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# Neuromelanin synthesis in rat and human substantia nigra

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**Summary.** A relation between neuromelanin synthesis and vulnerability of dopaminergic neurons is suggested by the fact that heavily pigmented cells are preferentially lost in aging and Parkinson's disease and that the dopaminergic neurotoxin MPP+ (1-methyl-4-phenyl-pyridine) binds to neuromelanin. To elucidate the mechanism of neuromelanin synthesis, we studied the formation of melanin in homogenates of human and rat substantia nigra tissue "in vitro". It was found that enzymatic processes accounted for 70% and 90% of the melanin formation in homogenates of human and rat tissue, respectively. The enzymatic synthesis was due to the activity of monoamine oxidase (MAO), since it was prevented by selective inhibitors of this enzyme. Both MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and MPP+ inhibited melanin formation, probably due to their ability to inhibit MAO. No evidence was found for involvement of cytochrome P-450 monooxigenases, which have been postulated to exist in central catecholaminergic neurons. Proadifen reduced melanin formation, not necessarily because it is an inhibitor of P-450 monooxigenases, but rather as it is also a potent inhibitor of MAO. Some antioxidants like ascorbic acid, but not agents destroying hydrogen peroxide, inhibited melanin formation. The findings suggest that the formation of neuromelanin in the substantia nigra involves MAO and non-enzymatic oxidative processes.

**Keywords:** MPTP, $MPP^+$, neuromelanin, Parkinson's disease.

## Introduction

Neuromelanin, a complex polymer bound to lipofuscin granules is found in human and other mammalian brains where it is located in catecholaminergic neurons in the substantia nigra, locus coeruleus and other brain stem nuclei (Bazelon et al., 1967; Bogerts, 1981; Lillie, 1957; Marsden, 1983). Cytophotometric studies have shown a linear increase in the pigmentation of catecholaminergic neurons with age until the seventh decade (Mann and Yates, 1974;

Mann and Yates, 1977). After this time the mean neuromelanin density of individual neurons diminishes. The heavily pigmented neurons are preferentially lost, suggesting a relationship between synthesis or accumulation of neuromelanin and cell degeneration (Mann and Yates, 1983; Hirsch et al., 1988).

Several studies have shown that dopa, dopamine, norepinephrine and serotonin serve as a substrate for neuromelanin synthesis. This is thought to proceed by polymerization of oxidated products via indole quinone intermediates (Barden, 1969; Das et al., 1978; Graham, 1978; Graham, 1979; Rodgers and Curzon, 1975; Van Woert et al., 1967). This pathway appears to be an alternative route for metabolism of catecholamines besides the well described pathways involving oxidative deamination (catalyzed by monoamine oxidase, MAO) and o-methylation (catalyzed by catechol-o-methyl transferase). Besides resulting in the accumulation of neuromelanin, oxidation of catecholamines is likely to produce potentially toxic products such as hydrogen peroxide, superoxide anions and hydroxyl radicals (Graham, 1978a, b). The quinone intermediates themselves react with sulfhydryl groups and are inhibitors of enzymes with cysteinyl residues at or near active sites (Graham et al., 1977; Tiffany et al., 1978).

The exact mechanism of neuromelanin formation is still controversial. Previous studies suggest that neuromelanin formation is catalyzed enzymatically, particularly by MAO (Fellman, 1958; Van Woert et al., 1967). On the contrary, Rodgers and Curzon (1975) denied any participation of MAO enzymes in neuromelanin synthesis, and suggested that the process occurs spontaneously. Given the fact that neuromelanin containing cells degenerate selectively in Parkinson's disease (Hirsch et al., 1988) and that neuromelanin is abundant in humans and other primates but not in rat substantia nigra, we decided to further study the "in vitro" synthesis of neuromelanin in tissue from both species. Due to the above mentioned conflictual data, in order to elucidicate the role of MAO in the process of neuromelanin synthesis we measured melanin formation in substantia nigra in the presence of specific MAO inhibitors.

Recently, NADPH cytochrome P-450 reductase was seen in central catecholaminergic neurons, suggesting the presence of cytochrome P-450 monooxygenases within these cells (Haglund et al., 1984). It was speculated that cytochrome P-450 participates in the metabolism of catecholamines and that it may be responsible for the formation of toxic products derived from them. In the present study the possibility that cytochrome P-450 monooxigenases participate in the synthesis of neuromelanin was tested.

Recent studies have suggested an involvement of neuromelanin in the neurotoxic action of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (D'Amato et al., 1986; Lyden et al., 1983; Wu et al., 1986). It has been shown that MPTP and MPP+ bind to synthetic melanin formed by polymerization of dopamine and norepinephrine, as well as to neuromelanin extracted from the substantia nigra of monkeys. On the basis of these studies it has been proposed (Snyder and D'Amato, 1986) that melanin may form a depot for MPP+ within

dopaminergic neurons, permitting its retention and consequent gradual release. Although binding properties of MPTP and MPP+ to melanin have been described their influence on melanin synthesis has not been studied. This question was addressed in the present report. Furthermore, in an attempt to overcome the "cascade" of oxidative reactins occurring in the synthesis of neuromelanin we studied melanin formation in the presence of the antioxidants, ascorbic acid and hydrazine.

## Methods

### *Materials*

Proadifen was a gift from Smith Kline and French Laboratories, Philadelphia, Deprenyl a gift of Dr. Heikkila (Rutgers Medical School, Piscataway N.J.). Clorgyline, MPTP and MPP+ were purchased from Research Biochemicals Inc.; ascorbic acid and hydrogen peroxide were obtained from Mallinckrodt Inc., St. Louis, Missouri, U.S.A. All other chemicals were purchased from Sigma Chemicals Co., St. Louis, Missouri, U.S.A. Human midbrain tissue containing the substantia nigra was dissected from two neurologically normal subjects. The tissue was obtained 10–12 h after death and was stored at −70 °C. The rat midbrain area containing substantia nigra was dissected from Wistar rats (250–300 g) killed by decapitation.

### *Assay of melanin synthesis*

Pooled material from human and rats (separately) was used for the experiments. Tissue was homogenized in 10 volumes (w/v) of 0.1 M sodium phosphate buffer pH 7.4 using a glass-teflon homogenator. Fifty µl of the homogenate were incubated at 37 °C with 10 µl of a solution containing 0.1 µmol of [7-$^{14}$C]dopamine (1.1 mCi/mmol). Standard incubation time was 5 h. Blanks consisted of homogenates incubated with labelled dopamine and kept for 5 h at 0 °C. (No difference was observed between homogenates kept at 0 °C for 5 h or homogenates processed immediately after addition of labelled dopamine.) The reaction was stopped by the addition of 100 µl of cold 10% trichloroacetic acid (TCA). The mixture was then filtered through a membrane filter (Milliposte type AA, pore size 0.8 µm). The filters were washed sequentially with 5% TCA, $H_2O$, 0.1 M HCl and $H_2O$. Filters were then put in scintillation vials and counted using Scintrex liquid scintillation cocktail for samples on solid supports (J. T. Baker) in a 1,211 LKB counter. Drugs used in the assay were dissolved in $H_2O$ and added to the incubation medium. The final volume of each sample was 100 µl. Samples were run in triplicate. Results were expressed as nmoles dopamine incorporated per hour, and mg protein [determined according to Bradford (1976)].

### *Assay of MAO activity*

A modification of the method of Roth and Feor (1978) was used. Two hundred µl of brain homogenate were incubated with 200 µl incubation solution containing 50 nmol ascorbic acid and 40 nmol of [7-$^{14}$C]dopamine (1.6 mCi/mmol) for 60 min at 37 °C. The reaction was stopped by adding 150 µl of 0.4 M perchloric acid. After removal of the precipitated proteins by centrifugation, the supernatant was loaded on small columns of Dowex-50 W (50X8-200). Columns were prepared in Pasteur pipets and were equilibrated with 0.1 M HCl. Deaminated dopamine was eluted with 2 ml of $H_2O$ and the effluent was collected in scintillation vials and counted with 15 ml of Quantafluor (Mallinckrodt).

## Results

### *Measurement of melanin synthesis*

The assay used in this study measures the incorporation of $^{14}$C-dopamine into TCA-precipitable polymers during incubation with homogenates of substantia nigra tissue. The following points justify the fact that this parameter reflects the formation of melanin. First, neuromelanin isolated from human substantia nigra has similar properties to melanin formed in vitro from dopamine (Das et al., 1978; Van Woert et al., 1967). Second, using an assay similar to ours, Rodgers and Curzon (1975) showed earlier that incorporation of dopamine into TCA-precipitable polymers parallels the increase in absorbance and is dependent on the presence of oxygen. Third, these authors ruled out that dopamine is incorporated into proteins by showing that dopamine-containing polymers are not degraded when heated in the presence of 6M HCl. The concentration of dopamine used in our assay (1 mM) was based on estimations of dopamine concentration in the cytoplasm of dopaminergic cells (Lichtensteiger, 1971; Lichtensteiger et al., 1976, 1979). The assay was linear with regard to time and concentration of homogenate in the range used in our studies (Fig. 1).

### *Non-enzymatic melanin synthesis*

Boiling of homogenates (100 °C for 30 min) reduced melanin formation in human and rat substantia nigra homogenates to 29% and 9% of control values, respectively (Table 1). This species-related difference was a consistent finding observed in all experiments and represented the only significant difference between rat and human tissue.

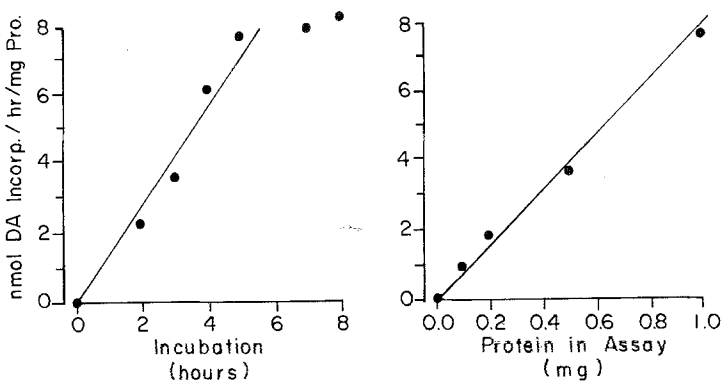


**Fig. 1.** Time and substrate concentration dependence of assay used to measure melanin synthesis (measured as nmol DA incorporated into "melanin"/n/mg protein). Homogenates of human substantia nigra were incubated at 37 °C with 1 mM of [7-$^{14}$C]dopamine (1.1 mCi/mmol). Dopamine incorporated into insoluble polymers ("melanin") was then separated with a membrane filter

**Table 1.** Effect of cytochrome P-450 oxidase inhibitor, MAO inhibitors, MPTP and MPP+ on melanin synthesis in homogenates of human and rat substantia nigra. Homogenates were incubated for 5 h with 1 mM [$^{14}$C]dopamine. Insoluble polymers were then collected on a membrane filter

| | Human | Rat |
|---|---|---|
| | (nmoles of [$^{14}$C]dopamine incorporated/h/mg protein) | |
| Controls | 7.65 ± 1.27 | 8.41 ± 1.34 |
| Boiled tissue | 2.21 ± 0.57* (29%) | 0.75 ± 0.34 (9%) |
| NADPH 0.5 mM + $MgCl_2$ 5 mM | 4.52 ± 0.71 (54%) | 6.18 ± 1.32 (73%) |
| Proadifen 10 mM | 3.57 ± 0.41 (47%) | 2.63 ± 0.58 (31%) |
| Proadifen + pargyline 1 μM | 2.84 ± 0.46 (37%) | 2.25 ± 0.52 (27%) |
| Pargyline 1 μM | 2.54 ± 0.37 (33%) | 2.63 ± 0.60 (31%) |
| Deprenyl 1 μM | 3.41 ± 0.62 (45%) | 3.26 ± 0.36 (39%) |
| Deprenyl 0.1 μM | 4.75 ± 1.01 (62%) | 4.98 ± 0.17 (59%) |
| Clorgyline 1 μM | 3.95 ± 1.80 (52%) | 4.33 ± 0.60 (51%) |
| Clorgyline 0.1 μM | 5.11 ± 1.30 (67%) | 6.24 ± 0.50 (74%) |
| Clorg. 1 μM + depr. 1 μM | 3.00 ± 0.32 (39%) | 2.71 ± 0.60 (32%) |
| MPTP 1 μM | 5.00 ± 0.31 (65%) | 5.20 ± 0.94 (62%) |
| MPP+ 1 μM | 3.54 ± 0.95 (46%) | 3.85 ± 0.59 (46%) |

The numbers express means ± S.D. of 4–8 experiments with triplicates (using pooled human and rat material separately); numbers in brackets indicate percentage of control values. Values of all treatment groups were significantly lower than corresponding control values ($p < 0.05$)

* Significantly different from corresponding value measured in rat tissue ($p < 0.05$)

### *Involvement of cytochrome P-450 monooxigenases*

Addition of NADPH and $MgCl_2$, which are necessary cofactors of cytochrome P-450 (Mayer et al., 1980) to the incubation medium, failed to stimulate melanin formation (Table 1). The presence of NADPH was even inhibitory, probably due to the antioxidant properties of this compound. Proadifen, an inhibitor of cytochrome P-450 monooxigenase (Mayer et al., 1980) reduced melanin formation. The $IC_{50}$ of this process was 0.9 mM (Fig. 2). However, since melanin formation was also inhibited by MAO inhibitors (see below), we tested for the possibility that proadifen inhibits the deamination of dopamine by MAO. Indeed, proadifen was found to inhibit MAO activity with an $IC_{50}$ of 0.2 mM (Fig. 2). Furthermore, proadifen added together with an inhibitor of MAO-pargyline, failed to reduce melanin formation to levels lower than those measured in the presence of pargyline alone (Table 1). These findings strongly suggest

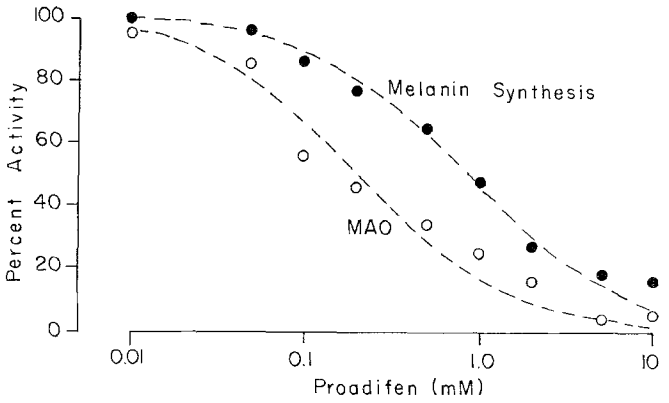


**Fig. 2.** Inhibition of melanin formation and MAO by proadifen (measured as nmol DA incorporated into "melanin"/n/mg protein). Aliquots of a homogenate of rat substantia nigra were taken for measurement of melanin synthesis or of MAO activity (using $^{14}$C-dopamine as substrate). 100% corresponds to 9.7 nmol DA incorporated into melanin/h/mg protein and 0.5 nmol DA deaminated/h/mg protein. Values shown are means of two independent experiments. $IC_{50}$ values for the inhibition of melanin synthesis and MAO activity by proadifen were 0.86 mM and 0.20 mM respectively

that the reduction of melanin formation mediated by proadifen was due to the ability of this compound to inhibit MAO, and not by inhibiting the P450 monooxigenases.

### *Effect of MAO inhibitors, MPTP and MPP+*

Melanin synthesis was inhibited by the MAO inhibitors deprenyl, clorgyline and pargyline in both human and rat substantia nigra homogenates, indicating that MAO is involved in the formation of melanin (Table 1). Maximal inhibition produced by MAO inhibitors reduced melanin formation to 30–40% of control values. Maximal inhibition was observed with pargyline, an inhibitor of both MAO-A and MAO-B (Heikkila et al., 1984) and with the combination of clorgyline and deprenyl, specific inhibitors of MAO-A and MAO-B respectively (Heikkila et al., 1984; Knoll, 1983). When used alone, clorgyline and deprenyl produced intermediate inhibitions. The addition of MPTP or MPP+, which are both inhibitors of MAO (Bochetta et al., 1985; Del Zompo et al., 1986; Heikkila et al., 1984; Parsons and Rainbow, 1984; Singer et al., 1985) also produced a marked inhibition in melanin synthesis in homogenates of both species (Table 1). The addition of MAO inhibitors MPTP or MPP+ did not affect non-enzymatic melanin formation occurring in boiled tissue homogenates.

### *Effect of hydrogen peroxide, catalyse, ascorbic acid, and hydrazine*

MAO might stimulate melanin formation by providing deaminated dopamine metabolites which are incorporated into melanin. An alternative possibility is that hydrogen peroxide formed as a by-product of MAO-catalyzed deamination,

**Table 2.** Effect of antioxidants, hydrogen peroxide and catalase on melanin formation in rat substantia nigra homogenates

| | S. nigra homogenate | Boiled tissue |
|---|---|---|
| | (nmoles of [$^{14}$C]dopamine incorporated/h/mg protein) | |
| Controls | 8.41 ± 1.30 | 0.75 ± 0.30 |
| Ascorbate 1 mM | 2.67 ± 0.35* (32%) | 0.26 ± 0.03* (35%) |
| Hydrazine 1 mM | 8.25 ± 0.28 (98%) | nd |
| $H_2O_2$ 1 mM | 12.55 ± 0.35* (149%) | 0.79 ± 0.03 |
| 0.1 mM | 11.47 ± 0.27* (136%) | 0.70 ± 0.02 (93%) |
| 0.01 mM | 10.67 ± 0.35* (126%) | nd |
| 0.001 mM | 10.25 ± 0.20 (121%) | nd |
| Catalase 2,500 U/ml | 7.55 ± 0.30 (89%) | nd |
| 5,000 U/ml | 7.48 ± 0.60 (89%) | nd |
| Pargyline 1 μM | 2.54 ± 0.87* (30%) | nd |
| Pargyline 1 μM + Catalase 5,000 U/ml | 4.75 ± 0.38* (56%) | nd |

The numbers express the means ± S.D. of 4–8 experiments with triplicates (using pooled rat material); numbers in brackets indicate percentage of control values

* Significantly different from control values ($p < 0.05$).

*nd* not determined

may promote the oxidation of dopamine. To test for this latter possibility, reagents which alter the concentration of hydrogen peroxide were included in the incubation medium. The addition of hydrogen peroxide produced modest increases in melanin formation (Table 2). Catalase, which inhibits hydrogen peroxide formation, failed to significantly inhibit melanin formation and did not reduce melanin formation below values measured when MAO was maximally inhibited. These findings fail to support the concept that hydrogen peroxide is involved in the formation of melanin in vitro.

Melanin formation in native and boiled homogenates was strongly inhibited by the addition of ascorbic acid, a strong well known antioxidant. Hydrazine, another antioxidant, was ineffective (Table 2).

## Discussion

Despite the fact that the selective loss of melanin-containing neurons in Parkinson's disease has been known for many years, the mechanism of neuromelanin synthesis is still poorly understood. Neuromelanin synthesis in the brain clearly differs from the synthesis of melanin as it occurs in pigmented

cells outside of the CNS. Synthesis of peripheral melanin which is catalyzed by the enzyme tyrosinase uses tyrosine or L-dopa as a substrate (Barden, 1969; Graham, 1979; Marsden, 1983; Prota, 1980; Rodgers and Curzon, 1975). The evidence that neuromelanin synthesis in the CNS does not involve tyrosinase includes the following points. First, homogenates of human and monkey substantia nigra tissue fail to oxidize tyrosine to melanin (Barden, 1969; Rodgers and Curzon, 1975). Second, the substantia nigra has been found to be pigmented in human albinos who lacked all peripheral tyrosinase (Foley and Baxter, 1958). Third, the addition of cyanide, which inhibits tyrosinase, does not affect neuromelanin synthesis (Pomerantz, 1958; Prota, 1980). Fourth, chemical characterization of neuromelanin revealed that its synthesis starts from L-dopa or other catecholamines rather than from tyrosine (Das et al., 1978; Van Woert et al., 1967).

### *Non-enzymatic synthesis of neuromelanin*

Rodgers and Curzon (1975) showed that melanin formation still occurred "approximately in the same amount" after heat inactivation of enzymes present in homogenate of human substantia nigra (100 °C for 30 min). On the basis of these findings, they concluded that "enzyme activity may play little direct part in melanin synthesis by brain tissue in vitro". In our study, homogenates of human substantia nigra denatured by heating, (the same procedure) synthesized approximately 30% of melanin when compared with untreated homogenates. Homogenates of rat substantia nigra denatured by boiling were able to form only 5–10% of melanin when compared with intact tissue. The discrepancy between our findings and those of Rodgers and Curzon is probably due to the fact the Rodgers and Curzon excluded the participation of MAO by adding inhibitors to their assay (see below). The reason for the difference in rates of non-enzymatic synthesis between rat and human tissue found in our study remains unexplained. This difference may be partly responsible for the difference in pigmentation between rat and human dopaminergic neurons (DeMattei et al., 1986; Marsden, 1983). Recently, a peroxidase which oxidizes dopamine has been isolated from canine brainstem (Grisham et al., 1987). This peroxidase might be present in other species and may be responsible for the enzymatic melanin formation in rat homogenates, not ascribed to the catalytic activity of MAO (Table 1).

### *Involvement of MAO*

The possibility of MAO playing a role in neuromelanin synthesis was proposed by Fellman (1958) and Van Woert (1967) based on their finding that melanin is formed during the incubation of amines with MAO from liver mitochondria. Rodgers and Curzon (1975) concluded that enzymes play a small role in neuromelanin synthesis in vitro, however, they rather arbitrarily excluded the participation of MAO by the addition of tranylcypromine (a non specific MAO

inhibitor) in all their measurements. Their data show that this MAO inhibitor reduced melanin formation to a small extent in the substantia nigra but by up to 50% in various other brain areas. In our study, combined inhibition of MAO-A and MAO-B (produced by pargyline or by the combined administration of deprenyl and clorgyline) inhibited neuromelanin formation by 60–70% (see Table 1). As we homogenized the tissue before incubation, dopamine contained in substantia nigra cells might have been exposed to both types of MAO, which are normally present in the region (Rainbow et al., 1985; Westlund et al., 1985). In vivo, intraneuronal dopamine might have access to MAO-A only, since, using immunohistochemical methods, only this form of the enzyme has been found to be located within dopaminergic cells of the substantia nigra (Westlund et al., 1985).

MAO might catalyze the formation of neuromelanin by at least two separate mechanisms. First, it may act directly by providing dopamine metabolites incorporated into neuromelanin. There is presently no evidence supporting this concept, however a "novel" catalytic function of MAO other than its well characterized deamination of monoamines has recently been found. The pyridine ring of MPTP is converted by MAO to 1-methyl-4-phenyl-2,3-dihydropyridine (MPDP), which is then further oxidated to MPP+ (Cohen et al., 1985; Heikkila et al., 1984). As a second possibility MAO might stimulate NM formation indirectly by producing reactive by-products like hydrogen peroxide which then catalyzes the auto-oxidation of DA (Kopin et al., 1986; Maker et al., 1981). We failed to find evidence in support of this concept since the addition of catalase, which destroys newly formed hydrogen peroxide, did not reduce melanin formation in our assays. Our findings are in line with those of an earlier study in which catalase failed to reduce the formation of melanin catalyzed by liver extracts (Blashko and Hellmann, 1953). The concept of another alternative, as yet undescribed mechanism of interaction, could be possible.

### *Involvement of cytochrome P-450 monooxigenases*

Using immunohistochemical procedures, the enzyme NADPH cytochrome P-450 reductase was seen in central catecholaminergic neurons (Haglund et al., 1984). This finding suggests that cytochrome P-450 monooxygenases are located within catecholaminergic neurons. It was speculated that these enzymes are involved in the metabolism of catecholamines and may be responsible for the formation of toxic products. In the present study, the possibility that cytochrome P-450 monooxigenases participate in the synthesis of neuromelanin was tested. We failed to obtain evidence supporting this hypothesis, since the addition of NADPH and $MgCl_2$, which are necessary cofactors of cytochrome P-450 (Mayer et al., 1980) did not facilitate the reaction. Proadifen, which is known to inhibit cytochrome P-450 monooxigenase (Mayer et al., 1980) reduced melanin formation. However, it was found to also be an effective inhibitor of MAO, suggesting that the reduction of melanin formation in our assay was probably

due to its ability to inhibit MAO. This property of proadifen may be responsible for the potentiation of MPTP toxicity on hepatocytes, which has been ascribed to cytochrome P-450 inhibition (Smith et al., 1987).

### *Interaction of MPTP and MPP+ with melanin formation*

Both MPTP and MPP+ were found to inhibit the synthesis of melanin in vitro (Table 1). The interference of these compounds may have occurred by interaction with MAO activity, since recent studies have shown that both these neurotoxic drugs can inhibit this enzyme (Heikkila et al., 1984; Singer et al., 1985). It seems unlikely that MPTP and MPP+ interfere with the non-enzymatic oxidation of dopamine, since they failed to affect melanin formation from dopamine incubated without tissue extracts (data not shown).

One of the theories concerning the toxic effect of MPTP postulates that its action is due to increased oxidation of dopamine with the production of superoxides and other cytotoxic free radicals in a quantity that exceeds the neutralizing capacity of the natural cellular protective enzymes and reducing substances (Fuller et al., 1985; Parsons and Rainbow, 1984). However, the toxicity of MPTP to dopaminergic neurons in vivo is not affected by treatments that affect intracellular dopamine levels (Melamed et al., 1985; Schmidt et al., 1985). The lack of interaction of MPTP and MPP+ with the non-enzymatic melanin synthesis, as observed in our study, sustains this notion.

### *Relation of neuromelanin synthesis to dopaminergic cell toxicity*

The number of pigmented nerve cells in human substantia nigra and locus coeruleus declines during aging. The melanin content of the remaining cells in the elderly also declines, indicating that neurons with the highest content of neuromelanin are preferentially lost (Mann and Yates, 1974, 1977, 1983). In Parkinson's disease there is a greater reduction in the amount of melanin, because of the severe loss of the heavier pigmented cells (Hirsch et al., 1988; Mann and Yates, 1983). These findings suggest that synthesis or accumulation of neuromelanin has deleterious consequences on the cells. Indeed, Mann and Yates (1977) demonstrated that the accumulation of neuromelanin is accompanied first by a reduction in cytoplasmic RNA content and finally by a decrease in nucleolar volume in substantia nigra neurons. These authors proposed a mechanical disruption of the microanatomy of intracellular membranes by neuromelanin as the mechanism mediating its toxicity. Other authors (Graham, 1979) have postulated that catecholamine neurons may degenerate as the result of lifelong injury from the by-products of catecholamine oxidation. Findings indicating that dopamine is much more cytotoxic than norepinephrine or epinephrine support this concept. Dopamine oxidation of the o-quinone derivative occurs more rapidly and the o-quinone derivative formed cyclyzes more slowly as compared to norepinephrine, and therefore is more likely to react with cisteinyl residues of enzymes and membranes (Graham, 1979).

If the synthesis of neuromelanin is deleterious to the cells because of production of oxidative products, the addition of potent biological antioxidants like ascorbic acid may have protective effects (Wagner et al., 1986). Ascorbic acid was a very effective inhibitor of melanin synthesis in our studies, both in the presence and absence of tissue homogenates. In contrast, hydrazine, an ingredient of cigarette smoke, proposed to be an effective antioxidant (Yong et al., 1986) failed to inhibit melanin synthesis. We do not know the cause of these different effects among both drugs. Although the rationale seems obvious for applying antioxidants to dopaminergic cells to avoid toxic deleterious effects of intrinsic or extrinsic compounds, antioxidants may also interfere with a system removing "waste" products of catecholamine metabolism. Such a treatment may have negative consequences on the integrity of dopaminergic neurons. Therefore, beneficial effects of antioxidants should be clearly demonstrated in animals before such compounds are administered in an indiscriminate way to humans, as has been proposed recently (Perry et al., 1985).

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