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INJURED INDEX *and* PATHOGENIC BACTERIA:

Occurrence and Detection in Foods, Water and Feeds

Bibek Ray

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PREFACE

Indicator and pathogenic bacteria are enumerated or isolated from foods, water, and feedstuffs by incubating a sample aliquot in specific selective agar or broth media. For some bacteria, additional selective environments, such as high or low incubation temperature and incubation under anaerobic or microaerophilic condition, are used. The selective conditions used to detect indicator or pathogenic bacteria presumably do not inhibit growth of physiologically normal cells of their species, but inhibit or restrict growth of other bacteria that are always present in foods, water, and feeds. It is assumed that in the selective environments, the indicator and pathogenic bacteria, even when present in low numbers and constitute only a minor population in comparison to the associated bacteria, will get selective advantage to multiply and be detected.

The selective media and other conditions, recommended for the detection of a specific indicator group or species and pathogen, are developed generally with physiologically normal cultures and in many instances are developed originally to test clinical samples. However, microorganisms present in food, water, and probably in feedstuffs, differ in several respects from those present in a clinical sample. In clinical samples the indicator and pathogenic bacteria are generally actively growing and usually present in high members. In contrast, in most food, water, and feed samples, the indicator and pathogenic bacteria are present in relatively low numbers in relation to the total microbial population. Also many of the associative microorganisms in foods, water, and feeds may be taxonomically closely related to the indicator and pathogenic bacteria and thus might not be inhibited by the selective conditions. Thus, selective media and incubation parameters developed with laboratory grown pure cultures and for clinical specimen may not be always effective for use in foods, water, and feedstuffs.

The other difference is that most foods and food ingredients, potable water, and feedstuffs are given one or more physical or chemical treatments that are known to inflict sublethal injury in bacteria. As a consequence the injured indicator and pathogenic bacteria lose their resistance to the selective media and incubation conditions and in many instances lose their viability when exposed to these environments. The selective methods thus will not detect these viable but injured indicator and pathogenic bacteria from the food, water, or feed samples. However, the injured cells are capable of repair relatively easily. The repaired cells regain resistance to the selective environments and can be detected by the specific recommended methods.

This book provides up-to-date information on the occurrence of injured indicator and pathogenic bacteria in foods, water, and feeds, and also the methods that could be used for their effective detection. This publication will be an invaluable source of information for the scientists in the food industries, food regulatory agencies, public health services, and microbiologists involved in developing methods for the detection of indicator and pathogenic bacteria from foods, water, and feeds. It will also serve as a useful guide to scientists interested in microbiological quality and standards of pharmaceutical and cosmetic products. Finally, the microbial ecologists, especially those who are interested in monitoring survival of released genetically engineered mircoorganisms in the environment, will find information in this book that could be helpful in developing effective methods.

THE EDITOR

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Prof. Ray obtained his B.S. and M.S. degrees in Veterinary Science from the University of Calcutta and University of Madras, India, respectively. He received his Ph.D. degree in Food Science from the University of Minnesota in 1970. His research for his dissertation was in the area of sublethal injury of *Salmonella* and its influence on their isolation from foods. In 1971 he joined the faculty in the Department of Food Science, North Carolina State University, Raleigh, and continued to conduct research in the area of sublethal injury of index and pathogenic bacteria and their detection from foods. In 1981 he joined the Department of Animal Science, University of Wyoming as Associate Professor of Food Microbiology.

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Chapter 1

INTRODUCTION

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I. HISTORICAL

"Death of microorganisms, exposed to many sublethal treatments, is a gradual process which could be reversed under proper conditions if the reaction has not progressed too far." This statement made by Rahn and Barnes[1] and Rahn[2] in 1932 clearly suggested that some physical and chemical agents in a sublethal dosage inflict reversible injuries in microbial cells. Since the beginning of this century many microbiologists recognized that pure cultures of both vegetative bacteria and bacterial spores subjected to a sublethal dosage of heat, UV light, mercuric chloride, and other agents suffered cellular damages and became more exacting in their nutritional need for subsequent growth.[3-8] Microbiologists involved in the development of methods and media for the quantitative evaluation of the microbiological qualities of heat-processed foods that contained different types of microorganisms observed that supplementing nonselective types of media with yeast extracts, milk, etc., improved recovery.[9-13] It was recommended that "This should be considered in the formulation of media for the enumeration of bacteria in heated food products and in experiments concerned with the effects of heat on microorganisms."[12] Other researchers also observed that indicator, pathogenic, and other bacteria in frozen foods also were not effectively detected, either by nonselective or selective media, due to reversible injury.[14,15] In 1959 Straka and Stokes[16] showed that certain fractions of *Escherichia coli* and *Pseudomonas* spp. that survived freezing and thawing were metabolically injured and needed several types of peptides to reverse their injury. From the 1960s to the early 1980s many laboratories, mainly in the U.S., the U.K., Japan, Canada, and the Netherlands, conducted research on the sublethal injury of indicator and food and water-borne pathogens.[17-19] These studies indicated that most physical and chemical treatments, when applied to sublethal dosages, could inflict injury on microbial cells found in food and water (see Tables 1 and 2). These cells, although constituted as part of the viable microbial population, have many altered physiological characteristics, and a specific method used in the microbiological evaluation of a sample could make the injured fraction detectable or undetectable.

The studies conducted during the last 25 years could be divided into separate stages. In the 1960s most studies were conducted to develop differential media to measure both "metabolic" and "structural" injuries.[17] In the 1970s, basic studies were conducted to identify the sites of cellular injuries, the mechanisms of cellular repair, and the methods to enumerate and isolate injured indicator and pathogenic bacteria from foods and water. In the later part of the 1970s and in the early 1980s, basic studies on the nature of cellular damages at the molecular level were studied.[17,18] Currently, very few laboratories are conducting research in the area of sublethal injury in bacteria associated with foods; however, research in bacterial injury still remains viable in the area of water.[19]

II. MAJOR EMPHASIS

Most studies on bacterial injury during the 1960s, 1970s, and early 1980s were conducted with indicator and pathogenic bacteria. Very limited basic studies were done on the injury of bacteria associated with either food spoilage or food bioprocessing.[17,18,20-22] However, the data presented in Table 2 revealed that not only index and pathogenic bacteria, but also food spoilage bacteria,[23-25] bacteria used as starter cultures[20,23] and in dietary adjunct,[21,22] as well as yeasts[26,27] and molds[28] found in foods are injured by different sublethal treatments. Although no report has yet been published on the existence of reversible injury of viruses present in foods, water, or feeds, freezing and thawing have been reported to cause reversible injury in T4 bacteriophages[29] and in southern bean mosaic virus virion[30] that affected their detection. It would be important to study the possibility of reversible injury in pathogenic viruses in foods and water, and probably in feeds.

Table 1
SUBLETHAL PHYSICAL AND CHEMICAL TREATMENTS KNOWN TO CAUSE REVERSIBLE INJURY IN MICROORGANISMS

Physical stresses
- Low temperature: refrigeration, freezing
- Heat: temperature and time below lethal treatment
- Drying: air drying, freeze-drying
- High solids: sugars, salts
- Radiation: UV, X-ray

Chemical stresses
- Acids: organic and inorganic
- Sanitizers: chlorine, QAC
- Preservatives: sorbate, benzoate
- Toxic chemicals: mercuric chloride

Partially adapted from Ray, B., *J. Food Prot.*, 42, 346, 1979.

Table 2
MICROORGANISMS KNOWN TO SUFFER REVERSIBLE INJURY FROM SUBLETHAL STRESSES

Microorganisms	Main importance in foods[a]
Escherichia coli	Indicator (some are pathogenic)
Enterobacter aerogenes	Indicator
Klebsiella sp.	Indicator (some are pathogenic)
Streptococcus faecalis	Indicator
Salmonella sp.	Pathogenic
Shigella sp.	Pathogenic
Vibrio parahaemolyticus	Pathogenic
Yersinia enterocolitica	Pathogenic
Campylobacter jejuni	Pathogenic
Staphylococcus aureus	Pathogenic
Clostridium perfringens	Pathogenic
Pseudomonas sp.	Spoilage
Bacillus sp.	Spoilage (*B. cerus* is pathogenic)
Streptococcus lactis	Bioprocessing
Lactobacillus bulgaricus	Bioprocessing
L. acidophilus	Dietary adjunct
Saccharomyces cerevisiae	Bioprocessing
Candida sp.	Spoilage
Aspergillus flavus	Spoilage

Note: Information on specific microorganisms can be obtained from references in this chapter[20-28] and from related chapters in this book.

[a] Partially adapted from Ray, B., *J. Food Prot.*, 42, 346, 1979.

In pure-culture studies, injured fractions of the indicator and pathogenic bacteria were detected by enumerating a sample simultaneously on nonselective and selective media. This was based on the observation that the injured cells of a species developed a sensitivity to many chemicals used in many selective media, and to which the uninjured or normal cells were resistant. It was assumed that the injured cells would rapidly multiply in the nonselective

Table 3
EFFICIENCY OF NONSELECTIVE PLATING MEDIA TO ENUMERATE HEAT-STRESSED BACTERIA

Bacterial species	Treatment	Colony counts in plating media: Nutrient agar	Milk agar	Beef infusion agar
Escherichia coli	Heat (57°C, 5 min)	183×10^3	87×10^3	233×10^3
Pseundomonas aeruginosa	Heat (55°C, 7 min)	204×10^2	187×10^2	44×10^3
Bacillus subtilis spores	Heat (100°C, 20 min)	53×10^3	164×10^3	32×10^3
Streptococcus liquefaciens	Heat (62°C, 15 min)	2×10^3	325×10^3	108×10^3
Staphylococcus aureus	Heat (57°C, 5 min)	117×10^3	56×10^3	66×10^3

Adapted from Nelson, F. E., *J. Bacteriol.*, 45, 395, 1943.

media and these media would enumerate all the survivors, while the selective media will enumerate only the normal cells. However, some earlier studies showed that even nonselective complex media did not facilitate the growth of all the survivors. Many surviving bacterial cells failed to form colonies equally well in different media (see Table 3). After the recognition of metabolic injury by Straka and Stokes,[16] the inability of injured bacterial cells to form colonies in a nonselective medium was thought to be due to the failure of the medium to supply the necessary nutrients. Later studies showed that supplementing a nonselective medium, such as tryptone-glucose-extract (TGE) agar, with compounds that degrade hydrogen peroxide or block its formation increased the enumeration of stressed bacterial cells by 2000- to 3000-fold over the counts, on nonsupplemented TGE agar.[31] It was proposed that these supplements reduced the formation and facilitated the degradation of hydrogen peroxide that could form spontaneously in the media and to which the injured bacterial cells are extremely susceptible. The supplementation of selective media with one or more of these compounds has considerably increased the enumeration of injured cells.[31] It would probably be important to study whether or not the incorporation of these compounds in the media could increase the enumeration of the surviving population of microorganisms from foods and water. If a significant and consistent increase is found, such a compound or compounds can then be included in the compositions of media recommended for the microbiological examination of foods and water.

III. THE FUTURE

Historically, the existence of reversible cell injury in microorganisms that have been exposed to sublethal physical and chemical treatments was conceived by the microbiologists who were studying the lethality of various agents on pathogenic and index bacteria.[1,2] It remained up to the food microbiologists to prove, with both pure culture and processed foods, the existence of reversible injury in microorganisms. Their studies also revealed the sites of damage, the nature of cellular damage, and the mechanisms of repair of injury.[17,18] From these studies, methods were developed that would allow the resuscitation and subsequent enumeration and isolation of the injured cells from food samples.[17] Similar studies by the water microbiologist also showed the presence of injured bacteria in water and the effectiveness of resuscitative methods on the detection of the injured cells.[19] As indicated previously, most studies on injury were conducted in indicator and pathogenic bacteria. The bulk of evidence, published during the 1960s and 1970s, was helpful in making the bacterial reversible injury cross the barrier of "mere academic interest" to possible "regulatory implication". Books dealing with the microbiological examination of foods either devoted new chapters[32,33] to or suggested precautionary steps[34-36] on the implications and methods

Table 4
SOME AREAS WHERE INFORMATION ON REVERSIBLE INJURY IN MICROORGANISMS COULD HAVE IMPORTANT APPLICATIONS

Areas of study	Application of information
Rapid detection methods	Detection of index and pathogenic bacteria from foods, water, and feeds
New method development	Detection of newly emerging pathogens from foods, water, and feeds
Irradiated foods	Resuscitation methods of pathogens, indicator, and other microbes
Comparison of several media (selective or nonselective)	To determine the relative efficiency of several media used to detect a specific group or species of microorganisms
Effectiveness of some current methods	To detect pathogenic viruses from foods and water and other bacteria and yeasts and molds from foods
Efficiency of cryopreservatives	Transport and storage of refrigerated food samples in a frozen state for microbiological analysis
Effect of residual chlorine in water	Lethal effect on indicator and pathogenic bacteria during transport and storage prior to analysis
Rates of thawing (of frozen) and rehydration (of dried) foods	Optimum rates for effective detection of microorganisms from foods
Preventing the repair of injured spoilage microorganisms and enhancing their death	Increased shelf life of refrigerated foods
Increased resistance to freezing and drying of starter cultures	Extension in storage time and use of cultures for direct fermentation
Stability of plasmids in genetically engineered bacteria and the hybridomas	Long-term preservation of economically important microbial cultures, including those used in food fermentation, and hybridomas used for the production of monoclonal antibodies
Injury of genetically engineered microbial cultures when released into the environment	Monitoring the survival rate of these laboratory cultures in soil and water, and in the presence of pesticides, insecticides, etc.

Partially adapted from Ray, B., *J. Food Prot.*, 49, 651, 1986.

of detection of injured bacteria from foods. In addition, two books were published on the importance and implication of bacterial reversible freeze-injury in 1984.[34,38]

Currently, very little research on microbial injury is being done in the area of food microbiology. In contrast, many important basic and applied studies are being conducted in the water microbiology area.[39,40] In the past, food microbiologists directed their research activities toward the effective detection of injured index and pathogenic bacteria. Very little or no studies were done in other areas that might also be important in food microbiology.[18] Also, basic information generated from these studies could be valuable in other areas of microbiology. These aspects are briefly discussed below (see Table 4).

Currently, the possible applications of "genetic probes" and "enzyme immunoassay" in the rapid detection of pathogens from foods are being investigated.[41] However, these methods are effective only when pathogens have efficiently multiplied during the preenrichment/enrichment stage. Unless they are properly resuscitated, the injured cells of pathogens fail to multiply. To make these rapid and expensive methods effective, studies should be done on the rapid resuscitation and the subsequent multiplication of injured pathogens. This should include the influences of optimum pH, temperature, and composition of a medium as well as any possible inhibitory effect of food components on the resuscitation of injured cells.[18] In the development of a selective method for the effective detection of any newly emerging pathogen, the selective agents, their concentrations, and other selective conditions should be evaluated for their noninhibitory effects on injured cells prior to their inclusion in a

recommended method.[42] The irradiation of certain foods has been in use in several countries and now is being used in selected food items in the U.S. The effectiveness of the currently available resuscitation methods on the detection of microorganisms injured by a low dosage of irradiation needs to be studied. Data on comparative studies about the efficiency of different selective media on the enumeration and isolation of index and pathogenic bacteria from semipreserved foods and water are published from time to time.[44] However, one should recognize that the different media, even when recommended for use on a bacterial group or species, contain different selective agents, and injured cells show different degrees of developed susceptibility to these agents. For a comparison of the efficiency of the selective media, the food or water sample should be incubated in a resuscitation medium prior to testing with selective media.[18] Studies on T4 phages and southern bean mosaic virus have indicated that sublethal stresses could cause reversible injury with the loss of their adsorption to host cells by altering conformation of the surface protein subunits.[29,30] As the current methods of detecting pathogenic viruses in foods and water are based on the ability of the virus to adsorb on host cells in a cell culture, it should be worthwhile to study the effect of sublethal treatment on the adsorption of pathogenic viruses to host cells. Also, the effectiveness of media containing oxgall to enumerate *Lactobacillus acidophilus*[21] and that of acidified media to enumerate yeasts and molds[27] from foods exposed to sublethal conditions should be evaluated. For microbiological quality evaluation, many foods and food ingredients are currently being transported to distant laboratories in frozen condition. Also, these samples may be thawed and refrozen for testing. This procedure, due to the lethal and sublethal effects of freezing and thawing, may not indicate the real microbiological quality during sampling of the products as well as it can introduce variability in the results. This could probably be reduced by freezing the sample in a suitable cryoprotective agent. Similarly, the presence of residual chlorine in water could alter the microbiological quality between the time of sampling and testing. The procedures for finding the optimum thawing rate of frozen products and the optimum rehydration rate for dried products need to be investigated to minimize microbial death and injury, as both these techniques are known to influence the detection of microbial cells from frozen and dried foods. The injury of spoilage bacteria, yeasts, and molds can also be used advantageously to extend the shelf life of sublethally processed (such as pasteurized, acidified, frozen and thawed, irradiated at low dosage, etc.), refrigerated, semipreserved foods. By maintaining a low enough temperature, the injured cells should be prevented from repair, and by adding suitable additives to which they have developed a sensitivity, the injured cells can be killed.[47] This will allow only the uninjured cells among the viable population of psychrotrophic microorganisms to multiply. With good sanitation to keep the initial population of psychrotrophic spoilage bacteria low and the use of a selective storage environment, the shelf life of many refrigerated, semipreserved foods could be prolonged.[17] There is a great interest in using concentrated cultures directly for the production of fermented foods and food ingredients. Although methods of preparing frozen starter concentrates of selected strains have been achieved, there are disadvantages in handling frozen cultures. Also, many of these cultures could not be stored in the food processing plants for long. Finally, these methods do not effectively preserve many starter strains, even though they carry desirable traits. The success of producing dried, concentrated starter cultures is much more limited. It is believed now that reversible injury is an intermediate state between normal and lethal or irreversible injury or death of microbial cells.[48] An understanding of the sites and nature of reversible injury due to the freezing and/or drying of microbial cells will help us to recognize the physical and biochemical basis of such injury and to develop procedures to prevent the changes associated with injury. This in turn will help us to develop freezing or drying methods for the long-term preservation of desired starter cultures with high survival rates.[17] Genetically engineered microorganisms with the desirable traits and hybridomas for the production of monoclonal antibodies are now being

developed for use in food fermentation and for the detection of pathogens in foods.[49] Also, many microbial strains and cell lines are being developed for use in controlling different microbial and genetic diseases in humans and animals. Successful methods for the preservation of these microbial strains and cell lines have to be developed so that they do not lose the newly acquired traits. Limited studies have shown that some bacterial strains during cryopreservation could lose their genetic materials associated with the engineered traits.[50] Finally, the rate of disappearance from the environment of genetically engineered laboratory cultures of microorganisms when released into the environment for some beneficial effect, such as the prevention of ice nucleation and frost damage by spraying nonice-nucleating variants, needs to be monitored. However, these laboratory cultures may suffer reversible injury by the environmental stresses (i.e., sunlight, temperature fluctuation, the lack of necessary nutrients, competition from associative microorganisms, insecticides, etc.) and develop the need for special nutrients as well as a sensitivity to some chemicals. This possibility should be considered in the methods used for monitoring these cultures.

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Chapter 2

ENUMERATION OF INJURED INDICATOR BACTERIA FROM FOODS

Bibek Ray

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I. INTRODUCTION

A. Significance of Indicator Bacteria in Food

The major objectives of using indicator bacteria in food are based on the ability of certain species or a group of bacteria to indicate the possible contamination of a food with fecal materials, the possible presence of pathogenic microorganisms in food, and the degree of sanitation used during processing and subsequent handling of foods. The selection of an effective indicator bacterial group or species is based on the following criteria:[1,2] its association with fecal matter and enteric pathogens; the levels or ratio at which it occurs in the fecal matter in relation to enteric pathogens; the ease of and time requirements for its detection, in relation to enteric pathogens from foods; the chances of its being a natural contaminant in the foods; its rate of growth, in relation to enteric pathogens in the food

environments; and its relative resistance to various processing and storage conditions of foods as compared to enteric pathogens. Several groups and species of bacteria have been suggested as indicator bacteria in foods, and include coliforms, fecal coliforms, *Escherichia coli,* total Enterobacteriaceae and fecal enterococci. However, it should be recognized that a single species or group of bacteria is not capable of satisfying all the criteria of indicator bacteria, either to evaluate the likelihood of the presence of enteric pathogens in a food or to determine the level of sanitation practiced in the processing, handling, and storage of foods. Even with these shortcomings there are general agreements that the indicator bacteria have an important place in Food Microbiology, especially when used with cautions to evaluate the microbiological quality of foods.

B. Characteristics of Indicator Bacteria

The bacterial groups and species included as indicator bacteria differ in many physiological and ecological characteristics. An understanding of these characteristics discussed in this section would be useful in their effective use as indicator bacteria.[3-7]

1. Coliform Group

This group includes several species of gram-negative, nonspore-forming, aerobic and facultative anaerobic, rod-shaped bacteria, capable of producing acid and gas at 32 to 37°C within 48 h. The species included in this group are *Escherichia coli, Citrobacter freundii, Enterobacter aerogenes, E. cloacae, Klebsiella pneumoniae,* and several other lactose-fermenting species. Among these, *Escherichia coli, C. freundii,* and *K. pneumoniae* are normal inhabitants of the intestinal tract of humans, warm-blooded animals, and birds, while *Enterobacter aerogenes* and *K. pneumoniae* inhabit the soil. Consequently, the coliform test is not intended to determine fecal contamination, but is aimed at determining the efficiency of sanitary practices used to reduce microbial contamination in a food, especially processed food. Caution should be used in the interpretation of the significance of coliforms in the raw foods, both of animals and plant origins and in foods that have been subjected to heat, freezing, drying, and low-pH treatment. Also, as some coliforms can multiply at refrigeration temperatures, their presence in large numbers in a food may be due to growth from a low initial population. As they are fairly sensitive to heat, their presence in pasteurized and other heat-treated products suggests postheat-treatment contamination. Similarly, in frozen, dried, and low-pH products, a low level of coliforms really does not indicate the original population levels, as coliforms are killed by these treatments. They also may not be reliable indicators for the presence of nonenteric pathogens, such as *Staphylococcus aureus* and pathogens of marine origins, such as *Vibrio parahaemolyticus* and *V. cholera.*

2. Fecal Coliform Group

This group includes the coliforms that are capable of fermenting lactose and producing gas at 44.5 ± 0.2°C within 24 ± 2 h. The main objectives are to detect the coliforms of fecal origin, especially *Escherichia coli* and to eliminate nonfecal coliforms. However, some nonfecal coliforms are capable of lactose fermentation during incubation at 44.5 ± 0.2°C for 24 ± 2 h. The major advantage of using the fecal coliform group as an indicator is that it provides better specificity of fecal contamination of a food. Their presence in a processed food indicates improper sanitation during the processing and handling of the product. However, their ability to multiply in many foods and on equipment surfaces, as well as their sensitivity to freezing, drying, and low pH, adversely affect their effectiveness as an indicator of the possible presence of enteric pathogens in a food.

3. E. coli

E. coli is a normal inhabitant in the intestinal tracts of humans, warm-blooded animals, and birds. Hence, there are direct or indirect associations between its presence in and fecal

contamination of a food. The presence of *E. coli* in a food thus implies that other fecal microorganisms, including enteric pathogens, could be present. Conversely, its absence in a certain amount of food does not necessarily imply that the whole amount of the product is free of enteric pathogens. There are several shortcomings in the use of *E. coli* as indicator bacteria. It is a poor indicator of nonenteric pathogens and pathogens of marine origin. It does not survive well in a marine environment. It is killed by pasteurization and rapidly dies off during storage under refrigeration, freezing, drying, and at low pH. It is also able to multiply in many foods and on the surfaces of improperly cleaned equipment. These aspects should be considered in the effective use of *E. coli* as an indicator of sanitary practices during the processing and handling of foods and of the possible presence of enteric pathogens in a food. The ability of certain psychrotropic enteric pathogens, such as *Yersinia enterocolitica* and *Listeria monocytogenes,* to grow at the refrigeration temperature at which *E. coli* does not multiply needs further evaluation as to the significance of *E. coli* as an indicator of the presence of these pathogens in refrigerated foods. In addition, due to the pathogenic nature of certain *E. coli* strains, such as enteropathogenic *E. coli* and the inability to ferment lactose by some of these pathogenic strains, the enumeration of *E. coli* in a food just as an indicator may not be justifiable.

4. Enterobacteriaceae Group

Some of the frequently occurring enteropathogenic bacteria in foods are from the family Enterobacteriaceae and include the *Salmonella* sp., the *Shigella* sp., and enteropathogenic *Escherichia coli* strains. In addition, many species from the genera included in the family Enterobacteriaceae are of fecal origin. Because of this, suggestions have been made to use the total Enterobacteriaceae group as indicator bacteria in foods. However, some species from this group are present in soil and vegetation. The value of this group as an indicator of either fecal contamination or the possible presence of enteric pathogens in a food is thus questionable. In processed foods, and especially in heat-treated products, they could be used to indicate recontamination and improper sanitation.

5. Enterococci or Fecal Streptococci Group[6,8-10]

The Lancefield group D streptococci found in the intestinal contents of humans, warm-blooded animals, and birds are included in this group. The group includes *Streptococcus faecalis* and its four subspecies, *S. faecium, S. bovis, S. equinus,* and *S. avium.* However, *S. faecalis* and *S. faecium* are the two most commonly found in foods. Some enterococci can establish colonies and multiply in soil and vegetation, and on equipment surfaces. In general, they survive better than the coliforms in refrigerated, frozen, dried, and low-pH environments. Because of their fecal origin and ability to survive in environments that are lethal to the coliforms, enterococci have been suggested as indicator bacteria for certain foods, especially those in which coliforms do not survive well. However, their ability to multiply in vegetation, on equipment surfaces, and in foods under refrigeration storage (by certain strains) have made them a poor candidate to indicate either fecal contamination or the possible presence of enteric pathogens in a food. In some processed and storage products they could be used as an indicator of sanitation and proper handling conditions.

In the current edition of *Bergey's Manual of Systematic Bacteriology,*[10a] *S. faecalis, S. faecium, S. avium,* and a separate species, *S. gallinarum* (which also belongs to Lancefield group D and is found in the intestinal tract of birds) have been listed in the enterococcus group. *S. bovis* and *S. equinus,* although both have the group D antigen, differ physiologically from the four species currently listed as enterococci and are thus grouped under "other streptococci" (see Table 1). The subspecies of *S. faecalis* are no longer recognized. In addition, a new genus *Enterococcus* has been proposed to include the four species listed above. This aspect needs to be considered in future publications of recommended methods for the microbiological examination of foods.

Table 1
SOME DISTINGUISHING CHARACTERISTICS OF ENTEROCOCCI AND *STREPTOCOCCUS BOVIS* AND *S. EQUINUS*[10,10a]

Characteristics[a]	Enterococci group				Other streptococci	
	S. faecalis	*S. faecium*	*S. avium*	*S. gallinarum*	*S. bovis*	*S. equinus*
Growth at 10°C	+	+	+	+	−	−
Growth in 6.5% NaCl	+	+	+	+	−	−
Growth in 40% bile	+	+	+	+	−	+
Arginine hydrolysis	+	+	−	d	−	−
Tetrazolium reduction	+	−	−	+	−	−
K-tellurite reduction	+	d	d	−	−	−

[a] (+): 90% or more positive; (−): 90% or more negative; (d): 11 to 89% positive.

C. Recommended Methods to Detect Indicator Bacteria from Foods

Several quantitative methods have been recommended by the regulatory agencies[3-8] and many researchers[11-22] to determine the level of indicator bacteria from foods. An understanding of these currently used methods will be helpful to recognize the principles used in the development of effective techniques to detect injured indicator bacteria from foods. A brief description of these methods is presented in this section.

As raw and semipreserved foods and food ingredients contain different species of microorganisms, some of which could be indicator bacteria, selective agar and broth media are recommended for the enumeration of the indicator bacteria from foods. In general, these media contain one or more selective ingredients that inhibit or restrict the growth of the associative microorganisms without affecting the growth of the indicator bacteria. These media usually contain ingredients that allow the differentiation of the colonies of the indicator and nonindicator bacteria. The composition of the media, the incubation time and temperature, and the confirmation methods differ considerably with a group or species of indicator bacteria. The recommended methods could be broadly grouped as the direct-plating (DP) methods and the indirect most probable number (MPN) methods. While the DP methods give direct numerical values, the MPN methods, based on statistical probability, give indirect estimates of the number of indicator bacteria in a sample. The accuracy of the MPN technique increases by increasing the number of replicates in each decimal dilution. For all practical purposes, three to five replicates per decimal dilution are found to be adequate. Although the basis of using either a DP or an MPN method depends upon several factors, the MPN method is recommended for a food that is expected to have less than 10 indicator organisms per gram and that could give "false-positive" results in the DP method.

The methods briefly described in this section do not include the preparation of the bacteriological media and sample. For this information a methodology book should be consulted.[3-8]

1. Enumeration of the Coliform Group

a. MPN Methods

The methods recommended by the U.S. Food and Drug Administration (FDA).[5] the American Public Health Association (APHA)[3,6] and the Association of Offical Analytical Chemists (AOAC)[7] for different types of foods other than dairy products and shellfish are basically the same and consist of two steps, a presumptive test followed by a confirmative test. The presumptive test consists of inoculating 1-ml portions, in triplicate, from each of at least three decimal dilutions into tubes containing 10 ml lauryl sulfate tryptose (LST) broth, incubating the tubes at 35 ± 0.5°C, and examining the tubes for gas formation first at the end of 24 ± 2 and then at 48 ± 2 h. Gas formation within 48 ± 2 h in the LST

broth tubes constitutes a positive presumptive test. From the number of positive tubes in three successive dilutions, a presumptive MPN of coliforms per gram or milliliter of a food is obtained from a 3-tube MPN table (see Table 2). For the confirmation test, a loopful of material from each positive-LST tube is inoculated in a tube containing 10 ml brilliant green lactose bile (BGLB) broth. These are incubated at 35 ± 0.5°C and examined for gas formation at the end of 24 and 48 h. From the number of positive tubes in three successive dilutions, the confirmed MPN of coliforms per gram or milliliter of food is calculated from the MPN table (see Table 2).

The method used to determine the MPN of coliforms in shellfish[6] includes presumptive, confirmed, and completed tests. In the presumptive test, five tubes of LST broth are used in each dilution and incubation at 35 ± 0.5°C for up to 48 ± 3 h. The confirmed test is carried out in BGLB broth as described above and the tubes are incubated at 35 ± 0.5°C for 48 ± 3 h. The completed test is carried out by streaking a loopful of material from each BGLB positive tube on a prepoured Levin's Eosine Methylene Blue® (EMB) agar plate. The plates are incubated at 35 ± 0.5°C for 24 ± 3 h and one typical colony, which is nucleated and with or without a metallic sheen from each plate, is transferred to a tube of lactose broth and to nutrient agar slant. The tubes are incubated at 35 ± 0.5°C; the lactose broth tube is examined after 48 ± 2 h for gas formation, and the culture from the slant is examined microscopically after 18 ± 2h from gram-negative rods. In the absence of typical colonies on the EMB plates, two atypical colonies (non-nucleated or pink) from each plate should be examined for gas formation in the lactose broth and the presence of gram-negative rods in the slant culture. Gas formation and the presence of gram-negative rods constitute a positive completed test. From the completed test, the MPN of coliforms per 100 g of the sample is calculated from the 5 tubes of the MPN table (see Table 2).

For dairy products the standard method[4] consists of inoculation of the sample aliquots in five replicates for each of three or more decimal dilutions in BGLB broth tubes. The tubes are incubated at 32°C for up to 48 ± 3 h, examined for gas formation, and from the number of positive tubes (with gas) in three successive dilutions, the MPN of coliforms per gram or milliliter of the product is calculated from Table 2. A completed test, similar to the one described for shellfish, has also been suggested; however, all incubations are at 32°C.

b. DP Methods

The pour plating of liquid foods and reconstituted solid foods with violet red bile (VRB) agar has been recommended for the enumeration of coliforms.[3,4] A sample aliquot is pour plated initially with 10 to 15 ml of boiled and tempered (48°C) VRB agar per plate. After solidification, another 3 to 4 ml of the same medium is overlaid to cover the entire surface. The plates are incubated at 32°C for dairy products and at 35°C for all other foods for 24 ± 2 h. The purple-red colonies of 0.5 mm or more in diameter with a zone of bile precipitation are counted as coliforms and the counts are reported as coliform counts per gram or milliliter of the products.

As some noncoliforms can mimic coliform colonies and coliform colonies in a crowded plate (more than 100 colonies) can produce atypical colonies, a confirmed test in the enumeration of coliform also has been recommended. This involves inoculation of representative typical and atypical colonies from the VRB agar plates into BGLB broth tubes, incubation up to 48 h at 35°C (32°C for dairy products), and examination for positive tubes with gas production but no surface pellicle. Materials from positive tubes with a surface pellicle should be tested to exclude gram-positive, lactose-fermenting bacteria. The number of coliforms per gram or milliliter of food is determined from the percentage of original colonies on VRB agar plates that gave a positive test in BGLB broth. A completed test, the same as the completed test in the MPN methods, has also been recommended for dairy products.

Food containers and equipment surfaces are examined for coliforms by rinsing or washing

Table 2
THREE OR FIVE REPLICATES MPN WITH 0.1, 0.01, AND 0.001 g OR ml SAMPLES[3-5]

No. positive in[a]			MPN per gram or milliliter sample	
0.1	0.01	0.001 g or ml	3 Replicates	5 Replicates
0	0	0	<3	<2
1	0	0	4	2
1	1	0	7	4
1	1	1	11	6
1	2	0	11	6
2	0	0	9	5
2	1	0	15	7
2	1	1	20	9
2	2	0	21	9
3	0	0	23	8
3	0	1	39	11
3	1	0	43	11
3	1	1	75	14
3	2	0	93	14
3	2	1	150	17
3	3	2	210	—
3	3	0	240	—
3	3	1	460	—
3	3	2	1100	—
3	3	3	≤2400	—
4	0	0	—	13
4	1	0	—	17
4	1	1	—	21
4	1	2	—	26
4	2	0	—	22
4	2	1	—	26
4	3	0	—	27
4	3	1	—	33
4	4	0	—	34
5	0	0	—	23
5	0	1	—	31
5	1	0	—	33
5	1	1	—	46
5	1	2	—	63
5	2	0	—	49
5	2	1	—	70
5	2	2	—	94
5	3	0	—	79
5	3	1	—	110
5	3	2	—	140
5	3	3	—	180
5	4	0	—	130
5	4	1	—	170
5	4	2	—	220
5	4	3	—	280
5	4	4	—	350
5	5	0	—	240
5	5	1	—	350
5	5	2	—	540
5	5	3	—	920

Table 2 (continued)
THREE OR FIVE REPLICATES MPN WITH 0.1, 0.01, AND 0.001 g OR ml SAMPLES[3-5]

No. positive in[a]			MPN per gram or milliliter sample	
0.1	0.01	0.001 g or ml	3 Replicates	5 Replicates
5	5	4	—	1600
5	5	5	—	>2400

[a] For other combinations see *Standard Methods for the Examination of Water and Wastewater* by the American Public Health Association.

the test material with a known volume of sterile water or a phosphate buffer and enumerating a portion for coliforms by pour plating with VRB agar. In case the volume of liquid is large and the coliform population is low, a larger portion can be filtered thorugh a membrane filter. The filter is then placed on the surface of a pad soaked with MF-Endo broth in a petri plate and incubated for 24 h at 32 or 35°C. Red colonies with dark nucleated centers and a metallic sheen are enumerated and expressed as coliform counts for the total volume.

2. Enumeration of Fecal Coliform Group

a. MPN Methods

The method recommended for the enumeration of fecal coliforms in shellfish and water from shellfish-growing areas consists of a presumptive test of five tubes in LST broth at 35 ± 0.5°C for 48 ± 2 h.[3,6] A loopful of material from each positive tube is transferred to a tube of EC broth, which is then incubated at 44 ± 0.2°C for 24 ± 2 h and examined for gas production. The MPN of fecal coliforms per gram or milliliter of the sample is estimated from the MPN table (see Table 2). Quadri et al.[11] studied a rapid method for the estimation of fecal coliforms in the oyster. Sample aliquots from three or more decimal dilutions were inoculated into tubes containing MacConkey broth in triplicate, incubated first at 37 ± 1°C for 2 h, then at 44 ± 0.1°C for an additional 22 to 24 h and examined for acid and gas production. The MPN of fecal coliforms was calculated from the positive tubes in three successive dilutions (see Table 2).

Another rapid MPN method to determine fecal coliforms in foods was evaluated by Andrews et al.[12] A serial dilution of blended food homogenate was inoculated into tubes of A-1 medium with three tubes in each dilution. The tubes were first incubated at 35 ± 2°C for 3 h before being placed in a 44.5 ± 2°C water bath. The fecal coliform MPN was calculated from the positive combination of gassing tubes.

b. DP Methods

Francis et al.[13] described a rapid DP method for the enumeration of coliforms from foods. The sample aliquot was pour plated with a selective medium (protease-peptone, 5.0 g; yeast extract, 3.0 g; lactose, 10.0 g; NaCl, 7.5 g; Na-lauryl sulfate, 0.05 g; bromo-thymol blue, 0.3 g; agar, 15.0 g; water, 1000 ml), the plates were incubated at 41.5 ± 0.5°C, and after 7 h enumerated for fecal coliforms with a colony counter. The colonies that were yellow to orange with yellow haloes against a bluish-green background were counted as fecal coliforms. Representative colonies were then subjected to further tests consisting of gas formation in LST broth and EC broth at 44.5°C.

Another DP procedure to enumerate fecal coliforms in foods was developed by Klein and

Fung.[14] The samples were pour plated with 12 to 14 ml sterile and tempered VRB agar and overlaid with 2 to 4 ml of the same agar. The plates were incubated in an air incubator at 45.5 ± 0.5°C for 24 h. The dark-red colonies 0.5 mm or larger in size and with a zone of 1 mm were enumerated as fecal coliforms. The colonies obtained in the plate gave 99% or higher accuracy for fecal coliforms in the completed tests.

3. Enumeration of E. coli

a. MPN Methods

The methods recommended by the FDA,[5] the AOAC,[7] and the APHA[3] are quite similar. In the FDA method, all gas-positive LST broth tubes from the presumptive coliform test are subcultured by transferring a loopful of material from each tube to a tube of EC broth, which is then incubated at 45.5 ± 0.05° C in a circulating water bath and checked for gas formation after 24 ± 2 and 48 ± 2 h. The same incubation temperature is recommended in the AOAC method, but in the APHA method a temperature of 45.5 ± 0.2°C is suggested. Materials from the gas-positive EC broth tubes are streaked on EMB agar plates. After a 24 ± 2 h incubation at 35°C, the plates are examined for the suspected *E. coli* colonies that are nucleated, dark-centered, and with or without a metallic sheen. Two suspected colonies from each plate are further subcultured in tryptone glucose extract (TGE) or plate-count agar slants at 35°C for 18 to 24 h, and cultures from each slant are examined for gram-stain characteristics, cell morphology, gas production in lactose or LST broth within 48 ± 2 h at 35°C, and biochemical patterns for the production of indole (I), methyl red reaction (M), Voges-Proskauer reaction (V), and citrate utilization (iC). A culture that has gram-negative, nonspore-forming rods, has produced gas from lactose, and has either + + − − (biotype or variety I) or − + − − (biotype or variety II) IMViC patterns is considered as *E. coli*. From these results the MPN of *E. coli* per gram or milliliter of sample, in relation to the gas-positive LST tubes in the presumptive test, is determined.

A relatively rapid MPN method has also been suggested by the FDA that consists of inoculating sample aliquots in triplicate from at least three successive decimal dilutions into LST-broth tubes, incubating the tubes at 44 ± 0.2°C up to 24 h, and examining for gas production. Materials from the gas-positive tubes are then streaked on EMB agar plates. The subsequent steps are the same as described before and consist of examining the suspected *E. coli* colonies for gram-strain characteristics, morphology, gas production in lactose, broth, and + + − − or − + − − IMViC patterns.

Andrews et al.[15] described the suitability of A-1 broth for the MPN of *E. coli* determination in shellfish by incubating the tubes at 44.5 ± 0.2°C, examining the tubes after 24 ± 2 h for gas production, and confirming the gas-positive tubes for the presence of *E. coli* with + + − − or − + − − IMViC patterns. This method was rapid and gave results comparable to the lengthier APHA method.[6] Powers and Latt[16] developed an agar plate method for the IMViC tests that reduced the time required in the conventional method for *E. coli* enumeration.

b. DP Methods

Anderson and Baird-Parker[17] described a rapid DP method for the enumeration of *E. coli* biotype I from foods. An aliquot of a food was distributed on the surface of a cellulose-acetate membrane placed on the surface of a prepoured tryptone bile (TB) agar plate and incubated at 44°C for 24 h. *E. coli* biotype I colonies could then be identified by staining the membrane with a specific reagent to color indole-producing colonies red. Although this method did not detect *E. coli* biotype II (indole negative), the researchers suggested that this group only constitute less than 5% of *E. coli* in foods. This method also detected the lactose-negative *E. coli* biotype I, many of which were pathogenic and were not detected by the conventional methods based on lactose fermentation by *E. coli*. The results of a

collaborative study[18] showed that this method was less variable and gave a consistently higher enumeration of *E. coli* from foods than the MPN method. Sharp et al.[19,20] suggested a modification of this method by using hydrophobic grid membrane filters (HGMF). An enriched lauryl sulfate-aniline blue agar medium, described by Wright,[21] was found to give a higher enumeration of *E. coli* compared to the DP method of Anderson and Baird-Parker. The food sample was surface plated and incubated at 44°C for 18 h during which *E. coli* biotypes I and II formed colonies that were 2 to 3 mm in diameter, blue to blue-green in color, and surrounded by a zone of clear yellow-green medium.

4. Enumeration of Enterobacteriaceae Group

a. DP Method[13,22]

In this method a food sample is pour plated with VRB agar supplemented with 1% glucose. After overlaying with the same medium, the plates are incubated at 35°C for 18 to 24 h and all colonies that are dark red or purple, are 0.5 mm or more in diameter, and are with or without a bile-precipitation zone are counted. The results are reported as total Enterobacteriaceae per gram or milliliter of food. As some non-Enterobacteriaceae may also form colonies, suitable differential tests may be necessary.

5. Enumeration of Enterococci (or Fecal Streptococci) Group

a. DP Methods

The APHA[9] has recommended the use of two selective agar media for the direct enumeration of enterococci in foods. One method consists of pour plating the samples with KF-strepococcal agar, followed by incubation at 35 ± 1°C for 48 ± 2 h, then counting all red to pink colonies. The results are reported as KF-streptococcal counts per gram or milliliter of food. The other method consists of surface plating a sample on prepoured GTC-streptococcal agar plates, incubating the plates at 35 ± 1°C for 18 ± 2 h, and enumerating colonies that have a dark halo. The results are reported as GTC-streptococcal counts per gram or milliliter of food. This medium is less inhibitory to *S. bovis* and *S. equinus* than KF-streptococcal agar.

Enterococci in dairy products[4] are enumerated by pour plating a sample with citrate-azide agar, followed by an overlay with the same medium, incubating the plates at 37°C for 72 h, and enumerating all blue colonies as enterococci.

For the confirmation of enterococci, five to ten colonies from the plates for each sample are subcultured in tubes containing brain-heart infusion (BHI) broth, incubated at 35°C for 18 to 24 h, and examined for gram-stain characteristics, morphology, catalase activity, and growth in bile-esculin agar, in BHI broth at 45°C, and in BHI broth containing 6.5% NaCl. Enterococci are gram-positive, spherical or elongated, in pairs or in short chains, catalase negative, bile resistant, capable of hydrolyzing esculin, capable of growing at 45°C and, except for *S. bovis* and *S. equinus,* are capable of growing in the presence of 6.5% NaCl.

b. Presence or Absence of Enterococci[8]

This test is recommended for the examination of infant foods and involves inoculation, in duplicates, a 1-ml portion from a series of decimal dilutions of a food into a tube containing 9 ml of a selective broth (composition: tryptone, 20.0 g; yeast extract, 5.0 g; ox-bile, 10.0 g; NaCl, 5.0 g; Na-citrate, 1.0 g; esculin, 1.0 g; ferric ammonium citrate, 0.5 g; Na-azide, 0.25 g; distilled water, 1000 ml; pH, 7.0 ± 0.1). After an 18- to 24-h incubation at 37 ± 1°C, the tubes are examined for blackening of the medium. A loopful of material from the tubes which turned black is streaked on a prepoured plate of the selective agar made by adding 1.5% agar to the broth described above. After incubating at 37 ± 1°C for 16 to 24 h, the plates are examined for colonies surrounded by black haloes. The presence of such a colony constitutes a positive test. Complementary tests, such as catalase activity, growth

Table 3
SELECTIVE INGREDIENTS IN SOME MEDIA USED FOR ENUMERATION OF INDICATOR BACTERIA

Media	Selective ingredient(s)	Conc per 100 ml
Lauryl sulfate tryptose broth	Na-lauryl sulfate[a]	0.01 g
Brilliant green lactose bile broth	Oxgall[a]	2.00 g
	Brilliant green[b]	1.33 mg
EC broth	Bilesalts[a]	0.15 g
A1 broth	Triton® X-100[a]	0.1 g
MF-Endo agar	Na-deoxycholate[a]	0.01 g
	Na-lauryl sulfate[a]	0.005 g
	Na-sulfite[c]	0.21 g
Violet red bile agar	Bilesalts[a]	0.15 g
	Crystal violet[b]	0.2 mg
Levin EMB agar	Eosine Y[b]	0.04 g
	Methylene blue[b]	0.65 g
MacConkey agar	Bilesalts[a]	0.15 g
	Crystal violet[b]	0.01 g
	Eosine Y[b]	0.04 g
Tryptone bile agar	Bilesalts[a]	0.15 g
KF-streptococcal agar	Na-azide[d]	0.04 g
	Bromocresol purple[b]	1.5 mg
Citrate azide agar	Na-azide[d]	0.04 g
GTC agar	Tween® 80[a]	0.075 g
	Gentamicin[e]	0.25 mg

[a] Surface-active agent.
[b] Antiseptic dye.
[c] Reducing agent.
[d] ATP-synthesis inhibitor.
[e] Protein synthesis inhibitor.

at 45 ± 1°C, glucose-fermentation pattern, and cell morphology of the cultures from the plates, can also be performed.

D. Selectivity of Media Used for the Enumeration of Indicator Bacteria in Foods

The principles used in the enumeration of indicator bacteria from raw and semipreserved foods, either in broth or agar media, are based on the ability of the indicator organisms to multiply selectively in these media as well as on the inhibition of growth of associative microorganisms. To obtain the selective environment, one or more chemical compounds that are capable of inhibiting the growth of many nonindicator organisms are incorporated in each medium. The nature or specificity of these chemicals differs with a particular medium (see Table 3). Different surface-active agents and dyes are generally used in media designed to enumerate coliforms, fecal coliforms, *E. coli,* and Enterobacteriaceae. These indicators are capable of multiplying in an environment of relatively low surface tension and are resistant to many surface-active compounds and dyes. Many other bacteria are relatively sensitive to these compounds. Similarly, enterococci are resistant to Na-azide, while many other bacteria fail to grow in its presence.

Several aspects need to be considered for the incorporation of a selective compound in a medium designed for the detection of indicator bacteria from foods:

1. The selective compound may not inhibit the growth of all associative microorganisms and allow the growth only of indicator bacteria. Surface-active compounds are generally

Table 4
INHIBITION OF COLONY FORMATION OF *ESCHERICHIA COLI* BY SELECTIVE COMPOUNDS USED IN MEDIA[23,24]

Selective compounds added to basal agar[a]	Basal agar[b]	% Inhibition
Brilliant green	MA	99.9
Neutral red	MA	5.0
Crystal violet	MA	25.0
Crystal violet + neutral red	MA	35.0
Bilesalts	MA	20.0
Na-deoxycholate	MA	46.0
NaCl	MVA	0.0
NaCl + bilesalts	MVA	37.0
NaCl + bilesalts + crystal violet + neutral red	MVA	48.0

[a] Added to basal agar in concentrations used in selective media.
[b] Basal agar: minimal agar (MA); modified violet red bile agar (MVA; contains all ingredients of violet red bile agar except NaCl, bilesalts, crystal violet, and neutral red).

not inhibitory to all gram-negative bacteria, including coliforms, fecal coliforms, *E. coli,* and other Enterobacteriaceae.

2. Indicator bacterial species and strains differ considerably in their relative resistance to a selective compound. *E. coli* NCSM is more resistant to 0.1% Na-deoxycholate than *E. coli* K12 or B.[26a]
3. Different compounds, even in the same concentration, differ greatly in their degree of inhibition to the same species or strain of indicator bacteria (see Table 4).
4. A selective compound may interact with other selective or nonselective compounds in the same medium, resulting in a greater level of inhibition (see Table 4).
5. The selectivity or inhibitory property of a medium, especially with a dehydrated medium, can differ among different lots and manufacturers.
6. The selectivity of a dehydrated medium may increase with an increase in storage time, high-heat treatment during preparation, and storage conditions after reconstitution.
7. Certain ingredients of foods may reduce or increase the selectivity of a medium.
8. A slightly higher temperature (such as 48 vs. 45°C) of a melted medium during pour plating of samples can increase selectivity.[25]
9. Finally, the physiological states of the indicator bacterial cells present in a food can be altered due to the processing and handling of foods in such a way that they will develop a sensitivity to the selective compounds present in the bacteriological media and to which the indicator bacteria are normally resistant. This is due to sublethal cell injury and will be discussed in greater detail in this article.

It appears from this discussion that in the formulation and use of recommended selective media, standardized procedures should be adopted for the effective detection of indicator from foods.

II. SUBLETHAL INJURY IN INDICATOR BACTERIA

Studies with pure cultures of several species of indicator bacteria have revealed that a bacterial population, exposed to a sublethal physical or chemical environment, contains many viable cells that are physiologically abnormal or injured.[26,27] Many semipreserved foods and equipment surfaces are exposed to such environments and thus could harbor injured bacteria.[28] Injured indicator bacteria could be present in samples that have been

Table 5
LEVEL OF DETECTION OF SUBLETHALLY STRESSED SURVIVING INDICATOR BACTERIA IN SELECTIVE MEDIA

Species/strains	Sublethal stress	Selective media[a]	%Survivors detected	Ref.
Escherichia coli K-12	Air drying	VRB agar	1	42
Escherichia coli ATCC 11775	Heating (69°C)	VRB agar	76	43
E. coli ATCC 27622	Chlorine	m-FC agar	<1	46
E. coli NCSM	QAC (10 μg/ml)	VRB agar	21	30
E coli K-12	Acid (pH 4.2)	VRB agar	<1	38
E. coli NCSM	Freezing	VRB agar	9	45
E. coli	Irradiation (170 krad)	VRB agar	40	47
E. coli NCSM	Freezing	BGLB broth	18	45
Enterobacter aerogenes	Air drying	VRB agar	15	42
Streptococcus faecalis 9790	QAC (10 μg/ml)	AZD agar	44	23
S. faecalis	Freezing	KFS agar	33	32
S. faecalis	Freeze-drying	KFS agar	29	32
S. faecium NRC 1261	Heating (55°C)	TYGS agar	17	43
S. faecalis R 57	Heating (60°C)	TSS agar	<1	30

[a] Selective media: VRB agar, violet red bile agar; m-FC, m-FC broth + 1.5% agar; BGLB broth, brilliant green lactose bile broth; AZD agar, azide (0.02%) dextrose agar; KFS agar, KF-streptococcal agar; TYGS agar, tryptone yeast extract glucose salt (6%) agar; TSS agar, tryptic soy agar + 6% NaCl.

exposed to low heat,[29,30] refrigeration,[30a] feezing and thawing,[31,32] drying and rehydration,[33,34] high osmotic environments, irradiation,[35,36] low pH,[37,38] preservatives,[39] and sanitizers.[23,24] The injured population could constitute as high as 99% of the total surviving indicator bacteria (see Table 5). However, it is dependent upon different factors, such as the species and strains of indicator bacteria,[40,41] the nature of the suspending medium,[41] the nature of sublethal stress,[42-46] the selectivity of the bacteriological media,[43] and other conditions. As the injured cells are viable and are potentially similar to normal cells, they should be enumerated in the microbiological examination of foods.[28] The selective media, recommended for the enumeration of indicator bacteria from foods, should ideally enumerate all the viable cells of a specific indicator group or species present. However, most selective agar and broth media fail to enumerate the injured fraction of the viable population from the samples. Although the normal cells of indicator bacteria are resistant to the different selective compounds in concentrations that are used in the medium, the injured cells develop a sensitivity to many of these chemicals and fail to be detected.[26,27] The fate of the injured cells, when exposed to a selective medium, is not properly studied. It is generally assumed that they fail to multiply in the selective environment and thus are not detected in the selective agar or broth media. However, there are some indications that exposure of the injured indicator bacteria, at least in some selective media, results in their loss of viability (see Table 6). That the injured *E. coli* cells lose viability in BGLB broth and LST broth was shown by the subsequent inability of these cells to form colonies in a nonselective agar medium.[40,45]

A population of indicator bacteria after a sublethal treatment contains three physiologically different types of cells: the uninjured that are capable of multiplying equally well in a selective and a nonselective medium; the injured, that are viable and are capable of multiplying in a nonselective medium, but not in a selective medium; and the dead, that are incapable of multiplication even in a nonselective and nutritionally rich medium.[40,41] The ability of the injured indicators to multiply in a nonselective, but not in a selective, medium has formed the basis for differentiating them from the uninjured or normal cells that are capable of multiplying equally well in both nonselective and selective media. Another

Table 6
VIABILITY LOSS OF FREEZE-INJURED *ESCHERICHIA COLI* FOLLOWING EXPOSURE TO BROTH CONTAINING SELECTIVE COMPOUNDS[40,45]

Suspending media	Selective compound	Exposure time (min) at 25°C	% Viability loss of survivors[a]
Water	None	2 to 60	<5
TSY broth	None	2 to 60	0
BGLB broth	Oxgall and brilliant green	2	60
		60	90
LST broth	Lauryl sulfate	2	55
		60	Not tested

[a] *E. coli* NCSM (10^8 cells per ml) in water suspension were frozen, thawed, and transferred to the following broths: trypticase soy-yeast extract (TSY), brilliant green lactose bile (BGLB), and lauryl sulfate tryptose (LST) and water and incubated at 25°C. At 2 and 60 min of incubation the samples were withdrawn, serially diluted, and pour plated with TSY agar. Colony counts on TSY agar from TSY broth at 2 min incubation were used as 100% to calculate the percent detected from each broth.

characteristic of the injured cells is their inability to regain a resistance to selective compounds when the cells are incubated in a nonselective medium prior to their exposure to a selective medium. This suggests that the developed sensitivity and other abnormalities of the injured cells are reversible.

The information generated from the basic studies on the sublethal injury and the repair of injury in indicator bacteria has formed the basis in the development of methods to enumerate injured indicator bacteria from semipreserved foods.[47] Those aspects will be discussed briefly in this section.

A. Measurement of Injured, Uninjured, and Dead Cells in a Population

The relative fractions of injured, uninjured, and dead cells in a pure culture of indicator bacteria following a sublethal treatment can be determined by plating the sample before and after the treatment simultaneously in a nonselective and a selective agar medium and enumerating the colony-forming units or colonies (see Table 7).[48,49] The untreated cells will form colonies equally well in both media, but the treated cells will show differences in their colony formation ability. As the dead cells lose the ability to form colonies, the differences in colony counts in the nonselective medium before and after the treatment are used to calculate the number of dead cells. The uninjured cell fraction is obtained from the colonies in the selective medium after the treatment. The injured fraction is determined from the differences in colony counts between the nonselective and the selective media after the treatment.[40] In a mixed culture that contains both indicator and nonindicator bacteria, such as in food, this method could not be used to determine the relative fractions of injured and uninjured indicator bacteria. A modification could be used under this circumstance that includes the incubation of the sample in a nonselective broth and the enumeration of the indicator bacteria in a selective agar medium, specific for the indicator species or group, initially and until the initiation of multiplication. The increase in counts is used to determine the fraction of injured cells among the total viable indicator bacteria. Similarly, a selective broth, such as BGLB broth, could be used to determine the level of injured indicator bacteria (coliforms) by enumerating the numbers initially and after 1 h resuscitation in a nonselective broth (see Table 7).

Table 7
MEASUREMENT OF INJURED INDICATOR BACTERIA FROM PURE AND MIXED CULTURES[48,49]

Enumeration media[a]	Culture type	Colony counts per milliliter[b]		Physiological cell types
		Before freezing	After freezing	
TS agar	Pure culture in water	276×10^6	175×10^5	Dead: 276×10^6—175×10^5 (93.7%) Survivors: 175×10^5 Uninjured: 35×10^5
TSD agar		267×10^6	35×10^5	Injured: 175×10^5—35×10^5 (80%)
		At 0 hr after thawing	After 1 h in TS broth	
VRB agar (DP method)	Mixed cultures in frozen foods	10×10^1	190×10^1	Survivors: 190×10^1 Uninjured: 10×10^1 Injured: 190×10^1—10×10^1 (95%)
		At 0 hr after thawing	After 1 h in TS broth	
BGLB broth (MPN method)	Mixed cultures in frozen foods	24×10^1	$\geq 110 \times 10^1$	Survivors: $\geq 110 \times 10^1$ Uninjured: 24×10^1 Injured: $\geq 110 \times 10^1$—24×10^1 (>78%)

[a] TS agar, trypticase soy agar; TSD agar, TS agar + 0.075% Na-deoxycholate; VRB agar, violet red bile agar; BGLB broth, brilliant green lactose bile broth; TS broth, trypticase soy broth.

[b] In a pure culture study, *E. coli* NCSM in water suspension was frozen to −20°C overnight, thawed, and plated on TS agar and TSD agar before and after freezing and thawing. In the mixed culture studies, commercial frozen deviled crab was thawed, resuspended in TS broth, and incubated at 25°C for 1 h. For the DP sample, aliquots were pour plated on VRB agar at 0 h and after 1 h. For the MPN method, samples were serially diluted at 0 h and after 1 h, and the 9-tubes MPN method was performed in BGLB broth.

B. Altered Characteristics of Injured Indicator Bacteria[50–59]

Techniques are not currently available to separate the dead, injured, and uninjured fractions of cells present in a sublethally treated population of indicator bacteria and to study their characteristic differences. However, experiments have been designed to help us understand the changes that result due to sublethal treatment in the injured and dead cells (see Table 8). One of the most studied altered characteristics is the development of a sensitivity of the injured indicator cells to many chemicals at certain concentrations to which the normal or uninjured cells are resistant. *E. coli* and other coliforms are resistant to many surface-active compounds, such as lauryl sulfate, bile salts, and deoxycholate, in concentrations that are used in selective media. Injured cells develop a sensitivity to these compounds and lose the ability to multiply (and probably viability) in their presence (see Table 9). Similarly, injured enterococci develop a sensitivity to azide, a compound most frequently used for their selective detection in bacteriological media. Injured indicator bacteria also develop a sensitivity to some toxic chemicals, dyes, NaCl, antibiotics, organic and inorganic acids, some fatty acids, some food preservatives, and some enzymes (such as RNase and lysozyme). The sensitivity of the injured cells can be determined by incorporating a specific compound in a plating media (a selective medium) and enumerating the untreated and sublethally treated indicator cells by simultaneously plating on a nonselective and a selective medium and comparing the colony-formation ability. Alternately, the untreated and treated indicator cells could be incubated in a broth or water containing the chemical compound and enumerated for the colonies in a nonselective agar medium at different intervals. The sensitivity of the injured cells to the selective compounds can be synergistic.[23,24]

Table 8
ALTERED CHARACTERISTICS OF INJURED INDICATOR BACTERIA

Altered characteristics	Ref.
Increased sensitivity to:	
Surface-active compounds: bile salts, lauryl sulfate, deoxycholate, glycocholate	24,26,27,40
Toxic chemicals: Cu^{2+}, azide, dinitrophenol	26,27,32,38,40,56
Antibiotics: actinomycin D, rifampin vancomycin, bacitracin	26,40,52
High chemical conc: NaCl, citrate, phosphates	24,26,27,30,40
Dyes: brilliant green, crystal violet, neutral red	24,40
Preservatives: benzoate, sorbate[a]	107
Fatty acids: octanoic, nonanoic, decanoic	30a,36,40
Other acids (low pH): acetic, lactic, malic, citric, HCl	26,37,40
Hydrolytic enzymes: lysozyme, RNase	26,27,34,40
Oxygen atmosphere	40,82,84,85
Loss of cellular materials:	
Proteins, peptides, amino acids, RNA, enzymes, ions	26,27,40,89
Change in surface properties:	26,40,52,60
Increased hydrophobicity	
Loss in phage adsorption	
Inability to form compact pellet[a]	
Longer lag phase	26,27,40
Inability to multiply until repair	26,27,40

[a] Frozen coliforms developed an increased sensitivity to benzoate and sorbate. Also, frozen *E. coli* cells on centrifugation failed to form a compact pellet as did the unfrozen cells.[26a]

Table 9
RELATIVE SENSITIVITY OF INJURED INDICATOR BACTERIA TO SEVERAL CHEMICALS

Indicator bacteria	Type of stress and condition	Chemicals (conc)	% Survivors inhibited	Ref.
Escherichia coli NCSM	Frozen, −78°C	Deoxycholate (0.1%)	95	50
E. coli B	Frozen, −20°C	Lauryl sulfate (0.1%)	97	50
		Lysozyme (10 μg/ml)	60	50
		Actinomycin D (10 μg/ml)	90	50
E. coli ML30	QAC,[a] 30 μg/ml, 60 sec	NaCl (1%)	33	24
		Bilesalts (0.15%)	65	24
		Deoxycholate (0.15%)	93	24
		Lauryl sulfate (0.01%)	19	24
E. coli SA603	Citric acid, pH 3.7, 15 min	Bilesalts (0.15%)	95	37
E. coli K12	Heat, 48°C, 30 min	Deoxycholate (0.075%)	37	52
	Irradiation, 120 kGy	Deoxycholate (0.075%)	77	52
	Drying, 43°C	Deoxycholate (0.075%)	>95	52
	Freezing, −10°C	Deoxycholate (0.075%)	86	52
Streptococcus faecalis	Freezing, −20°C	NaCl (6%)	93	32
	Freezing, −20°C	NaN_3 (0.05%)	33	32
	Freeze-drying	NaCl (6%)	89	32
	Freeze-drying	NaN_3 (0.05%)	29	32
S. faecalis 9790	QAC,[a] 100 μg/ml, 30 sec	NaN_3 (0.02%)	56	23
S. faecalis NRC1261	Heat, 55°C, 15 min	NaCl (2.5%)	95	44

[a] Quaternary ammonium compounds.

Another characteristic of injured cells is the loss of cellular materials from the cytoplasm, membrane, wall, and periplasmic space in the environment. However, as this characteristic is common to both the injured and the dead cells, it could not be used to quantitate the fraction of injured cells in a population of indicator bacteria. Similarly, other characteristics, such as an increase in surface hydrophobicity, the inability to absorb phages on the cell surface, and the inability to form a compact cell pellet by centrifugation are probably common to both the dead and injured cells in a population and could not be used to quantitate only the injured cells. However, techniques could be developed to increase the fraction of injured cells and reduce significantly the fraction of dead cells in a population. The above characteristics could then be used for the quantitative estimation of injured cells.

The sublethally injured indicator cells also have a longer lag phase as compared to the normal cells under a similar growth condition. This is because the injured cells need a period of repair. By designing suitable experiments, the metabolic activities of the injured cells during the repair phase could be studied. However, we should recognize that during this time the uninjured cells in the population will multiply.

The injured indicator bacteria are capable of reparing their injury in a nonselective environment. In general, a nutritionally rich medium offers a relatively rapid and higher level of repair. Some cells are able to repair in a minimal medium or even in a solution of inorganic phosphate salt. In a given population of sublethally treated indicator bacteria, the injured cells differ in the degree of injury. The cells with a very little injury repair in a simple environment while the cells with a high degree of injury need a nutritionally complex medium. The differences in the degree of injury among cells in a population is further shown from the rate of repair following the incubation of the cells in a repair broth. Some cells are able to repair in seconds while others need hours under similar conditions of repair. The injured indicator cells repair optimally at 35 to 37°C. The rate of repair decreases at lower temperatures and stops below 15°C, while a temperature above 40°C is lethal to the injured cells. The repair process enables the cells to regain a resistance to selective compounds and other characteristics of the normal cells, including cell multiplication. The repair phase of the injured indicator bacteria should precede the cell multiplication.

C. Factors Influencing Injury in Indicator Bacteria

Many factors can influence the fraction of injured cells in a population (the quantitative aspect) and also the extent or level of injury (the qualitative aspect) among the injured cells. This information is not available for different types of physical and chemical treatments that are known to produce injury in different indicator bacteria, except probably for freezing and thawing in some indicator bacteria. These aspects will be discussed briefly here. This information will not only help in developing a method to detect injured indicator bacteria, but also help to clarify some discrepancies in data.

1. Factors Inherent to Indicator Bacteria[40]

The following factors in this category influence the sublethal injury due to the freezing and thawing of indicator bacteria:[40] the species and strains, the growth phase, the growth environments, and the cell concentrations during stress (see Table 10). Limited studies have provided information that the enterococci could be less sensitive to many stresses than *E. coli* and other coliforms. Among other factors, strains could differ greatly in their sensitivity to a stress, and the exponential phase cells are more sensitive to a stress than the early stationary phase cells of the same strain.

2. Conditions and Type of Sublethal Treatments

Among the factors in this category that have great influence on the injury of indicator bacteria are the composition of a suspending medium,[40,50] the type of sublethal treatments,[47,51,52] and the conditions during and after a treatment (see Table 11). A suspending

Table 10
INHERENT FACTORS INFLUENCING FREEZE-INJURY OF INDICATOR BACTERIA[32,100]

Factors[a]	Indicator bacteria	% Survivors injured
Species	*Escherichia coli* NCSM	90[a]
	Enterobacter aerogenes	74
	Streptococcus faecalis	95
Strains	*Escherichia coli* NCSM	90[a]
	E. coli K 12	99[a]
	E. coli B	85[a]
Growth phase:		
Exponential	*E. coli* NCSM	90[a]
Stationary	*E. coli* NCSM	70[a]
Growth media:		
TS broth	*E. coli* NCSM	99[a]
Minimal broth	*E. coli* NCSM	88[a]
Growth temperature:		
35°C	*E. coli* NCSM	88
25°C	*E. coli* NCSM	92

[a] All *Escherichia coli* strains were frozen to −20°C in water suspension and the injured among the survivors were enumerated in agar media containing 0.075% deoxycholate.[40] *Enterobacter aerogenes* was frozen to −14°C in a food and *Streptococcus faecalis* was frozen to −20°C in water; injured cells were detected in agar media containing 0.15% bile salts and 0.02% NaN_3, respectively.

medium that includes milk, sugars, or proteins can greatly reduce injury in *E. coli* and other indicators due to freezing, freeze-drying, and heat treatment. In contrast, the presence of salt, acid, and sanitizers can increase the amount of injury by other stresses by acting as a second stress. The rates of freezing and thawing (slow rates being more injurious), the dose of irradiation, and the concentration of salt and sanitizers also directly influence the amount of injury. Repeated freezing and thawing, a higher storage temperature, a longer storage period,[53] and exposure of the cells to oxygen increase the injury and mortality rate of the cells.

3. Conditions of Detection of Injured Indicator Bacteria

The methods used in the preparation of a sample and for the enumeration of indicator bacteria from the sample could adversely affect the injured cells and prevent their detection. In general, a slow thawing rate, rapid rehydration of a dried sample, exposure to high temperature, the presence of Cu^{2+} and other toxic ions in dilution blanks,[54,57] a low pH, and ingredients to which the injured cells are sensitive will cause the loss of the viabilty of the injured cells (see Table 12). Conversely, rapid thawing, slow rehydration in a high osmotic environment, rehydration in a mesophilic temperature range, the use of cysteine or peptone or similar compounds in the dilution blanks, reduced exposure to oxygen or to low pH, reduced exposure to chemicals to which the cells are sensitive, allowing the cells to repair prior to exposure to selective media, and the addition of compounds that degrade hydrogen peroxide[58] will increase the chances of detection of injured indicator bacteria.

D. Site of Injury in Indicator Bacteria due to Sublethal Treatments

Abnormal physiological characteristics manifested by the sublethally injured indicator bacteria have been related to the damages in many structural and functional components in

Table 11
ENVIRONMENTAL FACTORS DURING STRESS INFLUENCING INJURY OF INDICATOR BACTERIA

Factors	Indicator bacteria[a]	% Survivors injured
Frozen suspension[40,50]		
Water	*Escherichia coli* NCSM	99
10% Milk	*E. coli* NCSM	46
TS broth	*E. coli* NCSM	92
20% Sucrose	*E. coli* NCSM	71
20% Glycerol	*E. coli* NCSM	70
5% Meat broth	*E. coli* NCSM	86
Liquid egg	*E. coli* NCSM	80
Crab meat	*E. coli* NCSM	78
Freezing temperature[40]		
−20°C	*E. coli* NCSM	94
−70°C	*E. coli* NCSM	97
−196°C	*E. coli* NCSM	94
Type of stress[52]		
Heat	*E. coli* K 12	37
Irradiation	*E. coli* K 12	77
Drying	*E. coli* K 12	>95
Freezing	*E. coli* K 12	86
EDTA	*E. coli* K 12	>97
Storage time at −20°C[32]		
1 d	*Streptococcus faecalis*	38
5 d	*S. faecalis*	80
10 d	*S. faecalis*	90
15 d	*S. faecalis*	94

[a] Injury was measured from the sensitivity of the survivors to 0.075% deoxycholate for *E. coli* strains and 6% NaCl for *S. faecalis*.

the cells.[59-90] Evidence accumulated so far has suggested that in many different types of stresses, the same structural and functional components of the cells are damaged.[26,27,47] This might be the reason why the indicator bacteria, injured by different types of sublethal treatments, manifest similar impaired characteristics (see Table 8). However, the extent or level of damage of a particular cell component may differ with the type of stress. The outer membrane of *E. coli* could be damaged by freezing and drying to a great extent, while heating might cause more damage to RNA and radiation to DNA.

The structural and functional components that have been demonstrated to be damaged by sublethal stresses in some indicator bacteria are listed in Table 13. The lipopolysaccharide (LPS) molecules in the outer membrane in *E. coli* is damaged by freezing, drying, and heating.[60-64] These molecules form a barrier and prevent the entrance of hydrophobic and large hydrophilic molecules from the environment. This provides the resistance of the cells to many surface-active compounds and other chemicals. The injured *E. coli* cells become sensitive to these chemicals as the surface LPS structure, due to damage caused by a stress, loses its barrier function. Some proteins from the outer membrane of *E. coli,* after freezing and thawing, could be lost in the environment.[65] A report by Chai and Lee[66] indicated that *E. coli* cells with a mutation in an outer-membrane protein were more susceptible to freezing as compared with the parental cells. However, they did not indicate whether the mutants had only this or other defects. The wall polysaccharides, including the teichoic acid, would be damaged by heating in some gram-positive bacteria,[67,68] but this aspect has not been studied in *S. faecalis* and other enterococci.

Table 12
ENVIRONMENTAL FACTORS DURING ENUMERATION INFLUENCING VIABILITY AND INJURY OF *ESCHERICHIA COLI*

Factors	% Survivors[a]	% Survivors injured[a]	Ref.
Rate of thawing			
Slow	50	98	40
Fast	38	99	40
Repeated freezing and thawing			
One cycle	57	95	40
Five cycles	99	96	40
Oxygen exposure			
Present	0.1	NT[b]	59
Absent	57	NT[b]	59
Temperature of suspension			
37°C	NT[b]	48	96
45°C	NT[b]	98	96
pH of suspension			
5	40	NT[b]	40
7	17	NT[b]	40
Diluent composition			
Phosphate buffer	NT[b]	67	54
Distilled water	NT[b]	84	54
Selective media[c]			
VRB agar	46	67	40,47
D agar	46	87	40,47
DL agar	46	61	40,47
EMB agar	46	9	40,47
BGLB broth	46	60	40,47
LST broth	46	55	40,47
Temperature of selective media			
45°C	NT[b]	45	25
48°C	NT[b]	18	25

[a] Under certain conditions, although the percent injured among the survivors are not different, there are considerable differences in the number of deaths between treatments. A higher death rate will result in lower enumeration.
[b] Not tested or not available.
[c] D, deoxycholate agar; DL, deoxycholate lactose agar.

The loss of cellular components and an increased permeability of the solutes from the environment in the injured indicator bacteria have been related to the damage of the cytoplasmic membrane.[69-71] Different sublethal treatments have also been found to cause degradation of RNA,[75-78] strand breaks in chromosomal DNA,[79-87] and the activation as well as inactivation of several cellular enzymes.[88-90]

The nature of the damage due to sublethal treatments in certain structural components of indicator bacteria has been studied (See Table 13). More detailed studies have been conducted on the nature of freeze-injury in the LPS molecules in *E. coli*.[41,47,73,74] The outer membrane of *E. coli* is composed of three types of macromolecules: LPS, several proteins, and phospholipids. The phospholipid molecules form a uniform lipid bilayer in which the protein molecules, on the basis of their relative hydrophobicity of the three dimensional structures, are embedded at different depths. The LPS molecules have a hydrophobic and a hydrophilic region and are located on the cell surface. The hydrophilic part contains several carbohydrates and phosphates with the phosphates providing the diester bonds and ionic bonds. The

Table 13
INJURY OF CELLULAR COMPONENTS DUE TO SUBLETHAL STRESSES IN INDICATOR BACTERIA

Cellular components	Stress	Nature of damage	Ref.
Outer membrane			
LPS	Freezing Drying	Conformation change; also break in molecule and loss from cells	26,27,34,41,47,48,60-64,73,74
Proteins	Freezing	Conformation alteration and loss from cells	65,66
Teichoic acid[a]	Heating	Loss of D-ala and Mg^{2+}	27,67,68
Cytoplasmic membrane	Freezing Drying Heating, etc.	Loss of components	26,27,47,53,69-71
Surface-layer proteins[b]	Freezing Drying	Destabilization and loss	72
Surface property	Freezing Heating Irradiation Drying	Increase in hydrophobicity and failure to form pellet	52
Ribosomal RNA	Heating Freezing Drying	Degradation	27,33,47,75-78
Structural DNA	Heating Freezing Drying Irradiation	Strand(s) break, damage to replication site, mutation	27,47,79-87
Plasmid DNA	Freezing	Loss	86
Enzymes	Heating Freezing Drying Low pH	Inactivation, activation, loss from cells	26,27,47,58,59,88-90

[a] Teichoic acid in the cell wall of gram-positive bacteria. The observation was not made in enterococci.
[b] Observation was made on gram-positive nonenterococci.

hydrophilic parts are maintained in a three-dimensional conformation stabilized by the divalent cations. The natural conformation and functions of the outer membrane are the results of the structural association between the same and the other two species of macromolecules (such as phospholipid to phospholipid, phospholipid to lipid A, etc.). Some specific conformations are necessary for the natural resistance of *E. coli* and other coliforms to bile salts, lauryl sulfate, brilliant green, lysozyme, and other chemicals. Sublethally injured *E. coli* and other coliforms lose their resistance to these chemicals because of the damage of LPS molecules in the outer membrane. Electron microscopic studies of the whole cells by negative staining and the thin sections by transmission electron microscopy did not show any visible damage in the outer membrane of frozen *E. coli* cells.[48] Biochemical analysis of the cell supernatant showed a loss of only 2 to 7% of the proteins, phospholipids, and LPS molecules from the outer membrane, even with about 99% of the cells being injured.[62] Phage adsorption studies with *E. coli* revealed that while the frozen *E. coli* cells did lose the adsorption of several LPS-specific phages, they did retain the adsorption of phages specific for outer-membrane protein, except for T5 (See Table 14). The adsorption sites of the LPS-specific phages are located on the different components of the LPS molecules. Further analysis showed that the individual carbohydrate components were not lost from the LPS molecules, except for a small amount of galactose moiety due to a breakdown in the phosphodiester linkage. The LPS molecules seemed to be retained intact in the outer mem-

Table 14
LOSS OF PHAGE ADSORPTION IN FROZEN *ESCHERICHIA COLI*[48,60]

Bacteriophages	Adsorption sites	Loss in adsorption efficiency (%)[a]	
		Frozen cells	LPS from frozen cells
T1	LPS	61	18
T2	Proteins	12	0
T3	LPS, except heptose	44	1
T4	LPS: on phosphodiester bridges	70	42
T5	Proteins	57	3
T6	Proteins	9	NT
T7	LPS, except heptose	35	9
φW	LPS: on heptose and glucose	54	10
FP1	LPS, except heptose	21	8
φ3	LPS: on KDO and lipid A	−8	−22

[a] Loss in adsorption efficiencies in frozen cells and LPS from frozen cells were compared with the values obtained in unfrozen cells and LPS from unfrozen cells, respectively. A negative sign indicated an increase in efficiency due to higher availability of adsorption sites in ketodeoxyoctonate (KDO) and lipid A.

brane of the frozen *E. coli* cells. Further proof that the different components of the LPS molecules were retained in the frozen cells came from the phage adsorption studies on the LPS molecules purified from the frozen *E. coli* cells (see Table 14). Although the frozen cells lost the ability to adsorb specific phages, the LPS molecules from the frozen cells retained the adsorption sites, except for T4. In the frozen cells the sites for most phages were not available for adsorption. The loss of adsorption of T4 in both the frozen cells and in the LPS purified from the frozen cells probably resulted from the loss of the phosphodiester bonds, the site of T4 adsorption. In contrast, the increase in the adsorption of phages φ3 in the frozen cells and in the purified LPS compared with the unfrozen cells was due to the availability of more adsorption sites in the ketodeoxyoctonate (KDO).[60] In the unfrozen cells the KDO moieties were embedded in the lipid bilayer, but became available both in the frozen cells and in purified LPS molecules. All these data suggest that the LPS molecules underwent conformational change when the *E. coli* cells were frozen, resulting in the loss of their barrier function against selective compounds and the adsorption of specific phages. Further studies revealed that the conformational alterations resulted from the loss of divalent cations that were associated with the stabilization of normal conformation of the LPS molecules.[73] The loss of divalent cations probably resulted from the solution effect occurring during the freezing and drying of bacterial cells. Supplementing freeze-injured cells with Ca^{2+} allowed the LPS molecules to revert, at least partially, to the original conformation.[73]

Some cryoprotective compounds were able to prevent the conformation alteration of the LPS molecules during the freezing of cells.[74] Although the nature of the damage of the LPS molecules due to the heating, drying, and exposure at low pH of *E. coli* cells is not known, there is a good possibility that conformational alteration of the LPS molecules in the injured cells occurs from these stresses. The destabilization of the other macromolecules of the outer membrane may also result from different sublethal stresses.[47,72]

The damage to the LPS molecules from different sublethal treatments may not be directly related to cell death, but makes the injured indicator cells susceptible to selective compounds, and indirectly causes the loss of viability in the injured cells. The results discussed here also showed that freezing and probably some other sublethal treatments can destablize the ionic

bonds of the macromolecules and produce a conformational and functional alteration of these macromolecules.[73]

In some gram-positive bacteria, sublethal heat treatment was found to cause a loss in D-alanine and Mg^{2+} from the wall teichoic acid with a loss in the functional property of the wall.[67,68] Similar changes are expected to occur in the enterococci and may be associated with the developed sensitivity of the injured cells to azide and other chemicals.

Freezing and drying have also been shown to destabilize the disulfide and hydrogen bonds in the outer-membrane proteins in some gram-negative bacteria and the surface layer proteins in some gram-positive bacteria.[41,72,72a] Although these aspects have not been studied in the indicator bacteria, these observations revealed that at least some sublethal treatments can destablize the weak and labile bonds that are so important in maintaining the conformation of the macromolecules and for their structural and functional integrity in the bacterial cells.

The damages of the cytoplasmic membrane, resulting in the loss of permeability, are thought to be the major cause of injury of the sublethally treated indicator bacteria. Electron microscopic studies of the negatively stained whole cells and the thin sections by transmission electron microscopy of the carefully prepared samples showed the presence of the intact membrane in the injured cells.[40,41,72] However, the sublethally injured cells are easily affected by the harsh treatments given during sample preparation. These aspects should be recognized in the interpretation of the results.

Damage of the rRNA from some sublethal treatments of bacterial cells has been thought to be due to the activation of endogenous RNase and the degradation of the RNA.[67,68] The dissociation of larger RNA into smaller subunits has been shown to be associated with the loss of Mg^{2+}:protein:RNA interactions. In the indicator bacteria, different sublethal treatments have been shown to cause both single- and double-strand breaks of the chromosomal DNA molecules, probably from the activation of the endogenous DNase.[80,81] Sublethal treatments of indicator bacteria can also result in the loss of some cellular enzymes and in the activation as well as inactivation of some enzymes.[88,89]

The manifestation of injury in the sublethally treated indicator bacteria is probably associated with the cumulative effect of the damages suffered in different structural and functional components of the cells. It is quite unlikely that the expression of injury is associated with a single vital structure in the cell.[41,47]

E. Repair of Sublethal Injury in Indicator Bacteria

One of the most important characteristics of the injured bacteria is their ability to reverse or repair the injury in a suitable environment.[27,41,47] The repair process allows the cells to regain the characteristics of the normal cells, such as resistance to many selective and toxic compounds, enzymes, and antibiotics and the initation of cell multiplication. The rate of repair can be studied during the incubation of injured indicator bacteria in a nonselective broth by simultaneously plating the sample on a nonselective and a selective agar medium (see Figure 1).[26,41] Initially, due to injury, the colony counts on the selective agar will be much lower than the counts on the nonselective agar. Repair is indicated by an increase in counts on the selective agar, while the counts on the nonselective agar remained unchanged. As the incubation continues, at one point the counts start increasing simultaneously in both agar media due to the initiation of cell multiplication. Basic information on the environment for optimum repair, the metabolic processes during repair, and the structures that are repaired are available. This information is important in developing effective methods for the enumeration of injured bacteria from foods and are discussed briefly in this section.

1. Environment of Repair

The following characteristics are desirable in a repair environment: (1) the environment should be nonselective, i.e., it should not contain substances to which the injured indicator

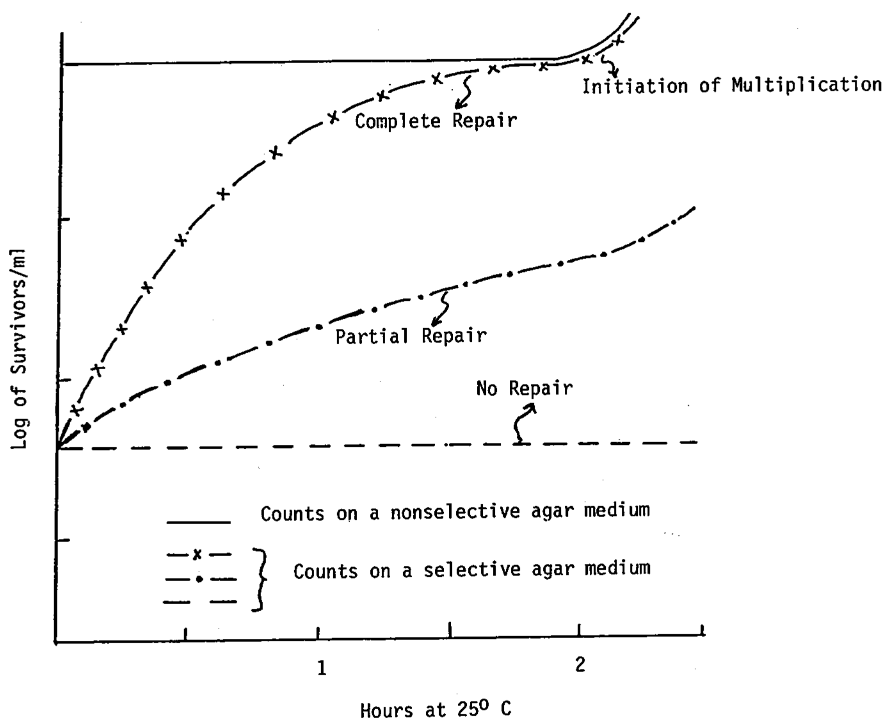

FIGURE 1. A hypothetical resuscitation curve of injured indicator bacteria. Repair is indicated by an increase in counts only in selective agar, but not in nonselective agar media during incubation in nonselective broths (—x— or —•—). Cell multiplication is indicated by a simultaneous increase in the counts in both selective and nonselective agar media.

cells are sensitive; (2) it should provide the nutrients necessary for the repair process; and (3) it should be optimum pH, temperature, and time for the repair.

The nonselective nature of the media is important both for a faster repair rate and high amounts of repair.[26] In the presence of selective compounds, the injured cells not only fail to repair, but also may be rapidly killed. The viability of freeze-injured *E. coli*[40,91,92] in the presence of dinitrophenol, actinomycin D, lauryl sulfate, and lysozyme and of heat-injured *S. faecalis*[75] in the presence of NaCl in the repair media was greatly reduced.

Many complex and simple organic compounds and some inorganic compounds are found to aid in the repair of injured indicator bacteria (See Table 15). In general, a medium rich in complex nutrients provides faster and higher amounts of repair than a minimal medium.[27,40,41,91] In addition, various compounds, such as yeast extract,[29] some amino acids,[57,93] protein digests,[94,95] organic molecules like ATP and pyruvate,[40] and inorganic molecules like K_2HPO_4,[40,91] facilitated the repair of freeze-injured *E. coli.* Repair in phosphate is enhanced in the presence of Mg^{2+}.[91] The supplementation of both nonselective agar and selective agar media with compounds that either block the formation of or degrade hydrogen peroxide, such as 3,3′ - thiodipropionic acid (TDPA), pyruvate, and to some extent catalase, enhanced the repair of freeze-injured *E. coli.*[58]

The rate and the total amount of repair of the injured indicator bacteria are dependent upon the pH of the media and the incubation temperature. In general, the injured indicator bacteria repair optimally at a pH near 7.0 and at a temperature range of 30 to 37°C.[50] At a pH below 5.0 and at temperature above 40°C, the injured cells may lose their viability.[96] At refrigeration temperature, freeze- and heat-injured *E. coli* do not repair.[92,96] The time of maximum repair differs considerably with the nature of sublethal injury. While *E. coli*[34,39,92]

Table 15
EFFICIENCY OF REPAIR OF INJURED INDICATOR BACTERIA IN SEVERAL MEDIA

Indicator bacteria	Type of stress	Medium	% Repaired	Ref.
Escherichia coli	Frozen (−20°C)	Water	3	40
		TSY broth[a]	90	40
		M broth[a]	70	40
		K_2HPO_4 (30 m*M*)[a]	40	40
		K_2HPO_4 (30 m*M*) + $MgSO_4$ (0.4 m*M*)[a]	75	40
		Na_2HPO_4 (30 m*M*)[a]	35	40
		Na_2HPO_4 (30 m*M*) + $MgSO_4$ (0.4 m*M*)[a]	64	40
		Na-pyruvate (10 m*M*)[a]	50	40
		ATP (10 m*M*)[a]	44	40
		Glucose (5%)[a]	3	40
E. coli	NaCl (0.15 *M*)	Tris buffer (10 m*M*) + Mg^{2+} (5 m*M*)[b]	50	39
E. coli	Acetate (0.3 *M*, pH 4.2)	TS-broth[c]	100	38
		K_2HPO_4 (40 m*M*)[c]	75	38
E. coli	Heated (57°C, 3 min)	EC-B broth[d]	95	96
Streptococcus faecalis	Heated (60°C, 15 min)	TS broth[e]	63	30

[a-e] Repair conditions: [a] 1 h at 25°C; [b] 30 min at 30°C; [c] 1 h at 32°C; [d] 6 h at 37°C; [e] 6 h at 35°C in specific media (TSY, trypticase soy with yeast extract; M, minimal; TS, tryptic soy; EC-B, EC broth without bile salts).

and *S. faecalis*[38,93] that were injured by freezing, drying, and acid or salt treatment repaired within 30 min to 2 h at 30 to 35°C, heat-injured cells[30] of the same species took 3 to 4 h at 37°C.

Repair starts almost instantly in a suitable environment by both gram-negative and gram-positive indicator bacteria injured by different sublethal stresses — i.e., there is no lag-phase.[30,34,91,93] In general, the initial rate of repair is very rapid. Freeze-injured *E. coli* cells repair most of their injury in trypticase soy broth with 1 h at 25°C.[91] The variation in the time of repair suggests that after a sublethal treatment a surviving population of indicator bacteria contains cells that differ greatly in the extent of cellular injury. The time a cell takes to repair is directly related with the extent of the injury it has suffered. The cells with extensive injury will need a much longer time or a different nutritional environment to repair as compared with cells that have very little injury. As the repaired cells regain resistance to different selective compounds, they could be exposed to such environments for their selective enumeration.

As the repaired cells become able to initiate multiplication, the cells that have repaired early will start multiplication even before the completion of repair by cells that need a longer repair time. During this period the uninjured cells in the population will also multiply. An enumeration method designed to detect injured indicator bacteria should differentiate between repair and multiplication.[26]

2. *Metabolic Processes During Injury Repair*[30,34,38,39,41,92,93]

To understand the metabolic events during the repair process, the injured indicator cells are allowed to repair in a medium supplemented with different metabolic inhibitors, and the progress of repair is determined from colony counts on a nonselective and a selective agar medium. The inability to regain resistance by the injured cells against a selective compound in the presence of a particular metabolic inhibitor in the repair medium is correlated with a specific metabolic process necessary for the repair. This method has some drawbacks that will be discussed later.

Table 16
EFFECTS OF METABOLIC INHIBITORS ON REPAIR OF INJURY IN INDICATOR BACTERIA

Indicator bacteria	Stress	Inhibitors used: Inhibited repair	Inhibitors used: Did not inhibit repair	Ref.
Escherichia coli	Air dry	Dinitrophenol[a]	Chloramphenicol[b]	34
			Actinomycin D[c]	34
			Penicillin G[d]	34
	NaCl (0.15 *M*)	Not tested	Chloramphenicol[b]	39
			Dinitrophenol[a]	39
			Na-azide[a]	39
	Acidified (0.3 *M* acetate, pH 4.2, for 60 min)	Dicyclohexyl Carbodiimide[e]	Chloramphenicol[b]	38
			Streptomycin[b]	38
			Cycloserine[d]	38
			Hydroxyurea[f]	38
			Fluorouracil[f]	38
			Actinomycin D[c]	38
			Dinitrophenol[a]	38
	Frozen (−20°C)	Dinitrophenol[a]	Rifampin[c]	40,91
		Na-cyanide[a]	Actinomycin D[c]	40,91
		Na-azide[a]	Fluorouracil	40,91
		Gramicidine[a]	Chloramphenicol[b]	40,91
		Oligomycine[a]	Kanamycin[b]	40,91
			Hydroxyurea[f]	40,91
Streptococcus faecalis	Heated (60°C, 15 min)	Actinomycin D[c]	Chloramphenicol[b]	30
			Penicillin[d]	30
	Frozen (−20°C)	Actinomycin D[c]	Chloramphenicol[b]	93
			Streptomycin[b]	93
			Puromycin[b]	93
			Cycloserine[d]	93
			Penicillin[d]	93

Note: Repair was measured by the ability of the injured cells to regain resistance against (as measured by the ability to form colonies): 6% NaCl;[30] lysozyme (50 g/ml);[34] 0.15% bile salts;[38] 0.15 *M* NaCl;[39] 0.1% Na-deoxycholate;[40,91] 6% NaCl.[93]

[a–f] Inhibit *de novo* synthesis of: [a] ATP synthesis by oxidative phosphorylation; [b] protein synthesis; [c] RNA synthesis; [d] wall mucopeptide synthesis; [e] energy synthesis by proton motive force; [f] DNA synthesis.

The results of these studies show that in *E. coli* cells[34,38,40] injured by the freezing or freeze-drying and acid treatments, the repair process does not involve *de novo* synthesis of proteins, RNA, DNA, or the wall mucopeptide, but involves the synthesis of ATP through oxidative phosphorylation and/or through the proton motive force (see Table 16). In NaCl-treated *E. coli,* ATP synthesis is not a requirement for repair. In the heat- and freeze-injured *S. faecalis,* the repair process is associated with *de novo* synthesis of RNA and not the synthesis of either proteins or mucopeptide.[30,93]

The use of metabolic inhibitors to study metabolic processes associated with the repair of sublethal injury in indicator bacteria needs cautious interpretation. Many inhibitors used in these studies prevent *de novo* synthesis of macromolecules, such as proteins, RNA, DNA, and wall mucopeptides. However, the repair of injury was measured by the ability of the cells to regain their resistance against selective compounds, such as bile salts, NaCl, etc. The two events may be different, i.e., the ability to regain resistance against bile salts or NaCl may not be related to the ability or inability of the injured cells to synthesize protein or other cell components. Also, the repair of the cellular structures by the injured cells, necessary to regain resistance against the selective compounds, may not require *de novo*

synthesis of macromolecules, but instead may need to reorganize the existing or reserved materials.

3. Structures Being Repaired

The developed sensitivity of the sublethally injured *E. coli* to surface-active compounds, lysozyme, and actinomycin D has been related to the damages in the LPS molecules on the outer membrane.[40,41,47,48] As discussed before, the injury in the LPS molecules, at least in freeze-injured *E. coli,* has probably resulted from the conformation change due to the loss of divalent cations.[73] Conversely, their regained resistance to these compounds results from the reversal of the conformation of the LPS molecules and their stablization by the divalent cations.[73] This is probably an energy-dependent process.[40] This could explain the ability of the injured *E. coli* cells to regain their resistance against bile salts after incubation in a solution of K_2 HPO4 + Mg^{2+}, and their inability to repair in the presence of inhibitors of ATP synthesis in the repair media.[40,41]

The sensitivity of the injured *E. coli* and *S. faecalis* cells to NaCl has been related to damage in the cytoplasmic membrane. How the cells repair this structure to regain their resistance against NaCl is not understood. Similarly, the relationship between the regain of resistance to NaCl and azide after repair by the heat- and freeze-injured *S. faecalis*[30,93] to RNA synthesis is also not known.

III. CONSEQUENCES OF INJURY IN THE DETECTION OF INDICATOR BACTERIA FROM FOODS

The injury of indicator bacteria from sublethal stresses was generally studied with pure cultures suspended in water, buffers, or broths. The results of these studies revealed that irrespective of the type of sublethal stresses and species or strains of indicator bacteria, a large proportion of the survivors could be injured and would not be detected by the selective media recommended for the enumeration of indicator bacteria. Limited studies have also revealed that different indicator bacteria, either inoculated in sterile or nonsterile foods prior to a sublethal treatment, or present naturally in semipreserved foods, could be injured and would remain undetected by many of the recommended selective enumeration procedures. This aspect is discussed in this section.

A. Injury of Inoculated Indicator Bacteria in Foods Subjected to Sublethal Treatments

In these studies, a pure culture of an indicator bacterial species was inoculated in either a sterile or a nonsterile food prior to subjecting the food to a sublethal treatment.[26,28,40] To detect the presence of injured indicator bacteria in an inoculated sterile food, an aliquot was serially diluted and materials from selected dilutions were enumerated for indicator bacteria by plating them simultaneously in a selective and a nonselective agar medium. The differences in colony counts in the two plating media were used to determine the fraction of injured survivors by the procedures described earlier. With inoculated nonsterile foods, an aliquot was incubated in a nutritionally rich broth for a specified time at a suitable temperature and enumerated for the indicator bacteria in a selective agar medium before and after the incubation period.[26,28] The differences in colony counts obtained before and after incubation gave the number of the injured indicator bacteria. For a precaution, the food before inoculation should not contain the specific indicator, or if present, it should be in a much lower level than the level of inoculation. Also, during "repair-incubation" the uninjured cells should not multipy, so that the increase in the count of indicator bacteria during incubation could result only from repair. In a different method for a nonsterile, inoculated food, an aliquot was first pour plated with a nonselective agar medium and incubated to facilitate repair of the injured cells. The plates were then overlaid with a selective agar medium,

Table 17
INABILITY OF THE SELECTIVE MEDIA TO ENUMERATE INDICATOR BACTERIA FROM INOCULATED FOODS

			% Survivors remained undetected		
Food	Nature of stress	Indicator	Selective broth[a]	Selective agar[b]	Ref.
Liquid egg	Freezing	*Escherichia coli*	Not tested	80	45
Meat broth	Freezing	*E. coli*	Not tested	86	45
Crab meat	Freezing	*E. coli*	Not tested	78	45
Milk	Freezing	*E. coli*	Not tested	75	45
Oyster	Freezing	*E. coli*	40	49	99
Shrimp	Freezing	Coliforms	35	17	99
Oyster	Freezing	Coliforms	12	21	99
Scallop	Freezing	Coliforms	4	4	99
Oyster	Freezing	Fecal coliform	63	60	99
Meatloaf	Freezing	*E. coli*	55	99	96
	Freezing	*E. coli*	90	97	96
Chicken chow mein	Freezing	*E. coli*	Not tested	48	100
	Freezing	*Enterobacter aerogenes*	Not tested	58	100
Milk	Freezing	Coliforms	Not tested	73	101
Cream	Freezing	*Streptococcus faecalis*	Not tested	20	33
Meat broth	Freezing	*S. faecalis*	Not tested	12	33
Starch	Freezing	*S. faecalis*	Not tested	30	33
Meat	Drying	*Escherichia coli*	77	75	102
Carrot	Drying	*E. coli*	53	59	102
Meat	Heating	Coliforms	20	NT	103
	Low pH	Coliforms	23	NT	103
Dry foods	Drying	Coliforms	8	NT	103
Meat	Freezing	Enterococci	8	NT	103

[a] As determined by MPN methods.
[b] As determined by colony counts.

incubated, and the characteristic colonies were enumerated. As a control, an aliquot was also pour plated with the selective agar medium, incubated, and enumerated for the characteristic colonies. The differences in colony counts between the two plating media were used to calculate the number of injured indicator bacteria.

Ray and Speck,[45] Warsek et al.,[49] Speck et al.,[98] and Hackney et al.[99] inoculated *E. coli* in sterile, reconstituted, nonfat dry milk, liquid egg, crab meat, and oyster prior to freezing at −20°C. Enumeration after thawing failed to detect 50 to 99% of the survivors on VRB agar and 40% in the BGLB broth. Gunerson and Rose[100] also failed to enumerate 52% of *E. coli* and 41% of *E. aerogenes* surviving in frozen chow mein on VRB agar. The inability of both the selective agar and the broth media to enumerate many surviving *E. coli* from inoculated frozen, dried, heated, or acidified foods has been observed by several other workers[32,96,101-104] (see Table 17). Brodsky et al.,[103] using the hydrophobic grid-membrane filtration technique, showed that up to 50% of coliforms, *E. coli*, and enterococci surviving in frozen, heated, or acidified, deboned poultry meat and a large variety of dried foods were not detected in selective agar media. Hackney et al.[99] inoculated *E. coli* in nonsterile crab meat, shrimp, oyster, and scallop and after freezing and thawing could not enumerate 25 to 60% of the survivors on VRB agar. Among the different types of seafoods, the largest number of surviving *E. coli* were not detected, on both VRB agar and BGLB broth, from the oyster samples.

Table 18
INABILITY OF SELECTIVE MEDIA TO ENUMERATE INDICATOR BACTERIA FROM COMMERCIAL FOODS

Food	Nature of stress	Indicator	% Survivors not detected: Selective broth[a]	% Survivors not detected: Selective agar[b]	Ref.
Deviled crab	Freezing	Coliforms	ca. 79	90	40,49
Crab cakes	Freezing	Coliforms	ca. 85	89	40,49
Breaded oyster	Freezing	Coliforms	81	88	40,49
Stuffed flounder	Freezing	Coliforms	99	93	40,49
Chicken chow mein	Freezing	Coliforms	14	12	40,49
Chocolate ice cream	Freezing	Coliforms	NT	62	40,49
Strawberry ice cream	Freezing	Coliforms	NT	83	40,49
Ice cream	Freezing	Coliforms	NT	26	58
Oyster	Refrigeration	Coliforms	NT	76	58
Sausage	Refrigeration	Coliforms	NT	22	58
Cheese	Acid	Coliforms	NT	26	58
Ice cream	Freezing	Coliforms	NT	81	98
Oyster	Refrigeration	Fecal coliforms	37	35	99
Shrimp	Refrigeration	Fecal coliforms	14	22	99
Fin fish	Refrigeration	Fecal coliforms	0	25	99
Oyster	Refrigeration	Enterococci	NT	48	99
Clam[c]	Refrigeration	Enterococci	NT	55	99
Scallop	Refrigeration	Enterococci	NT	54	99
Shrimp	Refrigeration	Enterococci	NT	85	99
Crab meat[c]	Refrigeration	Enterococci	NT	45	99
Fin fish	Refrigeration	Enterococci	NT	22	99
Meat	Freezing	*Escherichia coli*	24	54	102
Raw milk	Refrigeration	*E. coli*	52	>99	102
Ice cream	Freezing	*E. coli*	17	>99	102
Buttermilk	Acid	Coliforms	NT	35	105
Cheese	Acid	Coliforms	NT	54	105
Ice milk	Freezing	Coliforms	NT	45	105
Ice cream	Freezing	Coliforms	NT	20	105
Vegetables	Freezing	Coliforms	NT	30	106
Cottage cheese	Refrigeration	Coliforms	NT	80	106

[a] Estimated by MPN method.
[b] As obtained from colony counts.
[c] Sample did not have injured fecal coliforms.

B. Presence of Injured Indicator Bacteria in Naturally Contaminated, Semipreserved Foods

Several researchers have examined different types of semipreserved foods for the presence of injured indicator bacteria among the natural microflora in the foods (see Table 18). Among the products tested, injured coliforms or *E. coli* could not be detected in the refrigerated crab meat and clam samples, although these samples had 45 to 55% injured enterococci. In other refrigerated, frozen, and acidified foods, the levels of injured coliforms per *E. coli* ranged from 12% in frozen chicken chow mein to >99% in refrigerated raw milk and ice cream. Many ice cream samples examined by several workers harbored 20 to >99% injured coliforms. In general, the frozen products had higher percentages of injured coliforms than the refrigerated and acidified products. Several other workers have also demonstrated the occurrence of injured coliforms in refrigerated ground beef[104] and injured *E. coli* biotype I in frozen beef and chicken.[102] Limited data also have revealed that enterococci, present naturally in many refrigerated seafoods, could be injured (see Table 18).

C. Factors to be Considered in the Enumeration of Injured Indicator Bacteria from Semipreserved Foods

Different groups or species of indicator bacteria either inoculated or present naturally in semipreserved foods could be injured from the sublethal treatments and not be detected by the selective plating and MPN techniques. These methods[3-9] however, would be effective for the detection of uninjured indicator bacteria in the food samples. In recent years, methods have been developed that would facilitate the enumeration of uninjured as well as injured indicator bacteria from semipreserved foods. A detailed description of these methods and their relative advantages and disadvantages will be presented in the next section. Many different factors could adversely reduce the detection efficiency of the injured indicators by these modified methods. Proper adjustment in some of these factors could greatly enhance their enumeration, while other factors are inherent in specific foods and thus could not be greatly changed. For some of these factors, the data with the food systems are not available and the results obtained with pure cultures will be cited.

1. Duration and Condition of Storage

A sublethally treated population of indicator bacteria, in either a pure culture or a food system, contains both normal and injured cells.[40,41] During storage, the injured cells become dead while the normal cells become injured. The rates of these shifts are generally very rapid initially and then become quite slow. Depending upon the storage interval after sublethal treatment, a food that has indicator bacteria will give different enumeration data due to these shifts in the physiological states by the selective methods as well as by the modified methods designed to detect injured indicator bacteria. To obtain information in the sanitary quality used in the processing and handling of a semipreserved food, a product probably should be tested immediately after processing. Exposure of dried foods to oxygen, thawing and re-freezing of frozen foods, and high storage temperature of dried foods could accelerate both the death and injury of indicator bacteria.

2. Effects of Two or More Stresses

In general, the level of injury among indicator bacteria increases as a population is exposed to two or more stresses as compared with a single stress, and the effect is sometimes synergistic.[40] Most processed, semipreserved foods are subjected to more than one stress to enhance their shelf life. These include refrigeration and freezing after low-heat treatment, refrigeration of some acidified products, refrigeration of products containing preservatives, and others. Similarly, a stress could be introduced in a product during the preparation of samples for the microbiological analysis. These include thawing of frozen products, thawing and refreezing of products (sometimes practiced during testing of the same sample more than one time), rehydration of dried products, exposure of dried products to oxygen for a relatively long time prior to testing, and others. Generally, a rapid rate of thawing, the absence of refreezing, a slow rate of rehydration or rehydration in a high osmotic environment, and the reduction of exposure of dried foods to oxygen will increase the survival and enumeration of injured bacteria.

3. Food Composition

The developed sensitivity of injured indicator bacteria is not confined only to the selective ingredients in the selective media, but is also extended to many ingredients that are present in different semipreserved foods.[26,28] These include many organic acids, low molecular fatty acids,[107] emulsifying agents, salt, lysozyme (present in egg white and oyster), and some other hydrolytic enzymes and many preservatives. These compounds could enhance the death of injured bacteria during storage and sample preparation. It would be difficult to prevent the action of these food components on injured indicator bacteria. However, their lethal

effect could be reduced by mixing the sample rapidly, diluting it, and transferring it to the suitable bacteriological media. Similarly, an acid product could be blended in a suitable buffer. Also, when a product that is expected to harbor a low population of indicator bacteria and thus should be enumerated without dilution or with very little dilution, the carry over of the selective ingredients in the bacteriological media and their inhibitory effect on the enumeration of the injured indicator bacteria should be recognized. Similarly, the carry over of ingredients, such as sucrose from sugared products, could give growth characteristics similar to coliforms by many noncoliforms, and should be further tested for their confirmation.

4. Composition of Enumeration Media

The methods that have been developed for the enumeration of injured bacteria employ the exposure of the injured cells initially in a nonselective medium and then in a selective medium. The cells are incubated in the nonselective media to allow the resuscitation of the injured cells. Although the injured indicator bacteria are able to repair in the food homogenates, the total amount of repair varies greatly with the type of food.[49] In general, the incubation of a food sample in a nutritionally rich broth provides a consistently higher level and faster rate of repair. The composition of the selective agar and broth media used in conjunction with a nonselective medium also influences the enumeration of injured indicator bacteria from a food sample. This is mainly because the injured indicator bacteria differ greatly in their susceptibility to the type and concentration of a selective compound. A selective agar medium used at a relatively higher temperature (48 vs. 45°C) during plating can reduce the detection of injured indicator bacteria from a food.[25]

5. Time and Temperature of Resuscitation

The optimum time and temperature for the repair of injury by different indicator bacteria vary greatly. Freeze-injured coliforms, either in a pure culture or in food, repair rapidly during incubation in a nutritionally rich medium, and most cells repair within 1 h at 35°C or 2 h at 25°C.[49,98,105] A longer incubation time up to 4 h does not increase the counts substantially. In contrast, heat-injured *E. coli* require as long as 6 h at 35°C for complete repair; only 50% of the cells repair in 2 h at 35°C.[96] *E. coli* biotype I, injured by freezing, drying, or heating, require at least 4 h at 37°C for a larger percentage of the injured cells to repair.[102] However, most heat-injured *E. coli* cells repair within 2 h at 35°C in a suitable medium.[43]

Injured indicator bacteria seem to repair more rapidly at temperature ranges between 30 to 37°C. Most studies, although limited, have shown that an incubation temperature higher than 40°C could be lethal to the injured indicator bacteria.[40,96,102] Injured indicator bacteria also do not repair at refrigeration temperature, and at 15°C the rate is very slow.[40]

6. Methods of Detection

Both selective DP and indirect MPN methods have been compared by several workers to determine their relative merits to enumerate injured indicator bacteria from semipreserved foods. The selective DP methods have been reported to give a higher and more consistent enumeration of repaired coliforms and *E. coli* from foods. The MPN methods that included an initial repair phase gave a higher enumeration than the selective methods, but the data showed considerable fluctuations.[18,102] Because of less variability, the DP methods with an appropriate repair step have been preferred over the MPN methods to enumerate indicator bacteria from semipreserved foods.[18]

IV. METHODS TO DETECT INJURED INDICATOR BACTERIA FROM FOODS

It is evident from many studies that the indicator bacteria present in semipreserved foods could be injured and the current selective DP and MPN methods would not detect these injured survivors. These methods will detect mostly the uninjured fraction of the surviving indicator population and thus may not be very effective in either quality control or regulatory programs. However, if the injured indicator cells are allowed to resuscitate first, then the selective procedures will enumerate all or the majority of the viable indicator cells in semipreserved foods. Information generated from the basic studies on the repair of bacterial injury are used to design methods to enumerate injured indicator bacteria from foods. The following general concepts are used in designing these methods:[26,28,47,108]

1. Injured indicator bacteria, if exposed to a selective environment prior to repair, might lose their viability.
2. A nutritionally rich, nonselective medium facilitates the repair of most injured indicator cells.
3. The time and temperature required for most cells to repair vary with the type of stress, but a large fraction of the injured cells repair within a short time at a mesophilic temperature range.
4. The repaired indicator cells regain their resistance to the selective compounds used in the enumeration of indicator bacteria.
5. The repair process must precede cell multiplication.

In the resuscitation-enumeration methods, a food sample is mixed or homogenized with a nonselective medium and incubated at optimum conditions for the injured cells to repair. Then the selective procedures are employed to facilitate the selective enumeration of the indicators in the sample.

A. Effectiveness of the Resuscitation-Enumeration Procedures for the Enumeration of Injured Indicator Bacteria from Foods

The effectiveness of different resuscitation parameters has been studied both in broth and agar media. Other areas which have become of practical importance are the rapidity and convenience of the methods developed for the enumeration of injured indicator bacteria. The development of these methods and their relative effectiveness will be briefly discussed here.

Ray and Speck[45] reported that about 90% of the injured *E. coli* from several inoculated frozen foods repaired within 1 h at 25°C in trypticase soy yeast extract (TSY) broth as indicated by an increase in colony counts on VRB agar. Their studies showed that the cells did not start multiplication until after 2 h at 25°C in TSY broth. So the increase in colony counts during the initial 1 h was due to the resuscitation of injured *E. coli*. The repaired cells could also be enumerated in BGLB broth. Incubation of the injured *E. coli* in the food homogenates resulted in about 50% repair during 1 h at 25°C. In a similar study with naturally contaminated frozen foods, Warseck et al.[49] showed that injured coliforms repaired more effectively in trypticase soy (TS) broth than in the respective food homogenates during a 1 h incubation period at 25°C (see Table 19). Their results also revealed that cell multiplication in either TS broth or in food homogenates did not start until after 2 h at 25°C. The DP method in selective agar gave a better and more consistent enumeration of the repaired coliforms than the selective MPN methods.[49] Both the pour plating and the surface-overlay plating methods were found to enumerate the repaired coliforms effectively.

Although the incubation of a food sample in a nonselective broth provided a suitable

Table 19
RELATIVE ABILITY TO REPAIR FREEZE-INJURY BY COLIFORMS IN A BROTH AND IN RESPECTIVE FOOD HOMOGENATES[40,45,49]

	Coliform counts (× 10^1) per gram in VRB agar at		
	0 h	1 h in TS broth (25°C)	1 h in food homogenates (25°C)
Commercial foods			
Crab cake	80	700	630
Deviled crab	10	190	120
Breaded oyster	31	182	84
Stuffed flounder	29	300	180
	% Detected in VRB agar at		
	0 h	1 h in TS broth (25°C)	1 h in food homogenates (25°C)
Inoculated foods			
Milk	25	90	80
Liquid egg	20	82	50
Meat broth	14	85	60
Crab meat	22	84	50

environment for the resuscitation of injured indicator bacteria, there is a possibility that the increase in enumeration at the end of incubation for 1 h at 25°C could be due partially to multiplication of the uninjured cells. The chances of cell multiplication become greater as the incubation time is extended beyond 1 h at 35°C.[96] This problem, associated with the "liquid-repair" method, was overcome by the development of the "solid-repair" method by two groups of workers.[98,106] Speck et al.[98] initially showed that surface plating an aliquot of a food homogenate on a prepoured TS agar plate, followed by incubation at 35°C for 2 h, allowed the injured *E. coli* and other coliforms to repair. The plates were subsequently overlaid with 10 to 12 ml VRB agar for selective enumeration of the coliforms. This method increased the enumeration of coliforms from commercial ice cream samples fivefold over direct VRB agar plate counts. Later, Ray and Speck[105] showed that the initial pour plating of a sample aliquot with 5 ml of TGE agar on TS agar and incubation allowed the injured indicator bacteria to repair effectively (see Table 20). For selective enumeration, the plates were then overlaid with 10 to 12 ml of VRB agar and incubated according to the recommended procedures. They examined the effectiveness of the incubation time (15 min to 3 h), the incubation temperature (25 to 40°C), the volumes of TGE or TS agar (5 and 10 ml), and the concentrations of selective compounds in VRB agar (single and double) and observed that for freeze-injured coliforms a combination of 1 ml sample, 5 ml TGE or TS agar, 1 h at 35°C or 2 h at 25°C, and 10 to 12 ml VRB agar recovered 85% or more of the injured cells. This "two-layer pour plate" method has several advantages over "surface-overlay" or "liquid-repair" methods. The cells are fixed in an agar matrix, so cell multiplication during longer resuscitation periods will not increase enumeration, but will help to recover more injured cells. Also, larger sample volumes, as compared with the "surface-overlay" method, could be used for samples with a low coliform population. Although prolonged incubation beyond 3 to 5 h at 35 or 37°C may increase the enumeration of injured indicators, the increase may not be much higher than the counts obtained at 2 h. However, a shorter incubation time will not only be convenient, but will reduce the extensive growth of non-indicator bacteria in TGE or TS agar and the possible interference later with the growth of indicator bacteria. In an examination of 193 different dairy products this repair-enumeration

Table 20
INFLUENCE OF RESUSCITATION ON THE ENUMERATION OF INJURED INDICATOR BACTERIA FROM FOODS

Food	Nature of stress	Indicator	Count per gram of food: Selective agar[a]	Count per gram of food: Two layers agar[b]	Ref.
Ice milk[c]	Freezing	Coliforms	86	125	105
Oyster[c]	Refrigeration	Fecal coliforms	80	1,200	26
Oyster[c]	Refrigeration	Enterococci	88	171	99
Scallop[c]	Refrigeration	Enterococci	124	273	99
Shrimp[c]	Refrigeration	Enterococci	750	5,300	99
Ice cream[c]	Freezing	Coliforms	3	11	98
Bisquick®[c]	Drying	Coliforms	28	46	26
Breaded shrimp[c]	Freezing	Coliforms	30	980	26
Onion ring[c]	Freezing	Coliforms	380	4,000	26
Cheese spread[c]	Acid	Coliforms	900	3,000	26
Ice cream[c]	Freezing	Coliforms	60	240	26
Buttermilk[c]	Acid	Coliforms	24	42	26
Meatloaf[d]	Freezing	*Escherichia coli*	60	2,200	96
	Freezing	*E. coli*	3	265	96
Ground beef[d]	Freezing	*E. coli*	29,580	59,566	104

[a] Pour plating with KF-streptococcal agar for enterococci and VRB agar for others.
[b] Pour plating with 5 ml TGE or TS agar and overlaying with KF-streptococcal or VRB agar after resuscitation.
[c] Commercial foods.
[d] Inoculated foods.

method gave coliform counts as high as 54% over the counts obtained by the DP method with VRB agar; some samples showed an increase in count of more than 200%. More than 95% of these colonies were confirmed as coliforms in BGLB broth. Hartman et al.[106] used the nonselective pour plate method for the same purpose, but used a medium that contained all the ingredients of VRB agar except the selective agents, bile salts, brilliant green, and neutral red. Immediately after solidification, the plates were overlaid with the same volume (12 ml) of VRB agar that had double the concentration of the selective compounds (VRB-2 agar). They recorded 30 and 80% increases in the coliform counts from frozen vegetables and cottage cheese, respectively, by this modified method over the DP method with VRB agar. Hackney et al.[99] modified the "solid-repair" method of Ray and Speck[105] for the enumeration of fecal coliforms by incubating the plates (TS agar overlaid with VRB agar) at 45°C and of enterococci by using TS agar overlaid with KF-streptococcal agar. These modified methods, when used with different types of refrigerated and frozen commercial foods, increased the fecal coliform counts by 24 to 35% and the enterococci counts by 22 to 85% as compared with the respective selective plating methods. In many samples, the repair-enumeration method gave higher fecal coliform counts than the MPN methods, and more than 95% of the colonies tested positive for fecal coliforms by the confirmative tests. This repair-enumeration method was also examined by other workers[43,96,109] for rapid enumeration of coliforms and fecal coliforms from foods; however, Rowley et al.[96] suggested a resuscitation time in TS or TGE agar of 2 h at 37°C for freeze-stressed and up to 6 h at 35°C for heat-stressed *E. coli* cells. A slightly different "two-layer agar media method" was shown by Rose et al.[110] to be effective for the enumeration of fecal coliforms from water samples. In this method, a plate was first pour plated with a selective agar medium and after solidification a layer of nonselective agar medium was overlaid on top of the selective bottom layer. The water or a suitable sample was filtered through a membrane

Table 21
INFLUENCE OF RESUSCITATION ON THE ENUMERATION OF INJURED *ESCHERICHIA COLI* BIOTYPE I FROM FOODS[102]

Food	Nature of stress	*E. coli* biotype I/g: MPN in selective broth[a]	Counts in selective agar[b]	Counts by resuscitative procedure[c]
Fish[d]	Freezing	702	712	932
Apple puree[d]	Freezing	67	<10	128
Strawberry puree[d]	Freezing	119	<10	187
Meat[d]	Drying	9	10	40
Carrot[d]	Drying	150	130	320
Swede[d]	Drying	460	40	610
Raw milk[e]	Refrigeration	1100	0	2300
Ice cream[e]	Freezing	56	0	43
Meat[e]	Freezing	527	320	698

[a] MacConkey broth at 37 and 44°C.
[b] Tryptone bile salt agar at 44°C.
[c] 4-h at 37°C on nutrient agar and 18 h on tryptone bile salts agar at 44°C.
[d] Inoculated foods.
[e] Commercial foods.

filter and the membrane was then placed on the surface of the top agar layer. The plates were first incubated at 35°C for 2 h for resuscitation and then at 44.5°C for 22 to 24 h for colony formation by the fecal coliforms. This method increased the enumeration of fecal coliforms by 93% from the natural samples over the selective conventional methods. Holbrook et al.[102] developed a modified DP method that could be used for the enumeration of injured *E. coli* biotype I from foods. A membrane was first placed on top of the prepoured nutrient agar plate and an aliquot of a food in liquid state was distributed uniformly over the membrane surface. The plates were incubated at 37°C for 4 h for resuscitation, after which the membranes were carefully removed, placed on top of a prepoured, selective TB agar plate, and the plates were then incubated at 44°C for 24 h. The membranes were then exposed to a specific reagent (*p*-dimethyl aminobenzaldehyde) that stained the *E. coli* biotype I colonies (due to their ability to produce indole) pink. Examination of artificially inoculated as well as naturally contaminated foods by this method showed that more *E. coli* biotype I could be detected within 24 h over the lengthy selective conventional methods (see Table 21). McDonald et al.[58] demonstrated that supplementing the nonselective TA or TGE agar with pyruvate, thiodiproprionic acid, or catalase increased the resuscitation of injured coliforms and their subsequent enumeration by the selective medium (TS or TGE agar overlaid with VRB agar). The increase in recovery was suggested to be due to the protection of injured coliforms against the lethal effects of hydrogen peroxide formed spontaneously in many bacteriological media. Some injured cells with defective catalase and/or peroxidase systems were otherwise killed even in the nonselective media. Commercial foods tested by this method showed a coliform count increased considerably over the counts obtained with unsupplemented TGE agar, as well as by the DP method with VRB agar (see Table 22). A convenient method that used "Petrifilm® VRB plate" was evaluated for its efficiency to enumerate injured coliforms.[111,112] The samples with injured coliforms were incubated in 1% milk for 2 h at room temperature and enumerated by plating in "Petrifilm® VRB plates". This method gave enumerations that were comparable to VRB agar counts.

Several workers have applied the resuscitation-enumeration technique to the MPN methods

Table 22
EFFICIENCY OF SUPPLEMENTATION OF COMPOUND THAT DEGRADE H_2O_2 ON THE ENUMERATION OF INJURED COLIFORMS FROM FOODS[58]

		Coliform count per gram		
Food[a]	Nature of stress	Selective plating[b]	Unsupplemented resuscitative plating[c]	Supplemented resuscitative plating[d]
Ice cream	Freezing	3.6×10^2	4.9×10^2	6.8×10^2
Hamburger	Freezing	1.3×10^4	1.2×10^4	1.9×10^4
Cheese	Refrigeration	3.7×10^2	5.2×10^2	6.7×10^2
Oyster	Refrigeration	7.8×10^4	3.3×10^5	3.9×10^5
Sausage	Refrigeration	6.3×10^5	8.5×10^5	7.6×10^6

[a] Average of three to nine samples in each category.
[b] Pour plating with VRB agar.
[c] Resuscitation in TGE agar for 2 h at 25°C and selective enumeration with VRB agar.
[d] TGE agar was supplemented with 1.0% thiodipropionic acid.

for the recovery of injured indicator bacteria from foods. Lanz and Hartman[113] and Hartman[108] described a procedure in which the two steps, the presumptive and the confirmed tests, were combined into a single step. In the 9-tubes MPN methods a 1-ml aliquot from serial dilutions was inoculated in 10 ml lactose broth along with a capsule containing brilliant green dye and bile salts in concentrations present in the BGLB broth, and the tubes were incubated at 37°C. During the initial stage the nonselective environment favored the repair of injured cells. Presumably, the selective agents would be released slowly from the capsules to make the environment selective but allow the repaired cells to multiply and give a positive reaction. Rowley et al.[96] included a resuscitation step in both radiometric and impedance techniques designed for rapid enumeration of fecal coliforms from foods. They inoculated the food aliquots from serial dilutions in vials containing EC-B broth (EC broth without the bile salts) and incubated the vials for 3 h at 37°C. The EC broth containing double-strength bile salts was then added to the vials. For the radiometric assay, ^{14}C-lactose was also added at the same time. After incubation for 17 to 18 h at 45.5°C, the vials were examined for $^{14}CO_2$ production in a radiometric assay and change in impedance for the impedance technique. With replicates of samples in each dilution and from the number of positive vials in each dilution, the results could be expressed as the MPN of fecal coliforms per g or ml of a food. Brodsky et al.[103] demonstrated that a resuscitation step could be incorporated into the HGMF method to enumerate injured coliforms, *E. coli*, and enterococci. The membrane filter, through which a food sample had been filtered, was placed on top of a prepoured plate containing TS agar supplemented with 0.15% $MgSO_4$ and incubated for 4 to 5 h at 35°C in the case of frozen, heated, or low-pH foods, but for 2 h at 25°C for dried products to enable the injured cells to repair. The filters were then transferred on top of a selective agar, specific for the species or group, and incubated at an optimum temperature for a suitable time. From the number of occupied squares in the membrane filter grid, the MPN of the specific indicator could be calculated. In an inoculation study, the resuscitative HGMF procedure gave a higher recovery of coliform, *E. coli*, and enterococci from foods over the conventional MPN methods. Two groups of workers[11,12] have suggested that the inoculation of food samples from serial dilutions directly in selective broths and incubation initially at a mesophilic temperature helped the injured fecal coliforms to resuscitate. Quadri et al.[11] used MacConkey broth and a 2-h incubation period at 37°C prior to another 22- to 24-h incubation period at 44°C, while Andrews et al.[12] used A-1 broth and a 3-h incubation period at 35°C followed

by a 21-h incubation period at 44.5°C. Both procedures were able to provide the MPN of fecal coliforms in foods within 24 h. However, any detrimental effect of the selective broths on the injured indicator cells during the initial incubation was not determined.

A fluorogenic assay technique, based on the hydrolysis of 4-methylumbelliferon glucuronide (MUG) by glucuronidase to yield a fluorogenic product, was tested by Feng and Hartman[114] for the rapid MPN enumeration of *E. coli* injured by heat and chlorine treatment. The injured cells from serial dilutions were inoculated in LST broth tubes that contained 100 μg/ml of MUG. The tubes were incubated at 35°C for 24 to 48 h and examined for fluorescence and gas production. The LST broth recovered 73% of the cells surviving heat treatment, but only 31% of the cells surviving chlorine treatment. In contrast, the "two-layers" agar medium using TS agar (10 ml) and VRB-2 agar (with double the concentrations of the selective agents of VRB agar) with a resuscitation period of 2 h at 35°C yielded only 0.5 and 11% *E. coli* cells surviving heat and chlorine treatment, respectively. The low recovery was suggested for the selective nature of the LST broth used in the MPN methods and for the high concentrations of selective compounds in VRB-2, as well as the incomplete repair of injured *E. coli* cells in the plating method.

B. Suggested Methods for the Enumeration of Injured Indicator Bacteria from Foods

The methods developed by different researchers showed that the injured indicator bacteria could be enumerated from the semipreserved foods by incorporating a relatively short resuscitation phase with the currently recommended selective plating or MPN methods. There are discrepancies in these methods with respect to the type of nonselective media and incubation time and temperature that would be optimum for the repair and subsequent enumeration of the injured indicator bacteria present in semipreserved foods. Most nutritionally rich, nonselective media probably enable a large percentage of injured indicator bacteria to repair. Repair in an agar medium, as compared with a broth, eliminates the possibility of any increase in counts from multiplication. A more rapid and higher level of cell repair occurs at a temperature of 35 to 37°C. The repair rate is very slow below 15 to 20°C, while temperatures above 40°C could be lethal to the injured cells. Although a longer incubation time will allow more injured cells to repair, it has several disadvantages. Prolonged incubation in a broth medium not only will facilitate the injured cells to repair, but also will allow uninjured cells to multiply. A longer resuscitation time in an agar medium will also allow the nonindicator associative bacteria to multiply, which can then interfere with the growth and enumeration of indicator organisms. In an 8-h day working schedule, a longer incubation time, transferring plates or tubes from room temperature to a 35 to 37°C incubator for resuscitation, and again removing them for adding selective media prior to subsequent incubation at a suitable temperature may be neither convenient nor economical for a food-testing laboratory.

Currently, several methods have been tested and recommended for the enumeration of injured indicator bacteria from foods.[18,28] These methods and their advantages and disadvantages will be discussed here. The applicability and suitability of a particular method by a food-testing laboratory have to be decided by the particular laboratory. Methods of sampling and the initial sample preparation (such as mixing, blending, diluting etc.) are not included in this discussion. For these methods a methodology book should be consulted.[3-6] The resuscitation-enumeration methods could be grouped as DP and MPN methods.

1. DP Methods

A nonselective broth (liquid-repair) or a nonselective agar (solid-repair) medium is used to enable the injured cells to resuscitate prior to their exposure to a selective environment. The selective environment will allow the selective multiplication of the surviving indicator bacteria. DP methods are economical, take less time, and give relatively consistent data.

a. Liquid-Repair Methods[26,28]

The food samples are first incubated in TS broth at 35 to 37°C, not exceeding 1 h. Following dilutions, aliquots from selected serial dilutions are then pour plated with a selective agar medium specific for the group. For coliforms, fecal coliform, and *E. coli*, pour plating with VRB agar and an overlay of the same medium could be used, and for enterococci, pour plating with KF-streptococcal agar could be used. The plates are incubated at 35°C for 24 h for coliform and for 48 h for enterococci, then the characteristic colonies are enumerated. For confirmation, representative colonies could be further tested in BGLB broth for coliforms and for morphology, gram characteristics, and biochemical reactions for enterococci. For fecal coliforms and *E. coli*, the VRB agar plates are incubated at 44 to 45°C for 24 h. Representative colonies are confirmed for gas production in EC or A-1 broth at 44 to 45°C in 24 h for fecal coliforms and gram-characteristics and IMViC reaction pattern for *E. coli*.

This method will allow good repair of freeze-injured indicator bacteria. However, the shorter incubation time might not enable some heat-injured indicator bacteria to repair. A longer initial incubation period, on the other hand, could increase the enumeration due to the multiplication of uninjured indicator cells. Thus, this method may not be suitable for regulatory purposes.

b. Solid-Repair Methods[26,28,58,96,102,108,109]

Several variations of the basic principle, which involves resuscitation in a nonselective agar medium, have been tested.

Two-layers agar method[26,28,43,108,109] — The sample from selected serial dilutions are pour plated (0.1 or 1.0 ml or more per plate) with about 5 ml of TGE or TS agar. The plates are incubated for 1 to 2 h at 35 to 37°C and then overlaid with 10 to 12 ml VRB agar for coliforms, fecal coliforms, and *E. coli* and KF-streptococcal agar for enterococci. Subsequent incubation, enumeration of characteristic colonies, and other confirmative tests for individual groups and species are the same as described before. In another variation of this method, the plates after resuscitation are overlaid with double-strength selective agar (such as VRB-2 agar for coliforms[106,108]) to reduce the dilution of the selective agents by the nonselective agar medium. The major advantage of this method is that it eliminates the possibility of a higher enumeration due to the multiplication of indicator bacteria. The disadvantages are the extra work associated with the incubation of the plates in an incubator and overlaying of the plates with the selective agar. Incubation at 35 to 37°C in an incubator could be modified by incubating the plates at room temperature (22 to 25°C) for 4 h or longer; but that again may not be advantageous under practical laboratory conditions, working on an 8-h day schedule. Overlaying of the selective agar immediately after solidification of the nonselective agar was suggested by Hartman,[108] but some studies have shown that it might not be a very effective modification for the enumeration of injured indicator bacteria.[43,105,114]

Membrane filter agar method for *E. coli* biotype I[17,18,28,102-103a] — This method is useful specifically for the enumeration of *E. coli* biotype I. A cellulose-acetate membrane (85 mm) is placed on top of a well-dried, prepoured nutrient agar plate[102] or a TS agar plate[18] and 1 ml of a food sample is spread over the membrane surface. The plates are incubated at 37°C for 4 h, after which the membranes are carefully removed and each one is placed on top of a prepoured plate of TB agar. The plates are incubated at 44 ± 0.5°C in a circulating air incubator for about 18 h. To detect indole positive colonies, the membranes are removed and placed onto 2 ml of indole reagent (5% *p*-dimethylaminobenzaldehyde in N-HCl) in the petri dish lid. After 5 min the excess reagent is removed and the membrane in the dish is dried under a long-wave UV lamp. The pink-stained colonies are enumerated for *E. coli* biotype I. This method is rapid for the enumeration of *E. coli* biotype I. Although this

method does not enumerate *E. coli* biotype II, this group constitutes less than 5% of the *E. coli* encountered in foods.[18] The disadvantages are that only a small volume of food could be tested (0.1 g per plate); thus a large number of plates have to be used for samples containing a low population of *E. coli* biotype I. It may not be very economical with respect to supplies and labor.

Supplemented-agar method[58] — This method is similar to the "two-layer agar method" described before. However, the TS or TGE agar is supplemented with 0.66% Na-pyruvate or 1.0% TDPA. These supplements are added to the agar medium before sterilization. Before adding the supplement, the pH of the supplement is adjusted to 7.3 with NaOH. This method has been tested for the enumeration of freeze-injured coliforms, but will probably be effective for other injured indicator bacteria. The main advantage of this technique is that it neutralizes the toxic effect of hydrogen peroxide and allows better enumeration of the injured indicator bacteria from a food. The disadvantages are the same as in the "two-layers agar method".

Fluorogenic assay of *E. coli*[114] — The sample aliquot is pour plated with 10 ml of TS agar and incubated for 2 h at 35°C for resuscitation. Each plate is then overlaid with 10 to 12 ml VRB-2 agar (VRB agar containing double the concentrations of bile salt, crystal violet, and neutral red) that has 200 μg/ml of MUG. The plates are incubated and examined for colonies with fluorescent haloes within a 12- to 20-h incubation at 35°C. This method is rapid for the enumeration of *E. coli* that produce B-glucuronidase (GUD). However, some other species in the family Enterobacteriaceae are also capable of producing this enzyme. Also, the plates have to be checked early, otherwise the fluorogenic compound produced by GUD will diffuse in the entire surface of the plating medium and make it difficult to differentiate between the positive and negative colonies.

2. *MPN Methods*

Both "liquid-" and "solid-repair" techniques could be used to enumerate injured indicator bacteria by the MPN methods. In general, the MPN methods are costly, take a long time, and give results that can fluctuate greatly.

a. Liquid-Repair Methods

Several variations of this procedure have been suggested.

Conventional method[26] — From the serial dilutions of a sample, a 1-ml portion is inoculated to 4 ml of TS broth for the 9-tubes MPN method. The tubes are incubated 1 to 4 h, and then each is inoculated with 5 ml of a double-strength, selective broth. The broths are BGLB for coliforms, EC or A-1 for fecal coliforms, and KF-streptococcal broth for enterococci. The tubes are incubated further at 35°C for 24 h for coliforms and 48 h for enterococci, and for 24 h at 44 to 45°C for fecal coliforms. Cultures from the positve tubes are confirmed by gram characteristics and biochemical reactions for enterococci and for an IMViC pattern for *E. coli*. This procedure will allow the resuscitation of injured indicator bacteria. However, a longer resuscitation time, as might be necessary for heat-stressed indicators, can make the method inconvenient.

Radiometric and impedance methods for fecal coliforms[28,96,112] — From the serially diluted sample, a 1-ml portion is inoculated to a 40 ml vial containing 10 ml EC-B broth (EC broth without bile salt) following the protocol of the 9-tubes MPN method. The vials are incubated for 3 h at 37°C and then each is inoculated with 10 ml EC broth containing double-strength bile salt. For a radiometric assay, each vial is also supplemented with ^{14}C-lactose. The vials are incubated at 45.5°C for 18 h and examined for the production of $^{14}CO_2$ in the radiometric assay and for a change in electrical impedance in the impedance technique. The MPN is calculated from the number of positive vials in three successive dilutions. Both techniques give a rapid enumeration of injured fecal coliforms; however, they need a costly and sophisticated instrument. A short resuscitation time may not allow some injured fecal coliforms to repair. The methods have not been tested against enterococci.

Fluorogenic assay of *E. coli*[111,114] — Sample aliquots from serial dilutions are inoculated in LST broth tubes containing 100 μg/ml MUG for the 5-tubes/3 dilutions MPN method. The tubes are incubated at 35°C for 20 h and examined for gas production and the presence of a fluorogenic compound released by GUD of *E. coli* to calculate the MPN. This method allows the rapid enumeration of *E. coli*, but the LST broth may not be efficient for enumerating injured *E. coli*.[26,40] Instead, lactose broth containing MUG could be used to facilitate repair as well as for the enumeration of injured *E. coli*.[114a]

b. Solid-Repair Methods

HGMF method[103,103a,111] — An aliquot of a homogenized food is filtered through a HGMF, the filter is placed on the surface of a prepoured plate of TS agar supplemented with 0.15% $MgSO_4$, and the plate is incubated for 2 h at 35°C. The filter is then transferred to the surface of a prepoured plate of a selective agar medium and incubated under appropriate conditions, which are, for coliforms, MFC agar without rosolic acid at 35°C for 24 ± 2 h; for *E. coli* biotype I, TB agar at 44.5°C for 24 ± 2 h; for enterococci, M-enterococcus agar at 35°C for 48 ± 2 h. For coliform and enterococci the filter is examined for the number of squares occupied by characteristic colonies. For *E. coli* biotype I, the membrane is treated with indole reagent[102] and examined for the number of squares occupied by indole-positive colonies. From the number of occupied squares, the MPN of a specific indicator is calculated. This method is rapid and could be used for different types of indicator organisms. However, a shorter resuscitation time may not allow all the injured cells to repair.

Microtitration plate-MUG assay for *E. coli*[114] — Wells in a microtitration plate are first filled with TS agar containing 100 μg/ml of MUG. For the 5-wells MPN analysis, 0.05 ml from a serially diluted sample is inoculated in each well. The plates are then sealed with tape, incubated at 35°C for 24 h, and examined for the number of wells that fluoresced in each dilution to estimate the MPN of *E. coli*. This method is rapid for *E. coli* enumeration, but has not been tested with food. Because of the small volume of sample used, it could be effective only in samples that have high *E. coli* counts.

V. CONCLUDING REMARKS

Both quantitative[3-7] and qualitative[8] estimations of the indicator bacteria have been recommended for the determination of the sanitary quality of semipreserved foods and food ingredients. However, several facts question the suitability of these groups or species as indicators of fecal contamination and the possible presence of enteric pathogens in a food. These include: some coliforms are of nonfecal origin; most are able to grow in many food systems; some are able to grow at refrigeration temperature; some are extremely susceptible to some food-storage environments; and some are able to establish colonies on equipment surfaces. Although fecal coliforms and *E. coli* are considered to be better indicators of fecal contamination, their value in indicating contamination of foods with pathogens of nonfecal origin, such as *Staphylococcus aureus* and *Vibrio parahaemolyticus*, needs to be considered. In addition, the ability of some newly emerging pathogens (such as *Yersinia enterocolitica, Listeria monocytogenes*, and *Aeromonas hydrophilia*) to grow at refrigeration temperature and the susceptibility of fecal coliforms and *E. coli* to low temperature should be considered in determining their suitability as indicator bacteria. Finally, the significance of their relative numbers in raw, processed, or stored products should be considered separately.

The major advantage of relating the level of indicator bacteria to the degree of sanitation used in the production, processing, and storage of food products is in the rapidity at which they could be enumerated, especially by some of the rapid methods that have been developed in recent years,[12-17] as compared with the time-consuming methods necessary for the detection of many enteric food pathogens. The other disadvantage of testing foods for pathogens is

that different procedures are recommended for different pathogens; this could be avoided by testing the foods for a suitable indicator. Among the two general types of methods recommended for the enumeration of indicator bacteria in a food, the indirect MPN methods have several inherent disadvantages. The one factor that can greatly restrict the use of MPN values for setting up indicator standards in foods is its wide confidence limits. These disadvantages could be overcome by the DP methods. DP methods could also be modified[17,26] to test a large volume of a sample, thus eliminating their ineffectiveness for testing foods with a low level of indicator bacteria. However, MPN methods might find their usefulness in some of the automated techniques now being developed to test a large number of samples simultaneously.[96,103,112,114]

The federal, state, and municipal regulatory agencies are interested in establishing microbiological criteria in the form of standards and recommended limits, that include indicator bacteria, to assist the agencies in making judgments on the sanitary quality and safety of foods.[115] Proper judgment can only be applied when the methods used in bacteriological analysis are effective and could be uniformly applied in different laboratories. Procedures that give an improper enumeration of indicator bacteria are ineffective for the practical interpretation of data and their use for regulatory purposes.[116]

The occurrence of injured indicator bacteria in many semipreserved foods and the inability of the selective detection methods to enumerate them present problems with respect to the assessment of the sanitary quality of semipreserved foods.[117] Their ability to repair rather quickly in food and nonselective environments poses another problem of obtaining uniform data for a sample between testing or in multiple testing, such as in a collaborative study. As a proper resuscitation technique allows the viable indicator bacteria to be enumerated by the recommended selective procedures, both in the development of new methods and in collaborative studies of a method, the possibility of the presence of stressed indicator bacteria in semipreserved foods should be taken into consideration.[118]

The ability of the injured indicator bacteria to resuscitate in a suitable environment within a short period of time could be advantageously used to incorporate a resuscitation phase with the selective enumeration procedures recommended for the detection of indicator bacteria from foods. The studies conducted by different workers indicated that effective resuscitation could be achieved in several different ways. However, the differences in the methods could create confusion as to the selection of a specific method by the users. A collaborative study that would compare the existing methods and advocate one that could be used beneficially and economically should probably be undertaken to overcome this confusion.

The use of a resuscitation-enumeration technique will definitely increase the detection level of indicator bacteria in semipreserved foods, over the level obtained by the conventional methods. This should be taken into consideration in evaluating the existing standards of indicator bacteria in some semipreserved foods or in setting up future standards for different semipreserved foods.

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Chapter 3

METHODS FOR RECOVERING INJURED "CLASSICAL" ENTERIC PATHOGENIC BACTERIA (*SALMONELLA, SHIGELLA*, AND ENTEROPATHOGENIC *ESCHERICHIA COLI*) FROM FOODS

Wallace H. Andrews

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I. INTRODUCTION

Members of the genera *Salmonella* and *Shigella* and enteropathogenic *Escherichia coli* organisms may be considered "classical" pathogens in the sense that their pathogenicity has been well documented and long recognized. The analytical methods originally developed to identify these pathogens in clinical specimens were subsequently modified or tailored for use with foods. Because many foods are subjected to processing, surviving cells may sustain various degrees of injury. This chapter discusses cell injury, the methods of cell resuscitation, and the effects of various factors on resuscitation.

II. THE GENUS *SALMONELLA*

A. Cell Injury and Resuscitation

Three steps are common to most methods for the resuscitative recovery of *Salmonella* from foods: preenrichment, selective enrichment, and selective agar plating. Preenrichment, the initial phase of the isolation procedure, ideally provides nutrients for multiplication, repair of cell damage, rehydration, and dilution of toxic or inhibitory substances. After incubation under prescribed conditions, a small preenriched portion is subcultured to one or more selective enrichment media. These media contain certain ingredients that allow *Salmonella* to proliferate while inhibiting, or at least restricting, the growth of competing non-*Salmonella* organisms. After incubation, cultures from the selective enrichment media are streaked to selective plating media, on which *Salmonella* may appear as discrete colonies while the growth of non-*Salmonella* organisms is suppressed. Colonies that appear to be *Salmonella* are then subjected to biochemical and serological confirmation.

Because of the occurrence of *Salmonella* organisms in a wide spectrum of foods, their role in producing human illness, and the relative ease of their cultivation under laboratory conditions, numerous methods have been developed for isolating these pathogens. Several authors have reviewed these methods (see Table 1). Fagerberg and Avens[1] discussed 11 selective enrichment and 8 plating media, and recommended the determination of optimum methods for individual types of foods. Validation by collaborative study was recommended as a "standard method" to be used by all analytical laboratories. Hartman[2] reviewed most probable number (MPN) procedures, membrane filter techniques, and direct plating methods for the recovery of injured coliforms and *Salmonella* with emphasis on methods that would not substantially alter normal laboratory routines. Harvey and Price[3] presented a comprehensive review of the methods and principles of preenrichment, selective enrichment, and selective plating. Unlike the recommendation of Fagerberg and Avens[1] for uniform standard methods, Harvey and Price[3] took the view that each laboratory should choose a method particularly suited for its own special purpose. D'Aoust[4] compared methods of preenrichment and selective enrichment recommended by governmental regulatory agencies and various national and international scientific organizations, whereas in a companion paper, Moats[5] reviewed selective plating media for the recovery of *Salmonella* from foods. Recently, Andrews[6] reviewed conventional methods and discussed their relation to rapid methods for the recovery of *Salmonella* from foods. In a subsequent review, Andrews[7] discussed the resuscitation of injured *Salmonella* during preenrichment. Fortunately, in the development of many of these methods, the phenomenon of cell injury was recognized and taken into account. Defined experimental conditions for producing cell injury in *Salmonella* and, in certain instances, the conditions for repair are given in Table 2.

1. Freeze Injury

Hartseli[8] was among the first investigators to define injured cells as those capable of forming colonies on nonselective media, but not on selective media. Using the formula,

Table 1
REVIEWS OF METHODS FOR RECOVERING *SALMONELLA* FROM FOODS

Year published	Authors	Content	Ref.
1976	Fagerberg and Avens	Selective enrichment and selective agar plating	1
1979	Hartman	Recovery of injured organisms without introducing major changes in routine laboratory procedures	2
1979	Harvey and Price	Preenrichment, selective enrichment, and selective agar plating	3
1981	D'Aoust	Preenrichment and selective enrichment	4
1981	Moats	Selective agar plating	5
1985	Andrews	Preenrichment with emphasis on rapid culture methods	6
1986	Andrews	Resuscitation of injured organisms during preenrichment	7

$$\left(1 - \frac{\text{number of colonies on selective agar}}{\text{number of colonies on nonselective agar}}\right) (100\%)$$

the percentages of injured *S. oranienburg* cells frozen and stored in peas and beef were determined. Raccach and Juven[9] demonstrated that freezing *S. gallinarum* cells in a phosphate buffer produced a population of injured cells, and this percentage was further increased when the frozen cell suspension was stored up to 7 d. Ray et al.[10] demonstrated that fast freezing and slow thawing of *S. anatum* cells resulted in injury to more than 90% of the cells. When allowed to recover in a rich medium, such as tryptic soy broth with 0.3% yeast extract or in a glucose-citrate-salts minimal medium, most of the injured cells were repaired within 1 to 2 h at 25°C, but repair was greater in the rich medium than in the minimal one. Phosphate or citrate at concentrations found in the minimal medium supported the repair of some of the injured cells, and this repair was further enhanced by magnesium sulfate.

A second definition of cell injury is that of Straka and Stokes,[11] who defined injured cells as those requiring nutrient supplementation. Using this definition, Gomez et al.[12] determined the percentages of injured cells by the formula

$$\left(1 - \frac{\text{number of colonies on minimal agar}}{\text{number of colonies on agar with nutrient supplementation}}\right) (100\%)$$

Populations of *S. typhimurium* cells injured by freezing or freezing and storage were enumerated by using trypticase soy (TS) agar with 0.5% yeast extract or M-9 agar, a modification of the minimal, chemically defined medium of Adams.[13]

Rather than defining cell injury by the inability to grow on selective media or the need for nutrient supplementation, a third characteristic, extension of the lag phase, has been associated with the metabolic damage of bacterial cells. According to Sinskey and Silverman,[14] it is during the extended lag phase that injured cells repair damage and synthesize the proteins and nucleic acids needed for growth. Postgate and Hunter[15] showed that *Enterobacter aerogenes* cells that survived freezing and thawing had extended lag phases. Working with *S. typhimurium*, Mackey and Derrick[16] demonstrated that the menstruum in which the cells were frozen affected the extent of injury. Cells frozen in meat extract were less damaged than those frozen in a saline solution under otherwise identical conditions. For cells frozen in either menstruum, however, repair occurred in tryptone soya broth or agar with 0.1% sodium pyruvate.

The effect of freeze injury on the pathogenicity of *S. gallinarum* was investigated by Sorrells et al.[17] The difference in chick mortality caused by uninjured cells and cells injured by freezing was insignificant. These results are similar to those of Szturm-Rubinsten et al.,[18]

Table 2
CONDITIONS FOR INJURY AND CELL REPAIR OF *SALMONELLA*

Type of stress	Experimental conditions	Menstruum or substrate	*Salmonella* serotype	Cells injured (%)	Media or additives for resuscitation	Ref.
			Freezing			
Freezing, storage, and rapid thawing	Frozen, stored 6 weeks at −9°C, and thawed at 37°C for 5 min	Peas	*S. oranienburg*	45		8
	Frozen, stored 4 months at −9°C, and thawed at 37°C for 5 min	Beef	*S. oranienburg*	96		8
Freezing and rapid thawing	Frozen at −40°C and thawed at 45°C for 1.5 min	Butterfield's phosphate buffer	*S. gallinarum*	36		9
Freezing, storage, and rapid thawing	Frozen at −40°C, stored for 7 d at −20°C, and thawed at 45°C for 1.5 min	Butterfield's phosphate buffer	*S. gallinarum*	96		9
Freezing and slow thawing	Frozen in a dry ice-acetone bath for 10 min and thawed at 4°C for 75 min	Water	*S. anatum*	>90	Tryptic soy broth with 0.3% (wt/vol) yeast extract or glucose-citrate minimal broth	10
Freezing and rapid thawing	Submerged in liquid nitrogen for 5 min and thawed at 37°C for 2 min	0.067 *M* phosphate buffer	*S. typhimurium*	13		12
Freezing, storage, and rapid thawing	Frozen in still air freezer at −20°C for 16 h and thawed at 37°C for 2 min	0.067 *M* phosphate buffer	*S. typhimurium*	33		12
	Frozen in polyethylene glycol bath at −10°C, stored for various periods, and thawed at 37°C	0.05% (wt/vol) NaCl	*S. typhimurium*	3.3[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16

	Frozen in polyethylene glycol bath at −10°C, stored for various periods, and thawed at 37°C	Meat extract in distilled water	*S. typhimurium*	1.3[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16
Freezing and rapid thawing	Frozen in dry ice-acetone bath at −75°C and thawed at 37°C for 4 min	Distilled water	*S. gallinarum*	29		17
Freezing, storage, and rapid thawing	Frozen in dry ice-acetone bath at −75°C, stored 1 d at −20°C, and thawed at 37°C for 4 min	Distilled water	*S. gallinarum*	42		17
Freezing and slow thawing	Frozen in dry ice-acetone bath for 10 min and thawed at 4°C for 45 min	Reconstituted nonfat dry milk	*S. cubana*	25		19
			S. senftenberg	17		
			S. infantis	16		
			S. anatum	15		
			S. derby	13		
			S. typhimurium	10		
			S. heidelberg	10		
	Frozen in dry ice-acetone bath for 10 min and thawed at 4°C for 45 min	Reconstituted nonfat dry milk	*S. anatum*	64[b]	0.12% Nonfat milk solids	20
				26[b]	1.2% Nonfat milk solids	
				15[b]	12.2% Nonfat milk solids	
				16[b]	17.7% Nonfat milk solids	
				10[b]	25.0% Nonfat milk solids	
	Frozen in dry ice-acetone bath for 10 min and thawed at 4°C for 45 min	Water	*S. anatum*	88[b]	Same as menstruum	
		Beta-lactoglobulin		73[b]	Same as menstruum	
		Whey protein plus milk salts		63[b]	Same as menstruum	
		Sodium caseinate		42[b]	Same as menstruum	
		Skim milk		22[b]	Same as menstruum	
		Whey		22[b]	Same as menstruum	
		10% nonfat milk solids		20[b]	Same as menstruum	
		Whole milk		16[b]	Same as menstruum	

Table 2 (continued)
CONDITIONS FOR INJURY AND CELL REPAIR OF *SALMONELLA*

Type of stress	Experimental conditions	Menstruum or substrate	*Salmonella* serotype	Cells injured (%)	Media or additives for resuscitation	Ref.
			Drying			
Drying	Dried at 20°C in an open container	Bovine albumin	*S. typhimurium*	2—3[c]	Tryptone soya broth or agar with 0.3% (wt/vol) yeast extract and 0.1% (wt/vol) sodium pyruvate	24
			Freeze-Drying			
Freeze-drying	Frozen at −40°C for 12 h and freeze-dried at a platen temp of 83°C	2% (wt/vol) Gelatin solution with 0.5% (wt/vol) nutrient broth	*S. typhimurium*	24		27
	Frozen at −40°C for 12 h and freeze-dried at a platen temp of 83°C	2% (wt/vol) Gelatin solution with 5% (wt/vol) glucose	*S. typhimurium*	65		27
	Frozen at −40°C for 12 h and freeze-dried at a platen temp of 83°C	2% (wt/vol) Gelatin solution with 0.5% (wt/vol) nutrient broth and 5% (wt/vol) glucose	*S. typhimurium*	71		27
	Frozen at −40°C overnight and freeze-dried at a platen temp of 49°C	Liquid egg	*S. typhimurium*	44		28
	Frozen at −40°C overnight and freeze-dried at a platen temp of 83°C	Liquid egg	*S. typhimurium*	65		28
	Frozen at −40°C overnight and freeze-dried at a platen temp of 49°C	Beef	*S. typhimurium*	40		28
	Frozen at −40°C overnight and freeze-dried at a platen temp of 83°C	Beef	*S. typhimurium*	34		28

	Frozen in dry ice-acetone bath for 10 min and freeze-dried for 40 h at room temp	Reconstituted nonfat dry milk	*S. anatum*	70	Rehydration in water with and without glycerol, lactose, sucrose, or milk solids	29
Freeze-drying and storage	Frozen in dry ice-acetone bath for 10 min, freeze-dried for 40 h at room temp, and stored 24 h at 25°C	Reconstituted nonfat dry milk	*S. anatum*	90—95	Rehydration in water with and without glycerol, lactose, sucrose, or milk solids	29
Freeze-drying	Frozen in dry ice-acetone bath for 10 min and freeze-dried for 40 h at room temp	Reconstituted nonfat dry milk	*S. anatum*	94 (91)[d]	Dinitrophenol, 75 μg/ml	30
				94 (46)[d]	Penicillin, 100 IU/ml	
				87 (36)[d]	Tetracycline, 25 μg/ml	
				88 (32)[d]	Chloramphenicol, 100 μg/ml	
				90 (31)[d]	Water	
				92 (19)[d]	Dodecyl sulfate, 0.01%	
				76 (11)[d]	Desoxycholate, 0.25%	
	Frozen at −40°C for 15 h in an air-blast freezer and freeze-dried at a platen temp of 49°C	2% (wt/vol) Gelatin solution	*S. typhimurium*	20	Pyruvate, hematin, and menadione	31
			Heat			
Heat	Heated at 48°C for 30 min	100 m*M* phosphate buffer	*S. typhimurium*	83[e]	Trypticase soy broth, nutrient broth, lactose broth, lauryl tryptose broth, selenite F broth, and tetrathionate broth	32
				81[f]		
				80[g]		
				77[h]		
				69[i]		
				39[j]		
				36[k]		
				2[l]		
				2[m]		
	Heated at 48°C for 30 min	100 m*M* phosphate buffer	*S. typhimurium*	>95	Trypticase soy broth	33
	Heated at 48°C for 30 min	100 m*M* phosphate buffer	*S. typhimurium*	99.9	Citrate salts minimal medium	34—36
	Heated at 48°C for 10 min	0.1 *M* potassium phosphate buffer	*S. typhimurium*	30	Magnesium, spermine, or sucrose in menstruum	37

Table 2 (continued)
CONDITIONS FOR INJURY AND CELL REPAIR OF *SALMONELLA*

Type of stress	Experimental conditions	Menstruum or substrate	*Salmonella* serotype	Cells injured (%)	Media or additives for resuscitation	Ref.
	Heated at 48°C for 30 min	0.1 *M* potassium phosphate buffer	*S. typhimurium*	86	Magnesium, spermine, or sucrose in menstruum	37
	Heated at 48°C for 60 min	0.1 *M* potassium phosphate buffer	*S. typhimurium*	98	Magnesium, spermine, or sucrose in menstruum	37
	Heated at 52°C[n]	0.1 *M* potassium phosphate buffer	*S. typhimurium*	9.7[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16
	Heated at 60°C[n]	0.5% (wt/vol) Meat extract in distilled water	*S. typhimurium*	11.0[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16
	Heated at 57°C[n]	0.5% (wt/vol) Meat extract in distilled water	*S. typhimurium*	8.6[a]	Tryptone soya broth agar with 0.1% (wt/vol) sodium pyruvate	16
	Heated at 52°C[n]	0.5% (wt/vol) Meat extract in distilled water	*S. typhimurium*	4.9[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16
	Heated at 48°C for 30 min	0.1 *M* potassium phosphate buffer	*S. typhimurium*	1.6—3.6 $\log_{10}$ units[o]	Lactate, mannitol, alpha-glycerophosphate, tryptic soy broth, proteose peptone, and plate count agar	38
			Other			
Acid	Exposed to 0.2 *N* acetic acid for 4 min	Saline solution with 0.5% (wt/vol) sucrose	*S. bareilly*	90%	0.5% (wt/vol) casamino acids, 0.5% (wt/vol) thiotone, brain heart infusion broth, lactose broth, nutrient broth, or tryptic soy broth	39
	Exposed to 0.3 *M* sodium acetic acid buffer at 37°C	Peptone-NaCl maintenance medium	*S. typhimurium*	9.7[a]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	16

Gamma radiation	Exposed to gamma radiation from ^{60}Co source at dose rate of approximately 160 krad/h	0.1 *M* potassium phosphate buffer	*S. typhimurium*	1.8[c]	Tryptone soya broth or agar with 0.1% (wt/vol) sodium pyruvate	24

[a] Extension of lag phase (h).
[b] Percentage of injured cells following resuscitation in the indicated menstruum.
[c] Duration of lag phase.
[d] Percentages of injured cells rehydrated with indicated antimicrobial agent immediately after freeze-drying; in parentheses are the percentages of injured cells after 1 h rehydration with indicated antimicrobial agent.
[e] Determined on MacConkey agar.
[f] Determined on Endo agar.
[g] Determined on bismuth sulfite agar.
[h] Determined on desoxycholate agar.
[i] Determined on brilliant green agar.
[j] Determined on eosin methylene blue agar.
[k] Determined on *Salmonella-Shigella* agar.
[l] Determined on eosin methylene blue agar plus 2% (wt/vol) NaCl.
[m] Determined on desoxycholate citrate agar.
[n] Duration of heat treatment not specified.
[o] For 15 compounds or for 8 complex media added to Levine's eosin methylene blue agar with 2% (wt/vol) NaCl, the mean level of injury ranged from 1.6 to 3.6 log_{10} units. Injury was defined as the logarithmic difference between counts on Levine's eosin methylene blue agar with and without 2% (wt/vol) NaCl.

who reported that *Shigella sonnei*, frozen in ice used in a patient's food, was responsible for an outbreak of dysentery.

Janssen and Busta[19] found significant differences in the percentages of injured cells of various *Salmonella* serotypes that had been frozen and thawed in reconstituted nonfat dry milk. The effect of the concentration of milk solids in the freezing and thawing menstruum on the injury of *S. anatum* was also studied.[20] The percentages of injured cells decreased as the concentration of milk solids increased. In another study, Janssen and Busta[21] froze and thawed *S. anatum* cells in a variety of milk and milk components. The protection against freeze injury given by many of these components is in agreement with Mazur's finding[22] that several substances in milk, such as proteins and lactose, prevented freeze and thaw injury in bacteria. Moreover, Davies[23] suggested that complex substances protect the bacterial cell from injury by regulating the sudden influx of water back into the cell during thawing.

2. Injury Due to Drying

For dry-injured *S. typhimurium* cells, Mackey and Derrick[24] observed short lag times, indicating mild injury, and that only short periods were needed for repair. van Schothorst and van Leusden[25] cautioned, however, that bacterial cultures are heterogeneous populations that contain individual cells with various degrees of injury. Cells sustaining the least amount of injury would be the first to repair and begin multiplication. Thus, measured lag times on any given bacterial population may underestimate the time needed for all injured cells to complete repair. van Schothorst and van Leusden[25,26] demonstrated that storage itself affected the recovery of dry-injured *S. utrecht* cells. In freshly prepared dried milk powder, injured cells recovered their resistance to the selective enrichment, tetrathionate broth, within 6 h. Upon storage of the powder, however, the repair time increased up to 18 h.

3. Injury Due to Freeze-Drying

Sinskey et al.[27] demonstrated that the menstruum used for freeze-drying *S. typhimurium* cells affected the extent of injury. A 0.5% concentration of nutrient broth added to a 2% gelatin solution gave the greatest protection against injury when the cells were freeze-dried at a platen temperature of 83°C. Substantially less protection was given by a 5% concentration of glucose added with and without nutrient broth to the gelatin solution. When the effects of two platen temperatures, 49 and 83°C, were determined for the injury of *S. typhimurium* freeze-dried in liquid eggs and beef, inconsistent results were obtained.[28]

The extent of injury of *Salmonella* cells freeze-dried in reconstituted nonfat dry milk was studied by Ray et al.[29] The lag phase of rehydrated freeze-dried cells was about 1 h longer than that of unstressed cells. A higher percentage of injury was observed in freeze-dried *S. anatum* cells that had been stored 24 h at 25°C than in freeze-dried cells that had not been stored. Rehydration of freeze-dried cells in solutions of glycerol, lactose, sucrose, or milk solids resulted in recoveries higher than those of cells rehydrated with water. When a 10% concentration of milk solids was present in the recovery medium, however, repair did not increase by the addition of other nutrients. It is possible that the milk may have supplied all of the nutrients needed for repair, and thereby masked any potential beneficial effect of one or more supplements.

In another study, Ray et al.[30] studied the effects of various antibiotics and other antimicrobial compounds on the repair of *S. anatum* cells that had been freeze-dried in reconstituted nonfat dry milk. The repair of injured cells was almost completely inhibited in the presence of dinitrophenol, which interferes with the synthesis of adenosine triphosphate through oxidative phosphorylation. Thus, the repair of these freeze-injured *S. anatum* cells required energy in the form of adenosine triphosphate. The repair of injury in the presence of antimicrobial compounds that inhibit the synthesis of other cellular components was not inhibited to this extent.

The effect of specific metabolites in the resuscitation medium was investigated by Lewicki and Silverman.[31] They observed that after freeze-drying *S. typhimurium* cultures, a certain portion of the cells required specific metabolites, which were not required by the uninjured cells. Increased cell recovery was observed when pyruvate, hematin, or menadione was added to the recovery medium. These results are similar to those of Sinskey et al.,[28] who demonstrated that freeze-dried microorganisms have an increased nutritional requirement as evidenced by their inability to grow on a minimal medium.

4. Heat Injury

The exposure of *S. typhimurium* cells to heating at 48°C for 30 min produced injured cells with different recovery rates that were dependent upon the selective plating agar used.[32] Injured cells were able to recover in various preenrichment media (TS, nutrient, lactose, and lauryl tryptose broths) and, to a lesser extent, in selective enrichment media (tetrathionate and selenite F broths). Using these same conditions for thermal injury, i.e., heating at 48°C for 30 min, it was shown that the recovery process was dependent upon the biosynthesis of lipids,[33] ribosomal ribonucleic acid,[34,35] adenosine triphosphate,[35] and new protein,[35] and on the function of selected enzymes of the tricarboxylic acid cycle.[36]

The effect of heating *S. typhimurium* in a phosphate buffer at 48°C for various periods was demonstrated by Lee and Goepfert.[37] Heating the cells in the buffer was more injurious to them than heating them in water. Although the buffer concentration played a significant role in inducing heat injury, there was no direct correlation between the buffer concentration and the degree of injury. The presence of magnesium, spermine, and sucrose in the heating menstruum each offered various degrees of protection against thermal injury.

Mackey and Derrick[16] compared the injury in *S. typhimurium* heated in phosphate buffer with that in an aqueous meat-extract suspension. The meat extract afforded some protection against injury since shorter lag times were noted for cells heated at 52°C in this suspension. As expected, the lag times of cells heated in the meat extract increased as the heating temperature increased to 60°C. In one instance, the measured lag time of low numbers of bacteria from the same population of heat-stressed cells varied from 16 to 70 h. The authors concluded that the use of a uniform resuscitation period, especially if abbreviated, may cause *Salmonella* to be unobserved in some food samples.

The effect of 32 compounds added to Levine's eosin methylene blue (EMB) agar with 2% sodium chloride for recovering heat-injured *S. typhimurium* was determined by D'Aoust.[38] Lactate, mannitol, and alpha-glycerophosphate were each able to recover more than 90% of the injured cells. Moreover, similar levels of recovery were given when this selective agar was supplemented with 1% concentrations of tryptic soy broth, proteose peptone, or plate-count agar.

5. Other Sources of Injury

The injury of *S. bareilly* by acetic acid increased with an increase in the acid concentration from 0.03 to 0.2 *N* and with an increase in the acid treatment temperature from 10 to 30°C.[39] The effects of various inhibitory compounds on this recovery indicated that protein and ribonucleic acid synthesis, as well as electron transport, were needed for the recovery of these injured cells.

The effects on *S. typhimurium* of sublethal injury by freezing,[16] drying,[24] and heating[16] as reported by Mackey and Derrick has been discussed previously. Two other forms of injury, acidification[16] and gamma radiation,[24] have been studied by these same investigators. After equivalent treatments by each of these five sources of injury, the duration and/or extension of the lag times, and hence, the repair times, were longer for cells injured by freezing, heating, or acidification than for cells injured by drying or gamma radiation. Accordingly, the authors concluded that the choice for an optimal recovery period for injured

Salmonella organisms from food was dependent upon the knowledge of the processing background of that food.

B. Preenrichment

1. Specific Media

A great deal of effort has gone into the development of optimal media and conditions for the recovery of *Salmonella* and other organisms from foods. Dijkman,[40] however, raised a somewhat unconventional question in an article entitled "The optimal medium — always the best choice?". If an optimal medium is defined as one which gives the most characteristic growth or the best recovery of the desired organism and inhibits unwanted types, then Dijkman answers the question with a clear "no" for two reasons. First, microorganisms are subject to changes, which means that the optimal medium of today may be useless tomorrow. Second, a medium is developed by a person or persons for a specific purpose. Specific conditions are established for the use of that optimal medium and, as is often the case, other persons using the medium give little attention to the specific circumstances that prevailed during the development of the medium. As a result, contradictory reports are given on the performance of the original optimal medium, and these conflicting reports are in turn responsible for the generation of additional media, which are invariably claimed to be optimal.

Although the issues raised by Dijkman have some basis for consideration, there has to be a compilation of media and procedures, not necessarily optimal or "the best", to be used as a reference point or standard. Such a compilation is found in the *Bacteriological Analytical Manual*[41] (*BAM*) of the Food and Drug Administration (FDA). Although the *BAM* procedures for *Salmonella* recovery have been used as a standard in numerous comparisons with alternative procedures, it would be presumptuous on the part of this author to claim that the *BAM* procedures are optimal in all cases. It would be more realistic to consider the *BAM* procedures as those that have given relatively higher recovery and have proven effective to the FDA in enforcing the provisions of the Federal Food, Drug, and Cosmetic Act.

The specific media used in the initial isolation step, as listed in the *BAM*, are shown in Table 3. Lactose broth is the most commonly used preenrichment medium, and the concept of preenrichment in this medium is usually credited to North.[42] Because most *Salmonella* organisms do not utilize lactose carbohydrate, the beef extract and peptone contained in the lactose broth apparently serve as sufficient sources of energy. van Schothorst and van Leusden[26] obtained equivalent recoveries of *Salmonella* from dried milk powder preenriched in lactose broth, dulcitol broth, and a medium containing neither lactose nor dulcitol carbohydrate. It was concluded that neither the lactose nor the dulcitol nor any carbohydrate at all was important for the resuscitation of these injured *Salmonella* organisms. North[42] concluded that when a mixed flora is present, lactose carbohydrate lowers the pH, thereby controlling the non-*Salmonella* bacteria. Thus, it appears that the lactose broth does not provide specific enrichment for *Salmonella*, but rather provides a self-limiting environment for non-*Salmonella* organisms. Silliker et al.,[43] however, reported that the efficiency of preenrichment in lactose broth was impaired by unfavorable coliform-*Salmonella* ratios. Even though lactose broth is the most widely used preenrichment medium in the *BAM*, other preenrichment media are used for specific foods. The composition of various preenrichment media and their use by government regulatory agencies and other organizations have been discussed by D'Aoust.[4]

2. Minimal and Complex Media

a. Evaluation with Pure Cultures

All of the preenrichment media listed in the *BAM* may be considered complex, or nutritionally rich. In the examination of foods for *Salmonella*, the practice of preenrichment in a nutritious, nonselective medium has been universally accepted. Gomez and Sinskey[44]

Table 3
PREENRICHMENTS RECOMMENDED BY THE *BACTERIOLOGICAL ANALYTICAL MANUAL*

Food	Preenrichment medium[a]	Preparation method
Dried eggs and egg products; pasteurized liquid and frozen eggs; oral or tube feedings containing egg; infant formulas; powdered bread and pastry mixes; sodium caseinate; coconut;[b] gelatin;[c] food dyes with 10% (wt/vol) aqueous solution having pH ≥ 6	Lactose broth	Mix
Nonfat dry milk (instant); nonfat dry milk (noninstant);[d] dry whole milk[d]	Distilled water with 1% (wt/vol) brilliant green (0.45 ml solution per 225 ml preenrichment medium)	Soak[e]
Soy flour;[d] lactic casein; rennet casein	Lactose broth	Soak
Pasta (noodles, macaroni, spaghetti); egg rolls; cheese; dough; prepared salads; fresh, frozen, or dried fruits and vegetables; nut meats; crustaceans; fish; processed meats, meat substitutes, meat byproducts, animal substances, and glandular products; meals (fish, meat, and bone)	Lactose broth	Blend
Candy and candy coating; milk chocolate	Reconstituted (10%, wt/vol) nonfat dry milk with 1% brilliant green (0.45 ml solution per 225 ml preenrichment)	Blend
Frosting and topping mixes	Nutrient broth	Mix
Dried active and inactive yeast; black pepper, white pepper, celery seed or flakes, chili powder, cumin, paprika, parsley flakes, rosemary, sesame seed, thyme, and vegetable flakes	Trypticase (tryptic) soy broth	Mix
Onion flakes and powder, garlic flakes and powder	Trypticase (tryptic) soy broth containing 0.5% K_2SO_3 (wt/vol)	Mix
Allspice, cinnamon, and oregano	Trypticase (tryptic) soy broth	Mix[f]
Clove	Trypticase (tryptic) soy broth	Mix[g]
Leafy condiments	Trypticase (tryptic) soy broth	Mix[h]
Food dyes with 10% (wt/vol) aqueous solution having pH <6; nonpasteurized frozen egg products	None	Mix directly with selective enrichments
Frog legs; rabbit carcasses	Lactose broth	Shake in preenrichment; decant and examine rinsings
Raw or highly contaminated meats and meat products	None	Blend directly with selective enrichment

[a] Samples (25 g) are preenriched in 225 ml preenrichment broth. Unless otherwise specified, up to 15 25-g samples may be composited and analyzed as a single unit, provided a 1:9 sample to broth ratio is maintained.
[b] Up to 2.25 ml Tergitol® 7 or Triton® X-100 is added to initiate foaming.
[c] 5 ml 5% (wt/vol) gelatinase solution is added.
[d] Only 25 g samples may be prepared by this method. They should not be composited.
[e] Procedure consists of slowly and gently adding the sample to the surface of the preenrichment medium and leaving the sample undisturbed during incubation.
[f] A sample to broth ratio of 1:100 is used.

Table 3 (continued)
PREENRICHMENTS RECOMMENDED BY THE *BACTERIOLOGICAL ANALYTICAL MANUAL*

[g] A sample to broth ratio of 1:1000 is used.
[h] A sample to broth ratio of >1:10 is used.

questioned this practice in view of their demonstration of a higher recovery of heat-injured *Salmonella* organisms with the chemically defined, minimal M-9 medium than with TS broth with 0.5% yeast extract (TSY) (see Table 4). This occurrence was similar to the "minimal medium recovery" observed earlier by Ganesan and Smith[45,46] in radiation-damaged *Escherichia coli* organisms. Gomez and Sinskey[44] observed a marked increase in DNA single-strand breakage along with a reduction in the number of viable cells when heat-injured cultures of *S. typhimurium* were incubated in TSY broth. When these damaged cells were incubated in M-9 broth before being incubated in TSY broth, DNA strand breakage decreased in the TSY broth. This decrease was attributed to the repair of heat-induced damage.

In a subsequent study, Gomez et al.[47] demonstrated minimal medium recovery with M-9 agar rather than M-9 broth. Levels of heat-injured *S. typhimurium* cells were higher on M-9 than on TSY or brain heart infusion (BHI) agar. The addition of yeast extract or casamino acids to M-9 agar resulted in a reduction in viable counts.

In another study, Gomez and Sinskey[48] compared the counts of unstressed and rifampin-stressed *S. typhimurium* cells on M-9 and TSY agars. After exposure to rifampin, the counts were significantly lower on TSY than on M-9 agar. As with the heat-stressed cells, this reduction was due to DNA strand breakage.

Amsden et al.[49] compared the growth of *S. typhimurium* on M-9 agar, supplemented with histidine and biotin, and on TSY agar. These strains included mutants with deficiencies in DNA repair systems. The greatest differences between the colony counts on these two agars occurred during the exponential phase of growth, with greater differences for some mutant strains than for strains having normal repair capability.

Not all evaluations of the M-9 media have demonstrated an enhanced minimal medium recovery of injured *Salmonella* cultures. Mackey and Derrick[50] found no significant difference in M-9 and nutrient agars for the enumeration of unstressed and heat-stressed *S. typhimurium* cells. Mossel et al.[51] compared M-9, tryptone soya broth, and Mueller-Hinton agars for the recovery of freeze-injured *S. brandenburg* organisms. The recoveries were virtually identical on all three agars, and it was concluded that minimal medium recovery of stressed bacterial populations is not a common phenomenon. Arpai[52,53] obtained similar results and suggested that the synthetic powers of bacteria are damaged by freezing. Thus, preformed peptides, bases, and proteins contained in nutritionally rich media must be supplied to the damaged cells for growth. Conversely, Stapleton et al.[54] demonstrated that part of the total damage in irradiated *E. coli* organisms resulted from an impaired synthetic ability. This impairment was less marked with cells grown in a minimal medium before radiation than with those grown in a rich medium. Cells grown in the minimal medium were induced to synthesize a wide range of enzymes. Thus, cells grown in a rich medium may be nutritionally exacting after damage, whereas those grown in a minimal medium were able to synthesize the substances needed for growth.

Minimal media, other than M-9 medium, have been evaluated for recovering pure cultures of damaged *Salmonella* organisms. Tang and Jackson[55] obtained significantly higher counts of chill-injured *S. heidelberg* on a minimal glucose-salts agar than on TSY agar. However, Ray et al.[10] compared a similar minimal medium with TSY (0.3% yeast extract) agar for recovering freeze-injured *S. anatum*, and obtained slightly higher counts on the rich medium. Comparing a minimal glucose-salts agar with tryptic soy agar for enumerating *S. gallinarum* cells injured by freezing and thawing, Raccach and Juven[56] obtained higher counts with the

Table 4
RECOVERY OF *SALMONELLA* WITH MINIMAL AND COMPLEX MEDIA

Food	*Salmonella* serotype	Type of stress	Preenrichment or recovery medium	*Salmonella* counts per milliliter	Ref.
None	*S. typhimurium*	Heating	M-9 broth	5.0×10^{7}	44
			TSY broth	7.0×10^{5}	44
None	*S. typhimurium*	Heating	M-9 agar	1.0×10^{-1}[a]	47
			TSY agar	1.0×10^{-4}[a]	47
			BHI agar	3.2×10^{-4}[a]	47
None	*S. typhimurium*	None	M-9 agar	3.0×10^{7}	48
			TSY agar	1.8×10^{7}	48
		Exposure to rifampin	M-9 agar	4.0×10^{7}	48
			TSY agar	4.8×10^{5}	48
None	*S. typhimurium*	None	M-9 agar with histidine and biotin	1.0×10^{8}	49
			TSY agar	5.0×10^{3}	49
None	*S. typhimurium*	None	M-9 agar	7.9[b]	50
			Nutrient agar	7.9[b]	50
		Heating	M-9 agar	5.2[b]	50
			Nutrient agar	5.9[b]	50
None	*S. brandenburg*	Rapid freezing	M-9 agar	8.1[b]	51
			Tryptone soya broth agar	8.2[b]	51
			Mueller-Hinton agar with "Polyvitex"	8.0[b]	51
None	*S. heidelberg*	Chilling	Glucose-salts minimal agar	8.2[b]	55
			Tryptic soy agar with yeast extract	6.4[b]	55
None	*S. anatum*	Freezing and slow thawing	Glucose-salts minimal broth	6.5[b]	10
			Tryptic soy broth with yeast extract	6.7[b]	10
None	*S. gallinarum*	Freezing	Glucose-salts minimal agar with leucine and thiamine	1.0×10^{8}	56
			Tryptic soy agar	1.4×10^{8}	56
Skim milk powder	*S. senftenberg*	Heating	M-9 broth	27[c]	57
			Lactose broth	2[c]	57
Dried whole egg	*S. senftenberg*	Heating	M-9 broth	27[c]	57
			Lactose broth	4[c]	57

Table 4 (continued)
RECOVERY OF *SALMONELLA* WITH MINIMAL AND COMPLEX MEDIA

Food	*Salmonella* serotype	Type of stress	Preenrichment or recovery medium	*Salmonella* counts per milliliter	Ref.
Fresh egg albumen	*S. senftenberg*	Heating	M-9 broth	83[c]	57
			Lactose broth	6[c]	57
Fresh whole egg	*S. senftenberg*	Heating	M-9 broth	66[c]	57
			Lactose broth	19[c]	57
Lactic casein	*S. cerro*	None	M-9 broth	9.3×10^3	58
			Buffered peptone water	4.3×10^3	58
			Nutrient broth	9.3×10^3	58
			Lactose broth	9.3×10^3	58
			TS broth	9.3×10^3	58
Lactic casein	*S. cerro*	Heating	M-9 broth	2.3×10	58
			Buffered peptone water	4.3×10	58
			Nutrient broth	9.3×10^0	58
			Lactose broth	7.5×10	58
			TS broth	2.3×10^2	58
Milk chocolate	*S. senftenberg*	Heating	M-9 broth	6.4×10^2	59
			Buffered peptone water	2.3×10^4	59
			Lactose broth	2.3×10^2	59
			TS broth	2.3×10^4	59
			Reconstituted (10%, wt/vol) nonfat dry milk with brilliant green	4.3×10^4	59

Note: TSY, trypticase soy broth or agar with yeast extract; BHI, brain heart infusion.

[a] Surviving fraction.
[b] Log_{10} counts.
[c] Percentage of recovery.

tryptic soy agar. The addition of catalase or vitamin-free, hydrolyzed casein to the minimal agar improved recovery by 32 and 34%, respectively.

b. Evaluation with Foods

The effect of food on minimal medium recovery of heat-injured *S. senftenberg* was investigated by Wilson and Davies.[57] The four foods investigated by these authors (see Table 4) were present in a 10% concentration in the liquid holding recovery media (M-9 or lactose broth) for thermally injured organisms. Even though a higher percentage of recovery was obtained with M-9 than with lactose broth for all four foods, the efficiency of both media was quite variable and dependent upon the food present in the recovery medium. Because the authors were able to demonstrate an enhanced minimal medium recovery with cells grown in a rich medium and a minimal medium before heat injury, they questioned the hypothesis[47] that minimal medium recovery may be simply a heat-induced exacerbation of the normal sensitivity of an injured cell to a sudden change to a higher nutritional environment.

Other evaluations of chemically defined media for the recovery of *Salmonella* from foods showed no advantage of minimal medium recovery. Poelma et al.[58] did not observe a higher recovery of unstressed and heat-stressed *S. cerro* organisms from lactic casein preenriched in M-9 broth as compared with preenrichment in four nutritionally rich media. Similarly, Wilson et al.[59] demonstrated higher recoveries of heat-stressed *S. senftenberg* cultures preenriched in rich media as compared with those preenriched in M-9 broth. Investigating the recovery of a wide variety of *Salmonella* serotypes from irradiated foods, Licciardello et al.[60] observed a slightly lower recovery of *Salmonella* on glucose-salts minimal agar than on TSY agar.

3. Temperature of Incubation

A preenrichment incubation temperature of 35 to 37°C is normally used in methods for recovering *Salmonella* from foods. Unless otherwise specified, this was the preenrichment incubation temperature for all entries in the subsequent tables. However, some studies have compared the effectiveness of various incubation temperatures for rehydration and/or preenrichment. Rehydration, defined as the restoration of water that was removed from a cell during drying or freeze-drying, may be a separate, distinct analytical step or it may be a function of preenrichment.

The effect of incubation temperatures on the rehydration of pure cultures of *Salmonella* was investigated by Lewicki and Silverman.[31] It was shown that the optimum temperature for the recovery of *S. typhimurium* cells that had been freeze-dried in a menstruum containing 2% gelatin ranged from 15 to 30°C, with at least a tenfold difference in the recovery of cultures rehydrated at 30°C as compared with cultures rehydrated at 45°C. Working with *S. anatum* organisms that had been freeze-dried in a menstruum of reconstituted nonfat dry milk, Ray et al.[29] observed that, although more cells were recovered by rehydrating the cultures at 15 to 25°C, growth was initiated earlier and was more rapid at 35°C.

The effect of incubation temperatures on the rehydration and/or preenrichment of foods as opposed to cultures has also been investigated. Ray et al.[61,62] compared two temperatures for the initial rehydration (25 and 45°C) and preenrichment (35 and 45°C) of dried-milk products and found that the number of *Salmonella*-positive samples was consistently higher at 45°C than at 25 (rehydration) or 35°C (preenrichment). Wells et al.[63] used a cotton-gauze swab for recovering *Salmonella* from liquid milk and reported that this method was more sensitive for swabs incubated at 43 rather than at 37°C. With samples of milk chocolate, however, Wilson et al.[59] demonstrated lower *Salmonella* MPN values in preenrichment media incubated at 43 than in those incubated at 35°C. From broiler chickens and their environment, Rigby and Pettit[64] reported an enhanced recovery of *Salmonella* from test

samples preenriched at room temperature for 7 to 10 d rather than overnight at 37°C. The presence of two variables (i.e., the period and the temperature of incubation), however, makes it impossible to determine to what extent, if any, the incubation temperature was responsible for this difference.

4. Period of Incubation

It is customary to incubate the preenrichment media of foods being analyzed for *Salmonella* for 18 to 24 h. The possibility of increasing the analytical sensitivity by extending this period, or the possibility of decreasing the analytical time by reducing the incubation time without compromising method sensitivity has been studied by several investigators.

a. Low-Moisture Foods

Taylor et al.[65] observed a slight increase in the recovery of *Salmonella* from dried egg albumen after preenrichment for 72 rather than 24 h (see Table 5). Other investigators, however, were concerned with reducing this preenrichment incubation period. With dried egg albumen, Sveum and Hartman[66] combined preenrichment and selective enrichment into a single step by using timed-release capsules, which gradually released selective agents into the nonselective basal medium. No significant difference in the recovery of *Salmonella* was observed between the timed-release capsule method and the conventional method using separate 24-h preenrichment and selective enrichment steps. In a comparison of 6- and 24-h incubation periods for a wide spectrum of low-moisture foods preenriched in various media, D'Aoust and Maishment[67] demonstrated a significant reduction in the recovery of *Salmonella* from preenrichments of the shorter period. Moreover, the components of the various preenrichment media were less significant than the incubation period for the recovery of *Salmonella*. These results are compatible with those of van Schothorst and van Leusden,[25,26] who reported that the resuscitation of injured *Salmonella* organisms in dried milk was not always completed within 6 h.

b. High-Moisture Foods

To determine whether an incubation period longer than 18 to 24 h would substantially increase the recovery of *Salmonella*, Rigby and Pettit[64] preenriched test samples from chickens and their litter for 7 to 10 d at room temperature before subculturing the samples to selective enrichments. Compared with overnight incubation at 37°C, the "delayed secondary enrichment" procedure produced more positives.

Other studies were concerned with determining the efficiency of reduced incubation periods. Using a modification of the membrane filter-disk immobilization technique of Swaminathan et al.,[68] LaRoche and Desai[69] observed significantly higher recoveries of *Salmonella* from raw whole eggs and egg albumen preenriched for 6 rather than 4 h. Sveum and Kraft[70] combined preenrichment and selective enrichment into a single step by adding selenite or iodine to a combined lactose-basal selenite broth or lactose-basal tetrathionate broth, respectively, after a 4-h incubation. No significant differences were observed in the recovery of *Salmonella* from ground beef and turkey roasts examined by the combined enrichment and conventional methods. Hawa et al.[71] preenriched rinsings from chicken carcasses for 2 h, then centrifuged and plated them on a dulcitol-novobiocin agar. This rapid method gave a substantially higher number of positives than did the conventional method, which used an 18- to 24-h preenrichment incubation. D'Aoust et al.[72] compared a 3-h preenrichment in lactose broth and a 16- to 18-h preenrichment in nutrient broth for raw shrimp. It was suggested that the higher recovery with the 16- to 18-h period was due to the incubation time rather than to compositional differences of the media, since an earlier study[67] demonstrated their equal productivity. In an expanded study of a wide variety of high-moisture foods preenriched in one of several media, D'Aoust and Maishment[67] clearly demonstrated the inferiority of a 6-h incubation period as compared with the 24-h period.

Table 5
RECOVERY OF *SALMONELLA* FROM FOODS PREENRICHED FOR VARIOUS PERIODS

Food	Preenrichment medium	% *Salmonella*-positive samples when foods were preenriched for various periods (h)[a]							Ref.
		0[b]	2—3	4	6—7	16—24	72	7—10 (d)	
Low Moisture									
Dried egg albumen	Nutrient broth					73/210 (35)	78/210 (37)		65
	Lactose broth					45/47[c,d] (96)			66
	Combined lactose broth-tetrathionate basal medium	44/47[d,e] (94)							66
Variety of low-moisture foods	Lactose broth				21/39 (54)	38/39 (97)			67
	Nutrient broth				20/39 (51)	39/39 (100)			67
	Tergitol® broth				15/39 (39)	38/39 (97)			67
High Moisture									
Raw liquid whole eggs and raw egg albumen	Lactose broth with Tergitol® 7			14/70 (20)	49/70 (70)				69
Frozen ground beef	Lactose broth					18/18[c,d] (100)			70
	Combined lactose broth-tetrathionate basal medium	18/18[d,e] (100)							70
Frozen turkey roasts	Lactose broth					11/15[c] (73)			70
	Combined lactose broth-tetrathionate basal medium	10/15[c] (67)							70
Chicken carcasses	Buffered peptone water		34/51 (67)			26/51 (51)			71

Table 5 (continued)
RECOVERY OF *SALMONELLA* FROM FOODS PREENRICHED FOR VARIOUS PERIODS

Food	Preenrichment medium	% *Salmonella*-positive samples when foods were preenriched for various periods (h)[a]							Ref.
		0[b]	2—3	4	6—7	16—24	72	7—10 (d)	
Chicken carcasses and specimens from chickens and their litter	0.1% Peptone water					282/2283 (12)		373/2283 (16)	64
Shrimp	Lactose broth		1/7[d,f] (14)						72
	Nutrient broth					6/7[d,g] (86)			72
Variety of high-moisture foods	Lactose broth				12/18 (67)	18/18 (100)			67
	Nutrient broth				12/18 (67)	18/18 (100)			67
	Tergitol® broth				12/18 (67)	18/18 (100)			67
Low and High Moisture									
Soy products	1% Proteose peptone				3475/3486[h,i] (>99)				74
	Varied with food[j]					3486/3486[k] (100)			74
Dried egg albumen, dried beef gravy, and vegetable beef soup	Lactose broth					10/11[c,d] (91)			70
	Combined lactose broth-tetrathionate basal medium	11/11[d,e] (100)							70
Dried egg albumen, pork, poultry, and meat and bone meal	Lactose broth					11/17[k] (65)			77

	Basal medium	11/17[l] (65)			77
Variety of low- and high-moisture foods	FAS broth[m]		34/347[n] (10)	52/347[o] (15)	75
Variety of low- and high-moisture foods and feeds	Modified M broth		101/106[i] (95)	106/106 (100)	

[a] Number of *Salmonella*-positive samples per total number of samples examined (%), unless otherwise specified.
[b] Does not represent the lack of a preenrichment phase, but rather a combination of preenrichment and selective enrichment phases into a single step.
[c] Lactose broth preenrichment media were incubated 24 h and then subcultured to tetrathionate brilliant green broth, which was incubated 24 h.
[d] Number of *Salmonella*-positive samples by a particular method per total number of *Salmonella*-positive samples.
[e] Just before initiation of analysis, selective ingredients were added to the combined lactose broth-tetrathionate basal medium, which was incubated 48 h.
[f] Lactose broth preenrichment media were incubated 3 h and then subcultured to selenite cystine broth, which was incubated 16 to 18 h.
[g] Nutrient broth was incubated 16 to 18 h and then subcultured to selenite cystine and tetrathionate brilliant green broths, which were incubated 16 to 18 h.
[h] Proteose peptone preenrichments were incubated 6 h and then subcultured to tetrathionate broth, which was incubated 18 h.
[i] Correctly identified as compared with the conventional culture method of the *Bacteriological Analytical Manual.*
[j] As recommended by the *Bacteriological Analytical Manual.*
[k] Lactose broth preenrichment media were incubated 24 h and then subcultured to selenite cystine and tetrathionate brilliant green broths, which were incubated 24 h.
[l] Basal medium was shaken for 4 h. Selenite cystine was added and combined preenrichment-selective enrichment media were shaken an additional 18 to 20 h.
[m] With the exception of milk products, which were incubated in brilliant green water.
[n] Confirmed positives by a rapid fluorescent antibody technique in which test portions were preenriched 7 h.
[o] Confirmed positives by conventional culture and fluorescent antibody techniques in which samples were preenriched 24 h.

c. Low- and High-Moisture Foods

Several rapid methods use abbreviated preenrichment periods for the recovery of *Salmonella*. Using a modification of the enrichment serology procedure of Sperber and Deibel,[73] Surdy and Haas[74] preenriched a variety of soy products for 6 h and accurately analyzed 99.7% of more than 3000 of them. Mohr et al.[75] and Insalata et al.,[76] however, observed a reduction of positives when abbreviated preenrichment periods were used with the fluorescent antibody technique for *Salmonella*.

In addition to the combined enrichment technique of Sveum and Kraft,[70] another method, introduced by Alford and Knight,[77] involved the combination of preenrichment and selective enrichment into a single step. After shaking in a basal medium for 4 h at 37°C, selenite and cystine are added, and the test samples are shaken an additional 20 h. Both of these combined enrichment methods gave *Salmonella* recoveries similar to those of the conventional method.

One reason for the seemingly contradictory results in Table 5 was offered by Mackey and Derrick,[24] who reported that the duration of the lag phase of injured *Salmonella* organisms was dependent upon the type of sublethal injury. Heat- and freeze-injured cells need longer repair times than cells injured by drying or gamma radiation. In addition to the type of treatment, the time needed for resuscitation may be affected by the menstruum in which the cells were injured and the methodology used for recovery.

5. Low- and High-Osmotic Environments

Although most of the damage to dried or freeze-dried cells seems to occur during rehydration,[78-80] this damage can be minimized by controlling the amount and/or the rate of the addition of diluent to the injured cells. Leach and Scott[78] reported higher counts of *Vibrio metchnikovi* cells when the cells were rehydrated by the dropwise, rather than the rapid, addition of water. Record et al.[80] reported an improved recovery of dried *E. coli* cells that were reconstituted and dialyzed with a 50% glucose solution slowly diluted with a buffer over a 6-h period. Similarly, Choate and Alexander[81] obtained optimum recovery of *Spirillum atlanticum* in a 24% sucrose solution and suggested that the sucrose molecules acted as an osmotic buffer, regulating the rehydration of these freeze-dried cells and thereby reducing rehydration damage.

The enhanced recovery of *Salmonella* from dried milk rehydrated gradually in a high-osmotic environment, resulting from a low sample to broth rehydration ratio, has been reported by several investigators (see Table 6). Ray et al.[61,62] obtained higher *Salmonella* MPN values in dried milk by rehydrating the MPN portions at an initial 1:2.5 sample to broth ratio, then diluting after 1 h to a final 1:10 sample to broth ratio, as compared with rehydrating at a 1:10 sample to broth ratio initially. Similarly, van Schothorst et al.[82] obtained the enhanced recovery of *Salmonella* from dried milk rehydrated at a sample to broth ratio of 1:2 and diluted 30 min later to a ratio of 1:9. This method was subsequently subjected to a collaborative study[83] and gave an improved recovery of *Salmonella*. The enhanced recovery of *Salmonella* from instant nonfat dry milk rehydrated by a soak procedure has been reported by Andrews et al.[84] and Poelma et al.[85] The soak procedure consisted of slowly adding the dried products to the diluent and letting them remain undisturbed during incubation. The result was a slow, gradual rehydration of the dried *Salmonella* organisms in a high-osmotic environment at the interface of the product and diluent. With noninstant nonfat dry milk and dry whole milk, the soak procedure (slow rehydration method) gave an increase in positive, 25-g test samples. However, the soak procedure was not recommended for test samples weighing more than 25 g because of the clumping of the material, even after overnight incubation.

In addition to testing with dried milk, the soak procedure has been evaluated for several related low-moisture dairy foods[86] as well as other foods.[87] The enhancement of the recovery of *Salmonella* from dried foods by the soak method is apparently not a widespread phe-

Table 6
RECOVERY OF *SALMONELLA* FROM FOODS PREENRICHED IN LOW- AND HIGH-OSMOTIC ENVIRONMENTS

Food	Preenrichment medium	%*Salmonella*-positive samples when foods were preenriched at two different rates		Limitation	Ref.
		Rapid	Slow		
Instant nonfat dry milk	Distilled water with brilliant green	16/79 (20)	62/79 (79)		84
	Distilled water with brilliant green	170/296 (57)	210/296 (71)		85
Noninstant nonfat dry milk	Distilled water with brilliant green	6/40 (15)	8/40 (20)	Not recommended for sample sizes >25 g	86
Dried skim milk powder[b]	Buffered peptone water	234/350 (67)	327/350 (93)		83
Dried skim milk powder[c]	Buffered peptone water	160/350 (46)	285/350 (81)		83
Dried condensed milk	Buffered peptone water	43/125 (34)	109/125 (87)		82
Dry whole milk	Distilled water with brilliant green	42/120 (35)	57/120 (48)	Not recommended for sample sizes >25 g	86
Dried milk products (nonfat dry milk, dry whole milk, and dry buttermilk	Lactose broth	31/81 (38)	33/81 (41)		61
	Lactose broth or distilled water with brilliant green	284/1080 (26)	357/1080 (33)		62
Lactic casein	Lactose broth	56/140 (40)	66/140 (47)		86
Rennet casein	Lactose broth	91/140 (65)	111/140 (79)		86
Sodium caseinate	Lactose broth	73/80 (91)	39/80 (49)		86
Brewers' yeast	Lactose broth	92/120 (77)	80/120 (67)		87
Dried active yeast	TS broth	42/60 (70)	8/60 (13)		87
Soy flour	Lactose broth	37/60 (62)	47/60 (78)	Not recommended for sample sizes >25 g	87
Onion powder	TS broth with final concentration of 0.5% K_2SO_3	84/140 (60)	40/140 (29)		87

[a] Number of *Salmonella*-positive samples per total number of samples examined.
[b] Samples stored for 6 months at 4°C.
[c] Samples stored for 6 months at room temperature.

nomenon, but is specific and dependent upon the food being examined. One major contributing factor to the success of the soak procedure is the physical nature of the food. Foods favorably examined by the soak procedure may have been provided with a slow, steady rate of rehydration of the *Salmonella* cells, thereby minimizing or reducing cell damage and/or death by osmotic shock.

C. Selective Enrichment

1. Specific Media

Selective enrichment media for *Salmonella*, under ideal conditions, allow the proliferation of this organism while restricting, or preferably inhibiting, the growth of competing organisms. In practice, however, this condition does not always prevail. A commonly used selective enrichment medium is selenite broth, originally formulated by Leifson.[88] North and Bartram[89] added cystine to this medium for improved *Salmonella* recovery in the presence of a large amount of organic material. Another amino acid, methionine, was shown to increase the toxicity of selenite to *E. coli*.[90] Other modifications affecting the selectivity of selenite broth have been the addition of brilliant green dye,[91,92] sulfapyridine,[91] gentian violet,[93] sterilized feces,[94] and yeast extract,[91,92,95] and the substitution of lactose with mannitol[91,92] or dulcitol.[95]

Another widely used selective enrichment medium has been tetrathionate broth, which was originally formulated by Mueller.[96] Like selenite broth, this medium also has undergone numerous modifications. The Kauffmann[97,98] modification with brilliant green dye and bile salts is widely used, but has been reported to be inhibitory for some *Salmonella* serotypes.[4,99-101] Other modifications have included the addition of sodium lauryl sulfate,[102] sodium sulfathiazole,[103] Tergitol®,[104,105] and novobiocin.[106]

A comparison of liquid and solid versions of selenite and tetrathionate media, as well as nutrient media, for determining the repair of heat-injured *Salmonella typhimurium* cells was made by Mackey and Derrick.[50] All solid media, including nutrient agar, were more inhibitory to the injured cells than the analogous liquid media. Thus, the physical state of the medium, i.e., solid or liquid, was just as significant as its composition for the recovery of injured cells. Pyruvate or catalase added to nutrient agar increased the counts to the level of those obtained in nutrient broth. Both of these agents are able to react with or catalyze the decomposition of the toxic substance, hydrogen peroxide. Thus, it is possible that peroxide is more inhibitory to cells on solid media than in liquid media because of the more limited diffusion away from the cellular site of production. A second possibility is that there may be a higher level of endogenous peroxide in agar media than in broth media.

Other selective enrichment media used for isolating *Salmonella* include gram-negative (GN) broth,[107] magnesium chloride-sodium cholate media,[108] modified lysine-iron-cystine-neutral red broth,[109] strontium chloride and strontium selenite media,[100,111] and Ruys' medium.[112] Rappaport et al.[113] introduced an enrichment medium containing magnesium chloride and malachite green as the selective agents. Two modifications were subsequently introduced by Vassiliadis et al.[114] in the composition and use of Rappaport's medium. One modification reduced the concentration of malachite green; a second incubated the medium at 43 rather than 37°C. The modified medium, known as Rappaport-Vassiliadis enrichment medium, was used successfully for the isolation of *Salmonella* from meats and meat products.[115-121]

2. Temperature of Incubation

The incubation of selective enrichment media at temperatures higher than 35 to 37°C has been recommended by several investigators. The efficiency of elevated-temperature incubation, however, is dependent upon the type of food being examined. Gabis and Silliker[122,123] did not observe any significant difference in the recovery of *Salmonella* from low-moisture foods when the selenite cystine and tetrathionate broth enrichments were incubated at 35 and 43°C (see Table 7). Moreover, the incubation at 43°C of media containing tetrathionate has been reported to be toxic to some *Salmonella* strains.[124,125]

With high-moisture foods, several investigations have clearly demonstrated the advantage of incubating the selective enrichment media at 42 to 43°C.[126-134] Based on preliminary data, however, Smyser et al.[132] cautioned that the incubation of selective enrichments at temperatures greater than 43°C was lethal for *Salmonella* organisms.

Table 7
RECOVERY OF *SALMONELLA* FROM FOODS ENRICHED AT VARIOUS TEMPERATURES

Food	Preenrichment medium	Selective enrichment medium	% *Salmonella*-positive samples[a]		Ref.
			35—37°C	42—43°C	
Low Moisture					
Dried dairy foods	Distilled water with brilliant green	SC	52/848 (6)	52/848 (6)	122
	Distilled water with brilliant green	TBG	52/848 (6)	60/848 (7)	122
Dried soya products	Lactose broth	SC	33/332 (10)	27/332 (8)	122
	Lactose broth	TBG	32/332 (10)	33/332 (10)	122
Low-moisture foods and feed ingredients	Varied with food	SC	42/98 (43)	40/98 (41)	123
	Varied with food	TBG	38/98 (39)	39/98 (40)	123
High Moisture					
Raw liquid egg products	Buffered peptone water	SC	14/55 (26)	30/55 (55)	134
	Buffered peptone water	Greenwood broth	22/55 (40)	30/55 (55)	134
	Lactose broth	SC	14/100 (14)	25/100 (25)	133
	Buffered peptone water	SC	9/100 (9)	24/100 (24)	133
Ground-meat filtrate	None	S	35/124 (28)	42/124 (34)	127
	None	TBG	45/124 (36)	55/124 (44)	127
Pork sausage	None	TBG with Tergitol®	17/68 (25)	18/68 (27)	129
	None	TBG without Tergitol®	8/68 (12)	22/68 (32)	129
Raw chicken	Nutrient broth	SC	6/45 (13)	28/45 (62)	130
	Nutrient broth	TBG	20/45 (44)	26/45 (58)	130
	Nutrient broth	RAP	23/45 (51)	31/45 (69)	130
Frozen raw meats	Lactose broth with Tergitol®	SC	67/120 (56)	81/120 (68)	131
	Lactose broth with Tergitol®	Selenite brilliant green sulfa	72/120 (60)	80/120 (67)	131
	Lactose broth with Tergitol®	TT	56/120 (47)	71/120 (59)	131
	Lactose broth with Tergitol®	TBG	56/120 (47)	76/120 (63)	131
Abattoir samples	None	S	53/501 (11)	105/501 (21)	126
Oysters	None	SC	1/30 (3)	25/30 (83)	128
	None	TT	9/30 (30)	9/30 (30)	128

Table 7 (continued)
RECOVERY OF *SALMONELLA* FROM FOODS ENRICHED AT VARIOUS TEMPERATURES

Food	Preenrichment medium	Selective enrichment medium	% *Salmonella*-positive samples[a]		Ref.
			35—37°C	42—43°C	
	Lactose broth	SC	0/30 (0)	3/30 (10)	128
	Lactose broth	TT	0/30 (0)	2/30 (7)	128
		Low and High Moisture			
Rendered marine by-products	None	TBG	89/340 (26)	104/340 (31)	132

Note: SC. selenite cystine broth; TBG, tetrathionate brilliant green broth; RAP, Rappaport broth; TT, tetrathionate broth; and S, selenite F broth.

[a] Number of *Salmonella*-positive samples per total number of samples examined, when selective enrichment broths were incubated at indicated temperatures (°C).

It is not certain why elevated incubation temperatures favor selective enrichments of high-moisture foods and not those of low-moisture foods. Presumably, any injured cells in both low- and high-moisture foods are fully recovered at the end of the preenrichment period and should have multiplied to levels that would readily adapt to the transition from a nonselective preenrichment medium to a selective enrichment medium. Thus, the influence of temperature on the growth rates of *Salmonella* organisms in selective enrichment media does not appear to be a contributing factor for the superiority of an incubation temperature of 42 to 43°C. A more logical explanation may reside in the different nature and levels of the associated microflora in low- and high-moisture foods. Unprocessed and/or raw, high-moisture foods such as those listed in Table 7 are likely to contain relatively high levels of competing organisms not belonging to the genus *Salmonella*. It is possible that the selectivity of selenite cystine broth, tetrathionate broth, and other selective media is enhanced against these competing organisms at the elevated temperature. Because processed low-moisture foods would be less likely than high-moisture foods to contain a large population of competing organisms, there would be less opportunity for the increased selectivity and, hence, performance, of the enrichment media to be demonstrated.

3. Period of Incubation

Selective enrichment media are usually incubated for 24 h. However, the relative productivity of other incubation periods has been studied by several investigators (see Table 8).[118,127,129,134-141] As in Table 5, with various incubation periods of the preenrichment step, the results shown in Table 8 are no less variable. The type of food being examined, the kind of processing to which the food was subjected, the particular selective enrichment medium used, the incubation temperatures, the particular *Salmonella* serotype(s) involved, and the level of contamination of both *Salmonella* and competing organisms may all play a role in determining the optimal incubation period of the selective enrichment media.

The selection of a particular selective enrichment incubation period may be as much an administrative as a scientific decision (see Table 8). If the intent is to select a method of

Table 8
RECOVERY OF *SALMONELLA* FROM FOODS ENRICHED FOR VARIOUS PERIODS

Food	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	%*Salmonella*-positive samples (h)[a]						Ref.
				8	24	48	72	96	120	
Low Moisture										
Dried egg albumen	None	SC	37		25/50 (50)	36/50 (72)				141
	None	TBG	37		2/50 (4)	2/50 (4)				141
	None	SC	37		4/40 (10)		5/40 (13)			141
	None	TBG	37		4/40 (10)		6/40 (15)			141
Milk chocolate and cocoa products	Reconstituted skim milk powder (10% wt/vol) with brilliant green	SC	35		10/152 (7)	9/152 (6)				136
Reconstituted skim milk powder (10%, wt/vol) with brilliant green		TBG	43		10/152 (7)	10/152 (7)				136
Dry whole milk, nonfat dry milk, whey powder, egg powder, cocoa powder, and milk chocolate	Sterile water with brilliant green for dry whole milk and nonfat dry milk; sterile nonfat milk with brilliant green for all others	TT	43	24/27 (89)	27/27 (100)					139
	Sterile water with brilliant green for dry whole milk and nonfat dry milk; sterile nonfat milk with brilliant green for all others	RAP	43	27/27 (100)	27/27 (100)					139

Table 8 (continued)
RECOVERY OF *SALMONELLA* FROM FOODS ENRICHED FOR VARIOUS PERIODS

Food	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	%*Salmonella*-positive samples (h)[a]						Ref.
				8	24	48	72	96	120	
High Moisture										
Raw liquid egg products	Buffered peptone water	SC	37		9/55 (16)	13/55 (24)				134
	Buffered peptone water	SC	43		29/55 (53)	29/55 (53)				134
	Buffered peptone water	TT	43		28/55 (51)	33/55 (60)				134
Ground-meat filtrate	None	S	37		35/124 (28)	41/124 (33)				127
	None	S	43		42/124 (34)	31/124 (25)				127
	None	TBG	37		45/124 (36)	42/124 (34)				127
	None	TBG	43		55/124 (44)	73/124 (59)				127
Minced meat[b]	None	TBG	43		214/913 (23)	459/913 (50)				137
	Buffered peptone water	TBG	43		767/913 (84)	858/913 (94)				137
Minced meats[c]	None	TBG	43		58/152 (38)	83/152 (55)				137
	Buffered peptone water	TBG	43		86/152 (57)	102/152 (67)				137
Pork sausage	None	TBG with Tergitol®	37		12/68 (18)	17/68 (25)				129
	None	TBG with Tergitol®	43		15/68 (22)	18/68 (27)				129
	None	TBG without Tergitol®	37		7/68 (10)	6/68 (9)				129

	None	TBG without Tergitol®	43		21/68 (31)	21/68 (31)				129
Poultry carcasses	None	SC	37	217/220[d] (99)	220/220[d] (100)					135
Meat products (pork sausages, chicken carcasses, bovine minced meat, and pig mesenteric glands)	Buffered peptone water	TT-SMM[e]	43		(5.1)[f]	(14.5)[f]	(4.6)[f]	(1.8)[f]	(0.7)[f]	118
	Buffered peptone water	RAP-V-SMM[g]	43		(2.6)[f]	(26.2)[f]	(4.0)[f]	(0.4)[f]		118
Water	Buffered peptone water	RAP-V	43		70/247 (28)	72/247 (29)				138
		Low and High Moisture								
Variety of foods, feeds, and environmental materials	Buffered peptone water	Selenite brilliant green-sulfa	42		117/211 (56)	116/211 (55)				140
	Buffered peptone water	Selenite brilliant green-sulfa-SMM[h]	42		113/211 (54)	91/211 (43)				140
	Buffered peptone water	TT	42		137/211 (65)	157/211 (74)				140
	Buffered peptone water	TT-SMM[h]	42		168/211 (80)	173/211 (82)				140

Note: SC, selenite cystine broth; TBG, tetrathionate brilliant green broth; TT, tetrathionate broth; RAP, Rappaport broth; and S, selenite F broth.

[a] Number of *Salmonella*-positive samples per number of samples examined, unless otherwise specified.
[b] Artificially contaminated.
[c] Naturally contaminated.
[d] Number of *Salmonella*-positive samples per total number of *Salmonella*-positive samples.
[e] TT was incubated at 43°C; at 48 h a subculture was made to the secondary selective enrichment medium (SMM) of Harper and Shortridge (*J. Hyg.*, 67, 181, 1969). Tubes of SMM medium were incubated at 37°C and examined daily for growth of *Salmonella*. Values indicate relative *Salmonella* productivities of SMM medium incubated for various periods.
[f] Percentage of SMM tubes positive for *Salmonella*.
[g] RAP-V, Rappaport-Vassiliadis broth, was incubated at 43°C; at 24 h a subculture was made to SMM medium. Tubes of SMM medium were incubated at 37°C and examined daily for a potential growth of *Salmonella*. Values indicate relative *Salmonella* productivities of SMM medium incubated for various periods.
[h] Selenite brilliant green sulfa and TT were both incubated at 42°C; at 24 and 48 h a subculture was made to SMM medium. Tubes were incubated at 37°C and examined daily for a potential growth of *Salmonella*. Values indicate relative *Salmonella* productivities of selenite brilliant green sulfa and TT broth incubated for 24 and 48 h.

the utmost sensitivity, with factors such as analytical expediency and expense being of secondary importance, then perhaps incubation periods of 24 h and longer should be considered. However, with the advent of new technologies for identifying *Salmonella*, e.g., hydrophobic grid-membrane filtration, enzyme-linked immunosorbent assay, and genetic probes, the emphasis has been to compare the 24-h incubation period with those of shorter duration.

D. Other Factors Affecting Preenrichment and Selective Enrichment

1. Surfactants

Surfactants have been recommended primarily as dispersing agents for both low- and high-moisture fatty foods. Ray et al.,[62] however, compared the recovery of *Salmonella* from dried milk powder, a low-moisture, nonfatty food that was preenriched in lactose broth with and without 0.04% Tergitol® 7, but observed no significant difference in recovery (see Table 9). Similarly, D'Aoust et al.[142] observed no significant differences in the recovery of *Salmonella* from a variety of low- and high-moisture fatty foods preenriched in nutrient broth with one of several added surfactants. Morris and Dunn,[129] however, observed that the incubation temperature of the selective enrichment media affected the efficiency of added Tergitol® 7. The recovery of *Salmonella* from pork sausage was improved by the addition of Tergitol® 7 when selective enrichment media were incubated at 37 but not at 43°C. Working with pure cultures, Mossel et al.[143] observed that the addition of sodium lauryl sulfate to Enterobacteriaceae enrichment medium had no significant effect on the growth of *S. oranienburg* and *S. typhimurium.*

The addition of surfactants to preenrichment and selective enrichment media is of limited usefulness (see Table 9). Surfactants should not be used indiscriminately in view of their reported toxicity to *Salmonella* under specified cultural conditions.[129,144,145]

2. Aerobic and Anaerobic Incubation

The importance of the oxidation reduction potential of the medium for the initiation of the growth of certain bacteria has long been recognized.[146-148] Quastel and Stephenson[149] suggested the use of thioglycollic acid for establishing anaerobic conditions in culture media. Subsequently, Nelson[150] demonstrated large increases in the recovery of heat-injured *E. coli* when 0.01% thioglycollic acid was present in the growth medium. In several instances the medium supplemented with thioglycollic acid gave higher counts of heat-injured bacteria than did tryptone glucose extract milk agar, a highly nutritional, complex medium. Thus, the mere presence of certain nutrients in the recovery medium may not always be the controlling factor in determining the ability of heat-injured bacteria to initiate repair. This conclusion is quite similar to that of investigators who advocate the minimal medium recovery of injured cells.[47,57]

Rather than using a supplement to alter the oxidation reduction potential of the medium, several investigators have modified environmental conditions to achieve anaerobic (and nonaerated) conditions. The recovery of *Salmonella* from foods incubated under aerated (preenrichment and/or selective enrichment media shaken) and nonaerated (not shaken) conditions has been compared in several studies (see Table 10). Ray et al.[62] observed no advantage of incubating preenrichment media of dried milk products under aerated conditions. When preenrichment media of cocoa powder were shaken, however, Busta and Speck[151] observed that with some brands the inactivation rate of *S. gallinarum* was greater than 99.9%. Agitation may have enhanced the release of one or more lethal components from the cocoa suspension; moreover, the presence of antimicrobial anthocyanin compounds in cocoa is well known. Similar results with cocoa powder were obtained by Zapatka et al.[152] Cocoa was preenriched in one of four media under aerated and static conditions. For each medium, the survival of heat-damaged *S. typhimurium* cells was higher under nonaerated

Table 9
EFFECT OF SURFACTANTS ON THE RECOVERY OF *SALMONELLA* FROM FOODS

Food	Preenrichment	Surfactant in preenrichment medium	Selective enrichment medium	Surfactant in selective enrichment medium	Incubation temp of selective enrichment medium (°C)	% *Salmonella* positive[a]	Ref.
			Low Moisture				
Dried milk powder	Lactose broth	None	TT	None	35	65/207 (31)	62
		Tergitol® 7, 0.04% (wt/vol)	TT	None	35	57/207 (28)	62
Variety of low-moisture foods	Nutrient broth	None	SC and TBG	None	35 for SC and 43 for TBG	8/8[b] (100)	142
		Arlacel® 80 plus Tween® 60, 10% (vol/vol)	SC and TBG	None	35 for SC and 43 for TBG	8/8[b] (100)	142
		Myrj® 525, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	7/8[b] (88)	142
		Tergitol® 7, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	8/8[b] (100)	142
		Triton® X-100, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	8/8[b] (100)	142
		Tween® 80, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	8/8[b] (100)	142
			High Moisture				
Pork sausage	None	NA	TBG	None	37	8/68 (12)	129
				Tergitol® 7, 0.6% (vol/vol)	37	17/68 (25)	129
			TBG	None	43	22/68 (32)	129
			TBG	Tergitol® 7, 0.6% (vol/vol)	43	18/68 (27)	129

Table 9 (continued)
EFFECT OF SURFACTANTS ON THE RECOVERY OF *SALMONELLA* FROM FOODS

Food	Preenrichment	Surfactant in preenrichment medium	Selective enrichment medium	Surfactant in selective enrichment medium	Incubation temp of selective enrichment medium (°C)	% *Salmonella* positive[a]	Ref.
Variety of high-moisture foods	Nutrient broth	None	SC and TBG	None	35 for SC and 43 for TBG	24/25[b] (96)	142
		Arlacel® 80 plus Tween® 60, 10% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	23/25[b] (92)	142
		Myrj® 525, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	25/25[b] (100)	142
		Tergitol® 7, 3% (wt/. vol)	SC and TBG	None	35 for SC and 43 for TBG	25/25[b] (100)	142
		Triton® X-100, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	25/25[b] (100)	142
		Tween® 80, 3% (wt/vol)	SC and TBG	None	35 for SC and 43 for TBG	25/25[b] (100)	142
Pure culture of *S. oranienburg*	EE broth Mossel	None	None	NA	NA	7.0[c]	143
		Sodium lauryl sulfate, 0.1% (wt/vol)	None	NA	NA	7.5[c]	143
Pure culture of *S. typhimurium*	EE broth Mossel	None	None	NA	NA 7.5[c]		143
		Sodium lauryl sulfate, 0.1% (wt/vol)	None	NA	NA 7.5[c]		143

Note: TT, tetrathionate broth; SC, selenite cystine broth; TBG, tetrathionate brilliant green broth; NA, not applicable.

[a] Number of *Salmonella*-positive samples per total number of samples examined (%), unless otherwise specified.
[b] Number of *Salmonella*-positive samples obtained by a particular method per total number of *Salmonella*-positive samples.
[c] Data expressed as the negative logarithm of the highest decimal dilution found positive.

Table 10
RECOVERY OF *SALMONELLA* FROM FOODS PREENRICHED OR ENRICHED UNDER AERATED AND NONAERATED CONDITIONS

Food or culture	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	Preenrichment (P) or selective enrichment (SE) phase studied	Condition of testing	Productivity[a]	Ref.
Dried milk products (nonfat dry milk, dry whole milk, and dry buttermilk	Lactose broth	TT	35	P	Aerated	53/234 (23)	62
					Nonaerated	61/234 (26)	62
Cocoa	TS broth	None	NA	P	Aerated	>99.9% inactivation relative to nonaerated samples	151
					Nonaerated	See productivity for aerated samples	151
	Nutrient broth	None	NA	P	Aerated	0[b]	152
					Nonaerated	2[b]	152
	Nutrient broth with 5% (wt/vol) casein	None	NA	P	Aerated	16[b]	152
					Nonaerated	50[b]	152
	Reconstituted nonfat dry milk (10% wt/vol)	None	NA	P	Aerated	21[b]	152
					Nonaerated	42[b]	152
	Lactose broth	None	NA	P	Aerated	0[a]	152
					Nonaerated	7[b]	152
Ground-meat filtrate	None	S	37	SE	Aerobic	35/124 (28)	127
					Anaerobic	47/124 (38)	127
	None	TBG	37	SE	Aerobic	45/124 (36)	127
					Anaerobic	40/124 (32)	127
	None	S	43	SE	Aerobic	42/124 (34)	127

Table 10 (continued)
RECOVERY OF *SALMONELLA* FROM FOODS PREENRICHED OR ENRICHED UNDER AERATED AND NONAERATED CONDITIONS

Food or culture	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	Preenrichment (P) or selective enrichment (SE) phase studied	Condition of testing	Productivity[a]	Ref.
					Anaerobic	52/124 (42)	127
	None	TBG	43	SE	Aerobic	55/124 (44)	127
					Anaerobic	37/124 (30)	127
Dried egg albumen, pork, poultry, and meat and bone meal	Basal medium or lactose broth[c]	SC	37	P and SE	Aerated	91/180 (51)	77
					Nonaerated	97/180 (54)	77
Pure *Salmonella* cultures challenged with natural mixed cultures from chicken carcasses	None	SC	37	SE	Aerobic	6.47[d]	153
					Anaerobic	6.88[d]	153
	None	SC	43	SE	Aerobic	8.31[d]	153
					Anaerobic	7.88[e]	153
Pure culture of *S. senftenberg*	Acidified basal medium	None	NA	P	Aerated	10^4 cells/ml[e]	154
					Nonaerated	$>10^6$ cells/ml[e]	154
Pure culture of *S. typhimurium*	TS broth with 0.5% (wt/vol) yeast extract	None	NA	P	Aerobic	7.5×10^3 [f]	155
					Anaerobic	3.2×10^7 [f]	155

Note: NA, not applicable; TT, tetrathionate broth; S, selenite F broth; TBG, tetrathionate brilliant green broth; SC, selenite cystine broth.

[a] Number of *Salmonella*-positive samples per number of samples examined (%), unless otherwise specified.
[b] Percentage survival of *S. typhimurium* in preenrichment media after 6 h.

[c] Basal medium preenrichment media were shaken (aerated) 3—4 h; selenite cystine was added and shaking was continued for 20 to 21 additional hours. Lactose broth preenrichment media were stationary (nonaerated) for 24 h and subsequently subcultured to selenite cystine broth, which was incubated for 24 h under stationary conditions.

[d] Log_{10} *Salmonella* counts per milliliter.

[e] Lowest number of cells capable of initiating growth.

[f] DNA molecular weight of heated *S. typhimurium* after incubation in preenrichment medium for 15 min under aerobic and anaerobic conditions.

conditions. The inhibitory effect of the cocoa for *Salmonella* organisms was also reduced in preenrichment media containing either casein or nonfat dry milk.

Kafel and Bryan[127] investigated the effect of the type of selective enrichment medium, the incubation temperature, and incubation in an aerobic or anaerobic atmosphere on the recovery of various *Salmonella* serotypes from ground-meat filtrate. Even though there was a statistically significant advantage with the 43°C incubation temperature, the results were not consistent with respect to the incubation atmosphere. Alford and Knight[77] combined preenrichment and selective enrichment into a single step and incubated this combined enrichment medium under aerated conditions. About 10% fewer *Salmonella* positives were obtained with this procedure than with the *BAM*[41] procedure, which includes separate preenrichment and selective enrichment steps, both of which are nonaerated.

The remaining comparisons of aerobic and anaerobic incubation were concerned with pure cultures. Because there was no significant difference in the growth of several *Salmonella* serotypes under aerobic and anaerobic conditions, Bailey et al.[153] recommended that *Salmonella* analyses continue to be performed under aerobic conditions. Chung and Goepfert[154] studied the interaction of oxygen, low pH, and inoculum level on the growth of *S. senftenberg*. At a pH of 4.1, an inoculum level of 10^4 cells/ml initiated growth under aerated conditions. Under static conditions, however, even an inoculum level of 10^6 cells/ml was unable to initiate growth. Gomez and Sinskey[155] studied the change in the DNA molecular weight of heat-injured *S. typhimurium* after incubation for 15 min in TS broth with 0.5% (wt/vol) yeast extract under aeration and in an atmosphere of nitrogen. The number of single-stranded breaks in DNA was less in the presence of nitrogen than in air, and the DNA breaks were repaired in nitrogen but not in air. Licciardello et al.[156] grew radio-sensitive and -resistant strains of four *Salmonella* serotypes under aerobic and anaerobic conditions, and observed no significant differences in the generation time, the lag time, and the maximal concentration of organisms. Thus, the results in Table 10 once again emphasize the importance of defining the analytical system used to generate data. It is only within such a framework that some explanation for seemingly contradictory results can be offered.

3. Preenrichment vs. Direct Selective Enrichment

It is generally acknowledged that any *Salmonella* organisms that survive the processing conditions of low-moisture foods will be present in a debilitated condition and will need to be resuscitated in a preenrichment medium before being subjected to the rigors of selective enrichment. Raw or otherwise unprocessed foods may be expected to contain large numbers of organisms that are competitive with *Salmonella* organisms. Such foods are placed directly into selective enrichment media which, at least theoretically, allow the *Salmonella* organisms to multiply while restricting the growth of the competing non-*Salmonella* organisms.

Studies comparing preenrichment and direct selective enrichment for recovering *Salmonella* from dried egg albumen generally support the concept of preenrichment (see Table 11).[43,141,157] However, Silliker et al.[43] demonstrated the effect of the contamination level on the relative performance of preenrichment and direct selective enrichment for dried egg albumen. Three methods of enrichment were compared: (1) lactose preenrichment, (2) direct enrichment in selenite cystine broth, and (3) direct enrichment in selenite cystine broth containing 10% (wt/vol) sterile feces. It had been reported (Silliker et al.[94]) that the addition of sterile fecal extracts to selenite broth increased significantly the ability of the medium to select *Salmonella* organisms in an environment containing relatively large numbers of competing coliform organisms. The data[43] in Table 11 show that with dried egg albumen containing a high number of *Salmonella* organisms, direct enrichments in selenite cystine broth and in selenite cystine broth with 10% sterile feces were superior to preenrichment in lactose broth. However, for low levels of *Salmonella* organisms, preenrichment in lactose broth was superior to direct enrichment in selenite cystine broth, but not to direct enrichment in

Table 11
RECOVERY OF *SALMONELLA* FROM FOODS BY PREENRICHMENT VS. DIRECT SELECTIVE ENRICHMENT

Food	Preenrichment medium	Selective enrichment medium	Incubation temperature of selective enrichment (°C)	% *Salmonella* samples[a] Preenriched	Directly enriched	Ref.
		Low Moisture				
Dried egg albumen	Lactose purple sugar broth	SC	37	24/40[b] (60)	5/40[c] (13)	141
	Lactose purple sugar broth	TBG	37	32/40[b] (80)	6/40[c] (15)	141
	Dulcitol purple sugar broth	SC	37	24/40[b] (60)	5/40[c] (13)	141
	Dulcitol purple sugar broth	TBG	37	39/40[b] (98)	6/40[c] (15)	141
	Mannitol purple sugar broth	SC	37	38/100 (38)	4/100 (4)	157
	Mannitol purple sugar broth	TBG	37	54/100 (54)	3/100 (3)	157
Dried egg albumen[d]	Lactose broth	SC	37	30/60 (50)	39/60 (65)	43
	Lactose broth	SC[e]	37	30/60 (50)	59/60 (98)	43
Dried egg albumen[f]	Lactose broth	SC	37	24/50 (48)	17/50 (34)	43
	Lactose broth	SC[e]	37	24/50 (48)	34/50 (68)	43
		High Moisture				
Liquid eggs, raw meats, and raw chicken[g]	Lactose broth	SC	35	137/273[h] (50)	93/273 (34)	159
	Lactose broth	TBG	35	137/273[h] (50)	65/273 (24)	159
Liquid eggs, raw meats, and raw chicken[i]	Lactose broth	SC	35	71/105[h] (68)	36/105 (34)	159
	Lactose broth	TBG	35	71/105[h] (68)	17/105 (16)	159
Minced meat[j]	Buffered peptone water	TBG	43	767/913 (84)	214/913 (23)	137
Minced meat[k]	Buffered peptone water	TBG	43	86/152[l] (57)	58/152[l] (38)	137
Fresh chicken carcasses[m]	Lactose broth	SC	37	29/60 (48)	24/60 (40)	160
Fresh chicken carcasses[n]	Lactose broth	SC	37	26/60 (48)	28/60 (47)	160
Frozen chicken carcasses	Lactose broth	SC	37	119/120 (99)	119/120 (99)	161
Raw meats and raw chicken livers	Lactose broth	SC	35	19/32 (59)	21/32 (66)	162
	Lactose broth	TBG	35	21/32 (66)	22/32 (69)	162
	Lactose broth	TBG	42	27/208 (13)	36/208 (17)	163

Table 11 (continued)
RECOVERY OF *SALMONELLA* FROM FOODS BY PREENRICHMENT VS. DIRECT SELECTIVE ENRICHMENT

Food	Preenrichment medium	Selective enrichment medium	Incubation temperature of selective enrichment (°C)	% *Salmonella* samples[a]		Ref.
				Preenriched	Directly enriched	
	Buffered peptone water	TBG	42	28/208 (14)	36/208 (17)	163
Oysters[j]	Lactose broth	SC	35	0/30 (0)	1/30 (3)	128
	Lactose broth	TT	35	0/30 (0)	9/30 (30)	128
	Lactose broth	SC	42	3/30 (10)	25/30 (83)	128
	Lactose broth	TT	42	2/30 (7)	9/30 (30)	128
Oysters[k]	Lactose broth	SC	35	2/21 (10)	4/21 (19)	128
	Lactose broth	TT	35	3/21 (14)	4/21 (19)	128
	Lactose broth	SC	41	2/21 (10)	9/21 (43)	128
	Lactose broth	TT	41	4/21 (19)	2/21 (10)	128

Note: SC, selenite cystine broth; TBG, tetrathionate brilliant green broth; TT, tetrathionate broth.

[a] Number of *Salmonella*-positive samples per total number of samples examined (%), unless otherwise specified.
[b] Lactose purple sugar or dulcitol purple sugar broth preenrichment media were incubated 18 to 24 h and then subcultured to selective enrichment media, which were streaked daily for more than 1 week.
[c] Direct enrichment media were incubated and streaked up to 72 h.
[d] High level of *Salmonella* contamination (10^5 to 10^6 cells per gram).
[e] Plus 10% (wt/vol) sterile feces.
[f] Low level of *Salmonella* contamination (one cell per test portion).
[g] 25-g samples.
[h] For test portions that were preenriched, the number of *Salmonella* positives obtained by subculture to SC and TBG enrichment media was reported as a single, combined result.
[i] Composited test portions weighing 108.3, 325, or 500 g.
[j] Artificially contaminated.
[k] Naturally contaminated.
[l] Number of *Salmonella*-positive samples obtained by a particular method per total number of *Salmonella*-positive samples.
[m] Destructive sampling of chicken carcass.
[n] Nondestructive sampling of chicken carcass.

selenite cystine broth with added feces. Another study demonstrating the significant influence of the coliform to *Salmonella* ratio on the efficiency of lactose preenrichment was conducted by Montford and Thatcher,[158] who reported the failure of this procedure for the examination of frozen egg products containing moderate numbers of coliforms.

The data in Table 11 comparing the efficiency of preenrichment and direct selective enrichment for the recovery of *Salmonella* from high-moisture foods are somewhat contra-

Table 12
RECOVERY OF *SALMONELLA* FROM REFRIGERATED PREENRICHMENT MEDIA AND SELECTIVE ENRICHMENT MEDIA

	% *Salmonella*-positive test portions[a]			
		Refrigerated		
Food	Unrefrigerated	Preenrichment	Selective enrichment	Ref.
Low moisture	34/34 (100)	34/34 (100)	34/34 (100)	164
	175/175 (100)	174/175 (99)	174/175 (99)	165
Poultry	105/105 (100)	93/105 (89)	96/105 (91)	164
High moisture	21/21 (100)	20/21 (95)	21/21 (100)	164
	291/291 (100)	257/291 (88)	265/291 (91)	165

[a] Number of *Salmonella*-positive samples obtained by a particular procedure per total number of *Salmonella*-positive samples (%).

dictory. Gabis and Silliker[159] demonstrated the superiority of lactose broth preenrichment for liquid eggs and raw meats. Similarly, Edel and Kampelmacher[137] obtained higher *Salmonella* recoveries from preenrichments of minced meat in buffered peptone water than from direct enrichments in tetrathionate broth. However, Cox et al.[160] obtained different results when they examined fresh chicken carcasses by destructive (entire carcass incubated with rinsing fluid) and nondestructive (rinsing fluid incubated with concentrated lactose broth or selenite cystine broth) procedures. There was no significant difference between lactose preenrichment and direct selective enrichment for the recovery of *Salmonella* by either procedure. Essentially, the same results were obtained in a subsequent study[161] using frozen rather than fresh chicken carcasses. Other foods which gave higher recoveries of *Salmonella* with direct selective enrichment rather than preenrichment include pork sausage,[162,163] fresh chicken livers,[162,163] and raw oysters.[128]

In view of the foregoing, the rationale of preenriching processed, low-moisture foods in nonselective media and of directly enriching raw, high-moisture foods in selective enrichment media may have to be reexamined. This concept was based on the reasoning that any *Salmonella* organisms surviving processing conditions would be debilitated and would have to be resuscitated before being placed in a selective enrichment medium. Raw, nonprocessed foods, it was reasoned, may contain large numbers of competing organisms that could overgrow the *Salmonella* organisms in a nonselective medium and, thus, should be placed directly in a selective enrichment medium. The data in Table 11 represent too many exceptions, however, for the above generalization to be accepted unquestionably.

4. Refrigeration of Sample after Preenrichment and Selective Enrichment

The recovery of *Salmonella* from foods by the conventional culture method is a continuous procedure that can be interrupted only after the selective agar plates have been incubated. Accordingly, the examination of foods requiring preenrichment cannot be initiated later than Tuesday if weekend work is to be avoided. D'Aoust et al. demonstrated in a preliminary study[164] and in a subsequent collaborative study[165] that incubated preenrichment and selective enrichment broth cultures of low-moisture foods could be refrigerated 72 h without any

significant decrease in the recovery of *Salmonella*, thereby making it feasible to initiate analyses as late as Thursday and still avoid weekend work (see Table 12). Refrigeration of incubated preenrichment and selective enrichment broth cultures of poultry and other high-moisture foods, however, gave fewer positives than did unrefrigerated broth cultures. The lack of complete agreement of results with high-moisture foods in the two studies was attributed to variations in laboratory performance, since most of the false-negative reactions occurred in only two laboratories participating in the collaborative study.[165]

E. Selective Plating Agars

Various selective plating media have been formulated to obtain pure, discrete colonies characteristic of the genus *Salmonella*, while the growth of competing non-*Salmonella* organisms is, under ideal conditions, inhibited. Some of these plating media are brilliant green (BG),[166] bismuth sulfite (BS),[167] MacConkey's,[168] desoxycholate citrate (DC),[169] Hektoen enteric (HE),[170] xylose lysine desoxycholate (XLD),[171] xylose lysine brilliant green,[171] Shanson's,[172] Rappold-Bolderdijk modified lysine iron,[173] lysine iron cystine,[174] and *S. arizonae*[175] agars.

In some instances antibiotics, sulfa drugs, surfactants, and other substances have been added to these agars to improve the selectivity and/or recovery of *Salmonella*. Jameson and Emberly[176] observed the inhibition of *Proteus* and an increase in the size of *Salmonella* colonies when the surfactant Teepol® was substituted for bile salts in MacConkey agar. Novobiocin added to HE agar or to XLD agar was reported by several investigators to improve media selectivity.[177-179] Sulfadiazine, sulfapyridine, or sulfanilamide improved the selectivity of BG agar.[91,105,180,181] Moats and Kinner[182] observed that the addition of a hydrogen sulfide system to BG agar facilitated the recognition of *Salmonella* colonies, especially in mixtures with other organisms. Gadberry and Highby[183] reported the inhibition of *Proteus*, *Providencia*, and *Citrobacter* on BG agar with dimethylchlortetracycline hydrochloride.

The relative performance of various selective agars for the recovery of *Salmonella* from foods is shown in Table 13. It would be impossible to draw any generalized conclusions from this table, since the relative productivity of the agars appears to be influenced by one or more of several factors: the type of food, the type of enrichment media, the incubation conditions, the modifications of agars, the commercial-brand variation, the dye-lot variation, the *Salmonella* serotype(s) present, the competitive microflora, the experience of the analyst, preference or bias of the analyst and the media preparation. With respect to media preparation, a variation may exist in the conditions of sterilization or storage. For example, both the FDA[41] and the Association of Official Analytical Chemists[195] recommend autoclaving BG agar for 12 min at 121°C, whereas two commercial manufacturers[196,197] of this medium recommend autoclaving for 15 min. A variation in the preparation of BS agar involves the age of these plates at the time of streaking. Difco Laboratories[197] recommends that the BS agar be made fresh, but McCoy[125] reported that freshly poured BS plates were too inhibitory for serotypes other than *S. typhi*. Cook[198] also reported that freshly prepared BS agar was inhibitory to several *Salmonella* serotypes, but felt that this inhibition could be reduced by refrigerating the prepared plates up to 5 d before use. In contrast to these results, D'Aoust[199] reported that inoculated plates of freshly prepared BS agar incubated for 48 h gave recoveries of *Salmonella* comparable to those of plates that had been refrigerated for up to 4 d.

Table 13 seems to represent many contradictory results; however, *defined* conditions do not always mean *uniform* conditions. This lack of uniformity is a direct result of a lack of standardization, which can inevitably lead to variation in the final results and conclusions.

III. THE GENUS *SHIGELLA*

Methods for recovering members of the genus *Shigella* in foods are relatively insensitive, since these organisms can be recovered only when they are initially present at a concentration

Table 13
RELATIVE PRODUCTIVITY OF SELECTIVE AGARS FOR THE RECOVERY OF *SALMONELLA* FROM FOODS

Food	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	Agar productivity[a]	Ref.
		Low Moisture			
Egg albumen powder	Purple broth base with 0.5% mannitol	TBG[b]	37	BS, 76/210 (36); DC, 76/210 (36); BG, 75/210 (36)	65
	Nutrient broth	None	37	BS, 73/210 (35); DC, 73/210 (35); BG, 33/210 (16)	65
Glucose-free egg powder	None	SC[c]	37	BG, 6/113 (5); BS, 6/113 (5)	184
Dried whole egg	None	S[d]	37	BS, 50/100 (50); DC, 41/100 (41)[e]	185
	Lactose broth	SC	35	BS, 7/225 (3); SS, 6/225 (3); BG, 2/225 (1)	186
Raw liquid eggs	None	SC	37	BS, 51/51 (100); BG, 34/51 (67)	184
Unpasteurized liquid egg albumen, egg yolk, and whole egg	Lactose broth	SC	37	BG, 14/100 (14); BS, 8/100 (8)	133
	Lactose broth	SC	43	BG, 25/100 (25); BS, 10/100 (10)	133
	Lactose broth	TBG	43	BG, 37/100 (37); BS, 26/100 (26)	133
Pasteurized, glucose-free liquid eggs	None	SC	37	BS, 14/39 (36); BG, 4/39 (10)	184
Frozen egg albumen	None	S	37	DC, 70/100 (70); BS, 51/100 (51)[e]	185
Frozen egg products	None	SC	37	BG, 86 isolates; BGS, 64 isolates; SS, 39 isolates; DC, 36 isolates; BS, 7 isolates	158
Frozen whole eggs	None	S	37	DC, 68/100 (68); BS, 49/100 (49)	185
Manchego cheese	0.1% Peptone water with brilliant green	SC	37	BS, 94/101 (93); SS, 89/101 (88); BG, 83/101 (82); XLD, 72/101 (71)[e]	187
	0.1% Peptone water with brilliant green	TBG	43	SS, 81/101 (80); BG, 74/101 (73); BS, 70/101 (69); XLD, 66/101 (65)[e]	187

Table 13 (continued)
RELATIVE PRODUCTIVITY OF SELECTIVE AGARS FOR THE RECOVERY OF *SALMONELLA* FROM FOODS

Food	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	Agar productivity[a]	Ref.
Milk chocolate and cocoa products	Reconstituted (10% wt/vol) skim milk powder with brilliant green	SC	35	BS, 10/152 (7); BGS, 8/152 (5)	136
	Reconstituted (10% wt/vol) skim milk powder with brilliant green	TBG	43	BS, 10/152 (7); BGS, 9/152 (6)	136
Low-moisture fatty foods (coconut, sausages, milk chocolate, cocoa beans, and chili powder)	Nutrient broth with and without various surfactants	SC	35	BS, 45/47 (96); BGS, 39/47 (83)[e]	142
	Nutrient broth with and without various surfactants	TBG	43	BS, 47/47 (100); BGS, 46/47 (98)[e]	142
Variety of low-moisture foods	Varied with food	SC	35	BS, 34/34 (100); BGS, 27/34 (79)[e]	164
	Varied with food	TBG	43	BS, 34/34 (100); BGS, 31/34 (91)[e]	164
	Varied with food	SC	35	BS, 421/469 (90); BGS, 380/469 (81)[e]	188
	Varied with food	TBG	43	BS, 442/469 (94); BGS, 430/469 (92)[e]	188
Variety of low-moisture foods and feed ingredients	Varied with food	SC	35	BS, 37/98 (38); BG, 22/98 (23); SS, 18/98 (18)	123
	Varied with food	SC	43	BS, 34/98 (35); BG, 23/98 (24); SS, 16/98 (16)	123
	Varied with food	TBG	35	BS, 33/98 (34); BG, 11/98 (11); SS, 11/98 (11)	123
	Varied with food	TBG	43	BS, 34/98 (35); BG, 25/98 (26); SS, 18/98 (18)	123

		High Moisture			
Roast beef	None	TBG	Unknown	TSXL, 14/31 (45); TSBG, 14/31 (45); HE-N, 13/21 (45); XLD-N, 14/31 (45); XLD, 12/31 (39); HE, 10/31 (32)	178
Pork sausage	None	TBG with Tergitol®	37	BG with sulfadiazine, 12/68 (18); BS, 5/68 (7)	129
	None	TBG with Tergitol®	43	BG with sulfadiazine, 15/68 (22); BS, 11/68 (16)	129
	None	TBG without Tergitol®	37	BG with sulfadiazine, 7/68 (10); BS, 4/68 (6)	129
	None	TBG without Tergitol®	43	BG with sulfadiazine, 21/68 (31); BS, 10/68 (15)	129
Deboned turkey meat	Lactose broth	SC and TBG[f]	Unknown	TSXL, 9/44 (21); TSBG, 8/44 (18); XLD-N, 7/44 (16); XLD, 4/44 (9); HE, 3/44 (7); HE-N, 3/44 (7)	178
Raw chicken carcasses	Nutrient broth	SC	37 and 43[g]	BS, 45 isolates; DC, 17 isolates; BG, 15 isolates	130
	Nutrient broth	TBG	37 and 43[g]	BS, 86 isolates; DC, 14 isolates; BG, 2 isolates	130
	Nutrient broth	RAP[h]	37 and 43[g]	BS, 105 isolates; BG, 45 isolates; DC, 44 isolates	130
	Buffered peptone water	Mannitol SC	42	BG with sulfadiazine, (19); SS, (19); XLD, (15); BS, (<1)[i]	71
	Buffered peptone water	BG selenite sulfonamide	42	BG with sulfadiazine, (6); XLD, (2); BS, (1); SS, (1)[i]	71
	None	SC	37	BGS, 95 isolates; HE, 89 isolates; BS, 60 isolates	189
Raw poultry carcasses	Nutrient broth	SC	35	BGS, 42/105 (40); BS, 42/105 (40)[e]	164
	Nutrient broth	TBG	43	BGS, 96/105 (91); BS, 82/105 (78)[e]	164
Shrimp	Nutrient broth	SC	35	BGS, 3/7 (43); BS, 3/7 (43)[e]	72
	Nutrient broth	TBG	43	BGS, 6/7 (86); BS, 6/7 (86)[a]	72
Frog legs	Lactose broth	SC	35	HE, 101/148 (68); XLD, 97/148 (66); BS, 94/198 (64); BG, 86/148 (58); SS, 63/148 (43)[e]	190

Table 13 (continued)
RELATIVE PRODUCTIVITY OF SELECTIVE AGARS FOR THE RECOVERY OF *SALMONELLA* FROM FOODS

Food	Preenrichment medium	Selective enrichment medium	Incubation temp of selective enrichment medium (°C)	Agar productivity[a]	Ref.
	Lactose broth	TBG	35	BS, 114/148 (77); XLD, 102/148 (69); BG, 99/148 (67); HE, 97/148 (66); SS, 72/148 (49)[e]	190
High-moisture fatty foods (raw poultry and giblets, pork and bovine organ meats, shellfish, lamb, liquid whole eggs, and blood pudding)	Nutrient broth with and without surfactants	SC	35	BS, 113/147 (77); BGS, 98:147 (67)[e]	142
	Nutrient broth with and without surfactants	TBG	43	BGS, 135/147 (92); BS, 132/147 (90)[e]	142
Variety of high-moisture foods	Nutrient broth	SC	35	BGS, 819/1,616 (51); BS, 811/1,616 (50)[e]	188
	Nutrient broth	TBG	43	BGS, 1,239/1,616 (77); BS, 1,226/1,616 (76)[e]	188
	Nutrient broth	SC	35	BS, 18/21 (86); BGS, 15/21 (71)[e]	164
	Nutrient broth	TBG	43	BS, 20/21 (95); BGS, 17/21 (81)[e]	164
Low and High Moisture					
Eggs and egg products, powdered foods, fish, bovine, porcine, and poultry carcasses and viscera	Varied with food	Varied with food	Varied with food	BG, 1,475/11,226 (13); HE, 1,315/11,226 (12)	191
Variety of low- and high-moisture foods	Varied with food	SC	35	HE, 388/492 (79); XLD, 386/492 (79); BS, 375/492 (76); SS, 365/492 (74); BG, 340/492 (69)[e]	192
	Varied with food	TBG	35	BS, 435/492 (88); HE, 416/492 (85); XLD, 403/492 (82); BG, 400/492 (81); SS, 362/492 (74)[e]	192

Meat products and animal feeds	Varied with food or feed	SC	37	BGS, 39/97 (40); BS, 39/97 (40); BG desoxycholate, 27/97 (28)[e]	193
	Varied with food or feed	TBG	43	BG desoxycholate, 78/97 (80); BGS, 69/97 (71); BS, 68/97 (70)[e]	193
	Varied with food or feed	RAP-V[j]	43	BG desoxycholate, 97/97 (100); BGS, 97/97 (100); BS, 93/97 (96)[e]	193
Variety of low- and high-moisture foods and animal feeds	Varied with food or feed	SC and TET[f]	35	HE, 288/288 (100); SS, 223/288 (77); BG, 160/288 (56); BS, 155/288 (54)[e]	194

[a] Number of *Salmonella*-positive samples obtained on a particular agar per total number of samples examined, unless otherwise specified. Agars compared were BG, brilliant green agar; BG desoxycholate agar; BG agar with sulfadiazine; BGS, brilliant green sulfa agar; BS, bismuth sulfite agar; DC, desoxycholate citrate agar; HE, Hektoen enteric agar; HE-N, HE agar with novobiocin; SS, *Salmonella-Shigella* agar; TSBG, tryptic soy-brilliant green agar; TSXL, trypic soy-xylose lysine agar; XLD, xylose lysine desoxycholate agar; XLD-N, XLD agar with novobiocin.

[b] TBG, tetrathionate brilliant green broth.

[c] SC, selenite cystine broth.

[d] Selenite F broth.

[e] Number of *Salmonella*-positive samples obtained on a particular agar per total number of *Salmonella*-positive samples.

[f] Results for two selective enrichment broths were combined.

[g] Results for selective enrichment broths incubated at two different temperatures were combined.

[h] RAP, Rappaport broth.

[i] Percentage of recovery of total number of inoculated *Salmonella* organisms.

[j] RAP-V, Rappaport-Vassiliadis broth.

of at least 10^6 cells/g of food.[200] Whereas methods for *Salmonella* may incorporate a preenrichment step, foods examined for *Shigella* are placed directly into a selective enrichment medium. Any naturally occurring *Shigella* organism may be expected to be vastly outnumbered by competing bacteria[201,202] and overgrown by them. Along with this disadvantage, the *Shigella* organisms would have to grow in the fermented acidic detritus resulting from the growth of the competitive organisms.

The antagonism between *Shigella* and coliform organisms is well documented. Halbert[203] reported that 18.3% of 1243 strains of coliform organisms demonstrated bacterial antagonism toward a single, arbitrarily chosen *Shigella* species. Hentges and Freter[204] reported a close correlation between the *in vivo* and *in vitro* antagonism of 22 strains of frequently transferred or continuous-flow broth cultures of *Escherichia coli, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa*, and *Alcaligenes faecalis* against *Shigella flexneri*. In a subsequent report, Freter[205] concluded that this antagonism was a result of the competition of fermentable carbon sources under reduced conditions. Hentges,[206] however, demonstrated that *Klebsiella* inhibited the growth of *S. flexneri* in an aerated medium that contained excess glucose at the time of inhibition, and concluded that mechanisms other than the exhaustion of fermentable carbon sources were responsible for this antagonism. It was demonstrated that neither nutrient depletion, adverse pH, nor anaerobiasis alone could account for this inhibition of *S. flexneri*. The remaining possibility, inhibition by metabolic end products, was investigated, and it was demonstrated that *S. flexneri* was inhibited by formic and acetic acids produced by *Klebsiella* under the reduced conditions of the medium. It is generally acknowledged that inhibition by these acids is due to undissociated, rather than dissociated, molecules.[207] Since pH determines the degree of dissociation, the activity of these acids is dependent upon the pH of the medium. Because the percentage of undissociated molecules is greater at an acid pH than at a neutral pH, the inhibitory effect of formic and acetic acids increases as the pH of the medium decreases. Thus, pH and oxidation reduction potential are significant factors in affecting the antibacterial activity of these acids. However, in a subsequent study[208] it was shown that the enhanced toxicity of these acids at low pH cannot be explained by the hydrogen ion concentration alone, since in a synthetic medium without formic and acetic acids, the *Shigella* population was the same at pH 6 as at pH 7. It was concluded that in a mixture of *S. flexneri* and coliforms, when enough formic and acetic acids are produced in a medium of a reduced oxidation reduction potential, the exponential growth phase of *S. flexneri* ceases and the organisms enter a stationary or death phase.[209] This effect appears to be selective for *Shigella*, since these acids demonstrated only a moderate inhibition of the coliform strains under anaerobic conditions. In yet another study, Hentges and Fulton[210] demonstrated that in a mixed culture, the *Klebsiella* multiplication rate always exceeded that of *S. flexneri*. Whenever interference occurred, the *Klebsiella* strain was always the antagonist.

Any proposed method for recovering *Shigella* from foods would have to take into account not only the environmental conditions discussed above, but also the nature of the cell injury and the need for resuscitation. Nakamura and Dawson[211] froze *S. sonnei* cells at −20°C in saline, nutrient broth, or skim milk. Recovery on nutrient or BHI agar was superior to recovery on a chemically defined medium.[212] The addition of complex substances (e.g., meat extract, peptone, or casamino acids) to the synthetic medium improved recovery, indicating an increase in nutritional requirements of the freeze-injured cells. Cells frozen in saline had a greater metabolic injury than cells frozen in nutrient broth or skim milk. Tollison and Johnson[213] demonstrated that *S. flexneri* cells sublethally heat-stressed at 50°C for 30 min became sensitive to 0.85% bile salts or 0.50% sodium desoxycholate, and that the cells needed a resuscitation period of about 6 h on a nonselective agar to regain their tolerance for these selective agents.

For the isolation of *Shigella* from foods, the *Compendium of Methods for the Micro-*

biological Examination of Foods[214] of the American Public Health Association recommends enrichment in GN broth and streaking to a plate of low selectivity (Tergitol® 7 agar or MacConkey's agar) and to either a plate of intermediate selectivity (XLD agar) or of high selectivity (DC, HE, or *Salmonella-Shigella* agar) with incubation at 35°C. A plate of low selectivity is recommended for the isolation of the more fragile *Shigella* strains, while a plate of high selectivity is recommended if a problem of overgrowth by competing organisms is expected. XLD agar (intermediate selectivity) is recommended because it has been reported[215-218] to be superior to other selective agars.

The procedure in the fifth edition of the *BAM* recommends the enrichment of food in GN broth and in selenite cystine broth, with the subsequent streaking of the incubated enrichment media onto plates of MacConkey, Levine's EMB, DC, and XLD agars at a 35°C incubation temperature. This procedure was subsequently modified in the sixth edition, based on the ability of *Shigella* to compete with coliforms under anaerobic conditions in media containing low concentrations of carbohydrate, the ability of *S. sonnei* to grow at 44°C, and the relative tolerance of *Shigella* to novobiocin at elevated temperatures. Essentially, the procedure for *S. sonnei* consists of enrichment in a complex medium, *Shigella* broth,[41] containing 0.5 μg novobiocin per milliliter. The test sample is periodically shaken in this broth for 10 min and the rinsings are incubated under anaerobic conditions at 44°C for 20 h. The enrichment media are then streaked to MacConkey agar and incubated at 35°C. The procedure for the other *Shigella* species is similar, except that novobiocin is incorporated into the *Shigella* broth at a concentration of 3 μg/ml and incubation is anaerobic at 42°C.

Analogous to the controversy of rich vs. minimal medium recovery of *Salmonella*, at least one study has reported the successful growth of six strains of *S. flexneri* in a simple, chemically defined medium.[212] The essential nutritional requirements of these organisms was met by a combination of mineral salts, glucose, L-aspartic acid, and two accessory growth factors.

Overall, the methods for *Shigella* are not nearly as developed as those for *Salmonella*, owing to the difficulty of growing *Shigella* under laboratory conditions. Although a few improvements for recovering *Shigella* have been made in recent years, the methods remain relatively insensitive.

IV. ENTEROPATHOGENIC *ESCHERICHIA COLI*

It has been acknowledged for some time that certain strains of *E. coli* were capable of causing illness in humans. Only recently, however, have reproducible methods been developed to identify these pathogenic strains. Kornacki and Marth[219] recognized four major categories of enteropathogenic *E. coli*:

1. Classical enteropathogenic strains historically associated with outbreaks of diarrhea in infants and young children
2. Facultatively enteropathogenic strains, which are associated with sporadic diarrhea and include many serogroups associated with normal intestinal microflora
3. Enterotoxigenic strains, which produce only a heat-stable (ST) enterotoxin, a heat-labile (LT) enterotoxin, or both
4. Enteroinvasive strains, which cause illness by their invasive infection of the gastrointestinal tract

Because many of the pathogenic strains of *E. coli* tend to be anaerogenic, traditional enrichment media such as lauryl tryptose broth, EC medium, and brilliant green lactose bile broth, all of which enumerate coliforms on the basis of lactose fermentation, are not suitable for the identification of enteropathogenic *E. coli*. Instead, the *BAM* recommends rinsing a

25 g portion of food with 225 ml of BHI broth and incubating the eluate 3 h at 35°C to resuscitate the damaged cells. After resuscitation, the enrichment medium is added to 225 ml of double-strength tryptone phosphate broth[41] and incubated 20 h at 44°C. After incubation, the enrichment medium is streaked to Levine's EMB and MacConkey agar and incubated 20 h at 35°C. The BHI resuscitative enrichment may be streaked directly to Levine's EMB and MacConkey agars if at least 25,000 *E. coli* cells/g are present and if *E. coli* represents at least 10% of the microflora capable of growing on these two agars. From each agar plate, ten typical and ten atypical *E. coli* colonies should be picked for further characterization.

The picked colonies are subjected to a series of biochemical tests, fully described in the *BAM*,[41] to screen potential enteropathogenic *E. coli* cells from the organisms of related species. The isolates are then serologically characterized by the procedure of Mehlman.[220] Routine serotyping of *E. coli* has been criticized, however, because of a lack of correlation between serotype and pathogenicity.[221] The production of enterotoxin is mediated by a plasmid, which can be transmitted from one *E. coli* strain to another without a change in the serotype of the donor or recipient.[222-224] Using 2 polyvalent and 12 monovalent sera, however, Merson et al.[225] reported 89% accuracy in the recognition of enterotoxigenic strains. Serotyping may also be useful in the epidemiological documentation of the spread of a particular strain in a foodborne outbreak.

The enteropathogenicity of an organism is finally demonstrated by determining its invasiveness and its production of LT or ST. The presumptive test for invasiveness is to determine the ability of the inoculum to infect a HeLa cell culture.[226] If at least 0.5% of the HeLa cells contain at least five bacteria, the culture is presumed positive and should be confirmed by the guinea pig eye test.[227] One drop of the bacterial suspension is placed on the guinea pig eye and the development of conjunctivitis ulceration and opacity in the eye is considered a positive confirmed test for invasiveness.

Enterotoxigenic *E. coli* may produce ST only, LT only, or both. LT is antigenically similar to the enterotoxin produced by *Vibrio cholerae* and is nondialyzable.[228-230] Fluid secretion in the small intestine is induced by LT stimulation of the adenylate cyclase-cyclic 3′,5′-monophosphate system.[231] ST is dialyzable and nonantigenic and stimulates a sharp increase in the concentration of cyclic guanosine 5′-monophosphate in the small intestine before fluid secretion.[231]

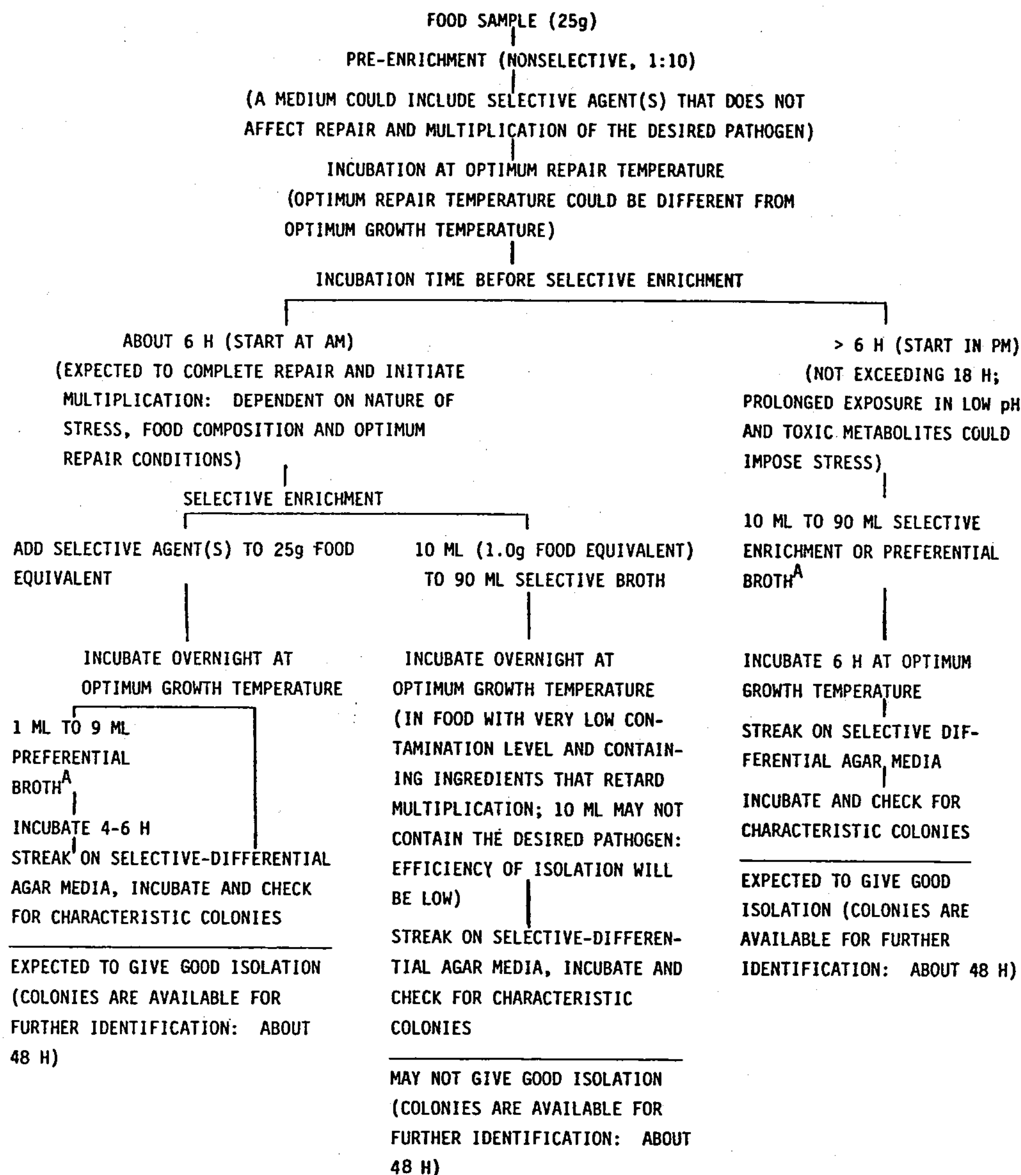
The presence of LT and ST may be demonstrated with animals. The infant rabbit test (LT and ST),[230] the vascular permeability test (LT),[232] the rabbit ligated loop reaction (LT and ST),[230] and the infant mouse test (ST)[230] have all been used with some success. Tissue culture assays of Chinese hamster ovary cells (LT),[233] intestinal epithelial cells (LT),[233] rat pituitary cells (LT),[233] and Y-1 adrenal cells (LT)[234] are also used.

Vero toxin is a heat-labile product that has been isolated from several *E. coli* strains. However, the exact role of this toxin in producing diarrhea in humans has not been established.[235] The presence of this toxin may be determined by its ability to produce cytopathic effects in tissue cultures of HeLa or Vero cells.

More recent techniques involving an immunological approach for the identification of enterotoxigenic *E. coli* include the solid-phase radioimmunoassay,[236] passive immune hemolysis,[237] the reversed passive hemagglutination test,[238] the staphylococcal coagglutination test,[239] and the enzyme-linked immunosorbent assay.[240,241] DNA colony hybridization has been used to identify enterotoxic *E. coli* isolates from both clinical specimens[242] and foods.[243-245]

V. CONCLUDING REMARKS

With the current emphasis on rapid methods for the detection of microorganisms in food, there will be a future tendency to reduce or minimize the time required for resuscitation.



[A] Preferential broth: for Salmonella sp. a broth containing dulcitol (0.5%) and phenyl-β-D-galactoside as carbohydrates could be used. Salmonella will show in dulcitol and lactose positive organisms will be killed by phenyl β-D-galactoside.

FIGURE 1. A theoretical model for the rapid isolation of injured pathogens from foods. (From Ray, B., *J. Food Prot.*, 49, 651, 1986. With permission.)

Ray[246] presented a model for the rapid detection of injured microorganisms in foods (see Figure 1). As Ray acknowledged, however, many aspects of this protocol are theoretical and need to be studied before being incorporated into any method.

Methods for the resuscitative recovery of the classical enteric pathogens from foods have been summarized in this chapter. Acknowledgment of the concept of cell injury is now universally accepted and has been instrumental in developing sensitive methods for recovering many of these organisms.

One unfortunate consequence of this proliferative development of methods has been the appearance of varying, even conflicting, reports and conclusions. This occurrence, in turn, has fostered the establishment of "pet" methods in some laboratories. Once a favored method has become entrenched in a particular laboratory, it may be difficult, if not impossible, to persuade that laboratory to use a standardized method that is nationally and/or internationally recognized. The use of nonstandardized methods may be expected to yield nonuniform results, which would be microbiologically unacceptable in government regulatory laboratories as well as in quality control laboratories in industry. The use of uniform, standardized methods, however, offers the advantage of reproducibility. The same results obtained in one laboratory can be obtained by competent analysts working in a different laboratory, provided there is a strict adherence to the method.

In recent years rapid methods have been developed and offered as alternatives to lengthier conventional methods for the identification of classical enteric pathogens. Some of these rapid methods have been subjected to collaborative study, a useful tool for validating the uniform performance of a method by competent analysts working in independent laboratories. The collaborative study could be the means for a harmonious resolution of existing analytical differences and, ultimately, method standardization.

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Chapter 4

INJURY IN EMERGING FOODBORNE PATHOGENS AND THEIR DETECTION

Samuel A. Palumbo

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I. INTRODUCTION

Any sublethal treatment or stress will cause injury to a microorganism. Virtually all of the physical treatments and chemicals used in food processing, such as heating, freezing, drying, acidification by either fermentation or the direct addition of acid, NaCl, $NaNO_2$, etc., have been shown to injure microorganisms associated with foods.[1-4] Injured microorganisms are most easily detected and quantitated by the use of differential plating agars. Heat injury in *Staphylococcus aureus* will serve as an example. Sublethal heating (50 to 55°C) of *S. aureus* causes the cells to become sensitive to NaCl (nonheated/noninjured cells are very tolerant of NaCl levels of up to 15%). A plating system was developed,[5-7] comprised of plating on trypticase soy agar (TSA, on which both injured and noninjured cells grow) and on TSA containing 7.5% NaCl (TSAS, on which only noninjured cells will grow). At any time during heating, the difference between TSA and TSAS represents the number of injured cells.

The types of cellular damage caused by sublethal stresses vary with the type of stress and the organism studied. Heat is the most common stress and *S. aureus, Escherichia coli,* and *Pseudomonas fluorescens* are the bacteria generally studied. The most common cellular lesion is damage to the cellular membrane. This causes leakage of various cellular components such as Mg^{++}, K^{+}, amino acids, 260 nm absorbing material (nucleic acid), and 280 nm absorbing material (protein).[8] Additionally, in *S. aureus* MF31, the degradation of ribosomes occurs as a consequence of heat injury.[9-11] The topic of injury, including the mechanisms and sites of cellular damage, repair with practical implications, and applications has been extensively reviewed.[1,3,8,12-14] It should be noted that an injured cell, as opposed to a dead cell, is one which is capable of repairing the cellular damage and regaining its ability to form a colony in the presence of the selective agent; a dead cell cannot form a colony under any condition.

II. EMERGING PATHOGENS

Food microbiologists have traditionally tested for and been concerned with the presence of the so-called "big three" food-poisoning bacteria, *S. aureus, Salmonella,* and *Clostridium botulinum* (types A and B).[15] In recent years, however, several "new" organisms have emerged into positions of prominence and importance and the list[16,17] now includes a mesophilic group consisting of *C. perfringens, Campylobacter jejuni, Bacillus cereus,*[18] *Vibrio parahaemolyticus,*[19,20] *V. cholerae,*[21] *V. fulvialis, Shigella,*[22] and *Plesiomonas shigelloides,* and a psychrotrophic group consisting of enterotoxigenic *E. coli,*[23] *Aeromonas hydrophila, Listeria monocytogenes, Clostridium botulinum* type E, and *Yersinia enterocolitica.* This psychrotrophic group was recently reviewed[24] and is of particular concern to food microbiologists because of its ability to grow at 5°C, a temperature once though to be adequate to protect food from the hazards of food poisoning.

The importance of the quantitative recovery of bacteria from processed foods is well known. Injury has not been studied as extensively in the emerging pathogens because they have not been known as long as the traditional organisms. Several organisms from the above list are the subject of chapters of their own. It is the purpose of this chapter to review the literature on the injury and recovery of *Campylobacter jejuni, Y. enterocolitica, A. hydrophila, P. shigelloides, L. monocytogenes, Vibrio,* and *Shigella.* Specifically, these organisms will be discussed in terms of their taxonomy and specific biochemical characteristics, the disease(s) they cause if other than gastroenteritis, the foods associated with the organism, and a review of injury studies and problems related to the ability of various media to quantitatively recover the organism from various sources and under various conditions.

III. INJURY IN EMERGING PATHOGENS

A. *Campylobacter jejuni*

The genus *Campylobacter* is part of a group of related aerobic/microaerophilic, motile, helical/vibroid, gram-negative bacteria.[25] Some distinguishing characteristics of the genus *Campylobacter*:[26] it has slender, spirally curved rods; it has a characteristic corkscrew-like motility; it is microaerophilic, with a respiratory type of metabolism; it requires a carbon dioxide concentration of 3 to 5%; it is oxidase positive; and carbohydrates are neither fermented nor oxidized. Members of the genus *Campylobacter* are found in the intestinal tract, the oral cavity, and the reproductive tract of humans and animals; many are pathogenic to humans and animals, especially domestic animals.

Though known in the older veterinary literature, *C. jejuni* has emerged to become a significant enteric pathogen of humans[27-31] and an important cause of food poisoning.[32,33] Recent surveys have indicated that *Campylobacter* is as prevalent as *Salmonella* in various foods, especially poultry[34,35] and red meats.[35,36] It is also found in raw milk and unchlorinated water.[33]

C. jejuni possesses certain unique characteristics which originally warranted the separation of the genus *Campylobacter* from the genus *Vibrio*. Palumbo[37] has discussed some of these characteristics and traits, especially as they have been used in various methods to isolate *C. jejuni* from foods and clinical specimens. *C. jejuni* is a slender (0.2- to 0.5-μm wide) rod; thus, a coarse filter can be used to eliminate other "fatter" bacteria. The organism is both microaerophilic and capnophilic (i.e., it requires CO_2), characteristics which can suppress many associative bacteria. *C. jejuni* is thermophilic, with an optimum of 42°C; this temperature will inhibit much of the psychrophilic/psychrotrophic bacteria in foods. Finally, the organism is resistant to specific antibiotics,[26,31] which can be added to plating media to suppress associative bacteria. *C. jejuni* is particularly sensitive to various peroxides and related forms of oxygen, which are generated in bacteriological media, and thus George et al.[38] developed an aerotolerant supplement consisting of ferrous sulfate, sodium metabisulfite, and sodium pyruvate (FBP). Good growth of *C. jejuni* in broth can be achieved with Brucella broth supplemented with FBP and gassed with a mixture of 5% O_2, 10% CO_2, and 85% N_2 with incubation at 42°C. Selective plating media containing antibiotic mixtures have been developed for the isolation of *C. jejuni*.[26,31]

The topic of *C. jejuni* in foods and injury to *C. jejuni* was recently reviewed by Palumbo,[37] and additional information has since become available. Several types of injury to *C. jejuni* have now been described and include heat,[39,40] freezing and cold,[40-42] and chlorine.[43]

In developing a plating system to detect heat-injured *C. jejuni*, Palumbo[39] formulated a medium, designated BGBS, containing bile and brilliant green supplemented with FBP. *C. jejuni*, heated at 46°C in a phosphate buffer (0.1 *M*, pH 7.3), was plated at intervals on BGBS and the colony counts were compared to those on Brucella agar (Difco) supplemented with FBP and designated BAS (see Figure 1). As can be seen, the counts on BAS remained fairly constant over the heating period, while the counts on BGBS declined. The difference between BAS and BGBS is represented by the number of heat-injured *C. jejuni* found on the respective agars. Heat-injured *C. jejuni* became sensitive to both brilliant green and bile.[39] Though these are not usually used in selective media to isolate *C. jejuni*, Ullmann[44] has indicated that there is a medium which does contain brilliant green. This would not be recommended for use with any heat-processed food. Tomlinson[45] has stated that *C. jejuni* will not grow on the commonly used selective/inhibitory enteric agars. This may be due to their content of bile salts or deoxycholate. However, Palumbo (unpublished observation) obtained a good growth of *C. jejuni* on violet red bile agar when it was supplemented with FBP. It has been reported[40,41] that *C. jejuni* exposed to either a freezing or a heating treatment became sensitive to the antibiotic rifampicin as well as sodium deoxycholate. Thus, even

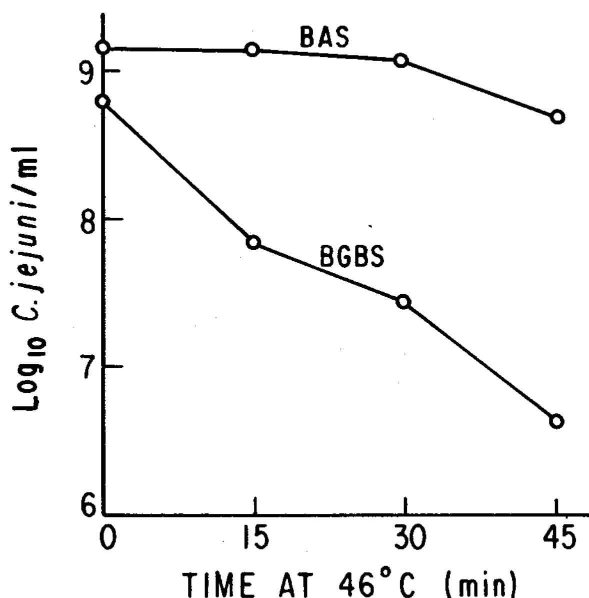

FIGURE 1. Heat injury in *C. jejuni:* effect of plating media. Cells were heated at 46°C in phosphate buffer (0.1 *M*, pH 7.3) and plated at intervals on BAS (Brucella agar supplemented with FBP) and BGBS (brilliant green 2% bile agar supplemented with FEB: ferrous sulfate, sodium metabisulfite, and sodium pyruvate). (From Palumbo, S. A., *Appl. Environ. Microbiol.*, 48, 477, 1984. With permission.)

enteric media supplemented with FBP would not be appropriate for isolating *C. jejuni* from heat-processed foods.

Ray and Johnson[42,46] observed that *C. jejuni*, after a freezing treatment, became sensitive to the antibiotic mixture commonly used in media to isolate this organism from food and clinical specimens (see Figure 2). This sensitivity of frozen cells was seen as a loss in the number of viable cells and a greater lag in the broth containing the antibiotic (see Figure 2). After their initial observation, Ray and Johnson[42,46] then determined which antibiotic in the mixture was responsible for this effect by inoculating freeze-stressed cells into broth containing each of the antibiotics. They found that freeze-stressed *C. jejuni* were sensitive to polymyxin B. In addition, the sensitivity to polymyxin B was increased when the freeze-stressed cells were incubated at 42°C as compared with 37°C. Supplementation of the recovery broth with sodium succinate (0.3%) and cysteine HCl (0.01%) (SCy) increased the recovery of freeze-stressed cells. Ray and Johnson[42,46] developed the following procedure for recovering freeze-stressed *C. jejuni:* enrichment in Brucella broth with SCy under a microaerophilic atmosphere, incubation at 37°C for 6 h, addition of the antibiotic mixture, followed by shifting the culture to 42°C. This procedure is suggested for isolating stressed *C. jejuni* from foods which have been frozen and thawed. Doyle and Roman[47] also found that the addition of SCy was useful in isolating *C. jejuni* from refrigerated foods such as raw milk, chicken skin, and hamburger. Stern et al.[35] determined that the enrichment broth of Doyle and Roman[47] functioned well during their survey of retail meats for the presence of *C. jejuni* and *C. coli.*

In contrast to freeze-stressed cells, both Palumbo[39] and Waterman[48] found that *C. jejuni,* heated in phosphate buffer and milk, respectively, were able to repair and form colonies in media containing polymyxin B. Waterman[48] incubated the plates at 42°C, and this temperature did not seem to be contraindicated for heated cells in contrast to freeze-stressed cells.

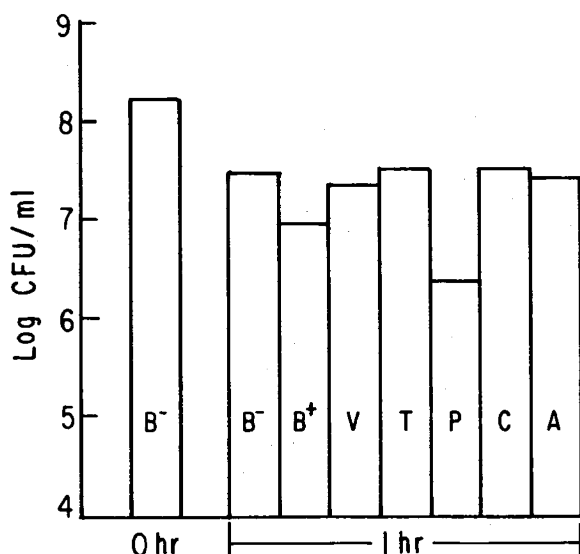

FIGURE 2. Effect of antibiotics in Brucella broth on survival of freeze-thaw stressed *C. jejuni* at 42°C. B^- = Brucella broth without antibiotic mixture; B^+ = Brucella broth with antibiotic mixture; V = Brucella broth plus vancomycin (10 mg/l); T = Brucella broth plus trimethoprim (5 mg/l); P = Brucella broth plus polymyxin (2500 IU/l); C = Brucella broth plus cephalothin (15 mg/l); and A = Brucella broth plus amphotericin (2 mg/L). (From Ray, B. and Johnson, C., *J. Food Safety,* 6, 183, 1984. With permission.)

Palumbo[39] found that heat-injured *C. jejuni* would repair in Brucella broth supplemented with FBP incubated at 42°C. Humphrey[40] has indicated that there are different degrees (amounts) of heat injury in *C. jejuni,* since cells heated for 15 min at 50°C were not able to repair when incubated at 43°C, as were cells heated only 5 min. Whether this lack of repair at 43°C observed by Humphrey[40] in contrast to repair at 43°C observed by Palumbo[39] represents differences in the strains used, the recovery media and procedures, or the temperature of injury (Palumbo used 46°C, while Humphrey used 50°C) is not known. Repair at elevated temperatures (42 or 43°C) is worthy of additional study, since elevated temperature is a very useful step in the isolation of *C. jejuni* from foods.

As indicated, bacteria can be injured by various chemicals. Blaser et al.[43] observed that *C. jejuni* exposed to 0.1 mg free chlorine per liter at 25°C was injured. This injury was manifested as a sensitivity to antibiotics in the plating medium. There was more injury when the pH of the chlorine-containing solution was 6 as compared with pH 8.

As can be seen, *C. jejuni* can be injured by numerous stresses and this injury is manifested by a sensitivity to antibiotics and bile derivatives. *C. jejuni* is also susceptible to another phenomenon that may be related to injury: the change in cellular morphology from curved, spiral rods to coccoid upon aging of the culture. Young, actively growing cultures of *C. jejuni* exhibit typical curved-rod, spiral morphology, while older cells and cultures contain mainly coccoid forms and donut-shaped cells.[49,50] Buck et al.[49] and Ng et al.[50] have designated the coccoid and donut forms as a degenerative state. Ng et al.[50] have found that the coccoid and donut forms are nonviable (nonculturable) in that they would not form colonies on Mueller Hinton (MH) agar. However, even though MH agar is a nutritionally complete and complex medium and should allow the growth of injured cells, these coccoid and donut forms may represent a specific type of injury that requires special nutrients for repair. This

Table 1
SUBLETHAL INJURY IN *YERSINIA ENTERACOLITICA* AND ITS REPAIR

Type of stress	Detection method	Conditions for injury	Conditions for repair	Ref.
Heat	Sensitivity to bile salts (TSA + 0.6% or 0.16% BS); counts compared to BHI	47°C — times varied with serotype in buffer or broth	BHI broth at 25°C	58
	TSA vs. BS agar	Roast beef heated to internal temp of 51°C	Not studied	57
Freezing	NaCl: TSA with 3% total NaCl vs. TSA	−20°C in broth or ground beef	Not studied	59
	NaCl: TSA with 2.5% total NaCl vs. TSA	Buffer −18°C and −75°C	Not studied	60
	TSA vs. BS agar	Frozen in beef roast at −34°C and stored at −23°C	Not studied	57
Radiation	NaCl: TSA with 3% total NaCl vs. TSA	60 krads at 25°C in ground beef or in TSB	Repair/recovery not specifically studied, but some cells could not form colonies on TSA at 5°C	59
	NaCl: TSA with 2.5% NaCl vs. TSA	Buffer −0°C 10 krads, −18°C 15 krads, and −75°C 25 krads	Not studied	60
Chlorine	TLY agar vs. TLY agar + 0.1% deoxycholate	1.07 mg/l free chlorine at 4°C, pH 6.5—7.0	Not studied	62
Copper	TLY vs. + 0.1% sodium deoxycholate	0.75 mg Cu/l, 4°C for 3 d	Not studied	61

Note: TSA, trypticase soy agar (BBL); BHI, brain heart infusion; BS, bismuth sulfite; TLY, typtic soy (Difco) + 1% lactose and 0.3% yeast extract.

would require an extension of the study of Ng et al.,[50] in which coccoid/donut forms are studied in terms of increasing viability by various supplements to MH agar. A further study would be an electron microscopy examination of heat-, freeze-, or cold-stressed *C. jejuni*, similar to the study of Jones et al.[51] on injured *S. aureus*. This type of study would allow the determination of whether stress would cause the formation of the coccoid and donut forms or other changes in cellular morphology.

B. *Yersinia enterocolitica*

Y. enterocolitica is a facultatively anaerobic, short, gram-negative rod which is currently classified as part of the family Enterobacteriaceae.[52] The organism is found primarily in foods of animal origin, though water may also be a vehicle. One characteristic of *Y. enterocolitica* of particular concern to food microbiologists is the ability of the organism to grow at 5°C, a temperature formerly considered adequate to restrain the growth of foodborne pathogens. The significance, the occurrence in foods, and the virulence and pathogenicity of *Y. enterocolitica* have been reviewed elsewhere.[53-56]

The topic of injury to *Y. enterocolitica* has received some study, and the organism has been reported to be injured by stresses such as heat,[57,58] radiation,[59] and freezing[54,60] and exposure to copper[61] and chlorine.[62]

Injury to *Y. enterocolitica* resulted in a sensitivity to either sodium deoxycholate or NaCl (see Table 1). Sensitivity of the organism to salt on freezing and thawing appears to be a phenomenon observed in other gram-negative bacteria as well as in *Streptococcus faecalis*.[63] The recommended medium for *Y. enterocolitica* is CIN (cefsulodin-irgasan-novobiocin) agar;

the formulation contains 2 g bile salts per liter[64] and thus should be used with caution for direct plating for injured *Y. enterocolitica*. Harmon et al.[64a] and Head et al.[65] have indicated that even nonstressed *Y. enterocolitica* are sensitive to sodium deoxycholate. The data in Table 1 suggest, as indicated above, that the quantitative recovery of stressed *Y. enterocolitica* cannot be achieved on media containing deoxycholate and bile salts.

In contrast to the work on injured *C. jejuni* and other bacteria, there have been virtually no studies on the repair and recovery of injured *Y. enterocolitica*. Restaino et al.[58] found that heated *Y. enterocolitica* would regain their tolerance to bile salts in BHI broth held at 25°C. El-Zawahry and Rowley[59] observed that radiation damage resulted in some injured *Y. enterocolitica* that could not form colonies on TSA at 5°C. According to the results from work with *C. jejuni*[39] and *E. coli*, a period of about 4 h should suffice for the repair of injured *Y. enterocolitica*. Preliminary incubation of injured cells in a nonselective medium followed by the addition of deoxycholate and bile salt should allow the recovery of both noninjured and repaired injured cells.

C. *Aeromonas hydrophila*

A. hydrophila, a gram-negative rod that is currently a member of the family Vibrionaceae,[66] has emerged as an organism of increasing significance to both clinical and food microbiologists.[67-80] Some distinguishing characteristics of the genus *Aeromonas:* it is facultatively anaerobic, with a glucose-based metabolism that is both respiratory and fermentative, it is oxidase positive, it is resistant to the vibriostatic agent 0/129, it is amylolytic, and the optimum temperature for growth is in the range 22 to 28°C.[81] This organism is known in the older literature as the cause of various diseases in fish, reptiles, and amphibians. Only recently has it begun to be recognized as a human pathogen.[82] Buchanan and Palumbo[83] have reviewed the topic of *A. hydrophila* as a foodborne pathogen and indicated that *A. sobria* should also be included as an organism of concern. Further, the third species included in the general classification of motile *aeromonads, A. caviae,* also has now been implicated as a human pathogen,[84,85] and thus, the presence of any motile *aeromonad* in a food or clinical specimen should be viewed with concern and alarm. In addition to diarrhea and other gastrointestinal symptoms, this organism can produce other disease syndromes, such as wound infections, sepsis, meningitis, various musculoskeletal infections,[86] and endocarditis.[68]

The isolation of the organism from clinical specimens was reviewed by von Graevenitz and Bucher.[87] When Palumbo et al.[88] began to evaluate various media described by von Graevenitz and Bucher[87] for isolating the organism from refrigerated foods of animal origin (i.e., seafood, fish, poultry, red meats, and raw milk), they found that the clinical media either did not provide ready differentiation of *A. hydrophila* from other gram-negative bacteria found as the normal flora of these foods, or did not give quantitative recovery data of the organism from inoculated foods. In response to these problems, Palumbo et al.[88] developed a new medium for the isolation of *A. hydrophila* from foods. This new medium contains starch as the differential substrate, as this organism is one of the few found in foods which is amylase positive. The medium also contains ampicillin (at 10 μg/ml) to suppress the associative bacteria found in foods of animal origin. Using this newly developed medium, Palumbo et al.[88] found *A. hydrophila* in virtually every food sampled and also observed that the count of this organism increased during storage for one week at 5°C.

Palumbo et al.,[88] in evaluating the various clinical media used for the isolation of the organism, observed that deoxycholate seemed particularly toxic to *A. hydrophila* and recovery on media containing 0.25% deoxycholate was 10^2- to 10^3-fold less than that on media without deoxycholate. Whether this represents a type of cold shock as described above for *C. jejuni* (where the foods were refrigerated at 5°C) or just simply an inherent sensitivity of this organism to bile components is not known.

Injury has not yet been studied in *A. hydrophila*. In any possible study, the apparent inherent sensitivity of the organism to deoxycholate should be taken into account. Media developed for the isolation of *A. hydrophila,* besides those evaluated by von Graevenitz and Bucher, include those of Shotts and Rimler[89] (deoxycholate), Rippey and Cabelli[90] (deoxycholate), Kay et al[91] (ampicillin), Figura[92] (deoxycholate), and Millership and Chattopadhyay[93] (deoxycholate and bile salts).

In addition, there has been a recent report describing the isolation of *A. hydrophila* on CIN agar,[94] originally developed for the isolation of *Y. enterocolitica*. Since CIN agar contains deoxycholate, it cannot be expected to yield a quantitative recovery of the organism. The quantitative detection of various pathogens is particularly important in foods, since the hazard from particular organism increases with the increasing concentration of the organism.

D. *Plesiomonas shigelloides*

P. shigelloides, also a member of the family Vibrionaceae,[66] is a facultatively anaerobic, gram-negative, motile rod often found in the aquatic environment.[95,96] Biochemically, the organism is similar to various Enterobacteriaceae; however, *P. shigelloides* is oxidase positive, and this test should routinely be included to ensure its recognition.[96] The bacterium is becoming recognized as an organism of importance in both clinical[70,73-78] and food microbiology.[96] In addition to being isolated from water, it is also associated with oysters, crabs, and fish as well as various animals.[96-98] In addition to the usual gastrointestinal syndrome (diarrhea, abdominal pain, and nausea), *P. shigelloides* is similar to *A. hydrophila* in that it, too, can cause various extraintestinal infections, including sepsis, meningitis, cellulitis, suppurative arthritis, cholecystitis, endophthalmitis, and pyometra.[86]

One characteristic found in many of the emerging foodborne pathogens is their ability to grow at what was once considered adequate refrigeration (5°C). This does not appear to be a prominent trait in the *P. shigelloides* tested. Miller and Koburger[98] observed that only 22% of their strains (both clinical and environmental) could grow at 8°C and none at 5°C; however, all grew at 10°C in 3 to 14 d. Rouf and Rigney[99] found that 80% of their strains grew at a minimum temperature of 10°C, 10% at a minimum of 5°C, and 10% at a minimum temperature of 0°C. While only two strains of *P. shigelloides* (out of a total of 50 studied by both groups) could grow at 5°C or below, even short periods of temperature abuse could support the rapid growth of this organism, and the temperature abuse of various foods does occur at all levels of the food-handling chain.[100-102]

Miller and Koburger[96] discussed the various media used for isolating *P. shigelloides* from environmental and clinical sources. In a recent report,[97] they evaluated in detail inositol brilliant green bile salts (IBB) and Plesiomonas (PL) agars for recovering *P. shigelloides* from aquatic samples. They found that, for nondamaged cells from an aquatic environment, IBB was more effective in terms of the percentage of positive samples recovered and the percentage of isolates confirmed as *P. shigelloides*. However, with heat-injured (20 min at 50°C) or cold-injured (frozen for 24 h at −20°C) cells, the use of IBB agar gave much lower recoveries compared to both plate count and PL agars. The lowered recovery of injured cells on IBB is not unlike the lowered recovery of heated *C. jejuni* cells reported by Palumbo.[39] In that study, heat injury was quantitated by the use of brilliant green bile broth and Brucella agars.

While *P. shigelloides* is only now becoming recognized as a possible foodborne pathogen, any attempted isolation of this organism from an environment which may contain damaged cells should take into account the fact that the use of media containing dyes such as brilliant green and inhibitory substances such as bile salts may lead to lowered or negative recoveries. Since these types of substances (dyes and bile salts) are relatively common in media used for the isolation of gram-negative bacteria, and since bacteria can be injured by heating, freezing, and other stresses, the actual incidence and level of *P. shigelloides* in foods may be much more extensive than currently thought.

E. *Listeria monocytogenes*

L. monocytogenes, a catalase-positive, facultatively anaerobic, motile, gram-positive rod, is part of a conglomerate group of regular, nonsporing, gram-positive rods.[103] *Listeria* can be differentiated from other closely related organisms by several biochemical characteristics.[103,104] The bacterium display one very unique, specific phenomenon which has permitted their ready differentiation from other bacteria: the colonies appear bluish gray by normal illumination and a characteristic blue-green is produced by obliquely transmitted light.[104] *Listeria* is widely distributed; it is found in water, mud, sewage, vegetation, and in the feces of animals and humans. Some species are pathogenic for humans and animals.[104,105]

L. monocytogenes is well known in the older literature, especially in veterinary references. It is also a pathogen of humans and can cause the typical food-poisoning syndrome of diarrhea, which in normal, healthy adults is often mild. In certain individuals, such as the immunocompromised patient or pregnant woman, an infection with *Listeria* can lead to various, often very severe, disorders: meningo-encephalitis; flu-like, low-grade septicemia *in gravida;* septicemia in the perinatal period; septicemia in adults (often imposed on other disorders); pneumonia; endocarditis; urethritis; and habitual abortion.[105] There is also a recent report involving *L. monocytogenes* in a case of cholecystitis.[106] Food was shown to be the vehicle in three recent major outbreaks of human listeriosis in the U.S. and Canada.[107-109] *L. monocytogenes* possesses, among others, two traits which make it an organism of concern to food microbiologists: (1) the ability to grow at low temperatures (5°C and below) in foods[110,111] and (2) an apparent ability to survive minimum high-temperature, short-duration pasteurization (16 s at 162°F) for milk in laboratory-scale pasteurization studies.[112]

The isolation of *Listeria* from clinical, environmental, and food samples has always been difficult and tedious.[105] The ability of the organism to grow at low temperature prompted the development of the effective, but lengthy, cold-enrichment procedure.[105,107,113] In this procedure, infected material is stored at 5°C and sampled at intervals (up to 3 months) for the presence of viable *Listeria.* Very often, viable *Listeria* can be isolated only after cold enrichment.[105,107,113]

A second approach for the isolation of *Listeria* is the use of media containing various selective agents, such as chemicals (potassium tellurite, phenylethanol, lithium chloride), antibiotics (guanofuracin, fuacin, chloramphenicol), and several dyes.[105] The most recent medium developed for the isolation of *L. monocytogenes* consists of modified McBride's agar containing 5 g/l lithium chloride and 20 mg/l moxalactam.[114] This medium functioned well in recovering the organism from beef.

The difficulty in isolating *L. monocytogenes* may be due to minor but significant differences in the media components and preparations or to the possibility that *L. monocytogenes* is stressed (injured) by the environment, food, or host. In one study, Beuchat et al.[115] has observed that two strains of *L. monocytogenes,* heated at 52°C in clarified cabbage juice (pH 5.6), because sensitive to NaCl (4% in TSA). Bradley[112] has suggested that with the *L. monocytogenes*-contaminated pasteurized milk, cold enrichment or incubation for 14 d was needed so that heat-damaged cells could repair themselves. Experimental support for this suggestion comes from the study by Beuchat et al.,[115] who studied the thermal inactivation of *L. monocytogenes* and observed an enhanced recovery (an increase in plate count) of the organism after exposure to the more severe heat treatment only when cold enrichment (21 d at 5°C) was used. Since the selective agents (chemicals, antibiotics, and dyes) present in the media used for the isolation of *Listeria* have been shown to be inhibitory for injured bacteria of other genera, it is possible that the poor recovery of *L. monocytogenes* may be due to the presence of injured cells and their inability to grow on the selective media. This is a topic for future research, and answers thus obtained may improve the methods used for isolating *L. monocytogenes.*

F. *Shigella* Species

Shigella, a member of the family Enterobacteriaceae,[116] is a facultatively anaerobic, anaerogenic, gram-negative, nonmotile rod.[117] *Shigella* is very closely related to *E. coli,* and, based on DNA homology studies, Brenner[116] has suggested that *Shigella* and *E. coli* are a single species. The organism is the cause of bacillary dysentery and water has traditionally been the vehicle of transmission. However, a recent review by Smith[22] has indicated that shigellosis outbreaks and cases in the U.S. have been much more frequently associated with food than with water. The foods most often implicated in foodborne shigellosis were potato salad and salads containing chicken or fish. This is probably due to the salads being prepared from raw ingredients or previously cooked ingredients that are not reheated after formulation. Additional foods associated with foodborne shigellosis include Mexican food, fruit compote, chili con carne, cream puff dessert, shrimp, and raw clams.[22]

The isolation of *Shigella* from clinical or food specimens is usually accomplished by the use of various selective media. Rowe and Gross[117] suggest the use of both relatively non-inhibitory media, such as MacConkey or EMB agars, and inhibitory media, such as deoxycholate citrate (DC) or *Shigella-Salmonella* (SS) agars. Since even very low numbers of *Shigella* can cause disease in humans,[22] media that maximize bacteria recovery should be employed.

The presence of injured or stressed cells can lower the recovery of *Shigella* from a food. In a study on injury to *Shigella,* Tollison and Johnson[118] observed heat injury in *S. flexneri* when the cells were heated at 50°C in 0.1 *M* phosphate buffer and plated on tryptic phytone glucose agar with and without bile salts or sodium deoxycholate. Cells heated for 30 min gave significantly lower counts on agars containing either bile salts or sodium deoxycholate. The heat-injured cells were able to regain their tolerance to the inhibitory substance during a repair period of 2 and 6 h in tryptic phytone glucose broth and agar, respectively. Tollison and Johnson[118] indicated that heat-injured *S. flexneri* required a longer resuscitation period than that observed for other gram-negative bacteria.

Nakamura and Dawson[119] studied the effect of different suspending and recovery media on the survival of *S. sonnei* cells frozen at −20°C. *S sonnei* was frozen in saline, nutrient broth, or milk, and after thawing plated on a synthetic agar medium, nutrient agar, and blood heart infusion agar. The synthetic medium was unable to recover cells injured by freezing or did so poorly (1 to 4 log cycle lower count) compared to the complex media. Since the addition of meat extract, peptone, or casamino acids to the synthetic medium improved its ability to recover injured cells, the authors termed this freezing stress "metabolic injury". Since the authors did not employ any of the commonly used selective *Shigella* media in their studies, it is not known whether bile salts or sodium deoxycholate would interfere with the recovery of metabolically injured *S. sonnei.*

There is relatively little data on the quantitative recovery of various media used to isolate *Shigella,* and the occurrence of injured cells could further limit quantitative recovery. Twedt[120] recommended the use of gram negative (GN) broth as an enrichment medium followed by plating on xylose lysine deoxycholate agar, EMB agar, MacConkey agar, and DC agar. However, since GN broth contains deoxycholate, this procedure may not give quantitative recovery if any of the cells are injured. The long incubation period in GN broth (16 h) may allow some repair to occur, but an enrichment broth that does not contain any substance inhibitory for injured *Shigella* needs to be developed.

G. *Vibrio* Species

The genus *Vibrio,* along with *Aeromonas* and *Plesiomonas* discussed above, is a member of the family Vibrionaceae.[66] The genus *Vibrio* contains several species of importance to both clinical and food microbiologists, including *V. cholerae, V. parahaemolyticus,* and several additional species that are just becoming recognized as medically important, including

V. minicus, V. flavialis, V. vulnificus, V. alginolyticus, V. metachnikovii, and *V. hollise.*[121] Some characteristics of the species in the genus *Vibrio* include the following.[122] The bacteria are facultatively anaerobic, capable of both fermentative and respiratory metabolism; molecular oxygen is the universal electron acceptor; sodium ions stimulate the growth of all species and are an absolute requirement for most; in liquid media, the bacteria are motile by monotrichous or multitrichous polar flagella, which are enclosed in a sheath; and most are oxidase positive. They are found in aquatic habitats with a wide range of salinities; they are very common in marine and estuarine environments and on the surfaces and in the intestinal contents of marine animals.

V. cholerae is the cause of cholera, characterized by a profuse diarrhea, which can be fatal because of the loss of electrolytes and marked dehydration.[123] *V. parahaemolyticus* causes gastroenteritis[19,20] and is associated with seafoods that are consumed raw or are lightly cooked. While many *V. parahaemolyticus* food-poisoning outbreaks have occurred in Japan, some have occurred in the U.S.[124,125] as well as in many other nations.[20]

Isolating *V. cholerae* or *V. parahaemolyticus* is relatively easy.[122] Alkaline peptone water is the simplest and most widely used enrichment medium. Although many selective agars have been developed for these two organisms, thiosulfate-citrate-bile salt sucrose agar (TCBS) seems to be the most suitable and is highly selective and differential. After incubation at 37°C for 18 to 24 h, *V. cholerae* normally appear as yellow, sucrose-fermenting, flatish colonies (about 2 mm in diameter), while *V. parahaemolyticus* are green or blue (sucrose negative) and 2 to 5 mm in diameter. Bacteria of other genera grow slowly or not at all in this medium. Several other media have been formulated,[121] and usually contain bile salts in some form, or other surface-active agents.

Because *V. parahaemolyticus* is most often associated with various seafoods, its behavior under conditions that simulate seafood processing has received extensive study. Injury to *V. parahaemolyticus* caused by refrigeration (cold shock), freezing,[126] and heat[127,128] has been studied. In many of these studies, injury was expressed as an increased sensitivity to NaCl in the plating medium (*V. parahaemolyticus* will grow in the presence of 8% NaCl).[122] NaCl, at a 3% level, is used in most media for isolating *V. parahaemolyticus* from foods.[129] Ray et al.[130] have shown that the use of a broth recommended for *V. parahaemolyticus* (glucose salt teepol broth, GSTB)[127] gave decreased recoveries of injured (stressed by cold, 4°C, or freezing, −20°C) *V. parahaemolyticus.* They then developed a procedure, designated the repair-detection method, for enumeration of *V. parahaemolyticus* from commercial seafoods. In their procedure, blended seafood samples are first inoculated into trypticase soy broth (TSB), incubated for 2 h at 35°C, and then sterile NaCl solution was added to yield a final concentration of 3%. The samples were then further incubated overnight at 35°C. The GSTB is inoculated from the 3% NaCl TSB and incubated overnight at 35°C; the TCBS plates are then inoculated from the GSTB and incubated overnight at 35°C and examined for typical *V. parahaemolyticus* colonies (see above). In a survey of commercial seafoods, Ray et al.[130] observed that 68% of the samples tested positive for *V. parahaemolyticus* when examined by the recommended procedure. When their procedure was used in an MPN method, they observed about a 50% increase in MPN *V. parahaemolyticus* per gram of product. The study by Ray et al.[130] indicates that injured *V. parahaemolyticus* actually do occur in commercial seafoods, that they can repair, and ultimately be recovered and quantitated.

IV. SUMMARY AND CONCLUSIONS

The phenomenon of injured cells is of particular importance to food microbiologists, since they are concerned with the quantitative detection of various foodborne pathogens. Quantitative detection is important for two reasons: (1) the risk associated with the ingestion of

different pathogens is proportional to the number of pathogens in the food ingested and (2) assessing the adequacy of a food-processing step or sanitation operation is predicated on detecting all viable cells.

Injury has long been known for the "traditional" pathogens, especially *Salmonella* and *Staphylococcus aureus*. It can be seen from the group of emerging pathogens discussed here that injury is also a phenomenon which can be applied to almost all of them. At this point, *A. hydrophila* is the only bacterium of the group in which injury has not yet been observed. Further, there has been only a minor observation of injury in *L. monocytogenes*. However, some of the notorious difficulties associated with the isolation of *L. monocytogenes* from processed foods may very well be due to injured cells.

Despite the fact that these "new" pathogens are just becoming known to the food microbiologists, many selective media are already available for their isolation. These media have generally been developed by clinical microbiologists. Since the cultures with which the clinical microbiologists deal are actively growing and the cells are usually present in large numbers, as well as the fact that clinical specimens have background flora that are different from foods, clinically developed media cannot always be expected to serve the requirements of food microbiologists. Six of the seven emerging pathogens discussed above are gram-negative, and, except for *C. jejuni*, the media developed for them contain bile salts. As can be seen from the studies on *Y. enterocolitica, P. shigelloides, Shigella,* and *V. parahaemolyticus,* injured cells become sensitive to bile salts and deoxycholate. In addition, Head et al.[65] have indicated that nonstressed *Y. enterocolitica* is sensitive to deoxycholate. Palumbo et al.,[88] in their evaluation of media for the isolation of *A. hydrophila,* observed that nonstressed cells of this organism are also sensitive to bile salts, and ultimately they developed a medium containing ampicillin and starch and no bile salts or deoxycholate for the isolation of this organism from food.

Another approach has been developed to circumvent the sensitivity of injured cells to the agent(s) found in selective enrichment broths or inhibitory media: a delay in the exposure of injured cells to the agent, until repair of the cellular damage. This approach has been successfully utilized in recovering injured coliforms from dairy products[4] and is the basis for the repair-detection method for enumerating injured *V. parahaemolyticus* from seafoods.[130] This approach was also used in the study of freeze injury to *C. jejuni* by Ray and Johnson,[42] in which they delayed the addition of polymyxin B to the enrichment broth until the injured cells had time to repair. This procedure can be used with either plates[4] or liquid media.[42,130]

Thus, emerging foodborne pathogens are no different from the traditional foodborne pathogens: they can be injured by the same stresses as traditional pathogens. Food microbiologists should be aware of this, and, depending on the medium, organism, and food environment or treatment background, a negative plate cannot necessarily be interpreted as negative.

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Chapter 5

DETECTION OF INJURED *STAPHYLOCOCCUS AUREUS* FROM FOODS

Scott E. Martin

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I. INTRODUCTION

Staphylococcal food poisoning is probably the most common cause of foodborne disease illness in the world. The presence of *Staphylococcus aureus* in food may indicate a potential public health problem, since many strains of *S. aureus* are capable of producing enterotoxin(s) which may result in food poisoning upon ingestion. In general, there are four reasons why a food or an ingredient is examined to determine the presence of *S. aureus:*

1. To confirm the presence of *S. aureus* following a food-poisoning outbreak
2. To determine whether or not a food is a potential source of staphylococcal food poisoning
3. To demonstrate postprocessing contamination
4. As part of a routine quality control program.

It has been observed in the past that when staphylococcal cells are sublethally stressed, many are no longer able to grow on selective media. The reasons for this failure, and the methods to overcome it, have been extensively studied in *S. aureus.*

II. INJURED CELLS

Sublethal injury and stressed-cell recovery have been examined in many review articles.[1-3] Physical and chemical stresses that may injure staphylococcal cells during processing include heating,[4,5] freezing and freeze-drying,[6] irradiation,[7] reduced water activity,[8] and exposure to different chemicals such as acids and salts.[9,10]

Several sites of damage have been observed in sublethally injured cells. Most commonly reported are damage to the cytoplasmic membrane with the resultant leakage of cytoplasmic constituents[4,8,11,12] and the degradation of ribosomes and ribonucleic acid (RNA).[13,14] Other sites of damage include protein denaturation and enzyme inactivation.[9,15]

The involvement of RNA in injury was first recognized when investigators found that mildly heated suspensions of *S. aureus* lost 260-nm absorbing material into the suspending medium. Several investigators have reported similar observations, especially in relation to heat injury.[16] Russell and Harris[17] found that heat-injured *Escherichia coli* leaked 260-nm absorbing material, but the cells were still intact. They concluded that this effect was due to a weakening of the cytoplasmic membrane. Iandolo and Ordal[4] found that the 260-nm absorbing material that leaked from heat-injured *S. aureus* was RNA or RNA-derived nucleotides. Later studies demonstrated that this material was due to the degradation of ribosomal RNA, in addition to cellular pool constituents.[13,18]

The stability of ribosomes both *in vivo* and *in vitro* is dependent upon the correct interaction of protein, RNA, and various ions, especially the magnesium ion.[19] Phosphate also has been shown to exert considerable influence on the stability of ribosomes *in vitro*. Natori et al.[20] observed that when *E. coli* ribosomes were incubated in various phosphate concentrations, the ribosomes unfolded with a significant loss of protein. These phosphate-treated ribosomes were also shown to be more sensitive to ribonuclease than ribosomes from untreated cells. However, the addition of Mg^{++} to the heating menstruum prevented the change. These *in vitro* studies illustrate the importance of ionic interactions and heat on the conformation and stability of ribosomes.

The degradation of ribosomes and ribosomal RNA *in vivo* as a result of thermal stress has been examined in *S. aureus.*[13,18,21] Sucrose gradient analysis of ribosomes from heated cells revealed that the 30S subunit was selectively destroyed. Polyacrylamide gel electrophoresis of ribosomal RNA extracted from heated cells demonstrated that the 16S RNA had been degraded, yet the 23S RNA appeared normal.[21,22] Using methylated albumin Kieselguhr

column analysis, Sogin and Ordal[18] showed that both the 16S and 23S RNA species were altered in heated *S. aureus*. In a later study, Rosenthal and Iandolo[13] compared the susceptibility of 23S RNA from heated and unheated cells with digestion by pancreatic ribonuclease and found that RNA from heated cells was more susceptible to digestion. In addition, titration with labeled formaldehyde indicated fewer paired bases in 23S RNA from heated cells. Therefore, during heating, 16S RNA was almost completely degraded and 23S RNA was altered.

Hurst et al.[23] showed that *S. aureus* lost 40% of its cellular Mg^{++} when the cells were heated in a potassium phosphate buffer. Hurst and Hughes[24] suggested that RNA destruction resulted from Mg^{++} loss rather than by heating and the action of a Mg^{++}-inhibited nuclease. Thus, the heating of bacterial cells in the presence of a phosphate ion produces conditions which are conducive to RNA degradation by the stimulation of ribonuclease activity[25] and by the disruption of ribosome stability due to the chelation of intracellular Mg^{++}. Consequently, this degradation of bacterial ribosomes was due to the combination of phosphate and heat, both of which destabilized the ribosome, resulting in a susceptibility to nucleolytic attack. Magnesium is required for the integrity of ribosomes and, moreover, serves to inhibit a ribonuclease(s). Haight and Ordal[26] showed that heating partially purified ribosomes in tris buffer containing phosphate or ethylenediaminetetraacetic acid caused a greater disruption of ribosomes than heating in tris buffer with 10 m*M* Mg^{++}. These authors suggested that the degradation was the result of polynucleotide phosphorylase and ribonuclease, and that heat stimulated the degradation.

Recovery represents a period of cellular rebuilding, during which time injured organisms repair damage produced by the sublethal stress. Since ribosome damage and ribosomal RNA degradation are major lesions within most heat-stressed organisms, it follows that ribosomal RNA synthesis and ribosome assembly should be major events in recovery. Iandolo and Ordal[4] first reported the need for RNA synthesis during recovery. They demonstrated that the recovery of heat-injured *S. aureus* occurred in the presence of inhibitors of cell wall synthesis, protein synthesis, and energy metabolism. However, recovery was completely suppressed by the RNA synthesis inhibitor, actinomycin D. A rapid incorporation of a label into the RNA during the recovery period also was observed. These results indicated that RNA synthesis was the major metabolic event required for repair.

Sogin and Ordal[18] substantiated the involvement of RNA in the heat injury and repair of *S. aureus*. Using injury and recovery conditions similar to those of Iandolo and Ordal,[4] they confirmed that RNA synthesis was required for recovery. They further demonstrated that RNA synthesis preceded protein synthesis, and occurred even when protein synthesis was complete inhibited. In addition, these investigators examined the ribosome and ribosomal RNA of heated *S. aureus* before and after recovery. The methylated albumin Kieselguhr column analysis of ribosomal RNA revealed an altered elution pattern following heating. The elution pattern of ribosomal RNA from recovered cells appeared normal, suggesting that ribosomal RNA was synthesized during the recovery period. These data were supported by sucrose gradient analysis of 70S ribosomes. The 70S peak on the gradient was completely absent following heat injury, but was regenerated during recovery.

The sucrose gradient conditions used in this study did not allow the analysis of the individual 50S and 30S subunits. In a subsequent study, Rosenthal and Iandolo[13] found that only the 30S subunit and 16S RNA were degraded during heating. However, the 50S subunit, as well as the 30S subunit, was regenerated during repair.[21] The incorporation of labeled nucleosides into the ribosomal RNA of regenerated ribosomes demonstrated that both 16S and 23S ribosomal RNA were synthesized during recovery. The addition of chloramphenicol did not inhibit the regeneration of ribosomal subunits, suggesting that proteins from the preinjury ribosomes were reutilized. This hypothesis was confirmed by highly labeling the ribosomal proteins prior to injury and recovering the cells in media containing labeled uridine,

under conditions in which protein synthesis was inhibited. The prelabeled proteins were associated with newly synthesized RNA in the regenerated subunits.

Flowers and Martin[27] examined the process of ribosome formation during the repair of sublethal heat injury in *S. aureus*. Cells recovering from sublethal injury were examined for changes with time in the sedimentation and electrophoretic properties of ribonucleoprotein particles and RNA, respectively. When the cells were allowed to recover in [^{3}H]uridine, the label could be followed into an RNA species that coelectrophoresced with 23S and 16S RNA. The ribonucleoprotein particles (49S, 36S, and 30S) were isolated from repairing cells by sedimentation through sucrose gradients. Polyacrylamide gel electrophoresis showed that the 49S particle contained 23S RNA, the 36S particle contained both 23S RNA and 16S precursor and mature RNA, and the 30S particle contained 16S and precursor 16S RNA. Particles with similar sedimentation properties were found in unheated cells.

III. OXYGEN TOXICITY

Molecular oxygen is only slightly soluble in water, with only 9 mg/l being dissolved at 20°C and 1 atm of pressure.[28] Only 7 mg O_2/l are dissolved in nutrient broth at 30°C. However, molecular oxygen is seven to eight times more soluble in organic solvents than in water, such that it probably becomes concentrated in the lipophilic cell membranes.[28] Molecular oxygen is paramagnetic, due to two of its valence electrons being unpaired. Molecular oxygen in this form is in the lowest energy configuration with the unpaired electrons in parallel spin, and is referred to as ground or triplet oxygen. Triplet oxygen may be energized to yield singlet oxygen, which has the two unpaired electrons in antiparallel spin. Two forms of singlet oxygen exist, and are of unequal energies. The first, which has the lower energy of the two, occurs when the unpaired electrons of the ground state become paired in the same orbital, and are of antiparallel spins. The second occurs when one electron undergoes a spin inversion, with the two unpaired electrons remaining in separate orbitals. Singlet oxygen is exceptionally reactive and therefore poses a threat to the integrity of all cellular components.[28] While it is very short-lived in aqueous media, it may be most damaging to those hydrophobic lipid/protein regions, such as membranes, where it reacts with carbon-carbon double bonds in polyunsaturated fatty acids, and where singlet oxygen would not be quenched and would be long lasting.[29]

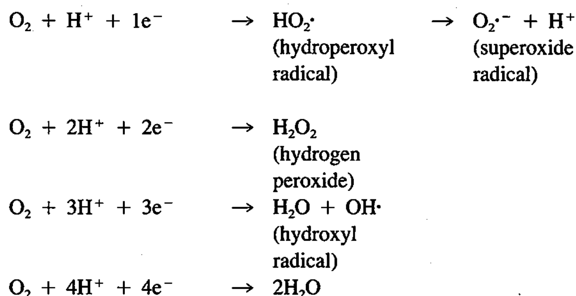
Triplet oxygen contains two unpaired electrons of parallel spin. These parallel electron spins forbid the direct entry of paired electrons.[29] Therefore, in order for a divalent reduction to occur, one electronic spin would have to be inverted in order to avoid the placement of two parallel spins in one orbital. As a result, whenever energetically feasible, univalent pathways of the reduction of oxygen are favored over divalent pathways. The first product in the univalent reduction of molecular oxygen is the hydroperoxyl radical in the protonated form, and the superoxide anion radical in the ionized form (see Table 1).

The superoxide anion radical ($O_2{\cdot}^-$) can act as a powerful reducing or oxidizing agent, and can also initiate free radical chain reactions. The superoxide radical and hydrogen peroxide (H_2O_2) can interact to form the hydroxyl radical OH·, the most potent oxidant known. Finally, singlet oxygen may be produced by the reaction of $O_2{\cdot}^-$ with OH·,[28] or by the interaction of two H_2O_2 molecules.[30]

H_2O_2 is formed by the two-electron reduction of molecular oxygen or by the dismutation of superoxide. It is a strong oxidant, and therefore has pronounced bactericidal effects, as a function of time, exposure, and concentration, on spore-forming and nonspore-forming bacteria.[31] H_2O_2 is produced by most aerobically growing cells (bacterial, plant, and animal) by the action of superoxide dismutase and by some of the flavin-linked enzymes.

H_2O_2 is the most stable or least reactive of the intermediates formed in the univalent pathway, and yet it remains a strong oxidant capable of causing irreversible damage to

Table 1
THE UNIVALENT REDUCTION OF MOLECULAR OXYGEN



$O_2 + H^+ + 1e^-$	→	$HO_2\cdot$ (hydroperoxyl radical)	→	$O_2\cdot^- + H^+$ (superoxide radical)
$O_2 + 2H^+ + 2e^-$	→	H_2O_2 (hydrogen peroxide)		
$O_2 + 3H^+ + 3e^-$	→	$H_2O + OH\cdot$ (hydroxyl radical)		
$O_2 + 4H^+ + 4e^-$	→	$2H_2O$		

various cell components. H_2O_2 is a bactericidal agent that has been used as a topical antiseptic for many years.[32,33] The effectiveness of H_2O_2 varies with the concentration, temperature, duration of treatment, and the type and number of organisms.[31,34] The modes of action of H_2O_2 on bacterial cells are numerous.

For example, H_2O_2 can deactivate enzymes by modifying amino acid residues.[35,36] The degradative effects of H_2O_2 on deoxyribonucleic acid (DNA) have been demonstrated. H_2O_2 degrades DNA by changing the primary and secondary structures. The following are the relative magnitude of degradation rates.[37-40]

Base destruction > single strand breaks > double strand breaks > cross linking

The third product produced in the univalent reduction of O_2 is the hydroxyl radical. The hydroxyl radical can theoretically be produced in a number of ways. Beauchamp and Fridovich[41] showed that the hydroxyl radical could be generated in significant amounts by a reaction between $O_2\cdot^-$ and H_2O_2 in an aqueous system. The $OH\cdot$ radical is the most potent oxidant known, and can cause numerous types of injury.

IV. ENZYMATIC DEFENSES

Oxidative enzymes have evolved to circumvent the spin restriction of oxygen and accomplish the divalent and tetravalent reduction of oxygen without the release of toxic intermediates.[29] In fact, most of the oxygen consumed by respiring cells is used by cytochrome C oxidase, which reduces the oxygen to water without the formation of $O_2\cdot^-$ or H_2O_2.[29] Nevertheless, $O_2\cdot^-$ and H_2O_2 are formed in respiring cells via the univalent pathway of oxygen reduction. The use of oxygen for respiring organisms would lead to their destruction if not for the enzymatic defenses against these toxic intermediates.

The superoxide radical is scavenged and converted to H_2O_2 and O_2 by the enzyme superoxide dismutase (SOD). SOD, discovered by Fridovich,[42] is a defense mechanism which is found in most aerobic and facultative anaerobic organisms.[28] SOD occurs in three metalloenzyme forms: dismutases containing both copper and zinc, and dismutases containing either manganese or iron as cofactors. These three forms segregate into two families, copper-zinc and manganese/iron dismutases, of dramatically different properties.[43]

Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide to oxygen and water. Thus far, all catalases studied are composed of four identical subunits, each have molecular weights of approximately 60,000 Da.[44] Each of the tetrameric units contains a protoheme group and an independent active site. Catalase is able to catalyze the decomposition of H_2O_2 by different reactions, and is dependent upon the concentration of H_2O_2.[45]

In our laboratory, we have isolated catalase from *S. aureus* MF-31. The optimal pH for *S. aureus* MF-31 catalase was pH 5 to 6.[46] The enzyme was stable over the range pH 4 to 9. The apparent isoelectric point was 5.3 ± 0.1 pH units. With respect to temperature stability, *S. aureus* MF-31 catalase was stable at 52°C after 45 min of heating. The enzyme was deactivated at 60°C for 10 min. However, this was dependent upon the concentration of the enzyme and the presence of protectants. The apparent subunit molecular weight was 64,000 ± 1000 Da, whereas the apparent native molecular weight was 235,000 ± 5000 Da. Amino acid analysis revealed that *S. aureus* MF-31 catalase was similar to amino acid analyses of catalase from other sources. The iron content of *S. aureus* MF-31 catalase was found to be 0.098%, comparing favorable to other published findings. The enzyme was inhibited by millimolar concentrations of sodium cyanide, sodium azide, and hydroxylamine, indicating the presence of a heavy-metal catalyst. The effects of salt, pH, and salt concentration with heating on catalase activity were also examined. The results indicated that of the variables tested, pH played a major role. The chloride anion inhibited catalase activity at low pH, but this inhibition was influenced by the cation. Sodium chloride was more inhibitory at low pH than either potassium chloride or magnesium chloride.

H_2O_2 can also be nonenzymatically degraded by pyruvate.[47] This reaction is common in microorganisms such as *Lactobacillus delbruckii* and *Lactobacillus acidophilus* which lack catalase.

V. SIGNIFICANCE OF INJURED *STAPHYLOCOCCUS AUREUS* CELLS

The food industry often employs some form of processing or low-temperature storage to extend the shelf life of perishable products. Food processing, which may consist of the use of chemical preservatives, drying, freezing, heating, radiation, or a combination of these methods, is designed to reduce or eliminate pathogenic and spoilage organisms in the food products. Although most microorganisms are destroyed, some sublethally injury or stressed cells may survive and thus may be able to recover and grow at a later time. These stressed cells are usually more susceptible to an adverse environment or secondary stress to which noninjured cells could readily adapt. Secondary stresses are also encountered in the form of selective agents used in the enumeration medium.

The choice of the method for the detection of *S. aureus* depends upon the purpose for conducting the test and upon the product involved. When food is suspected as the source of a staphylococcal food-poisoning outbreak, large numbers of *S. aureus* are frequently present, and sensitive methods may not be required. More sensitive methods are required to detect small populations of *S. aureus,* which may be present as the result of postprocessing contamination. In many cases, *S. aureus* is not the sole or even the predominant organism present in a sample. For this reason, selective inhibitory media are employed for isolation and enumeration.

Selective media utilize a number of different toxic chemicals to achieve selectivity. Some of the ingredients used include sodium chloride, tellurite, lithium chloride, and various antibiotics. Most selective media are suitable for the enumeration of normal or unstressed *S. aureus*. However, due to processing, preservation, or other adverse conditions, sublethal stress may occur, resulting in the increased sensitivity of *S. aureus* to the selective agents. Because injured cells exhibit an increased sensitivity to selective agents, *S. aureus* may go undetected in conventional selective enumeration procedures. Lancette[48] recently reviewed current enumeration methods for the recovery of stressed *S. aureus*.

Baird-Parker and Davenport[49] demonstrated that the recovery of heated or dried cells of *S. aureus* was best on media containing blood or sodium pyruvate. They suggested that catalase of *S. aureus* may be destroyed or its activity reduced by heating or drying and that blood, which contains catalase, or the addition of pyruvate, helped in the enumeration by

Table 2
EFFECTS OF CATALASE ON THE ENUMERATION OF UNSTRESSED AND THERMALLY STRESSED *STAPHYLOCOCCUS AUREUS* MF-31

	Unstressed		Stressed[a]	
Medium	CFU/ml	Enumeration[b] %	CFU/ml	Enumeration[b] %
B-P	3.9×10^9	100	2.4×10^9	100
VJ	3.5×10^9	90	6.2×10^8	26
VJ + catalase[c]	4.2×10^9	108	2.9×10^9	121
TSA	3.4×10^9	87	1.9×10^9	79
TSA + catalase[c]	3.9×10^9	100	2.5×10^9	104
TSAS	3.1×10^9	79	1.8×10^7	0.8
TSAS + catalase[c]	3.5×10^9	90	1.2×10^9	50
MSA	3.0×10^9	77	2.5×10^6	0.1
MSA + catalase[c]	3.4×10^9	87	7.2×10^8	30
S110	2.2×10^9	56	2.3×10^6	0.1
S110 + catalase[c]	2.5×10^9	64	3.7×10^8	15
TPEY	4.2×10^9	108	8.0×10^8	33
TPEY + catalase[c]	4.3×10^9	110	2.5×10^9	104

Note: CFU, colony-forming units; B-P, Baird-Parker agar; VJ, Vogel and Johnson agar; TSA, tryptic soy agar; TSAS, tryptic soy agar + 7% NaCl; MSA, mannitol salt agar; S110, staphylococcal 110 agar, TPEY, tellurite polymyxin egg-yolk agar.

[a] Cells were heated in 100 m*M* potassium phosphate buffer (pH 7.2) at 52°C for 15 min.

[b] Percentage of enumeration was calculated by dividing colony-forming units per milliliter on the various media by colony-forming units per milliliter on B-P and multiplying by 100.

[c] Catalase activity was about 780 units per plate.

destroying H_2O_2 produced by recovering cells. Amin and Olson[50] demonstrated the increased sensitivity of *S. aureus* to heat treatment in milk treated with 0.05% H_2O_2. The increased catalase activity of the surviving populations following heat and H_2O_2 treatment and the addition of sodium pyruvate to the medium were shown to be beneficial in the enumeration of *S. aureus*.[34] Several workers have found that Baird-Parker (B-P) agar is most satisfactory in enumerating injured cells when compared with other staphylococcal selective media.[51,52,53]

In our laboratory, we have found that the addition of catalase to tryptic soy agar plus 7% NaCl (TSAS; see Table 2) and other selective media increased the enumeration of thermally stressed *S. aureus* cells, while the addition of heat-inactivated catalase had little effect on enumeration[9,10] (see Figure 1). In a later study, the addition of catalase to other selective media was found to increase the enumeration of both heat-stressed and unstressed cells.[10] The beneficial effects of catalase were most pronounced when the stressed cells were plated on a selective high salt-containing medium (see Table 2). The addition of catalase or pyruvate (another agent causing the spontaneous decomposition of H_2O_2) to tryptic soy broth with 10% NaCl also increased the enumeration of normal and thermally stressed cells of *S. aureus* using a most probable number (MPN) technique[54] (see Tables 3 and 4). Hurst et al.[8] examined 45 substances for their effects on the enumeration of heat-injured *S. aureus*. Among others, pyruvate was effective in increasing enumeration. The accumulation of superoxide radicals and H_2O_2 in various media used for the enumeration of anaerobic bacteria has been observed after exposure to atmospheric oxygen.[55,56] Carlsson et al.[56] suggested that when phosphate and glucose were autoclaved together in a culture medium at neutral or alkaline pH, products resulted which rapidly autooxidized, forming these reactive species. The level of H_2O_2 in

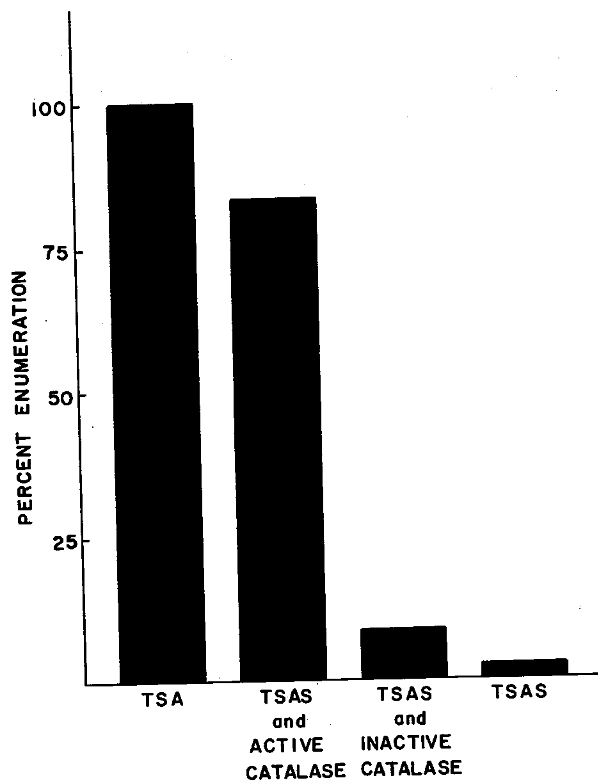


FIGURE 1. Effect of catalase and heat-inactivated catalase on the enumeration of heat-treated *Staphylococcus aureus* MF-31. Catalase was inactivated at 80°C for 30 min. TSA, tryptic soy agar; TSAS, TSA plus 7% NaCl.

ordinary anaerobic culture media exposed to air is not expected to exceed 100 m*M*.[56,57] The addition of catalase to such media resulted in the increased enumeration of *Clostridium perfringens, Mycobacterium tuberculosis,* and *Shigella dysenteriae.*[55,58,59] Similarly, the addition of catalase to selective media increased the enumeration of *Salmonella typhimurium, Pseudomonas fluorescens,* and *Escherichia coli* subjected to a variety of stresses, such as heating, chilling, freezing, and freeze-drying, but did not increase the enumeration of thermally stressed *Streptococcus faecium,* an organism lacking catalase.[9,60]

The decreased activity of catalase and the subsequent accumulation of H_2O_2 have been implicated as factors in the decreased enumeration of thermally stressed cells on selective media. Bucker et al.[61] demonstrated the synergistic effect of heat and salt in reducing the catalase activity of *Staphylococcus aureus*. The decrease in catalase activity allowed the accumulation of H_2O_2, which led to cell death. In an effort to test this hypothesis, Bucker et al.[61] thermally stressed *S. aureus* cells and recovered them under anaerobic conditions. Since most H_2O_2 production in cells is due to aerobic respiration, one would expect a greatly reduced production of H_2O_2 under anaerobic conditions. These authors found a 200-fold increase in enumeration when thermally stressed cells were incubated anaerobically on tryptic soy agar containing 6.5% NaCl over aerobic counts on the same medium.

Several attempts have been made to develop a staphylococcal-selective medium that gives an enumeration equal to that of B-P agar while overcoming difficulties in the use of B-P agar (e.g., with milk products) and its expense. Staphylococcal-selective media formulations are shown in Table 5. Andrews and Martin[62] modified Vogel and Johnson agar by the

Table 3
ENUMERATION OF LOW NUMBERS OF *STAPHYLOCOCCUS AUREUS* STRAINS USING A 5-TUBE MPN PROCEDURE

		Unheated cells		Heated[a] cells	
Strain	Medium	MPN/ml	% Enumeration[b]	MPN/ml	% Enumeration[b]
MF-31	TSB	7,900	100	2,300	100
	TSBS	4,900	62	NG	
	TSBS + catalase[c]	7,000	89	2,300	100
	TSBS + 1% pyruvate	7,000	89	2,300	100
196E	TSB	46,000	100	34,500	100
	TSBS	33,000	72	23	0.07
	TSBS + catalase[c]	33,000	72	17,200	50
	TSBS + pyruvate	46,000	100	17,500	51
181	TSB	7,000	100	3,300	100
	TSBS	4,900	70	NG	
	TSBS + catalase[c]	6,300	90	490	15
	TSBS + 1% pyruvate	4,900	70	700	21
210	TSB	4,600	100	3,300	100
	TSBS	3,300	72	14	0.4
	TSBS + catalase[c]	4,900	107	1,720	52
	TSBS + 1% pyruvate	4,600	100	1,720	52

Note: TSB, tryptic soy broth; TSBS, TSB with NaCl (10%); NG, no growth in any tubes.

[a] Cells heated at 52°C for 20 min in 100 m*M* potassium phosphate buffer (pH 7.2).
[b] Percent enumeration calculated using MPN/ml in TSB as 100%.
[c] Catalase added to a level of 200 U/ml.

Table 4
ENUMERATION OF *STAPHYLOCOCCUS AUREUS* FROM NATURALLY CONTAMINATED, LOW-TEMPERATURE RENDERED GROUND BEEF

Sample	MPN/g[a] in TSB	MPN/g[a] in TSBS	MPN/g[a] in TSBS + C[b]	MPN/g[a] in TSBS 1% pyruvate	CFU/g on B-P
1	23	23	230	230	400[c]
2	150	230	930	930	700[c]
3	24,000	230	1,500	2,100	5,600
4	2,100	2,400	24,000	24,000	17,200
5	930	430	930	930	600[c]
6	930	430	2,300	2,300	2,000
7	2,300	240	2,300	1,500	2,100

Note: Positive MPN tubes were plated on Baird-Parker agar (B-P), from which typical colonies were picked and tested for coagulase production and heat-stable deoxyribonuclease activity. The reported MPN is for tubes showing the presence of confirmed *S. aureus*. TSB, tryptic soy broth; TSBS, TSB with NaCl (10%); CFU, colony-forming units.

[a] Three-tube MPN procedure.
[b] Catalase, added to a level of 200 U/ml.
[c] Estimated.

Table 5
STAPHYLOCOCCAL SELECTIVE MEDIA COMPOSITION

Component	B-P (g/l) pH 7.0	PCVJ (g/l) pH 7.2	VJ (g/l) pH 7.2
Peptone	10	10	10
Yeast extract	1	5	5
Beef extract	5	5	—
LiCl	5	5	5
Glycine	12	10	10
Sodium pyruvate	10	—	—
Potassium phosphate dibasic	—	5	5
Mannitol	—	10	10
Phenol red	—	0.025	0.025
Phosphatidyl choline	—	2	—
DNA	—	2	—
Agar	18	16	16
Tellurite (1%)	10 ml	10 ml	20 ml
Egg yolk (50%)	53 ml	—	—
Catalase	—	780 units per plate	—

Note: B-P, Baird-Parker agar; PCVJ, phosphatidyl choline-Vogel and Johnson agar; VJ, Vogel and Johnson agar.

addition of 0.5% beef extract, 0.2% DNA, 0.2% phosphatidyl choline (lecithin) and 780 units of catalase spread on the agar surface prior to inoculation. They found this phosphatidyl choline-Vogel and Johnson agar (PCVJ) gave an equivalent enumeration of stressed *S. aureus* cells and staphylococci from naturally contaminated food samples, to that found for B-P enumeration. Enumeration with this medium was easier than with B-P and allowed ascertaining the production of DNase. Idziak and Mossel[63] modified the B-P agar formulation by replacing the egg yolk with pig plasma (B-PP). These authors found that, based on selectivity, diagnostic characterization, and increased sensitivity, B-PP agar was superior to B-P agar.

Lachia[64] described a simplified method for the enumeration of *S. aureus* from food. He replaced the egg yolk in B-P agar with Tween® 80 (0.05% wt/vol) and $MgCl_2$ (0.1%). When these compounds were added to the egg yolk-free B-P agar, the recovery of stressed cells was comparable to recovery on complete B-P agar.

Mentzer-Morgenstern and Katzenelson[65] developed a single-step staphylococcal-selective medium identified as 4-S agar. This medium permitted the isolation and identification of staphylococci and was achieved in a single step. Coagulase-positive staphylococci form small, grey to dark-grey colonies surrounded by a dense, white opacity. Unfortunately, heat-stressed cells were inhibited by this medium, and a 3-h preincubation period in brain heart infusion was required to enumerate these injured cells. This medium is reported to be very selective for *S. aureus*.

When low numbers of *S. aureus* are expected in a food sample, a MPN procedure is generally employed. The MPN technique is considered more efficient in the enumeration of low numbers of organisms, or when high levels of competing organisms are present.[66,67,68] A MPN value is an estimate of the population and not a precise enumeration of viable organisms. Microbiological counts are reported as "number of microorganisms per quantity of sample by MPN method". Strict interpretation of the confidence limits for a MPN value of 20/g, for example, asserts that the true population density lies between 7 and 89/g in 95% of all samples.[69]

Because of its selectivity, NaCl (10%) has been incorporated into tryptic soy broth (TSBS) in a MPN procedure. After 48 h in TSBS, suspected tubes must be streaked onto B-P agar for an additional 48 h for confirmation.[48] The enumeration of injured *S. aureus* cells in TSBS has been shown to be greatly depressed.[48,54] Brewer et al.[54] found that the addition of 1% pyruvate or catalase significantly increased the enumeration of stressed cells (see Tables 3 and 4). Because of the requirement to add catalase after autoclaving, pyruvate is considered more desirable.

Other staphylococcal MPN media have been examined. Van Dorne et al.[70] proposed a liquid modification of B-P agar, followed by streaking on B-P agar plates and incubation at 43°C for 24 h. This medium has demonstrated good recovery of stressed cells. However, Lancette[48] described several disadvantages; tellurite and paraffin plug addition; small colonies after 24 h at 43°C; and completing bacteria may outgrow *S. aureus* cells. Giolitti and Cantoni[67] developed a MPN medium to recover *S. aureus* cells from dried milk. This medium contains pyruvate and Tween® 80. Chopin et al.,[71] in a collaborative study, found good correlation between Giolitti-Cantoni broth results and direct enumeration on B-P agar. However, Lancette[48] reported that this medium is inadequately selective and its use is labor intensive.

VI. DETECTION METHODS

A. High Numbers of *Staphylococcus aureus*

When a sample is thought to contain ≥1000 *S. aureus* cells per gram, the most widely recommended enumeration medium is B-P agar. The samples are suspended in sterile diluent and 1.0 ml is spread-plated in triplicate on B-P agar plates. The plates are dried, inverted, and incubated for 48 h at 35°C. Typical *S. aureus* colonies are black to dark gray, circular, smooth, convex, moist, frequently with a light-colored margin, surrounded by an opaque zone of precipitation, and frequently with an outer clear zone. The colonies have a buttery to gummy consistency.[72] Unfortunately, the zones are not always apparent, causing some *S. aureus* cells to be missed. Further tests are necessary to confirm the suspected colony as *S. aureus*. These tests include coagulase, catalase, anaerobic utilization of mannitol, and lysostaphin sensitivity.

B. Low Numbers of *Staphylococcus aureus*

The Association of Official Analytical Chemists (AOAC) procedure for the detection of low numbers of *S. aureus* is a MPN procedure which utilizes MPN tubes containing TSBS and 1% sodium pyruvate.[73] In a collaborative study, this method was shown to significantly increase the enumeration of low, middle, and high levels of *S. aureus* from naturally contaminated products. This method is highly selective for *S. aureus*. The MPN tubes are inoculated from appropriate dilutions and are incubated at 35°C for 48 h. The tubes are confirmed positive by streaking on B-P agar incubated at 35 to 37°C for 48 h. Lancette et al.[74] reported that the addition of 1% pyruvate to TSBS in a MPN procedure gave a significantly better enumeration of both artificially and naturally contaminated foods than did TSBS alone. They also found that the addition of pyruvate to TSBS increased the recovery of heat-stressed and nonstressed staphylococci. Their results are similar to those of Brewer et al.[54] as shown in Tables 3 and 4. As can be seen, both catalase and pyruvate improved recovery. For confirmation, the positive MPN tubes should be used to inoculate B-P agar, followed by the previously described biochemical tests.

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Chapter 6

DETECTION OF INJURED SPORE-FORMING BACTERIA FROM FOODS

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I. INTRODUCTION

Processing treatments, such as the application of heat, cold, irradiation, and chemicals, used for microbial destruction or control to preserve foods, can cause injury to microbial cells or spores when applied at sublethal levels. Microbial damage or injury is expressed as an inability to produce detectable growth under conditions suitable for the proliferation of uninjured organisms. This inability is manifested as more complex or different nutritional and cultural requirements for spore germination and/or growth, or as increased sensitivities to environmental and chemical conditions, such as incubation temperature and time, and selective or inhibitory agents.

Injured microorganisms are distinguished from those that are dead or mutated by their ability to regain normal physiological activity when placed in the appropriate environment. A return to normal physiological function has been termed resuscitation or repair.[1] The extent and severity of sublethal injury, the mechanisms of damage, and the mechanisms and degree of recovery vary with the processing treatments, the species, the strains, the condition of the microorganism, and the methods of resuscitation.

The injury of spore-forming bacteria has been detected at different stages of the spore cycle, including germination, outgrowth, and cellular growth, while the sites of injury include damage to enzymes and germination systems, membrane disruption, and damage to genetic material. Investigations on microbial injury, however, have been more extensive with nonspore-forming than with spore-forming bacteria, while the injury of spores has been studied more extensively than injury to vegetative cells of spore-forming bacteria. Thermal injury of bacterial spores has also been studied to a greater extent than other types of injury.

Information on the sublethal injury and recovery of bacterial spores is very important in evaluating the lethality of existing and new food processing procedures. While injured spores and cells may be undetected during the routine microbiological examination of processed foods, they may repair during food storage and result in potential spoilage and quality and/or safety problems.

The subject of bacterial injury and repair has been reviewed extensively in books and journal publications.[1-30] The main objective of this chapter is to summarize information dealing with the repair, resuscitation, and detection of injured spore-forming bacteria. An overview of bacterial spore injury, its manifestations, mechanisms, and significance is also included.

II. SIGNIFICANCE OF INJURY

The injury of spore-forming bacteria by various processing treatments, especially by those most commonly used, such as heat, is a common occurrence.[16] Injured microorganisms are of significance in food preservation, spoilage, safety, food fermentations, and laboratory research.[1,4-7,16,24]

Inadequate detection of injured spores in a food can be a potential spoilage and/or health hazard if the injured spores undergo repair or germination by an alternative pathway, and proliferate during product storage. It is therefore very important to employ techniques that will optimize the detection and enumeration of both injured and uninjured organisms. If the

injured spores, however, remain inactive indefinitely, then injury is a beneficial phenomenon in food processing and preservation, because no health or spoilage problems will develop. An added benefit is achieved if lower levels of preservatives (e.g., salt or nitrite) will be adequate to inhibit injured organisms without the need of more severe processing treatments that may damage the nutritional and eating quality of the food. If this is the case, however, then development of sensitive methods for the detection of injured spores would not be necessary.

Bacterial spore injury is also important in developing and modifying methodologies and in evaluating the lethality of food processes, such as thermal and irradiation treatments. Ignorance or poor recovery of injured organisms may lead to an overestimation of lethal effects, which can be expressed as low heat or irradiation resistance values.

An understanding of injury can help researchers and industry personnel to optimize the proliferation and maintenance of useful microbial cultures. Injured spores can be valuable experimental tools to study various resistances and mechanisms of inhibition and physiological function. In the long run, sublethal damage may serve as a selection process to eliminate sensitive forms and enhance the domination of resistant groups.[1,4]

In general, it is important to understand the resistance, injury, repair, and detection of spore-forming bacteria of importance to food and other industries. This can help scientists and processors optimize the lethal effects with the minimum damage possible to food, which can lead to a shelf life of a known and acceptable length. The knowledge of microbial injury and repair will also prevent damage to useful microbial cultures, improve present analytical methods in food microbiology, and may be valuable in improving our understanding of microbial physiology and inhibition.

III. BACTERIAL SPORE FORMERS AND THEIR LIFE CYCLE

Bacterial spores are formed in the vegetative cell during sporulation and are released into the environment during cell lysis. Spores are able to withstand high doses of heat, irradiation, and chemicals during the processing of foods. Spore-forming bacteria of importance to foods include species of *Bacillus* and *Clostridium,* as well as *Desulfotomaculum* and *Sporolactobacillus.*[31,32]

The basic structure of bacterial spores consists, from the inside to the outside, of a protoplasmic core, an inner plasma membrane, a cell wall, a cortex, an outer membrane, and the spore coats. The spore coats protect the spores and respond to germinants. The cortex and the cell wall are comprised of peptidoglycan of different structures. The spore cell wall forms the cell wall of the germinated cell. The dehydrated core consists of deoxyribonucleic acid (DNA), ribosomes, enzymes, and other cellular components, including calcium, magnesium, manganese, and dipicolinic acid.

Bacterial spores present in foods in the injured, dormant, or superdormant states are not considered as threats to future quality and safety, unless they germinate and become metabolically active. The objectives of food processing methods for the manufacture of shelf-stable products are to destroy or inhibit bacterial spores, rendering the products safe while retaining acceptable quality for long periods of time. The repair and proliferation of injured or dormant spores, however, may lead to product quality loss or food safety problems, or both. Thus, it is very important to be able to detect undamaged as well as injured, dormant spores in a food.

Since processing treatments designed to inactivate the resistant bacterial spores will certainly inactivate their more sensitive cells, most of the information available on the injury and recovery of spore-forming bacteria deal with spores instead of vegetative cells. The recovery and enumeration of normal or injured, dormant spores which are susceptible to injury and repair involve the completion of the various stages in their spore cycle.[16,23,26,27,33-37]

A. Activation

Activation is generally accomplished by a sublethal heat or other treatment (e.g., alcohol), which does not cause significant changes in the properties of the dormant spore, but accelerates the germination process. This treatment also may inactivate the vegetative cells that may be present in the substrate.

B. Germination

During this stage of the spore cycle, the dormant state of the spore is irreversibly terminated. This complex series of events leads to a stress-sensitive, metabolically active form, which is ready for the next stage or outgrowth. When spores germinate they no longer reflect light; they become stainable and lose their characteristic resistance to heat, radiation, chemicals, and other stresses. The events of this stage are degradative in nature and involve the rapid depolymerization and excretion of spore constituents, resulting in a loss of about 30% of the dry weight of the spore.

Germination is initiated with several agents which bring about the first, or trigger, reaction. Germinants include nutrients (e.g., amino acids and sugars), nonnutrient germinants (e.g., metal ions, bicarbonate, and calcium dipicolinate), enzymatic germinants (e.g., lysozyme, spore enzymes, and initiator protein), and physical treatments (e.g., abrasion or hydrostatic pressure).

Although the mechanism of spore germination is not well defined, it is believed that nutrient-mediated germination occurs in two stages, identified as microlag and microgermination.[16] Microlag is the period between exposure to the nutrient germinant and the first visible sign of germination. Microgermination leads to the complete loss of refractility.

In general, germination is a multistage process involving several pathways in each stage. Each of these pathways may have specific cultural requirements, which may be involved in bypassing injury of the bacterial spore.

C. Outgrowth

This is a transitional stage, during which the germinated spore is transformed into the first vegetative cell. It is a metabolic step involving the synthesis of ribonucleic acid (RNA), protein, DNA, and cell wall and membrane, which differentiate the spore into a vegetative cell.[16] Outgrowth may be inhibited at any of these metabolic steps, and, thus, injury affecting outgrowth may take place at several potential sites. The effects of injury on outgrowth, however, have received only limited study.

D. Growth

This stage involves metabolic growth and an increase in the number of vegetative cells. The inhibition of growth or the doubling process will prevent detection of the organism. Of course, this would prevent potential spoilage and health hazards.

IV. SPORE INJURY

One major difference between bacterial spores and cells is the high resistance of the spore to environmental stresses, which makes their destruction difficult to achieve under ordinary or mild conditions. For complete destruction or inhibition, severe processing or chemical treatments should be used, or treatments may be combined to minimize damage to the quality of the food.[28]

The injury of bacterial spore formers has been observed for decades, and it has been considered an important factor in evaluating the extent of the destruction accomplished by various treatments.[2,4,5,16,21] Several types of injury have been reported for bacterial spores, involving both germination and outgrowth.[16] Spore injury is more complex than injury to

vegetative cells[6,7] due to the fact that any of the several steps in the spore cycle can be independently affected.

Overall, the injury and repair mechanisms of bacterial spores are different from those in vegetative cells due to the dormant and resistant state of spores, the several stages involved in the transformation of a spore into a vegetative cell, and the possibility of a spore being defective in nature during sporulation.[23]

Treatments and substances that injure bacterial cells may also damage spores if they are increased in severity or concentration. Mild treatments, however, such as mild heating, cation exchange, or mild heating in the presence of calcium dipicolinate, can also injure spores or increase their dormancy.[23]

Some treatments may injure spores by mechanisms different than those involved in damage to vegetative cells. Furthermore, treatments such as freezing and drying, which often injure vegetative cells, are not usually implicated in the injury of spores. Chemicals such as antimetabolites, which injure vegetative cells by disrupting their metabolic function, are usually ineffective in injuring the dormant spores, except when they bind to the spores and result in injury expressed at a later stage of development involving metabolic activity,[23] or when they are used in combination with other treatments.[28]

In general, the physical treatments involved in damage to bacterial spores include wet, dry, and ultrahigh-temperature heating; ionizing and UV radiations; and, possibly, hydrostatic pressure. Chemical injury of spores is caused by extreme pH values, cation-exchange treatments, and disinfectants such as hypochlorite, phenols, hydrogen peroxide, and ethylene oxide. Enzymatic treatments are active when spore coat permeability is altered and include lysozyme and other hydrolytic enzymes. Metabolic factors may be active during sporulation and include the lack of important nutrients and altered levels of divalent cations. Mutations interfering with sporulation can also lead to altered or injured spore crops.[23] Injury is also caused by exposure to combinations of physical treatments and chemicals,[28] including food preservatives.

Injury to bacterial spores is expressed in various forms. These were classified by Adams[16] as a need for nonnutrient germination stimulants by the injured spores, modified optimum incubation temperatures for the enumeration of survivors, an increased sensitivity of the survivors to inhibitors and selective agents, and altered nutritional requirements by the survivors. Other manifestations of spore injury, required for recovery of the injured survivors, include changes in pH, the oxidation-reduction potential, the recovery medium, the incubation time, etc.

A. Heat Injury

The use of elevated temperatures in food processing may stress and injure bacterial spores.[4-6,16,20,23] Heat injury is the most widely studied damage to bacterial spores, and it has been observed since 1916.[16] The exact effects and mechanisms of action in heat injury, however, are still not completely known.

Bacterial spore injury by heat has been observed in the temperature range 50 to 170°C and on spores of both aerobic and anaerobic bacteria,[16,21] including *Bacillus cereus,*[38,39] *B. subtilis,*[40,41] *B. stearothermophilus,*[50,52-54] *B. pumilus,*[50] *Clostridium perfringens,*[55-64] *C. botulinum,*[65-68] and *C. sporogenes.*[61,62,67,69-72]

Heat-injured spores are unable to develop visible signs of growth under conditions that are optimal for unheated spores. They exhibit sensitivities to antibiotics and curing agents, such as sodium chloride and sodium nitrite; fastidious growth requirements; the need for otherwise nonessential agents, such as starch, activated charcoal, lysozyme, and other lytic enzymes; altered incubation temperature requirements; and an extended lag phase.[16,20,23,73]

B. Radiation Injury

Exposure to both ionizing and UV radiation treatments has damaged both aerobic and

anaerobic bacterial cells and spores. Spore injury caused by irradiation has been reported[20,21] for *B. megaterium,*[74,75] *B. stearothermophilus,*[50] *B. subtilis,*[50,76-83] *B. pumilus,*[50,84] *C. botulinum,*[77,80,81,85-87] *C. perfringens,*[61,62,88,89] and *C. sporogenes.*[90]

Spores injured by irradiation can generally initiate germination, and in some cases even faster than unirradiated controls. This makes irradiation injury appear different from that caused by heat, which is frequently expressed as damage to germination.[23] An explanation for this is that irradiation damages spore DNA that is not needed for the initiation of germination. Thus, irradiation injury is usually expressed during outgrowth,[87] even though high doses of irradiation can interfere with or initiate germination-like changes in the spores.[23,91]

Thermorestoration is the phenomenon of increased viability of irradiated spores by heat, if both heating and irradiation are performed under anoxic conditions.[20] The phenomenon has been observed with *B. megaterium*[74,75] and *B. subtilis*[82] spores, and is believed to reflect a reversal of DNA damage.[82]

C. Injury by Hydrostatic Pressure

Spores of some species are affected by hydrostatic pressures that are lower than those needed to injure vegetative cells.[23] The application of pressure results in germination-like changes in spores similar to those caused by chemical germinants.[92-95] Since pressure initiates spore germination, it should be considered irreparable because the spores do not regain their dormancy. It is debatable, however, if these changes caused by hydrostatic pressure can be classified as spore injury. Certain gases under pressure also injure spores, but this type of injury is partly reversible because the spores regain their normal condition when the pressure is removed.[23]

D. Injury by Cold Temperatures

Although freezing and thawing can injure vegetative cells,[9,15] there is little research on the effect of cold temperatures on potential spore injury.[20,21] Important factors in potential spore injury at cold temperatures should include the rates of cooling, freezing, and thawing; the medium composition; the microbial type, strain, and phase of growth; and the temperature and length of storage at cold temperatures.[25]

Refrigeration and freezing temperatures, however, have induced injury to cells of *C. perfringens,* since their numbers declined by as much as fivefold even in protective environments.[4,25,96] The storage of *C. botulinum* at 7 to 8°C in several substrates and in the lyophilized form also resulted in a decreased recovery, which was attributed to DNA damage.[97] Although spores are considered resistant to freezing, *B. stearothermophilus* lost viability and became sensitive to heat after freezing and freeze-drying treatments.[25,98] In general, however, it is debatable whether or not cold temperatures can actually cause major damage to bacterial spores.

E. Chemical Injury

Various chemical agents used in disinfection and food preservation can injure bacterial spores, especially through their action on spore coats.[23] Spores with damaged spore coats are even more sensitive than intact organisms to chemical disinfectants. Chemical agents also injure spores when used in combination with physical treatments.[28] Resistance to chemicals, however, does not correlate well with physical resistance. Also, resistance to one chemical agent may not correlate well with resistance to others.[23,99]

Bacterial spore injury through the action of chemical agents[20,38] has been reported for adverse pH conditions,[23,60,100] ethylene oxide,[61,62,101] hydrogen peroxide,[102,103] hypochlorite,[104-109] alcohols,[110] nitrite,[23,111] and other compounds of lesser significance in food processing. Bacterial spore formers injured by chemical treatments include *B. subtilis, B. stearothermophilus, C. perfringens* and *C. botulinum.*

Chemical agents such as hypochlorite, used to sanitize equipment and other items in food processing, can injure bacterial spore formers, such as *C. botulinum*.[4,104-109] Hydrogen peroxide, which is used for the treatment of materials for aseptic packaging of foods, has injured spores of *B. subtilis* var. *niger*,[103] *B. cereus*, and *C. sporogenes*.[102,112] In general, however, few studies have examined the injury and recovery of spores treated with hydrogen peroxide.

Ethylene oxide also injures bacterial spores,[12] as indicated with *C. perfringens (welchii)*,[61,62] *B. subtilis*, and *B. stearothermophilus*.[101] Alcohol treatment can reduce the temperature required for the activation of *C. perfringens* spores, and higher levels also injure the spores. This injury was manifested as a dependence on lysozyme for germination and recovery.[110] Ozone has also been reported as an effective sporicide, especially at low pH, and the removal of spore coat proteins in *Bacillus* and *Clostridium* spores enhanced their inactivation by ozone.[113] Spores lacking intact coats were also significantly more sensitive to inactivation by chlorine dioxide than strains with intact spore coats.[114]

The concentrations of sodium nitrite used in meat curing have no effect on spore germination, but they can interfere with outgrowth and cell division.[115,116] Higher concentrations, however, can result in germination-like changes.[23,117,118] Nitrite can induce bacterial injury through the reaction of nitrous acid with the cortex peptidoglycan and the hydrolysis of glycosidic bonds adjacent to muramic lactan residues.[23,111]

Other chemicals that injure spores have been discussed by Gould.[23] Trichloroacetic acid has increased the spore sensitivity of *B. cereus* to alkali treatment, which resulted in decreased germinability, the loss of spore components, and lower heat resistance.[119] Copper increases spore sensitivity to hydrogen peroxide.[120,121] Alkyl-*p*-hydroxy benzoates at low concentrations inhibit germination.[122] Mercuric chloride allows solubilization of peptidoglycan during germination of *B. megaterium* spores, but inhibits the loss of refractility,[123] while *p*-chloromercuri benzoate increases the range of germinants acting on spores of the same species.[124] The formation of aberrant cells through the treatment of *Clostridium* and *Bacillus* spores with sorbate and certain phosphates may also be some form of spore injury.[125-128]

Defective spores can also be produced by mutants which undergo abnormal sporulation. The wide range of these mutants with different deficiencies can be used to study and better understand spore germination, injury, and repair,[23] but mutation cannot be classified as injury.

F. Injury by Combined Treatments

Combined chemical and/or physical treatments causing bacterial spore injury (see Table 1) have been tabulated and discussed by Waites and Bayliss.[28] The influence of such combined treatments on spore resistance, destruction, and injury should be considered in developing food processing treatments and methods for the recovery of survivors to evaluate these processes.

Hydrostatic pressure treatment reduced the heat[92,95] and irradiation resistance of spores.[23] This is due to the influence of pressure in initiating spore germination and the reduced resistance of germinated spores. Other destructive agents should also be more efficient against spores treated with hydrostatic pressure. Also, the agents activating the spores should reduce their resistance to pressure, heat, irradiation, and other destructive treatments.[28]

Interactions have also been observed between gamma-irradiation, heat, and chemical agents.[129] Irradiation reduced heat resistance,[89,130] and preheating reduced radiation resistance.[131] Increased irradiation doses also may increase the sensitivity of spores to chemical agents such as sodium chloride[50] and hydrogen peroxide.[132,133] These interactions, however, are not classified as spore injury.

The presence of free-radical producing compounds (e.g., iodide, iodate, or iodoacetate) during radiation treatments result in strong synergistic effects expressed as the inhibition of spore germination. This is believed to be due to inactivation by the free-radicals of spore proteins or enzymes involved in germination.[23,121,134]

Table 1
SPORE-FORMING BACTERIA INJURED BY COMBINED TREATMENTS

Treatment combinations	Injured spore-forming bacteria
Heat + hydrostatic pressure	*Bacillus pumilus, B. coagulans, Clostridium sporogenes*
Heat + irradiation (γ)	*B. cereus, C. perfringens*
Heat + ultrasonic waves	*B. cereus, B. licheniformis, B. subtilis*
Heat + chlorine	*B. cereus, C. bifermentans*
Heat + iodine	*B. cereus*
Heat + phenol	*B. cereus, B. anthracis*
Heat + glutaraldehyde	*B. anthracis*
Heat + hydrogen peroxide	*B. subtilis* var. *globigii, B. subtilis* var. *niger*
Heat + peracetic acid	*B. subtilis* var. *niger*
Heat + ethidium bromide	*B. subtilis, B. megaterium*
Heat + Cu^{2+}	*C. bifermentans*
Heat + sodium chloride	*B. stearothermophilus, B. pumilus, B. subtilis, B. subtilis* var. *niger, C. perfringens*
Heat + nitrite	*C. perfringens*
Heat + polymyxin and neomycin	*C. perfringens*
Heat + KOH and NaOH	*B. anthracis*
Heat + ethylene oxide	*B. subtilis* var. *globigii*
Irradiation (γ) + hydrostatic pressure	*B. pumilus*
Irradiation (γ) + sodium chloride	*B. stearothermophilus, B. pumilus*
Irradiation (UV) + hydrogen peroxide	*B. cereus, B. licheniformis, B. pumilus, B. megaterium, B. stearothermophilus, C. sporogenes*
Ultrasonic waves + glutaraldehyde	*B. subtilis, B. cereus, C. sporogenes*
Hydrogen peroxide + iodophor	*C. sporogenes*
Ethylene oxide + methyl formate	*B. subtilis*
Methanol + hypochlorite	*C. sporogenes*
Glutaraldehyde + formaldehyde	*B. subtilis*

Modified from Waites, W. M. and Bayliss, C. E., in *The Revival of Injured Microbes*, Andrew, M. H. E. and Russell, A. D., Eds., Academic Press, London, 1984, 221.

Irradiated spores can be more sensitive to subsequent heat treatment than unirradiated controls.[76] This increased heat sensitivity, however, was not observed when the spores were heated in the presence of sucrose or glycerol, which indicated that damage of an osmoregulatory mechanism of rehydration was involved in the development of heat sensitivity in irradiated spores.[20,88,89] The heat sensitivity of irradiated spores was increased even further with acidification to pH 1.0 to 1.5. Since reloading the spores with cations increased their heat resistance, it can be concluded that acid treatment sensitizes spores through the removal of cations.[8,76]

Spore damage is greater in the presence of both heat and chemical treatments, and either one of these treatments can be used to sensitize the spores to the other.[28,135] The pretreatment of spores with hydrogen peroxide, peracetic acid,[136] and ethidium chloride[137] made them more susceptible to damage by heat. Heated spores are also more sensitive to inhibition by chemicals such as chlorine,[138] iodine, phenol,[139] hydrogen peroxide,[140] glutaraldehyde,[141] and curing agents.[118]

Combinations of various chemicals are also more effective in destroying or damaging bacterial spores.[28] Such combinations include methanol with hypochlorite,[142] hydrogen peroxide with iodophors,[143] and methyl formate with ethylene oxide.[144]

V. INJURY OF OUTGROWTH

Although injury to spores has been better documented, the outgrowth stage of spore-

forming bacteria may also be the site of damage by physical and chemical treatments. Heat injury has often been linked to outgrowth, and outgrowth is the stage of inhibition by curing salts in heated spores.[4,5,16,73,145] Shifts in optimal temperatures to lower levels for the recovery of heated spores have been suggested as favoring outgrowth because germination can occur in a wider temperature range.[5,146,147]

Since outgrowth is a complex metabolic phase, injury during this stage may involve any of several structures and metabolic pathways.[16] In addition to damage to spore membranes, injury may also involve damage to DNA or other vital components and structures of the cell. Results with *C. perfringens* and *B. cereus* have implicated the inner spore membrane as the site of thermal injury and sensitivity to antibiotics, nitrite, nitrate, and sodium chloride. After injury, the spores were germinated with lysozyme but they retained their injury because they continued to be sensitive to antibiotics.[56,59,64,148] The results, therefore, suggested that injury was occurring at the outgrowth step of the life cycle of spore-forming bacteria.

Similar conclusions have also been reached with *C. botulinum* spores injured with irradiation treatments. Examination of injured spores by phase contrast microscopy at 30°C indicated the germination and emergence of both injured and uninjured spores, but the elongated cells from injured spores failed to form colonies.[86] At 40°C the injured organisms grew and formed colonies. These results also suggested that injury was on the spore membrane and affected outgrowth.[5]

An interesting type of injury involving outgrowth was suggested by the results of Johnson et al.[149] and Johnson and Busta[150] with *B. cereus* spores. During heating (up to 90°C) the total population of the spores did not decline, but there were two phases of decline during cooling (down to 10°C at 10°C/h). The first phase of decline occurred between 90 and 80°C and the other between 50 and 38°C. The first phase of decline indicated the inactivation of spores at a lethal temperature and time. The second phase, however, coincided with spore germination. This second phase of decline indicates that injury involved the stage of outgrowth rather than germination.[27] Further cooling indicated repair of the injury in rice, but not in trypticase soy broth (TSB).

VI. CELL INJURY

The vegetative cells of spore-forming bacteria may be injured in ways similar to the vegetative cells of other bacteria,[6,8,73] but additional studies are needed to confirm the proposed injury and repair of the vegetative cells of spore formers. The injury of spores, however, is more important in the group of spore-forming bacteria than that of cells, because food processing treatments designed to destroy spores should certainly destroy less resistant vegetative cells. Research also is needed on the sporulation of injured vegetative cells to determine any inherent changes in the resulting spores that may influence subsequent destruction, injury, and repair processes.[6]

The injury of vegetative cells may be expressed as a sensitivity to selective agents and antimicrobials, modified metabolic activity, and leakage of intracellular material.[4] Damage to permeability barriers has also been observed in the injured cells of spore formers.[5,24,151] Electron microscopic observations have indicated holes and fractures in the membranes of heated *B. cereus* cells.[5] Other observations included the disappearance of ribosomes and the coagulation of cytoplasmic protein. Studies with *B. subtilis* and *B. cereus* have indicated reversible damage to cell membranes, DNA, and RNA.[41,49,152]

Damage to vegetative cells of bacterial spore formers has also been reported for *C. perfringens* by rapid cooling and freezing,[96,153] which was expressed as a sensitivity to neomycin. Also, the sensitivity of heated *C. botulinum* type E cells to bile salts in peptone yeast extract agar indicated possible injury to the vegetative form of the organism.[68]

Heating *C. perfringens* cells at 51.7°C resulted in the "Phoenix Phenomenon".[154] This

potential injury was expressed as a decrease in plate count (phase I), followed by an increase in count to the initial level (phase II), and a continued increase above the initial count (phase III). Under strictly anaerobic conditions, the phenomenon was observed at 52.3°C, but it was eliminated at the same temperature when a combination of strict anaerobic conditions, prereduced media, and prereduced veal diluent were employed. The results suggested that phases I and II were an injury-recovery process.

VII. FACTORS AFFECTING INJURY

The various factors that affect bacterial spore destruction can also be expected to affect injury.[20] These factors have been divided inherent to the spore population and environmental parameters, which may be active during sporulation, exposure to treatment, or repair and recovery processes.[20,30]

The conditions influencing resistance to stress, injury, manifestations, mechanisms of injury, and repair may vary with the genera, species, and strains of bacterial spore formers. Spores of *B. subtilis* are less resistant to irradiation injury than spores of *C. botulinum* strain 33A, which are also more resistant than spores of *C. botulinum* strain 53B.[77,85] The mechanism of DNA damage by heat was different between the spores and cells of *B. subtilis*,[49] and, of course, bacterial spores are more resistant to stress than cells.

Resistance and injury of bacterial spores and cells is influenced by the conditions present during sporulation and vegetative cell growth, respectively. Conditions active during these stages include culture media and ingredients, gas atmosphere, microbial types and other contaminants, inoculum handling, and storage.

Factors important during exposure to stress include the phase of growth, the type and extent of stress, and the substrate composition and properties.[20] Actively growing cells are more susceptible to injury than spores or cells in the stationary phase.[25,30] Important properties of the substrate include the water content, the concentration and type of ions and pH, the oxidation-reduction potential, the gas atmosphere, the presence of antimicrobials and protective agents, and contamination with other microorganisms.

Handling, storage, and treatment of the injured spore suspension after exposure to stress will also affect the manifestation of injury and its recovery. Important considerations include the recovery media, the germinants, the nutrients, the selective agents,the antimetabolites, the antibiotics, the oxidation-reduction potential, the gas atmosphere, the pH, the water activity, the incubation temperature, and the time.[20]

Additional factors may be active during preparation of the culture for examination because it may involve exposure of the organism to additional stress.[30] They include the storage temperature, which may induce cold shock; the diluents and their temperature, which may interfere with recovery; and the methods of sampling and blending.

VIII. MANIFESTATIONS OF SPORE INJURY

Manifestations and symptoms of injury have been grouped into classes or categories of related effects,[11,16,25] and have been discussed in several reviews.[6,8,20,21] These are based on the requirements and sensitivities of the survivors during recovery and enumeration, and include their sensitivity to chemicals (e.g., selective agents or additives), their altered nutritional requirements (e.g., rich or minimal media and special nutrients), the requirement for nonnutrient germinants (e.g., lysozyme, other lytic enzymes, and initiation protein), the requirement for osmotic stabilization, the altered environmental conditions for recovery (e.g., lower incubation temperature and longer incubation time); and the loss of biological properties. Other changes listed by Adams[16] include the sensitivity to pH and the oxidation-reduction potential.

Different treatments can result in similar symptoms of injury, especially when they act on a common site or through the same mechanism of injury. Sometimes, however, the sites may be similar, but the mechanisms of injury are different.[25] The site, manifestations, and mechanisms of injury can be influential in the repair, recovery, and enumeration of survivors.

Common manifestations of spore injury by heat include the requirements for sucrose, glycerol, or sodium chloride for colony formation; starch; charcoal; sterile culture supernatants; amino acids; calcium chloride; dipicolinate; lysozyme; and initiation proteins. Thermally injured spores have demonstrated sensitivities to sodium chloride and other curing salts (i.e., sodium nitrate or sodium nitrite), antibiotics, other chemical inhibitors, certain culture media, varying lots of culture media, the pH of the recovery medium, the oxidation-reduction potential, and the gas atmosphere during recovery. In addition, thermally injured spores have demonstrated delayed germination, a sensitivity of germination to media components, the need for a modified incubation temperature, and the need for a longer incubation time for optimum colony formation.[6,8,16,20,21]

Manifestations of spore injury by irradiation include a sensitivity to sodium chloride and other components in the plating media; a sensitivity to sublethal heat treatment, which was reversed by the inclusion of sucrose or glycerol in the recovery medium; sensitivity to pH, certain gas atmospheres, and dilution and incubation temperature; and a need for longer incubation times during recovery.[20,21]

Injury by chemical agents has been manifested as a sensitivity to various recovery media, pH, gas atmosphere, plating media, and incubation temperature. Recovery has also been improved with lysozyme, high alanine levels, lactate, and malate, which improved germination; with ferrous sulfate, manganous sulfate, yeast extract, glucose, and vitamin-free casamino acids; and with a longer incubation time.[20,21,104]

IX. SITES AND MECHANISMS OF SPORE INJURY AND REPAIR

Sites and mechanisms of spore injury have been tabulated by Foegeding and Busta[20] and Busta et al.,[21] and have been discussed in several review publications (see Table 2). Reported sites and mechanisms of spore injury by heat include damage or alterations to germination systems; the loss of cortex lytic activity through enzyme inactivation or inactivation of the mechanism involved in enzyme release; damage to the spore membrane (plasma or cortical) structures, as indicated by the need for suitable osmolarity or water activity during recovery; and damage to DNA, although heat has not caused single-strand DNA breaks. Chemical injury has been associated with the inactivation or alteration of spore-germination systems, while injury by irradiation is almost universally associated with single-strand DNA breaks.

A. Injury to Germination Enzymes

The germination mechanisms of bacterial spores may be inactivated or altered by heat and chemical treatments.[5,6,8,16,20,21,23] Reports of damage to germination systems by wet, dry, or ultrahigh-temperature heat treatments have been published for *B. subtilis*,[40,43,45,47-49,155,156] *C. botulinum*,[65,157] *C. perfringens*,[55-57,60,89,158-160] and *B. cereus*.[39]

Heat damage to germination systems has often been characterized by a need for specific germinants or enzymes in the recovery medium, which are believed to help bypass the injured germination system and allow germination and recovery through alternate germination mechanisms.[16,20,23] Damage to the main germination system has been demonstrated with *B. subtilis, C. perfringens,* and *C. botulinum* spores, and has been bypassed with the use of calcium dipicolinate,[42,43] initiation factors, egg yolk emulsion, lactate,[107] lysozyme,[45,48,55,57,58,60,65,158] and mixtures of amino acids.[40,49,155,161]

Since most chemicals that promote improved recovery (e.g., lysozyme, other lytic enzymes, calcium dipicolinate, and amino acids) can be involved in promoting spore germination,[23,162,163] it is believed that they act by helping the spores bypass the injured L-alanine

Table 2
SITE OF BACTERIAL SPORE INJURY BY COMBINED TREATMENTS

Site of injury	Treatments
Coat	Heat + chlorine
	Heat + hydrogen peroxide
	Glutaraldehyde + formaldehyde
Membrane	Heat + neomycin and polymyxin
	Glutaraldehyde + formaldehyde
Cortex	Heat + hydrostatic pressure
	Heat + irradiation (γ)
	Heat + chlorine
	Heat + hydrogen peroxide
	Heat + Cu^{2+}
	Irradiation (γ) + hydrostatic pressure
	Ultrasonic waves + glutaraldehyde
Exosporium	Ultrasonic waves + hydrogen peroxide
Proteases	Heat + Cu^{2+}
Calcium removal	Glutaraldehyde + ionizing cation
DNA	Heat + irradiation (γ)
	Heat + hydrogen peroxide
	Heat + ethidium bromide
	Irradiation (γ) + hydrostatic pressure
	Irradiation (UV) + hydrogen peroxide

Modified from Waites, W. M. and Bayliss, C. E., in *The Revival of Injured Microbes,* Andrew, M. H. E. and Russell, A. D., Eds., Academic Press, London, 1984, 221.

germination system.[16,23] Several studies have indicated that the recovery of heated spores in the presence of lysozyme is improved even more dramatically when the heated spores are treated with alkali compounds or ethylenediaminetetraacetic acid (EDTA) before plating in lysozyme-containing media.[56,58,160,164] Thus, alkali or EDTA treatment appear to sensitize spores to action by lysozyme. This is accomplished through the action of alkali treatment in removing alkali-soluble protein layers from spores.[20,23,56,58,159,165]

Adams and Busta[48] observed that the optimal germination temperature of 45°C for *B. subtilis* type A spores in L-alanine was shifted to 30°C and was independent of L-alanine. This indicated that heating the spores resulted in damage to one germination system and the activation of a second with a lower optimum temperature, which was inactive in unheated spores.[16,47] This, however, may not be the case in every situation, because other studies have indicated that incubation temperature was more critical in the outgrowth stage than in germination.[146]

Since the exact mechanism(s) of spore germination is not well defined, the actual mechanism of injury to germination systems is also unknown.[6,16,23] Several studies, however, have indicated that one mechanism of injury to germination involves the inactivation of lytic enzyme systems, or the mechanism that releases these lytic enzymes, which are involved in degradation during germination.[16,60] This is supported by the above stated effect of lysozyme, other lytic enzymes, and dipicolinate in allowing the injured spores to bypass the damage and germinate.

The inactivation of germination enzymes has been supported by results indicating large losses of cortex lytic enzyme activity in thermally injured *B. cereus* spores,[39] and by thermodynamic values for the inactivation of the L-alanine germination system of *B. subtilis* type A spores that were consistent with protein denaturation.[48] Improved recovery through germination by a second system at a lower temperature of incubation, however, suggests

that damage in this situation occurred in the trigger reaction which is operative before the activation of the lytic enzyme system.

Increased recovery by the use of complex amino acid mixtures may be effective through the generation of NADH and ammonium, since these could be unavailable in the L-alanine germination system through the inactivation of alanine dehydrogenase.[40,155,161] Certain of the amino acids that enhanced recovery, however, may be active in the stages of outgrowth and cell division.

Treatment with chemical agents may also render the germination process sensitive to lysozyme, which enhances the germination of such spores.[5,6] Such effects have been observed after treatment of a slowly germinating mutant of *C. bifermentans* with sodium hydroxide and chlorine.[166,167] As indicated above, treatment with alkali removes spore coat proteins and renders germination sensitive to lysozyme. Chlorine treatment also removes coat proteins from spores.[104,168]

The mechanisms of the injury of germination systems have been reviewed recently by Gould.[23] Lytic enzymes, including lysozyme, are believed to enhance germination by hydrolyzing the β,1-4 linkages of peptidoglycan in the spore cortex, which then permits the hydration of the core of the spore. The actual mechanism at the molecular level, however, is still unknown.[23] Existing evidence suggests that it may involve interference with membrane function, as indicated by the increased sensitivity of heated *C. perfringens* spores[59] to surface active agents, including antibiotics.[23] This is also supported by the observation that in the absence of osmotic stabilizers, cells from these injured spores tended to lyse during outgrowth.

B. Damage to Membrane Structures

In addition to the potential inactivation or neutralization of spore lytic enzymes involved in spore germination, damage to spore membranes and related structures has also been proposed as the site of injury.[6,8,14,16,20,23] In fact, it has been suggested that even when lysozyme is needed to germinate injured spores, the actual repair may be taking place during outgrowth, which could indicate that injury may be associated not with a germination system, but with membranes.[20,55,59]

Evidence used to support the theory of membrane damage includes the sensitivity of ultrahigh-temperature heated *C. perfringens* spores to certain antibiotics and other chemicals with surface-active properties;[55,59] their sensitivity to chemicals such as sodium chloride, nitrate, nitrite, and fatty acids;[59,64] the lack of repair during germination, which occurred only when transferred in a medium supporting outgrowth;[55,59] the completion of repair during outgrowth even in the presence of inhibitors of RNA, protein, DNA, and cell wall synthesis;[59,64] the observation that injured spores were osmotically fragile;[38,76,89] and electron microscopic observations of heat-induced separation of the plasma membrane and the core, as well as convolution, blistering, and puffing of the normally smooth membrane of *C. botulinum* 33A.[169,170] It has thus been concluded that spore injury, at least by ultrahigh temperature, may also cause damage to the plasma or cortical membrane, which become the vegetative cell membrane and cell wall, respectively.[8,16,20]

Extreme changes in pH may also cause damage associated with spore membranes.[23] Alkali treatment dissolves protein components in the spore coat,[100] which increases its permeability and allows lysozyme to act on the cortex peptidoglycan. This also happens after treatments that rupture disulfide bonds.[23,60,171] Milder alkali treatments increase the sensitivity of spores to various germinants,[166,172] probably by improving access of the germinants to receptor sites.[23] The effect of alkali treatment on spore coat permeability may also be responsible for the increased susceptibility of the spores to disinfectants and other chemicals, some of which (e.g., hypochlorite) also remove coat protein.[13,14,23,104,173]

Reduced pH may activate spores and enhance germination,[135] while drastically low pH values may induce dormancy.[23,172] This action of high acidity is linked with the removal of

cations, especially calcium,[174,175] from the spores, which is believed to cause a reversible injury of the germination mechanism.[23,176]

The exchange of cations caused by acid treatment also reduces the heat resistance of the spores,[177-180] which is also reduced by irradiation treatments.[76,89] Reduced heat resistance catalyzed by acid is reversed when the spores are reloaded with cations.[23] The mechanism for the acid-catalyzed loss of heat resistance may be related to changes in spore hydration caused by changes in the ionic state of the spore cortex, which affects its osmotic and contraction state.[23,177] Thus, the spore germination mechanism is unprotected.[160]

Certain other chemical treatments may also injure spores through their action on membrane structures.[23] An example of this is trichloroacetic acid, which increases the sensitivity of *B. cereus* spores to alkali treatment and decreases their germinability and heat resistance by increasing the loss of spore components, such as dipicolinic acid, during heating.[119]

C. Injury to Genetic Material

Irradiation injury and some heat-induced injuries may also be due to changes in the genetic material of the cell, especially its DNA.[8,16,20,23] Injury by irradiation treatments has been especially associated with the damage of DNA, which is not necessary for the initiation of spore germination.[181]

Several studies with irradiated spores have identified breaks in one strand of the spore DNA.[77-81,83] Radiation-resistant strains (e.g., *C. botulinum* 33A) undergo fewer DNA breaks than the less resistant strains (e.g., *C. botulinum* 53B).[80,81] Spore DNA (e.g., *B. subtilis*) is more resistant to single- and double-strand breaks than the DNA of vegetative cells.[83] The ionizing and UV-radiation resistances of DNA isolated from cells and spores are similar.[182] Thus, the increased resistance of spore DNA appears to be due to either increased structural integrity[83] and/or its ability to repair single-strand DNA breaks after the initiation of spore germination, but before the initiation of DNA replication.[77,79-81,98]

The repair of DNA also appears to take place in dormant, irradiation-resistant spores (e.g., *C. botulinum* 33A) during dormancy and irradiation, but not after germination. The repair of DNA during dormancy is apparently catalyzed by an enzyme being active at this nonmetabolic state.[80] This enzyme appears to be a magnesium-dependent DNA ligase.[23,80,183-185]

Spore injury by UV irradiation also involves DNA, but it appears to be different from the DNA breaks described above for ionizing radiation treatments. Damage of spore DNA by UV irradiation also appears to be different from that found in vegetative cells. Spore damage is believed to be due to DNA being changed to the "A" configuration, instead of the "B" type.[23] The "A" form is associated with low water-activity systems, while the "B" form is characteristic of higher water activities.

Injury and repair by thermorestoration also is believed to occur at the site of DNA.[82] The synergistic effect of irradiation and heat treatments, which is expressed as an increased heat sensitivity of preirradiated spores,[88,129,186] is probably due to the increased fragmentation of spore DNA, and the inactivation of DNA repair enzymes by heat, which remain active in spores treated only with irradiation.[23,184] Another theory, however, suggests that the increased heat sensitivity of preirradiated spores may be due to damaged peptidoglycan in the spore cortex, which can allow the hydration of the core and result in reduced heat resistance.[6,8,23,76]

In addition to germination enzymes and membrane damage, DNA also has been suggested as a target of spore injury by heat treatments.[6,8,16,20,23,49,73,156,184] Some of the more complex nutritional requirements of heat-injured spores have been attributed to heat-induced mutations.[16,156] These autotrophic mutations may be due to the depurination and depymimidation of DNA.[8,187] The single-strand DNA breaks observed in irradiated spores, however, were not found in the heated spores of *B. subtilis*.[49,187]

The very high temperatures involved in dry heating may also be involved in the depurination of DNA.[23,156] The need for amino acid supplementation to induce the recovery of

injured spores has also suggested that heat may be damaging spore DNA,[49] since amino acid requirements are believed to be genetically inherited. This would indicate that the alteration of amino acid metabolism or amino acid-stimulated germination may be secondary effects of thermal injury, assuming that permanent damage to DNA is the primary mechanism.[23]

Dry heat also caused mutations which were expressed as reduced sporulation instead of auxotrophy.[188] Chemical damage by agents such as ethidium bromide and daunomycin may also be on DNA and result in lower heat resistance.[137] Rappaport and Goepfert[152] reported that heat injury of *B. cereus* cells appeared to be RNA related, because rifampicin interfered with recovery, and because DNA replication and protein synthesis were not required for recovery.

X. REPAIR AND RECOVERY OF INJURED SPORES

Several review publications have discussed various aspects of the repair and recovery of injured bacteria. General steps in repair and recovery of injured spores were discussed by Foegeding and Busta,[20] while Adams[16] presented steps in the recovery of heat-injured spores. Brief introductions and overviews of recovery have been presented by Busta,[1,4] Hurst,[8] and Gould.[23] Recovery of injured spores has also been discussed by Roberts,[12] Russell,[13] and Ray and Adams.[189] Waites and Bayliss[28] discussed the recovery of spores injured by combinations of treatments; Mossel and Van Netten[30] reviewed the harmful effects of selective media on stressed microorganisms and discussed their nature and remedies; Mackey[25] discussed the lethal, sublethal, and revival effects of cold temperatures; and Gilbert[24] examined the revival of microorganisms sublethally injured by chemicals. The role of medium constituents in the recovery of injured spores was discussed by Blocher and Busta,[26] who also reviewed the effects of inhibitors on recovery and the modified metabolic requirements of injured spores. Finally, Johnson and Busta[27] considered the various aspects of spore injury that should be of concern to those involved in the detection and enumeration of these organisms.

Microbial injury was first recognized by cultural inadequacies observed in organisms exposed to various stresses. Most of these inadequacies should be restored before the organism presents any evidence of vitality through cell division. Restoration to the original undamaged condition is accomplished through the process known as resuscitation, repair, or recovery.[4]

Since injured bacterial spores are of major concern in processed foods, methods of analysis should include procedures capable of recovering and enumerating uninjured as well as damaged spore-forming bacteria.[189] Research on the recovery of injured bacterial cells of nonspore-forming bacteria is more extensive than information on the enumeration of injured bacterial spores. Thus, there is a need for the development of standardized methods for the recovery and enumeration of both injured and uninjured spores, if possible, simultaneously.[27]

As indicated earlier, there are several complex steps (i.e., activation, germination, and outgrowth) to be completed before the dormant spores can grow and be enumerated. The same set of events should be completed for the detection and enumeration of injured spores. The culture media and environmental conditions employed should support these events for appropriate spore recovery. Injured spores, however, may need modified conditions for their recovery and enumeration compared with uninjured entities.

The sublethal damage of spores results in various sensitivities and additional requirements for the injured spores to repair their damage and continue their life cycle. Factors to be considered include sensitivity to activation treatments, germinants, selective agents, chemical additives, other inhibitors, oxidation-reduction potential, pH, water activity and osmolarity, nutritional requirements, and incubation requirements including gas atmosphere, temperature, and length of incubation time. The repair and recovery of injured cells usually requires

richer culture media, the removal of selective agents and inhibitors, and optimum incubation conditions, including pH, temperature, and extended incubation time.

The influence of these factors in specific situations, however, appears to be dependent on the type of microorganism, the nature of the stress, and the type and extent of injury.[27] For example, a wide variation in recovery was obtained with *C. perfringens* spores injured by heat, depending on the strain and the recovery media employed.[190]

Although an activation treatment usually consists of heating at 80°C for 10 min, specific requirements may vary with the strain, the injury, the suspending medium, and the recovery medium.[27] In addition to heat, other treatments (e.g., alcohol treatment) may be more desirable for the inactivation of vegetative cells before the enumeration of damaged and uninjured spores.

Some specific factors that may need to be monitored during recovery include the condition of the population before exposure to stress, handling and storage of the sample before enumeration, the composition of the recovery medium (e.g., the type of medium, nutrients, selective agents, and detoxifying compounds), the dilution procedures and diluents, the mode of inoculation, the enumeration procedure (e.g., plating or MPN [most probable number]), and the conditions of incubation.[30] It should be emphasized that recovery requirements may be different with the type and extent of injury; the species, strains, and individual spore suspensions (e.g., the conditions of sporulation and handling before and after exposure to stress); storage and handling after exposure to stress and before recovery; and, of course, the conditions of recovery.

Research on specific recovery media and constituents for injured spores is limited and not systematic (see Table 3). The work actually done indicates that responses may be different with varying strains and the types of injury.[26,191] Thus, specific media may be appropriate for recovery under certain conditions, and different individual components may be necessary for enumeration under different conditions of injury.

Variations in media, their constituents, purity, and age may be influential in the repair of injured spores. Media components that may enhance recovery under certain conditions include potential nutrients such as sugars, yeast extract, glucose, and amino acids; absorbants such as starch; reducing agents such as thioglycollate and cysteine; bicarbonate; lysozyme and egg yolk; cultural filtrates; and divalent cations.[26] In addition to the media and their constituents, the appropriate pH, gas atmosphere, oxidation-reduction potential, incubation temperature, and incubation time should be selected for the recovery of individual strains under specific types of injury.

Additional studies are needed to evaluate recovery under various conditions, including injury by combined treatments[28] in order to facilitate the recommendation of more standardized conditions for the recovery of injured spores. Understanding the sensitivities expressed by injured spore-forming bacteria is essential in devising testing procedures which may lead to standardized recommendations for the recovery of spores injured under similar conditions.

Injured spore formers have exhibited sensitivities to sodium chloride,[50,118,152,192] nitrate and nitrite,[59,64,115-117] surface-active agents including antibiotics,[55,59,64] pH,[62,152,192] water activity and osmolarity,[38] gas atmosphere,[62,192,193] culture media and their lots,[54,69] and incubation temperature and time.[61,62,192] The type and extent of these sensitivities, of course, vary with the species and the type of injury. Irradiation-injured spores, for example, were less affected by pH than by heat-damaged suspensions.[61]

As indicated in the *Compendium of Methods for the Microbiological Examination of Foods*,[189] only two modifications have been recommended widely and adopted in the specific detection of injured and uninjured suspensions. They include the use of starch as a binder of potential inhibitors in the recovery medium and extension of the incubation period to up to 12 months for the detection of survivors in inoculated packs. Other potential requirements

Table 3
EFFECTS OF THE INGREDIENTS OF CULTURE MEDIA ON THE RECOVERY OF INJURED BACTERIAL SPORES

Ingredients	Microorganisms		
	Clostridium	*Bacillus*	*Desulfotomaculum*
Nutrients			
Glucose	0, +	0, +	
Fructose		+	
Sucrose	0	+	
Galactose		+	
Maltose		0, +	
Egg yolk	+	+	
Yeast extract	+	0, +	
Casamino acids		+	
Lactate	+		
Absorbants			
Starch	0, +	+	
Charcoal	+	+	
Serum albumin	+		
Reducing agents			
Thioglycollate	−, +	0	
Cysteine	+	0	
Cations			
Magnesium		+	
Iron		+	+
Other			
Bicarbonate	+		
Lysozyme	+	0	
Cultural filtrate	+	+	

Note: 0 = No effect on recovery; − = reduced recovery; + = increased recovery.

Modified from Blocher, J. C. and Busta, F. F., *Arch. Lebensmittelhyg.*, 33, 138, 1982.

and modifications which may be valuable in the recovery of injured spore-forming bacteria are discussed in more detail in the following paragraphs.

A. Culture Media

The kind of culture medium used to detect injured spore formers will influence their recovery.[27,69,103] Several media have been used for the recovery of injured spores (see Table 4). The recovery of *B. pumilus* spores injured by UV irradiation was greater in double-strength TSB used with the MPN technique than trypticase soy agar used with the conventional plating method.[84] Heat-injured *C. sporogenes* P.A. 3679 spores were recovered better in Anderson's pork-pea infusion than in trypticase peptone agar, yeast extract agar, pork infusion agar, and T-Best agar.[194] This study also suggested that the pour-plate procedure was not adequate for the recovery of heat-stressed spores. The repair rate of *C. perfringens* was much higher in trypticase yeast extract broth than in 0.1% peptone.[96] The recovery of heat-stressed *C. perfringens* was also better on tryptone-sulfite-cycloserine medium without egg yolk than on sulfite-polymyxin-sulfadiazine medium.[195] More heat-stressed *C. sporogenes* P.A. 3679 spores were recovered on modified P.A. 3679 agar than on yeast extract agar and peptone trypticase agar.[196] *C. botulinum* spores treated with hypochlorite were

Table 4
CULTURE MEDIA USED FOR THE RECOVERY OF INJURED BACTERIAL SPORES

Stress	Organism	Recommended medium	Ref.
Heat	*Clostridium sporogenes*	Yeast extract starch bicarbonate agar	233
		Yesair's pork infusion + thioglycollate	210
		Pork-pea agar	197
		Pork-pea or beef infusion extract	234
		Thioglycollate milk + bicarbonate	90
		Beef infusion or yeast extract agar	235
		Anderson's pork-pea infusion	194
		Modified P.A. 3679 agar	196
	C. botulinum	Yesair's pork infusion + starch	236
		Yeast extract starch bicarbonate agar	233
		Yeast extract agar	218
	C. perfringens	Tryptone-sulfite-cycloserine, or Shahidi-Ferguson perfringens agar	55
		Trypticase yeast extract broth	96
		Sulfite-polymyxin-sulfadiazine medium	195
	Bacillus cereus	Mannitol egg yolk polymyxin	237
	B. stearothermophilus	Antibiotic assay medium A	207
		Eugon broth	101
	B. subtilis	Beef infusion	238
		Columbia broth	101
	Desulfotomaculum nigirificans	Soytone-sulfite agar	199
Irradiation	*C. sporogenes*	Eugon agar cystine	197
		Pork-pea infusion	90
	C. botulinum	Pork-pea infusion	197
	B. pumilus (UV)	Double-strength trypticase soy broth	84
Ethylene oxide	*B. subtilis*	Columbia broth	101
Hydrogen peroxide	*B. subtilis*	Yeast extract, glucose, casamino acid agar + $FeSo_4$ + $MnSO_4$	103
Hypochlorite	*C. botulinum*	Modified yeast extract agar	106

Modified and updated from Blocher, J. C. and Busta, F. F., *Arch. Lebensmittelhyg.*, 33, 138, 1982.

sensitive to components in the recovery medium and could not be enumerated by a five-tube MPN technique in modified peptone colloid medium. They were enumerated, however, in modified yeast extract agar medium in Lee tubes.[104] Treatment of *B. megaterium* with chlorhexidine resulted in a sensitivity to potassium chloride during recovery in tryptone soy agar, but was repaired within 30 to 60 min in TSB.[151]

Recovery is influenced not only by the kind and constituents of the culture medium, but also by the state (infusion vs. dehydrated) of the medium,[54,69] the quality of its constituents,[27] and the manufacturer and the lot of the medium.[54,69,197-199] Fresh infusions are usually better for the recovery of injured spores than commercial dehydrated media.[54,69] Different lots of soybean casein digest, even from the same manufacturer, resulted in different recoveries of heated *B. stearothermophilus* spores.[54] This lot to lot variation may be due to the presence of inhibitors in higher or smaller quantities, or to slight differences in the nutrient composition among lots of the same medium. New lots of media and components should be examined for their influence on damaged spores and cells before their use is approved.[27]

B. Nutrient Supplements

Injured bacterial spore formers have exhibited fastidious requirements and may need

nutrient supplementation in the recovery media.[12,16,20,25] This is especially true for heat-injured spores. Nutritional requirements, however, vary with the species and strains of uninjured microorganisms and should also be expected to be variable in their injured state.[27]

The recovery of heated *Bacillus* spores was improved when glucose or blood was added to the nutrient agar.[200] A variety of compounds (yeast extract, liver extract, glucose, fructose, mannose, galactose, sucrose, maltose, soluble starch, pyruvate, glycerol phosphate, amines, amino acids, and vitamins) increased the recovery and apparent heat resistance of *B. natto* spores.[201,202] Other compounds, however (xylose, arabinose, trehalose, lactose, glycerol, mannitol, glycogen, lactate, acetate, and succinate) did not improve recovery. Several individual and some combinations of amino acids (glycine, homoserine, threonine, valine, glutamine, arginine, isoleucine, aspartic acid, and methionine) also improved the recovery of damaged *B. subtilis* spores.[40,49,155,161]

Cold injury of *B. subtilis* has been repaired with the addition of casein hydrolysate to a tris-magnesium buffer.[203] The recovery of hydrogen peroxide-damaged *B. subtilis* var. *niger* spores was improved with yeast extract, glucose, and vitamin-free casamino acids.[103]

C. Germinants

Agents able to initiate spore germination have been reported to improve the recovery of injured spores, because they are useful in bypassing damage to germination systems. Useful agents in this group include lysozyme, egg yolk emulsion, initiation protein, and calcium dipicolinate. Treatment with alkali or EDTA sensitizes injured spores to germination agents such as lysozyme.[56,164,204]

Lysozyme is the most common additive that has improved the recovery of heat-injured *C. perfringens*[55,57,58,60,158] and *C. botulinum* spores.[65,157,205] The effect of lysozyme on *C. botulinum* spores is more pronounced with spores of lower heat resistance, such as type E[65,205] and nonproteolytic type B strains.[205] Increases in the recovery of heated spores with the use of lysozyme have been as dramatic as 100,000-fold.[16]

Egg yolk emulsion is believed to be active through the lysozyme it contains.[65,157] An initiation protein produced by *C. perfringens* also has increased the measured heat resistance of the same species.[60] An initiation factor was also detected during the growth of *B. stearothermophilus* in certain media, and promoted the recovery of the same organism when thermally injured.[53] This factor, however, appeared to be influential on outgrowth instead of germination. Calcium dipicolinate has improved recovery, and thus has increased the apparent heat resistance of *B. subtilis* A spores.[42,43,45,47] The use of lactate, malate, or higher L-alanine (10- to 100-fold) concentrations in the recovery medium restored the ability of *C. botulinum* spores, injured by hypochlorite, to germinate. Tested individually, these compounds did not support germination.[107,108]

D. Detoxifying Agents

The increased sensitivity of stressed microorganisms to various inhibitors is well recognized.[16] Traces of inhibitory agents (e.g., fatty acids) which may be present in the recovery media are thought to be absorbed and neutralized through the addition of starch, activated charcoal, and serum albumin to the medium. Increased recovery with the inclusion of such compounds in the medium has been well recognized for a long time with various spore formers, including *C. botulinum, C. sporogenes, C. thermosaccharolyticum,* and *B. stearothermophilus*.[51,53,66,67,206,207] Several other studies, however, have reported no improved recovery with starch in the culture medium.[208-210] These discrepancies, however, may be due to the possible presence of inhibitors not absorbed by starch, the presence of inhibitor concentrations higher than those absorbed by starch, or the lack of sensitivity of certain strains to inhibitors absorbed by starch.[26]

E. Chemical Inhibitors and Selective Agents

Injured spores are sensitive to selective agents, such as antibiotics with surface-active properties (e.g., polymyxin, kanamycin, neomycin, and streptomycin) and other surface-active agents (e.g., sodium lauryl sulfate, sodium deoxycholate, and quaternary ammonium compounds), as well as sodium chloride, nitrite, nitrate, acids, alkali, fatty acids, and glycerol and sucrose esters of fatty acids.[55,59,64,115,116,211-213] Injury to vegetative cells also is often manifested as a reduced recovery in media with selective agents, which are perfectly tolerated by undamaged cells. In these cases, the recovery substrate should be modified so that it can support the recovery of both injured and normal organisms.[26] Modifications may include adjustments in pH, the removal or reduction of amounts of selective or inhibitory agents, the use of different selective agents, or the use of chemicals that will support recovery under adverse conditions. These modifications will depend on the microbial strains[190] and the type of injury.

Injured spores are often sensitive to pH variations in the recovery medium.[61] In general, heat-injured spores are recovered optimally at neutral pH values.[214,215] Higher pH values have improved the recovery of heat-damaged *C. botulinum* spores, while the addition of sodium chloride has enhanced recovery at lower pH values.[216] In general, however, bacterial spores are more sensitive to chemicals such as sodium chloride at lower pH values.[103,118] Sodium chloride was also detrimental to the recovery of hypochlorite-injured *C. botulinum* spores.[108]

In addition to heat-injured spores, those damaged by irradiation are also more sensitive to the pH of the recovery medium than untreated controls.[217] Although antibiotics such as polymyxin and neomycin inhibit the recovery of heat-damaged spores, Rowley et al.[87] reported that the repair of irradiated *C. botulinum* type E spores was accomplished during storage at 30°C in an agar medium containing these antibiotics.

Other chemicals that improve spore recovery include sucrose and glycerol,[38] bicarbonate,[90,218] and thioglycollate.[218] Sodium bicarbonate is useful in the recovery of *C. botulinum* spores because it stimulates germination.[108,218] Several cations, such as iron, magnesium, copper, and manganese, also can improve recovery by enhancing germination or cell growth.[147,219]

F. Gas Atmosphere and Redox Potential

The gaseous environment and the oxidation-reduction potential of the medium can be very influential on the recovery of normal as well as damaged anaerobic spore formers.[193] Obligate anaerobes can grow only in media with very low or nondetectable oxygen levels. Methods of eliminating oxygen (e.g., anaerobic jars and chambers, and the use of reducing agents), the handling of cultures in oxygen-free environments, and the use of prereduced diluents and media have been discussed in the review of Shoesmith and Worsley.[193]

Since anaerobic atmospheres usually consist of mixtures of nitrogen, hydrogen, and carbon dioxide,[220] it appears useful to examine the information on the influence of these gases on recovery. Spores of *C. perfringens* damaged by heat were recovered best in pure nitrogen or in hydrogen atmospheres than in their mixture, while unheated controls showed good recovery in pure, as well as in mixed, atmospheres. Irradiated spores, however, were recovered better under nitrogen than under hydrogen or mixtures of the two. Spores damaged by ethylene oxide also preferred nitrogen in their recovery environment, but they could tolerate up to 50% hydrogen.[62]

Certain levels of carbon dioxide are known to enhance the germination and growth of *Clostridium* sp.[221] Hydrogen, in combination with carbon dioxide or nitrogen, however, did not alter germination, compared with carbon dioxide or nitrogen atmospheres, even though hydrogen reduced the oxidation-reduction potential.[221] Carbon dioxide levels at atmospheric pressure (1 atm), however, inhibited the germination of the aerobe *B. cereus,* but enhanced

the germination of clostridia. Increased carbon dioxide levels (10 to 25 atm), however, caused the complete inhibition of clostridial spore germination.[222] More studies are needed to detect the potential injury of spores by gases such as carbon dioxide.

Although the type of gas atmosphere is important in the recovery of anaerobes, the oxidation-reduction potential of the substrate has been presented as more important than the gas composition of the environment.[27] Improved recoveries with sodium thioglycollate have been attributed to its reducing properties.[218] Reducing agents, however, including thioglycollate, may be inhibitory, depending on the type, the concentration, the strain, the recovery medium, and the exposure time.[26,193,223-225] Thus, the use of cysteine as a reducing agent, which is noninhibitory to clostridia, has been recommended.[223]

G. Incubation Temperature

The optimal incubation temperatures for the enumeration of various species and types of spore-forming bacteria vary. Often, these optimum temperatures for injured spores are different than for uninjured spores. This has been observed with heat-injured spores, which often require lower or more restrictive temperature ranges for optimal recovery.[16,61,67,226] The lower or more restrictive temperature range for better recovery of injured spores may be influential either on germination[16] or on outgrowth.[146] This phenomenon has been observed with a variety of heat treatments (at regular and ultrahigh temperatures), heating substrates, and recovery media. The species of spore formers that have required a lower or more restricted incubation temperature range include *B. subtilis*,[43,146] *B. stearothermophilus*,[207] *C. perfringens*,[61] and *C. botulinum*.[67,226,227]

Spores of *B. subtilis* type A heated at ultrahigh temperatures were recovered more effectively at 32°C, while uninjured spores had an optimum recovery at 45°C.[43] Heated spores of *B. subtilis* were also recovered better at 30°C, while uninjured spores were enumerated equally well at temperatures in the range 15 to 50°C.[146] Maximum counts for heated *B. stearothermophilus* spores were obtained at 45 to 50°C, compared with 50 to 65°C for unheated spores.[207] In contrast to this, however, Feehery et al.[192] reported that heated spores of the same species were recovered better at 55 than at ≤45°C. The maximum enumeration of heated *C. perfringens* spores was achieved at 26[61] and 37°C[190] compared with 21 to 34°C and 45°C for the controls, respectively. Injured spores of *C. botulinum* were recovered best at 25°C compared with 31 to 37°C for uninjured controls.[16]

Changes in the optimal incubation temperature have also been observed with irradiation-treated spores. Unirradiated spores of *C. botulinum* 62A were enumerated equally well at temperatures in the range 20 to 45°C, while the detection of irradiated spores showed a maximum enumeration at 40°C.[86] Elongated cells lysed at 30°C, but they repaired and formed colonies at 40°C. Incubation for 8 to 10 h at 40°C resulted in repair, which improved recovery during subsequent incubation at 30°C.

H. Incubation Time

Injured spore-forming bacteria may exhibit a longer lag phase during recovery, which can be as short as a few hours or as long as several months. Thus, the incubation period should be adjusted for optimum recovery.[12,26,30] Delayed germination and growth of injured spores may result in the underestimation of heat resistance if the incubation time has not been extended.[228]

Longer incubation times have been required for heat-, irradiation-, and chemically damaged spores.[43,61,86,103,112] In some instances, repair may be accomplished during storage at refrigeration (4°C) temperatures.[43] Injury caused by cold temperatures, however, is usually repaired in a short (i.e., minutes) period of time.[25,96] The incubation time may also be shortened by the inclusion of certain ingredients (e.g., yeast extract) in the recovery medium.[103]

XI. FUTURE NEEDS

The injury and repair of spore-forming bacteria is now a well-recognized phenomenon and has been addressed by a group of excellent researchers worldwide who have conducted very important studies and published several review papers and books. The food industry and government agencies are also well aware of the potential problems that can arise due to the presence of injured microbial spores in foods.

Research efforts, however, should continue even more intensely than before, because there are many aspects of spore destruction, injury, repair, and detection that need clarification. As indicated by Johnson and Busta,[27] there is a need for comprehensive studies to determine spore injury under actual food processing conditions and to determine the influence of various foods and ingredients on spore destruction, injury, and repair. For example, the resistance of bacterial spores is greatly enhanced by lipid materials,[229-231] but it is largely unknown how variations in lipid content may affect destruction or injury in a food system.

The phenomenon of superdormant spores[232] and their relationship to injury needs additional testing. The relationship of tailing in survivor curves to spore injury also needs evaluation. Foods preserved with combinations of physical (e.g., heat) and chemical (e.g., sodium chloride, nitrite, or sorbate) treatments become spoiled or toxic after a given period of time, longer than that for control treatments. Could this delay involve some type of injury and repair process?

Although heat injury has been studied more extensively than injury by other processes, there is a need for additional work on injury caused not only by heat, but also by other, less well-investigated, injuries, such as those caused by chemicals, drying, and cold temperatures.

Future research will be more productive and useful if its objectives are formulated by many researchers from several institutions. These researchers can examine the existing knowledge and determine the additional needs which can be met with future comprehensive and well-coordinated research efforts. Some specific questions that need answering include: Why is injury expressed in various ways among species, strains, and processing treatments?, Why does the same treatment sometimes result in injury that is expressed differently in various species or even within the same strain?, Can knowledge on the repair requirements for some species be applied to others under similar conditions of injury?, What is the extent and importance of injury in real food systems?, etc.

The studies to answer these and other pertinent questions should be designed only after existing knowledge is well evaluated. These studies should be coordinated to avoid unnecessary duplication. An order of priorities should also be developed. If such efforts are successful, then scientists in the future may be able to formulate guidelines and standardized procedures for the recovery and enumeration of both normal and injured spore formers in culture media, as well as in complex food systems.

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Chapter 7

DETECTION AND SIGNIFICANCE OF INJURED INDICATOR AND PATHOGENIC BACTERIA IN WATER

Gordon A. McFeters

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I. INTRODUCTION

The concept of bacterial injury has been widely recognized for many years relative to indicator organisms in foods. Bacteria associated with food products are exposed to stressful chemical and physical conditions during processing and preservation that result in cellular debilitation. For example, the application of sublethal levels of disinfectants and temperature extremes during food processing cause cellular lesions resulting in bacteria that are frequently more difficult to cultivate and enumerate. As a consequence, the application of common selective media to enumerate gram-negative bacteria in testing the microbiological quality of foods can result in a significant underestimation of the actual number of viable indicator bacteria that are present. This, in turn, can lead to an overly optimistic estimation of the microbiological quality of the product, since many of the injured organisms will not be detected. Intensive research spanning more than 2 decades has described this phenomenon and its implications. This has resulted in an extensive literature on the subject that has been discussed in earlier reviews.[1-6] The significance of bacterial injury is also commonly acknowledged by food microbiologists and policy makers dealing with the food industry,[4,7] and the enumeration of injured indicator bacteria associated with food products is commonly attempted with the use of media and methods specifically designed for this task. In addition, the potential presence of injured pathogens in foods is regarded as important. However, the occurrence, detection, and significance of injured bacteria in water has only recently gained recognition among water microbiologists.

The microbiological quality of treated water and wastewater is determined by the enumeration of indicator organisms. In addition, a number of related pathogenic bacteria are transmitted through water. In general, these enteric indicator and pathogenic bacteria are not well adapted to conditions in water where a variety of chemical, biological, and physical stressors are usually present. These factors impose stress upon the bacteria as they are shed from the gut, where conditions are favorable for their reproduction, into aquatic environments that are inhospitable for these organisms. In this process the bacteria are exposed to factors that can lead to injury. These include sublethal concentrations of disinfectants and other chemical as well as physical and biological antimicrobial influences associated with the treatment of sewage and drinking water. Additional environmental factors that are harmful may also be encountered in natural bodies of water.

Another factor of importance in the consideration of bacterial stress within aquatic systems is the composition of the media used to enumerate indicator bacteria in water samples. Generally speaking, the selective media that are commonly used in this way were originally developed for the analysis of clinical samples. However, these are now known to be highly restrictive for the growth of stressed bacteria. As pointed out by Litsky[8] and others,[9-11] environmental conditions that stress gram-negative allochthonous bacteria are a prominent feature of most aquatic systems. Consequently, it should not be surprising that water quality assessments with currently accepted media are frequently inaccurate. Detailed studies that describe bacterial injury in aquatic systems span only a little more than the past decade. In two recent reviews, LeChevallier and McFeters[9,10] have documented much of this literature. Therefore, our understanding of stressed indicator bacteria associated with water and the resulting inaccurate enumeration of indicator bacteria with accepted media has emerged more recently than in the area of food microbiology, but the two situations are similar in many ways. Further, it should be remembered that the etiology of many food- and waterborne diseases is similar and that water is ingested as a fluid as well as being used to prepare many foods and beverages.

The injury of waterborne bacteria can be defined in ways that are similar to those used previously by food microbiologists.[1-4] Injury is considered to be the sublethal physiological and structural consequence(s) resulting form exposure to injurious factors within aquatic

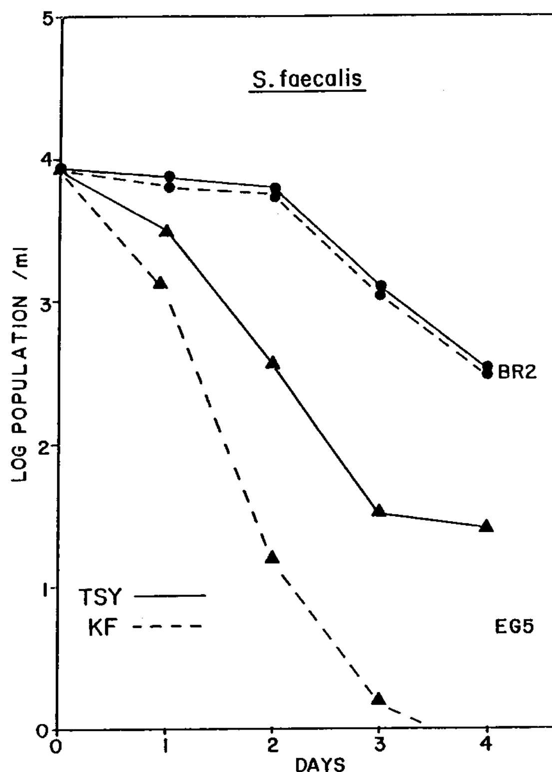

FIGURE 1. Comparative recovery of *Streptococcus faecalis* RS1009 in membrane filter chambers located at sites BR2 (●) and EG5 (▲) during a 4-d exposure period. Samples were pour plated using TSY (———) and KF(- - -) agar.

environments. This is manifested in the inability of injured cells to reproduce under conditions that are suitable for the growth of uninjured cells. These restrictive conditions can include media that contain certain selective ingredients, defined minimal media, or restrictive temperatures. Therefore, a medium that is nonselective or complete must be used as a reference in determining the extent of injury. This is illustrated in Figure 1, where a suspension of *Streptococcus faecalis* was exposed to the water in two streams using membrane diffusion chambers.[12] Injury is seen in one location (EG5) as a progressive difference in the recovery using TSY (nonselective) and KF (selective) media.[13] It is usually assumed that both injured and noninjured cells are capable of growth on the reference medium, while only the noninjured bacteria can form colonies on the test medium. The following equation has been used to quantify the degree of injury in specified populations.

$$\% \text{ injury} = \frac{\text{CFU nonselective} - \text{CFU selective}}{\text{CFU nonselective}} \times 100$$

It must be stressed that calculations of the percentages of cells that are injured must be viewed as relative values, since, as discussed later, different selective media recover injured bacteria with varying efficiencies. However, this kind of calculation can provide useful information concerning the relative level of injury within a waterborne population, since bacteria in natural systems are thought to respond to stress individually, as pointed out by Mossel and van Neeten.[14] That is, following stress, the bacteria are present in at least three states; those bacteria that are irreversibly inactivated, those that are not injured, and those

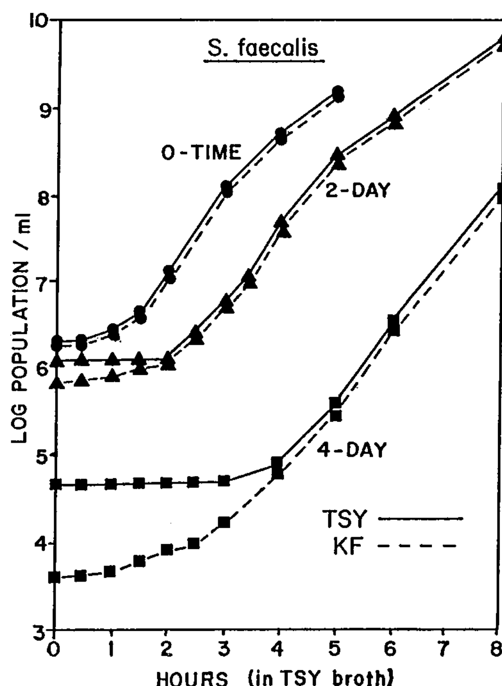

FIGURE 2. Repair of injury in TSY broth for *Streptococcus faecalis* RS1009 cells which were exposed to the stream environment of site EG6 for 2 and 4 d. Control or 0-h cells (●), 2-d exposed cells (▲), and 4-d exposed cells (■) were enumerated over an 8-h growth period in TSY broth, using TSY (————) and KF (- - - -) media with membrane filtration procedures.

that are injured to varying degrees. The observation of viable but nonculturable bacteria in some aquatic and marine systems adds another dimension to this consideration.

The definition and concept of injury includes the capability of debilitated cells to repair the cellular damage and regain the tolerance for growth under restrictive or selective test conditions. This is illustrated in Figure 2. Suspensions of *S. faecalis* that were uninjured (0 time) and injured to varying degrees (2 d and 4 d) were incubated in a nonselective medium, and the recovery from injury followed as a convergence of the plate counts on TSY and KF media, as well as the resumption of growth.[13] Although this definition of waterborne bacterial injury was borrowed from the food microbiology literature,[1-4] another somewhat different approach was taken by Zaske et al.[15] who showed that injured bacteria from aquatic environments display an extended lag phase and have an abnormal susceptibility to lysozyme.

The purpose of this chapter is to discuss injured indicator and pathogenic bacteria in water. It is not intended that the review of the literature will be all-inclusive, but much of the existing knowledge describing the causes and cellular consequences of injury will be presented. The development of methods to detect injured coliform bacteria in water will also be discussed with some information on the occurrence of stressed coliforms in potable water. Finally, recent studies describing injury and its influence on properties relating to the virulence of some waterborne enteric pathogenic bacteria will be summarized, along with a discussion of the significance of injured bacteria in water.

II. CAUSES OF INJURY

A wide variety of chemical, physical, and biological factors can cause sublethal bacterial

injury. For example, antimicrobial chemicals that are used in the processing and treatment of foods and water are eminently capable of causing sublethal cellular damage if the concentrations of the chemicals is low or the conditions for antimicrobial activity are suboptimal. Factors associated with the processing of foods that have been implicated in causing cellular stress include elevated temperatures, freezing, freezing and thawing, freeze-drying, moisture reduction, exposure to diluent, salt deprivation, nutritional environment modifications, radiation, gases, antimicrobials, and preservatives.[2-6] In addition, combinations of these factors that might be thought of individually as inconsequential in terms of antimicrobial activity can also cause injury by additive effects.[14] Stressors are also found in aquatic environments, but fewer factors have been identified than in the case of food products. This is probably a reflection of the shorter time aquatic microbiologists have been studying injury in water and because of the relative simplicity of the processes used to treat and disinfect water and wastewater. In any case, additional stressors are likely to be found in aquatic systems.

Many factors associated with water have the potential to cause bacterial injury. This is particularly true in the case of potable water and wastewater that has been treated with disinfectants.[16-19] However, natural aquatic systems can also be stressful environments for many enteric bacteria, due to the presence of subtle, and often multiple, antimicrobial factors. These may have an additive effect, as pointed out by Mossel and van Netten,[14] in the case of foods. Furthermore, the characteristics of the enteric bacteria that render them sensitive to conditions outside the gut also predispose them to injury.[20] For example, exposure to reagent-grade water for 3 d can lead to injury in some coliforms.[20]

A. Chemical Stressors

1. Disinfectants

Disinfectants and other biocides used to treat water probably represent the major cause of bacterial injury in aquatic systems. While bacterial lethality is a consequence of the disinfection process, the application of disinfectants in ways that lead to suboptimal biocidal activity promotes injury. For example, the level of injured coliforms in treated drinking water can increase significantly when the disinfectant contact time is reduced for the purpose of minimizing trihalomethane formation.[20a] Variations in other discretionary operational variables in water treatment can likewise promote injury. In addition, the concentration of disinfectant in drinking water usually varies widely from >1 mg/l at the point of application to below the level of detection at the extremities of the distribution system, and will fluctuate temporarily.[21,22] Thus, suboptimal levels of disinfectant may occur during and after water treatment and lead to injury.

Chlorine is widely used as a disinfectant for both potable water and wastewater. As early as 1935, there was evidence that chlorine caused a reversible form of bacterial inactivation,[23] and somewhat later, Heinmets[24] reported the usefulness of metabolic intermediates in the cultivation of chlorine-inactivated *Escherichia coli*. These developments were followed by a period of disagreement among various laboratories concerning these observations. In 1971, Schusner et al.[25] convincingly described the reversible inactivation of *E. coli* by chlorine in foods, followed a few years later by two reports of the same process associated with tap water and wastewater.[16,17] These publications highlighted the need for growth media that would allow the recovery of the injured subpopulation, since accepted media (i.e., m-Endo and m-FC) were found to be very inefficient in the enumeration of stressed coliforms. Lin[26] extended these observations by describing chlorine-injured fecal streptococci in chlorinated sewage effluents. More recently, others have also documented the presence of chlorine-injured coliforms in water and wastewater.[27,28]

Studies were initiated in our laboratories to further characterize the process of chlorine injury under conditions similar to those in drinking water and to study the recovery process from the physiological standpoint.[19,20] The results of these studies revealed that reproducible injury could be attained at low chlorine concentrations (about 0.5 mg/l), and the process

was a function of both concentration and time of exposure. We further demonstrated that there was an extended lag phase, similar to that seen in different examples of injury by others,[13,20,25] when chlorine-injured bacteria were placed in a nonrestrictive medium to allow recovery and growth.

Alternative antimicrobial agents, such as ozone, chloramines, chlorine dioxide, and UV radiation, have been suggested for use in the disinfection of water because of growing concerns regarding toxic substances that are produced by chlorination.[29] Currently, there is little specific information concerning the tendency of these treatments to injure bacteria in water, although a recent report by Blazer et al.[30] described the injury of *Campylobacter jejuni* by chloramines. Literature on the effects of various antimicrobial agents used in the processing of food products supports the contention that these alternative treatments would also injure bacteria in water.[7,25,31]

2. Metals

The results of field studies performed in our laboratory suggested that metals may act as stressors in natural aquatic systems. Suspensions of coliform bacteria and fecal streptococci were exposed to natural surface water in a number of streams in southwest Montana using membrane diffusion chambers.[12] When the aliquots were removed and the extent of injury assessed, it was clear that some factor or combination of factors in certain streams caused the bacteria to become highly injured in 3 to 5 d, while the water in other streams had very little effect.[13,15] Later analysis of these waters revealed that the primary difference in the chemical and physical composition was the content of metals (unpublished results). Streams in which the greatest levels of injury were observed consistently contained higher concentrations of metals, including copper, zinc, lead, and nickel. This is consistent with earlier findings that metals affect metabolic damage within bacteria.[33,34] These results further suggested that bacteria within the drinking water and natural water of many geographic regions might be injured due to naturally occurring metals within the aquatic systems. This hypothesis was supported by the findings of two surveys indicating that the concentration of copper in the drinking water of the majority of U.S. cities tested is sufficiently high to cause >90% injury in *E. coli* within 2 d of exposure.[35,36] Copper can be found in source water in areas where it is naturally abundant in geological formations, and is frequently used in plumbing fixtures as well as an algicide in drinking-water reservoirs.[37]

Subsequent laboratory studies investigated this form of chlorine-independent injury in more detail. It was determined that low concentrations of metals in chlorine-free distribution water caused 64% injury.[37,38] Also, statistical modeling of the chemical and physical characteristics of 30 water samples was done, and predicted that copper concentration and factors such as temperature, pH, and alkalinity, which determine copper availability, influenced the extent of injury. These findings were confirmed in controlled laboratory studies.[37] In addition, a concentration-injury response relationship was established with copper. While it is likely that other metals also affect injury to some degree, the results of these studies indicate that copper is the primary stressor among metals that are commonly found in potable water.[37,38] These findings have important implications concerning the advisability of routinely adding ethylenediaminetetracetic acid (EDTA) to water samples. *Standard Methods for the Examination of Water and Wastewater*,[39] as well as the two USEPA books describing the microbiological analysis of water,[40,41] specify the addition of EDTA to chelate metals in waters, such as wastewater and industrial effluents, where concentrations of metals may be high. However, the results outlined here indicate that metal concentrations are sufficiently high in many types of water, including drinking water from most major cities in the U.S., to warrant the routine addition of EDTA to all water samples destined for microbiological analysis at the time of collection, as suggested by Domek et al.[37]

B. Physical Factors

A wide variety of physical factors have been implicated in the injury of bacteria associated with foods.[1-4,14] These factors are of obvious practical importance, since they are closely monitored and are often deliberately altered in the processing and preservation of many food products. By contrast, most physical parameters are not manipulated or monitored in the treatment of water and wastewater. However, source and natural waters are subject to ambient conditions that determine their physical properties.

1. Sunlight and UV

Although UV radiation has not been widely exploited in the treatment of water, it has been studied for use in the disinfection of wastewater effluent[42] and is occasionally employed for the disinfection of drinking water. The work of Fujioka and Narikawa[43] in Hawaii indicated that sunlight is a potent bacteriocidal agent, inactivating 90% of the fecal coliforms in sewage diluted with seawater after 13 to 32 min of radiation. Chamberlin and Mitchell suggested that sunlight is probably the most effective bacteriocidal factor in natural waters. Solar radiation was further noted as the cause of injury in *E. coli* suspended in seawater in an earlier study,[45] and in fecal coliforms as well as fecal streptococci by the workers in Hawaii.[43] The latter report also indicated that fecal streptococci were much less susceptible to sunlight inactivation than were coliforms, and that sewage bacteria diluted in seawater were more sensitive than when freshwater was used as the diluent. Other investigators also noted the injury of aquatic bacteria by UV light. Moss and Smith[46] found that following UV injury in seawater, *E. coli* recovery was more efficient when a lower nutrient medium was used. Somewhat later, Bailey et al.[47] described solar radiation-induced injury in a natural bacterial population from Chesapeake Bay. Dutka[48] described variable recovery efficiencies with different media used in the detection of *Legionella pneumophila, Pseudomonas aeruginosa, E. coli,* and *S. faecalis* following exposure to sunlight. These findings indicate that sunlight is bacteriocidal and injurious with respect to both indicator bacteria and pathogens in source waters and marine systems receiving sewage.

2. Acidic pH

The antibacterial influence of acidic environments has been appreciated for some time, and a 1971 publication documented injury caused by low pH in *E. coli.*[49] This injury resulted in the increased sensitivity of *E. coli* to a selective medium containing violet red and bile. More recently, Hackney and Bissonnette,[50] as well as Double and Bissonnette,[51] have demonstrated that coliforms exposed to acid mine water became injured and were more sensitive to media containing desoxycholate. These findings have obvious implications for monitoring low-pH environments, such as acid mine streams and bodies of water influenced by acid precipitation.

3. Temperature Extremes

Food microbiologists have described the influence of temperature extremes causing bacterial injury.[3] A recent review by Hurst[52] discussed the revival of vegetative bacteria after sublethal heating, while Mackey[53] reviewed the lethal and sublethal effects of refrigeration, freezing, and freeze-drying on microorganisms. All three articles mentioned indicator as well as pathogenic bacteria. Although some of these factors probably cause bacterial injury in aquatic systems, there is very little information available describing such stress. For example, bacteria in water used in heat exchangers are subject to alternate cycles of extreme heating and cooling, and temperatures below freezing can seasonally influence waterborne bacteria in some regions.

4. Other Physical Factors

Other physical factors that have been implicated as bacterial stressors associated with

foods include osmotic pressure, ionizing radiation, drying, and extremes in redox potential.[2,4] However, there appears to be no information describing the sublethal influence of these factors on bacteria in water.

C. Biological Factors

Biological interactions provide another potential mechanism to explain the injury of enteric bacteria in aquatic systems. Unlike the previously described causes of injury, there is little precedent for this type of stressor in the food microbiology literature. It is generally appreciated that excessive populations of noncoliform heterotrophic or heterotrophic plate count (HPC) bacteria can suppress the detection of coliforms in water.[54] To investigate this possibility, the interaction between waterborne pseudomonads and *E. coli* was examined in our laboratory.[55] The results of this study demonstrated that pseudomonad populations caused injury in *E. coli* (about >55%) when the pseudomonads exceeded the coliforms by a ratio of 100,000 to 1. At the same time there was little decrease in the total viable population of *E. coli*. Competition for limiting organic nutrients was suggested as the mechanistic explanation of this phenomenon, since no evidence of phage or bacteriocins could be demonstrated. However, coliform inhibition by bacteriocin-like substances has been reported in drinking-water systems.[56]

The significance of this observation lies in the oligotrophic character of the pseudomonads as well as their ubiquitous presence in water. Pseudomonads proliferate to reach high numbers in unrefrigerated water during storage or shipment, and have been observed in populations exceeding 500 cfu/ml in instances where coliform enumeration was suppressed.[54,57] Another situation in which this phenomenon could be important is reflected in the findings of two studies where elevated numbers of HPC bacteria have been found in water associated with waterborne disease outbreaks when coliforms were not detected.[58]

III. CELLULAR CONSEQUENCES OF INJURY

Reports in the food microbiology literature contain numerous examples of injury where the mechanisms of cellular damage have been studied. Much of this information has been discussed recently in review articles.[1,3,4,6,14,60-62] Among various physiological responses to different causes of sublethal injury, the cytoplasmic membrane is frequently described as the site of cellular damage.[6] The physiological consequences that have been observed were leakage of intracellular material, including macromolecules, amino acids, and specific ions; increased lipid synthesis; altered nutrient transport; increased sensitivity to NaCl; and uncoupling of oxidative phosphorylation.[3] Although these findings suggest that lesions in the cytoplasmic membrane are common to injury resulting from different stressors, it is not easy to identify precisely the primary site of cellular damage. The overall cellular response is likely to be the cumulative effect of a number of interrelated events, as pointed out by Russell.[60] However, the cell envelope is an obvious target of injurious chemicals in general, and particularly in the case of highly reactive agents such as chlorine. In support of the hypothesis that lesions in the cell membrane are common to many forms of bacterial injury, it is important to note the central structural and physiological role of the cytoplasmic membrane in processes such as the maintenance of the membrane electrochemical potential, the generation of ATP, and solute transport. It would not be surprising if injury involving some compromise(s) of membrane-related functions or structure leads to a wide range of altered phenotypic responses.

The highly variable characteristics of different types of water make it difficult to draw generalizations concerning the mechanisms of injury in waterborne bacteria. Bacterial damage in aquatic systems probably results from a number of concurrent antimicrobial factors, such as chlorine, metals, and biological stress. Even uncontaminated surface water usually contains several factors with antimicrobial potential, plus solar radiation. This picture is

further complicated by the nutrient limitation in oligotrophic waters which must also function as a stressor.

The following discussion will examine the mechanisms of cellular injury in the few water systems that have been studied in detail.

A. Cellular Damage from Chemical Stressors

1. Disinfectants

The mechanism of chlorine damage has been studied by a number of groups over many years because of the importance of this agent in the disinfection of water and wastewater. Despite the early disagreement regarding the mode of action of chlorine, most workers studying the sublethal consequences of exposure to low (about <2 mg/l) concentrations of chlorine now agree that it affects alterations in membrane-related functions.[3,4,18] As the chlorine concentration becomes lower with time in systems such as drinking-water distribution, the chances of cellular damage to the envelope increase. However, higher levels of chlorine can affect a number of intracellular alterations, including disruption of protein synthesis,[63] reactions with nucleic acids,[64,65] and the creation of chromosomal aberrations.[66] It should be noted that some of the effects on nucleic acids[65,66] were determined after exposure to chloramines. Other cellular responses to chlorine dioxide have been described.[63] However, there is insufficient information to make generalized conclusions regarding the mode(s) of action of the various forms of chlorine and other disinfectants that have injurious properties.

Detailed physiological studies of chlorine-injured cells were carried out in our laboratory, and the results of this work provide support for the hypothesis that membrane-related functions, including respiration and nutrient transport, were dramatically reduced by chlorine.[18] This was supported by an immediate decline in intracellular ATP concentration following injury, while the activity of aldolase, i.e., an intracellular enzyme, was unaffected.[18] The findings of other studies have provided further strength to this argument.[67,68] A number of additional effects have been noted following chlorine injury, which include the modification of amino acids and mutagenesis.[19] However, it is still generally accepted that the primary cellular location of chlorine damage resulting from interaction with low concentrations of this disinfectant is the cytoplasmic membrane and cell surface. Hence, physiological processes associated with the cell envelope become compromised by low levels of chlorine, and it is axiomatic that other physiological activities dependent upon the membrane functions are also affected in the chlorine-mediated injury process.

2. Metals

An investigation was also recently done in our laboratory to study the physiological mechanism(s) of copper-induced injury. These studies employed respirometry, gas-chromatography, and nuclear magnetic resonance (NMR) spectroscopy. The results of these experiments revealed that copper injury caused major metabolic alterations and a >75% reduction in aerobic respiration.[37,38] This study also defined copper as the primary stressor among the metals present in Bozeman, MT drinking water. Subsequent experiments using NMR spectroscopy to identify differences in metabolism between healthy and copper-injured *E. coli* revealed major alterations, including reduced rates of glucose transport and the utilization as well as accumulation of lactate, ethanol, acetate, and glutamine and other manifestations of altered carbon flow in injured cells. These findings collectively indicate that the physiological and metabolic characteristics of copper-injured enteric bacteria are similar to those of chlorine-injured cells. This further suggests that copper, and perhaps other metals, cause major lesions within the cytoplasmic membrane which are reflected in the reduction or loss of certain metabolic processes associated with this structure. Most of the work that described the effects of metals on bacteria was done with concentrations that were far in excess of those detected in natural aquatic systems or drinking water. However,

supportive evidence for our conclusions is given by Lamb and Tollefson,[69] who showed that 5.0 mg/l copper reduced the rate of glucose mineralization in activated sludge by 90%.

Earlier studies by Zaske et al.[70] also relate to the cellular consequences of metal-induced injury. These experiments used membrane diffusion chambers to expose *E. coli* suspensions to natural oligotrophic surface waters with an elevated metal content. Progressive injury was observed as an increased susceptibility to lysozyme and a prolonged lag phase, as well as differential counts on selective and nonselective media. Electron micrographs also revealed that water-exposed bacteria produced large numbers of envelope blebs that were rarely seen in control cells. These findings support the hypothesis that metal-induced injury also results in alterations within the cell envelope, although many of the same changes were also seen in cells suspended in reagent-grade water.

B. Cellular Damage from Physical Stressors

1. Solar Radiation

Kapuscinski and Mitchell[45] and Fujioka and Narikawa[43] documented the injurious effect of sunlight on waterborne bacteria. They noted that the addition of pyruvate or catalase enabled bacteria to overcome or repair the injury. As a consequence, they proposed that sunlight caused damage to the catalase system, which resulted in the accumulation of peroxide. Hence, the addition of peroxide scavengers or hydrolytic enzymes reversed the observed sensitivity of the cells to restrictive cultivation procedures. This also points to the cytoplasmic membrane as a site of damage, since peroxide is a product of aerobic respiratory activity, which is a membrane-associated function. The possibility that other enzymes could be damaged by sunlight exposure was suggested by Krinsky.[71]

2. Temperature Extremes

The antibacterial effects of excessive heat have been studied for some time, and a few of the proposed physiological effects are listed by Haight and Morita.[72] Some of these effects relate to alterations in the normal structure and function of the cytoplasmic membrane. Damage to the cytoplasmic membrane upon heating a marine psychrophile resulted in the abnormal leakage of intracellular materials.[72,73] The release of cytoplasmic components in response to heating and freezing was also observed by Witter and Ordal.[74] The injurious effects of heating *P. aeruginosa* and *Staphylococcus aureus* suspensions in dilute buffer solutions could be reversed by the addition of catalase.[75,76] These effects suggest that the effects of elevated temperatures result in membrane damage. The envelope and its function seem also to be altered by reduced temperature. Goodrich and Morita[77] showed that temperatures below the growth range for an *E. coli* amino acid auxotroph resulted in the cessation of amino acid uptake. In addition, Ray and Speck[78] demonstrated that $>$90% of *E. coli* cells surviving freezing in water were injured and were unable to form colonies on a selective medium.

3. Acidic Environments

Although there is a paucity of detailed physiological information on the effects of low pH, at least two groups have demonstrated that the enumeration of *S. aureus* and *E. coli* following acid-mediated injury was significantly increased when catalase was added.[75,76] The same effect was noted when media containing peroxide scavengers, such as Baird-Parker medium, were employed. These findings are of interest, since the enumeration of *S. aureus* following injury from reduced water activity and freeze-drying could also be stimulated by catalase,[76] while this effect was seen only in the case of acid-injured *E. coli.*[75] Therefore, there appears to be a distinction in the way catalase and peroxide scavengers aid in the recovery of injured bacteria, based upon the gram-staining reaction and the character of the stressor. This may be explained by the differences in cell envelope structure between gram-positive and gram-negative bacteria, as well as the differences in the physiological

mechanisms of injury resulting from various stressors. This is supported by the unpublished observation in our laboratory that the addition of catalase did not assist in the recovery of coliforms following injury caused by chlorine and copper in water.

C. Cellular Responses to Other Aquatic Stressors

The availability of organic nutrients is highly variable in aquatic environments; hence, this factor often limits the growth of heterotrophic bacteria.[79] This is an important consideration in bacterial persistence, although some organisms (oligotrophs) are capable of reproduction in very low substrate concentrations. However, most enteric bacteria are heterotrophs which require high levels of nutrients (copiotrophs) and are stressed by nutrient limitation in aquatic systems where suitable organic matter is periodically or persistently low.

Heterotrophic bacteria respond to nutrient limitation in a number of ways that have been reviewed recently by others.[79,80] Many enteric bacteria are capable of carrying on a maintenance level of metabolism by hydrolyzing endogenous macromolecular reserves.[81-83] Some bacteria respond to nutrient limitation by attachment to the nutrient-enriched microzone found at solid-liquid interfaces.[84,85] Also, the miniaturization of specific marine bacteria is an interesting physiological and morphological response to nutrient limitation that appears to extend viability with time. In this progression of events, the bacteria become smaller and their physiological activity decreases markedly.[79]

Some bacteria seem capable of entering a stage that has been termed "viable but nonculturable" in simulations of natural aquatic environments.[86-88] In each of these cases, however, the decline in culturability was paralleled by an increase in the sensitivity of the bacteria to certain media, resembling the injury phenomenon. The "nonculturable" state that is eventually reached satisfies many of the criteria of dormancy as proposed by Sussman and Halvorson,[89] Morita,[90] and Stevenson.[91] This dramatic response is likely to be seen only after extended nutrient limitation, whereas the first observable change is the progressive increase in sensitivity to certain media (i.e., injury).

IV. DETECTION OF INJURED BACTERIA

Injured bacteria can represent a numerically important fraction of the total microbial population in foods[1,4,14] and water.[20] The importance of this finding in the microbiological analysis of foods has been widely recognized,[1-4,14] but is only beginning to gain acceptance among the individuals and agencies involved in water quality testing.[9,10] Injured bacteria are viable and recoverable under the appropriate cultural conditions, and there have been significant developments in the formulation of media specifically for the enumeration of injured indicator bacteria in both foods and water. Early efforts to design methods for the enumeration of injured indicator bacteria in foods concentrated on the composition of the media, since it has been known for over 70 years that certain selective and nutritionally deficient media restrict the growth of damaged indicator bacteria, while undamaged cells are not affected in this way.[92,93] Therefore, the approaches followed by food microbiologists in the development of these media and methods considered selective ingredients, nutrients, and detoxifying constituents.[14] Developments in the area of aquatic microbiology have followed similar approaches. The primary objectives of this effort were to minimize secondary stresses associated with the manipulations of water samples following collection and to optimize the enumeration of injured bacteria.

A. Influence of Laboratory Manipulations on the Enumeration of Injured Bacteria from Water

Laboratory handling can significantly influence the outcome of efforts to enumerate injured bacteria once a water sample has been collected. For example, it is important to add sodium

thiosulfate[39-41] to neutralize the residual chlorine that may be present, since disinfectants are probably the major cause of injury in treated water. The addition of EDTA will prevent the further injurious effects of metals.[39-41] Both of these measures are obviously of more importance when sample transportation or storage is protracted, since their absence will allow a continuation of the injury process.

The use of diluents can also influence the recovery of injured bacteria. Studies in our laboratory involving suspensions of various coliforms injured in treated drinking water revealed that diluent composition is less important if diluted samples are maintained at refrigerator temperatures and that the addition of low concentrations of organic materials (about 0.1%) augmented the enumeration of stressed coliforms from water.[94] The addition of certain inorganic salts can also be helpful. It should be noted that most of these concepts were taken from the earlier food microbiology literature and adapted to aquatic applications.

An additional factor of importance in the analysis of water samples containing injured bacteria is the selection of the membrane filter. General awareness of the variable recovery of waterborne coliforms with different types and brands of membrane filters reached a critical level in the mid-1970s, and a meeting was organized to discuss this problem.[95] The difficulty was largely resolved when it was discovered that only the recovery of fecal coliforms at 44.5°C was influenced by filter properties,[94] and that the surface-pore morphology was the physical characteristic of primary importance in this phenomenon.[96] This group at Millipore Corporation learned that membranes with a larger surface opening of conical geometry allowed the enhanced recovery of fecal coliforms at 44.5°C as compared with conventional filters with more cylindrical openings. Consequently, filter manufacturers began to produce filters such as the HC filters from Millipore specifically for the purpose of recovering injured fecal coliforms from water.

B. Methods to Enumerate Injured Indicator Bacteria in Water

Early efforts to develop methods and media to enumerate injured indicator bacteria have been reviewed previously.[5,14,16,97] These reports primarily represent the food microbiology perspective, although that approach overlaps the concerns of water microbiologists. Advances in coliform methodology and the enumeration of injured coliform bacteria in drinking water have also been reviewed recently by LeChevallier and McFeters.[9,10] The following discussion will concentrate on the development of media for the recovery and enumeration of injured indicator bacteria in water using the membrane filtration technique.

Highly variable coliform recovery efficiencies have been observed for some time in natural samples.[17,98-100] This may be explained by the data in Figure 3, which show a progressive discrepancy between enumerations on various selective media as a pure culture of *E. coli* was exposed in a natural stream for 4 d.[13] A more recent study[94] showed that the media most commonly used in the microbiological analysis of water, including m-Endo and m-FC media, were in the lower half of those evaluated in terms of recovering coliforms that were injured by exposure in flowing drinking water, while freshly cultured bacteria were largely unaffected (see Table 1). In addition, early studies noted that the selective ingredients in some media used for the enumeration of gram-negative bacteria were very inhibitory for injured bacteria.[25,93,99,101] Some of these reports also described bile salts as inhibitory to damaged *E. coli,* and one[25] noted that sodium deoxycholate was the most suppressive of the selective agents tested. Other studies have also described the inhibitory effect of surface-active selective ingredients in various media which were evaluated for recovery of injured coliforms.[17,25,99] A recent study[94] supported this observation by reporting that media that were most inhibitory for the enumeration of water-injured coliforms contained one or more of the bile salts in concentrations that ranged from 0.01 to 2.0% (see Table 1). That study also demonstrated that control suspensions of *E. coli* were virtually unaffected by 0.1% deoxycholate, while injured cells were 80% inhibited (see Figure 4). This series of studies formed the principal rationale that guided the development of specific media and methods for the improved recovery of injured coliforms in aquatic systems.

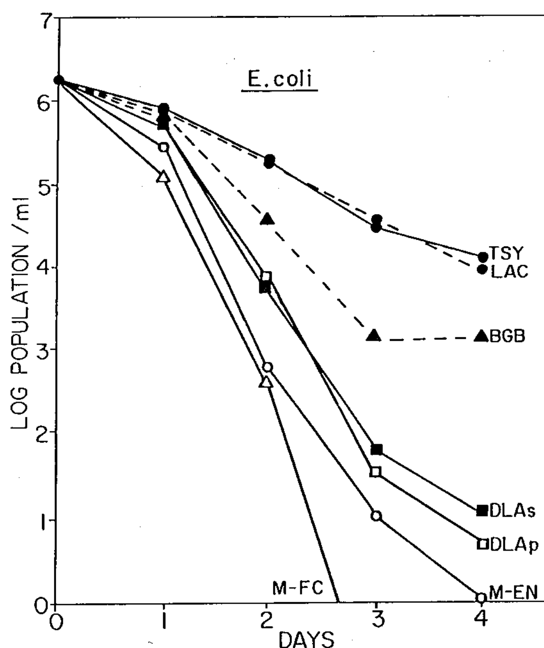

FIGURE 3. Comparative recovery of *Escherichia coli* C320MP25 in a membrane filter chamber located at site EG6 with various media, which included LAC (lactose broth) MPN (●); BGB (brilliant green lactose bile broth) MPN (▲); DLAs (desoxycholate lactose agar) surface-overlay plate (■); DLA pour plate (□); m-Endo MF membrane filtration (○); and m-FC membrane filtration (△). TSY counts (●) represent average values with TSY medium using MPN, pour plate, surface-overlay plate, and membrane filtration procedures.

Methods that were developed early for the detection of injured indicator bacteria, primarily in foods, were published concurrently in 1975.[102,103] Both procedures employed a period of incubation in the presence of a nonselective medium to allow the resuscitation of the injured cells, followed by the addition of a selective agent. This development, exploited by food microbiologists, also resulted in increased plating efficiencies of coliforms from surface waters.[103] Workers interested in the microbiological analysis of wastewater had proposed a membrane filtration enrichment procedure several years earlier,[104] although it attracted little attention. This procedure, which was devised to provide for "enrichment or resuscitation" of damaged bacteria, incorporated a 2-h preincubation of the membrane filter on a pad saturated with lauryl tryptose broth (LTB) at 35°C followed by a 22-h incubation on Endo agar medium at 35°C. The results of this study revealed that the preincubation procedure yielded more than twice the number of coliforms from a number of polluted waters than the conventional MF method using Endo medium. This observation is somewhat surprising in view of the more recent finding that lauryl tryptose medium recovered only 56% of the water-injured coliforms in a controlled study (see Table 1).[94] Perhaps the lack of agreement in the performance of this medium is due to different specific causes of injury in the respective studies. It is noteworthy that this early enrichment procedure is listed in the 17th edition of *Standard Methods for the Examination of Water and Wastewater* (909A.5c and 920.2),[39] although results from two laboratories indicated only a limited advantage over the conventional method.[105,106] In addition, one report demonstrated that this medium was less efficient than m-Endo medium in the enumeration of injured coliforms.[94] Lin[26] showed somewhat later that the enrichment procedure appeared to be comparable to the multiple-tube most

Table 1
MEDIA AND THE RECOVERY OF INJURED AND HEALTHY COLIFORMS FROM WATER

Medium[a]	% Recovery (range)[b]		% Deoxycholate or related compounds
	Injured	Uninjured	
Group I			
Triple sugar iron	181	106	0
Nutrient alginate	125	88	0
Minerals modified glutamate	99	106	0
Tergitol® 7	86 (71—101)	99	0
Boric acid	84	92	0
TLY + 0.1% Tween® 80	72	ND	0
Group II			
Lactose broth	72 (47—98)	102	0
m-Endo	66 (30—102)	93	0.1; 0.005[c]
Lauryl tryptose	56 (34—79)	98	0.1[c]
Levin's eosin methylene blue	42 (37—47)	119	0
3V	39	95	NA
Purple serum	38	56	0
EE	38	106	2.0[d]
Brilliant green bile 2%	34 (18—51)	106	2.0[d]
Deoxycholate lactose	26	94	0.05
Group III			
Levin eosin methylene blue	24 (7—42)	102	NA
Violet red bile	12	99	1.5[e]
m-FC at 44.5°C	7 (4—10)	105	1.5[e]
MacConkey	5	97	0.1[e]
GN	4	71	0.05
TLY-D	2	82	0.10
XLD	0	40	0.25

Note: ND, not done; NA, not available.

[a] Coliforms tested include: *Escherichia coli* (two strains), *Klebsiella pneumoniae, Citrobacter freundii,* and *Enterobacter aerogenes.* See reference 94 for experimental details and media composition.
[b] (Percent recovery) = {[(CFU selective medium)/(CFU TLY)] × 100}. Injury was between 90 and 99%. The range for injured coliforms is calculated from seven repetitions, using five coliforms over a 1-year period.
[c] Lauryl sulfate.
[d] Oxgall.
[e] Bile salts.

probable number (MPN) method for the enumeration of total coliforms from sewage, and, hence, was superior to the membrane filter (MF) procedure with Endo medium. In that report Lin also proposed a method for the improved enumeration of injured fecal coliforms. Membrane filters were preincubated on pads saturated with a dilute m-FC medium for 2 to 6 h at 25°C, followed by incubation on full-strength medium for 25 h at 44.5°C. This technique resulted in somewhat improved recovery efficiencies. Some time later, the notion of a preincubation attracted additional attention by aquatic microbiologists when Stevens et al.[107] suggested an extended resuscitation period at 35°C. Results with that procedure compared favorably with the MPN technique for fecal coliform enumeration. These studies collectively indicate the early progress that was made in the development of new methods for the improved recovery of injured coliforms from aquatic systems through the use of a pre-enrichment period under nonrestrictive conditions to allow reversal of the cellular damage.

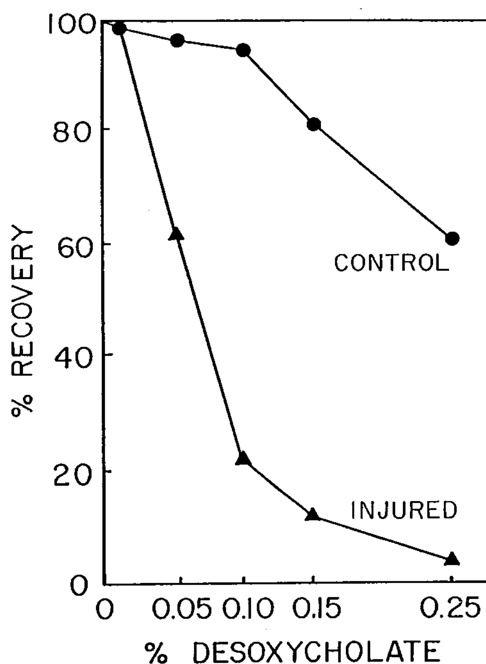

FIGURE 4. Effect of various concentrations of desoxycholate on the recovery efficiency of injured and control *Escherichia coli* suspensions. Desoxycholate was added to TLY agar, and the spread-plate method was used.

In 1975, a modification of this approach was proposed by Rose et al.[108] for the detection of fecal coliforms. This method involved a two-layer medium composed of an enriched, nonselective agar medium overlying conventional m-FC medium, prepared immediately before use, in addition to a 2-h preincubation period at 35°C. This approach was designed to allow recovery of the injured bacteria at the lower temperature before the selective ingredients reached the membrane. The evaluation of this procedure using a variety of waters indicated that it yielded almost twice the number of fecal coliforms than the conventional m-FC medium incubated at 44.5°C and that it was particularly beneficial in the analysis of waters that had received disinfection or contained metals. A modification of that procedure was proposed by Green et al.[109] This procedure, which omitted the two-layer medium but retained a 5-h preincubation period, is probably the most widely used technique for the enumeration of injured fecal coliforms because of its simplicity and commercial availability (Millipore Corp., Bedford, MA) of an incubator that is programmed to regulate the temperature at 35°C for the prescribed time period and then shift it to 44.5°C. Another medium developed specifically for the enumeration of fecal coliforms in water was proposed at about the same time by Stuart et al.,[110] employing the additional feature of nutrient enrichment. This procedure, called the injury-mitigating membrane filter (IM-MF) procedure, incorporated a layer of enriched lactose agar containing reducing agents overlying a modified m-FC medium and a three-temperature incubation sequence. Evaluation of this technique using chlorinated sewage effluents revealed that it yielded fecal coliform counts comparable with results obtained by the MPN test and significantly greater numbers than the conventional MF technique using m-FC medium. Although this rather cumbersome procedure has not gained wide acceptance, it has potential for future development and application in circumstances that present particular analytical difficulties. This is because the IM-MF method

Table 2
FORMULATION OF M-T7 MEDIUM[a]

Ingredient[b]	Quantity per liter of distilled water
Proteose peptone 3	5 g
Yeast extract	3 g
Lactose	20 g
Tergitol® 7 (25% solution)	0.4 ml
Polyoxyethylene ether W1	5 g
Brom thymol blue	0.1 g
Brom cresol purple	0.1 g
Agar	15 g
Penicillin G[c]	1.0 mg

[a] The medium was autoclaved at 121°C for 15 min, and the final pH was adjusted aseptically to 7.4 with 0.1 *N* NaOH.

[b] All ingredients were manufactured by Difco Laboratories, Detroit, MI, except polyoxyethylene ether W1, penicillin G, and brom cresol purple, which were manufactured by Sigma Chemicals, St. Louis, MO, and Tergitol® 7, which was obtained from Baker Chemical Co., Phillipsburg, NJ.

[c] Penicillin G (1650 units) should be added to the medium after autoclaving (when tempered). Media prepared with penicillin G should be used within 1 week when stored at 4°C.

includes all of the known strategies for enhancing the recovery of injured cells, including nutrient enrichment with metabolic intermediates, temperature acclimatization, and a non-selective enrichment period with a two-phase medium. These methods and media provide a number of effective approaches to enumerate fecal coliforms from aquatic environments where the bacteria are injured. This is acknowledged by the inclusion of a section (#920) in *Standard Methods for the Examination of Water and Wastewater,* entitled "Stressed Organisms".[39] This section briefly outlines problems associated with the recovery of injured indicator bacteria from water and lists some of the techniques described here.

More recently, a new medium was developed in our laboratory to enumerate injured indicator bacteria from water, including both total and fecal coliforms.[111,112] This medium has been designated as m-T7, since it is a modification of the established Tergitol® medium. The need for a new coliform medium grew out of dissatisfaction with Endo media,[94,111] the difficulty in distinguishing coliforms from noncoliforms on existing media,[106,113,114] low coliform verification rates,[106,115,116] uncertainty concerning the availability of acceptable basic fuchsin,[116a] as well as a growing need for a reliable method to recover injured coliforms. The formulation for the medium is given in Table 2, and it is now commercially available (Difco, Detroit, MI). It should be stressed that the addition of penicillin after sterilization, at a concentration of 1.0 mg/l, is important to provide sufficient selectivity. This medium was initially evaluated for enumerating total coliforms in drinking water, since that was its intended application. m-T7 performed very well,[111] recovering 43% more coliforms in the initial testing of 67 surface and treated drinking-water samples and performing significantly better than m-Endo with or without the LTB preincubation. This medium was also tested for the enumeration of fecal coliforms from stressful waters with an 8-h acclimatization step

at 35°C. This did not reduce the selectivity of the medium, and it recovered more than 3 times more fecal coliforms than the standard m-FC medium and 1.7 times more than the two-layer, temperature-acclimatization procedure.[112]

A number of other new media that have been developed for the enumeration of coliforms from water also might be useful in the recovery of injured bacteria. A medium designated mTECM has been formulated by Cabelli. Although this medium had not been described or extensively evaluated in the literature at the time of this writing, the results of a survey of small, community drinking-water systems in Oregon has recently demonstrated that it performed much better than m-Endo, but well below m-T7. However, comparative testing revealed that a similar medium (mTEC) recovered comparable numbers of fecal coliforms in marine water samples collected along the coasts of New York and New Jersey as m-FC with fresh-water samples.[117] The status of a coliform medium developed by Haines (unpublished) and one by Geldreich and Rice,[118] designed to be selective for members of the genus *Klebsiella*, is somewhat similar. It should also be noted that an earlier report from England demonstrated that Tergitol® 7 (T7) performed slightly better than lauryl sulfate (LS) and Teepol 610 media in the recovery of coliforms from chlorinated waters.[119] This difference might be explained by the ability of T7 to detect injured bacteria, but it is noteworthy that the British Joint Committee of the Public Health Laboratory Service and the Standing Committee on Analysis recommended LS as the standard medium for the enumeration of coliforms in water by the membrane filtration technique.[119] Although there will almost certainly be more studies to compare the performance of various media relative to their recovery of injured coliforms in water, the data presently available indicate that m-T7 is superior to established media.[111,112,120] However, it is worth a note of caution that such comparative studies should examine distribution water samples in the field as opposed to diluted sewage, in order to adequately validate any medium that is intended for the analysis of drinking water. This is because laboratory studies frequently omit some environmental conditions which injure bacteria under *in situ* conditions; hence, the bacteria are not stressed as they are in the field. A secondary consequence of these studies will be the development of an expanded data base describing the occurrence of injured bacteria in various waters and wastewaters.

V. OCCURRENCE OF INJURED WATERBORNE BACTERIA

Information available on the incidence of injury within waterborne bacterial communities is somewhat limited. This might be explained by at least two factors. First, injury of indicator bacteria within aquatic systems has only been studied in systematic ways by microbiologists within the past decade. As a result, the awareness and acceptance of this relatively new concept have only recently started to gain momentum as more reports describing the process and its consequences are being published. Second, methods of documenting injured bacteria were unavailable until fairly recently. The media now available, including the commercial availability of the m-T7 medium, should be of assistance to workers interested in enumerating injured coliforms in water.

Numerous early reports of higher bacterial counts determined with MPN methods over membrane filtration procedures provided evidence of injury within waterborne bacterial communities.[6,12,26,35,95,100,120-122] Our awareness of this discrepancy, which can be partially explained by injury, became evident as MF procedures gained increasing acceptance and workers wanted to know the comparative efficiencies of MPN and MF procedures. Most of these studies reported that the MPN technique detected higher numbers of coliforms when samples were analyzed by both methods.[123] As a result, the MPN method is usually regarded as the standard with which other procedures are compared.[39] Although there are flaws in the MPN methods that have been described elsewhere,[124,125] the use of LTB or lactose broth

appears to be less restrictive to the growth of injured coliforms than the MF technique with the m-Endo medium, and this difference may be the consequence of injured cells. However, this observation is not fully understood, since LTB is somewhat restrictive for the growth of injured coliforms.[94]

A form of injury, described as "weakened cells", was used in 1954 to interpret the results of an early study comparing the MF and MPN procedures to enumerate coliforms from natural waters.[126] In addition to reporting that the MPN technique usually yielded higher counts, these workers found that this discrepancy was consistently greater when samples from the Wautauga River were analyzed. As possible explanations of these results, they suggested that toxic substances present in that river accumulated on the filters or that "weakened cells" might recover more readily in the MPN broth. A similar rationale was also used somewhat later to explain similar observations in a comparison of MF and MPN coliform enumerations in chlorinated sewage.[127] Other workers reporting the cellular consequences of UV, chemical, and heat inactivation about that time used the term "injury" to specifically signify a form of reversible bacterial inactivation.[24,128] In addition, the injury of aquatic *E. coli* suspensions resulting from exposure to low levels of chlorine was described in 1959 as a differential plate count using complete and minimal agar plating media.[129] However, the practical consequences of injury in microbiological applications and systematic studies of the phenomenon did not emerge until the 1960s. Much of the subsequent work of bacterial injury was carried out by food microbiologists, as described elsewhere,[1-3,5,14] and it was not until the 1970s that aquatic microbiologists again became interested in the process.

A renaissance of interest among aquatic microbiologists in the phenomenon of injury was evidenced in the early 1970s by a number of reports. Klein and Wu[11] described the increased sensitivity of standard plate count bacteria (SPC) in water to secondary warming. These investigators also proposed that it should be possible to evaluate the degree to which microorganisms have been stressed by measuring the effects of secondary stress. This report confirmed the earlier suggestion by Postgate[130] that the exposure of allochthonous bacteria to environmental stresses such as starvation results in hypersensitivity to secondary stresses. Braswell and Hoadley[16] further revealed that *E. coli* exposed to chlorine in sewage became incapable of growth on m-FC medium or gas formation in lactose broth. Another report at approximately the same time described the injury and recovery of *P. aeruginosa, E. coli,* and *Streptococcus faecalis* in tap water.[17] Each of these bacteria became progressively more sensitive to selective media with increasing incubation time in water. These authors suggested that low concentrations of copper and other metals could have been responsible for the observed effect and that media used in the microbiological analysis of water should be less restrictive for the growth of injured bacteria. These papers are significant, since they described the injurious character of some aquatic systems for a variety of bacteria in water and pointed to the feasibility of recovering injured cells through the modification of media.

Previous information from the food microbiology literature, in addition to the reports described above, prompted a series of studies on the aquatic injury of indicator bacteria in our laboratory. In 1975, Bissonnette et al.[20] examined the problems associated with the recovery of *E. coli* and *S. faecalis* from surface waters. Membrane diffusion chambers[12] were used to expose these bacteria to various natural streams near Bozeman, MT. It was observed that these bacteria became reversibly injured to different degrees in the various streams, as illustrated in Figure 1, and that the injured populations regained a tolerance for selective media with incubation in a nonselective medium (see Figure 2). Injury in *E. coli* ranged between 10 and 97% after 4 d of aquatic exposure in the various streams with an average value of 61%. The results with *S. faecalis* were somewhat similar with an average of 67% injury after 4 d of exposure to stream water. It is interesting to note that both bacteria reacted similarly at the same sites; some streams proved to be highly injurious for both species, while others caused little effect. Additional experiments using a different strain of

Table 3
INJURED COLIFORMS IN DRINKING WATER

Location date	Type of water	No. samples	Mean coliforms per 100 ml		% Injured	% False negatives[a]
			m-Endo	m-T7		
Midwest/1986	After filtration	9	1.1	3.9	71	55
Midwest/1986	Distribution	13	2.4	7.9	69	23
Northeast/1985—1986	Distribution	86	1.9	4.8	64	54
Northeast/1985—1986	Raw	86	14.5	16.8	14	—
Northeast/1985	During treatment	320	1.4	1.9	26	24
Caribbean/1985	Cisterns	13	15.2	20.5	26	—
East/1986	After treatment	4	2[b]	209	99	100
Northwest/1984—1986	Small systems	552	62[c]	139[c]	55[d]	—

[a] % False negatives represents the percentage of coliforms that failed to produce colonies on m-Endo medium but were enumerated on m-T7.
[b] MPN value.
[c] Values are % positive for coliforms.
[d] Estimate.

E. coli, Klebsiella pneumoniae, S. faecalis var. *liquefaciens,* and *S. durans* yielded similar results.[13] The injury of these last four species was also examined in well water, and it was found that very little injury (i.e., <20%) occurred within 3 d of aquatic exposure. As discussed earlier, the highly variable injury was thought to be a reflection of the differences in the chemical composition of the various streams and wells examined. A subsequent report by Bissonnette et al.[100] described the influence of various media and methods in the recovery of injured coliforms and prompted later efforts to develop more efficient media for this purpose.

Experiments to validate m-T7 as an effective medium for the enumeration of total coliforms also yielded information on the incidence of injured waterborne bacteria. An average of 65% of the coliforms were injured in 44 samples of Montana drinking water, while 30% of the coliforms in 11 samples of untreated surface water and 34% in 12 samples of chlorinated surface water were injured.[111] When a similar study was done to compare m-T7 and m-FC media using chlorinated wastewaters, 61% of the fecal coliforms were injured.[112] A survey of 254 samples of chlorinated and unchlorinated drinking water from four systems in Montana and Massachusetts showed a similar degree of injury.[10] The mean level of injury found in 31 coliform positive samples was 58%. A more recent study examined treated drinking water in a northeastern U.S. city for injured coliforms using m-T7 and m-Endo media.[120] Coliforms found in 71 samples of distribution water and 46 samples of water leaving the treatment plant were 97% injured. Injured coliforms were also found (87 to 97%) to be associated with the backwash water from filters used in the treatment process and in samples of distribution water following a pipe break (97%). All 11 of the samples taken 1 week following the repair and disinfection of that pipe break were found to be negative for coliforms using m-Endo, while all were positive on m-T7 medium. Unpublished results from a number of systems using m-T7 medium are shown in Table 3 and reveal a similar trend. Injury values in most of the chlorinated systems is high, while raw and cistern water contain fewer injured coliforms. This is consistent with the results cited earlier.[111] The percentage of samples in which injured coliforms were detected in chlorinated water with m-T7 but not with m-Endo is also striking.

As mentioned previously, SPC bacteria became sensitive to secondary warming with time in aquatic environments.[11] Likewise, *P. aeruginosa* can become more sensitive to selective media with aquatic exposure in the absence of chlorine.[17] It would be of interest to learn

Table 4
INJURED WATERBORNE BACTERIA

Bacteria	Stressor	Environment	Ref.
Coliforms	Unknown	Natural water	123
	Unknown	Sewage	124
Escherichia coli	UV, chemicals	Lab study	16, 125
E. coli, fecal coliforms, fecal streptococci	UV	Seawater and sewage	39, 57, 40
E. coli	Chlorine	Lab study	126
SPC bacteria	Unknown	Natural water	127
E. coli, fecal coliforms, fecal streptococci	Chlorine	Sewage	9, 23, 25
E. coli, Pseudomonas aeruginosa, Streptococcus faecalis	Unknown	Tap water	10
E. coli and *S. faecalis*	Unknown	Natural surface water	13
E. coli, S. faecalis, Klebsiella pneumoniae	Unknown	Natural surface water	18
Coliforms	Unknown	Drinking and surface water	86, 24, 109
	Unknown	Drinking water	86, 109, 110
Clostridium perfringens (veg.)	Unknown	Tap water	129
E. coli, Shigella sonnei, Vibrio cholerae	Unknown	Estuarine water	78, 79, 80
E. coli, Yersinia enterocolitica, Salmonella typhimurium, Shigella spp., ETEC[a]	Copper and chlorine	Lab study	32, 130, 131
E. coli	Chlorine	Lab study	11, 12
Legionella pneumophila	UV	Fresh and marine waters	58
Coliforms	Acid pH	Acid-mine streams	43, 44
	Biological	Lab study	47

[a] ETEC, enterotoxigenic *Escherichia coli.*

the additional effect(s) of chlorine on these bacteria. Injury might also affect the vegetative cells of *Clostridium perfringens,* since there was a significant difference in the plating efficiencies with different selective media when these bacteria were incubated in tap water.[131]

The exposure of *E. coli* to brackish water also appears to injure this bacterium, since suspensions exposed to Chesapeake Bay water became progressively unable to form colonies on selective media.[86] This process might also encompass a number of other organisms, including pathogens such as *Shigella sonnei* and *Vibrio cholerae,* as they progress, with aquatic exposure, from cells that are fully viable and tolerant of selective media following cultivation to a form that is viable but not culturable.[86-88] This effect in pathogenic bacteria is consistent with recent results from our laboratory which have demonstrated that enteric pathogens, including *Yersinia enterocolitica, Salmonella typhimurium, Shigella* spp., and enteropathogenic *E. coli* became injured following aquatic exposure to levels of copper and chlorine that resemble those found in drinking water.[132,133]

Table 4 gives some of the representative organisms, stressors, and environments where injured bacteria of interest in water have been demonstrated. It is clear that a wide variety of bacteria can be injured by a number of stressors of importance in different aquatic systems. With a growing awareness of the injury concept and the availability of new methods and media that are capable of enumerating injured bacteria, it is likely that this list will continue to grow and extend to other environments, such as air and soil. For instance, one group has

obtained evidence that certain bacteria become injured in soils.[133a] Such information will provide insights concerning the microbiology of each system studied.

VI. INJURY AND PATHOGENIC BACTERIA IN WATER

Previous studies of injury and waterborne bacteria have focused largely on the indicator organisms. As a result, a growing body of information has accumulated concerning the occurrence of injured coliforms in various waters. This, in turn, has been used to help explain some of the microbiological problems associated with the treatment and distribution of drinking water.[9,10,120] Previous studies on the persistence of waterborne bacteria have related the population dynamics of indicator bacteria with that of some of the enteric pathogens that can be transmitted through water.[134-139] Most of these studies have concluded that the survival of many enteropathogenic bacteria in water resembles that of the indicator bacteria, although differences in the experimental design used by the various investigators make comparisons of the data difficult.

Most of these studies did not address important questions relative to the injury of enteropathogenic bacteria and their ability to cause disease following aquatic exposure. However, it might be assumed that these bacteria retain virulence properties to some degree, since they are transmitted via the waterborne route of infection. In addition, it is logical that they should be subject to injury resulting from stressors in water in a way that is similar to the coliforms, since many enteric waterborne pathogens are likewise Enterobacteriaceae. Until very recently, there has been a paucity of data in this area of investigation. The fundamental questions that have recently been addressed in this context concern the susceptibility of pathogens to injury and the reaction of virulence properties following aquatic stress and injury. Recent studies from our laboratory and elsewhere have followed these bacteria, which had been exposed to the potentially damaging influences in water, and described the effect of these stressors on their virulence. We have also examined the ability of injured pathogens to recover from injury *in vitro* and *in vivo,* following the natural succession of ingestion by a susceptible host.

The results of these studies have a number of important implications within the field of drinking-water microbiology. For instance, this kind of information could be useful in explaining outbreaks of waterborne illness where coliforms were not detected,[10] or the converse situation where no observable waterborne morbidity accompanies the detection of coliforms. Such data would also be of value in addressing the health significance of injured coliforms in drinking water. In addition, this information would provide an expanded understanding of the ecology and epidemiology of waterborne pathogens in aquatic systems.

Preliminary experiments performed in our laboratory[10a] addressed these concerns and questioned the concomitant decline of pathogenicity and viability of enteric pathogenic bacteria in water. In this study, suspensions of enterotoxigenic *E. coli* (ETEC) were exposed to chlorinated tap water and sampled at daily intervals. Virulence was determined as the ability of the bacteria to cause significant weight loss due to diarrhea following oral administration of the test bacteria into infant mice. As in the previous studies with nonpathogenic coliforms,[137] the viable population declined at about 0.5 log/d, while the virulence was not detected after 4 d of aquatic exposure. At this time, the viable bacterial population remained sufficiently high to provide a positive virulence response if they were uninjured. This was determined with a freshly grown suspension of the ETEC containing an equivalent number of viable cells. More recently, Walsh and Bissonnette[140] reported similar results with a strain of ETEC that became less capable of attachment to human peripheral leukocytes with chlorine-induced injury.[140] These findings suggested that sublethal aquatic stress reduced the pathogenicity of ETEC.

A subsequent, more detailed, investigation of injury and the virulence of enteropathogenic

Table 5
SUSCEPTIBILITIES OF COLIFORM BACTERIA AND PATHOGENS TO INJURY BY CHLORINE

Organism	No. of strains tested	Free chlorine conc causing >90% injury (mg/l)[a]
Coliform bacteria[b]	11	0.38 (0.25—0.50)
Enterotoxigenic *Escherichia coli*	3	0.33 (0.25—0.50)
Yersinia enterocolitica[c]	7	1.07 (0.50—1.50)
Salmonella typhimurium	7	1.50 (1.00—2.00)
Shigella spp.[d]	3	0.92 (0.50—1.50)

[a] Values are means. The values in parentheses are ranges.
[b] The coliform isolates included five strains of *Escherichia coli,* two strains of *Klebsiella oxytoca,* two strains of *Enterobacter cloacae,* one strain of *Enterobacter aerogenes,* and one strain of *Citrobacter freundii.*
[c] For a description of the serotypes used, see the text.
[d] The *Shigella* species used included *Shigella dysenteriae, Shigella flexneri,* and *Shigella sonnei.*

bacteria stressed in water was carried out in our laboratories.[132,133,141,142] The initial experiments were designed to develop a reproducible procedure to injure pathogens in water. Bacterial suspensions in diffusion chambers[12] were immersed in flowing city (Bozeman, MT) drinking water that was chlorinated. The results of these experiments using ETEC, *Salmonella typhimurium,* and *Y. enterocolitica* demonstrated a high degree of injury within a few days of aquatic exposure. However, the kinetics were highly variable with successive repetitions of the experiment. Although these findings were encouraging, since they indicated that the pathogens became injured in drinking water within a relatively short time, this method was not acceptable as greater precision was needed in generating populations of highly injured (>90%) bacteria for subsequent analysis of virulence phenotypes. In retrospect, these findings were not surprising, since they indicated that key chemical and physical properties of the tap water, including such things as the levels of chlorine and metals, were variable with time. This necessitated the use of an alternative approach to produce injured pathogens to be used in these studies in a reproducible and predictable manner. The alternate experimental approach that was adopted utilized controlled concentrations of chemical components known to be involved in the injury of enteric bacteria in drinking water.

The first series of experiments revealed that the pathogens tested became reproducibly injured when exposed to aqueous solutions of chlorine under controlled conditions.[132] However, it is important to note that pathogens, including *Y. enterocolitica, S. typhimurium,* and *Shigella* spp., required significantly higher levels (0.9 to 1.5 mg/l) of chlorine to cause injury (>90%) than the nonpathogenic coliforms and ETEC (0.25 to 0.5 mg/l) as seen in Table 5. In addition, the virulence (intraperitoneal [i.p.] injection into mice and the observation of 50% lethality levels) of injured *Y. enterocolitica* was 20 times less than in uninjured control cells. A parallel series of experiments using copper-stressed *Y. enterocolitica* yielded similar results.[133] Again, higher levels of copper (about 0.75 mg/l) were required to injure (>90%) this pathogen than a nonpathogenic *E. coli* (0.05 mg/l) treated under the same controlled experimental conditions.[37] The virulence of the injured cells was 18-fold less than with control bacteria. Studies performed to determine the mechanism(s) of injury demonstrated a loss of invasive properties in *Y. enterocolitica,* while injured ETEC and *S. typhimurium* became incapable of attachment to appropriate cell cultures.[132] Additionally, copper-induced injury led to increased liver clearance of bacteria *in vivo* (in mice) as well as promoting *in vitro* aggregation in the presence of mouse liver membranes.[133] These results

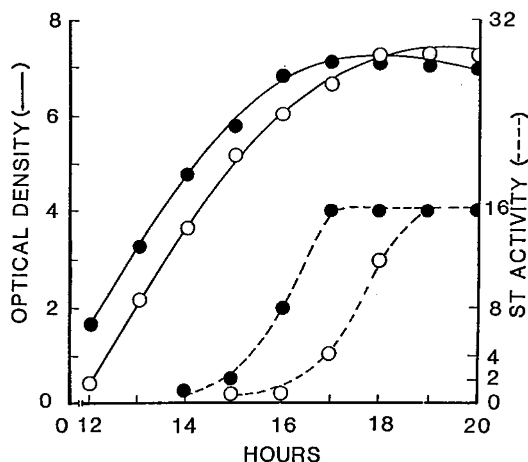

FIGURE 5. Comparison of the growth (________) and heat-stable toxin (ST) production (- - - -) of uninjured (●) and copper-injured (○) cultures of *Escherichia coli* H10407 in brain heart infusion medium. The OD was measured at 600 nm, and ST activity was determined by intragastric inoculation of culture filtrate into infant mice.

suggest that representative aquatic stressors, such as chlorine and copper, affect different determinants of the virulence phenotype. Therefore, generalizations concerning the mode(s) of action of the stressors studied and their effects upon various pathogens should be avoided, with the exception that surface properties appear to be altered in all the cases examined.

Experiments were also performed to examine the recovery, growth, and synthesis of enterotoxin following copper-induced injury in ETEC.[141] *In vitro* recovery of desoxycholate tolerance was demonstrated both in rich (3 h) and minimal (6 h) media. The growth kinetics of injured and control cells were similar in the rich medium, although there was a somewhat extended lag phase in the case of the injured populations as seen in Figure 5. The synthesis of heat-stable enterotoxin (ST) was followed in these growth experiments. It was also shown that the injured cells produced comparable levels of ST, although somewhat later, when compared with the control suspensions (see Figure 5). This indicated that the enterotoxigenic potential was fully retained, but delayed somewhat in expression following copper-induced injury. Another set of experiments was also done to follow the *in vivo* revival, growth, and pathogenicity of ETEC following chlorine- and copper-induced injury.[142] Following injection into ligated, ileal loops in mice, injured ETEC suspensions recovered in 3 to 4 h, with only a small increase in total bacterial numbers, as shown in Figure 6. This process was also seen *in vitro* when the injured bacteria were incubated in saline containing mouse intestinal mucosal homogenate, but not in its absence. In addition, the *in vivo* pathogenicity of injured and control cells was followed in ligated rabbit ileal loops. These experiments demonstrated that the enterotoxigenic activity of copper-injured cells was somewhat reduced when compared with that of chlorine-injured or uninjured control cells. These results indicate that injured pathogenic *E. coli* can recover both *in vitro* and *in vivo* (within the gut) and retain virtually full potential for enterotoxin synthesis. Therefore, injury processes resulting from copper and chlorine at levels approximating those found in drinking water do not significantly reduce the enteropathogenic potential of the pathogens examined. It is tempting to speculate that the subsequent ingestion of such cells could lead to human enteric disease.

The results of studies from another laboratory on the survival of several enteric pathogens in simulated aquatic environments also support the persistence of their health threat in natural

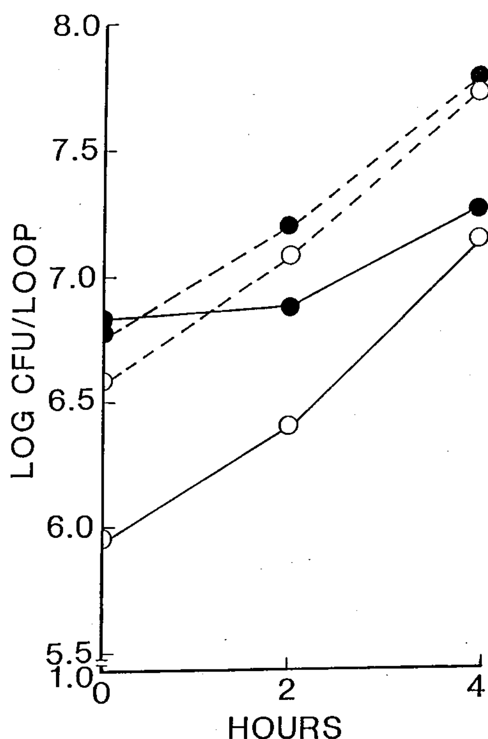

FIGURE 6. Comparison of the growth of uninjured (- - - -) and copper-injured H10407 (————) cells after intraluminal inoculation in mice. ● = CFU on TLY; ○ = CFU on TLY-D.

systems.[86-88] Various plating and enumeration procedures were used to follow their population dynamics and viability with time. The results of these studies indicate that enteropathogenic bacteria appear to enter a "viable but nonculturable" state upon extended incubation in simulated marine, estuarine, and freshwater systems. The pathogenicity of *V. cholerae* cells was demonstrated following this transition using the rabbit ileal loop assay. In addition, the findings of Palmer et al.[143] suggested the reversible loss of plasmids thought to be associated with the virulence in an ETEC strain upon exposure to water containing sublethal concentrations of toxic chemicals. These results provide additional evidence that allochthonous human enteric pathogenic bacteria retain both their viability and their pathogenic potential within various stressful aquatic environments that simulate natural systems.

VII. SIGNIFICANCE OF INJURED BACTERIA IN WATER

It is difficult to reach firm conclusions concerning the significance of injured enteric bacteria in water, since the true importance of uninjured coliforms in water is often debated. However, it is the intention in this section to discuss the significance of injured waterborne coliforms and enteropathogenic bacteria in light of the available information and common practice within the drinking-water industry in the U.S.

There is little definitive information describing the correlation of coliforms and enteric pathogens in water, although the latter is afforded great importance in the determination of water potability. Most justification for this position is based upon the earlier published findings of Kehr and Butterfield,[144] as well as Petersen and Hines,[145] both of whom found

a relationship between coliforms and waterborne morbidity. Clearly, more information is needed in this area, and it is not surprising that dissatisfaction with the present coliform indicator concept is frequently expressed.[86,146] In part, this position is fueled by the rising number of waterborne illness outbreaks in the U.S.,[147,148] as well as specific instances where coliforms were either undetected or found in very low numbers associated with waterborne morbidity.[59,149-151] Some of these occurrences might be explained by diseases caused by viruses and protozoa capable of forming cysts and the realization that these agents are much more persistent in water than the indicator bacteria.[35,152] However, it should not be overlooked that the coliform indicator concept has been of value despite its limitations, and that additional specific information is needed on the levels of coliforms associated with waterborne disease outbreaks, as pointed out by McCabe.[153]

In addition to its role as an index of public health risk and as a regulatory tool concerning water, the presence of coliforms is useful in determining problems associated with the operation of drinking-water treatment plants. Here again, more data are needed. The awareness of laboratory personnel and supervisors within that industry also needs to be expanded concerning the usefulness of more sensitive monitoring methods in the detection and solution of operational problems within treatment plants and distribution networks, regardless of whether the data are to be used in a regulatory context.

A. Coliforms

There are a number of justifications for the enumeration of injured coliforms and the recognition of their significance. Most of these relate to treated drinking water, where their presence is of regulatory importance. Hence, the detection of coliforms is unwelcome to those responsible for the operation of drinking-water systems. Understandably, such individuals view the use of methods that detect both injured and uninjured cells as a threat, since the practice could lead to coliform estimates that are as much as tenfold greater than when accepted media and methods are used. The finding that injured coliforms can comprise between 50 and >90% of coliforms present in treated drinking water, as discussed earlier, indicates that their detection is a more sensitive analytical approach to examining the microbiology of water. Therefore, the detection of the entire viable population of coliforms, including injured cells, increases analytical sensitivity and provides a greater capability to detect developing problems within the system. This could allow the initiation of remedial action before the difficulty becomes a crisis. Under these circumstances, such an analytical capability would provide the operator of the system a clear advantage in the early detection of developing problems. This increased sensitivity could also be used to good advantage in epidemiological investigations of waterborne disease outbreaks. For example, it is possible that instances of waterborne morbidity, where coliforms are either undetected or detected in low numbers, might be explained by injured bacteria. In such a case the use of media that enumerate injured cells, such as m-T7, might show that coliforms are in fact present in those waters. The use of methods that detect injured coliforms would provide a more sensitive and earlier warning of public health threats from pathogens in such cases.

There are also significant regulatory implications relating to injured coliforms. As pointed out previously, some within the drinking-water industry regard the enumeration of injured coliforms in an adversarial context, since it could result in detection of higher numbers of coliforms that might lead to the possibility that the system would violate existing regulations. That fear, however, is largely without substance, since systems with high-quality drinking water are likely to see little change in coliform recoveries.[122,125] Another important regulatory concern relates to the probable adoption of the presence-absence (PA) concept[154] into the federal drinking-water regulations. This could result in water quality test findings that could be erroneous in some circumstances. This issue is raised since, at the time of this writing, the exact way in which the PA test will be written into the regulations is not known. It is possible that sufficient latitude would be provided in the selection of the PA medium/method

to allow the analyst to use a combination that would not detect injured cells. Such a circumstance could result in an absence test result between 50 and >90% of the time when viable coliforms are actually present. This estimate, which is based upon the results presented earlier, indicates that such applications of the PA concept could lead to a significant level of false negative coliform tests, as shown in Table 3. On the other hand, if appropriate media/methods, such as m-T7 broth in an MPN test or m-T7 agar in the MF test, are used, more sensitive and accurate results would be obtained.

B. Pathogens

Much of the available data describing the virulence of enteropathogenic bacteria following aquatic exposure indicate that pathogenicity is retained.[85-87,132,133,140-143] These reports provide evidence that enteropathogenic bacteria exposed within aquatic systems become injured, and some may enter a viable but nonculturable stage. However, such altered populations appear capable of resuming normal growth under suitable conditions like injured populations, including the mammalian gut, where virulence properties are expressed and the bacteria appear capable of causing disease. On the other hand, not all of the evidence supports this concept, since it has been suggested that certain pathogens may loose virulence properties under some waterborne circumstances.[140,143]

It is clear that more information is needed in this important area of investigation before more definitive conclusions may be reached. However, existing data support the concept that enteropathogenic bacteria are less sensitive to injury than coliforms and retain pathogenicity upon aquatic exposure. These conclusions underscore the significance of injured enteropathogenic bacteria following aquatic stress.

VIII. CONCLUSIONS

The findings of these studies indicate that (1) injury of indicator and pathogenic bacteria can occur within drinking water systems; (2) methods are available that effectively detect injured coliforms; (3) the vast majority of coliforms within these systems may be undetected because sublethal stress leads to decreased detection on conventional media; (4) pathogenic bacteria, except ETECs, also become injured, but at higher levels of stressing agents; and (5) injured pathogens express virulence determinants following recovery. As a result, injured coliforms and pathogens are of public health significance and the detection of injured indicator bacteria affords an added measure of sensitivity to assist in the early detection of treatment deficiencies or contamination within potable-water systems.

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Chapter 8

METHODS FOR RECOVERING INJURED PATHOGENIC ORGANISMS FROM ANIMAL FEEDS

Wallace H. Andrews

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I. INTRODUCTION

Methods for recovering pathogenic bacteria from animal feeds have not been studied to the same extent as methods for recovering these organisms from foods intended for human consumption (see Chapter 3). This relative lack of information is somewhat unexpected, since the role of animal feeds as potential reservoirs of pathogens in the chain of human infection has been well documented.[1-3] Moreover, unlike internationally recognized methods for the examination of foods for the presence of pathogens, there are no universally accepted methods for the microbiological analysis of animal feeds.

II. ORGANISMS FOUND IN ANIMAL FEEDS

One of the earliest microbiological surveys of commercial poultry feeds in this country was conducted by Erwin.[4] Of 206 samples, 73 contained *Paracolobactrum*, one was positive for *Proteus mirabilis*, and three contained *Salmonella oranienburg*. Subsequently, in its initial report to the Nomenclature Committee at the Seventh International Congress of Microbiology, the *Enterobacteriaceae* Subcommittee recommended that the genus *Paracolobactrum* not be officially recognized.[5] According to *Bergey's Manual of Determinative Bacteriology*,[6] most of the strains formerly placed in this genus have now been redistributed among related enteric genera in the family Enterobacteriaceae. Even though Erwin originally described the *Paracolobactrum* as producing colonies resembling *Salmonella* on eosin methylene blue and bismuth sulfite agars, it would be speculative to conclude that they were in fact *Salmonella*.

Morehouse and Wedman[7] conducted a survey of 5712 samples of a wide variety of animal by-products in the U.S. that were used in the preparation of poultry, swine, and laboratory animal feeds. *Salmonella* spp. were found in 718 of these samples; the number of samples in which other isolates were found included *Staphylococcus* spp. (17), *Bacillus anthracis* (14), Newcastle disease virus (3), *Candida albicans* (2), *Klebsiella* spp. (1), and *Mycobacterium tuberculosis* (1).

Recently, Cox et al.[8] conducted a survey of *Salmonella* and other Enterobacteriaceae in commercial poultry feed produced in the southeastern U.S. *Salmonella* spp. were found in 92, 58, and 0% of the meat and bone meal, mash (finished feed), and pelleted feed samples, respectively. Overall, the most frequently encountered species were *Enterobacter agglomerans*, *E. cloacae*, and *Klebsiella pneumoniae*.

Surveys outside the U.S. further document the widespread occurrence of *Salmonella* in commercial feeds and feed ingredients. Stott et al.[9] conducted a survey of meat and bone meals, fish meals, and poultry feeds in Great Britain. *Salmonella* spp. were found in 19% of the meat and bone meal samples, but not in the other products. Jacobs et al.,[10] however, reported that 26% of the bags of fish meal imported into The Netherlands from Chile and Peru were contaminated with *Salmonella*. Of 306 samples of animal feed ingredients produced in Japan, Yoshimura et al.[11] found *Salmonella* in meat meal (68%), mixed meal (43%), and fish meal (21%) and in 31% of the imported samples of meat and bone meal. Bensink[12] found *Salmonella* in 70% of 164 samples of meat and bone meal from rendering plants in Australia.

Working with animal by-products used in commercial feeds, Tompkin and Kueper[13] demonstrated a linear relationship between the incidence of *Salmonella* and total plate count values between 10^4 and 10^7 cells/g. The incidence of *Salmonella* increased from 12.7 to 70.6% as plate count values increased from 10^4 to 10^7 organisms/g. The decreased occurrence of *Salmonella* for plate counts in excess of 10^7 organisms/g were attributed to overgrowth of the *Salmonella* by the other organisms during enrichment or plating, or both. Animal by-products were one of only a few product types in which a linear relation existed between plate count values and the presence of *Salmonella*.

The results of the preceding surveys indicate that members of the genus *Salmonella* are the pathogens of greatest concern in animal feeds. Accordingly, the remainder of this chapter will be concerned specifically with these pathogens.

III. TRANSMISSION OF *SALMONELLA* BY CONTAMINATED ANIMAL FEEDS

Several studies have demonstrated a direct correlation between the presence of *Salmonella* in poultry feeds and the isolation of these pathogens from poultry carcasses. In addition to feed, however, Bains and MacKenzie[1] cited four other sources of contamination for poultry flocks: carrier birds, litter, nest boxes, and the environment. All five sources of contamination were monitored throughout the 9-month laying cycle of a breeder flock. The investigators clearly demonstrated the transmission of *Salmonella* from the feed grains to the breeder feed, to breeder parent stock, to day-old chicks, and then to the dressed broilers. In a subsequent study, MacKenzie and Bains[3] demonstrated a significant correlation between the *Salmonella* organisms isolated from the raw feed ingredients and those from the poultry carcasses. Of the 17 serotypes isolated from the poultry carcasses, 14 could be traced to the original isolations from the feeds or feed ingredients.

The transmission of *Salmonella* by contaminated feeds to animals other than poultry has been demonstrated.[2] When an explosive outbreak of *S. newport* infection occurred in one mouse- and two guinea pig-breeding colonies, an investigation of the source of contamination led to the identification of *S. newport* and other serotypes in feeds supplied to the animals. It was suggested that fecal droppings from rodents in grain bins or even the bodies of mice pressed in bales of hay were the ultimate source of *Salmonella* contamination of the animal feeds.

These studies underline the importance of animal feeds and feed ingredients in spreading *Salmonella* organisms to both humans and animals. To minimize this potential, however, the presence of these pathogens in animal feeds must be monitored with the most effective analytical methods available.

IV. ANALYTICAL METHODS

For most low-moisture foods, a resuscitative step is needed to recover those *Salmonella* organisms that may have been injured during processing. Typically, this resuscitation occurs during the initial preenrichment phase of the isolation procedure. Because many of the ingredients of animal feeds may be highly contaminated with competing organisms not belonging to the genus *Salmonella*, however, there is some basis for considering the bypass of this preenrichment step. Several investigators have compared preenrichment with direct selective enrichment for the recovery of *Salmonella* from animal feeds. D'Aoust et al.[14] compared overnight preenrichment in nutrient broth with direct enrichment in selenite cystine or tetrathionate broth for the recovery of *Salmonella*. Of 269 samples of animal feeds and feed ingredients, 58 were positive with preenrichment, whereas 68 were positive with direct selective enrichment. Similar results were obtained by Cox et al.,[15] who compared 24-h preenrichment in lactose broth with direct enrichment in selenite cystine or tetrathionate broth for the analysis of commercially pelleted poultry feed. Preenrichment was significantly counterproductive when compared with direct selective enrichment. In a subsequent study, these same investigators[16] examined the effect of pyruvate on the recovery of *Salmonella* from samples that were preenriched or directly enriched in selective media. A total of six broths were compared: lactose, selenite cystine, and tetrathionate, each with and without pyruvate in a final 1% concentration. This comparison was based on an investigation by Brewer et al.,[17] who reported that the addition of 1% pyruvate to trypticase soy broth increased the recovery of heat-injured *Staphylococcus aureus* cells. Apparently, the addition of py-

ruvate aids in retaining cell viability by preventing hydrogen peroxide accumulation, which is responsible for cell death. Cox et al.[16] found a significantly higher number of positive *Salmonella* samples that were preenriched rather than directly enriched in selective media. However, the addition of pyruvate to any of the three media compared was highly inhibitory and resulted in a significant reduction of *Salmonella*.

Notwithstanding the above results, other studies have demonstrated the merits of preenriching samples of animal feeds and feed ingredients. In a collaborative study involving 12 laboratories, Edel and Kampelmacher[18] compared a standardized preenrichment method with the method of choice of each participating laboratory for determining the presence of *Salmonella* in swine feed. With one exception, the participating laboratories recovered a higher number of positive *Salmonella* samples with the standardized preenrichment method than with the nonstandardized method of choice.

Kafel[19] compared various preenrichment media (lactose broth, lactose broth with defibrinated horse blood, and lactose broth with fresh egg yolk), incubation times (24- and 48-h), temperatures (25, 37, and 43°C), and atmospheres (aerobic and anaerobic) for the recovery of *Salmonella* from fish meal. Lactose broth gave the lowest number (282) of isolations, but this recovery was improved by the addition of blood (321 isolations) or egg yolk (357 isolations). In general, there was some advantage in incubating preenrichments for 48 h (504 isolations) rather than 24 h (456 isolations). Within the range of temperatures studied, the number of *Salmonella* isolations decreased as the incubation temperature increased: 25°C (345), 37°C (324), and 43°C (291). Finally, the isolation of *Salmonella* was favored by the incubation of preenrichments under anaerobic (510 isolations) rather than aerobic (450 isolations) atmospheres.

In a comparison of methods for the isolation of *Salmonella* from bone meal, Gerichter and Sechter[20] compared the efficiency of lactose, dulcitol, or mannitol broth as preenrichments. They reported that even though preenrichment was necessary, the nature of the sugar added to the preenrichment medium was of no significance. When mannitol broth was used as the preenrichment medium, the efficiency of this medium was improved by the addition of desoxycholate to a final concentration of 1%. Grinding of the bone meal to a fine powder also increased the recovery of *Salmonella*.

The various *Salmonella*-selective media and their development and modifications have been discussed in Chapter 3. Several independent studies have demonstrated the advantage, or perhaps more appropriately, the necessity, of using more than one of these selective enrichment media for isolating *Salmonella* from animal feeds. In their analysis of animal feeds, feed ingredients, and related samples, Leistner et al.[21] demonstrated that 35% of the positive *Salmonella* samples were exclusively positive in either selenite or tetrathionate broth. Moreover, some of the serotypes demonstrated a definite preference for one or the other of the two media. In another study, Tompkin and Kueper[13] reported that 24 and 19% of the positive *Salmonella* samples of animal by-products used for feeds were exclusively positive in selenite cystine or tetrathionate broth, respectively.

The use of these selective enrichment media at various incubation temperatures has been studied by several investigators. Gabis and Silliker[22] observed no difference in the recovery of *Salmonella* from meat meal, bone meal, and fish meal enriched in selenite cystine or tetrathionate broth at 35 or 43°C. Similar results were obtained by D'Aoust et al.,[14] who reported no significant difference in the recovery of *Salmonella* from animal feeds and feed ingredients enriched at 35 or 43°C, and by Cox et al.,[15] who reported no difference between these two incubation temperatures with artificially contaminated poultry feeds.

In contrast, other investigators have demonstrated an advantage for the incubation of selective enrichment media at 43 rather than 37°C. Stott et al.[9] obtained higher recoveries of *Salmonella* organisms from meat and bone meal enriched in mannitol-selenite-brilliant green broth at 43 rather than 37°C. Smyser and Snoeyenbos[23] reported that the incubation

of selenite-brilliant green-sulfapyridine broth at 43°C was superior to incubation at 37°C for the recovery of *Salmonella* from animal by-products, animal feeds, and feed ingredients.

In comparing various incubation periods for selective enrichment media, several investigators have observed no significant differences in the recovery of *Salmonella*. Leistner et al.[21] observed no significant increase in meat and bone meals positive for *Salmonella* when selective enrichment media were incubated for 48 rather than 24 h. Working with this same type of product, Huhtanen and Naghski[24] obtained essentially the same results. Cox et al.[15] compared 24- and 48-h incubation periods for selective enrichments of poultry feeds and found no significant difference in the number of samples positive for *Salmonella*. However, in comparing 24-, 48-, and 72-h incubation periods for selenite broth enrichments of animal feeds, Harvey and Price[25] observed that the number of samples positive for *Salmonella* increased as the incubation period increased.

The final step of the isolation procedure for *Salmonella* involves streaking incubated selective enrichment media to selective plating agars. The selective agars available, the incorporation of various modifications, and the influence of variables, e.g., their preparation, have already been discussed in Chapter 3. The performance of these selective plating media used to determine the presence of *Salmonella* in animal feeds has not been studied as extensively as their performance with other products. Even so, a limited number of evaluations comparing these agars for the analysis of animal feeds, feed ingredients, and animal by-products have been made. Tompkin and Kueper[13] found a higher number of rendered animal by-products positive for *Salmonella* with *Salmonella-Shigella* agar than with brilliant green-sulfa agar. Bensink[12] reported that bismuth sulfite agar was superior to brilliant green-sulfadiazine agar for the recovery of *Salmonella* from meat and bone meal. Similar results were obtained by Gabis and Silliker,[26] who reported that bismuth sulfite agar was superior to brilliant green agar or to *Salmonella-Shigella* agar for the identification of *Salmonella* in animal feed ingredients. D'Aoust et al.,[14] however, observed that bismuth sulfite and brilliant green-sulfa agars were equally productive for recovering *Salmonella* from animal feeds and feed ingredients. Even more contradictory were the results of Cox et al.,[15] who found that bismuth sulfite agar was the least productive in a comparison of MacConkey, brilliant green-sulfa, and modified brilliant green agars for the recovery of *Salmonella* from poultry feed.[27]

V. CONCLUDING REMARKS

Attempts to break the chain of *Salmonella* contamination in poultry, swine, and other animal reservoirs have not been altogether successful. In the absence of establishing and maintaining flocks or herds free of *Salmonella* on a large-scale basis, one recourse to minimize the *Salmonella* hazard has been to rely on a program of consumer education and increased monitoring. The continuing high incidence of *Salmonella* in these animal reservoirs indicates that this program should be augmented with other measures.

The chain of *Salmonella* contamination/infection of food animals and products made from them will not be broken until the contamination of animal feeds is controlled. This control can be realized only by using highly sensitive analytical methods that can recover injured, as well as uninjured, pathogenic organisms.

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Chapter 9

IMPORTANCE AND REGULATORY IMPLICATIONS OF THE RECOVERY OF INJURED MICROORGANISMS FROM FOODS AND WATER

Wallace H. Andrews and Bibek Ray

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I. INTRODUCTION

There appears to be no shortage of methods used by various regulatory agencies for the recovery of foodborne and waterborne microorganisms having sanitary significance or posing a threat to the health of humans and other animals. Instead, the problem is one of having an abundance of microbiological procedures. This abundance, in turn, has led to a lack of method uniformity, with the resulting differences in analytical results. Despite the differences in the procedures themselves, there appears to be general agreement that some provision should be made for recovering injured organisms, if their presence is suspected, in any sample.

From a regulatory point of view, there are two primary considerations for recovering injured organisms. First, it should be determined what effect cell injury has on the expression of virulence by the pathogenic organism. Second, one must know too the extent to which the enumeration of injured cells will affect existing microbiological standards, particularly if those standards have been established with procedures that provide no resuscitation of stressed cells.

II. THE PATHOGENICITY OF INJURED CELLS

There are only a few published studies reporting the effect of injury on the expression of virulence by normally pathogenic microorganisms. With few exceptions, these reports indicate that injured pathogenic organisms are no less virulent than their uninjured counterparts. Sorrells et al.[1] investigated the effect of freezing (−75°C) and storage (−20°C) on the pathogenicity of cell suspensions of *Salmonella gallinarum*. Pathogenicity was demonstrated by observing the percentage of mortality of separate sets of 6-week-old White Plymouth Rock chicks inoculated with injured or uninjured cells. The absence of any significant difference between the effects of injured and uninjured cells on chick mortality indicates that the pathogenicity of *S. gallinarum*, under the conditions defined in this study, was not affected by freezing. It was suggested that the repair of injury could have occurred while the cells were in the intraperitoneal cavity of the chick. This repair might be expected, since replica plating of frozen and thawed cells indicated that cell injury was repairable in a nutritionally adequate environment.

The effect of a different type of stress, freeze-drying, on the stability of virulence was reported by Simon et al.[2] The virulence of three strains of *S. typhimurium* for mice did not change significantly when freeze-dried inocula had been stored for up to 2 years at 5°C. The authors, however, emphasized the importance of the type of host animal in determining pathogenicity. Relatively large inocula of freeze-dried cells were required to produce fatal injections in white Swiss mice, whereas extremely small inocula were needed to produce the same effect in another type of inbred mice.

The effect of radiation on the pathogenicity of *Salmonella* spp. organisms was investigated by Licciardello et al.[3] The chick embryo was considered more sensitive as a host than the mouse, since only a few viable cells were needed to kill the chick embryos, but a very large number of cells was necessary to kill the mice. Thus, the death of the chick embryos was most likely due to an infection, whereas the death of the mice may have been caused by intoxication. Of four *Salmonella* serotypes subjected to either 15 or 20 cycles of irradiation, the virulence of three serotypes remained unchanged, whereas a reduction in pathogenicity occurred with the fourth serotype. Working with *Pasteurella pestis*, Leshkovich[4] observed a marked decrease in the pathogenicity of cells exposed to X-ray treatment.

The occurrence and pathogenicity of sublethally injured, waterborne enteric pathogens have been studied to a great extent in recent years by several workers (see Table 1). Walsh and Bissonnette[5] showed by *in vitro* studies that enterotoxigenic *Escherichia coli*, injured

Table 1
INFLUENCE OF SUBLETHAL INJURY ON VIRULENCE OF SEVERAL PATHOGENS[a]

Pathogen	Type of stress	Study method	Observations
Salmonella gallinarum	Freezing	*In vivo* LD_{50} in chicks	No loss of virulence
Staphylococcus aureus	Heat	*In vitro* synthesis of toxin	Resumed after repair
	Freeze-drying	*In vitro* synthesis of toxin	Resumed after repair
Enterotoxigenic *Escherichia coli*	Chlorine	*In vitro* adherence to leukocytes	Reduced adherence
		In vitro adherence to intestinal epithelial cells	Reduced adherence and loss of invasiveness
	Copper	*In vitro* synthesis of toxin	Slow initial rate
	Chlorine or copper	*In vivo* ileal loop reaction in mice	Resuscitation followed by virulence
Salmonella typhimurium	Chlorine	*In vitro* adherence to intestinal epithelial cells	Reduced adherence and loss of invasiveness
Yersinia enterocolitica	Chlorine	*In vivo* LD_{50} in mice	Reduced virulence
		In vitro invasiveness in HeLa cells	Reduced invasiveness
	Copper	*In vivo* LD_{50} in mice	Reduced virulence
	Chlorine or copper	*In vivo* feeding to mice	Resuscitation followed by virulence in small intestine

[a] Results are from the studies listed in the references.

by sublethal doses of chlorine, had reduced attachment to human peripheral leukocytes. Electron micrographic studies suggested that the reduced adhesive ability of the injured cells was due to the loss of surface structures resulting from sublethal chlorination. They implied that the chlorine-injured cells will not be detected by the *in vitro* adherence methods and also, because of their reduced adherence to host cells, they might not be able to cause disease. Later, LeChevallier et al.[6] studied the pathogenicity of chlorine-injured cells of enterotoxigenic *E. coli, Salmonella typhimurium, Shigella flexneri, S. sonnei, S. dysenteriae*, and *Yersinia enterocolitica* strains. Injured *Y. enterocolitica* strains showed 20 times less virulence than uninjured cells in the mouse LD_{50} assay (3300 vs. 160 cfu, respectively). *In vitro* studies revealed that this reduced virulence was not due to reduced attachment to intestinal epithelial cells, but to a loss of invasiveness. In contrast, the reduced pathogenicity of injured *Salmonella typhimurium* and enterotoxigenic *E. coli* was due to their inability to attach to epithelial cells. Singh and McFeters[7] reported that enterotoxigenic *E. coli* cells were injured by copper in concentrations that could be present in drinking water. *In vitro* studies indicated that both growth and heat-stable enterotoxin production by the injured cells, as compared with the uninjured cells, were initially slow. However, maximum heat-stable enterotoxin levels in injured cultures were comparable to those produced by uninjured controls, suggesting that the enterotoxigenic potential of copper-injured cells was fully retained. The slower initial rate could be due to the extended lag phase necessary for the resuscitation of the injured cells. These observations are inconsistent with the reports of Collins-Thompson et al.[8] and Fung and Vanden Bosch.[9] They reported that foodborne *Staphylococcus aureus* cells, which were injured by either heat or freeze-drying, retained their ability to synthesize enterotoxins; synthesis occurred during the growth phase following resuscitation. An *in vitro* study with enteroinvasive as well as enterotoxigenic strains of *E.*

coli, injured by sublethal doses of chlorine and copper, was reported by Singh et al.[10] Injured cells were capable of repair within 3 to 4 h in the ligated ileal loops of anesthetized mice. The enterotoxigenic activity in rabbit ileal loops was less for copper-injured cells than for chlorine-injured cells. Their results showed that injured pathogenic *E. coli* were able to resuscitate in the small intestine and produce their enterotoxigenic activity. These observations are similar to the observations of Sorrells et al.,[1] who reported that freeze-injured *Salmonella gallinarum* cells were as pathogenic as the uninjured cells to chicks.

Copper-injured *Y. enterocolitica* cells,[11] like chlorine-injured cells, also had higher LD_{50} values in mice than did uninjured cells (2700 CFU vs. 150 CFU, respectively). *In vivo* studies showed that the reduced virulence of the injured cells, as compared to uninjured cells, was due to the rapid clearance of the injured cells from the blood of mice and their accumulation in the liver of hosts due to an increased interaction of the injured cells with a hepatic lectin(s). In contrast, chlorine-injured *Y. enterocolitica* cells had reduced virulence, even when they retained their ability to attach to tissue culture cells.[12] The reduced virulence was due to the reduced invasiveness of chlorine-injured cells resulting from some changes in their surface structure(s), which normally were necessary for triggering engulfment by the epithelial cells. Studies of mice that had been fed both copper- and chlorine-injured *Y. enterocolitica* cells showed that the gastric pH was partially lethal to the cells, which could be neutralized by feeding bicarbonate. However, some injured cells reached the small intestine, where they resuscitated within 3 to 4 h and produced virulence effects.[13]

It is evident from these studies that injured enteropathogens present in water supply or food systems can show a temporary reduction or inability to express pathogenicity. However, in a nonselective *in vitro* environment or in a suitable *in vivo* system, resuscitation takes place, followed by growth and the expression of pathogenic properties. Injured pathogens, when ingested through water or food, can survive in the stomach, and in the small intestine can resuscitate, multiply, and become virulent.

The regulatory, as well as the public health, significance of the findings above are evident. Injured pathogenic organisms may survive for considerable periods of time in processed foods and in water. Given the right environment, these injured organisms could undergo resuscitation and readily express their virulence. One such environment may be the intestinal tract of the human host. The stomach, with its hydrochloric acid-pH environment, may act as a barrier to these ingested organisms in many instances. Under certain conditions, however, the natural defense mechanism of the stomach may be compromised. Individuals who have had gastrectomies, who are achloric, or who are taking antacids appear to be especially susceptible to infection by *Salmonella* spp.,[14-17] *Vibrio cholerae*,[18] or *Giardia lamblia*.[18] Moreover, one possible explanation for the greater susceptibility of infants and the elderly to salmonellosis may be due to the fact that children less than 2 months of age produce little hydrochloric acid,[19] while the incidence of achlorhydria is greater for persons over 60 years of age.[20]

In addition to receiving protection because of compromised clinical conditions of the human host discussed above, ingested pathogens may be protected by the food bolus itself as it passes through the stomach and into the intestinal tract.[21] This concept seems especially plausible when one considers the low infective dose needed to initiate symptoms of salmonellosis in humans.

The earliest studies that were conducted to experimentally induce salmonellosis in humans involved the feeding of adult male prisoners large doses of *Salmonella* spp. organisms. It was observed that 10^5 to 10^{10} organisms were able to initiate clinical symptoms. If this were actually the lowest number of *Salmonella* spp. needed to produce illness in humans, then it would be difficult to justify the zero tolerance enforced by many regulatory agencies for this pathogen. These earlier studies were flawed, however, in that the minimum infective dose was not determined, the same subjects were used in repeated testing, and too few volunteers were used.

More recently, Blaser and Newman[22] reviewed outbreaks of human salmonellosis. The estimated number of ingested organisms for a variety of foods responsible for salmonellosis outbreaks was much lower than that reported in the earlier volunteer studies. The most probable number of *Salmonella* spp. organisms per gram in various processed foods involved in outbreaks was calculated as follows: pancreatin powder ($4.4 \times 10^1 - 2 \times 10^2$), carmine dye capsules ($1.5 \times 10^4 - 6 \times 10^4$), chocolate balls ($1 \times 10^2 - 2.5 \times 10^2$), cheddar cheese ($1 \times 10^2 - 5 \times 10^2$), goat cheese ($1.5 \times 10^5$), and imitation ice cream (1.1×10^4). With these types of foods, it can reasonably be assumed that a large segment of the population of surviving organisms would have been injured due to exposure to one or more kinds of stress: drying (pancreatin and carmine dye capsules), heating and subsequent storage at low water activity (chocolate), heating and exposure to various enzymes (cheddar cheese and goat cheese), and freezing (imitation ice cream). Nonetheless, in these instances, *Salmonella* spp. organisms were able to survive the exposure to one or more kinds of stress during processing and storage, survive exposure to the hostile environment in the stomach, reach the intestine where favorable conditions allowed the pathogenic organisms to resuscitate, if necessary, and multiply to the levels needed to initiate clinical symptoms. The above documented cases thus provide sufficient justification for the lack of a regulatory distinction being made between the significance of injured and that of uninjured pathogenic organisms.

III. THE ROLE OF INJURED MICROORGANISMS IN ESTABLISHING MICROBIOLOGICAL STANDARDS

According to Ray,[23] damage to the surface cellular structures (lipopolysaccharide in gram-negative bacteria and teichoic acid in gram-positive bacteria) and to the cytoplasmic membrane, as manifested by impaired permeability and increased sensitivity to many compounds, is perhaps the single most important reason why injured cells are not detected by many microbiological media. Injured cells are no longer able to prevent molecules of selective compounds from entering the cells, and the normally resistant cells become sensitive to these selective agents. Moreover, the selective environment offers no opportunity for cellular repair, and the injured cells are unable to multiply and are thus rendered undetectable by selective media. Once this damage has been repaired and the resistance to selective agents has been reestablished, the still-injured cells would be capable of repairing other cellular damages. This phenomenon forms the basis for the definition most commonly used to distinguish injured from uninjured cells. Injured cells are considered as those which have the ability to multiply on a nonselective but not on a selective medium, whereas uninjured cells are regarded as those which have the ability to multiply on selective, as well as nonselective, media. The major implication of this distinction is that, unless there is some provision in a method for the resuscitation of injured organisms in situations where their presence is expected, they will not be detected and/or enumerated. The situation becomes even more involved when it is considered that in any series of resuscitative methods for enumerating a particular analyte, it is not improbable that various degrees of repair are furnished by each of these methods. Thus, different percentages of stressed organisms will be recovered by the various methods. From a regulatory point of view, this variation in analytical results would be unacceptable.

In a study of methods for recovering injured cells, Ray[23] used the terms "liquid repair" and "solid repair". With liquid-repair methods, the food sample is mixed with a nonselective medium at a 1:10 sample to broth ratio and incubated to facilitate resuscitation. The time and temperature of incubation is dependent upon the analyte being recovered. This method can also be used to enumerate pathogens and indicator organisms by streaking the incubated broth media onto a selective agar. The major objection to the use of this method for regulatory

analyses is that the increase in counts could be due not only to the resuscitation of injured cells, but also to the multiplication of uninjured cells. This problem is overcome by the use of solid-repair methods. With these methods, the sample is mixed or blended with phosphate buffer as a diluent, and a pour plate is made with 5 ml of trypticase soy agar or plate count agar. The cells are rapidly immobilized by solidification of the medium. Thus, any cell multiplication of uninjured cells will not affect the final count. At the end of the repair phase (usually 1 to 2 h), the plates are overlaid with 10 to 12 ml of the appropriate selective plating medium and subsequently incubated under conditions appropriate for the analyte desired. During the incubation period, the ingredients, selective as well as nonselective, from the selective medium diffuse into the nonselective medium and create a selective environment. Since the injured cells have already undergone repair, they are not affected by the selectivity of the medium and thus are able to multiply and form colonies.

The problem that has evolved for many regulatory agencies is what to do when a particular method has been used to establish microbiological standards or guidelines and, subsequently, an improved method, often a resuscitative one, has been developed which provides for a higher recovery of organisms than does the existing method. This dilemma has confronted regulatory agencies for many years. It would be scientifically unrealistic to presuppose that all existing methods used to establish microbiological standards are optimal. Instead, it would seem reasonable to acknowledge the inevitability of a "better" method always emerging. In a discussion of the implications of the detection of stressed microorganisms on regulatory monitoring, Read[24] emphasized that if any method providing resuscitation of injured cells is adopted for regulatory purposes, then any microbiological standard that was established using the existing method being replaced must be considered. For example, a resuscitative method for coliforms in dairy foods might require a revision in the already established standards. However, the problem of concurrent change in the microbiological standards with method revision for the analysis of nondairy foods would be a problem of a lesser magnitude because of the relatively smaller number of microbiological criteria that exist.

IV. CONCLUDING REMARKS

From a regulatory point of view, the recovery of injured pathogens, indicator organisms, and other microbiological analytes may be as potentially significant as the recovery of their uninjured counterparts. Methods for the detection and/or enumeration of various microorganisms should therefore be developed with this thesis in mind.

Since the presence of any pathogen is unacceptable at any level in foods and drinking water intended for human consumption, the use of methods resulting in qualitative, or presence/absence, data is usually sufficient for regulatory purposes. Thus, any resuscitative method, proposed as an alternative or replacement to an existing regulatory method for detecting pathogenic organisms, must provide at least equivalent or, preferably, higher recovery results.

Methods for detecting nonpathogens such as indicator organisms, as well as for other organisms reflecting the sanitary quality of foods and water, usually provide quantitative data which allow the analyst to determine whether or not microbiological quality standards are being met. If a proposed, resuscitative method does not provide microbiologically equivalent results to those given by an existing method for determining regulatory compliance with existing quality standards, then the most likely option would be to revise those standards or guidelines.

In any event, the greater the degree of accountability of all the viable organisms, injured and uninjured alike, present in a food or water sample, then the more valid will be the results of the microbiological analysis. Based on this information, a valid regulatory decision can be made regarding the most appropriate disposition of any food or water sample.

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