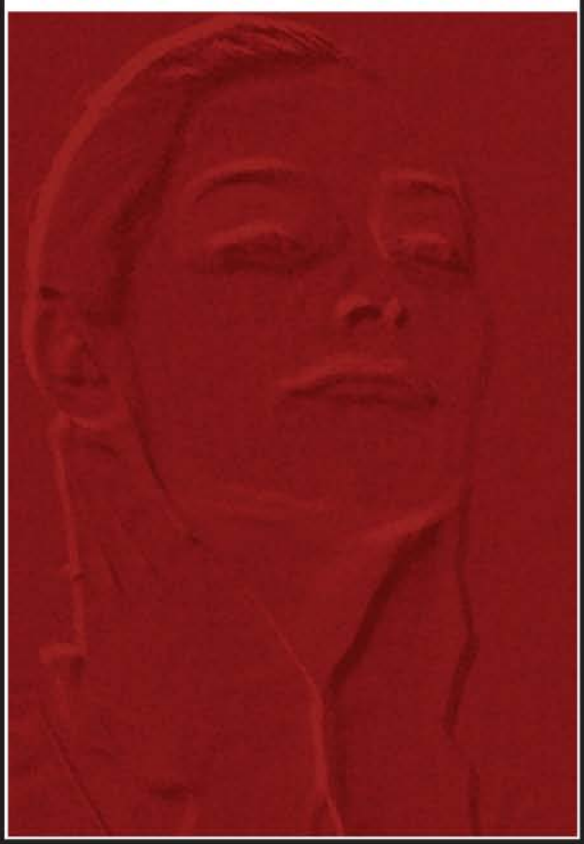
# COSMETIC MICROBIOLOGY

## *A Practical Approach*

SECOND EDITION



Edited by

Philip A. Geis, Ph.D.

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Taylor & Francis
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New York London

Taylor & Francis is an imprint of the
Taylor & Francis Group, an informa business

Published in 2006 by
Taylor & Francis Group
270 Madison Avenue
New York, NY 10016


Printed in the United States of America on acid-free paper
10 9 8 7 6 5 4 3 2 1

International Standard Book Number-10: 0-8493-1453-4 (Hardcover)
International Standard Book Number-13: 978-0-8493-1453-7 (Hardcover)
Library of Congress Card Number 2005052137



**Library of Congress Cataloging-in-Publication Data**

Cosmetic microbiology : a practical approach / edited by Philip A. Geis.-- 2nd ed.
p. cm.
Includes bibliographical references and index.
ISBN 0-8493-1453-4
1. Cosmetics--Microbiology.

QR53.5.C76C67 2006
668'.55--dc22 2005052137

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# *Preface*

Cosmetics are unique products, as diverse as foods without the practical limits of controlled shelf lives; they overlap with drugs but without the near-sterile manufacturing systems enjoyed by that category of product. The microbiology of cosmetics is also a unique and arcane science and practice that, unlike food or drug microbiology, is not supported by established academic programs or a significant body of publication. Rather it is empirical — learned through experience with principles and practices rarely documented in the literature. The field has few mentors beyond a small fraternity whose secrets are rarely shared.

The purpose of this text is to share the unique knowledge of a small group of cosmetic microbiologists and cover all aspects that are critical to providing consumers with microbiologically safe products in a focused discussion that allows immediate application. The first edition of this text was well received, and we have attempted to strengthen this second edition with expanded discussions of manufacturing hygiene and preservation. We have also expanded the regulatory review section to include a global perspective and cover current safety concerns for preservatives consistent with the most recent data.

# *Acknowledgments*

The Editor thanks the following individuals and institutions whose contributions and support made this book possible:

Dr. Dan Brannan, my colleague, coauthor and dear friend of many years, who edited the original version of this text and gave me the opportunity to edit this second edition.

Contributors to this second edition who took time from their busy scientific and technical careers to document and share their unique knowledge and experience. Their efforts will guide readers to the production of cosmetics that are microbiologically safe for consumer use.

The Procter & Gamble Company for providing decades of practical experience as well as immediate time and resources needed for this effort.

Lastly, the U.S. Army's 45th Field Hospital that, decades ago, entrusted to me its clinical microbiology laboratory responsibilities and initiated a career in applied microbiology.

# *Editor*

**Philip A. Geis, Ph.D.,** is a native Texan who earned bachelor and doctor of philosophy degrees in microbiology from the University of Texas. He is currently a section head with The Procter & Gamble Company (P&G) in Cincinnati, Ohio, and has responsibilities for global microbiology within its Beauty Business Unit.

Dr. Geis joined P&G in 1981 as a divisional microbiologist for the development of preservative systems and disinfectants and the study of household and skin microbial ecologies. His subsequent work focused on microbiological method development, design and maintenance of sanitary manufacturing systems, and microbial ecology associated with underarm odor, dandruff, toilet soil, and bathroom mildew. Responsible for the technical efforts of multiple operations around the globe, Dr. Geis has acquired unique experience involving diverse regulatory, manufacturing, and product quality realities. His experience and responsibilities with P&G have also included product development and formulation as well as regulatory and environmental affairs.

Dr. Geis publishes and lectures on microbiological and environmental subjects, served as an associate editor for the *Society for Industrial Microbiology News*, and represented the technical interests of P&G to professional organizations, regulatory bodies, and scientific societies. He is a member of MENSA, the American Society for Microbiology (ASM), and the Society for Industrial Microbiology (SIM). He is also a trustee of the Ohio Academy of Science and vice president of ASM's Ohio branch and works to promote science as an ad hoc reviewer for scientific journals and academic programs of ASM, Ohio EPA, and a number of state science standards academic programs.

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***Janet C. Curry, Daniel K. Brannan, with Philip A. Geis***

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## *Introduction*

Humans have a basic need to change their appearance. The vastness of today's cosmetics and toiletries industry clearly indicates this widespread and basic need for cosmetics. Perhaps this need arises because cosmetics allow us to make ourselves unique for rituals or societal reasons. We apparently have a primal need for cosmetics to provide for our well-being. Perhaps cosmetics serve as cures for the disease of being someone we prefer not to be. This chapter covers the history of cosmetics and of microbiology

and how they merged into the new field of cosmetic microbiology. The intent of this chapter is to provide insight into this unique field of cosmetic microbiology.*

## *The first cosmetics*

Archaeological evidence suggests that we have been decorating our bodies for nearly a half-million years. Archaeologists discovered sharpened red ocher sticks, presumably for coloring the body, at one *Homo erectus* site.[1] Some paleoanthropologists believe that the transition from *H. erectus* to *H. sapiens* began about this time. In fact, considerable discussion focuses on whether the mixed-trait fossils of *H. erectus* from a half million to two million years ago should be considered a separate species or a variety of modern man (e.g., *Homo sapiens* var. *erectus*).[2] Most anthropologists agree that humans of the last 200,000 years fall completely into the *H. sapiens* species even if some were of the Neanderthal variety.

*Homo erectus* was a hominid that ventured from the forest onto the African savannah. According to anthropologists, this hominid shared many characteristics with us. Current scientific thought is that *Homo erectus* had twice the brain size of other hominids, a body adapted to upright posture, sweat glands, and less hair. *H. erectus* also developed a workable social life, providing an advantage over other hominids. The men were hunters who first used stones and clubs as tools and weapons. Later on, they used chipped stones and spears. The women were gatherers who took care of the children while sharing their food at day's end. They moved around as family units and joined with other families as needed. The adventurous families moved from the tropics to the colder climates of West Asia, Europe, India, and China during several ice ages. Clothing and shelter became necessary. *H. erectus* then captured fire, worshipped it, and kept it alive for generations. Fire was very important because it provided fuel for warmth, cooking, and hardening of wooden spear tips.

The life of *H. erectus* was difficult. Nature was frightening. Magic, wizardry, supernatural pursuits, and ritualistic ceremonies were popular male pastimes. Those mysterious red clay sticks and charcoal mentioned earlier may have been used for body painting — perhaps a component of secret male rites performed deep within caves. Alternatively, the sticks and charcoal may have been used to enhance female sexuality. Other species of female primates develop reddened and swollen breasts or genitals when occasionally sexually receptive. Human females who are capable of being continually sexually receptive have permanently swollen buttocks, breasts, and enlarged pelvises without the reddening. Perhaps the ocher was used to enhance sexuality by reddening those body parts.[1]

* For more detail about the field of cosmetic microbiology, see Brannan, D.K., Cosmetic microbiology, in *Encyclopedia of Microbiology*, 2nd ed., vol. 1, Lederberg, J., Ed., 2000, Academic Press, Inc., San Diego, 887–898.

Even after *H. erectus* disappeared from the record, the red ocher sticks remained popular throughout succeeding Stone Ages. The Neanderthals, a variety of *Homo sapiens*, lived from 30,000 to 125,000 years ago. They painted their dead with red ocher before burial. This practice may have been done to bring the blush of life back to the body. *Homo sapiens sapiens* (e.g., Cro-Magnon) appeared around 40,000 years ago and had a skull shape more conducive to brain development. Cosmetic use became widespread. This enlarged use of cosmetics can be traced back to the urban civilizations of southern Iraq and Egypt where both men and women painted kohl around their eyes. This made the eyes look larger; they may have also thought it protected them from the "evil eye." We now know that kohl is made of ground lead sulfide or antimony sulfide and constitutes a safety risk![3]

Cro-Magnons were modern humans. Cro-Magnons, like modern humans, had round heads and high brows. They moved quickly throughout the world and replaced the Neanderthals. Unopposed, they spent the next 40,000 years evolving the social structures now enjoyed by modern humans.

The Cro-Magnons left a rich legacy of cave art, animal carvings, baked clay sculptures, and the ever-present red clay sticks. They lived together in clans or tribes and trained and bred animals to serve as beasts of burden and pets. The Cro-Magnons spun natural fibers into cord for baskets, bows and arrows, fishing lines, and animal snares. When the last Ice Age ended nearly 10,000 years ago, they no longer moved from place to place in search of food. The Paleolithic Age ended and the nomadic family life style ceased. The construction of villages and towns began and with it came the need for specialized occupations. The nomadic culture was replaced by complex communal cultures.

The start of farming marked the beginning of what we call the New Stone (or Neolithic) Age that developed at different times in different parts of the world. It started in Southwest Asia nearly 10,000 years ago. In Mexico and South America, it began as late as 1500 B.C.E. The discovery of copper and tin heralded the Bronze Age that also occurred at different times in different places; the earliest Bronze Age developments in Southwest Asia and Egypt appeared around 3500 B.C.E.

The need for highly specialized occupations in these emerging cultures may have led to the development of professional classes and upper and lower social classes. It may even have led to territorial wars. Civilization's arrival was not very civil. Experts define civilization as the time when man developed a written language. The first written language was used about 3200 B.C.E. in Sumer, an area in present-day Iraq between the Tigris and Euphrates Rivers. The people there developed cuneiform symbols carved into clay tablets. This was near the site of the Jewish story of the creation of Adam and Eve in Eden. According to the story, once their eyes were opened and they knew what they looked like, they began adorning themselves. This story emphasizes the long history of the human desire to alter appearance.

First settled around 4500 B.C.E., Sumer became a land of cities within about 1,000 years. The Sumerians were hardworking, cooperative people who refined farm practices to include large networks of irrigation canals. They had more food than they needed. As a result, they developed a brisk trading network with Indian and Egyptian merchants and this trading created a need for a written language.

By 2700 B.C.E., the ruling class of Egyptians in the lush Nile Valley enjoyed luxurious and leisurely lives at the expense of slaves. Their cities were elaborate. They built great pyramids to house the royal dead. Upper and lower classes were well defined. The wealthy wore sheer linens instead of sheepskins. Their hieroglyphic language was artistically beautiful and they wrote elegantly on papyrus instead of clay tablets. They remain famous, even today, for their cosmetics, hair dyes, wigs, and other adornments.

Although over time cosmetics were refined into luxuries, the lower classes had no leisure time for such frivolities. We also could view this progression as the emergence of the basic need of some humans to separate themselves from the lower classes by changing their appearance. Elegant kohl, henna, pomades, and hair dyes were used by wealthy Egyptian women 5,000 years ago. Apparently painting our skin is a part of human nature whether as a ritual, to enhance sexuality, or to advertise social status.

If red ocher was used 500,000 years ago as our first cosmetic and cosmetic use has become a human proclivity, then cosmetics have been with us throughout the development of culture and it appears that not much has changed. Women still signal reproductive value by using make-up to signal youth — our desire to rid ourselves of wrinkles is rampant — and health. Both rouges and lipsticks allows the blush of sexual arousal to be mimicked, thus stimulating sexual interest.[4] We still use plenty of cosmetics on corpses and display them under rose-colored lights at funeral homes. And, of course, cosmetics are costly, thus indirectly providing clues as to social status.

## *The first bacteria*

Some scientists think that bacteria were the first living things on Earth. According to current scientific thinking, they either came from reducing organic chemicals in watery soups or were bound to crystalline clay surfaces about 3.5 billion years ago. From that point, they evolved into different forms and adapted to whatever environment came their way. They experienced lots of changes. Earth cooled, land masses and oceans formed, and algae produced oxygen. Bacteria continued adapting as they formed consortia of organisms that acted as if they were single entities. Over time, these consortia, according to the endosymbiont theory, developed into plants, animals, and eventually humans.[5]

Bacteria still control the Earth as we know it. They are in and on every environmental niche where water is liquid on Earth. They even inhabit humans. Some are disease-producing, but most are not. Some are even

needed for our existence. If all bacteria were suddenly to die, all other living creatures would also die. However, if all the plants and animals were to die, the bacteria would still survive and adapt to their new environment to start a new cycle. This is a humbling concept and is well worth remembering as we approach the short 300-year history of microbiology, and the even shorter 70-year history of cosmetic microbiology.

## *Microbiology as a scientific discipline*

In the 13th century, Roger Bacon, an English friar, claimed that "invisible creatures" caused disease. It was not until the late 17th century that a wealthy linen merchant, Anton van Leeuwenhoek, developed the microscope and revealed the existence of an entire universe of tiny living creatures. He was the first to see microorganisms. Because of his careful drawings of these microorganisms, most microbiologists consider him the Father of Microbiology.

About 100 years later (1798), a country doctor named Edward Jenner noticed that farmers and milkmaids in contact with cows infected with cowpox were less apt to contract smallpox. Jenner reasoned that exposure to cowpox would strengthen the body's defenses. He then made history by rubbing cowpox exudate into the skin of an 8-year old boy, proving that immunization worked. As a result, he laid the foundations of modern-day immunology. Another 82 years passed before Louis Pasteur improved the vaccination process (1880). He used attenuated cultures in his work with chicken cholera. He named the process vaccination based on vaca, the Latin word for cow to honor Jenner's use of cowpox. Soon after this, Theobald Smith, an American, improved the process by using heat-killed cultures that remain the bases of modern vaccine prophylaxis.

Microbiology was primarily a curiosity for 200 years after van Leeuwenhoek's discovery and did not explode into a scientific discipline until the late 1800s. This surge in interest was mainly due to the spontaneous generation controversy and Koch's postulates for infectious disease. In the 20 years from 1880 to 1900 — the Golden Era of microbiology — scientists discovered causative organisms of almost every important disease. They isolated, described, and controlled the major bacterial pathogens and even a few viruses. Thus began the taming of killers such as anthrax, diphtheria, tetanus, typhoid fever, yellow fever, rabies, syphilis, and tuberculosis.

The major scientists of this period were Pasteur (France), Robert Koch (Germany), and Joseph Lister (England), who attracted students and scientists from all over the world. Their collective accomplishments are staggering. Within 20 years, microbiologists began understanding bacteria well enough to use and control them. American students who worked in the European and English laboratories of the masters brought microbiology to the United States. In 1899, the Society of American Bacteriologists (SAB) was founded. It is now known as the American Society of Microbiology (ASM), and is the largest biological scientific organization in the world.

The next 20 years served as a time of maturation. Many investigators recognized the need to bring order out of the chaos generated by the earlier activity. For example, in 1914 the Digestive Ferments Company (now known as Difco) was established. This company introduced dehydrated media, which saved long hours of preparation time and also provided much-needed uniformity to culturing conditions.

Uniform methods and taxonomic descriptions became standards. The major contributors were the American Public Health Association (APHA) and SAB. The APHA published the first editions of *Standard Methods for Water* and *Standard Methods for Dairy Products* in the early 1900s. The first edition of *Recommended Methods for Microbiological Examination of Foods* was published in 1958. The latest editions are included in the literature cited section below; however, be aware that these publications are frequently revised.[6–8]

Other essential texts needed by today's cosmetics and pharmaceutical microbiologists are the *U.S. Pharmacopeia* (XXIII, 1995)[9] for drugs, the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (1992),[10] and the Cosmetic, Toiletry, and Fragrance Association (CTFA) *Technical Guidelines* (1993)[11] for cosmetics and toiletries. Without uniformity of media, methods, and data expression, we could not understand or reproduce test results. We owe a debt of gratitude to those before us who brought order out of chaos.

## *Cosmetic microbiology*

The fields of cosmetics and microbiology did not meet much before the 1930s. For this reason, we will start this discussion with the 1930s and proceed by decades.

### *1930 to 1939*

The cosmetics industry turned the economic disaster of the stock market crash and World War II in Europe to its advantage. Throughout history, cosmetics were expensive luxuries and the province of the wealthy. When Wall Street toppled, many cosmetic companies failed, and many others such as Merle Norman, Almay, Revlon, Clairol, Shulton, and Avon took their place. The new companies rapidly adjusted to the times by introducing increased advertising, new marketing methods, and unique packaging that allowed them to supply their products in smaller, more affordable sizes. Because the industry was dependent on imported raw materials unavailable due to the war, they conducted intensive research to find less expensive synthetics. This led to the development of many new and less expensive products.

Obvious proof that cosmetics had arrived was the revision in 1938 of the old Food and Drug Law of 1906. It became the Food, Drug, and Cosmetic Act and the FDA established in 1931 became its enforcement arm. The worst

microbiological problem then was visible mold growth; this led to research on preservatives. The parabens became well known for their antifungal activity and became the preservatives of choice.

A unique discovery during the mid-1930s was Reneé Dubos' finding of a bacterial enzyme that dissolved the polysaccharide coats of Type III pneumococcus organisms. He isolated the coat material and used it to enrich a soil sample from a New Jersey cranberry bog. This enrichment selected one organism able to use the polysaccharide. Within 3 years, Dubos isolated the enzyme responsible and proved that it cured infected animals. The enzyme removed the polysaccharide capsules so that pneumococci were no longer protected from the human body's phagocytic defense system. Unfortunately, the shift to antibiotics (needed in World War II) eclipsed further development of the enzyme.

What is fascinating about Dubos' discovery is that we are just now returning to his concept with the use of antimicrobial peptides. Could enzymes serve as preservatives for cosmetic products? *Pseudomonas, Klebsiella*, and *Enterobacter* species also have protective polysaccharide coats. By removing the polysaccharide coat, the organisms might die before sticking to surfaces and adapting to products.

The 1930s also saw major microbiological advances in the medical field. The introduction of the sulfa drugs was the first advance in chemotherapy since Ehrlich's arsenical treatment of syphilis 50 years earlier. Dubos also discovered tyrothricin, a mixture of two polypeptides — tyrocidine and gramicidin — from the *Bacillus brevis* soil microorganism. Tyrothricin was the first combination antibiotic produced commercially and employed clinically. Gramicidin proved to be the most useful. Dubos used it to cure his first patient, Elsie, the Borden cow, after she contracted mastitis at the 1939 World's Fair in New York City. The 1930s represent the dawn of the antibiotic era.

## *1940 to 1949*

World War II wreaked havoc on the battlefields at a cost of about 10 million lives between 1939 and 1945. As often happens, however, disasters create needs. Science, medicine, and technology took giant strides forward. The search for antibiotics continued with Waksman's discovery of streptomycin in 1942. Avery's discovery of deoxyribonucleic acid (DNA) as the molecular basis of heredity in 1944 had the most far-reaching significance.

As in World War I (1914–1918), most people perceived the cosmetic industry as nonessential. Raw materials were needed for the war effort or they were completely unavailable. In response to this shortage, new materials were developed and the industry underwent another healthy period of change.

The cosmetic business continued to flourish, primarily because women went to work in factories. They became wage earners and their spending habits included cosmetics. Thus, an important economic change ensued after cosmetics were perceived as staples rather than luxuries. The industry began

to encounter some of the problems of high volume manufacturing: isolated cases of spoilage and toxicity began to appear.

One reaction to this was the establishment of a Scientific Section by the CTFA (then known as the Toilet Goods Association or TGA) in 1943. M. DeNavarre published the first edition of *The Chemistry and Manufacture of Cosmetics* in 1941 and founded the Society of Cosmetic Chemists in 1945. These measures proved to the industry that scientific discussions of formulating technicalities, chemical compatibilities, and preservatives were educational, not proprietary. This was no small accomplishment, considering the thickness of the secrecy cloak enveloping the industry then.

Microbiology became more important than ever to cosmetics during the 1940s. This increased interest arose from a more scientific approach to cosmetic manufacturing and was also due to growing availabilities of test organisms. Mold spoilage remained the prime microbiological product defect to avoid. Parabens remained the preservatives of choice, but the quest for new ones continued. For the first time, preservative test procedures included bacteria.

### *1950 to 1959*

The Korean War (1950–1953) marred the early 1950s, but the late 1940s and early 1950s were times of prosperity, accented by the arrival of television. Television greatly affected communication generally and advertising of cosmetics particularly. The 1950s also became the decade of antimicrobial overuse that continues today. Antibiotics worked miracles during the war. Physicians prescribed them for just about everything. Infections were relics of the past — or so we thought then. After the appearance of antibiotic-resistant *Staphylococcus aureus* in hospitals, the drug industry discovered several alternatives and the antibiotic euphoria continued unabated.

The same euphoria occurred when antibacterial compounds were included in toiletries. Chemists discovered halogenated bisphenolics, salicylanilides, carbanilides, and pyridinethiones, which led to a new generation of products intended to kill germs. New antibacterial skin creams, toothpastes, mouthwashes, deodorants, deodorant soaps, antidandruff shampoos, and surgical scrubs appeared on the market. These products contained active ingredients for reducing or altering the bacterial flora on skin or in the mouth. Sometimes the antimicrobial was hidden in a compound as a preservative instead of as an active ingredient. Many people still consider such products well preserved simply because they contain antibacterial compounds. Not even the FDA's annual list of antibacterials used in products differentiates active ingredients from preservatives.

We list them separately because we define them by the target organisms they affect. Antimicrobials that are active ingredients primarily kill Gram-positive flora on body surfaces. Antimicrobials that are preservatives primarily kill molds, yeasts, and Gram-negative flora in products. While a product may

contain a biocide, one should never expect the active antibacterial to act also as a preservative or vice versa.

As a result of efficacy testing of new antimicrobial products and increased attention to preservatives, microbiologists of the 1950s were hyperactive. Microbiologists conducted studies on biocides used as antimicrobials and preservatives to determine their spectra of activities and the concentrations needed. Efficacy studies included various bacteriostatic, bactericidal, substantivity, and in-use panel tests. Microbiologists developed most of these tests specifically to support claims. Preservative studies tested incompatibilities with product ingredients along with the effects of pH, temperature, packaging, and accelerated storage conditions. There were as many preservative challenge test protocols as there were companies.

By the end of the 1950s, both industry and medicine were more aware of the growing problem of bacterial resistance. Up to this point, most control of pathogens was achieved by using antibiotics against Gram-positive cocci. The key organisms to kill were the pneumococci (*Diplococcus pneumoniae*, now called *Streptococcus pneumoniae*), β-hemolytic streptococci (*Streptococcus pyogenes*), and staphylococci (*Staphylococcus aureus*). By the end of the decade, control of Gram-negative bacilli also became important. Both industry and the medical field began noticing increased problems with *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and most importantly, *Pseudomonas* organisms.

## *1960 to 1969*

Many remember this period as the Vietnam era and a time of social unrest. It also produced a back-to-nature movement. The accent was on safe products with natural ingredients. Of course, no one ever defined "natural." Environmentalists, consumerists, and female activists profoundly influenced the cosmetic industry. Cosmetics were always the least regulated of all consumer products. During the 1960s, they became the targets of various special interest groups. Government agencies also became more interested in them. The industry had to shed its last vestiges of secrecy. It was forced to open its doors voluntarily or face forced compliance with potentially unfair or impractical regulations. The FDA recognized that enforcement of regulations would be difficult without industry cooperation. Therefore, the CTFA and FDA established a liaison and agreed on a unique program of industry self-regulation.

In 1969, a report from Sweden by Professor Kallings showed contamination in nonsterile drugs and cosmetics. He was one of the first to recognize possible microbiological safety problems in the cosmetics industry. An FDA report on a survey of cosmetics from the New York area soon followed. Both reports showed a frequency of contamination of about 25% in cosmetics and a high incidence of Gram-negative bacteria. CTFA quickly established microbiology and quality assurance committees to look into the situation and develop technical guidelines for the industry.

During this period, clinical microbiologists saw more patients with Gram-negative infections. They attributed some of these infections to poor

quality water supplies or lax sanitation practices. Other developments were even more alarming to the healthcare and cosmetics industries. Contaminated disinfectants and hand cream dispensers were showing up in hospitals.

The 1960s also saw the emergence of G-11 toxicity. G-11 is a halogenated bisphenol known by its nonproprietary name: hexachlorophene. The toxicity was discovered after a product containing an exceptionally high level of G-11 due to a manufacturing error caused the deaths of 49 infants in France. The 1960s ended with a sense of urgency and with the industry on the defensive. We needed more effective and more responsible preservation practices to assure the microbiological and toxicological safety of products.

### *1970 to 1979*

The flurry of activity by the CTFA during the late 1960s quickly developed into a storm of contributions. The CTFA issued technical guidelines covering all aspects of good manufacturing and microbiological practices. The industry took note of the urgency of "cleaning house" as shown when a CTFA national survey of almost 4,000 marketplace cosmetics and toiletries over a 3-year period (1972 to 1975) showed minimal contamination. Instead of the 25% contamination incidence shown in the late 1960 surveys, the new survey proved the industry achieved a contamination rate of only 2%. Marketplace products made by reputable companies were clean although some small companies may continue to make products without attention to microbiological control or good manufacturing practices. Both industry and the FDA continue to emphasize the need for product cleanliness and safety.

During the latter half of the 1970s, eye cosmetics came under special scrutiny after several cases of blindness were caused by *Pseudomonas*-contaminated mascaras. The finding was that most products reached consumers in good microbiological condition. The question was whether they could withstand the insult of organisms added to the product during use and it was addressed for the next 15 years.

The FDA contracted with Georgia State University in 1975 to develop eye area challenge tests. The double membrane technique resulted and was further tested via another FDA contract with the University of California at San Francisco in 1977. Again, the CTFA acted quickly by setting up an Eye Area Task Force. This group arranged a collaborative study comparing Dr. Ahern's surface membrane preservative test with CTFA's direct inoculation procedure. Both tests proved equally satisfactory for measuring preservative activity. The industry then took steps to extend its product responsibility from manufacturing through consumer use.

The pharmaceutical industry continued developing antibiotics to combat increasing Gram-negative infections. However, both industrial and clinical microbiologists were gaining new respect for microbial resistance and adapt-

ability. Renewed attention to asepsis, hygiene, and sanitation began tempering our over-dependence on antibiotics and preservatives.

## *1980 to 1989*

The 1980s saw people facing the devastation of AIDS, the fatal virus-induced immunodeficiency disease that continues to affect people from all walks of life. On the positive side, the 1980s also engendered major advances by molecular biologists. Armed with knowledge of the double helix structure of DNA discovered by Watson and Crick in 1953, they successfully constructed recombinant DNA in the laboratory by 1975. With the groundwork laid, the 1980s saw explosive research effort focused on gene therapy, chromosome mapping, and agricultural trials of genetically engineered plants. The work of molecular biologists has had and will have profound effects on all future microbiological endeavors.

Directly applicable to our industry is the research for *in vitro* alternatives to animal testing. The industry and the private sector are aggressively pursuing this avenue either through grants to universities (Procter & Gamble) or direct research (Johns Hopkins). When (or if) we can end animal testing remains to be seen. Microbiological and tissue culture methods are starting to replace traditional toxicology testing performed with animals. This is helping to reduce the number of animals needed to produce sufficient data to support safe human use.

The FDA also continued its campaign to develop preservative challenge methods that were predictive of consumer contamination risks. The FDA's chief criticism was that existing methods did not predict whether consumers could contaminate products during use. In 1985, the FDA contracted with Schering-Plough and the University of North Carolina to perform research comparing preservative challenge testing methods used for creams and lotions that could predict consumer contamination. The FDA published the results, but they were compared to a simulated in-use regimen that was not valid as a true in-use comparison.[12]

In 1987, the CTFA published the results of a survey to determine whether companies tried to correlate their challenge test data with consumer use data. Nearly all companies claimed that they had correlation programs in place. None presented their data, however. There are only two challenge test methods that have been the subjects of published validation data proving their abilities to predict the in-use potential for consumer contamination.[13,14] Both tests are modifications of the CTFA method.

## *1990 to 1999*

The cosmetics and toiletries industry began to face the ramifications of the European Community (EC) open market in 1992. Multinational companies remained concerned about requirements for distributing their products from country to country. Their concerns included uniformity of test methods,

manufacturing practices, labeling requirements, and other requirements of the EC Commission. The 1990s were accompanied by a worldwide emergence of the need for industry to assume responsibility for protecting the environment. Some key issues were clean air and water, understanding the effects of hydrocarbons and fluorocarbons on the UV protecting ozone layer, saving the diversity of flora and fauna in rain forests and other natural habitats, and properly disposing of or recycling wastes. Stricter regulations and laws for a world facing ever-shrinking resources will be major concerns to our industry and others for some time to come.

Research to enable us to understand and control AIDS continues. The FDA even eased its rules concerning clinical trials for new drugs in an attempt to restrain this devastating disease. During this decade, microbiologists saw the returns of diseases we thought were under control: tuberculosis, syphilis, and cholera. Antimicrobial resistance to antibiotics continued to develop.

## *2000 and beyond*

The 21st century finds cosmetic microbiology on the verge of a sea change in its practices. The science is now taking its first cautious steps away from the agar paradigm that has supported its efforts for 150 years. Although its parent science began almost a decade earlier, cosmetic microbiology has begun to apply many technical and scientific advances including the techniques of molecular biology to products.

New rapid methods have reduced the time from the 5 days required for the classic agar culture plate confirmation of product quality to 48 hours or less, resulting in profound economic changes related to inventory costs and production times.[15–17] Advances such as those developed in response to bioterrorism offer potential in the areas of product release and monitoring and maintaining manufacturing hygiene in what approaches real time.[18–20] Preservative testing, although still cast primarily in the context of 28-day durations, may become much faster and efficacious based on the application of modified luminescent challenge bacteria, and may eventually provide more profound insight into the role of preservation itself. [21–24]

One remarkable benefit arising from progress in cosmetic microbiology is the opportunity to advance our knowledge and understanding of skin microbiology. This is a recent development and the advances continue as the tools of modern molecular biology reveal a world as remarkable as the one the ancient agar plate cultures of Hess and Petri offered a century ago.[25] An excellent example that comes to mind is the *Pityrosporum* genus formerly composed of two or three species vaguely differentiated on the basis of very subjective cellular morphology, one of which was even more vaguely associated with dandruff. In a revolutionary series of reports, it was found by exploitation of 16S ribosomal DNA sequences and confirmed through additional molecular and physiologic techniques that this group of fungi consisted of at least seven species, correctly categorized as the basidiomycetous

*Malassezia* species, all of which are uniquely resident in distinctive locations on human and animal bodies.[26–30] Furthermore, the *M. restricta* species is considered the most reasonable etiologic agent for dandruff.

In both its practical and scientific aspects, cosmetic microbiology is advancing and its earlier methods will soon be unrecognizable to its 21st century practitioners. Although methods, nomenclature, and even principles may change, aspects of the science that will not change are the essential practices necessary to manufacture microbiologically hygienic products; these will be discussed in Section Two.

## *Conclusion*

We now face an era of bacterial supremacy. The Gaia hypothesis that all the microorganisms on Earth will behave as one organism almost seems tenable. Pathogens are no longer easy to define. Even innocuous spoilage organisms are potential pathogens especially if present in high numbers. In 1990, the FDA established a joint program with CTFA and AOAC (formerly Association of Official Agricultural Chemists) to develop standard preservative challenge tests that are predictive of consumer contamination. Over years, perhaps this program will provide an improvement over existing test methods. Regardless of the outcome, all preservative tests should aim for self-sterilization of product units, rather than simple reduction of numbers of microorganisms to so-called safe levels. There is no safe level if survivors adapt and increase to pathogenic levels. Bacteria have been honing their adaptation skills for billions of years. They are ready and willing to show off those skills in the industry's new milder, safer, environmentally friendly, so-called natural, products of the future. We must adjust our activities accordingly.

We must remember that no amount of laboratory testing or monitoring can assure product quality in a climate of bacterial adaptation. In-house action standards and control programs give us an edge only if we know what actions to take. Preserved products should show fast-acting bactericidal activity (e.g., a 7-log reduction in 4 days). If it is not possible to accomplish such rapid kills, data could be generated to show that the packaging will protect a product adequately during use. Meeting these goals will enable us to achieve self-sterilization of product units during consumer use.

We should anticipate some degree of preservative failure and identify at least two suitable preservative systems for each new product early in the development process. We can protect cosmetics from contamination during manufacturing by strict enforcement of sanitation procedures. We should rigidly require and monitor cleanliness of process water, other raw materials and manufacturing processes, and maintain high standards of personal hygiene. Our jobs will be increasingly more difficult in the future. Today's bacteria have declared war. We must continue developing weapons and honing our fighting skills to win future battles of that war.

## References


1. Rhawn, J., *Sexuality: Female Evolution and Erotica*, 2nd ed., University Press, San Jose, CA, 2004, p. 24.
2. Ember, C.R. and Ember, M., *Anthropology: A Brief Introduction*, 4th ed., Prentice Hall, Englewood Cliffs, NJ, 2000, p. 25.
3. http://www.smith.edu/hsc/museum/ancient_inventions/eyeliner2.html
4. Low, B.S., *Why Sex Matters: A Darwinian Look at Human Behavior*, Princeton University Press, Princeton, NJ, 2000, p. 86.
5. Dyall, S.D., Brown, M.T., and Johnson, P.J., Ancient invasions: from endosymbionts to organelles, *Science* 304, 253, 2004.
6. *Standard Methods for the Examination of Dairy Products*, 16th ed., American Public Health Association, Washington, D.C., 1993.
7. *Standard Methods for the Examination of Water and Waste Water*, American Public Health Association, Washington, D.C., 1992.
8. *Compendium of Methods for the Microbiological Examination of Foods*, 3rd ed., American Public Health Association, Washington, D.C., 1992.
9. *U.S. Pharmacopeia XXIII*, U.S. Pharmacopeial Convention, Rockville, MD, 1995.
10. *FDA Bacteriological Analytical Manual*, 7th ed., Association of Official Analytical Chemists, Washington, D.C., 1992.
11. *CTFA Microbiology Technical Guidelines*, Cosmetic, Toiletry, and Fragrance Association, Washington, D.C., 1993.
12. Farrington, J.K., Martz, E.L., Wells, S.J., Ennis, C.C., Holder, J., Levchuk, J.W., Avis, K.E., Hoffman, P.S., Hitchins, A.D., and Madden, J.M., Ability of laboratory methods to predict in-use efficacy of antimicrobial preservatives in an experimental cosmetic, *Appl. Environ. Microbiol*. 60, 4553, 1994.
13. Brannan, D.K., Dille, J.C., and Kaufman, D.J., Correlation of *in vitro* challenge testing with consumer-use testing for cosmetic products, *Appl. Environ. Microbiol*. 53, 1827, 1987.
14. Lindstrom, S.M., Consumer use testing: assurance of microbiological product safety, *Cosmet. Toilet*. 101, 71, 1986.
15. Flickner, B., Making dollars and sense with rapid microbiology, *Pharm. Formul. Qual*. 4, 22, 2000.
16. Flow cytometry (e.g., Chemunex) www.chemunex.com
17. ATP-based technology (e.g., Celsis) www.celsis.com
18. Hietela, S.K. and Ardans, A.A., Molecular weapons against agricultural vulnerability and the war on terror, *J. Vet. Med. Educ*. 30, 155, 2003.
19. Sapsford, K.E., Shubin, Y.S., Delehanty, J.B., Golden, J.P., Taitt, C.R., Shriver-Lake, L.C., and Ligler, F.S., Fluorescence-based array biosensors for detection of biohazards, *J. Appl. Microbiol*. 96, 47, 2004.
20. Deisingh, A.K. and Thompson, M., Biosensors for the detection of bacteria, *Can. J. Microbiol*. 50, 69, 2004.
21. Gavin, J., Button, N.F., Watson-Criak, I.A., and Logan, N.A., Observation of soft contact lens disinfection with fluorescent metabolic stains, *Appl. Environ. Microbiol*. 66, 874, 2000.
22. Applegate, B.M., Nivens, D.E., Moser, S.A., Carroll, R.A., Sanseverino, J., Simonich, M.T., Pettigrew, C.A., Larson, R.J., and Bartolo, R.M., Determining disinfection efficacy using bioluminescent bacteria, 101st Annual Meeting, American Society for Microbiology, Orlando, FL, 2001, p. 604.

23. Kelana, L.C. and Griffiths, M.W., Use of autobioluminescent *Campylobacter jejuni* to monitor cell survival as a function of temperature, pH and sodium chloride, *J. Fd. Protect.* 66, 2032, 2003.
24. O'May, G.A., Allison, D.G., and Gilbert, P., A rapid method for evaluation of both extrinsic and intrinsic contamination and resulting spoilage of water-in-oil emulsions, *J. Appl. Microbiol*. 96, 1124, 2004.
25. Brock, T.D., *Robert Koch: A Life in Medicine and Bacteriology*, American Society for Microbiology, Washington, D.C., 1999.
26. Gueho, E., Boekhout, T., Ashbee, H.R., Guillot, J., Van Belkum, A., and Faergemann, J., The role of *Malassezia* species in the ecology of human skin and as pathogens, *Med. Mycol*. 36, Suppl. 1, 220, 1998.
27. Gemmer, C.M., DeAnglis, Y.M. Theelen, B., Boekheut, T., and Dawson, T.L., Fast noninvasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology, *J. Clin. Microbiol*. 40, 3350, 2002.
28. Duarte, E.R., Lachance, M.A., and Hamdan, J.S., Identification of atypical strains of *Malassezia* spp. from cattle and dog, *Can. J. Microbiol*. 48, 749, 2002.
29. Hirai, A., Makimura, R., Duarte, E.R., Hamdan, J.S., Lachance, M.A., Yamaguchi, H., and Jasegawa, H., *Malassezia nana* sp. nov., a novel lipid-dependent yeast species isolated from animals, *Int. J. Evol. Microbiol*. 54, 623, 2003.
30. Sugita, T., Saito, M., Ito, T., Kato, Y., Tsuboi, R., Takeuchi, S., and Nishikawa, A., The basidiomycetous yeasts *Cryptococcus* and *C. liquefaciens* colonize the skin of patients with atopic dermatitis, *Microbiol. Immunol*. 47, 945, 2003.

*chapter two*

# *Biology of microbes*

***Daniel K. Brannan***

*Contents*

# *General biological concepts*

## *Philosophy of biology*

The purpose of this chapter is to present to the nonscientist the critical information needed to understand microbiology so that it does not seem to be such a "black art." The black art aspect of science is often confused with fanciful stories that some science promoters tell about what science can do.* Scientists are humans too and enjoy a good story — but we must be critical of anecdotes. Most anecdotes are little more than collections of factoids of questionable provenance that masquerade as science. Although individual scientists make such mistakes, the collective process of science usually corrects them over time as a result of skepticism and critical thinking.

Far more insidious are the few scientists who make pronouncements regarding what should rightly be left to philosophers. Science deals with natural phenomena and has no ability to make claims about the non-natural or the supernatural. It is not that individual scientists do not accept such possibilities; it is simply that the process of science has no ability to test such claims. Consequently, when someone claims that spontaneous generation or a life force causes foods to spoil, the scientist actively engaged in science chooses to look for natural mechanisms such as microbes rather than an *élan vital*. However, when scientists claim that science proves or disproves the existence of some supernatural force, we need to realize that science — which focuses on naturalistic mechanisms — simply has no way to adjudicate such matters. If scientists would eliminate their philosophical musings, or at least admit that they are dealing in metaphysics instead of science, our schools could get on with teaching true science and not inserting religious views (either secular humanism *or* intelligent design) into curricula.

However, the elimination of philosophical questions does not eliminate the rather non-scientific attitude that Barbara McClintock once called "a feel for the organism." A microbiologist often learns so many technical details that he or she does not have time to simply become familiar with a microbe by growing and caring for it. Every microbiology student should, at some point in his or her career, have the experience of acting as a curator for a culture collection to learn "how to think like a bug." Such experiences allow microbiologists to develop an empathy for the microbes that often allows them to intuitively reach decisions based on thousands of data points that are processed subconsciously based on a gestalt not developed

* Richard Dawkins is a classic example of a promoter who espouses secular humanism and claims to support it with science. See his books, *Selfish Gene* and *Unweaving the Rainbow,* and Francis Crick's *The Astonishing Hypothesis*.

in those who never acquired such understanding. It is this empathy that separates the real microbiologist from the mere technician. It starts with a belief that a common bond exists between humans and even the most bizarre of microbes — a bond that allows us to appreciate the organism simply because it represents life itself and a bond that makes us realize that we all have the same origin.

A real biologist is interested in studying an organism simply because it allows him or her to gain an understanding of the creature. A real biologist studies life simply because of the desire to understand the incredible adaptability that has enabled nature to develop the various species that today occupy every conceivable available niche. And, in the process, the real biologist is struck with an incredible awe of life itself. One individual who was very much filled with this type of awe arising from the creatures around him was Charles Darwin[1] who wrote:

> To my mind it accords better with what we know of the laws impressed on matter by the Creator, that the production and extinction of the past and present inhabitants of the world should have been due to secondary causes, like those determining the birth and death of the individual. There is grandeur in this view of life, with its several powers having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.

Thus, the real biologist will experience a somewhat non-scientific emotion of awe when he or she studies life. Science, after all, is still a very *human* endeavor.

The cosmetic microbiologist must add to this appreciation of life and feel for the organism the details of technical training and balance a variety of factors to achieve safe, unspoiled, quality products. In addition to understanding sanitation procedures and use of preservatives, the cosmetic microbiologist must understand microbial physiology, pathogenic microbiology, and microbial ecology. In addition, he or she must understand organic and physical chemistry, toxicology, engineering, manufacturing and processing, and regulatory and environmental law. The cosmetic microbiologist must use all this education and knowledge within the context of the business needs of his or her employer and be able to balance risks of and benefits to consumers using products. The microbiologist who best fits this description has a good liberal arts undergraduate education followed up with post-graduate training in microbiology. Finally and most importantly, this person must have the highest of ethical standards, and consider himself or herself a member of the cadre of healthcare providers dedicated to serving humankind via the mission of providing them with microbiologically safe and efficacious products.

## *Evolution and adaptation*

Dobzhansky once stated that nothing in biology made sense outside the light of evolution. This is understandable when we see evolution only as change, variation, and adaptation of organisms to various environments. This adaptation is easily seen in the human's *artificial selection* of domesticated animals and can even be seen in nature's *natural selection* during one lifetime by looking at the beak of the finch as Rosemary and Peter Grant have done.[2] Natural selection is observable, testable, and, when models are based on it, predictable. This is what makes evolutionary theory so rich and transforms biology from a descriptive science to a predictive, explanatory science.

Admittedly, nature has no sentience and therefore cannot actually select or make a choice. Therefore the idea of *natural* selection is more of an analogy or metaphor akin to a human's sentient choice of artificial selection or breeding. But metaphors and analogies are powerful ways of thinking and evolution provides an enormous amount of explanatory sense, especially in light of the neo-Darwinian syntheses of such biologists as Ernst Mayr, Sir Ronald Fisher, and Edward Wilson. Nature is not actually "selecting" in the sense of making sentient choices. Instead, it "chooses" purely by serendipity, with the "goal" of any creature being simply to proliferate as best it can whether that occurs through competition or cooperation. Perhaps a far more useful idea than Herbert Spencer's Victorian idea of *survival of the fittest* or its equally Victorian corollary of progress via the *scala naturae* is a more modern concept that takes into account the serendipitous stochasticity of evolution — an idea I shall call Brannan's corollary: *proliferation of the slightly better.* Whatever allows a creature to proliferate slightly better is what really matters; not survival and not even being the best!

Certainly, as Stephen Gould noted, many abuses have arisen from attempts to explain evolution, for example, the "just-so" Panglossian-like explanations for why nature is just the way it is. We still have missing pieces to find, more data to gather, and better explanations to offer before the general theory of organic evolution can advance beyond more than a simple non-supernatural metaphysics based on materialistic naturalism combined with randomness and blind chance. Darwin saw this and decided to reject a completely naturalistic explanation for life's origin (with his reference to a Creator). However, there are just as many other cases where great insights and explanations have illustrated the way a species adapts to changing environments and changes sufficiently to form another species, for example, transitional fossils between genera, families, orders, and classes. The transitions between phyla and kingdoms are less well-known and a lot of gaps remain to be filled. Nevertheless, evolution is as much a fact as gravity, a spherical Earth, and a heliocentric solar system are facts today. (The latter two issues sparked religious controversies from the 15th through the 19th centuries.)

Further research will refine evolutionary ideas and show that evolutionary processes represent far more than a mere selection by nature. For exam-

ple, current evidence indicates that organismal change and even what is thought of as *complexification* over time is a result of lowered thermodynamic constraints and self-assembly governed by physical and chemical forces.[3] Regardless of what mechanisms are finally determined to be operative for causing evolution, Futuyma's definition[4] of what evolution actually is seems sufficient at present:

> … biological evolution is a change over time of the proportions of individual organisms differing genetically in one or more traits; such changes transpire by the origin and subsequent alteration of the frequencies of alleles or genotypes from generation to generation within populations, by the alterations of the proportions of genetically differentiated populations of a species, or by changes in the numbers of species with different characteristics, thereby altering the frequency of one or more traits within a higher taxon.

## *Biocide tolerance*

Every microbiologist is well aware of the power of biological evolution that requires us to constantly develop new biocides and antibiotics at a frenetic pace just to keep one step ahead of the microbes. Anyone who believes in the fixity of species in the microbial world needs only to work in an environment where microbes adapt very rapidly to the newest biocide or antibiotic. Microbes change so rapidly that a minor type of punctuated equilibrium has been shown by Richard Lenski's lab and was found in *Escherichia coli* in a matter of years.[5]

One of the key areas that requires understanding is the way that microorganisms evolve in order to become tolerant to biocides. While some may use the terms *tolerance* and *resistance* interchangeably, the idea that tolerance mechanisms against biocides are similar to those mechanisms found in antibiotic resistance is incredibly naive. Antibiotic resistance develops as a result of molecular changes at a single specific site of attack by the antibiotic (e.g., at the P sites of 50s ribosomes). Biocides attack at multiple sites (e.g., chlorine oxidizes all proteins, carbohydrates, and fats in a cell). Therefore, minor mutational events or acquisition of some plasmid containing a resistance gene that codes for some specific r-factor is an inappropriate model for describing biocide tolerance.

The development of tolerance for many biocides is more likely a result of phenotypic changes by which the population shifts to higher capsule production. This approach is a directional selection mechanism: the biocide forces a population bottleneck that allows survival of only a few hardy cells that pass on the genes for survival to their offspring (e.g., genes that express more frequently for capsule production). This is analogous to the proposal that the hairy elephant population gets hairier during an advancing ice age. Another mechanism allows cells to begin a primitive form of communication as in quorum sensing to stimulate physical community developments within

biofilms in which a form of "cooperation" that can provide protection for the populations within the biofilm exists.[6]

Certainly, specific biochemical mechanisms (e.g., formaldehyde dehydrogenase) of resistance to biocides exist in the case of formaldehyde tolerance. Such phenomena are discussed in detail by Dr. Chapman in Chapter 8 of this text. However, tolerance to biocides that act at multiple sites must not be assumed to occur *only* at the gene or even at a single cell level. In many cases, the tolerance claimed may be a result of population factors such as capsule formation or existence within a clump of organisms or within a biofilm. This population selection concept is not group selection because most of the organisms within a biofilm are closely related and share genetic information rather indiscriminately (a form of kin selection).

Regardless of the adapting mechanisms extant in a microbial population, the key idea to get across to non-biologists is that nature is never stable. Microorganisms change and adapt constantly. They are not simply little sacs of enzymes suspended in a liquid as the reductionist-minded analytical chemist thinks; they are not just another ingredient to eliminate as the pragmatic process engineer thinks. They are adapting little sparks of life containing information in the form of genes representing eons of proliferation and this allows them to survive no matter what we throw at them. The new biocides we develop today will not work tomorrow. The microbiologist will always have work to do to keep one step ahead of the ever-adapting, ever-changing, ever-evolving microbes.

## *Bacteria*

The bacteria play a major role in the world's ecosystems. They are so ubiquitous and important that they are considered by some to be the lifeblood of the planet. According to the Gaia hypothesis, they are involved in biogeochemical cycling to maintain Earth's homeostasis.[7] They occupy every conceivable niche and some that we may not have even dreamed possible. Their ubiquity means they are found throughout cosmetic and drug manufacturing plants. An area the size of a single pinhead may contain well over a billion bacterial cells. A single handful of soil represents an entire universe of bacterial possibilities, all capable of adapting to even the harshest environments. Well-preserved products and scrupulously clean manufacturing environments help prevent these organisms from establishing niches within consumer products, but their genetic adaptability and remarkable evolutionary capability present moving targets that are difficult to control without constant surveillance. This chapter presents an introduction to microbiology intended to enable even non-microbiologists to understand at least the basics of the very complex and ever-changing world of microorganisms.

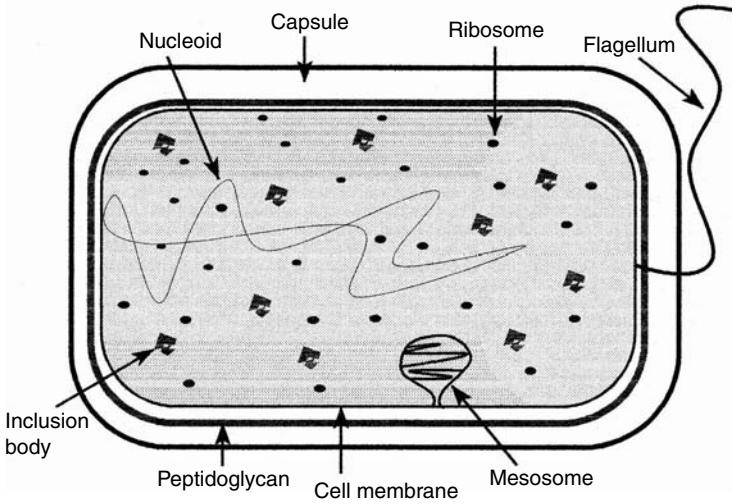


***Figure 2.1*** Typical Gram-positive bacterial cell.

## *Physiology and biochemistry of microorganisms*

### *Size, shape, and organization*

Prokaryotic cells such as bacteria lack internal membrane-bound organelles. They are much simpler than eukaryotic cells such as the cells of the human body that contain internal membrane-bound organelles (nucleus, mitochondria, endoplasmic reticulum; see below). Because bacteria are so simple, our first inclination to is believe that they should be simple in shape. In fact, most *are* either cylindrical (rod-shaped) or spherical (cocci), but they can also be shaped as spirals (spirilla and spirochetes), curves (vibrios), and even squares and triangles. The shape of the cell is determined by its wall.

Bacteria are also smaller than most eukaryotes. Some bacteria are as very small as 100 nm (*Mycoplasma* spp.); some are as large as 60 to 800 mm (*Epulopisicum fishelsoni*). The large size of *E. fishelsoni* contradicts the small size assumption by overcoming the hypothetical diffusion limits through a plasma membrane that is highly invaginated into the cytoplasm.

### *Bacterial organelles*

A variety of structures exist within prokaryotes. Figure 2.1 illustrates a typical bacterial (Gram-positive) cell. Table 2.1 describes the functions of cell structures. Not all bacteria are identical or contain all the structures shown.

In general, a cell wall and plasma (or cytoplasmic) membrane surround prokaryotic cells. Some prokaryotes lack cell walls but all have cytoplasmic membranes. The membrane can invaginate to form mesosomes and other internal membranous structures within the cytoplasm. In all prokaryotic cells, however, the cytoplasm is very simple and has no membrane-bound internal organelles. The genetic material (e.g., DNA) is in a region called the

***Table 2.1*** Functions of Various Prokaryotic Cell Structures

| Structure | Function |
|---|---|
| Capsule/slime layer | Resistance to phagocytosis; adherence to surfaces |
| Fimbria/pilus | Attachment to surfaces; bacterial conjugation |
| Flagellum | Movement |
| Cell wall | Provides shape and protection from lysis in dilute solutions |
| Plasma membrane | Serves as selectively permeable barrier and boundary of cell; location of respiration and photosynthesis; serves as receptor for chemotaxis |
| Periplasmic space | Site of hydrolytic enzymes and proteins for nutrient uptake and processing |
| Ribosome | Protein synthesis |
| Inclusion body | Storage |
| Gas vacuole | Provides buoyancy for certain bacteria |
| Nucleoid | Site of genetic material (DNA) |
| Endospore | Allows survival under harsh environments for certain bacteria |

nucleoid. This region is not surrounded by a nuclear membrane in a nucleus as it is in eukaryotes. Ribosomes and inclusion bodies are found throughout the cytoplasm. One organelle that extends outside the cell wall is the flagellum. A capsule and pili or fimbriae surround the cell wall. These structures help a bacterium adhere to surfaces and form biofilms. They also allow bacteria to stick to each other and clump, especially when exposed to biocides or other adverse conditions.

*Cell walls and cytoplasmic membranes.* Nearly all prokaryotes have cell walls. The only exceptions are the mycoplasma and a few archaeobacteria. A cell wall gives a bacterium its strength and shape. Without the rigid wall, a bacterium would lyse (explode) because of the effect of osmosis. Most bacteria exist in a dilute external environment. In fact, the environment inside the cell is more concentrated in solutes (dissolved materials) than the outside. Therefore, water flows into the cell continuously. A whimsical analogy is the water balloon. If a balloon is placed into a rigid box before it is filled with water, it cannot be filled so full that it will pop. If filled outside a box, it will pop when filled too full.

Between the cell wall and cytoplasmic membrane is the periplasmic space — a gel-like area filled with hydrolytic enzymes and binding proteins that digest nutrients and transport them into the cell.

The next structure for consideration is the cytoplasmic membrane. Simplistically, a novice would claim that this structure is not all that different from any other cell membrane — eukaryotic or prokaryotic. After all, it has proteins and lipids and surrounds the cytoplasm. It is the main point of contact between the cell and its environment. The cytoplasmic membrane is selectively permeable and will allow only certain molecules to enter the cell.

It transports large molecules that cannot diffuse and also carries on metabolic functions such as respiration, photosynthesis, and some biosyntheses. This membrane is composed of lipids that arrange into a bilayer (because of a lipid molecule's amphipathic nature — one end is hydrophilic and the other is hydrophobic). However, the few differences that do exist between eukaryotic and prokaryotic membranes are significant. The bacterial membranes of prokaryotes do not contain cholesterol as eukaryotic cells do. Their proteins are different. Although membranes have a common basic design, they differ widely in their various structural and functional capacities.

The model of a membrane that is widely accepted by most scientists is called the fluid mosaic model. One can think of this model as a layer of oil (lipid membrane) on top of an ocean (the cytoplasm) into which are interspersed ships of protein, except that our model includes dilute water on top of the oil layer (the external environment of the cell) as well. Like ships on the ocean, the proteins move laterally around the surface. The protein ships are peripheral (loosely connected to the membrane and soluble in water) or integral (hard to extract from the membrane and insoluble in water).

*Peptidoglycans.* The cytoplasmic membrane of any cell is delicate and prone to rupture unless the cell exists in an isotonic state. This is why physiological saline is used instead of distilled water to replace body fluids: to prevent rupture of red blood cells. However, bacteria rarely have the luxury of floating in an isotonic environment unless they are living inside a bloodstream. Most of the time, they exist in a hypotonic environment where water pours into cells. Peptidoglycan makes up the structure of the cell wall to provide it with rigidity and strength and combat the hypotonic environment.

Peptidoglycan (also known as murein) is a net-like structure comprising the cell wall. It is a polymer composed of two amino sugars (N-acetylglucosamine and N-acetylmuramic acid) and several amino acids (some unique only to prokaryotes and not even found in most eukaryotic proteins). Figure 2.2 shows the structure of peptidoglycan.

Cell walls of Gram-negative and Gram-positive bacteria exhibit considerable differences. One of the major differences is the type of peptidoglycan. A Gram-positive cell has a pentaglycine bridge between the d-alanine and l-lysine of the tetrapeptide that comes off the N-acetylmuramic acid (see Figure 2.2). Gram-negative cells exhibit a direct link between the alanine and the lysine.

The definition of Gram-positive and Gram-negative cell walls relates to two concepts: the color of the cell after the Gram stain and the physiological structure of the wall. Christian Gram developed a technique that allowed some bacteria to stain pink and others purple. When Gram devised the method, he did not know the reason for the staining. As a result of this differential staining, microbiologists began calling purple-stained bacteria "positive" and pink-stained bacteria "negative." Later on, they found out that the purple-staining Gram-positive bacteria usually had thicker peptidoglycan layers and the pink-staining Gram-negative bacteria had thinner

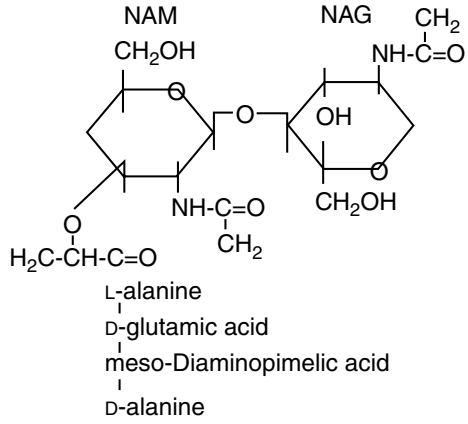


***Figure 2.2*** Peptidoglycan subunit. The crosslink between two NAG–NAM strands is direct via d-alanine and diaminopimelic acid (DAP) for Gram-negative peptidoglycan. In Gram-positive cell walls, the crosslink is indirect via a pentaglycine bridge. NAG = N-acetylglucosamine. NAM = N-acetyl muramic acid. A tetrapeptide is attached to NAM.

peptidoglycan layers. We will explore additional differences between Gram-positive and Gram-negative bacteria next.

*Gram-positive walls.* Gram-positive bacteria have very thick peptidoglycan layers. The peptidoglycan has a pentaglycine bridge between the d-alanine and the l-lysine of the tetrapeptide coming off the N-acetylmuramic acid (NAM). The NAM is polymerized to N-acetylglucosamine (NAG). Gram-positive cell walls also contain teichoic acids (Figure 2.3). These are ribitol and glycerol phosphate polymers. Coming off the ribitol and glycerol may be amino acids or sugars. The teichoic acids attach to

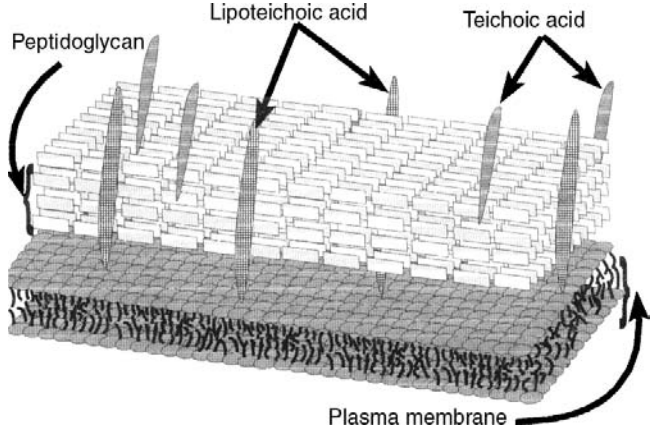


***Figure 2.3*** Gram-positive cell wall.

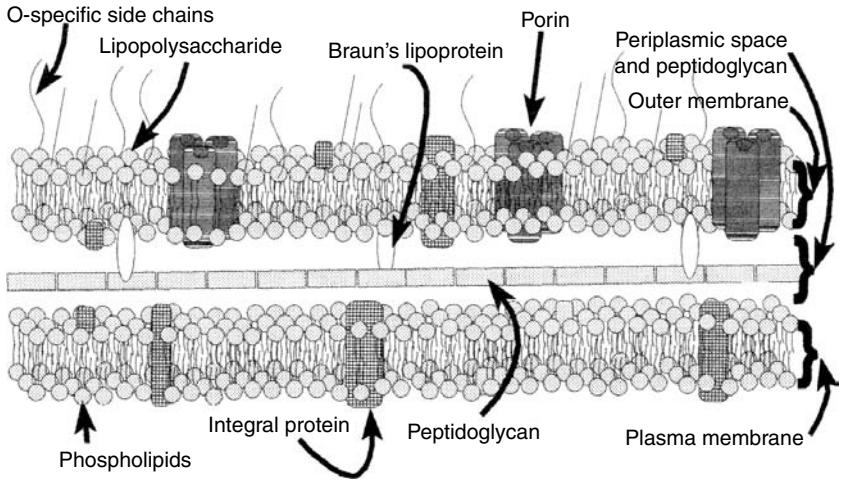


***Figure 2.4*** Gram-negative cell wall.

the peptidoglycan layer and extend to the outside of the cell where they give the cell a negative charge. They can even extend all the way down into the cell membrane and attach to lipids (lipoteichoic acids). They are like tie rods that hold the peptidoglycan to the membrane, but their true function has not been entirely clarified yet. They are not present in Gram-negative cell walls.

*Gram-negative walls.* A Gram-negative bacterium has a thin peptidoglycan where the tetrapeptide coming off the NAM is directly linked to the one coming off a NAM on an adjacent strand of NAG–NAM polymer. The complexity of Gram-negative cell walls is astounding. They have outer membranes on the outsides of their thin peptidoglycan layers. Linking this outer membrane to the peptidoglycan is a compound called Braun's lipoprotein. It is covalently linked to the peptidoglycan with its hydrophobic end stuck in the lipids of the underlying surface of the outer membrane (Figure 2.4).

The outer membrane of a Gram-negative bacterium is made of a lipid bilayer, as is the case with most classical membranes. Its uniqueness comes from the lipopolysaccharides (LPSs) that extend from the outer layer of the outer membrane into the environment. LPSs are very large molecules made of lipids and carbohydrates (see Figure 2.5). The O-region of the LPS elicits the most antibody response during an infection and the O-region rapidly changes to avoid antibody attack. The LPS also confers a negative charge to the bacterial surface and helps stabilize the outer membrane. It is also the component that acts as the endotoxin.

The outer membrane of Gram-negative cell walls really does not act as a selectively permeable membrane in the same way that a cytoplasmic membrane does. Instead, it offers protection and slows entry of toxic substances

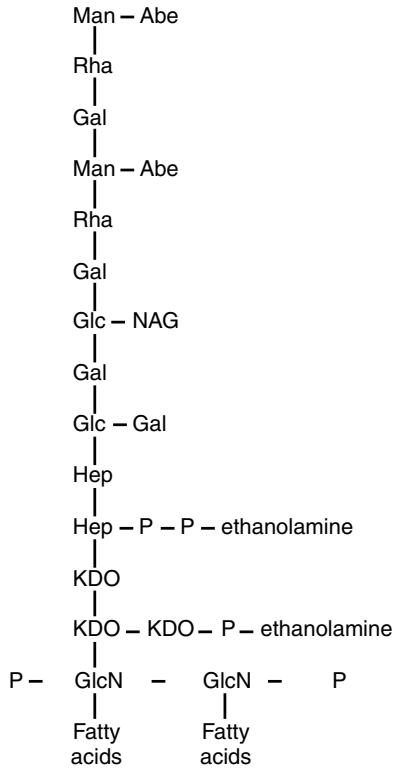


***Figure 2.5*** Lipopolysaccharide from *Salmonella* species. Abe – abequose. Gal = galactose. Glc = glucose. GlcN = glucosamine. Hep = heptulose. KDO = 2-keto-3-deoxy octonate. Man = mannose. NAG = N-acetyl glucosamine. P = phosphate. Rha = L-rhamnose.

into the cell. It does, however, allow small molecules such as monosaccharides to pass to the cell membrane via porin proteins. The porins form a channel through which these smaller molecules (<700 daltons) pass. Large molecules are transported by specific carrier proteins. The outer membrane of a Gram-negative cell wall has some selective permeability about it, but should never be thought of as another cytoplasmic membrane.

*Gram stain.* When performing a Gram stain, start by spreading a thin suspension of bacteria on a glass slide, let the suspension dry on the slide, and heat it gently to fix the bacteria onto the slide. The next step is to put a crystal violet solution on the slide for about a minute and rinse the slide with water. The bacteria absorb the dye and turn purple. Then put Gram's iodine on the slide. The iodine acts as a mordant that "sets" the purple dye. Next, rinse with an acetone–alcohol mixture to try to remove the set dye. Finally, counterstain with safranin, a pink dye. If the crystal violet dye cannot be removed with the acetone–alcohol mixture, then the safranin counterstain is not even seen; the bacterium is so dark purple that

the pink dye does not contribute to the color. If the crystal violet is removed, the bacteria are colorless until the pink safranin counterstain is added. Bacteria that stain purple are called Gram-positive (positive because they retain the first dye). Bacteria that stain pink are designated Gram-negative.

The key to understanding how the Gram stain works is in the cell wall. One hypothesis is that the alcohol shrinks the molecular pores of the thick peptidoglycan of Gram-positive cells where the crystal violet is trapped. In the Gram-negative cells, the thin peptidoglycan is not highly cross-linked. The pores are bigger and more permeable, allowing the crystal violet to leak out quicker when decolorized. The alcohol also dissolves the lipid of the outer membrane to allow the stain to escape.

*Cytoplasm, mesosomes, ribosomes, and other inclusions.* A bacterial cell minus its wall is a protoplast. A protoplast includes the plasma membrane, the cytoplasm, and everything within it. The prokaryotic cytoplasm, however, does not have typical unit membrane-bound internal organelles. Within the cytoplasm is the nucleoid where the DNA genetic material is localized. Also, within the cytoplasm are the enzymes needed for growth and metabolism, the machinery for manufacturing those enzymes (ribosomes), and some internal membrane structures called mesosomes. Mesosomes are actually invaginations of the plasma membrane. Finally, some bacteria also contain inclusion bodies consisting of polyphosphate, cyanophycin, and glycogen. These inclusions are not usually membrane-bound. Other bacteria have inclusions bound by a single-layered nonunit membrane. These consist of poly-b-hydroxybutyrate, sulfur, carboxysomes, hydrocarbons, and gas vacuoles.

Mesosomes are rather enigmatic. Their exact function remains uncertain despite their presence in both Gram-positive and Gram-negative bacteria. They are often found close to the septa of dividing cells; sometimes they even appear to be attached to chromosomes. Perhaps they are involved in cell wall formation or play a role in chromosome separation during cell division. This role would make them somewhat analogous to spindle fibers in eukaryotic cell mitosis. Maybe they are involved in secretory processes. Some microbiologists dismiss them entirely, claiming they are artifacts of the fixation process used in preparing samples for electron microscopy. Since we also see them in freeze etches where few or no artifacts develop from chemical fixation, it is doubtful that this concept will prevail.

Other more complex membrane systems of invaginated plasma membranes are seen in the complex and extensive foldings within cyanobacteria, nitrifying bacteria, and purple bacteria. The current thinking is that these organisms require the larger surface area that these infoldings provide in order to carry on greater metabolic activity.

Ribosomes are packed into the cytoplasm; some are loosely attached to the plasma membrane. They are made of both ribonucleic acid and protein and serve as sites where RNA is translated into protein after it is transcribed from DNA. In bacterial systems and in mitochondria, ribosomes are com-

posed of two components: 50S and 30S subunits (the S indicates a Svedberg unit — a measure of sedimentation based on a particle's volume, shape, and weight). Together, the 50S and 50S subunits comprise a single ribosome and together weigh 70S (the numbers are not supposed to add to an expected total because a Svedberg unit is based on more than weight alone).

*Nucleoids.* The nucleoid is another structure that is not surrounded by a membrane. Eukaryotes always contain their genetic material within a nucleus surrounded by a membrane; they have two or more linear chromosomes. A prokaryote has only one circular chromosome located within a region called a nucleoid. The nucleoid is apparently attached to the cell membrane that may be involved in cell division via the aid of mesosomes.

Bacteria also contain plasmids. These are extrachromosomal pieces of DNA (also circular) that replicate independently of the chromosome. They can also be incorporated into the chromosome. They rarely have genes that are absolutely required by the organism for growth and metabolism, but often carry genes that are very useful for survival: resistance genes that make them able to withstand antibiotics, genes that allow the organism to produce a toxin, or genes that provide some other selective advantage.

*Organelles outside the wall. Flagella, fimbriae, and pili.* Fimbriae (or pili) are thin protein hairs on the outer surfaces of Gram-negative bacteria that cause the bacteria to stick to surfaces. If it were not for pili, bacteria would be less able to cause infections by attaching to host tissues. They would not be as able to form biofilms on pipes of water systems to create reservoirs of contamination or endotoxins. They would not be able to attach to ship hulls to produce further corrosion. Some pili are even involved in providing a passage for DNA to travel from one bacterium to another during conjugation.

Flagella allow bacteria to move. Unlike eukaryotic flagella, the prokaryotic flagellum rotates rather than moving from side to side. Also, it is a relatively simple thread-like appendage of protein extending from the plasma membrane and cell wall (about 20 mm long and 20 nm thick). This portion is the filament. Compared to the 9 + 2 filament arrangement of eukaryotic flagella, this structure is simple.

A little more complexity appears when we look at the basal body of the bacterial flagellum. It is embedded within the bacterial cell wall and consists of a number of rings that vary, depending on whether the bacterium is Gram-negative or Gram-positive. A hook links the filament and the basal body. The basic difference between Gram-negative and Gram-positive flagella is the number of basal body rings. Gram-negative bacteria usually have four basal body rings. The first two rings are attached to the outer membrane (L-ring) and peptidoglycan layer (P-ring). The inner two rings contact the periplasmic space (S-ring) and the plasma membrane (M-ring). The Gram-positive flagellum has only two rings: one attached to the plasma membrane and the other attached to the peptidoglycan.

*Capsules and slime layers.* Outside the cell wall, a bacterium may have a layer of material that can be fairly well-organized and not easily washed away. This layer is known as the capsule. If the material is easily washed off and not really organized, the layer is a slime. Many scientists prefer to not worry about these distinctions and so refer to both types of layers as the glycocalyx. In both cases, the layers are made from polysaccharides or polyamino acids (in some bacteria) surrounding the outer cell walls. The capsule helps a bacterium resist phagocytosis when it infects a host. Encapsulated bacterial pathogens are usually much more virulent than varieties without capsules. The capsule also helps the bacterium avoid drying, acts as a biofilm to aid it in attachment to surfaces, helps it avoid predation from zooplankton, and protects it from detergent and biocide actions. From a practical standpoint, encapsulated bacteria are more likely to develop tolerance to preservatives and biocides in manufacturing conditions.

### *Metabolism*

In order to understand metabolism fully, the student should understand the basics of thermodynamics, chemical reactions coming to equilibrium, and oxidation–reduction reactions. He or she should also understand the roles of enzymes in biochemical reactions and the central activity of adenosine triphosphate (ATP) in mediating the flow of energy from one process to the next. This section will provide only rudimentary information to convey an understanding of these points.

All organisms require energy to perform work. This work falls into three categories: (1) chemical work in which complex biological molecules are synthesized from simpler molecules; (2) transport work in which molecules are transported into or taken out of cells; and (3) mechanical work in which energy is used for movement by the organism or enables the structures of the organism to change their positions. Most ecosystems receive their energy to do work from sunlight and through photosynthesis.

A few ecosystems receive their energy from geothermal means or through chemosynthesis using inorganic energy sources such as sulfides (e.g., oceanic thermal vents). The light energy from the sun (e.g., photosynthesis) and the geothermal energy (sulfide) from Earth (e.g., chemosynthesis) are trapped in the form of chemical energy stored in complex compounds. The organisms involved in this primary production of chemical energy from light, heat, or inorganic compounds (producers) serve as energy sources for chemoheterotrophs (consumers) that use complex organic molecules as sources of material and energy for building their own cellular structures.

In transforming light or chemicals into usable energy for cellular processes, cells use ATP as an energy exchange molecule to make free energy available to do work. ATP is a high energy molecule that is energetic because of repulsive forces that are overcome by covalent bonds between the highly charged triphosphates. At pH 7.0, the linear triphosphates are completely ionized, giving the ATP molecule four negative charges. These negative charges are in close proximity and repel each other. If the covalent bond that

overcomes these charges is hydrolyzed, this electrostatic stress between the phosphates is relieved and the difference in the energy of the products (ATP and water) and the reactants (adenosine diphosphate [ADP] and inorganic phosphate) releases sufficient energy for use in coupling to another reaction. When ATP is hydrolyzed, the reaction releases a $\Delta G^{O\prime}$ of –7.3 kcal/mole (free energy available for work).

Energy flow may be described in terms of chemical reactions that proceed to equilibrium such as we described for ATP hydrolysis. However, free energy changes are also involved in oxidation–reduction reactions that occur when electrons move from a donor to an acceptor. Each process is described using two slightly different thermodynamic equations.

The first equation relates to chemical energy:

$$\Delta G^{O\prime} = -RT(lnK_{eq})$$

where $R$ is the gas constant (1.9872 cal/mole–degree), $T$ is the absolute temperature of the reaction, and $K_{eq}$ is the equilibrium constant for the particular reaction.

The second equation relates to redox energy:

$$\Delta G^{O\prime} = -4\text{nF}(\Delta E_{O\prime})$$

where $n$ is the number of electrons transferred, $F$ is the Faraday constant (23,062 cal/mole–volt), and $\Delta E_{O\prime}$ is the difference between the reduction potentials of the coupled redox reaction (the reduction of the chemical that is oxidized to give electrons to the chemical that accepts them is known as the coupled redox reaction).

Chemical energy is one factor involved in substrate-level phosphorylations such as the step from 1,3 bisphosphoglycerate to 3-phosphoglycerate during glycolysis. Redox energy plays a role in the production of reduced NAD via the Krebs cycle. Subsequently, these electron carriers donate their electrons to a cytochrome chain in which energy is produced by creating a hydrogen ion gradient across the cell membrane that permits the production of ATP via ATP synthase as hydrogen ions flow back into the cell.

None of these processes would work effectively without the presence of enzymes that catalyze the reactions. Enzymes are simply protein catalysts that are specific for making a chemical reaction proceed from reactants to products. Enzymes do not change the equilibrium of the reaction nor do they cause more products to form. As catalysts, the enzymes simply speed up a reaction to its final equilibrium. They achieve this by lowering the activation energy required to bring the reacting molecules together in the correct way so they can reach what is called a transition-state complex that then can decompose into the products, assuming the energy states of the products are lower than those of the reactants.

The methods by which enzymes bring the reactants together to form the transition state represent an area of considerable research. Enzymes bring substrates together at an active site where the activation is lowered because far more molecules of the reactants are concentrated in the area for the reaction to take place. However, this concentration alone does not explain the whole process. In addition, the molecules are oriented into the correct positions for the molecules to react to form the transition state complex.

*Carbohydrate metabolism.* Metabolism refers to all the chemical reactions going on in a cell to allow the production of energy (catabolism) and the use of that energy to allow for synthesis of complex molecules to create the ordered cell or organism (anabolism). Catabolism involves the breakdown (oxidation) of organic compounds to provide energy. Catabolism can also involve the oxidation of inorganic compounds (chemolithotrophy) or the use of light (photosynthesis) to provide energy.

For heterotrophs, catabolism can easily be conceptualized as three interrelated stages. The first stage breaks down large molecules and polymers such as carbohydrates, proteins, and lipids. The chemical process is usually via hydrolysis. Carbohydrates are broken down into monosaccharides, proteins into amino acids, and lipids into glycerol and fatty acids. During the second stage, these breakdown compounds are further broken down into even simpler molecules such as acetyl CoA and pyruvate (during monosaccharide, glycerol, and fatty acid breakdown), and tricarboxylic acid cycle intermediates (during amino acid breakdown). During this second stage, the production of ATP and electron carriers such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) occurs. Finally, these simple molecules are completely oxidized into $CO_2$ and additional amounts of ATP, NADH, and $FADH_2$ are produced. The electron carriers are further oxidized via the electron transport chain to yield large amounts of ATP, producing water and $CO_2$ in the process.

What is fascinating about these reactions is that they all converge into three similar and common catabolic pathways: glycolysis, the tricarboxylic acid cycle, and the electron transport chain. What is even more fascinating is that these pathways exist as amphibolic pathways. This means that they can function both ways: anabolically and catabolically. Most of the reactions in glycolysis and the tricarboxylic acid cycle are reversible. Thus the molecules produced along the pathways can also be used as precursors for synthesizing macromolecules needed by cells for growth and repair. A few irreversible catabolic steps use special enzymes to catalyze the reverse reaction (i.e., phosphofructokinase) in order to regulate whether the amphibolic pathway will function as a catalytic or an anabolic pathway.

*Glycolysis.* Glycolysis, also known as the Embden–Meyerhof pathway, is one of three major pathways that break down sugar to pyruvate. The other major pathways are the Entner–Doudoroff and the pentose phosphate. Glycolysis is considered the most common pathway because it is found in

all major groups of microorganisms and functions regardless of the presence of oxygen. Glycolysis is usually divided into two main phases, to better explain how it works. The first is referred to as the 6-carbon sugar stage and involves two phosphorylating steps to convert glucose to fructose-1,6-bisphosphate. Two molecules of ATP are used to prime this step. The second step is the 3-carbon sugar stage that cleaves the fructose-1,6 bisphosphate into two 3-carbon molecules that are converted to form a total of two pyruvate molecules, two NADH molecules (electron carriers), and four ATP molecules. Because two ATP molecules are used to prime the reaction in step 1, we get a net of two ATP molecules from the reaction. The ATP is formed by a process known as substrate-level phosphorylation since ADP phosphorylation is coupled with the exergonic breakdown of a high-energy substrate molecule.

*Pentose phosphate pathway.* The pentose phosphate pathway (also known as the hexose monophosphate pathway) uses a different set of reactions to form 3-, 4-, 5-, 6-, and 7-carbon sugar phosphates that are used to produce the ATP and NADPH (used in biosynthesis). The pathway is primarily used to provide the carbon skeletons for the synthesis of amino acids, nucleic acids, and other macromolecules. If biosynthesis is not needed, the NADPH will be converted to NADH to feed the electron transport chain for the production of more ATP. A unique reaction in this pathway is the production of 3-ribulose-5-phosphate from 6-phosphogluconate.

*Entner–Douderoff pathway.* This pathway also produces pyruvate as an end product as glycolysis does, but it produces a lower yield of only one ATP, one NADPH, and one NADH. The key intermediate unique to this pathway is 2-keto-3-deoxy-6-phosphogluconate derived from 6-phosphogluconate.

*Tricarboxylic acid cycle.* The tricarboxylic acid (TCA) cycle starts after acetyl-CoA is produced from pyruvate. The steps required to produce acetyl-CoA from pyruvate involve a system of multienzymes known as the pyruvate dehydrogenase complex. In this process, just before the TCA cycle, pyruvate is coupled with coenzyme A after forming acetate from pyruvate by removing one of the carbon atoms as carbon dioxide. Thus, the acetyl-CoA formed is composed of coenzyme A bound to acetic acid via a high energy thiol ester bond.

The acetyl-CoA formed then reacts with the 4-carbon compound oxaloacetate to produce the 6-carbon molecule, citrate. In regenerating oxaloacetate from citrate, two carbon dioxide molecules are removed. The single ATP produced is from substrate phosphorylation when succinyl-CoA converts to succinate during part of the cycle. Also produced is one $FADH_2$, and three NADHs for each acetyl-CoA molecule that enters the cycle. These electron carriers can then enter the electron transport chain to produce more ATP as described further in the section covering electron transport and oxidation phosphorylation.

*Fermentations.* In the absence of oxygen, pyruvate — the product of glycolysis — cannot be completely oxidized to carbon dioxide and water via the Krebs cycle to produce the copious quantities of NADH that become oxidized via the electron transport chain because no terminal electron acceptor (e.g., oxygen) is available. In fact, the NADH produced during glycolysis must be oxidized in order to have more $NAD^+$ available for reuse during glycolysis. Basically, all fermentations are reactions that regenerate $NAD^+$ from NADH when an electron acceptor for the electron transport chain is unavailable.

The usual mechanism for regenerating $NAD^+$ is to oxidize the NADH produced using pyruvate as an organic electron acceptor. Thus, instead of completely oxidizing pyruvate, it becomes reduced in order to make $NAD^+$ available to keep glycolysis operating. The goal is to keep up a meager production of ATP when oxygen is absent (only two ATP molecules per glucose molecule) and continue the production of intermediates for anabolism.

When pyruvate acts as the electron acceptor, it may experience several fates. It may be decarboxylated into an acetaldehyde that then accepts NADH to form ethanol via catalysis by alcohol dehydrogenase. Pyruvate also may be directly reduced to lactic acid using lactate dehydrogenase to catalyze the oxidation of NADH. Because only lactic acid is formed, the process is called homolactic fermentation.

When several other products (including ethanol and $CO_2$) are formed in addition to lactic acid, the reaction is known as heterolactic fermentation. Lactic acid fermentors include various *Lactobacillus* spp. and some *Streptococcus* spp. Pyruvate may also be reduced to form formic acid, ethanol, and a variety of other acids (e.g., acetic, lactic, succinic). In this case, the reaction is designated mixed acid fermentation and is typical of many enteric bacteria such as *Escherichia*, *Salmonella*, and *Proteus*. If formate hydrogenylase is present, the formate will be further converted to hydrogen and carbon dioxide. Alternatively, the pyruvate may be converted to acetoin that is then reduced to butanediol using NADH. Organisms that perform this fermentation are *Enterobacter*, *Serratia*, and *Bacillus* spp. These processes help identify the various organisms that perform them and can be used as diagnostic tools. Other fermentations may also take place, for example, the formation of propionic acid (*Propionibacterium*), isopropanol, butanol, and butyrate (*Clostridium*). These latter fermentations may be applied to the manufacture of foods and fuels.

*Protein metabolism.* Many of the spoilage organisms and pathogens are efficient protein degraders. They secrete protease exo-enzymes to hydrolyze proteins into amino acids that can then be transported into cells. After the amino acids enter cells, they can be deaminated to remove their amino groups and produce tricarboxylic acid intermediates from which intermediates for either biosynthesis or energy may be obtained from the NADH produced that is coupled to electron transport.

*Fat metabolism.* Lipids are common energy sources for microbes. They exist as triglycerides, phospholipids, or other complex esters and are hydrolyzed into simpler compounds such as glycerol and fatty acids by lipases. Many of the fatty acids are then catabolized by β-oxidation that produces acetyl Co-A, a participant in the tricarboxylic acid cycle. Considerable energy and reducing power can be generated with each turn of the β-oxidation pathway.

*Electron transport and oxidative phosphorylation.* The key to generating considerable energy is to use the reducing power of NADH and FADH generated from the metabolic pathways outlined above. This is accomplished through a series of redox reactions by passing electrons from NADH and FADH via several electron carriers (e.g., cytochromes) to a final or terminal electron acceptor. This process occurs in the bacterial plasma membranes of prokaryotes, whereas it occurs in the inner mitochondrial membranes of eukaryotes.

During this process of transporting electrons along cytochromes, the considerable amount of energy produced is used to pump protons to the outsides of cells. This sets up a gradient with protons on one side of the membrane and electrons on the other. When the protons diffuse back into the cells to equalize the gradient, they flow back through an enzyme complex that includes ATP synthase, which synthesizes ATP as a result of the energy from this proton-motive force.

An alternative hypothesis has gained considerable weight: the conformational change hypothesis. This idea is that the energy released during electron transport causes the ATP synthesizing enzyme to change conformation such that it can bind ADP and phosphate more efficiently and thus better catalyze the reaction to ATP. The whole process of coupling redox reactions to the formation of ATP by the mechanism of an electron transport chain is known as oxidative phosphorylation. This is contrasted with the substrate level phosphorylation that occurs in glycolysis.

*Anaerobic respiration.* Many students of microbiology consider anaerobic processes to be synonymous with fermentation. In fact, many anaerobic processes still may have ATP formed as a result of oxidative phosphorylations if they replace only the terminal electron acceptor oxygen with an inorganic form of oxygen. For example, nitrate ($NO_3^-$), sulfate ($SO_4^-$), and carbonate ($CO_3^-$) all serve as inorganic salts of oxygen in order for anaerobic respiration (the use of a cytochrome chain) to take place. Use of the *respiration* term in microbiology does not mean that a microbe breathes air. Instead, it means that a microbe has a cytochrome chain capable of oxidative phosphorylation. In the case of anaerobic respiration, we use inorganic salts of oxygen rather than oxygen itself as the terminal electron acceptor.

*Oxidation of inorganic molecules.* The organisms that gain energy using inorganic molecules such as sulfide, iron, ammonia, and hydrogen do not

contaminate consumer products. These are known as chemolithotrophs and usually exist in extreme environments. Since they do not use organic molecules for energy, a carbon-rich environment such as a consumer product is not a milieu in which these organisms will grow.

### *Biosynthesis*

Our discussion of biosynthesis will be limited to heterotrophic organisms. We will not cover photosynthesis and chemosynthesis (fixation of carbon dioxide) because autotrophic organisms that utilize these processes would not be expected to contribute significantly to consumer product contamination.

*Synthesis of carbohydrates and polysaccharides.* The synthesis of glucose rather than its breakdown is achieved via the process of gluconeogenesis — which is essentially a reversal of glycolysis. Only three of the ten steps involved in glycolysis are different and require separate enzymes to catalyze the reverse reaction. Otherwise, the remaining seven steps that allow glycolysis are simply reversed. In addition, fructose is made via the same pathway. Glucose and fructose serve as the foundations for many other sugars and for phosphorylated derivatives of those sugars. Polysaccharides are then made from the phosphorylated derivatives of sugar nucleosides and used to construct cell walls of bacteria.

*Synthesis of purines, pyrimidines, and nucleic acids.* Since purines and pyrimidines make up the DNA and RNA of cells, they are critical for survival and reproduction. They are also involved in the formation of phosphorylated compounds like ATP used to produce energy.

*Purines and pyrimidines.* Purine (adenosine and guanosine) biosynthesis involves eleven steps, seven other cofactors, and folic acid. The requirement for folic acid allows us to capitalize on sulfonamides as antibiotics because they block the ability of bacteria to synthesize folic acid. We get all our folic acid from our food and can absorb it directly, whereas bacteria must manufacture their own. The first few steps involve production of inosinic acid that is then formed onto the ribose-5-phosphate sugar. After this structure has been formed, the steps for producing adenosine and guanosine monophosphate and then phosphorylating them into triphosphates are fairly simple and involve vitamin $B_{12}$ as a cofactor and thioredoxin as a reducing agent.

Pyrimidine biosynthesis begins by reacting aspartic acid (an amino acid) with carbamoyl phosphate (a high energy molecule formed from $CO_2$ and glutamine) to form orotic acid, the first pyrimidine product. The ribose sugar is then added. A simple decarboxylation of the orotidine monophosphate yields uridine that can be phosphorylated to uridine triphosphate (UTP); a transamination of the UTP produces cytidine triphosphate (CTP). If the ribose of the UTP is decarboxylated and the uridine is methylated with the use of folic acid, we form deoxythymidine monophosphate.

*DNA replication.* Now that we have the basic building blocks of RNA (UTP, CTP, ATP, and GTP) and DNA (dTTP [thymidine triphosphate], dCTP, dATP, and dGTP), we can begin DNA replication. RNA synthesis is covered in the next section. DNA is a polymer of four nucleotides: dTTP, dCTP, dATP, and dGTP (the *d* stands for *deoxy*, signifying that one less hydroxyl than is found in RNA is gone from the sugar portion of the nucleotide). As DNA is made, two complementary strands coil around each other in a double helix form. The easiest way to visualize the structure is to picture a ladder whose two railings represent the two deoxyribose-phosphate strands. The steps serve as crosslinks of purines and pyrimidines between the two strands. The ladder is twisted or coiled into a spiral. Bacteria carry enough information in their DNA to equal about 40 novels of 400 pages of words. A single human cell carries enough information in its DNA to equate to about 40,000 novels.

When DNA replication begins, the double strand comes apart. A useful metaphor to help envision this process is the unzipping of a zipper. Once the strands are "unzipped," free floating nucleotides present in the cell match with their complements on both sides of the two strands. Thus, a new strand is formed along each of the open strands and a single DNA molecule becomes two.

To understand the process in detail requires far more effort. In reality, at least seven enzymes are involved in the process described in the paragraph above: initiator protein, helicase, polymerases, repair nucleases, topoisomerase, single-strand DNA-binding proteins, and DNA ligase. The initiator protein first finds the right place to begin copying and guides the helicase to the correct position (an origin of replication site) on the nucleic acid. The helicase separates the DNA by breaking the weak bonds between the nucleotides to unwind the two strands of DNA. Then the polymerases arrive to join the free nucleotides to their matching complements on the old strands using the phosphate bond energy from the nucleotide to help form the new bond to the other nucleotides as they are added to the existing chain. These polymerases work along with primases that first synthesize a short (one to five nucleotides long) RNA primer. This primer allows DNA polymerase to begin catalyzing the addition of nucleotides to a new strand complementary to the existing template upon which the new DNA synthesis is based.

Because DNA replication must follow a specific direction and the two strands of DNA are antiparallel, two polymerases are involved. One is continuously adding new nucleotides to what is called the leading strand as the DNA separates due to the helicase. The other is called the lagging strand; it runs in the opposite direction of the leading strand. However, the polymerase attached to the lagging strand cannot advance in the opposite direction and must follow in the same direction as the polymerase attached to the leading strand. As a result, the solution to this apparent dilemma is to have the polymerase make a loop in the lagging strand and add nucleotides along the bottom half of the strand. As the lagging strand polymerase finishes a

length, it drops the completed end and attaches to a new loop to continue linking nucleotides along a new stretch of DNA in a stop-and-start fashion.

This process leaves a series of completed double helix fragments of DNA called Okazaki fragments (named after their discoverer), separated by incomplete gaps along the DNA strand. These gaps are filled in by the DNA ligase enzyme. Meanwhile, leading strands are made continuously. A variety of single stranded DNA binding proteins keeps the single strands apart until the polymerase and ligase can complete the addition of complementary nucleotides. The topoisomerases relieve the tension on the double helix in advance of the gyrase. Repair nucleases recognize errors in replication and remove incorrect nucleotides to allow the polymerases and ligases to replace them with the correct nucleotides.

*RNA synthesis (transcription).* Information storage is useless unless some mechanism is available to act on the information. Cells have such mechanisms.

The mechanism can be understood by considering the manufacturing metaphor of the use of a standard operating procedures (SOP) manual. Imagine a task such as sanitizing a large piece of equipment. The technician's first step is to consult the SOP manual (the equivalent of DNA) to obtain the instructions covering the sanitization method. He copies the information (the equivalent of ribonucleic acid [RNA]) and carries it to the factory floor instead of carrying the entire book or even the original page to the factory floor. This is only the first step, of course. The information taken to the factory floor must be acted upon. This step will be discussed when we cover synthesis of amino acids and proteins in the next section. The process of copying information from DNA to RNA is known as transcription.

Many of the mechanics of DNA replication are involved in transcription. The DNA double helix is opened up and a new nucleotide chain is made, for example. But many significant differences also exist. In transcription, only one of the DNA strands is used as a template; it is called the sense strand. Only a few genes are copied at a time, not the entire genome as occurs in replication.

The most significant difference, however, is that the nucleic acid produced is RNA, not DNA. This compound is basically the same as DNA but it has a different sugar, ribose, instead of deoxyribose, and it uses a uracil nitrogenous base rather than thymine. Thus, the process involves the RNA polymerase enzyme.

This is a versatile enzyme that finds the starting point of the gene (the Pribnov box), opens the double helix, copies it, and then closes it, releasing messenger RNA (mRNA) to be acted upon in the next step of protein synthesis or translation. The system knows when to stop transcribing because an ending point of the gene carries a sequence of the code instructing the mRNA to form a hairpin loop that causes the polymerase to stop transcribing. Transcription also allows for the production of two other types of RNA polymers: transfer RNA (tRNA) and ribosomal RNA (rRNA). Transfer RNA

carries amino acids during protein synthesis. Ribosomal RNA is the material of which ribosomes and proteins are made; ribosomes are critical for synthesizing protein.

There are a few differences in the processes by which eukaryotes and prokaryotes transcribe DNA to RNA. For the most part, they are limited to post-transcriptional modifications. In prokaryotes, no additional modification of the message is needed (except for some post-transcriptional modification in certain cyanobacteria and archeobacteria). However, in eukaryotes, a significant amount of mRNA is removed and not expressed since these are intervening sequences (introns); the sequences that are expressed (exons) are spliced together to leave the nucleus as a single mRNA molecule composed of several exons.

*Synthesis of amino acids and proteins (translation).* The final act in expressing the genes of DNA is to translate the transcribed message where the cell makes sense (or translates) from the mRNA. This process involves ribosomes to translate the information in the mRNA into proteins (polymers of amino acids). These strings of amino acids twist and turn and fall into shape on their own just because of their sequence to form the enzymes and other proteinaceous structural components of the cell. The first question to address, though, is where do the amino acids come from?

*Amino acid synthesis.* Simply put, the amino acids come from the digested polymers ingested by the cells. They can come from protein catabolism or from the intermediates of carbohydrate catabolism that are diverted from the catabolic process into the anabolic process of making amino acids. The production of amino acids requires energy in the form of ATP. Thus, these synthetic processes only occur when plenty of ATP is available from catabolism and the intermediates can be diverted away from the process of energy generation.

The first step in synthesizing amino acids is to assimilate nitrogen. One mechanism to do this is nitrogen fixation. The process occurs predominantly in ecologically related bacteria such as *Rhizobium*, *Azotobacter*, and the cyanobacteria. It also occurs in certain *Klebsiella* and *Clostridium* organisms.

The latter two are sometimes contaminants of personal care products, so we will touch briefly on nitrogen fixation. Realize, however, that nitrogen fixation is primarily a function of organisms that are not of concern to the cosmetic or drug microbiologist. Nitrogen fixation is the reduction of atmospheric gaseous nitrogen into ammonia by nitrogenase. It requires at least 6 electrons for reducing power and 12 ATP molecules.

Other more common mechanisms for assimilating nitrogen are ammonia incorporation and assimilatory nitrate reduction. Ammonia is easily incorporated into amino acids by forming the alanine amino acid directly by amination of pyruvate using the alanine dehydrogenase enzyme. Alternatively, a cell can form glutamate (an amino acid) by aminating α-ketoglutarate (a TCA cycle intermediate) using the glutamate dehydrogenase

enzyme. Once these two amino acids have been formed, the ammonia they carry (now called an α-amino group) can be transferred to other carbon skeletons of other catabolic intermediates by transamination to form several other amino acids.

Other mechanisms of assimilating nitrogen include use of two enzymes in tandem (glutamine synthetase and glutamate synthetase) with the same net result of the formation of glutamate from α-ketoglutarate. Nitrate can also be assimilated by forming ammonia using the nitrate reductase enzyme and electrons from the reaction of NADPH + $H^+$; the ammonia formed is then assimilated using the processes described above.

Most bacteria assimilate nitrogen by the processes described above. Once assimilated, the nitrogen is then transformed into the 20 amino acids that constitute the proteins organisms use. Other microbes assimilate nitrogen already in the form of amino acids via the breakdown of proteins. The details about biosynthesis of amino acids from basic carbon skeletons can be found in introductory biochemistry textbooks.

We will limit the discussion here to cover the general concepts of amino acid biosynthesis. We have already highlighted the formation of two amino acids (alanine and glutamate) from amination of pyruvate and α-ketoglutarate. Simply put, the rest of the amino acids are derived from similar processes but start with other intermediates of glycolysis and the TCA cycle. For example, aspartate is formed by transamination of oxaloacetate. Lysine, threonine, isoleucine, and methionine are then synthesized from modification of aspartate. Phenylalanine, tyrosine, and tryptophan are formed from intermediates of the pentose phosphate pathway. Glutamine, proline, and arginine are formed by modifying glutamate. Serine, glycine, and cysteine are formed from 3-phosphoglycerate. Valine and leucine are from pyruvate and acetyl-CoA.

*Ribosome and tRNA involvement.* Now that we understand the manufacture of amino acids, we need to put them together into proteins via a step known as translation. It is carried out by ribosomes that read (translate) the mRNA and involves tRNA (transfer RNA) that carries the amino acids to the ribosomes (composed of ribosomal RNA and protein). Before going further, however, we should understand the nature of the triplet code of nucleic acids that codes for each of the 20 amino acids required to make a protein.

The DNA molecule is composed of four nucleotides composed of a nitrogenous base and a sugar. The bases that make each of the four nucleotides different are guanine, cytosine, thymine, and adenine. When mRNA is made, the sequence of nucleotides is transcribed into the bases that pair with the DNA message: adenine pairs with thymine, but uracil is used instead in RNA. Thymine pairs with adenine, guanine pairs with cytosine, and vice versa. Each of the 20 amino acids is represented by a three-letter code of nucleotides. For example, AUG means methionine. However, the code is degenerate in the sense that the nucleic acid in the third position can

be any of several choices that produce the same result. Thus, for example, CCC, CCU, CCA, and CCG all code for proline. This sloppy base pairing is referred to as wobble and helps in decreasing the ill effects of DNA mutations. These same codes are also present in tRNA; specific tRNAs carry specific amino acids.

Protein synthesis is the final step in gene expression. In the following process known as translation, the mRNA nucleotide sequence is translated into the amino acid sequence of a protein. The first stage is amino acid activation by which the amino acids are attached to tRNA molecules. At one end of the tRNA molecule is a three-nucleotide anti-code that is able to match up with a complementary three-nucleotide code on the mRNA. On the other end, an attached amino acid is specific for the tRNA carrying the appropriate three-nucleotide code. An enzyme known as amino acid activating enzyme (or aminoacyl-tRNA synthetase) energizes each amino acid in order to attach it to the opposite end of the tRNA. The specificity (which amino acid goes with which tRNA) is determined by the specificity of the synthetase. Once the amino acid has been attached to one end of an adaptor, it must be linked into a chain with other amino acids in a specific order to produce a specific protein.

The first tRNA carrying a start codon binds to the free 30S subunit at the peptidyl site. Then the mRNA with the correct start anticodon attaches to the 30S subunit followed by attachment of the larger 50S ribosome subunit. In some prokaryotes, the mRNA binds first to the ribosome followed by the binding of the initiator tRNA to the ribosome. The second tRNA enters the second aminoacyl site. The amino acid carried by the first tRNA is shifted over and bound to the amino acid carried by the second tRNA. The mRNA then shifts to the right and the first tRNA drops off while the second tRNA shifts into the peptidyl site leaving the aminoacyl site free to accept another tRNA carrying another amino acid. One by one, the triplets are read and the protein chain grows until the final triplet that lacks an adaptor signals "stop." The ribosome then separates and releases the mRNA. The protein chain formed then undergoes self-assembly as it folds and associates with other protein chains to form enzymes and structural proteins.

*Synthesis of lipids.* Fatty acid synthesis is catalyzed with fatty acid synthetase using acetyl-CoA and malonyl-CoA as the substrates and NADPH as the reductant. Malonyl-CoA comes from an ATP-driven carboxylation (addition of $CO_2$) of acetyl-CoA that comes from glycolysis. Both acetate and malonate are transferred from coenzyme A to the sulfhydryl group of the acyl carrier protein (ACP) that carries the growing fatty acid chain during synthesis. The synthetase adds two carbons at a time to the carboxyl end of the growing fatty acid chain in a two-stage process. First, malonyl-ACP reacts with a fatty acyl-ACP and yields $CO_2$ and a fatty acyl-ACP that is now two carbons longer. Glycerol can be esterified to fatty acids to result in phosphatidic acid and then to phospholipids that are essential to membrane function.

*Synthesis of peptidoglycan.* Peptidoglycan consists of long polysaccharide chains made of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG). Pentapeptide chains are attached to the NAM groups. The chains are then attached to each other either directly by the pentapeptide chains (Gram-negative bacteria) or via a pentaglycine bridge (Gram-positive bacteria). During synthesis of this complex cell wall constituent, two key carriers participate: uridine diphosphate (UDP) and bactoprenol. Bactoprenol is a large 55-carbon alcohol that attaches to the NAM by a pyrophosphate. It moves peptidoglycan subunits back and forth through the hydrophobic membrane.

Eight stages occur in the synthesis of peptidoglycan. UDP derivatives of NAM and NAG are made in the cytoplasm followed by the addition of five amino acids to the UDP-NAM molecule with the expenditure of ATP. This NAM-pentapeptide is then transferred from UDP to the bactoprenol carrier at the inner membrane surface. UDP-NAG then adds NAG to the NAM-pentapeptide to form a peptidoglycan unit.

In Gram-positive organisms, the pentaglycine bridge may also be added at this time. The fifth step involves transporting this unit across the membrane to the outer surface of the membrane by the bactoprenol carrier. The peptidoglycan unit is attached to the growing end of a peptidoglycan chain to lengthen it by one repeating unit, and the carrier, now free, can return to the inside of the membrane to accept another NAM-pentapeptide. Finally, peptide cross-links form between the peptidoglycan chains by transpeptidation.

Peptidoglycan synthesis is one of the main sites of attack by antibiotics. There are several sites of attack available. Penicillin stops the transpeptidation reaction and bacitracin blocks dephosphorylation of bactoprenol pyrophosphate.

## *Growth*

Growth refers to an increase in cellular constituents that results in either an increase in a microorganism's size, population number, or both. In bacterial growth, it usually refers to population increases due to binary fission.

### *Growth curve*

When organisms are grown in batch cultures, they are incubated within a closed flask with a fixed amount of medium. During growth, the available nutrients are used and the wastes from metabolism build. Growth of a bacterial population can be determined by plotting the logarithm of live cell numbers against the incubation time to yield a typical growth curve with four distinct phases: lag, exponential, stationary, death.

When microorganisms are first introduced into fresh media, no immediate increase in cell numbers occurs. This is referred to as the lag phase. All cultures go through a lag phase if the medium is different from the one the organisms were transferred from or when the organisms are not in exponen-

tial phase. The lag phase may be the result when cells are old and depleted of ATP or it may be due to essential cofactors or ribosomes that must be synthesized before binary fission can occur. The lag phase is especially long if an inoculum is from an old culture. Sometimes the lag phase can be avoided entirely if an inoculum is transferred during log phase into a fresh medium of the same composition.

During the exponential phase, the population divides at its maximal rate possible by doubling at regular intervals. Because the population is composed of individuals that divide nonsynchronously, the curve rises smoothly. However, the individuals are remarkably uniform during this phase, and for this reason cultures should be harvested during this stage for use in most studies including preservative challenge tests. Waiting longer (until the stationary phase) allows the cells to become more resistant to a biocide or preservative.

Ultimately, the population reaches the stationary phase in which the total number of live microorganisms remains constant due to cell death rate equaling the cell division rate. Alternatively, the phase may be reached because the cells simply cease dividing despite remaining metabolically active. Organisms enter the stationary phase for several reasons: nutrient limitation, oxygen limitation, or the accumulation of toxic waste by-products such as acids. These factors may arise singly or in concert.

During death phase, the number of viable cells decreases exponentially as they die. The rate of death may decrease over time due to the extended survival of particularly resistant cells.

### *Mathematics of growth*

During the exponential phase, the population divides at constant intervals and doubles during a specific period called the doubling or generation time. Because the population doubles with every generation, the increase in population is always $2^n$ where $n$ represents the number of generations. To understand the concept of exponential increase, consider a pay scale by which an individual receives a penny the first day of work; the penny doubles to two pennies on the second day of work, then the amount received doubles every day thereafter. After 30 days, the individual's daily pay will amount to $10,737,418.24. The equation by which money is compounded daily at 100% interest for 30 days is the same as the equation for determining the rate at which a single bacterium is "compounded" every 20 minutes at the equivalent of 100% interest:

$$N_T = N_O \times 2^n$$

where $N_O$ is the initial population number, $N_T$ is the population number after some time $T$, and $n$ is the number of generations in time $T$.

Knowing this rate of growth allows us to appreciate the concern when a laboratory finds that a single organism exists in a mix tank of 30,000 gallons

(75,000 liters). Over a weekend, the division of that one organism every 20 minutes could result in $1.05 \times 10^{65}$ organisms in the tank or $10^{57}$ organisms for every milliliter in the 75,000-liter tank. Of course, the culture would have already reached stationary phase and entered the death phase with no more than $10^9$ cells per milliliter as a maximum long before such high numbers were reached. In fact, the $10^9$ cells in each of the 75 million milliliters in the 30,000-gallon mix tank was reached after 50 generations or just sooner than 17 hours after the discovery of the lone organism in the tank.

### *Measurement of cell numbers*

One of the easiest ways to determine the numbers of microorganisms in a sample is to count them directly under a microscope. In order to count them accurately, a Petroff–Hauser counting chamber can be used since such devices have chambers of known volume. One disadvantage of using microscopic counts is that a count may not be accurate if a population is dilute. Furthermore, operator fatigue can be a significant factor and may be overcome by the use of image analysis and recording films. Finally, the ability to distinguish live and dead cells is difficult but can be overcome somewhat with the use of viability stains.

Particle counters such as a Coulter Counter can be useful for quantifying protozoans, algae, and nonfilamentous yeasts. Bacteria are not countable with this method because of interference from particles and filaments. As an organism passes through a small hole, an electrical current is interrupted and the interruption sends a signal to be counted.

Perhaps the most common microorganism counting technique, especially for bacteria, is the plate count. A diluted sample containing the bacteria is spread over a medium of nutrient agar contained in a Petri plate. After incubation, the organisms grow into large collections of several million that can be seen and counted and an estimate of the numbers in the original sample can be made based on colony-forming units. This estimate is based on the fact that bacteria appear as clumps of cells and rarely exist as separate cells in nature. Thus, when they are spread out on a plate, the units that form a colony are often composed of several organisms stuck together rather than individual organisms. Membrane filters can also be used to trap microbes as they are filtered. The filter is then placed onto an agar surface and the nutrients are absorbed by the filter to provide the organisms an opportunity to grow into colonies.

### *Continuous cultures*

In batch cultures, the wastes are not removed and the nutrients are not replenished. The typical growth curve exists when the exponential phase lasts only a few generations. In nature, however, many systems are open — nutrients flow into the system and wastes flow out of the system. Such systems can be built in a laboratory as continuous cultures. These systems are very useful for studying interactions of microbial species and for mim-

icking natural lake and stream ecosystems. They are even useful for understanding growth in manufacturing systems that resemble plug-and-flow continuous cultures.

In continuous cultures, the flow rate can be varied as it flows into a constant volume container such that for every drop of fresh medium that drips into the culture vessel, a drop of spent medium drips out. A microbial population can be maintained in the exponential phase and at a constant biomass as long as the medium continues to flow into the culture vessel. The rate of dilution is controlled entirely by the flow rate and the volume of the vessel. In such a system that mimics natural systems as well as manufacturing systems, growth is controlled by the limiting nutrient. The dilution rate (D) controls the rate of nutrient provided and is related to the flow rate in ml/hr (f) and the volume of the culture vessel (V) by the following relationship:

$$D = f/v$$

The dilution rate is expressed as units per hour. As the dilution rate increases, the population's growth rate increases to match it until the dilution rate exceeds the growth rate and the organism washes out faster than it can reproduce. At dilution rates that are just below the wash-out rate, the organisms increase in both cell density and growth rate compared to dilution rates that are lower where cell density is lower and growth rate is also slower.

At slow dilution rates, only a limited amount of nutrient is available and nearly all the energy derived from the nutrient is used quickly for cell maintenance alone rather than for growth and reproduction. As the dilution rate is raised and more nutrients are provided, energy becomes available for growth as well as maintenance. Thus a certain amount of maintenance energy must be exceeded before growth can take place.

### *Environmental growth conditions*

Often the only way we control microbial growth is through biocides and preservatives. However, we should also consider all the chemical and physical parameters that must be provided to allow for growth to occur. Understanding these parameters may allow us to rely less on biocides and more on creating products that are inherently hostile to microbial growth because of their physical and chemical composition even if no preservatives are added. Thus, we may be able to limit growth in a manufacturing plant with these same parameters and reduce our reliance on chemical biocides.

*Solutes and water activity.* Most microorganisms exist in hypotonic solutions in nature. Thus, water enters cells through their semi-permeable membranes and would cause cells to burst if it were not for the rigid cell walls that maintain the shape and integrity of the organism. Clearly, organisms without cell walls such as *Mycoplasma* require isotonic conditions for growth. Many bacteria actually concentrate the contents of their protoplasm

in order to keep water flowing into them, thus keeping their plasma membranes pressed firmly against the cell walls. Bacteria increase their osmotic concentration by synthesizing compatible solutes such as choline, betaine, amino acid, and potassium ions.

A far more devastating condition for many bacteria is hypertonicity. Whenever solutes are more concentrated on the outsides of cells than they are on the insides, water leaves the cells and the plasma membranes shrink away from the walls; this is known as plasmolysis. It effectively constitutes dehydration and may damage cells, especially the membranes where energy processes occur. The cells then become metabolically inactive and stop growing. Duplication of such conditions can serve to control the growth of organisms.

We can actually reduce the amount of water available to microorganisms by interaction with solute molecules or surfaces. In the former, the water is bound by the solutes in the environment; whereas in the latter, the water is adsorbed to surfaces of solids. Thus, one cannot simply assume that an environment that has plenty of water will make the water available to organisms. Expressing the amount of water available to an organism quantitatively is done by relating the vapor pressure of a solution to the vapor pressure of water:

$$A_W = P_{soln}/P_{water}$$

Another way of thinking is to realize that water activity is a measure of the relative humidity of a solution if the solution were placed in a sealed chamber after the chamber came to equilibrium. Water activity is inversely related to the osmotic pressure of a solution. The lower the water activity, the higher the osmotic pressure and the less likely organisms are to grow. Most organisms prefer growing at water activity rates of 0.98 to 1.0. A few are osmotolerant and can survive water activities as low as 0.6, but these organisms are generally nonpathogenic.

The addition of a wide variety of water binding molecules in a formulation can actually tie up the water, resulting in a low water activity ($A_W$) rate. Thus, this avenue of preservation should not be overlooked. For example, many antiperspirants and dry make-up powders have extremely low $A_W$ levels and do not support growth unless water present in skin debris is placed into the products during use. As a result, preservative challenge tests are meaningless for these products and should be replaced with water activity measurements and consumer use data. Table 2.2 lists key water activity points, organisms inhibited by low water activity, and the effects of pH, a controlling factor described further below.

*pH and temperature.* The pH term refers to the measurement of hydrogen ion concentration of an environment on a logarithmic scale from 0 to 14. A pH of 0 represents the high concentration of 1.0 M hydrogen ions ($H^+$)

***Table 2.2*** Water Activity and Potential for Growth

| Water Activity | pH Level | Problem Organisms | Examples |
|---|---|---|---|
| 0.98 to 1.00 | 5 to 9 | Gram-positives and -negatives | Shampoos and emulsions |
| 0.95 to 0.97 | 5 to 9 | Gram-positives and -negatives; *Pseudomonas* limited | Liquid make-up; eye area products |
| | Below 5.5 | Gram-positives and -negatives; *Pseudomonas* limited | Hair conditioners |
| 0.92 to 0.95 | Above 5.5 | Gram-positives; few negatives | Pressed powders |
| | Below 5.5 | Gram-positives | |
| 0.90 to 0.92 | 5 to 9 | Lactobacilli and *Staphylococcus* | Rouges |
| 0.80 to 0.90 | 5 to 9 | *Staphylococcus,* molds, yeasts | Lipsticks |
| 0.70 to 0.80 | 5 to 9 | Molds, yeasts | Some talcs |
| 0.65 to 0.70 | 5 to 9 | Osmotolerant yeasts | Some antiperspirants |
| 0.60 to 0.65 | 5 to 9 | Osmotolerant molds | |
| Below 0.60 | 5 to 9 | None | |

and a pH of 14 is the low concentration of $10^{-14}$ hydrogen ions. Microorganisms grow in environments ranging from a pH of 1 to 10.

The only microbes of concern for microbiologists involved in keeping consumer products free of microorganisms are the neutrophiles that grow at pH 5.5 to 8.0 because they are also potential pathogens. Unless spoilage organisms that grow below 5.5 or above 8.0 are present in a product, they are of minimal concern to the consumer product microbiologist. Table 2.3 shows the consumer product and environment types and organisms present at all pH values from 0 to 14.

Many products do not support the growth of potential pathogens if the pH value is below 4.5 or above 10. The only reason for adding biocides to these products is to control potential spoilage organisms.

Temperature affects microorganisms because they have no way to control their own temperatures and because enzymatic reactions required for growth are affected by temperature in the same way any other chemical reaction is affected: the rate of reaction roughly doubles for every 10°C rise in temperature. At low temperatures, the enzymes operate more slowly and thus growth is also slower. Up to a certain temperature, growth increases until the point is reached where high temperatures are lethal because they damage cells by denaturing proteinaceous enzymes, destroying transport proteins, and destroying lipid membranes. The key or cardinal temperatures to consider for each species of microbe are the minimum, optimum, and maximum. Temperature ranges for the major groups of organisms of concern are shown in Table 2.4.

***Table 2.3*** pH Related to Types of Environments, Consumer Products, and Types of Organisms Present

| pH | Environment/Consumer Product Example | Organism Present |
|---|---|---|
| 0 | Concentrated acids | A few Thiobacilli |
| 1 | Acid thermal springs; gastric acids | Thiobacilli and thermoacidophiles |
| 2 | Lemon juice | Thiobacilli |
| 3 | Vinegar | Some Lactobacilli and *Leuconostoc* |
| 4 | Tomatoes, orange juice, hair conditioners | Lactobacilli and some *Staphylococci* |
| 5 | Cheese, bread, skin lotions, make-ups | Some pseumonads, *Lactobacillus* |
| 6 | Beef, chicken, milk, shampoos | *Pseudomonas aeruginosa* |
| 7 | Water, blood, shampoos, mascaras | *Staphylococcus aureus, E. coli* |
| 8 | Seawater, some shampoos | *Nitrosomonas*, bacilli |
| 9 | Alkaline soils and lakes; some deodorants, antiperspirants, skin cleansers | *Anabaena*, some bacilli |
| 10 | Soap | *Microcystis*, some bacilli |
| 11 | Household ammonia | Some bacilli survive |
| 12 | Drain openers | Spores survive |
| 13 | Bleach | Spores sometimes survive |
| 14 | Saturated sodium hydroxide | Spores sometimes survive |

***Table 2.4*** Temperature Ranges for Key Microorganisms Found in Consumer Products in Comparison with Key Extremophiles

| Microorganism | Min. Temp. (°C) | Opt. Temp. (°C) | Max. Temp. (°C) |
|---|---|---|---|
| *Micrococcus cryophilus* | –4 | 10 | 24 |
| *Pseudomonas cepacia* | 4 | 25 to 30 | 40 |
| *Staphylococcus aureus* | 6.5 | 30 to 37 | 46 |
| *Enterococcus* spp. | 0 | 37 | 44 |
| *Escherichia coli* | 10 | 37 | 45 |
| *Pyrodictium occultum* | 82 | 105 | 110 |

Microorganisms can be placed into four categories based on their temperature ranges. Psychrophiles grow between 0 and 20°C and are found throughout Arctic and Antarctic regions as well as in man-made refrigerated environments. In alpine snowfields, the psychrophilic alga, *Chlamydomonas nivalis*, produces pink snow. Psychrophilic bacteria include members of the genera *Pseudomonas*, *Flavobacterium*, *Achromobacter,* and *Alcaligenes*. Facultative psychrophiles grow optimally at 20 to 30°C and can spoil refrigerated foods. Few, if any, of these organisms present problems for consumer products because they only act as spoilage organisms and consumer products are rarely kept at

low temperatures. Psychrophiles and psychrotrophs are not pathogens, although some produce toxins that, if ingested, may cause problems.

Mesophiles can grow from 20 to 45°C although their optimum temperatures are from 25 to 40°C. Nearly all human pathogens are mesophiles because human body temperature is 37°C. Mesophiles are the key problem organisms for a consumer product microbiologist to control. The key mesophile species that require control in manufacturing environments include *Pseudomonas cepacia*, *Pseudomonas maltophilia*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Enterobacter agglomerans*, *Enterobacter gergoviae*, *Escherichia* spp., *Staphylococcus* spp., and some yeasts and molds. Thermophiles can grow between 45 and 110°C although the typical range is 55 to 85°C.

*Oxygen.* Many organisms encountered in a manufacturing environment are obligate aerobes — organisms that grow in the presence of atmospheric oxygen ($O_2$). Oxygen is critical for these organisms since it serves as the terminal electron acceptor for the electron transport chain that generates ATP during aerobic respiration. Other organisms are microaerophiles that require some oxygen but not at levels encountered in the atmosphere.

Some organisms are facultative anaerobes; they do not require oxygen but fare better when they have it. Aerotolerant anaerobes grow whether or not oxygen is present because they are not affected by it. Occasionally encountered, but relatively rarely in a manufacturing environment are obligate anaerobes, organisms that grow in the absence of oxygen. Although strict anaerobes are killed by any exposure to oxygen, they can be recovered from habitats that appear to be aerobic. This is especially true for organisms that associate with facultative anaerobes in communities where the facultative anaerobes serve as oxygen scavenging guilds to deplete any available $O_2$. Table 2.5 summarizes oxygen status of certain bacteria.

One of the key reasons for these differences in oxygen tolerance among organisms is the effect of toxic oxygen by-products of metabolism. Oxygen accepts electrons and then becomes reduced into superoxide radical, hydrogen peroxide, and hydroxyl radical prior to its breakdown into water. Obligate aerobe organisms need enzymes that protect against these toxic $O_2$ products. Such organisms evolved the superoxide dismutase and catalase enzymes as they developed the more efficient mechanisms of energy gener-

***Table 2.5*** Oxygen Requirements and Presence of Key Enzymes in Selected Bacteria

| Oxygen Tolerance | Superoxide Dismutase | Catalase | Examples |
|---|---|---|---|
| Obligate aerobes | + | + | *Pseudomonas, Bacillus* |
| Facultative anaerobes | + | + | Staphylococci, *Enterobacter* |
| Microaerophiles/aerotolerant anaerobes | + | – | *Enterococcus* spp. |
| Obligate anaerobes | – | – | *Clostridium* spp. |

ation using oxygen-based metabolisms. The relationship between these enzymes and oxygen tolerance is also shown in Table 2.5.

## *Diversity*

One of the fascinating and somewhat bewildering aspects of the microbial world is its extreme diversity. The microbes, it seems, have the ability to adapt to nearly any environment they encounter. As a result, a variety of physiologies have evolved such that some species break down sugar while others use sulfur and iron for energy. Some live at ambient temperatures and others live at either freezing or boiling temperatures. Some need organic compounds for growth, while others fix carbon dioxide.

No less amazing is the ability of these organisms to adapt to biocides. The diversity of these organisms is due to their ability to evolve so rapidly because their generation times are so short and their fecundities are so great.

Several factors contribute to the ability of microorganisms to adapt and become tolerant to biocides. The classic tolerance mechanism is based on mutation and plasmid resistance, which we discuss below. However, the physical mechanisms that protect organisms from biocides include growing within a biofilm and even growing within a community of several species where some organisms within the community provide protection for others. Additionally, the classic principle of Darwinian selection of some phenotypic trait that contributes to tolerance is a factor.

A bacterium may even over-express a specific gene for the production of some trait that confers tolerance against a biocide without changing its genotype. These mechanisms are simple "hairy elephant" stories: when the weather gets colder, only the elephants that have more hair — a characteristic distributed along the normal curve — will survive to reproduce. During exposure to biocides, only those bacteria that have thicker capsules will survive to achieve subsequent reproduction and the population will shift to those that produce plenty of slime.

### *Role of mutation*

Mutations are usually detrimental to organisms. Typically, for life to exist with stability, the nucleotide sequences of genes must not be disturbed. However, occasional sequence changes that are not detrimental occur. These changes generate new variability and, if they by chance confer some selective advantage to the organism, they allow for evolution of the population into a new strain or even a new species that can better survive the new environment.

If the new environment includes a biocide, then a tolerant population results. Mutations occur either spontaneously in the absence of any mutation-inducing agents or as a result of exposure to a mutagen. In the latter case, if the biocide also shows mutagenic properties, then it may actually "direct" the microbe to mutate (however, in a completely random way) and thus evolve resistance in what appears to be an almost Lamarckian mechanism.

Spontaneous mutations may be transition mutations in which the wrong base is paired with the template base. For example, the purine base (adenine or guanine) may pair with the wrong pyrimidine (cytosine or thymine), respectively, due to tautomeric shifts. Although very rare, mutations may also be transversion mutations in which a purine is substituted for a pyrimidine or vice versa. Spontaneous mutations may also result from frameshifts caused by the deletion of DNA segments that results in an altered codon reading frame.

Induced mutations are caused by agents that: (1) damage the DNA, (2) alter its chemistry, or (3) interfere with its repair mechanisms. These agents, known as mutagens, act by using base analogs in place of the required base, mispairing of the base, or intercalating a base into the DNA. If the damage occurs to the DNA before it can be repaired and a complete DNA replication cycle takes place before the site is repaired, then the mutation can become stable and inheritable if it confers some selective advantage for the organism.

*Recombination in prokaryotes*

The ability to recombine genetic information in sexually reproducing organisms is an efficient way to insure that diversity occurs in order that adaptation to changing environments (e.g., evolution) can have a chance. The use of a biocide represents a drastic change in the environment for a microorganism. Fortunately for the bacterium, adaptation mechanisms are in place to overcome the environmental disturbance caused by the biocide. Many of these adaptation mechanisms arise from mutations as described above. However, these mechanisms are relatively ineffective in maintaining the diversity needed.

Certainly, once a mutation occurs in a single bacterium and it reproduces rapidly, the favorable gene is spread throughout the progeny of the asexually reproducing clone. But the bacteria need a way to transmit the favorable mutation in more efficient ways. Because bacteria do not reproduce sexually, how do they reproduce to enhance their diversity? How do they adapt to changing environments? Keeping in mind that the key goal in sexual reproduction is recombination of genetic material, just how do bacteria achieve gene recombination without sex?

The most common way bacteria accomplish this task is via general recombination involving a reciprocal exchange between pairs of homologous DNA sequences. It occurs anywhere on the chromosome where a homology exists with another DNA strand where a break and subsequent cross-over can occur. Many of the enzymes involved in carrying out this recombination are the same as those involved in DNA repair. In bacterial transformation (detailed below), a nonreciprocal recombination occurs where sections of genetic material are inserted into the chromosome by incorporating a single strand of DNA that forms a stretch of heteroduplex DNA. Although the one strand is homologous, it is inserted into the genome as a complementary strand to the original and not duplicated.

A second type of recombination is site-specific, typified by the integration of a virus genome into its host cell's genome. The genetic material is not homologous with the bacterial chromosome and in fact works as a mechanism to integrate the genome into the bacterial chromosome using enzymes unique to the virus and its host. A third type is replicative recombination typified by transposons. A gene is replicated and then moved about the chromosome without regard to homology.

Movement of DNA from a donor bacterium to a recipient can occur in three ways: (1) direct transfer of DNA between bacteria by physical attachment via a pilus (conjugation), (2) transfer of DNA from an environmental source to a bacterium (transformation), and (3) transport of DNA from one bacterium to another via bacteriophages (transduction). Once the DNA enters a cell, it may be degraded (host restriction), become integrated into the chromosome especially if homologous, replicate outside the cell's normal genome, or not replicate.

*Plasmids and transposons.* Plasmids are small (usually less than 30 genes), circular DNA molecules that exist independently of main bacterial chromosomes. They replicate completely autonomously from chromosomes and are stably inherited. Some bacteria have multiple copies of the same plasmid, presumably allowing the genes to be transcribed more often if a need for the proteins for which they code is great. Bacteria can survive without their plasmids because, for the most part, plasmids carry information that is not critical for the bacterium's survival.

The classic examples are plasmids that carry R (resistance) factors that code for enzymes to destroy or modify antibiotics. If these resistance genes are within a transposon, bacteria can rapidly develop multiple resistance plasmids. If the plasmids are conjugative, they can spread throughout a population and even between species of bacteria. Some are virulence plasmids that cause the bacterium to better resist host defenses or produce toxins. Others are metabolic plasmids that carry genes for enzymes to degrade specific substances.

Transposons are pieces of DNA that can move around the genome without regard to homologous sites on chromosomes. They can originate in one location on a chromosome and move to a new location on the same chromosome. The actual mechanism of this process has been studied in detail and we will not cover it here.

Transposons produce a variety of important effects. They can insert into a gene to cause a mutation that destroys the expression of certain genes of the host or the loss of those genes. In other cases, they stimulate over-expression of the gene if they carry promoters and activate the genes near the site where the transposon inserts. Thus, they can turn many genes throughout a chromosome on or off, depending on where they insert. In fact, the many colors of Indian corn and the mosaic of colors on the skin of an apple appeared when these jumping genes moved from one spot on a chromosome to the next and told it which colors to express for its particular cell.

Transposons also carry antibiotic resistance genes and can even cause a plasmid to accept additional resistance genes if the transposon carrying the genes transfers into the plasmid. Because the transposons also jump from plasmids to chromosomes, they can cause drug resistance genes to exchange from the plasmid to the bacterial chromosome.

*Conjugation.* This mechanism of recombination in bacteria looks like sex but it is not. In conjugation, bacteria exchange portions of DNA via a pilus from one bacterium to the next. There are even positive "mating" types that are more capable of transferring copies of portions of their genes to another bacterium. Since they are haploid, however, their chromosome number (a single circular chromosome) is never halved and then recombined with another chromosome from the opposite sex as is the case for true sexual reproduction. For a bacterium, there is no opposite sex! Conjugation is an efficient way to transfer genes from one bacterium to another.

*Transduction.* Bacteria, like all living creatures, have viruses that attack them. Viruses are particles of protein and nucleic acid that use the cell's biochemical factory to replicate more viruses. The nucleic acid injected into a bacterial cell from a virus directs the cell to stop performing cell activities and produce viruses instead.

Viral replication, however, is rather sloppy. It is a process of self-replication in which all the viral parts are built and then they come together on their own to form a viral particle simply because the parts are chemically attractive to one another. This is almost a case of putting all the watch parts together in a box, shaking it, and finding that the box contains a complete watch after all the parts simply joined together on their own. The only difference is that the viral parts can fit together only in one way and they have chemical properties that cause the parts to be attractive to each other in very specific ways. Some viruses, however, are very sloppy in this random shuffling together of parts and sometimes incorporate bits and pieces of the bacterial host's own genome rather than their own.

When this happens and the sloppy viruses move on to the next cell, their bacterial host, instead of forming new viruses, will acquire a piece of the genome from the previous bacterium. Because the DNA for making the virus is incomplete, no new viruses are made in the bacterium but it now has a new gene from another bacterium. The virus was an unwitting "patsy" in transferring genes from one bacterium to another. If those genes confer some useful property on the recipient, then it is more likely to survive and reproduce to spread the newly acquired gene throughout the population.

*Transformation.* Transformation is one of the more unusual mechanisms of sharing genes that bacteria have. They simply absorb DNA from the environment. This requires a cell to lyse and spill its genes into the environment, and it also requires a competent cell to absorb naked DNA from the environment. Oddly enough, the cells are competent only during

the exponential phase of their growth cycles when they are also most susceptible to biocides.

The DNA that can be absorbed can be either linear or exist as plasmids. The efficiency of absorbing plasmid DNA is greater than the efficiency of absorbing linear DNA because plasmids are not degraded as easily.

### *Selected bacteria of industrial and medical importance*

The organisms most likely to be encountered in contaminated cosmetic products are those that are likely to be present in an ordinary household. Based on a study of contaminants of cosmetics, the typical organisms involved are those detailed in Table 2.6.

*Pseudomonas.* *Pseudomonas aeruginosa*, *Pseudomonas maltophilia*, *Pseudomonas paucimobilus*, and *Pseudomonas cepacia* (current classification *Burkholderia cepacia*) are ubiquitous microbes found in a variety of environments. They are also very versatile in their nutritional capabilities and adaptational abilities. They seem to adapt quickly to any new exposures to toxic chemicals including biocides. The pseudomonads are infectors of wounds and burns. They also cause pneumonia in patients who take immunosuppressive drugs. In cosmetics, the organism has been implicated in eye infections and loss of sight. When found in a cosmetic manufacturing plant, they usually arise from failure to control and monitor water systems, formation of biofilms in the equipment, ineffective or infrequent sanitization, and dead legs (short lengths of pipes with closed or "dead" ends) or other sources of stagnant product.

Metabolically, the genus *Pseudomonas* is aerobic, using oxygen (and sometimes nitrate) as a terminal electron acceptor. They are Gram-negative rods; they all use the tricarboxylic acid cycle to oxidize substrates to carbon dioxide. Most of the hexoses are degraded by the Entner–Douderoff pathway rather than by glycolysis. Perhaps their most interesting metabolic feature is the ability to degrade complex materials including biocides, various hydrocarbon ring structures, and even chlorinated pesticides with ease.

*Serratia.* The *Serratia* organism is not typically considered a pathogen because infections caused by it are rare. *Serratia* is a member of the Enterobacteriaceae family that includes *Escherichia*, *Enterobacter*, and *Proteus*, and comprises the largest of the three families cited in Section 5 of *Bergey's Manual* (1984) focusing on the anaerobic Gram-negative rods (Vibrionaceae and Pasteurellaceae are the other two families). Like most members of the Enterobacteriaceae, *Serratia* degrade sugars to pyruvate by means of the Embden–Meyerhof pathway (glycolysis), then use the pyruvate as the terminal electron acceptor to yield a variety of end products in a fermentation process. Pyruvate is further reduced to butanediol, ethanol, and carbon dioxide.

*Serratia* has been found to contaminate disinfectants and surfactants. When found in a cosmetic manufacturing plant, the sources to consider first

***Table 2.6*** Types of Microorganisms Isolated from Cosmetics after Use or Found in Contaminated Cosmetics

| Gram-Negative Nonfermentors | Gram-Negative Fermentors | Gram-Positives | Yeasts | Molds |
|---|---|---|---|---|
| *Acinetobacter* | *Citrobacter freundii* | *Bacillus* | *Candida* | *Absidia* |
| *Alcaligenes* | *Enterobacter cloacae* | *Staphylococcus aureus* | *Saccharomyces* | *Alternaria* |
| *Burkholderia cepacia* | *E. agglomerans* | *S. epidermis* | *Torula* | *Aspergillus* |
| *P. putida* | *E. aerogenes* | *Enterococcus* | *Zygosaccharomyces* | *Citromyces* |
| *P. fluorescens* | *E. gergoviae* | *Micrococcus* | | *Cladosporium* |
| *P. paucimobilis* | *Klebsiella oxytoca* | *Sarcina* | | *Dematium* |
| *P. aeruginosa* | *K. pneumoniae* | *Streptocococcus* | | *Fusarium* |
| *P. maltophilia* | *Proteus* | *Propionibacterium* | | *Helminthosporium* |
| | *Serratia liquefaciens* | | | *Hormodendrum* |
| | *S. marescens* | | | *Mucor* |
| | *S. odorifera* | | | *Geotrichum* |
| | *S. rubidaea* | | | *Paecilomyces* |
| | | | | *Penicillium* |
| | | | | *Phoma* |
| | | | | *Auerobasidium* |
| | | | | *Rhizopus* |
| | | | | *Thamnidium* |
| | | | | *Trichothecium* |
| | | | | *Verticillium* |

are stagnant product in the system due to dead legs or a breakdown in sanitization frequency or technique.

*Escherichia.* This organism, like *Serratia*, belongs to the Enterobacteriaceae. It utilizes a mixed acid fermentation pathway to produce lactate, acetate, succinate, and either formate or hydrogen, carbon dioxide gas, and ethanol. *E. coli* is perhaps the most studied and experimentally used bacterium. It is a major inhabitant of the human gut and is a presumptive positive for the presence of fecal contamination in water. Some strains cause gastroenteritis or urinary tract infections. *E. coli* can grow in the gut, producing enterotoxins that cause the hypersecretion of chloride and water in the small intestine; this is colloquially referred to as "traveler's diarrhea."

Typically, this organism can be found in older water systems or systems whose pipes contain considerable biofilm (corrosion). *E. coli* is also a naturally occurring organism and, despite its use as an indicator of fecal contamination, its presence in older water distribution systems does not necessarily indicate the presence of feces in the water.

*Enterobacter.* *Enterobacter*, like *Serratia* and *Klebsiella*, produces butanediol, ethanol, and carbon dioxide. It can be isolated from contaminated surfactants and particularly from quaternary-containing conditioners. It is also a typical contaminant in households and can grow in poorly preserved products. Some of the key species contaminating cosmetics include *Enterobacter agglomerans*, *Enterobacter gergoviae*, and *Enterobacter cloacae*. These organisms are found in soil and often invade plant tissues, thus causing a variety of necroses. They are not generally considered human pathogens unless they are directly introduced into the bloodstream. *Enterobacter* is a Gram-negative fermentative rod.

*Klebsiella.* This organism is very widespread in the environment. *Klebsiella* is a human pathogen; some species are commensals. They are found in soil and water and are plant pathogens. *Klebsiella pneumoniae* causes a severe fulminating pneumonia in people who are debilitated physically or due to alcohol abuse. Unlike other members of the Enterobacteriaceae family, *Klebsiella* is nonmotile. It is found routinely in households and can contaminate cosmetics during consumer use. *Klebsiella* is a Gram-negative rod.

*Proteus.* *Proteus* organisms are motile, produce hydrogen sulfide gas, and often exhibit a type of motion called swarming. They decompose urea to produce ammonia and carbon dioxide. *Proteus* is usually found in the intestinal tracts of humans and animals and in areas contaminated with feces. *Proteus* is typically associated with urinary tract infections; however, it can cause pyogenic infections in other parts of the body if accidentally introduced (e.g., wound infections). It is a Gram-negative rod. Its presence in a cosmetic would indicate either contamination of a raw material such as a surfactant or the use of water containing high levels of contamination.

*Staphylococcus.* These Gram-positive organisms are nonmotile cocci that can grow aerobically or anaerobically. Some of the species (e.g., *S. aureus*) cause boils, are involved in impetigo, cause conjunctivitis, and cause food poisoning. A common manifestation of infection is the production of pus.

When cultured on blood agar, *S. aureus* produces a clear zone known as β-hemolysis caused by the production of a toxin that lyses red blood cells. The role of the hemolysin in disease is not entirely known. The staphylococci also produce a variety of enzymes such as hyaluronidase, proteinases, lipases, coagulase, and penicillinase. The production of coagulase can be used to confirm in the laboratory that an organism is *S. aureus*, a potential pathogen. When *S. aureus* produces coagulase, it clots the serum. Performing this test in a laboratory is relatively simple: the addition of a culture to human or rabbit plasma in a tube; after a brief incubation, the coagulase-positive *S. aureus* clots the plasma through the formation of a fibrin clot. Its presence in a cosmetic indicates human contamination.

*Streptococcus.* Streptococci are Gram-positive; they appear in chains, sometimes in pairs and can grow either aerobically or anaerobically. Some produce an α-hemolysis (green zone) on blood agar plates; others produce a β-hemolysis (clearing of the blood around the colony); and others produce a γ-hemolysis (no effect). The β-hemolytic organisms include *S. pyogenes*, a human pathogen causing strep throat. Streptococci can also be dangerous infective agents in wounds and in blood poisoning after childbirth (puerperal sepsis). The organism's natural reservoir is in the throats of carriers, usually children. At one time, it was a scourge that progressed to scarlet fever (producing erythrogenic toxin) and rheumatic fever because of the lack of antibiotics to control it. Now, it continues to break out occasionally and sporadically.

*Streptococcus* spp. rarely appear in cosmetic products. Their presence in a cosmetic would be a result of employee failure to follow good sanitary practices, for example, by placing a hand into a product or container.

*Bacillus.* Members of the genus *Bacillus* are saprobes distributed widely throughout all the Earth's habitats. They are aerobic and catalase-positive. Living primarily in the soil, they are varied nutritionally, but are not fastidious. They are also common sources of antibiotics. They are spore-forming organisms that continuously disperse spores into the water and onto plants.

In the cosmetics industry, some of the more common raw materials to be contaminated with *Bacillus* spores include *Aloe vera* and a variety of thixotropic agents such as quaternized clays. Pasteurization of aloe vera gel will not eliminate *Bacillus* because its spores are not susceptible. Instead, tyndallization (a repetitive heating process) is required. Tyndallization is repeated several times on three successive days to allow the spores to germinate and become susceptible to the heating processes. Alternatively, irra-

diation can be used, particularly for the clays because they are not harmed by the process.

Very few *Bacillus* spp. are pathogenic. The exceptions are *B. anthracis* and *B. cereus*. *B. anthracis* can cause anthrax, a cutaneous disease caused by spores that enter the skin through small cuts and abrasions. The organism is invasive because of the production of virulence factors that include polysaccharide capsules and exotoxins that produce edema and cell death. The initial disease presents as a papule that becomes increasingly necrotic and then ruptures to form a painless black scab called an eschar. *B. anthracis* can also cause pulmonary anthrax from inhalation of airborne spores. The onset of this form of anthrax is rapidly fatal within a few hours after septicemia occurs. The toxins produced cause capillary thrombosis and cardiovascular shock. At one time, when natural animal hair was used, shave brushes were significant sources of *B. anthracis*. The transmission by this route was ideal. The spores on the bristles were deposited directly into the skin when an individual cut himself in the course of shaving.

*B. cereus* is a common air- and dust-borne contaminant that grows well in cooked foods, particularly rice, potatoes, and meats. The organism itself is not pathogenic, but it produces enterotoxins that cause nausea, vomiting, abdominal cramps, and diarrhea that resolve within 24 hours of eating the food contaminated with the toxin. Although not a common organism of concern to cosmetic microbiologists, all species of *Bacillus* are undesirable because their spores originate in soil and they serve as indicators of filth. Now that the immuno-compromised population is on the increase, we must continue to be aware of the problems that even so-called harmless organisms can offer.

*Clostridium.* *Clostridium* is another Gram-positive, spore-forming rod. It is also widely distributed in soils. Unlike *Bacillus*, it is anaerobic and catalase-negative. Many of its members are saprobes that grow in sewage, on vegetation, and even as commensals on humans. They are not ordinarily communicable. Instead, they cause infections when soil containing their spores is introduced into the body via puncture wounds or other skin injuries. They can also produce exotoxins that cause intoxication when the material containing the toxin is ingested.

Wound infections, primarily from *Clostridium perfringens*, *Clostridium novyi*, and *Clostridium septicum*, give rise to gas gangrene. Because Clostridia are not highly invasive, they usually gain access to the body via dead or damaged tissues that provide an ideal anaerobic and growth factor-enriched environment. Rapid vegetative growth occurs, and the exotoxins along with a variety of enzymes are produced. The toxins released cause extensive tissue destruction. As the organism ferments the carbohydrates in tissues, gas is formed. Thus, the organisms can easily weaken the tissues with enzymes and toxins and then tear tissues apart via the production of gas, thus enabling the organism to slowly continue its way into the infected patient.

*Clostridium tetani* causes tetanus, a neuromuscular disease involving the production of a toxin by the organism once it enters the body. It is poorly invasive, requiring not only a wound that allows access, but the wound must also be necrotic and poorly supplied with blood because *Clostridium* is a strict anaerobe. Although it initially causes a localized infection, the toxin that is produced can spread to nearby motor nerves and then travel to the ventral horns of the spinal cord where it binds to target sites on the spinal neurons responsible for controlling skeletal muscle contraction by inhibiting the release of neurotransmitters. As a result, muscles contract uncontrollably.

Food intoxication can be caused by *C. perfringens* and *C. botulinum*. However, this route of infection as a result of cosmetic use is rare unless the product somehow permits growth and the organisms produce toxins in an oral-care product that is accidentally swallowed (e.g., toothpaste swallowed by a toddler). Infant botulism can occur through ingestion of spores by children below 6 months of age whose gut environments cannot kill the spores as do the gut environments of adults and older children. The spores survive and germinate and then produce the toxin that causes a condition known as "floppy baby syndrome."

Of the Clostridia, the main species of possible concern are *C. perfringens* and *C. tetani* because, like Bacilli, they represent a potential source of filth (from soil) in product and the product they enter would be deemed adulterated. Clostridia would also be considered a potential threat to consumer health if product contaminated by these microbes might be used in such a way that the bacteria could infect customers. Classic examples are products associated with shaving and applied to skin (e.g., shave creams, after-shave products and colognes, lotions, antiperspirants, and deodorants).

## *Molds and yeasts*

### *Physiology and biochemistry*

Fungi are typical contaminants in a few products that contain limited water and have low pH values; many of these products are lotions and creams. Fungi are eukaryotic: they contain membrane-surrounded organelles, particularly membrane-bound nuclei within which is the DNA comprising the chromosomes.

Other membrane-enclosed organelles include the mitochondria (which once were bacteria according to the endosymbiont hypothesis), the Golgi apparatus, endoplasmic reticula, lysosomes, and nucleoli (where ribosomes and ribosomal RNA are made). The term *organelle* is used to emphasize the parallel between the organs of animals and the structures within cells, each of which performs a very specific function. The extensive membrane systems in eukaryotes are needed because of their large volume and their need for regulation and transport as a result of that large volume.

### *Cytoplasmic matrix, microfilaments, and microtubules*

Within the eukaryotic cell is also a homogeneous, somewhat bland structure known as the cytoplasmic matrix. It is actually the most important and complex structure of a cell because it is the site where organelles exist and where a majority of the important biochemical processes take place. The cytoplasm is composed of 70 to 85% water — both free and bound to the surfaces of proteins. The pH is about 6.8 to 7.1, except in the digestive vacuoles and lysosomes where it can be as low as 3 to 4.

Within the cytoplasm are microfilaments of about 4 to 7 nm in diameter that provide a structure to aid in cell motion and shape. These filaments contain networks or parallel arrangements and have the same basic structure as actin found in human muscle protein. The other filamentous organelle is a thin cylinder about 25 nm in diameter and known as a microtubule. These structures along with microfilaments help cells maintain their shapes and aid movement, but their major role is in intracellular transport of substances throughout the complex cytoplasm.

### *Organelles*

*Endoplasmic reticulum.* The endoplasmic reticulum (ER) is an irregular network of branching and fusing membranous tubules around 40 to 70 nm in diameter with flattened sacs called cisternae interspersed along the way. A large part of the function of the ER is synthesizing protein from the ribosomes located along the surface of the ER. Since the ER is covered with ribosomes, it is known as rough endoplasmic reticulum. The other type known as smooth ER lacks ribosomes and is perhaps involved in lipid synthesis. The ER transports proteins, lipids, and other materials throughout cells and serves as the site of cell membrane synthesis.

*Golgi apparatus.* This organelle is made of flattened sacs, called cisternae, that are stacked upon one another. The origin of the Golgi is the ER. The sac that forms from the ER and faces it is called the *cis* side; the opposite and already formed maturing face is called the *trans* side. The membranes composing this organelle lack ribosomes. About eight or more cisternae are contained in a sac; each one is about 15 to 20 nm thick and they are separated from each other by 20 to 30 nm. At the edges of the cisternae are tubules and vesicles.

The main function of the Golgi is to package materials and prepare them for secretion. Apparently, material is transported from the ER to the *cis* side of the cisternae and then transported to the *trans* side and on the next cisternae by vesicles that bud off the edges and move to the next sac. Most of the proteins that enter from the ER into the Golgi are glycoproteins that are modified in the Golgi by the addition of specific groups; they are then sent on their way to their proper locations for use.

*Lysosomes.* Lysosomes are synthesized by the ER and the Golgi apparatus. The digestive enzymes they contain are manufactured by the rough endoplasmic reticulum and then packaged by the Golgi apparatus. Lysosomes are spherical, single membrane-bound particles about 50 to 500 nm in diameter. They contain all the enzymes (hydrolases) needed to digest the macromolecules on which cells thrive. Oddly enough, the internal pH of lysosomes is acidic (3.5 to 5.0) because the unit membranes around the lysosomes pump hydrogen ions into their interiors for the proper functioning of the hydrolases.

The key beneficiaries of lysosomes are the cells that obtain their nutrients via endocytosis: the cells engulf materials by enclosing them in vacuoles surrounded by "pinched-off" portions of cell membranes. These vacuoles (known as vesicles if they are particularly small) are created by phagocytosis (engulfment of particles) or pinocytosis (ingestion of liquid and the solute molecules). The vacuoles or vesicles are also called phagosomes or pinosomes, depending on the method of creation; collectively they are called endosomes. The membranes of the lysosomes fuse with those of the endosomes to become food vacuoles as the digestive enzymes of the lysosome mix into the materials carried within the endosomes. Once digestion takes place, the nutrients diffuse into the cytoplasm, leaving behind residual bodies containing indigestible materials.

*Ribosomes.* The basic structure of the eukaryotic ribosomes is similar to that of prokaryotes, except that the subunits are larger in the eukaryotes. A ribosome is composed of a large subunit of size 60S and a smaller unit of size 40S for a total size of 80S (S refers to a Svedberg unit, a measure of how quickly a particle sediments in a centrifuged gradient). The primary role of a ribosome is translation of the messenger RNA (mRNA) transcribed from the gene within the nucleus of a eukaryotic organism. This translation process is protein synthesis. Ribosomes are manufactured within the nucleoli of cells.

Within the cytoplasm of a cell, the ribosomes appear as tiny particles that give the cytoplasm a stippled appearance. However, ribosomes are also intimately associated with the rough endoplasmic reticulum, studding its membranes, somewhat like a biker's black leather jacket is covered with silver studs.

*Mitochondria.* Mitochondria are currently thought by some scientists to be derived from endosymbionts that were once prokaryotic invaders of large progenitor cells. A symbiosis developed with the large cells that could not manage energy distribution very well. The symbiotic invaders could supply the cells with energy in the form of adenosine triphosphate (ATP). There is considerable support for this theory that, during the early formation of life, mitochondria were simply bacterial invaders turned good. Mitochondria contain circular genome-like bacteria; mitochondria replicate independently of the cell and even have prokaryotic-like 70S ribosomes.

*Nuclei.* The nucleus of a eukaryotic cell, as opposed to the nucleoid of a prokaryotic cell, is bound by a membrane called the nuclear envelope. This double-layered membrane is very porous in order to allow macromolecules easy access in and out of the cell. The nucleus contains several linear chromosomes compared to the single circular chromosome of a prokaryote. It also contains the nucleoplasm and the nucleolus that manufactures RNA for synthesizing ribosomes.

The *nucleoplasm* term is used to describe all the enzymes and proteins in the nucleus that are involved in replication of the genome. The key item within the nucleus is the chromatin that makes up the chromosomes, which are composed of DNA and proteins called histones. The nucleus is not visible until the cell undergoes mitosis and the chromosomes are duplicated and condensed by forming coils and supercoils around the histones just before being separated into the daughter cells.

### *External cell organelles*

Motility and protection are useful characteristics for any organism. Eukaryotic cells enjoy the benefits of motility by means of flagella or cilia. The presence of a glycocalyx (described below) provides the benefit of protection from the external environment.

*Cilia and flagella.* The eukaryotic flagellum is composed of microtubules arranged in what is referred to as a 9 + 2 arrangement (nine pairs of hollow tubules surrounding a single pair of tubules in the center). The whole arrangement is surrounded by an extension of the cell membrane. The movement can either be a back-and-forth, whip-like motion or a twirling motion resulting when the tubules slide past each other in an almost muscle-like fashion; all movement is coordinated by the cell membranes. In contrast, bacterial flagella are simple. Each flagellum consists of a protein strand and undergoes a spinning motion due to a basal body connected into the cell wall. Cilia are composed of the same basic architectures as flagella, but they are shorter. Usually they cover a large portion of a cell and beat in a regular fashion

*External cell coverings.* A eukaryotic cell may have a structure known as a glycocalyx that serves as an external boundary layer beyond the membrane (in protozoa and a very few algae) or the cell wall (in most algae and fungi) that comes in direct contact with the environment much like the glycocalyx of a prokaryote. The structure is composed of polysaccharides that comprise a slime layer for protection, provide for attachment to surfaces, or even serve as a mechanism for communications with other cells.

The cell walls of fungi and algae provide shape, support, and protection. A fungal cell is composed of a thick inner layer of chitin or cellulose fibers and a thin outer layer of mixed glycans covered by the glycocalyx. The cytoplasmic membrane is the typical bilayer of lipids into which proteins are free to move. The key difference between eukaryotes and prokaryotes is

the presence of sterols in the membranes of eukaryotes in addition to phospholipids. The sterols add some stability to the membrane by making it less flexible.

## *Growth and reproduction*

Reproductive strategies of eukaryotes are more complex than the simple process of binary fission carried out by prokaryotes. Fungi can propagate by simple growth of hyphae or fragmentation of the hyphae. They can also produce spores. If the spores are results of mitotic division of the parent cell, they are asexual; if they are formed by the fusion of two parental haploid nuclei and then undergo meiosis, they are sexual.

Algae reproduce asexually through fragmentation, binary fission, mitosis, and even by producing motile spores. They can also reproduce sexually, like fungi. Protozoans reproduce asexually by mitosis, although a few utilize a sexual reproduction method known as syngamy in which two haploid gametes unite to form a diploid zygote that undergoes meiotic division to produce a number of haploid trophozoites (the motile feeding stage). Protozoans can also undergo encystment in which a resistant stage much like a bacterial spore is formed. Like a bacterial spore, a cyst is not a means of reproduction; it is simply a dormant stage of the organism.

### *Asexual reproduction of fungi*

The key to identifying fungi is to observe their reproductive structures, the spores. In contrast to the bacteria and some protozoans, a fungal spore is a mechanism of reproduction. There are two types of asexual spores: (1) sporangiospores formed by successive cleavages within a sac-like head (sporangium) attached to a stalk; and (2) conidiospores. These spores are free and not enclosed by sacs, but still develop by segmentation of the vegetative hyphae. Conidiospores are found in a variety of forms:

Arthrospores (rectangular; septate hypha fragment at the cross walls)
Chlamydospores (spherical; hyphal cell thickens, fragments, and is released)
Blastospores (produced by budding from a parent that can be either a yeast cell or another conidium)
Phialospores (produced via budding of a spore-bearing structure called a phialide that looks like a vase with flowers on top of a conidiophore)
Porospores (grow through small pores in spore-bearing cells)
Microconidia and macroconidia (small and large conidia formed by the same fungus, depending on growth conditions).

### *Sexual reproduction of fungi*

Sexual reproduction gives an organism the opportunity for increased gene sharing and increased diversity. With increased diversity comes increased adaptability and thus increased survivability. The big difference between

sexual reproduction in fungi and in most other eukaryotic organisms is that the fungi are already haploid and thus ready to fuse their nuclei to form a temporary diploid state. The most common sexual spores formed are zygospores, ascospores, and basidiospores.

Zygospores are formed when hyphae of two strains fuse to create a diploid zygote that then swells, becomes coated by a spiny covering, and is released. When it germinates, it forms a sporangium that then produces haploid sporangiospores containing genetic material derived from both parents due to crossing over events occurring in the diploid state.

Ascospores and basidiospores follow the same initial steps of haploid strain fusing. However, in ascospores, a terminal diploid cell forms at the end of a single ascogenenous hypha as a result of the fusion of two parental hyphae (ascogonium and antheridium). The diploid cell at the tip of this hypha will then undergo meiosis to reduce the genome number down to the haploid state. The haploid cells can then undergo mitosis to produce additional spores, all contained within a sac.

In basidiospores, a basidium or pedestal is formed and the spores are formed on the basidium. The same processes described above take place, but here the terminal diploid cell forms the basidium that then produces through meiosis four haploid cells borne at the surface of the basidium. This is characteristic of mushroom reproduction in which the basidia are located along the gills of the fleshy part of the mushroom that is consumed as food. Remember that the next time you are eating mushrooms. Effectively, you are consuming an organism's sexual organs and its progeny at the same time!

## *Diversity: fungi of industrial and medical importance*

At one time, the fungi did not occupy their own kingdom, the Myceteae. They were classified along with the algae and bacteria as Plantae and were later classified with the motile alga and protozoa as Protista. They are unique enough to eventually warrant placement into their own kingdom, at least by Whittaker's approach. The confusion that these classification decisions caused still arises when one tries to explain why some of the slime molds are not protists. Superficially, the fungi can be subdivided into macroscopic varieties such as mushrooms and puffballs; there are also the microscopic molds and yeasts.

Most fungi are unicellular or colonial. A few exhibit cellular specialization that approaches tissue differentiation. Some fungi exhibit both cellular and mycelial stages when they form hyphae; these are called dimorphic fungi and are most often associated with pathogenic fungi, changing from the mycelial form at low ambient temperature to the unicellular form as they infect an animal at a higher temperature.

Most fungi are not pathogenic; they are saprobes and live free without the need for hosts. Thus, they are rarely problems for humans who have high resistance to them. A few fungi are frank pathogens that can attack healthy people but most are opportunists that require the right combination

of accidental exposure and weakened immunity for infection to begin. As more humans are kept alive by artificial means, contract AIDS, and have cancer or uncontrolled diabetes, the number of immunocompromised individuals capable of contracting fungal infections will increase. Thus, a cosmetic manufacturer must be aware of the use patterns of its products in these populations and plan accordingly. Alflatoxins produced by *Aspergillus* fungi represent an area of concern related to products that are ingested, especially foods (e.g., peanut butter) whose raw ingredients can support the growth of this mold.

The fungi comprise a very diverse kingdom subdivided into two subkingdoms: the Myxomycota and the Eumycota. The Myxomycota or slime molds play only a small direct role in cosmetic microbiology. They do, however, play a central role in decay processes of wood, chitin, lignin, and other cellulosic products in nature. Thus, wherever cellulosic products are used as raw materials in cosmetics, manufacturers should be aware that they can serve as food sources for slime molds in certain environments. Typically, the slime molds are found on moist forest floors and appear, as their colorful common names suggest (witches' butter, rutting deer seed), as macroscopically visible (10 to 50 cm) slimy organisms oozing along a rotting log. The Eumycota or true fungi include the yeasts and molds we most often associate with human disease and, therefore, play a more direct role in cosmetic microbiology.

The Eumycota are divided into four phyla: Zygomycota, Ascomycota, Basidiomycota, and Deuteromycota. The Zygomycota produce zygospores and sporangiospores or conidia. They include *Rhizopus* and *Mucor* and a variety of others. The Ascomycota produce ascospores as sexual elements and conidiospores in the asexual mode. This phylum includes a variety of pathogens including *Microsporum*, a causative agent of ringworm. Also in this group are *Penicillium*, a common source of antibiotics and *Saccharomyces*, a yeast used in beer and bread making. The last of the phyla that produce sexual spores are the Basidiomycota; they produce basidiospores sexually and conidia asexually. This phylum includes the mushrooms, the bracket fungi attached to trees in the woods, and the rusts and smuts that infect plants. The key human pathogen in this phylum is *Cryptococcus neoformans* that causes invasive systemic infections.

It is interesting to note that *Penicillium*, a cosmetic product contaminant, was once included in Deuteromycota phylum, as are certain species of *Microsporum*. Once a sexual stage is observed, a mold is moved from the Deuteromycota to one of the other three phyla named above.

Deuteromycota are fungi whose sexual stages have not yet been observed. They produce a variety of conidia; some are dimorphic, and a few are parasitic. The human pathogens include *Candida albicans*. A mildew fungus, *Cladosporium*, is in the Deuteromycota phylum and is capable of infecting cosmetic products kept in bathrooms. *Absidia*, *Rhizopus*, and *Mucor* can cause mucormycosis, usually via inhalation of dust and soil contaminated by these molds. The molds are found as contaminants in some prod-

ucts. Several other fungi can cause health problems and contaminate cosmetics. Clearly, antifungal properties of preservatives are important for their control.

Few people can relate to the microbial world because it is so alien and foreign to everyday experiences. Hopefully, this chapter makes clearer what can appear a very obscure world: the microbial universe.

## *Additional resources*

This chapter provides a brief overview of the microbial world in a simple and informal way so that both experts and novices can gain insight and practical information. For more extensive treatments of this subject, see Bauman, R.W., *Microbiology,* Pearson Benjamin Cummings, San Francisco, 2004; Pommervile, J.C., *Alcamo's Fundamentals of Microbiology,* 7th ed., Jones & Barlett, Sudbury, MA, 2004; Prescott, L.M., Harley, J.P., and Klein, D.A., *Microbiology,* 6th ed., McGraw Hill, New York, 2005; and Talaro, K., *Foundations in Microbiology,* 5th ed., McGraw Hill, New York, 2005.

## *References*

1. Darwin, C., *On the Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life,* John Murray, London, 1859.
2. Weiner, J., *The Beak of the Finch,* Vintage Press, New York, 1994 (outlines Rosemary and Peter Grant's work on Darwin's finches).
3. Brooks, D.R. and Wiley, E.O., *Evolution as Entropy: Toward a Unified Theory of Biology,* University of Chicago Press, Chicago, 1988.
4. Futuyma, D.J., *Evolutionary Biology,* 2nd ed., Sinauer, Sunderland, MA, 1986, p. 551.
5. Lenski, R.E. The *E. coli* long-term experimental evolution project site. http://myxo.css.msu.edu/ecoli 2004.
6. Kreft, J.U., Biofilms promote altruism, *Microbiology,* 150, 2751, 2004.
7. Margulis, L., *Symbiotic Planet: A New Look at Evolution,* Basic Books, New York, 1998.
8. *Bergey's Manual of Systematic Bacteriology* (volumes 1–4) Williams & Wilkins, Baltimore, 1984.

*part two*

# *Sanitary practices in cosmetic manufacturing*

*chapter three*

# *Microbial environment of the manufacturing plant*

***Richard Mulhall, Edward Schmidt, and Daniel K. Brannan***

*Contents*

## *Introduction*

Control of the microbial population within a plant environment is the goal of a plant sanitation program. Good plant sanitation is essential to protect raw materials, the manufacturing process, and the finished product. At least eight critical factors affect the microbial ecology of the plant environment. The three working factors are the cleaning and sanitation practices, personal hygiene of the staff, and raw material handling. The five engineering factors include good sanitary design of the equipment, plant, warehouse, water system, and air system. Current good manufacturing practices (cGMPs) address the various aspects of plant sanitation. Establishment of sanitation standards should include each area and task-related function in the plant.

We discuss all these factors and their significance in this chapter. Although we discuss them separately, all the factors relate to each other. Each functions as a part of a complete sanitation and quality assurance program. Inattention to any of these factors will alter the microbial ecology of the plant environment. When this occurs, the material or product becomes susceptible to contamination.

## Cleaning and sanitization basics

Every company should set up good housekeeping, cleaning, and sanitization programs for its entire plant environment that meet the specific needs in each area. Good manufacturing practices depend on the cleaning and sanitization practices used and their frequencies of performance within the plant. The specific procedures and schedules for the cleaning and sanitization of equipment and the physical plant should be written.

Several factors determine the effectiveness of cleaning and sanitization. Three of these factors are (1) the use of well-designed equipment, (2) the use of good cleaning and sanitizing products, and (3) the use of validated processes. Finally, management must provide training and supervision of employees. A company must include timely cleaning and sanitization schedules in its quality assurance program. When it does, it will benefit from the insignificant levels of microorganisms present in its manufacturing equipment and plant facilities.

### Cleaning

Cleaning is the physical removal of product or ingredient residues. Cleaning removes grease, dust, and contaminants from the surfaces of manufacturing equipment and the environmental surfaces of buildings. Physical removal requires the application of energy in some form such as scrubbing, spraying, or turbulent flow. Most cosmetic manufacturers use cleaning agents to aid physical removal of such materials. These agents break down soil in order to allow both visible and invisible foreign matter to rinse away. An ideal cleaning agent should be readily soluble and should provide good penetration and emulsifying action. It should be compatible with equipment, noncorrosive, and possess good wetting and rinsing properties.

Detergent formulations are ideal to use for most cleaning activities. Technically, a detergent is any cleaning agent. However, in popular usage, detergents are washing and cleaning agents with compositions other than metal salts of acids derived from fat (soaps). They react basically by the same mechanisms as soaps. All detergents possess the basic properties of penetration, wetting, dispersion, and emulsifying action.

Detergents may contain surfactants, builders, agents for penetrating, wetting, deflocculating, foaming, emulsifying, sequestering or chelating, and soil dispersants. The surfactants may be ampholytic, anionic, cationic, or nonionic. The choice of a detergent should be based on its intended use and the type of soil to be removed.

#### *Detergent ingredients and properties*

In describing detergents and their properties, a vocabulary unique to the industry has evolved. For that reason, we include definitions or explanations for a variety of common terms.

**Detergency** is defined as the ability to clean soil or other unwanted material from a surface. A detergent accomplishes this task through a combination of processes including the lowering of surface and interfacial tension, solubilizing or emulsifying, inactivating water hardness, and neutralizing acid soil.

**Builders** are materials that upgrade or protect the detergency of a surfactant. Builders perform several functions including inactivation of water hardness, increasing alkalinity to aid cleaning, providing a buffer, suspending soil, and emulsifying oily or greasy soils. Some builders help deflocculate or break up solid masses into smaller particles and disperse them through a liquid medium.

**Dispersing agents** are chemicals that increase the stability of particles in a liquid. These agents help remove soil particles by keeping them in a dispersed or suspended state during equipment rinsing. The mechanism of dispersal may be the process of emulsification involving dispersal or suspension of fine particles or globules of one or more liquids in another liquid. Similar terms for dispersing agents are soil suspending agents or inhibitors of soil redeposition. Dispersing agents are ingredients of detergents intended to keep soil suspended and dispersed in the cleaning solution. They reduce redeposition of the soil on surfaces by detaching soil globules from a surface and dispersing them through the cleaning solution. Surfactants are the principal emulsifying agents.

**Foaming agents** are chemical agents that increase the foaming or sudsing characteristics of a cleaning agent. Penetration refers to the characteristic that permits the cleaning solution (water) to get under the soil and loosen it from a surface. It also helps the solution to work its way through the soil.

**Chelating agents** are organic sequestering agents that inactivate water hardness and other metallic ions in water. A commonly used chelating agent is EDTA (ethylene diamine tetraacetic acid and its salts). A sequestrant is a chelating compound in an aqueous solution that combines with a metallic ion to form a water-soluble combination. The ion in the combination is inactive. Sequestrants soften water without the precipitation associated with other methods like lime softening. For example, complex phosphates are sequestrants that inactivate divalent metal ions such as calcium, magnesium, iron, and manganese without precipitation.

**Surfactants** (surface active agents) are organic chemicals that are added to liquids to change the surface-active properties of the liquids. Surfactants and soaps perform the important function of lowering the surface tension of water. Surfactants help remove fatty and particulate soils. They also keep soils emulsified, suspended, and dispersed to prevent settling at the surface.

Surfactants are also wetting agents. They increase the ability and speed with which liquid displaces air from a solid surface. This property improves the process of wetting surfaces. Surfactants lower the surface and interfacial tensions. This enables a cleaning solution to more quickly wet a surface. The chemical action of a surfactant can be enhanced by employing mechanical action to more readily remove soils.

### *Types of surfactants*

Amphoteric (ampholytic) surfactants may be either anionic or cationic, depending on pH. They are useful because of their wide compatibility with builders, acids, and alkalis. The properties of an anionic surfactant depend in part on the negatively charged ion (anion) of the molecule. This property accounts for the anionic designation. The detergent industry uses a wide range of anionic surfactants that are highly sudsing. Excess foaming is undesirable for surface cleaning. It leaves a residue from excess foam. This residue produces a tacky surface that presents a likely resoiling problem.

A cationic surfactant has a positively charged ionic group. Quaternary ammonium compounds are the most widely used cationic surfactants. They are used as sanitizers and disinfectants, fabric softeners, and static electricity dissipaters. They are not typically used alone as cleaning agents.

Nonionic surfactants do not contain positively or negatively charged functional groups. They are particularly effective for removing oily soil and many are low sudsing. They do not ionize in water as do anionic and cationic surfactants.

The surfactant industry also has a unique vocabulary for cleaning. We will describe these terms as well. A general purpose cleaner contains sodium hydroxide or sodium carbonate for alkalinity along with a sequestering agent. Some cleaners also include low-foaming wetting agents and silicates to inhibit corrosion. These cleaners are different from clean-in-place (CIP) cleaners that have the same formula as general purpose cleaners but contain nonfoaming wetting agents. A manual washing cleaner will exhibit lower alkalinity and contain a high-foaming agent. An acid cleaner contains an organic or mineral acid to remove hard water and mineral deposits. It may also include a heterocyclic nitrogen compound to inhibit corrosion and a wetting agent to allow penetration. Alkaline cleaners are of the general purpose type but they contain very high levels of sodium hydroxide or sodium carbonate. They are ideal for very difficult cleaning jobs.

Personnel should limit the use of cleaning agents in a plant to those necessary for accomplishing the assigned tasks. No single all-purpose cleaner serves every cleaning need, but use of duplicate products should be avoided wherever possible.

## *Sanitization*

Sanitization is the adequate treatment of surfaces by a process that is effective in destroying vegetative cells of pathogenic bacteria. It also should reduce spoilage microorganisms to insignificant levels. Chemical residuals from such treatment should not adversely affect products and should be safe for consumers.

Use of a sanitizer should be preceded by a cleaning procedure. An alternative is to use a combination disinfectant–detergent or detergent–sanitizer in a single-step procedure — a practice usually reserved for environ-

mental surfaces. Typically, combinations exhibit compromised detergent or disinfectant activity and are not as effective as using both agents separately.

Sanitizers are EPA-registered chemicals that reduce viable microbial contaminants on surfaces to safe levels. The definition of *safe levels* is dependent on public health or product requirements. The choice of a sanitizer should consider the characteristics, efficacy, and applicable regulations. An ideal sanitizer should:

- Kill microorganisms rapidly (within 30 to 60 seconds)
- Provide adequate microbial reduction (about 99.9%, a 3 log reduction)
- Be effective against a broad spectrum of microorganisms
- Be safe and nontoxic to employees handling it
- Be safe for consumers at use levels
- Be acceptable to regulatory agencies
- Exert no adverse effects on the product
- Be economical to use
- Be rinsable
- Leave no objectionable odor or residue
- Be stable in its concentrated form and at use levels
- Be noncorrosive
- Exhibit compatibility with equipment and other chemicals
- Possess ready solubility in water
- Be biodegradable at concentrations expected in a waste receiving stream

Furthermore, tests to detect a sanitizer in solution should be easy to conduct.

### *Chemical sanitizers*

Several chemical sanitizers are appropriate for various sanitization procedures within a manufacturing facility. A variety of reference books and product bulletins will provide detailed information about the numerous chemical sanitizers and specific product formulations. As a rule, experience with a sanitizer is usually the only reliable benchmark for its usefulness.

Chlorine is usually provided as a component sodium or calcium hypochlorite. Dichloro- or trichloro-isocyanuric acid and its salts are also chlorine-releasing sanitizers. Chlorine mixed with ammonia forms chloramine — which provides a good residual chlorine source for disinfecting water. Chlorine is useful for disinfecting water, process systems, and environmental surfaces.

An iodophor is an iodine-releasing formulation contained in a carrier. Depending on the intended use of the product, the carrier may or may not contain a nonionic detergent. Iodine is useful for surface and skin disinfection. In some cases, it is useful for water disinfection but this effect of chlorine use is secondary.

Quaternary ammonium compounds consist of cationic surfactants. The most frequent use for this type of chemical sanitizer is in combination with a nonionic detergent as a disinfectant–detergent or detergent–sanitizer. However, nonionic detergents usually do not clean as well as more caustic cationic detergents. Typically, one can achieve more effective cleaning and sanitizing by separating the cleaning and sanitizing steps rather than combining them. This is because most cleaners operate best at high pH levels and most disinfectants operate best at acidic to neutral pH.

Ethyl alcohol has limited use as a chemical sanitizer because of flammability risks. However, it can be diluted to 50 to 70% final concentration with water partially to mitigate this concern while providing good antimicrobial activity. It is effective at cutting the films of surfactant-containing products while providing some sanitizing impact. Ethyl alcohol does not kill spores. It is useful as a hand antiseptic for short periods. Many antiseptic products are now available that provide alcohol in an emollient form to reduce drying and chapping of the hands. Ethyl alcohol is more pleasant to use than isopropyl, which encourages plant personnel to use it for cleaning spills and tank tops.

While phenolics have value as sanitizers when used alone, they are more frequently combined with anionic detergents to produce disinfectant–detergents. Formalin is also useful. However, it should always be used in a closed system that does not permit it to escape into the air. Tighter restrictions on formalin use are likely because of OSHA regulations. Formaldehyde is a suspected carcinogen.

Phosphoric acid has only limited use in a plant environment. It is combined with other chemical products such as iodophors and used in tile and bathroom cleaners. Hydrogen peroxide is not used extensively as a chemical sanitizer. It is primarily useful for cleaning deep puncture wounds of the skin. Its bubbling action helps to lift out dirt deep within a wound but its disinfection capacity is minimal. Pine oil also has limited use in a manufacturing plant.

Peracetic acid is a combination of peracetic acid, acetic acid, hydrogen peroxide, and water that works as a very strong oxidizer. It is a broad spectrum disinfectant that has demonstrated good level of activity against biofilms. Peracetic acid has a pungent odor and is very irritating to the eyes and nose. It is noncorrosive to stainless steel.

### *Physical sanitizers*

Heat is the most efficient and thorough physical sanitizer. In our plant, we provide it as steam (100°C/212°F) or hot water (80 to 100°C/176 to 212°F). If the steam is under pressure, the temperature can be even higher (121 to 132°C/250 to 270°F). In cosmetic manufacturing, we usually do not use dry heat. Table 3.1 defines the temperatures required for sanitizing via dry heat and steam heat. Note that these exposure times and temperatures are not effective for sterilization.

***Table 3.1*** Comparison of Exposure Times and Temperatures for Wet and Dry Heat Sanitization

| Exposure Time | Wet Heat | Dry Heat |
|---|---|---|
| 1/2 hour | 180°F/82°C | 355°F/179°C |
| 2 hours | 160°F/71°C | 320°F/160°C |
| 4 hours | 140°F/60°C | 285°F/141°C |
| 24 hours | 120°F/49°C | 250°F/121°C |

Heat has several advantages over other sanitizing agents — chemical and physical. These advantages include (1) ability to penetrate into small cracks and crevices, (2) noncorrosive action, (3) nonselective treatment of microbial groups, (4) absence of residues on surfaces, (5) ability to be easily measured, and (6) reasonable cost.

The use of steam or hot water poses some challenges. Heat may cause condensation problems because of the high humidity created. Only thermostable materials can withstand the heat generated by steam or hot water. High energy costs are usually offset by reduced labor costs when compared to the costs of chemical sanitizers. When heat treatment is not cost effective, we must consider the proficiency of heat treatment compared with chemical sanitization treatments.

Ultraviolet (UV) light is of very limited use as a sanitizer in cosmetic microbiology practice. It can damage the eyes of personnel in the area if used as a local sanitizer. It has poor penetrating power and so must be used near the material to be treated. The UV intensity decreases by the square of the distance from the source. The typical use is for water sanitization, normally in combination with ozonated water systems. One use of UV lights is as point source polishers to remove the ozone. They also kill microorganisms that may have survived the ozonation process. UV is ineffective as an air sanitizer in large, open areas.

One example of the use of combined chemical and physical sanitization is the application of a cleaner or sanitizer with steam or hot water. This approach will usually increase the effectiveness of the chemical agent. However, the chemical agent must be compatible with the heat application. For example, heat does not significantly enhance hypochlorite activity except to improve the wetting characteristics of the water. When using chlorine gas as the chlorinating agent, heat may actually drive out the chlorine. This occurs when the chlorine is not converted to hypochlorous acid, especially at low pH. This reduces the effectiveness of the chlorine sanitizer and also presents a major safety hazard.

*Proper use of chemical agents*

Chemical cleaners and sanitizers should be used only according to label directions. All appropriate employees should be thoroughly trained in the proper use of chemical agents. Improper use of an agent will both reduce

the effectiveness of the product and produce unsatisfactory or less-than-optimal results. Overuse of a product will increase toxicity, increase corrosiveness, and may adversely affect equipment or product. In addition, federal law prohibits use of an EPA-registered product in any manner inconsistent with its label instructions. Therefore, misuse of an EPA-registered sanitizer is an illegal act.

## *Cleaning and sanitizing equipment*

The types of equipment used to clean or sanitize production equipment and environmental surfaces include pressure sprayers, compressed air systems, and steam and foam generators. Also included are wet and dry vacuum systems, ultrasonic cleaning devices, and clean-in-place (CIP) systems. These are described further below.

### *Portable and fixed pressure sprayers*

Low pressure systems are suitable for removing gross or loosely adhered product residues and for rinsing away cleaning solutions. A high pressure system delivers a high velocity stream of water or chemical solution and is efficient for removing adhered residues. Low and high pressure systems often use chemical additives to help in removing soil.

High pressure systems operate at nozzle pressures that may be well above 1000 psi. The advantages include low water and chemical solution consumption and high pressure impingement. Some use temperatures above 82.2°C (180°F), thus adding the sanitizing power of heat. Low pressure and high volume systems may attain nozzle pressures of 400 to 600 psi, but they use large quantities of water and chemical solutions.

An ideal hose-spray system should have an on–off valve near the wand and an automatic shut-off to be activated when the wand is not used or when the water pressure drops. The automatic shut-off is required for steam–water mixing systems. Such systems should also have easily interchangeable nozzles that deliver droplets rather than fog sprays. The hoses should be long enough to do the job and strong enough to withstand use pressures, temperatures, chemicals, and external abrasion. The system should have premixing mechanisms to provide economical use of chemicals and water and it should be composed of internal parts that will be resistant to corrosion and scaling. It should have enough capacity to maintain required use temperatures and pressures, keep maintenance costs low, and prove reliable over several hours of continual use. Finally, such systems should operate quietly and provide adequate safety features for employees.

No single system will combine all these features, but any system should be rated based on them. A centralized or decentralized fixed system with lines running to fixed, multiple locations throughout a plant is preferable to multiple portable units.

*Portable and fixed steam generators and sprayers*

These steam generators allow delivery of high velocity steam or sprays of steam with or without chemical additives. They provide very effective removal of product residues, films, oils, and fats. They also achieve some sanitizing effects. Steam, however, can be very hazardous to workers. Personnel must use it with extreme caution. Steam units may consist of centralized or decentralized fixed systems or portable units.

*Portable and fixed foam generators and applicators*

Foam generators provide a stream of chemical foam at ambient temperature under air pressure of about 100 to 400 psi. Foam provides a longer contact time between a cleaning agent and the soil. The generators reduce the amount of cleaning solution required. Foam application prevents splashing of cleaning solution. Thus, it also reduces irritating fogs and vapors. Foams help clean the undersides and other hard-to-reach surfaces of equipment by clinging to surfaces. Finally, they enable employees to clearly see the equipment areas covered.

The suitability of a foam for a particular job depends on its formulation, wetness, and contact time. A foam system requires a compressed air source and may be permanent or portable. Foams are not suitable for removing attached soil that requires turbulence for shear cleaning.

*Compressed air systems*

These systems deliver compressed air to work stations. The air can be used to blow away product residues, remove excess cleaning solutions, dry surfaces or components, and aid in applying foam. A compressor should have an air filtration device. Air compressors may be centralized, decentralized, portable, or fixed. The risk of creating aerosols and thus spreading the material throughout the plant should be balanced against usefulness.

*Portable and fixed wet–dry vacuum systems*

Wet vacuum systems are employed to remove excess or pooled cleaning solutions and water from equipment, floors, and other surfaces. Dry vacuum systems are necessary in areas where dry ingredients or products are processed. They are used for dry removal of residues, powders, and soils and are required in situations where wet methods cannot be used. Most plants centralize their vacuum systems. However, they may be portable as well.

*Ultrasonic cleaning systems*

An ultrasonic cleaning system consists of an immersion tank, a cleaning solution maintained at 65.6°C (150°F), an ultrasonic generator, and ceramic transducers. An ultrasonic generator produces waves in the frequencies of 30,000 to 40,000 hertz (cycles per second). The ceramic transducers convert the ultrasonic energy into mechanical vibrations. Ultrasound devices are

useful for cleaning small pieces of equipment that are delicate or hard to clean.

*Recirculation wash tanks*

This type of system consists of an immersion tank in which jets recirculate a cleaning solution at an elevated temperature across and through the tank. The combination of cleaning agent, hot water, and the physical shear force of circulation removes soil. This removal system is effective only for pieces of equipment that are small enough for immersion into the tank.

*Automatic washers*

An automatic washer includes a cabinet or tunnel washer that can prerinse, wash, rinse, sanitize, and dry items, and can warm or cool items, if required. Automatic washer systems are often used to recycle containers for product fills.

*Clean-in-place (CIP) systems*

Clean-in-place systems depend on the high velocity circulation of proper cleaning and sanitizing solutions. These solutions are circulated for a specified time at a specified temperature in a closed system designed for that purpose. No dismantling of equipment is necessary. A CIP system can apply to an entire production line or to individual parts of the line. Clean-in-place systems are useful for product-carrying lines, fittings and valves, tanks, production vessels, centrifugal pumps, heat exchangers, evaporators, and conveyor belts.

The advantages of CIP systems include reduced labor costs, automation, and the ability to use strong alkaline cleaners (1000 to 1500 ppm active alkalinity). Other advantages include faster clean-up, fewer leaks, and less damage from frequent disassembly that is common to other systems. Coupled with the ability to recirculate cleaning solutions (thus adding a cost savings), these advantages add up to a better and more cost-effective cleaning system.

*Miscellaneous equipment and supplies*

Manual or special cleaning of equipment and its parts may require special types of cleaning devices and supplies including scrapers, nonmetallic abrasive pads, squeegees, soft or stiff bristle brushes, brooms, wiping cloths, buckets, sinks, pallets, ladders, and stools. Wire brushes may scratch and damage equipment. Steel wool and metal scouring pads can shed metal particles that can become incorporated in product and also scratch the equipment. Wire brushes, steel wool, and metal scouring pads should never be used on production equipment, especially on the surfaces that contact product. Even minute scratches barely visible to the eye represent Grand Canyons in the microbial world and constitute perfect places for growth.

## *Cleaning and sanitization procedures*

Decisions about cleaning and sanitization frequencies, procedures, products, and equipment depend on the area and the equipment to be cleaned and sanitized. Every operation should establish and follow written instructions covering cleaning and sanitization procedures and frequencies. Continuous monitoring by supervisory personnel is necessary to assure the adequacy of schedules and procedures. Monitoring also provides data on which to base decisions that will ensure timely remedial action, revision of procedures, and adjustment of schedules when necessary.

The three areas to consider when establishing these procedures are warehouses, manufacturing and production facilities, and manufacturing and filling equipment. In addition, a production operation requires the establishment of appropriate monitoring, training, and waste disposal practices.

### *Warehouses*

Warehouses are used to store raw materials, packaging materials, finished products, equipment, and miscellaneous supplies. Environmental conditions within a warehouse must be sanitary to prevent microbial contamination of raw materials, finished products, and the environment. The following actions are vital for maintaining acceptable environmental conditions in warehouses:

- Ensure that janitorial personnel receive proper training to maintain acceptable environmental conditions.
- Keep aisles neat and clean by sweeping or vacuuming followed by damp mopping or machine scrubbing as needed.
- Clean up spills promptly using methods appropriate for the type of spill.
- Store all materials under clean conditions in an orderly fashion.
- Properly identify all raw materials.
- Protect raw materials from contamination.
- Assure that all containers are clean and are kept in clean, segregated areas before they enter manufacturing areas.
- Clean the exteriors of containers before transporting materials into manufacturing areas.
- Maintain routine pest (both insect and rodent) control programs to avoid vector-borne microbial contamination.
- Inspect warehouses routinely to determine the adequacy of sanitation and pest control programs.

### *Manufacturing and production areas*

Each area of a manufacturing plant requires specific cleaning and sanitization procedures and schedules. The procedures depend on the type of activities conducted and each area has specific needs. However, some general guidelines apply to all production facilities:

- Ensure that personnel receive proper training in cleaning procedures and use of chemical products.
- Prepare written standard operating procedures (SOPs) that adequately cover cleaning procedures and schedules.
- Clean floors daily by sweeping or vacuuming followed by damp mopping or machine scrubbing. One also may apply the right sanitizer when needed.
- Clean walls, ceilings, pipes, and fixtures on an appropriate schedule (monthly, quarterly, or whenever visibly soiled).
- Cleaning can be accomplished by vacuuming to remove dust and loose debris, but no airborne dust should be generated during clean-up operations.
- Clean up spills promptly using the correct method for the type of spill.
- Surfaces may need to be wet cleaned occasionally; use caution when spraying water and cleaning agents because it is essential to keep a manufacturing area as dry as possible to limit microbial growth.
- Use appropriate wall or ceiling cleaning equipment, or pressure sprayers only when necessary.
- Maintain all cleaning equipment in a clean and sanitary condition.
- Wherever possible, provide separate equipment for each area.

*Manufacturing and filling equipment*

As noted above with regard to the physical plant, cleaning and sanitizing procedures must be specific for each type of manufacturing and filling equipment. A written schedule covering the cleaning and sanitizing of each separate piece of equipment should be devised and followed. However, certain common basic cleaning and sanitization practices are applicable to all types of equipment.

- Determine cleaning frequencies for continuous process or consecutive batch equipment by appropriate testing.
- Thoroughly clean and sanitize all compounding and filling equipment between batches of product and after operations cease.
- Clean manufacturing and filling equipment by soaking, spraying, CIP, or any other method that uses appropriate chemical agents and cleaning equipment.
- Use the least labor-intensive cleaning procedures suitable for the task provided they are effective.
- Thoroughly rinse all cleaning solutions from equipment surfaces using an acceptable water supply.
- Properly protect cleaned and sanitized equipment to prevent contamination.

*Monitoring*

Supervising personnel should monitor work practices and compliance with established procedures and schedules. Quality assurance personnel should check the adequacy of the cleaning and sanitizing processes by visual observation and by appropriate microbial sampling. All monitoring required for GMP (good manufacturing practice) compliance should be documented.

*Training*

Training of all personnel who perform cleaning and sanitizing tasks must be thorough. This training should include the proper use of the chemicals, equipment, and procedures for each task. Cleaning staff should be thoroughly familiar with written procedures used for each piece of equipment for which they are responsible. This training should include proper disassembly of equipment as needed.

All employees who handle chemical agents or work in areas where chemicals are used must receive hazard communication training according to the hazard communication standard (worker right to know) of the U.S. Occupational Safety and Health Administration (OSHA). All requirements of the standard must be addressed and all training related to hazardous chemicals should be documented.

*Waste disposal*

The following general waste disposal guidelines apply:

- Place all refuse in covered leak-proof, rust-proof containers lined with plastic inserts.
- Empty all refuse at least daily — more frequently if needed.
- Waste containers should be cleaned as needed after emptying and before they are reused.
- Store refuse waiting for disposal in a protected area maintained in a sanitary condition consistent with a pest control program.
- Dispose of product and process wastes and all other types of wastes generated according to applicable local, state, and federal regulations.
- Train waste handlers in the proper methods of handling and disposal of waste.
- Handling should be in compliance with applicable plant policies and procedures and regulatory requirements.

## *Personal hygiene*

Good personal hygiene on the part of employees is a significant means of controlling microbial contamination in a manufacturing facility. Personnel who do not practice good personal hygiene will nullify all the training, cleaning, and regulatory compliance measures described above. Contamina-

tion can occur despite efforts to maintain the best facilities, equipment, and processes and implement effective cleaning and sanitization practices.

Personal hygiene is the one microbial control factor that is the least controllable by supervisory and quality assurance personnel. For that reason, a manufacturer must provide workers with proper training in good personal hygiene habits and also motivate them to want to practice good personal hygiene. Behavioral changes may be necessary. The most effective way to encourage cleanliness is through education about basic hygiene practices that should be observed, as discussed in the following sections.

## *Hand washing*

Personnel must always wash their hands after using the bathroom or whenever they return after leaving the production area. All employees' hands should be clean before they work with any product or production equipment. Signs reminding employees to wash their hands after using toilet facilities and before returning work should be prominently posted in relevant areas.

Proper hand washing requires readily accessible and adequate facilities that encourage the practice. Facilities should include hot and cold running water, a pleasant-to-use hand cleansing agent, and single-use towels or noncontact electric hand dryers.

Use of an antimicrobial hand cleansing agent is not as important as using a cleaning agent that encourages the hand washing process. A cleaning agent should be pleasant and feel good during use. It should impart a soothing smooth texture to the hands after use and leave a pleasant lingering odor on the hands. These signals will promote a behavior modification process that will lead to increased hand washing. Use of an emollient hand cleansing agent will also help prevent skin irritation, chapping, and cracking that can discourage hand washing. Cracking of the skin provides tiny foci for microbial growth. Hot air dryers also may cause excessive skin drying and cracking.

If hand-dip basins or antiseptic sprays are used, locate them immediately inside the area where employees re-enter the production area. Select the antiseptic agent used for these rinses based on both antimicrobial activity and mildness.

## *Dining and break areas*

Food should never be permitted or eaten in production areas. Dining facilities and break areas should be located near work areas and they should be maintained in sanitary condition. Provide ample trash disposal to encourage proper use of dining and break areas and hand washing facilities to encourage personal hygiene. Cleaning supplies to be used to remedy spills should be available. Dining areas should be cleaned and disinfected at least daily.

Smoking should be permitted only in designated smoking areas. Tobacco products should not be permitted in production areas even if they are not used there.

### *Wearing apparel*

Only authorized apparel should be worn in manufacturing areas. Personal clothing (if permitted) and company-provided uniforms should be clean when a shift starts. Generally, employees should cover their street clothes with lab coats or other clean cover-ups. All plant policies dictating the wearing of coats, smocks, aprons, and jumpsuits should be followed. These work clothes should never be worn outside the production area and should be changed as often as necessary.

Hair coverings (hats and beard covers), masks, and gloves should be worn correctly and changed as often as needed. Production personnel should not wear loose jewelry, earrings, brooches, high-crowned rings, or wrist watches. Wearing of plain-band rings (such as wedding bands) is permissible. Fingernail polish and long nails are not permissible.

Personnel who handle product or work with production equipment should not wear badges, decorative buttons, or identification cards. If they must wear identification badges, they should be securely attached to closing so they do not accidentally fall into the product or equipment.

### *Personal habits*

People who work around product or production equipment must not scratch their bodies or place their fingers in or around their noses and mouths. They should not sneeze or cough on product or equipment. Supervisors should be on the alert for any other personal habits of employees that could result in contamination of product or the equipment.

## *Raw material handling*

Raw materials for use in cosmetics do not have to be sterile. However, proper handling is important to reduce the potential for microbial growth in the materials and to prevent introduction of microorganisms.

The first step upon receipt of raw material should be inspection of package integrity. Bags should not be torn or wet. Drums should not be rusty or have too many dents. If it is necessary to accept materials that are not in good condition, segregate them into an area for special handling.

Place raw materials that are sensitive to microbial contamination into a segregated quarantine area until released by the microbiology laboratory. Once a raw material is proven acceptable, remove it from the holding area and place it into the warehouse for use by manufacturing. Store all raw materials in an orderly manner; date codes should be visible to ensure proper rotation.

Sample incoming raw materials in an aseptic manner in order to reduce the potential for introduction of microorganisms into the material that may result in contamination. Train the manufacturing personnel in the proper methods of opening containers and handling raw materials. Clean the exteriors of all containers before opening. Properly reseal partially used containers of raw materials to prevent contamination during storage. Repackage damaged containers of raw materials before returning them to storage.

## *Sanitary equipment design*

Sanitary equipment design is frequently neglected in the course of developing and purchasing equipment. Equipment can be one of the major sources for contamination of product during manufacturing. Properly designed machinery in operation will release microorganisms from locations harboring biofilms. These areas are usually not accessible for proper cleaning and sanitizing. Processes such as milling, sieving, centrifuging, and mixing may release contaminants into the environment. These contaminated aerosols can then transfer to other areas of the plant to cause contamination of other products.

The microbiologist plays an important role in determining proper cleaning and sanitizing procedures. He or she should also play a central role in approving sanitary designs of equipment before purchase or construction. Issues to be considered include the materials used to make the equipment, installation of sanitary fittings and valves, and providing appropriate slopes for proper drainage of the equipment components.

Alkaline and acid detergents and sanitizers may affect internal equipment surfaces. Heat sanitization may exert detrimental effects on construction materials too. Some of the effects including swelling of gaskets, expanding pistons and then freezing them into place, and shorting out electrical circuitry.

A microbiologist should consider an equipment design that permits ease of cleaning and inspection. This will help reduce critical hazard areas. Some of the key potential problem issues to consider are dead legs, inadequate drainage, and pipes that slope back into pumps and tanks.

Another area to consider when designing and constructing process equipment and systems is to ensure that materials used are compatible with the product. Systems should also be able to withstand the temperatures and pressures encountered during the cleaning, sanitation, and manufacturing processes. The most common material used to construct process equipment is stainless steel of at least grade 304; grade 316 is ideal. Surfaces should be ground smooth along welds and joints. Materials to be cautious of are cast materials because of surface roughness, aluminum because of reactivity, and plastic because of porosity.

The cleanability of the equipment is very important. The people who work with the equipment must be able to disassemble it easily to allow for proper cleaning, prevent cross-contamination from previously run products,

and help to control microbial growth. Surfaces must be easily accessible for inspection and mechanical cleaning. Equipment should be located in an area that permits easy cleaning and maintenance. The area should also be sanitary in order to avoid possible cross-contamination.

Compounding equipment must be of a sanitary design from the water inlet through the outlet valve of the mixing tank. It should be equipped with covers to prevent airborne contamination and bottoms should be rounded to promote proper drainage. Process control equipment such as flow meters, thermometers, pressure gauges, viscosity monitors, and level meters need special attention to prevent product build-up.

Pumps used to move liquids to various locations or to homogenize or recirculate product should be easily accessible for dismantling and cleaning. The inspection of pumps should include checks of bearings and shaft seals for evidence of leaking or product retention. The integrity of gaskets and impellers should also be inspected. All pumps should empty completely. Screw-threaded connections should be avoided as they can serve as sources of contamination.

Transfer hoses should be made of a material that is compatible with product and cleaning agents. Hoses should have sanitary fittings and be as short as possible to aid maintenance. Hoses should be cleaned and hung properly to drain adequately when not in use. After hoses have properly drained and dried, the ends should be covered or capped to prevent post-cleaning contamination.

Transport pipelines should be made with stainless steel and fitted with sanitary (or dairy) fittings. Other materials used in transfer piping have shown the potential for biofilm build-up and are difficult to clean. All pipelines should be sloped to drain properly. Sagging horizontal lines prohibit proper cleaning and may become sites of product build-up. Avoid dead-ends, "air-hammer" pipes, right angles, and vertical bends that can become contamination reservoirs. In-line filters may also serve as sources for contamination if not cleaned routinely. Filters should be designed for easy cleaning, sanitizing, and inspection.

Filling equipment used today in the cosmetic manufacturing environment is very diverse. However, certain common important features are critical when filling equipment is designed or selected. These features allow for proper cleaning and sanitization. The equipment should be designed for fast and easy dismantling. Sanitary detection switches should be used to determine proper product levels in filling equipment hoppers. Dead ends in product transfer piping should be eliminated. Bacteriological filters and air line dryers should be installed in order to prevent air line condensates from contaminating product. All critical control points should be regularly monitored to reduce contamination of product during the filling process.

Product containers are other items associated with filling equipment that require consistent monitoring. They should be protected from environmental dust and moisture. All portable tanks and hoppers that contain product while in use should have covers.

## Sanitary plant design

The design and construction of a plant will directly influence the ability to maintain clean and sanitary conditions within and outside the plant. The design and construction should include consideration of environmental surfaces, drainage, location of pipes and utility systems, and pest control. Plant design and construction should also include convenient traffic and material flow, proper lighting, and features that will allow efficient cleaning. Finally, every area of a manufacturing plant should be kept in good repair to meet the sanitary design parameters.

### Environmental surfaces

The environmental surfaces (floors, walls, ceilings, and windows) should be designed so as to limit the deposition of dust and debris and facilitate cleaning and sanitization.

#### Floors

Floors should be smooth and nonporous. They should be easily cleanable and able to withstand the traffic loads they will bear without sustaining damage. Vinyl flooring is easily cleanable but may not withstand heavy traffic. Concrete, quarry tile, and terrazzo flooring may be more porous but these materials are sealable with durable sealants such as epoxy coatings. Sealing makes a floor readily cleanable and protects against the corrosive actions of cleaning agents. The subflooring over which any flooring lies should be stable to prevent cracking of the flooring material. Because cracking interferes with floor cleanability, any cracks that appear should be sealed or the flooring should be replaced.

Floors should have slight slopes leading to drains. The drains should be constructed and maintained so they will remain unclogged and will not backflow. They should dry out completely between exposures to water without creating traps of dried materials and they should not create odors. Floor drains should be kept clean to prevent cross-contamination. All sink and plumbing fixtures should also be kept clean and in good repair and include back siphonage prevention devices. Rounded concave coves should be installed along all junctions between floors and walls to restrict the buildup of debris and to ease cleaning. The flooring material should extend for a short distance up every wall it touches.

#### Walls, ceilings, and windows

All walls and ceilings should be made of smooth, nonporous, easily cleanable materials that are resistant to the corrosive actions of cleaning agents. Often used wall coverings include vinyl, durable paints, and ceramic tiles. All seams and joints found in walls and ceilings should be sealed. Any cracked, damaged, or peeling wall and ceiling surfaces should be repaired or replaced

as soon as possible. Use kick plates or wall shielding to prevent damage where doors, corners, and walls are subject to contact from heavy equipment.

Projections on walls and ceilings should be kept to a minimum to maintain smooth, easily cleaned surfaces. Lighting fixtures and air vents should be flush with ceiling surfaces. Keep suspended piping extending from ceilings to a minimum. Locate piping behind ceiling overheads where possible, but all piping and utility systems should be readily accessible and cleanable.

Windows should be nonopening types and should be installed flush with wall surfaces. Window ledges should be eliminated because they are subject to dust and moisture deposition. When using hot water or steam for cleaning and sanitizing or where the humidity is high, installation of thermal windows reduces moisture condensation.

### *Traffic and material flow*

Plant design should allow a one-way, sequential flow of materials. Raw materials should enter the manufacturing area from a single set entry area. Any materials brought into the manufacturing area should be clean prior to entry. Outer secondary packaging materials should be removed before they enter either the raw materials dispensing area or the manufacturing area. A one-way sequential flow of materials will restrict any cross-flow of clean and contaminated materials. It also adds a measure of assurance that the required sanitation and product quality standards are being met.

Another important issue is restriction of personnel traffic. Do not allow unnecessary traffic in the plant. Provide separate pathways. Only authorized personnel should be allowed in the manufacturing area. Enforce restricted access through set entryways and designate a location where plant personnel can don any special protective apparel required for entry.

### *Lighting*

It is important to provide adequate lighting in all plant areas, particularly around machinery. Sufficient lighting allows personnel to accomplish their assigned tasks in a safe manner and meet performance requirements. Applicable OSHA lighting requirements and illumination standards should be met. Safety lights should be installed wherever flammable products are present and in all areas where lights are subject to contact with water.

### *Pest control*

Integrated pest control is a combination of sanitation first and the use of pesticides second. Sanitation in this contest includes designing a manufacturing plant with pest control in mind. Design and construct all areas of the plant to restrict access to insects, rodents, birds, and other pests. We have already discussed design and construction factors that will provide for min-

imum debris build-up and maximum cleanability. This section covers control of insect, bird, and rodent pests.

All unnecessary openings into the plant should be sealed and all required openings to the outside should be screened. All doors should be kept closed when not in use. Loading dock doors or other closures suitable for loading operations should be installed. Vertical air curtains can be used to restrict the entry of flying insects through doorways that must remain open for lengthy times. All areas that have potential to harbor pests must be eliminated. Pay special attention to warehouse and storage areas not subject to the strict cleaning and sanitization practices required in the production areas. Also, correct any conditions on the exterior of the plant that can serve to attract or harbor pests.

### *Plant exteriors*

Plant exterior areas should be maintained in clean and orderly fashion. Exterior maintenance includes controlling excessive growth of weeds and decorative plants to prevent infestation by insects or vermin. Areas used for waste storage should be maintained in a sanitary manner to control pests and for aesthetic reasons. All spills should be cleaned promptly. Exterior areas must be kept in good repair to help control environmental conditions within the plant and restrict pest entry. Roofs must be constructed to provide good drainage and be kept in good repair to prevent leakage of water into the building.

Good drainage must be provided around the plant to eliminate standing water that could serve as breeding places for insects or allow prolific microbial growth. Drainage must also accommodate run-off from rain or melting snow to avoid flooding and to allow for spill clean-up.

All above-ground bulk chemical storage areas should be diked so that they contain spills. Construct below-ground chemical storage areas to be impervious to contaminating ground water. Provide drains if the spilled chemicals can be safely discharged to a sanitary sewer or storm drain.

## *Sanitary design of warehouses*

The Federal Drug and Cosmetic Act has set the general requirements for the design and construction features of storage areas. These regulations specify the minimum requirements for current good manufacturing practices (cGMPs). Products not stored according to cGMPs may become adulterated. Therefore, warehouses must provide conditions that will not exert harmful effects on materials stored in them.

Sanitary design of a warehouse encompasses the building and the outside grounds. Grounds surrounding a warehouse should have good drainage to prevent the breeding of insects. Regular removal of discarded equipment, lumber, litter, waste, and weeds is necessary to prevent breeding and harboring of insects, rodents, and other pests. Outside waste disposal containers

should be located in a well-drained area and kept covered between uses. They should also be kept clean and in operable condition.

Warehouse walls, ceilings, and floors should have finishes that are resistant to normal use and accessible for maintenance and cleaning. The cleanability of a surface is an important microbiological issue. Soil and dirt accumulation on a surface has the potential to become airborne and release contaminated particles through a warehouse environment. Conversely, clean, dry surfaces will not retain dirt particles under normal use conditions.

Adequate lighting is needed in warehouse areas designed for handling, processing, and examination of stored materials, and also to allow inspection, clean-up, and repair of the structure and equipment. All warehouse equipment such as trucks, jacks, and dollies, must be cleanable. Equipment must never contaminate materials or products with lubricants, metal fragments, or water. The ventilation must be adequate to prevent condensation from developing on ceilings, fixtures, ducts, pipes, and products. Other sanitary design features include sanitary plumbing that will provide enough potable water throughout the warehouse and properly remove sewage and wastes. As a further measure to prevent contamination, washing and toilet facilities should not be located in areas in proximity to warehouse contents.

## *Sanitary design of water systems*

It is critical to design water systems that permit effective sanitization. Holding tanks and associated piping should be made of stainless steel. Galvanized, copper, polyvinyl chloride (PVC), and black iron piping should never be used. Water lines entering mixing tanks should not permit back-siphonage of the tank contents into the water system. This is easily accomplished by installing shielded air gaps. Prevent back-siphonage in the system when pressure drops in water lines by avoiding cross-connections. Construct filters in the water system in parallel. This allows isolation of a filter needing service from the rest of the system.

Supply water treatment systems with chlorinated city water. The treated water should go directly into stainless steel storage tanks and be heated periodically to 180°F. Circulate the heated water through the system and into the storage tank. An alternative to heat treatment of water is ozonation. Ultraviolet lights at the point of use can be used to remove the ozone and further sanitize the water.

## *Sanitary design of air systems*

The designs of air systems must be specific for each area served and should also consider the air quality needs for the operations performed in each area. This will require several different air handling systems based upon the different air quality needs in the areas served. These systems are commonly known as HVAC (heating, ventilation, air-conditioning) systems.

HVAC system design must consider several factors including the quality of the incoming air, temperature, humidity, air exchange rate, and desired air purity. Other issues that require consideration are locations of incoming and exhaust air vents and ducts and the duct work for the control of air flow patterns.

## *Air filtration*

All incoming air supplies should be filtered. The degree of filtration required depends on the air quality desired in the area served and the quality of the incoming air. Raw material areas may require low efficiency air filtration while manufacturing and filling areas usually require more highly purified air and air filtration. Most systems require sequential filtration: a primary filter providing lower level filtration and secondary and tertiary filters providing higher level filtration. Install filters downstream from the air handling units (fans). Electrostatic precipitators are sometimes useful in place of final filters to remove fine particulate matter. Servicing filters or changing them on a regularly scheduled basis is preferable to changing them only when they are dirty and restrict air flow (as determined with a manometer).

## *Air treatment*

Many manufacturing plants must humidify or dehumidify the air. This need depends on area requirements, the time of the year, and the geographical location. Humidifying and dehumidifying systems must be maintained in a clean and sanitary manner. The water in humidification systems and the collection pans and drains of dehumidification systems must be monitored and properly maintained to prevent the proliferation of microorganisms.

## *Air exchange*

Air supply systems must be balanced in order to provide proper air exchange rates for the areas served. It is preferable to have cleaner areas under slightly higher air pressure than adjoining dirtier areas. The positive pressure in the clean area will restrict the flow of contaminated air from dirtier areas via openings where air exchange occurs. Unserviced filters become overloaded and restrict incoming airflow, thereby lowering the air exchange capacity. Any nonessential or unplanned openings, such as cracks in walls can alter the exchange rate. This makes good building maintenance essential to maintain the desired airflow.

## *Energy conservation*

It is the practice of some manufacturing plants to turn off the HVAC systems at night, on weekends, or holiday periods for energy conservation purposes. When this is done, the systems must be turned on again early enough to

bring the air quality back into conformance with requirements. This will usually require a few hours, depending on the size of the area and the efficiency of the air-handling system. In certain critical areas or in facilities where production is almost continuous, it will be necessary to leave the HVAC system on full operation.

### *Make-up air*

Some HVAC systems use 100% make-up air. Many HVAC systems use recirculated air or provide only partial fresh air make-up supply. Locate air intakes for incoming air supplies away from exhaust air ducts and loading docks. Keep them away from any other areas where chemical air pollutants may enter the fresh air make-up supply. The usual filtration used in HVAC systems does not remove chemicals from the air.

### *Monitoring*

Microbial or particulate air monitoring and simple airflow measurements are usually within the capabilities of plant personnel. However, the services of a ventilation engineer or industrial hygienist will permit more intensive and extensive evaluation of HVAC systems.

## *Summary*

Maintaining a manufacturing plan in sanitary condition is one of the critical preventive measures for mitigating contamination of product. In fact, in a well-controlled, sanitary plant where all raw materials are carefully controlled and equipment is kept industrially sterile, even marginally preserved products can be manufactured.

## *Resource*

For additional information beyond the brief overview contained in this chapter, consult Troller, J.A., *Sanitation in Food Processing*, Academic Press, Orlando, FL, 1983.

*chapter four*

# *Hazard analysis and critical control point (HACCP) protocols in cosmetic microbiology*

***Laura M. Goolsby and Harry L. Schubert***

*Contents*



## *Introduction*

Protocols for microbiological monitoring and control of manufacturing have been established for categories of products regulated by the U.S. Food and Drug Administration (FDA). They are designated the HACCP (Hazard Analysis and Critical Control Point) protocols.[1] Considerable documentation describes appropriate environmental sampling and control of manufacturing systems for drugs,[2,3] but cosmetics are not as precisely regulated as foods and drugs. While guidelines covering cosmetic manufacture exist,[4] they do

not include functional details for protocols specific to the cosmetics industry. Such details are essential because cosmetics indeed constitute the most unique group of consumer products.

Each cosmetic product includes up to hundreds of ingredients combined precisely; manufacture may involve various types of chemical reactions and fermentations conducted at different geographic locations. While they are not intended to be sterile, cosmetic manufacturing systems involve production demands unrivaled by other categories of consumer products. The unique product forms and packages distinct to cosmetic products are often accompanied by implements (e.g., applicators) of various conformations and compositions. Cosmetic products are distributed and marketed without refrigeration or shelf-life controls. Finally, depending on purpose, they are applied by consumers to all body surfaces and orifices.

Clearly, protocols for foods and drugs are insufficient to serve the cosmetics industry. It is the purpose of this discussion to present new HACCP concepts tailored specifically to serve the cosmetics industry.

## *What are HACCP protocols?*

Hazard analysis critical control point (HACCP) is a program for monitoring manufacturing processes to identify potential microbial contamination hazards and ensure the integrity of overall systems. This monitoring program measures the health of a system in its operational entirety and encompasses equipment design, microbiological control procedures, microbiological awareness of operators, operational training, product susceptibility, plant and equipment maintenance, processing parameters, and material flows.

All areas must be in microbiological control to sustain the integrity of the system and prevent microbial contamination. Identifying the hazards and determining the critical control points in order to manage the hazards will be the focus areas of this chapter. A critical control point can be described as any point at which a production control can be applied to minimize, eliminate, or control a hazard. For successful implementation of a HACCP program, operational involvement is key and the program must be integrated among all operations. The seven basic steps to implementation of a HACCP program are:

1. Conduct an assessment to determine hazards and identify preventative measures to minimize or eliminate each hazard.
2. Identify all critical control points.
3. Establish critical limits for each control point.
4. Establish an ongoing monitoring program.
5. Establish corrective action when a critical control point is outside predetermined limits.
6. Establish a documentation system.
7. Establish an ongoing system to re-evaluate the program and/or verify control.

## *Why apply HACCP to cosmetics?*

The ultimate benefit of implementing HAACP is monetary because the program serves as a primary means to avoid microbiological contamination problems that can bring a business to a screeching halt. The cost of a product contamination can be extreme and run well into tens of millions of dollars. Brands have been lost, images tarnished, and manufacturing locations closed as results of product contamination incidents. Potential cost elements include product loss, scrapping costs, and loss of production while the plant operations organization attempts to recover.

Another issue is significant impact on customer service. Most major microbial contamination incidents have involved exposure of an organism to a product preservative system and resistance by the organism as it becomes adapted over time after repeated exposures to a preservative typically in a diluted state. As illustrated in Figure 4.1, the ideal state of operation is in the first quadrant where a manufacturer proactively addresses any breaks or trends in integrity. HACCP is a way to maintain an operation in the ideal state of the first quadrant. Product quality is assured by information from the HAACP system followed by confirmation via finished product testing (quality control).

If operations are consistent with the parameters of the second or third quadrant, intervention is a necessity. In the second quadrant, substantial numbers of microbes are present but do not survive due to process controls and product preservation. Most major contaminations of cosmetic products occurred because entry into the third quadrant may not have been detected

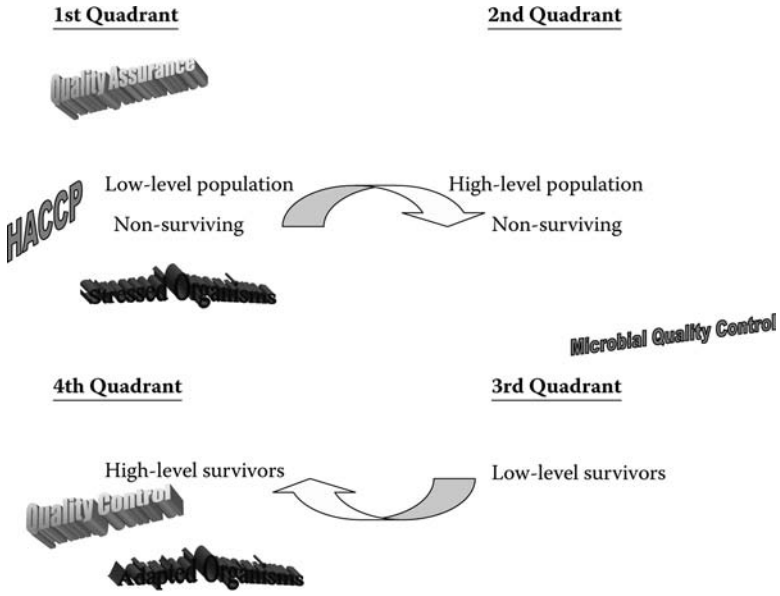


***Figure 4.1*** Cycle for building microbial resistance.

initially due to certain quality control method parameters (e.g., test sensitivity, preservative neutralization, or sampling frequency) coupled with low microbial numbers. However, once low numbers become detectable, almost any method will recover the organism if it can survive in the product because neutralization effectiveness will not be a barrier to recovery. Under these conditions and with transition into the fourth quadrant, product quality is only determined by finished product testing (quality control).

Areas to be assessed for hazards should include the following at a minimum:

| Ancillary Systems | Formulations | Production Systems |
|---|---|---|
| Water system | Raw materials | Hold tanks |
| Environmental air system | Premixes or intermediates | Delivery and/or transfer lines |
| Compressed air system | Finished formulations | Processing tanks |
| Steam system | | Bulk storage |
| Clean-in-place (CIP) system | | Packing transfer systems |
| Wastewater removal system | | Filling process systems |

## *Wastewater removal and CIP systems*

Wastewater removal systems and CIP systems are not normally considered when a HACCP program is established, but effective wastewater removal and CIP systems can contribute greatly to the integrity of an overall production system and should be evaluated.

Wastewater removal systems pose hazards because they usually maintain populations of organisms adapted to certain concentrations of the preservative systems used at a site. One needs to consider whether to eliminate, kill, or contain organisms when dealing with wastewater removal systems to control operational exposure of a potentially resistant strain. System control must include drain lines from systems, sumps, and associated lines. Control of this ancillary system is critical.

Another factor rarely considered as a risk to microbiological quality is a clean-in-place (CIP) system by which stand-alone components maintain and deliver cleaning or sanitization medium. These systems are typically seen as tools for cleaning and sanitizing and not as a potential hazards or sources of inoculation. Sanitary design may appear as less of a priority; drainability is not seen as a necessity because most systems use some type of cleaning chemical thought to be hostile.

CIP systems can very quickly become inoculation sources rather than means of effective cleaning and sanitization. For this reason, the design and management of these systems can be even more critical than the process systems they support. Inoculation occurs because production systems often remain idle between uses and the last fluid contained is typically purified

water. Because such systems are non-drainable and present the risk of building biofilms, they can become ideal sources of organisms adapted to the cleaning and sanitizing media and also to product preservative systems. These biofilms aid the development of resistance to cleaning agents and continue to build protective shields into the process systems they are intended to clean. Control of this ancillary system is critical.

### *Selecting critical control points*

In considering the selection of critical control points for an HACCP protocol, validation coupled with assessment (formulation, equipment, process flow) is the key to determining the points within a system and should be based on review of the following factors:

- Areas of low turbulence
- Poor equipment design
- Undrainable sites
- Areas of poor heat penetration (where heat is used for sanitization)
- Human intervention sites
- Low usage or idle pathways

## *Parameters of an effective HACCP program*

Any parameter that directly controls the integrity of the system should be the subject of monitoring and measurement. Examples include temperature of water in hot water storage tanks, ozone concentrations in cold water storage tanks, pH levels of product intermediates, moisture of raw materials, chemical concentrations of sanitizing solutions, equipment surface temperatures during heat sanitization, and the presence of diluted formulation in dead legs.

Not all parameters that can contribute to the control of systems are easy to measure, but they still warrant consideration, for example, execution of process steps that have microbial control significance, maintenance of equipment (o-ring and gasket replacement), housekeeping, etc. All physical measurements should be supported with biological monitoring throughout a system to verify that the control parameters are indeed effectively controlling microbiological quality.

Biological examination of systems can involve two approaches based on HACCP objectives:

1. **Maximize product volume without complete neutralization of the formulation.** The method objective is to recover organisms capable of surviving in system and product. Basically, this step is intended to prevent release of product with a potentially adapted organism that could subsequently proliferate in the marketplace.
2. **Optimize product volume to achieve complete neutralization.** The method objective is to recover stressed organisms and/or organisms

that cannot survive in product. This approach prevents release of product with a non-adapted organism and signals and alerts manufacturing personnel of a potential compromise of the microbial integrity of a system.

In the context of the cycle-for-building resistance diagram (Figure 4.1), the first approach above will typically result in a window of time of initial contamination during which a product may meet specification because the number of microbes is below the limit of detection determined by typical methods. Upon reanalysis, the product may fail to meet specifications if organisms capable of surviving and growing in the product have had time to grow to detectable levels. Previously tested clean samples may now be confirmed as contaminated. Unfortunately, the saleable product that enjoyed the window of low microbial detection will be typically out on the market, placing both consumers and the manufacturer at risk. The power of the second HACCP approach cited above will eliminate this scenario.

With the second approach, low levels of organisms can be detected before resistance to the product preservative system is established. The source of contamination can be addressed and the risk eliminated. The cycle of resistance will be broken and operations can be maintained in a preventative versus reactive mode. The ultimate result is that both the consumer and the manufacturer are protected against contamination. This approach is consistent with the preventative strategies behind HACCP programs.

When selecting biological sites for monitoring, other areas to be considered include all sites where diluted product and/or preservative is present, areas surrounding functional pieces of equipment such as heat exchangers containing chilled water that can serve as potential sources of contamination, and all other conditions deemed critical based on the principles listed previously. Types of biological samples range from raw materials, product intermediates, final formulations, and cleaning solutions, to swabs and/or equipment rinses. Most effective HACCP programs cover a combination of all these types of samples. Swab samples offer the benefits of testing localized areas considered high risk and offer limited surface area accessible to swabbing. Rinse samples offer greater surface area contact, but can dilute a localized contamination source simply by the volume of water passing through the system or subsystem.

Frequency of measurement should be based on the significance of the control point and reflect the executional frequency and control of any related procedure. For example, in a purified water distribution system, microbial control may consist of maintaining 82°C temperatures at all points via recirculation. In this case, monitoring the ongoing temperature and installing alarms to detect malfunctions (temperature drops and/or pump outages) coupled with minimal microbial content monitoring would be appropriate.

If sanitization of a certain piece of equipment is a requirement, the potential monitoring could constitute measurement of microbial contents at all critical points by rotating through them during the sanitization produc-

tion window. Specific monitoring may be warranted after major maintenance or construction to verify that no microbiological integrity break of the system occurred when the work was conducted.

Critical limits for each control point must be established through assessment and validation data. Once limits are set, an ongoing monitoring program for a continuous determination of control is established and may be modified subsequently if necessary as a function of ongoing observations and change controls. The HACCP program thus becomes a dynamic, data-based system.

A documentation process should be in place to track generated data, aid decision making, allow corrective action, and provide other information relevant to the HACCP program. The HACCP program should also be re-evaluated on an ongoing basis to maintain effectiveness and verify that the systems are under control.

Common manufacturing issues and potential approaches to eliminate or minimize microbiological risk hazards focus on the following areas.

Formulation susceptibility:

- Make susceptible premix and intermediate materials inherently hostile by maintaining extreme pH levels, lowering water activity, moving preservative additions upstream in the manufacturing process, adding preservatives to premixes and intermediates
- Maximize process temperatures of premixes and intermediates
- Optimize sanitary equipment design
- Increase sanitization frequency

Equipment:

- Optimize sanitary equipment design and/or eliminate unnecessary equipment or flow paths
- Optimize construction materials (chemical or heat tolerance for exposure time required for biological control)
- Increase sanitization time and/or frequency

Process flow:

- Work toward one common flow path
- Manage idle equipment and flow paths
- Plan for major maintenance and/or construction

## *Losses preventable via an effective HACCP program*

### *Tri-blender operation*

A tri-blender is a device that incorporates powders into a liquid product stream using a high-shear mixer. It is much more efficient in dispersing

materials than an agitator in a standard mixing tank. The device is particularly useful for incorporation of gelling and suspending agents.

In this scenario, powder was introduced through a hopper into a tri-blender and hot purified ingredient water was used to incorporate the powder into the batch. The tri-blender flow path was tied into the recirculation line of the mix tank and retained residual powder diluted in purified water until the next batch of product was produced through this flow path.

The HACCP program identified this as a hazard. The incoming water temperature was a critical control point and the flow path was monitored biologically during normal production and after the longest planned downtime to represent the worse case scenario. This monitoring occurred during the week and then immediately on Monday morning after a prolonged weekend of downtime. The biological monitoring detected sporadic recovery of low levels of *Burkholderia cepacia* on Monday mornings. The hazardous conditions present were:

1. The water temperature could only be maintained prior to the addition and would drop in the tri-blender flow path when the device sat idle.
2. The combination of the residual powder and purified water provided a suitable environment for organisms to grow. This became an inoculation source within the process.
3. The *Burkholderia cepacia* organism acquires resistance very easily and is well known as the causative microbial agent involved in cosmetic product recalls.

The hazard was eliminated by recirculating the final preserved formulation back through the tri-blender pathway to eliminate any residual powder diluted in purified water that accumulated when the system was idle. The recirculation step became part of the batch process and was implemented every time a batch was produced.

## *Retention of diluted product in low point strainer*

For pump protection, a basket strainer was located on a transfer line. The basket strainer was the low point of the transfer line and consisted of a ball valve on the bottom of the strainer. The cleaning and sanitization procedure was dependent on the manual cycling of this ball valve.

The filling procedure consisted of the manual purging of product through the low point until full strength product had filled the low point strainer. The HACCP program identified this as a hazard. The low point was a critical control factor for the operation. It was monitored for visually clean rinse water after each cleaning, for temperature during each sanitization, for dilution in the purged product samples, and biologically throughout the sanitization window. The visual inspection of the purged product sporadi-

cally revealed dilution indicating failure during the filling procedure to purge through the low point valve manually. The hazardous conditions present were:

1. The ball valve was not sanitary and was considered high risk due to its design.
2. The effectiveness of cleaning and sanitization depended on manual intervention by the operator.
3. The elimination of trapped diluted product was dependent on manual intervention by the operator during the purging procedure. The environment allowed organisms to adapt or build resistance to the preservative system.

Hazards were eliminated by the following actions:

1. Short-term:
   Visual inspections of the purged product after filling the transfer line increased in frequency.
   Procedural reviews involved the entire team.
   Greater accountability was implemented.
2. Long-term:
   An in-line strainer was installed.
   The low point ball valve was replaced with a more sanitary butterfly valve.
   Cleaning, sanitization, and purging procedures were automated to eliminate the need for manual intervention.

## *Susceptible premix operation*

Two types of vitamins were mixed with purified water in a premix tank prior to transfer to the final mix tank. The final formulation became heavily contaminated with *Burkholderia cepacia* and the loss to the company was estimated at $350,000 based on scrapped batches and production time lost while decisions about future production were made. The hazardous conditions present but not identified were:

1. The premix formulation was highly susceptible: the combination of vitamins and water allowed microbial growth.
2. Equipment design was not sanitary for this type of application.
3. The sanitization frequency was inadequate based on the susceptibility of the premix.
4. The premix system was not subjected to biological monitoring to ensure that the overall integrity of the system was maintained.

The hazard was eliminated by process modification. The manufacturing process that caused susceptibility of the vitamin premix to microbial growth was eliminated and the vitamins were added directly to the final mix tank.

## *Inoculating CIP system and multiple processing flow paths*

A CIP system was utilized to introduce purified water into a process system for purposes of cleaning and sanitization. The CIP system was not drainable, not sanitary, and not monitored for integrity against microorganisms during use. The system became heavily contaminated and was inoculating the process system during cleaning and sanitization.

The process system included multiple flow paths that were not drainable and the cleaning procedure was not effective because diluted formulation was retained within some of the pathways. This provided an environment for *Burkholderia cepacia* organisms to adapt to the final product formula. The contamination resulted in a $20 million loss to the company based on the following factors:

- Packed product containing contamination had to be scrapped.
- A national recall covered product that may have been manufactured during the window of contamination.
- Three contract packing sites were contaminated by materials from the supplying manufacturing site.
- The extent of clean-up to remove the adapted organism from the supplying sites and contract packing sites was massive. Equipment had to be replaced, caustic washes were performed, gaskets and other items had to be replaced.
- Equipment at the contract packers' sites was upgraded to sanitary design.
- Moist heat sanitization capability was installed at contract packers' sites.
- The manufacturer paid to have the three contract packing systems put on hold status so that they could start up as soon as the plant sourcing the product was ready.
- The manufacturer lost revenue from more than 30 days of production.
- Product reformulation was required; the manufacturer was out of stock for over 30 days.
- Manufacturing was moved to another site where the adapted organism was not present.

The hazardous conditions that were not identified were as follows:

1. The CIP system was not designed to be sanitary and/or drainable.
2. The CIP system was not sanitized prior to use on the process system; it was not managed via heat and/or recirculation processes that would have maintained the microintegrity of the system when not in use.
3. The cleaning procedure used for the process system was inadequate.

4. Multiple flow paths were not managed when idle and contained diluted product, thus providing an environment in which organisms could adapt.
5. No biological monitoring of the CIP system, the process, or the bulk product was conducted; such monitoring would have ensured acceptable microbial levels prior to packing.

Hazards were eliminated by taking the following actions:

1. Moving production to another site.
2. Designing a sanitary system without multiple flow paths.
3. Implementing a cleaning and sanitization procedure that was effective.
4. Eliminating need for a separate CIP system.
5. Eliminating dilute product environment within the process.

## *Conclusion*

Cosmetic products are unique in the diversity of formulations, forms, packaging, distribution, and consumer use. Importantly, they are also unique in microbiological risks associated with their manufacture and an effective HACCP program will pay for itself in maintaining control of these risks. Just as preservation measures are intended to cover the life of a product, HACCP programs are perpetual and sustained during active manufacturing and should continue when equipment is idle. With effectively preserved materials, cosmetic HACCP programs provide the ultimate ways to provide microbial quality assurance.

## *References*

1. Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, HACCP: A State-of-the-Art Approach to Food Safety, 2001, http://www.cfsan.fda.gov/~lrd/bghaccp.html
2. Reich, R.R., Miller, M.J., and Reich, H., Developing a viable environmental monitoring program for nonsterile pharmaceutical operations, *Pharm. Technol.*, March, 92, 2003.
3. Parenteral Drug Association, Fundamentals of an environmental monitoring program, Technical Report 13, Revised, *PDA J. Pharm. Sci. Technol.*, 55, 1, 2001.
4. Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Cosmetic Good Manufacturing Practice Guidelines, 2001, http://www.cfsan.fda.gov/~dms/cos-gmp.html

*part three*

# *Cosmetic microbiology test methods*

*chapter five*

# *Antimicrobial preservative efficacy and microbial content testing*

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*Contents*

## *Introduction*

Cosmetics do not need to be sterile, but they must be adequately preserved or otherwise protected from microbial contamination and spoilage. When consumers use cosmetic products, they repeatedly challenge the cosmetics with microorganisms in saliva, on dirty hands, and in tap water. Microbial growth may occur in cosmetics and toiletry articles kept in a bathroom and subjected to heat and humidity.[1] These products include mascaras, eye shadows, shampoos, facial powders, and facial lotions (foundations and moisturizers).

Cosmetics intended for use in the eye area are of particular concern. The cornea, especially if compromised, can be extremely vulnerable to infection. Several cases of mascara contamination from *Pseudomonas aeruginosa*[2] have occurred. Microbes in products can lead to infection, discoloration, production of gas, and odor formation. Typical contaminants of cosmetics include *Enterobacter* spp., *Klebsiella* spp., *Serratia* spp., and *Pseudomonas* spp.[1,3,4]

Although cosmetics occasionally are contaminated with spoilage microorganisms, the biggest threat of contamination is the presence of pathogens that may potentially pose health threats.[5] Even nonpathogenic spoilage microbes in a cosmetic may cause disease under appropriate conditions. For example, a microbe contaminating a cosmetic product may be invasive if one applies the cosmetic to cover a blemish or break in the skin.[6] With the increasing numbers of immunocompromised individuals in the population due to the pandemic of AIDS, the problem becomes even more acute.

The microbiologist uses a variety of chemical preservatives to prevent contamination by pathogens or spoilage microorganisms. This use extends the shelf life of products. The preservatives include benzyl alcohol, boric acid, sorbic acid, chlorhexidine, formaldehyde, parabens, quaternary ammonium compounds, phenol, imidazolidinyl compounds, and others. Several excellent reviews cover this topic.[1,7–9]

The survival and growth of microorganisms in cosmetic products is well known. The purpose of this chapter is to review the technologies of two methodologies central to cosmetic microbiology: preservative efficacy determination and microbial content testing.

## *Preservative efficacy methods*

### *Current preservative efficacy test methods*

Three organizations serve as sources for guidelines covering testing of preservation efficacy in cosmetic and toiletry products in the United States. These sources are the Cosmetic, Toiletry, and Fragrance Association (CFTA), the American Society for Testing and Materials (ASTM), and the U.S. Pharmacopeia (USP). All three involve challenging cosmetic formulations with microorganisms. However, specific differences among the procedures are intended to address the concerns of the parent organizations.[10–15]

The U.S. Food and Drug Administration has revised the microbiological methods for cosmetics, Chapter 23, of its *Bacteriological Analytical Manual*.[6] A preservative challenge test that preferably is predictive of consumer contamination is the FDA's goal but it has not yet released a final version of such a challenge test.

#### *Preservation efficacy testing: general procedure*

The CFTA, ASTM, and USP have developed their own specific recommendations for organisms, media, growth and storage conditions, and test methods (Table 5.1, Table 5.2, and Table 5.3). Standardization of all the requirements of a challenge must be achieved in order to obtain reproducible data.

The preparation of the challenge organisms is of special importance. The growth and preparation of a challenge organism determines the physiological state of cells that exert a direct influence on the results of any assay of disinfection efficacy.[16,17] It is essential to maintain cultures of microorganisms that are subcultured on appropriate media to assure viability and resistance. Standard conditions for organism preparation and storage are essential for reproducible results.

It is important to remember that these tests use populations of cells — not individual cells. The data generated from these tests will be less variable if the cell population assayed is homogeneous. Liquid cultures or confluent growths on solid media are adequate for the reproducible growth of inocula, but reports about which mode provides the most reproducible results are conflicting.[17] Whenever growth is slowed due to stress (including exposure

***Table 5.1*** Suggested Organisms for Testing

| Organism | ATCC | CTFA | ASTM | USP |
|---|---|---|---|---|
| **Bacteria** | | | | |
| **Gram-Negative** | | | | |
| *Enterobacter aerogenes* | 13048 | | + | |
| *Escherichia coli* | 8739 | | | + |
| | N/A | + | | |
| *Proteus* spp. | N/A | * | | |
| *Pseudomonas aeruginosa* | N/A | * | | |
| | 9027 | | + | + |
| | 15442 or 13388 | + | | |
| **Gram-Positive** | | | | |
| *Bacillus subtilis var. globigii* | N/A | * | | |
| *Staphylococcus aureus* | 6538 | + | + | + |
| *Staphylococcus epidermidis* | N/A | * | | |
| **Yeasts and Molds** | | | | |
| *Aspergillus fumigatus* | N/A | * | | |
| *Aspergillus niger* | 16404 | | + | + |
| | 9642 | + | | |
| *Candida parapsilopsis* | N/A | * | | |
| *Candida albicans* | 10231 | | + | + |
| | N/A | + | | |
| *Fusarium solani* | N/A | * | | |
| *Eupenicillium levitum*[a] | 10464 | | + | |
| *Penicillium pinophilum*[b] | 9644 | + | | |

Asterisk = _____. N/A = no ATCC number cited in method.

[a] ATCC name; name listed in method is *Penicillium levitum*.

[b] ATCC name; organism previously known as *Penicillium luteum*.

to biocides) or because of some nutrient limitation, microbes have the potential to change their responses to a biocide.[18]

Bacteria used as an inoculum are usually at a concentration of $1.0 \times 10^8$ colony forming units per milliliter (CFU/ml). This allows for a 1:100 ratio of inoculum to product dilution, giving a final recommended concentration of bacterial challenge of $1.0 \times 10^6$ CFU/ml or gram. The high initial concentration of the challenge organism reduces the dilution of product upon inoculation. It is typical to use molds and yeasts at a final concentration of $1.0 \times 10^4$ mold spores or yeast/ml or gram (CTFA). The USP recommends $1.0 \times 10^5$ mold spores or yeast/ml or gram (ASTM). The USP calls for standardization of the yeast or mold inoculum to $1.0 \times 10^8$ yeast/spores per ml. This translates to an initial inoculation of $1.0 \times 10^6$ microorganisms per milliliter or gram of product (see Table 5.2). Note that the dilution of the test product should not exceed 1%.

***Table 5.2*** Comparisons of Three Challenge Systems

| Parameter | CTFA | ASTM | USP |
|---|---|---|---|
| Detailed passage information | No | Yes | Yes |
| Harvesting solution | N/A | Sterile water | Sterile saline |
| Use of actual contaminants | Yes | No | No |
| Specifics for mold harvesting | No | Yes | No |
| Mold harvesting solution | N/A | N/A | Polysorbate 80 |
| Standardization procedures | No | Yes | Yes |
| Mixed culture inoculum | No | Yes | No |
| Pure culture inoculum | Yes | Permitted | Yes |
| Standardized inoculum CFU: | | | |
| Bacteria | N/A | $1 \times 10^8$/ml | $1 \times 10^8$/ml |
| Yeasts/molds | N/A | $1 \times 10^7$/ml | $1 \times 10^8$/ml |
| Challenge CFU: | | | |
| Bacteria | $1 \times 10^6$/ml | $1 \times 10^6$/ml | $10^5$ to $10^6$/ml |
| Yeasts/molds | $1 \times 10^4$/ml | $1 \times 10^5$/ml | $10^5$ to $10^6$/ml |
| Inoculum amount | 1% final volume | 1 ml | 0.5 to 1% final volume |
| Sample size | 20 g or ml | 100 g | N/A |
| Sample times (days) | 0, 1, 2, 7, 14, 21, 28 | 0, 7, 14, 21, 28 | 7, 14, 28 |
| Use of neutralizers addressed | 3 general broths | Separate method: E1054-85 | Yes, and in separate guidance chapter* |
| Neutralizer evaluation | No | No | Yes |
| Controls | No | Yes | No |

*Note:* Table 5.1 lists organisms.

N/A = not applicable.

* CFU per milliliter or gram of product.

The challenge inoculum for the test consists of inoculating a known amount of test sample with the test organism. The technician then removes aliquots from the test sample and dilutes them in a neutralizing broth. Neutralization of the preservative must occur in the broth to avoid inflated estimates of efficacy. In these procedures, some residual preservative will carry over into the recovery medium and a neutralizer should be used to inactivate this residual and permit the organisms to grow. The CTFA and the ASTM both address the neutralization of the preserved test solution.

One determines the number of surviving CFUs by plating the broth dilutions on the proper agar. Table 5.3 lists several of these media and their uses. Thereafter, the inoculated plates are inoculated at the optimal temperatures until the colonies grow large enough to be countable. The number of

*Table 5.3* Media Used in Preservative Efficacy Testing

| Agar | Microorganisms | Purpose | Procedure | Ref. |
|---|---|---|---|---|
| Tryptic soy agar | Bacteria | General | CTFA | 21 |
| Nutrient agar | Bacteria | General | CTFA, ASTM | 20 |
| Eugonagar | Variety | General | CTFA | 22 |
| Sabouraud dextrose agar | Yeasts and molds | General | CTFA, USP | 23 |
| Potato dextrose agar | Yeasts and molds | General | CTFA | 24, 25 |
| Mycological (mycophil) agar | Fungi | General | CTFA | 26, 27, 28, 37, 38 |
| Mycophil agar, pH 4.7 | Molds<br>Acidic Bacteria<br>Yeast (saprophytic) | General | CTFA, ASTM | 26, 27, 28, 37, 38 |
| Letheen agar | Bacteria<br>Yeast, molds, anaerobic microorganisms | Neutralization/general | CTFA, ASTM | 19, 29, 31, 33, 34 |
| Tryptic digest of casein and soy agar | Bacteria<br>Yeasts and molds | General | CTFA, USP | 32 |
| Infusion agar | Bacteria | General | CTFA | 19 |
| Thioglycollate agar | Bacteria | Neutralization/general | CTFA | 19 |
| Tryptone–glucose–yeast extract agar | Bacteria | General | CTFA | 24, 25 |
| Dey–Engley agar | Bacteria | Neutralization/general | CTFA | 35, 57 |
| Trypticase soy agar with lecithin and Polysorbate 80 | Bacteria | Neutralization/general | CTFA, USP | 23, 32 |
| Soybean–casein digest agar | Bacteria, yeasts, and molds | General | USP | 23, 32 |
| Letheen broth with lecithin | Bacteria | Neutralization/dilution | CTFA | 19, 31 |
| Letheen broth with lecithin and Triton X-100 | Bacteria | Neutralization/dilution | CTFA | 19 |
| Thioglycollate broth | Bacteria<br>Yeast/mold | Neutralization/dilution | CTFA | 19, 31 |
| Williamson buffered suspending fluid (modified) | Bacteria, yeasts, and molds | Neutralization/dilution | CTFA | 19, 31 |

surviving CFUs is then calculated from the dilution, yielding countable plate growth (traditionally 30 to 300 CFU/plate).

Criteria for passage of these tests vary. Surviving organisms are evaluated either as *percent survivors* (i.e., no more than 0.1% of inoculum surviving) or as *$log_{10}$ unit reductions*. In practice, these are not different measures since the log reduction is calculated by subtracting the $log_{10}$ unit of the survivors at a time point from the $log_{10}$ unit of the inoculum concentration.

### *CTFA method*

These guidelines[19] were issued in 1981 by CTFA's Preservation Subcommittee.

*Challenge organism inoculum.* The challenge organisms suggested for testing cosmetics are listed in Table 5.1. In addition, it is often useful to include organisms that may be acquired from indigenous microflora around the eye, clinical isolates, or isolates from contaminated products.

One should culture the organisms on suitable media. Recommended media for bacteria include nutrient agar,[20] tryptic soy agar,[21] or Eugonagar.[22] Culture media for fungi include Sabouraud dextrose agar,[23] potato dextrose agar,[24,25] or mycophil agar.[26–28]

The storage of the working cultures is another concern. Once growth occurs, it is possible to maintain bacteria and yeast at 5°C on slants for a period dependent on cell type. For example, most bacteria and *Candida* spp. remain viable for up to 1 mo in refrigerated conditions, while *Pseudomonas aeruginosa* may not be useful after 2 wk (depending on specific conditions).

One effective means of maintaining mold spores is to store them at room temperature on slants. Weekly or periodic subculturing can be done to assure the viability of the microorganisms, but this practice raises the risk of loss of resistance. Cultures may also be frozen or lyophilized as an alternative to maintain the stability of the microorganism and end frequent subculturing. The main advantage to these latter storage means is that they prevent loss of genetic resistance factors. These factors, as well as those requiring phenotypic expression, are sometimes lost with frequent subculturing in media without the selective pressure of a biocide to maintain resistance due to genotypic and phenotypic characteristics. One may also maintain contaminating microorganisms in the same marginally preserved products from which they were isolated. This practice maintains continued resistance to the preservatives.

*Conducting test.* The microorganism challenge is usually done in at least 20 g or ml of product. A recommended challenge level is $1.0 \times 10^6$ bacteria or $1.0 \times 10^4$ molds and yeast per gram of product. The CTFA prefers use of single cultures for the challenge or pooling of similar organisms to provide specific data for each organism or category of organism.[29] Inoculum volume should not be more than 1% of the final volume.

These challenge levels represent a larger microbial challenge of the product than might be expected from normal consumer use. Therefore, most

challenge tests are far more stringent than they need to be in order to ensure that a product withstands contamination during consumer use. This is especially true when we consider that many products also have protective mechanisms for delivering the product without contact with the consumer; such protective mechanisms would be metered dosages or a closure system that encourages separation of the consumer from the product. Challenge tests do not measure the protection afforded by these delivery mechanisms.[30]

Once the sample is inoculated, the technician mixes the contents thoroughly. A sample is taken and diluted in the proper neutralization broth to inactivate the preservative. If the neutralizer's effectiveness cannot be established, then physical dilution or membrane filtration may aid in inactivating the preservatives (see next section).

Most procedures require pour plating of 1 g or 1 ml of the inoculated product. Some laboratories use an alternate streaking method to estimate the number of microorganisms present. Additionally, one may use spread plating, especially when the organisms are sensitive to temperatures required for tempering agar (45 to 47°C). The inoculated samples are incubated at room temperature or at a temperature that encourages proliferation of the test organisms used in the challenge. The incubation temperature for the plates is typically 32 to 37°C for bacteria and 25 to 30°C for fungi.

Most test method development groups such as CTFA, USP, and ASTM recommend sampling on the following days after each challenge: 0, 1, 2, 3 (eye cosmetics only), 7, 14, and 28 (other products). Some tests may need more than 28 days, depending on a product's intended usage. Rechallenges (multiple, sequential inoculations) are often conducted to estimate preservative adequacy in certain products. Repeatedly challenging the product with a particular organism will show the number of challenges needed to inactivate the preservative system.[19]

*Neutralization and recovery.* The CTFA recommends diluting the inoculated sample with the following neutralizers: Letheen broth with lecithin, Letheen broth with lecithin and Triton X-100, thioglycollate broth, and Williamson buffered suspending fluid.[19,31] Lecithin or Polysorbate 80 added to a medium is usually enough to neutralize most preservative carry-over and disperse the product when using the pour plate method.[19] Letheen agar is a standard recommendation for bacteria, yeasts, and molds.[29] This medium was originally intended as an anaerobic growth medium,[33] but it is also effective for neutralizing quaternary ammonium compounds.[34] Other media recommended by the CTFA[19,29] include tryptic digest of casein and soy agar,[32] nutrient agar,[20] thioglycollate agar,[33] infusion agar (brain, heart, veal, or combinations), Eugonagar,[22] tryptone–glucose–yeast or trypticase–glucose–yeast extract agars,[24,25] Dey Engley (D–E) medium,[35,36] and trypticase soy agar with lecithin and Polysorbate 80. Other media that may produce more luxuriant growths of fungi include Sabouraud dextrose agar,[23] mycophil agar, mycological agar,[26–28,37,38] and Eugonagar.[22]

A neutralizer should be incorporated in the plated agar when recovering bacteria by plating.[34,39–41] Most of these methods recommend Letheen agar.[19,31] However, an agar with sodium thioglycollate should be used with a preservative containing mercury or other heavy metals. Alternately, recovery of microorganisms can be facilitated by dilution of a preservative, if appropriate for that preservative.[42] The critical concern is the removal of residual antimicrobial activity to allow quantitative recovery of surviving microorganisms.

### *ASTM method*

*Challenge organism inoculum.* This method[43] is used to test preserved samples compared to nonpreserved samples. The ASTM provides a list of challenge organisms (Table 5.1). The method recommends maintaining the microorganisms on nutrient agar[14] for bacteria and on mycophil agar at pH 4.7 for molds, yeasts, and acidophilic bacteria.[26–28,37,38] Transfers should be performed monthly, with bacteria incubated at 32°C and fungi at 25°C.

One prepares fresh cultures for the inoculum. This is done by growing them on the proper solid medium for 18 to 24 h at 37°C (bacteria) or 48 h at 25°C (yeasts). Mold cultures should grow for 7 to 14 days (until full sporulation) at 25°C on proper media.

The method provides for harvesting the organisms with a sterile inoculating loop and transferring them to sterile distilled water. The optical density is measured at 425 nm to yield $1.0 \times 10^8$ bacteria/ml. Mold spores are dislodged from mycelial cultures by rubbing gently with a sterile inoculating loop or removal with a sterile glass hockey stick. Then the spores are filtered with sterile nonabsorbent cotton to remove the hyphae and break up any clumps. One may use a hemocytometer count to adjust the spore level to $1.0 \times 10^7$/ml.

*Conducting test.* ASTM allows two types of challenges: a mixed culture or single culture method. The mixed culture challenge permits three separate inocula preparations. These preparations usually include equal portions of (1) Gram-positive bacteria, (2) Gram-negative bacteria, and (3) yeast and mold suspensions. To determine the number of CFUs, the method uses serial dilution. Plating is done in duplicate using Letheen agar.[19,31] Incubation is done at 32°C for 24 h for bacteria and yeasts and at 25°C for 72 h for molds.

ASTM suggests preparing three 100-g samples in glass containers with lids and inoculating each sample with 1 ml of each microorganism suspension (final concentration of $1 \times 10^6$ bacteria/ml or $1 \times 10^5$ yeasts or spores/ml). The inoculated samples are then mixed and stored at ambient temperature. At the proper times (0, 7, 14, 21, and 28 days), one part test sample is mixed with nine parts Letheen broth. Additional 10-fold dilutions are done and duplicate plating of each dilution is performed with Letheen agar. Incubations of bacteria and yeasts are done at 32°C (molds at 25°C) for at least 72 h. One counts the CFU per plate to determine the number of surviving microorganisms per gram of test product.

Some cosmetics are subjected to repeated exposure or contamination. Where this is possible, ASTM allows a rechallenge with the microorganisms at 28 days. ASTM, however, does not specify an inoculum level. The test would then continue for an additional 28 days.

*Neutralization and recovery.* The ASTM procedure provides a method to check for neutralization of the preservative if no growth is seen on any plates. This is done by streaking plates from the $10^{-1}$ and $10^{-2}$ plates with a $10^{-3}$ dilution of nutrient broth culture of mixed inoculum. This mixed inoculum may be Gram-negative bacteria, Gram-positive bacteria and yeast, or a mixed culture of mold incubated 18 to 24 h. Lack of growth after incubation at 32 and 25°C for 72 h suggests that neutralization of the biocide did not take place. Unfortunately, this procedure does not prove that neutralization occurred at the time of plating.

The original plates used for the test have already been incubated at least 3 days. Therefore, the preservative is not in the condition it was in at the time of sampling. Growth in this system cannot be taken as evidence of effective biocide neutralization at the time of the sampling. Another far more appropriate method offered by ASTM is detailed below.

*Criteria for passage.* The ASTM criteria require that Gram-positive and Gram-negative bacteria and yeasts should show at least a 99.9% decrease within 7 days following each challenge and no increase should occur after that for the remainder of the test. Within 28 days, fungi should show a 90% decrease and again show no increase within the remaining test period. Unpreserved controls should fail both these criteria.

### *USP method*

The USP method[21] has undergone a great number of changes in recent years in an attempt to harmonize with the European Pharmacopeia and the Japanese Pharmacopeia. In addition, many changes have been introduced in an effort to minimize variability between tests and between laboratories.[44]

*Challenge organism inoculum.* Table 5.1 lists the recommended test organisms. One may also include any other organisms that are likely contaminants. A medium such as soybean–casein digest agar supports vigorous growth and is recommended for initial cultivation of such organisms.[21,32]

Freshly grown stocks of a particular culture are prepared by inoculating a solid agar medium. Incubate bacterial cultures at 30 to 35°C for 18 to 24 h. Incubate yeasts at 20 to 25°C for 48 h, and molds at 20 to 25°C for 1 wk, then harvest the bacteria and yeast using sterile saline (0.9% NaCl) and dilute the suspended cells to $1 \times 10^8$ CFU/ml. The mold is harvested with sterile saline containing 0.05% Polysorbate 80, adjusting the spore count to $1 \times 10^8$ CFU/ml. The number of colony forming units per milliliter determines the amount of inoculum to use in the test. The viability of the suspension should be monitored, especially if not used promptly.

*Conducting test.* A 20-ml sample of the product is transferred to a sterile, capped bacteriological tube if it is not possible to inoculate the product container and sample it aseptically. Inoculation of the test sample with the suspension is done using a ratio of 0.01 ml inoculum to 20 ml test sample. The concentration of microorganisms in solution should be between $1 \times 10^5$ and $1 \times 10^6$ CFU/ml. Numbers of viable microorganisms in the inoculum suspension are determined by the plate count method. Use this value to calculate the initial concentration of CFU/ml in the test product.

The inoculated containers are incubated at 20 to 25°C and examined 7, 14, 21, and 28 days after inoculation. Microbial numbers (CFU/ml) are determined by the plate count method at each of these intervals, and percentage change is estimated by comparison to initial viability.

*Criteria for passage.* According to this method, an effectively preserved system will reduce the viable bacteria level to less than 0.1% of the initial concentration by the 14th day. For yeasts and molds, the viable level must decrease or remain the same. The concentration for all microorganisms must remain at or below these designated levels for the remainder of the test.

## *Comparison of methods*

Of the three methods discussed, the ASTM includes the most detail. The CFTA procedure leaves more room for customizing a test for a specific target. The USP recognizes that many drugs are not subjected to the same rigors of consumer use and abuse as are cosmetics. Table 5.1 provides an overview of the three methods along with American Type Culture Collection (ATCC) data.

### *Challenge microorganisms*

Challenging a product with appropriate organisms is a major concern in determining how effective a preservative must be. Organisms representing possible contaminants obtained following consumer use or manufacture failures are ideal for challenge testing. Because organisms can develop resistance to preservatives and cause opportunistic infections,[1,5] we must always be looking for new sources of challenge organisms.

*Staphylococcus aureus* (ATCC 6538) is a common skin organism.[19] Most preservative challenge test methods use it to challenge frequently used cosmetic products because it is a common contaminant that may pose threats to consumers.[5] It represents Gram-positive cocci in many tests. Since its nutrient needs are comparatively demanding, it does not always seem to be a logical choice as a challenge inoculum.

*Pseudomonas aeruginosa* is a non-fermentative Gram-negative rod suggested by all three testing groups. The ASTM and USP recommend ATCC strains 9027 and the CTFA recommends strains 15442 and 13388. *P. aeruginosa* is a well-known, highly ubiquitous pathogen. It also shows high resistance to many preservatives.[29]

Both the CTFA and the USP methods recommend *Escherichia coli*, ATCC 8739, a fermentative Gram-negative rod. It is a member of one of the largest bacterial families, the *Enterobacteriaceae*, and is considered an indicator of fecal contamination.[29] Like most of the coliform bacteria, it can easily develop biocide resistance. *Enterobacter* (*Aerobacter*), *Klebsiella*, and the *Proteus* spp. are sometimes substituted for *E. coli*. ASTM even recommends *Enterobacter aerogenes* (ATCC 13048) instead of *E. coli*.

Methods of all three institutions recommend *Candida albicans* as naturally occurring representative yeast. It can be pathogenic[29] and also represent the resistance of yeasts to preserved systems. The ASTM and USP recommend ATCC strains 10231 while the CTFA does not recommend a specific ATCC strain.

A major cause of product decomposition is contamination by filamentous fungi such as *Penicillium* or *Aspergillus* spp.[29] All three methods recommend the use of *Aspergillus niger*. The ASTM and USP use ATCC strains 16404, while the CTFA uses strain 9642. In addition, the CTFA also suggests *Penicillium pinophilum** ATCC 9644.

Microorganisms indigenous to the normal eye, clinically significant isolates, and product isolates are recommended by CTFA for challenging eye cosmetics. These include the organisms detailed in Table 5.1. Gram-positive spore formers are represented by *Bacillus subtilis*. Eye cosmetics and inadequately preserved systems may allow Gram-positive spore formers to survive, germinate, and actively proliferate. Using spore formers such as *B. subtilis*, for a challenge inoculum should be done carefully in order to evaluate susceptibility of the vegetative forms (versus spores) to the preservative. If the inoculum preparation procedure promotes sporulation, then the challenge may be too rigorous for a product to pass until it is over-preserved and toxicologically unsafe for use.

### *Maintenance and harvesting of organisms*

The ASTM and USP give specific recommendations for maintenance of the microorganisms. The CTFA specifies the media but recommends only periodic subculturing. This is an important consideration as standardization of culture preparation is a critical concern for achieving reproducible results. Considerable work has been done by Peter Gilbert and his laboratory to show that a great deal of phenotypic variance occurs simply due to growing organisms under nutrient-limited conditions such as occur when grown to late log or early stationary phase.[17,18] The ASTM and USP methods also cover harvesting and standardizing conditions that may involve filtration of mold spores.[43]

Harvesting methodology differs for the ASTM and USP methods and this factor may influence the viability of organisms.[44] Neither method specifies buffering the solutions to the pH or ionic range of the culture medium. Suspension of the cells in a solution that is at an incorrect pH or osmolarity

* Previously known as *Penicillium luteum*.

compared with the culture medium may produce inhibitory toxic effects.[45] Orth found that broth inocula decreased the rate of inactivation of the test organisms compared to the use of saline inocula prepared from surface growth on agar media.[46] Although not specifically mentioned by Orth, this result was likely due to the broth medium serving as a preservative inactivating agent.

*Preparation and standardization of inoculum*

Dilution of the cell suspension should not occur in an unfavorable environment. Diluted cells are more susceptible to harm than denser cell concentrations. A buffered solution will protect against a pH change after the cells have been suspended.[45] The ASTM uses sterile water while the USP uses sterile saline when washing the organisms from the transferred stock culture. The CTFA does not specify any recommendations for harvesting.

The ASTM is specific as to standardization of bacteria, yeasts, and molds. It recommends a certain spectrophotometer, spectrophotometric tube, and a specific absorbance wavelength. Neither the USP nor CFTA are as specific. They require only a final inoculum level. The inoculum level is different for each of the three methods. Before inoculation, the ASTM recommends that the concentration of a microbial suspension be $1 \times 10^8$ CFU/ml for bacteria and $1 \times 10^7$ CFU/ml for yeasts and molds. The USP recommends adjusting the microbial or spore count to $1 \times 10^8$ CFU/ml while the CTFA does not address standardization of the suspension.

The resulting challenge level of the product is more similar between the methods than the inoculum levels. All three methods recommend a level of $1 \times 10^6$ CFU/ml or gram of product for bacteria. The USP actually gives a range between $10^5$ and $10^6$. For yeasts and molds, the CFU levels per milliliter or gram are slightly different. The ASTM recommends $1 \times 10^5$, the CTFA $1 \times 10^4$, and the USP again gives a range of $1 \times 10^5$ to $1 \times 10^6$ CFU/ml. The CTFA challenge level is comparatively low. As a result, this level limits the measurable reduction to less than four $\log_{10}$ units.

*Pure versus mixed cultures*

The ASTM recommends using a pure or mixed culture inoculum, while the CTFA and USP methods typically involve pure cultures. Mixed cultures may more accurately reflect the normal contamination profile of a product used by a consumer. However, pure cultures may exhibit more resistance to a preservative than a mixed culture.[5,15] One recommendation is that organisms should be related mixed cultures are used.[5] For example, one would combine Gram-positive or Gram-negative species.

*Incubation conditions, interpretation, rechallenge*

All three methods specify sample and inoculum size, and the ASTM and USP methods also define storage conditions for the samples. All three methods require sampling after inoculation at 0, 7, 14, and 28 days. The CTFA

also suggests sampling at days 1 and 2, with a 3-day sampling for eye cosmetics. By 7 days, according to the CTFA and ASTM, the vegetative bacterial counts should reach a 3 log or 99.9% reduction. The USP allows 14 days for this reduction of bacteria. The yeast and mold counts must be reduced by 90.0% at day 7 for the CTFA method and 28 days per ASTM. The USP only specifies that by 14 days the levels of yeasts and molds remain the same or below the initial concentrations within a certain tolerance level (e.g., 0.5 log).

In discussing these criteria, it is interesting to note that an apparent increase in the number of microorganisms recovered may be noted under conditions where no increase is expected. This can happen even with well-characterized formulations in tests performed by experienced technicians. Orth characterized this as the "Phoenix Phenomenon" and argued that it is to be expected in tests of this type.[47]

Only the ASTM recommends a rechallenge with microorganisms at 28 days. Repeat challenges may provide better indications of potential problems of product contamination while in consumer use.[1] For example, rechallenge may be important for assessing hand and body lotion contamination or testing mascaras that are repeatedly used. Rechallenging or repeating the inoculations may indicate how the preservative system of a particular product will withstand insult before failure.[30]

## *Other published methods*

### *D-value methods*

Orth proposed a rapid method for estimating preservative efficacy.[46,48] This method uses short sample times and estimates the final response at 28 days by linear regression as a D-value. Orth claimed that each organism has a characteristic rate of death. When this rate is multiplied by the log of the inoculum challenge, it can predict the time required to inactivate the entire challenge.[49]

This method is not a new development, but an adaptation from food microbiology heat destruction D-values. One weakness inherent in this method is the extrapolation of kill beyond the measured data. This is not valid for linear regression analysis in the absence of data showing the linear nature of such data.[50]

A second weakness of the method is that it assumes a linear relationship between time of exposure to the biocide and number of surviving microorganisms. One can usually analyze the logarithmic nature of biocide killing by log transforms. However, even this relationship does not exist for a variety of microorganisms and biocides.[51]

If one performs all the D-value assays in precisely the same manner, it may be possible to show reproducible results. This would allow a rapid preliminary screening of preservatives, but should not be relied upon as the sole method of testing.

*Capacity tests*

The capacity test assesses the efficacy of concentration and antimicrobial spectrum of a preserved cream, suspension, or solution. Mixed bacterial cultures are grown in nutrient broth for 48 h. Yeasts and molds are grown in 2% v/v malt extract in distilled water.[52] The test method permits storage of mixed mold spore suspensions in distilled water at 4°C. These suspensions are from cultures grown on malt extract agar plates.

In a capacity study by Barnes and Denton,[52] the mixed cultures consisted of: (1) Gram-negative bacteria (*Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, P. fluorescens*), (2) Gram-positive bacteria (*Staphylococcus aureus, S. albus, Micrococcus flavus, Sarcina lutea*), (3) aerobic spore formers (*Bacillus subtilis, Bacillus cereus, Bacillus megaterium*), (4) mold spores (*Mucor plumbeus, Aspergillus niger, Cladosporium herbarum, Penicillium spinulosum, Trichoderma* spp.), and (5) yeasts (*Saccharomyces cerevisiae, Sporobolomyces* spp., *Schizosaccharomyces pombe, Candida albicans*). The preservatives tested included benzalkonium chloride, Bronopol, chlorhexidine gluconate, chlorocresol, Dowicil 200, methyl parabens, Phenonip, propyl parabens, thimerosal, and "Preservative C." Barnes and Denton incorporated the preservatives into creams, suspensions, or solutions at recommended use levels and also tested two lower concentrations.

The test protocol required a thorough mixing of 1 ml inoculum into 20 g of the cream, suspension, or test formula. They stored the formulae at room temperature for 48 h, then sampled the creams and suspensions with a sterile loop; 1 ml was removed from each of the solutions. Barnes and Denton dispersed the samples into either 5 ml (for creams and suspensions) or 9 ml (for solutions) of nutrient broth containing Lubrol® and lecithin as neutralizers for all preservatives except thimerosal, which required sodium thioglycollate as the neutralizer.

A sample of this dispersion was then plated on nutrient agar (bacteria) or malt extract (molds and yeasts) containing neutralizers. The authors incubated the plates at 37°C for 24 h (bacteria) or for 25°C for 48 h (molds and yeasts). They repeatedly cycled through the reinoculation and sampling 15 times or until 3 consecutive positive results occurred.

A preservative must reduce the number of viable organisms in an inoculated formulation by $10^3$ within 48 h for creams and suspensions to produce a single negative result according to Barnes and Denton.[52] This ability diminishes gradually due to dilution and biocide absorption by the added organisms.

Bean[8] cited the need for a performance test rather than a typical preservative efficacy test. He felt that a performance test would measure "the ability of the contaminating organisms to destroy the product." Such a test may be more rapid than a typical preservative efficacy test and more quantitative in assessing the ability of a product to handle contamination.

*Tests predictive of consumer contamination*

Brannan et al.[53] conducted a study designed to validate the CTFA preservative efficacy test as a predictive model of consumer contamination. This is a critical concern. One can control the microbial insults from the manufacturer by sanitary processing, but a product must also provide consumer protection from pathogens during use.

In a later study, Brannan evaluated two cosmetic formulations, a lotion, and a shampoo, at three different preservative levels using a modified CTFA preservative efficacy test method. They first challenged products diluted to four concentrations (30, 50, 70, and 100%) with mixed cultures of bacteria known to contaminate cosmetics. After 28 days, they used these results to classify the formulations as (1) poorly preserved, (2) marginally preserved, or (3) well preserved. The formulae were then used by consumers and evaluated after use for contamination.

For the consumer contamination part of the study, the products were packaged in containers to allow direct contact with consumers. This assured that package design was not a significant factor in preventing exposure to microbial contaminants from the consumer or the environment. Brannan's group defined contamination as recovery of >100 CFU/g or the presence of Gram-negative bacteria at initial receipt and 4 to 7 days post-receipt.

No samples of the well-preserved products were contaminated after consumer use, while 46 to 90% of the poorly preserved products were contaminated after consumer use; 0 to 21% of the marginally preserved products were contaminated. Thus, the method accurately predicted the potential for consumer contamination. It did not, however, account for the significant role that containers play in preventing consumer contamination.

Farrington et al.[54] evaluated the abilities of nine antimicrobial systems to preserve an experimental water-based cosmetic formulation in a round-robin format among several manufacturers. Six microbiological challenge tests were evaluated: the USP, the British Pharmacopeia (BP), the CTFA, an experimental rapid screen test, a test that incorporated a rechallenge after 14 days, and a simulated use/post-use test (product was challenged with $10^7$ CFU/g *P. aeruginosa* following simulated use). The simulated products used in these assays were several combinations of varying amounts of two parabens and a quaternary compound.

The products were then also used to evaluate maintenance of product purity in an 8-week simulated in-use study. The level of contamination in the simulated in-use study was then determined to be the accurate measure and all laboratory tests were compared to it. The experimental rapid screen test was determined to be the best for predicting differences at intermediate levels of preservation, although the authors determined that:

> Statistically, all of the tests were equivalent predictors of preservation efficacy in the in-use test (P = 0.05). At the P = 0.10 level, only the U.S. Pharmacopeia, British Pharmacopeia, rapid screen, and Cosmetic, Toiletry, and Fragrance Association tests were sig-

nificantly predictive. The results of prediction by a test, based on the preservative levels used, agreed well with the in-use test results (P = 0.01).

Susan Lindstrom presented a different design in an attempt to model contamination occuring through consumer use.[55] While Brannan and the CTFA round-robin study both attempted to correlate laboratory tests to consumer use, Lindstrom focused on designing repeatable consumer panels to use in product testing. Four parameters are essential to the consumer use test: (1) consumer panel selection, (2) product selection, (3) test design, and (4) product evaluation. The panel selection is designed to be representative of the target consumer, the product selection to evaluate products of interest. The test design is important in that Lindstrom recommends a double-blind cross-over design, with the duration of use dependent on the product under test (mascaras tested for up to 8 weeks, for example) and the use conditions reflecting actual use. The evaluation phase is determined by the desired level of antimicrobial efficacy, but testing should be done within 24 h of last use to prevent loss of viable microorganisms.

The pharmaceutical industry has also been concerned about in-use testing, particularly in Europe. In this regard, Urban et al.[56] presented a laboratory test utilizing multiple low level challenges. The Committee for Proprietary Medicinal Products (CPMP)* has published standards on testing methodologies,[57,58] and the entire subject has been recently reviewed.[59] Fundamentally, the approach resembles that recommended by Lindstrom.[55]

## *Tests for factors affecting preservative efficacy*

A number of factors can influence the results of assays of preservation efficacy. Russell provides a review of some of these factors,[60,61] correctly noting that the condition of the challenge microorganism and the conditions of the assay can play large roles in the measured activity of an antimicrobial. While directing their discussion to antibiotic test methodologies, Gilbert et al. presented an excellent discussion of preservation issues in 1991.[62] The central theme of their discussion is that the treatment received by an inoculum will affect a microorganism's susceptibility to antimicrobial agents, particularly the manner in which it was harvested and the medium in which it is suspended.

### *Inoculum preparation and handling*

A recent review of the role inocula play in the apparent activities of disinfectants was prepared by Bloomfield et al.[63] They argue that much of the lab-to-lab and day-to-day variability seen in disinfectancy testing is due to

* The reference standards from the CPMP are available through the European Medicines Evaluation Agency (EMEA) Web site: http://www.emea.eu.int/index/indexh1.htm

poor control over the challenge microorganism cultures and inocula preparation and offer specific suggestions to address these sources of variability. Many of their suggestions have found their way into the current USP chapter (51) on antimicrobial effectiveness testing.[21]

The USP recommends the use of freshly prepared inoculum, a recommendation taken to encourage harmonization with the Pharmacopeia Europaea.[44,64] However, Muth and Casey present data suggesting that there is no difference in response to three different antimicrobial agents (sodium hypochlorite, benzalkonium chloride, and propyl paraben) if the challenge inoculum is freshly prepared or a frozen preparation, recently thawed.[65] It should be noted, however, that the activities of the agents were not measured in the standard antimicrobial efficacy test. Rather than measuring surviving CFUs after exposure, Muth and Casey measured the length of time required for challenge microorganisms to grow in trypticase soy broth (TSB) containing different concentrations of the antimicrobial agent. While this study is of interest, it is not clear whether it is relevant to questions of bactericidal activity or relates only to inhibition of growth.

Orth recommended standardized conditions of inoculum preparation in 1989.[66] Using the linear regression model for data analysis, he reported on two different methods for inoculum preparation: growth in broth and growth on solid agar with a saline wash. Upon addition of 0.2% broth inocula (0.1 ml in 50 ml of sample), he saw a decrease in the antimicrobial activity compared to that seen with a saline rinse of cells grown on agar. Not investigated were the effects of media constituents on antimicrobial activity and it would be of interest to see whether similar effects would be seen with broth-grown microorganisms that had been centrifuged and then resuspended in saline, eliminating the potential inhibition of the organic load introduced by medium carry-over.

The size of the inoculum can also have an effect on the apparent activity of the antimicrobial as reported by Van Doorne and Vringer[67] in 1994 after a study conducted with *Candida albicans* and *Aspergillus niger* and by Steels et al. in 2000.[68] When conducting a laboratory preservative efficacy test, it is necessary to have a large inoculum so that the microbiologist can measure the required reduction. It is difficult to measure a 3-$\log_{10}$ reduction if an inoculum is smaller than 100,000 ($10^5$) CFU/ml. However, these authors showed microbial growth in a cream formulation when the inoculum was relatively small, a growth not observed with greater inocula. They note that these results are not consistent with earlier work that employed prokaryotic challenge organisms.

Other authors have argued that the laboratory tests are so far removed from the real world situation that they are all but meaningless.[69] Factors seen as critical in this regard are the prevalence of biofilm as a contaminating agent, the extremely slow growth (and subsequent physiological changes) of naturally occurring microorganisms, and the extraordinary post-growth treatments to which inocula are subjected (including putative damage from centrifugation).

In a somewhat opinionated article, Gilbert et al. describe several aspects of inocula preparation of concern for reducing variability in antimicrobial tests through tight control of the inocula.[70] Of particular interest in this article are the discussions about growth on solid agar and the difficulties in generating reproducible inocula due to differences in growth rates. Liquid growth is recommended, however, in another article.[71] Gilbert et al. describe injury to the challenge organism *Pseudomonas aeruginosa* by centrifugation. One is left with a dilemma: how to grow organisms in liquid media (and so enhance reproducibility) while avoiding problems of media carry-over into the product tested. The pharmacopeias have elected to allow centrifugation.

## *Recovery conditions*

Media used for the recovery of microbial survivors will have direct effects on estimates of antimicrobial activity. It is well established that some media, while capable of supporting growth of healthy microbial cells, are unable to support the growth of sublethally injured microorganisms.[72,73] In addition to the nutritive properties of the media, the temperature and duration of incubation are of particular concern in providing opportunity for cell recovery and proliferation.[74]

## *Plating concerns*

It must be stressed that a significant amount of variation occurs strictly as a result of the methods used to count microorganisms. Standard practice utilizes duplicate plating to provide an estimate of the number of viable CFUs present in the suspension. This is acknowledged to be a different measure from the number of viable cells present as a colony could arise from 100 cells in a cluster or chain as easily as from a single cell. However, this consideration is not the only element of the variability introduced by the plating technique.

Wilson and Kullman looked at the advisability of increasing the number of replicate plates.[75] Using *Rhizobium trifolii*, they evaluated the errors of estimates in cell density using three or four parallel plates to derive an estimate of plating error. Based on this study, the authors recommended the use of at least three plates and preferably more to decrease the inherent variability and allow elimination (without bias) of plates containing markedly different colony numbers. Jennison and Wadsworth described two components to variability in plating: sampling error and dilution error.[76] While Wilson and Kullman assumed that dilution error was a small contributor to variability, Jennison and Wadsworth argued that errors in volume of dilution blanks, variability in pipettes, and the number of dilutions made were large contributors to variability in the derived microbial counts. These errors were examined in greater detail by Hedges[77] who recommended larger numbers of replicate plates as well as the preparation of tables estimating the errors

particular to the pipettes used in each laboratory and the method used for enumeration.

The other source of plating variability comes, of course, from the actual number of colonies on a plate. If too few colonies are present, random sampling error becomes too great to allow accurate estimates. Too many colonies on a plate will mask each other and compete for nutrients, thus depressing the observed numbers.[78,79] Breed and Dotterrer determined the most accurate counting range for milk-borne bacteria to be between 30 and 400 colonies/plate.[80] The FDA's *Bacteriological Analytical Manual* notes a specific range of 25 to 250 colonies per plate, citing Tomasiewicz et al.[81]

## Neutralizer evaluation

### Functions of neutralizers

A neutralizer should inactivate a preservative or biocide and allow for unrestrained microbial growth.[82] If a biocide is not inactivated, the antimicrobial activity of the biocidal agent will be overestimated because killing will continue in the recovery medium.[36] Included in the evaluation of a neutralizer should be controls to measure neutralizer toxicity toward the microorganisms.[83]

### Types of neutralization

#### Chemical neutralization

Lecithin, Polysorbate 80, and sodium thiosulfate are examples of chemical inactivators for quaternary ammonium compounds, phenolics and halogens, respectively. The type of neutralizer and the effective concentration must be determined for each biocide and microorganism permutation (see Table 5.4). Disinfectants and preserved products are typically tested by inoculating either directly or via contaminated carriers. When sampling an inoculated disinfectant or preserved product, one must stop the killing activity of the biocide immediately as the disinfectant or product is sampled.

Some biocides are difficult to inactivate. For example, sodium bisulfite is the neutralizer for formaldehyde and glutaraldehyde. Unfortunately, sodium bisulfite also inhibits the growth of bacteria and the germination of spores.[16] Thus, in addition to efficacy, it is equally important that a neutralizer be nontoxic to the microorganisms.

Neutralizer toxicity can be determined by comparing growth in the neutralizing medium alone to growth in a typical medium such as tryptic soy agar or Sabouraud dextrose agar.[39] Most test procedures require the chemical neutralizers to be included as part of the dilution broths into which the samples are placed. One may also include the chemical neutralizer in the plating agar.

One effective general neutralizing medium is Dey–Engley. It contains sodium thioglycollate, sodium thiosulfate, sodium bisulfite, lecithin, and

***Table 5.4*** Neutralizing Agents in Disinfectant Testing[46]

| Substance or Group | Neutralizing Agent or Dilution |
|---|---|
| Aldehydes | Glycine |
| | Sodium sulfite |
| | Dimedone-morpholine? |
| Phenolics | Dilution |
| | Polysorbate 80 |
| Mercury compounds | Sodium thioglycollate |
| | Cysteine |
| Hydrogen peroxide | Catalase |
| Alcohols | Dilution |
| Organic acids and esters | Dilution |
| | Polysorbate 20 or 80 |
| Acridines | Nucleic acids |
| Quaternary ammonium compounds (QACs), biguanides | Lecithin + Lubrol W |
| | Lecithin + Polysorbate 80 |
| Tego compounds | Polysorbate 80 |
| EDTA and related chelating agents | Dilution |
| | $Mg^{2+}$ |
| Hypochlorites | Sodium thiosulfate |
| | Nutrient broth |
| Iodine | Polysorbate 80 |
| | Sodium thiosulfate |

Polysorbate neutralizing agents[35,36,39] and is available in broth and agar preparations. Some common diluting fluids are in the *USP XXII*: diluting fluid A with 0.1% meat peptone, diluting fluid D with meat peptone plus Polysorbate 80, and diluting fluid K with meat peptone, Polysorbate 80, and beef extract. Another variant on this broth has been described for use in membrane filtration testing.[82]

### *Dilution and membrane filtration*

Some preservatives are sensitive to concentration and are effectively neutralized by dilution (see Table 5.4). Several excellent papers discuss this subject.[16,36,42] Another method of neutralizing a preservative is membrane filtration. Bacteria are isolated on a filter and then rinsed free of preservative. One then transfers the surviving bacteria to a growth medium. Bloomfield[16] suggests washing the membrane with nutrient broth and transferring it to the surface of an agar plate to count surviving colonies. The nutrients from the agar diffuse through the membrane to support the microorganism's ability to grow into visible colonies.

Another test for neutralization of a biocide on a membrane filter involves placing a known number of microorganisms on the membrane without the biocide, then placing the same number of microorganisms on another membrane that has been exposed to disinfectant. The disinfectant is rinsed through (and thus off) the membrane filter by placing neutralizing agent

onto the filter and allowing the neutralizer to go through the filter using vacuum or pressure. If the two membranes show equal counts, then the disinfectant has been neutralized. If the disinfectant is bound to the membrane, then counts will be significantly lower than counts on the membrane not exposed to the biocide.[16]

### *Methods for testing biocide neutralizers*

The ASTM methods[84] actually comprise a series of experiments to show whether a neutralizer is nontoxic and effective. Testing is first done to determine the maximum tolerated concentration (MTC) of the inactivator using the correct liquid test medium. Often, the medium will be buffered peptone water. After adding the target organism, one determines the microbial counts at 0 and 30 min. The peptone water should not cause a significant decrease in microbial survival over the 30 min.

One can add several concentrations of the neutralizer to the peptone water system, then compare the number of microbial survivors of the 30-min exposure in the neutralizer–peptone solution to the number of 30-min peptone control survivors. This comparison yields the MTC of neutralizer that does not decrease the microbial survivors of the targeted microorganism.

The next procedure determines the effect of the MTC on the antimicrobial. It involves adding specific concentrations of the antimicrobial to the MTC of the neutralizer and comparing the numbers of microbial survivors at the 30-min point. The number of survivors of the neutralizer–antimicrobial system should not differ significantly from the counts of the peptone–neutralizer controls.

The ASTM method uses square-root transforms of the plate count data for the different treatment groups and controls. It assumes that the plate count data are Poisson-distributed. The method then uses the T test to determine significant mean differences.

The USP dedicated an entire guidance chapter[85] to demonstration of neutralizer efficacy and toxicity. This chapter clearly distinguishes methods useful for demonstrating recovery on agar and in liquid. It also provides a method for determining the maximum number of colonies allowable on a plate, which is especially useful information if you encounter an unusual microorganism with odd colonies. Finally, the chapter highlights concerns about the accuracy of very low numbers of colonies on a plate.

## *Rapid methods used in preservative testing*

Two approaches noted in the literature make the preservative test less labor-intensive and time-consuming. The first is to design a screening test that will allow predictions of the activity of the test formulation when tested by the official method. An example of this is Orth's linear regression method discussed previously. Mulberry et al. discuss other options in an excellent review[86] in which they reinforce the point that these methods must be vali-

dated against the accepted method and should not be relied upon without evaluation.

A second approach is to utilize an alternate technology (avoiding the use of plate counts). Relatively little has been published on this topic, perhaps as the need for a 28-day sampling period lessens the impact of a rapid enumeration of survivors. However, studies of several technologies have been reported in the literature. Impedance techniques are the most commonly reported for this application.[87,88] Connolly et al. compared impedance, direct epifluorescence (DEFT-MEM), and ATP bioluminescence for applicability to preservative testing and found that only impedance yielded results similar to the traditional method.[89] A final approach that shows promise, at least for *E. coli*, is the use of green fluorescent protein to monitor viability. Casey and Nguyen[90] utilized a genetically engineered strain of *E. coli* that contained the green fluorescent protein, which provided an easily distinguished marker for viable cells. Using this strain, determination of viable cells required less than 2 min per sample.

## *General considerations for formulating preserved products*

### *Interactions of preservatives*

The chemical and biological activities of a preservative may be influenced by the overall formulation of the product. For example, a minor alteration of pH may change the ionic character of a preservative, change the chemical groupings on the bacterial surface, or increase the partitioning of the preservative between the product and microbial cells.[8]

Some preservatives can bind to surfactants. Nonionic surfactants in particular can impair the antimicrobial activities of some preservatives such as the parabens. Other preservatives will be inactivated by proteins and exhibit reduced antimicrobial activity as a result.[91] In oil-based emulsions, most of the emulgen disperses throughout the water phase as emulgent micelles. This redistributes the preservative and changes the concentration level in the aqueous phase.[8]

Additionally, the buffer system may affect the activity of the preservative. For example, borate buffered formulations are more easily preserved than phosphate buffered formulations, presumably because the boric acid also acts as an inhibitor of membrane potential. One must understand all factors of the formulation, not simply the ingredients recognized as preservatives.

The level of solids present in a formulation can also impact the effectiveness of a preservative. Inorganic solids (carbonates, silicates, and oxides) and organic solids (cellulose and starch) absorb preservatives such that one must use higher concentrations. Talc, for example, decreases the antimicrobial activity of methyl parabens by as much as 90%.[91] Preservation strategies are reviewed in more detail by Dr. Geis in Chapter 7.

### *Water activity*

As reviewed in detail by Daniel Brannan in Chapter 2, microorganisms require available water for metabolism. Water activity ($A_w$) is the measure of available water, expressed as the ratio of the vapor pressure of a sample to the pressure of pure water at a constant temperature. An $A_w$ value of 1.0 (100% relative humidity) is only obtained with pure water. Restriction of this necessary component for life is an effective means of preserving a formulation.[92–94] The consideration of water activity in product formulation may well allow for a decrease or total elimination of conventional preservatives.[95]

### *Container considerations*

The type of container used for packaging a cosmetic will influence the concentration and activity of a preservative.[30] Generally, the more lipid-soluble preservatives are associated with greater risk of absorption by containers and their closures.[8] Containers must be tested in order to determine the actual preservative effectiveness under actual storage conditions. Adsorption, complexation, and volatility can erode antimicrobial activity. Certain containers are not compatible with certain preservatives, such as nylon and parabens or polyethylene with certain phenolics, mercurials, and benzoates.[91]

Dispensing closures can also be important considerations in preventing microbial contamination during consumer use. A study by Brannan and Dille[96] showed that, during consumer use, unpreserved shampoo in bottles with flip-type caps exhibited the greatest degree of protection from contamination (0%). For an unpreserved skin lotion, a pump-top dispenser afforded the best protection from contamination (10%). Other types of closures tested included the standard screw cap and slit cap. The screw cap closure provided the least amount of protection, while the slit cap provided moderate protection from contamination. This study underscores the need for considering preservation as an attribute of an entire product, not simply a characteristic of an active preservative. Brannan and Dille's study also points out the fallacy of the argument that a preservative test in a laboratory can predict final product performance. A preservative test cannot fulfill this broad function. Its intended purpose is to provide an indication of the ability of a formulation to withstand microbial challenge.

## *Microbial content testing*

The survival and growth of microorganisms in cosmetic products are known quantities.[97–100] Some incidents of microorganism growth involved health risks, as in the case of eye infections caused by mascara contaminated with microorganisms.[2,101,102] Other incidents only led to product spoilage.[99,103–105] Whatever the outcome, the knowledge that microbes can grow in cosmetics means the manufacturer is responsible for detecting them.

The currently used methods include traditional plate counting and enrichment testing.[6,106,107] These methods share the limitations of plate counts as estimates of viable cells discussed earlier in this chapter. An additional limitation is that counts at very low CFU/plate numbers rapidly approach errors of 100% relative to the mean as the number of colonies per plate drops below 10 CFU.[85]

The plate count is a pragmatic and practical method that has survived because of its simplicity and long acceptance, not because of its scientific validity. Thus, we must continue using it until new methods supersede it. A variety of new methods are currently used for microbial content testing, and more will be developed.[108,109] This will be interesting because virtually all raw material and product specifications are currently stated as CFUs per unit of measure.

Two concerns surround the current use of CFU measurements in our specifications as the alternative methods grow in prominence. The first is that the number of CFUs per milliliter in a suspension is at best an estimate of the number of cells in the suspension because many bacteria clump or grow in chains, and thus confound counts — a single colony may arise from a single cell or hundreds of cells in a clump. The second issue is that many of the new technologies do not require bacterial growth, and may allow us to "see" organisms in ways not achievable with present methods.[110]

As discussed above for the Antimicrobial Effectiveness Test (AET), care must be taken to ensure that any residual preservative or antimicrobial property of the material tested should be completely neutralized. This "method suitability" study should be done prior to establishment of the material test method and specifications.

## *General product and raw materials tests*

The aim of these tests is to obtain an estimate of the aerobic population of potential contaminants in a product. The product is sampled aseptically (usually in 1-g amounts) into a diluent meant to both neutralize the product's antimicrobial nature and dilute the organisms present to countable values. The diluted and neutralized product is then plated onto a medium that promotes growth and recovery of injured organisms.

In order to conduct any of the content tests, a well-supplied microbiology laboratory is needed. Many lists of the needed equipment and supplies are available and will not be enumerated here. Each laboratory should document in its SOPs how the equipment should be used, how the materials and reagents (media, dyes, biochemical test solutions) should be prepared, and how the tests should be conducted (e.g., aseptic technique, room features, air circulation, and handling should be clearly defined).[111]

Most products and raw materials received are packed for consumer use or delivered in sterile containers. Typically, if a product or raw material is in aqueous form, 10 g of product or raw material is placed into 90 ml of a dilution blank with appropriate neutralizing media. The bottle is then

capped and shaken thoroughly. Anywhere from 0.5 to 1.0 ml of this dilution is pour-plated or 0.1 ml is spread-plated on the recovery medium.

The plates are incubated at 33 to 37°C for anywhere from a few days to a week if lower temperatures are employed for detection of yeasts and molds. Some microbiologists prefer using a specific medium for recovery of yeasts and molds (e.g., potato extract agar or Sabouraud). The colonies on the plate are counted and the value is multiplied by the dilution factors required.

If a product is a powder or a solid, then it should be dissolved or resuspended into the diluent using a solubilizing agent such as Polysorbate 80. If the material cannot be put into solution, an alternate for conducting the test is the most probable number (MPN) method that utilizes a dilution series approach with the sample suspended in growth media. This approach allows the testing of samples that cannot be plated and samples that are so full of particulate matter that plate counts are uncertain. Interested readers are referred to several excellent treatments of the subject.[6,112–115]

## *USP tests*

The USP microbial limits test (Chapter 61) is in the process of harmonization with the Pharmacopeia Europaea and the Japanese Pharmacopoeia. This process and the work of the USP for the past 10 years have been summarized in the literature.[116,117] As part of the harmonization, three new chapters have been proposed to replace the current Chapters 61 and 62:

61 — Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests
62 — Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms
1111 — Microbial Contamination Limits for Nonsterile Products

### *Chapter 61 —Microbial enumeration tests*

The harmonization of the microbial limits tests that began in the 1995–2000 revision cycle and reached official Stage 4 harmonization document for chapter 61 was last published in 2003.[118] The proposed chapter provides a good amount of detail on generation of the inocula, growth promotion testing of microorganisms, and controls.

### *Chapter 62 — Tests for specified microorganisms*

The 2001 publication of the Stage 4 harmonized Chapter 62[119] placed a good deal of emphasis on the absence of "objectionable" microorganisms. After years of debate, the three major pharmacopeia organizations decided that this objective was too vague and indeed outside the scope of the pharmacopeia. A revised Stage 4 document was published[120] with renewed emphasis on the absence of "specified" microorganisms, simplification of the identification scheme, and more detailed information about controls, growth promotion, and incubation conditions.

*1111 — Microbiological contamination limits for nonsterile products*

This chapter is intended to provide some guidance covering nonsterile pharmaceutical products, but also can be used as general guidance related to certain cosmetics. The chapter is very brief, consisting mainly of several tables providing recommended target values for bioburdens and absence of specified microorganisms.

## *Package tests*

These tests are usually conducted on empty packages received from suppliers that are not expected to be free of microorganisms. Typically, the first step is pouring a specific amount of sterile diluent into a bottle (or other container) that is then capped with the type of cap used in ordinary production. The bottle is then shaken thoroughly and the diluent is plated or diluted further with sterile diluent and then plated. Frequently, the numbers of CFUs in containers, particularly molded plastic containers, is very low and the entire volume of the rinse is filtered through a 0.45-μm (nominal) pore size filter, laid on a plate, and incubated for growth.[121,122]

The plates are incubated and counted as described previously. The CFUs per sample are determined by multiplying the count by the appropriate dilution factors employed in the test.

## *Environmental tests and monitoring*

A variety of environmental tests may be conducted to determine bioburden in the air or on equipment. A good review of the topic has recently been published for the pharmaceutical industry[123] in response to the industry's great concern about maintenance of total clean room environments for aseptic processing.[124] However, environmental monitoring is also a consideration for nonsterile facilities.[125] The primary concern in cosmetic manufacture is the number of organisms present on product contact surfaces inside a plant.

A simple way to test this issue is to moisten a swab with Letheen broth (or any other neutralizing diluent that would support growth) and swab the equipment. The portion of the swab that is not contaminated by technician handling is broken off into the medium and incubated. If turbidity occurs after incubation, then sterility has not been achieved on the product contact areas. Alternatively, the diluent may be immediately plated or diluted further and plated to determine the actual numbers of contaminants per surface area.

A second way to these tests is through the use of RODAC (replicate organism detection and counting) plates. A RODAC plate is slightly overfilled so that the medium projects above the lip of the plate.[126,127] The medium is then pressed on the surface to be sampled, removed, and the cover is returned to its position for incubation. A replica of the viable counts that were on the flat surface can then grow on the surface of the agar.

Whichever way the test is conducted, the operator must be aware of the sampling efficiency of the method used in that facility. Even the best tech-

nician will only succeed in sampling a relatively small proportion of the microorganisms present in any location, and studies should be conducted in the facility to estimate this efficiency.[128–130]

## *Identification of microbes*

Considerable space in the literature is typically devoted to the identification of the microbes isolated from the above tests. A thorough resource for identification of microorganisms is the *Manual for Clinical Microbiology* of the American Society for Microbiology (ASM). Use of rapid identification techniques is replacing many of the traditional culture methods. Although the techniques can be adapted from their clinical uses to cosmetic microbiology, it must be kept in mind that most of the organisms encountered in cosmetic microbiology are environmental isolates. Thus, rapid ID systems geared for environmental isolates should be preferred over those developed primarily for clinical use.

There are two major categories of commercially available microbial identification systems: those that rely on phenotypic characteristics for identification and those that rely on genotypic characteristics. Phenotypic identification systems are more commonly found in industrial microbiology labs. The archetype is the collection of test tubes we dealt with as undergraduates trying to determine whether an unknown was urease positive or negative, able to ferment different sugars, etc. The first improvement on those was the API strip, followed by the Vitek, and now the Vitek 2 Compact. A different approach to phenotypic identification is incorporated in the Biolog systems that rely on a pattern of carbohydrate utilization among a standard set of carbohydrates to identify microorganisms. A final approach is the Sherlock fatty acid analysis system that uses gas chromatographs of cellular fatty acids to identify microorganisms.

Genotypic methods are fundamentally different in that they look directly at nucleic acids to achieve identification. DuPont Qualicon markets the Riboprinter that uses the restriction endonuclease fragment patterns of sequences homologous to 16S rDNA as the identifying characteristics. Diversilab markets the Bacterial Barcode system that utilizes re-PCR (polymerase chain reaction) technology in a similar fashion. The other general identification system in this category is the MicroSeq that sequences the first 500 base pairs of the unknown organism's 16S rDNA to identify it. Of course, if you are looking for a specific microorganism, several choices of PCR technologies are directed to organisms of interest.

All of these systems are completely dependent on the adequacies of their databases to make correct identifications. Genotypic methodology is a fascinating and rapidly changing field, and the reader is referred to several recent review articles on identification of environmental isolates and the different methodologies currently available.[131–134]

## *Summary*

This chapter has attempted to provide an overview of preservative efficacy and microbial content tests used by the pharmaceutical and personal care industries. Several points should have been made clear to readers:

1. The classical antimicrobial efficacy test is a control test of a formulation. It does not do a good job at predicting contamination during use because it does not include the effects of the container in preventing contamination nor does it utilize all possible microbial contaminants that might be introduced into the product. Additional test designs that mimic consumer use conditions to varying degrees are described in the literature.
2. No single test design meets all needs. Each user must determine his or her company's needs (regulatory compliance, rapid answer, etc.) and use a design or a group of designs that address those needs.
3. The goal of antimicrobial efficacy tests is to provide reproducible results to assist in determination of product quality. Therefore, unrealistically high inocula are used to assist the technician in determining kill over time, and a limited range microorganism species is used under tightly controlled conditions to minimize variability of test results.
4. Estimates of preservative efficacy are dependent upon the accurate counting of surviving microorganisms over time. Therefore, complete neutralization of the preservative system during plating to avoid residual inhibition of growth is essential to obtaining accurate results.

## *References*

1. Durant, C. and Higdon, P., Methods for assessing antimicrobial activity, in *Society for Applied Bacteriology Technical Series 27: Mechanisms of Action of Chemical Biocides*, Denyer, S.P. and Hugo, W.B., Eds., Blackwell Scientific, Bedford, U.K., 1991.
2. Wilson, L.A. and Ahearn, D.G.. *Pseudomonas*-induced corneal ulcers associated with contaminated eye mascaras, *Am. J. Ophthalmol*., 84, 112, 1997.
3. Block, S.S., *Disinfection, Sterilization, and Preservation*, 4th ed., Lea & Febiger, Philadelphia, 1992, pp. 18, 887, 1009.
4. Tenenbaum, S., Pseudomonads in cosmetics, *J. Soc. Cosmet. Chem*., 8, 797, 1967.
5. Cowen, R.A. and Steiger, B., Antimicrobial activity: a critical review of test methods of preservative efficacy, *J. Soc. Cosmet. Chem*., 27, 467, 1976.
6. U.S. Food and Drug Administration, *Bacteriological Analytical Manual*, 8th ed., 2001. http://www.cfsan.fda.gov/~ebam/bam-23.html
7. Kabara, J.J., *Cosmetics and Drug Preservation, Principles and Practices*. Marcel Dekker, New York, 1984.
8. Bean, H.S., Preservatives for pharmaceuticals, *J. Soc. Cosmet. Chem*., 23, 703, 1972.

9. Chapman, D.G., Preservatives available for use, in *Society for Applied Bacteriology Technical Series 22, Preservatives in the Food, Pharmaceutical and Environmental Industries*, Board, R.G., Allwood, M.C., and Banks, J.G., Eds., Blackwell Scientific, Bedford, U.K., 1987.
10. Brannan, D.K., Cosmetic preservation, *J. Soc. Cosmet. Chem.*, 46, 199, 1995.
11. Orth, D.S., *Handbook of Cosmetic Microbiology,* Marcel Dekker, New York, 1993.
12. Ray, B., *Injured Index and Pathogenic Bacteria*, CRC Press, Boca Raton, FL, 1989.
13. Madden, J.M., Microbiological methods for cosmetics, in *Cosmetic and Drug Preservation: Principles and Practices*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 573.
14. Orth, D.S., Principles of preservative efficacy testing, *Cosmet. Toilet.*, 96, 43, 1981.
15. Cosmetic, Toiletry, and Fragrance Association Preservation Subcommittee, Evaluation of methods for determining preservative efficacy, *Cosmet. Toilet. Frag. Assoc. J.*, 5, 1, 1973.
16. Bloomfield, S., Methods for assessing antimicrobial activity, in *Society for Applied Bacteriology Technical Series 27, Mechanisms of Action of Chemical Biocides*, Denyer, S.P. and Hugo, W.B., Eds., Blackwell Scientific, Oxford, U.K., 1991, p. 1.
17. Gilbert, P., Brown, M.R.W., and Costerton, J.W., Inocula for antimicrobial sensitivity testing: a critical review, *J. Antimicrob. Chemother.*, 20, 147, 1987.
18. Gilbert, P., Collier, P.J., and Brown, M.R.W., Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response, *Antimicrob. Agents Chemother.*, 34, 1865, 1990.
19. Cosmetic, Toiletry, and Fragrance Association Preservation Subcommittee, *A Guideline for the Preservation Testing of Aqueous Liquid and Semi-Liquid Eye Cosmetics*, Washington, D.C., 1981.
20. American Public Health Association, *Standard Methods for the Examination of Water and Wastewater,* 1st ed., Washington, D.C., 1980.
21. U.S. Pharmacopeia Convention, Antimicrobial preservatives: effectiveness, in *U.S. Pharmacopeia 2005,* Rockville, MD, 2004, chap. 51.
22. Bacto® Eugon agar, in *DIFCO Manual*, 11th ed., DIFCO Laboratories, Detroit, MI, 1986, p. 190.
23. Odds, F.C., Sabouraud['s] agar, *J. Med. Vet. Mycol.*, 29, 355, 1991.
24. American Public Health Association, *Standard Methods for the Examination of Dairy Products*, 14th ed., Washington, D.C., 1978.
25. American Public Health Association, *Compendium of Methods for the Microbiological Examination of Foods*, Washington, D.C., 1976.
26. Von Reisen, V.L. and Jensen, T., Acid mycophil agar for the selective isolation of yeasts, *Am. J. Med. Technol.*, 24, 123, 1958.
27. Beneke, E.S. and Rogers, A.L., *Medical Mycology Manual*, 3rd ed., Burgess Publishing, Minneapolis, 1971, p. 23.
28. Mycological media, in *DIFCO Manual*, 10th ed., DIFCO Laboratories, Detroit, 1985, p. 589.
29. Cosmetic, Toiletry, and Fragrance Association Preservation Subcommittee, A guideline for the determination of the adequacy of preservation of cosmetics and toiletry formulations, in *CTFA Technical Guidelines*, Washington, D.C., 1981.
30. Brannan, D.K., The role of packaging in product preservation, in *Preservation-Free and Self-Preserving Cosmetics and Drugs*, Kabara, J.J. and Orth, D.S., Eds., Marcel Dekker, New York, 1996, chap. 10.

31. el-Falaha, B.M., Furr, J.R., and Russell, A.D., Quenching of the antibacterial activity of chlorhexidine and benzalkonium by Letheen broth and Letheen agar in relation to wild-type and envelope mutant strains of Gram-negative bacteria, *Microbios,* 49, 31, 1987.
32. U.S. Pharmacopeia Convention, Microbial limit tests, in *U.S. Pharmacopeia 2005*, Rockville, MD, 2004, chap. 61.
33. Brewer, J.H., A clear liquid medium for the "aerobic" cultivation of anaerobes, *JAMA*, 155, 598, 940.
34. Quisro, R.A., Gibby, I.W., and Foster, M.J., A neutralizing medium for evaluating the germicidal potency of the quaternary ammonium salts, *Am. J. Pharm*., 118, 320, 1946.
35. Engley, F.B., Jr., and Dey, B.P., A universal neutralizing medium for antimicrobial chemicals, in *Proceedings of the 56th Mid-Year Meeting of the Chemical Specialties Manufacturers Association*, New York, 1970, p. 100.
36. Dey, B.P. and Engley, F.B., Jr., Methodology for recovery of chemically treated *Staphylococcus aureus* with neutralizing medium, *Appl. Environ. Microbiol*., 45, 1533, 1983.
37. Martinelli, S.D., Media, *Prog. Ind. Microbiol*., 29, 829, 1994.
38. Microbial limit test for cosmetics. EU/ASEAN Document TH-02, 2004.
39. Sutton, S.W.V., Wrzosek, T., and Proud, D.W., Neutralization efficacy of Dey–Engley medium in testing of contact lens disinfecting solutions, *J. Appl. Bacteriol.,* 70, 351, 1991.
40. MacKinnon, I.H., Use of inactivators in evaluation of disinfectants, *J. Hyg. Cambr.*, 73, 189, 1974.
41. Singer, S., The use of preservative neutralizers in diluents and plating media, *Cosmet. Toilet*., 102, 55, 1987.
42. Hugo, W.B. and Denyer, S.P., Concentration exponent of disinfectants and preservatives (biocides), in *Preservatives in the Food, Pharmaceutical and Environmental Industries*, Board, R.G., Allwood, M.C., and Banks, J.G., Eds., Blackwell Scientific, Boston, 1987, p. 281.
43. American Society for Testing and Materials, Standard method for water-containing cosmetics (ANSI/ASTM E640-78), in *Annual Book of ASTM Standards, Vol. 11.04*, Philadelphia, 1991, p. 290.
44. Sutton, S.V.W. and Porter, D. Development of the antimicrobial effectiveness test as USP Chapter 51, *PDA J. Pharm. Sci. Tech.,* 56, 300, 2002.
45. Gerhardt, P., Murray, R.G.E., Costilow, N., Nester, R., Eugene, W., Wood, W.A., Krieg, N.R., and Phillips, G.B., *Manual for Methods for General Bacteriology,* American Society for Microbiology, Washington, D.C., 1981, pp. 67, 174, 504.
46. Orth, D.S., Lutes, C.M., and Smith, D.K., Effect of culture conditions and method for inoculum preparation on the kinetics of bacterial death during preservative efficacy testing, *J. Soc. Cosmet. Chem*., 40, 193, 1989.
47. Orth, D.S., Putting the phoenix phenomenon into perspective, *Cosmet. Toilet*., 114, 61, 1999.
48. Orth, D.S., Linear regression method for rapid determination of cosmetic preservative efficacy, *J. Soc. Cosmet. Chem*., 30, 321, 1979.
49. Orth, D.S., Preservative efficacy testing of cosmetic products: rechallenge testing and reliability of the linear regression method, *Cosmet. Toilet.,* 97, 61, 1982.
50. Zar, J.H., Simple linear regression, in *Bio-Statistical Analysis*, Prentice-Hall, Englewood Cliffs, NJ, 1984, p. 261.

51. Sutton, S.V.W., Franco, R.G., Mowrey-McKee, M.F., Busschaert, S.C., Hamberger, J., and Proud, D.W., D-value determinations are an inappropriate measure of disinfecting activity of common contact lens disinfecting solution, *Appl. Environ. Microbiol.*, 57, 2021, 1991.
52. Barnes, M. and Denton, G.W., Capacity tests for the evaluation of preservatives in formulations, *Soap Perf. Cosmet.*, Oct., 729, 1969.
53. Brannan, D.K., Dille, J.C., and Kaufman, D.J., Correlation of *in vitro* challenge testing with consumer use testing for cosmetic products, *Appl. Environ. Microbiol.*, 53, 1827, 1987.
54. Farrington, J.K., Martz, E.L., Wells, S.J., Ennis, C.C., Holder, J., Levchuk, J.W., Avis, K.E., Hossman, P.S., Hitchins, A.D., and Madden, J.W., Ability of lab methods to predict in-use efficacy of antimicrobial preservatives in an experimental cosmetic, *Appl. Environ. Microbiol.*, 60, 4553, 1994.
55. Lindstrom, S.M. and Hawthorne, J.D., Validating the microbiological integrity of cosmetic products through consumer-use testing, *J. Soc. Cosm. Chem.*, 37, 481, 1986.
56. Urban, S., Hecker, W., and Shiller, I., Low-level challenge test for the examination of the microbiological susceptibility, during the period of use, of liquid and semi-solid dosage forms in multiple-dose containers [in German], *Zbl. Bakt. Hyg. I. Abt. Orig. B.,* 172, 478, 1981.
57. European Union, EMEA/CVMP/127/95, Note for Guidance: In-Use Stability Testing of Veterinary Medicinal Products, Brussels, 1995.
58. European Union, EMEA/QWP/2934/99, Note for Guidance: In-Use Stability Testing of Human Medicinal Products, Brussels, 2001.
59. Sutton, S.V.W., In-use stability testing: what data are required and when? *Reg Affairs J.*, 9, 728, 1998.
60. Russell, A.D., Factors influencing the activity of antimicrobial agents: an appraisal, *Microbios,* 10, 151, 1974.
61. Russell, A.D., Neutralization procedure in the evaluation of bactericidal activity, in *Society for Applied Bacteriology Technical Series 16, Disinfectants: Their Use and Evaluation of Effectiveness*, Collins, C.H., Allwood, M.C., Bloomfield, S.F., and Fox, A., Eds., Academic Press, London, 1981, p. 45.
62. Gilbert, P. and Brown, M.R.W., Out of the test tube into the frying pan: post-growth, pre-test variables, *J. Antimicrob. Chemother.,* 27, 859, 1991.
63. Bloomfield, S., Development of reproducible test inocula for disinfectant testing, *Int Biodet. Biodegrad.*, 38, 311, 1996.
64. Matthews, B.R., Preservation and preservative efficacy testing: European perspectives, *Eur. J. Parenteral Pharm. Sci.,* 8, 99, 2003.
65. Muth, H., The effects of antimicrobial preservatives on organisms derived from fresh versus frozen cultures, *Pharmacop. For.,* 26, 51, 2000.
66. Orth, D.S., Effect of culture conditions and method of inoculum preparation on the kinetics of bacterial death during preservation efficacy testing, *J. Soc. Cosmet. Chem.,* 40, 193, 1989.
67. Van Doorne, H., Effect of inoculum size on survival rate of *Candida albicans* and *Aspergillus niger* in topical preparations, *Lett. Appl. Microbiol.*, 18, 289, 1994.
68. Steels, H., James, S.A., Roberts, I.N., and Stratford, M., Sorbic acid resistance: the inoculum effect, *Yeast,* 16, 1173, 2002.
69. Gilbert, P., Some perspectives on preservation and disinfection in the present day, *Int. Biodeter. Biodegrad.,* 36, 219, 1995.

70. Gilbert, P., Brown, M.R., and Costerton, J.W., Inocula for antimicrobial sensitivity testing: a critical review, *J. Antimicrob. Chemo.*, 20, 147, 1987.
71. Gilbert, P., Coplan, F., and Brown, M.R., Centrifugation injury of Gram-negative bacteria, *J. Antimicrob. Chemo.*, 27, 550, 1991.
72. Mossel, D.A. and Van Nette, P., Harmful effects of selective media on stressed microorganisms: nature and remedies, in *The Revival of Injured Microbes*, Academic Press, New York, 1984, p. 328.
73. Gilbert, P., The revival of microorganisms sublethally injured by chemical inhibitors, in *The Revival of Injured Microbes*, Academic Press, New York, 1984, p. 175.
74. Harris, N.D., The influence of the recovery medium and the incubation temperature on the survival of damaged bacteria, *J. Appl. Bact.*, 26, 387, 1963.
75. Wilson, P.W. and Kullman, E., Statistical inquiry into methods for estimating numbers of Rhizobia, *J. Bacteriol.*, 22, 71, 1931.
76. Jennison, M. and Wadsworth, G., Evaluation of the errors involved in estimating bacterial numbers by the plating method, *J. Bacteriol.*, 39, 389, 1940.
77. Hedges, A.J., Estimating the precision of serial dilutions and viable bacterial counts, *Intl. J. Food Microbiol.*, 76, 207, 2002.
78. Jennison, M.W., Relations between plate counts and direct microscopic counts of *Escherichia coli* during the logarithmic growth period, *J. Bacteriol.*, 33, 462, 1937.
79. Jennison, M.W. and Wadsworth, G.P., Evaluation of the errors involved in estimating bacterial numbers by the plating method, *J. Bacteriol.*, 39, 389, 1940.
80. Breed, R. and Dotterrer, D.W., The number of colonies allowable on satisfactory agar plates, *J. Bacteriol.*, 1, 321, 1916.
81. Tomasiewicz, D.M., Hotchkiss, D.K., Reinbold, G.W., Read, R.B., Jr., and Hartman, P.A., The most suitable number of colonies on plates for counting, *J. Food Prot.*, 43, 282, 1980.
82. Proud, D.W. and Sutton, S.V.M., Development of a universal diluting fluid for membrane sterility testing, *Appl. Environ. Microbiol.*, 58, 1035, 1992.
83. Sutton, S.V.M., Proud, D.W., Rachui, S., and Brannan, D.K., Validation of microbial recovery from disinfectants, *PDA J. Sci. Technol.*, 56, 255, 2002.
84. American Society for Testing and Materials, Evaluating Inactivators of Antimicrobial Agents in Disinfectant, Sanitizer, and Antiseptic Products, Philadelphia, 1991, p. 1054.
85. U.S. Pharmacopeia Convention, Validation of microbial recovery from pharmacopeial articles, in *U.S. Pharmacopeia 2005*, Rockville, MD, 2004, chap. 1227.
86. Mulberry, G., Entrup, M.R., and Agin, J.R., Rapid screening methods for preservative efficacy evaluations, *Cosmet. Toilet.*, 102, 47, 1987.
87. Basa, E., Preservative efficacy testing for stability using impedance, *Cosmet. Toilet.*, 115, 61, 2000.
88. Zhou, X. and King, V.M., An impedimetric method for rapid screening of cosmetic preservatives, *J. Indust. Microbiol.*, 15, 103, 1995.
89. Connolly, P., Study of the use of rapid methods for preservative efficacy testing of pharmaceuticals and cosmetics, *J. Appl. Bacteriol.*, 75, 456, 1993.
90. Casey, W.M and Nguyen, N.T., Use of the green fluorescent protein to rapidly assess viability of *E. coli* in preserved solutions, *PDA J. Sci. Pharm. Tech.*, 50, 352, 1996.
91. Sabourin, J.R., Evaluation of preservatives for cosmetic products, *Drug Cosmet. Ind.*, December 24, 1990.

92. Friedel, R.R. and Cundell, A.M., The application of water activity measurement to the microbiological attributes testing of nonsterile over-the-counter drug products, *Pharmacop. For.,* 24, 6087, 1998.
93. Curry, J., Manipulation of water activity for developing products intrinsically hostile to microbial growth, *Cosmet. Toilet. Manuf.*, 107, 53, 1993.
94. Curry, J., Water activity and preservation, *Cosmet. Toilet.,* 100, 53, 1985.
95. Cowen, R., Why a preservative system must be tailored to a specific product, *Cosmet. Toilet.,* 92, 15, 1977.
96. Brannan, D.K. and Dille, J.C., Type of closure prevents microbial contamination of cosmetics during consumer use, *Appl. Environ. Microbiol*., 56, 1476, 1990.
97. Baird, R.M., Microbial contamination of cosmetic products, *J. Soc. Cosmet. Chem.,* 28, 17, 1977.
98. Baird, R.M., Bacteriological contamination of products used for skin care in babies, *Int. J. Cosmet. Sci.*, 6, 85, 1984.
99. Ashour, M.S. et al., Microbial contamination of cosmetics and personal care items in Egypt, *Cosmet. Toilet.,* 102, 61, 1987.
100. Goldman, C.L., Microorganisms isolated from cosmetics, *Drug Cosmet. Ind.,* 117, 40, 1979.
101. Wilson, L.A., Julian, A.J., and Ahearn, D.G., Survival and growth of microorganisms in mascara during use, *Am. J. Ophthalmol*., 79, 596, 1975.
102. Gabriel, H., Gable, E.M., Sauser, K., and Rice, J., Testing the inhibitory effects of mascara life on bacterial growth in mascara, *Optometry,* 72, 251, 2001.
103. Wolven, A. and Levenstein, I., Cosmetics: contaminated or not? *TGA J. Cosmet*., 1, 34, 1969.
104. O'May, G.A., Allison, D.G., and Gilbert, P., Rapid method for the evaluation of both extrinsic and intrinsic contamination and resulting spoilage of water-in-oil emulsions, *J. Appl. Microbiol*., 96, 1124, 2004.
105. Smart, R. and Spooner, D.F., Microbiological spoilage in pharmaceuticals and cosmetics, *J. Soc. Cosmet. Chem*., 23, 721, 1972.
106. Tran, T.T. et al., Adequacy of cosmetic preservation: chemical analysis, microbial challenge and in-use testing, *Int. J. Cosmet. Sci*., 16, 61, 1994.
107. Yablonski, J. and Mancuso, S.E., Personal care wipes: manufacturing practices and microbiological control, *Cosmet. Toilet*., 119, 53, 2004.
108. Jimenez, L., Molecular diagnosis of microbial contamination in cosmetic and pharmaceutical products: a review, *J. AOAC Intl*., 84, 671, 2001.
109. Swaminathan, B., Rapid detection of foodborne pathogenic bacteria, *Ann. Rev. Microbiol*., 48, 401, 1994.
110. Rappe, M. and Giovannoni, S.J., The uncultured microbial majority, *Ann. Rev. Microbiol.,* 57, 69, 2003.
111. U.S. Pharmacopeia Convention, Microbiological best laboratory practices, Chapter 1117, *Pharmaco. For.,* 30, 1713, 2004.
112. Garthright, W.E. and Blodgett, R.J., FDA's preferred MPN methods for standard, large, or unusual tests with a spreadsheet, *Food Microbiol.,* 20, 439, 2003.
113. Roser, D.J. et al., Application of most-probable-number statistics to direct enumeration of microorganisms, *Appl. Env. Microbiol.*, 53, 1327, 1987.
114. Loyer, M. and Hamilton, M.A., Interval estimation of the density of organisms using a serial dilution experiment, *Biometrics,* 40, 907, 1984.
115. Aspinall, L.J. and Kilsby, D.C., Microbiological quality control procedure based on tube counts, *J. Appl. Bacteriol.,* 46, 325, 1979.

116. Sutton, S.V.W. et al., Activities of the USP microbiology subcommittee of revision during the 1995–2000 revision cycle, *PDA J. Pharm. Sci Tech.*, 55, 33, 2001.
117. Sutton, S.V.W. et al., Activities of the USP microbiology subcommittee of revision during the 2000–2005 revision cycle, *PDA J. Pharm. Sci. Tech.*, 59, 157, 2005.
118. U.S. Pharmacopeia Convention, Microbial limits tests, Chapter 61, *Pharmaco. For.,* 29, 1714, 2003.
119. U.S. Pharmacopeia Convention, Microbiological examination of nonsterile products: microbial enumeration tests, Chapter 61, *Pharmaco. For.,* 27, 2299, 2001.
120. U.S. Pharmacopeia Convention, Microbiological examination of nonsterile products: tests for specified microorganism harmonization, Chapter 62, *Pharmaco. For.,* 29, 1722, 2003.
121. U.S. Pharmacopeia Convention, Microbiological quality of nonsterile pharmaceutical products, Chapter 1111, *Pharmaco. For.,* 29, 1733, 2003.
122. Means, E.G. et al., Evaluating mediums and plating techniques for enumerating bacteria in water distribution systems, *J. Amer. Water Works Assn.*, 72, 585, 1981.
123. Tran, T.T. et al., Direct contact membrane method for evaluating preservative efficacy in solid cosmetics, *Int. J. Cosmet. Sci.*, 12, 175, 1990.
124. Parenteral Drug Association, Fundamentals of an Environmental Monitoring Program, Technical Report 13 (Revised), 2001.
125. U.S. Food and Drug Administration, Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice, 2004.
126. One, J., An approach to developing a microbial environmental control program for non-sterile pharmaceutical manufacturing, *Amer. Pharm. Rev.*, 8, 32, 2005.
127. Rohde, P.A., A new culture plate: its applications, *PDA Bull. Parenteral Drug Assn.*, 17, 1, 1963.
128. Bruch, M., Improved method for pouring RODAC plate, *Appl. Microbiol.*, 16, 1427, 1968.
129. Whyte, W., Methods for calculating the efficiency of bacterial surface sampling techniques, *J. Hosp. Infect.*, 13, 33, 1989.
130. Poletti, L., Comparative efficiency of nitrocellulose membranes versus RODAC plates in microbial sampling on surfaces, *J. Hosp. Infect.,* 41, 195, 1999.
131. Salo, S. et al., Validation of microbiological methods: Hygicult Dipslide, contact plate, and swabbing in surface hygiene control: a Nordic collaborative study, *J. AOAC Intl.,* 83, 1357, 2000.
132. Sutton, S.V.W. and Cundell, A.M., Microbial identification in the pharmaceutical industry, *Pharm. Forum,* 30, 1884, 2004.
133. Meays, C.L., Source tracking fecal bacteria in water: a critical review of current methods, *J. Environ. Mgt.,* 73, 71, 2004.
134. O'Hara, C.M., Manual and automated instrumentation for identification of Enterobacteriaceae and other aerobic Gram-negative bacilli, *Clin. Microbiol. Rev.*, 18, 147, 2005.

*chapter six*

# *Validation of methods*

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*Contents*



## *Introduction*

Within this chapter, we offer general guidelines for validating and documenting microbial methods for the personal care product industry. Both plants and laboratories include areas that require validation. By instituting a rigorous program of validation, we can ensure that the methods and systems used do what they purport to do and thus merit a high level of confidence.

Validation should be an integral part of continuous quality improvement in order to provide customers with high quality finished products. Validation of microbiological procedures within the personal care product industry simply establishes their efficacy, accuracy, and reproducibility. Validation is performed whenever a new procedure is developed and whenever signifi-

cant changes are made to existing systems, methods, and procedures. Validation without documentation, however, is useless; the results of validations should be documented in organized and permanent records.

## *Model for validation*

Every validation program includes five basic steps. In order to allow better understanding of these steps, the validation of the preservative efficacy test (PET) is used as an example.

1. Define what the system, method, or procedure is supposed to do (e.g., a PET should predict the rate of consumer contamination of a product).
2. Identify and control, if possible, the variables in the system, method, or procedure (e.g., a PET cannot predict the abuse of the product during use).
3. Establish acceptance criteria for the system, method, or procedure before beginning the validation process (e.g., a PET can rate well-preserved products and one would not expect them to be contaminated under ordinary use and foreseeable abuse).
4. Develop and execute a protocol to determine whether the procedure meets the criteria (e.g., Do products rated as well preserved not become contaminated?).
5. Document the procedures and results.

Documentation should include detailed information about how the procedure works and how the data were obtained. For example, when validating a sanitization procedure, one would document the date of sanitization, the equipment sanitized, the product for which the equipment was sanitized, the sanitizer and concentration or method of sanitization used, the performance criteria for acceptance, whether the specifications were met, appropriate signatures of witnesses, and proof that the results were properly reviewed by qualified supervisory personnel. Records are generally kept from 3 to 7 years, although retention criteria may be variable.

The three approaches to validation are prospective, concurrent, and retrospective. To illustrate examples of these approaches, we can again cite the PET. A prospective approach involves qualification of the system and subsystems using an approved protocol with appropriate acceptance criteria. The protocol is executed and the data are analyzed and reported for approval or rejection. Reference 1 is a published example of this approach. A concurrent approach to validation is based on information gathered during actual implementation of the process or system. This type of data is generated by periodically allowing consumer panels to use a product that has passed the preservative efficacy test and determining whether the product is still uncontaminated. The last approach is retrospective validation that involves the review and analysis of historic data. For example, one could determine

whether a PET was valid by noting whether consumer comments and complaints were related to microbial growth in the product. This assumes, of course, that the product samples that may have engendered comments potentially related to microbial contamination were assayed for microbial content.

## *Validation of equipment cleaning and sanitization*

Validation, as applied to cleaning and sanitization, is the evaluation of data gathered under a validation scenario (prospective, concurrent, or retrospective) that assures that the procedures produce acceptably cleaned and sanitized equipment surfaces that are in contact with the product. Most prospective validation schemes of sanitization validation require that a test liquid be used to mimic the product during the validation run.

After cleaning and sanitization, the sterile unpreserved liquid is introduced into the equipment and subjected to the same conditions as the product manufactured on the system. After contacting the same surfaces to which the product will be exposed, an aliquot of the test liquid is taken and examined for microbial content. Decisions are made regarding the adequacy of the sanitization procedure based on the content data; a range of 10 to 100 CFU/ml is the common limit. Some organizations intentionally contaminate their systems with known numbers of organisms. Contamination is followed by sanitization and processing the test liquid (sometimes a microbiological medium) through the system.

Aside from the obvious risk of introducing microorganisms unnecessarily into a system, another danger exists when the system passes a prospective validation: it gives the personnel a false sense of security. Validation is a snapshot of a system subjected to a continuous process of biological adaptation and evolution. Thus, when a validated system becomes contaminated, people are incredulous and wonder how a problem occurred on a validated system. The missing pieces of information are the two variables that can never be controlled: (1) biological evolution that allows microbial adaptation to the biocides used and (2) the human element.

In conducting a concurrent validation of sanitization, one generally relies on swab samples of critical control points in the system before and after a sanitization during actual production. One can even analyze final wash-out water to remove chemical sanitizers as in prospective validations if the water is free of microorganisms. The uncontrolled variable here is whether all the critical control points have really been sampled. This approach assumes that no dead legs are in the system.

In conducting a retrospective validation of sanitization, one relies on the historical data related to clean product and correlates that production data with sanitization documentation. The uncontrolled variable here is that this approach does not account for the biocidal activity of the product that may mask sanitization deficiencies.

The details of sanitization validation include the cleaning method used before sanitization, the sanitization method, the type of sanitizer employed

(if chemical), its use concentration, temperature, and contact time, type of equipment or surface sanitized, and acceptance criteria. Generally, the best sanitizer to use is heat. A temperature of at least 180°F (80 to 85°C) for up to a half hour is highly effective, assuming the heat penetrates through the pipes entirely. Heat effectively destroys biofilms. Free chlorine at a concentration of 200 ppm is the next most effective measure, assuming the system has been thoroughly cleaned.

Implementing a validation program and selecting sanitization procedures are not necessarily the tasks of a microbiologist alone. Instead, he or she should facilitate validation by including people such as safety officers, engineers, production staff, quality assurance technicians, and upper management. The microbiologist should drive the process but should involve the others in order to ensure complete acceptance and compliance with the standards. Revalidation should also be considered whenever a substantive change takes place in the sanitization procedure, equipment, or process.

Documentation is the most difficult step simply because of the general dislike of paperwork. However, without documentation, validation in essence was not accomplished. A properly documented sanitization validation notes the date of sanitization, a description of the equipment sanitized, the name and batch number of the product run through the equipment, the type of sanitizer and/or procedure used, sanitizer concentration, signature of the person performing the procedure, and a countersignature of the supervisor of the shift during which the equipment was used.

## *Validating cleanliness of plant environment*

Two areas are usually analyzed for determining the microbial quality of the plant environment: air and surfaces. Air sampling equipment is fairly complex, and the need for it in a cosmetic plant is rarely warranted. If used, the ability of the sampler to recover organisms of interest without hindering their growth should be validated.

Settling plates are more commonly used. The important issue when they are used is the effect of drying on the ability of the plates to support growth. Known numbers of organisms in an aerosol could be sprayed on the plates to validate them. Surface monitoring can be validated by attempting to recover known numbers and types of microorganisms from the various types of surfaces. Various monitoring techniques such as swabs or Rodac plates may be used.

Documentation of an environmental control program involves tracking trends in contamination levels in the environment using process control charts. Significant spikes or deviations may indicate a need for action. The data needed to ensure proper documentation are date, time, and location of the sample, method of sampling, name of person who performed sampling, descriptions of laboratory procedures including the growth medium and the time and temperature of incubation, resulting data, and any pertinent comments such as general activity at the time of sampling, presence of visible

dirt or airborne dust, presence of air currents from air-conditioning vents, and date of last surface cleaning.

## *Validation in microbiology laboratory*

Laboratory-generated data are used in quality control and assurance. The data must be reproducible and reliable. This is accomplished by controlling laboratory operations via well-documented and validated procedures that permit safe, efficacious, and reliable production of the consumer product.

### *Media*

Microbiological culture media must contain available sources of carbon, nitrogen, and appropriate trace elements. Fastidious organisms may also require vitamins or other growth factors. The growth of microorganisms in a medium depends on proper preparation. Documentation should include dates of receipt and preparation, pH, lot number, and any deviations from performance standards outlined by the manufacturer in order to provide reliable test results.

As each batch of media is made, a record should be kept including such information as the manufacturer's batch number, the date and amount made, the final pH and any adjustments needed, method of sterilization and results, ingredient lot numbers for media requiring separate ingredients, expiration date of medium, test organisms used to show the medium promotes growth and the results, and the signature of the preparer.

Perhaps the one raw material most rarely checked or validated is the water used in media making. Purified water obtained by distillation or ion exchange treatment should be used. The quality of this water should be checked chemically at regular intervals to determine its suitability for media preparation.

Growth support checks of a medium should consider which organisms are to be detected. Selective and enrichment media need to support and detect their appropriate organisms. Media for identification of microorganisms should include positive and negative control organisms compared to uninoculated controls. Validation also includes establishing storage conditions and shelf life limits through appropriate performance testing.

The above principles should be applied when devising systems for validation of media. The purpose of a medium is to detect organisms in a sample. Some of the variables to control are sterilizing time, preparation methods, storage time and temperature, and incubation time and temperature. The key criterion for acceptance is that the medium support the growth of the organisms to be detected without inhibition (even in the presence of the product) of growth; the medium should also be sterile. To conduct the validation protocol for sterility, one can rely on the validation of the autoclave (see below) and a demonstration that uninoculated media do not show growth.

In validating a microbial content test, for example, one can combine the validation of neutralization and growth support in one test. In the sample data provided below, distilled water was used as a control for the particular product validated against. The procedure calls for the organisms to be added to 90 ml of Letheen broth neutralizer such that they will be suspended at a concentration of about 100 CFU/ml. Then the product or the water control (10 g) is added to the broth and mixed immediately. This order of addition is important. We must ensure that the neutralizer eliminates the biocide activity of the product before the biocide activity can eliminate the organisms. After the mixing, 0.1-ml aliquots may be spread-plated onto an agar surface or 0.5-ml aliquots may be put into melted agar deeps, vortexed and poured.

Alternatively, 0.5- to 1.0-ml aliquots may be placed into plates and melted agar poured on top of the aliquots and swirled to ensure complete mixing. Whichever method of plating is chosen, it should validated against the same method using the water control and compared to the other methods to show equivalency if various labs use different plating protocols. Data such as those in the Table 6.1 validation register are typical of spread-plating. The data are expressed as CFU/g product (or water). The data can be subjected to statistical testing using a standard T-test, provided the count values (CFU/g product) are first transformed logarithmically (log x + 1) and the data are worked using the log transformations.

One of the classic ways of validating growth support and neutralization (ASTM method) is streaking with test organisms a plate that shows no growth after product has been added to it. If the streak grows, the medium is considered validated with respect to growth support. This approach is not valid. Growth only indicates that the biocide was finally inactivated at the time the streak was done, not at the time the product was plated. No growth on the plate does, however, prove that the product was never inactivated to allow microbial growth.

## *Microbial content tests*

Microbial content tests are needed to determine the bioburden of raw materials, package components, water, and the final product. The microbial content tests typically use plate count procedures that should be evaluated for their reproducibility. In particular, the procedures, materials, and equipment used must be proven effective. The procedures should be written in a standard operations manual in sufficient detail that will allow someone educated in the science of microbiology to follow them.

Some microbiological procedures are compendial in nature (e.g., U.S. Pharmacopeia [USP]), others are guidelines (e.g., Cosmetic, Toiletry, and Fragrance Association [CTFA]), and some are collaborative laboratory methods (e.g., Association of Analytical Communities [AOAC]). Regardless of their apparent "official" nature, the procedures are still subject to considerable human error and opinion on the part of the panels that developed them.

*Table 6.1* Microbiology Validation Register

**DATE:** 12/96

**PRODUCT:** XYZ Brand Shampoo Batch No. 96AA

**MEDIA:** Letheen broth and TSA-Tween

**OBJECTIVE:** To ensure that the neutralizer and plating media used recover organisms whether they are in the presence of biocides or not.

**CONDITIONS AND PROCEDURES:** Media autoclaved at 121°C for 20 min. Incubation conducted at 30°C for 48 h. Add organisms to 90 ml neutralizer at 100 CFU/ml concentration. Add 10 g product (or water as control) to the 90 ml of inoculated neutralizer. Mix thoroughly and remove ten 0.1-ml aliquots using an Eppendorf pipet into plates to be spread plated.

**DATA AND RESULTS:**

| *Escherichia coli* | | | *Pseudomonas aeruginosa* | | | *Staphylococcus aureus* | | |
|---|---|---|---|---|---|---|---|---|
| Product | | Control | Product | | Control | Product | | Control |
| 138 | | 149 | 100 | | 119 | 60 | | 28 |
| 188 | | 149 | 120 | | 115 | 51 | | 41 |
| 102 | | 134 | 119 | | 109 | 40 | | 47 |
| 99 | | 166 | 115 | | 118 | 93 | | 46 |
| 131 | | 113 | 132 | | 163 | 21 | | 47 |
| 117 | | 137 | 124 | | 134 | 28 | | 76 |
| 146 | | 137 | 108 | | 133 | 53 | | 17 |
| 115 | | 147 | 122 | | 131 | 49 | | 58 |
| 138 | | 120 | 97 | | 108 | 56 | | 53 |
| 152 | | 124 | 125 | | 77 | 31 | | 50 |
| 133 | Avg. | 138 | 116 | Avg. | 121 | 48 | Avg. | 46 |
| $p = 0.62$; $t = 0.512$ | | | $p = 0.58$; $t = 0.570$ | | | $p = 0.814$; $t = 0.232$ | | |

**CONCLUSIONS:** Counts recovered from the test diluent and medium in the presence of the product did not differ significantly from those obtained in the presence of water. Thus the methods are valid for use.

*Note*: Plate counts to validate recovery of organisms by spread plate technique using TSA-Tween and Letheen broth neutralizer. Data shown in CFU/g.

As a result, they should not be trusted as validated methods, particularly because none of the published methods are designed specifically for a product of a particular manufacturer.

Thus, all methods used for determining microbial content must be validated by the manufacturer for each product or class of product. When validating a microbial content test, the elements to be validated include plating methods (e.g., spread or pour), dispersion techniques (mechanical or manual), incubation time and temperature, dilution factors, and neutralizing agents. An example was provided in the discussion above about media and growth support validation.

### *Identification*

Organisms from plate counts are identified based on colony morphology, Gram reaction, pigment production, motility, and biochemical characteristics including nutritional requirements and reactions on selective and differential media. Validation of methods used to identify these organisms includes demonstration of the ability to conduct the Gram stain and biochemical assays properly. The use of standard slides containing known Gram-positive and -negative organisms can help validate a technician's ability to perform a Gram stain. Use of reference cultures on the media used to identify the organisms is appropriate for validating biochemical assays. In many cases, microbiology laboratories rely on the more modern rapid identification methods. Despite their extreme reliability, they must still be validated using known reference cultures to prove they are capable of identifying the organisms of interest.

### *Laboratory equipment*

Laboratory equipment should be maintained so that it will perform properly. All performance deficiencies and corrective actions taken should be documented. Some of the items requiring validation and documentation include buffer solutions for pH meters, thermometers, temperature gauges, and sterility indicators such as spore strips for autoclaves. A file should be kept for each piece of equipment to be validated and should contain the following information: model and serial numbers, purchase date, manufacturer, maintenance and operation manuals, and information for contacting technical service representatives.

The operator's manual on each piece of equipment should establish control steps for carrying out validation. Documentation should include the frequency and criteria for acceptance, performance standards, deficiencies, and corrective actions taken. Specialized checks, such as calibration of centrifuges, balances, and scintillation counters, should be performed by an authorized representative of the relevant manufacturer.

Installation of a piece of equipment should be validated in order to establish basic operational requirements, specifications, and tolerances. Most equipment manufacturers provide recommended installation procedures, but do not sup-

ply qualification instructions to validate proper installation of their equipment. The user is then left to determine what validates proper installation.

In validating installation, a user should identify the critical features of operation that might affect function, variability, data, and records and also consider the effects that usage patterns have on the equipment; usage may be continuous, intermittent, variable, or involve long down times between uses. Idle equipment usually needs more attention than devices in constant operation.

Calibration of the installed equipment should involve particular care in selecting the reagents used with the machine, the techniques for detection of variances, and the measurement of what constitutes control. Wherever possible, a calibration program should use recognized standards, especially those from recognized standard-setting agencies or associations.

After a piece of equipment is validated as operational, it will require periodic monitoring to detect variation in set standards of performance. Major variations of these standards may require shut-down and revalidation. Use of control charts is a good statistical control process to verify that the equipment continues to operate within limits. Written standardized procedures are required for all laboratory equipment. Of course, all validation procedures require attestation of at least two signatures (with dates) to the records kept in a bound laboratory notebook or on appropriate forms. These validation records should be placed on the equipment or instrument or retained in a centralized location.

Also included in validation of equipment is the need for preventative maintenance to provide evidence that the equipment is in a state of control. Preventative maintenance helps minimize malfunction and performance variation. It allows stable laboratory operations and is preferable to sporadic, uncontrolled major maintenance. Routine maintenance involves daily, weekly, or monthly activities such as lubrication and cleaning and replacement of recording paper. A written record should show the date and time when the maintenance was performed. When more skill and technical knowledge is required for maintenance activities than an average technician can handle, specially trained individuals should perform this function.

The key element to controlling and validating refrigerators and freezers is that these devices should maintain the temperatures required. The sophistication of their electronic controls will decide the validation steps needed. Simple temperature controlling rheostat devices only set the instrument at a certain temperature point and provide no feedback.

Refrigerators and freezers with these controls are dependent somewhat on the ambient temperature of the environment into which they are placed. Validation must take into account the potential for variation of the ambient temperature, the frequency with which the doors are opened and closed, and the temperature gradients that may exist within the refrigerator or freezer when empty and full.

Validation procedures should include a check of the internal chamber temperature with a calibrated thermometer traceable to a standard of the NBS

(National Bureau of Standards) or with calibrated temperature sensors independent of the equipment's operational devices. Alarms are helpful for warning of equipment malfunctions. When very precise temperatures are needed, more highly specialized control devices and feedback systems can be used.

Centrifuges may be used to harvest inocula for preservative efficacy tests or cells required to set up seed-lot cultures for maintaining the organisms. One of the key areas for control is the velocity of spin. This velocity should be kept even and consistent at each of the speeds to be used. Therefore, tachometers must be kept calibrated in order to check the dial settings of the centrifuge against the actual spin velocity. In refrigerated centrifuges, the temperature variability within the chamber must be determined. In addition, procedures that specify which materials and solvents may be used in a centrifuge and which are prohibited should be followed, along with procedures that ensure that such materials do not remain in the centrifuge head after use. Use of timing devices on a centrifuge must also be validated for reproducibility and reliability (see below).

The elements of blending and mixing devices that require control include velocity and timing. The measurement of blending and mixing capabilities should be performed at several velocities. Equipment timers should be checked against calibrated timers.

Maximum–minimum thermometers may be used to control and monitor equipment temperatures. An operator can use this type of thermometer to manually check the thermometer and determine the maximum temperature reached in the instrument over a period of time. Maximum–minimum thermometers may be used in rooms, chambers, freezers, refrigerators, incubators, and water baths. One criticism is that these thermometers do not show how long a maximum temperature is held. A thermometer can be calibrated by comparison with an NBS-calibrated thermometer at two control temperatures that span the range desired.

Balances used in microbiology labs range from simple triple beam types to electronic balances that can tare and calibrate at the push of a button. Regardless of type, all balances must be properly calibrated with certified weights. Some balances must be calibrated daily. Modern electronic balances require calibration less frequently, but usually at least monthly with a single weight.

pH meters also come in a diversity of designs. The manufacturer's recommendations for calibration should be followed. At a minimum, daily calibration against two buffer solutions should be done, one at pH 7 and the other (the slope) at either pH 4 or 10, depending on whether the solutions to be measured are acidic or basic.

Timers are used in all kinds of laboratory processes and for all kinds of equipment and instruments. Calibration of timers is often overlooked. However, certified calibrating stop watches can be used to check timers.

Instruments that measure turbidity include spectrophotometers, Klett meters, and nepholometers. These instruments are complex enough to demand that the operations manuals are carefully followed regarding instal-

lation, operation, and calibration. Typically for a spectrophotometer, a blank is used to provide the standard for 100% transmittance (or 2.0 optical density) and complete blocking of the light at a specific wavelength is provided as the standard for 0% transmittance (or 0 optical density).

Incubators provide controlled temperatures and moisture for the promotion of bacterial growth in inoculated media. They have specific requirements for precise operation. Installation, operation, and calibration should consider the variability acceptable for reproducible reliable data collection. For example, temperature variations within an incubator should be documented because extremes from the top to the bottom of an incubator can occur and can differ based on how full or empty the incubator is. Many incubators resolve this problem via fan circulation of the air inside the incubator. Use of multiple thermocouples installed throughout the incubator and hooked to a computer is an efficient way of determining the temperature profile of an incubator. If this type of monitoring is beyond the ability of a laboratory, the manufacturer of the incubator should have such capacity. In sophisticated incubators, temperature data can be recorded continuously on charts or via computer data systems.

In a sense, water baths are similar to incubators. The amount of permissible variation in temperature depends upon the intended use of the bath. Another area where the analogy to incubators is accurate is that circulation of water in a water bath (rather than air circulated in an incubator) will provide better temperature control; calibration of the thermal sensors and fluid flow control devices are the basic elements of control. In addition, thermal mapping should also be performed during installation. Alarm systems are available and continuous recording systems can be integrated into a water bath.

Colony counters require minimal controls. Nevertheless, written procedures covering proper use and maintenance are required. The most critical element of a colony counter is the mechanism for recording the counts. Manual tabulators are still very common but are being replaced by touch probes that eliminate human variability. With manual tabulation, a plunger is pressed with the thumb each time a colony is counted. The touch probe records a count each time a probe is pressed to a colony. Variability is controlled by providing a standard plate with a known number of colonies and having a technician count them.

The more sophisticated automated surface colony readers eliminate the human elements of fatigue and errors due to poor visual acuity. They are used where high numbers of plate counts are routine. The automated colony counters are based on either image analysis or interruption of a laser beam as it passes through a colony. These counters must be standardized using plates with painted patterns of spots to mimic colonies. Installation qualification and employee training are critical for operating scanning recording colony counters.

Laminar air flow hoods are used for culture transfers and for putting up cultures permanently to ensure purity. Standards for these types of hoods

focus on maintaining laminarity and, of course, sterility. Both of these parameters can be checked daily. In addition, detection of leaks in HEPA filters is critical to operation of laminar flow hoods. A simple mechanism to validate the sterility of a hood is to use either settling plates or air samples from within the hood. In addition to having written procedures for validation and the operation of laminar air flow hoods, detailed instructions for the personnel operating the hoods should be provided so the operators do not compromise the flow of air.

## *Sterilizers*

Laboratories require the use of sterilizers to provide sterile media and materials for assaying the microbial contents of products. The most commonly used sterilizer is the autoclave. It operates by killing the microbes through exposure to moist heat generated by steam under pressure. The critical function is to ensure that this moist heat penetrates throughout the autoclave load to destroy all organisms. The two key variables to consider are size and type of load. Thus, validation of an autoclave should include instrument or component calibration, installation qualification, operational qualification, and certification under a diversity of loads.

During installation and operational qualification, thermal profiles of the empty chamber and during performance under various loads obtained with thermal sensors and biological indicators will provide the validation needed. As these operations are performed, one should identify the chamber area that takes the longest to reach temperature before sterilization takes place. The simplest way to do this is to use thermocouples that are passed into the autoclave through the chamber door. These are connected to microprocessor recording devices with computer tie-ins.

After this initial thermal mapping, one should mimic typical loads expected in the planned sterilizer operations. The type of load will impact the thermal profile. Absorptive loads may cause steam to condense and thus increase the time required for the chamber to become saturated with steam. Fluid and solid loads may take longer to heat up. The type of container surrounding the material to be sterilized will also affect the efficacy of an autoclave. For example, a plastic flask will not permit penetration of heat to the media as quickly as a glass flask.

The most common standard in microbiological laboratory operations is the one for making microbiological media in an autoclave: sterilize for 15 min at 121°C. Unfortunately, this standard does not ensure sterility. The neophyte often misinterprets this standard as a sterilizing cycle of 15 min at 121°C instead of 15 min of thermal exposure of the center of the item to be autoclaved.[2]

This problem is especially acute during sterilization of large volumes of liquids. These have a high capacity for absorbing heat and must be heated to the boiling point before being autoclaved. For smaller volumes, a single liter for example, a microbiologist can often incorporate this step into the

autoclave cycle by extending it to 30 min. More often, however, the volume is so great that incorporating the heating time into the sterilizing cycle is not possible because once inside an autoclave, a material cannot be stirred. As a result, uneven heating and burning of the medium may take place.

The most expedient way to validate a sterilizing cycle in a microbiology laboratory is the use of a biological indicator (BI).[3] We have not discussed sterilizers other than autoclaves, but BIs are useful for all sterilizer cycle validation methods including steam, dry heat, and ethylene oxide. For more extensive discussion of various other sterilizing devices, refer to Gardner and Peel.[4] Biological indicators come in a variety of forms, including inoculated products with sterilant-resistant spores, spore paper strips, and disks and commercially available self-contained BI systems. The BIs should be placed in each corner of a load and in the center of an autoclave. The BIs are then incubated after exposure to the autoclave to see whether sterilization was effective.

### *Decontamination*

Relatively little attention is given to decontamination procedures for microbiological cultures and lab benches and materials that may become contaminated during testing of products. The goal of decontamination is usually defined as rendering an area and materials within it safe to handle. When validating the ability of an autoclave to sterilize spent cultures, the same types of guidelines used to validate the autoclave can be employed: using the cultures or using a BI in the center of the load or area.

Essentially, the same principles used to attain sterility should be applied to decontamination processes. Decontaminating the lab benches can be validated using contact plates or swabs to monitor the efficacy of the process used. In all cases, documentation is required in order to track the results.

## *Summary*

While this chapter has of necessity been very general, the guidelines described should provide at least a model for developing a laboratory's own specific procedures for validation. There really is no single way to accomplish validation. However, a five-step process can be followed to validate any system:

1. Define what the system, method, or procedure is supposed to do.
2. Identify and control the variables in the system, method, or procedure.
3. Establish acceptance criteria for the system, method, or procedure before beginning the validation process.
4. Develop and execute a protocol to determine whether the procedure meets the criteria.
5. Document the procedures and results.

Regardless of the methods used to validate a process, the one common requirement is documentation. Documentation provides the evidence that justifies the methods used. A simple way to accomplish this aim is to use a validation register. External documentation in the form of published, peer-reviewed scientific journal articles should be gathered whenever possible.

## References

1. Brannan, D.K., Dille, J.C., and Kaufman, D. J., Correlation of *in vitro* challenge testing with consumer-use testing for cosmetic products, *Appl. Environ. Microbiol.*, 53, 1827, 1987.
2. Halleck, F.E., Thermal solution sterilization, *Pharm. Technol.*, 2, 48, 1978.
3. Pflug, I.J. and Smith, G.M., The use of biological indicators for monitoring wet-heat sterilization processes, in *Sterilization of Medical Products*, Gaughran, E.R.L. and Kereluk, K., Eds., Johnson & Johnson, New Brunswick, NJ, 1977, p. 19.
4. Gardner, J.F. and Peel, M.M., *Introduction to Sterilization, Disinfection, and Infection Control*, 2nd ed., Churchill Livingstone, Melbourne, 1991.

*part four*

# *Preservation of cosmetics*

*chapter seven*

# *Preservation strategies*

***Philip A. Geis***

*Contents*



## *Introduction*

In order to explain the pathways to cosmetic microbiological quality including the strategies for preservation, it is necessary to identify the unique nature of cosmetic products. The most appropriate starting place is the definition established in the U.S. Food, Drug, and Cosmetic Act.[1] The term *cosmetic* covers:

> (1) articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance, and (2) articles intended for use

> as a component of any such articles; except that such term shall not include soap.

The great diversity of intended uses identified by the act requires that successful preservation can only be established if one considers all aspects of development from concept and design through manufacturing to the last consumer use before disposal. Complexity is immediately encountered with the wide range of cosmetic ingredients that includes components as refined as reagent quality chemicals and as unknown as obscure plant extracts. The microbiological quality of each component must be maintained under control.

This is no easy task — a reading of a typical product label reveals tens of ingredients, including a "perfume" that itself is composed of a score or more compounds. Ingredients are combined in unique ways including controlled chemical reactions, partial precipitation and crystallization processes, precise blending, uniquely applied temperature gradients, and particle sizing and emulsification to form solutions, suspensions, emulsions and mixtures. Products are dispensed as fluids, pastes, creams, sticks, loose and pressed powders, and solids at rates of hundreds of units or more per minute and in quantities ranging from a fraction of an ounce to more than a pound. Packaged units such as bottles, tubes, sachets, pens, pans, paper-wrapped bars, and aerosols may be combined with implements such as brushes, wands, pads, sponges, and other microbiologically sensitive articles, each necessitating its own microbiological quality controls.

Once packaged in final product form, these cosmetic units are expected to remain stable through the vagaries of temperature and humidity during distribution and ultimate consumer acquisition, use, and storage. Finally, once they arrive in the hands of consumers, the microbiological quality of cosmetics is subjected to special risks because consumers intentionally add water, spit on mascara wands, share cosmetics, and store them under conditions potentially hostile to preservative stability — in hot cars, damp bathrooms, and wet showers.

Each of the steps and events involved in taking raw materials through product formulation and consumer use is unique to the cosmetics industry, both in complexity and risk for microbiological compromise. When such compromise occurs, it is not trivial and affects consumer health and product integrity and carries regulatory compliance consequences.[2–4]

Of these, clearly the consumer health risk is the most important. Infection, illness, and even death have been attributed to microbes in a contaminated cosmetic. Hopfer et al.[5] reported infections and one death attributable to contaminated shampoo used by neutropenic patients about to undergo chemotherapy. They visited a hospital salon where their hair was washed with a shampoo product diluted with tap water and their heads were shaved to address anticipated alopecia. The diluted shampoo used was heavily contaminated with *Pseudomonas aeruginosa* that infected small nicks caused

by shaving. These infections fulminated in these immunosuppressed patients and proved fatal to one.

In other exposures to microbially compromised products, exposure to contaminated mouthwash in an intensive care unit resulted in 74 nosocomial infections.[6] Infections secondary to indirect exposure to contaminated cosmetics have also been reported after a number of hospital infections were traced to a nurse's use of a contaminated hand cream.[7]

Cosmetic-related infections can also occur in consumers' homes. Significant eye infections and even blindness have resulted from ocular infections following the use of contaminated mascara products; mascara brushes facilitated infection by abrading eye surfaces.[8,9] In each of the above cases, a form of physical or immune compromise-facilitated infection occurred and such conditions are not unusual. About 20% of the U.S. population is immunocompromised.[10]

While consumer health is the primary concern of the cosmetic microbiologist, product functionality and esthetics are subject to direct risk of microbiological contamination. The phenomenal degradative capabilities of typical cosmetic contaminants place almost every cosmetic ingredient at risk, from surfactants to antimicrobial solutions that may be incorporated into products.[11–13] The clouding of clear fluids represents visible degradation, but also at risk are opaque products such as emulsions. These products are subject to microbe-mediated phase separation, a phenomenon that can result from the mere presence of dead bacteria.[14–16]

Ironically, some isolates of the common *Pseudomonas aeruginosa* cosmetic contaminant have been found to form emulsions from otherwise distinct lipid–water combinations.[17] Microbial contamination can also compromise product appearance via the pigmentation effects characteristic of contaminants such as the red prodigiosin of *Serratia marcescens*[18] and the greenish pigments of *Pseudomonas aeruginosa*.[19] Finally, product perfume aroma is a uniquely important quality parameter of cosmetics and frequently serves as an effective trademark for a product. While product aroma entails great formulation effort, it is readily compromised by microbially synthesized volatile organic compounds that have thresholds of detection at parts-per-million if not parts-per-trillion levels.[20] These compounds include skatole, indole, hydrogen sulfide, and diverse fungal volatiles.[21,22]

Sharing manufacturers' concerns for consumer health, regulatory bodies are alert to contaminated cosmetics. A review of FDA records finds about 150 recalls of cosmetic and related over-the-counter (OTC) drug products in the United States for microbial contamination reasons since 1990.[23,24] The majority of these recalls were based on the presence or possible presence of Gram-negative *P. aeruginosa* and *Burkholderia cepacia* bacteria and involved products of all types from shampoos to mascaras to eye drops. The literature extends these observations to include a greater diversity of products from aerosol saline eye washes[25] to antiseptics and antibiotics[26,27] and validating the issue that microbiological contamination of cosmetics is of global concern.[28–30]

## Scope and microbiological targets of preservation

Compelling reasons drive the cosmetics industry to maintain microbiological integrity of its products, and preservation in its broadest meaning is the pivotal element of this effort. Because consumer use provides the ultimate relevant microbiological challenge for manufacturers, preservation is targeted to control post-manufacturing events. Nevertheless, cosmetic are rarely produced under sterile conditions and some degree of microbial control is required as a necessary aspect of maintaining manufacturing quality. In fact, certain products such as aerosol mousses packaged in pressurized barrier containers are reasonably preserved solely to help address potential in-process contamination, and the regulations recognize that ingredients playing no roles in finished product quality may be added as "processing aids."[31]

Manufacturing processes and materials may compromise the stability of preservation. For that reason, the following discussion, while focused primarily on finished product preservation, will consider cosmetic preservation as the application of chemical, physical, procedural, and energetic means to finished products, manufacturing intermediates, and raw materials to maintain effective microbial quality.

In Chapter 2 of this text, Dr. Brannan provided a complete historical review of microbial contaminants recovered from cosmetics. Those microbial contaminants from manufacturing sources have been fairly well documented over recent decades[23,24] but have not been so well reported in the primary context of consumer use. Certainly microorganisms potentially significant to product quality can be found in any nonsterile consumer environment and associated with household and human surfaces and fluids.[32–34] However, the biological range of consumer product contamination is not so well known.

One of the few detailed reports describing the range of biological elements of consumer contamination was written by Brannan et al.[35] The authors placed sterile shampoo and lotion cosmetic products of differing preservative capacities (described as unpreserved, marginally preserved, and effectively preserved) in the hands of consumers, then recovered the products after extended consumer use, cultured product samples, and identified all contaminating microbes recovered. From the unpreserved and a smaller number of marginally preserved products, they recovered a wide range of microorganisms including many types of bacteria and fungi. Table 7.1 lists consumer use-derived and manufacturing-derived microbes and represents a comparison of limited additional data[36,37] and the better documented list of microbes recovered as pre-consumer contaminations.[23,24] Many microbes appear on both the manufacturing-derived and consumer use-derived lists. One very notable absence from the list of consumer microbes is *B. cepacia*, one of the most common microbial contaminants encountered in manufacturing environments. Differences of less significance included as consumer contaminants likely skin and oral microbes (especially *Staphylococcus*, *Streptococcus*, and

***Table 7.1*** Cosmetic Microbiological Contaminants [3,22,23,35–37]

| Consumer-Derived | Manufacturing-Derived |
|---|---|
| **Bacteria** | |
| *Pseudomonas aeruginosa* | *Burkholderia cepacia* |
| *Pseudomonas* spp. | *B. picketii* |
| *Stenotrophomonas maltophilia* | *Pseudomonas aeruginosa* |
| *Acinetobacter* spp. | *Pseudomonas* spp. |
| *Moraxella* spp. | *Acinetobacter* spp. |
| *Enterobacter* spp. | *Moraxella* spp. |
| *Citrobacter freundii* | *Enterobacter* spp. |
| *Klebsiella pneumoniae* | *Citrobacter freundii* |
| *K. oxytoca* | *Klebsiella pneumoniae* |
| *Serratia* spp. | *K. oxytoca* |
| *Staphylococcus aureus* | *Serratia marcescens* |
| *S. epidermidis* | *Serratia* spp. |
| *Sarcina* spp. | *Salmonella* spp. |
| *Propionibacterium* spp. | *Ochrobacterium anthropi* |
| *Corynebacterium* spp. | *Proteus* spp. |
| *Streptococcus mutans* | *Aeromonas sobria* |
| *Bacillus* spp. | *Bacillus* spp. |
| *Clostridium perfringens, C. tetani* | |
| **Fungi** | |
| *Candida albicans* | *Candida lipolytica* |
| *Rhodotorula sp.* | *Saccharomyces cerevisiae* |
| *Penicillium* spp. | *Penicillium* spp. |
| *Aureobasidium pullulans* | *Aureobasidium pullulans* |
| *Scopulariopsis sp.* | *Paecilomyces variotii* |

*Corynebacterium* spp.). Apparently unique to manufacturing contaminants were microbes attributed to earth-derived materials such as talcs (e.g., *Clostridium* spp.).

## *Preservation of finished products*

Effective preservation of cosmetics is not a simple process of adding one or more antimicrobial ingredients to a formula. Rather, successful preservation is a function of appropriate consideration of factors that affect efficacy and stability including overall formulation chemical and physical characteristics, specific ingredients, preservative efficacy and stability *in situ*, product packaging, manufacturing processes and materials, and anticipated consumer use. This process not only identifies and addresses pitfalls that may compromise efficacy but also exploits those factors that can facilitate successful preservation.

## *Formula parameters*

A number of cosmetic chemical and physical characteristics directly affect preservative efficacy and stability. Product pH is one of the more recognizable parameters. While highly acidic or basic formulations are intrinsically hostile to microbial contamination, relatively minor changes within the pH range that permit microbial growth can have profound effects on preservative efficacy and stability. For example, the activities of organic acids are profoundly compromised in neutral and alkaline formulations as are the stabilities of isothiazolinone preservatives in the presence of amines.[38–40]

For some products, consideration of the pH effect should go beyond the monitoring of the bulk product to the microenvironments provided by some product constructs. Pongcharoenkait et al.[41] described pH-mediated paraben hydrolysis at the alkaline interfaces of emulsified lipid droplets in a product of reportedly neutral pH in its continuous phase.

Another general moderator of efficacy is water activity or $A_w$ — a measurement of the level of water available to contaminating microorganisms for growth. It is important to note that water activity does not correlate directly to formula percentage of water. Rather, it is a measure of water that is not complexed with solutes, especially salts or glycols. As water activity is reduced, a product becomes more hostile and in a manner selective for groups of microbes.[42] At slightly limited $A_w$ levels (<0.9), Gram-negative bacteria, particularly pseudomonads, are inhibited; lower $A_w$ conditions (<0.8) are required to inhibit Gram-positive bacteria such as staphylococci. Even lower water availability is required to inhibit yeast-like fungi, and mycelial fungi are the most tolerant, some growing at substantially reduced water activity levels.[43] While it may not be feasible to formulate $A_w$ values prohibitive for all microbial growth, control of this product characteristic may allow simplification of preservation by focusing on those microbial groups capable of growth.

Knowledge of specific ingredient–preservative interactions derives primarily from empirical observations of cosmetic microbiologists and largely exists as unique and internal proprietary information. The relatively small amount of relevant published information describes both interference and synergy of preservative efficacy through interactions with product components such as sugars, sugar alcohols, polymers, and sunscreen actives.[44–48]

General product type also is a controlling element of preservative efficacy. While preservation of simple solutions would appear to correlate most directly to the presence of effective preservatives, preservation of other product forms can prove more challenging. For example, it has long been known that the commonly used paraben preservatives in lipid–water emulsions can partition into the lipid phase, leaving the water phase and the product effectively unpreserved.[49]

Newer forms of cosmetics casually referred to as "atypical"[50] have come from the inventive minds of cosmetic chemists. These include broader appli-

cation of relatively small product categories (e.g., wipes) as well as novel chemical compositions (e.g., high water powders) and physical conformations (e.g., water-in-silicone emulsions) that reportedly do not lend themselves to conventional approaches to preservation.[51,52] Schnittger et al.[52] described preservation of water-in-silicone oil emulsions as unlike traditional water-in-oil emulsions in that the products failed conventional preservative tests but preservation was effectively achieved as judged by the results of practical consumer-use evaluation.

Investigation determined that test microbes offered in an aqueous-based inoculum failed to come into contact with preservatives in emulsified aqueous droplets, and no effective microbial kill resulted. However, in extended in-use testing (thousands of units), no consumer-derived contamination was found in any unit. The authors concluded that the conformational character of the product prevented contamination by consumers. These authors and other workers[14] have suggested that preservation of water-in-silicone oil emulsions may have value in manufacturing but may be of limited utility in preventing development of consumer contamination. Similarly divergent from expectations, preservation of a lotion used to impregnate a wetted substrate-based wipe product may not translate to a preserved finished product because partitioning of preservatives within the stacked substrate may compromise efficacy *in situ*.[39,51]

## *Commercial preservatives*

The neophyte cosmetic microbiologist searching for effective preservatives may feel daunted by the few texts that review the general field of cosmetic preservation. Potential preservative chemicals are often documented in extensive lists that do not discriminate between the most useful compounds and those of more limited applications (see Wallhauser,[38] for example). In fact, relatively few effective preservatives are available and only a handful offer efficacy in common application. A more utilitarian and focused list is presented in this volume in the appendix following Chapter 10. The list is based on the frequency of preservative applications.

As reported in the FDA's voluntary cosmetic registration program,[53] the U.S. cosmetic industry employs about 60 preservatives. Fewer than 20 enjoy high frequency of use and they can be categorized into five general groups (Table 7.2); some have focused applications. As indicated in Table 7.3, preservative systems differ among product forms and often target different potential contaminants. For example, make-up powder products typically contain relatively limited water and preservation is largely targeted at fungi and other microbes that can grow under such selective conditions. Therefore, powder preservative systems differ from preservatives used for highly aqueous shampoos, the contamination of which is typically limited to pseudomonad and enteric bacteria.[35]

If the reader were to examine product labels in detail, he would find that cosmetics typically use preservatives in combinations (Table 7.3) to

*Table 7.2* Commonly Used Cosmetic Preservatives

| |
|---|
| **Esters of para-hydroxybenzoic acid (parabens)** |
| Methyl parabens |
| Ethyl parabens |
| Propyl parabens |
| Butyl parabens |
| **Formaldehyde releasers[a]** |
| Imidazolidinyl urea |
| Diazolidinyl urea |
| Dimethyl dimethylol hydantoin (DMDM hydantoin) |
| Quaternium 15 (triaza-azoniaadamantine chloride) |
| **Isothiazolinones** |
| Chloromethyl isothiazolinone/methyl isothiazolinone |
| **Organic acids (including their metallic salts)** |
| Benzoic acid |
| Sorbic acid |
| Dehydroacetic acid |
| **Organic alcohols** |
| Ethyl alcohol |
| Benzyl alcohol |
| Phenoxyethyl alcohol |
| **Other** |
| Benzalkonium chloride |
| Benzothenium chloride |
| Chlorhexidene digluconate |
| Iodopropenyl butylcarbamate |

*Note*: Derived from Sedlewicz, L.B., http://www.nyscc.org/news/archive/tech0903/html

[a] Preservatives grouped under this heading are known to release some level of formaldehyde in aqueous solution. Efficacy is reported to be partially attributable to the parent molecule. Some are correctly termed formaldehyde adducts based on their means of synthesis. For reference here, they are designated formaldehyde releasers.

effect broad antimicrobial action against a range of potential contaminating bacteria and fungi as well as to establish some degree of synergy in activity.[54–56] Combinations include preservatives of broad efficacy such as the isothiazolinones, hydantoin derivatives, and imidazolidinyl ureas combined with one or more compounds of more selective efficacy. This latter group includes organic acids and salts (e.g., sorbic acid, benzoic acid, and sodium dehydroacetate), organic alcohols (e.g., benzyl and phenoxyethyl), and esters of para-hydroxybenzoic acid and iodopropenyl butyl carbamate. More extensive combinations of this second group may also establish necessary efficacy.

***Table 7.3*** 2005 Label Survey: Preservation Systems of Selected Major Brands

| Product Category/Preservative Systems |
|---|
| **Shampoo** |
| DMDM hydantoin, methyl paraben, EDTA |
| DMDM hydantoin, MI/MCI/EDTA |
| Diazolidinyl urea, benzyl alcohol, IPBC, EDTA |
| MI/MCI, sodium benzoate, EDTA |
| **Conditioner** |
| MI/MCI, benzyl alcohol |
| Methyl paraben, chlorhexidine |
| DMDM hydantoin, MI/MCI, EDTA |
| MI/MCI, benzyl alcohol, EDTA |
| **Body wash** |
| DMDM hydantoin, EDTA |
| DMDM hydantoin, sodium benzoate, EDTA |
| MI/MCI, EDTA |
| Quaternium 15, EDTA |
| **Lotion** |
| DMDM hydantoin, IPBC, EDTA |
| Benzyl alcohol, phenoxyethanol |
| DMDM hydantoin, methyl paraben, EDTA |
| Benzyl alcohol |
| **Mascara** |
| Methyl paraben, propyl paraben |
| Diazolidinyl urea, methyl paraben, propyl paraben |
| Parabens (methyl, ethyl, propyl, isopropyl, butyl, isobutyl), phenoxyethanol, EDTA, sodium |
| Dehydroacetic acid |
| Diazolidinyl urea, methyl paraben |
| **Mousse** |
| DMDM hydantoin |
| Diazolidinyl urea |
| Phenoxyethanol, methyl parabens |
| Sodium benzoate |
| **Make-up powder** |
| Sorbic acid, parabens (methyl, propyl, butyl), EDTA |
| Methyl paraben, propyl paraben |
| Phenoxyethanol, parabens (methyl, propyl, butyl) |
| Sorbic acid, methyl parabens, propyl parabens |

*Note:* MI/MCI = methylisothiazolinone, chloromethylisothiazolinone; IPBC = iodopropenyl butylcarbamate.

## *Preservative adjuncts*

In addition to common preservatives, the cosmetic industry uses a number of compounds that facilitate preservative activity.[57] Some may be effective alone if used at sufficiently great concentrations. These include ethanol that at >15% exerts a direct preservative effect and at lower levels complements the activities of better known preservatives.[58]

At very high concentrations, glycols may control $A_w$ levels sufficiently to inhibit microbial growth but lower levels may facilitate preservation. This group includes propylene, butylenes, and longer chain glycols.[58,59] Mixtures of longer chain organic acids with long chain glycols and related combinations may have usefulness at intermediate concentrations and have recently been commercialized to serve this facilitating function.[39,60]

An adjunct whose activity warrants special mention is ethylenediaminetetraacetic acid (EDTA). This ingredient is thought to sequester divalent cations such as calcium and magnesium, compromising microbial cell wall and capsular integrity in a manner that allows better penetration of antimicrobial agents into cells.[60] Its widespread use (Table 7.3) testifies to its efficacy in preservative applications.

## *Perfumes and natural preservatives*

Selected perfume components and natural materials have been shown to have some degree of preservative efficacy.[61,62,63] These are of limited use[61] because their activity is often lost upon dilution in formulation and efficacy may be pH-dependent.[64,65] For natural materials, the active principles are rarely known, making consistency among recipes difficult to assure. Although some so-called natural or naturally derived preservatives have been commercialized, at least one of these materials has been shown to include unreported synthetic antimicrobials.[66] As of this writing, the cosmetics industry has not shown significant interest in these materials for preservative application.[67]

## *Process considerations*

Successful use of primary preservatives with relevant facilitating components must be viewed in the contexts of product manufacturing and final consumer use. It is important to consider compatibility and long term stability with other ingredients, relevant manufacturing events and exposures, packaging components, and consumer use. Ingredient incompatibilities are typically indicated in supplier literature and technical summaries (see appendix) and may often be found in supplier-recommended means of preservative neutralization.[67]

Similarly, suppliers can provide data concerning processing risks — especially the consequences of transient exposure to high temperatures or extremes of pH. Although not so well documented, physical stability con-

cerns derive from other processing parameters and ingredients should also be considered. For example, highly water soluble preservatives may be sensitive to order of ingredient combinations such that transient conditions of limited solute may provoke partial precipitation. Conversely, a highly lipid-soluble preservative combined with a lipid premix may upon final emulsification remain partitioned to that phase, leaving suboptimal levels in the critical water phase.[68] Such interactions may be accelerated by heat involved in processing.[69] Physical incompatibilities, especially partitioning or migration of preservatives into processing and even packaging components, have been described[70–72] and may guide both preservative selection as well as choice of materials.

### *Role of packaging*

The final parameter to be considered here is packaging — as an effective physical element preventing consumer-introduced contaminants and as a means of preservation. Packaging clearly is a necessary barrier to microbial contamination and an essential if not obvious part of any preservative system. For aerosol products, packaging establishes a complete barrier to consumer challenge, and the aerosol propellant itself offers some limited preservative function.[73] Similarly, single- or limited-use packaging types such as sachets and sample size bottles limit opportunities for contamination.

As with control of volume, closure and pump designs that limit ingress of contaminants have been shown to maintain product quality.[74,75] However, as will be described for manufacturing systems, contamination need not spread throughout an entire product to be considered significant. Donzis[76] associated the development of a *P. aeruginosa* corneal ulcer with the use of a preservative-free aerosol saline product. Although the product contents were found to be sterile, the microbe was recovered from a reservoir of product that remained in the tip after use.

Finally and despite their functional roles in product preservation, some packaging applications require additional preservative consideration. Cellulosic packaging and labels may require preservation against mold growth if conditions of condensed moisture are anticipated.[77] Such conditions may be established by the environment in which the product is distributed or by the product itself. Paper packaging such as that used for bar soaps has been reported as a target for preservation measures and methods have been documented for evaluation.[78,79]

## *Preservation during manufacturing*

The primary purpose of preservation is clearly to address contamination risks associated with consumer use. However, because cosmetics are rarely produced on sterile systems, microbiological control is a necessary consideration for hygienic manufacturing. From raw materials through process

intermediates and flow streams to finished product, preservation in its broadest sense is a necessary quality parameter.

## *Raw materials preservation*

The great number and diversity of cosmetic raw materials clearly make microbiological control a necessary element of manufacturing hygiene and finished product quality.[11] While microbial issues may be associated with almost any raw material, the number for which preservation is an important quality parameter is relatively small. Most raw materials lack sufficient water to require preservation and are most appropriately addressed through hygienic manufacturing, delivery, and storage.

Raw materials, especially if derived from natural or earth sources, may include significant numbers of *Bacillus* spp. Although such bacteria would not be expected to increase numerically during manufacturing or in finished products, sufficient numbers may remain in finished products to exceed industry standards and regulatory expectations.[80,81] The same concern can be applied to product implements such as brushes, pads, and wipe substrates.[82,83] Sterilization by irradiation or ethylene oxide treatment may be appropriate for such implements.[84,85]

Raw materials such as surfactants and solutions of plant-derived materials that require preservation typically include substantial amounts of water.[11] Although manipulation of pH and $A_w$ values may offer limited opportunities for microbiological control, the use of chemical preservatives is probably a more common means of raw material preservation. The chemicals of choice are similar if not the same as those used for finished product preservation. Water is the exception.

Although it is usually the ingredient of greatest concentration, it is also the only ingredient whose microbiological quality is determined only after formulation. Microbiological control of process water is important because microbes such as *Burkholderia cepacia* can multiply to millions of colony-forming units per milliliter in purified water without obvious changes in clarity.[86] Persistent challenge of any product with such high levels of microorganisms provides substantial risk of microbiological failure (contaminant survival and growth). Means of process water preservation and treatment include maintenance at high temperature, for example, 80°C, ozonolysis, ultraviolet irradiation, and filtration.[11]

## *System Preservation*

Manufacturing hygiene is discussed in previous chapters, and it should be evident from those discussions that the level of hygiene identified as appropriate and attainable for cosmetic manufacturing does not demand sterility. Therefore, materials transiting such systems in cosmetic production are subject to some limited risk of microbiological presence. Such failures jeopardize

not only the finished product, but also the integrity of the system and its components.[87]

Therefore, from the initial introduction of formulation water to the final pack-out of finished product, it is critical that a certain degree of preservative capacity be maintained throughout the process flowpath. This is especially true for premixes, captured intermediates, and flowpaths through which effectively unpreserved materials are transferred. This risk is substantially resolved at the process point of preservative addition. Therefore, judicious scheduling of formulation will help address process preservation. The remaining unpreserved (and vulnerable) process elements can be protected through control of temperature, pH, $A_w$, and even time between cleaning and sanitization efforts.

## *Conclusions*

The objective of cosmetic preservation is clearly to maintain microbiological quality and most microbiologists would see consumer contamination as its primary challenge. What is often not so obvious is that the act of preservation is not limited to the addition of one or more of a fairly small number of commercial chemicals. Preservation includes application of these specific ingredients in the context of clear understanding and microbiological control of the cosmetic product from raw material through processing ingredients, package design, and anticipated consumer use. Only through appropriate control of these elements can a cosmetic microbiologist ensure that consumers enjoy products containing stable and effective preservative systems that function effectively against the microbes of risk and in the context of consumer use.

In concept and as considered by regulatory authorities, cosmetics are intended to achieve benefit more discretionary than essential to survival, and it is laudable that the industry has so successfully maintained microbiological quality of what arguably constitute the most complex and diverse of consumer products.

## *References*

1. 75th Congress of the United States, Federal Food Drug and Cosmetic Act, 1983 and as later amended, 21 U.S.C. 201(i), p. 2.
2. Spooner, D.F., Hazards associated with the microbiological contamination of cosmetics, toiletries and non-sterile pharmaceuticals, in *Microbial Quality Assurance in Cosmetics, Toiletries and Non-Sterile Pharmaceuticals*, 2nd ed., Baird, R.M. and. Bloomfield, S.F., Eds., Taylor & Francis, Bristol, PA, 1996, p. 9.
3. Geis, P.A., Preservation of cosmetics and consumer products, *Dev. Indust. Microbiol.*, 29, 305, 1985.
4. Brannan, D.K., Cosmetic preservation, *Cosmet. Toilet.*, 111, 69, 1996.
5. Fainstein, V., Andres, N., Umphrey, J., and Hopfer, R., Hair clipping: another hazard for granulocytopenic patients, *J. Infect. Dis.*, 158, 655, 1988.

6. Anon., Nosocomial *Burkholderia cepacia* infections and colonization associated with intrinsically contaminated mouthwash: Arizona, *MMWR*, 47, 926, 1998.
7. Passaro, D.J., Waing, J., Armstrong, L., Bolding, F., Bouvier, B., Rosenburg, J., Reingold, A.W., McQuitty, M., Philpott, S.M., Jarvis, W.R., Werner, S.B., Tompkins, L.S., and Vugia, D.J., Postoperative *Serratia marcescens* wound infections traced to an out-of-hospital source, *J. Infect. Dis*., 175, 992, 1997.
8. Taub, S.J., Contaminated cosmetics as a cause of eye infections, *Eye Ear Nose Throat Mon*., 54, 81, 1975.
9. Wilson, L.A. and Ahearn, D.G., *Pseudomonas*-induced corneal ulcers associated with contaminated eye cosmetics, *Am J. Ophthalmol*., 84, 112, 1997.
10. Rose, J.B., Haas, C.N., and Gerba, C.P., Linking microbiological criteria for foods to quantitative risk assessment, *J. Food Protect*., 15, 121, 1995.
11. Russell, M., Microbiological control of raw materials, in *Microbial Quality Assurance in Cosmetics, Toiletries and Non-Sterile Pharmaceuticals*, 2nd ed., Baird, R.M. and Bloomfield, S.F., Eds., Taylor & Francis, Bristol, PA, 1996, p. 31.
12. Oie, S. and Kamiya, A., Contamination of antiseptics and disinfectants, *Am. J. Infect. Control*, 24, 389, 1996.
13. Frank, M.J. and Schaffner, W., Contaminated aqueous benzalkonium chloride: an unnecessary infection hazard, *JAMA*, 236, 2418, 1976.
14. O'May, G.A., Allison, D.G., and Gilbert, P., A rapid method for the evaluation of extrinsic and intrinsic contamination and resulting spoilage of water-in-oil-emulsions, *J. Appl. Microbiol*., 96, 1124, 2004.
15. Valderama, M.J., Marquina, D., and Peinado, J.M., Isolation, characterization and effect of *Candida albicans* isolated from a deteriorated cosmetic, *Int. J. Biodeter. Biodegrad*., 40, 151, 1997.
16. Bento, F.M. and Gaylarde, C.C., The production of interfacial emulsions by bacterial isolates from diesel fuels, *Int. J. Biodeter. Biodegrad*., 38, 31, 1999.
17. Grimont, P.A.D. and Grimont, F., Family I, Genus VII, *Serratia* bizio, 1823, in *Bergey's Manual of Systematic Bacteriology*, Krieg, N.R. and Holt, J.G., Eds., Williams & Wilkins, Baltimore, 1984, p. 477.
18. Palleroni, N.J., Family I, Pseudomonadaceae, Genus I, *Pseudomonas migula*, 1894, in *Bergey's Manual of Systematic Bacteriology*, Krieg, N.R. and Holt, J.G., Eds., Williams & Wilkins, Baltimore, 1984, p. 141.
19. Nagata, Y., Measurement of odor threshold by triangle odor bag method, *Odor Measure. Rev.*, 2003, 118, 2003.
20. *Bergey's Manual of Systematic Bacteriology*, Krieg, N.R. and Holt, J.G., Eds., Williams & Wilkins, Baltimore, 1984.
21. Wheatley, R.E., The consequences of volatile organic compound-mediated bacterial and fungal interactions, *Antonie Van Leeuwenhoek J. Microbiol*., 81, 257, 2002.
22. Wong, S., Street, D., Delgado, S.I., and Klontz, K.C., Recalls of food and cosmetics due to microbial contamination reported to the U.S. Food and Drug Administration, *J. Food Protect.*, 63, 1113, 2000.
23. FDA Enforcement Report Index. http://www.fda.gov/opacom/enforcement.html
24. Riordan-Eva, P., Eykyn, S.J., and Kerr-Muir, M.G., *Pseudomonas aeruginosa* ulcer associated with an aerosol can of preservative-free saline, *Arch. Ophthalmol*., 106, 1506, 1988.
25. Oie, S. and Kamiya, A., Bacterial contamination of aerosol solutions of antibiotics, *Microbios*, 82, 109, 1995.

26. Oie, S. and Kamiya, A., Microbial contamination of antiseptics and disinfectants, *Am. J. Infect. Control*, 24, 289, 1996.
27. Okeke, I.N. and Lamikanra, A., Bacteriological quality of skin-moisturizing creams and lotions distributed in a tropical developing country, *J. Appl. Microbiol.*, 91, 922, 2001.
28. Ashour, M.E., Abdelaziz, A.A., El-Tayeb, O.M., and Hefnai, H., Microbial contamination of cosmetics and personal care items in Egypt I: contamination of toothpastes and mouthwashes, *J. Soc. Cosm. Chem.*, 38, 435, 1987.
29. Taguchi, F. and Amemiya, K., Bacterial contamination of hair washing liquids, *Kansen. Zass.*, 68, 177, 1994 (translated).
30. U.S. Food and Drug Administration, 21 C.F.R. 701.3(h)1.
31. Scott, E., Hygiene in the home, *Am. J. Infect. Control*, 27, S22, 1999.
32. Larson, E.L., Gomez-Duarte, C., Lee, L.V., Delia-Latta, P., Kain, D.J., and Keswick, B.H., Microbial flora of hands of homemakers, *Am. J. Infect. Control*, 31, 72, 2002.
33. Darwazeh, A.M., Al-Refai, S., and Al-Mojaiwel, S., Isolation of *Candida* species from the oral cavity and fingertips of complete denture wearers, *J. Prosth. Dent.*, 86, 420, 2001.
34. Chaidez, C. and Gerba, C., Comparison of the microbiological quality of point-of-use (POU)-treated water and tap water, *Int. J. Environ. Health Res.*, 14, 253, 2004.
35. Brannan, D.K., Dille, J.C., and Kaufman, D.J., Correlation of *in vitro* challenge testing with consumer use testing of cosmetic products, *Appl. Env. Microbiol.*, 53, 1827, 1987.
36. Dawson, N.L. and Reinhardt, D.J., Microbial flora of in-use, display eye shadow testers and bacterial challenge of unused eye shadows, *Appl. Env. Microbiol.*, 42, 297, 1981.
37. Svanberg, M., Contamination of toothpaste and toothbrush by *Streptococcus mutans*, *Scand. J. Dent. Res.*, 86, 412, 1978.
38. Wallhauser, K.H., Antimicrobial preservatives used by the cosmetic industry, in *Cosmetic and Drug Preservation*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 605.
39. Sedlewicz, L.B., Preservatives demystified: a guide to choosing the correct preservative for your product, New York SCC Technical Note, 2005. http://www.nyscc.org/news/archive/tech0903/html
40. Collier, P.J., Ramsey, A., Waigh, R.D., Douglas, K.T., Austin, P., and Gilbert, P., Chemical reactivity of some isothiazolinone biocides, *J. Appl. Microbiol.*, 69, 578, 1990.
41. Pongcharoenkait, N., Narsimhan, G., Lyons, R.T., and Hem, S., The effect of surface charge and partition coefficient on the chemical stability of solutes in O/W emulsions, *J. Pharm. Sci.*, 91, 559, 2002.
42. Curry, J., Water activity and preservation, *Cosmet. Toilet.*, 100, 53, 1985.
43. Morris, C. and Leech, R., Natural and physical preservative systems, in *Microbial Quality Assurance in Cosmetics, Toiletries and Non-Sterile Pharmaceuticals*, 2nd ed., Baird, R.M. and Bloomfield, S.F., Eds., Taylor & Francis, Bristol, PA, 1996, p. 69.
44. Ma, M., Lee, T., and Kwong, E., Interaction of methyl paraben preservative with selected sugars and sugar alcohols, *J. Pharm. Sci.*, 91, 1715, 2002.
45. Kurup, T.R., Wan, L.S., and Chan, L.W., Interaction of preservatives with macromolecules I: natural hydrocolloids, *Pharm. Acta Helv.*, 67, 301, 1992.

46. Scalzo, M., Orlandi, C., Simonetti, N., and Cerreto, F., Study of interactive effects of polyacrylic acid polymers (Carbopol 940) on antimicrobial activity of methyl parahydroxybenzoate against some Gram-negative, Gram-positive bacteria and yeast, *J. Pharm. Pharmacol.*, 48, 1201, 1996.
47. Orth, D.S., Lutes-Anderson, C.M., Smith D.K., and Milstein, S.R., Synergism of preservative system components: use of survival curve slope method to demonstrate anti-Pseudomonas synergy of methyl paraben and acrylic acid homopolymer/copolymer *in vitro*, *J. Soc. Cosmet. Chem.*, 40, 347, 1989.
48. Rassat, F., Gonzenbach, H., and Pittet, G.H., Use of sunscreen and vitamins in daily-use cosmetics, *Drug Cosmet. Ind.*, December, 16, 1997.
49. Bean, H.S. and Herman-Ackah, S.H., Influence of water:oil ratio on activity of some bactericides against *Escherichia coli* in liquid paraffin and water dispersions, *J. Pharm. Pharmacol.*, 16 (Suppl.), 58, 1964.
50. Yablonski, J.I. and Mancuso, S.E., Preservation of atypical products, *Cosmet. Toilet.*, 117, 1, 2002.
51. Cosmetic Toiletry and Fragrance Association Microbiology Committee, *Preservation of Atypical Products*, Washington, D.C., in press, 2005.
52. Schnittger, S., Sabourin, J., and King, D., Preservation of water-in-silicone emulsions, *Annual Meeting Society of the Cosmetic Chemists,* New York, 2001.
53. Steinberg, D.C., Frequency of preservative use, 2003, *Cosmet. Toilet.*, 119, 55, 2004.
54. Rosen, W.E. and Berke, P.A., Germall 115: a safe and effective preservative, in *Cosmetic and Drug Preservation*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 191.
55. Hall, A.H., Cosmetically acceptable phenoxyethanol, in *Cosmetic and Drug Preservation*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 79.
56. Richards, R. and McBride, R., Phenylethanol enhancement of preservatives used in ophthalmic preparations, *J. Pharm. Pharmacol.*, 23, 141S, 1971.
57. Anon., Compounds that contribute to preservative activity, *Cosmet. Toilet.*, 105, 63, 1990.
58. Happel, J.A., Antimicrobial preservatives in pharmaceuticals, in *Disinfection, Sterilization and Preservation*, 3rd ed., Block, S.S., Ed., Lea & Febiger, Philadelphia, 1983, p. 579.
59. Branna, T., A preservative update, *HAPPI*, 41, 89, 2004.
60. Lambert, R.J., Hanlon, G.W., and Denyer, S.P., The synergistic effect of EDTA/antimicrobial combinations on *Pseudomonas aeruginosa*, *J. Appl. Microbiol.,* 96, 244, 2004.
61. Kalemba, D. and Kunicka, A., Antibacterial and antifungal properties of essential oils, *Curr. Med. Chem.*, 10, 81, 2003.
62. Nostro, A., Cannatelli, M.A., Morelli, I., Musolino, A.D., Scuderi, F., Pizzimenti, F., and Alonzo, V., Efficiency of *Calmintha officicinalis* essential oil as preservative in two topical product forms, *J. Appl. Microbiol.*, 97, 395, 2004.
63. Ibrahim, Y.K. and Ogunmodele, M.S., Growth and survival of *Pseudomonas aeruginosa* in some aromatic waters, *Pharm. Acta. Helv.*, 66, 286, 1991.
64. Morris, C. and Leech, R., Natural and physical preservatives, in *Microbial Quality Assurance in Cosmetics, Toiletries and Non-Sterile Pharmaceuticals*, 2nd ed., Baird, R.M. and Bloomfield, S.F., Eds., Taylor & Francis, Bristol, PA, 1996, p. 69.

65. Takeoka, G., Dao, F., Wong, R.Y., Lundin, R., and Mahoney, N., Identification of benzothonium chloride in commercial grapefruit seed extracts, *J. Agric. Food Chem.*, 49, 3316, 2001.
66. Dweck, A.C., Natural preservatives, *Cosmet. Toilet.*, 118, 45, 2004.
67. Singer, S., The use of preservative neutralizers in diluents and plating media, *Cosmet. Toilet.*, 102, 55, 1987.
68. Haag, T.E. and Longrini, D.F., Esters of para-hydroxybenzoic acid, in *Cosmetic and Drug Preservation*, Kabara, J.J, Ed., Marcel Dekker, New York, 1984, p. 63.
69. Zhang, X. and Kirsch, L.E., The physical stability of thermally stressed phospholipids-based emulsions containing methyl, propyl and heptyl parabens as model drugs, *Int. J. Pharmacol.*, 265, 133, 2003.
70. Bahal, S.M. and Romansky, J.M., Sorption of parabens by flexible tubing, *Pharm. Dev. Technol.*, 6, 431, 2001.
71. Bahal, S.M. and Romansky, J.M., Sorption of benzoic acid, sorbic acid, benzyl alcohol and benzalkonium chloride by flexible tubing, *Pharm. Dev. Technol.*, 7, 49, 2002.
72. McMarthy, T.J., Storage studies of preservative solutions in commonly used plastic containers, *Cosmet. Perf.,* 88, 41, 1973.
73. Ibrahim, Y.K. and Sonntag, H.G., Effect of formulation pH and storage temperatures on the preservative efficacy of some gases used as propellants in cosmetic aerosols, *J. Appl. Bacteriol.*, 74, 20, 1993.
74. Brannan, D.K. and Dille, J.C., Type of closure prevents microbial contamination of cosmetics during consumer use, *Appl. Env. Microbiol.*, 56, 1476, 1990.
75. Klocker, N., Kramer, A., Verse, T., Sikora, C., Rudolph, P., and Daeschlein, G., Antimicrobial safety of a preservative-free nasal multiple-dose drug administration system, *Eur. J. Pharmacol. Biopharmacol.*, 57, 489, 2004.
76. Donzis, P.B., Corneal ulcer associated with contamination of aerosol saline spray tip, *Am. J. Ophthalmol.*, 124, 394, 1997.
77. Narcisco, J.A. and Parish, M.E., Endogenous mycoflora of gable-top carton paperboard used for packaging fruit juice, *J. Food. Sci.*, 62, 1917, 1997.
78. Block, S.S., Preservatives for industrial products, in *Disinfection, Sterilization and Preservation*, 3rd ed., Block, S.S., Ed., Lea & Febiger, Philadelphia, 1983, p. 608.
79. TAPPI, Fungus resistance of paper and paperboard, Classic Method T487cm-93, 1993.
80. Cosmetic, Toiletry, and Fragrance Association, Microbiological limits for cosmetics and toiletries, in *CTFA Microbiology Guidelines,* Curry, A.S., Graf, J.G., and McEwen, G.N., Eds., Washington, D.C., 1993, p. 1.
81. Madden J.M. and Jackson, G.J., Cosmetic preservation and microbes: viewpoint of the Food and Drug Administration, *Cosmet. Toilet.*, 96, 75, 1981.
82. Oie, S. and Kamiya, A., Microbial contamination of brushes used for preoperative shaving, *J. Hosp. Infect.,* 21, 103, 1995.
83. Jacobs, G.F., Gamma radiation decontamination of cosmetic raw materials, in *Cosmetic and Drug Preservation*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 223.
84. Richards, C., Furr, J.R., and Russell, A.D., Inactivation of microorganisms by lethal gases, in *Cosmetic and Drug Preservation*, Kabara, J.J., Ed., Marcel Dekker, New York, 1984, p. 209.

85. Carson, L.A., Favero, M.S., Bond, W.W., and Peterson, N.J., Morphological, biochemical and growth characteristics of *Pseudomonas cepacia* from distilled water, *Appl. Microbiol*., 25, 476, 1973.
86. Flaherty, J.P., Garcia-Houchins, S., Chudy, R., and Arnow, P.M., An outbreak of Gram-negative bacteria traced to contaminated O-rings in reprocessed dialyzers, *Ann. Int. Med*., 119, 1072, 1993.
87. Busscher, H.J., deBoer, C.E., Verkerke, G.J., Kalicharan, R., Schuette, H.K., and van der Mei, H.C., *In vitro* growth of yeasts into medial grade silicone rubber, *Int. J. Biodeter. Biodegrad*., 33, 383, 1994.

*chapter eight*

# *Antimicrobial mechanisms of selected preservatives and the bacterial response*

***John S. Chapman***

*Contents*



## *Introduction*

The mechanisms by which cosmetic preservatives exert their antimicrobial effects and the strategies adopted by bacteria to avert those effects are topics of significant importance to maintaining biological control in the various microbial habitats available within the cosmetic industry. A basic understanding of the chemistry and biological interactions of the chemicals used as preservatives allows a microbiologist to make reasoned estimations of the environments in which a particular preservative would work best and identify possible sources of trouble when a preservative system fails to perform as desired. Further, understanding the resistance mechanisms bacteria

employ against preservatives provides a solid basis for a microbiologist to recommend measures to counteract the resistance mechanism.

## *Overview of biocide mechanism and suitability for use in cosmetic preservation*

Biocide mechanisms of action can be grouped into several broadly defined classes. One large class, the reactive biocides, has been so named because the active species participates in a chemical reaction with target molecules. This class contains the oxidants and their less extreme cousins, the electrophiles. Oxidants comprise chemicals such as the hypohalous acids (chlorine, bromine) and molecules that deliver them (such as bromochlorodimethyl hydantoin) as well as the various peroxides. These molecules are rarely used in cosmetic preservation because they are so reactive they tend to degrade formulation components such as fragrances, and their half-lives within products are too short to deliver effective preservation.

The major electrophilic preservatives are represented by formaldehyde (FA), formaldehyde-releasing biocides, and chloromethylisothiazolone (CMI). The electrophiles are less reactive than the oxidants and they are widely used as cosmetic preservatives. The details of their mechanisms will be discussed later in this chapter.

Membrane-active biocides, as their name implies, attack the integrity or function of the microbial cell membranes. Mechanistically, the membrane-active biocides can be divided into three major groups: cationic biocides, solvents, and protonophores. Cationic biocides include molecules such as biguanides and quaternary ammonium compounds and their cationic nature is crucial to their mechanism. The initial event in their antimicrobial mechanism is the interaction of the biocide-borne cations and the anionic surfaces of microbial cells, followed by the subsequent insertion of the biocide into the membrane — when sufficient numbers of molecules are dissolved in a membrane, it cannot maintain proper structure and the cells lyse.

The widespread use of anionic surfactants that inactivates the cationic biocides precludes the use of this class of biocides as cosmetic preservatives and their mechanisms will not be further discussed. Solvents such as ethanol and phenoxyethanol can be used as cosmetic preservatives when present at high levels (>1%). Their presumed mechanism is the solvation of hydrophobic molecules from the cell membrane, leading to a loss of membrane integrity and lysis. Formulation component compatibility problems limit the use of this class of preservative, although they certainly have their uses.

The last class of membrane-active biocides consists mainly of weak acids such as the parabens and citric and sorbic acids. These compounds have been in use as food preservatives for many years (or centuries, one might argue) and their inherent low toxicity make them attractive candidates for preserving cosmetics.

A great deal of work supports the idea that these compounds exert an antimicrobial action via their ability to alter intracellular pH and interfere with the pH gradient across the cell membranes, qualifying them for description as protonophores. However, unlike protonophores such as valinomycin or lasalocid that have minimal inhibitory concentration (MIC) values in the micromolar range, the weak acids are quite inefficient and require concentrations up to several tenths of a percent to inhibit microbial growth. The poor solubility of these compounds often makes formulating them into a product matrix difficult; they are nonetheless quite commonly used.

Thus there are three classes of biocides whose chemistry and antimicrobial mechanisms are appropriate for use as cosmetic preservatives: electrophiles, solvents, and weak acids. Of the three, the electrophiles are the most versatile and widely deployed, and the remainder of this chapter will be devoted to a detailed examination of their mechanisms of action and the strategies bacteria use to avoid their antimicrobial effects.

The successful understanding of biocide mechanisms of action requires the ability to live with compromise and an appreciation of multiple perspectives. In some respects, those who study antibiotic mechanisms of action have it far simpler — the molecules they study are restrained by their very complexity to interact with a single (or at most two or three) targets, and the environment in which they are expected to perform is relatively uniform and easily (and predictively) modeled in the laboratory.

In contrast, the seemingly simple molecules employed as preservatives interact with a diversity of molecules within the cell and thus exhibit multiple biological effects. They are required to perform in a wide variety of matrices where simple laboratory tests such as MIC or killing experiments are not predictive and where the antimicrobial interactions of the matrix components and the preservative are largely unknown. Thus, associating a single mechanism of action with a particular preservative is often a difficult task and while much progress has been made, plenty of room for deeper understanding remains.

## *Preservative targets*

Electrophilic preservatives do not possess the exquisite target site specificity associated with antibiotics because they are too reactive and have insufficient steric character to allow them to interact with target sites in the "lock-and-key" manner of specific inhibitors. Instead, their targets are the reactive nucleophilic moieties found on biological molecules, such as thiol groups on proteins, enzyme cofactors, and small peptides.

Traditional dogma has long held that the targets of biocides such as FA include the amine groups on proteins, but in terms of physiological relevance and chemical considerations thiols are probably the major relevant targets. Within the physiological range of pH, amines are not considered good nucleophiles and not until they have been protonated do they become reactive.

Thiols are sufficiently nucleophilic at physiological pH that they react readily with electrophilic compounds. In addition, while amines are abundant within proteins and contribute to their secondary and tertiary structures, thiols are often directly involved in the catalytic mechanisms of enzymes, and it is enzyme inhibition that triggers the cascade leading to cell death both with antibiotics and biocides. Thus while some reaction of electrophilic biocides with amines may occur, the targets of greater physiological significance are likely to be thiols.

## *Target access*

The routes by which the electrophilic preservatives arrive at their targets are very poorly understood. Considering their generally small size, uncharged nature, and reasonable hydrophobic–hydrophilic balance, it has long been assumed that these molecules can traverse the Gram-negative outer membrane by porin-mediated diffusion and then simply diffuse through the cell membranes to access the cell interiors.

Precious few studies support or dispute that assumption. Porin-deficient bacteria have been shown to exhibit reduced susceptibility to parabens[1] and isothiazolone-resistant bacteria isolated from consumer products were shown to lack porins.[2] These observations support the role of porins in preservatives gaining access to the periplasm, at least. Once within the periplasm, several possibilities exist. The molecules may diffuse through the cytoplasmic membrane, either in a passive manner driven by mass action or in a facilitated manner in which the cell has some role in aiding the diffusion of the preservative through the membrane. The preservatives may be actively transported by the cell using an energy-requiring process with specific or general transport mechanisms. Finally (and possibly heretically), the possibility exists that the preservatives do not have to enter cells to exert their antimicrobial action. The periplasm may contain targets whose inhibition may be sufficient to inhibit cell growth, or for which the downstream effects of reacting with the preservative result in growth inhibition and cell death.

Two studies examining the association of chloromethylisothiazolone (CMI) have been performed with several different species. It is worthwhile to note the complexities of interpreting transport studies using reactive molecules. It is very difficult to distinguish binding to the outer surfaces of the cell from transport into the cell and subsequent binding, and it is thus more accurate to refer to these experiments as studying "association" and not "transport." Collier et al.[3] administered radio-labeled CMI to *Escherichia coli* and the *Schizosaccharomyces pombe* fission yeast. Although the experimental format made a kinetic interpretation of the data difficult, it is clear that the labeled biocide associated with the cell in a time- and concentration-dependent manner. Further, the authors separated the proteins from labeled cells by gel electrophoresis and performed autoradiography on the resultant gels. Their conclusion was that the proteins of the cells in both species were more or less uniformly labeled, illustrating that almost every protein in a cell can

be considered a target for isothiazolones and strongly suggesting that the preservative does cross the cytoplasmic membrane.

Similar studies performed with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*[4] using a format more akin to traditional transport studies were able to kinetically distinguish two association processes, one active at low concentrations of CMI and the other at higher levels. They were also able to show the process active at low concentrations requires an energized cell membrane. Although this suggests an active transport process, that conclusion requires further verification. The result is, nevertheless, intriguing. To this author's knowledge, no studies have addressed to the mechanisms by which FA or FA-releasing biocides associate with or enter cells.

## *Reactions with thiols*

The reaction of FA with thiols is relatively straightforward. The situation is complicated slightly by the need for the parent FA-releasing structure to hydrolyze and release free FA. The rate and extent of FA release from a parent molecule are influenced by the structure of the biocide and matrix factors such as temperature, pH, salt, and myriad other ingredients used in industry. A good discussion of FA release from parent biocide structures can be found in References 5 through 7. It is not known whether the significant or effective release of FA occurs in the cell or in the product matrix, although free FA can be measured in preservative formulations.

Once the FA is released and is at the target site, it participates in a simple electrophilic attack on the nucleophilic thiol (R-SH) (Figure 8.1). The double bond opens, the thiol is added, and the oxygen is reduced. If the thiol involved is a critical one at an enzyme active site, the enzyme is inhibited.

The reaction of CMI with thiols is more complex (Figure 8.2). CMI can undergo a series of reactions with reduced thiols such that one mole of CMI can theoretically consume multiple moles of reduced thiol, while FA is restricted to a simple reaction mechanism (Figure 8.1). The first reaction is a ring opening with the ring cleaving between the sulfur and the nitrogen. This ring-opened species (II) can undergo one of two fates: the chlorine can be displaced by another reduced thiol to form structure III or can undergo a disulfide exchange that generates structure IV. Structure IV can undergo a rearrangement to generate a thioacyl chloride — an extremely reactive electrophilic species that can readily combine with thiols, amines, and even hydroxyls. Reaction with a thiol generates V, which can also be formed by another disulfide exchange with III.

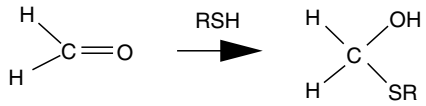


***Figure 8.1*** Reaction of formaldehyde with a thiol.

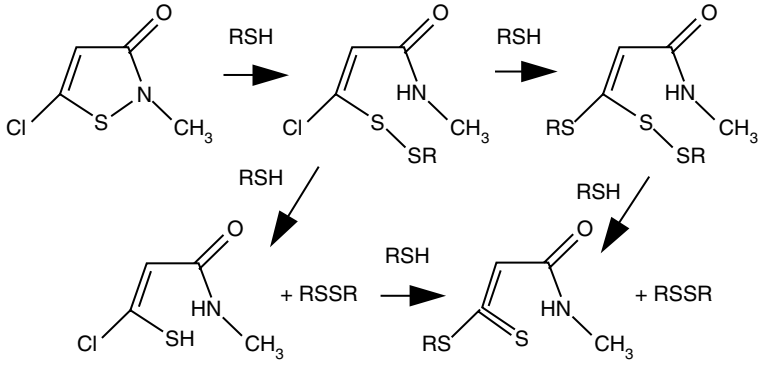


***Figure 8.2*** Reactions of chloromethylisothiazolinone with thiols.

Methylisothiazolone (MI), used as a co-biocide with CMI and recently introduced as a stand-alone preservative, lacks the strong electron-withdrawing chlorine atom at the 5 position. This restricts its reaction with thiols to the initial ring-opening reaction and subsequent disulfide exchange. This difference may be reflected in the relative potencies of CMI and MI; CMI is a far more potent biocide.

## *Consequences of thiol binding: growth inhibition and death*

Merely reacting with thiols is unlikely to directly result in growth inhibition. Small molecule thiols like glutathione, cysteine, lipoic acid, and many others are abundant within the cytoplasm and their reactions with electrophiles are unlikely to lead directly to enzyme inhibition. These molecules serve to protect the critical thiols from reactions with electrophiles and, indeed, cells lacking glutathione are hypersusceptible to electrophilic biocides.[8] Critical thiols are located within an enzyme active site and participate in the enzyme's function; if they are sterically accessible to an electrophilic biocide, their oxidation results in enzyme inhibition. Growth inhibition results when sufficient numbers of essential enzymes are inhibited to significantly reduce the flux of substrate through a pathway.

Growth inhibition alone is not sufficient to cause cell death. Bacteria can remain viable in a growth-inhibited or dormant state for months or even years. In order for a cell to die, the rate of damage infliction must outpace and eventually overwhelm the rate of repair. Bacteria exposed to subinhibitory doses of a biocide have time to repair the damage and even induce additional repair systems to cope with the damage. However, when high levels of biocide are administered, the cell cannot keep up repairs or induce protective systems and eventually the accumulation of damage exceeds the capability of the cell to repair itself and the cell dies.

Thus, *E. coli* organisms exposed to subinhibitory levels of CMI undergo a transient state of growth inhibition before resuming growth.[9] Bacteria exposed to growth inhibitory levels of CMI are inhibited within seconds but

display a delayed onset of lethality, while cells exposed to high levels are rapidly killed. Cells in a growth-inhibited state can be rescued and death can be prevented by the administration of reduced thiols during the period between growth inhibition and the onset of death. The ability to prevent death in an inhibited population implies the existence of a mechanism that causes cell death after the initial events resulting in growth inhibition. Evidence exists that this lethal mechanism is the generation of toxic radicals.

*Escherichia coli* has evolved two overlapping regulons controlled by *oxyR* and *soxRS* whose function is to detoxify and defend against intracellular radicals.[10,11] These regulons control the expression of enzymes such as superoxide dismutase and catalase. The ancient electrophilic biocides, mercury[12] and copper,[13] are efficient inducers of the radical defense mechanisms, and mutants deficient in superoxide dismutase are hypersusceptible to killing by copper.

Expression of the *oxyR* regulons in *E. coli* substantially reduces killing by FA, other aldehyde biocides,[14] and CMI.[9] Chang et al., in a poster presentation,[15] reported that biocide-specific radicals are found in *E. coli* when treated with a set of electrophilic biocides including FA and FA-releasing biocides, CMI, and MI. They also reported that induction of the *oxyR* regulon reduced killing by nine of ten electrophilic biocides used in the study. Further studies revealed that induction of the *oxyR* regulon reduces the level of radicals observed in CMI-treated cells (J.S. Chapman, unpublished observation).

The mechanisms that generate these radicals have not yet been determined. Several potential routes are the widespread disruption of metabolism resulting in reductant imbalances, inhibition of mechanisms that detoxify radicals, aberrant functioning of biocide-modified enzymes, and (for some biocides) the homolytic scission of disulfides. Other mechanisms are certainly possible, although classic redox cycling seems unlikely due to the structure and reactivity of the biocides.

## *Preservative resistance*

Microbes have limited but effective repertoires of resistance strategies developed over the last billion years or so. They can deploy resistance mechanisms effective at protecting individual cells or they can utilize the strategy of the biofilm growth mode to protect the community. There are three major cell-based resistance strategies: alteration of the target site so that it no longer interacts with the inhibitor, reduction of inhibitor concentrations at the active site, and enzymatic or covalent inactivation of the inhibitor. Combinations of these mechanisms, including biofilm growth, are very effective.

The relatively non-specific nature of preservative–target interactions (compared to antibiotics) makes the use of target site alteration as a resistance strategy very difficult. In the case of antibiotics that attack only one or two targets, the cells can often alter the target that is generally an enzyme's active site. The target site must be altered such that it cannot interact with the inhibitor and the enzyme maintains its function. It is difficult to conceive of

a mechanism whereby the cell can alter all its critical thiols and maintain viability. With the possible exception of triclosan there have been no reports of cells attaining resistance to cosmetic or consumer product preservatives by target site alteration.

A reduction in the concentration of inhibitor at the target site can be achieved by excluding the inhibitor from interior cell compartments or by actively removing the inhibitor from the cell interior via efflux pumps. Efflux pumps are multicomponent transmembrane assemblages that bind and remove small antimicrobial molecules from the cytoplasm.[16] They may utilize a small related set of substrates such as tetracyclines or they may have a broader substrate specificity such as lipophilic cations. Efflux pumps have been implicated in resistance to quaternary ammonium biocides,[17–19] but no convincing evidence as yet indicates that this resistance strategy is employed against cosmetic preservatives.

Excluding preservatives from the cytoplasm can be done at the outer membranes of Gram-negative bacteria by the loss of porins or at the cytoplasmic membranes of both Gram-positive and Gram-negative bacteria by eliminating specific transport proteins or otherwise altering membrane structures. In a study using bacteria isolated from isothiazolone-preserved consumer products, a diverse range of Gram-negative bacteria with reduced susceptibility were shown to be uniformly lacking porins.[20] These bacteria displayed reduced susceptibility to CMI as well as cross-resistance to FA-releasing biocides, indicating these compounds share a common route across the outer membranes. Susceptibility could be restored by treating the cells with EDTA, which disrupts the integrity of the outer membrane.

Previous studies[1] using a related series of parabens with a defined set of porin-deficient mutants of *E. coli* revealed that these mutants had reduced susceptibility to parabens, indicating these weak acids also traverse the outer membranes via porins. These studies indicate that reduction in permeability to preservatives via porin loss can be a successful strategy for the bacteria.

The extent to which bacteria can reduce their susceptibility by altering the composition of their cytoplasmic membranes is largely unknown but not surprising given the paucity of knowledge about the mechanisms by which preservatives traverse the cytoplasmic membrane.

Resistance to FA and FA-releasing preservatives is well documented, occuring in the metal-working, cosmetics and consumer products industries, and in laboratories.[5,6,21,22] FA is a by-product of carbohydrate metabolism and bacteria have evolved an enzyme, formaldehyde dehydrogenase, that utilizes FA as a substrate. Overexpression of this enzyme results in enzymatic inactivation of FA and resistance in a variety of bacteria.

The mechanistic commonality of FA-releasing preservatives results in widespread cross-resistance to them as a class when bacteria over-express FA dehydrogenase.[5,6] Similar results to those obtained by Sondossi were demonstrated in bacteria isolated from cosmetics and consumer products treated with FA-releasing preservatives (J.S. Chapman and M.A. Diehl, unpublished observations). The bacteria characterized in that study retained

their sensitivity to isothiazolones and other mechanistically unrelated biocides and had normal outer membrane protein profiles.

## Summary

The mechanisms by which cosmetic preservatives inhibit and kill bacteria are in many respects very different from those associated with antibiotics. However, at a very basic level, they have similar characteristics. They must interact with cellular components to inhibit essential functions, gain access to their targets, and exert their effects at low enough concentrations so as not to interfere with the matrix in which they are required to perform.

Both cosmetic preservatives and antibiotics are susceptible to resistance development, and the strategies bacteria employ to thwart their antimicrobial activities are similar. They are also sadly similar in yet another respect — the discovery and development of new preservatives and antibiotics have been largely neglected. Whether the strategy of relying on a static repertoire of antimicrobial compounds will be successful in the long run is an experiment whose outcome we await — nervously.

## References

1. Russell, A.D. and Furr, J.R., Susceptibility of porin- and lipopolysaccharide-deficient mutants of *Escherichia coli* to a homologous series of esters of p-hydroxybenzoic acid, *Int. J. Pharmaceut.*, 27, 163, 1985.
2. Chapman, J.S., Characterizing bacterial resistance to preservatives and disinfectants, *Int. Biodeter. Biodegrad.*, 41, 241, 1998.
3. Collier, P.J., Austin, P., and Gilbert, P., Uptake and distribution of some isothiazolone biocides into *Escherichia coli* ATCC 8739 and *Schizosaccharomyces pombe, Int. J. Pharmaceut.*, 66, 20, 1990.
4. Diehl, M.A. and Chapman, J.S., Association of the biocide 5-chloro-2-methyl-isothiazol-3-one with *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, *Int. Biodeterior. Biodegrad.*, 44, 191, 1999.
5. Sondossi, M., Rossmore, H.W., and Wireman, J.W., Induction and selection of formaldehyde-based resistance in *Pseudomonas aeruginosa, J. Indust. Microbiol.*, 1, 97, 1986.
6. Sondossi, M., Rossmore, H.W., and Wireman, J.W., The effect of fifteen biocides on formaldehyde-resistant strains of *Pseudomonas aeruginosa, J. Indust. Microbiol.*, 1, 87, 1986.
7. Rossmore, H.W. and Sondossi, M., Applications and mode of action of formaldehyde condensate biocides, *Adv. Appl. Microbiol.*, 33, 223, 1988.
8. Chapman, J.S. and Diehl, M.A., Biocide susceptibility and intracellular glutathione in *Escherichia coli, J. Indust. Microbiol.*, 12, 403, 1993.
9. Chapman, J.S. and Diehl, M.A., Methylchlorisothiazolone-induced growth inhibition and lethality in *Escherichia coli, J. Appl. Bacteriol.*, 78, 134, 1995.
10. Demple, B., Redox signaling and gene control in the *Escherichia coli soxRS* oxidative stress regulon: a review, *Gene*, 179, 53, 1996.
11. Storz, G. and Imlay, J.A., Oxidative stress, *Curr. Opin. Microbiol.*, 2, 188, 1999.

12. Fuentes, A.M. and Amabile-Cuevas, C.F., Mercury induces multiple antibiotic resistance in *Escherichia coli* through activation of *soxR*, a redox-sensing regulatory protein, *FEMS Microbiol. Lett*., 154, 385, 1997.
13. Kimura, T. and Nishioka, H., Intracellular generation of superoxide by copper sulfate in *Escherichia coli, Mutat. Res*., 389, 237, 1997.
14. Nunoshiba, T., Hashimoto, M., and Nishioka, H., Cross-adaptive response in *Escherichia coli* caused by pretreatment with $H_2O_2$ against formaldehyde and other aldehyde compounds, *Mutat. Res*., 255, 265, 1991.
15. Chang, H.R., Westmoreland, D.G., Fearnside, K.B., and Chapman, J.S., A role for free radicals in the mechanism of action of electrophilic biocides, in *Abstracts of the 95th Meeting of the American Society for Microbiology,* Washington, D.C., 1995.
16. Lewis, K., Hooper, D.C., and Oulette, M., Multidrug resistance pumps provide broad defense, *ASM News,* 63, 605, 1997.
17. Littlejohn, T.G., Paulsen, I.P., Gillespie, M.T., Tennent, J.M., Midgely, M., Jones, I.G., Purewal, A.S., and Skurray, A.W., Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus, FEMS Microbiol. Lett*., 95, 259, 1992.
18. Paulsen, I.T., Littlejohn, T.G., Radstrom, P., Sundstrom, L., Swedberg, G., and Skurray, R.A., The 3′ conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants, *Antimicrob. Agents Chemother*., 37, 761, 1993.
19. Heir, E., Sundheim, G., and Hollock, A.L., Resistance to quaternary ammonium compounds in *Staphylococcus* spp. isolated from the food industry and nucleotide sequence of the resistance plasmid pST827, *J. Appl. Bacteriol*., 79, 149, 1995.
20. Chapman, J.S., Characterizing bacterial resistance to preservatives and disinfectants, *Int. Biodeter. Biodegrad*., 41, 241, 1998.
21. Kuemmerle, N.F., Feucht, H.H., and Kaulfers, P.M., Plasmid-mediated formaldehyde resistance in *Escherichia coli*: characterization of resistance gene, *Antimicrob. Agents Chemother*., 40, 2276, 1996.
22. Chapman, J.S., Diehl, M.A., and Fearnside, K.B., Characterization of preservative-resistant bacteria, in *Abstracts of the 95th Meeting of the American Society for Microbiology,* Washington, D.C., 1996.

*part five*

# *Global regulatory and toxicologic aspects of cosmetic preservation*

*chapter nine*

# *Consumer safety considerations of cosmetic preservation*

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*Contents*

## *Introduction*

Preservatives are ingredients used to limit microbial growth and survival by exerting adverse effects upon various biological and cellular processes in contaminating microorganisms. While these effects are helpful for protecting a product from microbial contamination, it is important that developers consider consumer exposure to these materials to minimize safety and toxicity concerns. Therefore, consumer product safety and regulatory assessment can ultimately determine the type of preservative and its level of use in a cosmetic product.

Consumer product safety evaluations of cosmetic products are based on the premise that the product should be safe for consumers under normal and foreseeable use. This means a cosmetics microbiologist must protect the product from significant microbial contamination within the context of a preservative system that is safe for human exposure. Other chapters of this book deal extensively with preservative efficacy issues.

This chapter will provide a cosmetic microbiologist with a background in toxicology intended to be helpful for the development of cosmetic preservatives. This information will not, however, prepare the microbiologist to develop his or her own independent preservative safety testing program. Instead, it will provide a basic understanding that will be useful when interacting with toxicologists responsible for preservative safety evaluations. Information contained in this chapter will also heighten awareness of preservative safety as a major element for consumer products today. This awareness will help decrease the time lost and expense associated with multiple development cycles to replace otherwise effective preservatives due to safety concerns.

## *Use of existing information*

Faced with the task of developing and qualifying a preservative system for a new product, a microbiologist typically first considers preservatives that are marketed specifically for cosmetic products. He or she will often contact the preservative manufacturer in an effort to obtain information about the efficacy of the preservative in question.

When obtaining efficacy information, the microbiologist should also specifically request data on toxicological testing that the manufacturer may have performed or have access to with regard to the preservative. Consid-

erable toxicological data may be immediately available at no cost. Unnecessary or duplicate preclinical toxicity testing may be avoided by reviewing the data available from the manufacturer. While the types of safety test data vary widely from manufacturer to manufacturer, at a minimum, the information should include acute toxicity screening, eye irritation, primary skin irritation, skin sensitization, and basic mutagenicity testing data.

More extensive safety test data are frequently available from manufacturers of well-established, high sales volume preservatives. When secured early in the development cycle, these data can help a qualified toxicologist determine whether significant safety issues may exist for the preservative and the specific usage requested.

A microbiologist can save considerable time and expense by not performing extensive microbiological evaluations with a preservative that may not be toxicologically suitable for the product application under consideration. Likewise, if a safety profile provided by a manufacturer is sufficiently robust and indicates no apparent toxicological problems, a microbiologist can commence efficacy testing with a higher degree of confidence that human safety toxicological issues will not preclude the use of the preservative.

## *Trade associations*

Other sources of information for various preservatives and their usage in cosmetics are the various trade associations; for example, the Cosmetic Toiletry and Fragrance Association (CTFA) in the United States issues safety reports known as Cosmetic Ingredient Reviews (CIRs) based on work of independent scientific panels. COLIPA (Cosmetic Toiletry and Perfumery Association) is a similar trade association in Europe.

A CIR report covers a specific ingredient or class of ingredients and includes the relative formulation levels for the ingredient's use across marketed cosmetic products along with a compilation of toxicity and safety data for the material of interest. The CIR report then formulates a conclusion as to whether (1) the current safety data support the usage of the material at its current level across cosmetic products, (2) more safety data are needed to support its current use, or (3) an ingredient is unsafe at its current use levels. While the conclusion of a CIR report is a recommendation and is not binding in a regulatory sense, the CIR serves as another good source of information on a preservative's current use frequency and formulation level across various cosmetic products.

## *Regulatory considerations*

The use of preservatives as chemicals and as ingredients in finished products is subject to stringent regulatory oversight in different regions. As ingredients, preservatives must comply with general chemical legislation in countries where they are marketed. One example of relevant legislation and regulatory body involvement in the regulation of cosmetic ingredients is the Dangerous

Substances Directive (67/548/EEC, 7th Amendment: Dir 92/32/EEC) of the European Union. Areas of regulatory focus involve classification and labeling of chemicals through an understanding of their physicochemical properties and identification of their hazards to human health and the environment.

Regulations can involve specified physicochemical and toxicity tests for certain ingredients, with implications for safety evaluations of finished products. Examples of relevant legislation and regulatory bodies that regulate cosmetic products include the Cosmetics Directive of the European Union (76/768/EEC), the Food and Drug Administration (FDA) in the United States, and the Ministry of Health, Labour, and Welfare (MHLW) in Japan.

Some of the most specific legislation related to cosmetic preservatives is defined in the European Union's Cosmetics Directive (76/768/EEC) stating that cosmetics should be free of harmful levels of microorganisms. The directive also contains in Annex VI a list of all preservatives that may be used in cosmetic products sold in the EU along with any restrictions of use such as level in product, product form (aerosol versus cream), and product type (leave-on product versus rinse-off product).

Entry into Annex VI of the directive is dependent upon a safety review (subsequently published on the agency's Web site) by the Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP), a body that advises the Scientific Committee for Consumer Products (SCCP) in accordance with their published guidelines for the safety evaluation of cosmetic ingredients and cosmetic products.[1] In essence, the regulatory requirements for a new preservative are very much dependent upon what is already known about the toxicity of the preservative, its regulatory status as a chemical, the type of product involved, and the region of sale for the preserved product.

## *External and technical considerations*

A second consideration is the external relations climate with regard to the use of a particular preservative in a cosmetic product. The rapid spread of information over the Internet and other media has furthered education about preservative safety but also in certain circumstances perpetuated misinformation about safety to the general public. Both peer-reviewed scientific journal articles and rumors generated by certain special interest groups are available to consumers today, often without a larger context or framework that would allow the general public to assess the information. Because preservatives are active against microorganisms, it is predictable that they will also have the potential to produce effects in humans as revealed by preclinical toxicity testing.

For cosmetic products, the human health effect that receives the most external attention is contact allergy. Several well-known cosmetic preservatives have been identified as having the ability to induce contact allergy and/or elicit allergic contact dermatitis (ACD) under certain exposure conditions. For many of the preservatives known to be contact allergens, use of

appropriate risk assessment approaches can assure that they are used safely and effectively in formulations. Nevertheless, the literature contains well-documented information about situations in which the use of preservatives in cosmetic products caused concern about contact allergy.

Such examples include methylchloroisothiazolinone/methylisothiazolinone (MCI/MI; one trade name is Kathon CG®) introduced in the early 1980s, and methyldibromoglutaronitrile (MDBGN; one trade name is Tektamer®) used in leave-on cosmetic products in the late 1990s.[2] Both cases involved inappropriately high exposures to these preservatives, resulting in a high contact allergy incidence in Europe.

Great care has been taken to demonstrate to the external community that these preservatives can be safely used in cosmetic products at certain lower levels.[3,4] For preservatives to be considered both safe and efficacious, strong collaboration involving the cosmetics industry, external scientific groups, and the dermatology community is essential. Strengthening this collaboration will help in the development and the continued safe and effective use of preservatives.

In the early 21st century, the paraben family of preservatives came under special external scrutiny because of concern over their potential estrogenicity or endocrine disruption capabilities and possible links of paraben-containing underarm cosmetics to breast cancer. While this issue attracted a great deal of public attention, no objective evidence has demonstrated any significant risk from current cosmetic usage of these preservatives.[5] The SCC published an extended opinion specifically focused on the breast cancer question and stated that, "in light of the present knowledge, there is no evidence of demonstrable risk for the development of breast cancer caused by the use of paraben-containing underarm cosmetics."[6]

Consumers have shown a growing trend and desire for products that are natural or naturally derived. This trend provoked a strong push by the cosmetics industry to identify natural ingredients that have antimicrobial efficacy and can potentially replace existing preservatives. The issue has received considerable media attention and will continue to develop in the future.

While most public relations issues do not often impact the actual safety or toxicological profile of a given preservative, it is important to be aware of these issues when selecting a preservative system for a cosmetic product. The geography or region where a cosmetic product is to be marketed may also play into any potential external relations issues related to preservative choices. A geographic region may also have unique regulations as cited above for certain preservatives and permissible levels in various cosmetic products.

## *Consumer safety considerations for preservatives*

When developing a new preservative system or choosing an existing preservative system for a cosmetic product, four primary areas related to consumer safety evaluation and risk assessment must be addressed:

1. Hazard identification: the potential toxic effects associated with a given material in preclinical and clinical evaluations
2. Dose–response assessment: understanding of the relationship between dose and incidence of effects
3. Exposure: actual consumer use of the product
4. Risk characterization: placing the known hazards of an agent in the context of human exposure

These concepts are developed further in the rest of this chapter. If safety test data are insufficient, it may be necessary to begin microbial efficacy testing of a preservative at the same time safety testing is conducted. This is acceptable if no human exposure during the efficacy testing is anticipated or if the available safety data support the requested human exposure. Explanations of typical safety tests conducted with cosmetic preservatives together with fundamental background in the principles of toxicology are outlined below.

## *Fundamentals of toxicology*

Toxicology is the study of deleterious effects of chemical, physical, or biological agents on living organisms. The degree to which these deleterious effects are manifested is dependent on a number of factors such as species or strain of the organism exposed, stage of development of the organism when tested, compound or physical effect to which the organism is exposed, duration of exposure, frequency of exposure, route of exposure, and site of exposure.

"The dose makes the poison" is a true statement made by Paracelsus, one of the founders of modern toxicology, in that every chemical has both a safe exposure level and a hazardous exposure level.[7] The central premise of toxicology is to define both the hazardous and safe exposure levels of a compound to the species of interest and to ensure that exposure from this chemical as a cosmetic ingredient is well within the range of safety.

The toxicity of a material such as a preservative can vary significantly from species to species. There are many reasons for this variance, but for the most part it is a product of an organism's physiology and anatomy, both of which tend to control the organism's actual exposure and response to a toxicant. For example, an animal with relatively thin, fragile skin is more likely to be subject to the effects of a topically applied toxicant than an animal with thicker, less penetrable skin. Similarly, if an otherwise emetic toxicant is orally administered, an animal incapable of complete regurgitation, for example, a rat, is more likely to display toxic effects than an animal such as a dog that can regurgitate more completely. An organism capable of rapid and complete metabolism or excretion of a toxicant is much less prone to exhibiting toxic effects than an organism incapable of effectively eliminating the toxicant from its system. For these reasons and based on expectations of the applicable regulatory community, it is important to judiciously select the species on which toxicological testing will be performed.

## Hazard identification

Toxicities of chemical and physical agents can vary over a wide range. For example, one of the most toxic compounds characterized is botulinum toxin. This material is produced by the *Clostridium botulinum* bacterium and has an $LD_{50}$ value (see explanation of $LD_{50}$ under "acute toxicity studies" heading below) of 10 ng/kg — extremely toxic. Sodium chloride, on the other hand, has an $LD_{50}$ of approximately 4 g/kg and is therefore substantially less toxic. The purpose of a hazard assessment is to determine whether exposure to the ingredient of concern can cause an increase in the incidence of a particular adverse health effect and determine whether the health effect is likely to occur in humans.[8]

## Dose–response assessment

Dose–response assessment is the process of quantitatively evaluating toxicity information for a particular ingredient. The process involves characterizing the relationship of the dose of the ingredient delivered in a toxicity study and the incidence of adverse health effects in the exposed population.[8] Dose–response assessment may involve extrapolation from high dosages in a given toxicity test to low doses to estimate the prevalence of a particular effect at very low exposures.

## Exposure considerations

The magnitude, duration, frequency, and route of exposure can all dramatically influence the toxicity exhibited by a compound. Major routes of exposure are topical or dermal (a particularly important exposure route for cosmetics), inhalation, and oral. Typically, the maximum toxic effect is observed in safety studies by the route (based on the chemistry of the toxicant and on the physiology and anatomy of the organism) that will gain the toxicant the quickest and most prolonged presence in the circulatory system.

For example, if the dermal route has the greatest potential for systemic exposure to a particular compound in humans, this agent would be more likely to cross the skin and become systemically available at a site where the skin is thin (e.g., the post-mandibular area) than it would at a site where the skin is thicker and less subject to absorption (e.g., the soles of the feet). It should be noted that the matrix in which the toxicant is applied can significantly affect toxicity by affecting absorption of the toxicant. Specifically, the chemical composition of the matrix, the amount of the matrix, and the concentration of the toxicant in the matrix also serve to influence dermal absorption.

These are also important considerations when assessing preservative safety in cosmetic products composed of complex formulations because these preservatives may enter the body via the dermal route. Duration of exposure can also influence toxic effects. Toxicologists group exposure in

***Table 9.1*** Durations of Exposures in Preclinical Studies

| Exposure Categories | Duration |
|---|---|
| Acute | < 24 hours |
| Subacute | 1 to 30 days |
| Subchronic | 30 to 90 days |
| Chronic | > 90 days |

preclinical tests into four exposure categories (Table 9.1) that are then used to predict and provide data to support the anticipated human exposures.

## *Risk characterization*

Risk characterization is the final step in the risk assessment process. In this step, the hazard identification and exposure assessments are summarized and integrated into quantitative and qualitative expressions of risk. Extrapolations of toxicity data from the model in which the compound was tested (typically an animal model) to humans are made. Within this process of extrapolation, uncertainty factors are commonly used.

Extrapolating from an animal model to a human typically requires a factor of 10 and extrapolation to sensitive populations within species requires an additional factor of 10.[9] Additional uncertainty factors may be required and are specific to each case. Risk characterization serves as a bridge between risk assessment and risk management and is therefore a key step in the ultimate decision-making process.[10,11]

## *Toxicity studies grouped by exposure duration*

### *Acute toxicity studies*

An acute toxicity study involves either a single administration of a test chemical or several administrations within a 24-h period. The animals are then observed for signs of toxicity for a defined period, often 14 days. Most acute toxicity studies are designed to determine the median lethal dose ($LD_{50}$) of a toxicant. The $LD_{50}$ has been defined as the median lethal dose of a material that will kill 50% of the animals. Acute studies may also indicate probable target organs and provide guidance on doses to be used in longer duration studies. Important considerations in determining acute toxicity are selection of animal species, route of administration, dosages, number of animals, and environmental factors. All these factors can affect the outcome of a study.

For preservatives and other chemicals that are applied to the body, the most common route of exposure is dermal. However, the most common route of exposure used for acute toxicity tests is oral because in most instances the systemic absorption from the oral route is significantly higher than absorption via the dermal route. Thus, the use of oral acute toxicity

data for a dermal exposure assessment would be a conservative estimate of exposure for most chemicals.

In acute toxicity testing, the mortality or frequency of other effects is plotted against the dose on a logarithmic scale that is sufficient for estimation of the $LD_{50}$. Guidelines for acute oral, dermal, and inhalation toxicity studies have been published by various national and international agencies. Previous classification systems have been simplified in recent years. Traditional acute toxicity studies are performed less frequently because refined methods have been developed to reduce animal usage. Due to inherent variability in acute toxicity testing caused by a number of factors (animal model, age, strain, weight, etc.), typically only $LD_{50}$ ranges are determined.[12]

Recently drafted guidance of the U.S. Occupational Safety and Health Administration (OSHA) classification system from the Hazard Communication Standard (29 CFR 1910.1200) cites classifications for acute toxicity testing. A chemical with an oral $LD_{50}$ below 50 mg/kg is considered highly toxic; one with an oral $LD_{50}$ between 50 and 500 mg/kg is classified as toxic. Additional acute toxicity classifications are provided both by OSHA and other federal agencies. For example, the Environmental Protection Agency (EPA) recommended acute toxicity testing of rats at oral dosages up to 2000 mg/kg or up to 5000 mg/kg, depending on product type and hazard labeling status.[13]

### *Subacute and subchronic toxicity studies*

Humans are more often exposed to chemical levels with much lower toxicities, but exposures occur over longer periods. Subacute and subchronic toxicity tests are conducted to assess such toxic effects, preferably with animals that can biotransform chemicals in a manner similar to biotransformation in humans. The rat is most often the species of choice, but regulatory considerations can be relevant in the model selection process.

Equal number of males and females are used for subacute and subchronic studies. Chemicals are usually administered by routes of exposure intended for human use or required by regulatory agencies. The preferred exposure is oral (incorporating the chemical into the diet of the animal). The dermal and inhalation routes may also be considered, depending upon the ingredient or type of product type in which it is to be used.

Because these studies aim to determine the no-observed-adverse-effect level (NOAEL), three doses are typically selected: (1) a dose high enough to induce toxic effects in the animals, (2) a medium dose, and (3) a low dose that does not cause any adverse effects. These doses are selected based on the information yielded by acute toxicity test results ($LD_{50}$ and the slope of the dose–response curve). Information about related chemicals and their biotransformation profiles should also be considered. Observations made during and immediately following testing include mortality, body weight, food consumption rate, various chemical parameters, hematology, and histology. These tests yield information about toxicity with respect to target

organs, types of effects, and reversibility of effects (if the study includes a recovery group) and help define dose–response relationships.

Subacute tests are usually conducted for short durations —14 to 28 days. Subchronic toxicity testing is of intermediate duration; a typical test lasts about 90 days and is preferred to subacute testing for understanding extended exposure to a given chemical. If little is known about the subchronic toxicity of a compound or product, an orally administered range-finding test may be conducted in rodents to determine the proper dosages for the subchronic testing.

#### *Chronic toxicity studies*

As the name implies, chronic toxicity testing is subchronic testing extended to a longer interval. The definition of *chronic* depends on the species, but is intended to cover at least a very significant portion of the lifespan of an animal — if not its entire lifetime. Test duration may vary from 180 days up to 2 years or more in animals, thus mimicking lifetime exposure in humans. The study considerations are similar to those for subchronic toxicity tests.

Chronic tests are usually conducted to determine the nature of the toxicity of a chemical and determine the NOAEL. Uncertainty factors are applied to the NOAEL when using the data for human risk assessment. These factors include but are not limited to (1) interspecies variation when extrapolating from animal studies, (2) variability or sensitivity differences among the human population, and (3) use of a subchronic study to assess chronic exposure. Chronic testing is especially useful for detecting carcinogens.

### *Toxicity studies grouped by toxic endpoint*

#### *Ocular irritation tests*

The eyes may be exposed to cosmetic products and their ingredients via use of products meant to be used around the eyes (e.g., mascaras and eye creams) or through accidental exposure to products that may enter the eyes in diluted form during normal use but are not intended to come into contact with the eyes in undiluted form (e.g., shampoos). The evaluation of eye irritation potential of a cosmetic product and its ingredients is essential to obtain reassurance that a product is safe for consumers to use through intended and foreseeable uses and also accidental exposures. The preservative used in a formulation can impact the irritation profile, depending upon the type of preservative, its use level, and the specific cosmetic of interest.

The best-known method of assessing ocular irritation is the Draize test that involves instilling the test compound into rabbit eyes and then grading the eyes for irritancy.[14] However, significant progress has been made in developing eye irritation testing that reduces animal stress and the number of animals used and major developments in the field of *in vitro* alternative modeling have appeared over the last decade.

Significant efforts to develop alternative methods continue and should yield methods that eventually will replace animals as models to evaluate eye irritation. Recent approaches that have reduced the number of animals such as tiered testing strategy of the Organization for Economic Cooperation and Development (OECD) are now included as part of OECD Guideline 405 for hazard identification and regulatory classification of new chemicals. However, *in vivo* testing is still required when the results of *in vitro* or *ex vivo* testing are negative.[15,16]

Many alternatives assays have been developed for the purpose of replacing the Draize test. Several are cultured tissue assays from different cell lines, for example, the tissue equivalent and/or various cell-based assays (including the neutral red, red blood cell lysis, and fluorescein leakage assays) that have been correlated to aspects of *in vivo* ocular irritation.[16] Other alternative assays involve target organ and *ex vivo* models, such as the bovine corneal opacity and permeability (BCOP) test, isolated rabbit eye (IRE) test, and chicken enucleated eye test (CEET). Such assays have proven useful for the evaluation of cosmetic product formulations. For example, certain assays are more predictive for surfactant-oriented products such as shampoos and others are more robust indicators of irritation for other types of cosmetics.[17–20] Unfortunately, none of the alternative methods as of 2005 has been deemed sufficient by regulatory authorities to completely replace the current animal test accepted by OECD.[21]

## *Introduction to skin*

Since most cosmetic products are directly applied to the skin, a brief overview of skin anatomy and function is presented. The skin is the largest organ of the body and serves as a barrier to protect the organism from external insult. As a result, the skin is exposed to a wide variety of chemicals, cosmetics, topical medications, and industrial pollutants. Topical exposure coupled with dermal penetration is the most common route of exposure. The skin is composed of the epidermis and the dermis, a structure that rests on the subcutaneous tissue. The two layers are separated by a basement membrane.

The living layer of the epidermis consists of a basal cell layer (stratum germinativum), which provides the outer layers with new cells. The epidermis also contains melanocytes that produce pigments and also contains cells such as macrophages and lymphocytes involved in the immune process. The epidermis provides a protective outer cover.

The dermis is mainly composed of collagen and elastin that form important matrices to support the skin. The dermis layer contains several types of cells; the most abundant are the fibroblasts that aid the biosynthesis of fibrous proteins. Subcutaneous tissue lies underneath the dermis. In addition, the skin contains sweat glands, hair follicles, and small blood vessels. These components are commonly known as skin "appendages."

A variety of toxic effects can result from dermal exposure to toxicants. Most effects involve the skin alone, but some affect the appendages such as

hair, sebaceous glands, and sweat glands. Absorption of chemicals (including preservative agents) by the skin is a primary factor contributing to both local and systemic toxicity effects. As a result, the absorptive characteristics of a chemical are often significant factors in determining whether it will produce dermal toxicity.

Many nonpolar, lipophilic compounds readily diffuse across the skin barrier.[22] Diffusion of hydrophilic polar compounds is highly influenced by the hydration state of the stratum corneum. Depending on environmental conditions, the stratum corneum can be hydrated with 10 to 70% water. As the stratum corneum becomes hydrated, hydrophilic compounds diffuse more easily across the barrier. Therefore, the conditions under which skin testing (e.g., closed patch, open patch, etc.) is conducted can significantly affect the outcome of the test.

The epidermal appendages (sweat glands, hair ducts, and sebaceous glands) also provide additional capabilities for diffusion of chemicals across the skin. This effect is probably secondary to transepidermal diffusion because these appendages comprise only a small fraction of the total surface area of the skin.

## *Skin responses to preservatives*

In general, two categories of skin responses can be expected from preservative agents: non-immunologically mediated irritant responses and immunologically mediated allergic responses. The mechanisms for these responses are different and they will be discussed separately.

## *Irritant responses*

Irritant responses caused by antimicrobial preservatives fall into one of two broad categories: (1) corrosion and (2) irritation. Corrosion is irritation resulting from the direct necrotic action of a chemical on skin with an irreversible disintegration of the skin tissue that often results in scarring. Chemical burns, such as those produced by strong acids or bases, are classic examples of corrosion. Irritation is typically divided into three categories: (1) acute irritation, (2) cumulative irritation, and (3) photoirritation.

Acute irritation occurs as a localized, reversible non-inflammatory response resulting from a single application of a toxicant.

Cumulative irritation results from repeated exposures to toxicants that, upon initial application, do not elicit acute irritation. The difference in development of acute versus cumulative irritation may be simply a function of exposing the skin to a certain concentration of a compound in a formulation.

Phototoxicity results from light-induced chemical changes in a compound that cause the compound to become an irritant after it is applied to the skin.

In practice, antimicrobial preservatives that elicit irritation responses act as either acute or cumulative irritants and a microbiologist should test for this type of irritancy with animal or human patch tests that involve placing

***Table 9.2*** International Contact Dermatitis Research Group Scoring Scale for Irritation Testing

| Description | Score |
|---|---|
| No reaction | 0 |
| Erythema | 1+ |
| Erythema and edema | 2+ |
| Marked erythema and edema | 3+ |

varying concentrations of the preservative under occlusive patches on the skin of an animal such as a rabbit or a human. Because of ethical concerns, humans should participate in irritation patch testing only when an evaluation is confirmatory in nature and not exploratory.

Most patch testing in humans is conducted on the arms or backs of volunteers. For acute irritancy testing, toxicologists often use a 2- to 4-day patch testing protocol.[23] The material to be tested is added to an occlusive patch and placed on the test subject's skin for 24 to 48 hours. At the end of the test period, the patch is removed and the skin is allowed to equilibrate for 30 to 60 minutes. The amounts of erythema and edema of the skin are then scored.

The International Contact Dermatitis Research Group Scoring Scale can be used to score the skin responses.[24] Table 9.2 summarizes this four-point scale grading. Toxicologists use the cumulative irritancy patch test to test chemicals for additive irritancy.[25] This test is a variation on the 2- to 4-day patch test outlined above except that testing typically continues for 14 to 21 days with repeated applications of the test material.[26] The interval for this type of test is a matter of choice and relevance to the cosmetic of interest.

## *Skin sensitization*

Skin sensitization is also known as delayed contact hypersensitivity, a type IV response according to the scheme of Combs and Gell.[27] The key factors characterizing type IV reactions are typically delayed, T lymphocyte- and macrophage-mediated reactions. This distinguishes type IV responses from the type I through type III categories of allergic responses in that the type I through type III reactions are primarily antibody-mediated.

Poison ivy-allergic contact dermatitis is a classic example of delayed type IV hypersensitivity.

One manner in which type IV sensitization can be distinguished from the irritation responses discussed above is that the poison ivy rash-like sensitization response will likely spread beyond the site of exposure of the chemical insult, while the irritation response (typically with less edema than sensitization) tends to be localized to the initial area of the insult and the skin response will typically persist for a longer period. In addition, induction on skin sensitization is a permanent change because memory cells within the immune system will remember the original exposure if it exceeds the threshold for

***Table 9.3*** Potential Contact Sensitizers Used for Preservative Applications in Cosmetics

| |
|---|
| Formaldehyde |
| Isothiazolinones |
| Organic mercurial compounds |
| Phenolics (e.g., hexachlorophene) |

induction and the individual may react to any future exposures to the allergen that are above the threshold for elicitation of allergic contact dermatitis.

From a simplistic standpoint, a type IV skin reaction has two stages: (1) induction in which the individual becomes sensitized to a chemical, and (2) elicitation in which the skin reacts to the chemical upon subsequent exposure. In addition to catechols from poison ivy, many other compounds are known to induce skin sensitization and they include several cosmetic preservatives that can induce hypersensitivity under testing and actual use conditions. Table 9.3 lists these sensitizers.[28,29]

The test of choice for toxicologists who want to understand the sensitization potentials of materials including preservatives is the local lymph node assay (LLNA).[30,31] The LLNA is especially useful for identifying contact allergens because it can determine whether a material is a contact allergen and also provide information about its potency. In addition, the LLNA involves less pain and distress than guinea pig methods and uses fewer animals. Other animal models for sensitization testing include the Buehler and guinea pig maximization tests (guinea pigs have the ability to produce a satisfactory type IV skin response).

After the contact allergy status of a material has been identified in a preclinical assessment, confirmatory human tests may be conducted to confirm the lack of sensitizing potential of the specific ingredient of interest or in the context of a finished product containing the preservative.

One typically uses animals for preliminary tests to make a gross judgment about sensitization potential of a novel chemical. If the compound proves acceptable during animal testing, the next step is sensitization patch testing with human volunteers, but only to confirm the absence of sensitization at the relevant concentration. It must be noted that such human tests are always confirmatory and not intended for hazard identification purposes. One example of such a confirmatory step is the human repeat insult patch test (HRIPT) that has two basic stages. The first is the induction period when patches containing the test material are applied typically for 24 hours to the upper arms or backs of 100 or more subjects 9 times over the course of 3 weeks.

The second stage is elicitation. This phase is conducted after a brief rest period of about 2 weeks after the final induction patch application. It consists of reapplying a challenge patch containing the same test material to the same site and/or an alternative skin site. After removal of the challenge patch, the test facility skin grader evaluates the responses of the subjects at several time

intervals (24, 48, 72, and 96 hrs) for signs of positive or negative sensitization responses. The skin reactions evaluated are erythema, edema, itching, and in some cases blistering. Defined criteria exist for the determination of sensitization responses based on the observed skin reactions.[32]

## *Use testing for sensitization*

Human patch testing, while confirmatory in nature, occasionally produces equivocal results. The technique employed to resolve equivocal results is use testing. This type of testing is conducted typically at exposure levels that more closely relate to the exposure that would be experienced by a consumer in normal use of the product rather than under the highly exaggerated exposure conditions of a patch test. This technique can be particularly useful in evaluating the elicitation potential of a preservative in a presensitized test population. An informed volunteer is given the product of interest and instructed to use it the same way he or she would typically use it in a personal (no-test) situation.

At the beginning of the test, panel members are told to report any erythema, edema, or unusual itching. Panelists are periodically examined during the test for signs of skin responses suggestive of skin sensitization. If the product containing the preservative elicits a response, it is likely that a substitute preservative with less sensitization potential will be needed. If no response is seen under actual test conditions with a large test population, then supporting evidence demonstrates that the preservative as used in the product is acceptable from the human safety viewpoint of contact allergy.

## *Post-marketing surveillance*

After a new cosmetic formulation has been launched on the market, post-marketing surveillance to ensure that no unexpected safety issues have arisen is very important. This surveillance is performed in a passive sense, through consumers who might contact the company that launched the product, but it also involves active collaborations with members of the dermatological community. Many dermatologists in Europe and elsewhere patch-test their patients with various preservatives as part of their standard allergy testing protocols.

This testing works to monitor sensitization levels to various preservatives in patients with skin reactions who present themselves at a dermatologist's clinic. A dermatologist then extrapolates these clinical data to the public at large and determine whether preservative allergy incidence rates are increasing.

Close collaboration between industry and the dermatological community has been instrumental in addressing increases in preservative allergy incidence in Europe, both with use of MCI/MI in the 1980s and MDBGN in the 1990s.[33] This cooperation has allowed safe usage of the MCI/MI preservative at certain lower levels by means of additional patch and use testing

described above. As a result, MCI/MI allergy incidence rates in Europe have stabilized and are consistently at low levels. MDBGN can still be used at low levels in rinse-off products.[33,34]

## Genetic and developmental testing

### Genetic toxicology tests

The assessment of mutagenic and genotoxic potentials of preservative compounds has special relevance to cosmetic preservatives. Mutagenicity or mutation is defined as a permanent change in the content or structure of the genetic material of an organism that may result in a heritable change in the characteristics of the organism.[35] These changes may involve many aspects of the genetic structure including a gene or gene segment, a block of genes, or whole chromosomes.

Genotoxicity is a broader term and includes harmful effects on genetic materials such as DNA strand breaks and DNA adducts that do not necessarily lead to mutagenicity.[35] Genotoxic effects can occur in both somatic and germ cells. Malformation, death, or a permanent heritable change in the resulting embryo can occur in germ cells, while somatic cell mutations could result in cancer since cancer often arises from a mutagenic occurrence. Thus, genotoxicity testing is important for evaluating the ability of a preservative to interact with genetic material and cause adverse effects that could produce cancer or heritable changes in offspring.

Mechanistically, one type of mutagenesis involves small changes in the DNA base pairs. The changes consist of addition or deletion of base pairs or base pair substitutions that can alter the reading frame. This anomaly is known as a frameshift mutation and specific changes are demonstrated in the tables below.

Base pair substitutions:

| Transversions | Transitions |
|---|---|
| GC to CG | AT to GC |
| GC to TA | GC to AT |
| AT to TA | |
| AT to CG | |

Base pair additions:

–GGGGGG– to –GGGXXGGG–
–CCCCCC– to –GGGYYGGG–

Although hundreds of genotoxicity assays exist, several standard *in vitro* and *in vivo* tests can detect chemically induced mutations. Many of these tests are indirect evaluations of mutational events. They detect phenotypic changes due to mutations versus the actual alterations of the DNA. Perhaps

the most common mutagenesis assay is the *in vitro* Ames test, also known as the salmonella test[36] that employs Dr. Bruce Ames' operon mutant strains of *Salmonella typhimurium*. The strains revert by addition/deletion mutations or base pair substitutions to a wild-type strain that does not require growth factors.

The degree of correlation with the Ames test in detecting mutagens is rather strong.[37] As a result of this high degree of correlation coupled with low cost, toxicologists often used the Ames test as a first screening test for chemical mutagens. However, certain types of carcinogens such as hormonal compounds, metals, and agents with non-mutagenic modes of action are not detected by these bacterial mutagenicity assays, and a battery of assays including measurements of other endpoints is typically required.[38]

Other standard tests for mutagenicity include measurements of structural chromosome aberrations (clastogenicity), and numerical chromosome aberrations (aneugenicity). Typically, *in vitro* genotoxicity studies are performed before *in vivo* studies due to increased cost, timing, and animal use considerations. Typical *in vivo* genotoxicity studies focus on somatic cells and include *in vivo* chromosomal aberration, micronucleus assay, and unscheduled DNA synthesis (UDS). *In vivo* genotoxicity tests in germ cells include the dominant lethal assay.[39] While most mutagenicity and genotoxicity tests have their respective vulnerabilities such as false positives, false negatives, and certain chemical classes for which a test may not be predictable, a battery of the aforementioned tests can produce a robust amount of information to reveal the potential of a compound to be a human carcinogen.

## *Regulatory guidance: mutagenicity and carcinogenicity testing of preservatives*

The SCCNFP (Scientific Committee on Cosmetic and Non-Food Products Intended for Consumers, the regulatory body with cosmetic oversight in the European Union) has published some of the most detailed guidance on mutagenicity and genotoxicity testing for cosmetic products.[40] The committee recommends hazard evaluation using tests for gene mutation (mutagenicity), chromosome breakage and/or rearrangements (clastogenicity), and numerical chromosome aberrations (aneugenicity). Since no single validated test is capable of addressing all these endpoints, the recommended *in vitro* battery consists of the Ames assay, *in vitro* mammalian mutation assay (which also detects clastogenicity), and the *in vitro* micronucleus test. If the *in vitro* genotoxicity tests reveal positive results, *in vivo* genotoxicity assays will be required. The regulatory aspects of cosmetic preservation are discussed in detail in Chapter 10 of this text.

### *Developmental toxicity testing*

Any agent that causes an adverse effect in a developing embryo or fetus is a developmental toxicant. Typical developmental toxicants are teratogens — agents responsible for birth defects. The number of known teratogens is extensive and includes biological entities (rubella virus), physical entities (x-rays), and a diversity of chemicals including pharmaceuticals like thalidomide and diethylstilbestrol.[41–45]

Many chemical agents cause teratogenic effects under very specific conditions. Biologically active molecules such as cosmetic preservatives are certainly subjects for teratology testing. Manson et al. provided a thorough review of teratogenic testing procedures. For the evaluation of potential short term exposures, a single generation animal test using rodents or rabbits is often used.[46] This type of testing is usually conducted in three parts:

1. Female animals are dosed before and during mating with continued dosing during pregnancy and lactation. They are then sacrificed during pregnancy and the unborn offspring are examined for abnormalities. Other animals are allowed to deliver and the delivered offspring are weaned and examined for abnormalities.
2. Pregnant females are dosed only during organogenesis and sacrificed just before delivery. The unborn offspring are examined for abnormalities.
3. Pregnant females are dosed during the final trimester of pregnancy and through the lactation period. Offspring may be examined for abnormalities immediately following weaning or be allowed to reach adulthood prior to examination. By modifying dosing, investigators may compensate for effects of exposure timing on the teratogenic potential of the test compound.

It is well known that exposure across very specific and often narrow windows can greatly affect teratogenicity of a compound. Furthermore, the timing of the exposure may influence the type of teratogenic effect observed. To evaluate the effects of longer term exposures to potential teratogenic agents, multiple generation studies proceeding through at least three generations are used. They are conducted much like single generation studies except that a portion of each generation is retained and mated to produce the next study generation. In this way, the effects of the test compound to provoke congenital abnormalities as well as affect fertility, and litter size, viability, and growth may be determined.

## *Conclusion*

Current toxicology utilizes the risk assessment paradigm described above to develop sound risk assessment decisions that allow safe use of cosmetic preservatives. One general principle is that hazard and exposure may be

inversely related. In theory, a low hazard–high exposure chemical may be safe, and a low exposure–high hazard chemical may be safe. Chemicals that present low hazards and low exposures are obviously safe, but those involving high exposures and high hazards would not be determined to be safe. Specific quantification of what constitutes high and low hazards and high and low exposures is highly variable and each case has specific parameters. A toxicologist should be requested to conduct an appropriate risk assessment and determine the safety of a preservative in its specific use context.

## References


1. The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation, 5th Revision. Adopted by the SCCNFP during the 25th plenary meeting of 20 October 2003. SCCNFP/0690/03 Final, 2003.
2. Wilkinson, J.D., Shaw, S., Anderson, K.E., Brandao, F.M., Bruynzeel, D.P., Bruze, M., Camarasa, J.M., Diepgen, T.L., Ducombs, G., Frosch, P.J., Goossens, A., Lachapelle, J.-M., Lahti, A., Menne, T., Seidenari, S., Tosti, A., and Wahlberg, J.E., Monitoring levels of preservative sensitivity in Europe: A 10-year overview. *Contact Dermatitis*, 46, 207, 2002.
3. Gray, J.E. and McNamee, P.M., *Scientific Review Series — Preservatives — Their Role in Cosmetic Products*. Munksgaard, Copenhagen, 2000.
4. The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) Opinion concerning Methydibromo Glutaronitrile, SCCNFP/0585/02, 2002.
5. CTFA Response Statement concerning Parabens, November 2003.
6. European Commission—The Scientific Committee on Consumer Products (SCCP) Extended Opinion on Parabens, underarm cosmetics, and breast cancer, SCCP/0874/05, 2005.
7. *Casarett and Doull's, Toxicology: The Basic Science of Poisons*, 6th ed., McGraw-Hill, New York, 2001.
8. Environmental Protection Agency, EPA Approach to Assessing the Risk Associated with Exposure to Environmental Carcinogens. Appendix B to the Integrated Risk Information System, 1989.
9. EPA, Health Effects Test Guidelines. Hazard Evaluation, *Code of Federal Regulations*, title 40, Part 792 and 798, 1989.
10. Environmental Protection Agency, Guidelines for carcinogen risk assessment, *Federal Register* 51, 33992, 1986.
11. Environmental Protection Agency, Guidelines for exposure assessment, *Federal Register* 51, 34042, 1986.
12. *Casarett and Doull's, Toxicology: The Basic Science of Poisons*. 6th ed., McGraw-Hill, New York, 2001.
13. Environmental Protection Agency, Health Effects Test Guidelines OPPTS 870.1100 Acute Oral Toxicity, 1996.
14. Draize, J. H. and Kelley, E. A., Toxicity to eye mucosa of certain cosmetic preparations containing surfactant active agents, *Proc. Sci. Sect. Toilet Goods Assoc*. 17, 1, 1952.

15. OECD, *OECD Guideline for the Testing of Chemicals, No. 405: Acute Eye Irritation/Corrosion*, 14 pp. OECD, Paris, 2002.
16. Daston, G.P. and McNamee, P., Alternatives to toxicity testing in animals: challenges and opportunities, *Essays on the Future of Environmental Health Research*, in press.
17. Gettings, S.D., Teal, J.J., Bagley, D.M., Demetrulias, J.L., DiPasquale, L.C., Hintze, K.L., Rozen, M.G., Weise, S.L., Chudkowski, M., Marenus, K.D., Pape, W.J.W., Roddy, M., Schnetzinger, R., Silber, P.M., Glaza, S.M. and Kurtz, P.J., The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test (Phase I) Hydro-alcoholic formulations; (Part 2) Data and biological significance. *In Vitro Toxicol*., 4, 247, 1991.
18. Gettings, S.D., DiPasquale, L.C., Bagley, D.M., Casterton, P.L., Chudkowski, M., Curren, R.D., Demetrulias, J.L., Feder, P.I., Galli, C.L., Gay, R., Glaza, S.M., Hintze, K.L., Janus, J., Kurtz, P.J., Lordo, R.A., Marenus, K.D., Moral, J., Muscatiello, M., Pape, W.J.W., Renskers, K.J., Roddy, M. and Rozen, M.G., The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test (Phase II) Oil/water emulsions. *Food Chem. Toxicol*. 32, 943, 1994.
19. Gettings, S.D., Lordo, R.A., Hintze, K.L., Bagley, D.M., Casterton, P.L., Chudkowski, M., Curren, R.D., Demetrulias, J.L., DiPasquale, L.C., Earl, L.K., Feder, P.I., Galli, C.L., Glaza, S.M., Gordon, V.C., Janus, J., Kurtz, P.J., Marenus, K.D., Moral, J., Pape, W.J.W., Renskers, K.J., Rheins, L.A., Roddy, M., Rozen, M.G., Tedeschi, J.P. & Zyracki, J., The CTFA evaluation of alternatives program: an evaluation of *in vitro* alternatives to the Draize primary eye irritation test (Phase III) Surfactant-based formulations. *Food Chem. Toxicol*., 34, 79, 1996.
20. SCCP Report for Establishing the Timetable for phasing out animal testing for the purpose of the Cosmetics Directive. URL: http://pharmacos.eudra.org/F3/cosmetic/AnimalTest.htm:
21. OECD (2002). *OECD Guideline for the Testing of Chemicals, No. 405: Acute Eye Irritation/Corrosion*, 14 pp. OECD, Paris.
22. Scheuplein, R.J. and Blank, I.H,. Permeability of the skin, *Physiol. Rev.* 51, 702, 1971.
23. Schwartz, L. and Peck, S.M., The patch test and contact dermatitis, *Public Health Report* 59, 2, 1944.
24. International Contact Dermatitis Research Group (1970). Terminology of Contact Dermatitis *Acta Dermatol*., 50, 287.
25. Lanman, B.M., Zelvers, W.V., and Howard, C.S., The role of human patch testing the product development program, in *Proc. Jt. Conf. Cosmet. Sci*., The Toilet Goods Association, Washington, D.C., 1968.
26. Berger, R.S. and Bowman, J.P., Early appraisal of the 21 day cumulative irritation test in man, *J. Toxicol. Cut. Ocular Toxicol*., 1, 109, 1982.
27. Combs, R.R.A. and Gell, P.G.H,. Classification of allergic reactions responsible for clinical hypersensitivity and disease, in *Clin. Aspects Immunol*., Gell, P.G.H., Coombs, R.R.A., and Lachman, P.J., Eds., Oxford Press, Blackwell, England, 1975.
28. Schorr, W.F. Cosmetic allergy. A comprehensive study of the many groups of chemical antimicrobial agents. *Arch. Dermatol*., 104, 459, 1971.
29. Bauer, R.L., Ramsey, D.L., and Bondi, E., The most common contact allergens, *Arch. Dermatol*. 108, 74, 1973.

30. Dean, J.H, Twerdok, L.E., Tice, R.R., Sailstad, D.M., Hattan, D.G., Stokes, W.S., ICCVAM Evaluation of the murine local lymph node assay: conclusions and recommendations of an independent scientific review panel, *Reg. Tox. Pharmacol*., 34, 258, 2001.
31. Haneke, K.E., Tice, R.R., Carson, B.L., Margolin, B.H., Stokes, W.S., ICCVAM Evaluation of the murine local lymph node assay: data analyses completed by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, *Reg. Toxicol. Pharmacol*., 34, 274, 2001.
32. Gerberick, G.F., Gilpin, D.A., McNamee, P.M., Critical factors in the conduct and interpretation of the Human Repeat Insult Patch Test. *Contact Dermatitis*. Submitted, 2006.
33. Wilkinson, J.D., Shaw S., Anderson K.E., Brandao, F.M., Bruynzeel, D.P., Bruze, M., Camarasa, J.M.G., Diepgen, G. Ducombs, Frosch, P.J., Goosens, A., Lachapelle J.-M., Lahti, A, Menne, T, Seidenari, S., Tosti, A., and Wahlberg, J.E., Monitoring levels of preservative sensitivity in Europe: A 10-year overview, *Contact Dermatitis*, 46, 207, 2002.
34. SCCP, The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP) Opinion concerning Methydibromo Glutaronitrile, SCCNFP/0585/02, 2002.
35. The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP). Recommended Mutagencity/Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC SCCNFP Notes of Guidance, 2004.
36. Ames, B.N., McCann, J., and Yamasaki, E., Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat. Res*. 31, 347, 1975.
37. Ames, B.N. and McCann, J,. Validation of the Salmonella test: a reply to Rinkus and Legator, *Cancer Res*. 41, 4192, 1981
38. *Casarett and Doull's, Toxicology: The Basic Science of Poisons*. 6th ed. McGraw-Hill, New York, 288, 2001.
39. United Kingdom Dept. Health Services Committee on Mutagenicity of Chemicals in Food, Consumer Products, and the Environment: Guidance on a strategy for testing of chemicals for mutagencity, December 2000.
40. The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP). Recommended Mutagencity/Genotoxicity Tests for the Safety Testing of Cosmetic Ingredients to be included in the Annexes to Council Directive 76/768/EEC (SCCNFP Notes of Guidance, SCCNFP/0755/03), 2004.
41. Alford, C.A., Rubella. in *Infectious Diseases of the Fetus and Newborn Infant*, Remington, J.S. and Klein, J.O., Eds., Saunders, Philadelphia, 1976.
42. Gregg, N.M., Congential cataract following German measles in the mother, *Trans. Ophthalmol*. Soc. NZ 3, 35, 1941.
43. Warkany, J. Development of experimental mammalian teratology, in *Teratology: Principles and Techniques*, Wilson, J.G. and Warkany, J., Eds, University of Chicago Press, Chicago, 1965.
44. McBride, W.G., Thalidomide and congenital anomalies, *Lancet*, 2, 1358, 1961.
45. Postkanzer, D. and Herbst, A., Epidemiology of vaginal adenosis and adenocarcinoma associated with exposure to stilbestrol in utero, *Cancer*, 39, 1892, 1977.

46. Manson, J.M., Zenick, H., and Costlow. R., Teratology test methods for laboratory animals, in *Principles and Methods of Toxicology*, Hayes, A. W., Ed., Raven Press, New York, 1982

*chapter ten*

# *Global regulation of preservatives and cosmetic preservatives*

***David Steinberg***

*Contents*

## Introduction

All countries require that cosmetics sold inside their borders be safe. Part of the safety issue for a cosmetic is freedom from microbial contamination when it is placed on the market and the ability to remain free from contamination under normal and foreseeable use by consumers. Preservatives are chemicals added to cosmetics to prevent the growth of and destroy microbes arising from contamination. Although the primary purpose of a preservative is to maintain a cosmetic in "clean" condition and keep it free from contamination during consumer use, the true purpose of adding preservatives is to eliminate accidental contamination during production.

Because preservatives are biologically active chemicals, they have the potential to injure users in addition to killing microbes. This potential for injury has resulted in their regulation by governments. Japan and the European Union have developed lists of permitted or positive preservatives and it is illegal in Japan or Europe to use a preservative that is not permitted or listed as approved by the respective governments. The U.S. Food and Drug Administration (FDA) does not have a similar positive list because the agency does not preapprove preservatives. Instead, the FDA utilizes a list of banned (or negative) preservatives. This chapter will review the regulations of these three major markets.

## United States regulations

### Regulation of preservatives

As noted above, the FDA does not approve preservatives for use in cosmetics or topical cosmetic drugs. However, the agency has prohibited the use of bithionol[1] and halogenated salicylanilide[2] preservatives and the use of hexachlorophene is restricted. The maximum allowed level of hexachlorophene is 0.1%, provided no other preservative has been shown to be effective. It may not be used in cosmetics that may be applied to mucous membranes. Mercury compounds[4] limited to 65 ppm as the free metal may be included in eye-area cosmetics only, provided no other effective and safe preservative is available.

The Cosmetic, Toiletry, and Fragrance Association (CTFA), with the support of the FDA and the Consumer Federation of America, established the Cosmetic Ingredient Review (CIR) system in 1976. Although funded by CTFA, CIR assessments are independent of CTFA and the cosmetics industry. The CIR thoroughly reviews and assesses the safety of ingredients used in cosmetics in an open, unbiased, and expert manner and the results are published in peer-reviewed scientific publications.[5] The CIR system has issued assessments on most preservatives used in cosmetics. A CIR opinion may conclude that an ingredient is (1) safe to use, (2) safe at maximum concentrations, (3) unsafe to use, or (4) data submitted are insufficient to allow a determination. Ingredients found unsafe through this review process are not used in the U.S. because no one would market or buy an unsafe product. Where review data are found to be insufficient, an ingredient may be required to carry a warning notice that "the safety of this product has not been determined."[6]

The CIR process determined that chloroacetamide is unsafe for use in cosmetics. The following preservatives presented problems and as of this writing have insufficient data status:

- Benzylparaben
- Sodium iodate
- Glutural
- 2-Bromo-2-nitropropane-1,3-diol and 5-bromo-5-nitro-1,3 dioxane when used where amines and nitrosamines could be formed
- Formaldehyde in aerosols

## *Regulation of biocides*

Chemicals used as preservatives in non-FDA-regulated industries must be preapproved for their respective applications by the U.S. Environmental Protection Agency (EPA) through authority granted by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). It is important to note that the majority of cosmetic preservatives are *not* EPA registered and their use is not permitted in non-FDA-regulated applications.

## *Regulation of antimicrobial ingredients*

Antimicrobial compounds can be active ingredients in drugs in the U.S. Drugs sold without prescriptions (known as over-the-counter or OTC drugs) are regulated under a monograph system published by the FDA. Monographs for the following compounds are in effect; permitted levels are listed where appropriate.

*Healthcare antiseptic drugs*

| Category I (Safe and Effective)[7] | Category III (Not Permitted) |
|---|---|
| Alcohol, 60 to 95% by volume | Benzalkonium chloride |
| Povidone–iodine, 5 to 10% | Chlorhexidine gluconate |
| Iodine tincture, USP | Chloroxylenol |
| Iodine topical solution, USP | Chloroxylenol |
| Isopropyl alcohol, 70 to 91.3% by volume | Hexylresorcinol |
| | Cloflucarban |
| | Hexylresorcinol |
| | Undecoylium chloride–iodine complex |
| | Mercufenol chloride |
| | Methylbenzethonium chloride |
| | Phenol |
| | Secondary amylcresols |
| | Sodium oxychlorosene |
| | Triclocarban |
| | Triclosan |

When the final monograph is issued for a Category III ingredient, the ingredient must be permitted or disallowed. Until that time, it may still be used. Three types of applications are currently permitted for healthcare antiseptic drugs: (1) patient preoperative skin preparations, (2) antiseptic handwashes or healthcare personnel handwashes, and (3) surgical hand scrubs. The CTFA and Soap & Detergent Association (SDA) are urging the FDA to allow additional applications and have proposed the following uses:

- Preoperative skin preparations
- Surgical scrubs
- Healthcare personnel hand wash
- Food handler handwash
- Antimicrobial handwash
- Antimicrobial bodywash

*First aid antiseptic drugs*

| Category I (Approved Active Ingredients)[8] | Category III (Active Ingredients) |
|---|---|
| Alcohol, 48 to 95% by volume | Benzyl alcohol |
| Chloroxylenol | Calomel |
| Benzalkonium chloride, 0.1 to 0.13% | Chlorobutanol |
| Benzethonium chloride, 0.1 to 0.2% | Merbromin |
| Camphorated metacresol[a] | Mercufenol chloride |
| Camphorated phenol[b] | Phenylmercuric nitrate |
| Eucalyptol[c] | Secondary amytricresols |
| Hexylresorcinol, 0.1% | Triclocarban |
| Hydrogen peroxide topical solution USP | Triclosan |
| Iodine tincture USP | |
| Iodine topical solution USP | |
| Isopropyl alcohol, 50 to 91.3% | |
| Methylbenzethonium chloride, 0.13 to 0.5% | |
| Phenol, 0.5 to 1.5% | |
| Povidone–iodine, 5 to 10% | |

[a] 3 to 10.8% camphor and 1 to 3.6% metacresol in a ratio of three parts camphor to one part metacresol.

[b] 10.8% camphor and 4.7% phenol in a light mineral oil USP vehicle.

[c] 0.091% eucalyptol in combination with 26.9% alcohol, 0.042% menthol, 0.055% methyl salicylate, and 0.063% thymol.

When the final monograph is issued for a Category III ingredient, the ingredient must be permitted or disallowed. Until that time, it may still be used in a first aid antiseptic application.

*Topical acne drugs*

| Permitted Active Drugs[9] |
|---|
| Resorcinol, 2%, with 3 to 8% sulfur |
| Resorcinol monoacetate, 3%, with 3 to 8% sulfur |
| Salicylic acid, 0.5 to 2% |
| Sulfur, 3 to 10% |

The above chemicals are permitted under a final monograph issued August 16, 1991. At that time, benzoyl peroxide was classified as a Category III compound at dosages of 2.5, 5, and 10% requiring special warnings. As of this writing, no date has been set for issuing a final ruling about its use.

*Antifungal drugs*

| Permitted Active Drugs[10] |
|---|
| Clioquinol, 3% |
| Haloprogin, 1% |
| Miconazole nitrate, 2% |
| Povidone–iodine, 10% |
| Tolnaftate, 1% |
| Undecylenic acid or its calcium, copper, or zinc salts, individually or in any ratio with a total concentration of 10 to 25% |
| Clotrimazone, 1% |

*Dandruff, seborrheic dermatitis, and psoriasis drugs*

| Permitted Active Drugs[11] |
|---|
| Coal tar, 0.5 to 5%[a] |
| Pyrithione zinc, 0.3 to 2% when formulated to be applied, then washed off after brief exposure |
| Pyrithione zinc, 0.1 to 0.25% when formulated to be applied and left on skin or scalp |
| Salicylic acid, 1.8 to 3% |
| Selenium sulfide, 1% |
| Selenium sulfide, micronized, 0.6% |
| Sulfur, 2 to 5% |

[a] When a coal tar solution, derivative, or fraction serves as the source of the coal tar, labeling shall specify the identity and concentration of the coal tar source and the concentration of coal tar present in the final product.

## *Regulation of cosmetic microbiology*

The U.S. Food, Drug, and Cosmetic Act prohibits the distribution of adulterated or misbranded cosmetics. Additionally, the FDA prohibits production of cosmetics under conditions that could cause contamination. Although not required, cosmetics should be manufactured in conformity with current good manufacturing practices (cGMPs). The *Cosmetic Handbook* issued by the FDA lists these guidelines.[12]

The FDA states that cosmetics and topical cosmetic drugs need not be sterile; however, they must not be contaminated with pathogenic microorganisms and the density of non-pathogenic organisms must be low. Finally, the FDA requires that:

1. Each batch of a cosmetic that is not self-preserving must be tested for microbial contamination before it is released for interstate shipment.
2. Each cosmetic, particularly each eye-area cosmetic, must be tested during product development for adequacy of preservation against

microbial contamination that may occur under reasonably foreseeable conditions of consumer use.

Because the FDA does not specify acceptable levels, the cosmetics industry generally follows CTFA guidelines with regard to microbial levels and the absence of pathogens:

1. For eye area and baby products, not more than 500 CFU/mL
2. For all other products, not more than 1000 CFU/mL

In reality, most companies set their own internal limits of less than 10 CFU/mL for all aqueous-based products and oil-in-water emulsions and set higher levels for atypical cosmetics.

## *European Union regulations*

Cosmetics in the European Union (EU) are regulated by Cosmetic Directive 76/768. The directive consists of fifteen articles and nine annexes. Article 4 requires that only preapproved preservatives listed on Annex VI may be used in cosmetics. Article 7a, Part 1(b) requires microbiological specifications for each ingredient used in finished cosmetic products.

### *Annex VI approved preservatives*

Annex VI is a list of preservatives that cosmetic products may contain. Each listing for a permitted preservative includes its COLIPA number, EU reference number, maximum permitted concentration, limitations, and finally, conditions of use and warnings that must be printed on the label in all languages.

Salts are defined as sodium, potassium, calcium, magnesium, ammonium, and ethanolamine cations. The anions are chlorides, bromides, sulfates, and acetates and the esters are methyl, ethyl, propyl, isopropyl, butyl, isobutyl, and phenyl. Finally, plus signs (+) follow the names of certain preservatives to indicate that the ingredient can be used at higher levels for non-preservation purposes. Since 2001, the EU has been systematically reviewing all preservatives with plus sign designations to determine the levels at which they can be safely used regardless of purpose.

### *Requirements for Annex VI submissions*

The Scientific Committee for Cosmetics and Non-Food Products (SCCNFP) examines the safety of new preservatives. The committee's requirements can be accessed at http://europa.eu.int/comm/health/ph_risk/committees/sccp/documents/out130_en.pdf. After a positive opinion is issued, a preservative is referred to the European Union Commission. If the commission concurs, a document known as an adaptation to technical progress (ATP) is issued and the new preservative is added to Annex VI. In 2004, SCCNFP was replaced with the Scientific Committee for Consumer Products (SCCP).

### *Prohibited preservatives*

Annex II of the EU cosmetic directive lists ingredients prohibited from use in cosmetics. The following preservatives are listed:

| Number | INCI* |
|---|---|
| 221 | Mercury compounds except those listed in Annex VI |
| 348 | Tetrachlorosalicylanilides |
| 349 | Dichlorosalicylanilides |
| 350 | Tetrabromosalicylanilides |
| 351 | Dibromosalicylanilides |
| 352 | Bithionol |
| 369 | Sodium pyrithione |
| 370 | Captan |
| 371 | Hexachlorophene |
| 373 | Tribromosalicylanilide |

* International Nomenclature Cosmetic Ingredient

Annex III lists ingredients and relevant restrictions. This list includes certain preservatives that can be used at higher levels than those permitted under Annex VI. As of this writing their status is under review.

| Number | Preservative | Use/Comments |
|---|---|---|
| 11 | Dichlorophen | Requires warning label at 0.5% |
| 13 | Formaldehyde | Nail hardeners up to 5% with warnings |
| 45 | Benzyl alcohol | Solvent; used in fragrances |
| 49 | Selenium disulfide | 1%, antidandruff shampoos; warnings |
| 54 | Phenoxyisopropanol | 2% |

### *Regulations concerning microbiology*

Article 7a1(b) of the European Cosmetic Directive contains two important regulations related to microbiology. The regulations require that microbial specifications for each raw material used and also for each finished cosmetic be included in product information packages.

## *Japan regulations*

### *Approved preservatives for cosmetics*

In 2001, Japan changed its cosmetic regulations to eliminate the need for preapproval of most cosmetic ingredients (see Table 10.1). The exceptions for which preapproval is still required are UV filters, colors, preservatives, and a few miscellaneous items. It is important to note the change covers cosmetics only and does not include quasi-drugs.

***Table 10.1*** Preservatives Approved for Use in Japan

| Preservative | Maximum concentrations for all cosmetics % | Rinse-off; no MM % | Leave-on; no MM % | Use on MM % |
|---|---|---|---|---|
| Benzoic acid and its salts | 0.2 as acid 1.0 as salts as total | | | |
| Salicylic acid and its salts | 0.2 as acid 1.0 as salts as total | | | |
| Sorbic acid and its salts | 0.5 as total | | | |
| o-Phenylphenol and its salts | | ∞ | 0.3 | 0.3 |
| Zinc pyrithione | | 0.1 | 0.01 | 0.01 |
| Chlorobutanol | 0.1 | | | |
| Para acid and its salts and esters (parabens) | 1.0 as total | | | |
| Dehydroacetic acid and its salts | 0.5 as total | | | |
| Triclocarban | | ∞ | 0.3 | 0.3 |
| Triclosan | 0.1 | | | |
| Chloroxylenol | | 0.3 | 0.2 | 0.2 |
| Imidazolidinyl urea | | 0.3 with warning[a] | NA | NA |
| Phenoxyethanol | 1.0 | | | |
| DMDM hydantoin | | 0.3 with warning[a] | NA | NA |
| Methylchloroisothiazolinone and methylisothiazolinone | | 0.1 (as sold) | NA | NA |
| Chlorhexidine and salts | | 0.1 (gluconate ∞ ) | 0.05 (chloride 0.1) | 0.05 (chloride 0.001) |
| Chlorphenesin | | 0.3 | 0.3 | NA |
| Benzethonium chloride | | | 0.05 | 0.05 |
| Benzalkonium chloride | | 0.5 | 0.2 | NA |

MM = mucous membrane. NA = not allowed. ∞ = any amount.

[a] Warning required for imidazolidinyl urea and DMDM hydantoin: should not be used by infants or by people who are hypersensitive to formaldehyde.

## *Requirements for new preservatives*

Obtaining approval and placement on the list requires a formal submission to the Japanese Minister of Health, Labor, and Welfare (MHW), Examination

and Administration Section, who, after reviewing a submission will approve or reject it or request additional data. A submission must include data on the chemistry of the preservative, method of production, purity, and efficacy. If the preservative has been approved in any other market, this information must be included along with maximum use levels and any restrictions. If the chemistry is similar to the chemistries of other preservatives, a comparison is also required.

Safety testing data should cover single administration toxicity, repetitive administration toxicity, reproductive development toxicity, skin primary irritation, continuous skin irritation, sensitivity, phototoxicity, photosensitization, eye irritation, genetic toxicity, human patch tests on Japanese subjects, absorption, distribution, metabolism, and excretion. All data must be submitted on official forms in the Japanese language.

### *Prohibited preservatives*

Japan now has a list of 31 ingredients that are prohibited from use in cosmetics. The list includes:

Dichlorophen
Mercury and its compounds
Halogenated salicylanides
Bithionol
Hexachlorophene
Formalin

## *Non-regulatory considerations*

### *Formaldehyde issues*

Formaldehyde may be used as a preservative everywhere in the world except in Japan. However, due to consumer pressure, certain countries including Germany and Denmark will not permit the use of formaldehyde or even the so-called formaldehyde releasers.

### *"Green" Party issues*

The environmentalist or green political parties are generally against all preservatives but they especially dislike formaldehyde and all halogen-containing products. These groups also do not approve of the use of ethylene oxide and other ingredients such as EDTA that are not readily biodegradable.

### *Triclosan issues*

In addition to Green Party objections to halogenated compounds, a very strong movement in the EU seeks to limit or even prohibit new uses for triclosan.

### *Biodegradation issues*

The non-biodegradability of EDTA has become an issue in the EU. Although currently not restricted, this could become a critical issue in the future.

## *Non-preservative preservatives*

When is a preservative not a preservative? Rationally, when a product acts as a preservative but is not listed on Annex VI of the EU Cosmetics Directive, it cannot be a preservative! Therefore, it must have a different function in the formulation. This principle does not include the addition of high levels of glycols, polyols, or salts to lower water activity levels. Non-preservative preservatives must show cidal properties and function as one of three chemical entities: essential oils, emulsifiers, and humectants.

**Essential oils** — We have known for years that many essential oils found in fragrances have antimicrobial properties. In general, the level of essential oil needed to preserve product far exceeds the levels used in fragrances and formulations. Sometimes simple emulsification of the oil will eliminate most microbial activity. For example, tea tree (INCI: *Melaleuca alternifolia*) leaf oil at 100% shows good activity. After emulsification, its activity cannot be measured. The critical issue for formulators is that their fragrances may have microbial activities and this can mean that smaller amounts of additional chemical preservatives are required.

**Emulsifiers** — One of the earliest emulsifiers promoted for its antimicrobial properties is the 90% monoester of glyceryl monolaurate (INCI: glyceryl laurate). This compound never achieved great popularity because its antimicrobial action and emulsifying activity were both weak.

**Humectants or hydroxy-containing compounds** — Although high concentrations of propylene glycol can act as preservatives, the safety of products that contain them would prevent their marketing. The esthetics of products with glycol levels high enough to reduce water activity to sufficiently low levels to prohibit microbial growth are unacceptable to consumers. Certain products are antimicrobial and fulfill this function when used as humectants in cosmetics. They include:

Farnesol, a long-chained unsaturated hydrocarbon with a terminal hydroxyl group; frequently found in fragrances; also shows activity against Gram-positive bacteria.

Pentylene glycol, a 5-carbon diol, shows good broad-spectrum activity when used at levels over 2%; functions as an excellent humectant at that level.

Caprylyl glycol, an 8-carbon diol exerting major activity against bacteria.

1,2 Hexanediol, a 6-carbon diol, has been shown to be synergistic with pentylene glycol to offer broad-spectrum coverage and adequate humectancy.

## Concluding comments

Regulatory constraints on global preservative use are varied, complex, and costly, and they will further constrain preservative choices in the future. Requirements for increasingly diverse formulations and packaging, consumer use and price pressures from cosmetic manufacturers, and limited market sizes for cosmetic preservatives are all factors that restrain producers from developing and qualifying new cosmetic preservatives. These constraints may make the effective global preservative system an obsolete concept. Clearly, cosmetic manufacturers will be faced with efforts to develop multiple formulations in order to satisfy various requirements based on geographic markets and regulatory activities. A company that can satisfy all these requirements with the least duplication of effort will maintain a significant competitive edge.

## References

1. 21 CFR 700.11.
2. 21 CFR 700.15.
3. 21 CFR 250.250.
4. 21 CFR 700.13.
5. www.cir-safety.org
6. 21 CFR 740.10.
7. Federal Register, Vol. 59, No. 116, pp. 31402–31452, June 17, 1994.
8. Federal Register, Vol. 56, No. 140, pp. 33644–33680, July 22, 1991.
9. 21 CFR 333.310.
10. 21 CFR 333.210.
11. 21 CFR 358710.
12. www.FDA.gov
13. U.S. Food and Drug Administration, *Cosmetic Handbook*, Washington, D.C., 1992. p. 10. (http://www.cfsan.fda.gov/~dms/cos-hdbk.html)

# *Appendix*

# *Common cosmetic preservatives*

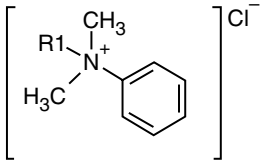
*Contents*

# *Benzalkonium chloride*
# *EEC #54*
# *CAS #139-07-1*

| | |
|---|---|
| Chemical names: | Mixture of alkyldimethylbenzylammonium chloride and N-dodecyl-N,N-dimethylbenzylammonium chloride |
| Trade names: | Zephirol, Dodigen 226, Barquat MB-50 |
| Type of compound: | Quaternary, cationic |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White or yellowish white amorphous powder |
| Odor: | Aromatic |
| Solubility: | Very soluble in water (about 50%) and alcohols |
| Optimum pH: | 4.0 to 10.0; 1% solution gives a pH of 6.0 to 8.0 |
| Stability: | Good, stable at 121°C for 30 min |
| Compatibility: | EDTA, non-ionic detergents; incompatible with anionics, soap, nitrates, heavy metals, citrates, sodium tetraphosphate, and hexametaphosphate; adsorbed by plastics |
| Neutralization: | Tween 80 and lecithin; reduced activity below pH 5 |
| Structural formula: | R is a mixture of the alkyls $C_8H_{17}$ to $C_{18}H_{37}$ |




Benzalkonium Chloride

### *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal germicidal concentration (mg/ml) suspension test for times shown | |
|---|---|---|
| | 24 hr | 5 min |
| *Staphylococcus aureus* | 4 to 10 | 50 |
| *Escherichia coli* | 10 | 80 |
| *Pseudomonas aeruginosa* | 10 to 100 | 200 |
| *Candida albicans* | 10 | 160 |
| *Aspergillus niger* | 100 to 200 | — |

### *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | Mouse: $LD_{50}$, 300 mg/kg;<br>Rat: $LD_{50}$, 450 to 750 mg/kg |
| Subacute oral toxicity: | Rat: daily oral application of 550 ppm over 3 months without toxic effects |
| Chronic toxicity: | Rat: dietary study with addition of 0.25% over 2 yr without toxic effects |
| Primary skin irritation: | 0.1% solution without effect on animals and humans; 0.5% caused irritation |
| Rabbit eye irritation: | 1:3000 dilution tolerated |
| Mutagenicity: | Negative Ames test |

### *4. Cosmetic and other applications*

In hair conditioners as a cationic, added as preservative; in deodorants as antibacterial compounds; antiseptic for preoperative skin preparation, wounds, burns; preservative in eyedrops.

### *5. Mode of action*

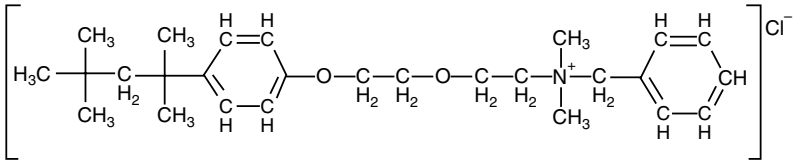
Solubilizes membrane lipids and other lipids by surfactant action to destroy cell integrity; may also denature proteins.

## *Benzethonium chloride*
## *EEC #53*
## *CAS #121-54-0*

| | |
|---|---|
| Chemical names: | N,N-Dimethyl-N-[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl] benzene methane ammonium chloride; diisobutylphenoxyethoxyethy]dimethy] benzyl ammonium chloride |
| Trade names: | Hyamine 1622 |
| Type of compound: | Quaternary compound |

## 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Thin, hexagonal crystals |
| Solubility: | Very soluble in water, alcohol |
| Optimum pH: | 4 to 10; 1% aqueous solution gives a pH of 4.8 to 5.5 |
| Compatibility: | Incompatible with soap, anionic detergents, mineral acids, and salts |
| Neutralization: | Tween 80 and lecithin |
| Structural formula: | |



Benzethonium Chloride

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 10 min) |
|---|---|---|
| *Staphylococcus aureus* | 40 | 0.5 |
| *Escherichia coli* | 50 | 332 |
| *Pseudomonas aeruginosa* | 800 | 250 |
| *Candida albicans* | — | 64 |
| *Aspergillus niger* | — | 128 |

## 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Mouse: | $LD_{50}$, 500 mg/kg |
| | Rat: | $LD_{50}$, 420 mg/kg; 765 mg/kg |
| Human toxicity: | Ingestion may cause vomiting, collapse, convulsions, coma | |

## 4. Cosmetic and other applications

In-use concentration (EEC), 0.1% as a preservative in deodorant; topical anti-infective, antiseptics, disinfectants, wound powders.

## 5. Mode of action

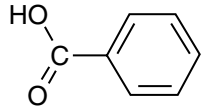
Solubilizes membrane lipids and other lipids by surfactant action to destroy cell integrity. May also denature proteins.

# *Benzoic acid*
# *EEC #I/1*
# *CAS #121-54-0*

Chemical names: Benzene carboxylic acid
Trade names: —
Type of compound: Organic acid

## *1. Structure and chemical properties*

Appearance: Monoclinic tablets, plates, leaflets, or white powder
Solubility: In water (20°C), 0.29%; in ethanol (20°C), 1 g in 2.3 ml; sodium salt in water (20°C), 1 g in 1.8 ml
Optimum pH: 2 to 5
Stability: Stable at low pH
Compatibility: Loss of activity in the presence of proteins and glycerol; incompatible with non-ionics, quaternary compounds, and gelatin
Neutralization: pH above pKa
Structural formula:




Benzoic Acid

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr; pH 6) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr; pH 6) |
|---|---|---|
| *Staphylococcus aureus* | 50 to 100 | 20 |
| *Escherichia coli* | 100 to 200 | 160 |
| *Pseudomonas aeruginosa* | 100 to 200 | 160 |
| *Candida albicans* | 500 to 1000 | 1200 |
| *Aspergillus niger* | 500 to 1000 | 1000 |

The undissociated form has antimicrobial activity, making it optimally effective below its pKa; migrates to oil phase. In oil–water emulsions, benzoic acid migrates to the oil phase; only the amount dissolved in water is effective.

### *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Mouse: | $LD_{50}$, 2.37 g/kg |
| | Rat: | $LD_{50}$, 1.7 g/kg |
| Subchronic toxicity: | Mouse: | 880 mg/kg/day per day over 3 months produced results in high mortality |
| Chronic toxicity: | 40 mg/kg/ per day (mice and rats) up to 18 months inhibited growth | |
| Acceptable daily intake: | Human: 0.5 mg/kg body weight/per day | |
| Human skin: | Toxic dose: 6 mg/kg | |

### *4. Cosmetic and other applications*

Use concentration, 0.1 to 0.2%; EEC maximum concentration, 0.5%. Preservative agent in foods and pharmaceuticals (oral dosage forms).

### *5. Mode of action*

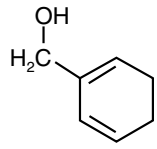
Destroys chemiosmotic balance across the cytoplasmic membrane by disruption of the membrane electrical potential through dissociation of protons from the compound into the cytoplasm of the cell. It may also denature proteins.

## *Benzyl alcohol*
## *EEC #51*
## *CAS #100-51-6*

| | |
|---|---|
| Chemical names: | Benzyl alcohol, benzenemethanol, phenylcarbinol, phenylmethanol |
| Trade names: | — |
| Type of compound: | Natural alcohol |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Liquid |
| Odor: | Faint aromatic odor |
| Solubility: | 4 g/100 ml water; 1 vol/1.5 vol 50% ethanol; miscible with absolute alcohol |
| Optimum pH: | Above 5 |
| Stability: | Slowly oxidizes to benzaldehyde; dehydrates at low pH |
| Neutralization: | Inactivated by non-ionics like Tween 80 and dilution |
| Structural formula: | |




Benzyl Alcohol

### *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 25 |
| *Escherichia coli* | 2000 |
| *Pseudomonas aeruginosa* | 2000 |
| *Candida albicans* | 2500 |
| *Aspergillus niger* | 5000 |

### *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 1.23 g/kg |
| | Mouse: | $LD_{50}$, 1.58 g/kg |
| | Rabbit: | $LD_{50}$, 1.94 g/kg |
| Acute dermal toxicity: | Guinea pig: | $LD_{50}$, 5.0 ml/kg |
| Percutaneous toxicity: | High percutaneous toxicity | |
| Human toxicity: | By dermal application; only a small amount resorbed by the dermis | |
| Toxicokinetic data: | Metabolized to hippuric acid | |

### *4. Cosmetic and other applications*

Use concentration, 1.0 to 3.0%; EEC guideline, 1.0%. Preservative in injectable drugs, ophthalmic products, and oral liquids.

### *5. Mode of action*

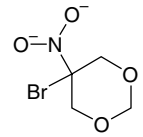
Disruption of membrane by solubilization of lipids; and possibly denatures proteins.

## *5-Bromo-5-nitro-1,3-dioxane*
## *EEC #18*
## *CAS #30007-47-7*

| | |
|---|---|
| Chemical name: | 5-Bromo-5-nitro-l,3-dioxane |
| Trade names: | Bronidox |
| Type of compound: | o-Acetal, o-formal |

## 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Solution, 10% (wt/vol); active ingredient in propylene glycol |
| Solubility at 20°C: | 25% in ethanol; 10% in isopropanol; 0.46% in water; 10% in propylene glycol |
| Optimum pH: | 5 to 7 |
| Stability: | Unstable at pH < 5 and temperature above 50°C; corrosive to metal containers |
| Compatibility: | Compatible with non-ionics |
| Neutralization: | Cysteine and protein |
| Structural formula: | |




5-Bromo-5-Nitro-
1,3 -Dioxide

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 75 |
| *Escherichia coli* | 50 |
| *Pseudomonas aeruginosa* | 50 |
| *Candida albicans* | 25 |
| *Aspergillus niger* | 25 |

## 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Mouse: | $LD_{50}$, 590 mg/kg |
| | Rat: | $LD_{50}$, 455 mg/kg |
| Subacute dermal toxicity: | Rat: | 100 mg/kg/day, no effect after 15 weeks; 200 mg/kg/day caused deaths |
| Primary skin irritation: | Irritation occurs above 0.5% | |
| Human skin: | Patch test at 0.1% without irritation; 0.5% in suspension and 0.25% in Vaseline showed irritation; partial resorption (cutaneous); Partial resorption (cutaneous), some metabolites in urine | |
| Rabbit eye irritation: | Ten applications of 0.05% produced no reaction; irritation threshold about 0.1% | |
| Guinea pig sensitization: | None | |

### *4. Cosmetic and other applications*

In use concentration, 0.1% in EEC guideline; only for rinse-off products. Preservative for technical products.

### *5. Mode of action*

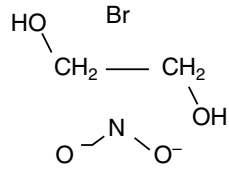
Converts protein thiol groups to disulfides, resulting in denaturation of proteins with sulfhydryl groups at the active sites.

## *2-Bromo-2-nitropropane-1,3-diol*
## *EEC #19*
## *CAS #52-51-7*

| | |
|---|---|
| Chemical name: | 2-Bromo-2-nitropropane-1,3-diol |
| Trade names: | Bronopol |
| Type of compound: | Alcohol |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White crystalline powder |
| Odor: | Faint characteristic odor |
| Solubility: | Water, 25% (wt/vol); ethanol, 50%; isopropanol, 25%; glycerol, 1%; propyleneglycol, 14% |
| Optimum pH: | 5.0 to 7.0 |
| Stability: | Stable at low pH; yellows and browns under alkaline conditions; unstable with iron and aluminum; stable with stainless steel and tin; nitrite evolved to form nitrosamine |
| Compatibility: | Not affected by anionic, cationic, non-ionic surfactants, or proteins |
| Neutralization: | 0.1% cysteine; thioglycolate and thiosulfate also inactivate |
| Structural formula: | |




Bromo Nitropropane Diol

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (serial dilution test: 24 to 72 hr incubation time) |
|---|---|
| *Staphylococcus aureus* | 62.5 |
| *Escherichia coli* | 31.25 |
| *Pseudomonas aeruginosa* | 31.25 |
| *Candida albicans* | 50 |
| *Aspergillus niger* | 50 |

## 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Mouse (male): | $LD_{50}$, 374 mg/kg |
| | Rat (male): | $LD_{50}$, 307 mg/kg |
| Intraperitoneal toxicity: | Rat (male): | $LD_{50}$, 22 mg/kg |
| Acute dermal toxicity: | Rat (acetone solution): | death at 160 mg/kg |
| Primary skin irritation: | 0.5% in acetone; 2.5% in aqueous methylcellulose; and 5.0% in polyethoxyleneglycol nonirritant | |
| Rabbit eye irritation: | 0.5% in saline or 2% in polyethyleneglycol nonirritant; 5% is irritant | |
| Guinea pig sensitization: | Three challenges for 2/10 animals sensitized. (Magnusson-Kligman) | |
| Human sensitization: | Human skin irritant at 0.25% to 1% in soft paraffin and at 0.25% in aqueous buffer at pH 5.5 | |
| Chronic toxicity: | In 90-day test, daily oral doses of 20 mg/kg/day to rats were tolerated; 80 to 160 mg/kg caused gastrointestinal lesions, respiratory distress, and some deaths | |
| Carcinogenicity: | 0.2 to 0.5% in 0.3 ml acetone applied to shaved backs of mice 3 times/week for 80 weeks did not increase spontaneous tumor profile; 10 to 160 mg/kg/day orally for 2 yr without tumor incidence | |
| Mutagenicity: | No mutagenic activity (Ames or host-mediated assay in mice) | |

## 4. Cosmetic and other applications

Used at 0.01 to 0.1% in hand and face creams, shampoos, hair dressings, mascaras, and bath oils, pharmaceutical products, household products (fabric conditioners and washing detergents).

## 5. Mode of action

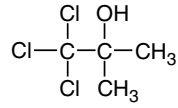
Forms disulfide bonds with thiol groups to denature proteins. For example, it inhibits dehydrogenase activity as a result.

# *Chlorobutanol*
# *EEC #I/11*
# *CAS #57-15-8*

| | |
|---|---|
| Chemical name: | 1,1,1- Trichloro-2-methylpropan-2-ol |
| Trade names: | — |
| Type of compound: | Chlorinated alcohol |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Colorless crystals |
| Odor: | Camphor-like odor |
| Solubility: | 0.8% in water; 1 g/ml ethanol or propylene glycol; 1 g/10 ml glycerin |
| Optimum pH: | Acidic pH (up to 4.0) |
| Stability: | Decomposed by alkali and heat |
| Compatibility: | Incompatible with non-ionics, alkalis; unstable in polyethylene |
| Neutralization: | Tween 80 and polyvinylpyrrolidone |
| Structural formula: | |




Chlorobutanol

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 625 |
| *Escherichia coli* | 625 |
| *Pseudomonas aeruginosa* | 1000 |
| *Candida albicans* | 2500 |
| *Aspergillus niger* | 5000 |

## *3. Toxicity*

Acute oral toxicity: Dog: $LLD_{50}$, 238 mg/kg

## *4. Cosmetic and other applications*

Use concentration up to 0.5%; prohibited in aerosols except for foams. Label must state "contains chlorobutanol."

### 5. Mode of action

Disruption of membrane by alcohol solubilization of lipids and possibly denatures proteins.

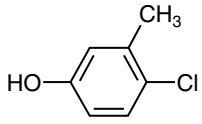
## *p-Chloro-m-cresol*
## *EEC #26*
## *CAS #59-50-7*

| | |
|---|---|
| Chemical name: | 4-Chloro-3-methylphenol, PCMC |
| Trade names: | Preventol CMK |
| Type of compound: | Halogenated phenolic |

### 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | White powder |
| Odor: | Odorless when very pure |
| Solubility: | 1 g/260 ml of water at 20°C; freely soluble in alcohol |
| Optimum pH: | Broad-spectrum activity at acidic pH |
| Stability: | Aqueous solution yellows in light and air |
| Compatibility: | Partial inactivation in the presence of non-ionics; discoloration with iron salts |
| Neutralization: | Dilution |

Structural formula:




Chlorocresol

### 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr incubation time) |
|---|---|
| *Staphylococcus aureus* | 625 |
| *Escherichia coli* | 1250 |
| *Pseudomonas aeruginosa* | 1250 |
| *Candida albicans* | 2500 |
| *Aspergillus niger* | 2500 |

### 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Mouse: $LD_{50}$, 4 g/kg |
| Guinea pig sensitization: | None |

### *4. Cosmetic and other applications*

Use concentration, 0.1 to 0.2% in protein shampoos and baby cosmetics; EEC maximum, 0.2%. Topical antiseptic, disinfectant, preservative in pharmaceutical products.

### *5. Mode of action*

As with many chlorinated phenolics, PCMC most likely uncouples oxidation from phosphorylation and inhibits active transport by disrupting the cell membranes through solubilizing lipids and denaturing proteins. Once membrane integrity is compromised, the cell is more permeable to protons and thus any potential gradient for running ATP synthetase is destroyed.

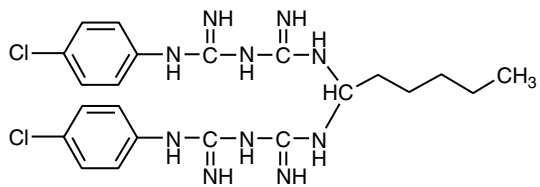
## *Chlorhexidine digluconate*
## *EEC #31*
## *CAS #55-56-7*

| | |
|---|---|
| Chemical name: | Bis(p-chlorophenyldiguanido)hexane |
| Trade names: | Hibitane, Novalsan, Rotasept, Sterilon, Hibiscrub, Arlacide |
| Type of compound: | Biguanide |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White crystalline powder |
| Odor: | Odorless |
| Solubility: | Digluconate in HOH, >70%; diacetate and dihydrochloride salts are less soluble |
| Optimum pH: | 5 to 8 |
| Stability: | Unstable above 70°C |
| Compatibility: | Compatible with cationics; incompatible with anionics, gums, soaps, inorganic anions, alginates, carboxymethylcellulose, cork seals |
| Neutralization: | Non-ionic surfactants |

Structural formula:




Chlorhexidine
Digluconate

### 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 0.5 to 1.0 | 100 |
| *Escherichia coli* | 1.0 | 100 |
| *Pseudomonas aeruginosa* | 5 to 60 | 400 |
| *Candida albicans* | 10 to 20 | 400 |
| *Aspergillus niger* | 200 | 400 |

### 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Mouse: $LD_{50}$, 2 g/kg (diacetate) |
| Chronic toxicity: | Rat: 0.05% in drinking water over 2 yr without toxic effects |
| Primary skin irritation: | No effect |
| Human sensitization: | Allergic tendency |
| Mutagenicity: | Positive Ames test; positive DNA repair test |

### 4. Cosmetic and other applications

Use concentrations 0.01 to 0.1%. Used in non-ionic creams, toothpastes, deodorants/antiperspirants; EEC maximum, 0.3%. Skin disinfectant, preservative in eye-care products at 0.01%; topical antiseptic at 0.02%.

### 5. Mode of action

General protein coagulant/denaturant that destroys membrane integrity and cytoplasmic enzymes. The molecule may become oriented within a lipid component of the membrane to cause a general disruption of membrane structure and function.

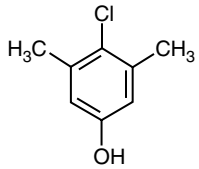
## *Chloroxylenol*
## *EEC #32*
## *CAS #88-04-0*

| | |
|---|---|
| Chemical names: | p-Chloro-meta-xylenol, PCMX, PCMX |
| Trade names: | Ottasept, Nipacide |
| Type of compound: | Halogenated phenolic |

### 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Crystalline powder |
| Odor: | Phenolic |

Solubility: 0.33 g in 1 liter HOH at 20°C
Optimum pH: Wide
Compatibility: Incompatible with cationics and non-ionics
Neutralization: Dilution
Structural formula:




Chloroxylenol

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 250 |
| *Escherichia coli* | 1000 |
| *Pseudomonas aeruginosa* | 1000 |
| *Candida albicans* | 2000 |
| *Aspergillus niger* | 2000 |

## 3. Toxicity

Primary skin irritation: Less than phenol or cresol
Guinea pig sensitization: None

## 4. Cosmetic and other applications

Used for protein solutions, hair conditioners, silicone emulsions; EEC, 0.5%. Maximum concentration in soap, 2%; used as an antiseptic, as a preservative for pharmaceutical products, and as an ingredient in disinfectants.

## 5. Mode of action

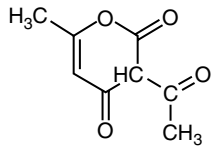
As with many chlorinated phenolics, chloroxylenol most likely uncouples oxidation from phosphorylation and inhibits active transport by disrupting the cell membranes through solubilizing lipids and denaturing proteins. Once membrane integrity is compromised, the cell is more permeable to protons and thus any potential gradient for running ATP synthetase is destroyed.

# *Dehydroacetic acid*
# *EEC #4*
# *CAS #520-45-6*

| | |
|---|---|
| Chemical names: | 3-Acetyl-6-methyl-2H-pyran-2,4(3H)dione, DHA |
| Trade names: | — |
| Type of compound: | Organic acid |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Sodium salt is a colorless, tasteless powder |
| Odor: | Odorless |
| Solubility: | The acid form is relatively insoluble; the salt (wt/wt at 25°C) is 1% in ethanol, 48% in propylene glycol, and 33% in water |
| Optimum pH: | 5 to 6.5; activity decreases with higher pH above the pKa |
| Stability: | Stable to heat |
| Neutralization: | pH above 6.5 |
| Structural formula: | |




Dehydroacetic Acid

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 10,000 | 20,000 |
| *Escherichia coli* | 10,000 | 20,000 |
| *Pseudomonas aeruginosa* | >20,000 | 20,000 |
| *Candida albicans* | 200 | ND |
| *Aspergillus niger* | 200 | ND |

Only undissociated dehydroacetic acid is active. This activity depends on pH being below the pKa. Data above is for pH = 6.

### *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 1.0 g/kg (sodium salt)<br>Human: Impaired kidney function; vomiting, ataxia, and convulsions |
| Chronic toxicity: | Rat: Daily dose with food, 0.1%; showed no toxic effect over 2 yr; no-effect level, >50 mg/kg |
| Primary skin irritation: | No irritation or sensitization in humans |

### *4. Cosmetic and other applications*

0.02 to 0.2%; EEC maximum, 0.6%. Preservative for pumpkins in the U.S.; not allowed in Europe for food preservation.

### *5. Mode of action*

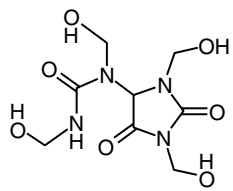
As with most organic acids, they destroy the chemiosmotic balance across the cytoplasmic membranes by disruption of the membrane electrical potential through dissociation of protons from the compound into the cytoplasm of the cell. DHA may also denature proteins.

## *Diazolidinyl urea*
## *CAS #78491-02-8*

| | |
|---|---|
| Chemical name: | N-(hydroxymethyl)-N-(1,3-dihydroxymethyl-2,5-dlo o-4-imidazolidinyl)-N-(hydroxymethyl) urea |
| Trade name: | Germall II |
| Type of compound: | Heterocyclic imidazolidinyl urea, formaldehyde donor |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Fine, white, free-flowing powder |
| Odor: | None or characteristically mild |
| Solubility: | Water soluble |
| Optimum pH: | Wide range |
| Stability: | Stable |
| Compatibility: | Compatible with ionics, non-ionics, proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |




Diazolidinyl Urea

### 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 250 | 1000 |
| *Escherichia coli* | 1000 | 4000 |
| *Pseudomonas aeruginosa* | 1000 | 4000 |
| *Candida albicans* | 8000 | 8000 |
| *Aspergillus niger* | 4000 | 8000 |

### 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 2.57 g/kg |
| Acute dermal toxicity: | Rabbit: | $LD_{50}$, >2.0 g/kg |
| Primary skin irritation: | Rabbit, 1 or 5% solution not an irritant | |
| Eye irritation: | Rabbit, 1 or 5% solution not an irritant | |
| Guinea pig sensitization: | None | |

### 4. Cosmetic and other applications

Use at 0.1 to 0.5% in combination with parabens or other antifungals.

### 5. Mode of action

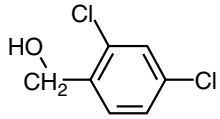
As chemical degrades, it donates formaldehyde to microorganism; formaldehyde denatures proteins by reacting with amino groups in proteins of cell walls, membranes, and cytoplasm.

## *Dichlorobenzyl alcohol*
## *EEC #24*
## *CAS #1777-82-8*

| | |
|---|---|
| Chemical names: | 2, 4-Dichlorobenzyl alcohol |
| Trade names: | Myacide SP, Unikon A-22 |
| Type of compound: | Chlorinated alcohol |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White to yellowish crystalline powder |
| Solubility: | Water (20°C), 0.1%, propylene glycol, 73.0%; soluble in ethanol and isopropanol. |
| Optimum pH: | Wide pH range (3.0 to 9.0) |
| Stability: | Can oxidize in aqueous solutions |
| Compatibility: | Incompatible with some anionics and non-ionics |
| Neutralization: | Dilution |
| Structural formula: | |




Dichlorobenzyl Alcohol

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 1000 |
| *Escherichia coli* | 500 |
| *Pseudomonas aeruginosa* | 1000 |
| *Candida albicans* | 500 |
| *Aspergillus niger* | 500 |

## *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Mouse: | $LD_{50}$, 2.3 g/kg |
| | Rat: | $LD_{50}$, 3.0 g/kg |
| Subchronic toxicity: | Rat: | 98-day test, 7.2 and 14.4 ppm daily with the diet — no toxic effect |
| Primary skin irritation: | Rabbit: | 0.5% solution to shaved flanks of rabbits for 5 days was not irritating |
| Rabbit eye irritation: | 0.8% in aqueous solution had no effect | |
| Guinea pig sensitization: | None | |
| Mutagenicity: | Negative Ames test | |
| Environmental toxicity: | Daphnia magna: | $LC_{50}$ = (24 hr), 22.0 ppm; (48 hr), 13.1 ppm |
| | Rainbow trout: | $LC_{50}$ = (24 hr), 18.9 ppm, (48 hr), 14.4 ppm; (72 hr), 13.3 ppm |
| | Mallard duck: | Acute oral $LD_{50}$ = 2.5 mg/kg |

### *4. Cosmetic and other applications*

Use concentration 0.15% in aqueous solutions, lotions, creams, and gel formulations. Topical antiseptic, disinfectant, antiseptic mouthwash, and gargle.

### *5. Mode of action*

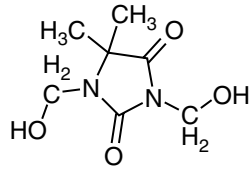
Disruption of membrane by alcohol solubilization of lipids and possibly denatures proteins.

## *Dimethylol dimethyl hydantoin*
## *EEC #50*
## *CAS #6440-58-0*

| | |
|---|---|
| Chemical names: | Dimethylol dimethyl hydantoin, DMDM hydantoin |
| Trade names (supplier): | Glydant, Dekafald, Mackstat DM, Nipaguard DMDMH |
| Type of compound: | Formaldehyde donor |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Clear solution |
| Odor: | Mild formaldehyde odor |
| Solubility: | Freely soluble in water (>50%) and ethanol |
| Optimum pH: | 3.5 to 10.0 |
| Stability: | Stable over wide pH range and temperature conditions (<90°C) |
| Compatibility: | Compatible with anionics, cationics, non-ionics, and proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |




DMDM Hydantoin

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 250 to 800 | 4000 |
| *Escherichia coli* | 500 | 4000 |
| *Pseudomonas aeruginosa* | 800 to 1000 | 4000 |
| *Candida albicans* | 725 to 1250 | 5000 |
| *Aspergillus niger* | 750 to 1500 | ND |

Broad spectrum mainly against bacteria. Should be combined with antifungal components (e.g., anionic surfactants, parabens, Kathon CG, or formaldehyde) for products needing protection from yeasts and molds.

## *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Female rat: | $LD_{50}$, 3.8 g/kg |
| | Male rat: | $LD_{50}$, 2.7 g/kg |
| Acute dermal toxicity: | Rabbit: | $LD_{50}$, >20 g/kg |
| Primary skin irritation: | No effect (rabbit) | |
| Human patch test: | 400 ppm in water over 9 to 24 h; occluded patch showed no irritation | |
| Rabbit eye irritation: | No irritation using 1.0% wt/vol solution | |
| Phototoxicity: | Not a photoallergen | |
| Sensitization: | None with 4000 ppm, on 50 people | |
| Mutagenicity: | Ames test shows nonmutagenic | |

## *4. Cosmetic and other applications*

Use concentration, 0.15 to 0.4%; shampoos, conditioners, hand creams. Preservative agent in detergents.

## *5. Mode of action*

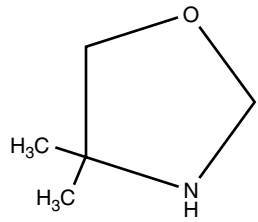
Mechanism of action is the same as for formaldehyde which reacts with proteins in the membranes and cytoplasm to denature.

# *Dimethyl oxazolidine*
# *EEC #51*
# *CAS #200-87-4*

| | |
|---|---|
| Chemical name: | 4.4-Dimethyl-1,3 oxazolidine |
| Trade name: | Oxadine A |
| Type of compound: | Cyclic substituted amine, oxazolidine, formaldehyde donor |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Colorless liquid |
| Odor: | Penetrating amine-like odor |
| Solubility: | Completely water soluble |
| Optimum pH: | 6.0 to 11.0 |
| Stability: | Unstable below pH 5.0 |
| Compatibility: | Compatible with cationic, anionic, and non-ionic systems over the pH range 5.5 to 11 |
| Inactivation: | Low pH, dilution and peptone |
| Structural formula: | |




Dimethyl Oxazolidine

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 125 to 500 | 500 |
| *Escherichia coli* | 250 to 500 | 500 |
| *Pseudomonas aeruginosa* | 250 to 500 | 500 |
| *Candida albicans* | 500 to 1000 | 1000 |
| *Aspergillus niger* | 250 to 1000 | 500 |

### *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Male rat: | $LD_{50}$, 950 mg/kg |
| Acute dermal toxicity: | Rabbit: | $LD_{50}$, 1400 mg/kg |
| Rabbit eye irritation: | 5000 ppm in water had no discernible effect | |
| Human sensitization: | $LC_{50}$, 11.7 mg/liter; no sensitization at use levels | |
| Mutagenicity: | Ames test nonmutagenic | |

### *4. Cosmetic and other applications*

Protein shampoos, hand creams: 0.05 to 0.2% use concentration; not in EEC guideline; antimicrobial preservative for cutting oils.

### *5. Mode of action*

Same as for formaldehyde; reacts with proteins in membranes and cytoplasm to denature. See DMDM hydantoin.

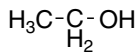
## *Ethanol or Ethyl alcohol*
## *CAS #64-17-5*

| | |
|---|---|
| Chemical names: | Ethanol refers to absolute ethyl alcohol. Alcohol refers to 95% (vol/vol) ethanol |
| Trade names: | — |
| Type of compound: | Alcohol |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Clear, colorless |
| Odor: | Characteristic, burning taste |
| Solubility: | Miscible with water, acetone, and glycerol |
| Optimum pH: | Acidic |
| Stability: | Absorbs water; volatile |
| Compatibility: | Incompatible with acacia, albumin, bromine, chlorine |
| Neutralization: | Inactivated by dilution to below 1%; may be inactivated by non-ionics |

Structural formula:



Ethanol

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (%) (Serial dilution test: 24 to 72 hr) | Minimal killing time (sec) 70% concentration (suspension test) |
|---|---|---|
| *Staphylococcus aureus* | 5% | 15 |
| *Escherichia coli* | 5% | 30 |
| *Pseudomonas aeruginosa* | 5% | 10 |
| *Candida albicans* | 5% | ND |
| *Aspergillus niger* | 5% | ND |

## 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 13.7 g/kg |
| | Guinea pig: | $LD_{50}$, 5.5 g/kg |
| | Rabbit: | $LD_{50}$, 9.5 g/kg |
| Chronic toxicity: | Daily tolerable dose, 80 g; acceptable daily intake, 7 g/kg/day | |
| Primary skin irritation: | Predictive skin sensitization test showed delayed allergic skin reaction with 50% solution | |

## 4. Cosmetic and other applications

Satisfactory preservation above 15%, better at 20%. Use at 60 to 70% for disinfection. Topical anti-infective, antiseptic.

## 5. Mode of action

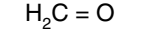
Disruption of membrane by alcohol solubilization of lipids and possibly denatures proteins.

# *Formaldehyde*
# *EEC #1/5*
# *CAS #50-00-0*

| | |
|---|---|
| Chemical names: | Formaldehyde, Formalin |
| Trade names: | — |
| Type of compound: | Aldehyde, 37% (wt/wt) in water; formalin has 10% methanol added to prevent polymerization |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Colorless liquid; powerful reducing agent especially in presence of alkali; density, 1.12 g/ml; keep in tightly closed container; density, 1.12 g/ml |
| Odor: | Pungent |
| Solubility: | Freely soluble in water |
| Optimum pH: | pH 3 to 10; 2.5 to 4.0 for Formalin |
| Stability: | May become cloudy in cold and form trioxymethylene (paraformaldehyde); oxidizes to formic acid |
| Compatibility: | Incompatible with ammonium, hydrogen peroxide, iodine, iron, gelatin, proteins |
| Neutralization: | Dilution, peptone, and ammonium ions |
| Structural formula: | |



Formaldehyde

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 125 | 62.5 |
| *Escherichia coli* | 125 | 31.25 |
| *Pseudomonas aeruginosa* | 125 | 62.5 |
| *Candida albicans* | 500 | 250 |
| *Aspergillus niger* | 500 | 500 |

### 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 800 mg/kg |
| | Guinea pig: | $LD_{50}$, 260 mg/kg |
| Acute subcutaneous toxicity: | Mouse: | $LD_{50}$, 300 mg/kg |
| | Dog: | $LD_{50}$, 800 mg/kg |
| Inhalation toxicology: | Rat: | LD, 250 mg/kg (4 hr) |
| | Man: | LD, 36 mg/kg |
| Human toxicity: | Current permissible exposure limit: 3 ppm (OSHA); irritation (eye, nose, throat): 0.03 to 4.0 ppm; allergy caused by inhalation exposure to 0.3% solutions; 1 to 4% people show positive patch tests with 2% formaldehyde; 44% people sensitized show positive patch tests to 30 ppm solution | |
| Mutagenicity: | Ames test negative; mouse lymphoma positive; sister chromatid exchange positive; chromosomal aberrations in bone marrow negative; Drosophila positive | |
| Rat carcinogenicity: | Inhalation of 2 ppm formaldehyde 6 hr/day, 5 days/week for 24 months had no effect; at 6–15 ppm, 1.5% to 43.2% tumor frequency | |

### 4. Cosmetic and other applications

Preservative for shampoos (0.1 to 0.2%). Label warning: "Contains Formaldehyde" when >0.05% free formaldehyde (EEC).

### 5. Mode of action

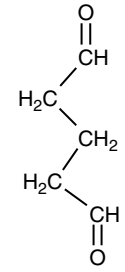
Denatures proteins by reacting with amino groups in proteins of the cell walls, membranes, and cytoplasm.

## *Glutaraldehyde*
## *CAS: #111-30-8*

| | |
|---|---|
| Chemical names: | Glutaraldehyde |
| Trade name (supplier): | Ucaricide |
| Type of compound: | Dialdehyde |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Oily liquid (25% solution) stabilized with ethanol, alkaline pH, or 0.1 to 0.25% hydroquinone |
| Odor: | Pungent odor |
| Solubility: | Slightly soluble in water |
| Optimum pH: | Broad pH range: optimal pH for bactericidal activity = 7.5 to 8.5, polymerizes at highly alkaline pH |
| Compatibility: | Inactivated by ammonia or primary amines at neutral to basic pH |
| Neutralization: | Sodium bisulfite |
| Structural formula: | |




Glutaraldehyde

## *2. Antimicrobial spectrum*

Compared with 4% aqueous formaldehyde, 2% aqueous glutaraldehyde is 10 times as effective as a bactericidal and sporicidal agent.

## *3. Toxicity*

| | |
|---|---|
| Inhalation toxicity: | Rat: 8 hr in saturated glutaraldehyde atmosphere caused no deaths |
| Acute oral toxicity: | Rat: $LD_{50}$, 60 mg/kg |
| Chronic toxicity: | Not mutagenic |
| Primary skin irritation: | $LD_{50}$ (25% solution), 2.38 mg/kg |
| Human contact dermatitis: | 550 ppm without effect (706 persons) |

## *4. Cosmetic and other applications*

Use concentration, 0.02 to 0.2%; disinfectant for instruments and equipment; preservative at 0.2% and pH about 5; corrosive.

## *5. Mode of action*

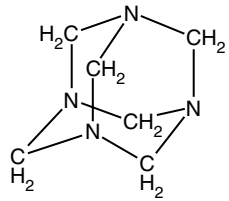
Denatures proteins by reacting with amino groups in proteins of the cell walls, membranes, and cytoplasm.

# *Hexamethylenetetramine*
# *EEC #44*
# *CAS #100-97-0*

| | |
|---|---|
| Chemical names: | 1,3,5,7-Tetraazatricyclo[3,3,1,13,7]decane |
| Trade names: | Aminoform, Formin, Uritone, Cystamin |
| Type of compound: | Formaldehyde donor, n-acetal |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Crystals, granules, or powder; hygroscopic; voIatile at low temperature |
| Odor: | Odorless |
| Solubility: | 1 g dissolves in 1.5 ml of water or 12.5 ml of alcohol |
| Optimum pH: | pH of 0.2 M aqueous solution = 8.4; forms formaldehyde at acid pH |
| Compatibility: | Compatible with anionics, cationics, non-ionic detergents, and proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |




Hexamethylenetetramine

## *2. Antimicrobial spectrum*

Hexamethylenetetramine alone shows no antimicrobial activity but is derived from the breakdown into formaldehyde. See the data for formaldehyde.

## *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | $LD_{50}$, 200 mg/kg |
| Chronic toxicity: | Rat, 90-day test with 0.4 g/day produced no toxic effect; 1% hexamethylenetetramine daily oral dose over 60 wk showed no effects on mice and rats |
| Carcinogenicity: | 0.15 mg/kg/day orally produced no carcinogenic effect |

### *4. Cosmetic and other applications*

Preservation of lotions and creams (0.2%); if the concentration exceeds 0.05% free formaldehyde, warning "Contains Formaldehyde" must appear on the label. Preservation of hides; phenol-formaldehyde resin; corrosion inhibitor for steel.

### *5. Mode of action*

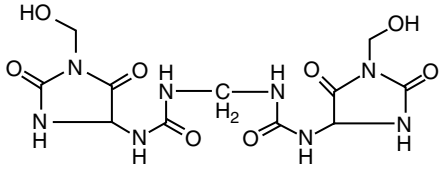
As formaldehyde is released from the breakdown of hexamethylenetetramine, it denatures proteins by reacting with amino groups in proteins of the cell walls, membranes, and cytoplasm.

## *Imidazolidinyl urea*
## *EEC #36*
## *CAS #39236-46-9*

| | |
|---|---|
| Chemical names: | N, N'-methylene-bis-N'-[1-(hydroxymethyl)-2,5-diox -4-imidazolidinyl] urea |
| Trade names: | Germall 115, Blopure 100, Euxyl K 200 |
| Type of compound: | Heterocyclic substituted urea; formaldehyde donor |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Stable white powder |
| Odor: | Odorless |
| Solubility: | Water, 200; glycerol, 100; isopropanol, 0.05 |
| Optimum pH: | Wide range |
| Stability: | Releases formaldehyde upon decomposition; decomposes >160°C |
| Compatibility: | Compatible with ionics, non-ionics, and proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |




Imidazolidinyl Urea

### 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 1000 | 2000 |
| *Escherichia coli* | 2000 | 8000 |
| *Pseudomonas aeruginosa* | 2000 | 8000 |
| *Candida albicans* | 8000 | 8000 |
| *Aspergillus niger* | 8000 | 8000 |

### 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 2.57 g/kg |
| Acute dermal toxicity: | Rabbit: | $LD_{50,}$ >2 g/kg |
| Primary skin irritation | Rabbit: | 1 to 5% solution not a primary irritant |
| Rabbit eye irritation: | 1 to 5% not an eye irritant | |

### 4. Cosmetic and other applications

Use concentrations, 0.1 to 0.5% in combination with parabens or other antifungal preservatives; used for lotions, creams, hair conditioners, shampoos, and deodorants.

### 5. Mode of action

As the chemical degrades, it donates formaldehyde to the microorganism. The formaldehyde denatures proteins by reacting with amino groups in proteins of the cell walls, membranes, and cytoplasm.

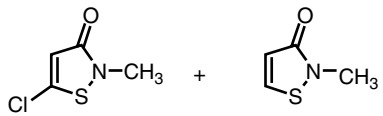
## *Isothiazolinones: chloromethyl and methyl*
## *EEC #45*
## *CAS #26172-55-4/2682-20-4*

| | |
|---|---|
| Chemical names: | 5-Chloro-2-methyl-4-isothiazolin-3-one; 2-Methyl-4-isothiazolin-3-one |
| Trade name (supplier): | Kathon CG |
| Type of compound: | Two isothiazolinones and inorganic magnesium salts in water |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Nonviscous liquid at 1.5%; light amber |
| Solubility: | Highly soluble in water, lower alcohols, and glycols |
| Optimum pH: | Optimum pH 4 to 8; stability reduced in systems of increasing alkalinitye pH (pH > 8) |
| Stability: | Loses activity upon storage in some formulations at elevated temperatures |
| Compatibility: | Compatible with anionic, cationic, and non-ionic surfactants and emulsifiers; inactivated by hypochlorite |
| Neutralization: | Use of dilution with protein-containing broths such as Letheen or peptone broth |

Structural formula:




Chloromethyl and Methyl Isothiazolinones

## *2. Antimicrobial spectrum (as Kathon CG)*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 150 | 500 to 1000 |
| *Escherichia coli* | 300 | 1500 |
| *Pseudomonas aeruginosa* | 300 | 1500 to 2000 |
| *Candida albicans* | 300 | 125 |
| *Aspergillus niger* | 200 to 600 | — |

Bactericidal activity: 5-chloro-2-methyl-4-isothiazolin-3-one, 1.15%; fungicidal activity: 2-methyl-4-isothiazolin-3-one, 0.35%

### 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Rat (male): $LD_{50}$, 3.35 g/kg<br>Rat (female): $LD_{50}$, 2.63 g/kg |
| Acute dermal toxicity: | Rabbit (male): $LD_{50}$, >5 g/kg |
| Subchronic toxicity: | In a 3-month dietary study with rats, 2 g/kg day nontoxic, no pathological findings; no effect level = 633 mg/kg/day. In a 3-month percutaneous study (1 application/day, 5 days/week at 0.40%, Kathon was nontoxic; no pathological findings |
| Primary skin irritation: | Rabbit: product diluted at 3.7% is a nonirritant |
| Human patch test: | 0.37% Kathon CG (10 times minimum dose) showed no primary irritation or sensitization |
| Guinea pig sensitization: | Skin sensitization (Magnusson-Kligman Test), 0.37%: Kathon CG showed no skin sensitization |
| Phototoxicity/ photosensitivity: | Neither phototoxic nor photosensitizing |
| Rabbit eye irritation: | 18% corrosive to the eye, 3.7% no irritation |
| Teratology: | Rat: No fetus toxicity; no teratogenicity in the 100 to 1000 mg/kg range |
| Mutagenicity: | Ames test, variable; cytogenetic study on male rats, no effect at 19 to 190 and 1900 mg/kg |

### 4. Cosmetic and other applications

Use concentrations, 0.035 to 0.15%; shampoos, hair conditioners, hair gels, body gels. Color dye solutions, bubble baths, skin creams and lotions, mascaras. Used in cutting oils.

### 5. Mode of action

Isothiazolinones inhibit active transport and glucose oxidation mainly by reacting with cellular thiol groups of proteins like ATPase and glyceraldehyde-3-phosphate dehydrogenase to denature them.

### 6. Comment

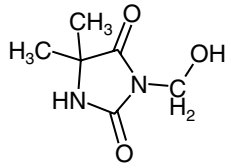
One supplier recently introduced methyl isothiazolinone as a stand-alone preservative (Neolone) with projected use levels of approximately 100 ppm in leave-on and rinse-off cosmetic products.

# *Monomethylol dimethyl hydantoin (MDM hydantoin) EEC #39 CAS # 116-25-6/28453-33-0*

| | |
|---|---|
| Chemical names: | 1-(Hydroxymethyl)-5,5-dimethylhydantoin, MDM hydantoin, MDMH |
| Trade names: | Dantoin 685 |
| Type of compound: | Formaldehyde donor |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Crystals |
| Odor: | Odorless |
| Solubility: | Water soluble; soluble in ethanol or methanol |
| Optimum pH: | 4.5 to 9.5 |
| Stability: | Stable at temperatures below 85°C; formaldehyde is split off at pH 6 in aqueous solutions |
| Compatibility: | Compatible with ionics, non-ionics, and proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |



Monomethyl Dimethylol Hydantoin

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 512 |
| *Escherichia coli* | 1024 |
| *Pseudomonas aeruginosa* | 2048 |
| *Candida albicans* | 2048 |
| *Aspergillus niger* | 2048 |

### 3. Toxicity

See data for dimethylol dimethyl hydantoin (DMDM hydantoin).

### 4. Cosmetic and other applications

Use concentration, 0.25%; EEC allows 0.2% for shampoos and as active compound in deodorants; biocide for cutting oils.

### 5. Mode of action

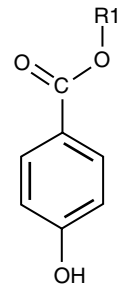
See data for dimethylol dimethyl hydantoin (DMDM hydantoin).

## *Parabens: methyl, ethyl, propyl, and butyl esters*
## *EEC #I/12*
## *CAS # 99-76-3/120-47-8/94-13-3/94-26-8*

| | |
|---|---|
| Chemical names: | Esters of para-hydroxybenzoic acid |
| Trade names (supplier): | Solbrol; Nipagin; Nipasol |
| Types of compounds: | Benzoic acid esters |

### 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | White crystalline powder |
| Odor: | Odorless |
| Solubility: | In water at 25°C (g/100 ml): 0.006, benzyl; 0.02, butyl; 0.04, propyl; 0.11, ethyl; and 0.25, methyl |
| Optimum pH: | 3 to 9.5 |
| Stability: | Stable |
| Compatibility: | Compatible with anionic and cationic emulsions, proteins; incompatible with polyoxy-40-stearate, polyvinylpyrrolidone, methylcellulose |
| Neutralization: | Any non-ionic emulsifier such as Tween 80 |
| Structural formula: | R may be methyl, ethyl, propyl, butyl, or benzyl |




Parabens

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Suspension test: 24 to 72 hr) | | | | | Minimal germicidal concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Methyl | Ethyl | Propyl | Butyl | Benzyl | Methyl | Ethyl | Propyl | Butyl | Benzyl |
| *Staphylococcus aureus* | 800 | 500 | 150 | 120 | 120 | 1250 | 625 | 180 | 160 | 50 |
| *Escherichia coli* | 800 | 600 | 300 | 150 | 160 | 1250 | 1250 | 360 | 160 | 125 |
| *Pseudomonas aeruginosa* | 1000 | 800 | 4400 | 175 | 160 | 1250 | 6625 | 625 | 160 | 175 |
| *Candida albicans* | 1000 | 800 | 250 | 125 | 250 | 5000 | 2500 | 625 | 625 | 100 |
| *Aspergillus niger* | 600 | 400 | 200 | 150 | 1000 | 5000 | 5000 | 2500 | 1250 | 125 |

## *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | Dog: Methyl $LD_{50}$ = 3.0; ethyl $LD_{50}$ = 5.0; propyl $LD_{50}$ = 6.0; butyl $LD_{50}$ = 6.0 (in g/kg) |
| Subchronic toxicity: | Rabbit: 500 mg/kg/day methyl ester for 6 days without effect; 3000 mg with toxicity |
| Human toxicity: | 2 g/day methyl and propyl ester over 1 month without effect |

## *4. Cosmetic and other applications*

Use concentration 0.1% benzyl ester (EEC); 0.4% for a single ester and 0.8% for a mixture of esters; most commonly used: 0.18% methyl + 0.02% propyl ester. Preservative for pharmaceuticals and foods.

## *5. Mode of action*

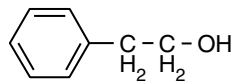
The most likely MOA supported by the data is disruption of membrane potential. This disruption interferes with membrane transport and energy generation. Cells exposed to parabens leak intracellular contents but show no overt changes in cell structure and can recover when exposed to preservative-free media. This suggests that cell lysis and membrane damage did not occur.

# *Phenethyl alcohol*
# *Merck Index #7094*
# *CAS #60-12-8*

| | |
|---|---|
| Chemical names: | 2-phenylethanol |
| Trade names: | — |
| Type of compound: | Natural alcohol |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Colorless liquid |
| Odor: | Rose or floral-like |
| Solubility: | 2 ml in 100 ml water; miscible with alcohol (1:1 in 50% alcohol) |
| Optimum pH: | Acidic pH |
| Stability: | Unstable in presence of oxidants |
| Compatibility: | Compatible with most surfactants; imparts a rose odor to formulae |
| Neutralization: | Partially inactivated with non-ionics and Tween 80 |
| Structural formula: | |




Phenethyl Alcohol

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 1250 |
| *Escherichia coli* | 2500 |
| *Pseudomonas aeruginosa* | 2500 to 5000 |
| *Candida albicans* | 2500 |
| *Aspergillus niger* | 55000 |

## *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 1.79 g/kg |
| Acute dermal toxicity: | Guinea pig: | $LD_{50}$, 5 to 10 ml/kg |
| Human eye irritation: | 0.75% | |
| Guinea pig sensitization: | None at sensitization in concentrations of 1 to 2% | |
| Teratogenicity: | No effect | |

### *4. Cosmetic and other applications*

Eye makeup at 1%; pharmaceuticals: eyedrops; cutaneous antiseptic, 0.3% combined with 0.01% benzalkonium chloride; oral pharmaceuticals, 0.3% to 0.5%.

### *5. Mode of action*

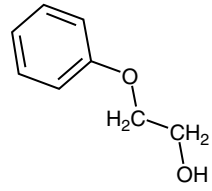
Disruption of membrane by solubilization of lipids and possibly by denaturation of proteins.

## *2-Phenoxyethanol*
## *EEC #43*
## *CAS #122-99-6*

| | |
|---|---|
| Chemical names: | 2-Phenoxyethanol |
| Trade names: | Dowanol EPH; Phenyl Cellosolve; Phenoxethol; Phenonip |
| Type of compound: | Phenolic |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Oily liquid |
| Odor: | Faintly aromatic odor |
| Solubility: | 2.67% in water; miscible with alcohol |
| Optimum pH: | Wide pH tolerance |
| Stability: | Highly stable |
| Compatibility: | Compatible with anionic and cationic detergents |
| Neutralization: | Inactivated by non-ionics and dilution |
| Structural formula: | |




2–Phenoxyethanol

### 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 2000 |
| *Escherichia coli* | 4000 |
| *Pseudomonas aeruginosa* | 4000 |
| *Candida albicans* | 5000 |
| *Aspergillus niger* | 5000 |

### 3. Toxicity

Acute oral toxicity: Rat: $LD_{50}$, 1.3 g/kg
Primary skin irritation: No irritation

### 4. Cosmetic and other applications

0.5 to 2.0% used in combination with parabens, dehydroacetic acid, or sorbic acid; may add with propylene glycol for improvement of water solubility. Used as a bactericide with quaternary ammonium compounds.

### 5. Mode of action

Disruption of membrane by solubilization of lipids and possibly denatures proteins.

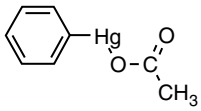
## *Phenylmercuric acetate*
## *EEC #11*
## *CAS #62-38-4*

Chemical names: Phenylmercuric acetate
Trade names: Advacide PMA 18; Cosan PMA; Mergal A25; Metasol 30; Nuodex PMA 18
Type of compound: Organic mercurial

### 1. Structure and chemical properties

Appearance: Crystalline
Solubility: Poorly soluble (1:600 in water); soluble in hot ethanol
Optimum pH: Neutral
Compatibility: Incompatible with iodine compounds, sulfides, thioglycollates, anionics, halogens, ammonia; compatible with non-ionic emulsifiers
Neutralization: Use thiol medium and dilution

Structural formula:




Phenylmercuric Acetate

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 0.1 |
| *Escherichia coli* | 0.5 |
| *Pseudomonas aeruginosa* | 1 to 5 |
| *Candida albicans* | 8 |
| *Aspergillus niger* | 16 |

## 3. Toxicity

| | |
|---|---|
| Acute oral toxicity | Rat: $LD_{50}$, 30 mg/kg (acetate salt); $LD_{50}$, 60 mg/kg (nitrate) |
| Chronic toxicity: | 0.1 mg Hg/kg animal feed over 1 yr, shows no effect in rats; 0.5 mg Hg/kg shows kidney effects in some animals |
| Primary skin irritation: | 0.1% solution is a skin irritant; 0.01% shows no effect |

## 4. Cosmetic and other applications

Limited to eye-area cosmetics that cannot be preserved with any other available preservative. Must be labeled, "Contains phenylmercurial compounds." Maximum concentration in EEC is 0.003% in creams and concentrated shampoos that cannot be preserved with any other option. Also used in pharmaceutical eyedrops and nasal sprays at 0.002 to 0.005%.

## 5. Mode of action

Protein denaturant of cytoplasmic and membrane-associated proteins by heavy metal reaction with thiol groups of proteins.

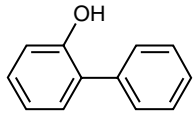
# *O-phenylphenol*
# *EEC #I/7*
# *CAS #90-43-7*

| | |
|---|---|
| Chemical names: | Ortho-phenylphenol |
| Some trade names: | Dowicide 1, Preventol O |
| Type of compound: | Phenolic |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White flaky crystals |
| Odor: | Mild characteristic odor |
| Solubility: | Virtually insoluble in water, except the sodium salt (tetrahydrate) is soluble at 122 g/100 g water. |
| Optimum pH: | 12 to 13.5 for the sodium salt |
| Compatibility: | Incompatible with non-ionics, carboxymethylcellulose, polyethylene glycols, quaternary compounds, and proteins |
| Inactivation/Neutralization: | Dilution |

Structural formula:




O-Phenylphenol

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) | Minimal germicidal concentration (mg/ml) (Suspension test: 24 to 72 hr) |
|---|---|---|
| *Staphylococcus aureus* | 100 | 625 |
| *Escherichia coli* | 500 | 1250 |
| *Pseudomonas aeruginosa* | 1000 | 1250 |
| *Candida albicans* | 50 | 125 |
| *Aspergillus niger* | 50 | 25 |

### *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 2.48g/kg |
| Subchronic toxicity: | Rat: | 200 mg/kg/day over 32 days without toxic effects |
| Chronic toxicity: | Rat: | 0.2% in feed over 2 yr without toxic effects |

### *4. Cosmetic and other applications*

Used in a variety of products where "medicinal" odor is not a problem. Maximum use defined in EEC as 0.2%.

### *5. Mode of action*

As with many phenolics, o-phenylphenol most likely uncouples oxidation from phosphorylation and inhibits active transport by disrupting the cell membranes through solubilizing lipids and denaturing proteins. Once membrane integrity is compromised, the cell is more permeable to protons and thus any potential gradient for running ATP synthetase is destroyed.

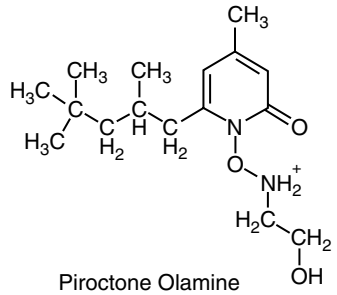
## *Piroctone olamine*
## *EEC #57*
## *CAS #68890-66-4*

| | |
|---|---|
| Chemical name: | 1-Hydroxy-4-methyl-6-(2, 4, 4-trimethyl-pentyl)-2(1H) pyridone ethanolamine salt |
| Trade name: | Octopirox |
| Type of compound: | Pyridone derivative |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White to slightly yellowish powder |
| Odor: | Odorless |
| Solubility: | 0.2% in water; 0.05 to 0.1% in various oils; 10% in alcohol; 1 to 10% in surfactants depending on solubilizing effects of surfactant |
| Optimum pH: | 5 to 9 |
| Stability: | Stable up to 80°C for 2 wk |
| Compatibility: | Compatible with anionic, cationic, and amphoteric surfactants; incompatible with some fragrances |
| Neutralization: | Dilution |

Structural formula:




Piroctone Olamine

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 32 |
| *Escherichia coli* | 64 |
| *Pseudomonas aeruginosa* | 625 to 1250 |
| *Candida albicans* | 64 |
| *Aspergillus niger* | — |

## 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 8.1 g/kg; mouse: 2.48g/kg |
| Acute dermal toxicity: | Rat and dog: NOEL, 100 mg/kg/day |
| Primary skin irritation: | Low under use conditions |
| Rabbit eye irritation: | Good tolerance |
| Human sensitization: | None induced |
| Guinea pig sensitization: | None induced |
| Teratogenicity: | Not a teratogen |
| Mutagenicity: | Not a mutagen (point and chromosomal mutation tests) |

## 4. Cosmetic and other applications

Antidandruff products, particularly transparent products; use concentration is 0.5 to 1.0% in rinse-off products, 0.05 to 0.1% in leave-on products; exhibits adsorbtion to keratin and thus good substantivity.

## 5. Mode of action

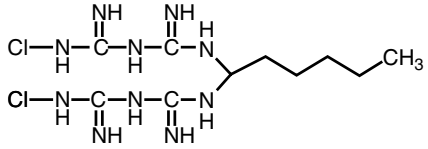
May disrupt membrane function by protein denaturation; other extant mechanisms extant may destroy membrane integrity and thus membrane transport.

# *Polyaminopropyl biguanide*
# *EEC #42*
# *CAS #270830-27-8*

| | |
|---|---|
| Chemical name: | Polyhexamethylene biguanide hydrochloride |
| Trade name: | Cosmocil CQ |
| Type of compound: | Cationic |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Clear, yellowish liquid |
| Odor: | Odorless |
| Solubility: | Water- and alcohol-soluble |
| Optimum pH: | 4 to 8 |
| Stability: | Stable below 80°C |
| Compatibility: | See chlorhexidine data |
| Neutralization: | See chlorhexidine data |
| Structural formula: | |




Polyaminopropyl Biguanide

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 20 |
| *Escherichia coli* | 20 |
| *Pseudomonas aeruginosa* | 100 |
| *Candida albicans* | — |
| *Aspergillus niger* | 375 |

### *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | Rat $LD_{50}$, 5 g/kg |
| Chronic toxicity: | 90-day feeding test, 3.1 ppm for 90 days showed no toxicity or abnormalities; at 6.2 ppm, rats showed retardation of growth and lower food intake; 2 -yr feeding test with pathogen-free Wistar rats at dietary levels of 3000, 5000, and 10,000 ppm; during first 3 mo, reduction in body weight and food intake; no-effect level, 5000 ppm. |
| Primary skin irritation: | Concentrated form is strong irritant; 50,000 ppm tolerated by rats with no irritation; no-effect level for mice was 100 mg/kg/day |
| Rabbit eye irritation: | No irritant effect with 0.1 ml of a 2000 ppm dilution |
| Photoirritation: | No significant photoirritancy |
| Guinea pig sensitization: | None |
| Environmental toxicity: | Rainbow trout: 96-h $LC_{50}$, 10 ppm |

### *4. Cosmetic and other applications*

Use concentration, 0.2 to 1.0% (of 20% solution); EEC, 0.3%; used as disinfectant and preservative for technical products.

### *5. Mode of action*

See chlorhexidine data.

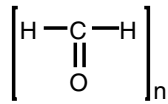
## *Polyoxy methylene*
## *EEC #I/5*
## *CAS #9002-81-7*

| | |
|---|---|
| Chemical name: | Polyoxymethylene |
| Trade names: | Triformol, Formagene, Foromycen |
| Type of compound: | Polymerized formaldehyde |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | White crystalline powder |
| Odor: | Formaldehyde |
| Solubility: | Soluble in water; insoluble in alcohol |
| Optimum pH: | 4 to 8 |
| Stability: | Unstable in alkaline solutions; formaldehyde given off in hot water; container must be kept tightly closed to prevent formaldehyde off-gassing |
| Compatibility: | Compatible with anionic and non-ionic detergents; not compatible with proteins, ammonia, oxidants, heavy metals |
| Neutralization: | Dilution and peptone |

Structural formula:




Polyoxy Methylene

### *2. Antimicrobial spectrum*

Same as data for formaldehyde.

### *3. Toxicity*

Same as data for formaldehyde.

### *4. Cosmetic and other applications*

Used at maximum of 0.2%; may be used as a disinfectant.

### *5. Mode of action*

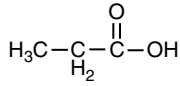
Same as formaldehyde.

## *Propionic acid*
## *EEC #I/2*
## *CAS #79-09-4*

| | |
|---|---|
| Chemical names: | Propionic acid |
| Trade names: | Mycoban |
| Type of compound: | Organic acid |

## 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Oily liquid |
| Odor: | Pungent, slightly disagreeable, rancid odor |
| Solubility: | Miscible with water; may come out of solution by addition of salts |
| Optimum pH: | 3.5 to 4.5; limit is pH 6 |
| Stability: | Stable |
| Neutralization: | pH above pKa; dilution |
| Structural formula: | |



Propionic Acid

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 2000 |
| *Escherichia coli* | 2000 |
| *Pseudomonas aeruginosa* | 3000 |
| *Candida albicans* | 2000 |
| *Aspergillus niger* | 2000 |

## 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 2.6 g/kg |
| Subchronic toxicity: | No effect in rat feeding study over several weeks at 1 to 3% sodium or calcium propionate in feed |
| Chronic toxicity: | 3.75% addition to rat chow administered to rats over 1 year without negative effects |
| Primary skin irritation: | Irritates skin and mucous membranes when concentrated |

## 4. Cosmetic and other applications

2% allowed in EEC; commonly used in bread at 0.15 to 0.4% to prevent ropiness.

## 5. Mode of action

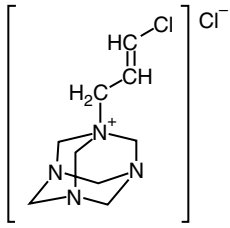
Destroys chemiosmotic balance across the cytoplasmic membrane by disruption of the membrane electrical potential through dissociation of protons into the cell cytoplasm of the cell.

# *Quaternium-15*
# *EEC #48*
# *CAS #4080-31-3*

| | |
|---|---|
| Chemical names: | Cis isomer of 1-(3-chloroallyl)-3,5,7-triaza-1-azoniaadamantane-chloride, N-(3-chloroallyl)-hexammonium chloride |
| Trade names: | Dowicil 200, Dowicide Q, Preventol D1 |
| Type of compound: | Quaternary adamantane |

## *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Cream-colored powder; hygroscopic |
| Odor: | Odorless |
| Solubility: | 127 g/100 ml water; less soluble in propylene glycol (18.7%), glycerol (12.6%), ethanol (1.85%) |
| Optimum pH: | 4 to 10 |
| Stability: | Unstable above 60°C; yellows in some cream formulations |
| Compatibility: | Compatible with anionics, non-ionics, cationics, and proteins |
| Neutralization: | Dilution and peptone |
| Structural formula: | |




Quaternium–15

## *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 200 |
| *Escherichia coli* | 500 |
| *Pseudomonas aeruginosa* | 1000 |
| *Candida albicans* | >3000 |
| *Aspergillus niger* | >3000 |

### 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 0.94 to 1.5 g/kg |
| | Rabbit: $LD_{50}$, 40 to 80 g/kg |
| Primary skin irritation: | Not a primary human skin irritant |
| Guinea pig sensitization: | Nonsensitizing up to 2% |
| Mutagenicity: | Nonmutagenic based on Ames test and unscheduled DNA synthesis |

### 4. Cosmetic and other applications

In-use concentration, 0.02 to 0.3%. In EEC up to 0.2% in shampoos and skin lotions. Preservative for paints.

### 5. Mode of action

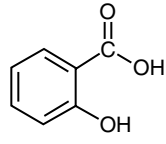
Donates formaldehyde without the release of gaseous formaldehyde. Formaldehyde alkylates amino and sulfhydryl groups of amino acids well as the ring nitrogens of purine bases resulting in protein and DNA denaturation.

## *Salicylic acid*
## *EEC #I/3*
## *CAS #69-72-7*

| | |
|---|---|
| Chemical names: | 2-Hydroxybenzoic acid |
| Trade names: | — |
| Type of compound: | Organic acid |

### 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Crystalline powder; upon heating, it decomposes into phenol and $CO_2$ |
| Odor: | Odorless |
| Solubility: | 1 g in 460 ml water; increased solubility of the salts |
| Optimum pH: | 2 to 5 |
| Stability: | Discolors with iron salts; discolors in sunlight and must be protected from light exposure |
| Compatibility: | Incompatible with iron salts and iodine |
| Neutralization: | pH above pKa; dilution |
| Structural formula: | |




Salicylic Acid

### *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml)* (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 1250 |
| *Escherichia coli* | 1250 |
| *Pseudomonas aeruginosa* | 2500 |
| *Candida albicans* | 2500 |
| *Aspergillus niger* | 2500 |

*Data are for pH of 3.2

### *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 891 mg/kg |
| | Rabbit: | $LD_{50}$, 1300 mg/kg |
| Toxicokinetic considerations: | Salicylic acid is quickly resorbed but slowly metabolized and secreted, running the risk of accumulation. | |

### *4. Cosmetic and other applications*

Used at 0.025 to 0.2%. The EEC concentration limit is 0.5% and use in products for children under 3 is prohibited. Also used as a preservative of food products in a few countries; it is forbidden for such use in others. Typically used as a topical keratolytic agent in antidandruff preparations.

### *5. Mode of action*

Destroys chemiosmotic balance across the cytoplasmic membranes by disruption of the membrane electrical potential through dissociation of protons from the compound into the cytoplasm of the cell. May also denature some enzymes.

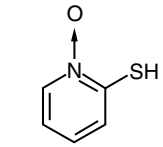
## *Sodium pyrithione*
## *EEC #40*
## *CAS #3811-73-2*

| | |
|---|---|
| Chemical names: | Sodium-2-pyridinethiol-1-oxide |
| Trade names: | Sodium Omadine, Pyrion-Na |
| Type of compound: | Cyclic thiohydroxamic acid salt; pyridine derivative |

## 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | White to yellowish powder |
| Odor: | Mild odor |
| Solubility: | 53 g/100 ml water; 19 g/100 ml ethanol |
| Optimum pH: | 7 to 10 |
| Stability: | Unstable to light, oxidizing agents, and reducing agents |
| Compatibility: | Inactivated by non-ionics; and chelated when reacted with metal ions |
| Neutralization: | Tween 80 or thiol broth and dilution |

Structural formula:




Sodium Pyrithione

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 1 |
| *Escherichia coli* | 8 |
| *Pseudomonas aeruginosa* | 512 |
| *Candida albicans* | 4 |
| *Aspergillus niger* | 2 |

## 3. Toxicity

| | | |
|---|---|---|
| Acute oral toxicity: | Rat: | $LD_{50}$, 875 mg/kg |
| | Mouse: | $LD_{50}$, 1172 mg/kg |
| Subchronic toxicity: | Rat: | Oral administration of 75 mg/kg/day over 30 days or intraperitoneal administration of 40 mg/kg/day over 30 days produced no pathological effects |
| Rabbit eye irritation: | No effect with 10% solution at pH 7.8 to 7.9 | |

## 4. Cosmetic and other applications

Use concentration, 250 to 1000 ppm (as active); EEC guideline is 0.5% for rinse-off products only. Used to preserve cutting oils at 0.005%.

## 5. Mode of action

Several studies have confirmed the hypothesis that pyrithiones destroy the chemiosmotic balance across the cytoplasmic membrane by disruption of

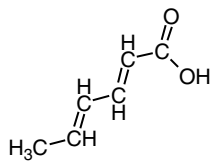
the membrane electrical potential through dissociation of protons into the cytoplasm of the cell. However, there are likely to be other mechanisms extant that may destroy membrane integrity and thus membrane transport. The presence of a reactive sulfhydryl suggests sodium pyrithione would denature membrane proteins.

## *Sorbic acid*
## *EEC #I/4*
## *CAS #110-44-1*

| | |
|---|---|
| Chemical names: | 2,4-Hexadienoic acid |
| Trade names: | — |
| Type of compound: | Organic acid |

### *1. Structure and chemical properties*

| | |
|---|---|
| Appearance: | Crystalline<br>Ca and K salts are white powders |
| Odor: | Faint characteristic odor |
| Solubility: | 0.25% in water; 0.29% in ethanol; 8.4% in isopropanol; 0.5 to 1.0% in oils<br>Potassium salt — 138 g/100 ml water, calcium<br>Calcium salt — 1.2% in water |
| Optimum pH: | Below 6.5 |
| Compatibility: | Incompatible with non-ionics |
| Neutralization: | pH above the pKa and Tween 80 |
| Structural formula: | |




Sorbic Acid

### *2. Antimicrobial spectrum*

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 50 to 100 |
| *Escherichia coli* | 50 to 100 |
| *Pseudomonas aeruginosa* | 100 to 300 |
| *Candida albicans* | 25 to 50 |
| *Aspergillus niger* | 200 to 500 |

### 3. Toxicity

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 7.36 g/kg (acid); $LD_{50}$, 5.94 g/kg (sodium sorbate) |
| Chronic toxicity: | 5% sorbic acid added to animal feed over a lifetime had no negative effects; 10% over 2 yr showed reduction in weight, but an increase in thyroid gland, kidney, and liver |

### 4. Cosmetic and other applications

Antifungal for creams and lotions. Preservative in oral pharmaceutical oral dosages, and in foods and wines.

### 5. Mode of action

Destroys chemiosmotic balance across the cytoplasmic membrane by disruption of the membrane electrical potential through dissociation of protons from the compound into the cytoplasm of the cell.

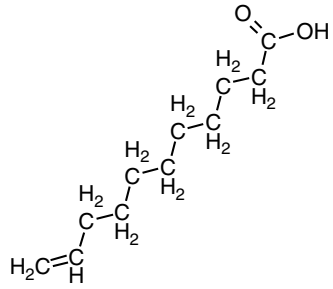
## *Undecylenic acid*
## *EEC #13*
## *CAS #112-38-9*

| | |
|---|---|
| Chemical names: | 10-Undecenoic acid |
| Trade names: | Declid, Renselin, Sevinon |
| Type of compound: | Organic acid; may also be used as the zinc salt or as a monoethanolamide-di-sodium-sulfosuccinate |

### 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | Liquid or crystals |
| Odor: | Sweaty odor |
| Solubility: | Insoluble in water; soluble in alcohol |
| Optimum pH: | 4.5 to 6.0 |
| Compatibility: | Compatible with boric acid and salicylic acid |
| Neutralization: | pH above its pKa; dilution |

Structural formula:



Undecylenic Acid

## *2. Antimicrobial spectrum*

Used as an antifungal agent at 2 to 15% in products for tinea pedis, tinea capitis, tinea cruris, moniliasis, and vulvovaginitis. Antifungal nature is enhanced in the zinc salt form; activity greatest at acid pH.

## *3. Toxicity*

| | |
|---|---|
| Acute oral toxicity: | Rat: $LD_{50}$, 2.5 g/kg |
| Primary skin irritation: | Irritant to mucous membranes at >1% |

## *4. Cosmetic and other applications*

EEC use limited to 0.2%. In the sulfosuccinate form (undecylenic acid monoethanolamide-di-sodium-sulfosuccinate), it is used at 1% in shampoos as an antidandruff agent at pH 5.0 to 6.5. Also used in pharmaceuticals as a topical antifungal at 2 to 15%, often in combination with boric acid and salicylic acid.

## *5. Mode of action*

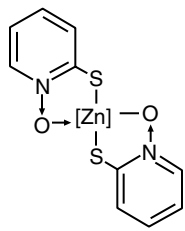
Like many organic acids, it probably destroys chemiosmotic balance across the cytoplasmic membrane by disruption of the membrane electrical potential through dissociation of protons into the cytoplasm of the cell.

# *Zinc pyrithione*
# *EEC #I/8*
# *CAS #13463-41-7*

| | |
|---|---|
| Chemical names: | Zinc bis-(2-pyridinethiol-1-oxide)bis-(2-pyridylthio) zinc-1,1'-dioxide |
| Trade names: | Zinc Omadine, Vancide |
| Type of compound: | Cyclic thiohydroxamic acid |

## 1. Structure and chemical properties

| | |
|---|---|
| Appearance: | White to yellowish crystalline powder |
| Odor: | Mild odor |
| Solubility: | Soluble in water, 15 ppm; in ethanol, 100 ppm; in PEG 400, 2000 ppm |
| Optimum pH: | 4.5 to 9.5 |
| Stability: | Forms an insoluble product with some cationics and amphoterics; unstable to light and oxidizing agents; unstable in acid or alkaline solutions at higher temperatures |
| Compatibility: | Incompatible with EDTA, which chelates out the zinc; slight inactivation by non-ionics |
| Neutralization: | Tween 80 and use of dilution in thiol broth |
| Structural formula: | |




Zinc Pyrithione

## 2. Antimicrobial spectrum

| Test organisms ($10^6$ CFU/ml) | Minimal inhibitory concentration (mg/ml) (Serial dilution test: 24 to 72 hr) |
|---|---|
| *Staphylococcus aureus* | 4 |
| *Escherichia coli* | 16 |
| *Pseudomonas aeruginosa* | 512 |
| *Candida albicans* | 0.25 |
| *Aspergillus niger* | 2 |

## *3. Toxicity*

| | | |
|---|---|---|
| Acute oral toxicity: | Rat : | $LD_{50}$, 200 mg/kg |
| | Mouse: | $LD_{50}$, 300 mg/kg |
| Subchronic toxicity: | Rat: | 10 ppm as food additive over 30 days showed no effect; higher doses toxic |
| Chronic toxicity: | No irritation to humans or rabbits when applied to skin in ointment form ion | |
| Primary skin irritation: | 48% dispersion and powder are irritating to the skin and extremely irritating to eyes | |
| Guinea pig sensitization: | Not an allergic sensitizer | |

## *4. Cosmetic and other applications*

Use concentration is 250 to 1000 ppm in various antidandruff products; EEC guideline is 0.5% for rinse-off products only. Used in sanitization of paper goods, woolens, hospital goods.

## *5. Mode of action*

Several studies have confirmed the hypothesis that pyrithiones destroy the chemiosmotic balance across the cytoplasmic membrane by disruption of the membrane electrical potential through dissociation of protons into the cytoplasm of the cell. However, there are likely to be other mechanisms extant that destroy the membrane integrity and thus membrane transport. The presence of a reactive sulfhydryl suggests it would denature membrane proteins.

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