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# NADPH-DEPENDENT MELANIN PIGMENT FORMATION FROM 5-HYDROXY-INDOLEALKYLAMINES BY HEPATIC AND CEREBRAL MICROSOMES

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SUMMARY

Incubation of 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxytryptophol and 5-hydroxyindoleacetic acid with rat liver or brain microsomes in the presence of an NADPH-generating system and molecular oxygen caused the formation of microsomal protein-bound melanin-like pigment. The free radical property of this pigment was proved by electron spin resonance spectroscopy. The formation of the pigment was inhibited by superoxide dismutase which indicates the participation of superoxide anion radical in this type of melanogenesis.

## INTRODUCTION

Three possible synthetic pathways for neuromelanin have been reported: MAO[1], peroxidase and non-enzymatic oxidation called pseudoperoxidation (1). Among the three possibilities, the peroxidase-mediated oxidation of tyrosine, DOPA and catecholamines, such as norepinephrine and dopamine (2,3), has been argued recently to be the most probable one. Besides catecholamines, however, another possible precursor substrate of neuromelanin is 5-HT (4) or its related hydroxyindoles which are present in the brain of many animals.

According to the scheme of the metabolism of 5-HT (5), 5-HIAAl formed from 5-HT by the action of MAO is converted partly to 5-HIAA by NAD-dependent aldehyde dehydrogenase and partly to pigment of which nature has not been

[1]Abbreviations: TP, tryptophan; 5-HT, 5-hydroxytryptamine, serotonin; 5-HTP, 5-hydroxytryptophan; 5-HTOL, 5-hydroxytryptophol; 5-HIAAl, 5-hydroxyindole acetaldehyde; 5-HIAA, 5-hydroxyindoleacetic acid; 5,6-DHI, 5,6-dihydroxyindole; DPPH, α,α-diphenyl-β-picrylhydrazyl; MAO, monoamine oxidase; SOD, superoxide dismutase

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clarified. There are also other reports of the contribution of mitochondrial enzyme to the formation of a melanin-like pigment from tryptamine (6-8) and 5-HT (6-9).

In this communication, we wish to report the characterization of the reddish-brown and black colour observed in the reaction mixture of 5-HT and 5-HTP, respectively, accompanied by the formation of their protein-bound metabolites when 5-HT or 5-HTP was incubated with liver microsomes in the presence of NADPH generating system (10).

## MATERIALS AND METHODS

Rat liver microsomes were prepared by the procedure of Mitoma et al (11). Male Wister rats (250-350 g) were fasted for 12 hrs before sacrifice and the livers were perfused with an ice-cold 1.15% KCl solution to remove as much haemoglobin as possible. Rat brain microsomes were prepared by the method of Lai and Clark (12) except that the microsomes were washed once with 0.1 M Tris-HCl buffer, pH 7.8.

To determine the free radical property and the NADH oxidizing property of the protein-bound pigments formed, large quantities of these chromogens were prepared enzymatically. 5-HT (2.02 mg), 5-HTP (1.1 mg), 5-HTOL (1.2 mg) or 5-HIAA (1.16 mg) was incubated in a 40 ml glass-stoppered centrifuge tube containing liver microsomes and NADPH generating system as described in the legend of Fig.1. After 30 min of incubation at 35°C, 5 ml of 0.5 M borate buffer (pH 10.1) and 25 ml ethyl acetate were added and the tube was shaken for 2 min. The precipitate collected by centrifugation was washed once with absolute ethanol. In the case of brain microsomes, a small scale of the incubation (10) was performed. 5-HT (0.2 mg) or 5-HTP (0.11 mg) was incubated in a 10 ml glass-stoppered centrifuge tube containing brain microsomes and NADPH generating system as described in the legend of Fig. 2. After 30 min of incubation at 35°C, 0.5 ml of 0.5 M borate buffer (pH 10.1) and 6 ml ethyl acetate were added and the tube was shaken for 2 min. The precipitate from 10 tubes was put together and collected by centrifugation, washed once with 6 ml absolute ethanol and stored at -70°C.

The resulting moist powder was inserted into an EPR tube. The reduction of the sample with $Na_2S_2O_4$ was performed prior to the insertion into an EPR tube. About 5 mg of $Na_2S_2O_4$ dissolved in 1 ml of distilled water was added to the sample. After the termination of the reduction (almost instantaneous disappearance of the colour of sample), 8 ml of $H_2O$ was added and the sample was centrifuged to remove excess $Na_2S_2O_4$ and the precipitate was inserted into an EPR tube. EPR spectra were measured at -196°C with a Varian E-12 spectrophotometer: modulation frequency 100 kHz, modulation amplitude 4 G, microwave frequency 9.1 GHz and microwave power 1 mW. Spin concentrations were determined by double integration with DPPH as standard.

The non-enzymatic oxidation of NADH in the presence of protein-bound pigment was measured by oxygen consumption (13) since the prepared protein-bound pigment of 5-HT or 5-HTP resuspended in 0.1 M Tris-HCl buffer, pH 7.8, precipitated easily during the spectrophotometrical measurement and the turbidity due to the use of highly concentrated suspension disturbed the measurement of the absorbance. Oxygen uptake was monitored at 35°C using an oxygraph with a Clark electrode in an oxygen monitor MOD. 53-YSI. Protein was measured by the method of Lowry et al (14).

Superoxide dismutase was purified from bovine erythrocyte according to the method of McCord and Fridovich (15). NAD(P)H were obtained from Oriental Yeast Co. Ltd., Japan.

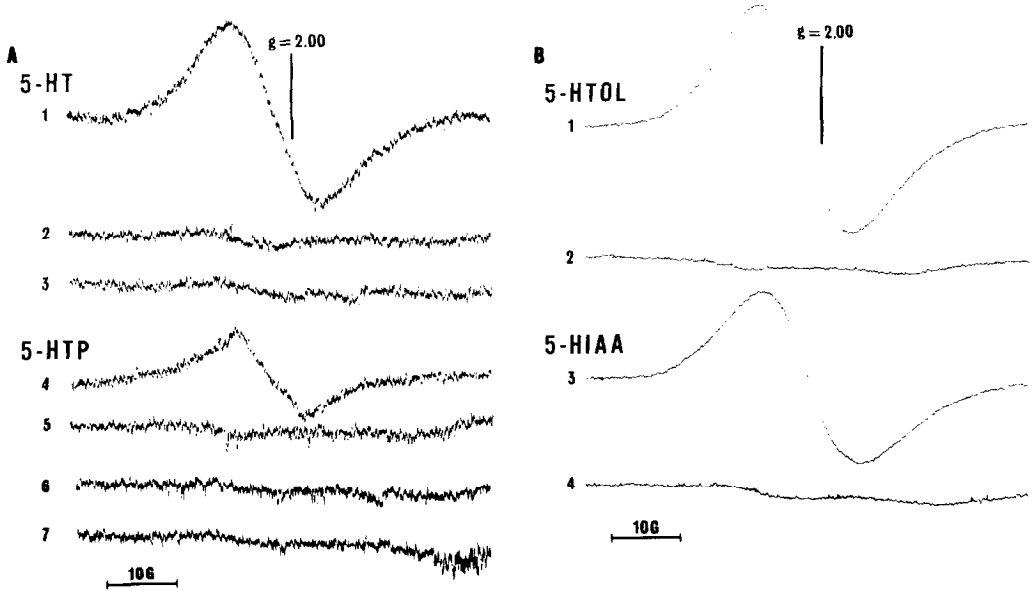



Fig. 1. EPR spectra of melanin prepared by incubating 5-HT or 5-HTP (A) and 5-HTOL or 5-HIAA (B) with rat liver microsomes in the presence or absence of NADPH generating system.
The reaction mixture, in a final volume of 10 ml, contained 15 mg of microsomal protein, 0.1 M Tris-HCl buffer, pH 7.8, 1.5 mM KCN, 0.5 mM 5-HT or 5-HTP (A), 0.6 mM 5-HTOL or 5-HIAA (B) and NADPH generating system composed of 0.6 mM NADP, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate, 35 U glucose-6-phosphate dehydrogenase, and was incubated at 35°C for 30 min with constant shaking in air.
A-1,4, in the presence of NADPH generating system (wet weight of sample: 1, 163 mg; 4, 177 mg); A-2,5, after reduction of melanin precipitate with $Na_2S_2O_4$; A-3,6, in the absence of NADPH generating system; A-7, microsomes + NADPH generating system alone. B-1,3, in the presence of NADPH generating system (wet weight of sample: 1, 144 mg; 3, 104 mg); B-2,4, in the absence of NADPH generating system.

## RESULTS

Fig. 1A shows the EPR signal with a g value of 2.00 displayed by incubating liver microsomes with 5-HT or 5-HTP in the presence of NADPH generating system. The line width of both signals derived from 5-HT and 5-HTP (lines 1 and 4) was about 8 G which agrees well with that of other melanins (6,16,17). A much stronger signal from 5-HT was seen than that from 5-HTP. When 5-HT or 5-HTP was incubated with microsomes without NADPH generating system, no signal appeared (lines 3 and 6). Nor did the incubation of microsomes with NADPH generating system alone give any signal (line 7). Therefore it can be said that the observed signals are derived from the protein-bound metabolite(s) of 5-HT and 5-HTP. A similar free radical signal was observed also when 5-HTOL or

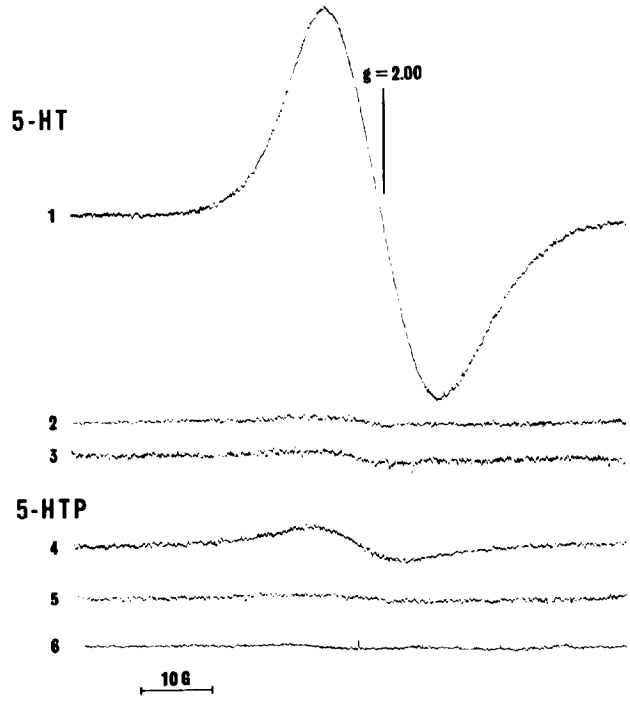



Fig. 2. EPR spectra of melanin prepared by incubating 5-HT or 5-HTP with rat brain microsomes in the presence or absence of NADPH generating system and the effect of SOD on the formation of free radical signals. The reaction mixture, in a final volume of 1.0 ml, contained 1.2 mg of microsomal protein, 0.1 M Tris-HCl buffer, pH 7.8, 1.5 mM KCN, 0.5 mM 5-HT or 5-HTP and NADPH generating system as described in the legned of Fig. 1, except for the use of 7 U glucose-6-phosphate dehydrogenase. 0.5 mg of SOD was added if necessary. The precipitated membrane protein from 10 tubes was put together into an EPR tube. 1,4, in the presence of NADPH generating system (wet weight of sample: 1, 103 mg; 4, 92 mg); 2,5, in the presence of NADPH generating system and SOD; 3,6, in the absence of NADPH generating system.

5-HIAA, two metabolites of 5-HT, was incubated with microsomes in the presence of NADPH generating system (lines 1 and 3 in Fig. 1B). No signal appeare without NADPH generating system (lines 2 and 4). The spin density calculated by double integration gave approximately $5.9 \times 10^{12}$, $2.3 \times 10^{12}$, $5.8 \times 10^{12}$ and $6.6 \times 10^{12}$ spins per mg wet weight of powder prepared from the reaction mixture of 5-HT, 5-HTP, 5-HTOL and 5-HIAA, respectively. Upon reduction with sodium dithionite, the signal intensity disappeared almost completely (lines 2 and 5 in Fig. 1A).

The free radical signal could be observed also by incubating brain microsomes with 5-HT or 5-HTP used as two representatives in the presence of NADPH generating system (lines 1 and 4 in Fig. 2). Without NADPH generating system,

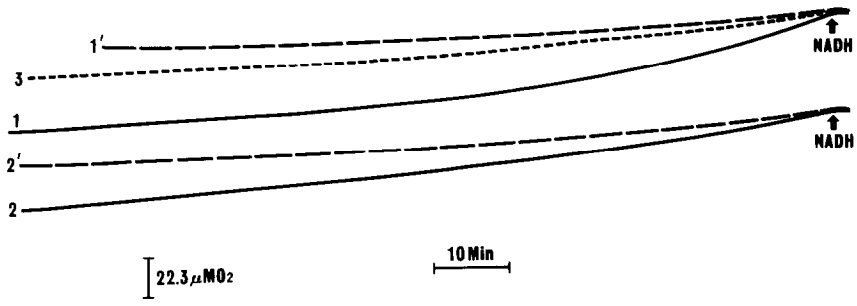
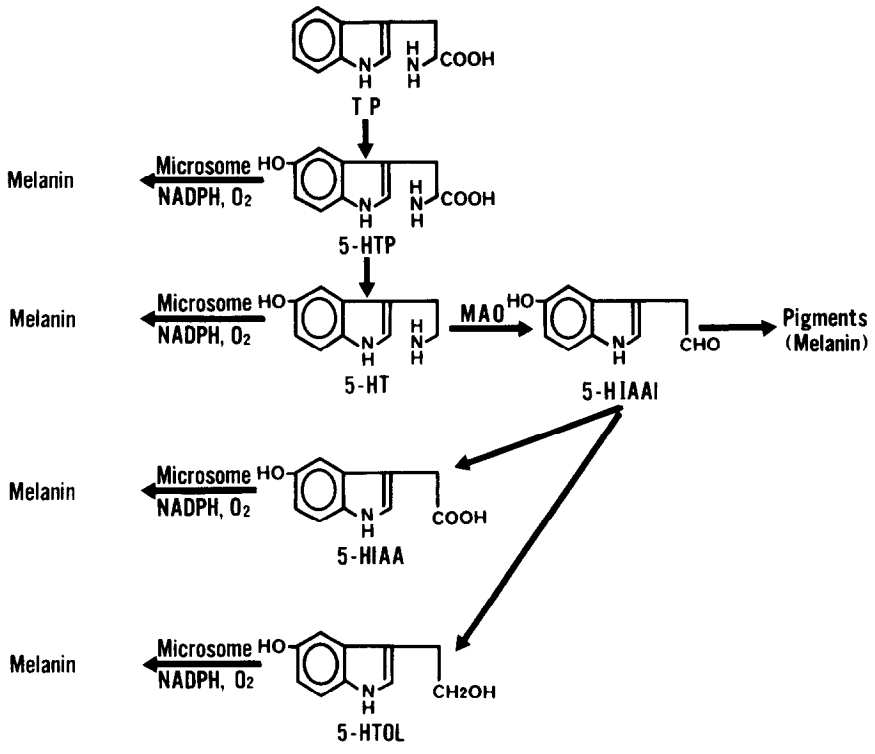



Fig. 3. The rate of oxidation of NADH by melanin-like pigment prepared by incubating 5-HT or 5-HTP with rat liver microsomes in the presence or absence of NADPH generating system.
Melanin-like protein-bound pigment washed again with hot absolute ethanol to complete the denaturation of microsomal protein was resuspended in 1.1 ml of 0.1 M Tris-HCl buffer, pH 7.8 and used as NADH-oxidizer. 1.0 ml of the sample (6.9-7.6 mg of protein) and 1.98 ml of the buffer were put into the sample chamber of oxygraph. 20 µl of NADH was added at the arrow at the final concentration of 4 mM. The temperature was kept at 35°C. 1,1', 5-HT; 2,2', 5-HTP; ————, in the presence of NADPH generating system; — — —,in the absence of NADPH generating system; 3, microsoems + NADPH generating system alone.

no signal appeared (lines 3 and 6). As shown previously, the inclusion of SOD in the reaction mixture inhibited almost completely the formation of protein-bound metabolite(s) of 5-HT and 5-HTP (10). Nor was the free radical signal produced when SOD was added to the reaction mixture (lines 2 and 5). These results indicate the participation of $O_2^-$ in the microsomal melanogenesis.

The bleaching characteristics of melanins were examined histochemically according to the procedure of Lillie (18). It was found that the reddish-brown and black colour of protein-bound metabolite(s) of 5-HT and 5-HTP, respectively, vanished with 10% hydrogen peroxide or with 0.25% $KMnO_4$ on subsequent treatment with 1% oxalic acid. They decolourized also by sodium dithionite but were restored when left in air.

The ability of melanins to oxidize or reduce non-enzymatically a number of redox systems has been often reported (13,19-21). If our coloured materials were indeed one type of melanins, they must have such properties as an electron transfer agent. NADH was chosen as the oxidizable substrate because it is oxidized most efficiently by melanin (21) and its oxidation proceeds linearly for a long period because of the reoxidation of NADH-reduced melanin by atmospheric oxygen (13,21). As shown in Fig. 3, the prepared protein-bound




Scheme 1. Pathway of Indoleaminergic Melanin Biosynthesis

chromogens from 5-HT and 5-HTP oxidized NADH as measured by oxygen consumption. This result is somewhat different from that of melanoma-melanin which can oxidize NADH only when protein moiety bound to melanin is removed by trypsin digestion or by acid hydrolysis (20).

## DISCUSSION

The present paper shows that melanin is synthesized from 5-HT not only by mitochondrial MAO (5,6,8,9) or lysosomal peroxidase (1) but also by microsomes. Moreover, the precursor substrate, 5-HTP, as well as the final two products, 5-HIAA and 5-HTOL, can be transformed to melanin by a microsomal system as depicted in Scheme 1. According to the broad classification of Nicolaus (22), melanin produced from 5-HT, 5-HIAA and 5-HTOL may be called as phaeomelanin (dusky reddish-brown) and that from 5-HTP as eumelanin (black). Almost all schemes generally accepted for the final polymerization step of melanogenesis is that of indole-5,6-quinone formed from 5,6-DHI whose remote

progenitor(s) is tyrosine or DOPA (23,24). The present microsomal melanogenesis from four 5-hydroxyindoles, however, does not seem to proceed through indole-5,6-quinone. The intermediate(s) of the present melanin polymer may be p-quinoneimine. Although we have not yet succeeded in the characterization of certain polymerization intermediate(s) because of the general rapid oxidative polymerization and of its instability, the property of our final protein-bound metabolite(s) satisfies well the criteria of melanin histochemically, paramagnetically and functionally: bleaching of their colours by $H_2O_2$, by $KMnO_4$ and oxalic acid, and by reduction with $Na_2S_2O_4$, the free radical signals (Fig. 1 and 2) and the catalytic property to oxidize NADH (Fig. 3) even though our pigments were neither digested by trypsin nor hydrolyzed in HCl, whereas melanoma-melanin oxidizes NADH only when digested or hydrolyzed (20). Probably the differences in structures of the melanin polymers and the difference in protein moieties between our preparation and melanoprotein in melanoma may explain the different capacities to oxidize NADH.

The biological function of melanin has not been clearly elucidated despite the reports of its biochemical redox function (13,19-21). Almost all of these studies, however, have been performed with DOPA-melanin and not with indoleaminergic melanins. The origin of neuromelanin in certain brain regions has been reported to correlate intimately with the presence of catecholamines, e.g., dopamine-melanin in substantia nigra (25) and norepinephrine-melanin in locus coeruleus (26). The localization and the function of indoleaminergic melanin remain to be elucidated.

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