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SHORT REPORTS

Electron Spin Resonance Studies on Phaeomelanins

C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren

Departments of Dermatology and Pharmacology, University of Lund, Sweden

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Abstract. ESR spectra of synthetic melanins formed from dopa and from 5-S-cysteinyldopa and of melanins isolated from human melanomas were studied. Alkali-soluble melanin synthesized from cysteinyldopa showed a signal similar to that of insoluble melanin synthesized from dopa, but also a simple fine structure. Trichochromes C and F synthesized from 5-S-cysteinyldopa had a broader signal than dopamelanin, and in addition a hyperfine structure that could be explained by the presence of manganous ions. An insoluble eumelanin from a human melanoma with a sulphur content of 5% showed an ESR spectrum consisting of one line like dopamelanin. An alkali-soluble phaeomelanin from a human melanoma with a sulphur content of 10% showed a very broad signal with a hyperfine structure that could be explained by the presence of manganous ions.

Key words: Electron spin resonance; Free radicals; Phaeomelanin; Trichochrome; Manganous ions

Human melanins are generally classified into two groups, eumelanins and phaeomelanins. *Eumelanins* are dark or black, insoluble in all solvents, and have been considered to contain little sulphur. *Phaeomelanins* are lighter in colour, yellow, brown or red, soluble in alkaline solutions, and some of them, the trichochromes, also in acid (10). They contain large amounts of sulphur. However, it has recently been demonstrated that dark melanin from human melanoma, insoluble in alkaline and acid solutions, may also contain large amounts of sulphur (11). It has previously been thought that some of the sulphur in isolated eumelanins could be attributed to sulphur bonds to proteins, but the large amounts of sulphur present in the eumelanin studied by us cannot be explained in this way. Instead it has been suggested that eumelanins with a high sulphur content also contain cysteinyldopa monomers (11).

The above findings complicate the classification of melanins. It is well known that eumelanins contain stable free radicals (2, 5, 8). Phaeomelanins have only recently been analysed in this respect (1). They have been found to have an ESR spectrum similar to eumelanins, but a hyperfine structure has also been registered. We now present an explanation for the hyperfine structure reported in phaeomelanins, which seems to be due to the presence of manganous ions in these pigments.

MATERIAL AND METHODS

Dopa melanin was prepared by air oxidation of 20 mg dopa in 25 ml water adjusted to pH 9–11 by NH_3 at room temperature for 4 days. All water used was triple glass distilled. In another experiment dopa melanin was prepared enzymatically by addition of 2 mg tyrosinase (2 750 U/mg) to 20 mg dopa in 25 ml 0.1 M phosphate buffer at pH 6.5 and incubation for 48 hours at 37°C.

Cysteinyldopa melanin was prepared by incubating 20 mg dopa and 20 mg cysteine with 2 mg tyrosinase (2 750 U/mg) in 25 ml 0.5 M phosphate buffer pH 6.5 at 35°C for 72 hours.

The melanins formed were centrifuged and dialysed against water. They were freeze-dried and kept in desiccator over P_2O_5 until ESR examination.

Insoluble and soluble melanins were isolated from two different human melanomas as previously described (11).

Trichochrome F was formed from 5-S-cysteinyldopa on a Dowex 50W-X4 column in H^+ form by elution with 2 M HCl. The pigment was chromatographed on paper

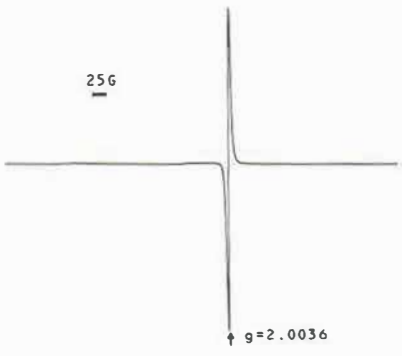

Fig. 1. ESR spectrum of an insoluble melanin prepared by incubation of dopa with tyrosinase.

Fig. 2. ESR spectrum of a solid sample of a synthetic alkali soluble melanin prepared by incubation of cysteine and dopa with tyrosinase.

(Whatman 3M) in isopropanol–formic acid–concentrated HCl (30:70:1, v/v), and eluted with 0.1 M NaOH. The eluate was evaporated to dryness and then kept in desiccator over P_2O_5 until ESR examination.

Trichochrome C was prepared from 5-S-cysteinyldopa as previously described (12), chromatographed on paper and eluted as trichochrome F.

Calculations from the ESR spectrum gave us reason to believe that the hyperfine structure previously reported in phaeomelanin could be explained by the presence of manganous ions in these pigments.

First derivative ESR spectra were recorded on solid samples with a Varian E-3 ESR spectrometer at room temperature. The g-values were obtained by measuring spectra with diphenylpicrylhydrazine (DPPH, g = 2.0036) in the form of small crystals as an internal standard. The g-value of DPPH is indicated by an arrow in the figures.

RESULTS

Dopa melanin prepared by incubation with tyrosinase and dopa melanin prepared by air oxidation showed identical ESR spectra with no hyperfine structure (Fig. 1).

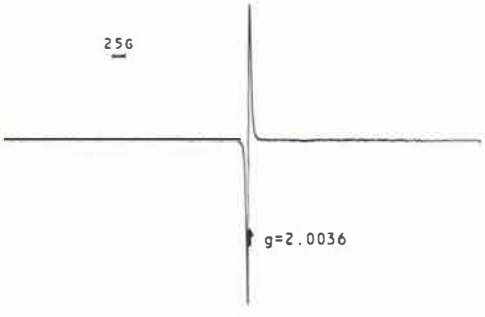

Fig. 3. ESR spectrum of an insoluble melanin containing 5% sulphur from a human melanoma.

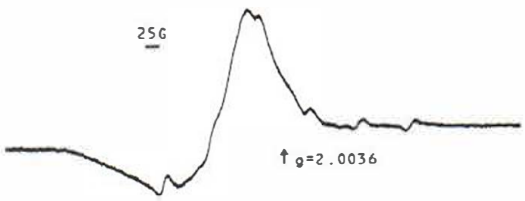

Fig. 4. ESR spectrum of a solid sample of an alkali soluble melanin containing 10% sulphur isolated from a human melanoma.

Cysteinyldopa melanin prepared by incubation of dopa and cysteine with tyrosinase gave a strong signal with a simple hyperfine structure (Fig. 2).

The melanin that contained 5% sulphur, isolated from a human melanoma, was insoluble in 0.1 M HCl and 0.1 M NaOH. With regard to solubility characteristics it was an *eumelanin*. It had the same ESR spectrum as dopa melanin, with no hyperfine structure (Fig. 3).

The melanin containing 10% sulphur, isolated from another melanoma from a patient with red hair, was soluble in 0.1 M NaOH but not in 0.1 M HCl, and was thus in all respects a *phaeomelanin*. It showed a strong broad signal with a hyperfine structure similar to that of trichochromes, described below (Fig. 4).

The trichochromes showed spectra with a broad signal with a g-value corresponding to that of melanin polymers.

Trichochrome F had in addition a hyperfine structure (Fig. 5).

Trichochrome C, too, showed a spectrum with a hyperfine structure (Fig. 6).

Manganous ions showed the hyperfine structures observed in the spectra of trichochromes and of

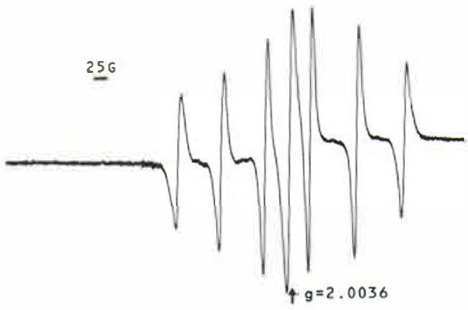

Fig. 5. ESR spectrum of solid trichochrome F.

Fig. 6. ESR spectrum of solid trichochrome C.

melanoma phaeomelanins. An atomic absorption analysis of trichochrome F gave a manganese content of 17 ng/mg melanin (9).

DISCUSSION

The synthetic dopa melanins showed stable free radicals of the type previously described. These radicals have been related to the ortho-semiquinone structure (Fig. 7*a*) within the melanin polymer which probably consists mainly of indol monomers (7, 8). The phaeomelanin monomers, the cysteinyldopas, are thought to give predominantly benzothiazine ring systems as their primary condensation product. (4). The further oxidation and polymerization of these phaeomelanin precursors would give an ortho-semiquinonimine structure in the melanin polymer and in the trichochromes (Fig. 7*b*). The synthetic cysteinyldopa melanins also showed stable free radicals with ESR characteristics similar to dopa melanins, but had also a simple type of hyperfine structure.

The simplest form of phaeomelanin, the trichochromes, which are dimers of benzothiazine derivatives, showed a broad signal similar to that of the melanin polymers. The free radical signal in dry melanins can thus also be found in the smallest melanin molecules. The ESR-signal for the ortho-semiquinone in eumelanin was narrower than that of the trichochromes and phaeomelanins.

The fact that trichochromes in dry state show free radicals is of special interest, since these are the only melanins with known molecular structure.

Free radicals in dried material mostly reflect the presence of radical precursors in the biological system which in the dry state react with oxygen to give radicals. They can therefore usually only be related indirectly to the original metabolic activity of the tissues.

Owing to the insolubility of eumelanins, we were obliged to perform our studies on solid samples. To enable us to carry out comparisons between melanins of different melanomas, the phaeomelanins were also studied in solid form.

After the purification procedure the phaeomelanic samples showed a hyperfine structure which could be related to manganous ions. On refluxing these melanins in 6 M HCl and then precipitating them at pH 7 and washing them repeatedly with water, the manganous signal disappeared. Manganese is known to be present in natural melanins (6), and these melanins are also known to have affinity to manganous ions (3). However, a hyperfine structure for manganous ions can only be expected in complexes showing a very high degree of symmetry (9). The differences in solubility make it impossible to purify phaeomelanins by the same methods as eumelanins. Concentration of manganous ions present in the tissue may also occur in the isolation procedure for phaeomelanins. The absence of hyperfine structure in eumelanins of biologic origin may therefore be due to the extraction of manganous ions originally present.

It should be noted, however, that a hyperfine structure observed in red phaeomelanic hair has not been found in black eumelanic hair (13). This may indicate that manganous ions are present in special complexes in phaeomelanin.

The complex formation capacity of eumelanins to metal ions have been studied in detail (7), whereas those of phaeomelanins and trichochromes are unknown. Possible differences in complex formation capacity of different melanins to essential metal ions may influence the enzymatic activity in the melanocyte.

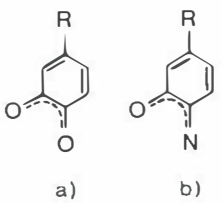

Fig. 7. (*a*) Ortho-semiquinone and (*b*) ortho-semiquinonimine.

ACKNOWLEDGEMENTS

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Diffuse Melanosis and Trichochromuria in Malignant Melanoma

G. Agrup, P. Agrup, C. Hansson, H. Rorsman, and E. Rosengren

Departments of Dermatology, Radiotherapy (Malmö) and Pharmacology, University of Lund, Sweden

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Abstract. A previous finding of pronounced excretion of trichochromes in a patient with metastasizing melanoma and diffuse melanosis prompted this study on trichochromes in another patient with these two features. The patient now reported also excreted large amounts of trichochromes. It is suggested that diffuse melanosis in metastatic melanoma patients is due to deposition of trichochromes in the tissue.

Diffuse melanosis is uncommon in metastasizing malignant melanoma. Among 161 patients with melanoma metastasis examined by us for 5-S-cysteinyldopa excretion, 3 were found to have diffuse melanosis. In a study on trichochromes in the urine of 16 melanoma patients with metastasis we observed a very high value in one patient, who also exhibited diffuse melanosis (1). Since, like diffuse melanosis, pronounced trichochrome excretion is rare, we assumed that the occurrence of both phenomena in the same patient suggested a biological relationship. The urine of another of the 3 patients who developed diffuse melanosis was also examined, and the findings support our assumption that trichochromuria and diffuse melanosis are closely related.

MATERIAL AND METHODS

The patient investigated was a 43-year-old brown-haired man suffering from widespread melanoma metastasis. A history of a pigmented, bleeding skin tumour which had healed spontaneously antedated the appearance of lymph node metastases by 8 years. The first metastasis appeared 2 years before our investigation, and during the intervening period the patient received surgical treatment, chemotherapy with DTIC® and Bleomycin, and radiotherapy. Diffuse melanosis became widespread during the 2 months before our investigation. At the time of our study the patient showed multiple lymph node metastases and a liver scintigram consistent with liver metastases. Histology and cytology of involved lymph nodes showed histiocyte-like melanoma cells, with most of the melanin in macrophages. He died 1 month after the investigation of trichochromes with symptoms of abdominal metastasis. Necropsy was not done.