Depigmentation of Black Guinea Pig Skin by Topical Application of Cysteaminylphenol, Cysteinylphenol, and Related Compounds

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Phenol and catechol were combined with sulfur to develop new melanocytotoxic agents. Among these synthetic compounds, 4-S-cysteaminylphenol (4-S-CAP) and 4-S-cysteinylphenol (4-S-CP), which showed an in vivo antimelanoma effect, were evaluated for cytotoxicity to normal epidermal melanocytes using hydroquinone (HQ) as the control. Topical application of 4-S-CAP on the skin of black guinea pigs revealed a marked depigmentation of black skin. 4-S-Cysteinylphenol also showed some depigmenting potency. 2-S-Cysteinylhydroquinone, which was made by combining cystine with HQ, on the other hand, did not show any depigmenting effect. Depigmentation of black skin by 4-S-CAP appeared to derive from: (1) a decrease in the number of functioning melanocytes; (2) a decrease in the number of melanosomes synthesized within the melanocytes and transferred to keratinocytes; and (3) destruction of the membranous organelles of the melanocytes. None of these degenerative changes was observed in the keratinocytes, indicating the selective effect of 4-S-CAP on melanocytes. *J Invest Dermatol 88:77–82, 1987*

Development of melanocytotoxic agents is important for establishing selective chemotherapeutic approaches to malignant melanoma and depigmenting agents of skin in various pigmentary disorders. We have previously shown that the synthesis of phenolic and diphenolic compounds may provide the basis for rational chemotherapeutic approaches to melanoma [1–4]. Phenols and diphenols were combined with cysteine or cysteamine [1] and the melanoma growth inhibition by these compounds was examined in mice [2,4]. Among 9 synthetic compounds, 4-S-cysteaminylphenol (4-S-CAP) and 4-S-cysteinylphenol (4-S-CP) showed a significant antimelanoma effect; 4-S-CAP was the most effective in prolongation of the lifespan of melanoma-bearing mice. This study is an extension of our previous reports, in which attempts were made to evaluate the cytotoxic effects of these compounds on normal epidermal melanocytes by topical application. It will be seen that: (1) 4-S-CAP is a potent depigmenting agent which causes a decrease in the number of functioning melanocytes as seen by split-dopa preparations; (2) there is a decrease in the amount of epidermal melanin pigments as seen by Fontana-Masson silver staining; and (3) there is degeneration and destruction of melanocytes as seen by electron microscopy.

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Abbreviations:

GHB: gamma-L-glutamyl-4-hydroxybenzene
HQ: hydroquinone
L-dopa: L-3,4-dihydroxyphenylalanine
4-S-CAP: 4-S-cysteaminylphenol
2-S-CHQ: 2-S-cysteinylhydroquinone
4-S-CP: 4-S-cysteinylphenol

MATERIALS AND METHODS

Chemicals The details of procedure for synthesis of chemical compounds have been described in our earlier reports [1,2]. Briefly, 4-S-CAP was produced by mixing phenol and cystamine dihydrochloride in the presence of HBr under reflux. Likewise, 4-S-CP and 2-S-cysteinylhydroquinone (2-S-CHQ) were produced by mixing L-cystine in the presence of HBr with phenol and hydroquinone (HQ), respectively. Hydroquinone was commercially obtained (Kishida Chem., Inc., Co., Osaka, Japan). The chemical structures of the synthetic compounds are shown in Fig 1.

Animals Black guinea pigs were supplied by the Research Animal Farm of our school. Guinea pigs with mottled black and white skin were mated to produce black guinea pigs. Black skin was found to contain numerous dopa-positive melanocytes by split-dopa preparation.

Method of Topical Application The synthetic compounds, 4-S-CAP, 4-S-CP, and 2-S-CHQ, were applied topically to the back skin of black guinea pigs for 4 weeks, once a day for 6 days in each week. Each compound was made in an oil-in-water emulsion at a 5% concentration. The oil-in-water emulsion alone was chosen as the control. Hair follicles were epilated a day before the experiment and once a week during the entire course of the experiment by a mixture of rosin and wax as described in our previous report [5].

Light and Electron Microscopy, and Split-Dopa Preparation Biopsy specimens were obtained after 4 weeks of topical application and processed for light microscopic examination of H & E and Fontana-Masson silver stains and split-dopa preparation. L-Dopa (L-3,4-dihydroxyphenylalanine) was dissolved in

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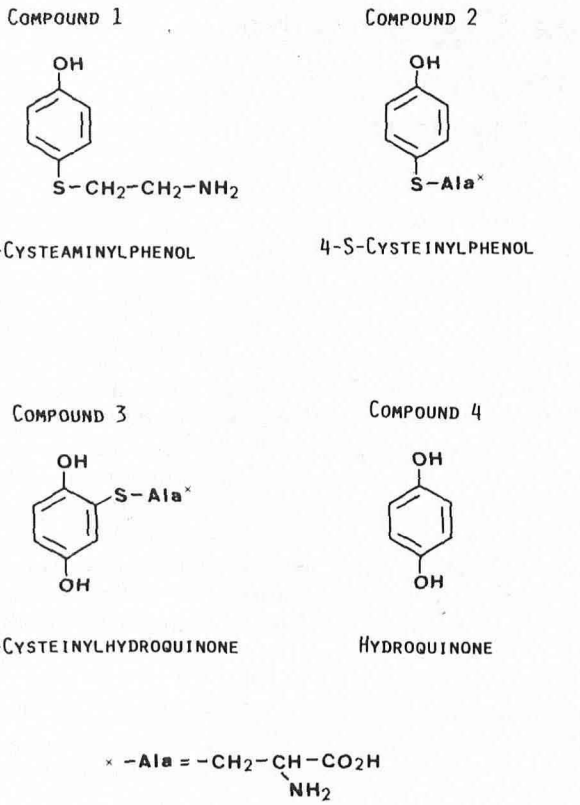

Figure 1. Chemical structure of synthetic compounds analyzed.

0.1 M phosphate buffer, pH 7.2, and processed by our previously described method [3]. The number of dopa-positive melanocytes per 0.04 mm^2 of split epidermis was counted.

Electron microscopic observation was carried out on the specimens treated with 4-S-CAP and with the base ointment alone. The specimens were prefixed with a mixture of 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and postfixed with 1% osmium tetroxide in cacodylate buffer, 0.1 M, pH 7.2. Then they were processed by the method described in our previous report [3].

RESULTS

Macroscopic, Visible Changes The depigmenting effect of synthetic compound was most prominent in the area where 5% HQ had been applied. Depigmentation became visible after as few as 7 topical applications. The degree of depigmenting potency was generally in parallel to the frequency of the topical application. Depigmentation with HQ was noted in all 7 guinea pigs examined.

4-S-Cysteaminylphenol also showed the depigmenting potency after topical application. The effect, however, was less than that with 5% HQ. With 4-S-CAP, depigmentation started by day 9 and reached the maximum at day 14 of topical application. It was prominently seen in 4 of 7 guinea pigs tested. 2-S-Cysteinylhydroquinone, however, did not reveal any apparent depigmentation. The control area where the ointment base alone was applied did not show any depigmentation of skin (Fig 2).

Besides depigmenting changes, 4-S-CAP and HQ showed inflammatory changes of skin that were most prominent in the area of HQ treatment and less prominent in the 4-S-CAP-treated area. Inflammatory changes included erythema, scaling, and erosion in the area of HQ and only mild scaling in the area of 4-S-CAP application. No appreciable changes were seen in the areas of 2-S-CHQ and 4-S-CP application as compared with the area of control.

Light Microscopic Findings Histologic sections of H & E and Fontana-Masson staining are shown in Fig 3. In the specimens treated with 4-S-CAP, melanin pigments decreased remarkably in the basal, malpighian, and horny layer areas (Fig 3*b,e*). Because of a marked reduction in the amount of melanin pigments, the structure of melanocytes, e.g., the dendritic processes, in the area of depigmentation became much more clearly visible than that of control, in which individual melanocytes were hardly recognizable due to heavily loaded pigments (Fig 3*a,d*). In the area treated with HQ, melanin pigments were barely visible even by Fontana-Masson staining (Fig 3*c,f*). Likewise, the structure of melanocytes was barely identifiable. In the HQ- and 4-S-CAP-treated specimens, mild acanthosis and hyperkeratosis were seen. Again, the areas treated with 2-S-CHQ and 4-S-CP showed no apparent changes of acanthosis and hyperkeratosis.

Split-Dopa Preparation The split-dopa preparations revealed more clearly the numerical and morphologic changes in the melanocytes during the course of depigmentation. In the control specimen (Fig 4*a*), numerous dopa-positive functioning melanocytes were seen, their mean number being 190.6/0.04 mm^2. Their dendritic processes were intricately intermingled and the structure of individual melanocytes was barely recognizable. In the specimen treated with 4-S-CAP (Fig 4*b,c*), there was a significant reduction in the number of functioning melanocytes; their mean number was 85.3 (55.3% reduction, $p < 0.001$). There were, however, regional differences in the number of melanocytes as well as in the degree of depigmentation as indicated in Fig 4*b,c*, in which the most extensively depigmented area and the least depigmented area are respectively shown. The area treated with 4-S-CP also showed a significant decrease in the number of functioning melanocytes; their mean number was 103.0 (46.0% reduction, $p < 0.001$) (Fig 4*d*). The HQ-treated area showed the most extensive depigmentation of skin and reduction in the number of functioning melanocytes; their mean number was 4.0 (97.9% reduction, $p < 0.001$) (Fig 4*f*). In these depigmented areas, there were numerous degenerating features of melanocytes, e.g., fragmentation of dendrites and large dense materials, which appeared to parallel the degree of depigmentation. The 2-S-CHQ-treated area showed the least change in the number of functioning melanocytes; their number was 168.1 (11.8% reduction, p = insignificant) (Fig 4*e*) (Table I).

Electron-Microscopic Findings Electron-microscopic observations were made on the specimens treated with 4-S-CAP, which

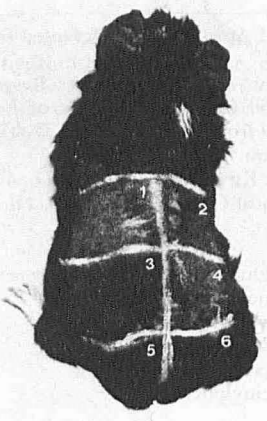

Figure 2. Depigmentation of black skin after topical application of synthetic compounds on the back of a black guinea pig for 4 weeks, once a day for 6 days in each week. Each compound was made in an oil-in-water emulsion at a 5% concentration. Biopsy specimens were obtained on the day of the epilation. *1*, 5% 4-S-CAP. *2*, 5% 4-S-CP. *3*, 5% 2-S-CHQ. *4*, 5% HQ. *5*, Base alone. *6*, No treatment.

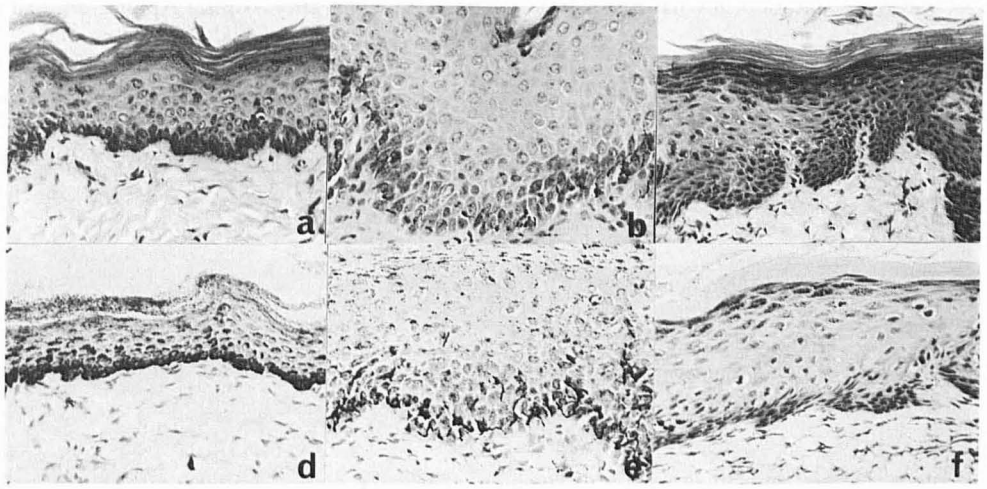

Figure 3. Light-microscopic findings of depigmented skin after topical application of 5% 4-S-CAP and 5% HQ. *a* through *f*, × 54. *a and d*, Control specimen treated with base alone. *a*, H&E stain; *d*, Fontana-Masson stain. *b and e*, 5% 4-S-CAP treated for 4 weeks. *b*, H&E stain; *e*, Fontana-Masson silver stain. *c and f*, 5% HQ treated for 4 weeks. *c*, H&E stain; *f*, Fontana-Masson silver stain.

showed the most significant depigmentation of skin. Compared with the control specimen (Fig 5*a,b*), a prominent change was noted in the number of melanosomes produced by the melanocytes and transferred to the keratinocytes. The melanosomes did not change in size. Similar to the control specimen, they were distributed as a single unit (Fig 6*a,b*). The melanocytes also showed a decrease in their population. Their dendritic processes and cytoplasm were poorly developed (Fig 6*b,c*). They appeared to show 2 basic patterns of cytoplasmic degeneration. In the first, the melanocytes showed the changes in structure of chromatin and nuclei that were diffusely dense and predominant with heterochromatin. They also possessed poorly developed organelles and a few melanosomes, but they did not show any apparent degeneration of whole cytoplasm (Fig 6*a*). Their Golgi apparatus was also poorly developed and consisted of a few cisternae. In the second pattern, there was a marked nuclear and cytoplasmic degeneration of the melanocytes (Fig 6*d*), which included the dense homogenous pattern of chromatin structure, condensation of nu-

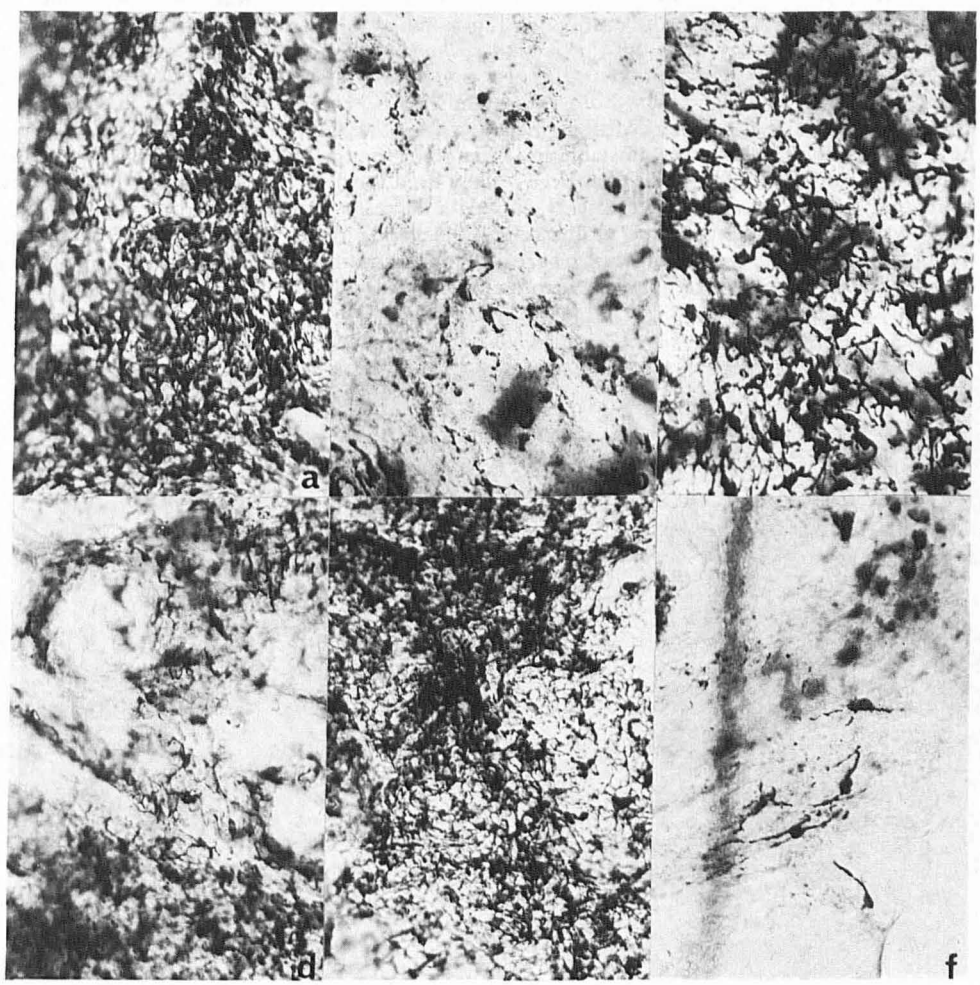

Figure 4. Split-dopa preparation of depigmented skin after topical application of 5% 4-S-CAP, 5% 4-S-CP, 5% 2-S-CHQ, and 5% HQ. *a* through *f*, × 54. *a*, Control treated with base. *b*, 4-S-CAP-treated skin, showing the lowest population of melanocytes. *c*, 4-S-CAP-treated skin, showing the highest population of melanocytes. *d*, 4-S-CP-treated skin. *e*, 2-S-CHQ-treated skin, showing no change in the number of melanocytes. *f*, HQ-treated skin, showing the lowest population of melanocytes.

Table I. Numerical Changes of Epidermal Melanocytes after Topical Application of Cysteinylphenol, Cysteaminylphenol, Cysteinylhydroquinone, and Hydroquinone[a]

Compounds	Number of Melanocytes[b] per 0.4 mm^2 of Epidermis	Percent of Reduction	*t*-test
4-S-Cysteaminylphenol	85.3 ± 20.0	55.2	7.2
4-S-Cysteinylphenol	103.0 ± 13.8	46.0	5.1 ($p < 0.001$)
2-S-Cysteinylhydroquinone	168.1 ± 34.2	11.8	1.1 (NS)
Hydroquinone	4.0 ± 1.2	97.9	8.9 ($p < 0.001$)
Base alone	190.6 ± 46.6	—	—

[a]The compounds were applied topically on the skin of a black guinea pig for 4 weeks.
[b]Number of melanocytes in the split-dopa preparation ± SD.

cleolar structure, and disruption and swelling of membranous organelles. The outer membrane of the melanosomes was often vacuolated. Large, swollen vacuoles were seen in certain foci of the cytoplasm. In addition, the entire cytoplasm was often diffusely electron-dense. A certain number of melanocytes, however, also displayed morphologic changes of the two basic forms mentioned above (Fig 6*b*,*c*).

In contrast, the keratinocytes showed no apparent degenerative changes. Several inflammatory cells, e.g., lymphocytes and macrophages, were identified in the epidermis.

DISCUSSION

In this study, we have shown that 4-S-CAP, which caused a significant growth inhibition of melanoma in mice in our previous experiments [2,4], is also a potent depigmenting agent of skin and it causes: (1) a decrease in the number of functioning melanocytes; (2) a decrease in the amount of epidermal melanin pigments; and (3) degeneration and destruction of melanocytes. It is well known that diphenolic or catecholic compounds of HQ and 4-isopropylcatechol cause a marked depigmentation of skin [6]. These compounds are, however, unstable and often toxic to non-melanin-forming cells, though HQ has been widely used for depigmenting the skin [5–11]. Pawelek [12] showed a decrease in the growth of in vitro mouse melanoma cells by elevating the level of tyrosine in the culture medium. Tyrosine administration caused an increased pigmentation of in vitro melanoma cells, and tyrosine was suggested to be melanocytotoxic. In contrast, 4-S-CAP and 4-S-CP appear to be unique compounds because they correspond electronically to tyrosine and because they are melanocytotoxic and cause depigmentation of skin.

Utilization of phenolic compounds as a potential source of chemotherapeutic agents against melanoma has been reported. In the studies of Vogel et al [13,14], a stable phenol, gamma-L-glutamyl-4-hydroxybenzene (GHB) isolated from common mushrooms, was shown to prolong the survival time of C57 BL mice inoculated with melanoma cells and to abolish or inhibit the growth of human and mouse melanoma cells. Interestingly, they found that GHB causes the depigmentation of black hair in non-melanoma-bearing mice after 2–3 weeks of i.p. injection. Furthermore, Burger et al [15] suggested that the melanocytotoxic effect of GHB is mediated through tyrosinase because of the absence of comparable cytotoxic alterations in adjacent keratinocytes, a lack of response by the melanocytes of albino mice, and patterns of deficient pigmentation produced by GHB in juvenile black mice. It was suggested at one time that GHB is oxidized by tyrosinase to a quinone which has highly reactive sulfhydryl-binding properties to curtail mitochondrial respiration and DNA polymerase alpha activity. In addition, the quinone is promptly oxidized to a second metabolic inhibitor that impairs ribosomal protein synthesis. It has, however, been shown that GHB is a poor substrate of mammalian tyrosinase, although it is a good substrate of mushroom tyrosinase, and that it is hydrolyzed in

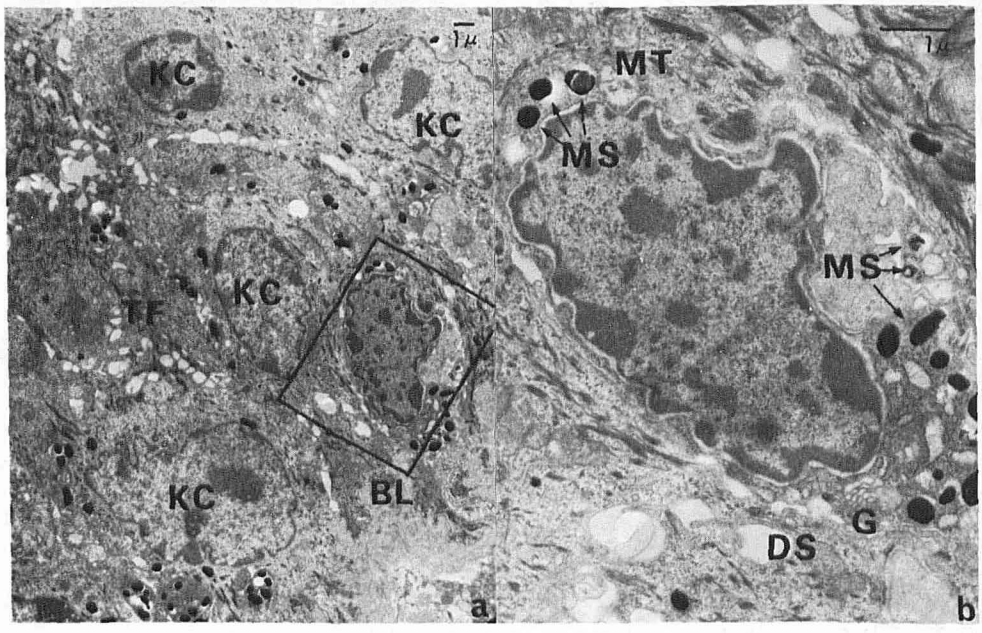

Figure 5. Electron microscopic findings of control skin of a black guinea pig. *a*, The keratinocytes and melanosomes in the basal and suprabasal layers. *KC*, keratinocyte; *TF*, tonofilament; *BL*, basal lamina. *b*, A high-power view of the boxed area shown in Fig 5*a*. A melanocyte in the skin treated with base alone. *MT*, mitochondria; *G*, Golgi apparatus; *MS*, melanosome; *DS*, desmosome.

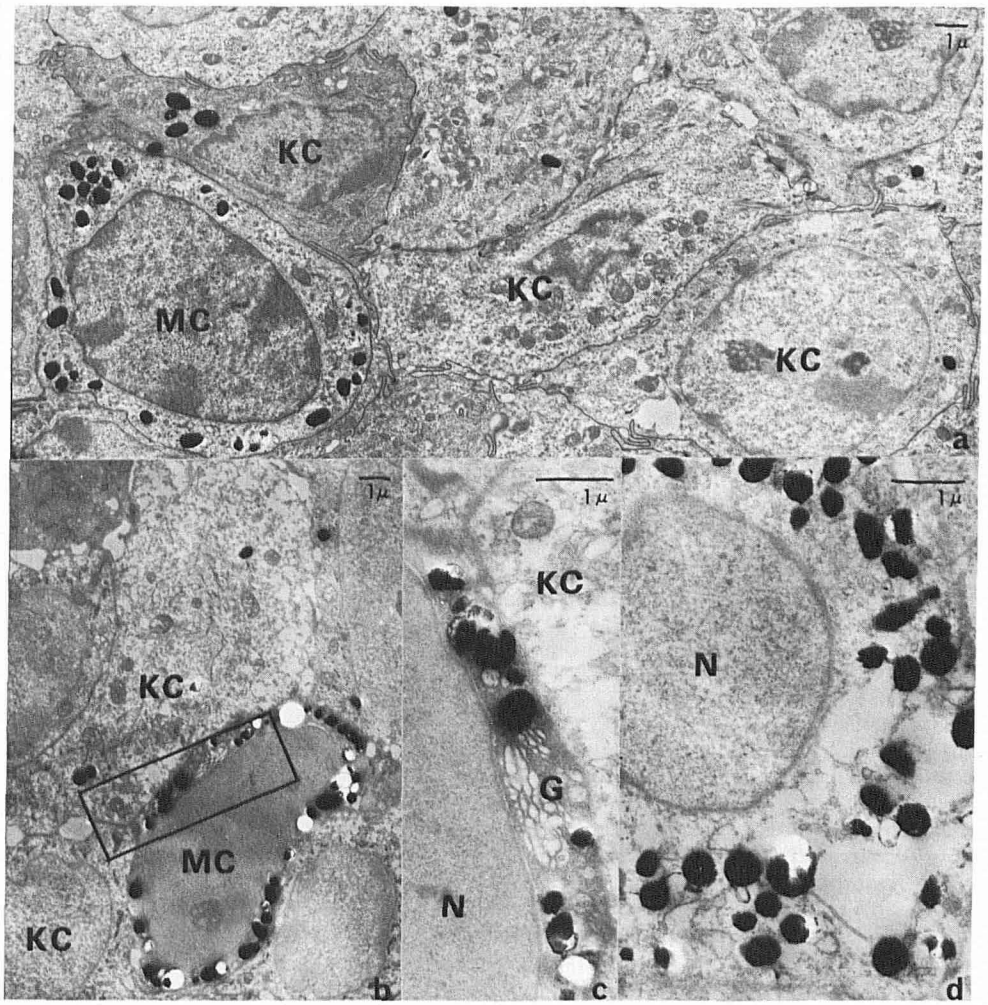

Figure 6. Electron-microscopic findings of depigmented skin after topical application of 5% 4-S-CAP. *a*, Low-power view showing a melanocyte (*MC*) and keratinocytes (*KC*). There is a marked reduction in the number of melanosomes in the keratinocytes. *b*, Low-power view of the melanocyte (*MC*) and keratinocytes (*KC*). *c*, High-power view of the melanocyte shown in the boxed area in Fig 6*b*. The Golgi apparatus (*G*) is poorly developed. *N*, nucleus. *d*, Vacuolation of a melanocyte treated with 4-S-CAP. *N*, nucleus.

animals by gamma glutamyl transpeptidase (γ-GTP) to release *p*-aminophenol as a product, which is responsible for depigmentation and melanocytotoxicity [16]. Our recent study indicated that normal human epidermis does not contain any appreciable amount of γ-GTP activity, and that only hair follicles (the inner root sheath) and sweat glands (the secretory portion) do contain this enzyme activity as judged by enzyme histochemistry [17]. It seems unlikely that GHB causes depigmentation of the skin when applied topically as was done in the present experiment. Our preliminary study also showed that 4-S-CAP and 4-S-CP are good substrates of both mushroom and mammalian tyrosinases, and that they are better than tyrosine with respect to K_m and V_{max} values [18]. Thus 4-S-CAP and 4-S-CP appear to be new compounds which are melanocytotoxic and cause depigmentation of skin. The detailed studies of the sequential processes of melanocytotoxicity by these compounds are in progress.

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