Biochimica et Biophysica Acta, 1116 (1992) 11–16
© 1992 Elsevier Science Publishers B.V. All rights reserved 0304-4165/92/$05.00

BBAGEN 23642

Antioxidative activity of synthetic melanins. Cardiolipin liposome model

Małgorzata Porębska-Budny [a], Natalya L. Sakina [b], Krystyna B. Stępień [a], Alexander E. Dontsov [b] and Tadeusz Wilczok [a]

[a] *Department of Biochemistry and Biophysics, Silesian Medical Academy, Sosnowiec (Poland) and* [b] *Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow (USSR)*

(Received 22 July 1991)
(Revised manuscript received 22 October 1991)

Key words: Catecholamine-melanin; DOPA-melanin; Antioxidant; Cardiolipin; Lipid peroxidation; Melanin

The inhibiting effect of melanin synthesized from dihydroxyphenylalanine (DOPA), dopamine, adrenaline and adrenolutin on the ultraviolet- or the Fe^{2+}-ascorbic acid-induced peroxidation of cardiolipin liposomes has been studied. All these melanins are able to inhibit both the ultraviolet- and the Fe^{2+}-ascorbic acid-induced lipid peroxidation. Antioxidative activity of melanins enhances in the order: dopamine–melanin < melanin synthesized from dopamine in the presence of Cu^{2+} < DOPA – melanin < melanin synthesized from adrenaline in the presence of Cu^{2+} ≅ adrenolutin–melanin, and correlates with their ability to scavenge superoxide anion radical. The optical screening effect of the investigated melanins in the inhibition of lipid peroxidation was not higher than 15% for the most active melanins.

Introduction

There are many types of natural melanins produced from different original substrates. Eumelanins are derived from the tyrosine or DOPA metabolism, whereas pheomelanins are mainly the products of cysteinyl-DOPA oxidation [1]. Melanins are usually located in the tissues of the eye, hair and skin. There are indications that melanins in these structures are involved in free radical reactions of lipid peroxidation, where they may act as natural radio- and photoprotectors [2–4]. The photoprotective role of the melanin pigment is realized by the ability to dissipate light energy either as heat or in chemical reaction, resulting in the consumption of molecular oxygen, and to scavenge active oxygen species (e.g., superoxide anion radical, singlet oxygen) [5–8]. It is known that melanin biopolymers easily react with free radical particles and form complexes with prooxidant Fe^{2+} [9–13].

However, only little is known about melanins formed from catecholamines. These pigments are basically located in the cells of the substantia nigra and locus coeruleus of human brain (neuromelanins) and in human blood plasma (rheomelanins) [14–16]. Their function is still unclear. However, it is speculated that neuromelanins may participate in bioelectronic processes in pigmented neurons of the human brain through phonon-electron coupling [17]. Neuromlanins are known to disappear from the middle brain structures during natural development of Parkinson disease [18] or artificially induced disease (Mn^{2+} or MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [19]. It is suggested that neuromelanin also participate in free radical processes in these structures of brain [20,21].

For this reason the comparison of the antioxidative activity of synthetic neuromelanins obtained from catecholamines and the suggested antioxidative activity of DOPA-melanin is of great interest. Such comparison may improve the understanding of the biological function of natural neuromelanins.

Materials and Methods

Preparation of melanins

Melanins were obtained by oxidative polymerization of DOPA, dopamine, adrenaline and adrenolutin solutions (5 mM) in Tris-HCl buffer (0.05 M, pH 7.4), in the absence or presence of copper ions, as described previously [22]. The melanins prepared from catecholamine/copper mixtures (catecholamine/Cu^{2+} ratio of 2:1) were treated with 0.1 M hydrochloric acid

Correspondence: T. Wilczok, Department of Biochemistry and Biophysics, Silesian Medical Academy, Jagiellońska 4, 41-200 Sosnowiec, Poland.

until the bound copper ws completely removed. The obtained preparations were stored as aqueous suspensions of known melanin concentration.

Liposome preparation and induction of the lipid peroxidation

Multilamellar liposomes from commercial bovine heart cardiolipin were prepared by the method described by Ostrovsky et al. [2]. Ethanol solution of cardiolipin was evaporated under vaccum in nitrogen atmosphere. The thin film of cardiolipin obtained was shaken with 0.05 M Tris-HCl buffer at pH 7.4 (control liposomes) or with melanin suspension in this buffer. The liposome suspension contained 2.5 or 1.25 mg cardiolipin per ml.

Lipid peroxidation in cardiolipin liposomes was induced by: (a) 15 μM Fe^{2+} and 0.8 mM ascorbic acid in 0.05 M Tris-HCl buffer (pH 7.4); (b) ultraviolet radiation (120 W mercury lamp; intensity 50 mW cm^{-2}). The accumulation of cardiolipin peroxidation products was estimated by the determination of 2-thiobarbituric acid (TBA)-reactive products in the incubation medium [23], and expressed as the increase of absorbance at 535 nm (ΔA 535).

The interaction of melanin with superoxide anion radical (O_2^-)

Generation of superoxide anion radical was determined by the method of Beauchamp and Fridovich [24] with modifications made by Sakina et al. [25]. The assay medium contained 5.15 μM riboflavin, 10 mM methionine, 0.009% cethyltrimethylammonium bromide (CTAB), 70 μM tetranitroblue tetrazolium (TNBT) in 0.05 M potassium phosphate buffer (pH 7.8) (control system) and melanin in a concentration of 50 μg/ml in this buffer. The reaction was initiated by illumination with halogen lamp (12 V/100 W) equipped with filter transmitting in the riboflavin absorption region ($\lambda_{max} = 380$ nm). Under these conditions the TNBT reduction caused by the superoxide anion and the formazan formation ($\lambda_{max} = 560$ nm) were observed. In order to investigate the effect of different melanins on the TNBT reduction, the reaction medium contained 70 μM TNBT, 0.009% CTAB and 50 μg/ml of melanin in 0.05 M potassium phosphate buffer (pH 7.8).

The result of superoxide anion interaction with various melanins was expressed as the increase of absorbance at 560 nm (ΔA 560) after the subtraction of this part of absorbance at 560 nm which is connected with the TNBT reduction by melanin.

Results and Discussion

The effect of melanins on the ultraviolet-induced cardiolipin peroxidation is shown in Figs. 1 and 2. Fig. 1 describes the influence of various concentrations of melanin synthesized from dopamine in the presence of Cu^{2+} on the TBA-reactive products accumulation. It is evident that this melanin has the inhibiting action on the cardiolipin peroxidation in liposomal membranes and that the extent of this inhibition depends directly on melanin concentration.

Comparative investigation of the inhibiting effect of different melanins on the ultraviolet induced cardiolipin peroxidation demonstrated that all melanins decrease significantly the rate of the lipid peroxidation. The relationship of the inhibition degree to melanin concentration is shown in Fig. 2. Among the analyzed biopolymers, the most effective inhibitors were melanins synthesized from adrenaline in the presence of Cu^{2+}, from adrenolutin and from DOPA, whereas the least active were melanins synthesized from dopamine both in the presence and in the absence of Cu^{2+}. As can be seen from Fig. 2, melanins prepared from adrenaline in the presence of Cu^{2+} and from adrenolutin, at a concentration of 100 μg/ml in the reaction medium inhibited cardiolipin peroxidation by about 65% and 56%, respectively, whereas melanins prepared from dopamine did not show any significant effect.

It is suggested that the inhibiting effect of melanin on the ultraviolet-induced lipid peroxidation is due to both passive screening of light and chemical interaction with products of lipid peroxidation [26]. It is well

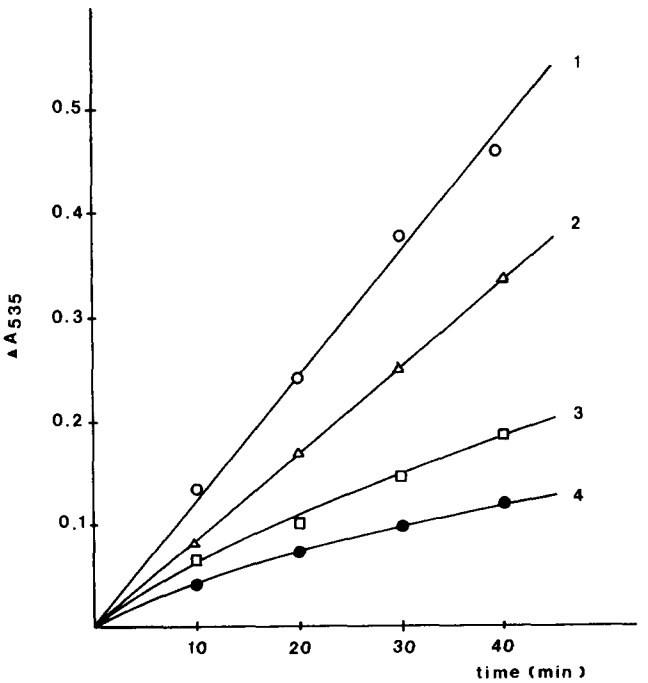

Fig. 1. The inhibiting effect of melanin synthesized from dopamine in the presence of Cu^{2+} on the ultraviolet-induced cardiolipin peroxidation. Melanin concentrations: control, 0 μg/ml (1), 200 μg/ml (2), 400 μg/ml (3), 800 μg/ml (4); cardiolipin concentration, 2.5 mg/ml.

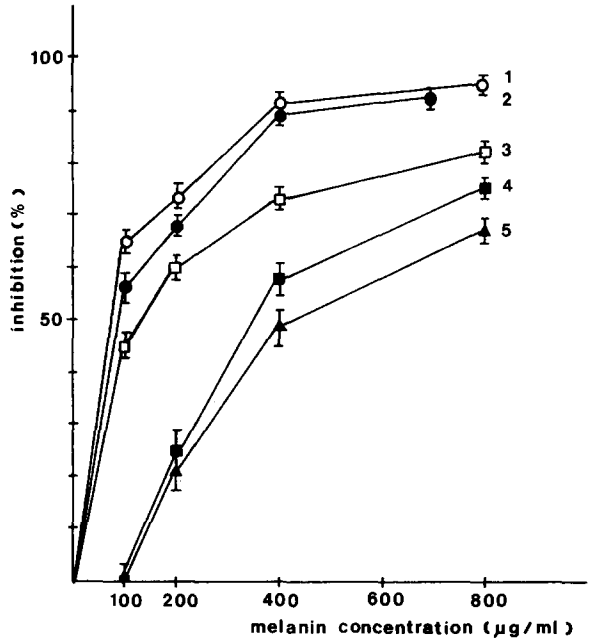

Fig. 2. Comparison of the inhibiting effect of synthetic melanins on the ultraviolet-induced cardiolipin peroxidation. Melanins obtained from: adrenaline in the presence of Cu^{2+} (1), adrenolutin (2), DOPA (3), dopamine in the presence of Cu^{2+} (4), dopamine (5). Cardiolipin concentration, 2.5 mg/ml; the ultraviolet light intensity, 50 mW cm^{-2}.

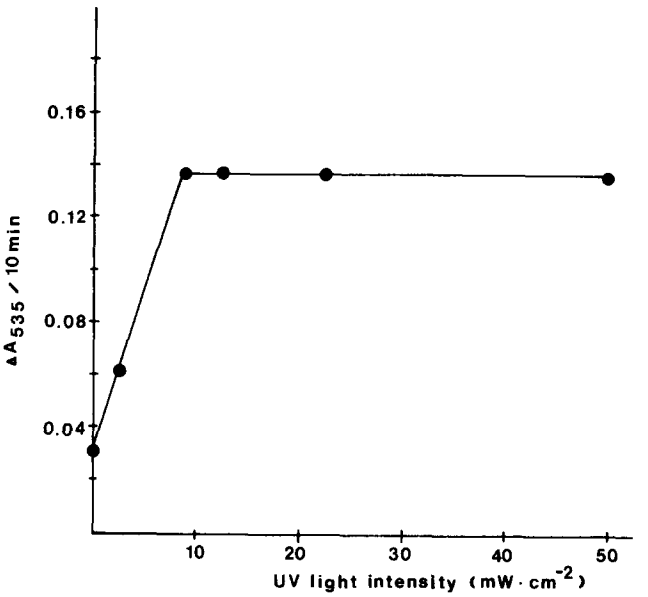

Fig. 3. Dependence of the cardiolipin photooxidation rate on the ultraviolet light intensity. Cardiolipin concentration, 2.5 mg/ml.

known that melanin absorbs the ultraviolet light and, thus, decreases the light intensity by simple screening action. In order to determine the optical screening effect of investigated melanins, the relationship of the ultraviolet-induced cardiolipin peroxidation rate against the ultraviolet light intensity was studied. As it is seen in Fig. 3, lower light intensity (up to 20% of original level) does not lead to alteration of the rate of TBA-reactive products accumulation under the described experimental conditions. The optical screening effect of melanins was calculated as previously presented [2]. It is apparent from Table I that the melanin screening effect (i.e., reduction in the amount of the ultraviolet irradiation reaching the cardiolipin) was not higher than 80% at almost all melanin concentrations under the experimental conditions used. When the screening effect was higher than 80% (i.e., for DOPA-melanin, melanin synthesized from adrenaline in the presence of Cu^{2+}, and adrenolutin-melanin in the highest concentrations) the correction for deceleration of lipid peroxidation products accumulation caused by decrease of the ultraviolet light intensity should be applied. It was estimated that the contribution of optical screening effect in the inhibition of lipid peroxidation process for the above-mentioned most effective melanins in the highest concentrations (700–800 μg/ml) is only about 15%.

Ferrous ions are known to be a strong stimulators of lipid peroxidation [27]. The inhibiting effect of different melanins on dark, Fe^{2+}-ascorbic acid-induced system of cardiolipin peroxidation in liposomal membranes is shown in Fig. 4. In this case, the most active inhibitors were melanins synthesized from adrenolutin, adrenaline in the presence of Cu^{2+} and DOPA, whereas the least inhibiting ability possessed melanins prepared from dopamine both in the presence and in

TABLE I

The screening effect of synthetic melanins

Melanin synthesized from	Melanin concentration (μg/ml)	Screening effect (% of ultraviolet irradiation) absorbed by melanin)
Adrenolutin	100	46.7
	200	63.6
	400	77.8
	700	86.0
Adrenaline in the presence of Cu^{2+} [a]	100	43.7
	200	60.8
	400	75.6
	800	86.1
DOPA	100	39.8
	200	57.0
	400	72.6
	800	84.1
Dopamine in the presence of Cu^{2+} [a]	100	25.2
	200	40.3
	400	57.4
	800	73.0
Dopamine	100	14.0
	200	24.5
	400	39.4
	800	56.5

[a] Copper was removed by treatment with 0.1 M HCl.

the absence of Cu^{2+}. As can be seen from Fig. 4, at low concentrations of melanin in the reaction medium (100 μg/ml) melanins prepared from dopamine did not show any significant effect on cardiolipin oxidation, whereas adrenolutin-melanin and melanin synthesized from adrenaline in the presence of Cu^{2+} inhibited cardiolipin peroxidation by about 30%. It is postulated that melanins chelate prooxidant Fe^{2+} and, thus, inhibit lipid peroxidation. The formed melanin-Fe^{2+} and melanin-Fe^{3+} complexes, even in the presence of ascorbic acid, have not ability to initiate the lipid oxidation reaction [2].

The obtained results demonstrate that catecholamine-melanins occupy a double position owing to their properties: antioxidative activity of melanins synthesized from adrenolutin and from adrenaline in the presence of copper ions is comparatible with or even above the antioxidative activity of DOPA-melanin. At the same time, the antioxidative ability of melanins prepared from dopamine both in the presence and in the absence of Cu^{2+} is significant lower than for DOPA-melanin.

The differences in the antioxidative activity of investigated melanins mainly depend on the differences in their ability to react with peroxide radicals. In order to determine the melanin interaction with superoxide anion radicals, the O_2^- mediated, reduction of TNBT with the formation of diformazan was studied. Superoxide anion was generated by illumination of riboflavin-methionine system. The photoreduction of nitroblue

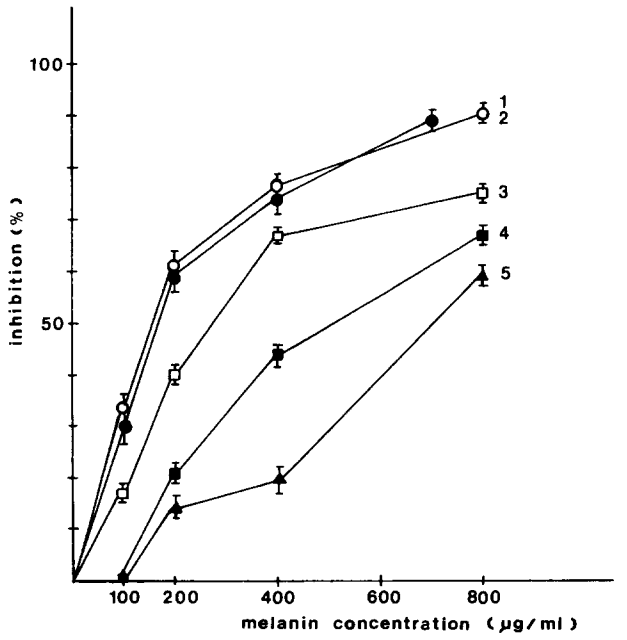

Fig. 4. Comparison of the inhibiting effect of synthetic melanins on the Fe^{2+}-ascorbic acid-induced cardiolipin peroxidation. Melanins obtained from: adrenolutin (1), adrenaline in the presence of Cu^{2+} (2), DOPA (3), dopamine in the presence of Cu^{2+} (4), dopamine (5). Cardiolipin concentration, 1.25 mg/ml.

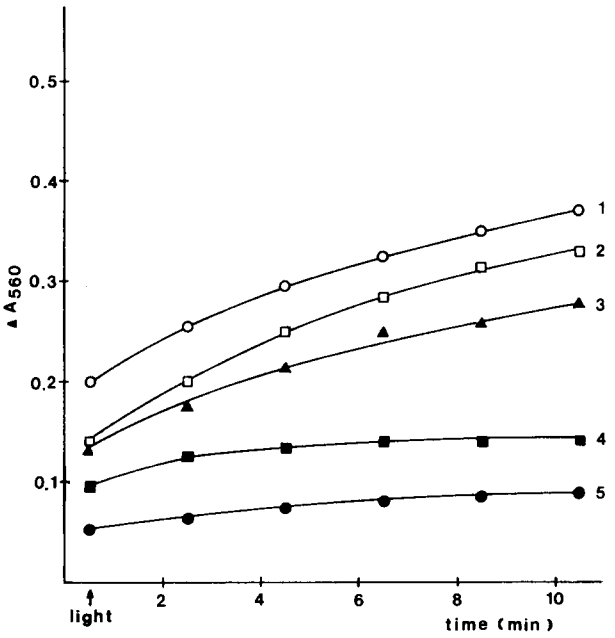

Fig. 5. Photoreduction of tetranitroblue tetrazolium by melanins (50 μg/ml) synthesized from adrenolutin (1), DOPA (2), adrenaline in the presence of Cu^{2+} (3), dopamine in the presence of Cu^{2+} (4), dopamine (5).

tetrazolium in the presence of riboflavin and methionine is known to be inhibited by SOD [24]. It was previously found that the xanthine-xanthine oxidase system is not applicable for studying the reaction of superoxide anion with melanins, as the enzymatic activity of xanthine oxidase is considerably inhibited by melanin [10].

It was found that all tested melanins reduced TNBT both during illumination of the reaction medium and without illumination, and the formation of diformazan was not, or only slightly, affected by SOD. As can be seen from Fig. 5, the largest activity in this respect is associated with melanins synthesized from adrenolutin, DOPA and adrenaline. The direct reduction of nitroblue tetrazolium by DOPA-melanin and black hair melanin was reported by Persad et al. [28] and Tomita et al. [29]. Melanins and superoxide anion radicals (O_2^-) may compete with TNBT which is reduced by both to diformazan [29]:

melanin-red. + TNBT → melanin-ox. + diformazan

O_2^- + TNBT → O_2 + diformazan

Fig. 6 illustrates the dynamic of diformazan formation during the TNBT reduction by O_2^- in the control system (curve 1) and in the presence of melanins (curves 2–6). It was demonstrated that all examined melanins caused the decrease of diformazan formation rate and, this effect is probably associated with scavenging of superoxide radicals by melanin. Among the analyzed melanin biopolymers, the preparation ob-

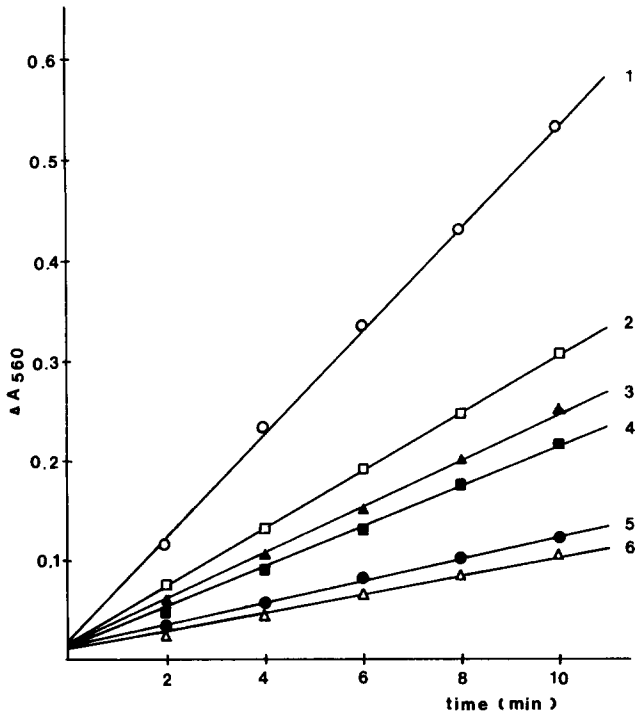

Fig. 6. The kinetics of diformazan formation during the TNBT reduction by superoxide anion radical (O_2^-) generated in the riboflavin-methionine irradiated system, in the absence (1) and in the presence of various synthetic melanins in concentration of 50 μg/ml (2–6). Melanins synthesized from: dopamine (2), dopamine in the presence of Cu^{2+} (3), adrenaline in the presence of Cu^{2+} (4), DOPA (5), adrenolutin (6). The result of reaction was expressed as the increase of absorbance at 560 nm after the subtraction of this part of absorbance at 560 nm which is connected with the TNBT photoreduction by melanins.

tained from adrenolutin was the most effective scavenger of superoxide radical, whereas melanins synthesized from dopamine were the least active scavengers. The obtained results demonstrate that there is a simple correlation between the inhibiting action of investigated melanins in both the ultraviolet and the Fe^{2+}-ascorbic acid-induced cardiolipin peroxidation and their activity in scavenging of superoxide radicals.

It is well known that melanins are redox polymers containing high concentrations of *o*-quinone (oxidizing) and *o*-hydroquinone (reducing) groups [30]. Since superoxide radical can act both as an oxidizing and a reducing agent, the following reactions should be assumed considering the melanin scavenging activity [10]:

$$O_2^- + \text{melanin-ox.} \rightarrow O_2 + \text{melanin radical}$$

$$O_2^- + \text{melanin-red.} \xrightarrow{2\,H^+} H_2O_2 + \text{melanin radical}$$

It has been calculated that approx. 30% of the reaction between superoxide anion and DOPA-melanin occurs via the reduction of superoxide to hydrogen peroxide, and the rest (approx. 70%) of the reaction occurs via oxidation of superoxide to molecular oxygen [31].

The results presented above indicate that the antioxidative activity of examined melanins depends on the nature of the precursor used for melanin polymerization, and in consequence on the molecular structure of the formed melanin polymer. The similar and the highest antioxidative effect was obtained for adrenolutin-melanin and melanin prepared from adrenaline in the presence of Cu^{2+}.

As it was shown previously on the basis of pyrolysis data [22], melanins formed from dopamine and adrenaline in the presence of copper ions and also from adrenolutin are mainly composed from indole type monomer units, whereas melanins prepared in the absence of Cu^{2+} contain additionally substantial amount of unindolized precursor-derived units, e.g., catecholamines and their quinones. A great similarity between the infrared spectra of melanins obtained from adrenolutin and adrenaline in the presence of copper ions was observed [22]. The investigation of semiconductor properties of catecholamine-melanin also revealed that the electric parameters (conductivity and photocurrent intensity) of melanin prepared from adrenaline in the presence of copper ions were very similar to those of adrenolutin-melanin [32]. The assumption of a similarity of chemical structure of both melanins is also supported by a study on the rearrangement of adrenochrome, the first isolable intermediate in the oxidation of adrenaline. Palumbo et al. [33] found that Cu^{2+} significantly directed the reaction course towards the formation of adrenolutin, while without of metal ions a dimeric compound consisting of an adrenolutin moiety covalently linked to the angular 9-position of adrenochrome was the major product of adrenochrome rearrangement.

Recently, d'Ischia et al. [34] have isolated and identified two hitherto unknown products of adrenolutin autoxidation: 5,6-dihydroxy-1-methyl-2,3-indoledione and its 4,4'-dimer. But in the literature no evidence exist that such compounds can be formed during melanin preparation by oxidative polymerization of adrenolutin.

Conclusion

Synthetic analogues of natural neuromelanins, catecholamine-melanins exert effective protection against both the ultraviolet- and the Fe^{2+}-ascorbic acid-induced lipid peroxidation. The protecting ability depends on conditions of melanin synthesis (presence or absence of copper ions) and on the nature of catecholamine precursors used, as well as on melanin concentration.

Intensive biochemical processes, accompanied probably by the appearance of free radical oxidation products, take place in the brain structures containing neuromelanins [20,35]. So far, it is not clear whether

neuromelanin is physiologically inactive product of free radical oxidation of neuromediators (dopamine, adrenaline, noradrenaline) or fulfils some physiological function in these structures.

The data obtained suggest that neuromelanins may actively participate in the inhibition of lipid peroxidation processes in brain structures.

References

1 Prota, G. (1980) J. Invest. Dermatol. 75, 122–137.
2 Ostrovsky, M.A., Sakina, N.L. and Dontsov, A.E. (1987) Vision Res. 27, 893–899.
3 Pathak, M. and Fitzpatrick, T.B. (1974) in Sunlight and Man (Fitzpatrick, T.B., Pathak, M.A., Haber, L.C., Seiji, M. and Kukita, A., eds.), pp. 725–750, University of Tokyo Press, Tokyo.
4 Porębska-Budny, M. and Stępień, K. (1990) Acta Biochim. Pol. 37, 89–92.
5 Sarna, T. and Sealy, R.C. (1984) Photochem. Photobiol. 39, 69–74.
6 Sarna, T., Menon, I.A. and Sealy, R.C. (1984) Photochem. Photobiol. 39, 805–809.
7 Goodchild, N.T., Kwock, K. and Liu, P.S. (1981) in Oxygen and Oxy Radicals in Chemistry and Biology (Rodgers, M.A.J. and Powers, E.I., eds.), p. 648, Academic Press, New York.
8 Sealy, R.C., Sarna, T., Wanner, E.J. and Reszka, K. (1984) Photochem. Photobiol. 40, 453–459.
9 Sławińska, D., Sławiński, J. and Cieśla, L. (1983) Physiol. Chem. Phys. Med. NMR 15, 209–222.
10 Korytowski, W., Kalyanaraman, I.A., Menon, I.A., Sarna, T. and Sealy, R.C. (1986) Biochim. Biophys. Acta 882, 145–153.
11 Sarna, T., Pilas, B., Land, E.J. and Truscott, T.G. (1986) Biochim. Biophys. Acta 883, 162–167.
12 Bruenger, F.W., Stover, B.J. and Atherton, D.R. (1967) Radiat. Res. 32, 1–12.
13 Bagirov, R.M., Stukan, R.A., Dontsov, A.E., Ostrovsky, M.A. and Lapina, V.A. (1986) Biofizika 31, 469–474 (in Russian).
14 Van Woert, M.H., Prasad, K.N. and Borg, D.C. (1967) J. Neurochem. 14, 707–716.
15 Hegedus, Z.L. and Altschule, M.D. (1970) Arch. Int. Physiol. Biochim. 78, 443–459.
16 Graham, D.G. (1979) Arch. Pathol. Lab. Med. 103, 359–362.
17 Lacy, M.E. (1984) J. Theor. Biol. 111, 201–204.
18 Adams, R., Van Bogaert, L. and Eachen, H. (1964) J. Neuropath. Exp. Neurol. 23, 584–589.
19 Donaldson, J., Mc Gregor, D. and La Bella, F. (1982) Can. J. Physiol. Pharmacol. 60, 1398–1405.
20 Mc Ginness, J.E. and Proctor, P. (1973) J. Theor. Biol. 39, 677–678.
21 Ostrovsky, M.A. and Dontsov, A.E. (1985) Physiol. Chelov. 11, 670–678 (in Russian).
22 Stępień, K.B., Dworzański, J.P., Bilińska, B., Porębska-Budny, M., Hollek, A.M. and Wilczok, T. (1989) Biochim. Biophys. Acta 997, 49–54.
23 Buege, J.A. and Aust, S.D. (1978) Methods Enzymol. 52c, 302–310.
24 Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.
25 Sakina, N.L., Dontsov, A.E. and Ostrovsky, M.A. (1985) Biokhimija, 50, 78–83 (in Russian).
26 Ostrovsky, M.A., Sakina, N.L. and Dontsov, A.E. (1987) J. Evol. Biochem. Physiol. 23, 575–581 (in Russian).
27 Barber, A.A. (1966) Lipids 1, 146–151.
28 Persad, S., Menon, I.A. and Haberman, H.F. (1983) Photochem. Photobiol. 37, 63–68.
29 Tomita, Y., Hariu, A., Kato, C. and Seiji, M. (1984) J. Invest. Dermatol. 82, 573–576.
30 Nicolaus, R.A. (1968) Melanins, Herman, Paris.
31 Korytowski, W., Pilas, B., Sarna, T. and Kalyanaraman, I.A. (1987) Photochem. Photobiol. 45, 185–190.
32 Jastrzębska, M.M., Stępień, K., Wilczok, J., Porębska-Budny, M. and Wilczok, T. (1990) Gen. Physiol. Biophys. 9, 373–383.
33 Palumbo, A., d'Ischia, M., Misuraca, G. and Prota, G. (1989) Biochim. Biophys. Acta 990, 297–302.
34 d'Ischia, M., Palumbo, A. and Prota, G. (1988) Tetrahedron 44, 6441–6446.
35 Lamale, A.A. (1971) Biochem. Biophys. Res. Commun. 43, 893–898.