Journal of the Society
of Cosmetic Chemists

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SYNOPSES FOR CARD INDEXES

The following synopses can be cut out and mounted on 127 × 76 mm index cards for reference without mutilating the pages of the Journal.

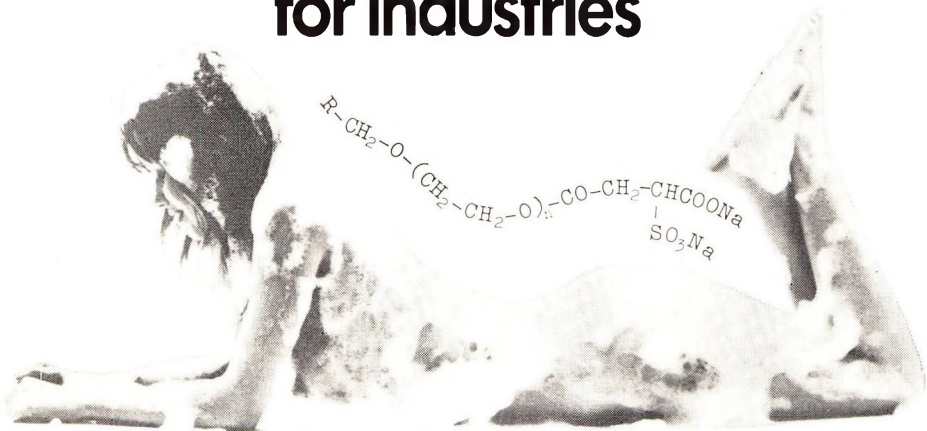**Preservatives for cosmetics and toiletries:** BETTY CROSHAW. *Journal of the Society of Cosmetic Chemists* **28** 3–16 (1977)

Synopsis—The need for preservation of cosmetics and toiletries and the function and selection of a satisfactory preservative system are discussed.

The properties of the ideal preservative are considered and the properties of some of the newer preservatives described.

Microbial contamination of cosmetic products: ROSAMUND M. BAIRD. *Journal of the Society of Cosmetic Chemists* **28** 17–20 (1977)

Synopsis—One hundred and forty seven cosmetic products purchased from five retail shops in 1974 were examined microbiologically. Viable bacteria were not recovered from ninety-nine products. Gram-negative rods were isolated from 6·1% of products and *Pseudomonas* spp. from 4·1% of products.

Microbiological applications of a novel replipad skin sampler: H. DIXON and A. K. PANEZAI. *Journal of the Society of Cosmetic Chemists* **28** 21–24 (1977)

Synopsis—A bacteriological skin sampler which can be used to obtain microbial prints of the axilla is described. Various applications include *in vitro* and *in vivo* evaluation of the antimicrobial properties of deodorant and other products using the subject's skin microflora.

The following papers have been accepted for publication in the *Journal*:

A spectroscopic study of the reaction products of dihydroxyacetone with aminoacids: A. MEYBECK. *Journal of the Society of Cosmetic Chemists* **28** 25–35 (1977)

Synopsis—Dihydroxyacetone (DHA) has been allowed to react with a series of aminoacids in water solution. The dark solutions or suspensions obtained were dialysed giving brown 'pigments' (melanoidins) which were subjected to instrumental analysis. Visible absorption spectra of their water solutions have no special features, while u.v. spectra show at best a slight maximum around 310–330 nm. I.R. spectra of all the products studied show broad bands around 3400, 1600 and 1400 cm^{-1}. All these melanoidins exhibit a strong Electron Spin Resonance (E.S.R.) signal. The E.S.R. spectra recorded in the solid state are single lines of 6–12 gauss linewidth, centred around $g = 2$, which correspond to rather inert unpaired electrons since they can be observed in water solution as well. These data suggest that the reaction products of DHA with aminoacids are conjugated polymers. The mechanism of skin tanning by DHA is discussed in view of these results.

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GENERAL NOTICES

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Preservatives for cosmetics and toiletries

*Presented on 23 February 1976 at the Symposium on Microbiological
Safety in Cosmetic and Toilet products, Birmingham*

B E T T Y C R O S H A W *The Boots Co. Ltd, Research Department, Microbiology,
Nottingham.*

Synopsis

The need for **preservation** of **cosmetics** and toiletries and the function and selection of a satisfactory preservative system are discussed.

The properties of the **ideal preservative** are considered and the properties of some of the newer preservatives described.

Introduction

It has been known for many years that some cosmetics and toiletries will support the growth of microorganisms (1) and that microbial action may cause spoilage problems (2) exemplified by visible growth or by chemical changes in the product. These changes may manifest themselves in a number of ways, e.g. by hydrolysis, oxidation, reduction causing off-odours, change in colour, adverse changes in pH, breakdown of the emulsion or change in texture of the product (2, 3, 4).

More recently the introduction of new ingredients such as proteins, gums, vitamins, herbs, beer, some of which may be merely 'promotional additives' (5), has increased the microbiological hazard. The widespread use over the last two decades of nonionic surfactants to replace anionic emulsifiers has resulted in superior and more stable cosmetics but this change has also increased the risk of microbial contamination since many of these nonionic surfactants will support the growth of Gram-negative bacteria and particularly pseudomonads (6). Bryce and Smart (7) found that contaminated shampoos invariably contained Gram-negative organisms and their survey coincided with the change from anionic to nonionic surfactants.

The degree of microbiological risk is affected by the physical state of the product. Aqueous solutions and oil-in-water emulsions are highly susceptible to microbial contamination. Water-in-oil emulsions are less susceptible because the continuous oil phase acts as a barrier to the penetration of microorganisms into the water phase and also impedes the spread of growth through the system (8). If, however, such a formulation is unstable and separation occurs, the separated aqueous phase is very vulnerable. Water-in-oil emulsions although less susceptible to microbial contamination are generally more difficult to preserve.

Water, the raw material common to many cosmetics and toiletries, may often be the prime source of contamination. The most frequently reported contaminants in recent years have belonged to such genera as *Pseudomonas, Klebsiella, Achromobacter* and *Alcaligenes*. These bacteria are common residents in water and it is now widely believed that the water used in the preparation of toiletry products is their likely source (9).

A consumer is unlikely to use a spoiled product but he or she could unknowingly use a contaminated cosmetic or toiletry. The possible health hazard is readily apparent since these products may be used on infants, by the old and the sick and by hospital staff and patients.

3

Nowadays, reputable manufacturers exercise a microbiological quality control programme to ensure that they do not market a product that is a spoilage or health risk. Recalls and loss of large batches of materials place a heavy economic burden on the manufacturer. The need to include a preservative, or a mixture of preservatives, in order to prevent microbial spoilage or contamination of susceptible cosmetics and toiletries is also now universally accepted. The alternative of manufacturing these products in a sterile form in single-use containers would greatly increase their cost and is unjustifiable since a safe product can be prepared by a combination of good manufacturing hygiene and the inclusion of a suitable preservative system.

FUNCTIONS OF PRESERVATIVES

Preservatives are chemical agents that prevent microbial growth in a product, rendering it safe and increasing its shelf life. Some workers would agree that a static action was all that was required of a preservative. Tenenbaum (10) suggested that the preservative ability of a product should be in excess of the minimum necessary to inhibit expected flora. He recommended that an ideal preservative system should be 'self-sterilizing'. By the latter phrase, it is presumed he meant lethal to vegetative cells. A preservative should never be used to replace good manufacturing hygiene with continuous control of raw materials, especially water. The preservative is an aid in good manufacturing hygiene to combat unavoidable contaminants introduced in raw materials, from the equipment and containers used and from the operatives and the environment. Its main function is to prevent in use contamination by the consumer and to prolong the shelf life of the product. Ideally the preservative should be capable of eradicating high numbers of vegetative bacteria in case good manufacturing hygiene breaks down.

Any cosmetic or toiletry must be free from pathogens even in small numbers. With the exception of eye cosmetics and baby products cosmetics and toiletries need not be free from non-pathogenic bacteria and fungi provided they are there in low numbers and in a static state. It must, however, be remembered that the line between pathogens and non-pathogens is a very tenuous one varying with different individuals, age-groups and state of health; many organisms may be pathogens in the right environment. It is the function of the preservative to prevent contaminating organisms from multiplying and, ideally, it will eventually cause their death. Low numbers of non-pathogenic spores are probably harmless but the preservative must prevent the germination of these spores.

The type of in-use contamination depends to a large extent on the presentation. Products supplied in tubes are less likely to become contaminated by the consumer than those supplied in jars. Some eye cosmetics incorporating applicators may be particularly hazardous unless adequately preserved since organisms from the skin are transferred to the preparation. The effective preservative should be capable of preventing the multiplication of any organism introduced during use whether this organism is a pathogen or a normal skin resident. Studies of the microflora of selected areas of skin (e.g. McConville and Anderson (11) on the outer eye) will help the microbiologist in consideration of in-use contamination.

SELECTION OF A PRESERVATIVE SYSTEM

There is still no satisfactory way of choosing a preservative system for a particular formulation on a theoretical basis. Nor can one from the many published lists

(12, 13, 14, 15) of available preservatives be merely chosen. In fact to the uninitiated these lists can be misleading since some of the agents may not be effective as preservatives because of their limited antimicrobial spectrum. Examples of these are the quaternary ammonium compounds, trichlorocarbanilide, the halogenated salicylanilides and bromochlorophen, all of which are potent antimicrobials as active agents for preparations such as soaps and scrubs where activity against Gram-positive bacteria and substantivity to the skin are the prime considerations. They are not effective preservatives. Every formulation must be considered on an individual basis although some general rules apply (3). The development of a new cosmetic or toiletry formulation involves consideration and adequate testing of the preservative system at an early stage and the formulators and the microbiologist should work in close collaboration. The ideal preservative does not exist and no one preservative system is satisfactory under all conditions. It may be that a combination of preservatives has to be used to obtain the desired effect in a particular formulation. The selection of a preservative depends on many factors (15); important considerations are the physical and chemical nature of the product, its potential use (e.g. on normal or broken skin, on infants, around the eyes), the type of container and the desired shelf life.

All too frequently the final formulation can only be achieved on the basis of compromise; safety, compatibility and efficacy are the most important factors influencing the preservative system. As Parker (16) points out although it is essential that the preservative capacity of a cosmetic is adequate to combat spoilage and in-use contamination it should not bestow the character of an antiseptic on the preparation. Many dermatologists regard the preservative as a necessary evil. Tronnier (17) makes the plea that cosmetic preservative levels should not be such as to upset the equilibrium of the biozone created by the microflora of the skin.

The search for new preservatives continues and new ones appear but no single example has yet matched up to the ideal. It has been suggested that it is perhaps unrealistic to expect any compound to possess all the properties attributed to the ideal preservative (3); this belief is supported by the author.

PROPERTIES OF THE IDEAL PRESERVATIVE

The properties of the ideal preservative have been listed by many workers (e.g. 3, 12, 18). The following factors must be taken into consideration.

Antimicrobial activity

Any preservative must have broad spectrum antimicrobial activity since Gram-negative and Gram-positive bacteria, fungi and yeasts may be involved as contaminants and/or spoilage agents. The use of a preservative with high activity against some organisms and much lower activity against others may lead to the selection of insensitive organisms. One of the major disadvantages in the use of 2, 4, 4'-trichloro-2'-hydroxydiphenyl ether (*Irgasan* DP 300) as a preservative is its *Pseudomonas* gap (19). Since pseudomonads are the most commonly encountered contaminants in aqueous products their importance cannot be overlooked (10, 20). They can render conditions suitable for less adaptable spoilage organisms; for example they can create conditions favouring the growth of anaerobes (2).

Coates (18) has reviewed the microbiological requirements of a preservative. While screening tests in solid or liquid media for the evaluation of the antimicrobial activity of potential preservatives are valuable in selecting agents worthy of further study they will not necessarily predict the activity in a formulation. This is readily understandable as other factors are involved, particularly the o/w partition coefficient since any effective preservative must be present in the aqueous phase. Thus, a compound whose m.i.c. is 1000 µg/ml may protect an emulsion at a concentration of 0·1–0·2% while another agent which inhibits growth at 20 µg/ml in screening tests can fail to protect an emulsion even at a level of 0·4% (21). On the other hand, Charles and Carter (22) found that the parabens, esters of *p*-hydroxybenzoic acid, were more effective in finished formulations than had been expected from their performance in component testing. Our own work with some formulations supports this; a potential preservative must be further evaluated in different formulations at various concentrations to determine its efficacy under different conditions.

Bactericidal, rather than bacteriostatic, activity is an added advantage although in most cases a preservative may be considered to be effective if it holds small numbers of non-pathogenic organisms in a quiescent state. Even a low level of preservative may be slowly bactericidal and a higher level, resulting in a more rapid bactericidal effect, may be undesirable because of skin irritancy and, indeed, may not be necessary.

The number of organisms challenging a preservative system must also be considered and the effect of inoculum size on the activity of any preservative should always be determined in the initial evaluation tests. In a formulated product an overwhelming microbial contamination may swamp the preservative system or, at best, may lengthen the time necessary to reduce the number of organisms to a safe, low level.

Resistance studies should also be carried out at an early stage on any potential preservative. Any agent which rapidly selects out insensitive strains of microorganisms is unlikely to be a satisfactory preservative.

Toxicological considerations

The ideal preservative must be safe in use; that is, it must be non-irritant, non-sensitizing and preferably non-poisonous.

No chemical can ever be listed as ever being totally non-allergenic or totally non-sensitizing (23). Even the parabens, which have been and still are widely used for the preservation of toiletries and cosmetics, cannot be regarded as completely safe. Paraben allergy was recognized in Europe in the early 1960s and later in the United States when a sensitization index of 0·8% was documented (24).

Sorbic acid, which was recommended as a preservative for formulations containing nonionic surface active agents (25) prior to the introduction of some of the newer preservatives, has a sensitization index probably slightly lower than that of the parabens (24). The phenolic group of preservatives also has its own problems (24). Two phenolics which are used as preservatives, chlorocresol (*p*-chloro-*m*-cresol) and especially chloroxylenol (*p*-chloro-*m*-xylenol), are known sensitizers and cross sensitization has been reported. The sensitization index for chlorocresol is 0·5% and in a survey of contact allergy by Calnan (26) chloroxylenol was second only to mercury in terms of sensitivity produced.

Formaldehyde, which has been widely used in shampoos (5, 7), constitutes a greater

sensitization hazard than the parabens and the organic mercurials present the greatest hazard (24). Quaternary ammonium compounds are relatively weak sensitizers.

The sensitization potential of some of the newer preservatives is not yet fully established from the clinical point of view. It has been suggested that 2-bromo-2-nitropropane-1, 3-diol (*Bronopol*) may be used safely at the concentrations normally used in formulations (27). This compound was found to be a mild irritant when applied in soft yellow paraffin on to 149 eczematous patients at a concentration of 0·25% but no evidence of sensitization was seen in this study nor was there any suggestion of cross-sensitization with any other substance particularly formalin. Imidazolidinyl urea (*Germall* 115) is reported to be non-toxic, non-irritating and non-sensitizing (28); *Irgasan* DP 300 is said to be free of allergenic or photoallergenic potential on the basis of animal and human tests (29). There is some evidence that 6-acetoxy-2, 4-dimethyl-*m*-dioxane (*Dioxin*) is a sensitizer (24).

Water solubility

The ideal preservative should be readily water soluble at the effective concentration since microbial growth occurs in the aqueous phase.

The low water solubility of the organic mercurials, *Irgasan* DP 300 and the parabens are marked disadvantages. Where water solubility is low, micellar solubilization of preservative molecules by compatible surfactants can be used to increase the amount of preservative in the system (18).

Effect of pH, temperature and storage times

The pH tolerance limits for microorganisms is between pH 2–11 and since cosmetic and toiletry formulations can cover a wide pH range the ideal preservative should be effective and stable in solution over this range. It should also be stable at temperatures likely to be encountered during the manufacture and storage life of the product.

It is well known that only the undissociated molecules of benzoic, dehydroacetic, salicylic and sorbic acids are active against microorganisms and that activity is lost with increasing pH. The parabens do not have the same pH dependence as benzoic acid but like phenols they show greater activity on the acid side of neutrality. Preservative dissociation as a function of pH has been reviewed by Wedderburn (3).

The ideal preservative should be non-volatile at temperatures used during manufacture as well as at normal storage temperatures. Volatility is a disadvantage of chlorocresol, chloroxylenol and formaldehyde, for example.

Effect of oil/water partition coefficient

Since microorganisms multiply in the aqueous phase of formulations the preservative must be available in an effective concentration in this phase. Aqueous solutions such as shampoos are therefore relatively easy to preserve since all the preservative is available providing that there is no chemical or physical incompatibility between it and the formulation or its container. The situation is obviously more complicated when the formulation is a cream or an emulsion; as Bean and his colleagues (6, 30, 31) have pointed out the failure of preservatives in this situation may frequently be attributed to the fact that

only a proportion of the total quantity of preservative is available in the aqueous phase where it is required. The remainder partitions into the oily phase or associates with the emulsifying agent, the other major component, and is thus inactivated. The ideal preservative should therefore have a low oil/water partition coefficient.

It is therefore desirable that the oil/water partition coefficient (K_w^o) of a preservative is known. The concentration in the aqueous phase (C_w) at equilibrium may then be calculated for any total concentration (C) and any oil/water ratio (ϕ) as follows (31):

$$C_w = \frac{C(\phi+1)}{K_w^o\phi+1}$$

Thus, when the oil/water partition coefficient is low, most of the preservative is in the aqueous phase and an increase in the oil/water ratio in an emulsion increases the aqueous phase concentration. When the oil/water partition coefficient is high most of the preservative is in the oil phase and an increase in oil/water ratio reduces the concentration in the aqueous phase (6). High partition coefficient values are usually observed for vegetable oil/water systems and low values for mineral oil/water systems. This means that vegetable oil/water systems usually require a higher total concentration of preservative than do mineral oil/water systems (31).

Attention has been drawn to the importance of the concentration of the preservative at the oil/water interface. In an emulsion partition of the preservative occurs between the oil, water and emulsifier and its effectiveness is determined largely but not completely by its concentration free in the water. The distribution of the emulsifier influences the distribution of the preservative. The above equation is modified to:

$$C_w = \frac{C(\phi+1)}{K_w^o\phi+R}$$

where R is the ratio of total/free preservative in the water (31).

Compatibility with other ingredients

The ideal preservative should be compatible with all the ingredients of modern cosmetics and toiletries. This is a very tall order in view of the many additives used in present day preparations.

Prior to the Second World War, almost all cosmetics and toiletries were stabilized by soaps, which made them alkaline, or by anionic surfactants; many anionics have some antibacterial action and also tend to potentiate preservative action (32). Formulations containing nonionic surfactants are usually formulated at neutral or slightly acid pH, a factor which enhances microbial growth. Incompatibility with newer cosmetic ingredients and particularly with nonionic surfactants has accounted for the failure of some of the older preservatives such as the parabens which at one time were considered to be ideal agents (3). Barr and Tice (25) and Wedderburn (32) examined the effect of some nonionic surfactants on preservatives in common use at that time and found that many of them, including the parabens, substituted phenols and quaternary ammonium compounds were inactivated to some extent when the ratio of the nonionics to preservative exceeded certain critical values. Benzoic and sorbic acids, formaldehyde and organic mercurials were affected to a much less extent. A vast amount of literature has accumulated on interactions between nonionic surfactants and preservatives (see reviews 3, 33, 34).

Other additives such as natural gums, thickeners, protein hydrolysates, carbohydrates, fruit and vegetable juices, which are now frequently used in cosmetics, present additional problems since they may support growth of microorganisms and interact with some preservatives (34, 35, 36). Protein hydrolysates, for example, are particularly difficult to preserve (37, 38).

The preservative should not complex with or be adsorbed by suspended solid ingredients of a cosmetic. Studies in this field have been carried out by Horn *et al* (39).

A number of workers have reviewed methods, both physico-chemical and microbiological, for studying interactions between preservatives and cosmetic ingredients (34, 40, 41, 42).

Compatibility with packaging

The ideal preservative must not be lost by passage through, or binding to, the packaging material. Interaction between preservatives and rubber has been well documented and the increasing use of plastic packaging has stimulated research into plastics/preservative interactions (see 3, 36). A satisfactory preservative should be non-corrosive to and retain its activity in the presence of metals and alloys such as aluminium and tin foil which are used as cap liners and in collapsible tubes.

Colour, odour, taste

The ideal preservative should be colourless, odourless and tasteless at use concentrations.

Cost

Finally, the ideal preservative should be cheap.

When all these properties are considered it is not surprising that the ideal compound does not exist and probably will never be found. The best use of the currently available preservatives must therefore be made and it cannot be emphasized too strongly that preservation should be the concern of both the formulator and the microbiologist at an early stage in formulation development. Each formulation must be regarded as a separate entity with its own preservation problems. The effectiveness of the preservative should be determined not only initially but throughout the shelf-life of the product. A preservative which is satisfactory when the formulation is first prepared may become ineffective during its shelf-life for a number of reasons. It is good practice to test the preservative capacity of formulations at their maximum intended shelf-life. It may well be that in many instances the most satisfactory results will be obtained by using mixtures of preservatives.

Various reviews of preservatives have been published, one of the most comprehensive is that of Gucklhorn (13). A list of some of the older preservatives which have been in use for some time so that their principal properties and uses have been established is shown in *Table I*.

NEW PRESERVATIVES

Over the last 10 years or so several new preservatives have appeared and some of these have yet still to be fully evaluated. These are considered in rather more detail here.

Table 1. The advantages and disadvantages of some of the established preservatives

Preservative	References	Advantages	Disadvantages
Alcohols, e.g. ethyl alcohol, isopropyl alcohol	(12, 13, 68, 69)	Broad spectrum	Volatile; high concentrations (15–20%) required.
Quaternary ammonium compounds	(12, 13)	Better as active agents, e.g. in deodorants.	Ineffective against some pseudomonads except at high concentrations which may be irritant; incompatible with proteins, anionics and nonionics.
Acids, e.g. benzoic, sorbic, dehydroacetic	(12, 13, 69, 70)	Active against fungi.	pH dependent because of dissociation.
Formaldehyde	(12, 13, 71)	Broad spectrum; cheap; water soluble. Retains activity in presence of surfactants.	Irritant (banned in some countries); volatile; unpleasant odour; highly reactive chemically; incompatible with proteins.
Parabens (p-hydroxybenzoates)	(12, 13, 69)	Low toxicity; relatively non-irritant at use concentrations; relatively effective over wide pH range.	More active against fungi and Gram-positive bacteria than Gram-ve bacteria; low water solubility; partition in favour of the oily phase; inactivated by nonionics, proteins.
Organic mercurials, e.g. phenyl mercuric salts	(12, 13, 72)	Broad spectrum; stable.	High toxicity and irritancy; inactivated by proteins, anionics but to much less extent by nonionics; low water solubility.
Phenolics	(12, 13)	Useful as packaging preservatives and as active agents.	Low water solubility; partition into the oil phase; volatile; incompatible with anionics above critical micelle concentration and with nonionics; may be irritant.

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

Dioxin has broad spectrum activity of a fairly low order (>625 μg/ml) so that its recommended use concentration is up to 0·2%. It is reported to be effective over a wide pH range and is not inactivated by nonionic surfactants (13, 19, 43, 44).

The compound is a water soluble liquid which is also miscible with oils, and there is a danger that it will migrate into the oil phase. In aqueous systems the ester group hydrolyses to produce acetic acid which results in a slight lowering of the pH. It is claimed to be non-irritant and non-sensitizing at 1·0% concentration although, as already mentioned, there is some more recent evidence that *Dioxin* may be a sensitizer (24). The main disadvantage of *Dioxin* is its strong odour which may affect the fragrance of the cosmetic product. *Dioxin*, at concentrations of 0·1–0·2%, was one of the compounds recommended for the preservation of anionic and nonionic lotions by Jacobs et al (45).

Cis isomer of 1-(3-*chloroallyl*)-3, 5, 7-*triaza*-1-*azonia-adamantane chloride* (Dowicil 200)

Dowicil 200, a type of quaternary ammonium compound, has broad spectrum (13) activity being rather more effective against bacteria (m.i.c. of 50–500 μg/ml) than against fungi and yeasts. Parker has found some fungi to be more tolerant of this preservative (46). It has very high water solubility and low oil solubility; the oil/water partition coefficient for all the oils tested was <1·0 (47). It is unaffected by pH, nonionic and anionic surfactants and is said to be non-irritant and non-sensitizing at concentrations up to 2·0%. Solutions of *Dowicil* 200 should not be held at high temperatures. It releases formaldehyde in aqueous solution (48), a factor which explains its broad spectrum activity over a wide pH range and its retention of activity in the presence of surfactants. However, the possibility of irritancy and sensitization due to the release of formaldehyde cannot be overlooked.

Dowicil 200 is used for the preservation of shampoos (5) and at 0·1% was found to be relatively effective against bacteria in a test cream, suspension and solution but ineffective against yeasts in the Barnes and Denton capacity test (49). It is recommended as an effective preservative at 0·1% for an anionic and a nonionic lotion although some discoloration occurred in the anionic lotion (45). *Dowicil* 200 has been used satisfactorily in Germany for the preservation of cosmetic creams, lotions and hair care preparations because of its wide pH range and good protein compatibility (50).

Imidazolidinyl urea compound (Germall 115)

Germall 115 is more active against bacteria than against fungi and yeasts. It is effective over a wide pH range and is claimed to demonstrate its maximum preservation potency not in screening experiments but in tests with formulations; it often shows synergism with other antibacterial preservatives (28, 51). *Germall* 115 is very soluble in water and almost insoluble in oils so that it remains in the aqueous phase. It is colourless, odourless, tasteless, stable and retains its activity in the presence of proteins and surfactants. It is said to be non-toxic, non-irritating and non-sensitizing.

A key property of *Germall* 115 is probably its ability to act effectively with other preservatives (51). This was confirmed by Jacobs *et al* (45) who found that *Germall* 115 with parabens preserved their nonionic and anionic lotions whereas *Germall* on its own did not.

2-*Bromo*-2-*nitropropane*-1, 3-*diol* (Bronopol)

Bronopol has broadspectrum antibacterial activity with somewhat lower activity against fungi and yeasts. It is effective over a wide pH range and inhibits most bacteria at 25µg/ml (52, 53, 54).

It is highly soluble in water, whereas its solubility in oils is very low and the oil/water partition coefficient is low; e.g. liquid paraffin/water 0·043; arachis oil/water 0·11. It is not adversely affected by anionic and nonionic surfactants (44, 52, 55) and synergism between *Bronopol* and some octyl or nonyl phenol nonionic surfactants has been described (55). Since it also maintains its activity in the presence of protein hydrolysates, it is a very useful preservative for all types of shampoos (5, 7, 37, 56).

Aqueous solutions slowly decompose when alkaline, sometimes with the production of a yellow colour. Decomposition is accelerated by increasing the pH and the temperature. In spite of this instability at alkaline pH, *Bronopol* can be very effective as a labile preservative in alkaline formulations.

Bronopol is odourless and colourless but it is incompatible with some metals, e.g. aluminium, so that the type of container and liner must be taken into consideration.

Thiol-containing enzymes are involved in the mode of action of *Bronopol* against bacteria (52, 57). The selectivity of the compound for microorganisms indicated by its very low mammalian toxicity, may be due in part to its rapid metabolism by the body tissues (58).

5-*Bromo*-5-*nitro*-1, 3-*dioxan* (Bronidox)

Bronidox has broad spectrum activity but its physical and chemical properties have not been fully described (59, 60). It is still under evaluation and its chemical stability and toxicological properties have not yet been reported. It is available as *Bronidox L* containing 10% of active agent in 1·2 propylene glycol.

Kathon 886 (*or* CG)

This new antimicrobial agent, consisting of a 12·8% aqueous solution of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one, also has broad spectrum activity (61). The product requires evaluation as a preservative in the cosmetic field.

Some preliminary data from the author's laboratory on the stability of aqueous solutions of *Bronidox L* and *Kathon CG* at various pH levels are shown in *Table II*.

Table II. Stability of aqueous solutions of Bronidox L and Kathon CG at 37°C*

| | Concentration (%) of active ingredient at time in weeks | | | | |
	0	1	2	4	14
Bronidox L pH 4	0·2	0·14	—†	0·14	0·09
6	0·2	0·16	—	0·14	0·09
8	0·2	0·14	—	0·12	0·05
Kathon CG pH 4	0·011	0·011	0·011	0·011	0·011
6	0·011	0·010	0·010	0·009	0·007
8	0·011	0·008	0·0075	0·005	<0·0011

* The buffer solution used was McIlvaine's 0·2M Na_2HPO_4 and 0·1M citric acid. A 2% solution of *Bronidox L*, corresponding to 0·2% of active ingredient, and a 1% solution of *Kathon CG*, corresponding to 0·011% of active ingredients, were examined by microbiological assay. † Not tested.

The spectra of antimicrobial activity of some established and of some newer preservatives are compared in *Tables III* and *IV*.

Table III. Bacteriostatic activity in agar of preservatives

Preservative	m.i.c. (µg/ml) of least sensitive strain*			
	Staphylococci	*Pseudomonads*	Other Gram-ve bacteria	Moulds and yeasts
Benzyl alcohol	>3200	3200	>3200	>3200
Benzalkonium chloride	3·1	3200	800	1600
Benzoic acid	100	1600	1600	800
Formaldehyde	100	200	100	200
Methylhydroxy-benzoate	3200†	3200	800	1600
Nipastat	800	3200	800	800
Phenylmercuric acetate	3·1	25	6·25	6·25
Chlorocresol	200	400	200	200
Phenoxyethanol	>3200	>3200	>3200	>3200

* These tests were carried out, using two-fold serial dilution of preservatives in Oxoid blood agar base for the bacteria and malt extract agar for the yeasts and moulds, by surface inoculation with a multi-point inoculator (73). The test organisms included several strains of *Staphylococcus aureus, Staph. epidermidis, Escherichia coli, Klebsiella aerogenes, Kleb. pneumoniae, Salmonella typhi-murium, Flavobacterium meningosepticum, Serratia marcescens, Proteus sp., Pseudomonas* sp., including *Ps. aeruginosa, Ps. fluorescens, Ps. cepacia, Ps. putida, Ps. stutzeri, Candida albicans, C. tropicalis, Aspergillus niger, Penicillium roqueforti* and organisms isolated from contaminated products. The minimum inhibitory concentrations (m.i.c.) were recorded after 48 h at 32°C for the bacteria and 5 days at 30°C for the moulds and yeasts.

Table IV. Bacteriostatic activity in agar of newer preservatives

Preservative	m.i.c. (µg/ml) of least sensitive strain*			
	Staphylococci	*Pseudomonads*	Other Gram-ve bacteria	Moulds and yeasts
Germall 115	800	>1600	800	>3200
Dioxin	1600	1600	1600	800
Dowicil 200	200	>1600	400	>1600
Bronopol	25	25	25	3200
Bronidox L	50†	100	50	200
Kathon CG	12·5†	12·5	6·25	12·5
Phenonip	1600	3200	3200	1600

* see footnote *Table III*. † µg/ml of active ingredients.

PRESERVATIVE MIXTURES AND COMBINATIONS

The use of preservative mixtures has been recently reviewed by Parker (19, 62) and Garrett (63) has discussed the rationales for the use of preservative combinations. These are; (i) the spectrum of activity can be increased; (ii) the toxicological hazard can be reduced by using lower concentrations of component preservatives; (iii) the development of the resistance of an organism to one preservative alone may be prevented; (iv) the response may exceed prediction from the separate preservative action or from

any concentration of one preservative alone; (v) convenience of use of smaller preservative amounts or economic savings may result.

The author suggests that the combination of a highly active labile preservative with a less active but more stable one to control prolonged in-use contamination is a further justification for preservative combinations.

Mixtures of parabens in place of a single ester have been used for a long time (64); claims for these mixtures, which are available commercially e.g. *Nipasept, Nipastat,* have varied from simple additive effects to potentiation (see 12). Probably the main advantage is that the solubilities of the individual esters in the mixture are independent of each other so that a higher total concentration can be used.

More recently, *Phenonip*, a combination of parabens and *Phenoxetol* (phenoxyethanol), has been evaluated and found to be a potent, wide-spectrum liquid preservative effective in the presence of anionic surfactants and proteins. There is some loss of activity in nonionic surfactants, in which *Phenonip* is very soluble, but this can be offset by utilizing the solubilization to create higher concentrations of the preservative in the product (65, 66, 67).

Examples of extension of the microbial spectrum are the combinations of parabens with compounds showing low antifungal activity such as *Dowicil* 200 and *Bronopol*. From our own experience a mixture of *Bronopol* and parabens can also be useful in some alkaline formulations where the *Bronopol* is effective as a labile preservative and the paraben will combat in-use contamination.

Germall 115 is reported to act synergistically with other preservatives (51); it is certainly useful in combination with parabens but true synergy is a rare occurrence.

The use of mercurials with parabens to reduce the toxicity of the former may be useful in some formulations (19).

Parker suggested in 1973 that the use of multicomponent preservative systems on a rational basis was probably the only way to preserve the complex formulations in use (62). This is still true today and may well be for some time to come since our knowledge of preservatives and their mode of action is still largely empirical. We cannot, therefore, hope to design an individual preservative or combination of preservatives that will match up to the ideal.

References

1   De Navarre, M. G. *The Chemistry and Manufacture of Cosmetics,* p. 145 (1941) (D. Van Nostrand Co. Inc., New York).

2   Smart, R. and Spooner, D. F. Microbiological spoilage in pharmaceuticals and cosmetics. *J. Soc. Cosmet. Chem.* **23** 721 (1972).

3   Wedderburn, D. L. Preservation of emulsions against microbial attack. *Adv. Pharm. Sci.* **1** 195 (1964).

4   Woodward, C. R. and McNamara, T. F. Microbiological considerations of cosmetic emulsions. *Am. Perfum. Cosmet.* **85** No. 3 73 (1970).

5   Yablonski, J. I. and Goldman, C. L. Microbiology of shampoos. *Cosmet. Perfum.* **90** No. 3 45 (1975).

6   Bean, H. S., Heman-Ackah, S. M. and Thomas, J. The activity of antibacterials in two-phase systems. *J. Soc. Cosmet. Chem.* **16** 15 (1965).

7   Bryce, D. M. and Smart, R. The preservation of shampoos. *J. Soc. Cosmet. Chem.* **16** 187 (1965).

8   Tice, L. F. and Barr, M. Factors to be considered in the preservation of cosmetic emulsions. *J. Soc. Cosmet. Chem.* **9** 171 (1958).

9   Malcolm, S. A. and Woodroffe, R. C. S. The relationship between water-borne bacteria and shampoo spoilage. *J. Soc. Cosmet. Chem.* **26** 277 (1975).

10   Tenenbaum, S. Pseudomonads in cosmetics. *J. Soc. Cosmet. Chem.* **18** 797 (1967).

11 McConville, J. F. and Anderson, D. W. Occurrence of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in the orbital area. *J. Soc. Cosmet. Chem.* **26** 169 (1975).

12 Manowitz, M. Cosmetic Preservatives in *Disinfection, Sterilization and Preservation* (Ed. by C. A. Lawrence and S. S. Block) 555 (1968) (Lea and Febiger, Philadelphia).

13 Gucklhorn, I. R. Antimicrobials in cosmetics series. *Manuf. Chem. Aerosol News,* **40–42** (1969–1971).

14 Bruch, C. W. Cosmetics: sterility vs. microbial control. *Am. Perfum. Cosmet.* **86** No. 4 45 (1971).

15 Gershenfeld, L. Antimicrobial agents in cosmetic preparations. *Am. Perfum. Cosmet.* **78** No. 10 55 (1963).

16 Parker, M. S. Microbiologically acceptable cosmetic. *Am. Perfum. Cosmet.* **86** No. 12 35 (1971).

17 Tronnier, H. (Editorial) The Preservation of cosmetics. *Soap, Perfum. Cosmet.* **41** 871 (1968).

18 Coates, D. Preservatives—microbiological and physical factors. *Manuf. Chem. Aerosol News* **44** No. 6 35 (1973).

19 Parker, M. S. A new generation of preservatives. *Soap. Perfum. Cosmet.* **45** 621 (1972).

20 Thomas, M. J. and Majors, P. A. Animal, human and microbiological safety testing of cosmetic products. *J. Soc. Cosmet. Chem.* **24** 135 (1973).

21 Manowitz, M. Preservation of cosmetic emulsions. *Developments in Industrial Microbiology: Proceedings* (Ed. by S. Rich) 2 65 (1961) (Plenum Press, New York).

22 Charles, R. D. and Carter, P. J. The effect of sorbic acid and other preservatives on organism growth in typical nonionic emulsified commercial cosmetics. *J. Soc. Cosmet. Chem.* **10** 383 (1959).

23 Schorr, W. F. Allergic skin reactions from cosmetic preservatives. *Am. Perfum. Cosmet.* **85** No. 3 39 (1970).

24 Schorr, W. F. Cosmetic allergy—a comprehensive study of the many groups of chemical antimicrobial agents. *Arch. Derm.* **104** 459 (1971).

25 Barr, M. and Tice, L. F. The preservation of aqueous preparations containing nonionic surfactants. II. *J. Am. Pharm. Ass. Sci. Ed.* **46** 445 (1957).

26 Calnan, C. D. Contact dermatitis from drugs. *Proc. R. Soc. Med.* **55** 39 (1962).

27 Marzulli, F. N. and Maibach, H. I. The use of graded concentrations in studying skin sensitizers: experimental contact sensitization in man. *Food Cosmet. Toxicol.* **12** 219 (1974).

28 Berke, P. A. and Rosen, W. E. *Germall,* a new family of antimicrobial preservatives for cosmetics. *Am. Perfum. Cosmet.* **85** No. 3 55 (1970).

29 Lyman, F. L. and Furia, T. Toxicology of 2, 4,4′-trichlorohydroxy-phenyl ether. *Ind. Med. Surg.* **38** 45 (1969).

30 Bean, H. S., Konning, G. H. and Malcolm, S. A. A model for the influence of emulsion formulation on the activity of phenolic preservatives. *J. Pharm. Pharmacol.* **21** Suppl. 173S (1969).

31 Bean, H. S., Konning, G. H. and Thomas, J. Significance of the partition coefficient of a preservative in cosmetic emulsions. *Am. Perfum. Cosmet.* **85** No. 3 61 (1970).

32 Wedderburn, D. L. Preservation of toilet preparations containing nonionics. *J. Soc. Cosmet. Chem.* **9** 210 (1958).

33 Coates, D. Interaction between preservatives and surfactants. *Manuf. Chem. Aerosol News* **44** No. 8 41 (1973).

34 Wedderburn, D. L. Interactions in cosmetic preservation. *Am. Perfum. Cosmet.* **85** No. 3 49 (1970).

35 Coates, D. Preservative/colloid interaction. *Manuf. Chem. Aerosol News.* **44** No. 10 34 (1973).

36 Coates, D. Interaction between preservatives, plastics and rubbers. *Manuf. Chem. Aerosol News* **44** No. 12 19 (1973).

37 Tuttle, E., Phares, C. and Chiostri, R. F. Preservation of protein solutions with 2-bromo-2-nitro-1, 3-propanediol (*Bronopol*). *Am. Perfum. Cosmet.* **85** No. 3 87 (1970).

38 Schuster, G., Modde, H. and Riepl, G. Experimental studies on chemical preservation of polypeptide-containing substances. *Am. Perfum. Cosmet.* **81** No. 6 39 (1966).

39 Horn, N. R., McCarthy, T. J. and Price, C. H. Interaction between preservatives and suspension systems. *Am. Perfum. Cosmet.* **86** No. 7 37 (1971).

40 Parker, M. S. and Barnes, M. The interaction of non-ionic surfactants with preservatives. *Soap Perfum. Cosmet.* **40** 163 (1967).

41 Coates, D. and Woodford, R. Methods available for studying antimicrobial interaction in cosmetics. *Cosmet. Perfum.* **88** No. 4 43 (1973).

42 Parker, M. S. The activity of preservatives in complex formulations. *Soap Perfum. Cosmet.* **47** 123 (1974).

43 Anonymous. *Dimethoxane,* a new preservative effective with nonionic agents. *Am. Perfum. Cosmet.* **77** No. 12 32 (1962).

44 Brown, M. R. W. Turbidimetric method for the rapid evaluation of antimicrobial agents:— Inactivation of preservatives by nonionic agents. *J. Soc. Cosmet. Chem.* **17** 185 (1966).

45 Jacobs, G., Henry, S. M. and Cotty, V. F. The influence of pH, emulsifier and accelerated ageing upon preservative requirements of oil/water emulsions. *J. Soc. Cosmet. Chem.* **26** 105 (1975).

46 Parker, M. S. The rapid screening of preservatives for pharmaceutical and cosmetic preparations. *Soap Perfum. Cosmet.* **45** 103 (1972).

47 Murray, E. A. A cosmetic preservative. *Deterg. Spec.* CT/7 31 (1971).

48 Scott, C. R. and Wolf, P. A. The antibacterial activity of a series of quaternaries prepared from hexamethylenetetramine and halohydrocarbons. *Appl. Microbiol.* **10** 211 (1962).

49 Barnes, M. and Denton, G. W. Capacity tests for the evaluation of preservatives in formulations. *Soap Perfum. Cosmet.* **42** 729 (1969).

50 Wallhaeusser, K. H. Die mikrobielle Kontamination von Kosmetika. *Parfuem. Kosmet.* **53** 305 (1972).

51 Rosen, W. E. and Berke, P. A. Modern concepts of cosmetic preservation. *J. Soc. Cosmet. Chem.* **24** 663 (1973).

52 Croshaw, B., Groves, M. J. and Lessel, B. Some properties of *Bronopol*, a new antimicrobial agent active against *Pseudomonas aeruginosa. J. Pharm. Pharmacol.* 16 Suppl. 127 T (1964).

53 Clark, N. G., Croshaw, B., Legetter, B. E. and Spooner, D. F. Synthesis and antimicrobial activity of aliphatic nitro compounds. *J. Med. Chem.* **17** 977 (1974).

54 Saito, H. and Onoda, T. Antibacterial action of *Bronopol* on various bacteria, especially on *Pseudomonas aeruginosa. Chemotherapy* (Tokyo) **22** 1466 (1974).

55 Allwood, M. C. Inhibition of *Staphylococcus aureus* by combinations of non-ionic surface active agents and antibacterial substances. Microbios **7** 209 (1973).

56 Schuster, G. Die Konservierung von Shampoos und Schaumbademitteln. *Seifen, Oele, Fette, Wachse* **99** No. 18 489 (1973).

57 Stretton, R. J. and Manson, T. W. Some aspects of the mode of action of the antibacterial compound *Bronopol* (2-bromo-2-nitropropane-1, 3-diol). *J. Appl. Bact.* **36** 61 (1973).

58 Moore, D. H., Chasseaud, L. F., Lewis, J. D., Risdall, P. C. and Crampton, E. L. The metabolism of the antibacterial agent *Bronopol* (2-bromo-2-nitropropane-1, 3-diol) given orally to rats and dogs. *Food Cosmet. Toxicol.* **14** 183 (1976).

59 Brit. Pat. 1250725.

60 Von Koppensteiner, G. Nachweis und Bestimmung von antimikrobiellen Wirkstoffen im turbidimetrischen Test. *Pharm. Ind.* **36** Nr. 6 442 (1974).

61 Anonymous. *Rohm and Haas trade literature* (1975).

62 Parker, M. S. Some aspects of the use of preservatives in combination. *Soap, Perfum. Cosmet.* **46** 223 (1973).

63 Garrett, E. R. A basic model for the evaluation and prediction of preservative action. *J. Pharm. Pharmacol.* **18** 589 (1966).

64 Boehm, E. E. Combination of the esters of *para*-hydroxybenzoic acid. *Am. Perfum. Aromat.* **73** No. 1 37 (1959).

65 Parker, M. S., McCafferty, M. and MacBride, S. *Phenonip,* a broad spectrum preservative. *Soap, Perfum. Cosmet.* **41** 647 (1968).

66 Boehm, E. E. and Maddox, D. N. Preservative failures in cosmetics with special reference to combatting contamination by *Pseudomonas* using binary and tertiary systems. *Am. Perfum. Cosmet.* **85** No. 3 31 (1970).

67 Boehm, E. E. and Maddox, D. N. Problems of cosmetic preservation. *Manuf. Chem. Aerosol News.* **42** No. 4 41 (1971).

68 von Fenyes, C. K. Alcohols as preservatives and germicides. *Am. Perfum. Cosmet.* **85** No. 3 91 (1970).

69 Grundy, W. E. Antimicrobial preservatives in Pharmaceuticals in *Disinfection, Sterilization and Preservation* (Ed. by C. A. Lawrence and S. S. Block) 566 (1968) (Lea and Fegiber, Philadelphia).

70 Woodford, R. and Adams, E. Sorbic acid *Am. Perfum. Cosmet.* **85** No. 3 25 (1970).

71 Henley, W. O. and Sonntag, N. O. V. Formaldehyde and its donors as preservatives in cosmetic formulations. *Am. Perfum. Cosmet.* **85** No. 3 95 (1970).

72 Eckardt, W. Phenylmercuric compounds as preservatives for cosmetics. *Am. Perfum. Cosmet.* **85** No. 3 83 (1970).

73 Hale, L. J. and Inkley, G. W. A semi-automatic device for multiple inoculation of agar plates. *Lab. Pract.* **14** 452 (1965).

Microbial contamination of cosmetic products

*Presented on 23 February 1976 at the Symposium on Microbiological
Safety in Cosmetic and Toiletry products, Birmingham*

ROSAMUND M. BAIRD *Department of Medical Microbiology,
The Medical College of St Bartholomew's Hospital, West Smithfield, London EC1A 7BE*

Synopsis

One hundred and forty seven cosmetic products purchased from five retail shops in 1974 were examined microbiologically. Viable bacteria were not recovered from ninety-nine products. Gram-negative rods were isolated from 6·1% of products and *Pseudomonas* spp. from 4·1% of products.

Introduction

In recent years increasing interest has been shown in the susceptibility of cosmetics and toiletry products to microbial contamination and the potential risk of infection to the user of those products. A number of workers have attempted to determine the incidence of contamination; the results from these surveys are conflicting and contamination rates have been found to vary from 2·5% to 43% (1–8). There is even less information on the possible health risk to the user of contaminated products. Two cases are often quoted from experience in hospitals (9, 10).

This paper presents the results of an investigation into the microbial contamination of unused cosmetic and toiletry products at the point of sale.

Materials and methods

A total of 147 unused cosmetic products were purchased from five retail shops near this hospital in July 1974. These comprised a wide range of preparations as shown in *Table I*. Where possible aqueous products were bought in preference to oily products.

One gram or 1 ml of product was homogenized in 20 ml of 4% Lubrol W in Nutrient Broth (Oxoid) using a Stomacher 80 (Seward). Products containing halogen compounds were homogenized in Nutrient Broth (Oxoid) containing 1% sodium thiosulphate. Phenolic products were diluted in 100 ml Nutrient Broth (Oxoid). All broths were incubated aerobically at 37°C overnight, then sub-cultured to Horse Blood Agar (Oxoid), MacConkey Agar and Cetrimide Agar 0·03% and incubated overnight at 37°C. Resulting colonies were identified; some of the Gram-negative rods were sent to the Computer Laboratory at Colindale for confirmation. Colony counts were only carried out on those products from which Gram-negative rods were isolated.

Table I. Type of products sampled and number contaminated

Product	No. of samples	No. contaminated	Type of contamination	Number
Creams and Lotions				
Handcream	27	9	asb	4
			g+	5
			g−	1
Cleanser	24	12	asb	6
			g+	3
			g−	3
Foundation	8	5	asb	4
			g+	2
Medicated topical	8	2	asb	2
			g+	1
Eye make-up				
Shadow	5	5	asb	3
			g+	3
Liner	3	1	asb	1
Mascara	6	4	asb	1
			g+	2
			g−	1
Non-medicated eye drop	8	0		
Baby				
Lotion and shampoo	12	1	g+	1
Dental				
Toothpaste	1	0		
Denture cleaning solution	2	0		
Denture powder and cream	2	2	g−	2
Teething gel	2	0		
Soaps and detergents				
Bath detergent	1	0		
Shampoo	9	0		
Conditioner	10	3	g+	1
			g−	2
Soap	1	1	asb	1
Miscellaneous				
Depilatory	6	1	g+	1
Deodorant	2	0		
Sun-tanning and after-sun lotion	10	2	asb	1
			g+	1
Total	147	48 (32·7%)	asb	23
			g+	20
			g−	9

Key, asb: aerobic spore bearer; g+ : non-haemolytic coagulase negative Gram-positive cocci; g− : Gram-negative rod.

Results

Of the 147 cosmetic preparations examined, ninety-nine showed no bacterial growth. A variety of contaminants were isolated from most types of products (*Table I*). Aerobic spore bearers were isolated from 23 preparations; *Staph. aureus* was not found but non-haemolytic coagulase negative Gram-positive cocci were isolated from twenty products.

On several occasions both these types of organism were isolated from the same product. Gram-negative rods were isolated from nine cosmetic products which included three cleansers, two hair preparations, two dental products, one hand-cream and one eye product, as shown in *Table II*. Several free-living bacteria were isolated from the dental powder. None of these preparations showed visible signs of bacterial contamination or degradation, despite the fact that the counts ranged from 10^3–10^6 organisms g^{-1} or ml^{-1}. When counts on these products were repeated a year later, in seven instances the counts were found to be unchanged; no evidence of contamination was detected, however, in the dental cream or in the moisture cream.

Table II. Gram-negative rods found in cosmetics

Contaminant	Product	No. of organisms (g^{-1} or ml^{-1})
Pseudomonas aeruginosa	Lanolin hand-cream	$1\cdot2 \times 10^3$
P. maltophilia	Mascara	$7\cdot0 \times 10^5$
P. pseudoalcaligenes	Cleansing milk	$3\cdot1 \times 10^4$
P. pseudoalcaligenes	Hair cream	$1\cdot9 \times 10^4$
P. fluorescens	Hair oil	$4\cdot0 \times 10^4$
P. putida	Cleansing jelly	$2\cdot5 \times 10^4$
Moraxella osloensis	Moisture cream	$1\cdot3 \times 10^3$
Enterobacter cloacae	Dental cream	$2\cdot3 \times 10^5$
Klebsiella aerogenes	Dental powder	$3\cdot4 \times 10^6$
K. oxytoca	Dental powder	
Erwinia herbicola	Dental powder	
Enterobacter cloacae	Dental powder	

Discussion

There are a number of explanations for the differing results from the surveys on the incidence of contamination in cosmetic products. Different types of product have been sampled; certain products, particularly aqueous products, are known to be more susceptible to contamination than others. In some surveys only one type of product has been sampled, such as cosmetics for the eye (5). Methods of sampling and cultivation have also varied; some have involved direct culture of the product, whilst others have used enrichment techniques. In the case of the latter, higher contamination rates have generally been found. Neutralization of antibacterial agents has also varied from survey to survey, or may have been overlooked.

With one exception (8), the results from all surveys, including this one, are based on the examination of a limited number of samples, mostly less than 250 products. Interpretation of results, expressed as percentages, is therefore restricted, but these results may be used for comparative purposes. Two recent studies have suggested that the incidence of contamination may be declining (2, 7). Evidence for an overall improvement in microbiological quality in cosmetic products is not, however, conclusive. Higher contamination rates in our survey may be partly explained by the use of a broth enrichment method and by the selection of aqueous, in preference to oily, products.

Our results support the findings of others in that some form of contamination in the finished product seems to be inevitable at present, particularly contamination caused by aerobic spore bearers and Gram-positive cocci. Of more concern, however, is the presence

of Gram-negative bacteria and especially *Pseudomonas* spp. In this study Gram-negative bacteria were isolated from 6·1% of cosmetics and *Pseudomonas* spp. from 4·1% of cosmetics. This interest in the cosmetic field has been stimulated by the increasing number of reports on the occurrence of these types of organisms in pharmaceutical products. Recently, attention has been drawn to the problem of contamination by some of the less generally considered pathogens. Use of such contaminated preparations in hospitals has in some cases been associated with the development of clinical infections in patients. Certain cosmetics, such as hand-cream and lotions, are frequently used in hospitals to prevent chapping of hands and in the control of cross-infection. Studies at this hospital have shown that these products may be an important source of contamination and that continued use of such products can contribute to the spread of nosocomial infections in the ward (11, 12).

Although much of the evidence for the occurrence and significance of contamination in cosmetics and toiletries is conflicting and inconclusive, it would be wrong to assume that the hazard does not exist. More information is required on the incidence of contamination in both used and unused cosmetics, using standardized methods of sampling and cultivation. This is a fruitful area for collaboration between the microbiologist and pharmacist in industry and their counterparts in hospital.

Acknowledgments

References

1   Wolven, A. and Levenstein, I. Cosmetics—contaminated or not. *T.G.A. Cosmetic J.* **1** 34 (1969).
2   Wolven, A. and Levenstein, I. Microbiological examination of cosmetics. *Am. Cosmet. Perfum.* **87** 63 (1972).
3   Heiss, F. Keimgehalt von Korperpflegemittela. *Fette Seifen Anstrichmittel* **69** 365 (1967).
4   Dunnigan, A. P. and Evans, J. R. Report of a special survey: microbiological contamination of topical drugs and cosmetics. *T.G.A. Cosmetic J.* **2** 39 (1970).
5. Wilson, L. A., Keuhne, J. W., Hall, W. and Ahearn, D. G. Microbial contamination in ocular cosmetics. *Am. J. Ophthal.* **71** 1298 (1971).
6   Myers, G. E. and Pasutto, F. M. Microbial contamination of cosmetics and toiletries. *Canad. J. Pharm. Sci.* **8** 19 (1973).
7   Jarvis, B., Reynolds, A. J., Rhodes, A. C. and Armstrong, M. A survey of microbiological contamination in cosmetics and toiletries in the U.K. (1971). *J. Soc. Cosmet. Chem.* **25** 563 (1974).
8   The 1974 Scientific Conference Committee Reports. CTFA. *Cosmetic J.* **7** 3 (1975).
9   Tremewan, H. C. Tetanus neonatorum in New Zealand. *N.Z. Med. J.* **45** 312 (1946).
10 Morse, L. J., Williams, H. L., Green, F. P., Eldridge, E. E. and Rotta, J. R. Septicaemia due to *Klebsiella pneumoniae* originating from a handcream dispenser. *New Eng. J. Med.* **277** 472 (1967).
11 Cooke, E. M., Shooter, R. A., O'Farrell, S. M. and Martin, D. R. Faecal carriage of *Pseudomonas aeruginosa* by new-born babies. *Lancet*, **ii** 1045 (1970).
12 Baird, R. M. *A study of the microbial contamination of pharmaceutical products and their possible role in dissemination of infection throughout hospitals. Ph.D. thesis,* University of London (1975).

Microbiological applications of a novel replipad skin sampler

H. DIXON *and* A. K. PANEZAI† *Beecham Products Research Department, Randalls Road, Leatherhead, Surrey KT22 7RX*

Synopsis

A **bacteriological skin sampler** which can be used to obtain microbial prints of the axilla is described. Various applications include *in vitro* and *in vivo* evaluation of the antimicrobial properties of deodorant and other products using the subject's skin microflora.

Introduction

The human axillary microflora is of special interest to the cosmetic microbiologist when testing deodorant products, since bacteria have been reported to have association with body odour (1, 2). Various methods have been used for sampling the skin microflora which include contact plate, agar sausage, sticky tape stripping, swabbing, scrub techniques and skin biopsy, but because of the peculiar shape of the axillary vault recoveries may be unreliable and few of these methods are suitable in practice.

The velvet pad method originates from the well-known replica-plating technique (3) which employs a sterile velvet pad to print bacteria from the master plate onto culture plates containing special media. This method has been extended by other workers and used for studying the bacterial flora of infected wounds and burns (4), for evaluating the relative efficiencies of common skin antiseptics (5), and as a means of making colony counts of pathogenic and commensal flora on the body surface (6).

AXILLARY SKIN SAMPLER (VELVET REPLIPAD)

The skin sampling device suggested by the authors was developed by *Strands Scientific specially to fit the axillary vault. Bacteria colonizing the skin surface are transferred by means of a sterile, moistened velvet pad to a suitable recovery medium. A sufficient number of organisms attach themselves to the velvet pad to enable culture plates to be successively inoculated. The sampling tool consists essentially of an anodised tapered holder and clamping ring which together hold the circular velvet pad (*Fig. 1*). When assembled, the sampler is enclosed in an autoclavable bag and sterilized in an autoclave at 121°C for 15 min.

EXPERIMENTAL

To obtain a microbial print, the sterile velvet pad is moistened and rendered slightly sticky, by bringing it into contact with the moist surface of an agar plate (the recovery

medium). The velvet head of the sampler is pressed firmly into the axilla, and then applied to the same area of culture plate. After suitable incubation a microbial print of the axilla surface is obtained.

Brain Heart Infusion Agar (B.H.I.A.) supplemented with 0·5% Tween 80 is recommended for the isolation of axillary bacteria. After 24–48 h, the common resident bacteria, staphylococci, pigmented micrococci and diphtheroids may be readily seen and identified.

TESTING A DEODORANT FOR ANTIBACTERIAL ACTIVITY IN VITRO

For this purpose a modified agar diffusion test is used. The method of evaluation departs from standard procedure by using freshly isolated resident axillary microflora as test organisms in place of type culture or stock culture strains which have been passaged several times artificially.

For economy, the test is carried out by using small disposable Petri dishes 60×13 mm (Sterilin). About 12 ml of B.H.I.A. are poured into each dish. When hardened the agar is inoculated by means of the axilla sampler as previously described. A 6-mm antibiotic assay disc (Whatman) is impregnated with the test deodorant and placed in the centre of the print. Blank discs impregnated with sterile water and a reference standard consisting of the test antimicrobial in simple aqueous solution should be set up at the same time. Dishes are incubated at 37°C for 24 to 48 h after which zones of inhibition are recorded (*Fig. 2*).

INTERPRETATION

A deodorant with good antibacterial potential should give a well-defined clear zone of inhibition. Antimicrobial activity is sometimes modified by interaction with other formulation ingredients. Such interaction can be dramatically revealed by comparing the activity of the antimicrobial in aqueous solution with the finished product containing the same active ingredient (*Fig. 3*).

For products which may exhibit antibacterial activity but diffuse poorly, the following direct contact method is recommended.

DIRECT CONTACT IN VITRO TEST

A sterile filter circle (Whatman 4·25 cm) is treated with test material, drained, and applied to an axilla print for 1 h after which it is removed. Suitable controls should be prepared at the same time consisting of sterile filter circles treated with sterile water, the appropriate solvent and the product without active ingredient. Circles are removed after 1 h as in the test procedure. Plates are incubated at 37°C and presence or absence of growth recorded after 24 and 48 h.

EVALUATION OF PRODUCT'S ANTIBACTERIAL ACTIVITY IN VIVO

Skin lipids are known to have the effect of greatly reducing the activity of some antimicrobial agents, so that for realistic results the product needs to be evaluated on the skin, and for deodorants, the axilla is the appropriate area for testing. Before commencing the trial it is important to establish that toiletries which contain antimicrobial agents (anti-

Figure 1. Velvet Pad Axillary Skin Sampler.
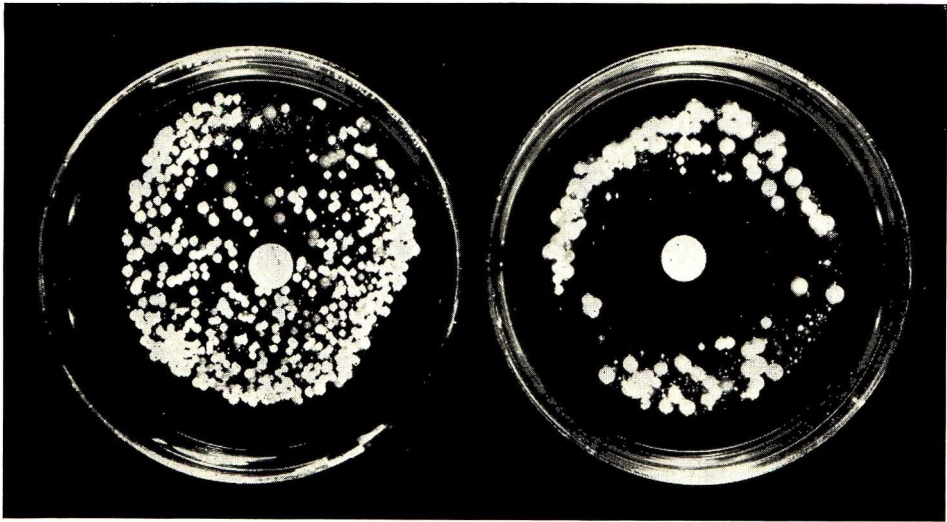
Figure 2. Disc diffusion test using axillary prints. A clear zone of inhibition surrounds the right hand disc which has been treated with deodorant, indicating satisfactory antibacterial activity *in vitro*. The blank control disc shows no activity against similar axillary microorganisms (staphylococci, micrococci and diphtheroids).

Figure 3. (a) Print showing axillary microflora with blank control disc. (b) Similar print in which the disc has been treated with an antimicrobial agent (Irgasan DP 300). (c) Demonstrates interaction of antimicrobial with Tween 80. Activity is seen to be much reduced.

Figure 4. Pre-treatment print shows normal density of axillary microflora compared with print showing a substantial reduction in microorganisms following treatment of the axilla with aluminium chlorhydrate. (Large colonies are due to small numbers and prolonged incubation.)
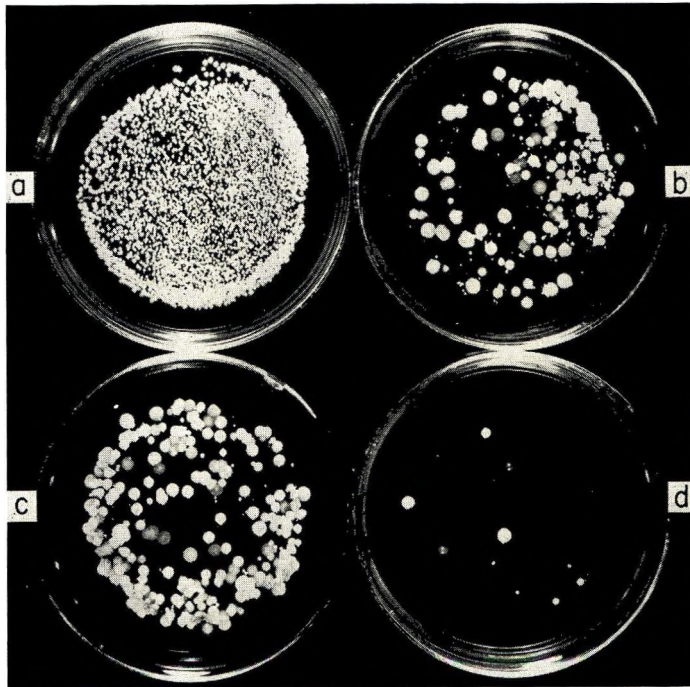
Figure 5. Prints showing microflora on different human skin surfaces. (a) Axilla ($+ + +$). (b) Forehead ($+$). (c) Cheek ($+$). (d) Inner aspect of forearm ($\pm$).

perspirants, deodorants, medicated soaps) have not been recently used by the subject undergoing the test. Bland soap only should be used for a period of 2 to 3 weeks before testing. The following protocol is intended only as a guideline and may be suitably modified as required.

Control pre-treatment samples to provide a base-line are obtained with the velvet Replipad at 24 and 48 h intervals, after which the test material is applied as in normal use once or twice daily for 3–6 days. Prints are taken 18 h after final application (*Fig. 4*). To follow duration of effect further prints may be taken at 24-h intervals. The effect of the antimicrobial may be assessed in terms of reduction in numbers of microorganisms (marked, moderate, slight reduction of surface bacteria). Changes in the ratio of member species (staphylococci, micrococci and diphtheroids) and introduction of new species or strains (Gram negative bacteria) may also be recorded. A suitable scoring system for the grading of growth is as follows:

Growth Scoring System

±	+	+ +	+ + +
		Colonies too	Heavy
<30 colonies	30–200 colonies	numerous	confluent
		to count	growth

Discussion

For test purposes the axilla offers a rich source of microorganisms and the velvet Replipad technique currently described provides a simple, rapid and convenient method of sampling this area of the skin. The fact that the test organisms represent the natural flora of the skin makes for a realistic method and one which is specially relevant to the testing of deodorant products. Useful information concerning residual activity can be obtained by taking prints for several days after treatment has ceased, since duration of antimicrobial effect may be short-lived or prolonged, depending on potency, skin substantivity and compatibility of the active ingredient.

The sampler may be used for investigating other skin sites, for example the scalp, forehead, face and hands; it will provide interesting information regarding the distribution, density and bacterial species colonizing the skin surface (*Fig. 5*).

The Replipad sampler may be used to monitor ecological changes in the skin microflora resulting from the application of cosmetic and toiletry products, skin antiseptics, occlusive dressings, etc. It may also be used as a replicating system to evaluate the sensitivity/resistance of resident microorganisms or clinical isolates, to antibiotics and other antimicrobial agents.

Main advantage of the print technique is that the microflora of the *stratum corneum* are probably less disturbed; and therefore more realistically represented in terms of ratio of member species. These can be more easily differentiated on the agar plate than by swabbing, or other methods where aggregates of cells are subsequently broken up.

A relatively large area of skin can be investigated with good reproducibility. Inaccessible areas of skin such as the axillary vault are easily sampled. A sufficient number of organisms may be picked up on the velvet pad to obtain four to six successive inoculations on plates of selective media.

Finally, when conducting a trial involving a large number of subjects not least in importance is the approval and acceptance of the sampling techniques by the participants; experience has shown that the velvet Replipad technique admirably suits this purpose.

References

1   Shelley, W. B., Hurley, H. J. and Nichols, A.C. Axillary odor; Experimental study of the role of bacteria, apocrine sweat and deodorants. *Arch. Dermat. and Syph.* **68** 430 (1953).
2   Shehadeh, N. H. and Kligman, A. M. The bacteria responsible for axillary odor II. *Jnl. Invest. Dermat.* **41** 3 (1963).
3   Lederberg, J. and Lederberg, E.M. Replica plating and indirect selection of bacterial mutants. *J. Bact.* **63** 399 (1952).
4   Gorrill, R. H. and Penikett, E. J. K. New methods of studying the bacteria flora of infected open wounds and burns. *Lancet,* **ii** 370 (1957).
5   Verdon, P. E. Efficiency tests on a series of common skin antiseptics under ward conditions. *J. clin. Path.* **14** 91 (1961).
6   Holt, R. J. Pad culture studies on skin surfaces. *J. appl. Bact.* **29** (3) 625 (1966).

A spectroscopic study of the reaction products of dihydroxyacetone with aminoacids

A. MEYBECK   *Laboratoires Jeanne Gatineau, 27 rue Salvador Allende, 95870 Bezons, France*

Synopsis

Dihydroxyacetone (DHA) has been allowed to react with a series of **aminoacids** in water solution. The dark solutions or suspensions obtained, were dialysed giving brown 'pigments' (**melanoidins**) which were subjected to instrumental analysis. Visible absorption spectra of their water solutions have no special features, while u.v. spectra show at best a slight maximum around 310–330 nm. I.R. spectra of all the products studied show broad bands around 3400, 1600 and 1400 cm^{-1}. All these melanoidins exhibit a strong **Electron Spin Resonance** (E.S.R.) signal. The E.S.R. spectra recorded in the solid state are single lines of 6–12 gauss linewidth, centred around $g=2$, which correspond to rather inert unpaired electrons since they can be observed in water solution as well. These data suggest that the reaction products of DHA with aminoacids, are conjugated polymers. The mechanism of skin tanning by DHA is discussed in view of these results.

Introduction

Although the first mention that dihydroxyacetone (DHA) makes human skin brown was published almost 50 years ago (1), still very little is known about the nature of the pigments formed. Soon after the first suntan-simulating cosmetic preparations appeared on the market, it was found that aminoacids were able to form brown or black products with DHA (2–4) and it is generally admitted now, that its tanning effect on skin is due to a condensation with amino groups of keratin and aminoacids released by sweat. The purpose of the present work was to isolate the ultimate coloured pigments formed in the reaction of aminoacids with DHA, which are very similar to the melanoidins formed in their so-called MAILLARD reaction with sugars (5), and to carry out a complete spectroscopic study of these products in order to gain more information about their structure.

Experimental and results

I. REACTIONS CARRIED OUT AT 100°C

Equimolar quantities (0.1 or 0.05 mol) of DHA and aminoacids usually in 25 ml of water, were allowed to react at 100°C on a steam bath for 4 h with refluxing. In some cases (especially when the aminoacid was not soluble enough) a greater amount of water was used (50 or 100 ml) and, or, an equimolar quantity of alkali (triethanolamine or sodium hydroxyde) was added. The reaction started almost immediately with formation of colour: yellow, then red and finally dark brown. In most cases a gas evolution was noticeable. Some experiments have also been carried out at 37°C using the same quantities of reactants but for longer periods, usually 24 h.

25

After cooling, the resultant dark brown viscous mixtures were diluted to about four times their volume and dialysed against tap water for at least 48 h. All the lower molecular weight coloured products were thus eliminated and only the highly polymerized pigments were retained for study. The dialysed solutions or suspensions were then evaporated to dryness by heating on a steam bath, and the black or brown residues were ground to powder form.

Yields

Yields have been taken as the amount of purified melanoidin collected, for 100 g of starting material (aminoacid + DHA). *Table I* gives the values thus calculated for a number of aminoacids. The best yields have been obtained for lysine, glycine, alanine, serine and the non-alpha aminoacids, especially 6-aminohexanoic acid. Arginine gave the poorest yield, a result which does not very well agree with the finding by Wittgenstein (4) that it was the most reactive aminoacid towards DHA and probably one of the most important in its tanning effect on human skin. It is also worth noticing that for glycine at least, less melanoidin was produced when triethanolamine and especially sodium hydroxyde, was added to the reaction medium. Only two aminoacids gave values in excess of 20% which is rather low, but it is to be remembered that at least some of the acids have probably been decarboxylated with production of carbon dioxide, that condensations and polymerizations leading to melanoidins imply at least some dehydration of the molecules, and finally that low molecular weight reaction products are eliminated by dialysis, all processes which can account for loss of solid matter.

Table I. Melanoidins recovered after reaction of DHA with aminoacids for 4 h at 100°C

Aminoacid	Alkali added	% Yield
Gly		16·4
Gly	triethanolamine	9·9
Gly	Na OH	5·8
Ala		12·3
Val		9·5
Leu		6·5
Ser		12·2
Asp		2·3
Glu		3·0
Glu	Na OH	5·2
Arg		1·7
Lys		29·7
Phe	Na OH	10·7
Tyr	Na OH	2·4
3-aminopropionic		11·7
4-aminobutyric		14·3
5-aminopentanoic		10·8
6-aminohexanoic		23·0

Ultra-violet and visible absorption spectra

U.V. and visible absorption spectra of 0·005 or 0·01% water solutions of the melanoidins have been recorded with regular equipment. In some cases the pH had to be raised with ammonia in order to fully solubilize the samples. No obvious change in the shape of the
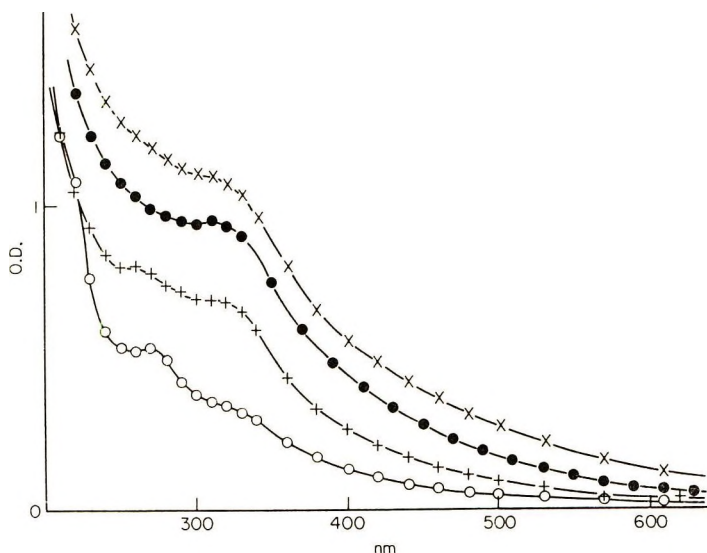
Figure 1. U.V. spectra in 0·005 % aqueous solution (1 cm cell) of melanoidins prepared by reacting DHA at 100°C with beta-alanine, glycine, glutamic acid and tyrosine, and purified by dialysis. The solution of beta-alanine has been brought to pH=9.8 with ammonia. ×, β Ala; ●, Gly; +, Glu; ○, Tyr.

Table II. U.V. absorption in 0·01 % water solution, of melanoidins obtained by reaction of DHA with aminoacids for 4 h at 100°C (1 cm cell)

| Aminoacid	pH	Optical density	
Gly	6·1		1·91 at 310 nm
	10·0		1·81 at 310
Gly + trieth			1·72 at 310
Gly + Na OH			1·28 at 320
Ala			2·02 at 320
Val	9·8		2·24 at 310
Leu	9·7		1·54 at 320
Ser			1·82 at 320
Asp			1·20 at 320
Glu			1·40 at 320
Glu + Na OH			1·36 at 320
Arg			0·38 at 320
Lys			1·17 at 330
Phe + Na OH		> 1·25 at 260,	> 0·99 at 310
Tyr + Na OH		0·86 at 270,	0·64 at 330
3-aminopropionic	5·9		1·86 at 310
	9·8		2·14 at 320
4-aminobutyric	9·7		1·84 at 320
5-aminopentanoic	9·7		1·68 at 320
6-aminohexanoic	9·7		1·63 at 320
8-aminooctanoic	9·7		1·35 at 310

Underlined wavelengths correspond to a maximum.

curves with pH was noticed. The spectra (*Fig. 1*) are all very similar, with no special features except for a shoulder (or an occasional maximum) around 320 nm. The melanoidins of phenylalanine and tyrosine however, show an additional maximum corresponding to the aromatic rings of the parent aminoacids (*Table II*). The optical densities

of 0·01% solutions at the wavelength of the maximum (underlined in *Table II*) or the shoulder near 320 nm, have been either measured directly or calculated from measures made on 0·005% solutions. They are practically all in the range 1–2, except for arginine again, which gave a much lower value. Although melanoidins are almost black in the powder form, their diluted solutions are light yellowish brown and their absorption spectra in the visible are rather flat.

Infra-red absorption spectra

I.R. absorption spectra have been recorded using the K Br pellet technique. All pellets were ground and pressed a second time to get a better dispersion of the melanoidins. The spectra, like those of polymers in general, are not very well resolved and their bands are very broad (*Fig. 2*). Nevertheless, some characteristic features appear in all of them,
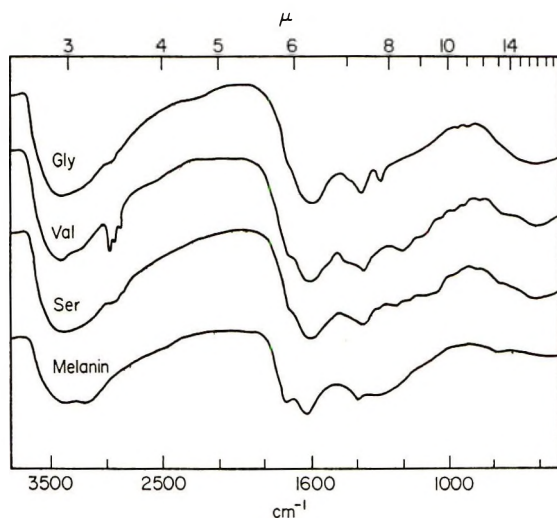
Figure 2. I.R. spectra in K Br pellets, of synthetic melanin and of melanoidins prepared by reacting DHA at 100°C with glycine, valine and serine, and purified by dialysis.
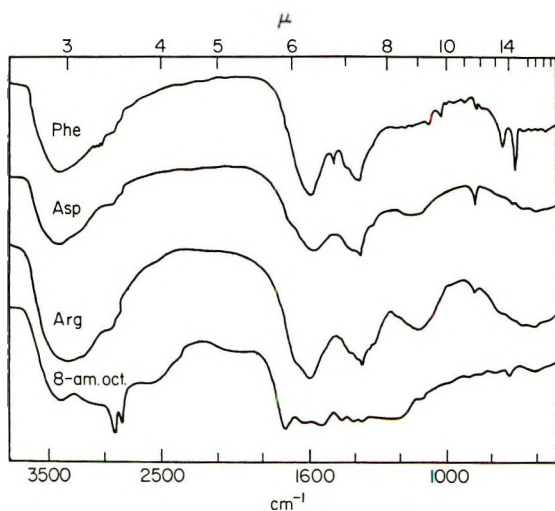
Figure 2. (bis)–I.R. spectra in K Br pellets, of melanoidins prepared by reacting DHA at 100°C with phenylalanine, aspartic acid, arginine and 8-aminooctanoic acid, and purified by dialysis.

namely strong absorption bands near 3400, 1600 and 1400 cm⁻¹. In addition to these the non-alpha aminoacid corresponding pigments show a band in the carboxyl group region around 1700 cm⁻¹, and bands characteristic of their hydrocarbon chains at 2930 and 2860 cm⁻¹. These last two bands are also found for melanoidins of alpha aminoacids with long hydrocarbon side chains like lysine, leucine and valine, and another band around 2970 is also noticeable for the last two as well as alanine which all bear methyl groups. Phenylalanine melanoidin also shows evidence that it still bears aromatic side chains, with well defined bands at 750 and 700 cm⁻¹. And the pigment prepared with glycine is the only one to have an I.R. absorption band at 1300 cm⁻¹, which can probably be attributed to its methylene group. Melanoidins prepared in alkaline medium gave spectra similar to those of the products obtained without addition of alkali.

Table III. I.R. absorption bands observed for melanoidins obtained by reaction of DHA with aminoacids for 4 h at 100°C

| Aminoacid	I.R. bands (cm⁻¹)
Gly	3400, 1600, 1380, 1300, 1120, 920, 600
Ala	3400, 2980, 2930, 1600, 1450, 1400, 600
Val	3400, 2970, 2930, 2870, 1610, 1380, 1220, 600
Leu	3420, 2960, 2930, 2870, 1620, 1400, 1170, 600
Ser	3400, 1600, 1380, 600
Asp	3400, 1570, 1410, 1160, 600
Glu	3400, 1570, 1410, 1160, 600
Arg	3350, 1600, 1400, 1130, 660, 600
Lys	3400, 2930, 2860, 1600, 1400, 540
Phe	3400, 1600, 1490, 1400, 750, 700
Tyr	3400, 1600, 1510, 1410, 1240, 1170, 600
3-aminopropionic	3400, 1700, 1560, 1400, 600
4-aminobutyric	3400, 2930, 1700, 1560, 1410, 600
5-aminopentanoic	3400, 2930, 2860, 1550, 1410, 600
6-aminohexanoic	3400, 2930, 2860, 1710, 1550, 1400
8-aminooctanoic	3400, 2930, 2860, 1710, 1630, 1550, 725

Electron spin resonance (E.S.R.) spectra

Although the DHA melanoidins were prepared and isolated at temperatures which could in no way exceed 100°C, they show a striking resemblance by their dark brown colour with polyacrylonitrile pyrolysates which we had the opportunity to study by E.S.R. spectroscopy some years ago. This led us to a first experiment in order to determine if the reaction product of DHA with glycine gave a signal and, after it did exhibit a high concentration of free spins, to a full study of all the melanoidins (*Table IV*). The equipment used was a Thomson T.H.N. 252 E.S.R. spectrometer.

All the spectra obtained are single lines with an almost Lorentzian line shape (*Fig. 3*). Their linewidths in gauss have been measured as the distance between the maximum and the minimum of the signals recorded as the first derivatives of the absorption curves. The numbers of free spins/g have been calculated for different melanoidins by comparison with a known standard (Varian Strong Pitch) assuming that they are proportional to the peak to peak height of the signals multiplied by their linewidths squared, with the same proportionality constant for curves having the same shape (an assumption which is reasonable in the present case). For alpha aminoacids, the linewidths are all within the

Table IV. E.S.R. characteristics of melanoidins obtained by reaction of DHA with aminoacids for 4 h at 100°C

Aminoacid	Linewidth G	Spins/g ($\times 10^{-18}$)
Gly	7·5	3·3
Gly+trieth.	7	4·5
Gly+Na OH	6.5	2·2
Ala	7	3·2
Val	7	3·2
Leu	7	5·9
Ser	7	3·5
Asp	6	2·1
Glu	6·5	1·5
Glu+Na OH	6	1·8
Arg	6·5	0·4
Lys	9	3·5
Phe+Na OH	7	5·6
Tyr+Na OH	6	2·7
3-aminopropionic	8·5	6·0
4-aminobutyric	11	10·0
5-aminopentanoic	7	4·0
6-aminohexanoic	11·5	15·0
8-aminooctanoic	9	2·6
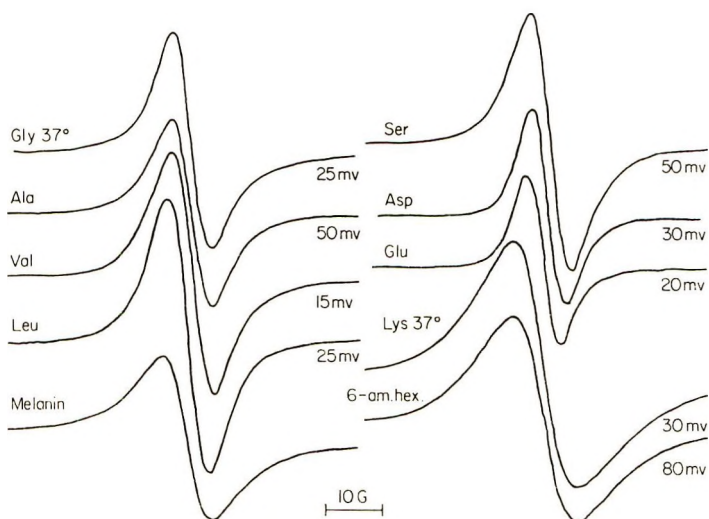
Figure 3. E.S.R. spectra of synthetic melanin and of melanoidins prepared by reacting DHA with glycine, alanine, valine, leucine, serine, aspartic acid, glutamic acid, lysine and 6-aminohexanoic acid at 100°C (37°C for glycine and lysine). All the samples have been purified by dialysis and have been examined as powders.

6–9 G range. Recording the spectrum of DHA and glycine melanoidin sample under vacuum, did not change the signal significantly and it is assumed therefore that these data, which were obtained in air, are representative of the actual linewidths without any line broadening by oxygen. As far as free spin contents are concerned, they fall in the 10^{18}

spins/g range except for arginine which gave a lower value. Two of the melanoidins obtained with non-alpha aminoacids have a slightly higher linewidth and a stronger signal.

II. REACTIONS CARRIED OUT AT 37°C

In order to better investigate the possible reactions occurring actually in the Stratum Corneum, a few experiments have also been made at 37°C with the same starting concentrations of DHA and aminoacids in water: 0·1 mole of each reactant (0·05 for Lys) in 25 ml. Only glycine and lysine did produce significant amounts of pigments after 24 h (*Table V*). Arginine reacted very poorly, as could be expected in view of the low yield obtained at 100°C. But what is more surprising is that serine and alanine which react readily with DHA at higher temperature, are almost inert at 37°C. It should be noted, however, that these two aminoacids were not entirely soluble at this temperature in the rather concentrated media used.

Table V. Melanoidins formed by reaction of DHA with aminoacids at 37°C

Aminoacid	Duration (h)	% Yield	U.V. absorption of 0·01% water sol.	Spins/g ($\times 10^{-18}$)
Gly	10	1·2	1·15 at 320 nm	2·5
Gly	24	1·7	1·40 at 310 nm	1·8
Gly	48	4·8	1·50 at 320 nm	3·1
Gly	72	4·7	1·46 at 310 nm	3·2
Lys	24	26·2	0·9 at 330 nm	7·2
Ala	24	0		
Ser	24	0		
Arg	24	0		

Underlined wavelengths correspond to a maximum.

The yield in melanoidin obtained for lysine as well as its free spin content, show by comparison with the results obtained at 100°C, that the reaction is already in an advanced stage after 24 h at 37°C. For glycine however, 48 h are necessary to obtain the same number of free spins/g, and after 72 h the pigment yield is still much lower than at higher temperature.

But in spite of these quantitative differences, the DHA melanoidins prepared at 37° or 100°C are very similar as far as their U.V., I.R. and E.S.R. spectra are concerned.

Discussion

Comparison with melanins

One of the most important characteristics of melanins, the natural pigments of hair, skin, squid ink . . . is to give an E.S.R. signal of 4.8 (6) to 8·8 G (7) linewidth depending upon the origin of the sample and perhaps also upon the conditions used in recording the spectra. It corresponds to free spin contents ranging from 5×10^{18}/g (6, 8) to 20×10^{18}/g (9) for Sepia melanin, and from $0·4 \times 10^{18}$/g (8) to 2×10^{18}/g (9) for synthetic melanin prepared by oxidation of DOPA. These data are strikingly similar to those of *Table IV*. Moreover, U.V. visible absorption spectra of melanins which have no special features

(6) or just a maximum at 327 nm (8), also resemble those of melanoidins. And as far as I.R. is concerned the same bands are found again for both types of pigments since melanins show, as melanoidins do (*Table III*), a maximum around 3400, and a maximum around 1620 with a shoulder near 1700 cm^{-1} (6, 7, 10, 11). Their percentage compositions are a little different, however, since a typical hair melanin contains 57·1% C, 3·5% H, 9·6% N and 29·4% O (11) while we found for the (DHA + Gly) melanoidin: 46·7% C, 5·5% H and 8·4% N, the remaining unaccounted for 39·4% being probably oxygen. All this shows that, although the pigments formed in the reaction of DHA with aminoacids are different by their preparation, they are nevertheless very similar to melanins by their properties and especially by their free spin content.

Hypotheses on the structure of melanoidins

Still another very interesting result is that E.S.R. spectra of melanoidins can be observed in their aqueous reaction media at room temperature (*Fig. 4*) showing that the free spin centres, if they were real free radicals, ought to be extremely inert. And it is particularly striking to remark that an unpurified solution of (DHA + Gly) pigment prepared at 37°C and never heated above this temperature, also exhibits a signal. The E.S.R. absorption lines observed in water have the same linewidths (7·5–9·5 G) and apparently the same intensity as the dry powders, although this last point is difficult to certify since a special flat cell of unknown volume had to be used in these experiments due to the high dielectric loss of aqueous solutions. These last results particularly, show that the E.S.R. behaviour of melanoidins is an intrinsic property of their macromolecule and is not due to some kind of free radicals trapped during polymerization.
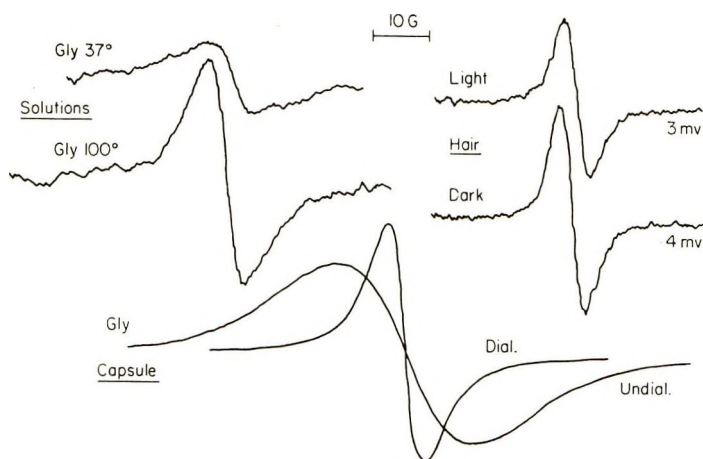
Figure 4. E.S.R. spectra of: solutions of melanoidins obtained by reacting DHA with glycine for 24 h at 37°C and 6 h at 100°C; natural dispersions of melanin pigments in light and dark hair; malanoidin obtained by action of DHA on glycine in an open capsule over a steam bath until the reaction medium was dry, and observed in the powder state before and after purification by dialysis.

In the following experiment, however, such species may have been formed. DHA (1·8 g) and Gly (1·5 g) were heated with 6 g water in a capsule on a steambath until the brown resultant mass was dry (1 h 30 min). A sample of this unpurified product was kept and

the rest of it was dialysed as usual, with a total yield of 35%. The E.S.R. spectrum of the purified pigment was similar to that of a melanoidin prepared by refluxing (linewidth $H=7$ G, spin content $=1 \cdot 8 \times 10^{18}$/g. But the signal given by the reaction product before dialysis was quite different (*Fig. 4*), with a linewidth of 25 G and an intensity corresponding to 21×10^{18} spins/g. These striking data can be explained if one assumes that some free radicals were trapped when the reaction medium became viscous by evaporation and finally dried to a vitrous state. These species would then have been lost by reaction with water upon dissolution prior to purification, thus explaining the high loss in free spins. The linewidth of 25 G can also be explained by real free radicals since the spectra of such localized unpaired electrons are likely to exhibit a hyperfine structure (by interaction with the protons of the molecule) which, although unresolved, would widen the signal significantly.

But if, in the general case, purified melanoidins do not contain real free radicals, how can one account for their E.S.R. properties? In recent years, a number of polymers with conjugated double bonds have been found to contain from 10^{16} to 10^{20} free spins/g (12–15). Their E.S.R. signal is generally a single line of 5–10 G width (12) although a hyperfine structure has been reported in some cases (14, 16) and it can be observed also in solution (12, 16) showing that it must be due to an intrinsic property of the molecule. This phenomenon has tentatively been explained by several kinds of theoretical considerations (12). It could be due to a semi-conductor effect such as the one proposed to account for the paramagnetism of melanins (17, 18). Or it could be, that conjugated polymers in their fundamental electronic state possess, at least partly, the character of triplet states (12). Or finally, the free spins observed may be bond alternation defects much like the ones predicted by calculation (19). According to this theory, the alternation between double (short) and single (long) bonds along a conjugated polymer chain, is disrupted by carbon atoms bearing a non-bonding molecular orbital occupied by a single spin. Such unpaired electrons can travel rapidly through long series of conjugated double bonds and this delocalization is responsible for the lack of hyperfine structure in the resulting E.S.R. spectra.

Two of these defects may be formed through the rupture of one double bond by thermal excitation or by lattice deformations (13), or even by photochemical excitation, a process which could explain the photoenhancement of melanin E.S.R. signals observed by Stratton and Pathak (20). In fact the paramagnetic behaviour of melanins should also be considered as an intrinsic property of their highly conjugated double bonds system, and not as a proof of the existence of so-called free radicals in their molecules.

In view of these theories, the E.S.R. properties of DHA melanoidins, together with their I.R. absorption around 1600 cm^{-1}, and their dark brown colour due to their non-specific absorption in the visible, point out to a highly conjugated double bond structure. The monomers of these macromolecules are still unknown, however, although they have to be derived from the Schiff base formed by condensation of DHA with aminoacids, probably after dehydration and eventually decarboxylation. In the case of the MAILLARD reaction of sugars, it has indeed been shown that the original condensation products lose water very easily to produce furfural derivatives (21–22). Only the alpha aminoacids are decarboxylated in the process and the non-alpha aminoacid corresponding pigments exhibit the characteristic absorption band around 1710 cm^{-1}. But in any case, I.R. spectra show that the rest of the parent aminoacid molecule is always retained in DHA melanoidin polymers.

Conclusion

The pigments formed by reaction of DHA with aminoacids are very similar to melanins, and like these they can be regarded as highly conjugated polymers bound to exhibit intrinsic E.S.R. properties due to bond alternation defects. They still bear the side chains of the parent aminoacids although decarboxylations occur to some extent with alpha derivatives.

The action of DHA on skin must start by condensation with the free aminoacids located on the surface and more probably within the horny cells (23), followed by polymerization and eventually linking to Stratum Corneum proteins through the participation of lysine side chains. It seems indeed very unlikely that this polymerization process could occur exclusively with basic aminoacids bound in polypeptide chains and therefore unable to come close enough to one another. But the fixation of an already grown pigment on protein macromolecules is possible and has similarly been found to occur when wool keratin is immersed in the reaction medium of glycine with glucose (24).

Finally, it should be noted that since DHA melanoidins possess the same free spin character as melanins, they too should be able to protect the skin from radiations through trapping of electrons and free radicals which are thought to be important intermediates in sunburn and ageing processes (25). Preliminary investigations of an eventual protective effect of DHA have been negative (26–27) but they should perhaps be resumed in view of these new results and those obtained by Fusaro *et al.* (28–32) with combinations of DHA and juglone or lawsone.

References

1   Von Noorden, C. and Isaac, S. *Die Zuckerkrankheit und ihre Behandlung* 8 Ausg. Julius Springer, Berlin, 415 (1927).
2   Butcher, J., Bandelin, F. and Kanas, N. The reaction of dihydroxyacetone with proteins. *Amer. Perf.* **75** (12) 46 (1960).
3   Wittgenstein, E. and Berry, H. K. Reaction of dihydroxyacetone (DHA) with human skin callus and amino compounds. *J. Invest. Dermatol.* **36** 283 (1961).
4   Laden, K. and Zielinski, R. The reaction of α-hydroxymethylketones with skin and aminoacids. *J. Soc. Cosmet, Chem.* **16** 777 (1965).
5   Ellis, G. P. The Maillard reaction. *Adv. Carbohydrate Chem.* **14** 63 (1969).
6   Blois, M. S., Zahlan, A. B. and Maling, J. E. Electron spin resonance studies on melanin. *Biophys. J.* **4** 471 (1964)
7   Blois, M. S. On the spectroscopic properties of some natural melanins. *J. Invest Dermatol.* **47** 162 (1966).
8   Mason, H. S., Ingram, D. J. E. and Allen B. The free radical property of melanins. *Arch. Biochem. Biophys.* **86** 225 (1960).
9   Tollin, G. and Steelink, C. Biological polymers related to catechol : electron paramagnetic resonance and infrared studies of melanin, tannin, lignin, humic acid and hydroquinones. *Biochem. Biophys. Acta.* **112** 337 (1966).
10   Green, D. B. and Happey, F. The infrared specta of melanins (natural wool pigments). IIIe *Congrès Int. de la Recherche Textile Lainière de France, Paris.* **I** 283 (1965).
11   Charle, R. Pigmentation naturelle et artificielle des cheveux. In: *Problèmes Capillaires.* Ed. by E. Sidi and C. Zviak), p. 79. Imprimerie Dermont, Paris (1966).
12   Nechtchein, M. Sur la nature des centres paramagnétiques détectés par RPE dans les polymères conjugués. *J. Pol. Sci.:* Part C, **4** 1367 (1963).
13   Holob, G. M., Ehrlich, P. and Allendoerfer, R. D. Electron spin resonance in crystallizable, high molecular weight polyphenylacetylene. *Macromolecules,* **5** 569 (1972).
14   Byrd, N. R., Kleist, F. K. and Stamires, D. N. Electric and magnetic properties of polymeric organic semiconductors. *J. Pol. Sci.:* Part A-2, **10** 957 (1972).

15 Huron, J. L. Etude cinétique des réactions de dégradation du polyacrylonitrile entre 200 et 300°C par résonance paramagnétique électronique, chromatographie en phase gazeuse et analyses thermiques. *D.Sc. Thesis*, Centre Universitaire du Ht-Rhin, Mulhouse, et Université de Strasbourg, France (1975).

16 Ehrlich, P., Mertzlufft, E. C. and Allendoerfer, R. D. Hyperfine structure in ESR spectra of polyphenylacetylene solutions. *Pol. Letters Ed.* **12** 125 (1974).

17 Longuet-Higgins, H. C. On the origin of the free radical property of melanins. *Arch. Biochem. Biophys.* **86** 231 (1960).

18 Pullman, A. and Pullman, B. The band structure of melanins. *Biochim. Biophys. Acta,* **54** 384 (1961).

19 Pople, J. A. and Walmsley, S. H. Bond alternation defects in long polyene molecules. *Molecular Phys.* **5** 15 (1962).

20 Stratton, K. and Pathak, M. A. Photoenhancement of the electron spin resonance signal from melanins. *Arch. Biochem. Biophys.* **123** 477 (1968).

21 Gottschalk, A. and Partridge, S. M. Interaction between simple sugars and aminoacids. *Nature,* **165** 684 (1950).

22 Dubourg, J. and Devillers, P. La réaction de Maillard, ses méfaits en sucrerie. *Industr. alim. agr.* **79** 625 (1962).

23 Tabachnick, J. and Labadie, J. H. Studies on the Biochemistry of Epidermis. IV. The free aminoacids, ammonia, urea, and pyrrolidone carboxylic acid content of conventional and germ-free albino guinea pig epidermis. *J. Invest. Dermatol.* **54** 24 (1970).

24 Burton, H. S., McWeeny, D. J., Pandhi, P. N. and Biltcliffe, D. O. Fluorescent compounds and non-enzymatic browning. *Nature,* **196** 948 (1962).

25 Meybeck, A. Electron spin resonance (ESR) in the study of proteins ageing. 8th *Int. Fed. Soc. Cosm. Chem. Congress,* paper D 8, London, 1974.

26 Goldman, L., Barkoff, J., Blaney, D., Nakai, T. and Suskind, R. Investigative studies with the skin colouring agents dihydroxyacetone and glyoxal. *J. Invest. Dermatol.* **35** 161 (1960).

27 Maibach, H. and Kligman, A. M. Dihydroxyacetone: a suntan-simulating agent. *Arch. Dermatol.* **82** 505 (1960).

28 Fusaro, R. M., Runge, W. J., Lynch, E. W. and Watson, C. J. Sunlight protection in normal skin by absorptive filter chemically induced in stratum corneum. *Archs Derm.* **93** 106 (1966).

29 Fusaro, R. M. and Runge, W. J. Sunlight protection in patients with chlorpromazine light sensitivity. *Int. J. Dermatol.* **10** 198 (1971).

30 Fusaro, R. M., Runge, W. J. and Johnson, J. A. Protection against light sensitivity with dihydroxyacetone/naphtoquinone *Int. J. Dermatol.* **11** 67 (1972).

31 Johnson, J. A., Czaplewski, R. P. and Fusaro, R. M. Protection against long ultraviolet light with dihydroxyacetone/naphtoquinone. *Dermatologica,* **147** 104 (1973).

32 Fusaro, R. M. and Johnson, J. A. Photoprotection of patients sensitive to short and/or long ultraviolet light with dihydroxyacetone/naphtoquinone. *Dermatologica,* **148** 224 (1974).

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