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Journal of the Society of Cosmetic Chemists

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Changes in sunburn and mechanisms of protection: B. E. JOHNSON. *Journal of the Society of Cosmetic Chemists* **29** 3–16 (1978)

Synopsis—A review and critical discussion is presented of the reactions of skin to sunlight and artificial sources of ultraviolet radiation. Particular attention is paid to the mechanisms involved in sunburn but mention is also made of premature ageing and skin cancer. Ultraviolet erythema is the most extensively studied reaction and it is possible that the primary molecular target for this and the other reactions is either DNA or lysosome membrane lipid. However, no definite conclusions may be drawn. Nor is it established at which level in the skin, the epidermis, where the most prominent histologic change, the appearance of sunburn cells, is found, or the dermis in which vasodilatation occurs, this primary target may be. Physiological protection against ultraviolet radiation is afforded by melanin pigment, the proteins of the horny layer and urocanic acid, but the mechanisms are poorly understood as is the possible involvement of naturally occurring anti-oxidants.

The activity and safety of the antimicrobial agent Bronopol (2-bromo-2-nitropropan-1,3-diol): D. M. BRYCE, B. CROSHAW, J. E. HALL, V. R. HOLLAND and B. LESSEL. *Journal of the Society of Cosmetic Chemists* **29** 17–38 (1978)

Synopsis—Recent work on the microbiological activity, chemistry and safety of the antimicrobial agent Bronopol is reported. Methods for the estimation of Bronopol are described, and the nature of its decomposition products is discussed. Animal metabolism and toxicology results are reported, together with animal and human studies on irritancy and sensitisation. The performance of Bronopol in a number of experimental formulations is described.

Towards objectivity in the assessment of eye irritation: R. HEYWOOD and R. W. JAMES. *Journal of the Society of Cosmetic Chemists* **29** 39–43 (1978)

Synopsis—The assessment of eye irritation is subjective. The eye test system is subject to such wide variation that it will never be possible to make precise measurement of irritancy. Clinical appraisal, supported by measurement of corneal thickness and intra-ocular pressure, is probably the best that can be achieved. The use of local anaesthetics should be considered when carrying out eye irritation tests in the rabbit.

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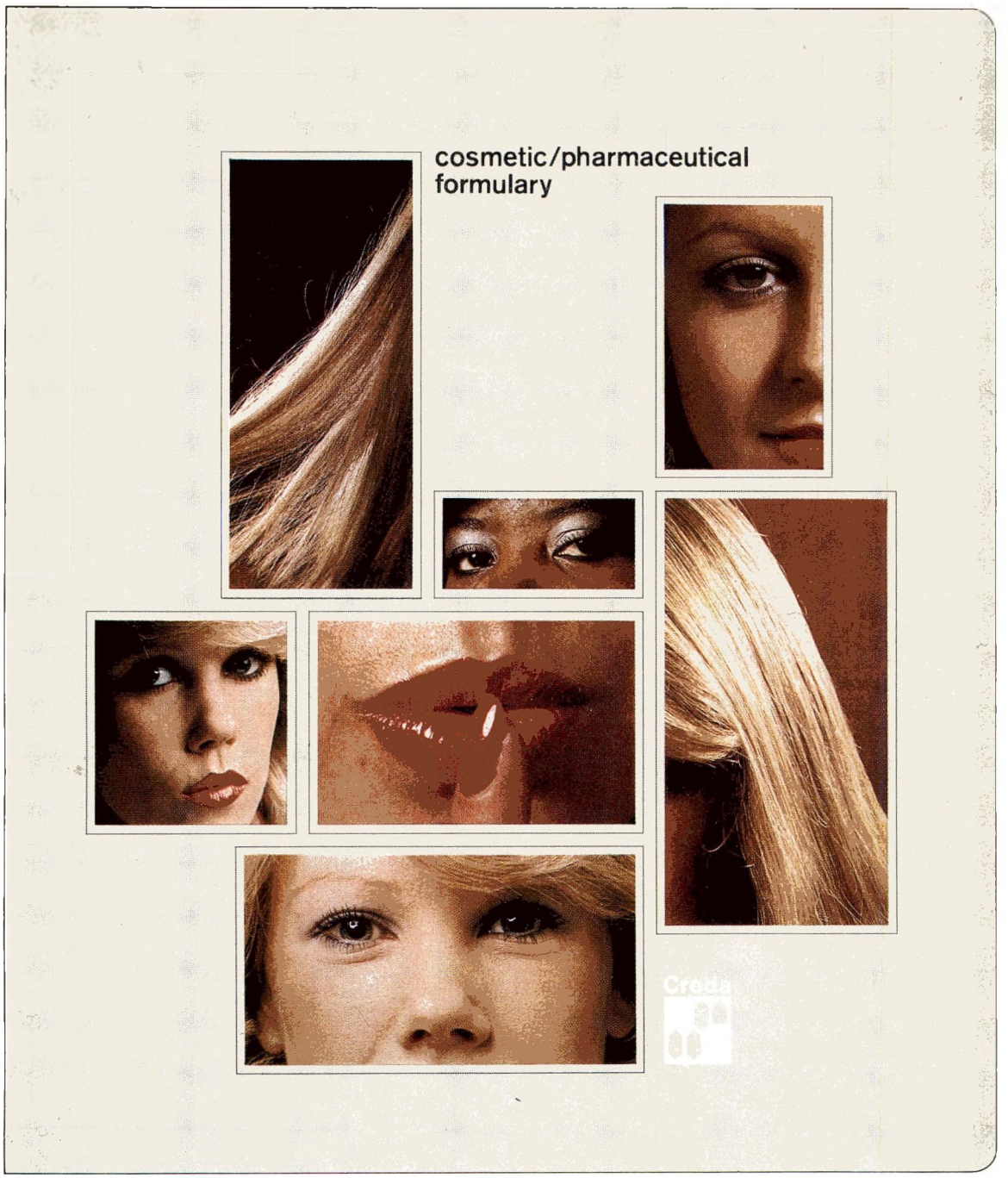

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The activity and safety of the antimicrobial agent Bronopol (2-bromo-2-nitropropan-1, 3-diol)

D. M. BRYCE, B. CROSHAW, J. E. HALL, V. R. HOLLAND *and* B. LESSEL *The Boots Company Limited, Nottingham NG2 3AA*

Synopsis

Recent work on the **microbiological activity, chemistry** and **safety** of the **antimicrobial** agent **Bronopol** is reported. Methods for the **estimation** of Bronopol are described, and the nature of its **decomposition** products is discussed. Animal **metabolism** and **toxicology** results are reported, together with animal and human studies on **irritancy** and **sensitisation.** The **performance** of Bronopol in a number of experimental formulations is described.

INTRODUCTION

Early work by Hodge, Dawkins and Kropp (1) and by Zsolnai (2) suggested that geminal bromonitroalkanes had antifungal activity. The broad-spectrum antibacterial properties of 2-bromo-2-nitropropan-1,3-diol (Bronopol) have been described in a preliminary communication by Croshaw, Groves and Lessel (3) and in comparison with other members of a series of antimicrobial aliphatic halogeno-nitro compounds by Clark *et al.* (4).

Bronopol is used as a preservative in various cosmetic, toiletry and household preparations particularly because of its high activity against Gram-negative bacteria, especially *Pseudomonas aeruginosa* and other pseudomonads. These organisms are common residents in water and as such can cause contamination and spoilage problems in cosmetics and toiletries (5, 6, 7, 8). Pseudomonads are frequently implicated, particularly in oil-in-water emulsions which contain a significant amount of nonionic surfactants (9, 7, 10).

Bronopol is an effective antibacterial preservative over a wide pH range. It is stable at acid pH's and is also useful as a labile antibacterial preservative in alkaline media. Because of its broad-spectrum antibacterial activity Bronopol can also be used as an active agent, for example, in aerosol formulations. Bronopol has been reported to show persistent activity on the skin by Marples and Kligman (11), this contrasts with the fact that *in vitro* it has been shown to have a weak growth-inhibitory effect on cultured human skin cells by Onoda and Saito (12).

A programme of experimental work was begun some time ago to extend our knowledge on the safety of Bronopol. This work is now completed and it is appropriate to review the results of these and other hitherto unpublished studies on the microbiology, chemistry, analysis and formulation of this compound.

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Table I. Antibacterial spectrum of Bronopol by agar-dilution in comparison with other preservative agents. Agar plates inoculated with 0·01 ml of 18 h cultures diluted 1/100 (except *Staph. aureus*); incubated for 24 h at 37°C

		Minimal inhibitory concentration (μg/ml)											
Test organism	No. of species or strains	Bronopol*	6-Acetoxy-2,4-dimethyl-*m*-dioxane [1]*	cis isomer of 1-(3-chloroallyl)-3,5,7-triaza-1-azonia-adamantane chloride [2]*	Methyl Hydroxybenzoate BP†	Propyl Hydroxybenzoate BP†	Substituted imidazolidinyl urea compound [3]*	Phenylmercuric Nitrate BP†	Benzalkonium Chloride*	8-Hydroxyquinoline sulphate†	Chlorocresol BP†	Phenoxyethanol BPC*	Formaldehyde*
Gram-positive bacteria													
Staphylococcus aureus	1	12·5	1600	400	800	800	200	1·56	3·1	3·1	200	> 800	100
Gram-negative bacteria													
Pseudomonas aeruginosa	2	12·5	1600	400	1600–> 1600	> 800	400–800	12·5–25	800	400	400	800–> 800	100
Proteus spp.	4	12·5	1600	100–400	800–1600	200	200–400	0·78–3·1	25–800	25–100	200	> 800	50
Escherichia coli	3	6·25–12·5	1600	100–400	400–800	800	200–800	3·1	25–50	50–100	100–400	> 800	50–100
Salmonella spp.	2	12·5	1600	50–200	800	800	200	3·1	200	50–100	200	> 800	50
Shigella spp.	2	12·5	1600	100–400	800	800	400	0·78	6·25–12·5	6·25–100	200	800–> 800	50

* Dilutions prepared in water.
† Dilutions prepared in 3: 1 acetone : water mixture.

[1] *Giv-Gard* DXN.
[2] *Dowicil* 200.
[3] *Germall* 115.

Table II. Antibacterial spectrum of Bronopol by agar-dilution in comparison with other antibacterial active agents. Agar plates inoculated with 0·01 ml of 18 h cultures diluted 1/100 (except *Staph. aureus*); incubated for 24 h at 37°C

		Minimal inhibitory concentration (μg/ml)														
Test organism	No. of species or strains	Bronopol*	Chlorhexidine Acetate BPC*	2,4,4'-Trichloro-2'-hydroxydiphenyl ether [1]†	3,4',5-Tribromo salicylanilide [2]†	N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide [3]†	Tetrabromo-*o*-cresol [4]†	Zinc salt of 2-mercapto-pyridine-1-oxide [5]†	Domiphen Bromide BP*	Cetrimide BP*	Hexachlorophane BP†	Bromchlorophene†	Dequalinium Chloride BP*	3,4,4'-Trichlorocarb-anilide	*o*-Phenylphenol†	Chloroxylenol BPC†
Gram-positive bacteria																
Staphylococcus aureus	1	25	1·56	0·19	1·56	200	1·56	1·56	3·1	6·25	0·78	3·1	1·56	0·4	200	100
Gram-negative bacteria																
Pseudomonas aeruginosa	2	6·25–12·5	25–50	> 800	100–> 800	200–> 1600	> 800	200–800	800	> 800	12·5–50	> 800	200	> 200	800	400–800
Proteus spp.	4	12·5	3·1–100	0·39	25–50	50–400	> 800	12·5–50	50–800	25–800	3·1–25	100–> 800	200	> 200	100–200	50–200
Escherichia coli	3	6·25–12·5	3·1–12·5	0·39	50–100	50–200	> 800	12·5–25	50–100	25–200	12·5–100	> 800	200	> 200	200–800	10–200
Salmonella spp.	2	12·5	3·1–12·5	0·19–0·39	50–100	100–200	> 800	12·5	200–800	200–800	25	> 800	200	> 200	200	200
Shigella spp.	2	6·25–12·5	3·1–6·5	0·39	50–100	200–400	800–> 800	6·25	6·25	25	6·25–100	> 800	200	> 200	100	100

* Dilutions prepared in water.
† Dilutions prepared in 3:1 acetone : water mixture.

[1] *Irgasan* DP 300.
[2] *Temasept* IV (TBS).
[3] *Vancide* 89 RE (highly purified grade of captan).
[4] Deodorant K.
[5] Zinc pyrithione.

RESULTS AND DISCUSSION

MICROBIOLOGY

Antibacterial Activity

The bacteriostatic activity of Bronopol in comparison with that of a range of other agents has been determined by serial dilution in 'Oxoid' nutrient agar (see *Tables I* and *II*). Plates were inoculated with a multi-point inoculator (13). The bacteriostatic activity against some pseudomonads, including *Ps. aeruginosa, Ps. fluorescens* and pseudomonads isolated from paints, water, cosmetics and unpreserved pharmaceutical formulations has been compared using a similar technique. Of the antibacterial agents compared only Phenylmercuric Nitrate BP and Chlorhexidine Acetate BPC had similar broad-spectrum activity to that of Bronopol (*Tables I* and *II*). 2,4,4′-Trichloro-2′-hydroxydiphenyl ether was more active than Bronopol against most of the organisms tested, but this compound was much less active against *Ps. aeruginosa*. Of the agents tested against pseudomonads only Phenylmercuric Nitrate BP was more active than Bronopol (see *Table III*).

Table III. Comparative activity of Bronopol and other agents against *Pseudomonas* spp. by agar-dilution. Agar plates inoculated with 0·01 ml of 18 h cultures undiluted; incubated for 48 h at 32°C

Preservative	No. of strains tested	No. of strains with m.i.c. (μg/ml) of:						
		12·5	25	50	100	200	400	>400
Bronopol	23	5	18	0	0	0	0	0
Propyl Hydroxybenzoate BP	23	0	0	0	0	0	0	23
Methyl Hydroxybenzoate BP	23	0	0	0	0	0	0	23
Phenoxyethanol BPC	23	0	0	0	0	0	0	23
Phenylmercuric Nitrate BP	23	19	2	2	0	0	0	0
Phenylethyl Alcohol BPC 1963	23	0	0	0	0	0	0	23
Benzalkonium Chloride	23	0	0	0	0	0	19	4
Chlorocresol BP	23	0	0	0	0	0	23	0
Chlorbutol BP	23	0	0	0	0	0	0	23
Chlorhexidine Gluconate	23	0	0	3	20	0	0	0
Chlorhexidine Acetate BPC	23	0	4	18	1	0	0	0
6-Acetoxy-2,4-dimethyl-*m*-dioxane [1]	12	0	0	0	0	0	0	12
cis-isomer of 1-(3-chloroallyl)-3,5,7-triaza-1-azonia-adamantane chloride [2]	12	0	0	0	0	5	3	4
Substituted imidazolidinyl urea cpd. [3]	6	0	0	0	0	2	1	3
N-Trichloromethylthio-4-cyclohexene-1,2-dicarboximide [4]	12	0	0	0	0	3	2	7
Zinc salt of 2-mercaptopyridine-1-oxide [5]	12	4	1	2	0	2	2	1
2,4,4′-Trichloro-2′-hydroxy-diphenyl ether [6]	6	0	0	0	0	0	0	6

[1] *Giv-Gard* DXN.
[2] *Dowicil* 200.
[3] *Germall* 115.
[4] *Vancide* 89 RE.
[5] Zinc pyrithione.
[6] *Irgasan* DP 300.

The effects of organic matter and some possible antagonists are shown in *Table IV*. Bryce and Smart (14) reported that nonionic surface active agents, e.g. polysorbate 80 and lecithin, have little or no effect on the antibacterial activity of Bronopol, although

such agents are known to antagonise the action of many preservatives and Brown (15) confirmed that a plot of activity *versus* the log phase concentrations of *Ps. aeruginosa* for solutions of Bronopol containing 1% polysorbate 80 showed that activity did not decrease. Sulphydryl compounds are markedly antagonistic to the *in vitro* activity of Bronopol (3). This has been confirmed by Stretton and Manson (16).

Table IV. The effect of organic matter and possible antagonists on the bacteriostatic activity of Bronopol by Agar dilution (strains of *Pseudomonas aeruginosa* test organism)

Additive	Decrease (-fold) in bacteriostatic activity*
10% ox serum	0–2
50% ox serum	4–8
10% human serum	2
50% human serum	4
10% oxalated horse blood	4–8
50% oxalated horse blood	32–64
10% milk	0
1% polysorbate 80	0
0·1% lecithin	0
0·1% cysteine hydrochloride	16–64
0·1% sodium thioglycollate	8–16
0·1% sodium thiosulphate	4–16
0·01% sodium metabisulphite	8–16

* 2-fold serial dilution.

Using the filter paper strip technique (17) with *Staphylococcus aureus* as the test organism, it has been shown that there was no inhibition of the activity of Bronopol by Cetrimide BP, Domiphen Bromide BP, Benzalkonium Chloride BPC or trichlorocarbanilide.

Further work has confirmed the report by Croshaw *et al.* (3) that there is no evidence of the development of Bronopol-resistant organisms after passage in the presence of Bronopol for 20 subcultures. In practice Bronopol-resistant organisms have not occurred.

Some insight into the mode of action of Bronopol has been obtained. Since Bronopol is more active against metabolising cells than resting cells and its antibacterial activity is reversed by thiol-containing compounds (3), thiol-containing enzymes would appear to be implicated. Bronopol forms disulphide bonds from thiol groups and these may account for the observed inhibition of dehydrogenase activity by the compound at concentrations approximating the minimum inhibitory value for each organism. Inhibition of microbial membrane-bound dehydrogenase enzymes may cause alterations in membrane structure and account for the cell leakage observed on Bronopol treatment (16). Thus thiol-containing enzymes are involved in the mode of action of Bronopol against bacteria. The selectivity of the compound for micro-organisms, indicated by its very low mammalian toxicity, may be due in part to the rapid metabolism of Bronopol by the body tissues.

CHEMICAL AND ANALYTICAL

Stability of Pure Bronopol

Experiments were conducted to establish the stability of Bronopol on storage in the pure

solid state. The results, shown in *Tables V* and *VI*, demonstrate no evidence of instability over a period of one year's storage at temperatures up to 45°, and at elevated humidity. No photodecomposition was observed at room temperature over this period. Samples of Bronopol stored in the dark at room temperature over periods up to 2 years also show no evidence of decomposition. All the assays were carried out by gas-liquid chromatography (g.l.c.) using the trimethylsilylation procedure described later in the section on analytical methods. Both the internal standard and normalisation methods were utilised. Initial assays were by g.l.c. using n-pentadecane as the internal standard after acetylation. Since that time, the trimethylsilylation procedure described later has been developed in order to achieve an improvement in precision. Assays after storage were done by this latter method, using the internal standard technique.

Table V. Stability of Bronopol during storage

	g.l.c. (internal standard method)	g.l.c. (normalisation method)	
Storage conditions	Bronopol %	Bronopol %	Impurity at R_T(rel) 0·62 %
Initial	100·0	99·6	0·45
4 weeks,			
at 20 to 25°C			
(a) at normal RH	99·5	99·6	0·43
(b) at 90% RH	100·3	99·6	0·41
at 37°C	99·9	99·6	0·39
at 45°C	99·7	99·6	0·39
in north window	100·4	99·6	0·43
8 weeks			
at 20 to 25°C			
(a) at normal RH	99·7	99·6	0·40
(b) at 90% RH	99·9	99·6	0·39
at 37°C	100·0	99·6	0·41
at 45°C	99·4	99·6	0·41
in north window	100·1	99·6	0·44
12 weeks			
at 20 to 25°C			
(a) at normal RH	99·8	99·5	0·49
(b) at 90% RH	100·1	99·6	0·41
at 37°C	100·2	99·5	0·46
at 45°C	99·6	99·6	0·44
in north window	99·8	99·6	0·44
52 weeks			
at 20 to 25°C			
(a) at normal RH	100·2	99·5	0·50
(b) at 90% RH	99·2	99·5	0·47
at 37°C	100·3	99·5	0·46
at 45°C	100·3	99·5	0·46
in north window	99·3	99·4	0·51

RH = relative humidity.
R_T(rel) = relative retention time.

Table VI. Stability of Bronopol after storage at room temperature in the dark

Initial assay Bronopol %	Time of storage	Assay after storage, Bronopol %
99·2	24 months	98·2
		99·2
101·1	22 months	101·5
		102·2
100·2	21 months	101·4
		102·4
99·9	18 months	99·7
		99·5

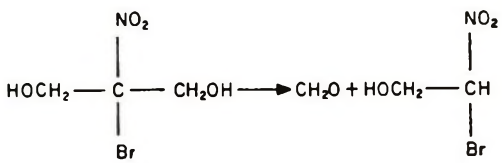
Stability in Aqueous Solution

Bryce and Smart (14) have shown that aqueous solutions of Bronopol are reasonably stable when acid. To investigate the stability of the compound in more detail, aqueous solutions of Bronopol (0·2% w/v) were prepared at pH 4 and 6 (McIlvaine buffer) and pH 8 (phosphate buffer). The solution at pH 4 was stored in the dark at 50°C, the solution at pH 8 at 30°C, and the solution at pH 6 at 30, 40 and 50°C. At appropriate time intervals aliquots were removed and examined microbiologically, polarographically, gas-chromatographically and for bromide ion, nitrate ion, nitrite ion and formaldehyde. Aqueous solutions of Bronopol (10% w/v) at about pH 6 were stored at temperatures ranging from 40 to 100°C, the pH being maintained by the addition of 5N sodium hydroxide. Aliquots were removed and examined by thin-layer chromatography (t.l.c.) and bioautography. Attempts were made to isolate the decomposition products from partially decomposed solutions by preparative layer and Sephadex column chromatography.

The decomposition of Bronopol was found to be accelerated by increasing the pH or the temperature of the solution. These effects are shown graphically in *Figs. 1* and *2*; using a factor of approximately 4 as the increase or decrease in the rate of decomposition per 10°C temperature change, the times for 50% decomposition extrapolated to 20°C, and based on the g.l.c. assay results, are as shown below:

pH	Time for 50% decomposition
4	>5 years
6	1½ years
8	2 months

The initial process in the decomposition of Bronopol appears to be a retroaldol reaction with the liberation of formaldehyde and the formation of bromonitroethanol:



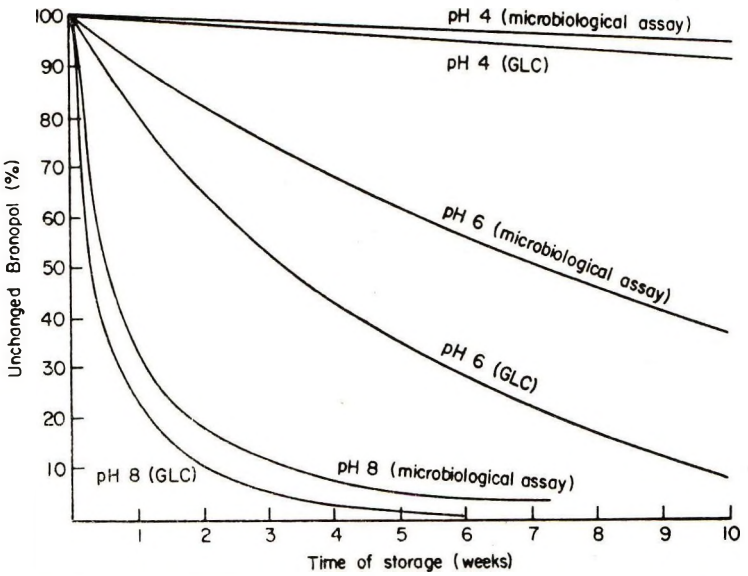


Figure 1. Effect of pH on stability of aqueous solutions of Bronopol at 40°C (initially 0·2% w/v)

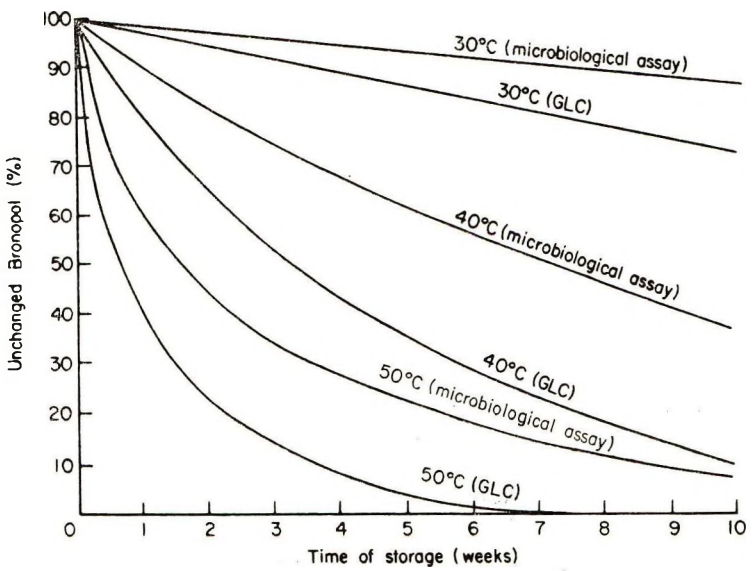
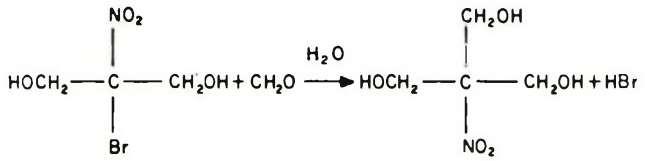


Figure 2. Effect of temperature on stability of aqueous solutions of Bronopol at pH 6 (initially 0·2% w/v)

Bromonitroethanol itself is considerably less stable than Bronopol and in the range of conditions investigated the maximal concentrations did not exceed 0·5% of the initial Bronopol concentrations.

Simultaneously a second-order reaction involving Bronopol and formaldehyde occurs to give 2-hydroxymethyl-2-nitro-1,3-propanediol:



2-hydroxymethyl-2-nitro-1,3-propanediol has been isolated from partially decomposed 10% w/v solutions of Bronopol by preparative layer and Sephadex column chromatography. The n.m.r. and i.r. spectra and the elemental analysis support the proposed structure. 2-Hydroxymethyl-2-nitro-1,3-propanediol itself decomposes with the loss of formaldehyde. This reaction is relatively slow, however, so that after 2–3 Bronopol half lives this compound accounts for 8–10% of the organic material, as shown by the thin layer chromatogram (*Fig. 3*).

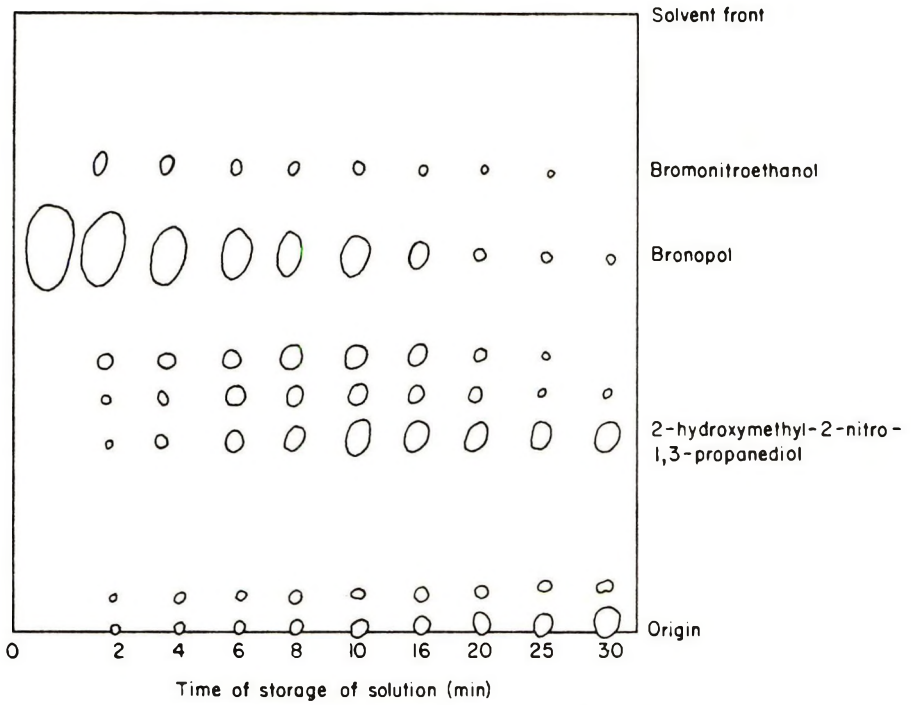


Figure 3. Thin-layer chromatogram of Bronopol aqueous solutions (initially 10% w/v) stored at 100° and maintained at pH 6

In more dilute solutions the second order reaction will be less important and the loss of bromine follows first order kinetics, the rate of loss being about one-half of the overall rate of decomposition of Bronopol.

A number of reactions involving formaldehyde occur simultaneously. The overall result is that the formaldehyde concentration tends to a maximum which is lower than an equimolar ratio. The rate of formation of formaldehyde relative to the rate of decomposition of Bronopol was not markedly affected by pH over the range investigated.

An additional mode of decomposition results in the formation of nitrite but not nitrate. The rate of formation of nitrite tends to follow second-order kinetics and is slower than the overall decomposition of Bronopol as measured by g.l.c. No information has been obtained on the route by which the nitro group is lost and the final organic products have not been identified. Their physical properties suggest, however, that some may be polymeric. It should be noted that in the presence of certain secondary and tertiary amines and amides, nitrite can form nitrosamines which may be carcinogenic. In the opinion of the authors, it is advisable that formulators using Bronopol, or any other substance giving rise to nitrite, take steps to ensure that if nitrosamines are produced, their presence does not represent a health hazard to the user.

The gaseous decomposition products of Bronopol have been examined by mass spectroscopy. Only three major peaks were found the first of which could be attributed to nitrogen plus a trace of ethylene at m/e 28 and the second to nitric oxide at m/e 30. The only constituent of the third peak, at m/e 44, which could be identified was the radical CH_2NO. Neither carbon monoxide nor carbon dioxide could be detected.

Thin-layer chromatograms of stored solutions of Bronopol were sprayed with starch/potassium iodide solution, which would locate components including those containing the aliphatic nitro group. At least seven components were detected (*Fig. 3*), three of which were identified. The origin contained sodium bromide and sodium nitrite. Bioautography, on the other hand, showed only two active zones corresponding to Bronopol and bromonitroethanol (*Fig. 4*).

It is difficult to explain the g.l.c. and microbiological results on the basis of the above observations since the bromonitroethanol and formaldehyde present are not sufficient to account for the difference between them.

Analytical Methods

The methods described in this section have been used to obtain the results recorded in the preceding sections, and to assay Bronopol in the types of formulations in which it is likely to be incorporated.

Pure Bronopol has been assayed by the determination of its bromine content, by the determination of its nitrogen content and by g.l.c. of the acetylated and of the trimethylsilylated material, the methods using g.l.c. being the most specific. In formulations, Bronopol has been estimated by t.l.c., by a polarographic procedure, by a microbiological procedure and by g.l.c. The procedure by t.l.c. has been applied to ointments (at a concentration of 0·1%), to barrier creams (at concentrations of 0·1 and 0·2%) and to aerosol concentrates (at a concentration of 0·05%). G.l.c. has been applied to aqueous formulations (at concentrations of from 5 to 50 ppm). The polarographic procedure has been applied to ointments, suppositories, creams and gels (all at a concentration of 0·2%) and has also been used to estimate Bronopol in buffered aqueous solutions and in blood serum. The microbiological procedure has been applied to creams, including barrier creams (at concentrations of 0·1 and 0·2%), to liquid shampoos and also to buffered aqueous solutions. The polarographic method estimates the alkyl nitro group and therefore, although an acceptable procedure for freshly-prepared formulations, is not

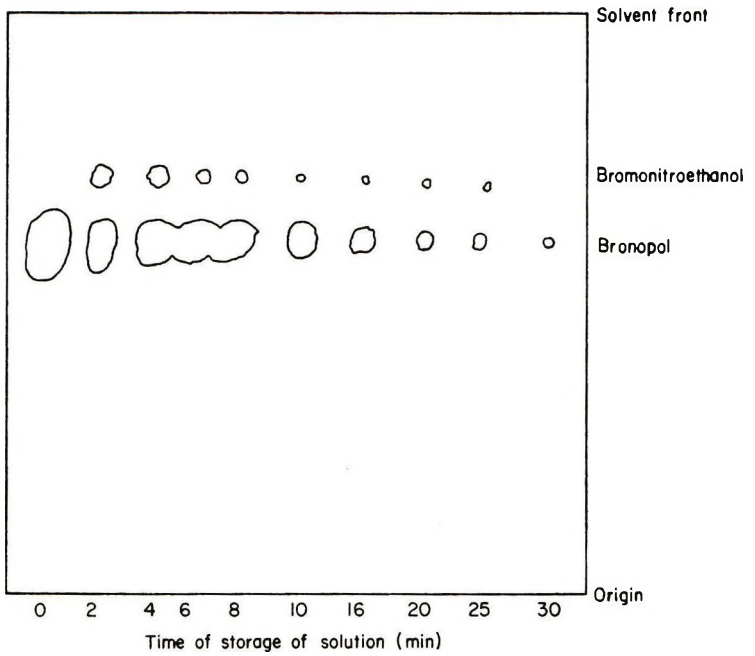


Figure 4. Autobiogram of Bronopol aqueous solutions (initially 10% w/v) stored at 100° and maintained at pH 6 (test-organism: *Pseudomonas aeruginosa*)

very specific and is subject to interference from breakdown products. In the absence of interfering substances, the precision of this procedure is about $\pm 2\%$. The method using t.l.c. is more specific, but the spot-comparison procedure that has been used is liable to relative errors of about 15%; errors of this magnitude may be acceptable, however, at the concentrations in which Bronopol is usually incorporated in formulations. The microbiological method had an error of about $\pm 10\%$ on aqueous solutions and about ± 10 to 20% on creams.

Polarographic Assay. The base electrolyte was McIlvaine's buffer solution containing 2% v/v of 0·2% v/v Triton X 200 as a maximum-suppressor. McIlvaine's buffer solution, pH 4, was prepared by mixing 12·29 parts by volume of 0·1M citric acid solution and 7·71 parts by volume of 0·2% disodium phosphate (Na_2HPO_4) solution. The test solution was prepared as follows. Aqueous solutions only required dilution with base electrolyte to a Bronopol concentration between 10^{-3} and 10^{-5}M. Bronopol in gels, creams and other fatty-base formulations could be extracted with base electrolyte by warming gently on a steam-bath, after which any insoluble matter in the aqueous phase was removed by centrifuging and the aqueous phase diluted to give appropriate concentrations of Bronopol. Other formulations were more appropriately treated by dissolving in chloroform and extracting with base electrolyte.

The determination was carried out by transferring a portion of the solution in base electrolyte to the cell of a suitable polarograph. A stream of oxygen-free nitrogen was passed through the solution for 10–15 min to remove dissolved oxygen. The height of the mercury reservoir was adjusted to give a constant drop rate appropriate to the apparatus, this drop rate being identical with that used for the preparation of the calibration curve.

The polarogram was recorded over the range 0 to −1 V relative to the quiescent mercury pool using the appropriate recorder or galvanometer sensitivity to give a suitable wave. The diffusion current at −0·8 V relative to the mercury pool was measured, and the concentration of Bronopol read from the calibration curve.

Since the polarographic response was affected by the composition of the test solution, it was necessary to prepare a calibration curve for each formulation examined. Such calibration curves were obtained by adding known amounts of Bronopol to blank formulations and processing in the required manner.

The following are examples of the assay method which have been used.

Microbioloical Assay. Bronopol can be assayed microbiologically by agar diffusion using *Ps. aeruginosa* in agar of the following composition:

	% w/v
Dextrose	0·1
Lemco beef extract	0·15
Difco yeast extract	0·3
Sodium chloride	0·5
Difco casitone	0·08
Magnesium sulphate ($7H_2O$)	0·004
Oxoid peptone	0·6
Davis agar	1·8
Distilled water	to 100, pH adjusted to 5·3.

Alternatively Difco Assay Agar No. 11 (pH 7·9) with *Bacillus subtilis* NCIB 8054 can be used. The minimum detectable level of Bronopol in water with *Bacillus subtilis* is 0·005%. A rapid diffusion method using *Bacillus stearothermophilus* has been described by Kabay (18).

Gas-liquid Chromatographic Assay. Although Bronopol is a water-soluble compound it can be extracted from aqueous solution into diethyl ether or ethyl acetate after the addition of sodium chloride. The extract can then be evaporated to dryness, the residue acetylated and the Bronopol estimated by means of g.l.c. with electron-capture detection. This procedure offers a means of determining Bronopol in aqueous formulations and has been applied to Bronopol concentrations down to 5 ppm.

In aqueous formulations containing concentrations of Bronopol down to 50 ppm, the Bronopol has been determined by a similar procedure, but using n-pentadecane as the internal standard, acetyl chloride in chloroform as an acetylating reagent, carbon disulphide as the final solvent and flame ionization detection.

The following are examples of the methods which have been used to assay Bronopol by g.l.c.

Based on the acetylated material. The sample (about 0·15 g accurately weighed) was dissolved in 15 ml of chloroform with the aid of minimum heating, 5 ml of a 2% solution of n-pentadecane (as internal standard) in chloroform was added and the solution diluted to 25 ml. To 1 ml of this solution in a vial was added 0·3 ml acetyl chloride and the vial was sealed and then heated on a steam bath for 3 h. The mixture (2 µl) was subjected to g.l.c. in a glass column (183 cm × 3 mm) packed with 10% of silicone JXR on Gas Chrom Z (70 to 80 mesh), operated at 150°C with nitrogen (20 ml min^{-1}) as carrier gas and flame ionisation detection. The ratio of the product of the peak height and retention

time for the Bronopol diacetate (relative retention time = 1·00) to that for n-pentadecane (relative retention time = 1·54) was calculated and compared with the ratio for a standard containing purified Bronopol which had been similarly treated. The relative standard deviation of the method was found to be 1·5%.

Based on the trimethylsilylated material. The sample (about 0·15 g, accurately weighed was dissolved in 15 ml of chloroform with the aid of minimum heating, 4 ml of a 1·4% solution of n-tridecane (as internal standard) in chloroform was added and the solution diluted to 25 ml. To 1 ml of this solution in a vial was added 0·1 ml of silylating reagent (prepared by mixing trifluoroacetic acid (1 part) and hexamethyldisilazane (2 parts) and filtering the mixture rapidly under dry conditions), the vial was sealed and then heated on a steam bath for 1 h. The mixture (1 µl) was subjected to g.l.c. in a glass column (152 cm × 3 mm) packed with 10% of silicone JXR on Gas Chrom Q (80 to 100 mesh), operated at 125° C. with nitrogen (40 ml min^{-1}) as carrier gas and flame ionisation detection. The ratio of the product of the peak height and retention time for Bronopol di(trimethylsilyl)ether (relative retention time = 1·92) to that for n-tridecane (relative retention time = 1·00) was calculated and compared with the ratio for a standard containing purified Bronopol which had been similarly treated.

Thin Layer Chromatographic Assay. 10% Bronopol solution was examined by t.l.c. and bioautography using 0·25 mm Kieselgel 'G', with chloroform/methanol (4 : 1) as developing solvent. 2 µl aliquots of the solution were spotted on the plates. A similar method for ointment formulations has been devised using an initial water:chloroform extraction system to remove excipients, followed by chromatography on Kieselgel GF_{254} using isopropanol as the developing solvent.

Determination of Bromide ion. Bromide ion was determined by potentiometric titration. Bronopol solution (5 ml) was acidified and titrated with 0·02M silver nitrate solution.

Determination of Formaldehyde. Formaldehyde was determined by reaction with chromotropic acid. 0·2% Bronopol solution (0·5 ml) was diluted to 25 ml with 12N sulphuric acid. To this solution (1 ml) was added a 5% solution of chromotropic acid in 12N sulphuric acid (1 ml) and the mixture heated at 100°C for 30 min. Concentrated sulphuric acid (2 ml) was added and the absorbance at 570 nm measured against the appropriate blank.

Determination of Nitrite and Nitrate. Nitrite and nitrate were determined by reaction with 2,6-xylenol before and after decomposition of nitrite with sulphamic acid. This method was not used after the preliminary work as the results were in good agreement with the polarographic estimation of alkyl nitro-groups.

TOXICOLOGY

Metabolism

After oral administration of [^{14}C]Bronopol, radioactivity was rapidly absorbed and evenly distributed in tissues of the rat and dog, Moore *et al.* (19). Excretion was also rapid, the majority of the dose being excreted within 24 h.

Bronopol was rapidly and extensively metabolised so that no unchanged compound was detected in plasma and urine. It has been shown *in vitro* that Bronopol is unstable in

plasma. The major urinary metabolite, accounting for more than 40% of administered radioactivity, was 2-nitropropane-1,3-diol. Other minor metabolites have not been identified. Complete metabolism was demonstrated by the finding of significant amounts of radioactivity in expired air and the appearance of a small amount of radioactivity in the tissues of dosed animals.

When applied in acetone solution to rat skin, a smaller proportion of the dose was absorbed than when dosed orally, Moore *et al.* (20). This may in part be due to the small area of skin to which the dose was applied.

When [^{14}C]Bronopol was applied in acetone solution to the skin of rabbits, the radioactivity was mainly localised on the epidermis around hair follicles, suggesting that limited percutaneous absorption may occur through the hair follicles.

The pattern of urinary metabolites was similar when the compound was administered orally or percutaneously, indicating no difference in metabolism related to the route of administration.

Acute Toxicity

Bronopol administered in single doses by the oral and intraperitoneal routes to rodents caused gastrointestinal lesions and peritonitis. The LD50 values are shown in *Table VII.* A small number of rats were injected subcutaneously with Bronopol and those that died had haemorrhage and oedema at the site of injection, stomach lesions and lung congestion and oedema. The LD50 was approximately 200 mg/kg. After dermal application to rats of acetone solutions of Bronopol using the procedure of Noakes and Sanderson (21), death occurred at 160 mg/kg or more.

Oral administration of single doses of 40 or 100 mg/kg to dogs caused gastric irritation but no permanent injury.

No methaemoglobinaemia was observed in cats over a 24 h period following a maximum single oral dose of 25 mg/kg of Bronopol, whereas a marked rise in blood methaemoglobin concentration followed 20 mg/kg of acetanilide.

Chronic Toxicity

In repeated-dose studies the observations and laboratory investigations generally included signs of poisoning, body-weight, food consumption, haematology, blood biochemistry, ophthalmoscopy, organ weights, macroscopic appearance at autopsy and histopathology, Gastrointestinal lesions, respiratory distress and some deaths resulted from daily administration of 80 or 160 mg/kg of Bronopol by oral intubation to male and female rats for 90 days whereas doses of 20 mg/kg were well tolerated. When Bronopol was given in the drinking water, rats maintained on 160 mg/kg/day for six weeks had a reduced water intake and slightly enlarged kidneys while among those given the highest dose level of 300 mg/kg/day a few deaths occurred. In dogs given a maximum daily dose of 20 mg/kg by oral intubation for 90 days, apart from some vomiting, there were no significant toxic reactions.

Aqueous 2·5% methyl cellulose solutions containing 0·2 or 0·5% Bronopol were applied once daily at a dosage of 1 ml/kg for 3 weeks to the clipped and abraded dorsal skin of rabbits. The vehicle alone and the 0·2% Bronopol solution elicited local skin erythema and the 0·5% Bronopol solution produced moderate erythema, oedema and scabbing, otherwise the rabbits showed no ill-effects clearly attributable to treatment.

Table VII. Acute toxicity of Bronopol to mice and rats

Species	Sex	Route	LD50 mg/kg
Mouse	male	oral	374
	female	oral	327
Mouse	male	i.p.	34·7
	female	i.p.	32·8
Rat	male	oral	307
	female	oral	342
Rat	male	i.p.	22·0
	female	i.p.	30·2

i.p. = intraperitoneal.

Carcinogenicity

A carcinogenicity study was carried out in mice by application of 0·3 ml of aqueous-acetone solutions containing 0·2 or 0·5% Bronopol to the shaved backs three times weekly for 80 weeks. The concentrations were selected after a preliminary tolerance study showed that 1% or more evoked a local skin reaction. Bronopol did not alter the spontaneous tumour profile either locally or systemically.

A 2-year toxicity and tumorogenicity test, in which rats received 10, 40 or 160 mg/kg daily in the drinking water, provided no evidence to suggest that the administration of Bronopol affected tumour incidence. There was no indication of toxicity at 10 mg/kg/day, whereas the higher dose levels adversely affected growth, food intake and survival rate. Renal changes associated with diminished water intake, histological reactions in stomach and gastric lymph nodes probably due to irritancy from prolonged exposure to Bronopol, and an exacerbation of spontaneous morphological alterations in the salivary gland were also observed at the higher dose levels in a dose-related manner.

Reproduction Studies

The effect of Bronopol on reproduction was investigated in rats and rabbits. In rats dosed from day 1 to 20 of pregnancy with 10, 30 or 100 mg/kg daily by oral intubation, no embryotoxic or teratogenic effects were seen even though the dams had a dose-related retardation in bodyweight gain and some died from gastric and lung lesions. A slight delay in calcification of the foetal skeleton was observed at the highest dose level. Daily application to the clipped dorsal skin of rats of 0·5 or 2% aqueous solutions of Bronopol thickened with 2·5% methylcellulose in a dose of 1 ml/kg from day 6 to 15 of pregnancy had no adverse effects on the dams or foetuses apart from causing local skin reactions at the site of application.

Oral administration of 1, 3·3 or 10 mg/kg daily to rabbits from day 8 to 16 of pregnancy also failed to produce embryotoxic or teratogenic effects though the highest dose level suppressed weight gain by the does during the dosing period.

Bronopol, 20 or 40 mg/kg daily, given orally to rats from day 15 of gestation and throughout lactation did not affect parturition, litter size or postnatal survival and development of the young. Fertility and general reproductive performance of rats were unimpaired by these dose levels given to males from 63 days before mating and females from 14 days before mating up to day 12 of pregnancy or until the litters were weaned

21 days *post partum*. In this study bodyweight gain of the males that received 40 mg/kg daily was slightly reduced.

Mutagenicity

Bronopol did not exhibit mutagenic activity under *in vitro* or *in vivo* conditions. It was tested using *Salmonella typhimurium* in the 'Ames' system and in the host-mediated assay in mice; in a dominant lethal assay in mice, the only noteworthy finding was anti-fertility arising from toxicity rather than dominant lethality.

Irritancy and Contact Sensitivity

Animal Studies. Preliminary studies on irritancy and contact sensitisation have been reported by Croshaw *et al.* (3).

Bronopol was tested for local effects to the mucous membrane of the eye in rabbits. A concentration of 0·5% in normal saline was non-irritant when applied once daily for four successive days, whereas solutions in polyethylene glycol 400 were irritant at 5% but not at 2% following a single application.

Skin irritancy was investigated by application of Bronopol in a variety of solvents to the non-abraded, clipped and shaved backs of rabbits for 6 h, with or without occlusion. Acetone solutions of Bronopol were non-irritant at 1% when given as a single application under occlusion though highly irritant at 0·5% on repeated application without occlusion. Similar results were obtained with Bronopol in 2·5% aqueous methylcellulose solution tested under these conditions at 0·5% concentrations. Bronopol in polyethylene glycol 300 was non-irritant at 5% as a single application with occlusion. These findings indicate that the irritancy of Bronopol to the skin is dependent upon the vehicle employed, thus it would be advisable to test each new formulation containing Bronopol for local effects on topical application.

Bronopol was without skin-sensitising activity in the guinea pig when tested as a 1% solution in acetone by the ear-flank method (Stevens (22)), whereas dinitrochlorobenzene was strongly positive.

Human Studies. The skin irritant effect of Bronopol was investigated both on volunteers and on patients attending a contact dermatitis clinic.

The volunteer study showed that Bronopol is slightly irritant to human skin at 1% in soft paraffin (petrolatum), and at 0·25% in aqueous buffer at pH 5·5. The study consisted of a closed patch test using 1 cm lint squares backed with Blenderm surgical tape on the forearms of ten subjects. Concentrations of 0, 0·5, 1 and 2% Bronopol in soft paraffin and of 0, 0·05, 0·1 and 0·25% in aqueous buffer at pH 5·5 were used. Any skin reaction after 24 h was graded from 0 (=normal skin) to 5 (=marked erythema with vesicles and induration). The results are shown in *Table VIII*. The study carried out on patients attending a contact dermatitis clinic showed that Bronopol is a mild irritant when applied in yellow soft paraffin (yellow petrolatum) at 0·25%. No evidence of sensitisation was seen in this study nor was there any suggestion of cross-sensitisation with any other substance, including formalin. The compound was applied as one of a battery of closed patch tests used in that clinic to screen the patients for a potential allergen. The patches were applied for 48 h and examined on the second and fourth days after the application. Of the 149 patients studied, three showed a slight erythema on the

Table VIII. The irritancy of Bronopol to human skin

Base	Bronopol concentration %	Positive skin response	Degree of reaction
Soft Paraffin	0	0/10	—
	0·5	0/10	—
	1	2/10	both slight erythema
	2	4/10	all moderate erythema
Aqueous buffer pH 5·5	0	0/10	—
	0·50	0/10	—
	0·1	0/10	—
	0·25	1/10	slight erythema

second day which had faded by the fourth day, and one a moderate erythema on the second day; this patient did not return for the second examination.

Marzulli and Maibach (23, 24) have studied the contact sensitisation in man of a number of commonly used biocides; and have concluded that, under the conditions of a closed patch test, Bronopol in yellow soft paraffin was a potential sensitizer. The challenge concentration in these studies was 2·5% which according to these authors was a non-irritant concentration. However, the studies reported above are not consistent with this view. The patch tests carried out by Marzulli and Maibach showed a dose–response relationship, and since the response decreased very rapidly to zero at an induction concentration of 2% which is considerably greater than that used in formulations, the authors inferred that Bronopol may be safely used in cosmetic formulations. In a further study, Maibach (25), has confirmed that Bronopol was a direct irritant to human skin at concentrations greater than 1% under these conditions. A subsequent sensitisation test included 93 normal subjects who were induced with 10 applications of 5% Bronopol in yellow soft paraffin under an occlusive dressing over a period of 3 weeks. After a rest period of 2 weeks the subjects were challenged at 0·25% Bronopol in yellow soft paraffin at a different site. No evidence of contact sensitisation was observed.

FORMULATION STUDIES

Bronopol has been in use for more than 10 years at a level of 0·01–0·02% or more in conventionally formulated shampoos based on sodium lauryl ether sulphates and alkanolamine alkyl sulphates with 2% or more of a foam-boosting alkanolamide. When a freshly prepared formulation is challenged with 1×10^6 pseudomonads per ml the bacterial count is reduced to <10 per ml within 24 h. The inclusion of protein-derived materials, e.g. 0·1–0·5% Crotein C (hydrolysed collagen, Croda Chemical Ltd) does not affect this result.

The use of Bronopol in the preservation of shampoos has been described by Bryce and Smart (14), Schuster (26) and in protein shampoos by Tuttle, Phares and Chiostri (27). Barnes and Denton (28) found Bronopol at 0·02% to be one of the most satisfactory preservatives against Gram-negative bacteria in a cream, suspension and solution in their capacity test.

Combinations of preservatives can be justified on several grounds, one of these being to increase the spectrum of antimicrobial activity. It is established that the antibacterial activity of Bronopol is greater than its antifungal activity and its spectrum can

be increased by the addition of parabens, Parker (29). Proserpio (30) and Jacobs, Henry and Cotty (31) have considered the combination of Bronopol with other agents in cosmetics and oil-water emulsions.

Medicated Skin Cream

Bronopol in an anhydrous base or in an aqueous formulation of low pH may have applications as an active antibacterial agent in skin care products. Experimental medicated skin creams containing Bronopol and Hexachlorophane BP showed activity on the skin against *Escherichia coli* whereas a cream without Bronopol did not. Although it is theoretically possible that synergism between these compounds is occurring, other experience suggests that this is unlikely. It is concluded therefore that Bronopol is exhibiting antibacterial activity *per se* in these formulations.

The composition of the cream was as follows:

	% w/w
Hexachlorophane BP	0·5
Bronopol	0·1 or 0·2
Sorbitol syrup	13·5
Arlacel 186 (ICI United States Inc.)	1·5
Cosmolloid wax 70H (Astor Petrochemicals)*	7·5
Light mineral oil	20
Aqueous citrate buffer pH 4·5	to 100

* The 70H grade is no longer available. Cosmolloid wax 70 grade has almost identical properties.

The forearms of eight subjects were washed, dried and swabbed with alcohol to remove the transient skin flora. The creams were applied in 0·05-g amounts to areas of 5 × 2·5 cm, and 0·025 ml of a 1 in 10 dilution in broth of an overnight culture of *Esch. coli* NCTC 5934 was applied on to each cream. After 30 min contact between cream and organism each area was swabbed with alginate swabs. The swabs were dissolved in 10 ml of quarter strength Ringer solution containing 1% of sodium hexametaphosphate and 1% of polysorbate 80. Aliquots (1 ml) of the swab diluents were plated in 'Oxoid' MacConkey agar No. 3 and plate counts were made after incubation for 48 h at 37°C. Results of a typical experiment are shown in *Table IX.*

Table IX. Antibacterial activity of medicated cream formulations on human skin. Viable organisms (per ml) from subjects (A–H) after contact time of 30 min

Cream	Subjects A	B	C	D	E	F	G	H
Base (no active agent)	+	+	+	+	+	+	+	+
Base + 0·5% Hexachlorophane BP	+	+	+	+	+	+	+	+
Base + 0·5% Hexachlorophane BP + 0·2% Bronopol	7	2	1	1	2	0	3	9
Base + 0·5% Hexachlorophane BP + 0·1% Bronopol	11	+	+	6	20	20	0	29

+ = uncountable numbers.

Alcoholic Deodorant Spray

An experimental alcoholic deodorant spray formulation containing 0·12% w/w Bronopol was found to be stable after one-year's storage at room temperature and 37° in a lacquered tinplate can, and showed a broad spectrum of antibacterial activity.

Composition of the spray was as follows:

	% w/v
Diethyl Phthalate BPC	0·330 (v/v)
Propylene glycol	0·167
Denatured ethanol B grade 750P	29·320
Perfume	0·083
Bronopol	0·100
Propellant 11/12	(1 : 1) to 100

This formulation was packed into internally lacquered tinplate cans soldered with solder 2/98 and fitted with a standard valve. The can fill was 128 g. Samples were then placed on storage test at room temperature and 37°C, and examined at regular intervals over a period of one year.

Chemical assays were carried out using an adaptation of the t.l.c. system described earlier. The results showed that no decomposition of the Bronopol had occurred. No corrosion of the container was observed.

Microbiological assessments were carried out by spraying the formulation on to a 13 mm Whatman A.A. disc and allowing to dry. The discs were then placed on to agar seeded with various test bacteria and *Candida albicans*. The zones of inhibition were recorded after 18 h at 37°C. The results obtained after one-year's storage are shown in *Table X*.

Table X. Antibacterial activity of Bronopol in an alcoholic spray formulation after one year storage at room temperatue and 37°C

	Diameter of zone of inhibition (+13 mm disc) in mm		
Test organism	Sample 1 (containing Bronopol initially at 0·12% w/w)		Sample 2 (containing no Bronopol)
Temperature of storage	Room temperature	37°	37°
Staph. aureus 8452	29	30	13
Staph. aureus FDA	35	35	13
Staph. albus NCTC 7944	28	29	13
Esch. coli NCTC 5934	26	26	13
Proteus vulgaris NCTC 4635	26	26	13
Ps. aeruginosa 10S	28	28	13
Candida albicans 239	13	13	13

Personal Hygiene or Foot Spray

An experimental hygiene spray formulation containing Bronopol was found to be stable in a lacquered aluminium can and showed more antibacterial activity than a similar formulation containing chlorhexidine, even after 3 months storage at elevated temperature.

The following formulation was chosen to represent a typical hygiene spray:

	% w/w
Talc 399 (Whittaker, Clark and Daniels Inc.)	0·500
Bronopol	0·075
Perfume	0·100
Propellant 11 (I.C.I. Ltd)	39·325
Propellant 12 (I.C.I. Ltd) to	100

Crystalline Bronopol was passed through a micro-mill to obtain the necessary reduction in particle size, the material subsequently passed through a 250 mesh screen sieve. The iron content of the Bronopol was 7 ppm and no evidence of discolouration was evident after 5 months storage at room temperature. Particle size analysis of the talc showed 98·7% less than 15 μm.

The above formulation was packed into an internally lacquered aluminium monobloc can fitted with a standard valve. The can fill was 64 g. Samples were placed on storage test at room temperature and 37°C, and were examined at regular intervals over a period of 3 months.

Chemical assays were carried out using an adaptation of the t.l.c. system described earlier. The results showed that no decomposition of Bronopol had occurred. No corrosion of the container was observed.

Microbiological assays were carried out using the method described for the alcoholic deodorant spray formulation. The results obtained after three months storage are shown in *Table XI.*

Table XI. Antibacterial activity of Bronopol and chlorhexidine in a hygiene spray formulation after 3 months storage at room temperature and 37°C

Test organism	Diameter of zone inhibition (+13 mm disc) in mm					
	Sample 1 (containing Bronopol initially at 0·075% w/w)		Sample 2 (containing chlorhexidine initially at 0·075% w/w)		Sample 3 (containing neither biocide)	
Temperature of storage	Room temperature	37°	Room temperature	37°	Room temperature	37°
Staph. aureus 8452	30	25	14	15	15	14
Staph. aureus FDA	32	22	15	15	13	13
Staph. albus NCTC 7944	31	24	14	14	13	13
Esch. coli NCTC 5934	21	21	13	13	13	13
Proteus vulgaris NCTC 4635	23	21	13	13	13	13
Ps. aeruginosa 10S	26	22	13	13	13	13
Candida albicans 239	13	13	16	16	13	13

Further microbiological tests were carried out with samples which had been stored at room temperature and 37°C for 6 months. Although these tests were only qualitative, a similar pattern of activity to that observed after 3 months was obtained.

CONCLUSIONS

Bronopol has been shown to possess a wide spectrum of antibacterial activity. Its activity against Gram-negative organisms, particularly *Ps. aeruginosa,* is greater than that of most other antibacterial and preservative agents.

Bronopol is most stable under acid conditions, although it demonstrates high antibacterial activity over a wide pH range. The mode of decomposition has been studied in detail, and a number of the decomposition products identified. The assay methods described are capable of estimating Bronopol in many formulations at its normally used levels, the sensitivity of the methods is dependent upon the nature of the formulations; in certain cases levels as low as 5 ppm can be assayed.

Bronopol is generally used as a preservative in formulations at levels between 0·01 and 0·1%. Animal toxicity studies and human patch tests have demonstrated the safety of Bronopol when used at these concentrations. In particular, no evidence of human skin sensitisation has been obtained at these levels.

Bronopol has been shown to be an effective antibacterial agent in a range of formulations including shampoos, skin creams and sprays and bath products. Many of the ingredients used in such products have been shown to have little or no effect on the antibacterial activity of Bronopol, although compounds containing sulphydryl groups are antagonistic to its activity.

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Towards objectivity in the assessment of eye irritation

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Synopsis

The assessment of **eye irritation** is subjective. The eye test system is subject to such wide variation that it will never be possible to make precise measurement of **irritancy.** Clinical appraisal, supported by measurement of **corneal thickness** and **intra-ocular pressure,** is probably the best that can be achieved. The use of local **anaesthetics** should be considered when carrying out eye irritation tests in the rabbit.

INTRODUCTION

The assessment of eye irritatation is basically subjective and it is not surprising that the literature abounds in results showing different inter- and intra-laboratory values. The need for objective assessment has been voiced by many investigators and some parameters for the measurement of functional and pathological change have been proposed. The purpose of this paper is to review the feasibility of some of these methods.

CLINICAL EXAMINATION

The assessment of irritants is based on the well-established Draize test – Draize, Woodard and Calvery (1) – which has been modified over the years, although most laboratories follow the standard procedure laid down by the U.S.A. Code of Federal Regulations (2).

Albino rabbits of either sex weighing between 2 and 3 kg are the usual test species. It must be pointed out that differences due to age, sex, management, strain, season and other environmental factors have received scant attention, although there is little doubt that the strain of rabbit is of considerable importance.

The degree of damage induced is dependent on the concentration of compound and the intimacy and duration of contact with the conjunctiva and cornea. The concentration of compound used should be that which induces a minimal measurable response. The intimacy of contact can be controlled by applying a known volume and standardising the application procedure. The other factor that could be varied is the period of contact between the test material and the conjunctival sac. It has been shown by Davies, Kynoch and Liggett (3) that 10 sec is the maximum delay time, after which irrigation is not beneficial. This initial contact time is too critical to control and it is inappropriate to introduce a washout technique and so add another variable to what is already a complicated situation. Another factor against initiating washout techniques is the fact that the washing procedure is not always beneficial. Sebaugh *et al.* (4) showed that the washing procedure shortened the onset of opacities produced by weak acids, but with 1%

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sodium hydroxide washing enhanced the initial eye irritation. With strong acids and alkalis, washing has no effect.

The response of the conjunctiva, cornea and iris are graded on a 0–4 scale. It is at this point that observer bias comes in. The reactions are best scored by technical staff trained to a common standard. Bayard and Hehir (5), investigating reader-scoring variability, found that readers were consistently able to produce a positive or negative reading more than 90% of the time. If clinicians, be they veterinary or medical, examine reactions, they are biased by their experience of clinical conditions in the eye and tend to score to lower grades than technical staff. Ballantyne *et al.* (6) suggested grading for lachrimation, blepharitis, chemosis, hyperaemia, sloughing, iritis, keratitis and for corneal vessels. It is common practice to manipulate the scores in order to express the total irritancy as a single number. Attractive as the idea may be to express a complicated biological situation as a single number, the practice must be viewed with caution. Ballantyne and Swanston (7) state that it is not possible to grade eye irritancy simply on the grounds of numerical values and that observed effects must be summarised verbally.

There is considerable variability between individual animals, whether one is working with rabbits or monkeys. To reduce the number of animals would decrease the ability of the test to differentiate degrees of irritation, but increasing the group sizes would not increase precision.

Comparison of the total Draize score and scores from the cornea only suggest that the conjunctiva makes the initial response to irritation and that the reaction persists for longer than it does in the cornea. The effect of this conjunctival response is not understood, but it could be considerable. The present trend in Draize testing is to reduce concentrations of the working solutions in the hope of being able to measure conjunctival response only.

Despite our concern about the Draize test, Marzulli and Ruggles (8) reported that reliable and reproducible results could be obtained in different laboratories in distinguishing an irritant from a nonirritant. They recommend a simple pass/fail criterion, monitoring four parameters.

SLIT LAMP EXAMINATION

In our experience, this examination, together with fluorescein staining, is the most sensitive indicator of corneal damage in the monkey. If fluorescein is instilled into the conjunctival sac of the rabbit, in nearly all cases staining patterns will result. Kikkawa (9) described intervals of 1–11 days between light and intense staining and correlated the pattern in both eyes. The stain pattern was attributed to physiological desquamation of the corneal epithelium. In our experience, this pattern of intense and light staining has not been established in the rabbit. The turnover rate of epithelial cells in the cornea is high and the vulnerability of the corneal epithelium to insult may reflect the different stages in the life-cycle of the cells. Nevertheless in monkeys this high turnover of cells is achieved without a break in the continuity of the epithelium. The fact that the corneal epithelium of the rabbit is not intact will generally increase the rate and amount of penetration, this being particularly true for water soluble and polar compounds.

CORNEAL THICKNESS

Corneal thickness can be readily measured following the technique defined by Mishima and Hedbys (10) using the Haag–Streit depth measuring attachments. Burton (11) must

be credited with first realising the usefulness of this parameter in assessing irritancy. Initially, it appeared to be a sophisticated and objective measure of irritancy, free from observer bias, but unfortunately, with experience the limitations become apparent. It is important that the measurement of thickness is made at the apex of the cornea. It is unfortunate that the cornea is not uniformly thick, nor does it uniformly respond to irritation. If sloughing of the epithelium occurs the measurement becomes meaningless. There is again an observer bias as to the point on the cornea which one chooses to measure. Nevertheless, it has been shown with commercial shampoos, Burton (11), and a variety of industrial solvents, Conquet *et al.* (12), that a highly significant correlation can be shown between the total Draize score and the corneal thickness measurement. It must be concluded that this measurement of corneal thickness can only supplement the clinical observations.

INTRA-OCULAR PRESSURE

Ballantyne, Gazzard and Swanston (13) showed that a rise in intra-ocular tension is one of the earliest indications that a compound is producing eye irritation. Furthermore, they suggested that there might be a relationship between the rise in tension and the severity of the subsequent reaction.

There are two indirect methods of measuring intra-ocular pressure. Firstly, there is the indentation tonometer, by which a weighted plunger is applied to the cornea, the most common piece of apparatus used being the Schiotz tonometer. The second and most applicable technique is that of applanation tonometry, which measures the force required to produce a degree of flattening. The use of the Schiotz tonometer is often impractical, because swelling of the cornea cushions the tonometer footplate and a satisfactory measurement cannot be made. To date, there is little published data to support the use of applanation tonometry for measuring ocular irritancy; but current investigations – Heywood and Walton (14) – will indicate the potential of this technique.

CORNEAL CURVATURE

Measurement of corneal curvature is precluded by the effects of irritants on the pre-corneal film, because any disturbance of this film and the superficial epithelial cells of the cornea prevents lining up of the mires of the ophthalmometer and making accurate measurements.

CONJUNCTIVAL AND CORNEAL WEIGHT

Conjunctival and corneal weights were found to be precise measurements of irritation by Laillier, Plazonnet and Douarec (15), who investigated many organic solvents. For control animals, the percentage dry weight was $26{\cdot}4\% \pm 0{\cdot}8\%$, but even with the most severe irritants, this never fell below 10%. The water content of the cornea does not show a direct relationship to opacity, Wright, Ulsamer and Osterberg (16).

EFFECTS ON CAPILLARY PERMEABILITY

Changes in capillary permeability in the conjunctiva and the blood/aqueous level can be demonstrated using the Evans blue dye technique. Laillier *et al.* (15) have shown change in capillary permeability to be one of the first events in eye irritation.

HISTOLOGICAL INVESTIGATION

At the end of each study, the eye and its adnexa should be taken for histological examination. The techniques used should be such as to reduce fixation artefact to a minimum.

The normal cornea should show microvillae in the superficial cells of the corneal epithelium, microtubules in Descemet's membrane and vesicles in the endothelium, indicating active transport systems. There should be no oedema and the collagen fibres in the stroma should be of normal size and show no distortion. In the conjunctiva, there should be normal microvillae and the epithelium cells should be rich in endoplasmic reticulum. Normal goblet cells are of considerable importance, as they are responsible for the mucin in the precorneal film. Alterations in any of these parameters must be considered of significance.

Tonjum (17) investigated the corneal epithelium of rabbits and vervet monkeys after treatment with benzylkonium chloride and demonstrated that the superficial cells were showing small holes and losing microvillae within 2 min of application of the irritant.

THE USE OF ANAESTHETICS

The present tests make no provision for the use of anaesthetics. Ulsamer, Wright and Osterberg (18), working with acetic acid, phenol, sulphuric acid, ammonia and sodium hydroxide, together with the anaesthetic butacaine sulphate (2%) measured the opacity, redness response, corneal water content, dry weight and electrophoretic protein patterns in groups of New Zealand White rabbits. Neither the opacity scores nor the moisture content produced clear-cut patterns of statistical differences, although with 10% acetic acid and 10% ammonia, the unanaesthetised eyes had lower scores. The non-anaesthetised cornea had a lower water content following the administration of 5% acetic acid and 1% ammonia and greater water content following the administration of 3% sodium hydroxide solutions.

Using 10% sodium lauryl sulphate as the irritant and 0·5% proparacaine as the anaesthetic in the New Zealand White rabbit, Heywood (19) showed that no statistically significant differences could be detected between the anaesthetised and unanaesthetised cornea. Although there was some evidence that the intensity of reaction was increased following anaesthesia, there was no evidence of prolonged effect. In the monkey, however, applying irritants to the anaesthetised corneal sac had a very marked effect in which all reactions were intensified and prolonged.

The results indicate that, in the rabbit, the use of a local anaesthetic should be considered when carrying out eye irritation tests, but in the monkey the use of anaesthesia is contraindicated on both scientific and humanitarian grounds.

DISCUSSION

On reviewing the literature, there appears to be no data which establishes convincingly a correlation between the Draize score and the objective irritancy measuring tests, although such a correlation has been established with certain shampoos and industrial solvents. However, Heywood (19), working with a variety of methods, treatment concentrations and regimes, showed that when correlations could be established these were between group mean values and could not be shown to occur consistently in individual animals. To draw any conclusion from the available data is difficult, for it would appear

that the eye test system is subject to such wide variation that it will never be possible to make precise measurements of irritancy and that it will always be a subjective clinical appraisal, supported by measurements of corneal thickness and probably of intraocular pressure.

Certain aspects of irritancy, such as the 'sting' phenomenon, which is probably of major commercial importance, cannot be measured in animal systems.

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Changes in sunburn and mechanisms of protection

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Synopsis

A review and critical discussion is presented of the reactions of **skin** to **sunlight** and artificial sources of **ultraviolet radiation.** Particular attention is paid to the mechanisms involved in **sunburn** but mention is also made of premature **ageing** and skin **cancer.** Ultraviolet **erythema** is the most extensively studied reaction and it is possible that the primary molecular target for this and the other reactions is either DNA or **lysosome** membrane lipid. However, no definite conclusions may be drawn. Nor is it established at which level in the skin, the **epidermis**, where the most prominent histologic change, the appearance of **sunburn cells**, is found, or the **dermis** in which vasodilatation occurs, this primary target may be. Physiological **protection** against ultraviolet radiation is afforded by **melanin pigment**, the proteins of the **horny layer** and **urocanic acid**, but the mechanisms are poorly understood as is the possible involvement of naturally occurring anti-oxidants.

INTRODUCTION

The skin is essentially a functional interface between a living organism and its environment, reacting to the various stimuli in that environment. Terrestrial solar radiation or 'sunlight' is a major component of the human environment and this paper is primarily concerned with the reactions of normal, lightly pigmented human skin to a single exposure to this radiation. These reactions, commonly known as sunburn, are a complex of inflammatory processes, the mechanisms involved being still largely unresolved. Some mention will be made of various abnormal skin reactions to sunlight, collectively termed the photodermatoses, and, for completion, the long-term effects of repeated exposures to high intensity sunlight will be briefly discussed. For more detailed discussions of the whole subject matter, the reader is advised to consult the reviews of Blum (1), Kimmig and Wiskemann (2), Johnson, Daniels and Magnus (3) and Magnus (4). Encyclopaedic accounts can be found in the proceedings edited by Urbach (5) and Fitzpatrick (6).

SUNBURN

The gross skin changes seen in sunburn are familiar to most lightly pigmented individuals and even Negroid types may experience discomfort as a result of injudicious exposure to early summer sunlight in the northern United States of America. In its mildest form, the reaction consists of a transient reddening, or erythema, of the exposed skin which may take some hours to develop and may fade rapidly. With a very slight increase in exposure time, a more intense erythema is produced, becoming visible during the exposure (7) reaching a peak of intensity 24 h or so later and persisting for up to 20 d (8). In individuals with a tendency to tan, hyperpigmentation begins to develop by 72 h after the exposure. With longer exposures, erythema may be accompanied by oedema, itching and pain.

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Blistering may occur in which case, any developing suntan will be lost in the blistered area. Even with a mild sunburn, the outer layers of the stratum corneum tend to be sloughed in sheets rather than as the normal minute scales. When larger areas of the body are exposed, the severe local reactions may be accompanied by nausea, headaches and muscle weakness and medical treatment may be necessary. In already tanned skin, in Asian caucasoids and in the less heavily pigmented negroids, the intensity of the inflammation produced by a given exposure dose is less than that in fair-skinned peoples and even in the latter, slight variations in degree of pigmentation produce differences in the intensity of reaction to sunlight (9). In pigmented skin, an immediate hyperpigmentation can be seen in exposed areas, a phenomenon known as immediate pigment darkening (IPD) which appears to result from an oxidation reaction in melanin already present in the outer layers of the skin but in a bleached form.

WAVELENGTH DEPENDENCE

Neither infrared radiation (beyond 700 nm) nor visible (390–700 nm) produces the characteristic sunburn reaction. Window glass, acting as a cut-off filter at 315–320 nm, prevents the reaction and this sets the upper limit for effective radiation at around 315 nm. The lower limit is set by the absorption of short wavelength ultraviolet radiation (uvr) by stratospheric ozone, the lowest value of wavelength recorded at the earth's surface being around 285 nm. It would seem that natural sunburn is produced by uvr between 280 and 315 nm, a minute fraction of the total solar spectrum. Because this wavelength region is so important for photobiology, the more conventional partition of the uv spectrum into far-uv (180–290 nm), and near-uv (290–390 nm) has been modified (10) into uv-A (315–390 nm), uv-B (280–315 nm) and uv-C (180–280 nm) a classification which has found considerable usage in dermatological literature. Artificial sources are essential for controlled studies of sunburn and other biologic effects of uvr. Low pressure mercury arcs emit principally the 254-nm line and this uv-C radiation does produce a sunburn-like reaction in human skin. There may be an additive or augmenting action of uv-A on the natural sunburn reaction (11, 12) and uv-B, uv-A and visible radiation are separately or collectively involved in the abnormal reactions seen in the various photodermatoses.

There are significant differences between the erythema produced by exposure to uv-C, uv-B and natural sunlight (7, 8, 13). The uv-C erythema is less intense and has a shorter duration than that produced by uv-B. While intense irritation and epidermal damage may be caused, the severe lesions, involving damage to the dermis, which may develop with uv-B irradiation cannot be elicited with uv-C. On the other hand, the uv-B erythema has a shorter duration than that produced by sunlight and the latent period for its development is relatively prolonged. Thus the natural sunburn response is not necessarily produced by exposure to a narrow band of uvr wavelengths and the reports of Hausser (14) that uv-A erythema, although requiring a very high exposure dose, may be observed very soon after the exposure and persists, and of Willis, Kligman and Epstein (11) and Parrish *et al.* (12) that the dose requirement for uv-B erythema may be reduced by exposure to uv-A, appear to have considerable relevance to the problem of the natural sunburn.

Detailed studies of the wavelength dependency for uvr erythema began with the work of Hausser and Vahle (15). An action spectrum for the just perceptible erythema in upper back skin of lightly pigmented individuals is shown in *Fig. 1*. This was plotted from data obtained by Dr John Anderson and myself at Dundee, using a small grating irradiation

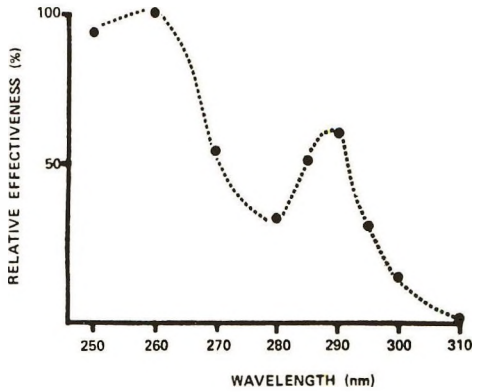

Figure 1. Action spectrum for just perceptible erythema in fair-skinned human subjects; back skin, readings at 7–8 and 24 h. The 100% level at 260 nm represents a mean MED of 4·3 $mJcm^{-2}$, standard deviation 1·4.

monochromator (Rank-Hilger, D330 with D335 grating) with a 450 W Xenon arc source (Osram, XBO 450 W). The half-maximum bandwidth used was 2·6 nm, giving minimal dispersion around the selected wavelength. Readings of the reactions were made at 7–8 h and 24 h after the exposures. The minimum dose required to produce a reaction, whether observed at 7–8 h or 24 h, was taken as the minimal erythema dose (MED). The action spectrum is plotted from the reciprocal of the MED at each wavelength and is a relative effectiveness curve. It is typical of the various action spectra published in the past (3) and resembles those of Cripps and Ramsay (16) and Nakayama *et al.* (17). Problems have arisen with regard to the shape of the action spectrum in the wavelength region below 285 nm which were foreshadowed in the work of Hausser and Vahle (18). In the action spectra published before that of Everett, Olson and Sayre (19) the wavelength region around 260 nm, although shown to have a peak of activity, was less effective than the 290-nm region. Where the reaction observed is a minimally perceptible erythema, it is obvious from *Fig. 1* that the shorter wavelengths are, in fact, more effective. Moreover, the trough in the action spectrum at 280 nm is much deeper in the earlier publications. Explanations for these discrepancies were proposed by Everett *et al.* (19) and by Berger, Urbach and Davies (20) and these are elegantly discussed by Leun (21). A minimal erythema produced by 260-nm radiation may develop by 8 h but fade before 24 h. Thus, for erythema readings at 24 h only, a higher dose is required for the shorter wavelengths' reactions and the activity of this wavelength region will appear to be relatively low. Moreover, the gradation of erythema redness produced by the two wavelength regions with increasing dose is different. While the intensity of the 260-nm reaction increases slowly and reaches a low plateau level, that for longer wavelengths increases rapidly to a much higher plateau level. If some degree of erythema other than the minimally perceptible is chosen as the end point for plotting an action spectrum, as was the case in the earlier studies, the longer wavelengths will again appear to be more effective. The trough in the action spectrum at 280 nm has always been ascribed to the filtering effects of the stratum corneum. This may be true as the trough is deeper in action spectra obtained with forearm skin with a relatively thick corneum, than in those obtained with back skin.

The prolonged arguments about the effects of uvr wavelengths not present in natural sunlight may appear superfluous. However, these shorter wavelengths are a prominent part of uvr photobiology, particularly in the investigations of cellular mechanisms of uvr damage. Nucleic acids have major absorption peaks in this wavelength region as do proteins through the aromatic amino acid constituents. Also, the total action spectrum

for erythema has allowed interesting theoretical treatments of the mechanisms involved in the sunburn reaction (22, 13). Leun (13) concludes that the uvr erythema is brought about by a dermal reaction to all effective wavelengths, the shorter wavelengths being more active, and that a more superficial reaction to the 290–300 nm region results in the formation of a diffusible mediator for erythema, this action being superimposed upon the dermal one to produce a double peak action spectrum. Although most of the incident radiation between 250 and 320 nm is absorbed in the epidermis, at least 1% may reach the dermis (23). The penetration of the longer uvr wavelengths is significantly greater than that of the 260-nm region and it appears more likely that the shorter wavelengths would act at a more superficial level. However, the theoretical treatment of uvr erythema published by Leun (13), Ramsay and Challoner (24) notwithstanding, has not been repudiated and at this time, the mechanisms suggested fit well with the majority of the experimental data.

All the published action spectra show similar features for uvr beyond 290–295 nm, i.e. a rapid fall off in effectiveness so that for all practical purposes, 320 nm and longer wavelength radiation are ineffective, confirming the filtering action of window glass. As the intensity of uvr in natural sunlight falls rapidly from 330 nm to almost zero at 290 nm, the most active wavelength region in natural sunlight will be at a higher value than that shown in *Fig. 1*. Schulze (25) has computed the most effective wavelength as around 307 nm using the data of a 'standard' erythema action spectrum and the solar radiation measurements of Bener (26). This peak of effectiveness would be moved further into the long-wavelength uvr in geographical locations in which a more rapid fall-off of intensity with decreasing wavelength occurs. As the gradation of severity of reaction increases so markedly with increasing dose at these wavelengths, it is not surprising that a severe sunburn may result from exposure to natural sunlight which appears to be very little beyond a minimal erythema dose. In fair-skinned individuals in Dundee, latitude 56·5N, back skin exposed to 20 min of mid-day June sunlight may show no reaction. A 30-min exposure, however, may result in an intense erythema accompanied by tenderness and oedema.

MECHANISMS FOR THE REACTIONS

Erythema is the most easily observed component of the sunburn reaction and has therefore been the most ardently studied. Mild erythema is a manifestation of vasodilatation in the superficial venous plexus of the papillary dermis. More intense erythema, accompanied by increased skin temperature, reflects arterial and arteriolar dilatation and increased blood flow (24, 27). With very high doses of uvr, vascular stasis may occur (28). Despite the intensive studies of the reaction, the nature and location of the primary molecular target for uvr damage to skin remain unknown. A direct effect of uvr on isolated blood vessels has been described (29) but the immediate contraction observed seems to have little relevance for the delayed onset, persistent erythema observed in irradiated skin. The effects of direct damage to blood vessels in the skin are seen in the photosensitised reaction obtained in the disease Erythropoietic Protoporphyria in which excess protoporphyrin production in the red blood cells leads to abnormal skin reactions to 400-nm radiation. The vascular endothelium is partially destroyed and perivascular deposits of a mucoid substance are laid down as the endothelium is repaired (30). There is no evidence of a similar process in the sunburn reaction and only when excessively high doses of uvr, in particular uv-A, are used, is endothelial damage observed (31).

HISTOPATHOLOGY

The histopathology of the sunburn reaction has been well documented by Rost and Keller (32), Uhlmann (33), Hamperl, Henschke and Schulze (34) and Daniels, Brophy and Lobitz (35). No changes are detected before the appearance of erythema and evidence of damage appears to be restricted to the epidermis. With the appearance of erythema, intracellular oedema in the epidermis and minimal leucocyte migration in the dermis may be observed. By 24 h after the exposure, cellular degeneration is evident in the epidermis which, with minimal erythema, is restricted to individual cells scattered throughout the upper stratum spinosum. With more severe reactions, the number of damaged cells is increased and basal layer cells may be involved. Complete epidermal necrosis is seen in blistering reactions and dermal connective tissue damage may be evident. Epidermal regeneration, as indicated by increased numbers of mitotic figures in the basal layer, occurs by 72 h. The sunburned epidermis is significantly thicker than normal by 6 d, a parakeratotic horny layer being present beneath the still retained, normally appearing original horny layer.

The early electron microscope studies of sunburn have been summarised by Nix (36). Cytoplasmic vacuoles appear in the basal and spinous cell layers shortly after exposure but are no longer observed at 72 h. Wilgram *et al.* (37) have described a decrease in number of keratinosomes in the upper malpighian and granular layer cells as early as 2 h after exposure, but no other changes are observed until 12 h when irregular dense bodies, presumed to be glycogen deposits, appear in the cytoplasm of the basal cells. Increased numbers of thickened tonofibrils are observed in the granular layer at this time. At 72 h, the granular layer is thickened and the cells contain numbers of vacuoles and irregular dense bodies. A parakeratotic horny layer, containing well preserved melanin granules and showing evidence of disordered keratinisation, is present between this granular layer and the original horny layer. Nucleolar enlargement, generally accepted as an indication of increased protein synthesis, in spinous cell nuclei, is a major feature of the E.M. picture at 72 h.

The isolated, damaged cells, scattered throughout the spinous cell layer and shown by light microscopy to have pyknotic nuclei and shrunken, deeply staining cytoplasm, have been studied at the ultrastructural level (37, 38). These so-called sunburn cells (39) the most specific feature of moderately sunburned epidermis, may be dyskeratotic cells, pushed into premature, and therefore abnormal keratinisation. Alternatively, they may be the epidermal equivalent of the general phenomenon known as 'apoptosis' or individual cell shrinkage necrosis (40, 41) by which damaged cells are removed from the general population and a stable cell population is maintained.

Histochemical studies of sunburn have mainly demonstrated secondary effects and have not been useful in determining the primary site for uvr damage in skin (42). Changes in enzyme activity generally and glycogen deposition (35) appear to be secondary events and decreased nuclear staining in epidermal cells of uv-irradiated skin is not evident until some 24 h after exposure (34, 43) and may represent a stain dilution effect as a result of nuclear swelling, rather than any photochemically induced alteration in DNA structure. There are exceptions to this general rule however. Autoradiographic studies have shown a uvr induced inhibition of DNA, RNA and protein synthesis in skin within the first hour after exposure (44). It may be inferred from these results that DNA and RNA are directly affected in skin by uv-irradiation *in vivo*. An earlier study (45) had demonstrated 'light labelling' with 3H-thymidine in skin irradiated *in vivo*, an indication of the

repair process by which uvr-induced thymine dimers are removed from DNA (46). This is perhaps the first report to show that DNA is a direct target for skin reactions to uvr and it may be inferred from the results that thymine dimers are the molecular lesions involved. In addition, fluorescent antibody techniques, using antibodies raised against uv-irradiated native DNA, have been used to demonstrate damage in DNA of skin irradiated *in vivo* (47, 48). As thymine dimers are the most prominent of the DNA photoproducts, these studies can be interpreted as demonstrating, although again indirectly, that thymine dimers are formed in skin irradiated *in vivo*. Wavelength dependency studies of this reaction show that it is restricted to the sunburn region of the uv-spectrum (49). Changes in intensity and distribution of the staining reaction for acid phosphatase activity in uv-irradiated skin have been interpreted as evidence that the lysosome membrane is a primary target for uvr damage and that lysosome enzymes released as a consequence of this damage may be involved in the secondary processes of sunburn (39, 50). The decrease in keratinosome number reported by Wilgram *et al.* (37) appears to support this hypothesis, but Honigsmann *et al.* (51) could find no evidence of early lysosome membrane disruption in epidermal cells of uv-irradiated skin using ultrastructural techniques.

BIOPHYSICS AND BIOCHEMISTRY

A specific free radical signal is produced in lightly pigmented skin on exposure to sunburning uvr (52) but the chemical nature of this excited molecular state has not been identified. Lipid peroxide, possibly derived from free radical induced reactions in unsaturated fatty acids and relatively long lived, has been identified in uv-irradiated skin (53, 54). Lipid peroxides have considerable biological activity which includes the inhibition of respiration and glycolysis, oxidation of sulphydryl groups and destruction of anti-oxidants such as vitamin E. Moreover, lysosome membranes are particularly susceptible to labilisation by lipid peroxides which have been incriminated in cell necrosis in carbon tetrachloride damaged liver and in inflammation of the intestinal mucosa. Peroxides isolated from uv-irradiated skin have been shown to be potent carcinogens (55) but the role, if any, of these compounds in uvr inflammation has still to be defined.

Under ideal conditions, the action spectrum for a photochemical reaction should fit the absorption spectrum for the molecule involved. In the photosensitive porphyrias the action spectrum for the abnormal response fits the soret band absorption characteristic of the porphyrins. The finding of a peak of erythemal activity at around 260 nm and general activity at longer wavelengths has focused attention on nucleic acids and proteins as the primary target molecules for skin reactions to uvr. However, skin is so heterogeneous a medium, uvr energy may be absorbed and dissipated as harmless quantities of heat or as fluorescence, that such a treatment is hopeful if not naive. The action spectrum presented by Everett *et al.* (19) might well be interpreted as representing an absorbance in unsaturated fatty acids to initiate the reaction, deviations from the shape of a smooth absorption curve being explained in terms of the filtering effects of the horny layer. Nonetheless, DNA appears to be the major molecular target for uvr lethality and mutagenesis in bacteria and mammalian cells in culture (56, 57, 58). Although DNA-protein binding may be involved in these effects, the most prominent and contributory molecular lesion is the cyclobutane type dimer formed between adjacent thymines on a single strand of the DNA double helix. Inhibition of DNA synthesis, cell death and mutation may result directly from the presence of thymine dimers or indirectly through faulty

repair. Excision repair has already been mentioned and is the best known mechanism in both bacteria and mammalian cells. It is a multi-enzyme process in which a segment of the DNA strand containing a dimer is removed and repair synthesis, based on the template of the sister strand, fills in the gap. The process is completed when the end of the newly synthesised segment is joined into the parent strand. A second repair mechanism, termed recombinational or post-replication repair (59, 60) involves the filling in of gaps left in daughter strand DNA after replication in which thymine dimers in the template are by-passed by DNA polymerase. A third form, photoreactivation, is a uv-A stimulated, enzymic splitting of the dimers. Although its action is well established in bacteria and most animal cells, higher mammals do not appear to possess the typical photoreactivation enzyme (61). Photoreactivation does not occur in higher mammalian cells in culture but some form of uv-A induced recovery of sunburn damage has been demonstrated, and Sutherland (62) has isolated a photoreactivation type enzyme from human leukocytes.

Until the late 1960's and the autoradiographic studies of Fukuyama, Epstein and Epstein (63), Epstein, Fukuyama and Epstein (64) and Epstein, Fukuyama and Epstein (45), few studies of the possible involvement of nucleic acids in the reactions of skin to uvr had been attempted. Tickner (64), although demonstrating a decrease in phospholipid content, a finding which drew attention to the uvr effects on cellular membranes, could find no change in DNA or RNA content of mouse skin exposed *in vitro* to massive doses of uvr. This is perhaps not surprising in view of the now known photochemistry of the nucleic acids. Baden and Pearlman (66) foreshadowed the autoradiographic studies by using chemical extraction methods to demonstrate that synthesis of RNA and protein is inhibited in mammalian epidermis shortly after uv-irradiation *in vivo*, recovering by 24 h and being increased at 72 h after exposure. A similar study of DNA synthesis (67) shows it to be inhibited shortly after exposure, the degree of inhibition and its duration depending on the exposure dose.

In 1970, Pathak and his co-workers reported the first isolation of thymine dimers from uv-irradiated skin, confirming this report later and showing that the effect was restricted to the sunburning wavelengths of uvr (68, 69). Bowden *et al.* (70) isolated thymine dimers from uv-irradiated mouse epidermis and appear to have demonstrated a form of repair replication to take place, although excision repair in rodent cells is thought to operate only at very low efficiency, if at all. Cooke and Johnson (71) have also isolated thymine dimers from mouse skin exposed to uvr *in vivo* but could find little evidence of excision repair, the dimers being retained in the skin for at least 24 h. Cooke's extensive studies of DNA involvement in mouse skin reactions to uvr have failed to demonstrate a clear cut relationship between thymine dimer formation and inhibition of DNA synthesis or the vascular reactions produced. For example, the dimer yield after exposure to 'sunlamp' fluorescent tube irradiation, peak emission around 315 nm, is very low but severe skin reactions are elicited and inhibition of DNA synthesis appears relatively efficient.

The involvement of DNA in human-skin reactions to uvr might be inferred from the findings that repair processes may be deficient in cells from patients with the various forms of Xeroderma Pigmentosum (72, 73, 74). The major feature of this autosomal recessive disease is an abnormal skin sensitivity to sunlight. Increased epidermal damage and accelerated development of the chronic effects of exposure such as premature ageing and cancer of the skin appear to be magnified normal skin responses to uvr, and for these reactions DNA may well be the primary molecular target. However, the vascular reactions

to uvr in Xeroderma Pigmentosum differ from those of normal skin, the abnormality is not simply a magnification of the normal response and the inference that uvr erythema is mediated through damage to DNA cannot be drawn. Similarly, the vascular reactions in the photosensitised response of 8-methoxypsoralen, almost certainly resulting from damage to DNA, differ from the normal uvr erythema (75, 76). At the present time, there would appear to be no involvement of DNA in the uvr induced lesions of photodermatoses such as polymorphic light eruption and actinic reticuloid (77).

PHARMACOLOGY

Ultraviolet erythema has come to be regarded as a typical inflammatory state (78, 79) although the degree of leucocyte migration is significantly less than in other forms of inflammation. Lewis (80) likened sunburn to the reaction obtained with histamine and suggested that the famous 'H' substance was the mediator involved. Histamine depletion and anti-histamine administration prior to irradiation fail to modify the uv-erythema however (81, 82). Serotonin may have a role in rat skin reaction to uvr (83) but does not appear to be involved in human erythema (84). Increased concentrations of vasodilator substances have been detected in blood and skin of uv-irradiated animals (1) but the kinins observed by Epstein and Winkelmann (85) in the perfusate of irradiated human skin are apparently not involved in the delayed erythema reaction. Greaves and Sondergaard (86) reported the presence of a prostaglandin-like substance in dermal perfusates of uv-irradiated human skin, the time course of its appearance approximating that of the erythema. Mathur and Gandhi (87) described an increase in prostaglandin content of rat skin after uv-irradiation and indomethacin, a potent inhibitor of prostaglandin synthesis, appears to prevent the development of uvr erythema (88, 89, 90). Greaves and his coworkers have recently shown that Prostaglandin E_2 is the major active constituent of uv-irradiated skin, but the site of its derivation has not been ascertained. If it is produced mainly in the epidermis, either as a result of photochemical changes in cellular fatty acids or by a process initiated by lysosomal enzymes, it may well be the mythical, diffusible mediator beloved of this field and so elegantly discussed by Leun (13).

CHRONIC EFFECTS

The long-term effects of repeated exposures to high intensity sunlight have been well reviewed by Blum (91), Epstein (92) and Urbach, Epstein and Forbes (93); a good summary is presented in the book of Magnus (4). The major change observed in the exposed skin of lightly pigmented peoples is an accelerated ageing. The skin loses its natural elasticity, there is marked epidermal atrophy and increased amounts of mucopolysaccharides are found in the dermis, along with deposits of an elastotic material. Focal, benign abnormalities of keratinisation develop to form small, crusty lesions called solar keratoses and these may be succeeded by basal and squamous cell carcinomas. Evidence for the role of solar uvr in skin carcinogenesis in humans is entriely epidemiologic but convincing. The majority of skin cancer, where no other direct carcinogenic influence is known, is found in the sun-exposed skin of lightly pigmented people and is rare in Negroids. There is an approximate correlation between the incidence of skin cancer and the degree of insolation in similar populations living in different areas and tumours develop mostly in areas of the skin most directly exposed to sunlight. This epidemiologic evidence is supported by data from numerous studies with experimental animals.

The carcinogenic action of uvr appears to derive from both the mutagenic effects and the hyperplasia inducing action. The uvr wavelength region involved is basically similar to that for uv-erythema but may well differ in detail and there is little reason to suppose that the mechanisms involved in uvr carcinogenesis are simply those in uv-erythema production repeated over a long term.

PROTECTION

The endogenous mechanisms for protection against the effects of uvr on skin may operate in three distinct ways. In the first place, uvr damage in the skin may be rapidly repaired: the lack of repair is held responsible for the increased sensitivity seen in Xeroderma Pigmentosum. Secondly, physiological processes may be present by which the energy of excited state molecules or the reactive oxidation products of uv-irradiation may be quenched or harmlessly dissipated. Natural anti-oxidants such as alpha-tocopherol may act in this way. However, the natural carotenoids do not appear to be effective in the normal sunburn reaction (94). The third and most obvious mechanism involves the attenuation of the incident radiation between the skin surface and the molecular target in the skin.

HORNY LAYER

In lightly pigmented skin, the major contribution to this attenuation comes from the horny layer of the epidermis. The multi-faceted surfaces present in this dead cell layer produce considerable reflection and scattering and the dense protein content provides a filtering effect by absorption. The importance of this attenuation is demonstrated by the difficulty experienced in producing uv-erythema in palmar or plantar skin, by the increase in intensity of erythema obtained in skin from which the horny layer has been removed, and by the fact that in the amelanotic skin of Vitiligo and Albinism thickening of the horny layer affords some protection against uvr damage. Bachem (23) showed that only some 15% of incident 280-nm radiation penetrated a 0·08-mm thickness of fair-skinned horny layer. At 300 nm, a value of 34% was obtained, while 80% of 400-nm radiation penetrated to the Malpighian layer. These results are typical of many obtained in studies designed to assess the protective function of the horny layer or to determine the anatomical site of uvr action in skin. Miescher (95) showed that the penetration of 297–320 nm radiation could be described as an exponential extinction with a half-value layer of about 9 microns.

UROCANIC ACID

The role of urocanic acid as a natural sunscreen for human skin remains debatable (96, 97, 98, 99, 100). This metabolite of histidine, found in human sweat and at higher concentrations in the epidermis, has an absorption spectrum which extends into the sunburn uvr. In unexposed skin, it is present mostly in the cis form, uv-irradiation resulting in a cis-trans isomerisation. It is this isomerisation with the absorbed energy being dissipated in the reverse direction which may account for any protective role ascribed. The urocanic acid content of epidermis in skin which has been previously exposed to uvr and shows a decreased sensitivity through accommodation, is found to be increased and it may be that the contribution of this protective mechanism to the overall decrease in sensitivity

of accommodated skin is greater than that of the protein constituents of the horny layer.

MELANIN

The major contributory factor in the natural protection of human skin against uvr is the complex, indole type polymer called melanin. The relationship between sunlight and melanin pigmentation has been most recently reviewed by Pathak *et al.* (9), and Quevedo *et al.* (101), Szabo (102), and Pathak (103) present good coverage of the subject matter as known at that time. The development of a suntan in normally lightly pigmented skin, resulting from a uvr-stimulated redistribution of preformed melanosomes in the epidermis and the tyrosinase controlled production of melanised melanosomes in the melanocytes with subsequent transfer to and distribution in the keratinocytes of the hyperplastic epidermis, obviously affords protection against further uvr exposure. The genetically controlled variation in number, form and distribution of melanosomes, mainly responsible for racial variation in skin colour, results also in variations in the skin responses to uvr. In fair-skinned Celtic peoples, Albinos and in vitiliginous skin, where little or no melanin is present, the skin reactions are severe. As the degree of melanisation increases, so the intensity of skin reactions decreases and in deeply pigmented Negroids and Australoids, it is difficult to elicit reactions other than the IPD and skin cancer due to uvr does not occur. It is thought that the racial variation in human skin colour may represent an evolutionary compromise between the need to protect against the carcinogenic action of uvr and the requirement of photochemically-produced vitamin D in the viable layers of the epidermis (104, 105). The transmission of 280–320 nm radiation through negro horny layer is significantly less than through that from white skin, but there is no significant difference in the thickness of this layer (106, 107). The only difference appears to be the presence of melanin as demonstrated by Kligman (108).

The mechanisms by which melanin exerts its protective function apparently vary. It may act as a neutral density filter in the outermost layers of the epidermis where it is present in a disperse, amorphous form. The absorbed energy is dissipated harmlessly as heat. Moreover, in this form it acts as an excellent scattering screen for the wavelengths involved. The scattering produced by the larger melanin granules, usually observed in a supranuclear distribution in the basal and suprabasal layers, is thought to involve a high degree of forward direction, and if this is so the function of the superanuclear caps in protecting the germinal DNA is a little difficult to accept. Stable free radicals are present in the melanin polymer and may act as electron traps in a uvr-excited system. However, the spatial relationship between the potentially susceptible biological substrate, for instance DNA, and the melanin would have to be rather more intimate than it appears in electron microscope pictures for this protective mechanism to function efficiently. It is also thought that melanin may act as a semi-conductor material, in this way diverting electron energy away from susceptible biological material. The exact mechanisms involved in melanin protection against uvr damage, apart from the action as a filter, are obviously not as yet worked out, but it would seem that they do exist and are effective.

CONCLUSION

In summary, the reactions of human skin to the uvr component of solar radiation may vary according to the intensity and wavelength of the incident radiation from the mildest

form of inflammation to severe blistering and necrosis. With repeated exposures, premature ageing and cancer of the skin may develop. The skin is probably a multi-target system for uvr effects and natural protection against these effects resides predominantly in the melanin content of the skin.

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Notice

10TH IFSCC CONGRESS

SYDNEY, AUSTRALIA 24–28 OCTOBER 1978

For the first time in its twenty-year history, the International Congress in 1978 will be held in the Southern Hemisphere, in Sydney, Australia. The major activity of the 10th Congress, to be held from 24–28 October 1978 will be the presentation of *original* papers relating to scientific and technological advances in cosmetics and toiletries. The emphasis will be on current and future trends under the theme: '*Cosmetic Horizons*'.

To have been eligible for consideration for the 1978 Awards, authors must have submitted titles and 400 word synopses to the Scientific Director, to be received by him, in Australia by 25 January 1978: Dr R. O. Hellyer, Scientific Programme Director, 10th IFSCC Congress, Box 2609 G.P.O., Sydney NSW, Australia 2001.

The Programme

Scientific Sessions

Wentworth Hotel Congress Centre, 25 October through 27 October 1978. Instantaneous interpretation from and to the official languages, English, French, German and Spanish. Original papers will be presented on the following topics: sunscreening and the effect of climate on skin, dermatological topics, perfumery ingredients and formulations, new products and raw materials.

Social Programme

Relaxed, informal, freiendly programme of events and functions, including: reception at the famous Sydney Opera House; Australiana Barbecue and Award Presentation; excursions to Sydney's surfing beaches, koala park, the Blue Mountains, to mention but a few places of scenic and historic interest.

Travel

For information on package tours, contact your local Society; details are available from the British, French, Swiss, American and Chilean Societies. For advice and assistance on travel and pre- and post-touring, contact your nearest office of QANTAS, the appointed Congress airline.

How to Register

Registration forms and Advance Programmes to be distributed in early 1978. Send your name and address for addition to the mailing list *now* to: The Congress Secretariat, GPO Box 2609, Sydney NSW, Australia 2001.

Starting on 6 November 1978, a week after the Congress, the *6th National Convention* of the *Royal Australian Chemical Institute* will be held at Surfers Paradise, Queensland. In addition to Plenary Sessions, most of the ten Disciplinary Divisions of the RACI will be holding specialist symposia.

For further information contact: 10th International Congress of Cosmetic Chemists, Office of the Secretariat, GPO Box 2609, Sydney NSW, Australia 2001 or IFSCC Office, 56 Kingsway, London WC2B 6DX, England.

J. Soc. Cosmet. Chem. **29** 47–48 (1978)

Book reviews

ADVANCES IN MODERN TOXICOLOGY VOL. 4 —DERMATOTOXICOLOGY AND PHARMACOLOGY

Eds. F. N. Marzulli and H. I. Maibach. Hemisphere Publishing Corporation

JOHN WILEY & SONS (1977). £26.00.

At last there is a book dedicated to the toxicology of skin. Although there are numerous texts on clinical dermatology, skin histopathology, the physiology, biochemistry and biophysics of the skin and even the occasional book on the pharmacology of the skin, never before has there been a single volume directed at the interests of those concerned with predictive toxicology. The Editors, Marzulli and Maibach, need no introduction to readers of this Journal, their experience and expertise are formidable and cover both clinical and experimental aspects of skin research.

The book contains 23 chapters each with its own list of references. The choice of authors for each chapter has been selective and the resulting amalgam has justified what must have been a big effort by the Editors.

For a book written with the toxicologist in mind, the chapter on 'Cutaneous Carcinogenesis' is rather abbreviated, but this brief treatment of the subject may be justified on the grounds that there are several other texts on the subject.

This book will fill an important niche in the office, laboratory or library of every person with a responsibility for safety evaluation of cosmetics and toiletries. Some of the figures, particularly the photomicrographs, would have been greatly improved by the use of colour and enlargement, but both of these would have increased the cost of the book, but at £26.00, just over £1 per chapter, it represents excellent value. The publishers have produced the book in a sensible, easily readable, format and it should withstand the extensive handling that its interest will engender.

V. K. BROWN

MARTINDALES EXTRA PHARMACOPOEIA

27th edn.

Ed. A. Wade. Asst. Ed. J. E. F. Reynolds. Pharmaceutical Press (1977). £30.00.

'Martindale' is surely well known to and much used by virtually every scientist in cosmetic R & D, even if it is primarily aimed at practising pharmacists and medical practitioners. We have come to appreciate its extraordinarily wide coverage, including a great number of ingredients actually or potentially suitable for inclusion in cosmetics and toiletries; the editorial lay-out giving information on both technological and biological features along with a range of abstracts of original papers, provides a unique source of everyday reference to aid formulators, analysts, toxicologists and others.

The first 'Martindale' appeared in 1883; in its 27th edition it is now thirty times the size of the original. Since commendable efforts have been made to present each item as concisely as possible, this is an interesting demonstration of the expanding universe of scientific information. It could also mean that finding the desired piece of information on a particular occasion is liable to become increasingly more difficult with each new and bigger edition. The editorial staff are clearly aware of the

problem and it would be difficult to find an encyclopaedic reference work with an indexing system to compare with 'Martindale'. Notwithstanding this provision, every copy is accompanied by a special card headed 'A guide to the use of Martindale – The Extra Pharmacopoeia', briefly explaining the system underlying the text and showing how various kinds of information may be located.

Looking through the latest volume for changes to distinguish it from its forerunners, its more international character becomes apparent. In the section headed 'Dermatological Agents,' a noticeable addition is a monograph dealing with tretinoin (retinoic acid) based on the U.S.P. and accompanied by abstracts from over 20 original references. The section for 'Supplementary Drugs and Ancillary Substances' includes various items of possible cosmetic interest such as sebacic acid and methylene chloride. Often this section is a repository for drugs that have ceased to justify their former acclaim as valuable medicaments; for example, it was a little saddening, in a nostalgic reflection on the origins of chemotherapy, to find the asaphenamines now relegated to supplementary or ancillary status. Of course, this should really be seen as a natural consequence of major developments in therapy during recent years, for which we should all be duly grateful. However, mention of this transfer of an item from one section to another prompts a question on the selection of Chapter Titles for successive editions of 'Martindale'; this has always seemed oddly arbitrary and still does seem so! Admittedly, though, it is virtually impossible to envisage any better arrangement.

Recognising the state of confusion in recent years over the safety-in-use of sucrose substitutes and synthetic colourants, it was intriguing to see how editorial policy coped with items like cyclamate, saccharin, amaranth and tartrazine. The relevant monographs give the essential facts in words delicately chosen to avoid offending readers in countries where attitudes to these controversial matters differ from those currently held in the U.K. This Chapter is immediately followed by one dealing with Fluorides; the important compounds are adequately, though briefly, discussed and principal references quoted but, in addition, the new Edition includes well chosen abstracts dealing with water fluoridation (including some indication of objections and alternatives).

It is tempting to thumb the pages of a new 'Martindale' picking out topics in which one is interested, looking to see the terms in which they are discussed, whether problem areas are mentioned and familiar references quoted. Of toiletries concern there are, for example, the aluminium and zirconium salts, hexachlorophene, chlorhexidine, organic mercurials, non-ionic surfactants. All receive attention and there is little reason to argue over what is or is not included. In general, technological claims made in Patents but not substantiated in the scientific literature are omitted; as such claims are not always well-founded, the editorial policy cannot really be criticised though it is probably true that many formulators obtain a considerable amount of helpful guidance from the Patent literature. With this reservation, the choice of references appears to be remarkably good considering the enormity of the task and also remarkably up-to-date.

The latest Edition of 'Martindale' is a competent production; in providing a reliable source for an enormous range of information, the technical feat of compilation and presentation could hardly be surpassed. Congratulations are due to the Editor and his staff and to the Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain.

N. J. VAN ABBE

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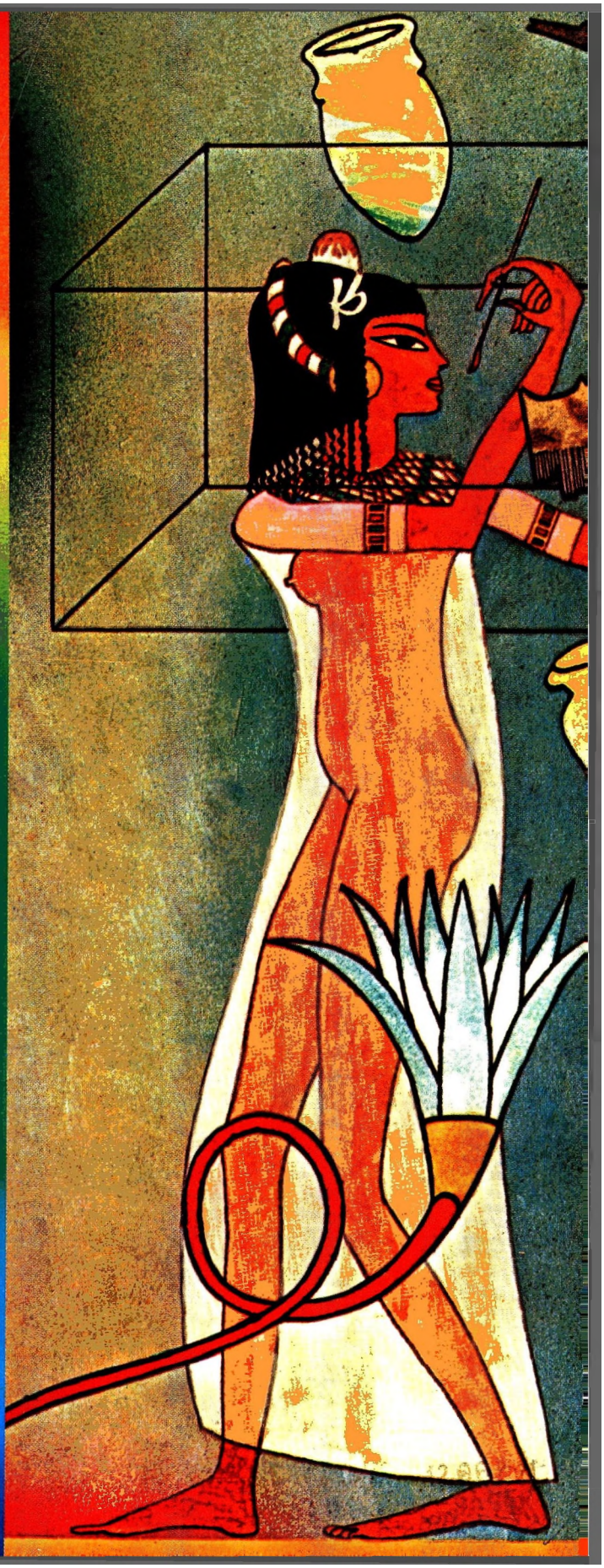

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