British Journal of Ophthalmology, 1987, **71**, 549–552

Is there any difference in the photobiological properties of melanins isolated from human blue and brown eyes?

I A MENON,[1] P K BASU,[2] S PERSAD,[2] M AVARIA,[2] C C FELIX,[3] AND B KALYANARAMAN[3]

From the [1]Clinical Science Division and [2]Department of Ophthalmology, Faculty of Medicine University of Toronto, Canada, and the [3]National Biomedical ESR Center Department of Radiation Biology and Biophysics Medical College of Wisconsin, Milwaukee, Wisconsin, USA.

SUMMARY Investigations were carried out to determine whether the melanin present in the blue and brown eyes were eumelanin, the melanin present in black hair and dark skin, or pheomelanin, the melanin present in red hair and the skin of people with red hair. Our results showed that UV-visible irradiation of blue or brown eye melanin did not produce any superoxide. Irradiation of ^{51}Cr-labelled Ehrlich ascites carcinoma cells in the presence of blue or brown eye melanin did not produce significant cell lysis. The electron spin resonance (ESR) signals of blue and brown eye melanins were very similar to those of eumelanin. Comparison of these findings with our previous results indicated that the blue and brown eye melanins are essentially eumelanin. The ESR signals further suggested that in the case of both blue and brown eye melanins the iris, ciliary body, choroid, and retinal pigment epithelium did not differ.

The melanin present in the black hair and in the skin of people with black hair is called eumelanin, whereas the melanin in the red hair and in the skin of people with red hair is termed pheomelanin.[1-3]. These melanins differ in their chemical and physical properties as well as in their biosynthetic pathways. Eumelanin is synthesised from dopa, and pheomelanin from cysteinyldopa. Eumelanin contains little or no sulphur, in contrast to pheomelanin which contains approximately 10% sulphur.[4-8] Various natural melanins are, however, neither pure eumelanin nor pure pheomelanin. They are copolymers of dopa and cysteinyldopa. These have sulphur contents and other chemical and physical properties intermediate between pure eumelanin and pure pheomelanin.[6 9-13]

Melanins contain stable free radicals, which can be detected by electron spin resonance (ESR). Both eumelanin and pheomelanin give one common ESR signal; but pheomelanin gives another ESR signal, which is not seen in the case of eumelanin. The above difference in the ESR signal is observed with melanins isolated from human black and red hair.[11 14]

Correspondence to Dr P K Basu, Department of Ophthalmology, University of Toronto, 1 Spadina Crescent, Toronto, Ontario, Canada M5S 2J5.

Certain other differences in the photochemical and photobiological properties of melanins from black hair and red hair have also been reported by us. Irradiation of the red hair melanin was found to produce considerable amounts of superoxide whereas irradiation of black hair melanin did not produce any detectable amounts of superoxide.[15-17] Further, when Ehrlich ascites carcinoma cells were irradiated in the presence of red hair melanin there was a greater extent of cell lysis than when they were irradiated in the presence of black hair melanin.[18]

Our previous studies[10 19 20] have shown that there are some qualitative differences in the physical and chemical properties of melanins isolated from human eyes having blue and brown irises (designated as blue and brown eyes respectively). These differences were observed in the oxidation properties of these melanins and the binding of some metabolites and drugs (e.g., protoporphyrin, chlorpromazine, and paraquat) to these melanins. The oxidation of reduced nicotinamide adenine dinucleotide (NADH) by the melanin-protoporphyrin complexes was also different for these two melanins from human eyes.

The present report is a part of our study on the comparison of the properties of the blue and brown eye melanins. In this investigation the photolytic

effects of the two melanins were studied. For this purpose we used an *in-vitro* cell system. We have also compared the ESR signals of these melanins. Furthermore, we also investigated in the course of this study whether the various pigmented tissues of the eye, namely, iris, ciliary body, choroid, and retinal pigment epithelium, contain the same type of melanin or not.

Materials and methods

Human donor eyes having blue and brown irises were obtained from the Eye Bank of Canada (Ontario Division). The colour of the iris was coded by one person using a standard magnification and illumination.[10] Only the eyes which had distinct blue and brown irises were selected. Eye donors were of both sexes and their ages ranged from 50 to 70 years. The average time between death and removal of the eyes was about six hours.

The eyes were dissected and the melanins were prepared from the iris, ciliary body, choroid, and retinal pigment epithelium according to the method previously described by us.[10]

The methods for the maintenance of Ehrlich ascites carcinoma cells in mice, the preparation of these cell suspensions, the labelling of the cells with ^{51}Cr, and the determination of ^{51}Cr release following cell lysis were carried out by the methods described previously.[18]

The cells were irradiated with a Westinghouse mercury vapor lamp. The cell suspensions were shaken in a waterbath at 37°C and irradiated by the UV light from the top. The lamp was placed at a height of 14 cm above the surface of the cell suspensions. The emission spectrum of the lamp and the irradiances with different filters were given in previous papers.[6,17]

The formation of superoxide was determined by measuring the reduction of nitroblue tetrazolium chloride (NBT). The NBT reduction, which was inhibited by superoxide dismutase (SOD), was taken as a measure of the superoxide formation.[17] Hydrogen peroxide (H_2O_2) was determined by the method of Wang and Nixon.[21]

The steps in the cell lysis experiments are summarised in Table 1.

The ESR measurements on melanins extracted from eye tissues were carried out with a Varian E-109 spectrometer operating at X-band (9·1 GHz) and employing 100 kHz field modulation. Measurements of magnetic field (H) and microwave frequency were made with a Radiopan MJ110-R gaussmeter and an EiP model 548 frequency counter respectively. The g values were calculated to ±0·0001 from $g=h/\beta H$, where h is Planck's constant and B is the Bohr magneton. Spectra were recorded on melanins previously incubated for 30 minutes in a solution of zinc sulphate (3 mM) at pH ca. 5. This procedure served to increase free radical concentrations and to accentuate any pheomelanin signals that were present.[11] Samples were maintained at −196°C in a Dewar flask inside the ESR cavity. For all ESR measurements the modulation amplitude was 4 G and the microwave power was 20 μW.

Table 1 *Steps in the cell lysis experiments*

1 Ehrlich ascites carcinoma was maintained in mice by serial transplantation.
2 The ascites fluid was withdrawn from the mice 7 to 10 days after the transplantation of the tumour.
3 The tumour cells were isolated and washed. The cells were suspended in medium 199 (Menon *et al*).[18]
4 The cells were mixed with ^{51}Cr and incubated.
5 The cells were sedimented, washed, and then suspended in medium 199.
6 The cells were either incubated in the dark or irradiated in the presence of melanin from the brown or blue eyes, or in the absence of melanin.
7 The cells were sedimented and the amount of ^{51}Cr released in the supernatant was determined with a gamma counter.

Table 2 *Release of ^{51}Cr from labelled Ehrlich ascites carcinoma cells during incubation in the dark or irradiation in the presence of blue eye melanin (B1EM) or brown eye melanin (BrEM)*

*Reagents added**	*^{51}Cr release (%) Mean ± SEM*	
	Incubation	*Irradiation*
None	2·3±0·5	1·0±0·6
B1EM	2·5±0·6	4·5±0·6
BrEM	2·0±0·4	2·7±2·0

*Concentrations: B1EM and BrEM, 200μg/ml. The means ± SEM were calculated from the results of four experiments, done in duplicates.

Results

RELEASE OF THE ^{51}CR

Ehrlich ascites carcinoma cells labelled with ^{51}Cr were irradiated in the presence of blue or brown eye melanin. As a control the cells were either incubated in the dark in the presence of melanin or irradiated in the absence of melanin. The amount of ^{51}Cr released under each of the above conditions was compared with the ^{51}Cr released from the cells which were frozen and thawed.[18] The results are given in Table 2. Cells which were incubated in the dark or irradiated in the presence of either the blue or brown eye melanin did not produce any greater ^{51}Cr release than the control cells.

Reduction of NBT. Incubation or irradiation of

Table 3 *Formation of hydrogen peroxide during incubation in the dark or irradiation of blue eye melanin (BlEM) or brown eye melanin (BrEM)*

*Reagents added**	*H_2O_2 formed (μM) Mean ± SEM*	
	Incubation	*Irradiation*
None	0·0	0·0
BlEM	0·7±0·3	2·4±0·2
BrEm	0·7±0·3	3·3±0·1

*Concentrations: BlEM and BrEM, 200 μg/ml. The means ± SEM were calculated from the results of four experiments, done in duplicates.

Table 4 *Magnetic parameters for eye melanins**

Sample		*Line width, G†*	*g‡*
Blue eye	1, iris	5·50	2·0042
	2, iris	5·50	2·0041
	1, ciliary body	5·50	2·0040
	2, ciliary body	5·50	2·0041
	1, choroid	5·50	2·0043
	2, choroid	5·50	2·0042
	1, pigment epithelium	5·75	2·0042
	2, pigment epithelium	5·75	2·0040
Brown eye	1, iris	5·75	2·0041
	2, iris	5·50	2·0041
	1, ciliary body	5·50	2·0042
	2, ciliary body	5·50	2·0043
	1, choroid	5·50	2·0042
	2, choroid	5·75	2·0042
	1, pigment epithelium	5·50	2·0042
	2, pigment epithelium	5·50	2·0041

*Measured in 3 mM zinc sulphate solution at pH 5. Temperature was −196°C.
†± 0·25 G.
‡±0·0001.

either blue or brown eye melanin did not show any reduction of NBT, indicating that no superoxide was formed under these conditions.

Formation of hydrogen peroxide. Incubation in the dark or irradiation of either blue or brown eye melanin showed the formation of only a small amount of H_2O_2 (Table 3).

ELECTRON SPIN RESONANCE

ESR spectra were obtained for melanins extracted from the iris, ciliary body, choroid, and retinal pigment epithelium of brown and blue eyes. Representative spectra are shown in Fig. 1. For each spectrum the magnetic parameters of line width and *g* value were measured as reported in Table 4. These spectral parameters (line width ca. 5·5 G, *g* ca. 2·004) were for the most part characteristic of eumelanins derived from dopa.[22] In terms of the ESR spectroscopy melanins from the different individual eye tissues were almost indistinguishable.

Discussion

Our results showed that irradiation of blue or brown eye melanin did not produce any detectable amounts of superoxide, (as tested by the reduction of NBT). Irradiation of the carcinoma cells in the presence of blue or brown eye melanin did not produce any cell lysis. Our previous reports with hair melanins[6 11 14–18 23] showed that irradiation of phenomelanin produced considerable amounts of superoxide, whereas irradiation of eumelanin did not produce any superoxide under similar experimental conditions. Irradiation of Ehrlich ascites carcinoma cells in the presence of pheomelanin produced significantly more cell lysis than irradiation in the presence of eumelanin. Comparison of these findings with our present results indicates that the blue and brown eye melanins are essentially eumelanin. This is in conformity with our previous reports[10 19 20] that these eye melanins had very low sulphur contents in contrast with the pheomelanins.

The ESR spectroscopy supports the conclusion that blue eye melanin and brown eye melanin are more like eumelanin than pheomelanin. The ESR signals further suggested that with respect to the quality of melanins, in the individual tissues from blue and brown eyes, namely the iris, ciliary body, choroid, and retinal pigment epithelium, these melanins did not differ.

Consideration of the present results and our previous results on the ocular melanins[10 19 20] and

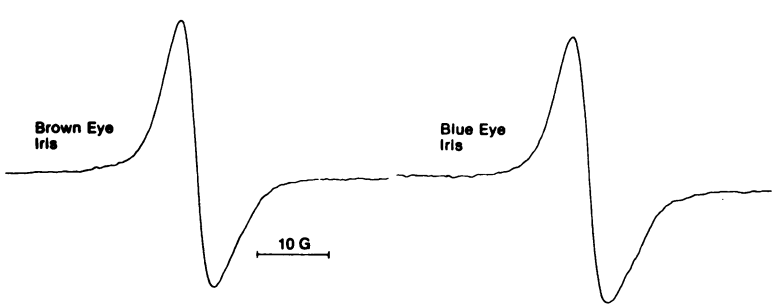

Fig. 1 *ESR spectra of melanins extracted from brown eye iris (left) and blue eye iris (right). Spectra were recorded at −196°C on samples incubated in 3 mM zinc sulphate solution at pH5.*

non-ocular melanins[6 11 14–18 23] lead to the possible conclusion that the classification of melanins into eumelanin and pheomelanin may be an oversimplification. It appears that there is a wide variety of melanins, two extreme prototypes of which may be represented by the melanins derived from dopa and cysteinyldopa. In between these extremes lie a number of melanins of a wide range of structures and properties. These intermediate melanins may be mixtures of pure eumelanin and pheomelanin, similar to dopa melanin and cysteinyldopa melanin, in diverse proportions or copolymers of dopa and cysteinyldopa.[11 14] It is highly possible that in all cases several intermediates in the synthetic pathway get incorporated into the melanins. The wide spectrum of melanins differing in colours and probably in other properties may be explained better on the basis of the above concepts of the chemical structure of the melanins.

Our previous results confirm that both blue and brown eye melanins are essentially eumelanins. However, the melanins from these two sources are not exactly identical; certain distinct differences exist between them as has been reported before.[10 19 20] From the information available at present these differences appear to be subtle when compared with the gross differences between the elemental composition and physicochemical properties of black and red hair melanins.[6 11 14–18 23] The factors responsible for the differences in the oxidation properties of the blue and brown eye melanins and the binding of certain drugs to these melanins as reported earlier[10 19 20] and the similarities in some of their other properties as reported in this paper are currently under investigation.

This research was funded by grants from the Medical Research Council of Canada, Lion's International District A-16 Ophthalmological Research Foundation, and the Independent Order of Odd Fellows and Rebekahs of Ontario, and grants from NIH (AM-26950 and DR-01008). We also wish to thank Dr R C Sealy for consultations in the ESR studies.

References

1 Jimbow K, Takeuchi T. Ultrastructural comparison of pheo-eumelanogenesis in animals. In: Klaus SN, ed. *Pigment cell.* Basel: Karger, 1979; **4:** 308–18.
2 Prota G, Thompson RH. Melanin pigmentation in mammals. *Endeavour* 1976; **35:** 32–8.
3 Thompson RH. The pigments of reddish hair and feathers. *Agnewandte Chemie* (international edition in English) 1974; **13:** 305–12.
4 Dryja TP, O'Neil-Dryja M, Albert DM. Elemental analysis of melanins from bovine hair, choroid, and retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 1979; **18:** 231–6.
5 Ito S, Novellino E, Chioccara F, Misuraca G, Prota G. Copolymerization of dopa and cysteinyldopa in melanogenesis in vitro. *Experientia* 1980; **36:** 822–3.
6 Menon IA, Persad S, Haberman HF, Kurian CJ. A comparative study of the physical and chemical properties of melanins isolated from human black and red hair. *J Invest Dermatol* 1983; **80:** 202–6.
7 Prota G. Structure and biogenesis of phaeomelanins. In: Riley V, ed. *Pigmentation: its genesis and biological control.* New York: Appleton-Century-Crofts, 1972: 615–30.
8 Rorsman H, Agrup G, Hansson C, Rosengren AM, Rosengren E. Detection of phaeomelanins. In: Klaus SN, ed. *Pigment cell.* Basel: Karger, 1979; **4:** 244–52.
9 Ito S, Jimbow K. Quantitative analysis of eumelanin and pheomelanin in hair and melanomas. *J Invest Dermatol* 1983; **80:** 268–72.
10 Menon IA, Persad S, Haberman HF, Kurian CJ, Basu PK. A qualitative study of the melanins from blue and brown human eyes. *Exp Eye Res* 1982; **34:** 531–7.
11 Sealy RC, Hyde JS, Felix CC, Menon IA, Prota G. Eumelanin and pheomelanin: characterization by electron spin resonance spectroscopy. *Science* 1982; **217:** 545–7.
12 Jimbow K, Ishida O, Ito S, Hori Y, Witkop CJ, King RA. Combined chemical and electron microscopical studies of pheomelanosomes in human red hair. *J Invest Dermatol* 1983; **81:** 506–11.
13 Jimbow K, Miyake Y, Homma K, *et al.* Characterization of melanogenesis by physicochemical properties of melanin and melanosomes in malignant melanoma. *Cancer Res* 1985; **44:** 1128–34.
14 Sealy RC, Hyde JS, Felix CC, *et al.* Novel free radical in synthetic and natural pheomelanins: distinction between dopa melanin and cysteinyldopa melanin by ESR spectroscopy. *Proc Natl Acad Sci USA* 1982; **79:** 2885–9.
15 Menon IA, Persad S, Ranadive NS, Haberman HF. Formation of superoxide and cell damage during UV-visible irradiation of melanin. In: Bors W, Saran M, Tait D, eds. *Oxygen radicals in chemistry and biology.* Berlin: Walter de Gruyer, 1984: 673–9.
16 Menon IA, Persad S, Ranadive NS, Haberman HF. Role of superoxide and hydrogen peroxide in cell lysis during irradiation *in vitro* of Ehrlich ascitic carcinoma cells in the presence of melanin. *Can J Biochem Cell Biol* 1985; **63:** 278–83.
17 Persad S, Menon IA, Haberman HF. Comparison of the effects of UV-visible irradiation of melanins and melanin-hematoporphyrin complexes from human black and red hair. *Photochem Photobiol* 1983; **37:** 63–8.
18 Menon IA, Persad S, Ranadive NS, Haberman HF. Effect of ultraviolet-visible irradiation in the presence of melanin isolated from human black or red hair upon Ehrlich ascites carcinoma cells. *Cancer Res* 1983; **43:** 3165–9.
19 Menon IA, Persad S, Haberman HF, Kurian CJ, Basu PK. Do the melanins from blue and brown human eyes differ? In: Seiji M, ed. *Pigment cell 1981: phenotypic expression of pigment cells.* Tokyo: University of Tokyo Press, 1981: 17–22.
20 Persad S, Menon IA, Basu PK, Haberman HF. Binding of imipramine, 8-methoxypsoralen and epinephrine to human blue and brown eye melanins. *J Toxicol Cutan Ocul Toxicol* 1986; **5:** 125–32.
21 Wang R, Nixon BT. Identification of hydrogen peroxide as a photoproduct toxic to human cells in tissue-culture medium irradiated with 'day light' fluorescent light. *In Vitro* 1978; **14:** 715–22.
22 Sealy RC. Radicals in melanin biochemistry. In: Parker L, ed. *Methods in enzymology, oxygen radicals in biological systems.* New York: Academic Press, 1984; **105:** 479–83.
23 Menon IA, Persad S, Ranadive NS, Haberman HF. Photobiological effects of eumelanin and pheomelanin. In: Bagnara J, Klaus SN, Paul E, Schartl M, eds. *Pigment cell 1985: biological, molecular and clinical aspects of pigmentation.* Tokyo: University of Tokyo Press, 1985: 77–85.

Accepted for publication 2 September 1986.