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Detection of environmental depigmenting substances

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We systematically screened the depigmenting capacity of several phenols, catechols and organic antioxidants. Clear-cut depigmentation was achieved with monomethyl ether of hydroquinone (MMH) and tertiary butyl catechol (TBC) using black guinea pigs and black mice as animal models. A goal was to establish a reliable *in vivo* method to demonstrate or to predict the depigmenting action of chemicals on mammalian melanocytes. There was no universal solvent or optimal body site, although all tested areas could be depigmented. Irritation induced by some vehicles and test materials produced false positive responses. False negative responses with known depigmenting chemicals were observed. Utilizing these observations, we propose a model for screening medicinal and industrial chemicals for depigmenting capacity.

Key words: Alkyl phenols – animal model – antioxidants – depigmentation – occupational leukoderma – phenolics.

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The depigmenting action of alkyl phenols has been firmly established (Calnan 1973, Hara & Nakajima 1969, Malten et al. 1971, Riley 1971). Clinical and experimental studies have corroborated that certain phenols and catechols can induce leukoderma on human skin of all races (Babanov & Chumakov 1966, Bleehen et al. 1968, Brun 1960, Calnan & Cooke 1974, Gellin et al. 1970a, Jimbow et al. 1974, Kahn 1970). The pattern may be indistinguishable from vitiligo (idiopathic acquired leukoderma (Bentley-Phillips 1974, Brun 1972, Dogliotti et al. 1974, Lerner 1971). Nonhuman mammalian hair and skin may also be depigmented (Bleehen et al. 1968, Brun 1960, Frenk & Ott 1971, Gellin 1975, Jimbow et al. 1974, Shelley 1974). For example, p-tertiary butyl catechol (TBC), an industrial antioxidant, depigments black guinea pig skin within 2–4 weeks of daily application, but not without variable antecedent inflammation, when the concentration and solvent are appropriate (Gellin et al. 1970b).

Earlier studies were expanded to answer the following questions:

1) Can a model *in vivo* system be established to reliably demonstrate the depigmenting capacity of known and suspect depigmenting chemicals?
2) Is there a universal solvent system for testing a variety of chemicals for their depigmenting action?
3) Can an optimal anatomical site be identified to simplify future similar investigations?
4) Can the mechanism involved in the pathogenesis of chemically-induced leukoderma be further elucidated?
5) Is there a standard concentration applicable to all test substances to induce depigmentation?

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6) Can the induction of depigmentation at one test site lead to pigment loss at distant untreated body sites?

Material and Methods

Guinea Pigs

A total of 158 randomly bred adult male and female black guinea pigs were studied. Groups of two to five guinea pigs were used for testing individual chemicals. Most observations were based on groups of five guinea pigs. Chemicals studied included industrially or commercially used phenols and catechols, their congeners, and antioxidants used as food preservatives. All animals were housed in individual cages and maintained on a commercial guinea pig diet. Adults averaged 675 g in weight.

Chemicals tested were: butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), catechol, monobenzyl ether of hydroquinone (MBEH), phenol, o-phenyl phenol, p-phenyl phenol, n-propyl gallate, p-

Table 1. Criteria to assess irritancy and depigmentation

A. *Scoring of Irritancy*

Observed reaction	Assigned gradation	Degree of irritancy
No reaction	0	Non-irritating
Erythema* and scaling	1+	Mildly irritating
Erythema and edema beyond area of application	2+	Moderately irritating
Eschar formation	3+	Severely irritating

* Erythema was difficult to detect on gray-black skin of the guinea pig.

B. *Scoring of Depigmentation*

Visual criteria	Assigned gradation	Depigmenting potency
No visible depigmentation; skin color similar to that of control areas	0	Absent
Small spots or speckles of depigmentation	±	Definite, but weak
Uniform hypopigmentation	+	Definite, but moderate
Complete depigmentation	++	Very strong

These criteria were adapted after Bleehen et al. (1968)

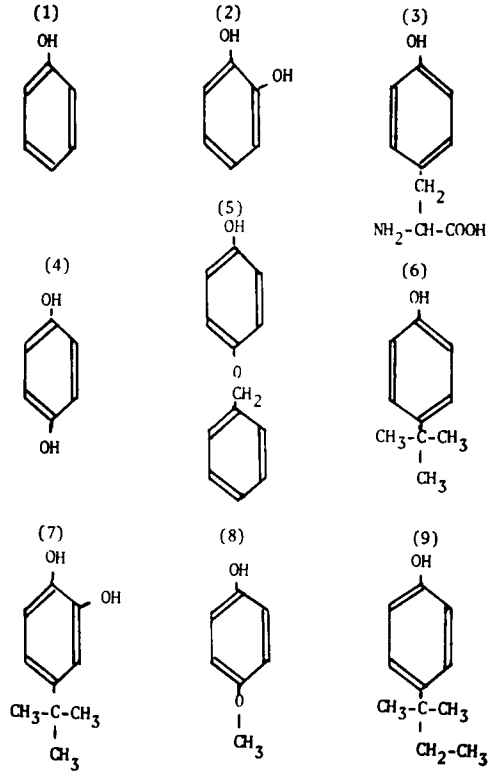

Fig. 1. Selected phenolic compounds including potent depigmenters. (1) Phenol (2) Catechol (3) Tyrosine (4) Hydroquinone (HQ) (5) Monobenzyl ether of hydroquinone (MBEH) (6) P-tertiary-butylphenol (TBP) (7) P-tertiary-butylcatechol (TBC) (8) Monomethyl ether of hydroquinone 4-hydroxyanisole (MMH) (9) P-tertiary amyl phenol (TAP).

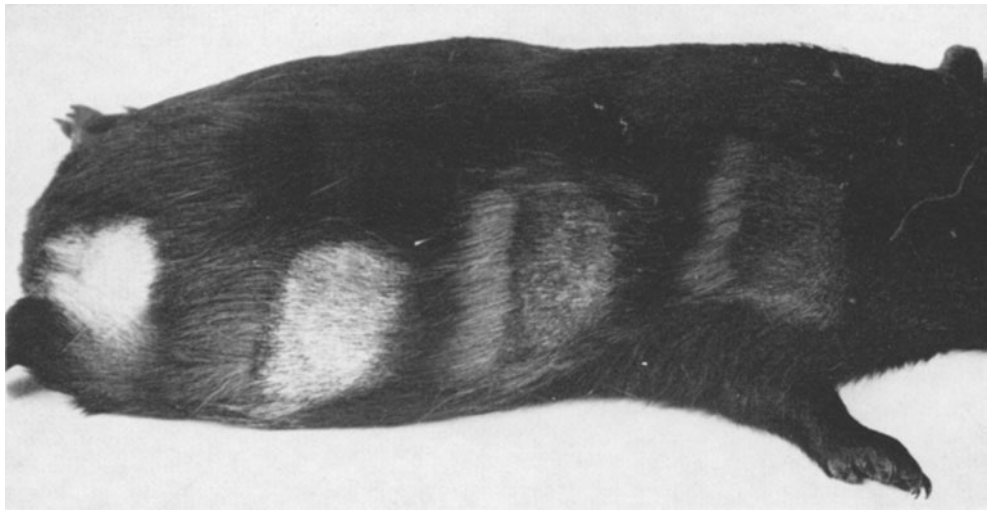

Fig. 2. Back of black guinea pig after 122 days of application of MMH in acetone in different concentrations. From left to right: 1.0 M, 0.5 M, 0.25 M, 0.1 M.

tertiary amyl phenol (TAP), p-tertiary butyl phenol (TBP), p-tertiary butyl catechol (TBC), isopropyl catechol (IPC), hydroquinone (HQ), mono-methyl ether of hydroquinone or p-hydroxy anisole (MMH), nordihydroguaiaretic acid (NDGA), dilauryl thiodiproprionate, nonyl phenol, octyl phenol, tocopherol (D-alpha tocopherol acid succinate), ethoxyquin, gum guaiac, diethylamine hydrochloride, and octyl gallate (Fig. 1).

Fig. 3. Ear of black guinea pig after 122 days of application of 1.0 M MMH in acetone. Acetone control on opposite ear.

The following solvents were used: acetone, petrolatum, hydrophilic ointment, Eucerin, dimethyl sulfoxide (DMSO) in concentrations of 100–30 %, propylene glycol, chloroform, and ethyl alcohol in concentrations of 100, 95 and 70 %.

The dorsal surface of the guinea pig was epilated weekly with an electric shaver. Eight dorsal sites, 3 cm × 3 cm, and the unepilated skin of ears and nipples were used. Each test area received 0.1 ml aliquots with either a micropipette or syringe. The solvent control sites included at least one sector on the back, an ear and one nipple. The test material was rubbed in with a glass rod or rubber-gloved finger. Applications and observations were made each weekday for 1–6 months. If significant irritation developed, application was curtailed until irritation subsided.

Chemical concentrations ranged from 0.01 M to 1.0 M for liquid solvents. Most

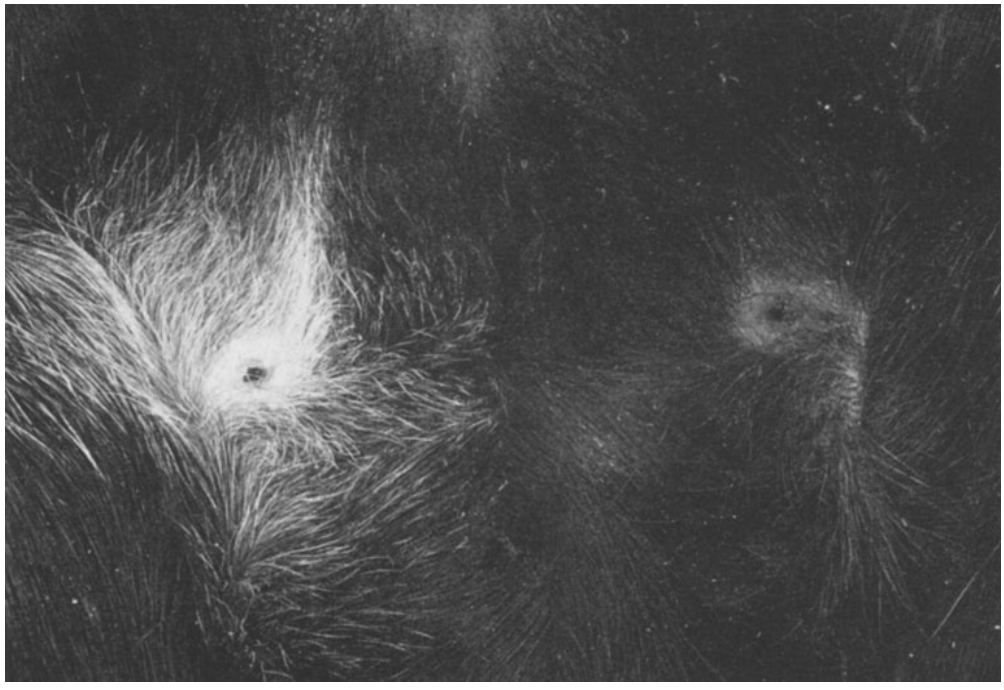

Fig. 4. Nipple of black guinea pig after 73 days of application of 0.1 M MMH in acetone. Acetone control on opposite nipple.

observations were on the molarities of 0.1, 0.25 and 0.5 M. For solid ointment bases, concentrations ranged from 0.1 to 10 %.

Table 2. Regional variation of maximal depigmenting activity of selected alkyl phenols in the black guinea pig

Chemical	*Body Site**		
	Back	*Ear*	*Nipple*
MMH	++	++	++
TBC	++	++	++
TAP	++	±	0
MBEH	0	±	++
HQ	+	±	+
TBP	+	+	0
Phenol	+	+	0
Catechol	+	+	+

* See Table 1 for scoring of depigmentation. Each data point represents the average of a minimum of five animals.

Most observations were based on concentrations of 1, 5 and 10 %.

The criteria for assessing depigmentation and irritation, as visually observed, are listed in Table 1.

Interval punch biopsies were performed on treated and control sites. The tissues were cut for light microscopy and stained with hematoxylin and eosin (H & E) and silver (Fontana).

Mice

A total of 240 black adult mice were used for comparing depigmenting potency as observed in guinea pigs, by using known depigmenters. Groups of 10 were tested for each substance and for solvent controls. The mice were housed five in a cage.

The chemicals compared were TBC, TBP, HQ, octyl phenol, MBEH, and o-

Table 3a. Vehicles and degree of irritation in association with complete depigmentation (++ gradation) at different body sites in the black guinea pig produced by selected alkyl phenols

Chemical	Site	Minimal eliciting concentration and vehicle	Grade of irritation #	Minimal interval in days for complete depigmentation to be observed
MMH	Back	0.25 M acetone	1	33
		0.5 M DMSO	2	18
		10 % hyd. ung.*	2	12
	Ear	1.0 M acetone	2	62
		5 % hyd. ung.	2	33
	Nipple	0.25 M acetone	1	49
TBC	Back	0.5 M PG**	3	64
		10 % hyd. ung.	3	29
	Nipple	0.25 M acetone	2	49
		5 % hyd. ung.	3	29
	Ear	1.0 M acetone	2	20
TAP	Back	0.25 M DMSO	2	18
MBEH	Nipple	0.5 M PG	2	45

* hyd. ung = hydrophilic ointment, USP
** PG = propylene glycol
# See Table 1 for gradation of irritation
Each data point represents the average of a minimum of five animals.

phenyl phenol. The concentrations used were 0.1, 0.25, 0.5 and 1.0 M. Solvents used were acetone and DMSO (in 10 % increments) from 30 to 90 %.

Limited by size, the entire dorsal surface of the mouse was the application site. Ears and nipples were not tested. The surface was unepilated, for the skin of black mice is white except for a few black spots. (The hair is black.) Each mouse received 0.1-ml aliquots with a micropipette. Applications and observations were made each weekday for 2–4 months. If significant irritation developed, application was curtailed until irritation subsided.

The criteria for assessing depigmentation and irritation were identical to the guinea pig (Table 1).

Biopsies were performed at the conclusion of treatment. Tissues were cut for light microscopy and stained with hematoxylin and eosin.

Results

Complete depigmentation on all test sites was achieved with MMH and TBC in the black guinea pig (Figs. 2–4 and Table 2). Less pronounced pigment loss was noted with these chemicals in black mice. TAP and MBEH were able to fully depigment only the back and nipple, respectively, of the black guinea pig. Moderate depigmentation followed the application of IPC, HQ, TBP, phenol and catechol (Tables 2 and 3). There was considerable variation in the duration of application to achieve partial or complete depigmentation (Table 3). Not only was this true from one chemical to

Table 3b. Vehicles and degree of irritation in association with uniform hypopigmentation (+ gradation) at different body sites in the black guinea pig produced by selected alkyl phenols

Chemical	Site	Minimal eliciting concentration and vehicle	Grade of irritation #	Minimal interval in days for uniform hypopigmentation to be observed
HQ	Back	0.5 M DMSO	2	30
	Nipple	5 % hyd. ung*	3	15
TBP	Back	0.25 M DMSO	3	23
		10 % hyd. ung.	3	74
	Ear	0.25 M DMSO	2	23
Phenol	Back	0.5 M DMSO	2	43
	Ear	0.25 M DMSO	1	43
Catechol	Back	10 % hyd. ung.	3	46
	Ear	5 % hyd. ung.	2	14
	Nipple	5 % hyd. ung.	2	14
IPC	Back	1 % petrolatum	3	30

* hyd. ung = hydrophilic ointment, USP
# See Table 1 for graduation of irritation
Each data point represents the average of a minimum of five animals.

another, but the concentration and solvent were important variables (Fig. 2).

Irritation was commonly observed with all phenols. It played a role in the production of pigment loss. Solvents affected the degree of irritation produced with and without subsequent depigmentation. For example, catechol and phenol produced uniform depigmentation in hydrophilic ointment and DMSO, respectively. However, no pigment loss was induced with these parent compounds, regardless of concentration, in acetone, DMSO and propylene glycol (for catechol) and acetone, hydrophilic ointment and propylene glycol (for phenol).

No depigmentation was induced with the following substances in black guinea pigs and black mice: BHA, BHT, octyl and propyl gallate, ethoxyquin, gum guaiac, diethyl amine hydrochloride, dilauryl thiodiproprionate, nonyl phenol, o-phenyl phenol, p-phenyl phenol, octyl phenol, NDGA, and tocopherol.

Onset of Depigmentation

The minimum time for the appearance of depigmentation at any site from daily applications was 7 days with 0.5 M TBC in DMSO, 12 days with 0.25 M MMH in acetone, and 23 days with 0.25 M TBP in DMSO – and all on the black guinea pig ear. In Tables 3a and b the minimal interval for complete or maximum depigmentation to be produced by the chemicals studied is recorded. Average figures are given. The longest time required to induce maximal depigmentation in a single guinea pig was 119 days for 0.25 M MMH in acetone on the ear and 112 days for 1 M TBP in acetone on the back.

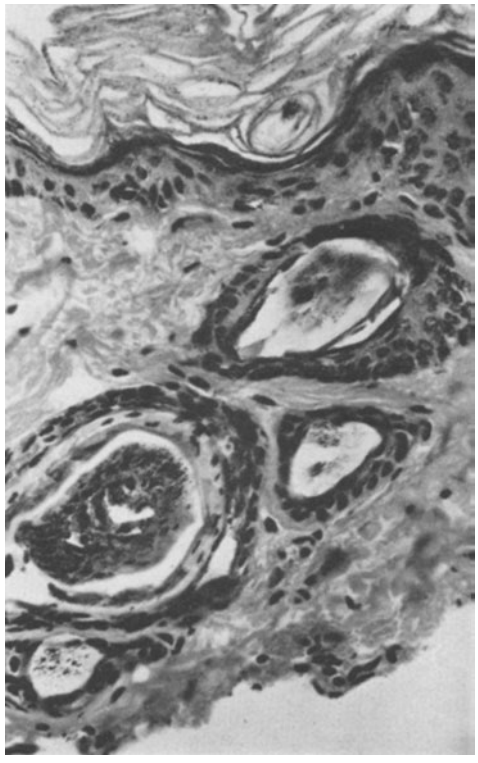

Fig. 5. Back of black guinea pig, untreated control site. 125 ×.

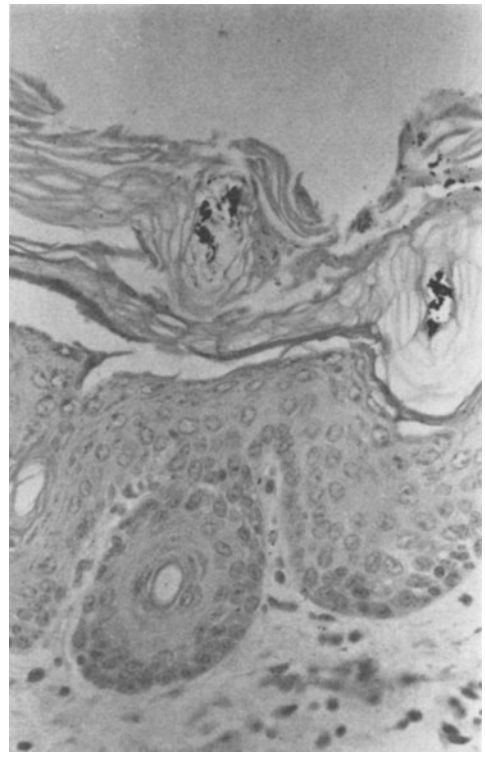

Fig. 6. Back of black guinea pig after 0.1 M TBC in DMSO. 125 ×. Hyperkeratosis, acanthosis and absent pigment in the basal layer, with increase in dermal mononuclear cells.

Depigmentation at Distant Sites

Despite the production of complete depigmentation with MMH in four black guinea pigs, repeated application at the three body sites chosen produced no pigment loss at any untreated area. In these animals depigmentation was first noted at 2 weeks; daily applications were continued for 119–175 days.

Histological Findings

Reduction to complete absence of melanin was noted with H & E and silver stains of skin specimens with clinically observed hypopigmentation and/or depigmentation (Figs. 5–10). Acanthosis was a concomitant finding. An increase of mononuclear-histiocytic cells appeared in the upper and middermis, some with engulfed pigment (melanophages). These findings were similar regardless of the chemical which produced the leukoderma.

Discussion

Confirmation of the depigmenting action of selected alkyl phenols was achieved. Unequivocal leukoderma using several solvents was noted with MBEH, MMH, TAP and TBC.

In view of the ubiquity of organic antioxidants in industry and the home, some of which are chemically similar to these potent depigmenters, a variety were studied. Commercial uses of the phenols and cate-

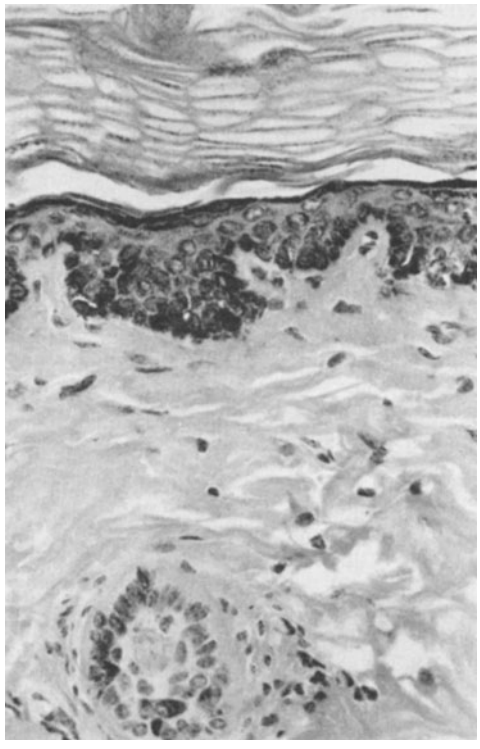

Fig. 7. Ear of black guinea pig, untreated control site. 125 ×.

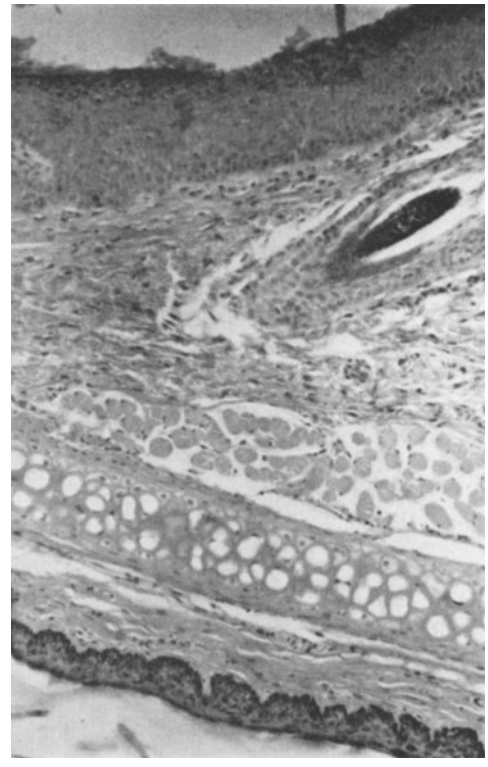

Fig. 8. Ear of black guinea pig treated with 0.25 M TBC in propylene glycol, 100 ×. Hypergranulosus, acanthosis, and absent pigment in the basal layers on treated dorsal aspect. Note normally pigmented untreated anterior surface.

chols in this study include manufacture of paint, plastics and varnishes, soaps and germicidal detergent disinfectants, de-emulsifiers of oil, motor and lubricating oil additives, synthetic rubber manufacture, insecticides and deodorants (Fisher 1976b, Gellin et al. 1970a, Kahn 1970). The antioxidants BHA and BHT are used in topical medications and foods (Fisher 1975, Fisher 1976a, Roed-Petersen & Hjorth 1976). Consumer products containing these substances are adhesive tapes, latex glues, rubber products ("falsies", condoms, stockings, girdles, bandages, cosmetic facial sponges), shoes, and wristwatch straps (Fisher 1976b). BHA and BHT (commonly used in pharmaceuticals and foods) did not induce leukoderma despite varying the concentration, solvent and test site, and continued application up to 10 weeks (Maibach et al. 1975). Failure to depigment mammalian skin was noted with another food antioxidant, n-propyl gallate, which Kahn et al. (1974) reported as a sensitizer.

Since there is widespread use of vitamin E in over-the-counter preparations for oral and topical use, this biological antioxidant was screened. With acetone as the solvent, no depigmentation was induced on the three test sites of the black guinea pig, although moderate irritation was seen. Brun (1960) also failed to induce depigmentation with

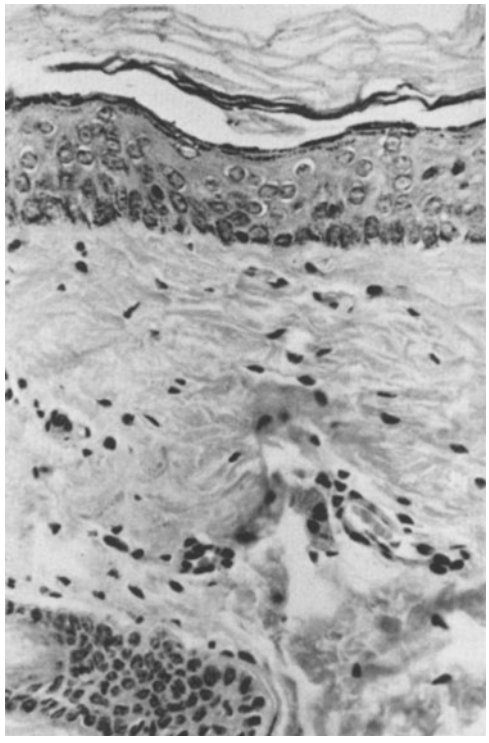

Fig. 9. Nipple of black guinea pig, untreated control site. 125 ×.

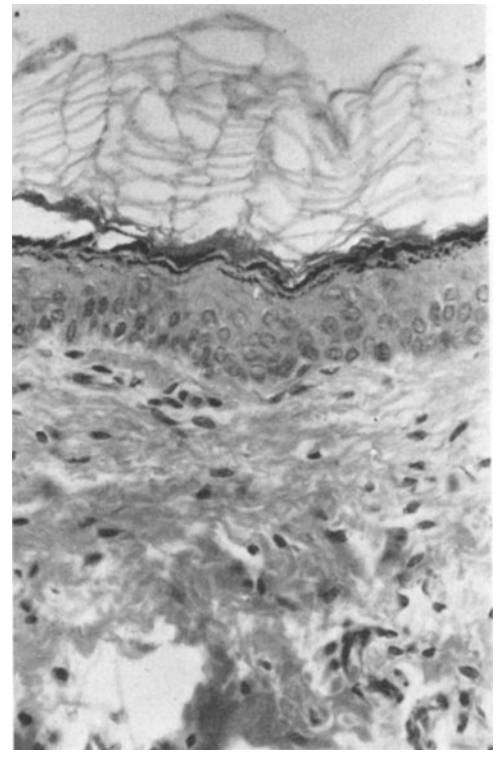

Fig. 10. Nipple of black guinea pig treated with 5 % TBC in hydrophilic ointment, USP, 125 ×. Hyperkeratosis, acanthosis and absent pigment in the epidermis.

tocopherol (vitamin E) on the nipples of brown guinea pigs.

No one solvent could be endorsed as appropriate for all substances. A certain solvent may be irritating with one chemical but not another, i.e. BHA in acetone is highly irritating while BHT in acetone is slightly irritating. As Kaidbey & Kligman (1974) noted in their study of suitable vehicles for topical photosensitizers, each substance has to be approached separately for its optimal solvent, should similar experimental studies be conducted. False positive results, i.e. decreased pigmentation resulting from marked irritation, per se, were seen with catechol, phenol and DMSO in concentrations over 70 %. False negatives or equivocal depigmentation were observed using known depigmenters: MMH in propylene glycol, hydroquinone in acetone and propylene glycol (Arndt & Fitzpatrick 1965, Jimbow et al. 1974); MBEH in acetone (Becker & Spencer 1962, Schwartz 1947, Snell 1964); TBP in acetone and propylene glycol (Babanov & Chumakov 1966, Kahn 1970, Malten et al. 1971); and octyl, nonyl and phenyl phenols in the same solvents (Ikeda et al. 1970, Ito et al. 1968, Malten et al. 1971). The fault lies with the solvent system, or possibly the animal model, per se.

Solvents. Almost all substances were tested in acetone, propylene glycol, hydrophilic ointment and 100 % dimethyl sulfoxide

(DMSO). DMSO was tested in 10 % increments from 30–90 % in mice only.

Acetone is easy to apply, readily absorbed into the skin, and dissolves most lipophilic chemicals. The disadvantages are that it evaporates readily if its container is not tightly closed, thereby raising the molarity of the solute; and that it has given false-negative results with known depigmenters, such as MBEH. MBEH was the first alkyl phenol demonstrated to depigment human skin (Oliver et al. 1939).

Propylene glycol permits some test substances to dissolve in it. It has disadvantages: difficult to apply to animal skin due to its viscosity, not quickly absorbed so that it tends to spread beyond application sites, and more irritating than acetone or hydrophilic ointment.

DMSO is readily absorbed through animal skin. Test materials are easily incorporated into it. Its disadvantages include its offensive smell to the technician, slower absorption on animal skin compared to acetone resulting in some of the solution rolling away from sites of application, and its irritant action, at concentrations of 70 % or more (Kligman 1965).

Hydrophilic ointment is mildly irritating. It is difficult to deliver exact amounts in replicate studies. It tends to spread away from sites of application by animal movements such as rubbing against cages and scratching, and it promotes retention of scales due to its occlusive nature.

Body sites: No one body site seemed preferential. However, nipples and ears were more susceptible than the back to depigmentation for most chemicals. For example, MBEH depigmented the nipples (in propylene glycol) but did not depigment the back (in either acetone or hydrophilic ointment). Depigmentation was induced at all three areas chosen. Brun (1960) studied the nipple almost exclusively in brown guinea pigs. Its histologic similarity to human skin is a major advantage. Most prior investigations have been performed on the back of the black guinea pig or mouse.

Back: Major advantages include the relatively large surface area, which is easily accessible for multiple chemical applications and biopsies. Disadvantages include the need for repeated shaving or epilation, and the confusion to grading that results from remaining hair and the tendency for gray scales to accumulate, mimicking mild depigmentation.

Ear: Its advantages are the absence of hair, simplicity of applying test substances, ease of rating depigmentation and taking biopsies.

Nipple: In addition to its resemblance to human skin, histologically, it is hairless. The disadvantages of using this area are its relative inaccessibility due to location, which is sometimes hidden by hair, and the limitation in the number of chemicals which may be applied – only one to an animal.

The *black guinea pig* is a satisfactory choice for studying chemically-induced leukoderma. The hair and skin are black, and multiple anatomical sites can be chosen for grading a series of dilutions or substances simultaneously. Problems encountered included the limited supply, since they are not widely bred; limitations of space since animals were housed individually; the need for shaving weekly; removal of test substance by shaking, rubbing or chewing off; and all too frequent premature deaths of animals in the first 2 weeks in the laboratory due to respiratory and/or gastrointestinal infection. Up to 20 % died in some lots received from commercial breeders. Salmonellosis affects some strains commonly.

Black mice have the following advantages: they may be housed in groups, are readily obtained from animal supply houses, and require no shaving, and the re-

sults are visually reproducible. Disadvantages of using them include the rubbing off or spreading of applied chemicals; only one substance and/or dilution can be applied to each animal; and the presence of hair may mask readings of irritation (although the hair was black the skin was unpigmented).

Mechanism of action: Chemically-induced depigmentation results from a selective melanocytotoxic action on functional melanocytes (Bleehen et al. 1968, Jimbow et al. 1974, McGuire & Hendee 1971). The potent depigmenters studied have a structural similarity to tyrosine, which is probably relevant (Brun 1972, Riley 1969a) (Fig. 1). Competitive inhibition of the enzyme tyrosinase has been suggested (Denton et al. 1952). These compounds are incorporated into melanogenic cells in culture (Riley 1969b). Riley showed that semiquinone free radicals are formed initiating lipid peroxidation, which is a chain reaction, leading to destruction of lipoprotein membranes of the melanocyte and its consequent death (Riley 1970, Riley 1971, Riley et al. 1975).

Optimal depigmenting action is seen when the 4 (or para) position is hydroxylated and there is a nonpolar side chain in the 1 position on the organic ring (Bleehen et al. 1968).

In vitro studies are being pursued in our laboratory to study isolated black guinea pig melanocytes incubated with the active antioxidants and depigmenters identified thus far. Routine and electron microscopic examinations of melanocytes and keratinocytes harvested *in vitro* are being performed to study these mechanisms further.

In addition to the hazard of skin irritation, sensitization and depigmentation induced by certain phenols and catechols, recent reports from West Germany have suggested that TBP has produced hepatosplenopathy and diffuse thyroid enlargement among workers (Rodermund et al. 1975). The route of exposure imputed was inhalational. The finding of abnormal liver function tests among six men with severe TBP-induced leukoderma has been noted recently (James et al. 1977).

The role of delayed hypersensitivity in chemically-induced leukoderma was explored. Numerous animals that were depigmented were skin tested. No evidence of delayed hypersensitivity was adduced (data unpublished). Further, that melanocytes can be destroyed *in vitro* suggests that sensitization may not be requisite for depigmentation (Mansur et al. 1978).

Proposed Model For Screening

On the basis of these studies, we believe that we know how to avoid certain pitfalls in proposing a model to be used in predictive screening. If many chemicals are to be examined, the back of the black guinea pig allows at least six chemicals to be assayed per animal. Although there is individual variation, five animals per compound would be adequate to identify any of the agents studied here.

Solvent variability was significant. With unknown chemicals we would utilize two solvents, such as acetone and hydrophilic ointment. Either alone might produce a false negative response. DMSO is useful, but the concentration must be less than 70 % to avoid irritation. Solvent controls should be run routinely as a background estimate of irritancy-induced depigmentation.

The duration of the assay should be a minimum of 60 days. This should be sufficient to identify all chemicals studied here.

Any animal model has the inherent risk of not being clinically relevant. False positive results seen in our studies serve to emphasize this. Any new agent found to be

a depigmenter in this type of predictive assay should not be discarded for commercial or industrial use as sufficient data do not exist to permit the assumption that this will occur in man. A cautiously performed human trial should offer minimal risk, as application can be limited to a small, non-cosmetic (covered) test area (Maibach et al. 1975).

We are not clairvoyant; yet, we suspect that when large scale screening assays are performed, additional compounds shall be found that depigment skin and have not been clinically suspected. We emphasize that chemically-induced leukoderma is clinically indistinguishable from vitiligo.

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