Elemental analysis of melanins from bovine hair, iris, choroid, and retinal pigment epithelium

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According to recent theory on the structure of melanin, the sulfur content of melanin is a reflection of the amount of cysteine used in its manufacture. We compared the sulfur contents of melanins from hair, choroid, iris, and retinal pigment epithelium from black-haired cattle and separately from red-haired cattle. In black-haired cattle, sulfur contents of hair and ocular melanins were all between 0.60% and 0.94%, however, in red-haired cattle, hair melanin had a sulfur content (8.66%) different from those of choroid (0.94%) or RPE (1.72%) melanin. One may conclude that in an individual animal, ocular melanins can have different chemical structures from hair melanins. The clinical importance of this finding is discussed with regard to sympathetic ophthalmia.

Key words: uveal tract, retinal pigment epithelium, hair, melanin, sympathetic ophthalmia

The color of the hair of mammals is primarily the result of the *type* of melanin present in that hair. On the other hand, iris color is thought to be due to the *quantity* of uveal pigment present in the iris stroma. This study examined the *type* of melanin in the uveal tract and retinal pigment epithelium (RPE) of the bovine eye. Specifically, we addressed the question of whether choroidal melanin and RPE melanin from an individual animal were identical to the hair melanin present in that animal. We took advantage of the fact that red melanin (phaeomelanin) has a high sulfur content whereas black melanin (eumelanin) is low in sulfur.[1] The melanins from the hair, irides, choroids, and RPEs were extracted from cattle with black hair (Holstein and Angus) and separately from cattle with red hair (Hereford and Ayrshire). We then compared the sulfur contents of the uveal and RPE melanins with those of the hair melanins.

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This work was supported by the Milton Fund and the National Eye Institute grants EY-01917-02 and EY-00089-04.

Submitted for publication May 30, 1978.

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Materials and methods

The procedure used to extract and purify the melanins was a modification of the one used by past investigators.[1, 2, 5, 10] It utilized melanin's insolubility in hydrochloric acid as a basis for the purification. The precise procedures are outlined below.

Purification of melanin from the hair of adult cattle. Hair from adult cattle was collected within 1 hr of the animal's death. Black hair was taken from Holstein and Angus cattle; red hair was cut from Hereford and Ayrshire cattle. All animals were adults (older than 18 months). Hair was cut from the head, neck, and shoulders of the animals. The hair was stored at room temperature for 3 days. It was then washed by being soaked in hot water and agitated for 10 min. The water was then

0146-0404/79/030231+06$00.60/0

discarded. The washing procedure was repeated for a total of three washes, followed by a last wash with 95% ethanol. The hair was spread to dry at room temperature overnight. After drying, there was a total of 204.1 gm of black hair and 119.3 gm of red hair. Samples were placed in flasks, and 600 ml of concentrated hydrochloric acid were added to each flask. The flasks were covered and stored at room temperature for 14 days. The mixtures were centrifuged at 500 × g for 14 hr. The supernatants were discarded. The sediment from the black hair mixture was resuspended in 400 ml of concentrated HCl, and the sediment from red hair was resuspended in 200 ml of concentrated HCl. The solutions sat at room temperature for 7 days, at which time fresh HCl was substituted in the same manner. After 7 more days fresh HCl was again substituted, and the solutions were brought to boiling, and were refluxed for 62 hr. The mixtures were centrifuged at 200 × g for 3 min. The supernatants were discarded, and the melanin pellets were washed by resuspension and centrifugation for a total of five washes with 1% HCl, four washes with double-distilled water, two washes with ethanol, and finally, two washes with anhydrous diethylether. (Each wash was with 50 or 25 ml of solvent for black or red hair melanins, respectively.) The melanins were dried. A total of 2.107 gm of black hair melanin and 0.546 gm of red hair melanin was collected.

Purification of iris melanin (method 1). Bovine eyes from adult black-and-white cattle and adult red-and-white cattle were received from a slaughterhouse on ice. Within 24 hr, each iris was removed by incising the cornea with a scalpel, grasping the iris with forceps, and tearing the iris at its root. The irides were stored for up to 4 weeks at −70° C before the melanin extraction procedure.

Irides from black cattle (9.1 gm) and 6.7 gm of irides from red cattle (wet weights) were placed in 100 ml of concentrated HCl each. Every 7 days the mixtures were centrifuged at 100 × g for 10 min, the supernatants were discarded, and the pellets were resuspended in concentrated HCl. After a total of 28 days under HCl, the pellets of melanin were washed in a Büchner funnel with five 25 ml portions of 1% HCl, five 25 ml portions of water, two portions of ethanol, and two portions of anhydrous diethylether. The melanins were dried, giving 0.127 gm of melanin from black cattle irides and 0.046 gm of melanin from red cattle irides.

Purification of iris melanin (method 2). Irides were collected and stored as in Method 1. Black cattle irides (25.5 gm) and 17.7 gm of red cattle irides were each dissolved in three times their weight of concentrated HCl (76 and 53 ml, respectively). As in method 1, the undissolved black melanin particles were resuspended in fresh concentrated HCl every 7 days; however, centrifugation was at 500 × g for 2 hr each time. After 28 days under HCl, the melanin pellets were washed by centrifugation exactly as the hair melanin pellets had been washed. After drying, 0.667 gm of black cattle iris melanin and 0.465 gm of red cattle iris melanin were obtained.

Purification of melanin from choroid and RPE. Over the course of 12 weeks, 98 eyes from adult black cattle and 110 eyes from adult red cattle were received, and within 24 hr of death the RPE and choroid were dissected. The eyes were opened at the ora serrata, and the vitreous and retina were dissected free. The remaining bowl-shaped posterior pole was filled with saline, rinsing the exposed RPE. After two rinses, the eye was again filled with saline. With a Pasteur pipette, the pigmented RPE was gently scraped from the underlying choroid, with care taken not to damage Bruch's membrane. (Histologic sections of typical samples of choroid with overlying RPE removed by the technique were examined microscopically and showed an intact Bruch's membrane.) Clumps of RPE were collected by aspirating the saline solution. After the major portion of pigmented RPE had been carefully collected and aspirated, the eye was inverted, and the remainder of RPE, excluding areas over the choroidal tapetum, was scraped from Bruch's membrane with the blunt edge of a pair of forceps. Bruch's membrane was repeatedly rinsed vigorously with saline. A piece of pigmented choroid (not including the tapetum), free of all overlying RPE, was cut from the globe. The RPE-saline mixture was centrifuged at 100 × g for 10 min, giving a pellet of RPE cells. These pellets, as well as the choroidal samples, were stored at −70° C for up to 3 months before the melanin was separated.

From the eyes of black cattle a total of 2.449 gm of choroidal tissue and 0.358 gm of RPE was collected; the eyes of red cattle yielded 2.635 gm of choroid and 0.759 gm of RPE. Each type of tissue was dissolved in three times its wet weight of concentrated HCl. The remainder of the procedure was exactly in the manner described in the iris melanin purification (method 2). The amount of melanin obtained from each specimen was as follows: black cattle choroid, 50.78 mg; black cattle RPE, 18.02 mg; red cattle choroid, 137.75 mg; red cattle RPE, 30.62 mg.

Elemental analyses. Melanin samples were sent

to Galbraith Laboratories, Inc., in Knoxville, Tenn., for elemental analyses.

Results

Color of melanins. The color of the melanins isolated from bovine hair correlated with the color of the hair itself. The black hair melanin was a very dark, blackish brown, whereas the red hair melanin was a reddish brown. The colors of the melanins from the iris, choroid, and RPE were all dark, blackish brown. Observers in the laboratory believed that the ocular melanins from the red-haired cattle were a lighter brown than those from the black-haired cattle, but this difference was minimal.

Elemental analyses. Elemental analyses of the purified melanins are listed in Table I. Duplicate analyses were performed in most cases, and the results of both analyses are presented. For comparison, the table gives the results of previously published analyses of melanins from black oxtail hair and ox choroid-RPE complexes. Also, predicted compositions for theoretic polymers of dopa and 5-S-cysteinyldopa are listed.

The amount of error introduced during the purification procedures is unknown. Comparison of the analyses obtained on the two separate batches of iris melanins gives one an idea of the degree of inaccuracy which can occur. Despite the inherent inaccuracies, sulfur content of the red hair melanin is higher than that from any other melanin. No noticeable difference can be detected in the sulfur contents of the other melanins studied (Table II).

Table I. Elemental analyses of various cattle melanins

	S (%)	*N (%)*	*C (%)*	*H (%)*
Black-and white cattle (Holstein, Angus):				
Hair	0.95	8.31	52.78	3.98
	0.93	8.25	52.99	3.94
Irides 1	0.82	9.33	54.89	4.20
Irides 2	0.38	9.06	55.77	4.94
	0.36	9.14	55.95	4.87
Choroid	0.54	8.01	51.56	4.47
	0.56	8.09	51.61	4.56
RPE	0.59	8.75	51.84	4.60
	0.62	8.85	52.19	4.48
Black oxtail hair (Nicolaus et al.)[2]	1.60	5.40	65.50	5.50
Ox choroid-RPE (Nicolaus et al.)[2]	0.90	8.70	60.40	4.60
Red-and-white cattle (Hereford, Ayshire):				
Hair	8.58	8.28	53.54	4.34
	8.74	8.56	53.69	4.33
Irides 1	1.06	9.60	53.02	4.36
Irides 2	1.48	9.51	52.17	5.01
	1.32	9.58	52.33	4.88
Choroid	0.93	8.86	48.13	4.84
	0.95	9.01	48.35	4.73
RPE	1.68	9.35	51.60	4.96
	1.77	9.41	51.42	4.84
Dopa polymer $(C_9H_9O_4N)_n$	0.0	7.17	55.33	4.61
5-S-Cysteinyldopa polymer $(C_{12}H_{14}O_6N_2S)_n$	10.18	8.91	45.81	4.45

Table II. Sulfur content of various cattle melanins*

	Average % S		
Cattle	*Hair*	*Choroid*	*RPE*
Black	0.94	0.55	0.60
Red	8.66	0.94	1.72

*Data are average values from duplicate sulfur analyses given in Table I.

Discussion

Despite the known physiologic and embryologic differences between hair bulb melanocytes and uveal and pigment epithelial melanocytes, ophthalmic scientists have assumed, without more than a few shreds of evidence, that the compositions of the various ocular and cutaneous melanins are similar. On the basis of this assumption, experiments on such various subjects as drug-melanin binding, autoallergy to uveal pigment, free radical properties of melanins, and ultrastructural stages of melanosome maturation have freely interchanged data on melanin from each of the different populations of melanocytes, without regard to possible major differences in the melanins involved. We can present new evidence and summarize old evidence which supports the contentions that (1) uveal melanin has an origin from dopa and cysteinyldopa similar to cutaneous

Invest. Ophthalmol. Visual Sci.
March 1979

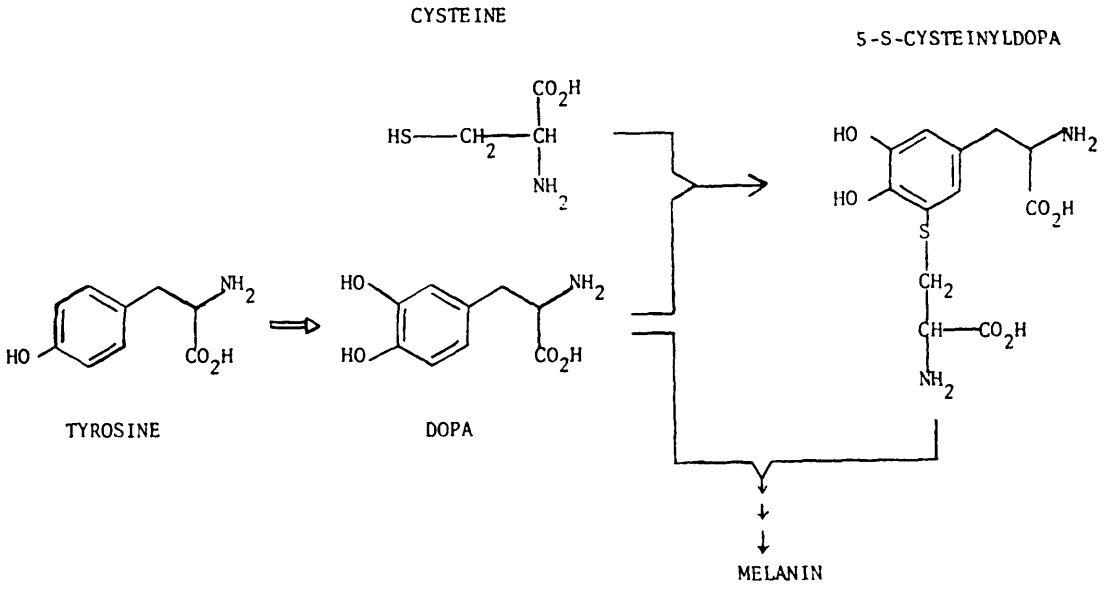

Fig. 1. Mechanism of melanin synthesis.

and hair melanin and (2) the ratio of dopa to cysteinyldopa in the melanin of the uvea or RPE need not be the same as that of the hair in a given individual.

Two populations of pigment cells are present in the eye, the uveal melanocytes and the pigment epithelial cells. The two cells differ in embryologic origin, time of initiation of pigment synthesis, and morphology of pigment granules.[3] The exact nature of the pigment in these cells has been an unanswered question. The enzyme tyrosinase is present in the eye in both cell populations.[4] However, tyrosinase is not a highly specific enzyme and can oxidize many types of polyphenols (e.g., epinephrine, norepinephrine, dopa, catechol,) to produce melanin.[1]

Rorsman[5] recently proposed a theory on the nature of mammalian hair melanins. His theory is based on Prota's[6] theoretical mechanism of melanin synthesis involving tyrosine being oxidized to dopa, followed by variable amounts of cysteine combining with an oxidation product of dopa to form 5-S-cysteinyldopa and lesser amounts of 2-S-cysteinyldopa and 2,5-S,S-dicysteinyldopa (Fig. 1). Cysteinyldopa is oxidized alone or with dopa to form melanin. Rorsman postulated that the proportions of cysteinyldopa and dopa available to premelanosomes determines the color and sulfur content of the melanin made. Pure cysteinyldopa melanin is red and has a high sulfur content (greater than 10%), whereas pure dopa melanin is black and has a low sulfur content (less than 0.3%). Intermediate ratios of dopa to cysteinyldopa produce melanins of intermediate colors between black and red, namely, the various shades of brown. The sulfur contents of brown hair melanins lie between 1% and 10%.[5]

The mechanisms which regulate the *type* of melanin synthesized by a single melanocyte are unknown. It is probably not a function of the enzyme tyrosinase which catalyzes the initial oxidations outlined above, for the same enzyme appears to catalyze the synthesis of either red or black melanins, depending on the substrates available.[1, 7] Rorsman's theory places control of the type of melanin synthesized on the amounts of cysteine and tyrosine present in the premelanosome during melanization. Assuming that melanins are polymers of a dopa-cysteinyldopa mixture, a ratio of dopa:cysteinyldopa of 90:10 would yield a melanin with a 1% sulfur content, and a ratio of 10:90 would yield a melanin with a 10% sulfur content.

Most of the above speculation is based on research on hair and feather melanins. With regard to the eye, little is known about the

nature of either uveal tract melanin or the melanin of the pigment epithelium of the retina, ciliary body, and iris. The data in this paper, together with those from previous studies, allow one some insight into the melanin(s) in the eye.

First, there is circumstantial evidence that ocular melanin, like hair and feather melanin, is made from dopa and cysteinyldopa. Dopa and cysteinyldopa have been found in the pigmented parts of the mature bovine eye,[8] and both compounds are present in high levels in the urine of patients with metastatic choroidal melanoma.[9] Furthermore, the ocular melanins analyzed in the present study, as well as those of Waelsch,[10] contained approximately 1% sulfur, an amount theoretically corresponding to a 90:10 ratio of dopa:cysteinyldopa.

The present data also show that in an individual animal, the melanin of the choroid and the RPE need not be identical with that of the hair. This is shown clearly by examination of the sulfur contents of melanins from red-haired cattle, where one finds that the sulfur content of hair melanin is very different from that of choroid and RPE melanin. Whether or not the same is true for black-haired cattle cannot be determined from the data, since the sulfur contents are close enough to be explainable by experimental error alone.

This last conclusion, that ocular melanin can be different from hair melanin in an individual, is important. The possibility that various melanocytes from one individual can produce different melanins should not be surprising to anyone who has observed the red and black patches on a calico cat, or the red, orange, and black stripes of a tiger. The clinical importance of the finding lies in its possible relevance to certain inflammatory diseases of the human eye, such as sympathetic ophthalmia and Vogt-Koyanagi-Harada syndrome. There has been speculation that the bilateral chronic inflammatory infiltrate of the uveal tract characteristic of these diseases is a manifestation of autoallergy to uveal pigment.[11] Vitiligo associated with sympathetic ophthalmia has been described,[13, 14] and it is a common occurrence with Vogt-Koyanagi-Harada syndrome.[15, 16] Histologic studies of the borders of vitiliginous patches have shown evidence of inflammation,[17] even in the absence of clinical signs of inflammation.[18] We speculate that the same proposed allergic response to uveal pigment which causes the uveitis may also be responsible for an inflammatory cell destruction of melanocytes.

If our data on melanin in cattle are applicable to humans, one might speculate that in an individual, uveal melanin can be different from hair melanin produced by hair bulb melanocytes. In those individuals where cutaneous and uveal melanins are similar, vitiligo would develop as a manifestation of Vogt-Koyanagi-Harada syndrome or sympathetic ophthalmia; in those where the melanins are different, the vitiligo would not appear.

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