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INJURED INDEX *and* PATHOGENIC BACTERIA:

Occurrence and Detection in Foods, Water and Feeds

Bibek Ray

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DEDICATION

To Purnima, Purbita, and Ranjan for their patience

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

Bibek Ray, Ph.D., is a professor of Food Microbiology in the Department of Animal Science, University of Wyoming, Laramie.

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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Prof. Ray has authored more than 50 scientific papers, reviews, and book chapters. He acted as a chapter chairman on "Repair and Detection of Injured Microorganisms" in the 2nd edition of *Compendium of Methods for the Microbiological Examination of Food* published by the American Public Health Association.

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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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