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Cosmetic preservation

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Synopsis

The proper use of preservatives to prevent microbial contamination of cosmetics is often viewed as an art rather than a science. This view is a result of the multifactorial thinking that has to go into preservative selection. In this general article, an historical and critical review of preservative efficacy tests (PETs) is provided to understand the assumptions inherent in designing PETs. A conceptual framework of microorganisms existing as communities in association with each other is also promoted, which provides a different understanding of how microorganisms contaminate cosmetics and why PETs are often misinterpreted. In addition, the mode of action of preservatives is discussed and contrasted with the mode of action of antibiotics. Finally, the role of the microbiologist is better defined in light of the fact that he or she must have expertise in far more than microbiology alone.

INTRODUCTION

Microbial contamination of cosmetics did not become an issue until about 50 years ago (1). The first microbial contamination observed was probably mold spoilage. Parabens provided adequate protection. During the 1960s, contamination of consumer products by *Escherichia, Klebsiella, Enterobacter, Serratia,* and *Pseudomonas* spp. occurred (2–5), demanding more effective and responsible preservation practices. By the mid-1970s, several cases of blindness due to *Pseudomonas*-contaminated mascaras caused eye cosmetics to be closely scrutinized (6–8). Most products reached the consumer in good microbiological condition, but they could not withstand contamination during use (9–14).

The main issue addressed over the next few years was to develop preservative efficacy tests (PETs) that predicted the risk of consumer contamination. In 1975 and in 1985 the Food and Drug Administration (FDA) gave contracts to develop such PETs. The FDA never published the data from these studies, and no FDA methods were developed. In 1990, the Cosmetics, Toiletries and Fragrance Association (CTFA) published the results of a survey to determine if companies had already correlated their PET data with consumer use data (15). Nearly all the companies claimed they already had correlation programs in place, thus validating the ability of their company's PET to predict consumer contamination potential (16). Inherent in this validation process is the question, "What level of consumer abuse must a manufacturer anticipate for his product?" This question is usually answered with a legal definition: "To a level that is safe under

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ordinary use and foreseeable misuse conditions." Unfortunately, what constitutes foreseeable misuse and ordinary use has never been defined. It is left up to the courts to decide when prosecuting specific cases. In the meantime, the company is left to decide for itself what "ordinary use and foreseeable misuse" really means.

A joint program with the FDA, the CTFA, and the Association of Official Analytical Chemists (AOAC) was established to develop a standard PET in order "to demonstrate the ability of products to withstand microbial insult which may occur during intended use." However, the CTFA/AOAC/FDA collaborative study conducted no consumer use studies to correlate with PETs; consequently, the collaborative study (publication expected in 1996) was not validated by in-use testing. Two PET methods have been published with data allowing prediction of the in-use potential for consumer contamination (17–20). However, neither of these methods is available for replication since they both used challenge organisms unique to the investigators conducting the PET.

THE IMPORTANCE OF PRESERVATION

The cosmetics that most need preservatives are those that contain water. Products with low water activity (non-water based lipsticks, rouges, talcs, and antiperspirants) usually need little more than methyl or ethyl parabens to protect against fungi. Table I provides the water activity and pH limits for microorganisms and relates these to product types in general (21,22). The only limit to microbial life is the availability of liquid water, with microbes being found to grow at extremes of temperatures and pH (23,24). However, most organisms of concern to the cosmetic microbiologist are not extremo-

Table I
Water Activity and the Potential for Growth

Water activity	pH	Problem organisms capable of growth	Examples of cosmetic products
0.98–1.00	pH 5–9	Most Gram positives and negatives	Shampoos and emulsion products
0.95–0.97	pH 5–9	Most Gram positives and negatives (*Pseudomonas* begins to be limited)	Liquid make-ups and eye area products
	Below 5.5	Some Gram negatives and most Gram positives (*Pseudomonas* limited)	Some hair conditioners
0.92–0.95	Above 5.5	Few Gram negatives and most Gram positives	Some pressed powders
	Below 5.5	Most Gram positives	
0.90–0.92	pH 5–9	Gram positive Lactobacilli and *Staph.*	Some rouges (non-water based)
0.80–0.90	pH 5–9	*Staph.*, molds, yeasts	Lipsticks (non-water based)
0.70–0.80	pH 5–9	Molds, yeasts	Some talcs
0.65–0.70	pH 5–9	Osmotolerant yeasts	Some antiperspirants
0.60–0.70	pH 5–9	Osmotolerant and xerophilic molds	
Below 0.60	pH 5–9	None	

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philes, and thus extremes of pH and A_W can be used to control them. Where these extremes cannot be met, a biocide is used to control growth.

Microorganisms metabolize product ingredients using a variety of hydrolytic enzymes to cause adverse changes in product odor, color, and viscosity. Even though health-related contamination incidences related to cosmetics are rare, a few have occurred and include infection from a hand lotion (3), eye infections from use of eye area cosmetics (25), and the death of one immunocompromised individual (26).

Aside from spoilage prevention and health-related concerns, cosmetics also need to be adequately preserved to withstand consumer use. Manufacturing contamination can be controlled with good sanitation. But consumer use and abuse cannot be controlled. Consumers may repeatedly challenge the cosmetic with microorganisms. The bathroom, where most cosmetics and toiletry articles are used, provides heat and humidity needed for microbial growth (27,28).

During use, cosmetics can be contaminated with a variety of spoilage organisms found in the household environment (29, 30). Table II lists some of the microorganisms that contaminate shampoos and skin lotions after consumer use (30). A few of these may invade and create disease (31). With more and more immunocompromised individuals in the population from the pandemic of AIDS, even spoilage organisms may be opportunistic pathogens. The biggest contamination concerns are pathogens that present a frank health risk such as the pseudomonads (32). Cosmetics intended for eye area use are particularly suspect since the cornea, when compromised, is highly vulnerable to infection, and several instances of mascara contamination from *Pseudomonas* spp. have been reported (6–10). Thus, choosing the proper preservative and package is critical to providing appropriate protection to the product.

Table II

Types and Percentages of Microorganisms Contaminating Cosmetics After Use (30)

Organisms	Isolated from shampoo	Isolated from skin lotion
Citrobacter freundii	18	0
Enterobacter spp[a]	37	9
Klebsiella spp.[b]	9	9
Pseudomonas spp.[c]	9	21
Serratia spp.[d]	18	4
GNR[e] (nonfermentative)	0	4
GNR (fermentative)	9	0
CDC serotype IVC2	0	4
Bacillus spp.	0	4
Staphylococcus epidermidis	0	4
Propionibacterium sp.	0	4
Sarcina sp.	0	4
Diphtheroid	0	4
Yeasts and molds	0	29

[a] *E. aerogenes, E. agglomerans,* and *E. cloacae.*
[b] *K. pneumoniae* and *K. oxytoca.*
[c] *P. putida, P. fluorescens, P. paucimobilis, P. aeruginosa,* and *P. maltophilia.*
[d] *S. liquefaciens, S. odorifera,* and *S. rubidaea.*
[e] GNR, Gram-negative rod.
Table adapted from Brannan and Dille (30).

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However, selection of preservatives for a cosmetic is complex. The ideal characteristics of a biocide are that it be safe, stable, and compatible with both the product and the container, be inexpensive, readily available, approved by appropriate regulatory agencies, have a positive consumer perception, and be environmentally friendly. Raw material quality, container and cap design, expected shelf life and exposure conditions, and even how the consumer will use and misuse the product are additional considerations in choosing the preservative system (30,33).

Compatibility of the biocide with other ingredients in the product requires the microbiologist to have knowledge of the art of formulation. Suspended solids in a formulation (e.g., carbonates, silicates, talc, metal oxides, cellulose, and starch) may adsorb preservatives (34). Minor pH changes inactivate other preservatives (35–37). Minor shifts in ionic strength or changes in the buffering system in a product can also alter a bacterium's susceptibility to a biocide or affect how a preservative partitions between the water matrix and the microbial cell (38,39). Parabens provide unique formulation challenges for water-in-oil emulsions because they have an affinity for the oil phase while the microbes live in the water phase (40). Even the surfactant system used can affect biocide performance (41–43). In fact, nonionic surfactants are used to neutralize some preservatives (44–46). However, these same surfactants enhance quaternary ammonium compounds (47). Finally, protein (often used in conditioner and lotions) may also reduce the antimicrobial activity of many preservatives (48–50); the presence of hydrophilic polymers will affect others (51).

Even simply choosing a container requires a microbiologist to check compatibility with the preservative (52,53). The preservative may either be absorbed into the container material in the case of lipid-soluble preservatives, inactivated because of complexation of the preservative with the dyes used in the plastic, or lost because of the volatility of the preservative (e.g., phenoxyethanol, formaldehyde, and ethanol). When considering containers, one should also not overlook the impact that dispensing closures have in preventing microbial contamination, especially during consumer use. Some closures provide more protection of products than others (30). Alternatively, some closures may inactivate the preservative (54).

PRESERVATIVE EFFICACY TESTING

DEFINING THE PURPOSE OF THE PET

Test protocols for determining preservation efficacy in cosmetics vary (55–58). The logic and arguments that go into establishing these protocols are primarily based on consensus. These compendial efforts, such as those developed by the CTFA, are "state-of-the-art," but they are not rigidly controlled protocols subjected to multiple laboratory replication and statistical analysis. Nevertheless, they have been useful. The CTFA/AOAC/FDA collaborative program mentioned previously may fill this gap despite not being a method that has been validated to predict consumer contamination potential. To develop such predictive tests, a company must employ a microbiologist who conducts a validated "in-house protocol" that is specific for the company's products. Alternatively, the protocol developed by the company may be contracted out to laboratories capable of conducting PETs.

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A major difference between PETs is due to a lack of understanding of the purpose of the PET. Defining the purpose of the test is critical. The entire experimental design for validating the PET will differ depending on the definition of purpose. The experimental design for validating use of a PET as a predictor of the potential for consumer contamination is different from that for validating use of a PET to demonstrate the presence of the preservative or as a predictor of potential for manufacturing contamination. To validate a PET as a predictor of consumer contamination requires prospective correlative consumer studies or retrospective validation, based on lack of consumer complaints, to corroborate the PET laboratory results. Regardless of which philosophy one adopts to define the purpose of a PET, at a minimum the goal should be to develop a data base to rank the antimicrobial hostility of the company's products.

The purpose of a PET as viewed by the FDA is to predict consumer contamination potential (59,60). The FDA has tried several times to develop a PET for this purpose without success. Products failing such a test would be subject to recall. Despite the collaborative work between CTFA, AOAC, and FDA to develop a standardized PET complete with multi-lab comparisons and statistical analysis, the method has not yet been demonstrated to have the ability to predict a product's ability to withstand microbial insult that may occur during intended use, since no correlative consumer studies were conducted using the same products for which the PET was conducted. Such an omission is fortunate. If such a standard PET were developed that was predictive of consumer contamination, then it could be used to enforce a recall on those products that fail it. One could counter the recall by pointing out that a PET does not account for consumer use and packaging parameters. One might also counter this argument with the observation that if the PET is done on freshly made product, then the PET data would only apply to freshly made product. Since most companies conduct PETs on shelf-aged product, such a statement would be admitting that they are out of line with the majority of reputable companies and have products that become a risk over time.

Several publications have already shown that a modified version of the CTFA preservative efficacy test is a valid predictive model of the risk of consumer contamination (17–20), but these all used proprietary "in-house" organisms unavailable to others. Thus, the CTFA test described by these publications does not provide a standard PET that could be used to enforce a recall as described above. Another study has compared several PETs for the ability to predict "in-use" contamination (61). The major criticism of this work is that the in-use test was merely simulated. The subjects dabbled with the product after rubbing their underarms with their fingers. The significance of ranking PETs against their ability to predict how well a product can withstand simulated consumer use does not represent validation against true in-use conditions. Nevertheless, since all the PETs were ranked against a single standard, one can still derive considerably useful information. For example, nearly all the compendial tests adequately separated poorly preserved from well-preserved products. Some of the more conservative tests classified marginally preserved products the same as poorly preserved ones, while the more liberal tests allowed marginally preserved products to rank with well preserved ones. The CTFA test exhibited the tendency to rank all three (poor, marginal, and well) correctly against the flawed but useful standard of a simulated in-use test. This study does not, however, support the use of the CTFA test to enforce recalls, since the comparison was against an invalid simulated in-use test.

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COMMON ISSUES SHARED BY PETS

PETs are conceptually simple. All the protocols involve introducing microorganisms into the cosmetic. All the protocols have the same general steps: product preparation, inoculum selection and preparation, inoculation, incubation, plating and estimating the surviving microorganisms, and interpreting the data. The differences are in how these steps are conducted. These differences are described below.

Product preparation. The product sample used in a PET should replicate all the parameters for national distribution, including formulation, packaging, and manufacturing conditions. Raw materials should be of the same quality and from the same source as expected for national distribution. Even minor changes for adjusting viscosity, color, perfume, pH—even changes in process water—may adversely affect a product's PET results. Scale-up from lab to plant also provides opportunity for variables that need to be identified and controlled so the final nationally delivered product will be adequately preserved.

Most published preservative methods test full-strength (100%) product (15). However, Brannan *et al.* (17,18) and Cooke *et al.* (62) added diluted product to the challenge test as well. The dilutions stress the product and also provide a means of ranking the product. For example, products that can be diluted and continue killing the inoculum may be classified as well-preserved, whereas products that kill the inoculum only when the product is at full strength may be adequately preserved if the packaging prevents consumer contamination during use. In addition, this approach is another way of mimicking expected use patterns. For example, products that are diluted during foreseeable misuse, such as shampoos, should be able to continue killing microbial challenges. Finally, dilution mimicks potential manufacturing errors, particularly those involving washouts where diluted product is accidently left in a line. If a product can remain hostile when diluted, then microorganisms are less likely to be selected to be resistant in the biocide. If not, then the organisms are selected for survival at diluted biocide concentrations and are just a minor step away from being selected for growth in full-strength product.

Inoculum: Selection and maintaining resistance. An appropriate microbiological challenge of the product is the most critical factor in determining the validity of a preservative efficacy test. All the tests currently specify a set of inoculum microorganisms. Some of the methods list specific strains from ATCC, while others also allow inclusion of other organisms the microbiologist chooses. These choices often include preservative-resistant strains from consumer-used product samples, raw materials, or manufacturing sites. However, use of these resistant organisms may be considered a form of abuse testing by some.

Use of these special strains, however, should be reevaluated if one has not maintained a rigorous program of preserving the originally isolated culture. More often than not, one maintains a culture collection by putting up an original set of vials. When the last remaining vial is subcultured, an isolated colony (obtained by streaking for isolation) is selected to grow up and harvest. This culture is preserved in another set of lyophilized vials. Unfortunately, this process represents a departure from the originally deposited culture because the progeny of only a single individual was selected to represent the original population.

Routine subculturing on nonselective growth media will also cause the loss of preservative resistance since the selective pressure of the preservative is no longer present. The

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likelihood of selecting organisms without the resistant factor is high when using traditional "streak for isolation" pure culture concepts. Instead, one should rely on assessing the purity of the population by its homogenous appearance on a lawned agar plate. Preferably this should be done on a medium that has the preservative in an active state (not neutralized) incorporated into the agar. Once the population is grown up as a lawn, the entire lawn should be harvested for freezing or lyophilization.

An area needing more research is the effect of growing the inoculum in broth or on solid media. Greater resistance to preserved product has been described for broth-grown cultures compared to cultures grown on solid medium. However, this result may have been due to the carryover of broth into the product acting as a neutralizing agent of the preservative in the product rather than to any intrinsic resistance gained by the bacteria by growing in broth (63,64).

Another area needing further research is the investigation of the importance of the growth phase of the challenge inoculum. The growth phase affects the physiological state of the organisms used as the inoculum. For example, Holm-Hansen found that ATP per cell is decreased as cells reach stationary phase (65). This physiological change and potentially other changes may affect an organism's resistance to preservatives.

Inoculum: Concentration and rechallenge. In the CTFA's PET, the recommended inoculum level for bacteria is 1×10^8 colony-forming units per milliliter (CFU/ml). If 20 grams of product are inoculated with 0.2 ml (a 100:1 ratio), then the final CTFA recommended concentration of 1×10^6 colony-forming units per gram (CFU/g) of product is obtained. Other PETs may use different inoculum levels. The key issue is to keep the dilution of product by the inoculum to a minimum; a good rule is to not dilute the product over 1% with the inoculum. In like manner, fungi and yeast are introduced into the product. However, their concentration is only 1×10^4 CFU/g of product in the CTFA method. The assumption that these counts represent fungal spores may not be valid since hyphae can also give rise to fungal colonies.

How to standardize the concentration of the inoculum is left up to the microbiologist in the CTFA method. A transmittance of 30–40% at 425 nm of bacteria suspended in buffer will usually yield 1.0×10^8 CFU/ml. However, any reference to standard microbiological methods will provide the specifics for determining microbial concentrations (66).

Rechallenge is the addition of fresh inoculum to a product that has already killed off the first challenge after an appropriate time. CTFA provides for a rechallenge if desired but does not require it. Some studies suggest this practice does not provide any more information than single challenges (67). The manufacturer, however, may be able to make a case for multiple challenges. For example, mascaras are commonly subjected to repeat insults by the consumer. In this case, the microbiologist should select a challenge level that is reasonable and likely from consumer use (1×10^2 CFU/gm) rather than the high levels recommended in the compendial methods. These levels could be determined by allowing people to use unpreserved products and analyzing the level of organisms introduced into the product after that use.

Another area of concern regarding the inoculum is whether or not to use pure or mixed challenges. This question refers to the use of several pure cultures that are mixed together after they were grown up and harvested. Use of this mixed inoculum may be more representative of actual conditions of contamination since microorganisms do not

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exist as pure cultures in nature but as interacting populations within communities of microorganisms. If one assumes that co-metabolism or synergism occurs within a community biology dynamic, mixed cultures may provide greater stress to the preservative system than pure challenges (68). In fact, cometabolism and vitamin and cofactor synthesis help stimulate mixed interactions within communities of microorganisms (69,70). The idea that such interactions occur during a PET is supported by the observations of Henriette *et al.* (71), who described a mixed-bacterial community that developed in disinfectants and antibiotics. None of the individual species were resistant to the antimicrobials. Only the community showed resistance.

In contrast to the above, however, it is the idea that mixed populations are more robust that forms one of the objections to their use. The claim is that it introduces the variable of microbial population dynamics into the challenge test. Alternatively, some feel that the mixed cultures may be less stringent than pure challenges because one organism may produce metabolic factors that are antagonistic against other microorganisms in the challenge (72) or that the organisms will compete with each other for limiting substrates and growth factors such as iron (73). Resolution of the issue will take more research.

Plate counts and other assumptions. There are two assumptions that microbiologists make that are false regarding plate counts . . . and yet we still rely on them: 1) one organism gives rise to one colony, and 2) organisms are evenly distributed as single cells and do not exist as clumps. A new paradigm of organisms existing as nonuniformly distributed clumps that later break up into individual cells may help to explain the anomalous results one occasionally gets in preservative efficacy testing. It must be emphasized that the following is only a model as it applies to preservative testing. It is, however, a valid model since it is based on a historically well known fact that organisms do exist predominantly in clumps rather than as single individuals, even in shake flask cultures (74,75). It is also based on reports about the clumping nature of bacteria due to hydrophobicity (76) and on the newer reports about biofilm and aggregate formation, particularly when exposed to biocides (77).

The following enigmatic scenario is sometimes seen during a PET. An initial kill occurs at 7 days (seen as a decrease in CFU) but is followed by an increase in CFU at 14 days, followed by another decrease at 21 days. Usually this is passed off as experimental error such as use of the wrong culture conditions or recovery system or incorrect dilution/ pipetting techniques. Occasionally one gets these results despite controlling all these factors. When this happens, the experimenter may pass off the result as an anomaly of biological systems. However, all these explanations assume that a CFU comes from single organisms that are evenly dispersed throughout the sample.

Let's explore the new paradigm that provides at least a hypothetical model that may help explain these results better. Most people working with bacteria exposed to disinfectants and antibiotics are very well aware that bacteria do not exist as uniformly distributed individuals but as biofilms and as Poisson-distributed or negative binomial-distributed clumps or aggregates (66,78–80). If one uses the paradigm of microbes existing in aggregates (or clumps), the enigma may be explained without having to claim "experimental error" (Figure 1). The initial kill at 7 days may have been due to killing of the cells in smaller clumps, where the entire clump of cells is killed but the larger clumps have a few cells within them that remain alive because they were protected. Our model is that CFU are really derived from clumps rather than individual cells. The surviving

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cells in clumps then disperse to result in single cells that give a higher CFU at 14 days but now are more susceptible to the biocide, and so a reduction follows at 21 days. Although this model needs further testing, it is satisfying that it logically describes what has heretofore been passed off as experimental error or biological variability or the development of resistance.

In doing plate counts, one can use either pour plates or spread plates to determine how many CFU/gm survive. In pour plates, the diluted product (about 1 ml) is vortexed into a test tube of about 15 ml of melted agar at 46 ± 2°C. The agar is then poured into a Petri dish. Alternatively, the dilution may be placed into the Petri dish and agar poured on top of it while the experimenter swirls the plate in a "figure 8" motion. With spread plates, the diluted product (about 0.1 ml) is spread onto prepoured solidified agar plates using a bent glass rod. Spread plating allows easy processing of samples. The main advantage is that it avoids exposing microorganisms to heated media. However, pour plating allows for more exposure to neutralizing agents in the agar. Some published information finds that the two methods give similar results (81,82).

One can also perform enrichments of the product to detect low levels of potentially

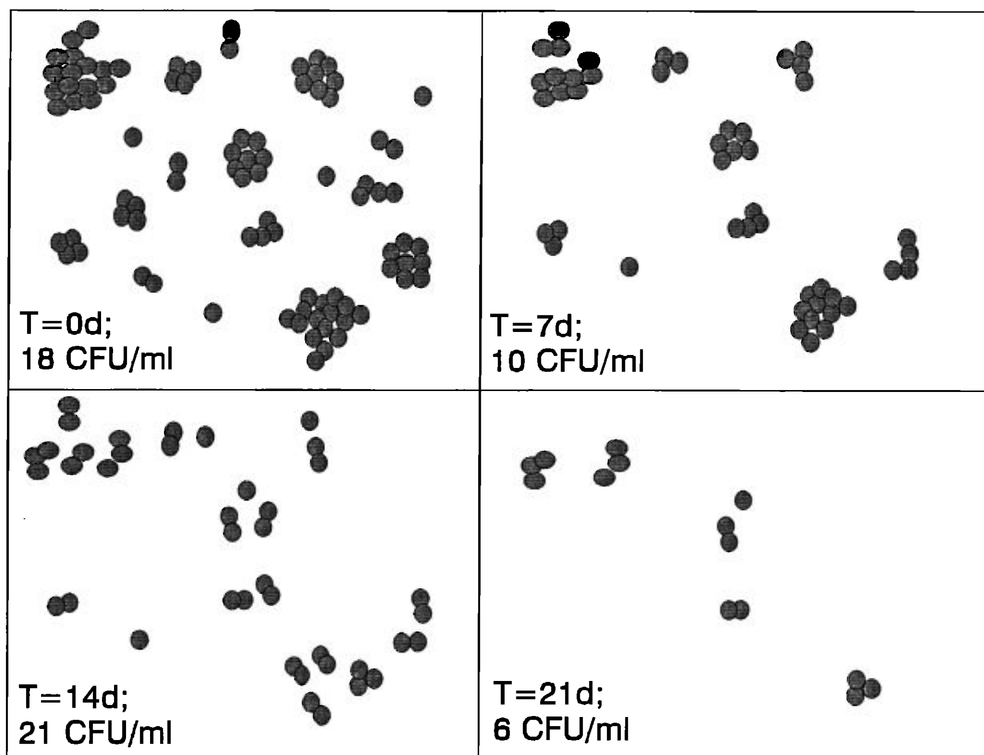

Figure 1. Bacteria exist as clumps in Poisson distribution. This model helps explain anomalous results in PET testing. Each clump gives rise to a colony rather than each individual doing so. After 7 days, the CFU/ml is reduced due to the death of organisms existing outside of the protection of the clump. A few of the organisms at the periphery of the clump are killed, but the clump still forms an individual colony. The fact that some of the organisms in the clump died is not detected upon plating. After 14 days, the clumps break up to provide more CFU. Now the individuals are no longer within the protection of the clump and are more susceptible to exposure to the biocide. Therefore, at day 21 the total CFU is decreased.

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recoverable organisms (83). The sensitivity or detection limit of typical dilution and plate count methods is usually from 10 to 20 CFU/gm of product. In enrichment, at least 10 g of product is put into 1 liter of broth and incubated. Any turbidity (or color change if one uses either a pH or redox indicator) indicates at least one organism was present in the 10-g sample. This approach makes the detection limit 1 CFU/10 gm of product (theoretically 0.1 CFU/gm). Thus the sensitivity is increased by 100× compared to traditional plate count methods. It is most useful in determining if, after the 28-day period, low levels of inoculum still exist that may still be capable of growing later on.

Adaptation and resistance. All of the recognized tests require long incubation periods (28–56 days). These long periods are supposed to account for the phenomenon of adaptation. After some lag phase the microbes "grow back" to high enough levels to be detected again. The mechanism(s) for this regrowth are not well understood. Perhaps it is due to survival and adaptation. Perhaps it is due to *in situ* recovery of injured organisms (84). It may be due to container-associated organisms that slough off into the product (85,86). It may even be due to inadequate mixing and inconsistent plating methods, since bacteria display Poisson distribution in the sample. The paradigm of organisms existing as clumps is also a possible explanation to help explain "grow-back," without needing to claim microbial adaptation, or recovery of injured cells or the "Phoenix Phenomenon" (87,88). These latter two explanations need not be the sole or even primary explanations; the clumping paradigm also explains what appears to be anomalous results when cells die off but then "recover" during a PET. Whether or not the clumping paradigm is a more valid explanation for these anomalous results than adaptation or the "Phoenix Phenomenon" remains to be shown empirically.

Certainly there are cases where adaptation occurs. However, where adaptation is claimed for preservatives that have multiple modes of action, resistance is rarely via an individual occurrence of plasmid acquisition, mutation, or lifting of repression (89), as is often found with antibiotics but rather is a result of enhancement of the expression of a characteristic within a population due to genetic drift. This may occur as a shift in the amount of capsule production, clumping, stimulation of production of glutathione, or even physical community developments within biofilms where certain organisms exist as protector guilds for other organisms (90–92). Such resistance is typified by whole-cell poisons such as chlorine (93,94). Few cases of true chlorine resistance occur (e.g., point mutations by a single mutant cell that survives). Instead, any "resistance" seen is really a population or community effect of cells existing within the protection of a biofilm or surrounded with a capsule composed of extracellular polymeric substances that excludes the chlorine or use of cellular energy to produce higher levels of glutathione (90,95). Perhaps in these examples a more proper term to use would be biocide "tolerance" rather than resistance. The establishment of a biofilm or clumps of organisms provides an adaptation mechanism for tolerance to biocides using extracellular polymers in the form of a capsule. This biofilm then leads to an inoculum source that is constantly being exposed to sublethal or subinhibitory levels of biocide. Once established, adaptation via increased production of glutathione or a slowdown of metabolism (or even perhaps mutation) can result in a resistant phenotype (or even genotype), and the problem becomes compounded (personal communication, J. S. Chapman, Rohm, and Haas).

Genetic adaptation to biocides at the individual rather than population level is a possibility in some cases (96). However, several papers claiming to have demonstrated this

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phenomenon are either a case of neutralization of the biocide (by carryover of the growth medium) or a case of saturating the biocide with more organisms than available biocide to the point of inactivating it (97,98). Specific genetic mechanisms (e.g., point mutations, plasmid acquisition, lifting repression) or the expression of formaldehyde dehydrogenase do exist in some cases (99, 100). The hallmark of whether or not a permanent genetic adaptation has occurred is the stability of the resistance in the absence of selective pressure from the presence of preservative, as apparently is the case for parabens (101). However, resistance to all biocides by permanent genotypic change must not always be assumed. The most naïve idea is that the resistance mechanisms against biocides are similar to those mechanisms found in antibiotic resistance. Whereas antibiotic resistance can be described based on specific molecular activity at specific sites, the resistance to biocides cannot. Often the resistance to biocides must be maintained at a population level by continuously culturing the organism in the presence of the preservative to maintain the selective pressure on the population. This selective pressure causes the population to develop higher capsule production, which enhances clumping associations, and the production of biofilms. Alternatively, enhancement of the expression of glutathione synthetase could also occur within the population to provide resistance to some biocides (102). Take the selective pressure away, however, and this expression stops, indicating that a permanent genetic change within individuals did not take place but rather that population shifts occurred.

Use of neutralizers. Appropriate use of neutralizers is often overlooked when conducting PETs. Some preservatives only require dilution in buffer to be inactivated. Others require chemical neutralizers used in the diluent or the plating medium, or both. Filtration is another approach but is limited to those products that can be filtered. The work of Sutton and others describes a number of neutralization methods for preservatives as well as a scientific basis for their evaluation (103–107).

The goal of a neutralizer is to inactivate the biocide before the biocide inactivates the microorganism in order to provide uninhibited microbial growth. Failure to inactivate the biocide immediately upon sampling causes one to overestimate the killing potential of the biocide. This failure is actually a measure of the kill that continues within the plating medium because the active biocide is carried over into the medium (108). A fairly effective all-purpose (universal) neutralizing medium is Dey-Engley broth (109). Dey-Engley broth is described further in Atlas' *Handbook of Microbiological Media* (110) and the *Difco Manual* (111). A thorough review of this and many other neutralizers may be found in the articles by Russell (84) and Sutton (112).

The ASTM provides a method to determine if a neutralizer is nontoxic and effective, using microorganisms as biological indicators (113). This method is a retroactive check for neutralization. It is done by streaking plates showing no growth with test organisms. The streak is done 48 hours or more after the inoculated product was originally plated. Since this streak is done so long after the initial plating, the retroactive test only proves that neutralization finally occurs after allowing the biocide to incubate in the medium for some length of time; it does not prove that neutralization occurred instantaneously when the product containing the biocide was mixed into the medium. Retroactive checks of neutralization, and thus the ASTM method of neutralizer validation, are invalid.

Interpretation of data. Interpretation of the data using the criteria set by the compendial

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tests is based on anecdotal evidence and opinion regarding how long a product should take to reduce the numbers of the challenge inocula. The best way of interpreting the data, however, is to compare how the test product performs against how well-preserved and poorly preserved control products perform. Well-preserved products are those that do not become contaminated during consumer use, and poorly preserved products are those that do become contaminated when used by consumers.

OTHER PET METHODS

D-value methods. Rapid tests are sometimes used for quick impressions of which preservatives to use in a product. One such method is the D-value method. Aside from one author, no one else claims D-value methods are valid for final testing of nationally distributed product (114). In fact, D-value methods are inappropriate for at least some consumer products (115). This method is actually an adaptation from food microbiology's heat or radiation destruction D-values.

Heat and radiation kills do indeed follow first-order rate kinetics, and therefore the D-values determined for them are quite valid. However, biocide kills follow second-order rate kinetics (115,116). The only case where a second-order reaction can approach pseudo–first-order rate kinetics is when the second reactant (biocide) is present in such large excess that it is virtually in constant concentration. Preserved products do not have an excess of preservative such that the biocide remains in constant concentration when contamination occurs (117). A biocide–organism reaction is stoichiometric; the biocide does not act like an enzyme that catalyzes a reaction where live organism goes to dead organism, but the biocide is not spent. Therefore, since the biocide–organism reaction is second order, with the biocide serving as the limiting reactant, D-value tests based on first-order rate kinetics are invalid (115,117).

The second criticism of the D-value technique is that it extrapolates beyond the measured data by falsely assuming a linear relationship between biocide exposure time and the number of surviving microorganisms. In defense of rapid D-value methods, however, one may find they allow a preliminary screening of preservatives. This approach assumes that appropriate reproducible controls are in place such that one will be able to rank the various D-values for a wide variety of products and be able to correlate that data to full-scale PET results on the same products.

Capacity tests. A capacity test determines how many bacterial challenges are needed before the product begins growing microorganisms (118). After each challenge, the products are sampled and challenged again until the product either receives 15 challenges without showing growth (a well-preserved product) or until three consecutive positive results occur (a lesser-preserved product). The goal is for the product to reduce the number of viable organisms by 3 logs (99.9%) in 48 hours. With each subsequent challenge, this ability diminishes as a result of dilution, neutralization, and reaction with the inoculum. The claim, by some studies, that multiple challenges provide no more information than single challenges (67) may actually be more pragmatically based than scientifically based. The reason why multiple challenges with low levels of organisms are not the same as one challenge with a high level is similar to the concept of the Danysz phenomenon in immunology (119), where when a high level of inoculum is used, the biocide combines with an equivalent amount of microbes, allowing the challenge to be killed, but when challenging multiple times, each challenge combines

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with more than its equivalent amount of biocide, leaving insufficient biocide to react with subsequently added microbes in the challenge (120). The value in such a test may be for multi-use products. However, for a more predictive test of consumer contamination potential, one should lower the challenge inocula to levels likely to be encountered during use. Once done, the capacity test may be a quantitative and valid way to understand a product's ability to handle contamination from use.

PRESERVATIVES AVAILABLE FOR USE

MODE OF ACTION OF PRESERVATIVES

The mode of action of antibiotics is known at the molecular level since they act via specific biochemical reactions. In contrast, the modes of action of preservatives and biocides are far more generalized,with numerous points of attack. Nearly all biocides work by denaturing cellular proteins or by affecting membrane permeability so that either transport or energy generation is blocked. For example, chlorine oxidizes reduced sites of organic compounds, including proteins, throughout the bacterial cell. Protein denaturants also include formaldehyde, formaldehyde releasers, isothiazolinones, and bromine compounds.

The parabens and weak acids (e.g., sorbic, benzoic, and dehydroacetic acids) disrupt control of membrane electrical potential to block energy generation and nutrient transport (121). Thus the parabens apparently inhibit nutrient uptake by shutting down permeases, disrupting porin channels, or by disrupting the membrane pH gradient or electrical charge potential across the membrane to prevent substrate transport and ATP generation. This inhibition is apparently reversible and is consistent with other observations that the mode of action of parabens is by disruption of the membrane electrical potential (122).

Organic acids probably work in the same fashion (123); however, they may even be enzyme inhibitors as well (124,125). Typically, they are only biocidal at pH values below their pK_a. In this protonated form, they pass through the membrane, and the hydrogen ion dissociates from the weak acid to decrease the cytoplasmic pH. As a result, both substrate transport and oxidative phosphorylation are uncoupled from the electron transport system. This effectively starves the cell of needed substrate and energy derived from ATP synthetase driven by hydrogen ions.

Phenolics disrupt the proton motive force of the cell membrane (126,127). They also have the ability to non-specifically denature cytoplasm, cell walls, and cell membranes (128). The more lipophilic phenolics have the greater antibacterial capacity perhaps because of a greater ability to partition out of the water phase and into the lipid membrane (129,130). Alcohols likewise disrupt the membrane, causing permeability loss (131), and they also appear to inhibit enzymes (132).

Perhaps some of the most widely used of the newer preservatives are the isothiazolinones. These are usually compounded into a single product composed of chloromethyl-isothiazolinone and methyl-isothiazolinone, but they can also include benzyl-type compounds (133,134). The isothiazolinones inhibit glucose oxidation and active transport without significantly affecting membrane integrity (135). In fact, these compounds denature enzymes and other proteins containing thiol groups (e.g., ATPase, glyceral-

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dehyde-3-phosphate dehydrogenase, and asparaginase). Initially, the isothiazolinone forms a disulfide link with the thiol group on the amino acid. Occasionally, the chloromethyl-isothiazolinone may facilitate linkage with another thiol group to establish a new disulfide linkage and release the biocide as a mercaptoacrylamide. This mercaptoacrylamide can tautomerize to a thioacyl chloride that may react again by denaturing nucleic acids (136).

Formaldehyde also denatures protein but by alkylating amino and sulfhydril groups; it can also alkylate the nitrogens of purine rings to denature DNA (137). Most of the formaldehyde donors (e.g., DMDM hydantoin, imidazolidinyl urea, Quaterium 15, polymethoxy bicyclic oxazolidine, etc.) act in this basic manner since these compounds release formaldehyde into the product or the microbial cell. Differences seen between the formaldehyde donors may exist as a result of when or what triggers the compound to release or "donate" formaldehyde. For example, a compound with a long hydrophilic chain connected to the formaldehyde-donating region (e.g., polymethoxy bicyclic oxazolidine) may release formaldehyde only when the long chain enters into the lipopolysaccharide portion of the membrane.

Brominated compounds such as bromo-nitropropanediol and bromo-nitrodioxane act by oxidation of thiol groups (138–141) or by causing thiols to convert to disulfides (142, 143) where the thiol group first becomes brominated and then reacts with another thiol group to yield a disulfide and free bromide. As a result, enzymes involved in respiratory activity (e.g., dehydrogenases) and nucleic acid synthesis are inhibited, cell membrane integrity is compromised, and the cell wall may even be affected (144).

One compound that is not technically a biocide but rather a biocide adjuvant is ethylenediamine-tetra-acetic acid (EDTA). This and other chelating agents remove magnesium and calcium divalent cations from the cell wall, which is needed for stability (145). Once destabilized, they permit easier access of biocides into the cell.

SELECTION OF PRESERVATIVE

The ideal preservative would be broad-spectrum, safe and completely free of any sensitization issues, completely water-soluble, completely stable to all extremes of pH and temperature, completely compatible with all ingredients and packages, and impart no color or odor to the product, be inexpensive, and comply with government regulations. This ideal does not exist. One must select a preservative based on empirical testing. The only approach bordering on a theoretical basis for choosing a preservative is a qualified microbiologist's intuition, finely honed by experience. Selection of preservative may also be from published lists of available preservatives (146, 147). These provide good sources for getting ideas of what might work in a formulation. Every formulation must be considered unique. Factors such as the physical and chemical nature of the product, how it is to be used, the container type and closure, and the shelf life must be considered when choosing the preservative (30). Often the selection of a preservative must be a compromise between efficacy, stability, and safety. More detail on the selection process of preservatives can be found by referring to several books and articles on the subject (148–150).

SAFETY CONSIDERATIONS OF PRESERVATIVES

One must always balance the risk of microbial contamination with the risk that a biocide

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may give to a product. For example, many eye area products were permitted to contain phenyl mercuric acetate because the risk of infection to the eye was greater than the risk of exposure to the compound. The key consideration is to judge whether the product will be safe for the consumer under normal use and foreseeable misuse conditions.

One of the first considerations of a preservative is its acute toxicity. Ocular irritation and subchronic and chronic toxicity tests are performed via the expected consumer exposure route to determine at what level the preservative can exhibit any irritant, toxic, or carcinogenic properties. Perhaps more important than these tests are the skin responses to biocides. Basic irritant responses can be a result of corrosion, acute irritation, cumulative irritation, or photoirritation.

Skin sensitization is another key concern when using biocides. Nearly all biocides used today will elicit sensitization. Sensitization testing is performed in much the same way as irritant patch testing, except that much lower concentrations and repetitive applications are used. Another concern for biocides is mutagenicity testing to determine if the biocide has the potential for mutating somatic or germ cells. In addition to this testing, embryological (or developmental) toxicity testing is done to determine if the biocide may be a teratogen capable of causing birth defects.

In all these tests, the results must be compared to the ordinary-use and foreseeable-misuse exposure levels to give us a reasonable risk assessment. The definition of reasonable risk must include considerations based on the benefits from using the biocide, the ability to use less risky biocides for the same use, the economic benefits from using the biocide (can the biocide help prevent costly recalls due to contamination?), even how the biocide may affect the quality of life, the environment, and public opinion of the company. More detail on the safety considerations of cosmetic ingredients may be found in books by Waggoner and Whittam (151,152).

CONCLUSION

This article does not detail or discuss the pros and cons of the various methods used in cosmetic microbiology. There are plenty of references available from which the serious student can get this information (56,153–156). Regardless of which methods are in use by any particular company, the fact that the cosmetic industry has been so successful in providing adequately preserved products for its consumers is commendable and reinforces the wisdom that we are capable of self regulation.

The cosmetic microbiologist must balance a variety of factors to provide for safe, unspoiled quality products (157). In addition to knowing preservatives, he or she must understand microbial physiology, pathogenic microbiology, and microbial ecology. In addition to microbiology, he or she must understand organic and physical chemistry, toxicology, engineering, manufacturing and processing, sanitation, and regulatory/environmental law. The cosmetic microbiologist must use all this education and knowledge within the context of the business needs of the company and be able to balance risk/benefit to the consumer using the product. Finally and most importantly, this person must have the highest of ethical standards, considering himself or herself as part of the cadre of health care providers in the world dedicated to serving humankind via the mission of providing microbially safe and efficacious products.

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