Preface

In December 1967, the Society of Cosmetic Chemists of Great Britain and the Toilet Preparations Federation set up a Select Committee of three members of each body with the following terms of reference:—

"To investigate and report upon the desirability and/or advisability of recommending legislative control in the interests of public safety, as to the ingredients in and composition of toilet preparations and the uses for which they are promoted and to recommend the form and nature of such control, bearing in mind, in addition, international harmonization of such control."

The Committee completed the production of a Code of Good Practice early in 1970. The original text of this forms Appendix A of this monograph.

The Select Committee will be publishing an appendix to the Code setting standards for hygiene and microbiological safety, and may amend these from time to time. Readers are urged to obtain a set of these as soon as they are available to use in conjunction with this monograph.

As the Select Committee felt it was not appropriate for it to give guidance on methodology, the Society of Cosmetic Chemists of Great Britain are publishing this monograph as an aid to their own members and to any other interested scientists in ensuring adherence to the standards laid down from time to time in the Code of Good Manufacturing Practice.

1. Introduction

1.1 *The problem of manufacturing hygiene*

Every responsible person concerned with the manufacture of toiletry and cosmetic preparations is aware of the need for clean operating conditions. The need to include a preservative in order to prevent microbial spoilage of susceptible products is also universally accepted. Good hygiene and efficient preservation call for detailed consideration of many complex factors. This monograph, prepared by a working party on behalf of the Society of Cosmetic Chemists of Great Britain, has been ratified by the Council of the Society. It is a guide to assist in solving the various problems likely to arise.

Fortunately there is little evidence that actual harm has ever been experienced by users in consequence of infection transmitted by a cosmetic or toiletry product. Nevertheless, complacency could be dangerous. Microbial deterioration does not always give rise to obvious changes in a product and identification of the source of skin infections is often difficult. The importance of recognizing and excluding product contamination is underlined by the fact that many species of micro-organisms once believed to be harmless are now known to be capable of assuming a pathogenic role. It is therefore incumbent upon the manufacturer to institute an adequate routine of microbiological quality control. Monitoring the microbiological status of toiletries and cosmetics should preferably be accorded comparable importance to the control of chemical purity. These contentions imply that personnel adequately trained in microbiology should be available to the control laboratory establishment of a toiletries or cosmetics manufacturer. A microbiologist engaged in quality control should have adequate authority to discharge his duty within the provisions of the Code of Good Practice for the toiletry and cosmetic industry. Products under test for microbial contamination may need to be held for several days before release for sale.

1.2 *Standards for microbial contamination*

If it were feasible to apply an unqualified requirement that every product should be sterile, or, in other words, totally devoid of living micro-organisms, the control problem would be relatively simple. Despite the theoretical attractions of this approach, however, it must be recognized that cosmetic preparations are not usually sterile and the achievement of such a standard would result in a great increase in production costs. There is no evidence that this would be justifiable in terms of any real additional

safeguard to the public. It is therefore necessary to examine whether special circumstances exist for which an absolute standard of sterility is warranted and, in other cases, to what extent the requirement may reasonably be relaxed.

Some authorities favour an acceptance target representing a particular level of microbial contamination such as 100 micro-organisms per gramme. Contamination levels are apt to vary widely in a comparatively short space of time and the particular species of micro-organisms present may be far more significant than the actual numbers. This approach without qualifications therefore has little to commend it.

Standards which apply the most stringent requirements when these are particularly appropriate and which are less rigid in other circumstances, will give adequate protection for the consumer without a prohibitive increase in manufacturing costs.

A set of Standards is being prepared by the joint Select Committee of the Toilet Preparations Federation Ltd. and the Society of Cosmetic Chemists of Great Britain. To avoid confusion, separate recommendations are omitted from this monograph, which therefore deals mainly with methods for achieving a satisfactory level of manufacturing hygiene and techniques for establishing preservation capacity and for monitoring the extent of microbial contamination.

1.3 *Definitions*

For the purpose of this monograph, "toiletry" and "cosmetic" are taken to be synonymous.

All temperatures are given in degrees Celsius.

2. General considerations

2.1 *Compliance with standards*

Standards need to be considered in relation to the efforts necessary to achieve them and the methods to be employed for verifying compliance. For example, the selection of raw materials and packaging components free from or having a low incidence of microbial contamination will materially help to avoid a high level of contamination in the finished product; the bacterial content of water used in manufacturing processes is of particular importance in this connection. Similarly, processing conditions should be carefully scrutinized to limit adventitious contamination. Factory design and lay-out, personnel training and arrangements for cleaning

equipment all contribute materially to the problem. It is impossible, however, to define in a single document exactly how every product should be manufactured and tested, if only because the contamination hazard is closely related to the scale of manufacture which differs greatly from one firm to another and from product to product.

In the process of verifying compliance with standards or of monitoring the microbiological condition of production arrangements, attention will have to be paid to the question of representative sampling. When there is a moderately high level of contamination in the material being sampled, whether or not the product is distributed in separate small units (e.g. the finished packs) and the contamination evenly spread throughout, the sampling problem is unlikely to be highly critical since precise knowledge of the level of contamination is seldom essential. If the contamination is at a low level and unevenly distributed, representative sampling may be more important, especially when its purpose is to detect the presence or absence of pathogenic micro-organisms. Another aspect of sampling is the extent of replication necessary when carrying out the appropriate tests on a particular sample.

Guidance on statistical sampling methods may be obtained from various publications such as:

Lowe, C. W. *Industrial statistics* **1** 259-273. (1968). (Business Books Ltd., London).
Thatcher, F. S. and Clark, D. S. *Micro-organisms in food.* **52-58** and Appendix V. (1968). (Toronto Press; Toronto).
Cowell, N. D. and Morisetti, M. D. Microbiological techniques—some statistical aspects. *J. Sci. Food Agric.* **20** 473. (1969).

Sampling theory is perhaps of greatest importance when the purpose of testing is to examine material on a pass-or-fail basis. Microbiological quality is preferably studied continuously as a preventive measure and the intelligent application of experience may provide a better safeguard than reliance on a rigid scheme of sampling. For example, a single manufacturing unit may be involved in virtually continuous production of some high-volume toiletry lines such as shampoos and in the preparation of batches of decorative make-up items at quite protracted intervals e.g. once per month. The sampling problems are hardly comparable but an experienced microbiologist will be able to devise testing procedures appropriate to the circumstances and still within the capacity of the available laboratory services.

For exceptional cosmetic preparations where sterility may be the unqualified aim, a recognized routine of sterility testing should be applied. Suitable methods are given in the Therapeutic Substances (Manufacture and Interpretation) General Regulations 1963; The United States Pharma-

copoeia, 18th Revision: British Pharmacopoeia 1968; and in the British Pharmaceutical Codex 1968.

For most cosmetic products a criterion of sterility is not appropriate and suitable testing procedures are described in various sections of this monograph. Generally, the microbiologist will not seek to identify every contaminant found but it is advisable to undertake detailed identification if there is any reason to suspect the presence of pathogenic micro-organisms. If the presence of potentially harmful micro-organisms is confirmed, the product should either be rejected as unfit for sale or treated in such a way that such micro-organisms are no longer detectable on further testing.

Test methods for verifying sterility and for enumerating viable micro-organisms should ideally be fully reproducible in different laboratories and should preferably be simple and quickly carried out. Unfortunately there are no available techniques capable of satisfying all these criteria. For example, the microbial count is liable to vary according to factors such as

(i) the size of sample and frequency of sampling,

(ii) the specified counting technique,

(iii) whether inactivation of any antimicrobial preservative is carried out,

(iv) the diluent and recovery medium used and the methods employed in mixing with the product, and

(v) the temperature and duration of incubation.

Appendix B gives a selection of recommended procedures from which an appropriate scheme for testing a range of cosmetic products may be chosen. As indicated above, the sampling procedure in particular manufacturing conditions can only be properly determined on the spot and deciding on the most suitable tests to apply similarly demands consideration of formulae and manufacturing techniques actually in use. For example, the criterion of excluding known pathogens might suggest the need for an elaborate programme of microbial identity tests on every batch of product. For a suitable testing programme in a given set of circumstances, however, it will probably suffice to devise a scheme capable of monitoring the overall product quality over the course of time; this is preferable to a prohibitively expensive and elaborate check on every batch, providing that a comprehensive study of product preservation has been undertaken at the research stage. If good provisions for hygienic manufacture are made and the preservative system is shown to be capable of dealing with likely levels of contamination, routine control can safely be restricted to a limited range of testing.

2.2 *The selection of a preservative system*

There is no satisfactory way of choosing preservative agents for a particular formulation on a theoretical basis or from a list of available compounds. Experimental determination of the efficiency of a preservative system is essential for satisfactory results. Furthermore, the choice and testing of preservatives should be regarded as an integral feature of product development; it should not be left until formulation work is thought to be complete. The formulation chemist, packaging technologist, physical chemist, and microbiologist will need to reach a compromise, because the ideal product, the ideal preservative, and the ideal pack may well prove to be incompatible with each other.

In the course of product development, the following aspects of preservation will be important:—

2.21 The preservative system should preferably act bactericidally and fungicidally in the actual product formulation concerned against a broad spectrum of possible contaminants. This activity should be achieved at all likely storage temperatures and in low concentration.

2.22 The preservative system should have adequate efficiency at a pH corresponding to that of the product formulation.

2.23 The preservative should have solubility and partition characteristics such that it is available in the *aqueous* phase of a biphasic system. Complete solubilization in an oily phase or in surfactant micelles can result in loss of bactericidal or fungicidal activity.

2.24 The selection of product ingredients providing carbon, nitrogen or sulphur sources which may be utilized as nutrients by contaminating microorganisms, will increase the difficulty of achieving preservation.

2.25 Blends of more than one preservative often ensure synergistic or complementary activity; certain blends in which only one compound has antimicrobial activity may also be of value, e.g. the saturation of micelles with a non-inhibitory compound having a partition coefficient higher than that of the inhibitor may limit the level of preservative needed.

2.26 Product ingredients tending to carry a significant level of contamination should be avoided unless they are easily sterilized by heating or by a gaseous inhibitor such as ethylene oxide.

2.27 The physical, chemical and biological compatibility of preservatives with the other product constituents must be examined. For example, toxic or irritant effects on skin or mucous membranes might be encountered; the

hazard of photo-sensitization should be taken into account. Other difficulties include instability on storage, odour deterioration, loss by volatilization and spoilage due to corrosion.

2.28 Association with biologically-active product constituents could result in unexpected changes in the antimicrobial properties of the product itself or in its toxicity towards the skin or mucous membranes.

2.29 The packaging should be designed to avoid

(a) access of contaminants,

(b) inactivation of preservative by the container or the closure, and

(c) diffusion of preservative through the container or closure.

As indicated above, a preservative agent should be selected for its broad spectrum of antimicrobial activity. The contamination problem can be exacerbated by using a narrow spectrum preservative which is itself susceptible to contamination by insensitive micro-organisms. This hazard must be precluded by appropriate testing.

Since "self-sterilizing" capacity is a desirable target, the preservative system should preferably exhibit bactericidal and fungicidal rather than bacteriostatic and fungistatic activity. In practice, the objective is to achieve rapid destruction of a reasonable number of contaminants, the "inoculum" being kept within bounds by sound hygienic manufacturing arrangements. To insist on "self-sterilizing" capacity against all possible degrees of contamination would call for unduly high concentrations of powerful antimicrobial compounds; the consequent hazard of eliciting adverse skin reactions would almost certainly exceed the risk of transmitting infection with a non-sterile product.

Whilst the suggestions given in this section have been mainly directed towards safety aspects, the same considerations naturally apply to the avoidance of product spoilage (e.g. unsightly mould growth, development of "off"-odour) from an aesthetic or economical standpoint.

The techniques required to deal with the problem of preservative selection are described in *Appendix B*.

2.3 *Bacterial contamination of cosmetics subsequent to purchase*

Products supplied in containers allowing for the intermittent withdrawal of material are exposed to contamination each time the closure is removed; in particular, they often acquire contaminants from the user's fingers. The health hazard to an individual user is probably limited but cross-infection could well occur owing to use by different members of a family unless the product has a relatively high level of "self-sterilizing"

activity. Particular attention should be paid to this problem in the case of products for use on newborn infants, on broken skin, or in the vicinity of the eyes.

3. Requirements for hygienic manufacture

3.1 *General principles*

Hygienic requirements differ according to the objective sought; if the final product needs to be sterile, the manufacturing process obviously differs from that required to attain a less rigorous standard. To achieve sterility, production must either

(a) employ sterile ingredients which are handled in such a way as to exclude all risks of contamination, along with the employment of sterilized plant and packaging materials, or

(b) utilize normal, clean manufacturing procedures, ending at the final stage (i.e. after applying the closure to the filled unit packs) with a reliable sterilizing procedure, such as an autoclaving process.

The first alternative, generally described as "aseptic technique", demands an exceedingly high standard of specialized knowledge and experience, along with complementary plant design to facilitate its successful achievement. It also calls for the most exacting sampling and control procedure so that any failure to achieve sterility is rapidly identified. Such an expensive and skilled method of operation is sometimes unavoidable in the preparation of, for example, certain injectable pharmaceutical formulations but would not normally be feasible in the cosmetic industry.

The more practicable approach to sterile manufacture for cosmetics will almost invariably be the conduct of operations in a clean manner, followed by a terminal sterilization process. Guidance on hygiene and sterilizing technique are given in this document and the principles discussed here can be adapted to a wide range of different types of product. Sterility is, however, a criterion of an absolute nature and, if the product is one that calls for such a standard, proof of its achievement will be an essential feature; that is, adequate sterility testing must be carried out by the control laboratory and the product should be isolated and only released for sale when the tests have been completed.

As previously indicated, cosmetic manufacture does not, and need not, usually aim at yielding a sterile product. The more limited target of "self-sterilizing" capacity or adequate preservation, calls for less demanding manufacturing requirements and control procedures appropriate to the

objectives. Nevertheless, hygienic operating procedures require a high standard of attention to a wealth of detail and many aspects will be discussed in this monograph. The extent to which the advice offered here needs to be applied in a particular manufacturing process will depend on the scale of production, the susceptibility to contamination of the formula concerned, and the extent of inoculation with contaminants to which it might be exposed if precautions are insufficient. For example, suggestions are given with regard to plant sterilization; whether or not the plant needs to be sterilized before each batch of product is manufactured should be determined by the microbial status of the resulting material. Microbiological quality control should always serve as the guide to the success of manufacturing hygiene and as the warning-light to show when more stringent precautions are needed.

3.2 *Hygienic methods*

Precautions should be taken to prevent micro-organisms from contaminating a product at the various stages of manufacture. Even if a preservative is incorporated, there is still a risk that its antimicrobial capacity may be overwhelmed. Hygienic methods, are, in essence, procedures for maintaining a high standard of cleanliness but the necessary precautions are not obvious to everyone and are easily overlooked or misunderstood. The foremost need is, therefore, to inculcate the correct attitude into all operative and supervisory staff, with periodic re-education in the subject so that slipshod habits do not develop. There are also numerous points concerning factory and plant design where attention to detail will help to limit contamination. A vigilant attitude needs to be persistently maintained to avoid a sudden upsurge of contamination; it often happens, for example, that current production needs call for modification of plant lay-out without delay and this may well result in serious contamination if the hazard is not recognised. The typical hygienic problems associated with cosmetic manufacture may be exemplified thus:

3.21 Air and water are often important sources of contamination.

3.22 Any arrangement of plant or operating procedure that would allow dust to enter the product will also encourage airborne microbial contamination.

3.23 Stagnant residues of product in the plant between the processing of successive batches may well lead to multiplication of micro-organisms.

3.24 Efficient cleaning and sterilization of plant is desirable, but bad plant design can make this difficult or virtually impossible.

3.3 *Factory and plant design*

Design of the building in which plant is to be housed should ensure that walls, floors and ceilings have smooth, non-absorbent and easily cleaned surfaces with no hidden corners or ledges in which dust and dirt will collect. Provision should be made for efficient ventilation, preferably by means of filtered air giving a positive pressure with respect to the surrounding atmosphere. The building should be designed as far as possible so that personnel moving from one area to another do not have to traverse the production floor. Changing rooms should be kept apart from the production area and lavatories should not connect directly with either changing rooms or production areas. Adequate washing facilities must be provided.

The design of manufacturing plant should be such that stagnation of product residues in pipes, joints, pumps, pressure gauges and valves cannot occur; these are some of the places where serious contamination is most likely to develop. Stainless steel of high quality is undoubtedly the most suitable material for the fabrication of pipe-work, but other construction materials may be satisfactory so long as they can be effectively cleaned and sterilized. Plastic hose is quite suitable when new but requires frequent examination for defects and for accumulation of residues on roughened surfaces. Pipe runs need to be kept as short as possible, avoiding right-angled joints in order to facilitate cleaning; they should never run completely horizontal but always slope towards a venting point. Complete and thorough removal of product residues is essential if machinery is to be left idle for even a short space of time. Joints in pipes should be made by welding or with plastic seals; interchangeable dairy-type fittings are satisfactory but the packaging of pipework joints with hemp and grease is highly undesirable. Flexible piping offers the advantage that it may be disconnected, cleaned and sterilized between runs. Dead ends in pipework and traps in the lines must be avoided as far as possible, e.g. a by-pass to another plant or filling machine will serve as a "dead end" and lead to stagnation if it is only used occasionally. Adequate provision should be made so that sterilization either by steam or chemical means can be accomplished; it is essential to provide a range of points at which steam or fluid may be vented, to ensure that air locks in the plant do not interfere with efficient cleaning and sterilization. All parts of manufacturing equipment likely to come in contact with the product should preferably be constructed of smoothly-finished stainless steel; vessels should be double-jacketed to allow for heat sterilization and equipped with well-fitting lids capable of excluding dust particles and bacteria from the surrounding atmosphere.

Drain cocks, at the lowest possible level on the equipment, should be readily accessible and easily dismantled for cleaning.

Pressure relief valves must be sited so that they are obviously visible and accessible for cleaning; when such a valve has operated, deposits of product tend to be trapped and to create a microbiological hazard. Plug valves and pressure glands on gear pumps should be avoided, if possible, as also should glycerine-filled U-tubes on pressure gauges. Manifolds may be used to limit the necessary number of joints; the number of right-angled internal bends will thus be limited, so that cleaning and sterilizing become easier and more effective. In choosing mixing, blending and homogenizing equipment, it is desirable to select designs where the product is unlikely to come in direct contact with glands which are often a serious source of contamination. Contamination may also result from inadvertent contact between the product and the lubricants for bearings.

In general terms, it is clearly desirable that the design of plant should take into account the need to avoid bacterial contamination; the main requirements are that it should be easily cleaned and not provide dead ends and corners where contamination can escape attention. Plant which is ideal from an engineering standpoint is sometimes totally inadequate with regard to cleaning and sterilizing but these operations are essential for the achievement of good hygienic standards.

3.4 *Water supply*

One of the most likely sources of microbial contamination is the water supply used in manufacture, which may be filtered from the mains, demineralised or distilled. Strict control is necessary since the bacterial population of water may reach 10^6 bacteria ml^{-1} or more. Bacteria typically encountered in water supplies include *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Achromobacter* and *Aerobacter*. The most common places for bacterial contamination to occur are intermediate storage tanks, pipework joints and the resin beds of demineralizing plant; trapped organic matter rapidly removes any chlorine in the mains supply and provides excellent growth conditions for micro-organisms. If filtered mains water is used, a bacteriological monitoring system is needed since organic matter collects in filters and renders the chlorine inactive. Filters should be renewed regularly in preference to attempting to sterilize them.

Sterilization of ion exchange beds with 0.25% formalin (0.1% formaldehyde) should be performed at regular intervals as necessary. Frequent bacteriological checks should be made since the count may be low on

one day and high on the next. When demineralised water has to be stored, it must be sterilized and stored in previously sterilized tanks fitted with lids capable of excluding aerial contamination. Sterilization should be carried out by boiling for 30 min, by the addition of formalin or chlorine or by ultraviolet irradiation. Care must be taken with ultraviolet sterilization to ensure that the water has a high uv transmission, for suspended matter will shield bacteria from radiation; the efficiency of disinfection depends upon the intensity of the uv source, the transmission depth, and the rate of water flow. It is necessary to check by bacteriological tests that the water flow is not too fast and that adequate mixing of water is taking place by careful arrangement of the water inlet and outlet. It is preferable if water is to be stored continuously to recirculate it under a uv source.

3.5 *Other sources of contamination*

Likely sources of contamination include sacks, bags, drums, vats, airborne dust, straw, cardboard and the materials in which products are eventually packaged. Damp patches on ceilings and under stacks of raw materials should be rigorously avoided, as bacteria and fungi thrive in these conditions and eventually reach the manufacturing equipment and the product.

Raw materials and packaging materials should be stored well away from the manufacturing area so that their flora cannot be disseminated into current production. All materials should be examined bacteriologically from time to time, preferably on a daily or batchwise basis; if they carry a heavy load of micro-organisms, they should be sterilized either by ethylene oxide treatment or by autoclaving before transfer to the manufacturing area. Raw materials of mineral earth origin, including talc and pigments, may need to be sterilized before incorporation into cosmetics, to ensure the absence of tetanus spores; in many instances, it may be preferable to purchase such ingredients already sterilized. Various forms of starch are also liable to be heavily contaminated.

3.6 *Effect of processing conditions*

The operational procedures for manufacturing a cosmetic are normally determined by the need to achieve the desired product characteristics at an appropriate rate and output. Whilst it is unlikely that a process will be designed specially with the microbial status of the finished product as a prime consideration, attention to certain details may render the achievement of good standards much easier than it would otherwise be. For ex-

ample, a hot process is likely to yield a product with a lower microbial count than a cold process.

When ingredients are not metered into the product within closed equipment, all weighing and measuring should be carried out in clean vessels, and materials should be exposed to the atmosphere for the shortest possible time. Dipsticks and stirring paddles should be fabricated in stainless steel, certainly not in timber, and should only be wiped with clean material, frequently replaced. All vessels such as mixers, storage vats, and transit containers should have well fitting lids and it is important to ensure that such vessels are kept closed, except when operations necessitate the temporary removal of lids.

The practice of returning product from earlier batches to current production, whatever the reason, is potentially hazardous from the microbiological standpoint. For example, "catch-pots" used on vacuum-operated liquid filling-lines to hold the overflow of product are liable to contain exposed, stagnant material unless a careful watch is kept, involving a strictly enforced routine.

Clarification stages in the production process should be scrutinized for risks of introducing contamination. When a filter-press with replaceable mats is used, the whole assembly including the mats should be subject to the plant sterilization procedure. The re-use of the same mats from batch to batch obviously creates the possibility of developing a serious reservoir of contamination, unless the mats are sterilized again before each batch is filtered. Similar considerations apply to other filtration systems.

The packaging operation can easily lead to microbial contamination; even though the product will usually include a preservative by the time it is ready for filling into packs, it is obviously desirable to limit subsequent contamination as much as possible. Filling machines with multiple product outlets are often difficult to sterilize and the contact parts may need to be dismantled at frequent intervals for steam or chemical sterilization. Hoppers should have well-fitting lids, kept in place whenever possible. Transit from the filling stage to the point where closures are applied should be rapid; if conveyors are necessary, these should be shielded or irradiated by means of suitable uv lamps.

Packaging components are, in many instances, sterile at the time of manufacture but they may well become highly contaminated before use through transport and storage in unfavourable conditions, e.g. in contact with straw. Cleaning and washing procedures should be monitored with the

aid of microbiological tests, to ensure that their hygienic objective is duly achieved.

Although aseptic technique is seldom necessary for cosmetic manufacture, one of the most important criteria is to avoid a cumulative increase of contamination in the plant from day to day. Detailed consideration is therefore given in the following sections to plant cleaning and sterilization.

3.7 *Plant cleaning*

Continuous production rarely takes place in any piece of equipment and interruptions in the use of machinery may vary from hours to weeks; the plant may, perhaps, be cleaned after production of the last batch of one product or just before the manufacture of the next. The idle time of the equipment is of great importance from the microbiological point of view, whether it occurs before or after the cleaning process. Undisturbed residues in damp, dark and warm regions of a plant are dangerous because the conditions are so admirably suited to the multiplication of bacteria and fungi.

In pumps, joints, pipelines, pressure gauges and valves, growth is quickly initiated where small amounts of liquid are left undisturbed for a few hours. Complete and thorough removal of product residues is essential for the plant to remain in a hygienic condition. Films of product trap and protect micro-organisms and diluted material often allows the rapid development of massive numbers of contaminants.

Hot detergent solutions should be used for cleaning, coupled with circulation of the solution, scrubbing and the dismantling of all valves, taps and joints.

Plant should be washed, sterilized and rinsed immediately before use and if operations are to be interrupted at some stage during this process, the safest point is after chemical sterilization and before rinsing. Leaving the plant idle after detergent washing or final rinsing is potentially hazardous from the microbiological point of view unless the machinery is thoroughly dried inside and out.

3.8 *Plant sterilization*

Cleaning and removal of product residues should be followed by sterilization, especially if the plant is not to be used immediately. Sterilization by heat is the most effective means of ensuring the destruction of all micro-organisms, but is not always convenient for manufacturing plants of moderate or large size unless special provision has been made in the

original plant design. Pasteurization is not recommended since it is ineffective against heat-resistant bacteria or spores.

Chemical sterilization is a possible alternative to heat treatment and is usually effective when it follows thorough washing to remove as much "soil" as possible; this is important because spores are not always destroyed by chemical disinfection and they may germinate in the residues. If contamination with spores is suspected, it is necessary to use strong concentrations of chemical disinfectants maintained at elevated temperature.

Chlorine is the best plant sterilizing agent in most circumstances. Cold solutions of hypochlorite yielding 200 to 250 ppm of available chlorine will sterilize glass and metal surfaces after 5 min if all organic matter has been removed previously.

Formaldehyde or formalin is a convenient sterilizing agent for use after plant washing but, like chlorine, its activity is greatly reduced by organic matter. Cold solutions should be used at a concentration of about 0.5% formalin (0.2% formaldehyde). This concentration will sterilize surfaces which are free from organic matter in 10 min. Cold solutions are recommended for both of these sterilants owing to the unpleasant or toxic effects that may result from inhalation of their fumes. Hot solutions may, however, be employed in totally-enclosed plant if proper precautions are taken.

Quaternary ammonium compounds, although weight for weight less effective than chlorine and formaldehyde, have advantages such as being almost odourless, relatively non-toxic to man, and less corrosive than chlorine. They do not have a wide spectrum of antibacterial activity, being more active against gram-positive than against gram-negative bacteria; they are also rendered less effective by organic matter and anionics. Since they are quite efficient surface-active agents, they can in some cases be used to clean the plant as well as to disinfect, although their detergent action would be inadequate for the removal of residues from w/o emulsions. A concentration of 0.5% of benzalkonium chloride at 60° will sterilize "soil"-free surfaces in less than 10 min.

Residues of disinfectant solutions must be removed from the plant after sterilization; in order to avoid re-contaminating the machinery, this should be done by irrigation with sterile water. The risk of recontaminating sterile machinery is high if cold water from a distilled or demineralized water storage tank is used. It is necessary, therefore, to use freshly boiled or autoclaved water, particularly if the plant is to be left idle before the next manufacturing schedule begins.

The detailed advice given in the foregoing sections on plant cleaning and

sterilization may well appear greatly to exaggerate the need for elaborate precautions in the manufacture of products which are not required to be truly sterile. The ultimate answer must depend on the results of microbiological control testing; if a consistently low level of contamination is already achieved, housekeeping is obviously adequate for the purpose but when high counts or potential pathogens are found, the production arrangements should be carefully re-examined. Important facets of this problem are whether the product tends to encourage microbial growth and whether the preservative is in danger of being overwhelmed. Quite small "pockets" of contamination, protected in one way or another from the usual cleaning or sterilizing procedures, can sometimes lead to a rapid and overwhelming increase of contamination in the bulk output; constant vigilance is therefore necessary, if only to avoid such catastrophes which can be exceedingly difficult to overcome quickly.

3.9 *Avoidance of contamination from human sources*

Personnel can play an active part in the introduction or in the prevention of contamination within manufacturing areas and they should be encouraged to have a continuing awareness of contamination problems. This should be done by instruction in the fundamentals of personal and factory hygiene. They should be encouraged to report minor ailments which may constitute a source of contaminating infection in the production process. Apparently healthy personnel may act as carriers for various microbial contaminants and bacteriological checks are advisable if difficulty is encountered in tracing sources of product contamination.

Much can be accomplished by providing ample modern washing facilities before beginning work and after using the lavatories. Taps should not be manually operated; the provision of a hand dip not exceeding 10 ppm of available chlorine may be useful. Disposable towels should be employed.

Suitable clean protective clothing should be supplied including, where appropriate, caps, hair nets, gloves, and masks. It is essential to insist that such clothing should be worn and changed frequently. Personal effects should not be allowed in the washing area. Access to washing facilities should be freely available and cleanliness in changing rooms should conform to the same standard as in the working area.

4. Microbiological quality control

Microbiological quality control serves four main purposes:

1. To check that plant hygiene is satisfactory.
2. To monitor the microbial contamination in raw materials.
3. To assess the microbial status of finished products.
4. To check that preservative capacity is adequate.

To carry out such testing on every raw material supply and every batch of finished product, with daily or even hourly testing of the manufacturing plant and using all the possible test methods would be prohibitively expensive and would cause unjustifiable production delays. It is therefore of great importance to establish a testing scheme appropriate to the actual circumstances in an individual production unit. The main aim will be to ensure gradually improving quality of output, along with early warning of adverse changes, rather than to "pass" or "fail" production batches in a purely routine fashion.

Microbiological quality control should be initiated at the start of production with regular examination of plant in which the products are manufactured and proceeding to cover raw materials, including the water supply and packaging components. Frequent checks are desirable at all stages of a manufacturing process so that if contamination does occur, its point of onset is easily established and action can be taken to prevent a recurrence. Full records of all microbiological examinations will prove invaluable if complaints are received some time after manufacture and sale.

The quality control programme for raw materials should include provision for quarantine on all items liable to be heavily infected until results are reported and evaluated, along with in-line testing on occasional product batches to determine microbiological changes in processing. Finished product testing should assess sterility or an acceptable microbial status for the particular product. Tests should be made for any pathogens previously encountered, unless the product has previously been shown to be self-sterilizing or unless production batches are found to be consistently sterile.

The plant should be examined for the presence of specific pathogens and for micro-organisms, including fungi, likely to cause spoilage of the product or harm to the consumer. All valves, traps, pressure-relief valves and working surfaces should be swabbed periodically and the swabs cultured and counts performed. Pipe-lines should be taken apart where possible and washed through with sterile physiological saline or Ringer's solution; viable counts should be performed on the washings, which should also be examined on selective media for the presence of potential pathogens. Manufacturing

vessels should also be swabbed or washings should be taken and the same examination procedure followed.

Particular attention should be paid to the bacteriological condition of filling equipment. Nozzles should be swabbed and pipe lines taken apart and washed through; pumps should receive particular attention. All types of packaging material should be examined including cap wads and cartons, especially for the presence of fungi; glass containers should be substantially free from bacterial or fungal contamination.

The detailed application of the principles outlined here will depend on the intrinsic liability of the product to microbial contamination and the scale of manufacture, type of plant and process. It is therefore virtually impossible to suggest exact routines appropriate for all circumstances. Detailed suggestions are, however, given in *Appendix B* which may be adapted according to the prevailing requirements.

Appendix A

Code of Good Practice for the toiletry and cosmetic industry

1. Application

The Code of Good Practice shall apply to the manufacture, packaging and storage of all cosmetic and toilet preparations (see Section 9—Definitions) intended for human use.

2. Standards

Preparations must comply with the standards established by Statutory Regulation and also the quality standards established by this code. Preparations manufactured for export must comply with any additional standards and procedures which may be required by the importing country.

3. Personnel

3.1 Each manufacturing establishment must employ at least one person possessing adequate technical and practical attainments, with the authority and responsibility to ensure and maintain the identity, purity and quality of the products manufactured.

3.2 All manufacturing, packaging personnel and those coming in contact with product or packaging must be clean and free from skin disorders or infections liable to contaminate the product or packaging.

3.3 Decisions concerning compliance or non-compliance of materials and finished goods with the standards defined in 2. above must rest with a key person responsible for quality control, who has adequate authority to discharge this duty and who is directly responsible to a chief executive.

4. Premises and facilities

4.1 Premises should be so laid out as to ensure:—

4.1.1. the clear identification of raw materials, bulk products, packaging materials and finished goods both in storage and during processing and packaging;

4.1.2. that cross-contamination of products cannot occur in manufacture and packaging;

4.1.3. that appropriate procedures can be carried out when specialised operations, e.g. handling sterile or highly inflammable materials, etc., form part of the manufacturing or packaging process.

4.2 Handwashing facilities must be provided within easy access of all manufacturing and packaging areas.

4.3 All staff working in processing and packaging areas must wear suitable garments laundered at least once weekly.

4.3.1. Food must not be brought into or consumed in areas where manufacturing or packaging processes are carried out.

4.3.2. Smoking must not be allowed in areas in which manufacturing or packaging processes are carried out.

4.4. Facilities for quality control must be provided through a laboratory functionally independent of manufacturing and provided with sufficent equipment to carry out all necessary assays and tests which are required for compliance with this Code of Good Practice.

5. Sub-contracting

5.1. If a manufacturer sub-contracts any work covered by this Code of Good Practice, then the sub-contractor must comply with the provisions of this code.

6. Equipment

6.1. Equipment must be maintained in a clean condition. It must not be deleteriously reactive to materials being handled and must be constructed so as to be capable of easy efficient cleaning and if necessary sterilization.

6.2. In relation to the work being carried out, weighing and measuring equipment for raw materials and intermediates should be accurate enough to ensure that weighings or measurings will not vary outside the limits $\pm 1.0\%$ of the quantity being weighed or measured. Equipment should be subject to regular skilled testing, on at least an annual basis.

7. Procedures

7.1. Systematic procedures must be established to control the identity and quality of each raw material, component and finished product at the appropriate stages of manufacture, filling, packaging and storage.

7.2. Each product, its raw materials and other components must be clearly identified at all stages of manufacture, storage and transportation.

7.3. Each product must have a recorded master formula which includes or appends the quality control parameters and processing details. Copies of the master formula required for working purposes shall be prepared in a manner which will minimise the possibility of transcription errors.

Deviation from standards must be authorised by a responsible person as specified in 3.1.

7.4. Records must be maintained which make it possible to ascertain the date of manufacture of any filled product.

7.5. All relevant records should be retained for a minimum of two years.

7.6. Stocks of approved raw materials and components must be differentiated from those untested.

7.7. Representative samples of raw materials and finished products should be retained for at least one year.

7.8. A procedure must be established for the issue of packaging materials including labels to ensure that those issued correspond with the product being packed. Safeguards against issued packaging materials becoming intermixed before and during use must be established.

7.9. A system of "first in first out" procedure of stock-keeping of all raw materials and finished goods must be established.

7.10. Procedures must be established to ensure that packaging lines, tables and equipment are clean and free from any materials and packs not relevant to the current operation.

8. Recommended standards and techniques

Where control by regulation does not exist, the Standards and Techniques given in the schedules for

(A) Hygiene—
Microbiological Safety (to follow)

(B) Safety in Use—
Toxicological and Dermatological Safety (to follow)

(C) Quality Standards—
Quality Control of Ingredients of Products (to follow)

will apply.

9. Definitions

"Cosmetic or Toilet Preparations" are substances or preparations

(i) intended to cleanse, beautify or modify the appearance of a person by external application to the skin, nails, hair, eyes or the oral cavity—but not intended to be swallowed; or

(ii) which exert a non-systemic action on or modification of local physiological functions so as to prevent, reduce or correct minor undesirable surface conditions, blemishes or defects of the skin, nails, hair, eyes or the oral cavity (but not intended to be swallowed) and are so sold.

The following definitions apply to (i) and (ii):—

"Non-Systemic"—Localised in or upon the skin, its appendages, its local blood capillary network or its own nerve supply and not mediated through the general blood circulation or the central nervous system.

"Minor"—Not requiring medical or dental diagnosis or treatment.

Explanatory notes

4.4. To be read in conjunction with 3.1. It recognises the use of outside consultants and laboratories that are adequately equipped and staffed.

6.1. Should be interpreted in its broadest sense: "deleteriously reactive" includes the possibility of contamination from all ancillary equipment.

6.2. Indicates an overall standard for measurement. It recognises the possibility of varying specific gravities and recommends weight measurement wherever possible.

7.7. Shelf life, turnover time and company policy will dictate practical representation.

Appendix B

This Appendix describes microbiological test procedures for use in product development and quality control, as well as giving detailed recommendations on preservatives. In order to produce a reasonably concise set of recommendations, it has been assumed that readers will have at least an elementary knowledge of microbiological laboratory technique. Whilst a fully-trained microbiologist should be thoroughly familiar with most of the contents of this and the following Appendices and may prefer various alternative procedures to those recommended here, the text has been prepared with a particular view to aiding the less experienced reader who is advised to follow the suggested techniques as closely as possible.

1. Microbiological test procedures

The information given below includes a selection of test media and conditions of incubation recommended for the isolation of microbial contaminants commonly found in cosmetic products and raw materials or which would represent a serious hazard if they were present. The list of test media is not exhaustive nor is it suggested that the entire range of media needs to be used routinely. Inoculation of samples on or in the media designated as 1.311, 1.312, 1.314, 1.361 and 1.363 together with gram staining and study of the morphology of micro-organisms found, will provide basic information on the extent and type of microbial contamination affecting the preparation. References dealing with methods of identification are given in *Appendix E*. Samples showing growth in nutrient broth or by an agar streak method should be re-examined *quantitatively*. According to the present recommendations, bacterial counts are performed in duplicate in Oxoid Plate Count Agar. Cultures are incubated at 28-32° and 37° and colonies counted after 48 h incubation. Mould counts are performed in malt extract agar or Sabouraud Dextrose Agar and incubated for five to seven days at 22-25°. Plates should be examined daily. New products, both at the formulation stage and with respect to the first large-scale batches, should be re-checked for micro-organisms after one month's storage at local ambient temperature.

1.1 *Qualitative examination*

Methods of isolation and enumeration of micro-organisms usually require some form of special treatment of the samples to release into a fluid

diluent or medium those organisms which may be embedded or trapped within the bulk of the material. Standardization of the preliminary procedure is important. Excessive speed of the cutting blades of a blender or unduly prolonged use of a mixer may cause injury to the microbial cells either mechanically or by heat generated. Care must be taken to minimize risk from aerosols created by a mechanical blender when pathogens are present or suspected.

1.11 *Preparation of sample for testing*

Mix the sample and transfer approximately 1 g or 1 ml to 9 ml of sterile Nutrient Broth to give an approximately 10% solution or suspension. Other dilutions may be prepared as necessary. The transfer and dilution technique must be carried out in such a manner as to avoid the introduction of extraneous contaminants. If the sample is not miscible with water, it should be dispersed with the aid of an emulsifying agent. *Lubrol W*, *Tween 80* and *Triton X100* are useful for this purpose. The following method may be adopted:

Prepare a supply of Universal bottles containing 4 ml aliquots of 4% *Lubrol W*. Sterilize by autoclaving. Add approximately 1 g or 1 ml of the test sample to 4 ml of 4% *Lubrol W*. Place in a 44° water bath for 10 min with intermittent shaking. At the end of the time add 5 ml of Nutrient Broth, previously warmed to 44°, to make a final volume of 10 ml.

1.12 *Inoculum*

Select a suitable range of agar and broth media and with a sterile Pasteur pipette inoculate each plate with 2 to 3 drops of suspension; spread with a sterile wire loop and add 0.5 to 1 ml of suspension to each bottle of liquid medium. Additionally, streak out on Nutrient Agar or other suitable medium a loopful of the *undiluted product* (or heavy suspension). Streak-plate cultures of the preparation in its original form should be made routinely, since spoilage organisms tend to become dependent on the product for their nutritional requirements and may fail to grow or show only sparse growth when first isolated from this environment. When spoilage organisms fail to grow satisfactorily on standard culture media, it may be advantageous to employ diluted, e.g. half-strength, media; this provides conditions more suitable for organisms adapted to an environment of poor nutritive status. On incubation, blood agar is lysed by prolonged contact with *Lubrol W* broth. If contamination with haemolytic

micro-organisms is suspected, an inoculum from a plain broth suspension should be used.

1.2 *Quantitative examination*

The problem of representative sampling has been discussed in *2.1* above and the value of quantitative studies will obviously depend to a considerable extent on the validity of the samples examined. Quantitative test procedures generally involve the replication of experiments on several aliquots derived from a single initial sample but it should be stressed that the validity of a test procedure is greatly strengthened by ensuring that replicate *samples* are included as well as replicate aliquots from a single sample.

1.21 *Plate count procedure*

All glassware and equipment must be chemically clean and sterile. When preparing serial dilutions use a fresh sterile pipette for each transference. A considerable saving in 1 ml pipettes can be achieved by using an automatic syringe connected to a sterile Pasteur pipette. The special syringe* is pre-set to deliver 1 ml. A fresh sterile disposable Pasteur pipette is used for each dilution but the same syringe can be used for any number of operations since the diluent is not sucked into the barrel.

1.211 Diluent

Use sterile 0.1 w/v peptone water at pH 7.0. Inoculation of media should be carried out within 30 min of the preparation of the dilution. When examining a sample for anaerobic bacteria use freshly prepared Reinforced Clostridial Broth as the diluent.

1.212 Liquid samples

Pipette aseptically 10 ml of the thoroughly-mixed sample into a sterile glass bottle fitted with a ground glass stopper and add 90 ml of diluent to give a 1:10 dilution v/v. Alternatively, weigh with aseptic precautions 10 g of the thoroughly-mixed sample into the bottle and add 90 ml of diluent to give 1:10 dilution w/v. Prepare further serial decimal dilutions in 0.1% sterile peptone water as necessary.

*R. B. Turner & Co. Ltd., Church Lane, London, N.2.

1.213 Powders

Weigh aseptically 10 g of sample into a sterile glass bottle which is marked at 100 ml capacity and fitted with a ground glass stopper, to give a 1:10 dilution w/v or weigh 1 g of sample and make up to 100 ml to give a 1:100 dilution w/v. In the transference of powder aseptic manipulation can be more readily carried out by using a sterile E-mil weighing scoop. To mix, shake the suspension 25 times.

1.214 Creams

Mix and transfer aseptically 1 g amounts of cream to sterile Universal bottles. Add 9 ml of sterile diluent and 6-8 sterile glass balls to each sample. Mix the contents on a Whirlimixer for 10 s. Prepare further serial tenfold dilutions as necessary. Mix the dilutions well and plate out immediately. For the treatment of emulsions with a hydrophobic continuous phase, *method 3.36* (p. 765) is recommended.

1.215 Plating out

Plate out in duplicate 1 ml of each dilution and add 10 to 15 ml of a suitable agar medium that has been liquefied and cooled to 45-47°. Mix well by rotating the plate clockwise and anti-clockwise several times, the plates being kept flat throughout the whole process. When the agar has set, invert the plate and transfer to an incubator. Do not stack plates more than six deep.

1.216 Colony count of bacteria

Use Tryptone Glucose Yeast Extract Agar, also known as Plate Count Agar. For routine colony counts incubate at 28-32° for 48 h intervals. Count the colonies. Actidione (cycloheximide) may be added to the medium at 0.001% to suppress moulds and yeasts. Actidione Agar (*Oxoid PM 118*) may also be used for this purpose.

1.217 Colony count of yeasts and fungi

Use Malt Extract agar or Sabouraud Dextrose Agar. The medium may be acidified with lactic or citric acid to pH 3.5 ± 0.1 to suppress the growth of bacteria. Once acidified, the medium should not be re-heated. See also *Appendix C*. For routine counts, incubate at 25° or other constant temperatures up to 30°. Inspect the plates at 24 h intervals. Count the colonies after five to seven days' incubation.

1.218 Counting of colonies

Colonies should normally be counted within 4 h of the end of the incubation period, or the plates may be stored overnight at a temperature not exceeding 4°. Use an illuminated, preferably electronic, colony counter. Alternatively, a lens of a magnification not exceeding $2\frac{1}{2}$ diameters, and a tally counter may be used to facilitate counting. Count all visible colonies on the plate including pin-point colonies beneath the surface. Where spreading organisms occur, count each "spreader" as one colony. Whenever possible, include only plates in which the dilutions have given colony counts between 30 and 300 for recording the results. To calculate the colony count per ml, multiply the number of colonies by the reciprocal of the dilution and determine the arithmetic mean for the replicates. Express results as colony count ml^{-1}. If "spreaders" cover more than half the area, discard the plate concerned. The result will be of doubtful validity if one quarter or more of the plate is covered by a spreading organism. In such cases an approximate estimate of the count may sometimes be obtained by examining a plate from another dilution. Multiplication of many organisms may be inhibited by high osmolarity or the presence of a preservative. Undissolved particles can complicate bacterial counts and must not be confused with colonies. It is sometimes more convenient to carry out a surface inoculation count (*Method 3.513*, p. 768).

REFERENCE

Postgate, J. R. Viable counts and viability, in Norris, J. R. and Ribbons, D. W. *Methods in microbiology* **1** (1969) (Academic Press, London).

1.219 Optimal temperatures

There is a wide divergency in the optimal temperatures for the propagation of various micro-organisms. Bacteria have been divided into three classes – psychrophiles, mesophiles and thermophiles – according to their optimum temperature-requirements for growth. Psychrophilic bacteria may show activity at 7° or below with optimal growth at 10° to 20°. They do not reproduce at 40°. Mesophiles grow optimally at 37° to 40°. Growth does not occur at 55° or at 20°. The optimal temperature for growth of thermophiles is 55°, but they may also grow at temperatures as high as 89°. They do not multiply at 40°. There is considerable overlapping in the divisions since some bacteria grow well over a wide range of temperatures whereas the optimal growth of others is restricted to a very narrow temperature range. It is advisable to incubate within the range of 22 to 32° as well as at 37°, for the isolation of spoilage organisms in cosmetics.

1.3 *Recommended test media and incubating conditions*

	Incubation temperature
1.31 *Detection of aerobic bacteria (Mesophiles)*	
1.311 Nutrient Agar or Plate Count Agar	28-32°
1.312 Nutrient Broth	28-32°
1.313 Blood Agar	37°
1.314 MacConkey Agar	28-32°

Examine at 24–48 h intervals and continue incubation up to 7 days.

1.32 *Detection of Escherichia coli (Type 1)*

1.321 Sub-culture in MacConkey Broth (with Durham tube) 44°

Examine for acid and gas at 48 h.

1.33 *Detection of Psychrophiles*

1.331 Tryptone Glucose Yeast Extract Agar 10°

Examine at 5 and 10 days. Continue incubation as necessary.

1.34 *Detection of Thermophiles*

1.341 Tryptone Glucose Yeast Extract Agar 55-63°

Incubate for 36 - 48 h in an atmosphere sufficiently humid to prevent drying of the medium. Continue incubation as necessary.

1.35 *Detection of Clostridia*

1.351 Cooked Meat Broth 37°

Incubate 3 to 4 days and sub-culture to suitable plate media and incubate anaerobically.

Tubes of cooked meat broth not used the day they are prepared should be placed in a boiling water bath or flowing steam for a few minutes to drive off dissolved oxygen then cooled to 37° before inoculating.

1.352 Thioglycolate Broth 37°

1.353 Reinforced Clostridial Agar and Broth 37°

Incubate anaerobically.

1.36 *Detection of yeasts and moulds*

1.361 Sabouraud Liquid Medium/Malt Extract Broth 22-25°

1.362 Sabouraud Dextrose Agar 22-25°

1.363 Malt Extract Agar 22-25°

Inspect at 24-48 h intervals and continue incubation up to 10 days.

1.37 *Selective enrichment media for the isolation of pathogens*

1.371 *Staphylococcus aureus*

(a) Salt Meat Broth 37°

Sub-culture to Nutrient Agar at 24 h. Carry out slide and coagulase test from Nutrient Agar (see *1.38* below).

(b) Baird-Parker Medium (Oxoid code CM275) 37°

For further details see 5.3 Ref. (6) (*Appendix E*).

1.372 *Pseudomonas aeruginosa* (*Ps. pyocyanea*) 37°

Plate on Cetrimide Agar from broth at 24 h.

1.373 *Salmonellae*

Selenite F broth 37°
43°

Subculture on brilliant green agar (Bacto) after 24 and 48 h. Incubate at 37° and examine the plates for colonies with Salmonella characteristics and identify by standard biochemical and serological methods [see Selected Bibliography. *Appendix E*, 5.3, Refs. (4, 6)]

1.38 *Staphylococcal coagulase test*

This test is used to differentiate pathogenic *Staph. aureus* from non-pathogenic staphylococci.

1.381 Test reagent

Human or rabbit plasma. Dried rabbit plasma, reconstituted and diluted 1:5 in isotonic saline is satisfactory.

1.382 Slide test

Use an 18-24 h Nutrient Agar Culture (not a broth culture). Mark a clean slide into two sections. Place a loopful of water (not saline) on each section and emulsify a colony or small amount of culture in each drop until a homogeneous suspension is obtained. If no clumping occurs in 10-20 s, add and mix a loopful of neat reconstituted plasma. Avoid excess plasma as this may give false positives. The second suspension serves as a control.

A positive result is indicated by visible clumping within 10 s. Delayed clumping does not constitute a positive result.

1.383 Tube test

Use an 18-24 h plain Nutrient Broth Culture. Place 0.5 ml of diluted plasma in each of two small test tubes. To one tube add 0.5 ml of an 18-24 h

broth culture. Incubate both tubes at 37° and examine after 1 h and at intervals up to 24 h.

A positive result is indicated by a definite clot formation. Coagulation usually takes place within 1-4 h. Granularity or ropiness is regarded as doubtful and the test should be repeated. Negative tubes will be clear or only faintly cloudy with no coagulation.

If the plasma has been stored in a refrigerator it may be sufficiently cold to delay coagulation; it is advisable to allow the plasma to attain room temperature before use.

The slide test detects 'bound' coagulase which acts on fibrinogen directly; the tube test detects 'free' coagulase which acts on fibrinogen in conjunction with other factors in the plasma. Either or both coagulases may be present. The slide test is a valuable presumptive test but negative results must be confirmed by a tube test.

Known coagulase-positive and negative strains must always be tested in parallel and, with the tube tests, an uninoculated control must also be set up.

Controls: Positive – *Staphylococcus aureus* (NCIB 9518, FDA 209, ATCC 6538 or NCTC 8532)
Negative – *S. epidermidis* (NCTC 7291 or 4276).

1.384 Additional screening test

The ability of coagulase-positive staphylococci to split deoxyribonucleic acid (DNA) provides the basis for a diagnostic laboratory test for the identification of potentially pathogenic organisms.

Test

Inoculate a plate of DNase Agar (Oxoid CM321) as follows:
Use an 18-24 h Nutrient Broth Culture. Place 1 drop onto the surface of the agar so that a thick plaque of growth is evident after 18 h incubation. Flood the plate with N/1 HCl and look for clearing around the colonies (DNase positive).

1.4 *Inactivators suitable for counting procedures in the presence of common antimicrobials and preservatives*

Special difficulties arise when the product being tested is inhibitory to bacterial growth or when it contains a bacteriostatic agent. In order to obtain a true bacterial count, the bacteriostatic effect must be overcome

by dilution with a sufficient volume of culture medium or by the addition to the medium of a substance of known capacity to neutralize the bacteriostatic effect. When assessing the ability of a preparation to prevent the multiplication of micro-organisms *within the confines of its container* (as distinct from sterility testing or evaluating bactericidal properties of an antimicrobial) then the normal dilution factors are usually sufficient to minimize bacteriostasis.

1.41 *List of recommended inactivators*

In the following list, the concentration level of preservative is taken to be within the range used in cosmetic preparations (*4.3*).

Antimicrobial agent	*Inactivator*	
Phenolic disinfectants	Polyoxyethylene sorbitan mono-oleate (*Tween 80*), charcoal, ferric chloride	(1)
Halogens	Sodium thiosulphate 0.05%	(2)
Hexachlorophane	*Tween 80*	(3)
Formaldehyde	Ammonium ions, 0.1% ammonia as cell-wash, *Tween 80*, 6% sodium sulphite	(4)
Quaternary ammonium compounds	2% Lecithin in 3% *Lubrol W* or *Lubrol W* up to 10%	(5)
Merthiolate Oxyquinoline sulphate Merthiolate and phenol	Sodium thiosulphate (2%)	(6)
Hexachlorophane Dichlorophene Povidone iodine	1% *Lubrol W* + 0.5% lecithin + 1% *Tween 80*	(7)
Benzalkonium chloride Chlorbutol Phenylethanol	1% *Lubrol W* + 0.5% egg lecithin . .	(8)

Alcohol Chloroform Chlorbutol Phenol and derivatives Esters of *p*-hydroxybenzoic acid Benzoic acid Sorbic acid	A general dilution of 1 part of sample to 100 parts of recovery medium	Addition of polysorbate 20 or 80 (*Tween*) to a level of 10% is an additional safeguard (9)
Hydrogen peroxide	Catalase, sodium thiosulphate	
Mercurials	Sufficient thioglycolate present in Brewer's or U.S.P. medium i.e. 0.05-0.1%	

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(2) Weber, G. R. and Levine, M. *Amer. J. Publ. Health* **34** 719 (1944).
(3) Lawrence, C. A. and Erlandson, A. L. *J. Am. Pharm. Assoc.* **42** 352 (1953).
(4) Nordgren, G. *Acta. Path. Microbiol. Scand.* **1** (Suppl. xl) (1939).
(5) *British Standard Specification* 3286: 1960.
(6) *Acta. Pharm. Hung.* **37** 235 (1967).
(7) Lowbury, E. J. L., Lilley, H. A. and Bull, J. P. *Brit. Med. J.* (2) 531 (1964).
(8) Anderson, K. and Crompton, D. *Lancet* (2) 968 (1967).
(9) Kohn, R. S., Gershenfeld, L. and Barr, M. *J. Pharm. Sci.* **52** 967 (1963).

GENERAL REFERENCE

A guideline for the determination of adequacy of preservation of cosmetic and toiletry formulations. *Toilet Goods Assoc. Cosmet. J.* **2**, 20 (Winter 1970).

1.5 *Routine bacteriological testing of water supply*

The necessary scale of testing depends on the general quality of the mains supply and the condition of the storage tanks and pipe-lines. Monthly examination will suffice if the whole system is in good order but, when this is not so, daily examination should be made. Special care is required if the preservative capacity of the formulation is limited with respect to its ability to cope with large inocula of waterborne organisms.

1.51 *Sampling*

A 250 ml sample is taken aseptically in a sterilized container at least once per month from the water inlet to each mixer in use. If samples are transmitted to a consultant for examination, they should be transported on ice with minimum delay. If the water to be examined contains, or is likely to contain, chlorine or chloramine, sufficient sodium thiosulphate to neutralize these substances should be added. The thiosulphate should be

added to the bottles before sterilization as a 3% solution to give a final concentration of 18 mg l^{-1} in the water sample, the amount depending on the size of the bottle, e.g. 0.1 ml is required for a 170 ml bottle. Sodium thiosulphate ($Na_2S_2O_35H_2O$) at a concentration of 18 mg l^{-1} has no significant effect on the coliform organisms and should neutralize up to 5 mg l^{-1} of residual chlorine.

1.52 *Colony count*

Water may contain a variety of micro-organisms having different optimum temperatures of growth. Most bacteria capable of growth in water will, in laboratory media, grow better at 22° than at higher temperatures. Organisms which grow best at 37° usually grow less readily in water and are more likely to have gained access from external sources. Since these two groups of organisms differ in their significance, it is desirable to count them separately. For this reason two sets of plate counts are usually prepared, one of which is incubated at 20-22° and the colonies counted after three days, the other of which is incubated at 37°, the count being made after 24 h. The number of dilutions used may be varied in the light of experience with waters of which the bacterial content is known. Normally original sample and dilutions of 10^{-1} and 10^{-2} are usually sufficient for this purpose. (*Appendix B, Section 1.215* p. 746 for method of plating out).

Details of special procedures relating to sampling, the membrane filter, the identification of *E.coli* (fermentation of lactose at 44°) and other appropriate biochemical tests, also special methods for the isolation of pathogens are described in the official report (1).

1.53 *Reporting plate count results*

Report the colony count as the number of colonies developing per ml of the original water on Plate Count Agar after 24 h at 37° or 3 days at 20-22° as the case may be.

REFERENCE

(1) *The bacteriological examination of water supplies.* Report No. 71 (1969). (H.M. Stationery Office, London).

1.6 *Microbiological status of atmospheric environment*

A high level of microbial contamination in the air of a cosmetic manufacturing plant is obviously undesirable as it may lead to contamination of the product. Aerial counts of bacteria, moulds and yeasts are liable to fluctuate widely in relatively short periods of time, e.g. due to air currents, opening of packing cases and the movement of personnel. Respiratory

infections affecting staff may well lead to localized high levels of atmospheric contamination.

Apart from the widely varying levels of atmospheric contamination actually present, assay techniques may lead to extremely variable findings depending, for example, on whether they record static or dynamic conditions of the air being sampled. For these various reasons, quantitative standards cannot be recommended for general application. It is, however, suggested that atmospheric contamination should be monitored along with other possible sources of contamination, in order to gain a general picture of the microbiological status of a production unit.

Suggested methods for use with a slit sampler* are impingement on selective media or membrane filtration after impingement in broth.

A suitable range of media would be Nutrient Agar and MacConkey Agar for the isolation of bacteria, Sabouraud and Malt Extract Agar for the isolation of yeasts and fungi.

2. Product development

2.11 *Assessing preservative capacity*

Whatever the preliminary methods of evaluating the effectiveness of a preservative, the final test should always be carried out on the complete formulation in the final pack. Most procedures are based on a dynamic or spoilage type of test in which the sample to be tested is challenged by inoculation with a selection of appropriate spoilage organisms. The contaminated sample is subsequently examined for evidence of microbial activity during a test period usually lasting several weeks or preferably, in the case of moulds, for several months. If micro-organisms fail to grow in such a test, the sample can be re-challenged to provide further information on the stability of the preservative system.

2.12 *Challenge organisms*

The range of micro-organisms examined in preservation tests is logically selected from those usually responsible for spoilage and others associated with common infections. Micro-organisms used in preservation studies must be vigorous strains and should include recent isolates from contaminated samples. Along with locally-isolated laboratory and factory contaminants,

*A suitable piece of equipment for testing air samples under standard conditions is Airborne Bacteria Sampler Mark II. From: C. F. Casella and Co. Ltd., Regent House, Britannia Walk, London, N.1.

the following typical spoilage organisms obtainable from Type Culture Collections have been recommended for the testing of preservatives (*Appendix E, 2.7,* p. 797):

Bacteria

Staphylococcus aureus, Staph. epidermidis, Micrococcus spp., Streptococcus spp, Corynebacterium pseudodiphtheriticum, Coliform, Coli-Aerogenes group, *Proteus, Pseudomonads* including *Ps. aeruginosa, Bacillus cereus,* and *B. Subtilis.*

Moulds and yeasts

Fungal organisms should include *Penicillium, Aspergillus, Mucor, Cladosporium, Alternaria, Trichoderma viride, Candida* and *Saccharomyces* species.

Naturally occurring bacterial contaminants will usually flourish more vigorously in a manufactured product than will the type culture strains of medical importance. It is advisable, therefore, first to inoculate the unpreserved formulation. Only those organisms showing vigorous growth and surviving for more than ten days in the product should be selected for testing preservative effectiveness. The selection of appropriate spoilage organisms and the number of strains to be used in a particular study is obviously important; the final choice and method of inoculation is left to the discretion of the microbiologist performing the test. An unpreserved formulation should always be included to serve as control; this should be checked for microbial content *before* commencing preservation studies. Inoculation with bacteria may be carried out with an actively growing broth culture by stirring 0.1 ml of culture into about 30 ml or the equivalent of product. (See also *methods 2.121 and 2.122.*) A viable count is performed on each suspension to estimate the number of live bacteria which has been mixed into each test sample. Inoculation with moulds and bacteria must be carried out separately.

Care must be exercised in the handling of preparations which have been deliberately infected with potentially dangerous micro-organisms. Strains of *Pseudomonas aeruginosa* recently isolated from clinical infections can be particularly harmful to the eyes and should only be used in preservation studies with great care to avoid disseminating infection.

The culture used for test purposes may be grown on solid or in liquid medium; a young and actively growing culture is essential. One of the following procedures will normally be applicable.

2.121 Culture on solid medium

Inoculate slopes from stock culture and incubate for 24 h, or longer if necessary, at the optimum temperature for the organisms. Maintain in continuous active culture by sub-culturing onto fresh solid medium at intervals of 24 h on three successive days.

2.122 Culture in liquid medium

Inoculate a tube of Nutrient Broth from the stock culture and incubate at the optimum growth temperature of the organism. Maintain in continuous active culture by subculturing into fresh medium at intervals of 24 h on three successive days. The cells should be well washed by centrifugation to remove surplus nutrient medium. A suspension should be prepared in sterile distilled water and standardized to an opacity or optical density calculated to produce a viable count usually in the order of 10^7 to 10^9 organisms. Yeasts and fungi are grown on slopes of Malt Extract Agar or Sabouraud Dextrose Agar for three and seven days respectively at 22-25°. Dry spores, rather than mycelium, suspended in small volumes of water are recommended for tests with fungi. One drop or a loopful of suspension should be applied to the surface of the product (usually a cream or emulsion) to be tested. In all tests, the inoculum should be in a form which interferes as little as possible with the properties of the product. The sample should be inspected visually for mould growth.

2.13 *Storage tests*

Long-term storage tests need to be conducted before finally approving a preservative for use in a particular product. These tests consist of exposing the finished product, processed under normal manufacturing conditions so as to include the usual environmental microflora, to a wide range of temperature and relative humidity conditions along with regular exposure to atmospheric contamination. The preparation should be inspected for visual and olfactory evidence of deterioration and samples periodically cultured for evidence of microbial contamination. Packaging materials may reduce the efficacy of preservatives by physico-chemical interaction. It is therefore important that the product be tested with the same type of container and closure that will actually be used. During the period of surveillance, which might be from six months to two years, resistant organisms may slowly develop in the product until they eventually flourish in what was previously a hostile environment. Ample time should be allowed for such adaptation. It follows that no final conclusion regarding the efficacy of a preservative in a given system can be reached for several months.

3. Product testing

3.1 *Toothpaste*

3.11 *Examination of samples in current production*

Extrude a small portion of paste from each tube and discard in order to eliminate chance contamination. Add approximately 1 g of paste to 9 ml of Peptone Water Diluent, mix thoroughly and plate out 6 aliquots each of 0.1 ml on Oxoid Nutrient Agar. Incubate the plates in triplicate at 28° or 37° and examine at 24 h intervals. Discard the plates after 7 days if no growth occurs.

3.12 *Plate-count technique for estimating viable bacteria in toothpaste*

Measure 1 g amounts of toothpaste into sterile bottles containing 9 ml of Peptone Water Diluent and mix thoroughly. Prepare decimal dilutions of the suspension in the same diluent down to 10^{-7}. Transfer four aliquots, each of 1 ml from each dilution to sterile Petri dishes and mix with Nutrient Agar. Incubate the plates in duplicate at 28° and 37° followed by colony counting after 48 h.

3.13 *Method for estimating survival of bacteria added to toothpaste*

Culture the test bacteria, preferably an isolate from a contaminated toothpaste, for 24 h at 28° on Nutrient Agar slopes. Wash the cells from the slopes and wash again twice before re-suspension to a final concentration of 10^9 ml^{-1}; use sterile distilled water throughout this procedure.

The paste for this test should have been made in the normal fashion and amounts of approximately 400 g placed in sterile glass jars (100 mm diameter) to a depth of 60 mm. Ensure that the surface is as smooth as possible. Layer 50 ml of the bacterial suspension on the surface of five such portions of toothpaste. During subsequent storage the cells become evenly distributed throughout the paste. Cover each jar to minimise evaporation but allow condensation to occur within the head space. Carry out viable counts at 28° (see *3.12*) immediately and after 1, 2, 3 and 4 weeks' incubation at 28°. Prior to each viable count, mix the contents of the jar thoroughly for 15 min. If a preservative is included in the toothpaste a suitable inactivator must be included in both the diluent and the Nutrient Agar.

3.2 *Shampoo*

3.21 *Microbial spoilage*

Shampoos based on detergents such as triethanolamine lauryl sulphate and sodium lauryl ether sulphate are usually able to support the growth of

bacteria if no effective preservative is present. Organisms responsible for shampoo spoilage consist largely of gram-negative, non-spore-bearing rods. Moulds and yeasts are rarely encountered. When contamination occurs, a sediment or "ropiness" may become visible but not always; the aroma may alter noticeably and also there may be a potential risk to the user. To avoid these undesirable consequences, it is theoretically possible either to employ sterile raw materials and handle them aseptically so as to avoid extraneous contamination altogether, or alternatively, to operate with a reasonably high standard of cleanliness and include a bactericide. Thorough aseptic technique would be unduly expensive and elaborate for shampoo manufacture and it is therefore customary to adopt the second course.

3.22 *Testing the preservative*

Before adding the preservative to the shampoo, set aside about 400 ml of the unpreserved product. Store at about 4° until ready for use as a control. After the preservative has been added to the shampoo, allow the product to stand at room temperature for at least 48 h. The length of time that should be allowed for chemical interaction between shampoo and preservative will largely depend on the stability of the preservative under test, e.g. formalin disappears very quickly from some formulations, but products containing less reactive preservatives should be retained before testing for at least 5-10 days. It is obviously best to allow a prolonged interaction time to be sure that the preservative will be stable in the shampoo formulation.

3.23 *Test inoculum*

The test inoculum should comprise representative strains of aerobic non-sporing gram-negative bacteria initially isolated from factory plant, spoilt shampoo, and contaminated detergent preparations. The inoculum should include a selection of *Pseudomonas* species. The organisms should be recent isolates and capable of vigorous growth in detergent solutions. The test bacteria should be grown on slopes of Nutrient Agar containing 5% of the unpreserved shampoo, for 18-24 h (see *methods 2.121 and 2.122*, p. 756). Experimental inoculation of the shampoo should be carried out in duplicate using a washed suspension of micro-organisms suitably diluted in sterile water. For test purposes a suitable inoculum of viable organisms is between 10^7 and 10^9 ml^{-1}.

To each 100 ml sample of shampoo add 1 ml of the standard bacterial suspension. Mix thoroughly but avoiding excessive froth. After inoculation, incubate the samples at 22° and 30°. Estimate bacterial counts in Tryptone

Glucose Yeast Extract Agar by plating out on the day of inoculation and after 1, 2, 3 and 4 weeks' incubation. To obtain more detailed information on bactericidal activity, viable counts can be performed more frequently. The following schedule may be used: viable counts are made on control and test samples immediately after inoculation, after 1 h, 6 h, and 24 h, then at daily intervals up to one week. If at any time there is an immediate decrease to a zero count which is maintained for two consecutive weeks, a second challenging inoculum should be added with further periodic culturing. This provides information on the sustained ability of the preservative to protect the product. The following controls should be included:

3.231 Test shampoo manufactured without preservative. This sample is not inoculated.

3.232 Shampoo manufactured as above, heated in a water-bath for 1 h at 65°, allowed to cool and inoculated with 1 ml of standard suspension. (The heat treatment is usually sufficient to destroy extraneous contaminants but before experimental inoculation the sample should be checked for viable bacteria).

3.233 Another shampoo formulation, known to be definitely self-sterilizing. Inoculated with 1 ml of standard suspension.

Findings on these controls should be interpreted thus:

3.231 Since this sample was not inoculated, the recovery of viable organisms will confirm the need for an antibacterial preservative.

3.232 The inoculated sample without preservative should normally demonstrate a colony count of at least 10^6 organisms ml^{-1} throughout the test period and thus serves as a check on the viability of the test inoculum.

3.233 Following experimental inoculation, the shampoo should normally demonstrate a self-sterilizing action within 7 days.

3.24 *Acceptance of shampoo preservative systems*

Incubation of the shampoo should proceed at 22° and 30° for at least one month. Plate count and sterility tests should be carried out during this time to ensure that the product remains sterile.

An effective shampoo preservative should be bactericidal in action so that, following experimental inoculation, the product becomes virtually sterile within 1-7 days. Although rapid bactericidal action is highly desirable, a slow-acting but stable preservative is, however, equally acceptable; it is most important that the preservative should not only yield a sterile

product but should continue to retain its activity within the product in order to cope with any subsequent contamination.

3.25 *Bacteriological examination of shampoo production (bulk samples.)*

Transfer a requisite amount of shampoo to a sterile bottle suitably calibrated, e.g. 30 g of shampoo made up to 300 ml with sterile diluent will give an initial dilution of 1:10. If the shampoo is highly contaminated it may be necessary to prepare a number of 10-fold serial dilutions by aseptically transferring 1 ml to 9 ml of Peptone Water Diluent. From each dilution remove 1 ml and add to separate sterile 20 × 100 mm Petri dishes. Add 15 ml of sterile liquefied Plate Count Agar which has been cooled to 45°-47°. Swirl the agar and allow to harden. Invert the Petri dishes and place in a 30° incubator for 48 h. Remove and count colonies.

In favourable circumstances, bulk shampoo will prove to be sterile. However, since the action of the preservative is not instantaneous, a *finite time* will be required for sterilization of shampoo initially bearing a reasonable level of contamination. The product will only fail to become sterile within 48 h if the initial contamination is exceptionally heavy or if resistant strains are encountered.

3.26 *Bacteriological tests on shampoo packs*

The schedule of testing given below should be regarded as a minimum requirement. The conduct of further studies from time to time will help to ensure that standards of hygiene are not falling below safe levels; for example, it is useful to keep a proportion of packed stock up to one month *before* testing to make certain that growth does not occur.

In conducting tests for sterility, aseptic precautions should be taken throughout all procedures. Testing should not be conducted under direct exposure to ultraviolet light or in areas under disinfectant aerosol treatment. Suitable environmental control tests, including plate counts, should be performed at regular intervals. Test each lot of culture medium for sterility and for its growth-promoting properties. The medium must be appropriately modified or precautions taken to neutralize the activity of any antimicrobial in the preparation tested (see *1.41*). Wide-bore pipettes are suitable for the transference of viscous liquids, e.g. H. J. Elliott Ltd. E-mil 1 ml disposable serological pipettes (Cat. No. D.P.1010, in sterile packs).

3.261 Broth sterility test

3.2611 Take two bottles or sachets for each half-day's filling from each filling line. Reserve one of these samples as a duplicate for examination only

if the first sample fails to satisfy the test. Take two separate 1 ml aliquots from the pack to be examined and dilute each aseptically with 9 ml Nutrient Broth in a 30 ml (1 oz) Macartney bottle. Incubate at 30° for at least 24 h.

When taking aliquots from a bottle pack, invert the bottle several times and transfer the sample aseptically, flaming the mouth of the bottle each time it is opened; the transference should actually be carried out in close proximity to the burner.

To take an aliquot from a sachet, snip the corner using a pair of scissors, flamed by dipping in alcohol and burning away the excess; cutting should be carried out while the scissors are still hot. Transfer aseptically as in the case of bottle packs.

If growth in the broth culture is difficult to detect owing to turbidity of the product, sub-culture a loopful onto Nutrient Agar, or use *method 3.35*.

3.2612 If the 24 h reading is negative (no visible turbidity), the batch may be passed *provisionally*. Tests should be continued, however, for at least a *further 48 h* for information. If the reading is positive (any degree of turbidity indicating bacterial growth), the tests should be repeated on the duplicate sample and on further aliquots taken from the same sample which will have stood at room temperature for 24 h. After 24 h incubation, negative cultures may be taken to show that contamination has died out and the batch would then be accepted as satisfactory. Material failing to pass the test under these conditions should be discarded, except that there would be no objection to repeated sampling after several days' storage to ascertain whether the contamination has eventually died out.

3.262 Membrane filtration methods for shampoo testing

Tests for coliforms, total counts, and sterility may be carried out by means of membrane filter techniques. Ability to filter the product (usually employing a membrane having a pore size of 0.45 μm) is only restricted by the amount of gross suspended matter present. The membrane filter technique offers certain advantages over the conventional methods of testing:

Larger volumes of fluid may be sampled than are conveniently handled with conventional plating techniques.

Small numbers of organisms can be detected, which might be missed with conventional counting methods.

Counts can be carried out in a shorter time.

To eliminate the effect of preservative or antimicrobial, the membrane can be washed free from inhibitory substance.

In principle, procedures similar to those used in water bacteriology may

be adopted for shampoo testing. The membrane filter should not, however, be used for testing shampoos containing preservatives which are not completely soluble in water. For the recovery of *Pseudomonads* and similar micro-organisms a membrane with a pore size of 0.22 μm is recommended. A wide range of membrane filtration systems, including disposable filter units, is now available commercially. For further technical information concerning membrane filter apparatus, filtration and dilution of sample, recovery media and incubation of membranes, the following technical references should be consulted:

GENERAL REFERENCES

Microbiological Analysis of Toiletries and Cosmetic Products. Application Report AR-16. (Millipore U.K. Ltd., Heron House, Wembley, Middlesex).
Membrane filtration with 'Oxoid' membrane filters and membrane media. Temporary leaflet (July 1967) (Oxoid Ltd., London).
Burman, N. P. *et al.* "Membrane Filtration Techniques" in Shapton, D. A. and Gould, G. W. *Isolation methods for microbiologists,* Technical Series 3. (Academic Press, London).
Collins, C. H. *Microbiological Methods* 2nd Edn. 150-152 and 313-314 (Butterworths, London).
Mulvany, J. G. Membrane filter techniques in microbiology, in Norris, J. R. and Ribbons, D. W. *Methods in microbiology* **1** (1969) (Academic Press, London).

3.3 *Creams and lotions*

3.31 *Assessment of preservative capacity*

When practical, test in the final pack and use the product without preservative as a control. Samples should be inoculated in triplicate with spoilage organisms (bacteria, moulds, yeasts and fungi). Incubate one set of samples at 22°, one set at 32° and another set at 35-37°. When testing for bactericidal action, steps should be taken to neutralise any residual preservative (see *Section 1.4,* p. 750).

3.32 *Inoculation with bacteria*

This is carried out by stirring about 0.25 ml of a bacterial suspension into each 25 g sample of the product, using a sterile spatula. The inoculum should be suitably diluted with Peptone Water Diluent to give a final concentration of about 10^6 organisms g^{-1} of product. The test inocula should include micro-organisms recently isolated from spoilt preparations. Additionally, the following strains obtainable from Type Culture Collections are recommended as test organisms: *Staphylococcus aureus, Streptococcus faecalis, Pseudomonas aeruginosa, Pseudomonas fluorescens, Escherichia coli, Klebsiella* spp. and *Proteus* spp.

Immediately after inoculating the samples and thereafter at regular intervals, estimate the number of viable organisms by plating out in Plate Count Agar. (See *methods 3.35 and 3.36*). A drastically reduced count or the

demonstration of an apparently sterile product which remains sterile on three successive samplings will suggest that the preservative is likely to give good protection against bacteria. However, it is prudent to keep the samples under test for 2-3 months or longer until it has been clearly established that any surviving bacteria do not increase in numbers with further incubation

3.33 *Inoculation with fungi*

The following fungi may be used as test organisms: *Aspergillus niger, Penicillium, Cladosporium, Alternaria, Fusarium, Mucor, Rhizopus, Phoma, Trichoderma* and *Verticillium* species. Since the samples are to be inspected visually, the dark pigmented fungi and green moulds are the most suitable. Inoculate only the surface of the cream with mould spores. This can be done by transferring a small inoculum of dry spores from the surface of a sporulating culture by means of a 2 mm loop, scattering the spores over the surface of the cream. Incubate in a moist atmosphere (95-100% RH) at 22-25°. Examine visually at regular intervals for evidence of mould growth during a six months' test period. In the absence of visible growth, subcultures to Malt Extract Agar and Broth can be made to establish whether the inoculum has been inhibited or killed.

3.34 *Inoculation with yeasts*

Suitable strains can be isolated from spoilt preparations. *Candida albicans* and *Saccharomyces cerevisiae* are also recommended. Allow the organisms to grow on slopes of malt extract agar for two days. The inoculation and plate count procedure is the same as for bacteria except that yeast counts are estimated in Malt Extract Agar.

REFERENCES

Wedderburn, D. L. *Advances in pharmaceutical sciences*. Preservation of emulsions against microbial attack 1 (1964) (Academic Press, London).
Tenenbaum, S. *Toilet Goods Assoc. Cosmet. J.* **2** 24 (1970).

3.35 *Method for detecting the survival of bacteria in emulsion systems*

The simple method given below is particularly useful when testing large numbers of samples. It can be used for spot checks on many emulsion systems or for following the development of bacterial contamination by daily tests on one system. The test is based on the growth of organisms in the emulsion on an indicator medium, keeping them within a defined area and noting their effect in mass. The indicator is 2:3:5-triphenyltetrazolium chloride (TTC) which becomes reduced to insoluble red formazan.

N.B. TTC is photosensitive in solution and should be stored in the dark in an amber bottle. The solution can be sterilized satisfactorily by membrane filtration or by autoclaving at 121° for 15 min. The solution cannot be incorporated in the medium and autoclaved. This causes a reduction to formazan.

3.351 Test medium (TCA)

The medium is made by adding 0.3% Oxoid Agar no. 1 to Oxoid Blood Agar Base no. 2 before making up according to the manufacturer's instructions in 200 ml amounts in screw-capped bottles. For use, add 0.5 ml of a 1% freshly prepared sterile aqueous solution of 2:3:5-triphenyltetrazolium chloride to 200 ml of Oxoid Blood Agar Base no. 2 which has been suitably liquefied. Mix the contents and pour plates. Allocate about 20 ml of medium to a standard 100 mm Petri dish. Store the plates in the dark at 4° and dry off before use at 37° for about 1 h. If only five plates are required, add 0.25 ml of 1% solution of TTC to 100 ml of medium.

3.352 Test

Imprint 5-6 circles in each plate of TCA medium with a sterile, lightly flamed no. 7 cork borer (10 mm diam.).

With a sterile 5 mm wire loop, transfer a sample of liquid emulsion to the centre of an imprinted circle. For the transfer of creams, take a small sample, about 0.1 g, on the end of a sterile wooden applicator* and apply to a fresh circle of agar. Incubate at 37°. Read tests within 18 h of incubation.

3.353 Results

Heavily contaminated emulsions give a red spot within the imprinted circle. Increasing density of red spots indicates various intensities of bacterial contamination. Fine reddish granules or dots (stipples) can be seen in positive creams. Also the area surrounding the cream is deeply coloured. Bacteria-free emulsions give no colour reaction, but a negative result does not necessarily mean the total absence of organisms. Negative samples may be re-checked in nutrient broth and agar in the usual way.

N.B. W/o emulsions tend to 'splutter' when heated in a naked flame. To prevent the spread of infected particles, wire loops charged with these materials should be sterilized in a burner which is equipped with a protective hood. A Kampff pattern micro-incinerator (Arnold R. Howell Ltd.) with a

*Obtainable from Arnold R. Horwell Ltd., London, N.W.6. The sticks should be autoclaved at 121° for 15 min before use and afterwards destroyed by incineration.

Pyrex glass chimney to contain the infected particles is useful for this purpose.

REFERENCES

Hill, E. C., Davies, I., Pritchard, J. A. V. and Byron, D. *J. Inst. Petrol.* **53** 275 (1967).
"*Tetrazolium salts*" publication by British Drug Houses Ltd., Poole, Dorset.

3.36 *Plate count technique for estimating viable organisms*

Aseptically transfer 1 g of emulsion to a universal bottle containing approximately six to eight glass balls, 8 ml of Peptone Water Diluent and 1 ml of sterile 1% *Tween 80*. Mix the contents on a Whirlimixer for 10 s. Prepare further tenfold dilutions, as necessary, with Peptone Water Diluent containing 0.1% *Tween 80*.

Plate out 1 ml aliquots in Nutrient Agar or Plate Count Agar. The tip of the pipette must be inserted well into the middle or lower layer of diluent when the sample is drawn to ensure that it is free from solid material.

Creams which are not miscible with water should be emulsified in *Lubrol W* broth according to the procedures previously described.

REFERENCE

Dunnigan, A. P. and Evans, J. R. *Toilet Goods Assoc. Cosmet. J.* **1** 38 (1969).

3.37 *Membrane filtration method for the detection of small numbers of organisms*

For water-based emulsions, prepare the sample in 0.1% sterile surfactant in Nutrient Broth but in the case of w/o emulsions, dissolve 0.1 g material in 25 ml of *iso*propyl myristate and proceed as for water-based emulsions as follows:

Prepare a 0.1% solution of *Triton X-100* in Nutrient Broth. Dispense the solution in 25 ml quantities and sterilize by autoclaving. Prepare the sample by aseptically weighing 0.1 g of product into one of the bottles of surfactant. Warm to 47° on a water bath and shake well to disperse the oil droplets thoroughly. There should be no visible clumping. (The use of a Vortex mixer at this point is an advantage.) Pour approximately 50 ml of the sterile 0.1% surfactant at 47° into a sterile filter holder containing a sterile membrane (47 mm diam. 0.5 μm pore). Add the sample solution to this as rapidly as possible, swirl and filter under vacuum. With the vacuum still applied, rinse the surface of the filter with 100 ml of sterile 0.1% surfactant solution at 47° to remove traces of oil from the filter. Follow this with a 100 ml rinse of sterile 0.1% peptone at 47°. When completely filtered place the membrane on Nutrient Agar for incubation at 28-32° and count colonies after 48 h or culture the membrane according to B.P. recommendations.

3.371 Materials

Millipore Sterifil Filtration System. Cat. No. XX1104700
Oxoid Membrane Filters Size 47 mm. Code No. MF47 or Millipore Filter Discs 47 mm Cat. No. HAEG047AO, 0.45 μm
Triton X-100. Nonionic wetting agent: Lennig Chemicals Ltd., 2 Mason's Avenue, Croydon, Surrey.

REFERENCE

Sterility testing of ointments and creams. Booklet published by Millipore U.K. Ltd., 109 Wembley Hill Road, Wembley, Middx.

3.4 *Powders*

Powders, derived from natural earths, should be examined for yeasts and fungi as well as for aerobic and anaerobic bacteria with special reference to pathogenic *Clostridia.* Talcs claiming deodorant or antiperspirant properties usually contain hexachlorophane, aluminium chlorhydrate or other antimicrobial compounds having a bacteria-inhibiting effect; a suitable inactivating agent must then be included in the test media.

3.41 *Detection of aerobic bacteria (qualitative)*

Using an aseptic technique shake 10 g of powder into 90 ml of Peptone Water Diluent. Plate out 0.1 ml aliquots on duplicate plates of Blood Agar, Nutrient Agar, and Malt Extract Agar. Spread the inoculum with a wire loop to obtain separate colonies. Incubate at 22° and 37°. Examine the Blood Agar and Nutrient Agar plates for sporing and non-sporing bacteria after 48 h incubation. Examine the Malt Extract Agar plates for moulds, yeasts and fungi at 24 h intervals up to seven days.

3.42 *Detection of Clostridia (qualitative)*

Plate out 0.1 ml of a peptone water suspension (as in *3.41*) on the surface of overdried Blood Agar, Nutrient Agar and Reinforced Clostridial Agar. Inoculate freshly prepared Thioglycolate Medium and Cooked Meat Broth with 1 ml of the peptone water suspension introducing the inoculum well into the meat layer. Inoculate further samples of Cooked Meat Broth and heat at 80° for 10 min in a water-bath to kill non-sporing organisms. Incubate all samples for *anaerobic* culture at 37°. Examine after 3-4 days' incubation. Sub-culture broth medium to Blood Agar. Check that isolates will not grow under aerobic conditions.

3.43 *Aerobic count*

Using a sterile E-mil weighing scoop, transfer 1 g of powder into 99 ml of Peptone Water Diluent. From this suspension (1:100) prepare a number

of tenfold serial dilutions by aseptically transferring 1 ml to 9 ml of Peptone Water Diluent. From each dilution remove 1 ml and add to separate sterile 15 × 90 mm Petri dishes. Plate out each dilution in triplicate. Add 15 ml of liquefied Plate Count Agar which has been cooled to 45-50°. Mix the agar and allow to harden. If necessary to prevent the swarming of bacteria, add a 5 ml overlay of Nutrient Agar and allow to harden (1). Invert the Petri dishes and incubate at 30° for 48 h. Remove plates and count colonies. Plate out as above in acidified Malt Extract Agar for the enumeration of yeasts and fungi.

3.44 *Anaerobic counts. Method 1*

Prepare dilutions as above but use Reinforced Clostridial Medium as a diluent. Plate out 1 ml aliquots in Reinforced Clostridial Agar and Plate Count Agar. Incubate for anaerobic culture at 30° and 37°. Count colonies after 48-72 h.

On ordinary agar the colonies of most *Clostridia* are large and spreading, but their size may be minimised by over-drying the plates or separate colonies ensured by plating out on Charcoal Agar (2). Concentrated agar e.g. 2.5–3% may also be used to prevent the spread of colonies.

3.45 *Anaerobic counts, Method 2*

If isolations are not being made and only counts are required, the following method may be used. Transfer 1 ml aliquots from serial tenfold dilutions of the suspension into sterilized, plugged Miller-Prickett tubes*. Cool freshly prepared reinforced Clostridial Agar to approximately 50° and, without shaking, add about 25 ml to each tube. Seal immediately with melted sterile paraffin wax and allow to set in a water-bath at about 15°. Incubate at 37° and count colonies after 48-72 h. Run at least one blank to detect contaminants occurring during the procedure. This method provides a simple means of culturing anaerobic bacteria without recourse to the use of an anaerobic jar (3,4).

REFERENCES

(1) White, M., Bowman, F. W. and Kirshbaum, A. *J. Pharm. Sci.* **57** 1061 (1968).
(2) Alwen, J. and Smith, D. G. *J. Appl. Bacteriol* **30** 389 (1967).
(3) Miller, N. J., Garrett, C. W. and Prickett, P. S. *Food Research* **4** (5) 447 (1939).
(4) *Oxoid Manual* 3rd edn. 222 (1965).

3.5 *Eye cosmetics: Solid, semi-solid and liquid preparations*

3.51 *Examination of samples in current production*

A representative number of samples should be examined for bacteria

*Miller-Prickett Anaerobic tubes may be obtained from Astell Laboratory Service Co. Ltd., London, S.E.6.

and moulds after a suitable period of storage in a moist atmosphere, e.g. after 5 days and again after one month.

3.511 Detection of bacteria

Add 0.1-0.2 ml of sterile distilled water to moisten dry preparations and incubate the samples in their containers at 22°. Culture for bacteria after 5 days' incubation, maintain in a moist atmosphere and re-check a proportion of stock after 1 month.

3.512 Swabbing technique (Qualitative examination)

When dealing with solid preparations it is sometimes convenient to culture from swab samples. Swabs are preferably prepared from calcium alginate (*Calgitex*) wool. The swabs are made by wrapping about 30 mg of calcium alginate (*Calgitex*) wool around the final 2 cm of a wooden applicator stick. The swabs are sterilized by autoclaving in glass tubes for 15 min at 122°. *Calgitex* swabs are also obtainable from a commercial source*. These are supplied ready for use in sterile packs. The preparation is sampled in its container. Moisten the swab in Peptone Water Diluent or nutrient broth before collecting a sample from a dry surface. The swab should be firmly applied and slowly rotated, thoroughly covering the exposed surface. Culture to Nutrient Broth and Agar, streaking out the inoculum on the solid medium with a wire loop to obtain separate colonies. Examine cultures after 48 h incubation at 30°.

3.513 Viable count (Bacteria)

Transfer 1 g of material aseptically to a universal bottle containing 8 glass balls, 8 ml of Peptone Water Diluent, and 1 ml of sterile 1% *Tween 80*. Mix on a Whirlimixer for 10 s. Prepare tenfold dilutions in peptone/*Tween* diluent from the 1:10 suspension. Plate out in duplicate and spread 0.1 ml aliquots of each dilution on the surface of dried Nutrient Agar plates. Allow the inoculum to dry, invert the Petri dishes and place in a 30° incubator for 48 h. Remove the plates from the incubator and count the colonies.

3.514 Detection of moulds

The following method may be used for the detection of mould contaminants in solid preparations, pressed powder eyeliners, mascara, etc.:

Transfer replicate samples with their coverings removed to glass Petri dishes which have been lined with filter paper (Whatman No. 1 filter

*Arnold R. Horwell Ltd., 2, Grangeway, Kilburn High Road, London N.W.6.

circles 9 cm diam. will conveniently fit into the lower half of a standard size dish). Prior to incubation saturate each filter circle with about 1.5ml of sterile distilled water and moisten the surface of the preparation with 0.1-0.2 ml of sterile water. Transfer the samples, in covered Petri dishes, to a plate box or other suitable container with a tight fitting lid and incubate at 18-22°. Alternatively, when dealing with a number of samples, large culture dishes (30 × 30 × 2.5 mm)* suitably lined with moistened filter paper and sealed with an air-tight covering may be used.

Periodically moisten the samples and inspect visually at regular intervals for evidence of fungal growth. Under favourable growth conditions, moulds may appear as discrete colonies visible to the naked eye within 10-14 days of incubation. Negative samples should continue to be incubated in a moist atmosphere for at least 6 weeks.

3.52 *Assessing preservative capacity*

It is recommended that all products intended for use in or around the eye should contain a preservative bactericidal to *Pseudomonas Aeruginosa.*

3.521 Preservation against bacterial contamination

When practical, test in the final pack and use the product without preservative as a control. Inoculate the preparation in triplicate with appropriate strains of bacteria at a level of 10^6 organisms g^{-1}. Allow the inoculum to soak into the surface of solid and semi-solid products or mix in with fluid products. The following test organisms are recommended: *Pseudomonas aeruginosa, Ps. fluorescens, Staphylococcus aureus, Micrococcus luteus, Streptococcus faecalis.* In addition spoilage organisms isolated from contaminated products may be used. Incubate the inoculated samples at 30° and 37°. To observe bactericidal action, carry out viable counts or do sterility checks on the test and control samples immediately after inoculation, after 1 h and 6 h, and then at daily intervals for one week, followed by counts and sterility checks at further weekly intervals. In conducting these procedures, it is important that effective preservative inactivation is carried out and that a sufficiently large volume of broth is used in sterility testing.

3.522 Preservation against mould growth

Studies on mould contamination should be carried out separately. When possible the preparation should be tested in its final pack. The method of

*Jencons (Scientific) Ltd., Mark Road, Hemel Hempstead, Herts.

inoculation and range of fungal organisms is generally the same as used in the testing of creams and lotions. Moulds isolated from spoiled products should also be used. After inoculation, incubate test and control samples at 22-25° in a moist atmosphere as previously described. Inspect regularly for evidence of fungal growth for a test period of not less than 3 months.

3.523 Saliva test

A test which simulates the customary practice of the user in moistening such products as eyeliners and mascara with saliva, thereby contributing large numbers of micro-organisms to the preparation, may be carried out in the laboratory.

For this purpose, pool together 4-6 samples of fresh saliva. The mixed sample will carry a rich microbial flora that is normally indigenous to saliva, including large numbers of *Streptococci*, *Staphylococci*, and *Neisseria*. Spread an inoculum of about 0.2 ml of the pooled saliva over the surface of duplicate preparations. Incubate the test samples, and a control, in a moist atmosphere at 22°. Periodically moisten dry samples with 0.1-0.2 ml of sterile distilled water. Observe bactericidal action by culturing the infected samples on Blood Agar at 37°. Sub-culture at regular intervals according to the schedule previously described for bacteria. If a complete kill has been achieved, the challenge test may suitably be repeated on the same sample with fresh saliva.

4. Choice of preservatives

4.1 *Requirements*

Modern cosmetic preparations, unless adequately preserved, often present a favourable environment for microbial growth. Apart from the health hazard, this may cause separation of emulsions, discoloration, changes in viscosity, the formation of gas and of off-odours. The extent of microbial growth depends largely on the type of formulation, water content, phase composition of an emulsion, pH, nutritive value, amount and type of perfume, and the type of container in which the product is packed. After manufacture, most products are likely to be exposed to further contamination from filling machines, containers and caps. Fungal growth on the surface of cosmetic creams has often been traced to infected cap-liners. Products packed in wide-mouth jars are easily contaminated by the user's fingers. Flexible bottles which draw air back into them are liable to suffer airborne contamination during use. Products packed in collapsible tubes and bottles with small apertures are less prone to contamination in use but

they may exhibit spoilage owing to the difficulties experienced in cleaning these packaged components effectively before the product is filled.

Creams and lotions generally need to be protected against attack by moulds, yeasts, aerobic and anaerobic bacteria by means of a suitable preservative. Organisms of particular concern from the public health aspect and which are most likely to be present as contaminants are *Pseudomonas aeruginosa*, *Klebsiella* species, and coagulase-positive *Staphylococcus aureus*. The dark pigmented fungi, such as *Cladosporium* and coloured moulds, can be a particular nuisance since they so obviously disfigure the product. Although they do not present a serious public health hazard, they could possibly serve as allergens.

Anionic detergents in many shampoos are particularly vulnerable to bacterial decomposition. Shampoo prepared from such materials must be protected against gram-negative bacteria generally, and *Pseudomonads* in particular.

Preparations intended for application to or near the eye such as solid or liquid eyeliners, eyelash materials, and pigmented materials for make-up around the eye require particular attention. These preparations should be sterile at the time of manufacture and should also contain a preservative known to be bactericidal to *Pseudomonas aeruginosa*. Similar precautions are necessary for products to be used on newborn infants or for application to broken skin.

Powders, such as raw talcs and mineral earth pigments, may contain large numbers of fungal spores, aerobic and anaerobic bacteria including *Clostridial* spore-bearing species; when used for cosmetic purposes they may need to be subjected to an efficient form of presterilization. Reliance, in this connection, should not be placed on the incorporation of a preservative.

4.2 *Recommendations*

A preservative should always be regarded as an additional aid in prolonging the shelf-life of a product. It must never be used to mask deficiencies in hygiene. In the avoidance of spoilage, good housekeeping is essential to cosmetic manufacture just as it is to food production. Notwithstanding good manufacturing conditions, unforeseen and exceptional contamination may occur during routine manufacture which will overwhelm an inadequate preservative system and render it ineffective. The preservative capacity of a product should therefore exceed the minimum requirements

necessary to inhibit or destroy anticipated levels of microbial contamination. The problem of product spoilage may be exacerbated by using antimicrobial agents which are themselves susceptible to contamination. Disinfectants and antiseptic solutions are sometimes metabolised by opportunist bacteria. This situation can result in an unexpected public health hazard; instances have, in fact, been recorded where clinical infections have been caused by a contaminated solution of an antibacterial. Given the opportunity, bacteria will often show a remarkable ability to adapt to a hostile environment.

Antimicrobial compounds used in industry today offer varying degrees of preservative action, depending upon such factors as pH, type of emulsifier, micellar solubilization, ease of migration into the oil phase of an emulsion system. There is still an unsatisfied need for the ideal antimicrobial agent that will remain in the aqueous phase of such systems, where microbial contamination is most likely to reach significant levels, continuing to exert biological activity over a broad pH range and throughout prolonged storage. The antimicrobial agent should be effective against a broad spectrum of micro-organisms, especially against the gram-negative bacteria which are most frequently involved in bacterial spoilage problems. Highly potent antimicrobial agents have sometimes proved to be ineffective as cosmetic preservatives whereas compounds with much weaker inherent antimicrobial properties have been quite effective in these compositions. Certain preservatives with good antimicrobial activity are liable to cause irreversible physical or chemical changes in a product and are therefore useless in practice.

Choice of a suitable preservative in adequate but non-toxic concentration can usually be made only after considerable experimental work. Consideration of preservatives should therefore be regarded as an integral facet of the initial formulation activity. Decisions should preferably be reached after close collaboration between the formulation chemist, packaging technologist, physical chemist, pharmacologist, and microbiologist. Safety, compatibility, effectiveness and cost are the over-riding factors influencing the choice of a particular preservative. If the preservative is a new one or used in unusually high concentrations, data to confirm safety-in-use will be required; the risk of sensitizing the skin must also be taken into account.

4.3 *List of preservatives*

This glossary discusses some of the more important antimicrobials which

have found general acceptance in the preservation of cosmetic preparations. The recommended level of preservative should be taken only as a general guide. The precise concentration required will depend largely on the type of preparation and the likely levels of exposure to micro-organisms. Each new formulation must be individually studied and the preservative rigorously evaluated by the appropriate method in the final pack.

4.31 *Hydroxybenzoates*

Methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid (often referred to as 'parabens') and their salts are widely used as preservatives in many preparations.

4.311 Properties

Sparingly soluble in water, the esters are tasteless, odourless, stable on storage and of low toxicity. Each ester is most active against a different range of organisms. Activity increases with increasing chain-length and water solubility decreases in the same direction. The higher esters are more effective against moulds and yeasts. Combinations of esters are likely to be more effective than a single ester. The parabens are active over a relatively wide pH range although their effect on specific types of micro-organisms varies with pH. They are usually well tolerated on the skin, compatible with most cosmetics containing proteinaceous ingredients, and unlikely to cause significant discoloration of the formulation.

4.312 Disadvantages

The parabens have low aqueous solubility; they have inhibitory activity but are not usually bactericidal or fungicidal, and quite often they need to be supplemented with an additional preservative. Activity may also be reduced in the presence of oils and nonionic emulsifiers (e.g. *Tweens*). An increase of paraben concentration above the level of inactivation is necessary for achieving efficient preservation. A reconsideration of the type of non-ionic employed in the product may, however, prove beneficial. *Tween 20* has the least damaging effect followed by *Tween 60*. *Tween 80* will reduce antimicrobial efficiency to the greatest extent. Skin sensitization due to the parabens has been reported, although it seems to be comparatively rare.

4.313 Uses

The methyl ester, which has the highest solubility in water, is commonly employed in concentrations of 0.1-0.3% while the higher esters are used in near-saturated solutions (0.01-0.025%). Combinations of the methyl, propyl and butyl esters are commonly employed (some of which are avail-

able as proprietary preparations). The esters are usually incorporated in the preparation as a solution in boiling water, but in non-aqueous preparations may be dissolved in acetone, alcohol, melted fats and oils, glycerine and triethanolamine, or incorporated in the emulsifier. The sodium salts of the parabens may be used to increase aqueous solubility. The soluble derivatives are suitable for preparing more concentrated solutions than is possible with esters but the solutions are strongly alkaline; they cannot therefore be used in preparations with an acid reaction, since the less soluble ester would be precipitated.

In nonionic systems, propylene glycol is reputed to form synergistic mixtures with *p*-hydroxybenzoic acid. Formaldehyde used at a relatively low level is claimed to be a useful supplement for the parabens and will increase activity generally.

4.314 Proprietary preparations based on *p*-hydroxybenzoates

The following blends are supplied by Nipa Laboratories Ltd.:

Nipasept. A mixture of the methyl, ethyl and propyl esters of *p*-hydroxybenzoic acid. Use up to 0.3%.

Nipasept Sodium. A water soluble form of *Nipasept.*

Nipastat. A mixture of the methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid. Use up to 0.3%.

4.32 *Bronopol (2-bromo-2-nitro propane 1,3-diol)*

4.321 Properties

Colourless, odourless, crystalline solid, highly soluble in water, only slightly soluble in oil. A good bactericidal agent unusually active against *Pseudomonads.* Bactericidal activity is not affected by nonionic surfactants.

4.322 Disadvantages

The compound is unstable to light, though it is stable in acid solution if protected from light. It is unstable in alkaline solution with formation of formaldehyde.

4.323 Uses

Bronopol should be used either in acid solution protected from light or in alkaline solution as a bactericide to achieve a rapid effect. In alkaline solution it may sometimes be used to greater advantage in conjunction with a more stable preservative. It is recommended particularly as a preservative for shampoos and may be used at concentrations of 0.01-0.02%. Dye fading or darkening may result if *Bronopol* is used at higher levels.

4.33 *Formaldehyde*

4.331 Properties

Freely miscible with water and alcohol. A powerful antibacterial and antifungal agent. Formalin solution contains 40% of formaldehyde.

4.332 Disadvantages

Formaldehyde is a volatile compound with a pungent odour and is liable to form insoluble polymers. It is highly reactive chemically and is irritant to the skin. It should only be used as a preservative in relatively low concentrations, and it is incompatible with ammonia, gelatin and other proteinaceous materials, phenols and oxidizing agents.

4.333 Uses

Despite the above disadvantages and the fact that sterilizing activity in the product is of short duration due to its chemical instability, formalin is used quite extensively as a preservative in shampoos. It is recognized generally that formaldehyde up to 0.05% is an acceptable preservative in such products from the toxicological standpoint (1).

4.34 *Organic mercurial compounds*

The relevant compounds comprise the phenylmercuric salts, such as the nitrate, borate and acetate.

4.341 Properties

These are colourless, odorless, crystalline powders, sparingly soluble in water (1:500 to 1:1 500). They are efficient antifungal and antibacterial agents.

4.342 Disadvantages

The phenylmercuric salts are potentially toxic and may sensitize the skin. Activity may be reduced in the presence of nonionic and anionic emulsifying and suspending agents, protein, and compounds having -SH groupings. They are precipitated in the presence of chloride. Some loss of activity may be experienced at high pH values and also through absorption by closure materials.

4.343 Uses

The phenylmercuric salts are specially exempted in the Third Schedule to the Poisons Rules (United Kingdom) provided that they are used (as preservatives) in cosmetic preparations containing not more than 0.01%. Local regulations vary from country to country and should be consulted when necessary. From the toxicological standpoint, the phenylmercuric salts are recommended for use in preparations, such as shampoos which

are applied transiently and then rinsed away (1). Phenylmercuric nitrate may be useful for preserving shampoos having a pH value below 7.0. At higher pH values it is less stable and may be irritating. The phenylmercuric salts are generally used in preparations at levels of 0.001–0.004%.

4.35 *Sorbic acid* (*Hexa-2,4-dienoic acid*)

4.351 Properties

Sorbic acid is a white crystalline powder with a mildly acrid odour, slightly soluble in water (1:600). It functions as an antimicrobial preservative, active against moulds and yeasts and to a lesser degree against bacteria. Antimicrobial activity is retained in the presence of nonionic surfactants.

4.352 Disadvantages

Sorbic acid is only effective in the form of the *undissociated* acid and therefore only of use in formulations below pH 6.5-7. In high concentrations, the sorbates oxidize under the influence of sunlight, which can result in product discoloration. Sorbic acid itself may cause slight itching and erythema, but there is little evidence that it has any adverse cutaneous effect at normal use-levels.

4.353 Uses

Sorbic acid is used primarily as an antifungal agent, with an optimum pH about 4.5. Its fungistatic activity is increased by the addition of acids and sodium chloride. It may be used in preparations at 0.1-0.2%.

4.36 *Dioxin* (*6-acetoxy-2, 4-dimethyl-m-dioxane*)

4.361 Properties

Dioxin occurs as a yellow to light amber liquid, soluble in or miscible with water and many organic solvents. It has a rather strong characteristic odour. *Dioxin* is active against gram-negative and gram-positive bacteria, yeasts and fungi at concentrations in the range of 0.03-0.1% and is active over a wide pH range. Activity is not reduced in the presence of nonionics. It has good bactericidal and fungicidal properties in proteinaceous cosmetic systems.

4.362 Disadvantages

The odour may be objectionable and difficult to mask in some formulations. *Dioxin* may cause some darkening or discoloration of a protein-

aceous product, which increases with time and is accelerated by elevated temperatures.

4.363 Uses

For preservation of emulsions, liquid soap and some proteinaceous cosmetic systems, *Dioxin* may be used at a level of about 0.1%.

4.37 *Phenols*

Phenolic compounds in general interact to form complexes with polyvinylpyrrolidone and certain glycols such as polyethylene glycol 6 000 or polypropylene glycol 1 200, resulting in precipitation or separation of oil, and loss of antimicrobial activity. These compounds have a high oil:water partition coefficient and are usually more active in acid than in alkaline conditions; they are unstable to light, sometimes causing product discoloration. Although many of these compounds are too irritant for use as preservatives in cosmetics, the halogenated phenols in particular have been employed for this purpose. Thus, *p*-chlor-*m*-cresol, *p*-chlor-*m*-xylenol, and dichlor-*m*-xylenol have been used in cosmetic preparations. *o*-Phenylphenol has been recommended for impregnation of paper liners for cosmetic jars. It should be borne in mind that the *p*-hydroxybenzoates are phenolic compounds, having similar physico-chemical properties to the phenols generally.

4.38 *Quaternary ammonium compounds*

The use of these compounds in cosmetics is limited by the frequency of inactivation by other ingredients, especially nonionic and anionic surfactants; they also interfere with the stability of some cosmetic formulations. However, they are readily water-soluble and inhibit bacterial growth at high dilution. Benzalkonium chloride has been found useful at 0.1% concentration. Whilst low concentrations of quaternary ammonium compounds appear to be quite safe to use, high concentrations are usually severe eye irritants.

4.39 *Alcohols*

Where appropriate, ethanol and *iso*propanol may be incorporated in liquid and other preparations in order to impart preservative activity. Concentrations of alcohol from 5-10% upwards generally inhibit moulds and bacteria, and give useful protection against further microbial contamination.

4.4 *New preservatives*

4.41 *Phenonip* (Nipa Laboratories Ltd.)

This is a proprietary preparation based on *p*-hydroxybenzoates. It is supplied in the form of a colourless solution miscible with water, claimed to be active against bacteria, yeasts and moulds. *Phenonip* is stable on storage, compatible with proteinaceous, anionic and cationic systems, and is stated to maintain its activity over a broad pH range. It is recommended by the manufacturers as a shampoo preservative at levels of 0.5-1.0%. Each formulation or composition, however, needs to be examined individually and bacteriological control tests should be carried out.

4.42 *Preservative mixtures*

The principle of blends of several preservatives has not been extensively explored. Dehydroacetic acid has been used with sorbic acid esters (2): studies on various cosmetic formulations containing nonionic surfactants have also indicated that combinations of sorbic acid and hexylene glycol may be effective preservatives (3). Other references to the beneficial use of synergistic combinations have been made (4-7).

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Appendix C
Recommended culture media

1. Formulae

Reference in this monograph to a culture medium identified by means of capital letters at the beginning of each word implies that the formula given in this Appendix should be employed and that the medium will have been sterilized at the stated temperature by autoclaving, unless otherwise directed. Water used for culture media should always be freshly distilled. Before use incubate samples from each batch of culture medium to check sterility and to test for growth promoting properties.

1.01 *Nutrient Broth*

Beef extract	10 g
Peptone	10 g
Sodium chloride	5 g
Water	1 000 ml

Dissolve the ingredients by heating in the water. Adjust to pH 7.6-8.0 with 10 N NaOH and boil for 10 min. Filter. Adjust to pH 7.2-7.4 and sterilize at 115° for 20 min.

Commercial form: *Oxoid* Granules CM67 or Tablets CM68.

1.02 *Nutrient Agar*

Nutrient broth (pH 7.2-7.4)	1 l
Agar powder	15 g

Dissolve the agar in the nutrient broth by autoclaving at 121° for 20 min. Adjust pH to 7.2. Filter through paper pulp. Distribute as required and sterilize at 121° for 20 min.

Commercial form: *Oxoid* Blood Agar Base, Granules CM55 or Tablets CM56.

1.03 *Blood Agar Base No. 2*

Proteose peptone (*Oxoid L46*)	15 g
Liver digest (*Oxoid L27*)	2.5 g
Yeast extract (*Oxoid L20*)	5 g
Sodium chloride	5 g
Oxoid agar No. 3	12 g
(pH 7.4 approx.)	

Add 40 g to 1 000 ml of water and soak for 15 min. Mix and sterilize at 121° for 15 min. Mix well before pouring.

For general purpose Nutrient Agar, Blood Agar Base no. 2 is recommended.

Commercial form: *Oxoid CM271*

1.04 *Blood Agar*

Add defibrinated sterile horse blood to sterile nutrient agar that has been liquefied by heating and then cooled to 52°. The final concentration of blood should be 5-10%.

Haemolysis is best observed in layered blood agar plates. For such plates, a layer of nutrient agar is poured into the petri dish and allowed to set; the blood-containing medium is then poured on top of the nutrient agar.

1.05 *MacConkey Agar*

Peptone	20 g
Sodium chloride	5 g
Sodium taurocholate	5 g
Water	1 000 ml

Dissolve the peptone, sodium chloride and sodium taurocholate in the water by heating. Adjust to pH 8.0. Boil for 20 min. Cool and filter.

Agar	20 g
Lactose	10 g
Neutral red, 1% aq. soln.	10 ml

Add and dissolve the agar by boiling and adjust to pH 7.4. Add the lactose and indicator solution. Mix and sterilize at 115° for 20 min. The quantity of indicator required depends on the depth of colour preferred. Sodium taurocholate, sodium tauroglycocholate or other satisfactory bile salt may be used.

The use of 0.1% *Teepol* (an anionic detergent) in place of bile salt in MacConkey agar has been recommended.

Commercial form: *Oxoid* Granules CM7 or Tablets CM8.

1.06 *MacConkey Broth (Modified)*

Peptone	20 g
Sodium chloride	5 g
Sodium taurocholate	5 g
Water	1 000 ml
Bromcresol purple, 0.2% soln.	5 ml
Lactose	10 g

Dissolve the peptone, sodium chloride and sodium taurocholate in the water by heating. Adjust to pH 8.0 and boil for 20 min. Cool, filter, and adjust to pH 7.4. Add the lactose and indicator solution, mix and distribute into tubes containing inverted Durham's tubes. Sterilize at 115° for 15 min.

Commercial form: *Oxoid* Granules CM5a or Tablets CM6a.

1.07 *Plate Count Agar* (*Tryptone Glucose Yeast Extract Agar*)

Tryptone	5 g
Yeast extract	2.5 g
D-Glucose	1 g
Agar	15 g
Water to	1 000 ml

Add the ingredients to the water and dissolve by steaming. Adjust to pH 7.0, dispense as required and sterilize at 121° for 15 min.

Commercial form: *Oxoid* Granules CM325 or Tablets CM326.

1.08 *Robertson's Cooked Meat Medium*

Minced meat	1 000 g
0.05 N Sodium hydroxide soln.	1 000 ml

Add the minced meat to the sodium hydroxide solution. Mix well and heat to boiling. Simmer for 20 min with frequent stirring. Skim off the fat and check pH which should be about 7.5. Strain through gauze or muslin, squeeze out excess liquor, and dry the meat particles at a temperature below 50°. For use, place sufficient dried meat in a screw-capped container to a depth of about 2.5 cm and add sufficient Nutrient Broth to give a depth of above 5.0 cm. Sterilize at 115° for 20 min. Avoid rapid release of pressure in the autoclave after sterilization. Although called Robertson's Cooked Meat Medium, the method of preparation differs considerably from the original.

Commercial form: *Oxoid* Granules CM81 or Tablets CM82.

1.09 *Sabouraud Dextrose Agar*

Mycological Peptone (*Oxoid L40*)	10 g
Dextrose	40 g
Agar	15 g
Water to	1 000 ml

Dissolve in a steamer and adjust pH to 5.0. Sterilize by autoclaving at 115° for 10 min.

Commercial form: *Oxoid* Granules CM41 or Tablets CM42.

1.10 *Sabouraud Liquid Medium*

Pancreatic digest of casein (*Oxoid L42*)	5 g
Peptic digest of fresh meat (*Oxoid L49*)	5 g
Dextrose	20 g
Water to	1 000 ml

Dissolve in a steamer and adjust pH to 5.7. Autoclave at 115° for 15 min.

Commercial form: *Oxoid* Granules CM147 or Tablets CM148.

1.11 *Malt Extract Agar*

Malt extract	30 g
Mycological Peptone	5 g
Agar	15 g
Water to	1 000 ml
(pH 5.4)	

Add all the ingredients to the distilled water and heat in a steamer until dissolved. Distribute as required and sterilize at 121° for 15 min. In order to inhibit the growth of bacteria, the medium may be acidified to pH 3.5. This is done by adding a sterile 10% lactic acid solution (or a sterile 10% citric acid solution) to the liquefied medium immediately before pouring the plates. The exact amount of acid to be added to adjust the medium to pH 3.5 will depend upon the make or batch of constituents used.

Commercial form: *Oxoid* Granules CM59 or Tablets CM60.

1.12 *Malt Extract Broth*

Malt extract (*Oxoid L39*)	17 g
Mycological Peptone (*Oxoid L40*)	3 g
Water to	1 000 ml
(pH 5.4 approx.)	

Prepare medium according to the manufacturer's directions.

Commercial form: *Oxoid* Granules CM57 or Tablets CM58.

1.13 *Salt Meat Broth*

Peptone (*Oxoid L37*)	10 g
Lab-Lemco beef extract	10 g
Neutral ox-heart tissue	30 g

Sodium chloride	100 g
Water to	1 000 ml
(pH 7.6 approx.)	

Commercial form: *Oxoid* Granules CM93 or Tablets CM94.

1.14 *Selenite F. Broth*

The selenite in this medium serves the same purpose as tetrathionate in tetrathionate broth and has been preferred to it.

Sodium acid selenite ($NaHSeO_3$)	4 g
Peptone	5 g
Lactose	4 g
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	9.5 g
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	0.5 g
Sterile water	1 000 ml

Dissolve the ingredients with aseptic precautions, and distribute the yellowish solution in 10 ml amounts in sterile screw-capped bottles, steam at 100° for 30 min. Excessive heat is detrimental to the medium and autoclaving must not be used to sterilize it. A slight amount of red precipitate may form but this does not interfere with the properties of the medium. The pH of the medium should be 7.1 and the phosphates may be varied slightly if necessary to attain this. Salts of selenium are very *toxic* to animals and man, and must be handled with care. Some organic compounds of selenium and hydrogen selenide are volatile and *toxic if inhaled.*

Commercial form: *Oxoid* Granules CM39 or Tablets CM40 (Modified).

1.15 *Bacto-brilliant Green Agar.* Difco Dehydrated Medium.

Bacto-Yeast extract	3 g
Proteose peptone no. 3, Difco	10 g
Sodium chloride	5 g
Bacto-lactose	10 g
Saccharose, Difco	10 g
Bacto-phenol red	0.08 g
Bacto-brilliant green	0.0125 g
Bacto-agar	20 g

Bacto-Brilliant Green Agar is a selective medium recommended for the isolation of *Salmonella,* other than *typhosa,* either directly or after preliminary enrichment.

To rehydrate the medium, suspend 58 g of Bacto-Brilliant Green Agar in 1 000 ml cold distilled water and heat to boiling to dissolve the medium completely. Distribute in tubes or bottles and sterilize in the autoclave for 15 min at 121°. A longer period of sterilization will tend to decrease the selectivity of the medium.

The final reaction of the medium will be pH 6.9.

Commercial form: Bacto-Brilliant Green Agar 0285-02 [Baird and Tatlock (London) Ltd.].

1.16 *Thioglycolate Broth (Modified Brewer)*

Peptone	15 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	1 g
Thioglycolic acid	1 g
Water	1 000 ml

Dissolve the solids in the water with the aid of gentle heat. Add the thioglycolic acid and adjust to pH 8.5 with N/1 sodium hydroxide solution. Sterilize at 115° for 10 min. To prevent darkening of the medium, screw-caps should be loosened during autoclaving.

Glucose	5 g
Methylene blue, 1% aq. soln.	0.2 ml

Adjust to pH 7.2. Add the glucose and dye solution. Mix well and sterilize at 115° for 10 min. This medium should be stored in screw-capped containers in the dark at 4°.

If more than 20% of the medium shows a green colour before use, it should be heated in a boiling water bath or steamed for 5-10 min, and allowed to cool undisturbed. This treatment must not be repeated.

Commercial form: *Oxoid* Granules CM23 or Tablets CM24.

1.17 *Cetrimide Agar*

Proteose peptone no. 3 (Difco)	20 g
New Zealand agar	15 g
Glycerol	10 g
Water to	1 000 ml

Adjust this basal medium to pH 7.2 and sterilize at 121° for 15 min. To

each 100 ml of the liquefied base add aseptically the following solutions, each made up in distilled water and then Seitz-filtered:

Dipotassium hydrogen phosphate (K_2HPO_4)	15% soln.	1.0 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	15% soln.	1.0 ml
Cetrimide B.P.	2% soln.	1.5 ml

Mix well by inversion several times and pour into Petri dishes.

Commercial form: Pseudosel Agar, Cat. No. 11553 (Becton, Dickinson U.K. Ltd.).

1.18 *Peptone Water Diluent*

Neutralized bacteriological peptone (*Oxoid L34*)	1 g
Water to	1 000 ml
(pH 7.0 approx.)	

Distribute as required and sterilize at 115° for 15 min.

1.19 *Reinforced Clostridial Medium*

Yeast extract (*Oxoid L20*)	3.0 g
Lab-Lemco beef extract	10.0 g
Peptone (*Oxoid L37*)	10.0 g
Soluble starch	1.0 g
Dextrose	5.0 g
Cysteine hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g
Water to	1 000 ml
(pH 6.8 approx.)	

Prepare according to directions given for *Oxoid* materials.

Commercial form: *Oxoid* Granules CM149 or Tablets CM150.

1.20 *Reinforced Clostridial Agar*

Yeast extract (*Oxoid L20*)	3.0 g
Lab-Lemco beef extract	10.0 g
Peptone (*Oxoid L37*)	10.0 g
Dextrose	5.0 g
Soluble starch	1.0 g
Sodium chloride	5.0 g

Sodium acetate	3.0 g
Cysteine hydrochloride	0.5 g
Agar	15.0 g
Water to	1 000 ml
(pH 6.8 approx.)	

Prepare according to directions given for *Oxoid* materials.

Commercial form: *Oxoid* Granules CM151 or Tablets CM152.

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Appendix D

1. Microbiological examination of plant and equipment

Control measures for checking plant hygiene, such as the sampling of containers and working surfaces, should be carried out regularly. The following methods are recommended:

1.1 *Swab technique* (1)

1.11 *Apparatus*

The following apparatus is required:

Test tubes, 250/25 mm of heavy borosilicate glass.

Stainless steel wire, BS 1554, rust, acid and heat resisting steel wire, 350 mm of 2.64 mm diameter, formed into a loop at one end leaving a straight length of 300 mm and notched at the other end to hold the ribbon gauze.

Ribbon gauze, unmedicated, 50 mm wide.

1.12 *Preparation of swab*

The swab should be 50 mm in length and consist of 175 mm of gauze wound round the notched end of the wire, secured with thread.

1.13 *Sterilization of swab*

Place the swab in 25 ml of Peptone Water Diluent in the test tube, plug with cotton wool or suitable rubber closure, cover the plug with aluminium foil or greaseproof paper, and sterilize by autoclaving at 121° for 15 min. To obtain a final quantity of 25 ml of solution, it is necessary to start initially with a larger amount to allow for evaporation during autoclaving. The actual quantity should be found by trial and error with each individual autoclave. The Peptone Water Diluent should contain sodium thiosulphate or other suitable inactivator.

1.14 *Swabbing technique*

Where possible, examine an area of 1 000 cm^2 (approx. 1ft^2). Press the swab with a rolling motion against the side of the test tube to remove excess liquid. Remove the swab and with heavy pressure rub back and forth over the area to be examined so that all parts of the surface are treated twice. Rotate the swab so that all parts of it make contact with the

surface under test. Return the swab to the test tube, and insert the cotton wool plug or rubber closure.

1.15 *Testing*

Test the swab samples as soon as possible. After not less than 5 min contact of swab and liquid, mix the sample by twirling the swab vigorously in the Peptone Water Diluent six times. Remove the swab, taking care to express the liquid by pressing against the side of the tube. After thorough mixing by rotation of the tube between the palms of the hands, plate out 1 ml and 0.1 ml quantities of the swab solution using Plate Count Agar and incubate at $30 \pm 1°$ for 72 ± 2 h. Carry out the preparation of the dilutions, inoculation, pouring, and incubation of the Petri dishes, and the counting of the colonies as previously described.

1.16 *Recording results*

Record the results as the colony count per 1 000 cm^2 of surface tested (colony count per ml $\times$ 25 $\times$ area factor, if not 1 000 cm^2).

1.2 *Agaroid sausage technique*

The principle introduced by Ten Cate (2) is to use an agar-filled plastic casing which, when the ends are sealed, resembles a sausage. The end of the agar and casing is cut off and the exposed agar surface is used to take an impression of the surface to be tested.

1.21 *Sampling technique* (3)

Swab the outside of the *Agaroid* casing with alcohol in the area to be cut and sterilize a very sharp, broad-bladed knife by swabbing with alcohol and flaming. Cut off the end of the *Agaroid* approximately 12 mm distal to the coloured band. Do not remove the coloured band; it is provided to prevent the agar slipping out of its casing during use. Applying light pressure at the base, push out about 1 cm of the agar column. Sample by pressing the cut end of the agar firmly on the test surface. Cut off a slice 4-6 mm thick and transfer, inoculated side uppermost, to a petri dish by supporting the agar slice on the blade of a knife. Do not put the exposed end of the *Agaroid* down on a bench or other contaminated surface. Once the plastic casing has been cut, do not re-seal. Incubate the agar slices in closed petri dishes at 37° for 18-24 h and then count colonies. A petri dish will hold 3 to 4 samples. The area of each sample is approximately 8.5 cm^2.

The bacterial flora of the test surface can be determined by examination of the colonies on the agar.

1.22 *Range of Agaroid media recommended for use*

Type	*Code*	*Application*
MacConkey	AG7	For the detection and count of coliform bacteria.
Plate count	AG183	General purpose media for the isolation of bacteria.
Sabouraud:		
Maltose	AG41A	For the detection of moulds/yeasts.
Malt extract	AG59	For the detection of moulds/yeasts.

With the seal intact, the *Agaroid* has a storage life of six months at 4°. *Agaroid* media can also be obtained with an inactivating agent incorporated. For sampling flat surfaces in particular, the *Rodac* plate can be recommended as an alternative to the *Agaroid* sausage.

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2. Summary of recommended sterilization conditions

Various statements appear in this monograph with regard to the sterilization of plant, raw materials, packaging components, culture media, etc. and appropriate sterilizing conditions are recommended. In order to achieve satisfactory results, however, it is most important that the general principles underlying the various sterilization techniques should be understood. Vegetative micro-organisms, i.e. those in an active state of growth and reproduction, are comparatively easy to kill with the aid of heat, by irradiation, or by other means. On the other hand, spores are produced in the life-cycle of certain species and some forms of spores are extremely resistant to all forms of environmental stress, including high temperatures. Hence it is often necessary to design a sterilization process capable of destroying heat-resistant spores.

2.1 *Dry heat*

Dry heat, employed in the form of hot air treatment or with *superheated* steam, is an effective sterilizing agent when properly applied. To ensure sterility by this means, it is essential to see that all the surfaces or materials concerned are maintained at 160° for at least 60 min or 180° for at least 20 min. Dry heat does not have good penetrating properties, and the requirement that the whole of the material to be sterilized should reach the stated temperature for the full period of heating is a stringent one, which cannot be relaxed without serious risk.

Equipment that will not be damaged by the prolonged high temperatures required may be sterilized by a dry heat technique. Raw materials may sometimes be sterilized in this way if decomposition will not occur; to ensure that the whole bulk reaches the correct temperature throughout the requisite time, powders should be spread in shallow layers. Dry heat sterilization may, for example, be used to sterilize talc, kaolin and other mineral earth constituents.

2.2 *Autoclaving*

A more practical method of achieving sterility is often to utilize the properties of *saturated* steam, usually as steam in the presence of water at the same temperature and pressure. When saturated steam comes in contact with the material to be sterilized, which is initially at a lower temperature, the steam condenses and gives up its *latent heat* of evaporation. Thus, the heat transfer to the material being sterilized is far greater than that achieved by dry heat treatment and the ability to destroy all microorganisms including spores, is much superior. A pressure of saturated steam 69 kN m^{-2} above atmospheric yields a temperature of 115° and will effectively sterilize in 30 min. At 103 kN m^{-2} above atmospheric pressure, the corresponding temperature will be 121° and sterilization will normally be achieved in 15 min. Saturated steam penetrates well, but manufacturing plant and some other items may take a substantial time to attain the full sterilizing conditions; whenever possible, it is therefore recommended to operate at 103 kN m^{-2} excess pressure for at least 30 min. In some systems for pressurized steam sterilizing or autoclaving, it is desirable to avoid leaving materials in a moist state after sterilization, and this can be avoided by the subsequent evacuation of the sterilizer. A preliminary evacuation of the autoclave before the admission of steam will also be helpful, by ensuring

that the correct conditions of steam saturation are rapidly achieved; where such evacuation cannot be arranged, *thorough venting is essential* for the first few minutes during which steam is being admitted to the equipment. Some models are now fitted with an automatic air purge which allows entrapped air to be displaced during the run-up and subsequently. Components of glass or metal being steam-sterilized may be wrapped in Kraft paper, so that they remain sterile after removal from the autoclave, until they are needed for use. Heat-stable, water-insoluble powders may be sterilized effectively by autoclaving, preferably with subsequent evacuation of the sterilizer to avoid a high residual moisture-content in the powder. Contaminated glassware and discarded laboratory cultures should be sterilized by autoclaving at 138 kN m^{-2} (126°) for 20 min.

2.3 *Ultraviolet irradiation*

The most effective bactericidal range of ultraviolet radiation lies between 280 nm and 240 nm. Whilst a radiation intensity of the order of 5 mW cm^{-2} may achieve a virtually 100% kill of micro-organisms in a few seconds, the exact requirements for a practical sterilization procedure always need to be established empirically. Specialist equipment suppliers should be consulted for apparatus of suitable design for the purpose envisaged; they will take into account, for example, whether an air-gap has to be irradiated above a conveyor belt or whether the irradiation can be arranged in the form of a continuous pipe-line system for clear liquids. *UV transmission* is probably the most important variable factor involved; for example, an arrangement capable of giving excellent results when newly-installed, may soon fail to achieve sterilization if the surfaces are not kept thoroughly clean. Even slight turbidity will markedly reduce sterilizing efficiency. UV irradiation is seldom used as the sole and final means of achieving sterilization, but it may be a useful adjunct to other procedures. However, monitoring by periodic microbiological tests is necessary to ensure that sterilizing efficiency is being maintained.

2.4 *Ethylene oxide treatment*

Ethylene oxide is highly toxic by inhalation at levels undetectable by smell, and is explosive unless mixed with carbon dioxide or nitrogen. It is, therefore, only suitable as a sterilant under strictly controlled operating conditions. A further complication is that ethylene oxide is a skin irritant

and, since it is soluble in rubber, may produce eruptions even when rubber gloves are worn. Concentrations of ethylene oxide used in practice have been given as 500 mg l^{-1} of air, or preferably nitrogen, for 4 h at room temperature and also 10-15% in air or nitrogen overnight. Special equipment is not essential, although chambers suitable for preliminary evacuation are sometimes employed.

2.5 *Filtration*

Numerous methods of filtration are available for sterilizing various types of liquids and for air sterilization. For large-scale treatment of water and aqueous solutions, it is usual to employ either diatomite filter candles in a specially-designed unit or special grades of filtering mats in a conventional filter-press. Filters intended to remove all micro-organisms from a liquid or vapour might appear to need a maximum pore-size below about 0.2 μm. In practice, adsorptive filtering materials do not rely solely on mechanical trapping of micro-organisms and will usually operate satisfactorily with a maximum pore-size of 1-2 μm. It is important to ensure that the correct *sterilizing grade* of filtering agent is used (not the coarser clarifying grades) and to see that the filter is properly installed without accidental damage or channelling, e.g. around the edges of the mats. Excess pressure intended to improve flow rates during use will often damage a sterilizing filter unit and allow micro-organisms to pass; careless cleaning efforts may have a similar effect.

Filters for sterilizing applications must be sterilized and operated within sterile equipment; in particular, pipe-lines and vessels taking the filtered material must obviously be presterilized with the aid of saturated steam or chemical sterilants, or the product will rapidly become contaminated again.

Sterilization by filtration is one of the most suitable and economical ways of handling large volumes of mobile liquids, such as shampoos, but it calls for considerable skill and experience to ensure routinely good results. Frequent microbiological tests are necessary to give assurance that sterilization is being achieved and that filters have not been damaged.

2.6 *Chemical sterilization*

References are made in this monograph to sterilization by means of chlorine (e.g. solutions of hypochlorite yielding 200-250 ppm of available chlorine) and formalin (e.g. 0.5% equivalent to 0.2% of formaldehyde). Used in accordance with the operating conditions prescribed, especially with respect to preliminary cleansing, these chemical sterilants will prove suit-

able for the maintenance of plant hygiene. In general, chemical sterilants cannot be incorporated as ingredients of products as they necessarily have a high degree of intrinsic chemical and biological reactivity; they are liable to alter the product characteristics to a marked extent or to elicit adverse cutaneous reactions. However, just as a chemical disinfectant will effectively sterilize *clean* surfaces of equipment, so a small percentage of a bactericide or fungicide may exert a sterilizing action in a cosmetic product which has been prepared in a clean and hygienic manner. The possibility of using preservatives to achieve self-sterilizing properties in cosmetics is dealt with in *Appendix B;* the principle, in brief, is that the preservative should not be employed to achieve sterility at the time of manufacture (which should depend on other measures) but should be aimed at the destruction of limited numbers of contaminants entering the product at a later stage.

2.7 *Flaming*

For microbiological test procedures, including the taking of samples in the production area, a rapid method of sterilizing hard surfaces of small pieces of equipment is often needed, e.g. temporarily-opened necks of bottles and also wire loops used for inoculating culture media. Direct heating in an open flame is employed and should be carried out thoroughly, in close proximity to the point where the flamed equipment will be used; quick passage through the flame gives reassurance but not sterility. Metal instruments (scissors, etc.) may be sterilized by swabbing with alcohol, which is then burned off; this is a less effective procedure than direct flaming but is not so detrimental to the cutting edges of instruments.

2.8 *After-care of sterilized materials*

Equipment and chemical products that have been sterilized will, in general, only remain sterile if the access of fresh contamination is rigorously avoided. This aspect is discussed in detail under *plant sterilization* as it affects production equipment and the same considerations apply to raw materials, intermediate and finished products. Even in circumstances where the aim is to achieve a low microbial count rather than absolute sterility, it is clearly desirable to restrict adventitious contamination as much as possible. Emphasis has therefore been given to matters, such as taking care to see that lids fit closely and that they are securely in place whenever possible.

The fact that contamination is a dynamic process should be borne in mind. With faulty storage, product bearing a low level of contamination

may rapidly acquire a massive count without obvious sign that any change is occurring. An energetic programme of microbiological surveillance at all stages of manufacture, packaging and warehousing is the only safeguard against disastrous consequences, such as the irrecoverable spoilage of complete batches of product.

Appendix E

1. Stock cultures of spoilage micro-organisms

Several references are made in this monograph to the use of spoilage micro-organisms in testing preservative systems. For this purpose, it is helpful to maintain cultures of various species and strains, isolated at various times, but some care is necessary to obtain consistent results in preservative studies.

Requirements of different laboratories vary as regards the size and type of collection. This may often be limited to a few essential strains, for which full records are kept. Test strains may be isolated from contaminated materials and either freeze-dried or maintained on agar. Freeze-dried cultures may also be purchased from various National Collections (see *Appendix E.2.*).

1.1 *Freeze-dried cultures*

Freeze-dried (lyophylized) cultures remain true to type and are preferred to stock cultures maintained continuously on growth media, since freeze-dried cultures are less liable to undergo changes in preservative resistance and other characteristics. For the preparation of freeze-dried cultures, appropriate publications should be consulted (1). Slope cultures for current use are prepared from freeze-dried cultures and stored at 5-10°; the sub-culture should be checked for purity and used preferably within a month. After making four serial sub-cultures a new freeze-dried tube should be opened and the old culture discarded.

1.2 *Other stock cultures*

When freeze-drying is not carried out, cultures may be maintained on a suitable nutrient agar medium by sub-culturing at appropriate intervals. The cultures should be stored in tightly-capped bottles in the dark at 5-10°. Cultures maintained continuously on agar should be specially checked for purity and to see that the required degree of preservation resistance is being maintained. To guard against accidental contamination or the development of an atypical strain, a duplicate set of master cultures should always be kept.

Suitable culture media and suggestions for minimal frequency of sub-culture needed to ensure survival of the organisms are listed in *Table 1.2.1.* (2).

Table 1.2.1.

Conditions for maintenance of test organisms

Genus	Medium	Incubation		Storage	Interval between sub-cultures (months)
		Temperature	Time		
Bacillus	Nutrient Agar	30°	48h	5-10°	6-12
Clostridium	Cooked meat*	37°	48h	5-10°	6-12
Citrobacter	Nutrient Agar	37°	18h	5-10°	3-6
Enterobacter	Nutrient Agar	37°	18h	5-10°	6
Escherichia	Nutrient Agar	37°	18h	5-10°	6
Klebsiella	Nutrient Agar	37°	18h	5-10°	3
Proteus	Nutrient Agar	37°	18h	5-10°	3
Pseudomonas	Peptone Water Agar	30°	18h	5-10°	3
Salmonella	Dorset egg*	37°	18h	5-10°	6-12
Staphylococcus	Nutrient Agar	37°	18h	5-10°	3
Streptococcus	Cooked Meat	37°	18h	5-10°	3

*Obtainable as a ready prepared culture medium from Oxoid Ltd.

1.3 *Fungi*

The yeasts and common moulds are grown on Malt Extract Agar slopes in screw-cap Universal bottles. To maintain growth, a few spores are transferred to a fresh slope using a sterile needle or small loop. Mould cultures are incubated at 22° for about a week. Yeasts are incubated at 30° for 3 days. If growth is satisfactory, the culture is transferred to a refrigerator (5-10°) or incubated at room temperature in the dark. To prevent drying, cultures may be preserved under oil and by this method will maintain their viability for a longer period of time. The cultures should be covered with sterile liquid paraffin oil (sterilized at 160° for 1-2 h in a hot-air oven). The screwcaps of the bottle should be tightened and the cultures stored vertically in the usual way. Sub-cultures may be taken at any time almost as easily as from an agar slope. To maintain viability, transfers should be made about twice a year although a few species will require to be sub-cultured after 3 months (3).

Sub-culture to corn meal agar (*Oxoid* Granules CM103, Tablets CM104) is recommended for the maintenance of most fungi, especially the black pigmented varieties.

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(2) Cowan, S. T. and Steel, K. J. *Manual for the identification of medical bacteria* 176 (1966) (Cambridge University Press, London).
(3) Smith, G. *An introduction to industrial mycology* 5th Edn. 300-313 (1960) (Edward Arnold Ltd., London).

2. Sources of stock cultures of micro-organisms

2.1 *Collection of fungus cultures:*

The Curator, Commonwealth Mycological Institute, Kew, Surrey.

2.2 *Industrial bacteria, bacteria for microbiological assay, etc.:*

The Curator, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

2.3 *Yeasts (non-pathogenic):*

The Curator, National Collection of Yeast Cultures, The Brewing Industry Research Foundation, Nutfield, Surrey.

2.4 *Bacteria pathogenic to man and animals:*

The Curator, National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

2.5 *Collection of strains of algae, bacteria, bacteriophages, fungi, and protozoa:*

The American Type Culture Collection, 12301 Parklawn Dri,ve Rockville, Maryland 20852, U.S.A.

2.6 *Deposition of cultures*

Research and industrial organizations may deposit cultures of micro-organisms which are of industrial or research interest with the curators of 2.1 and 2.2. The name of the micro-organism (genus and species) along with details of special properties and applications should be listed, e.g. spoilage organisms suitable for preservation testing.

2.7 *Recommended strains of bacteria obtainable from type culture collections for use as test organisms*

	NCTC(2.4)	*ATCC*(2.5)	*NCIB*(2.2)
Escherichia coli	8 196		
Escherichia coli		11 229	9 517
Klebsiella aerogenes	418	15 380	
Proteus morganii	10 041		
Proteus vulgaris	4 635		

*Pseudomonas aeruginosa**	6 749		
*Pseudomonas aeruginosa**		15 442	
Pseudomonas fluorescens	10 038	13 525	
*Staphylococcus aureus**	4 163		
*Staphylococcus aureus**		6 538	9 518
Staphylococcus epidermidis	4 276		
Staphylococcus epidermidis	7 291		
Streptococcus faecalis	8 213		

*These strains have been recommended for their known high resistance to antimicrobial agents but it is important to first check the individual strains for their ability to utilize the *unpreserved* product as a satisfactory growth medium before commencing detailed preservative studies (See *Appendix B 2.12.* p. 754).

3. Addresses of Suppliers of Apparatus

Supplier	*Equipment*
Astell Laboratory Service Co. Ltd. 172 Brownhill Road London S.E.26.	Miller-Prickett anaerobic tubes
C. F. Casella & Co. Ltd. Regent House Britannia Walk London N.1.	Airborne bacteria sampler
Fisons Scientific Apparatus Ltd. Loughborough Leicestershire.	Whirlimixer
Arnold R. Horwell Ltd. 2 Grangeway Kilburn High Road London N.W.6.	Kampff micro-burner Grahams Calgiswabs Wooden applicator sticks (for swabs)
Medical Alginates Wadsworth Road Perivale, Middx.	Alginate wool
Millipore (U.K.) Ltd. 109 Wembley Hill Road Wembley, Middx.	Membrane filters *Sterilfil* filtration systems, etc.
Oxoid Ltd. Southwark Bridge Road London S.E.1.	Membranes (bacteriological)
Sterilin Ltd. Richmond Surrey.	*Rodac* plates
R. B. Turner & Co. Ltd. Inocula House Church Lane London N.12.	Disposable Pasteur pipettes

4. Addresses of suppliers of reagents

Supplier	*Reagent*
Boots Pure Drug Co. Ltd. Nottingham	*Bronopol*
British Drug Houses Ltd. B.D.H. Laboratory Chemicals Division Poole, Dorset.	2, 3, 5-triphenyltetrazolium chloride (TCC)
Burroughs Wellcome & Co. The Wellcome Building Euston Road London N.W.1.	Rabbit plasma (for coagulase test)
Givaudan & Co. Ltd. Whyteleafe Surrey CR3 OYE	*Dioxin*
Honeywill & Stein Ltd. Mill Lane Carshalton, Surrey.	*Tween 80*
Imperial Chemical Industries Ltd. Millbank London S.W.1.	*Lubrol W* flakes
Lennig Chemicals Ltd. 2 Masons Avenue Croydon, Surrey.	*Triton X 100*
Nipa Laboratories Ltd. Treforest Industrial Estate Pontypridd, Glamorgan.	*Nipa* preservatives
Oxoid Ltd. Southwark Bridge Road London S.E.1.	Dehydrated culture media *Agaroid* media

5. Selected bibliography

5.1 *Biodeterioration of cosmetic materials*

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5.3 *Identification of bacteria*

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5.4 *Identification of yeasts and moulds*

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6. SI UNITS AND CONVERSION FACTORS

SI UNITS

physical quantity	*name of unit*	*symbol for unit*	*definition of unit*
length	metre	m	
mass	kilogramme	kg	
time	second	s	
	hour	h	
force	newton	N	$kg\ m\ s^{-2} = J\ m^{-1}$
power	watt	W	$kg\ m^2\ s^{-3} = J\ s^{-1}$
customary temperature, t	degree Celcius	°C	$t/°C = T/°K - 273.15$

Symbols for units do not take a plural form.

FRACTIONS AND MULTIPLES

fraction	*prefix*	*symbol*	*multiple*	*prefix*	*symbol*
10^{-2}	centi	c			
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	micro	μ			
10^{-9}	nano	n			

UNITS CONTRARY TO SI, WITH THEIR EQUIVALENTS

physical quantity	*unit*	*equivalent*
length	angström	10^{-10} m
pressure	pound (f)/sq.in.	6.894 76 kN m^{-2}
temperature	degree Fahrenheit	$t/°F = \frac{9}{5}T/°C + 32$

(Received: 21st July 1970)