetables and dried foods. The higher percentages of false-positives obtained by LST broth in the frozen vegetables and dried foods compared to the percentages obtained with LST broth in raw milk and hamburger, indicated that the inhibitory qualities of LST broth were also reduced when double strength medium was inoculated with 10 ml of the 1:10 sample inoculum.

Of the six broth media used with the MPN test to detect coliforms in the 24 food samples, the results obtained were similar with all media, based on the percentages of inoculated presumptive tests that were confirmed. The geometric means of coliform counts obtained in raw milk samples by using lactose, LST, and BE2% broths and DL and VRB agars, indicated a discrepancy in the numbers of injured coliforms in raw milk. Heat-injury investigations (Table 5) showed
that VRB agar was more inhibitory to heat-injured coliforms than LST or BGB2% broths. The counts in lactose, LST, and BGB2% broths indicated that a large number of injured cells were present in raw milk. The counts in DL and VRB agars, however, indicated that a smaller number of injured cells were present in raw milk than suspected with the broth media. Counts in DL and VRB agars, and LST and BGB2% broths on the hamburger samples, indicated that injured coliforms were probably absent from the hamburger samples. The discrepancies between the MPN and pour plate counts in the raw milk and hamburger samples may be due to what Ray and Speck (1972b) referred to as wide variations and poor reproducibility of the MPN method; a greater degree of variability was encountered using the MPN method than with DL or VRB agars in the detection of *E. coli* from milk, egg, meat, and frozen food samples (Ray and Speck, 1972a, b). The counts obtained in the "parent" broth, BGB2%, were slightly higher than counts in the three capsule media used for the enumeration of coliforms from the raw milk and hamburger samples. It appeared therefore, that the capsule method needed improvement before studies are continued on the examination of food. Several modifications are suggested to decrease the diffusion rate of brilliant green from the capsules into the basal media and allow the injured cells to fully recover. The modifications include reducing the "window" size and possibly
adding magnesium stearate to the capsule.

The geometric means of the coliforms detected in the frozen vegetables and dried foods by the six MPN broths and the two agar pour plates were low (0 to 12/g) and no comparison was made. Statistical studies were not made with the coliform counts from the food samples because the total number of samples for each variety of food was small (6) and the counts detected in the frozen vegetables and dried foods, which comprised half of the food samples, were low.

The success obtained with the capsule method as a combination of pre-enrichment and presumptive test in the examination of raw milk and hamburger indicated that the capsule method could be readily adapted for use in the examination of foods. Modifications and further confirmatory studies are needed before the capsule method could be recommended for routine use. The emphasis, again, is on the principle of the capsule method. This is a new principle. We have extended the studies of Matelova, et al. (1972) and included the suggestion of Ray and Speck (1972b) that a period of treatment in a non-selective liquid medium was necessary for better enumeration of injured coliforms in food. The capsule method saved time and materials because transfer from an enrichment medium to a selective medium was avoided. The purpose of this research was to develop a method that was reasonably successful and expose this method
to others who could adapt it for commercial use. The capsule method principle has also been adapted for use in the isolation of salmonellae from foods (William Sveum, Iowa State University, Ames, Iowa, personal communication); a combined pre-enrichment and enrichment test is being studied.
SUMMARY

New methods using MPN (Most Probable Number) procedures were developed to enumerate injured coliforms in foods and water. The methods involved resuscitation of injured bacterial cells in non-selective media followed by release of selective inhibitors from capsules into the same media. The inhibitors, brilliant green and oxgall, were added to the media by diffusion from timed-release, wax-coated capsules.

Capsules were prepared by dispensing 0.5-ml aliquots of a boiled and cooled (60°C) suspension of agar, brilliant green, and oxgall (capsule I) or agar and brilliant green (capsule II) into #0 gelatin capsules. The capsules were immersed in distilled water, dried overnight at 25°C, and coated (except for a 1.5 to 3.0 mm "window") with melted beeswax. When a capsule was added to a tube of basal broth, water entered through the "window", rehydration occurred, and the selective ingredients were released. Tests showed that concentrations of brilliant green did not reach inhibitory levels until 2 or more hr had elapsed, which presumably allowed sufficient time for repair of injured cells.

The efficiencies of recovery of heat-injured (6 min at 57°C) E. coli B by several media, in decreasing order of recovery, were: lactose broth, LST (lauryl sulfate tryptose) broth, TGE (tryptone-glucose-extract) agar, LP (lactose-
peptone) broth plus capsule I, LPO (lactose-peptone-oxgall) broth plus capsule II, lactose broth plus capsule I, BGB2% (brilliant green bile 2%) broth, and VRB (violet red bile) agar. Resuscitation of the injured cells probably occurred in the capsule-containing media but not in the two most highly selective media, BGB2% broth and VRB agar.

The capsule method was compared to standard methods for enumeration of coliforms in two groups of river water samples. No significant differences (95% level) were noted between MPN (lactose, LST, and BGB2% broths, lactose broth plus capsule I, LP broth plus capsule I, and LPO broth plus capsule II), membrane filtration (M-Endo-MF broth), and pour plate (VRB agar) methods. When LP broth (capsule I) and LPO broth (capsule II) were used, 97% of the positive presumptive tests were confirmed, compared to 93% and 94%, respectively, for lactose and LST broths. The capsule method could be used, therefore, as a combined presumptive and confirmed test for water analysis.

Higher percentages (93 to 100%) of positive presumptive tests were confirmed when the capsule methods were used than when lactose broth (84 and 94%) or LST broth (87 and 98%) were used for analyses of raw milk and hamburger samples. Presumptive counts of coliforms/ml were higher, however, when the standard methods were used than when the capsule methods were used.


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