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Role of Thiol Compounds in Mammalian Melanin Pigmentation: Part I. Reduced and Oxidized Glutathione

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Evidence for the postulated role of glutathione reductase in melanin pigmentation has been obtained by determinations of the glutathione concentrations in Tortoiseshell guinea pig skin of different colors (black, yellow, red, and white). As expected, the lowest levels of reduced glutathione (GSH) were found associated with eumelanin type pigmentation, whereas the highest ones were found in the skin with phaeomelanin producing melanocytes. On the other hand, white skin of guinea pig having no active melanocytes showed GSH levels which were intermediate between those of the black and yellow areas.

These results are consistent with the view that the activity of the enzyme glutathione reductase, though not primarily related to pigmentation, plays an important role in the regulation and control of the biosynthetic activity of melanocytes leading to various types of melanin pigments.

During the 1940's Flesch and Rothman [1,2] reported experimental evidence suggesting that sulfhydryl compounds, such as glutathione and cysteine, are capable of inhibiting the biosynthesis of melanin by combining with the copper present in tyrosinase. Since then this view has been widely accepted as a regulatory mechanism in melanin pigmentation receiving additional support through subsequent studies by Halprin and Ohkawara [3,4]. These investigators showed that the concentration of reduced glutathione in human skin is some 100 times the minimum concentration necessary to observe inhibition of melanin formation. They also showed that Negroid skin contains less reduced glutathione and glutathione reductase than does Caucasoid skin.

However, as our knowledge of the chemistry of melanogenesis has improved in recent years [5,6], it has become increasingly clear that sulfhydryl compounds have little or no effect on the catalytic activity of tyrosinase but they act as chemical scavengers for dopaquinone formed within melanocytes to give colorless adducts, e.g., the cysteinyldopas [7,8]. These may be either oxidized with production of yellow or reddish-brown phaeomelanins, or take part in the biosynthesis of certain sulfur-containing eumelanins [9,10], or released from melanocytes to be eventually excreted in the urine [11-13].

Though these facts are well known, no attempt has been made in recent years to correlate the sulfhydryl content of the skin with the type of pigmentation. We have therefore begun such a study, and our preliminary observations on the glutathione levels in guinea pig skin of different colors constitute the subject of this report.

MATERIALS AND METHODS

Tortoiseshell guinea pigs $e^{p}e^{p}$ with white spotting [14] showing a 3 colored coat were studied. Two different groups of animals were examined: one showing a mixture of white, black, and red areas; the other with white, black, and yellow patches. These animals were obtained from the breeding laboratory of Institut National des Sciences Appliqués de Lyon (Service du Professeur Laviolette). Reduced (GSH) and oxidized (GSSG) glutathione, N ethylmaleimide O. phtalaldehyde were obtained from Sigma chemical compagny (St. Louis, Mis.). All determinations of fluorescence intensity were performed with a fluorocolorimeter Aminco.

Tissue Preparation

Guinea pigs were killed by a blow on head then sheaved. The skin was removed, used immediately or frozen and kept at −80° C until use. 150-250 mg of tissue was homogenized at 2-3° C using a polytron homogenizer in a phosphate buffer, 0,1 M, EDTA, 5 mM; pH 8.0. 4% HPO_3 was used as a protein precipitant. The homogenate was centrifuged (3×10^6 g min at 4°C). GSH and GSSG were measured in the supernatant.

Light Microscopy

For light microscopy solubility test, skin biopsies were frozen with liquid nitrogen. Sections (8 μm thick) were cut on a cryocut. Solubility tests were carried out under a light microscope by treating sections in a sodium hydroxide solution 0.25 M [15].

Split DOPA Studies

Biopsies of the skin of different colors (red, yellow, black, white) were incubated in 2 N NaBr solution at 37°C for 45 min and the separated epidermis and hair follicles thus obtained were incubated with 0.1% DOPA solutions in 0.1 sodium phosphate buffer (pH 7.4) for 4 hr at 37°C. After the dopa reaction, the tissues were fixed with 10% neutral formalin, dehydrated in alcohol and mounted.

Electron Microscopy

Skin biopsies of the different colors were fixed in 3% glutaraldehyde for 1 hr, postfixed in osmic acid for 1 hr, dehydrated in alcohol, embedded in Epon and sectioned on a Reichert OM U3 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined with an Hitachi HU 12 A electron microscope.

Glutathione Assays

Both reduced and oxidized glutathione (GSH and GSSG) were measured by a specific and very sensitive fluorometric method described by Cohn and Lyle [16] and later modified by Hissin and Hilf [17]. This method takes advantage of the reaction of ortho phtalaldehyde at pH 8.0 with GSH and at pH 12.0 with GSSG. N ethylmaleimide is used to prevent interference of GSH with the measurement of GSSG.

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Abbreviations:
DTNB: 5,5′-dithiobis (2 nitrobenzoic acid)
EDTA: ethylene diamine tetracetate
GSH: reduced glutathione
GSSG: oxidized glutathione
HPO3: metaphosphoric acid

Assays of Other Thiols

Cysteine and cystine were measured according to Gaitonde [18] using acid ninhydrin reagent forming a pink product at 560 mn.

Total thiols and nonprotein thiols were measured using D.T.N.B. reagent as described by Ellman [19] modified by Jocelyn [20]. Statistical analysis of the data was performed using Student's *t*-test. Differences were considered significant at values less than or equal to 0.05.

RESULTS

Solubility Tests

On frozen sections of yellow and red skin, pigment granules characteristically located in the cytoplasmic particles of epidermal and follicular melanocytes have a yellow or and reddish coloration under a light microscope. On the other hand, in the black skin, pigment granules appear as dark brown particles.

When sections of yellow and red skin are rinsed in a NaOH 0.25 M for 1 minute at room temperature pigment granules disappear. Little change, on the other hand, was observed after similar treatment of pigment granules in black skin (Figure).

From their coloration and their solubility in NaOH 0.25 M one may assume that: (i) pigment granules in the yellow and red skin are different in chemical properties from dark granules. (ii) pigments deposited in pigment granules of yellow and red skin are a kind of phaeomelanin.

Split DOPA

In the white skin, no Dopa positive melanocytes are observed in the epidermis. In the colored areas, either yellow, red, or black, numerous epidermal melanocytes, with prominent dendrites and intense Dopa oxidase activity are present. The density per mm^2 of epidermis of epidermal melanocytes is higher in the black (396 ± 41) than in the red (208 ± 37) and yellow skin (192 ± 34).

Electron Microscopy

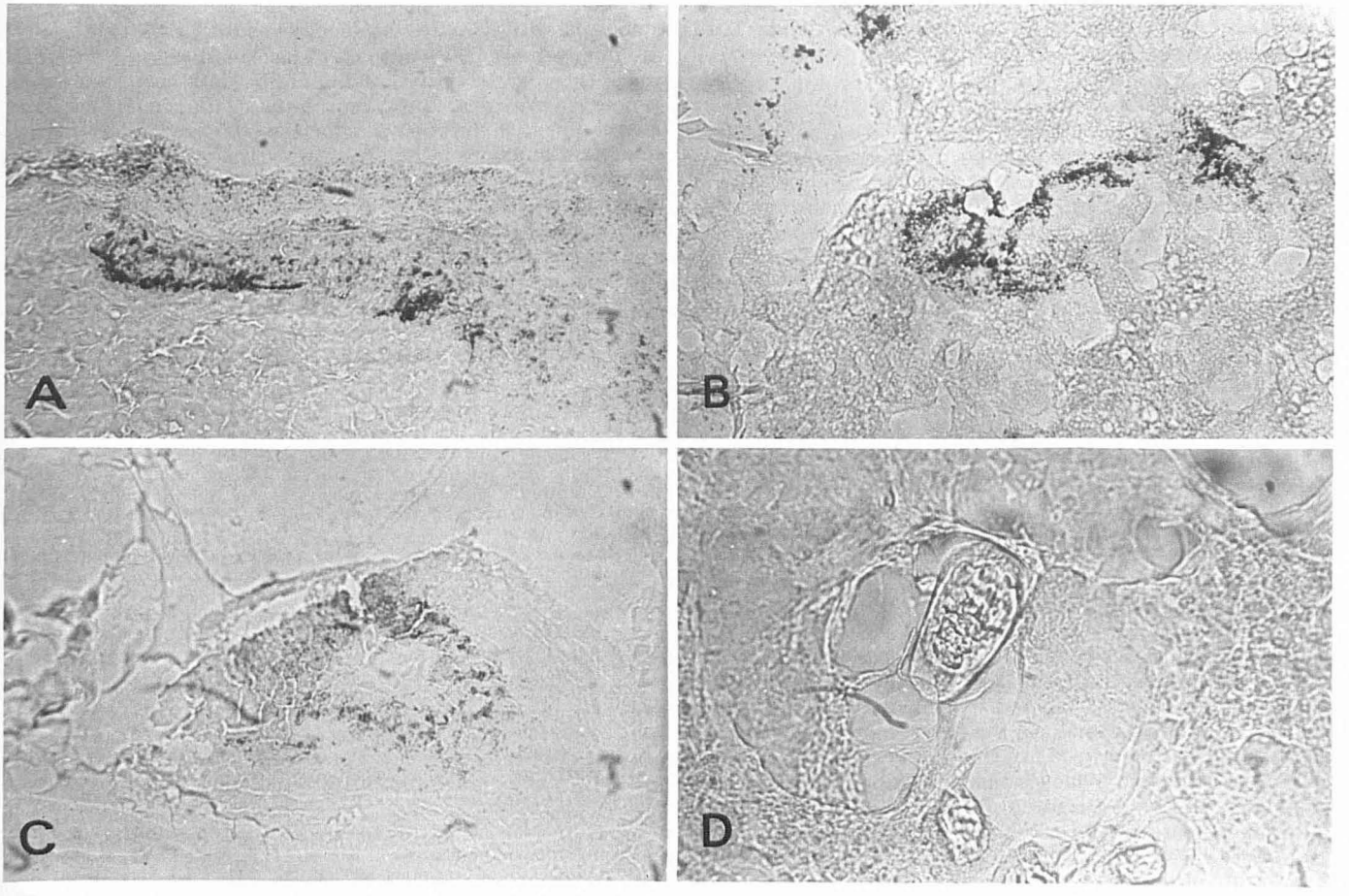
In the black skin, melanosomes have the morphology of typical eumelanosomes. On the other hand, in yellow and red skin, most of the melanosomes are round containing coarse granular material ressembling the particles described as phaeomelanosomes [21].

Biochemical Analysis

Table (A) and (B) show that reduced gluthathione represents the main nonprotein sulfhydryl compound present in the skin. According to the sensitivity of the assay, cysteine and cystine represent less than 5% of reduced (GSH) and oxidized (GSSG) glutathione.

It can be seen that GSH levels found in guinea pig skin are lower than those reported from human skin by Halprin and Ohkawara (1.47 μmoles/g in black skin and 1.83 μmoles/g in white skin) [4]. In this connection, it should be noted, however, that these investigators measured GSH and GSSG levels by indirect methods, while in the present study a specific fluorometric assay [15,16] was used which allows more sensitive and accurate determinations of both reduced and oxidized glutathione in the same tissue preparation.

As shown in the Table, GSH levels in guinea pig skin of different colors vary with the type of pigmentation; in particular



Solubility test of different colored areas of tortoiseshell guinea pig skin: black pigments before (*A*) and after (*B*) NAOH 0.25 M treatment: yellow pigments before (*C*) and after (*D*) NAOH 0.25 M treatment.

Thiols groups and glutathione levels in different colored areas of guinea pig skin (5 animals)

Colored area	Total SH groups	Nonprotein SH groups	Reduced glutathione GSH	Oxidized glutathione GSSG	GSH/GSSG	Total glutathione
			(A) red, black, white skin[a]			
Red	1.82 ± 0.33	1.50 ± 0.28	0.96 ± 0.16	1.08 ± 0.18	0.90	3.12 ± 0.36
Black	1.68 ± 0.31	1.26 ± 0.17	0.63 ± 0.08	1.21 ± 0.15	0.51	3.05 ± 0.38
White	1.78 ± 0.36	1.32 ± 0.42	0.74 ± 0.06	1.38 ± 0.21	0.55	3.50 ± 0.34
			(B) Yellow, black, white skin[a]			
Yellow	4.15 ± 1.09	3.20 ± 0.58	1.74 ± 0.40	1.60 ± 0.33	1.01	4.75 ± 0.85
Black	3.28 ± 0.86	2.30 ± 0.42	0.92 ± 0.26	1.42 ± 0.30	0.65	3.76 ± 0.56
White	3.58 ± 0.63	2.74 ± 0.51	1.13 ± 0.29	1.50 ± 0.19	0.75	4.14 ± 0.61
			(C)[b]			
Difference between black and yellow areas	$p < 0.10$	$p < 0.05$	$p < 0.05$	$p < 0.10$		$p < 0.05$
Difference between black and red areas	NS	$p < 0.10$	$p < 0.05$	$p < 0.10$		NS

[a] Thiols groups reduced, oxidized and total glutathione are expressed in micromoles per gram of fresh skin. Total glutathione is determined assuming that 1 micromole of GSSG represents 2 micromoles of GSH.

[b] The significance of differences between black and red areas means (Table (A)) black and yellow areas means (Table (B)) was calculated by the Student *t*-test. T-values are given, the T values > 2.77 are significant with $p < 0.05$.

they are lower in black skin than in yellow skin, while intermediate values are found in the unpigmented area with no active melanocytes. Differences in the levels of nonprotein SH groups and of reduced glutathione between black versus yellow areas and black versus red areas are statistically significant ($p < 0.05$) (Table (C)). On the other hand, total glutathione found in skin does not differ significantly with the type of pigmentation, ranging from 2.7 to 4.0 μmoles/g (Table (A)) and 3.13 to 6.23 (Table (B)).

DISCUSSION

Tortoiseshell guinea pig can be regarded as an excellent model for comparative studies on eu- and phaeomelanogenesis owing to the presence of melanocytes producing both types of pigments in different regions of the same animal. Individual variations such as those usually observed in the sulfhydryl content of mammalian skin are thus avoided, making it possible a direct comparison of the glutathione levels associated with different types of pigmentation.

In living tissues, glutathione is normally present in 2 chemically distinct forms, GSH and GSSG, the relative levels of which depending mostly upon the activity of glutathione reductase [21]. As pointed out recently by Prota and Searle [22] this enzyme should play a key role in the regulation and control of pigment cell metabolism by favoring the formation of glutathionedopas as well as of the corresponding cysteinyldopas involved in the biosynthesis of phaeomelanins. Indeed, glutathionedopa has been found in melanin producing cells [13], and more recently Hannson et al have shown that cysteinyldopa levels are some ten times higher in red skin than in white skin of uniformly colored guinea pigs [23].

The results of our assays on the GSH and GSSG levels in Tortoiseshell guinea pig skin of different colors are consistent with the postulated involvement of the glutathione system in melanogenesis. Despite marked individual variations among the various animals examined, the observed differences in the sulfhydryl content between black and yellow or red pigmented skin provide indirect evidence that the ability of melanocytes to produce eumelanins instead of phaeomelanins is associated with an increase in the activity of the enzyme glutathione reductase in the environment of the pigment cell. At first glance, one might consider that the observed differences in the GSH content between black and red skin are not particularly striking. It should be noted, however, that all cells contain relatively large amounts of GSH and even in the phaeomelanocytes, the specific uptake of dopaquinone by GSH probably represents only a small portion of the total GSH content in the region. And more over, one should consider that much of the GSH may be in the bound form [24] and relatively inaccessible to interaction with the dopaquinone formed within melanocytes. In this connection it is noteworthy that unpigmented skin of guinea pig with no active melanocytes shows GSH levels which are intermediate between those of black and yellow skin, thus suggesting that the activity of the enzyme glutathione reductase is not primarily related to pigmentation. This is not surprising considering that glutathione reductase is known to play several important functions including, notably, the maintenance of the redox state of the cell, and the detoxication of unstable cytotoxic compounds such as radicals and quinones [25]. In order to further understand the correlation between glutathione levels and type of pigmentation a detailed study of glutathione reductase and peroxidase activities in the skin of different mammals including man are in progress.

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