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OXIDATIVE STRESS AND MITOCHONDRIA:

IMPLICATIONS FOR PARKINSON'S DISEASE

by

PETER WERNER

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

OXIDATIVE STRESS AND MITOCHONDRIA:
IMPLICATIONS FOR PARKINSON' S DISEASE

by

PETER WERNER

Advisor: Professor Gerald Cohen

Oxidative stress as a mechanism of disease has been implicated in a wide variety of chronic and acute degenerative disorders, including disorders of the central nervous system (CNS), ischemic heart damage and rheumatic diseases. In recent years, oxidative stress has also become a prominent working hypothesis in a number of neurodegenerative disorders, such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). The work described here focused on the hypothesis that the chemical and biochemical characteristics of dopamine (DA) and its physiological precursor, L-dopa, may lead or contribute to an oxidative stress in Parkinson's disease. Prior evidence for this possibility was found in this and other laboratories. The interest in these two molecules stems from the fact that the neurons mostly affected in PD use DA as their neurotransmitter, and that the major therapy for PD is DA replacement therapy via L-dopa. The research presented here was to investigate the effect of DA and L-dopa on

cellular respiration and its organelle, the mitochondrion. The experimental systems used were isolated mitochondria from rat brain or liver or mouse liver (studies with DA) and cultured human embryonic fibroblasts (L-dopa and DA). DA is catabolized by monoamine oxidase (MAO), generating H_2O_2 in the process. Experiments in isolated mitochondria showed that MAO catalyzed turnover of DA increases the levels of glutathione disulfide (GSSG) and protein mixed-disulfides (PrSSG) in mitochondria. This demonstrates that DA turnover by MAO can affect the thiol-redox state in mitochondria. Furthermore, the increases in GSSG and PrSSG were accompanied by a reduction in the activity of aldehyde dehydrogenase, an enzyme localized to the mitochondrial matrix. In the experiments with human fibroblasts, exposure of cells to L-dopa partially inhibited cellular respiration, which was attributed to reactive species generated during autoxidation of the catechol function of L-dopa. L-dopa is commonly used in the treatment of Parkinson's disease, as DA replacement therapy. Both DA and L-dopa had negative effects on mitochondrial function in the experiments conducted for this thesis. Since a major form of treatment for Parkinson's disease is L-dopa supplementation, these findings raise again the issue of preventing or ameliorating oxidative stress as a therapeutic approach in the treatment of Parkinson's disease.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

5-HT: 5-Hydroxytryptamine (serotonin)
AA: L-Ascorbic acid
AADC: Aromatic Amino Acid Decarboxylase
ALS: Amyotrophic Lateral Sclerosis
BA: Benzylamine
CNS: Central Nervous System
DA: Dopamine
DNA: Deoxyribonucleic acid
DOPAC: Dihydroxyphenylacetic acid
L-dopa: L-Dihydroxyphenylalanine
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol-*bis*-(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid
FCCP : Carbonylcyanide p-trifluoromethoxyphenylhydrazone
GPx: Glutathione peroxidase
GR: Glutathione disulfide reductase
GSH: Glutathione
GSSG: Glutathione disulfide
HPLC-EC: High Performance Liquid Chromatography with Electrochemical Detection
HVA: Homovanillic acid
LPO: Lipid peroxidation
MAO: Monoamine Oxidase
MEM: Minimal Essential Medium
MOPS: 3-(N-morpholino)propanesulfonic acid
MPP^+ : 1-Methyl-4-phenylpyridine
MPTP: 1-Methyl-4-phenyl-1,2,3,6,-tetrahydropyridine
nA: Nanoampere

NAD:	Nicotinamide adenine dinucleotide
NADH:	Nicotinamide adenine dinucleotide, reduced
NADP:	Nicotinamide adenine dinucleotide phosphate
NADP(H):	Nicotinamide adenine dinucleotide phosphate, reduced
nL:	Nanoliter
nm:	Nanometer
PCA:	Perchloric Acid
PD:	Parkinson's Disease
PrSSG:	Protein-Glutathione mixed disulfide
R-OOH:	Hydroperoxide, were R usually stands for an organic (hydrocarbon) Rest
SOD:	Superoxide Dismutase
TBARS:	Thiobarbituric acid reactive substances
µL:	Microliter
µM:	Micrometer

Also: complex I = complex I of the respiratory chain,
(NADH:ubiquinone oxidoreductase, rotenone sensitive).

1: INTRODUCTION

1.1: Oxidative Stress

The potentially harmful or lethal effect of oxidative stress on a variety of organs and tissues is well established (Fridovich, 1976). Oxidative stress is usually thought of as a situation in which the affected organism, organ, cell or cell compartment experiences an unphysiologically high level of reactive oxygen species, such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) or hydroxyl radicals ($\cdot OH$). This can occur either by increasing production of reactive oxygen species, or by decreasing cellular antioxidant defenses, e.g. inhibition of the synthesis of glutathione (Martensson et al. 1991, Slivka et al. 1988). Oxidative stress has been implicated in a vast number of acute and chronic neurodegenerative processes (for review, see e.g. Cohen and Werner, 1993; Cohen 1985). Acute brain injury in ischemia/reperfusion situations has been tied to the generation of reactive oxygen species and consequential oxidative stress (e.g. Yamamoto et al., 1983; Goldberg et al., 1984; Lazzarino et al., 1992), with the production of superoxide ($\cdot O_2^-$) being a prominent factor (Kontos and Wei, 1986).

Oxidative stress is also involved in the toxicity of such neurotoxins such as 6-hydroxydopamine (6-OHDA) and MPP^+. 6-OHDA generates $\cdot O_2^-$ and H_2O_2 via redox-cycling (Heikkila and Cohen, 1971, Heikkila and Cohen, 1973). MPP^+ is a neurotoxin which inhibits mitochondrial respiration on the level of complex I (e.g. Nicklas et al., 1985). MPP^+ toxicity seems to involve an oxidative stress which requires the activity of the mitochondrial

respiratory chain; the toxic action of MPP^+ can be prevented by presence of catalase or ascorbate in experiments with submitochondrial particles (Cleeter et al., 1992).

1.2: Formation of reactive oxygen species

Reactive oxygen species are derived from molecular oxygen, usually via consecutive single electron transfer to oxygen (reduction). Radicals are defined by having one or more unpaired (single) electrons. Oxygen itself is a diradical, which reflects the fact that its two outer electrons occupy two orbitals (two single electrons). Despite its nature as a diradical, its reactivity is moderate. It can, however, accept one (single) electron, and form the anionic radical superoxide ($\cdot O_2^-$, eqn 1a). The single electron can be donated e.g. from ferrous iron (Fe^{2+}), which is oxidized to ferric iron (Fe^{3+}) in the process (eqn. 1b).

$$O_2 + e^- \longrightarrow \cdot O_2^- \qquad \text{(eqn 1a)}$$

$$O_2 + Fe^{2+} \longrightarrow \cdot O_2^- + Fe^{3+} \qquad \text{(eqn 1b)}$$

Superoxide itself has two possible fates: it can be reduced further to form hydrogen peroxide, or it can be oxidized back to molecular oxygen. Both reactions occur in the dismutation of two molecules of superoxide, a reaction which is also catalyzed by the enzyme superoxide dismutase (SOD; eqn 2; McCord and Fridovich, 1969).

$$\cdot O_2^- + \cdot O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{(eqn 2)}$$

H_2O_2, which can be formed by this and other reactions, is detoxified via two major pathways in mammalian cells; one is the dismutation of two H_2O_2 catalyzed by catalase (eqn. 3).

$$H_2O_2 + H_2O_2 \text{ ------> } H_2O + O_2 \qquad \text{(eqn. 3)}$$

1.3: Glutathione peroxidase

The other is the reduction of H_2O_2 by glutathione peroxidase (GPx, eqn. 4). Glutathione peroxidase was initially discovered as an enzyme which protects hemoglobin from oxidative damage in red blood cells (Mills, 1957). The major detoxification pathway for H_2O_2 is its reduction by glutathione peroxidase, as shown, for example, by the insensitivity of red blood cells challenged with H_2O_2 to the poisoning of catalase (Cohen and Hochstein, 1963).

Glutathione peroxidase contains a selenocysteine in its active center (Flohé et al. 1973). The selenocysteine in the active center of GPx is oxidized in the process. The enzyme is regenerated and the selenocysteine is reduced by oxidizing two molecules of glutathione (GSH), which results in the formation of one molecule of GSSG (eqn. 5). The GSSG formed is subsequently reduced by GSSG reductase, which utilizes NADP(H) to provide the reduction equivalents (eqn. 6).

Unlike catalase, glutathione peroxidase also detoxifies organic hydroperoxides (R-OOH), which occur, for example, as intermediates during

lipid peroxidation (see below). GPx serves as a scavenger system for these reactive peroxide intermediates of membrane damage. The product of the peroxidase reaction is here an alcohol (R-OH; eqn. 7).

$$H_2O_2 + \text{GPx-Se-H} \text{ ------> } H_2O + \text{GPx-Se-OH} \quad \text{(eqn. 4)}$$

$$\text{GPx-Se-OH} + 2\ \text{GSH} \text{ ------> } \text{GPx-Se-H} + \text{GSSG} + H_2O \quad \text{(eqn. 5)}$$

$$\text{GSSG} + \text{NADPH} \text{ ------> } 2\ \text{GSH} + NADP^+ \quad \text{(eqn. 6)}$$

$$\text{R-OOH} + 2\ \text{GSH} \text{ ------> } \text{R-OH} + H_2O + \text{GSSG} \quad \text{(eqn. 7)}$$

1.4: Role of iron

One of the reasons why the speedy removal of H_2O_2 is of importance for the maintenance of cellular integrity is the potential for the formation of the hydroxyl radical (·OH), which is one of the most reactive radicals known. The formation of ·OH can be catalyzed by accessible ferrous iron (Fe^{2+}). This reaction is known as the Fenton reaction (eqn. 8; reviewed e.g. in Aisen et al., 1990).

Since the reaction consumes ferrous iron, it would come to a standstill after all accessible Fe^{2+} has been used up. However, in the presence of a reductant such as ascorbate (vitamin C), the ferric iron (Fe^{3+}) produced (eqn. 8) can be reduced back to Fe^{2+} (eqn. 9), which makes it again available for the Fenton reaction.

$H_2O_2 + Fe^{2+}$ -----> $\cdot OH + OH^- + Fe^{3+}$ (eqn. 8)

Fe^{3+} + ascorbate -----> Fe^{2+} + ascorbyl-radical (eqn. 9)

Due to its high reactivity, ·OH can attack virtually every type of molecule in the cell, including proteins and unsaturated fatty acids. The results of these reactions are often used as markers of oxidative stress. Proteins exposed to ·OH exhibit increased concentrations of carbonyl groups (Stadtman 1988). Polyunsaturated fatty acids modified by free radical attack can form aldehydes, such as 4-hydroxynonenal or malondialdehyde (for review, see Esterbauer 1990).

The presence of malondialdehyde or its formation via the decomposition of lipid peroxides during heating forms the basis of the thiobarbituric acid reactive substances (TBARS) test. The TBARS test is one of the most commonly used ways to determine lipid peroxidation.

Other molecules which can cause or participate in oxidative stress are quinones and semiquinones, which can produce superoxide and hydrogen peroxide by redox cycling in the presence of a reductant, such as ascorbate. The mechanisms involved are discussed below.

1.5: Dopamine, L-dopa and oxidative stress

The neurotransmitter dopamine (3, 4-dihydroxyphenylethylamine, DA), and its metabolic precursor, L-dopa (3,4-dihydroxyphenylalanine) have a number of chemical and biochemical properties in addition to neurotransmission which are either due to their catechol moiety or their being a substrate for certain enzymes, e.g. monoamine oxidase (MAO). Figure 1 shows the biosynthesis of DA from tyrosine via L-dopa.

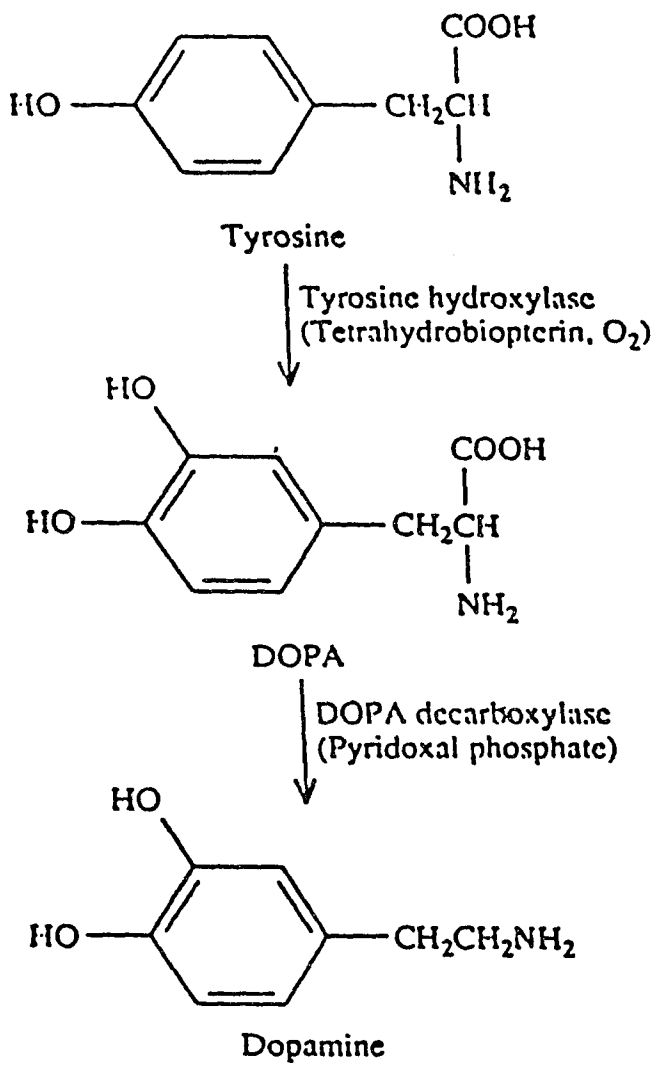
1.5.1: Autoxidation of catechols

The chemical nature of 3,4-dihydroxyphenols (catechols), a group to which both dopa and dopamine belong, enables them to undergo redox cycling reactions if both an electron acceptor (oxidant) and an electron donor (reductant) are present. In eukaryotic cells, the oxidant would most likely be molecular oxygen or its derivatives such as superoxide, whereas ascorbate and glutathione (GSH) would play the role of the reducing agent.

Equation (10) shows the generation of the radical anion superoxide from molecular oxygen, leaving the catechol in the semiquinone state, and equation (11) the reduction of this semiquinone back to the catechol by ascorbate. It should be noted that the semiquinone generated herein possesses an unpaired electron, and is thus also a radical. The generation of $\cdot O_2^-$ in the reaction shown in equation 10 is a critical step in the autocatalysis of catechol autoxidation, since the $\cdot O_2^-$ formed can subsequently react with another catechol, which produces H_2O_2 and semiquinone.

Figure 1

Biosynthesis of L-dopa and Dopamine from Tyrosine



Tyrosine is first hydroxylated to L-dopa by tyrosine hydroxylase. L-dopa is subsequently decarboxylated by DOPA decarboxylase (aromatic amino acid decarboxylase) to form dopamine.
(after Siegel, 1989, modified)

The autocatalytic effect of $\cdot O_2^-$ on catecholamine oxidation was successfully employed to assay for the activity of superoxide dismutase (Misra and Fridovich, 1972), which, by removing $\cdot O_2^-$, reduces the formation of adrenochrome from epinephrine. The ascorbyl radical generated in the reduction of semiquinone by ascorbate (eqn 11) is considered benign, due to its low reactivity. The semiquinone can however undergo further oxidation by oxygen or superoxide to the quinone (Q, eqn 12); if the latter occurs, hydrogen peroxide (H_2O_2) is formed; H_2O_2 will also be formed from the dismutation of $\cdot O_2^-$ (eqn 2). If ascorbate or a comparably potent reducing agent are present, the quinone has several possible fates: it can then either be reduced back to the catechol (over the semiquinone intermediate; equations 13a and 13b), form intramolecular addition products or participate in the formation of melanin.

If ascorbate is present in higher concentrations, it can intercept $\cdot O_2^-$ by reducing it to H_2O_2 (eqn 14), thereby slowing the rate of catechol autoxidation (Nishikimi, 1974). Nishikimi (1974) further estimated that ascorbate at levels commonly present in cells removes $\cdot O_2^-$ as efficient as the cellular SOD.

The ascorbyl radical formed in the reactions listed above dismutes into ascorbate and dehydroascorbate (eqn.15). In in-vitro experiments with dopamine, Tse et al. (1976) found that the reduction of dopamine-quinone by equimolar ascorbate is faster then intramolecular cylization to "dopamine-

chrome" and faster then the reaction of dopamine-quinone with aniline, which has a pseudo-first-order rate constant of 14.5 $^{-s}$.

catechol + O_2 ------> $SQ^{\cdot}$ + $^{\cdot}O_2^-$ (eqn. 10)

$SQ^{\cdot}$ + ascorbate -----> catechol + ascorbyl-radical (eqn. 11)

$SQ^{\cdot}$ + O_2 -----> quinone + $^{\cdot}O_2^-$ (eqn. 12)

$^{\cdot}O_2^-$ + $^{\cdot}O_2^-$ + 2 H^+ -----> H_2O_2 + O_2 (eqn. 2)

Q + ascorbate -----> $SQ^{\cdot}$ + ascorbyl-radical (eqn. 13a)

$SQ^{\cdot}$ + ascorbate -----> catechol + ascorbyl-radical (eqn. 13b)

$^{\cdot}O_2^-$ + ascorbate -----> H_2O_2 + ascorbyl-radical (eqn. 14)

2 x (ascorbyl-radical) -----> ascorbate + dehydroascorbate (eqn.15)

1.5.2: Iron and hydroxyl radicals

In the presence of catalysts such as iron or other transition metals, hydrogen peroxide can form the hydroxyl-radical, $^{\cdot}OH$ in a Fenton reaction, (eqn 8; Fenton, 1894, 1899; also Haber and Weiss, 1934). As mentioned before, this reaction is dependent on the availability of ferrous iron (Fe^{2+}),

which is readily formed from ferric iron (Fe^{3+}) in the presence of reducing agents such as ascorbate (eqn 9).

$$H_2O_2 + Fe^{2+} \quad \text{-----> } \cdot OH + OH^- + Fe^{3+} \qquad \text{(eqn. 8)}$$

$$Fe^{3+} + \text{ascorbate} \quad \text{-----> } Fe^{2+} + \text{ascorbyl-radical} \qquad \text{(eqn. 9)}$$

In most biological systems, such as the mammalian CNS, ascorbate is abundant and can fulfill this role. Concentrations of ascorbate have been reported to be in the range of 1-2 mM in rat brain (Milby et al., 1982) and 0.4-1.3 mM in major divisions of the human brain (Mefford et al., 1991).

1.6: Oxidative Stress and Parkinson's disease

The disease which is primarily of interest here is Parkinson's disease, which was described by James Parkinson in 1817 as a disorder characterized by an " involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported, with a propensity to bend the trunk forward, and to pass from a walking to a running pace, the senses and intellect being uninjured". The disease is characterized neuroanatomically by a massive loss of dopaminergic neurons in the basal ganglia (Ehringer and Hornykiewicz, 1960). The cause for this loss of dopaminergic neurons is still unknown. However, more and more evidence points to an involvement of oxidative stress in the etiology of this chronic neurodegeneration (reviewed e.g. in Cohen, 1983; Fahn and Cohen, 1992;

Jenner, 1991; Olanow et al., 1992; Cohen, 1994). Currently, the main therapy is dopamine replacement via L-dopa, which crosses the blood-brain barrier; dopamine cannot be given directly because it does not cross the blood-brain-barrier. L-dopa can partially alleviate the motor dysfunction present in PD, but does apparently not address the underlying cause of the disease, the degeneration of DA neurons.

Among the points of evidence indicating an increased oxidative load on the dopaminergic cells in the basal ganglia are the findings of increased TBARS upon autopsy, which indicates increased lipid peroxidation in these regions (reviewed in Jenner, 1991; see also references cited above).

1.6.1: Increased DA metabolism in Parkinson's disease

Another interesting finding is that of Hornykiewicz and Kish (1985), who reported increased DA turnover by monoamine oxidase (MAO) in the remaining DA neurons of PD patients, shown by a strong shift in the ratio of DA/MAO-derived metabolites towards the metabolites, especially homovanillic acid (HVA). This shift towards increased DA turnover could indicate increased activity of the remaining dopamine neurons, resulting in more free DA being accessible to MAO, or decreased fidelity of the DA uptake and storage system, maybe resulting from decreased cellular ATP levels.

1.6.2: Mitochondrial function in Parkinson's disease

A remarkable change in PD patients compared to controls is the apparent loss of mitochondrial complex I (NADH : ubiquinone oxidoreductase, rotenone sensitive) activity. Complex I plays an essential role in oxidative phosphorylation. This observation was initially made in a small pilot study (Schapira et al., 1989), and later confirmed independently by several investigators (Schapira et al. 1990, Parker et al. 1989). While the initial reports suggested a regional localization of this defect to some areas of the basal ganglia (Schapira, loc cit.), several reports now indicate a generalized deficiency in the activity of this mitochondrial enzyme beyond the CNS (e.g. platelets, muscle; Parker et al, 1989, Shoffner et al., 1991).

The increase in markers indicating the presence of oxidative stress and the decrease in the activity of a key enzyme involved in oxidative phosphorylation may be interconnected, since oxidative stress can influence mitochondrial activity, while damaged or dysfunctional mitochondria can also serve as a source for reactive oxygen species (Boveris and Chance, 1972; Zoccarato et al., 1989, Boveris, 1994).

1.6.3: Iron and Parkinson's disease

The possible involvement of iron in the pathogenesis of Parkinson's disease is strongly suggested by reports on increased iron levels in parts of the basal ganglia affected in PD (Riederer et al 1989; Dexter et al 1991; Good et al., 1992). Recent studies have also shown that infusion of iron into the substantia nigra leads to a dose-dependent loss of dopaminergic

neurons (Sengstock et al., 1993). The increase in lipid peroxide levels found may also be due to the increased levels of unbound iron present in affected brain areas of PD patients (e.g. Riederer et al., 1989, Dexter et al., 1991). Iron can cause or accelerate oxidative damage (see above).

The formation of H_2O_2 and quinones as final products of these initial and resulting reactions by catechols has been reported (see review in Cohen, 1983). The occurrence of reactive oxygen species such as superoxide (eqn 1), $^{\cdot}OH$ and semiquinone radicals make the auto-oxidation of catechols a source of oxidative stress. Although L-dopa has been found to have antioxidant activities on lipid peroxidation in rat brain homogenates (Zaleska and Floyd 1985; Dostert et al. 1991), this role is reversed in the presence of lower concentrations of ascorbate, where L-dopa has pro-oxidative effects on lipid peroxidation (Li et al. 1995).

1.7: DA and monoamine oxidase

Dopamine (DA), the metabolite of L-dopa and the active neurotransmitter in dopaminergic neurons, is subject to a second, enzymatically catalyzed, pathway which results in the formation of H_2O_2 . This pathway is its degradation by monoamine oxidase (MAO), a flavin-dependent enzyme of the outer mitochondrial membrane. MAO exists in two isoforms, MAO A and MAO B (e.g. Neff and Goridis, 1972; Tipton et al., 1976, for review). In contrast to dopamine, L-dopa, being an amino acid, is not a substrate for monoamine oxidase. Equation (16) shows the formation of H_2O_2 and dihydroxy-phenylacetaldehyde resulting from the oxidative

deamination of dopamine by MAO. The aldehyde formed in this reaction is further oxidized by aldehyde dehydrogenase to the corresponding acid.

In vivo, the final products are 3,4-dihydroxyphenylacetic acid (DOPAC) and an O-methylated derivative, homovanillic acid (HVA). The amount of H_2O_2 corresponds to the amount of DA undergoing deamination. As mentioned above, H_2O_2 can give rise to the formation of the hydroxyl-radical, $^{\cdot}OH$. It can also directly damage cellular structures. Therefore, the deamination of DA by MAO (eqn 16) represents a significant possible source of oxidative stress on dopaminergic neurons and neighboring cells.

$$\text{dopamine} + O_2 + H_2O \xrightarrow{\text{MAO}} \text{aldehyde} + H_2O_2 \quad \text{(eqn. 16)}$$

A large number of physiological and non-physiological aromatic amines undergo oxidative deamination by either or both isoforms of MAO, MAO A or MAO B (e.g. Johnston, 1968; Goridis and Neff, 1971).

1.7.1: Indicators of oxidative stress: Glutathione disulfide

The presence of elevated levels of H_2O_2 can be monitored by measuring a product of its major detoxification enzyme, glutathione peroxidase (GPx). Equations (4) and (5) show again the reduction of H_2O_2 by glutathione peroxidase, which results in the formation of one molecule of glutathione disulfide (GSSG), which can be used to indicate the magnitude

of the H_2O_2 load: That is, the level of GSSG can be used as an index for oxidative stress.

$$H_2O_2 + GPx\text{-}Se\text{-}H \quad \text{-----}> \quad H_2O + GPx\text{-}Se\text{-}OH \qquad \text{(eqn.4)}$$

$$GPx\text{-}Se\text{-}OH + 2\ GSH \quad \text{-----}> \quad GPx\text{-}Se\text{-}H + GSSG + H_2O \qquad \text{(eqn. 5)}$$

Glutathione peroxidase has been shown to be a major detoxification pathway for H_2O_2 generated by MAO activity. In experiments with perfused liver and benzylamine, GSSG formation was not affected by the inhibition of catalase with 3-amino-1,2,4-triazole (Oshino and Chance, 1977). The authors concluded that glutathione peroxidase, and not catalase, was responsible for the removal of MAO-generated H_2O_2.

1.7.2: Protein-mixed disulfides

The physiological fate of the GSSG formed in equation 5 has two possible outcomes: it can be reduced back to 2 GSH by glutathione reductase (eqn 6), or, alternatively, it can undergo disulfide interchange with a cysteine side chain of a protein (eqn 17), which can render the protein biologically inactive, if its function depends on the presence of free thiol functions. The protein-glutathione disulfides shown in (eqn 17) are also referred to as protein-mixed-disulfides (PrSSG).

$$GSSG + NADPH \text{ -----> } 2\,GSH + NADP^{+} \quad \text{(eqn. 6)}$$

$$GSSG + Pr\text{-}SH \text{ -----> } PrSSG + GSH \quad \text{(eqn. 17)}$$

Since the studies cited above link PD to changes in energy metabolism, my experiments addressed several connected questions on possible interactions of dopamine/dopa metabolism and auto-oxidation with mitochondrial function. The question was whether evidence could be found to link oxidative stress to the degeneration of the dopaminergic neurons in PD.

2: EXPERIMENTS WITH ISOLATED MITOCHONDRIA

2.1: Rationale of the experiments

The experiments with isolated mitochondria addressed the impact of oxidative stress generated by MAO catalyzed oxidative deamination of dopamine on this organelle. Parkinson's disease, which is histologically characterized by the progressive loss of dopaminergic neurons of the basal ganglia, mainly in the substantia nigra/pars compacta (Ehringer and Hornykiewicz, 1960), has intrigued many by the selectivity for its cellular target. Surviving dopaminergic neurons are suspected of being under increased oxidative stress as the disease progresses (e.g. Cohen, 1990). The shift in the ratio between DA and its MAO-generated metabolites towards the metabolites in PD brains (Hornykiewicz and Kish, 1986) lends support to this hypothesis. Since MAO activity results in the production of H_2O_2, this shift towards the MAO-generated DA-metabolites reflects an increased oxidative load on the cells that are left.

Furthermore, experimental stimulation of DA turnover, either by supplying L-dopa (Spina and Cohen, 1988), or by disabling the autofeedback regulation of DA terminals by haloperidol (Spina and Cohen, 1989), have resulted in increased levels of glutathione disulfide (GSSG), reflecting increased MAO-generated H_2O_2. In some of those experiments, MAO inhibitors were used to successfully suppress the rise in GSSG (Spina

and Cohen, 1989), giving further support to the role of MAO in the generation of an oxidative stress via the turnover of dopamine.

Monoamine oxidase, which catalyzes the oxidative deamination of aromatic amines , is localized in the outer mitochondrial membrane. This is true for both isoforms, MAO A and MAO B. I was interested in the impact of the generation of H_2O_2 by MAO activity on the inner compartments of the mitochondrion .

Increased levels of GSSG can lead to the formation of protein-mixed disulfides (PrSSG). The matrix space of mitochondria, which is the space enclosed by the inner mitochondrial membrane, contains several enzymes whose function depends on the presence of free thiol groups (Brigelius 1985), which may thus be affected. The thiol redox state, which refers to the ratio between reduced glutathione and glutathione disulfide as well as the ratio of Pr-SH to PrSSG present, could therefore be indicative of possible functional consequences of the H_2O_2 produced by the MAO catalyzed monoamine turnover. This hypothesis was addressed by measuring the activity of aldehyde dehydrogenase (aliphatic), a thiol-dependent enzyme localized to the mitochondrial matrix (Vallari and Pietruszko, 1982), after the exposure of mitochondria to MAO generated H_2O_2.

2.2: Listing of experiments

a.) Measurement of GSSG levels in brain and liver mitochondria following exposure of isolated mitochondria to various MAO substrates. MAO activity generates H_2O_2 (eqn. 16), which should result in increased amounts of GSSG (eqn. 18).

$$\text{dopamine} + O_2 + H_2O \xrightarrow{\text{MAO}} \text{aldehyde} + H_2O_2 \quad \text{(eqn. 16)}$$

$$H_2O_2 + 2\,GSH \longrightarrow 2\,H_2O + GSSG \quad \text{(eqn. 18)}$$

The measurements addressed the thiol redox status changes which are brought about by mitochondrial MAO activity. Rising GSSG levels would indicate that the H_2O_2 produced by oxidative deamination of MAO substrates can indeed penetrate into the mitochondrion. Data suggesting such responses to MAO generated H_2O_2 were obtained using mouse liver mitochondria (Werner and Cohen, 1991). MAO exists in two isoforms, MAO A and MAO B, in the mitochondrial membrane. The two isoforms differ in their substrate preference and sensitivity to certain inhibitors. The incubations were done in the presence or absence of selective MAO inhibitors, to identify and verify MAO as the source for the H_2O_2 formed. The use of selective MAO A or MAO B inhibitors permits the evaluation of the relative contribution of the two isoforms of the enzyme. The GSSG assays were conducted using a modification (Slivka et al., 1987) of the original enzymatic cycling assay as described (Tietze, 1969).

As a part of this thesis, the assay was later modified further to reduce the costs associated with the use of C-18 reverse phase cartridges (see methods), and adapted to the use of a microplate reader, which allows simultaneous analysis of multiple samples (Werner & Cohen, in preparation).

b.) Measurement of protein-mixed-disulfides (PrSSG) in mitochondria challenged as described under a.). As described above, PrSSG can be formed when GSSG accumulates(eqn. 17).

$$GSSG + Pr\text{-}SH \quad \text{-----}> \quad Pr\text{-}SSG + GSH \qquad \text{(eqn. 17)}$$

PrSSG formation reflects changes in the thiol redox status of the cysteine thiol functions of proteins, as opposed to the cysteine thiol in glutathione. Therefore, an increase in PrSSG levels may lead to altered biological function in the S-thiolated proteins. As part of this thesis, an assay for the measurement of PrSSG was devised (Werner & Cohen, in preparation), which allows the use of the enzymatic cycling assay for GSH (after Tietze, 1969) following the reduction of the disulfide bond by $NaBH_4$. Previously described assays rely on HPLC analysis of the released GSH, and require derivatisation of the GSH prior to assay (e.g. Kumari et al., 1994, Chai et al., 1994). The experiments conducted included dose-response experiments with DA, comparison of the MAO substrates

dopamine (DA), benzylamine (BA) and 5-hydroxytryptamine (5-HT) and the effect of MAO inhibitors on PrSSG build-up. In some experiments, the turnover of DA was monitored using HPLC with electrochemical detection (HPLC-EC).

The rationale for the measurement of protein-mixed disulfides is that the modification of essential thiol functions in enzymes by PrSSG formation can reduce the activity of the affected enzyme, and thus affect mitochondrial function (see below).

c.) Measurement of the activity of a thiol dependent enzyme, namely aldehyde dehydrogenase, in rat brain and liver mitochondria following exposure to MAO substrates. Aldehyde dehydrogenase (AldDH) was used as a "sensor", representing thiol-dependent mitochondrial enzymes.

As mentioned above, changes in the thiol redox status of enzymes could affect their function, e.g. reduce their activity. Since the alteration of mitochondrial function has been implicated in a variety of neurodegenerative disorders, changes due to the formation of PrSSG in mitochondria were of particular interest. In addition, experiments were conducted with direct addition of an organic hydroperoxide (R-OOH), t-butylhydroperoxide. ROOH are detoxified by GPx, but not by catalase. As mentioned earlier, the result of this detoxification is an alcohol (eqn. 7).

$$R\text{-}OOH + 2\ GSH \text{ -----> } R\text{-}OH + H_2O + GSSG \quad \text{(eqn.7)}$$

2.3 METHODS

2.3.1: List of materials

DTNB (5,5'-dithio-bis-2-nitrobenzoic acid), Ficoll 400, glutathione reductase type III (from bakers yeast), dipotassium EDTA (ethylenediaminetetraacetic acid), NAD (nicotine-adenine-dinucleotide), NADH (nicotine-adenine-dinucleotide, reduced), NADPH (nicotine-adenine dinucleotide phosphate, reduced), dopamine, benzylamine and 5-hydroxytryptamine were from Sigma. Clorgyline was from May and Baker, England. Deprenyl was from Medipex, Hungary. Sodium borohydride and potassium phosphate (mono- and dibasic) were from Fisher . Perchloric acid was from Mallinckrodt. Rats were from Charles River and housed in the Mount Sinai animal care facilities.

2.3.2: Equipment used and statistical tests

Centrifugations were carried out using a Sorvall RC 2 refrigerated superspeed centrifuge equipped with an SS 34 rotor. Analyses for GSH, GSSG, PrSSG and aldehyde dehydrogenase activity were carried out using a Tecan SLT 340 ATTC microplate reader equipped with a kinetics cartridge (Tecan US, Research Triangle Park, NC) or a Gilford Stasar III spectrophotometer (Gilford, Oberlin, OH) with a flow-through cell. Additional assays for aldehyde dehydrogenase activities were conducted using a Gilford 240 UV/VIS spectrophotometer.

Statistical evaluation of the data was carried out using single or multiple analysis of variance (ANOVA or MANOVA), followed by the Tukey-Kramer post-hoc test, or the Student two-tailed t-test.

2.3.3: Isolation of brain mitochondria

All experimental steps were carried out at 0-4 °C except where noted. The buffer used for isolation of mitochondria consists of 0.225 M mannitol, 0.075 M sucrose, 1mM EGTA (ethylene glycol-*bis*-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) and 5 mM MOPS (3-(N-morpholino) propane-sulfonic acid), adjusted to pH 7.4 with sodium hydroxide (NaOH).

The protocol used for the preparation of liver or brain mitochondria was that of Clark and Nicklas (1970). To obtain brain mitochondria, 4 male adult Spraque-Dawley rats were decapitated with a guillotine, the brains immediately removed and chilled in ice-cold Krebs buffer. The cerebellum and the striatum were removed for other experiments and the rest of the brains transferred into 40-50 mL of ice cold isolation buffer. The brain parts were minced and rinsed and then homogenized in 10-12 x volumes (w/v) of isolation buffer with 10-12 gentle up-and-down strokes in a Potter-Elvejhem homogenizer cooled with an ice water bath. The resulting homogenate was centrifuged in a Sorvall SS 34 rotor at 1900 x g for 3 min., the supernatant again centrifuged under identical conditions and the pellet discarded. The resulting supernatant was centrifuged for 8 min. at 12200 x g. The pellets from this centrifugation were resuspended in 3% Ficoll 400 (in buffer) and carefully layered over 6% Ficoll (in isolation buffer). After centrifugation at

11200 x g for 30 min. (brake off), the resulting hard brown pellet was again resuspended in isolation buffer and centrifuged for 10 min. at 11200 x g. The pellet was then resuspended in buffer, the protein concentration determined according to Lowry et al. (1951), and the concentration of the mitochondria adjusted to a final concentration of 1 mg of protein (bovine serum albumin equivalent) per tube.

2.3.4: Isolation of liver mitochondria

To obtain mouse or rat liver mitochondria, the livers of 2-3 mice (adult, male Swiss-Webster) or liver lobes from 2-3 rats were rapidly removed following decapitation of the animals, cut into smaller pieces, and immediately immersed in ice-cold isolation buffer. All further steps were carried out at 0-4 °C except where noted. The liver pieces were then thoroughly minced and rinsed with buffer, until the supernatant was clear and void of red blood cells. The minced and washed liver was then homogenized in 10 volumes of buffer in a Potter-Elvejhem homogenizer at 750 rpm with 6-7 up-and-down strokes while cooling in an ice water bath. The resulting homogenate was centrifuged at 580 x g for 10 min. The supernatant was carefully removed and recentrifuged at 8000 x g for 10 min. The second supernatant was discarded and the white fluffy layer removed by gentle shaking with the last portion of buffer. The pellet was then transferred into fresh buffer with care not to resuspend the occasional red spot, which consists of traces of erythrocytes at the bottom of the tubes.

The transferred pellet was resuspended by gentle homogenization in a hand operated wide tolerance glass-glass homogenizer in a total of approximately 35 mL of buffer. The resulting suspension was centrifuged at 9200 x g for 10 min. This step was repeated once. The resulting mitochondria were suspended in buffer.

2.3.5: Incubation protocol

In earlier experiments, the mitochondria were immediately incubated with or without MAO substrates at 25 °C while shaking and then analyzed for GSSG content. The GSSG values obtained were then normalized for the protein content determined after the experiment. In most of the experiments with both liver and brain mitochondria, the protein content was first adjusted and then the mitochondria were incubated at 30 °C for 10 min or 20 min in the presence and absence of MAO substrates and/or inhibitors while shaking, and the mitochondria then worked up as described below. In some experiments, mitochondria were incubated with t-butylhydroperoxide in lieu of MAO substrates.

2.4: Analyses for GSSG

GSSG was determined by a modification of the method used by Slivka et al. (1987), based on the recycling assay by Tietze (1969). The incubations of mitochondria as described above were stopped by addition of perchloric acid (0.4 N final concentration), the samples were immediately chilled in ice-water and 450 μL transferred into microcentrifuge tubes (on ice). Each tube was vigorously vortexed for 10 sec. and subsequently centrifuged for 15 min. at 16,000 g (0-4 °C). 200 μL of the supernatant was removed, 50 μL of 4 M potassium phosphate buffer (containing equal parts of mono- and dibasic potassium phosphate and 200 mM dipotassium EDTA) added, mixed and chilled on ice for 5 min. The resulting precipitate of potassium perchlorate was pelleted by centrifugation at 16,000g (0-4 °C) for 3 min. 200 μL of the resulting supernatant were transferred into small glass culture tubes, and 350 μL of 14.5 mM NEM in 10 mM H_3PO_4 to remove any GSH present. The pH was adjusted with 0.4 N KOH to 7.4 while vortexing and the solution incubated for 10 min. at room temperature.

2.4.1: Modifications to the assay protocol

The protocol was modified to enable the re-use of the cartridges and the use of a microplate reader. Following the incubation (described in the previous paragraph), the excess NEM (not bound to GSH) was extracted from the solution by reversed phase chromatography using SepPak C-18 cartridges (described in Slivka et al. 1987). The removal of excess NEM is pivotal , since it can irreversibly inhibit the GSSG reductase used in the

assay procedure. The cartridges were initialized by washing with 1 mL of methanol, then rinsed with 3-4 mL of distilled water, and then rinsed with 1 mL of 100 mM potassium phosphate buffer. Following drainage of the cartridges, 600 μL of the sample was applied, and eluted from the cartridge slowly (drop wise). The cartridge was further rinsed with two times 200 μL distilled water and the rinses combined with the initial filtrate. To recycle the cartridges, they were washed with 3-4 mL of distilled water, drained, rinsed with 1 mL methanol and again drained. The cartridges were then washed by slowly rinsing them two times with 1 mL 100% ethanol. Ethanol is among the best solvents for NEM (Beilstein, Richter 1935). The cartridges were then re-initialized with methanol and used as before. Up to ten NEM extractions were obtained from one cartridge without any NEM break-through.

After NEM removal, 750 μL of combined filtrate were removed, DTNB, NADPH (as 2.5 mM and 1.1 mM, respectively, in 100 mM phosphate buffer) and GSSG standard added and the volume made up to 1 mL with distilled water (dH_2O). Samples were then analyzed in duplicate per tube on a SLT 340 ATTC microplate reader using the standard wide-band filter at 405 nm and a kinetics cartridge. 250 μL were pipetted into each well, and the reaction started by adding 25 μL of glutathione reductase in 100 mM phosphate buffer (13 U/mL).

Readings were taken every 30 sec. and the resulting reaction rate calculated via regression analysis of 4 measurement points with the best linearity. The amount of GSSG was then calculated. The minimum linearity required to be acceptable for the rate calculation was set at r=0.95.

2.5: Assay for PrSSG

The protein-glutathione assay employed here was developed as part of the thesis. The goal was to obtain an analytical method that allowed the assay of small samples (1 mg of protein and below) without the need for derivatization and HPLC analysis. Protein glutathione mixed disulfide analysis was carried out as follows:

The proteins were pelleted following acidification of the incubation mixture with perchloric acid (0.4 N final). The supernatant was aspirated with great care not to leave any visible fluid behind and not to aspirate part of the pellet. The pellet resulting from acidification was rinsed by resuspending it using sonication in 500 μL ice-cold 0.4 N perchloric acid.

The resulting fine suspension had a milky appearance with no observable particles. An additional 750 μL of ice-cold 0.4 N perchloric acid was added, each tube vortexed for 10 sec. at highest setting and recentrifuged at 16,000 g for 15 min. (0-4 °C). This step was repeated once, again with care not to leave any fluid behind in the aspiration steps. Two washes were found to be sufficient for the removal of all soluble, i.e. not covalently bound glutathione. This was verified by measuring glutathione in

the supernatant of the second wash. The pellet resulting from the second wash step was resuspended in 75 μL of ice-cold 0.2 N NaOH by sonication as above, but only for 5-8 sec. at low setting. 25 μL of a 0.1 M $NaBH_4$ solution in 0.2 N NaOH was added, the samples mixed and eventual fluid droplets united by quick spinning in a microcentrifuge for 10-15 sec. The reduction was carried out at room temperature for 30-45 min.

To remove excess borohydride and precipitate the protein, 25 μL of 4.0 N perchloric acid was added by spinning a droplet of acid simultaneously into all tubes using a Speed-Vac centrifuge. Spinning as a way of adding the acid was necessary to avoid sample loss by foaming, and the Speed-Vac provides the explosion-proof centrifuge which was needed due to the release of hydrogen gas, which prevents the use of a common microcentrifuge. After 10 min., the tubes were transferred onto ice, chilled for 5 min., and then centrifuged for 30 min. at 0-4 °C at 16,000 g with the brake off. 75 μL of the resulting supernatant was transferred to fresh microcentrifuge tubes and 50 μL of the 4.0 M potassium phosphate buffer added. The samples were mixed, chilled for 5 min. to allow the maximum precipitation of potassium perchlorate, and then spun at 16,000 g in the cold for 4 min.

Of the resulting supernatant, 90 μL were transferred into small glass culture tubes (12x75 mm), 525 μL of dH_2O added, mixed and neutralized with 135 μL 0.4 N KOH to pH 7. Blanks and standards were assayed under identical conditions by acidifying a 3+1 mix of 0.2 N NaOH + 0.1 M sodium

borohydride in NaOH with 1 part 4.0 N perchloric acid. The resulting solution undergoes the identical perchlorate precipitation as the samples, thus assuring the presence of the identical mixture of anions and cations in blank and standard.

2.6: HPLC Assay for DA

HPLC analysis of DA and metabolites was carried out using reversed phase HPLC with electrochemical detection. The HPLC consisted of an ESA 5500 16 electrode in-line HPLC system equipped with an ESA/Kontron refrigerated autosampler 465. The mobile phase was 75 mM NaH_2PO_4, 1.7 mM 1-octanesulfonic acid (ion pairing reagent) (HPLC grade, Kodak), 0.01 % triethylamine and 10 % v/v acetonitrile (EM Science), made up in HPLC grade water (18 mΩ resistance). The NaH_2PO_4 was dissolved in H_2O (about 50% of the final volume), and filtered through a 0.2 μM nylon 66 membrane, water and the other chemicals added, the pH adjusted to pH 3.0 with H_3PO_4 and the volume brought up to final in a graduated cylinder. The measurements were carried out with the electrodes set in increments from 420-660 mV (channel 1-16). DA and dihydroxyphenylacetic acid (DOPAC) retention times were established by analyzing standards.

2.7: Assay for aldehyde dehydrogenase activity

2.7.1: General protocol

Rat brain and liver mitochondria were prepared as described above according to Clark and Nicklas (1970). Protein content was determined according to Lowry and adjusted to 1 mg/tube. MAO substrates were added at 1 mM final and aliquots of brain mitochondria were incubated in isolation buffer in a final volume of 500 μL. Incubations were performed at 30 °C while shaking 150/min. Following the incubation, the incubation mixtures were immediately chilled in ice water and the contents of the test tubes transferred into microcentrifuge tubes cooled on ice. The test tubes were then rinsed with 750 μL of chilled isolation buffer and this wash also added to the respective samples. The microcentrifuge tubes were spun at 16,500 g at 4 °C for 15 min. and the supernatant carefully aspirated so as not to disturb the pellet. The pellet was then rinsed with 1 mL of cold isolation buffer by gentle inversion and again centrifuged at 16,500 g for 10 min. Following the aspiration of this supernatant, the pellet was sonicated on a low setting using a micro tip for 10 sec. in 125 μL of ice-cold 30 mM potassium phosphate buffer containing 1 mM EDTA adjusted to pH 6. The sonicated samples were spun for 20 min at 16,500 g at 4 °C and the supernatants used for the assay

2.7.2: Measurements in brain mitochondria

For the measurement of aldehyde dehydrogenase activity in brain mitochondria, 100 μL of the supernatants were pipetted into a quartz glass cuvette containing 475 μL of 100 mM sodium pyrophosphate buffer pH 9.0,

25 μL 12 mM NAD in dH_2O added , the solutions mixed by inversion and an initial reading taken for 60-90 s. The assay was then started by adding 10 μL of 60 mM propionaldehyde in 3 mM potassium phosphate buffer pH 6.0 containing 0.1 mM EDTA, resulting in a final substrate concentration of 1 mM.

The rate was calculated by subtracting the slope before aldehyde addition from the slope after addition of the substrate, and the absolute amounts of NADH formed was calculated based on the readings following subsequent additions of NADH to water in a cuvette, corrected for the volume error. In preliminary experiments, a range of substrate concentrations (0.5 - 2 mM) was used to ensure that neither NAD nor aldehyde were rate-limiting. A final concentration of 1 mM for both NAD and propionaldehyde was found to be not rate-limiting throughout the assay. The data were then expressed as nmoles NADH/mg protein x min.

2.7.3: Measurements in liver mitochondria

Assays for aldehyde dehydrogenase activity in rat liver mitochondria were conducted on a SLT 340 ATTC microplate reader using the kinetic program cartridge and standard, flat bottom 96 well microtiter plates.

The reaction rate was obtained by monitoring the increase in absorption at 340 nm, with the instrument calculating the maximum slope of

the 4 measurement points with the biggest linear increase. The linearity was set at r = 0.95, to ensure first order kinetics.

100 μL of the sonicated supernatant obtained as described above were mixed with 475 μL of 100 mM sodium pyrophosphate buffer pH 9.0. To this was added 25 μL of 12 mM NAD in dH_2O, the mixture gently vortexed for 2-3 sec. and 2 wells of the microtiter plate filled with 225 μL per well. The assay was then started by adding 25 μL of freshly prepared 11 mM propionaldehyde in 3 mM potassium phosphate buffer pH 6.0 containing 0.1 mM EDTA. For liver mitochondria, the average of the controls was set at 100% activity, and the activity of treated mitochondria calculated in percent of corresponding control average.

The data obtained were analyzed for statistical significance using either the Student's t-test or one or multiple analysis of variance (ANOVA/ MANOVA), followed by the Tukey-Kramer post-hoc test.

3: RESULTS

3.1: Experiments with liver mitochondria and BA

The question of whether or not the H_2O_2 generated by the outer mitochondrial membrane enzyme MAO can affect the inner compartments of mitochondria was addressed by incubating isolated mouse liver mitochondria with the MAO B substrate benzylamine. The results of 6 experiments in triplicate are shown in **Table 1a**. Incubation with 100 μM BA for 5 min. at 25 °C while shaking resulted in a GSSG level of 290% of control (Werner and Cohen, 1991).

3.1.1: Effect of catalase

Catalase (10 μg/mL) had only a slight effect on the rise in GSSG. These data indicate that H_2O_2 generated by MAO activity gains access to the inside of mitochondria. The lack of effect of added catalase may be due to the fact that liver mitochondria are usually already contaminated with peroxisomes, which contain catalase and could act as a sink for H_2O_2. Therefore, added catalase may not have been effective. Part of the H_2O_2 formed may have been detoxified by catalase, and part by glutathione peroxidase.

3.1.2: Effect of MAO inhibitors

To verify that the GSSG increase in the BA-treated mitochondria was indeed due to MAO activity, mitochondria were incubated with the MAO A inhibitors clorgyline and the MAO B inhibitor deprenyl.

TABLE 1a

Effect of benzylamine, with and without catalase, on GSSG levels

	GSSG(pmoles/mg protein	
	Without catalase	With catalase
Control	27.6 ± 5.2	27.9 ± 5.4
Benzylamine	79.9 ± 10.3[a]	68.6 ± 8.7[a]

Mouse liver mitochondria were incubated in the presence or absence of benzylamine (100 μM) with or without added catalase (10-12 μg/mL) for 5 min. at 25 °C. Results are the mean ± S.E.M. from six experiments performed in triplicate. [a] $p < 0.005$ compared to the corresponding control (paired t-test).

TABLE 1b

Effect of deprenyl and clorgyline on the rise in GSSG evoked by benzylamine

	GSSG(pmoles/mg protein	
	Experiment 1	**Experiment 2**
Control	21.5 ± 3.4	14.3 ± 2.8
Benzylamine	40.2 ± 3.4[a]	43.5 ± 3.4[a]
Clorgyline + benzylamine	39.6 ± 3.2	36.9 ± 1.9
Deprenyl + benzylamine	16.0 ± 4.2[b]	5.5 ± 3.2[b]

Mitochondria were added to tubes containing 20 μM MAO inhibitor and 100 μM benzylamine (final concentrations) and incubated as in table 1a. Results are mean ± S.E.M. of triplicates. $p < 0.01$ compared to control; $p < 0.01$ compared to benzylamine alone.

Deprenyl is a widely used as a MAO B inhibitor, whereas clorgyline preferentially inhibits MAO A. **Table 1b** shows the results of 2 experiments conducted in triplicate. The MAO B inhibitor deprenyl abolished the rise in GSSG seen in the presence of the MAO B substrate BA. Clorgyline had little effect on the rise in GSSG, which is in keeping with its specificity for MAO A (Werner and Cohen, 1991). These results are as expected for an MAO B substrate (BA).

3.2: Experiments with brain mitochondria - GSSG

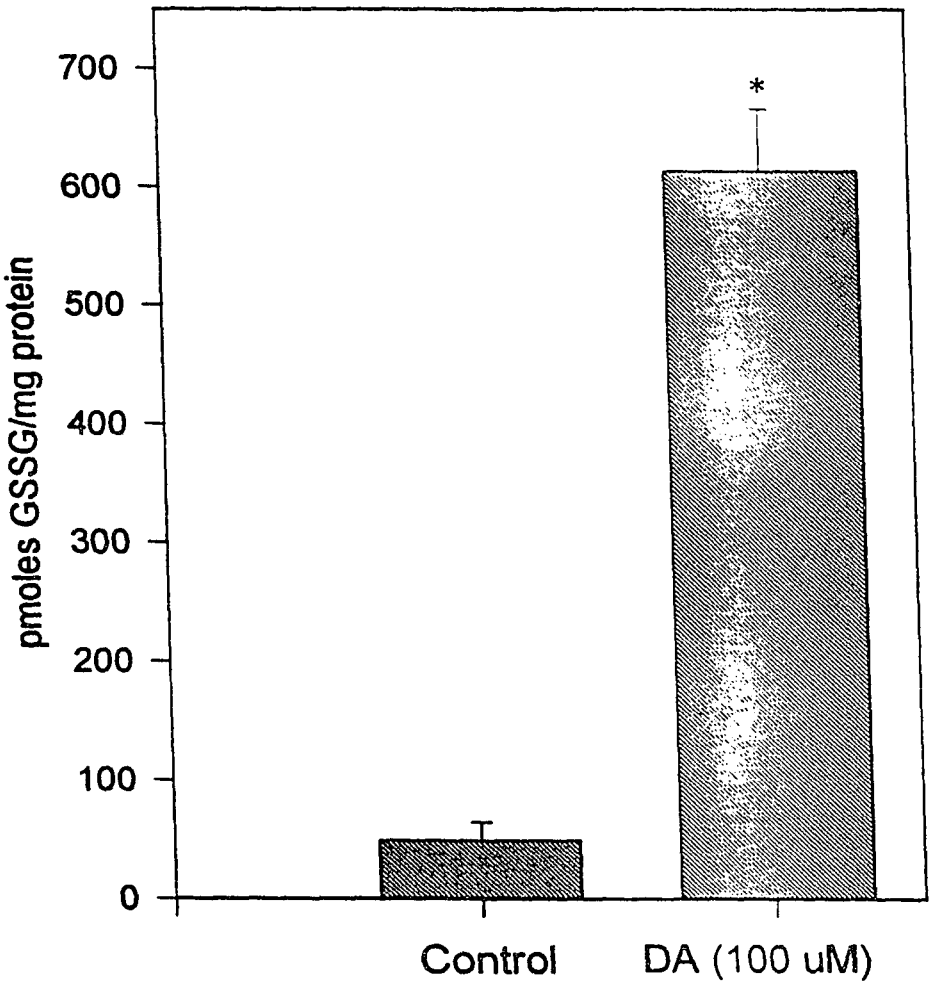
Following these initial observations, the studies were extended to brain mitochondria. This was done in order to confirm that the initial observations with liver mitochondria applies also to the organ of interest, the CNS.

3.2.1: Effect of DA

Incubation of rat brain mitochondria with 100 μM dopamine led to a marked, 11-fold increase in mitochondrial GSSG (**Figure 2**). These results extended prior observations with mouse liver mitochondria plus benzylamine.

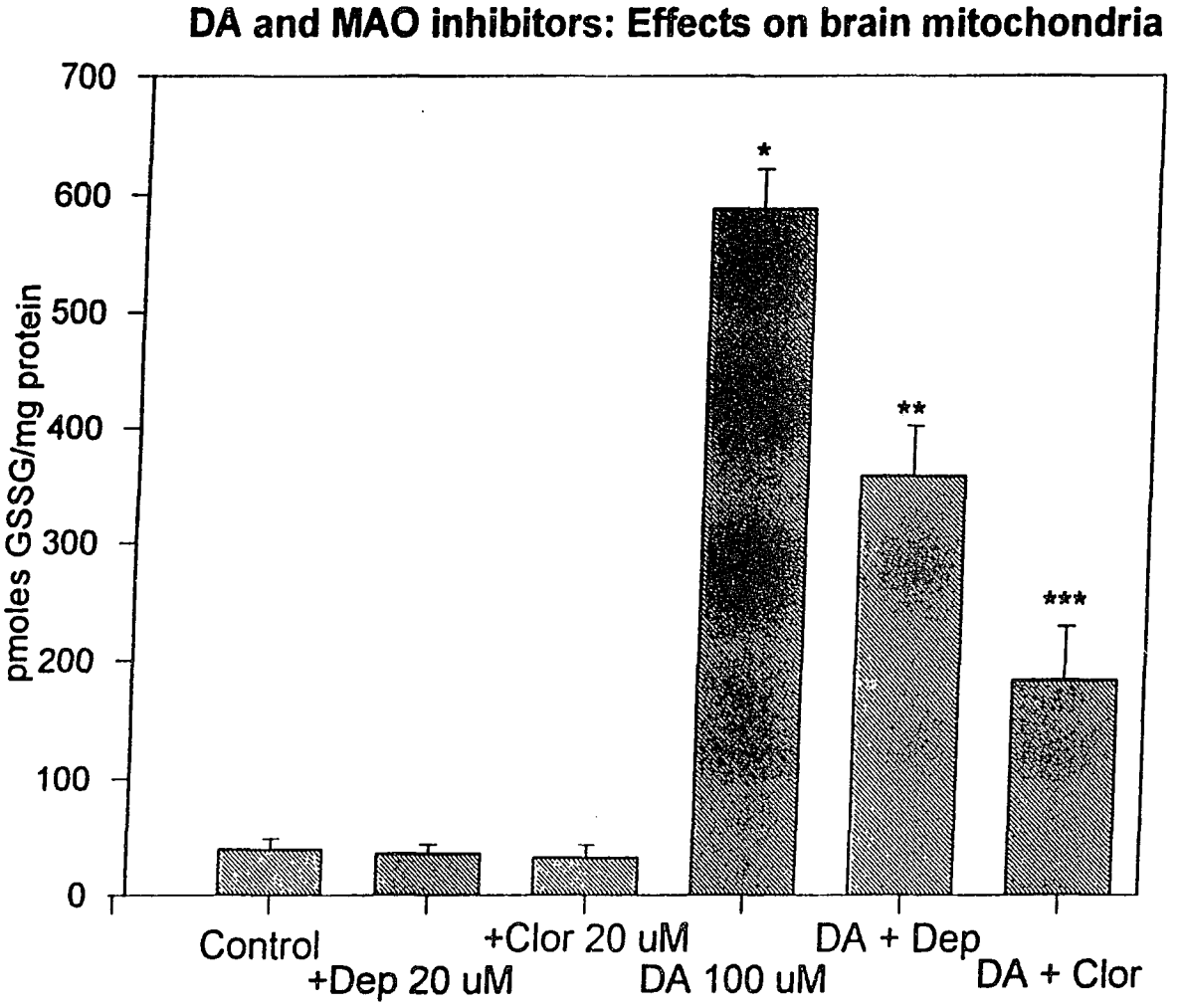
Figure 2

Effect of DA turnover by MAO on brain mitochondria



Shown are mean and S.E.M. of 4 experiments in triplicate. Brain mitochondria were incubated for 10 min. at 30 °C while shaking. * $p < 0.001$ compared to control; paired t-test.

Figure 3



Shown are means and S.E.M. of two experiments in triplicate. Brain mitochondria were incubated for 10 min. at 30 °C while shaking and then analyzed for GSSG. * $p < 0.001$ compared to all other conditions; ** $p < 0.001$ vs. deprenyl alone; *** $p < 0.01$ vs. DA plus deprenyl, $p < 0.05$ vs. clorgyline alone; ANOVA followed by Tukey post-hoc.

3.2.1a: Effect of MAO inhibitors

DA is a mixed substrate, i.e. it is deaminated by both MAO-A and MAO-B. Therefore, inhibitory effects on GSSG levels are expected for both MAO A and MAO B inhibitors. The rise in GSSG was partially prevented by 10 μM of the MAO A inhibitor clorgyline or 10 μM of the MAO B inhibitor deprenyl (**Figure 3**). Deprenyl suppressed the rise in GSSG by 39.1% compared to DA alone. Clorgyline was more effective, suppressing the rise in GSSG by 68.9%. Addition of both inhibitors simultaneously essentially blocked the rise in mitochondrial GSSG observed in the presence of dopamine, with GSSG levels of 70.8 ± 5.1 pmoles/mg protein. The data indicate that the rise in GSSG was provoked by MAO activity on DA, rather than autoxidation of the catecholamine. These results concur with prior observations,(see above) where similar effects were observed upon incubation of mouse liver mitochondria with benzylamine.

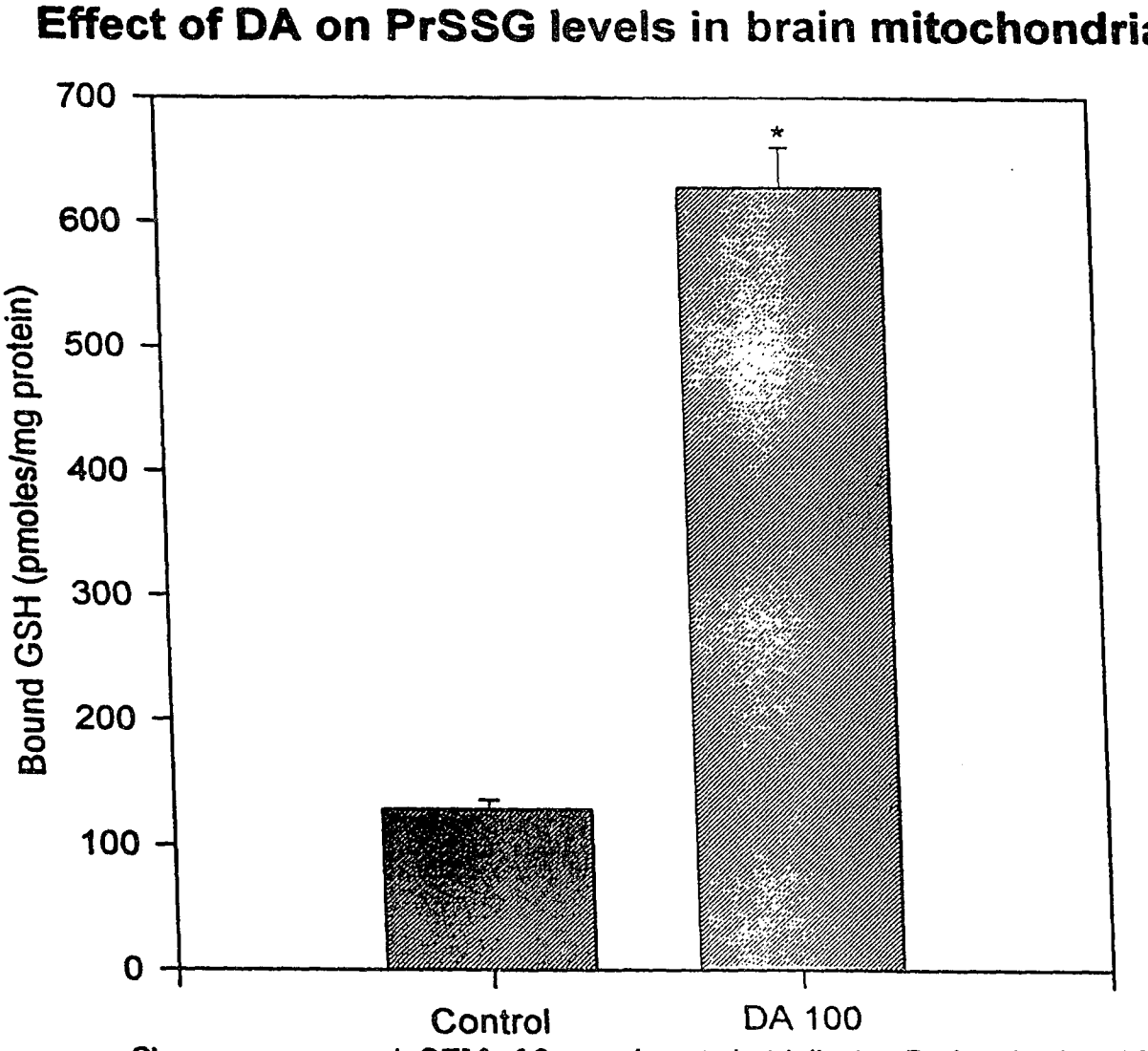
3.3: Experiments with brain mitochondria - PrSSG

3.3.1: Effect of DA

In order to address the question whether the build-up in GSSG was paralleled by changes in the thiol-redox state of proteins in mitochondria, changes in the levels of protein-glutathione mixed disulfides following MAO catalyzed deamination of DA and other MAO substrates were investigated. Covalently bound GSH (= PrSSG) in brain mitochondria was released by

reducing the mixed disulfides with sodium borohydride and assayed as described in "Methods". The incubation of rat brain mitochondria with 100 μM DA led to a 390 % increase in PrSSG, from 128.6 ± 7.6 (SEM) pmoles releasable GSH/mg protein for control to 628.1 ± 32.3 (SEM) pmoles/mg protein for DA treated mitochondria (**Figure 4**). This increase mirrors the rise

Figure 4



Shown are mean +/- SEM of 3 experiments in triplicate. Brain mitochondria were incubated as in figure 3, and protein bound GSH determined.
*** $p < 0.001$ compared to control (t-test).**

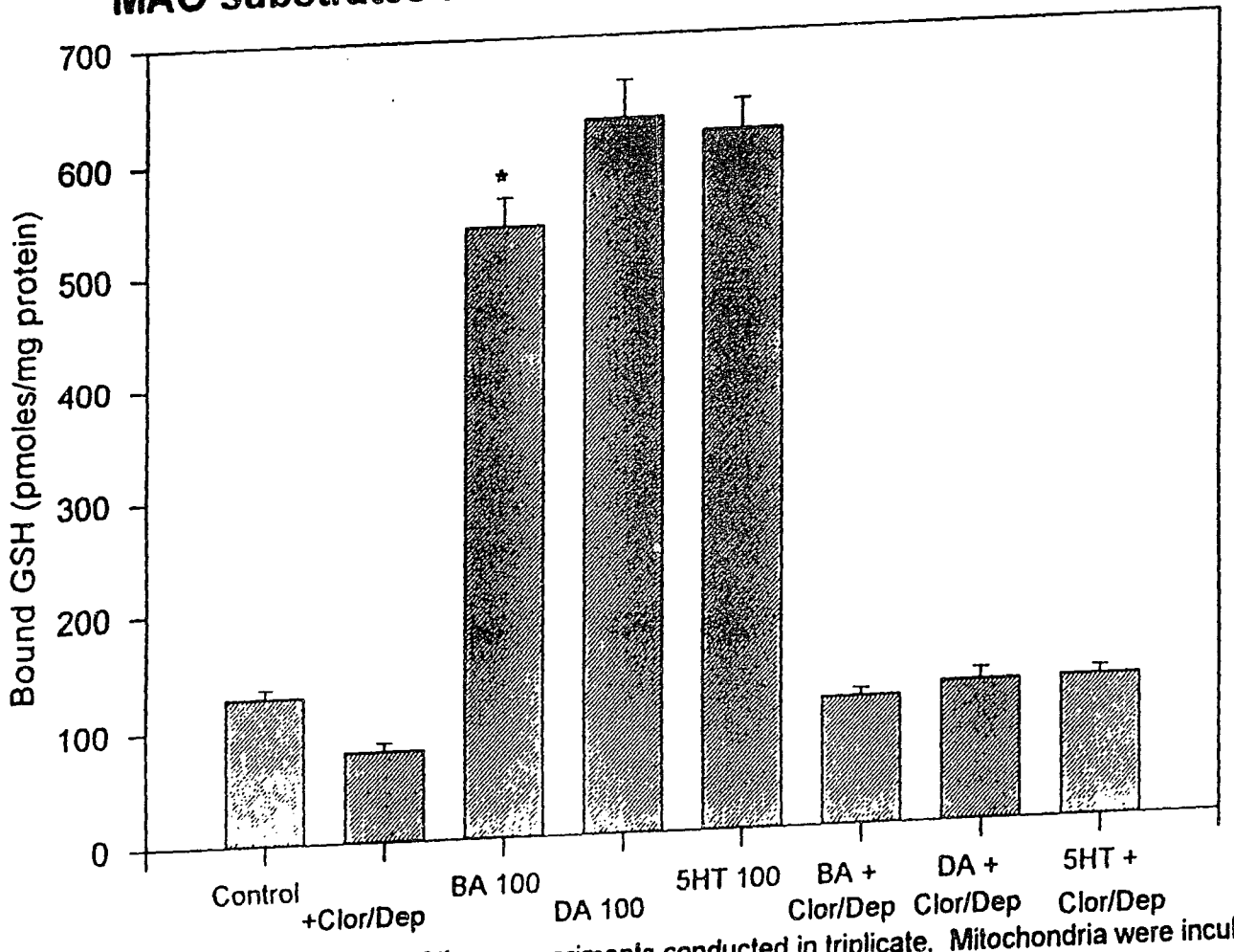
in GSSG in previous experiments carried out under identical conditions (**Figure 3**).

3.3.2: Effect of other MAO substrates and inhibitors

A second set of experiments was conducted to investigate the response of mitochondria to selective MAO-A and MAO-B substrates. The experiments were carried out using the preferred MAO-A substrate 5-hydroxytryptamine (5-HT, serotonin) and the preferred MAO B substrate benzylamine (BA) in addition to DA. The conditions were identical to the experiments shown in **Figure 4**, with the MAO substrates present at 100 μM. In addition, the MAO A inhibitor clorgyline and the MAO B inhibitor deprenyl were present at 20 μM each in some samples to prevent MAO catalyzed turnover of the monoamines. Data are again expressed in pmoles releasable GSH (borohydride reduction) normalized for protein. As in the experiments conducted with DA alone, MAO turnover of either DA, BA or 5-HT led to a rise of equal magnitude in PrSSG (**Figure 5**). Incubation with 100 μM BA led to 535.7 ± 26.5 (SEM) pmoles GSH/mg protein, compared to 128.6 ± 7.6 (SEM) pmoles GSH/mg protein for control, whereas 100 μM 5-HT resulted in 615.3 ± 25.8 pmoles GSH/mg protein.

Figure 5

MAO substrates and inhibitors: Effect on brain mitochondria



Shown are means and S.E.M. of three experiments conducted in triplicate. Mitochondria were incubated for 10 min at 30 °C with 100 uM MAO substrates and with or w/o 20 uM of both MAO inhibitors. All MAO substrates were significantly different from control ($p < 0.001$) and substrate plus inhibitor. * $p < 0.05$ compared to DA. ANOVA followed by Tukey post-hoc test.

As expected, the inhibition of both MAO A and MAO B prevented the rise of PrSSG levels over control regardless of which MAO substrate was present. The data show that the formation of PrSSG derives from the activity of mitochondrial MAO. These results are in keeping with our previous observation, where addition of both MAO A and MAO B inhibitors abolished the DA provoked rise in GSSG observed.

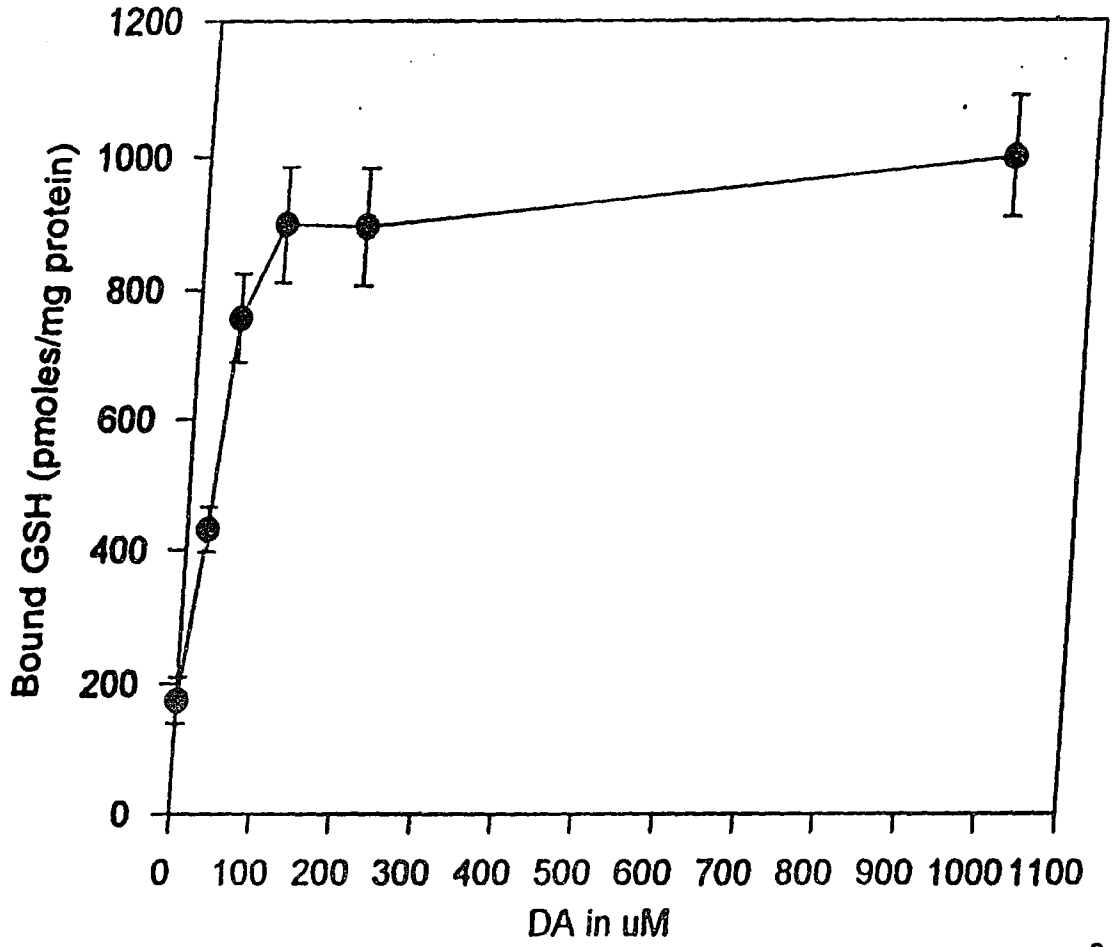
3.3.3: DA dose-response

In subsequent experiments, the dose dependence of PrSSG formation resulting from MAO catalyzed DA turnover was investigated. Rat brain mitochondria were incubated with various concentrations of dopamine, ranging from 25 μM to 1 mM for 20 min. and PrSSG levels determined.

All DA concentrations led to significant ($p < 0.001$) increases in PrSSG levels when compared to control, which was 174.3 ± 35.4 pmoles GSH/mg protein (**Figure 6**). DA at 25 μM increased PrSSG levels significantly ($p < 0.001$) to 431 ± 34.6 pmoles GSH/mg protein, while 50 μM DA resulted in PrSSG levels of 755.7 ± 68.3 pmoles GSH/mg protein. The PrSSG values obtained with 100, 200 and 1000 μM DA were 898.1 ± 86.7, 894.4 ± 88.6 and 1000.3 ± 91 pmoles GSH/mg protein, respectively. In one group of samples, 50 μM of both clorgyline and deprenyl (MAO A and MAO

Figure 6

PrSSG formation after incubation with various DA concentrations



Rat brain mitochondria were incubated with various concentrations of DA for 20 min. at 30 °C while shaking and then assayed for PrSSG. Shown are the means +/- SEM of two experiments conducted in quadruplicate. All concentrations of DA were significantly different from control ($p < 0.001$). 25 uM DA was significantly different from all other DA concentrations used ($p < 0.001$). 50, 100 and 200 uM were not significantly different from each other. 50 uM DA was significantly different from 1000 uM DA ($p < 0.001$); MANOVA with Tukey post-hoc test.

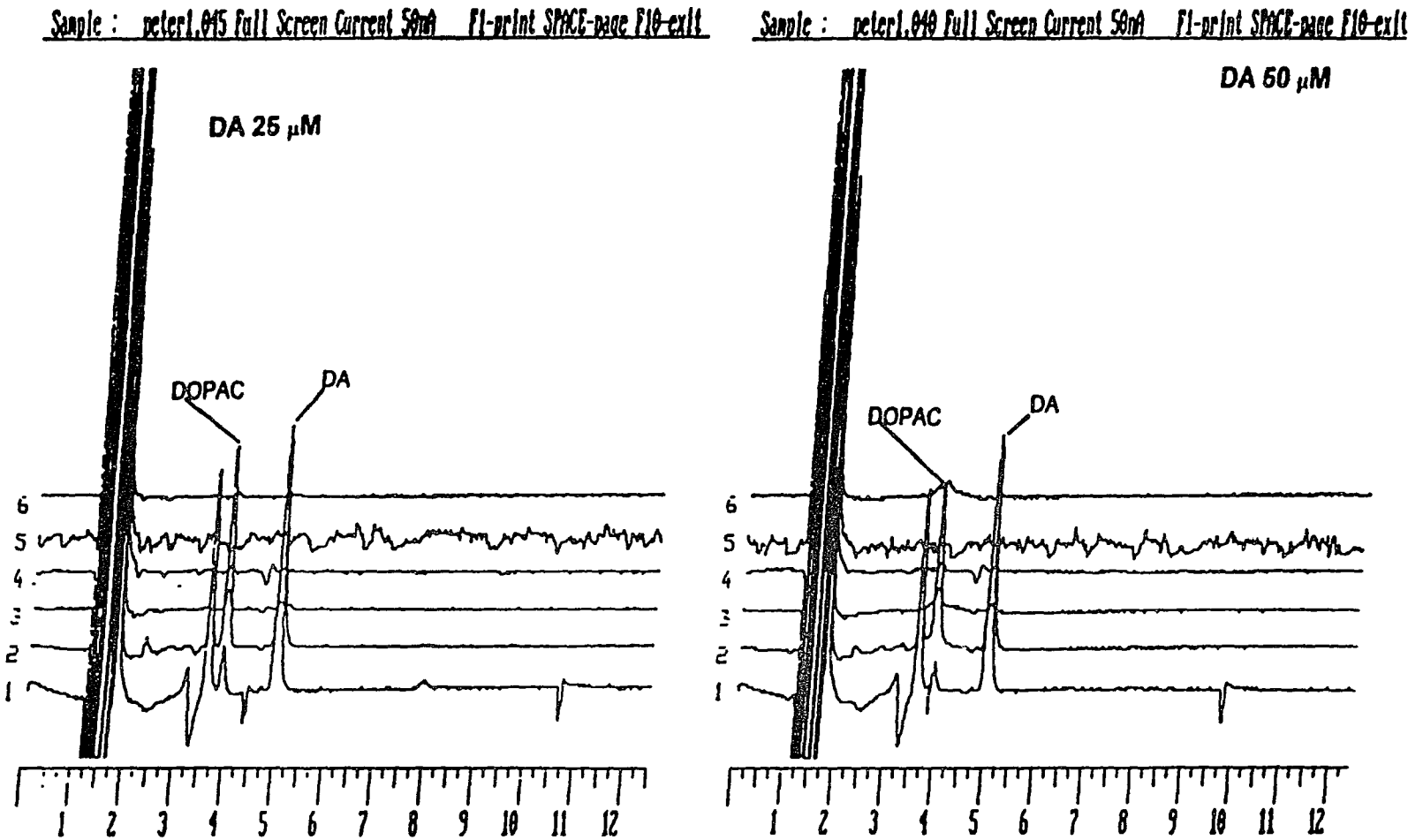
B inhibitors, respectively) were added together with 1000 μM DA. The level of PrSSG here was 172 ± 22.8 pmoles GSH/mg protein, which is equal to control.

3.3.3a: DA turnover - HPLC analysis

The turnover of DA by MAO was monitored using HPLC-EC, to measure residual DA and the appearance of dihydroxyphenylacetic acid (DOPAC). The HPLC-EC data were used to investigate whether the differences between the results of the low (25 μM) and the higher concentrations of DA was due to depletion of DA during the incubation at lower concentrations of DA. Acidified supernatants were obtained from the mitochondrial incubations, and all samples equalized to the same theoretical DA concentration and then analyzed. All chromatograms were analyzed at identical attenuation, with the full scale set at 200 nA. **Figures 7a** and **7b** show 4 representative chromatograms obtained from samples incubated with 25, 50, 100 and 1000 μM DA, respectively. As shown in **Figures 7a** and **7b**, DA was not the limiting condition in the MAO generated H_2O_2 production with subsequent PrSSG formation.

The fact that the PrSSG levels are essentially unchanged at concentrations above 100 μM can probably be explained with a saturation of

Figure 7a


Sample : peter1.045 Full Screen Current 50nA F1-print SPACE-page F10-exit
DA 25 µM
DOPAC
DA
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
Sample : peter1.040 Full Screen Current 50nA F1-print SPACE-page F10-exit
DA 50 µM
DOPAC
DA
6
5
4
3
2
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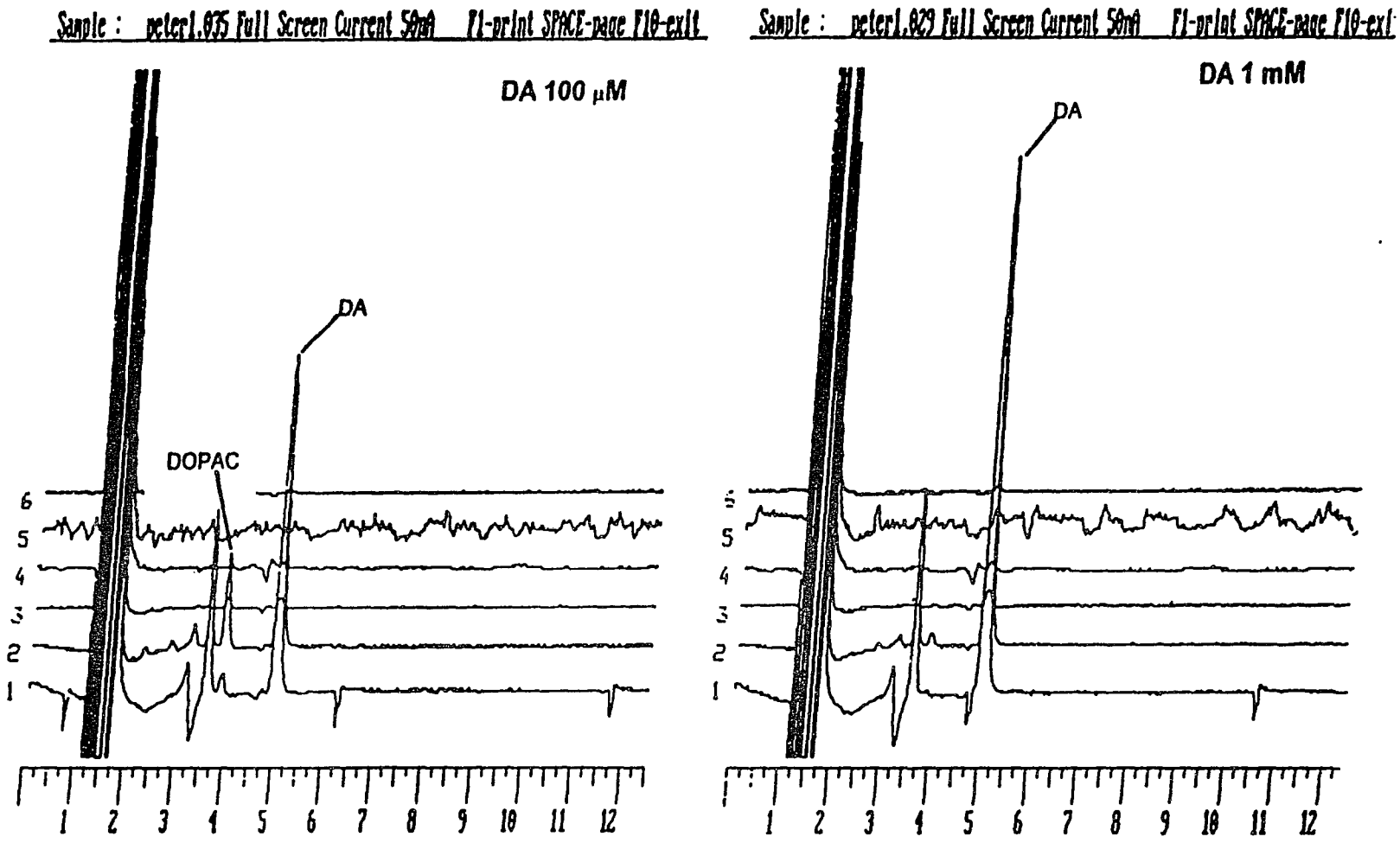
1 2 3 4 5 6 7 8 9 10 11 12

Figure 7b


Sample : peter1.035 Full Screen Current 50nA F1-print SPACE-page F10-exit
DA 100 µM
DA
DOPAC
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12
Sample : peter1.029 Full Screen Current 50nA F1-print SPACE-page F10-exit
DA 1 mM
DA
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12

accessible protein-SH groups, since the concomitant incubation of brain mitochondria (n = 4 samples, 1 experiment) with BA at 1 mM and with t-butylhydroperoxide at 100 μM resulted in equal PrSSG values.

3.4: Oxidative Stress and Activity of thiol dependent enzymes

3.4.1: Introduction

The increase in protein-mixed disulfides observed in the experiments described above drew our attention to the potential implications on protein function in these scenarios. Alteration of enzyme activities after formation of protein-mixed disulfides have been well established, although more for cytosolic enzymes (for review: Brigelius, 1985). We therefore addressed the question, whether MAO-activity related thiol-redox states observed affect the function of mitochondrial enzymes, especially within the mitochondrial matrix. We studied the function of the mitochondrial matrix enzyme aldehyde dehydrogenase, an enzyme proven to depend on its free thiol groups for its function (Pietruszko, 1986). The isoform studied here is the one preferentially oxidizing aliphatic aldehydes. Both rat brain and rat liver mitochondria were used.

Mitochondria were incubated as described above, except that the incubation was carried out for 20 min., and the substrate was added at 1 mM. DA turnover by MAO has been implicated previously in the inhibition of aldehyde dehydrogenase in liver mitochondria. However, the effect was attributed to the formation of adducts of the enzyme with autoxidation products of DA, shown by the covalent binding of radiolabeled DA to the

protein (Turan et al, 1989a, Turan et al, 1989b) . To account for possible interference by the formation of quinoidal compounds from dopamine, we conducted some experiments with both DA and BA. BA cannot form quinones, due to its lack of hydroxyl-groups on its benzene group, so any inhibition resulting from BA being present in the incubation is highly likely to be due to MAO activity generating H_2O_2 with subsequent disturbances of the thiol-redox state in the mitochondria.

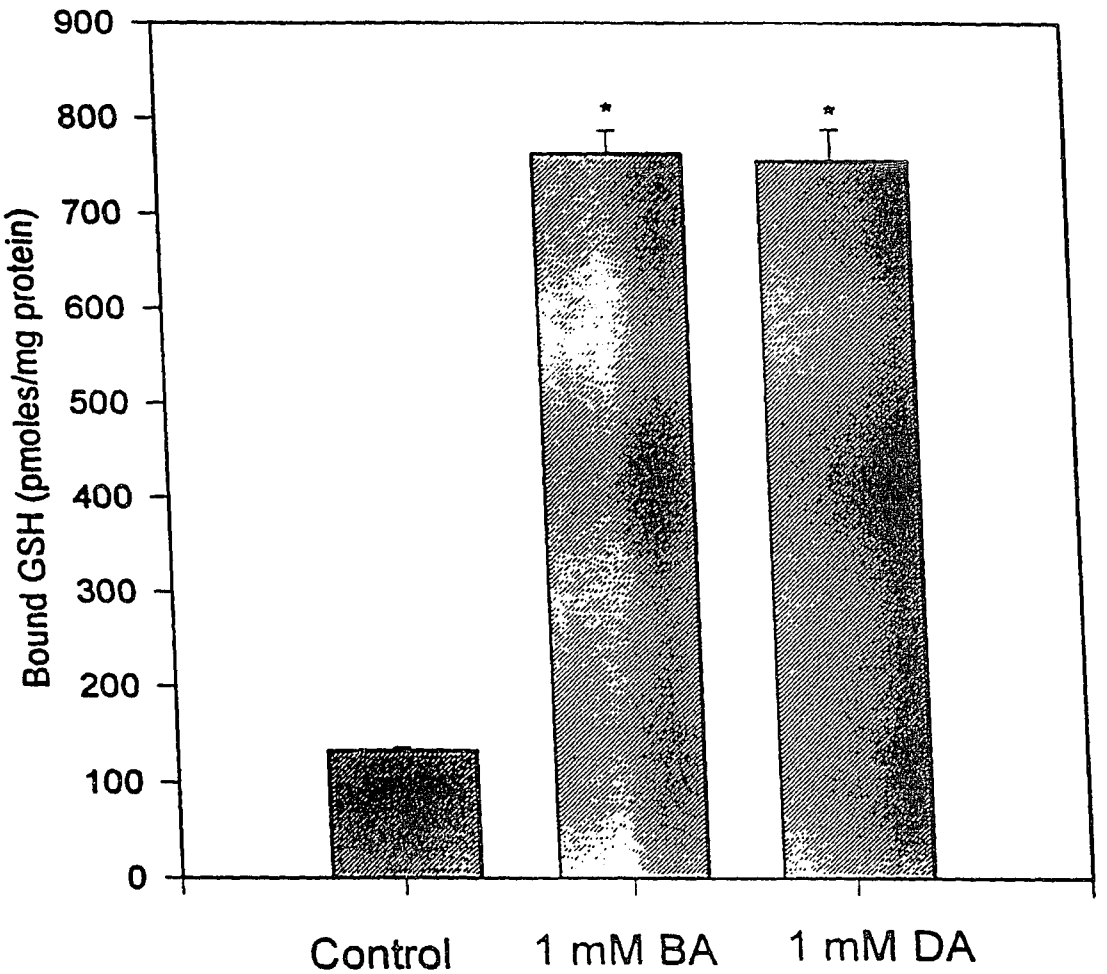
3.4.2: Experiments with BA and DA

Rat brain mitochondria were incubated for 20 min. at 37 °C with 1 mM DA or 1 mM BA, and the levels of PrSSG and aldehyde dehydrogenase activity (aliphatic) was determined as described in materials and methods. Data for PrSSG are again expressed as GSH releasable by reductive treatment with $NaBH_4$. Control mitochondria had 133.4 ± 3.3 pmoles releasable GSH/mg protein, whereas mitochondria challenged with DA had 754.6 ± 33.2 and BA 762.5 ± 24.1 pmoles releasable GSH/mg protein (**Figure 8**). Aldehyde dehydrogenase activity was reduced to 77.1 ± 3.4 (SEM) % compared to control after incubation with BA, and to 63 ± 4.4 (SEM) % after incubation with DA, respectively (**Figure 9**). Since challenging mitochondria with BA resulted in essentially equal inhibition of

the sulfhydryl-dependent enzyme aldehyde dehydrogenase, the formation of PrSSG is highly likely to have affected the function of this enzyme.

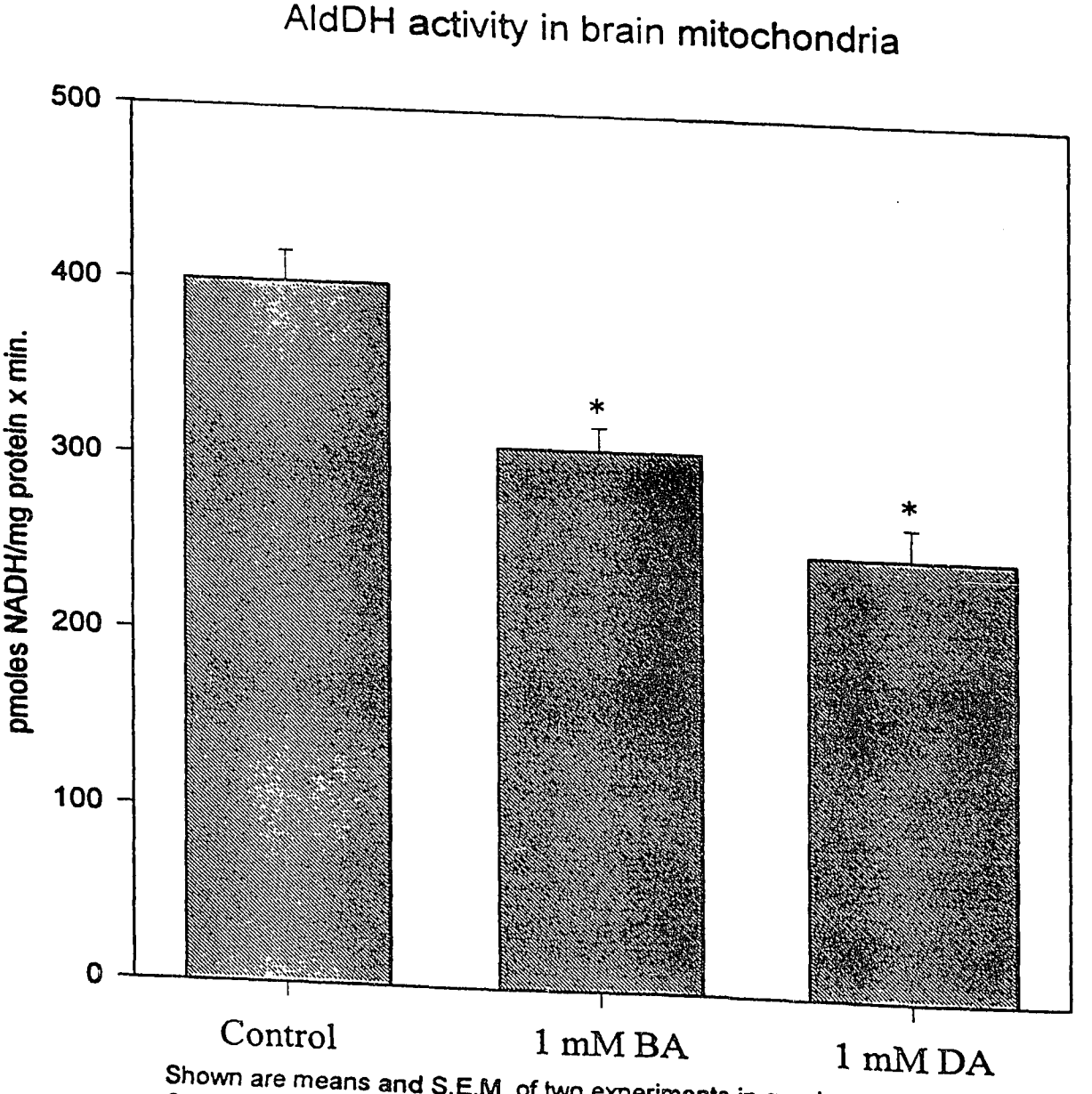
Figure 8

Protein mixed disulfides (PrSSG) in brain mitochondria



Shown are the means and S.E.M. of 2 experiments conducted in triplicate. Brain mitochondria were incubated for 20 min. at 30 °C while shaking. * significantly different from control ($p < 0.001$); not significantly different from each other. ANOVA followed by Tukey post-hoc.

Figure 9



Shown are means and S.E.M. of two experiments in quadruplicate. Conditions were as in figure 8. * $p < 0.01$ compared to control. ANOVA followed by Tukey post-hoc test.

3.4.3. Experiments with DA and MAO inhibitors

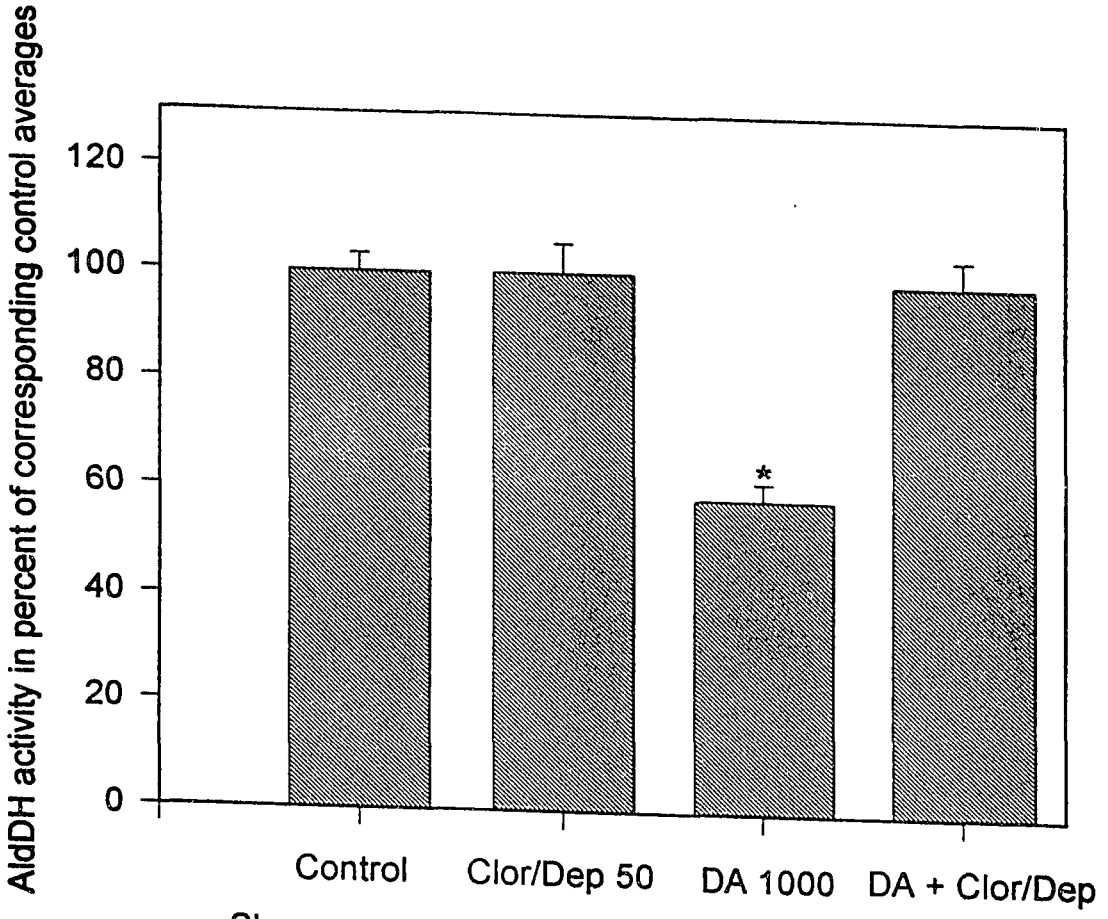
In additional experiments with rat brain mitochondria, the effect of inhibiting both MAO A and MAO B was investigated. This was to address the question whether MAO activity is indeed responsible for the decrease in aldehyde dehydrogenase activity. To this end, rat brain mitochondria were incubated for 20 min. with or without 1 mM DA in the presence or absence of 50 μM clorgyline (MAO A inhibitor) plus deprenyl (MAO B inhibitor) as described above. **Figure 10** shows the results of 4 experiments conducted in quadruplicate. Only mitochondria treated with DA alone showed a reduction of aldehyde dehydrogenase activity, which was reduced by 41.5%. All other conditions, including DA plus MAO inhibitors, showed no difference in activity from control.

In experiments conducted in parallel, both acid soluble glutathione (GSH and GSSG) and protein-bound glutathione (PrSSG) were determined in rat brain mitochondria. As predicted from previous experiments and in keeping with the hypothesis that PrSSG formation is the main mechanism of inactivation of aldehyde dehydrogenase in these experiments, only the samples treated with 1 mM DA alone showed a significant increase in the GSH bound to protein as PrSSG. **Figure 11** shows the effect of DA turnover by MAO on the fraction of protein-bound GSH (PrSSG) compared to total

glutathione (GSH + GSSG + PrSSG). Incubation with 1 mM DA for 20 min. led to a six-fold increase , to 24.1 ± 1.4 %, of the portion of GSH bound to protein. All other conditions, including DA + MAO inhibitors, remained essentially unchanged. Furthermore, as shown in **Table 2**, the amount of total glutathione was practically unchanged between control and DA-treated mitochondria, only the distribution between free and protein-bound glutathione was shifted towards the latter in the DA samples. This suggests that oxidation of the catechol function of DA with the subsequent formation of quinone compounds did not play a role in the inactivation of aldehyde dehydrogenase, since catechol-derived quinones bind to thiol groups rapid and indiscriminately (Tse et al., 1976), and should have led to an appreciable loss of GSH.

Figure 10

Aldehyde Dehydrogenase Activity in Brain Mitochondria

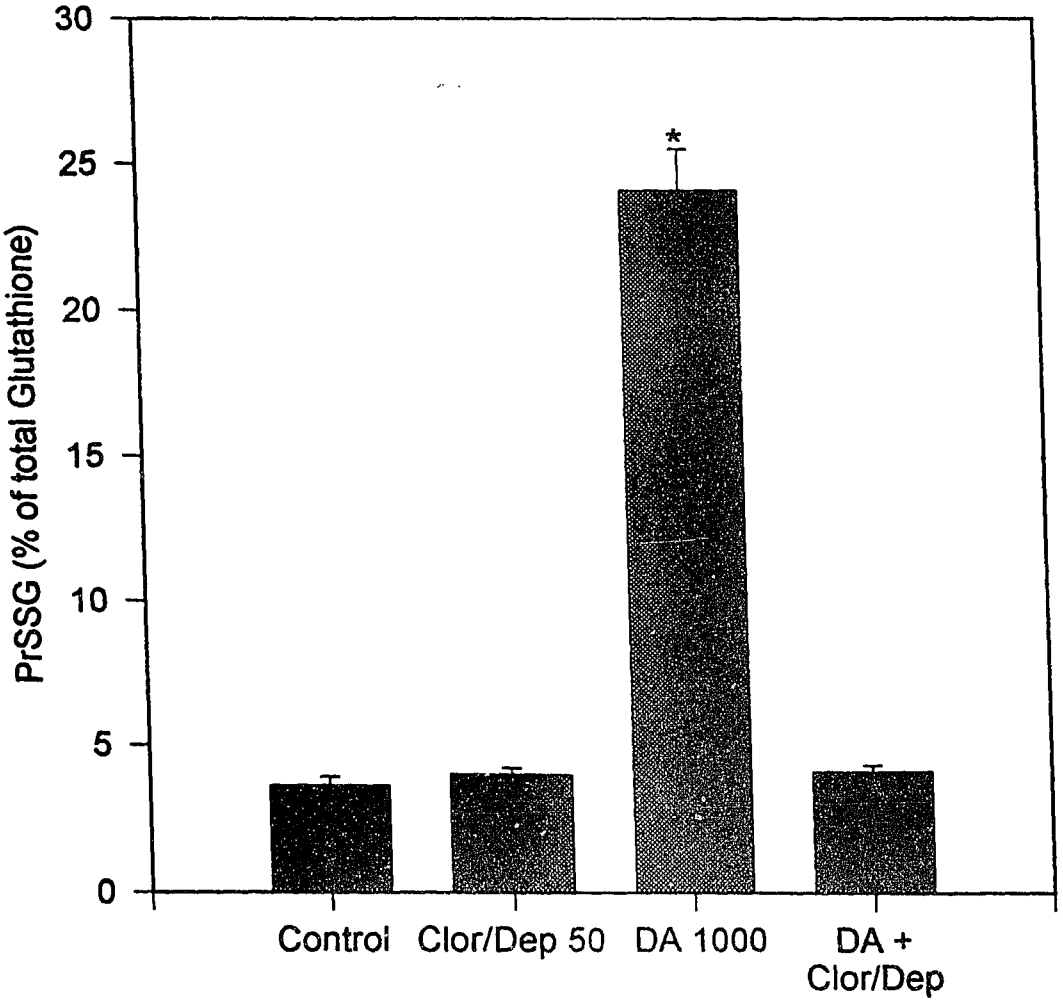


Shown are mean and SEM of 4 exp. in quadruplicates. rat brain mitochondria were incubated for 20' w. or w/o 1 mM DA and w. or w/o 50 uM Clor/Dep. Activity is expressed in % of corresponding control average.

*significantly different from all other conditions, $p < 0.01$ (ANOVA followed by Tukey-Kramer post-hoc)

Figure 11

Percentage of GSH bound in PrSSG after incubation of mitochondria with DA



Shown are mean +/- S.E.M. of 4 experiments conducted in quadruplicate. PrSSG is expressed in perecent of total glutathione (GSH + GSSG + PrSSG). * significantly different ($p < 0.005$) from all other conditions.

No significant differences between all other conditions (ANOVA followed by Tukey-Kramer post-hoc test).

TABLE 2

Soluble and protein-bound glutathione (PrSSG) in brain mitochondria after incubation with 1 mM DA

	GSH (pmoles GSH/ mg protein)	**PrSSG** (pmoles GSH/ mg protein)	**Total** (pmoles GSH/ mg protein)
Control	5103 ± 77	190 ± 14	5281 ± 74
DA 1 mM	4144 ± 56	1328 ± 92	5361 ± 125
[DA] in percent of Control	81%	698%	102%

Shown are mean ± S.E.M. of three experiments in quadruplicate. Rat brain mitochondria were incubated for 20 min with or without 1 mM DA while shaking, and then analyzed for acid-soluble glutathione (GSH + GSSG) and for PrSSG. [a] Significantly different from control, $p < 0.001$; [b] significantly different from control, $p < 0.0001$; no significant difference between control and DA total glutathione (Students unpaired two-tailed t-test).

3.5: AldDH activity in rat liver mitochondria

3.5.1: Experiments with BA and DA

In additional experiments, male rat liver mitochondria were used under identical circumstances, incubating with MAO substrates at 37 °C for 20 min., and then assayed for AldDH activity using a microplate reader. Incubation of rat liver mitochondria resulted in the reduction of aldehyde dehydrogenase activities to 86.5 ± 2.6 (SEM) % for DA and 79.3 ± 1.8 (SEM) % for BA of the corresponding control averages of each experiment (**Table 3**). This mirrors similar results by Turan et al. (1989a), who observed a reduction in aldehyde dehydrogenase activity to 80% of control upon incubation of male rat liver mitochondria with 1 mM DA for 90 min.

TABLE 3

Effect of MAO substrates on mitochondrial aldehyde dehydrogenase activity (% of control)

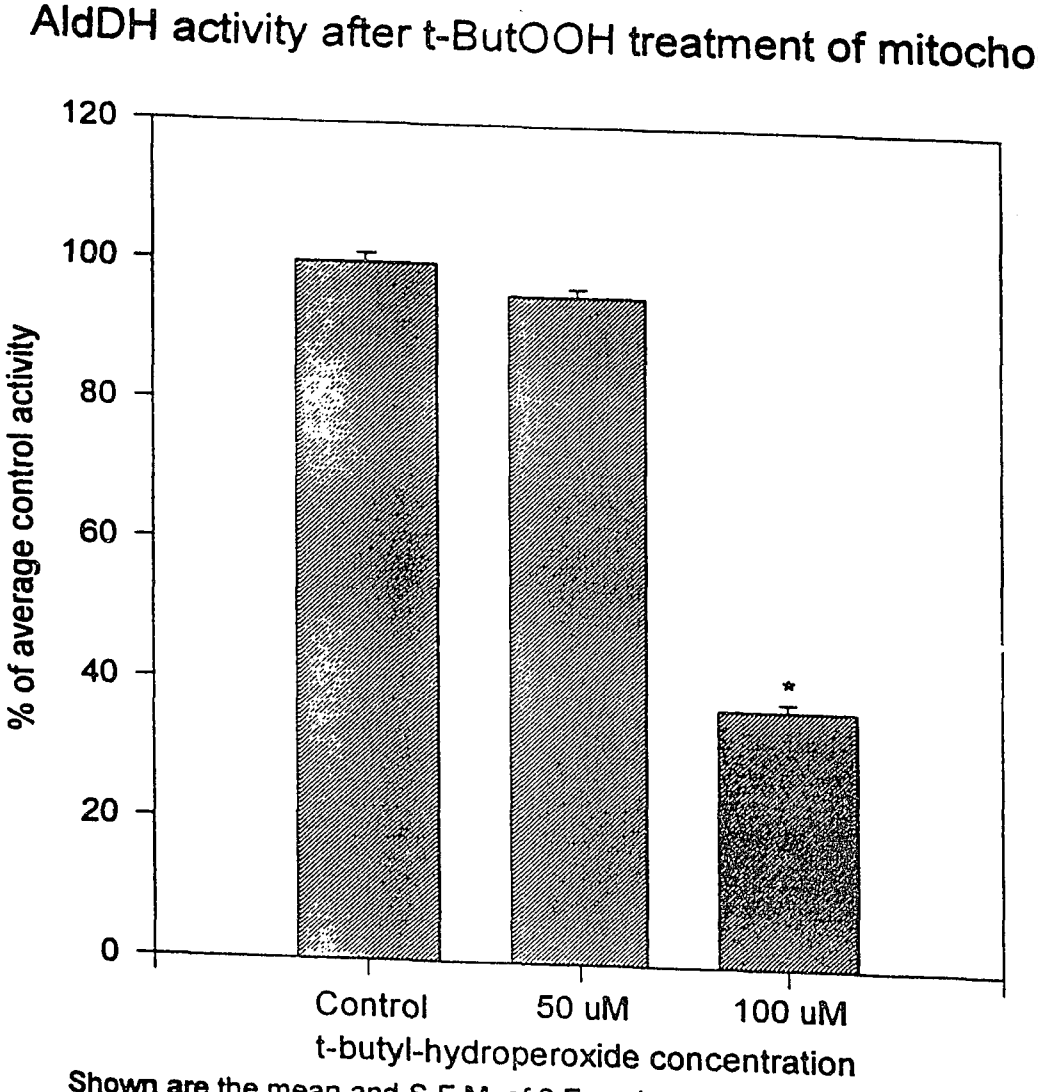
	AldDH activity (% of control)
Control	100 ± 2
Dopamine 1 mM	86.5 ± 2.6[a]
Benzylamine 1 mM	79.3 ± 1.8[b]

Rat liver mitochondria were incubated with benzylamine or dopamine for 20 min. and then analyzed for aldehyde dehydrogenase activity (aliphatic). Shown are the results of 5 (dopamine) and 7 (benzylamine) experiments in triplicate. [a] $p < 0.005$ compared to corresponding controls; [b] $p < 0.005$ compared to corresponding controls, $p < 0.01$ compared to corresponding DA experiments.

3.5.2: Experiments with t-butylhydroperoxide

In further experiments, male rat liver mitochondria were also challenged with t-butylhydroperoxide, an organic hydroperoxide, at concentrations of 50 and 100 μM (**Figure 12**). Incubation of liver mitochondria with 50 μM t-ButOOH resulted in aldehyde dehydrogenase activities of 95.6 ± 1.1 (SEM) % compared to control, while incubation with 100 μM t-ButOOH reduced AldDH activity to 36.9 ± 1.1 (SEM) % compared to control. Apparently, the liver mitochondria were capable of "buffering" a certain amount of oxidative stress; once this threshold was reached, a drastic effect on enzyme activity ensued.

FIGURE 12



Shown are the mean and S.E.M. of 6 Experiments in quadruplicate. Rat liver mitochondria were incubated for 20 min. at 30 °C while shaking. Aldehyde dehydrogenase activity is expressed as % of corresponding controls. 50 uM was not significantly different from control; * p < 0.001 compared to control and 50 uM; ANOVA followed by Tukey post-hoc.

3.6: DISCUSSION

Glutathione (GSH) is the main non-protein thiol in mitochondria (Jocelyn and Kamminga, 1974). GSH is taken up into mitochondria in an energy requiring manner (Kurosawa et al, 1990). Glutathione homeostasis is essential for mitochondrial function (Martensson et al., 1990). On the other hand, inhibition of oxidative phosphorylation has been shown to deplete cellular GSH (Mithöfer et al., 1992).

Glutathione peroxidase, for which GSH is the essential cofactor, is, according to the majority of investigators, the only H_2O_2 detoxifying enzyme in mitochondria (see review by Reed, 1990). In addition, GSSG is not exported from the mitochondria (Olafsdottir and Reed, 1988). Therefore, GSSG is a good indicator for oxidative stress elicited by H_2O_2 inside of the mitochondria.

Mitochondria are both sources and targets of oxidative stress. One prominent source of both $\cdot O_2^-$ and H_2O_2 is from electrons that by-pass the respiratory chain of mitochondria (Boveris and Chance, 1972). This happens especially when the respiratory chain is compromised (e.g., mitochondrial toxins, ischemic damage), although intact mitochondria can generate reactive oxygen species even when idling in state 4 respiration (Boveris and Chance, 1972). It has been estimated that 2-5% of mitochondrial O_2 consumption generates H_2O_2 (Chance et al., 1979).

Other enzymes localized to the mitochondrion are also capable of generating reactive oxygen species. One of these enzymes is monoamine oxidase (MAO). Although the production of H_2O_2 by MAO (eqn. 16) has long been recognized, its potential for the generation of an oxidant stress has only been realized over the past decade (e.g. Cohen, 1983).

$$\text{dopamine} + O_2 + H_2O \xrightarrow{\text{MAO}} \text{aldehyde} + H_2O_2 \quad \text{(eqn. 16)}$$

Studies by Maker et al. (1981) showed that the addition of dopamine to brain homogenates led to the increased formation of GSSG, a product of the detoxification of H_2O_2 by glutathione peroxidase (eqn. 18). A subsequent report by Spina and Cohen (1989), showed increased levels of GSSG upon release and metabolism of DA in synaptosomes by reserpine (Spina and Cohen, 1988). In experiments carried out in vivo with mice, GSSG increased upon stimulation of DA turnover with haloperidol (Spina and Cohen, 1989).

$$H_2O_2 + 2\ GSH \text{ -----> } 2\ H_2O + GSSG \qquad \text{(eqn. 18)}$$

Increased levels of GSSG are often accompanied by increased levels of protein-mixed disulfides (PrSSG), which are formed from cysteine residues in proteins and GSSG (Eqn 17). The equilibrium constant between glutathione disulfide (GSSG) and protein thiols is close to 1. (Creighton, 1983). In addition, reports on GSSG-independent formation of PrSSG exist (Chai et al., 1994); the latter authors suggest that thiyl radicals ($GS^{\cdot}$), generated in the reaction between GSH and other radicals, may be responsible.

GSSG + Pr-SH -----> PrSSG + GSH (eqn. 17)

Protein mixed disulfide formation has been observed upon treatment of liver mitochondria with t-butylhydroperoxide (Ravindranath and Reed, 1990). This is in keeping with prior reports on the retention of GSSG by mitochondria (Olafsdottir and Reed, 1988), which allows for with subsequent reaction to form PrSSG. The formation and decomposition of PrSSG may also be catalyzed enzymatically, at least in the cytosol (Mannervik and Axelsson, 1980).

The formation of PrSSG can have repercussions for cellular metabolism. The activity of SH-dependent enzymes can be altered by the formation of disulfides. Early work in this area has been summarized by Guzman Barron (1951). Modig (1968) found PrSSG formation as a cysteamine releasable GSH pool in Ehrlich ascites cells. Brigelius (1985) and Ziegler (1985) have reviewed the role of protein mixed-disulfide formation in altering the activity of sulfhydryl-dependent enzymes. Among the enzymes which are negatively affected in their activity by the formation of PrSSG are glycogen synthase I (Lau and Thomas, 1983), creatinine kinase (Collison and Thomas, 1987), phosphorylase phosphatase (Shimazu

et al., 1978), and phosphofructokinase (Gilbert, 1982). Gilbert has speculated on the potential role for formation of protein mixed disulfides as a "third messenger" system (Gilbert, 1982). Offermann et al. (1984) found that fructose-1,6,-bisphosphate aldolase is not only inhibited by PrSSG formation, but becomes also more susceptible to proteolysis, giving further weight to this hypothesis.

However, the formation of glutathione-protein mixed-disulfides does not always cause reduction of enzymatic activity. Glucose-6-phosphate dehydrogenase, which catalyzes the rate-limiting step in the pentose phosphate cycle, is activated by physiological concentrations of GSSG; this occurs even in the presence of otherwise feedback-inhibiting concentrations of NADPH (Eggleston and Krebs, 1974). Since NADPH is a necessary cofactor for the reduction of GSSG, this regulation provides for increased NADPH production in situations of an oxidative stress accompanied by increased levels of GSSG.

In the experiments described in this thesis, the question of the impact of H_2O_2 generated by MAO on the mitochondrion itself was addressed. Initial experiments with mouse liver mitochondria showed that levels of GSSG were doubled upon incubation with 100 μM BA (Table 1a).

Therefore, these experiments show that mitochondria can be at risk during increased oxidation or turnover of monoamines. This rise in GSSG with BA was sensitive to the inhibition of MAO. BA is a selective MAO-B substrate; in these experiments, the rise in GSSG was prevented by inhibition of MAO-B (with deprenyl), while inhibition of MAO-A (with clorgyline) was ineffective (Table 1b).

The same was true for experiments with rat brain mitochondria (Figures 2 and 3). Incubation of rat brain mitochondria with 100 μM DA for 10 min. resulted in an 11-fold increase in GSSG (Figure 2). In separate experiments, a 3.9-fold increase in protein-mixed disulfides (PrSSG) was observed (Figure 4). The fact that both GSSG and PrSSG increases were diminished or abolished when MAO was inhibited (Figures 3 and 5) underlines the predominant role of MAO in the generation of H_2O_2 in these experiments. The concentrations of DA used in the experiments described here are well within the estimated concentration ranges of DA in the cell bodies of DA neurons. Anden et al. (1966) estimated the DA concentration in the cell bodies to be between 400 and 1300 μM, while the total concentration of DA in the terminals, where DA is mainly present in storage vesicles, was estimated at 55 mM.

My findings on the rise in GSSG and PrSSG are in keeping with results reported by Sandri et al. (1990), who observed a disappearance of GSH in brain mitochondria following MAO catalyzed turnover of dopamine. Sandri et al. (1990) found a 30% decrease in GSH, which is in accord with the conversion of GSH to GSSG and PrSSG. The build-up of GSSG strongly suggests the involvement of GPx in at least part of the disappearance of GSH observed in the experiments by Sandri et al. (1990). The build-up of PrSSG led to another question. Previous experiments with rat liver mitochondria by Turan et al. (1989a, b) had shown that aldehyde dehydrogenase, a thiol-dependent enzyme (Vallari and Pietruszko, 1982), is inhibited by the addition of 1 mM DA. The authors noted, however, a dependency on the circumstances of the inactivation (Turan, 1989 a). While liver mitochondria prepared from male animals showed only a 20 % loss of aldehyde dehydrogenase activity after incubation with 1 mM DA for 1.5 h, mitochondria prepared from female rats in the metestrus part of the estrous cycle showed almost complete (97.5%) inactivation of the enzyme (Turan et al., 1989a). Furthermore, the authors reported that the inactivation of aldehyde dehydrogenase correlated strongly with the blackening of the medium, which is due to DA catechol oxidation resulting in the formation of melanin. In the experiments with mitochondria of female origin (metestrus part of the estrous cycle), the blackening of the medium and the inactivation

of aldehyde dehydrogenase was accompanied by binding of DA to the enzyme. Turan et al. (1989a) also demonstrated that a heat-labile component, possibly an enzyme, was necessary for the inactivation of aldehyde dehydrogenase in mitochondria of female origin. Some experiments on aldehyde dehydrogenase activity conducted for this thesis addressed the question whether the conditions which were used to study GSSG/PrSSG would also lead to the inactivation of the enzyme over a different mechanism, namely S-thiolation (PrSSG formation, eqn. 17).

GSSG + Pr-SH -----> Pr-SSG + GSH (eqn. 17)

Several points indicate this mechanism (PrSSG formation) in the partial inactivation of aldehyde dehydrogenase, rather than binding of quinones (Turan et al., 1989a) resulting from DA oxidation .

- The changes in aldehyde dehydrogenase activity were accompanied by the build-up of protein-mixed disulfides.
- No blackening of the medium was observed during the 20 min. period of the experiment, indicating the absence of the DA catechol oxidation products which were observed by Turan et al. (1989a).
- Inactivation of aldehyde dehydrogenase was also observed with benzylamine as a substrate, and the inactivation was of identical

magnitude as observed for DA. BA cannot form quinones, since it lacks the OH groups on the benzene ring. Therefore, the production of H_2O_2 by MAO with the resulting build-up of GSSG and PrSSG is the most likely mechanism of aldehyde dehydrogenase inhibition with both BA and DA as MAO substrates.

- Only mitochondria of male origin were used, both for liver and brain mitochondria. Turan et al. reported the essential absence of visible autoxidation and only a slight enzyme inactivation when incubating male mitochondria, which my experiments confirmed.
- The oxidation of the catechol function of DA with ensuing quinone formation should have also, at least partially, depleted the GSH in the mitochondria, since quinones react rather rapidly with thiol compounds, resulting in the formation of thiol-ethers (e.g. Tse et al., 1976). However, when total GSH (GSH + GSSG + PrSSG) was calculated, no difference was observed between control mitochondria and mitochondria incubated with 1 mM DA. This strongly suggests that the oxidation of the catechol moeity of DA did not play a significant role in the inactivation of aldehyde dehydrogenase in my experiments .
- A major difference between the experiments conducted by Turan et al. (1989a, b) and my experiments is in the respective incubation buffers used. Turan et al. (1989a, b) used an isotonic potassium phosphate

buffer, while I employed a mannitol/sucrose medium buffered with MOPS. Phosphate buffers are usually contaminated with iron. For example, the iron contamination of KH_2PO_4 (Fisher, ACS grade) is 0.0005%, which translates to approximately 1.5 μM in a 100 mM buffer. This iron may have played a key part in catalyzing the oxidation of the catechol moiety of dopamine and generating ·OH in the presence of MAO-generated H_2O_2 in the experiments by Turan et al. (1989a).

Therefore, both the formation of protein-mixed disulfides and the formation of adducts with quinone compounds derived from DA are possible pathways for the reduction of mitochondrial enzyme activities in vivo.

Finally, the finding that the reduction in aldehyde dehydrogenase activity was more pronounced in brain mitochondria compared to liver mitochondria may imply an increased suszeptibility of brain mitochondria to oxidative stress by MAO activity: Incubation with 1 mM DA resulted in twice the loss of aldehyde dehydrogenase activity in brain mitochondria (ca. 40%) then in liver mitochondria (ca. 20%) .

These new observations show that the activity of MAO, which generates H_2O_2, can affect the mitochondrion via the formation of GSSG and PrSSG, and have functional consequences for mitochondrial enzymes.

4: EXPERIMENTS WITH HUMAN FIBROBLASTS

4.1: Introduction

The effect of L-dopa on cellular respiration in intact human fibroblasts

As mentioned previously, Dopa is used in the treatment of PD, serving as a precursor for dopamine. Treatment of patients with L-dopa, which is usually given in conjunction with carbidopa (a peripheral aromatic amino acid decarboxylase inhibitor), provides for increased DA levels in brain by bypassing the limiting step in DA biosynthesis, the hydroxylation of tyrosine by tyrosine hydroxylase. However, the catechol function of dopa can undergo auto-oxidation, and producing $\cdot O_2^-$ and H_2O_2 in this process, as has been shown for epinephrine (Misra and Fridovich, 1972). Quinones, formed via auto-oxidation, have been shown to be effective inhibitors of mitochondrial respiration in vitro (Bironaite et al., 1992), and compounds that can form the catechol-oxidation product melanin have been shown to be cytotoxic (Hochstein and Cohen, 1963). It is of interest that L-dopa, because of its toxicity for melanoma cells in culture, was considered as a treatment of for this disorder (Wick, 1980a; 1980b).

The experiments presented here assayed for respiratory activity in human fibroblasts after exposure to L-dopa. Since L-dopa can autoxidize

and form semi-quinones and quinones, a negative effect on cellular respiration was expected.

Initial studies indicated that oxidation of pyruvate, but not succinate, was impaired when human fibroblasts were challenged with 100 μM L-dopa or higher for 48 hours (Werner et al., 1994), which confirmed the initial assumption.

4.2: List of experiments

a.) Comparison of L-dopa with a non-auto-oxidizable dopa analog, 3-O-methyldopa. 3-O-Methyldopa is a methoxyether derivative of dopa, which prevents its autoxidation. These experiments investigated the importance of auto-oxidation of dopa for its effect on cellular respiration.

b.) Comparison of dopamine and L-dopa. These experiments were to address the role of the different side chains of these catechols. L-dopa is an amino acid, being derived from tyrosine by hydroxylation of the phenolic ring in the 2 position, whereas dopamine, its main metabolite *in vivo*, is a monoamine.

c.) Measurement of the effect of inhibition of aromatic amino acid decarboxylase (AADC) on the L-dopa provoked inhibition of respiratory activity. The inhibition of this enzyme prevents the conversion of L-dopa to DA. These experiments addressed the question whether the intracellular conversion of L-dopa to DA is required for the inhibitory effect of L-dopa on cellular respiration. These experiments were conducted using carbidopa, a suicide substrate for AADC, used clinically to prevent the conversion of L-dopa to DA in the periphery.

One of the possible mechanisms of inducing oxidative stress on mitochondria is the deamination of monoamines such as DA (see experiments with mitochondria, above). Therefore, the MAO A and MAO B inhibitor pargyline was used in a subset of experiments to investigate the possible role of this mechanism. The inhibition of MAO investigated whether MAO catalyzed deamination of DA produced from L-dopa was involved in the reduction in cellular respiration. These experiments complemented the experiments with carbidopa.

Since L-dopa did visibly autoxidize in the medium, the inhibitory effect on respiration observed could also be due to the extracellular generation of reactive oxygen species, namely the formation of $\cdot O_2^-$ and H_2O_2. Catalase

and superoxide dismutase was added in conjunction with L-dopa to reduce the levels of $\cdot O_2^-$ and H_2O_2 produced extracellularly.

d.) Experiments were conducted with the antioxidant ascorbic acid (AA) added in the presence or absence of L-dopa. The rationale was that the preventing the autoxidation of L-dopa could prevent its detrimental effect on cellular respiration.

4.3: Methods

4.3.1: Materials

MEM (Minimum Essential Medium) was from GIBCO. Fetal bovine serum was from HyClone. 3-OMethyldopa, catalase (cell culture grade), L-dopa, pargyline, superoxide dismutase (CuZn form; cell culture grade) and Tris (Tris(hydroxymethyl)aminomethane) were from Sigma. Carbidopa was from Merck. Culture flasks were either from Corning or Falcon. Plastic pipettes were from Falcon. Trypsin inhibitor was either from GIBCO or Sigma. [2- ^{14}C]pyruvate and [1,4-^{14}C]succinate were from New England Nuclear.

4.3.2: Fibroblast cell lines

Two human embryonic fibroblast cell lines (called 93-8 and 93-17) were obtained from the skin of two human fetuses, and prepared according to standard procedures using skin explants. The cultures were maintained in Minimum Essential Medium containing 20% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. A third fibroblast line from a twelve week old fetus (93-15) was purchased from the Aging Cell Repository of the National Institute on Aging.

4.3.3: Treatments of fibroblasts

Confluent fibroblast cultures from passages 2-14 were treated with L-dopa, in the presence or absence of ascorbic acid, for 48 h with a medium change after 24 h. In some experiments the effect of the non-specific monoamine oxidase inhibitor pargyline, or catalase and superoxide dismutase (SOD) were examined by adding each of the compounds simultaneously with L-dopa. The cultures were analyzed either at the end of the L-dopa treatment or, in some experiments, after varying periods of washout. For the washout, the L-dopa containing medium was removed and the cultures were rinsed once with warm balanced salt solution before the addition of normal feeding medium.

3-O-Methyldopa, a non-autoxidizable analogue of L-dopa, or DA were also added in various concentrations and cellular respiration measured as described. In addition, carbidopa, an inhibitor of aromatic amino acid decarboxylase (AADC), was used in conjunction with L-dopa, to investigate whether the formation of DA from L-dopa was required for L-dopa's inhibitory effect on pyruvate oxidation.

4.3.4: Assay

Before harvesting, cells were washed twice with balanced salt solution and phosphate buffered saline and were then suspended with trypsin (0.04% for 7 min), which was subsequently inactivated with a 2-fold excess of trypsin inhibitor (GIBCO or Sigma). The cell suspension was further washed with Tris-Krebs buffer (0.15 M; pH 7.4) without glucose. The cells were pelleted by centrifugation (350 x g for 10 min) and resuspended in Krebs buffer. The rate of oxidation of [2-^{14}C]pyruvate (New England Nuclear; 13.5 mCi/mmol) and [1,4-^{14}C]succinate (New England Nuclear; 59 mCi/mmol) was assayed according to the method described by Slipetz et al. (1991) with modifications. The use of pyruvate labeled on the 2-position insured that the $^{14}CO_2$ released originates from decarboxylase activity in the Krebs cycle, rather than non-specific decarboxylase activity, because more than one full passage through the cycle was required before the $^{14}CO_2$ can be released. Cells were incubated for 2 h or 90 min. at 37 °C while shaking (100/min) in sealed tubes containing 1 mL or 0.6 mL Tris-Krebs buffer (pH 7.4) with 0.25 mM or 0.5 mM pyruvate (0.25 μCi) and 0.1 mM succinate (0.5 μCi). Throughout the incubation and 60-90 min. following the termination of the reaction by the injection of 100 μL 25% (v/v) H_2SO_4, the CO_2 was collected in plastic wells suspended from the rubber stoppers and containing a filter paper wet with 1 M KOH to collect released CO_2. The plastic wells

were then added to vials containing 10 mL Ecoscint (National Diagnostics) and 2 mL water and assayed in a Beckman scintillation spectrometer.

For cell homogenate experiments, cell pellets from fibroblast cultures were obtained by scraping and resuspended in 3 mL of ice-cold mannitol/sucrose/MOPS(4-morpholinepropanesulfonicacid)/EGTA (ethylenebis (oxyethylenenitrilo)tetraacetic acid) buffer pH 7.4 (225, 75, 5 and 1 mM, respectively) and homogenized at 0-4 °C with 10-12 strokes in a Potter-Elvejhem homogenizer at 2000 rpm. The volume was adjusted as needed with buffer and aliquots were incubated in a final volume of 500 µl with either [2- ^{14}C]pyruvate or [1,4-^{14}C]succinate, adjusted with cold substrate to a final concentration of 1 mM. Additionally, homogenates were incubated with or without FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone) as an uncoupler of mitochondrial respiratory control. Quadruplicate samples were incubated at 37 °C while shaking and the reaction was stopped after 30 or 45 min. by injecting 100 µL 25% H_2SO_4. The oxidation of the substrates was determined as described above. Proteins were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

4.3.5: Statistics

Statistical analysis was carried out using single or multiple analysis of variance (ANOVA or MANOVA), followed by the Tukey-Kramer post-hoc test.

5: RESULTS

5.1: Effect of L-dopa on cellular respiration

In initial experiments, a deleterious effect of L-dopa treatment on pyruvate but not succinate oxidation was observed using a human embryonic fibroblast cell line established in the laboratory (termed 93-8). Incubation of intact fibroblasts with 200 μM L-dopa for 48 h caused a significant (46%; $P<0.005$) reduction in pyruvate oxidation (**Table 4**). In some experiments 0.1 μM rotenone, an inhibitor of complex I activity, was added during the assay and resulted in greater than 95% inhibition of pyruvate oxidation (not shown). However, L-dopa had no effect on the oxidation of succinate in intact cells. To investigate whether L-dopa interference with cellular respiration can be remedied by antioxidant treatment, ascorbic acid (200 μM) was also added to the cell cultures. Treatment for 48 hours prevented the reduction of pyruvate oxidation by L-dopa. Ascorbic acid by itself also caused a significant increase in pyruvate oxidation (**Table 4**). In contrast to the observations with pyruvate oxidation, ascorbic acid did not increase the oxidation of succinate.

TABLE 4

Effect of pre-treatment with L-dopa with or without ascorbic acid on respiratory activity in human fibroblasts

	$^{14}CO_2$ formed (nmol/mg protein hour)			
SUBSTRATE	Control	L-dopa	Ascorbate	Asc.+L-dopa
^{14}C-pyruvate	14.22 ± 0.64	7.73 ± 0.40[a]	28.55 ± 1.48[b]	25.14 ± 0.78[b]
	(100 %)	54 %	201 %	177 %
^{14}C-succinate	0.26 ± 0.02	0.254 ± 0.009	0.207 ± 0.004	0.192 ± 0.005
	100 %	98 %	80 %	74 %

Human embryonic fibroblasts (93-8) were incubated with or without 200 μM L-dopa in the presence or absence of 200 μM ascorbate. values are means ± S.E.M. from 2 experiments in triplicate. [a] $p < 0.005$ compared to control; [b] $p < 0.001$ control (ANOVA followed by Tukey post-hoc).

5.1.1: Effect of FCCP

Addition of 1 μM FCCP (carbonylcyanide p- trifluoromethoxy-phenylhydrazone) to uncouple oxidative phosphorylation resulted in the expected increase in pyruvate oxidation (CO_2 formed in nmol/mg protein/hour: Control: 14.17 ± 0.46; FCCP: 27.46 ± 0.63). With uncoupled mitochondria, the reduction in pyruvate oxidation by L-dopa was 47 ± 2 % ($p< 0.001$, n=6); in addition, ascorbate increased the rate of pyruvate oxidation (162 ± 16 % of FCCP control; $p< 0.001$, n=3) and protected against the effect of L-dopa (100 ± 0.1% of ascorbate alone).

5.1.2: Experiments with cell homogenates

To ensure that the lack of an effect of L-dopa on succinate oxidation was **not** due to limited permeability of the cell membrane to succinate during the assay, succinate and pyruvate oxidation was determined in fibroblast homogenates (see methods). As with the intact cells, L-dopa caused significant inhibition of pyruvate but not succinate oxidation in cell homogenates (**Table 5**). The lower rate of pyruvate oxidation in cell homogenates compared to intact cells may be due to an increased intracellular concentration of pyruvate within the intact cells, due to the presence of monocarboxylic acid transporters in the cell membrane (Poole and Halestrap, 1993).

TABLE 5

Effect of pre-treatment with L-dopa on respiratory activity in homogenates of human fibroblasts

	$^{14}CO_2$ formed (nmol/mg protein hour)	
	^{14}C-pyruvate	**^{14}C-succinate**
Control	1.90 ± 0.07 (100%)	3.15 ± 0.15 (100%)
L-dopa	0.56 ± 0.12[a] (30%)	3.52 ± 0.64 (112%)

Human embryonic fibroblasts were pretreated with 200 μM L-dopa for 48 h. After harvesting, the cells were homogenized and the homogenates incubated with ^{14}C-pyruvate or ^{14}C-succinate for 30 min.

[a] $p < 0.001$ compared to control, two-tailed t-test.

5.1.3: Experiments with homogenates: FCCP

In separate experiments, both succinate and pyruvate oxidation were stimulated by 0.1 μM FCCP in fibroblast homogenates (in nmol/mg protein/hour; for succinate from 3.49 ± 0.09 to 17.28 ±0.66 and for pyruvate from 1.66 ± 0.04 to 6.18 ± 0.10). Furthermore, 10 nM rotenone inhibited pyruvate oxidation by more than 98%, indicating that the measurements in the homogenates represented indeed mitochondrial respiratory activity.

5.1.4: Comparison of different cell lines

In subsequent experiments, two additional human embryonic fibroblast cell lines were subjected to L-dopa treatment alongside the 93-8 fibroblasts. This was done to ensure that the sensitivity to L-dopa of the cell line raised in the laboratory was representative of the general response of this cell type to L-dopa exposure. The two additional cell lines, termed 93-15 and 93-17, were obtained from the NIA aging cell repository (93-15) or established in the laboratory as described above (93-17). Following incubation with 200 μM L-dopa for 48 h, all three fibroblast cell lines showed significant inhibition of pyruvate oxidation compared to their corresponding control (**Table 6**).

This observation shows clearly, that the initial observations with the fibroblast cell line 93-8 was indeed representative of the effect of L-dopa on cellular respiration in this experimental system.

TABLE 6

The effect of treatment with L-dopa on the oxidation of [2-^{14}C] pyruvate in 3 different human embryonic fibroblast cell lines.

CELL LINE	$^{14}CO_2$ formed (nmol/mg/protein/hour) CONTROL	L-DOPA
93-8	14.8 ± 0.8 (100%)	8.2 ± 0.2[a] (56%)
93-15	10.5 ± 0.3 (100%)	6.8 ± 0.5[a] (65%)
93-17	22.6 ± 0.5 (100%)	12.7 ± 0.6[a] (56%)

The fibroblasts were treated with 200 μM L-dopa for 48 hours with a change of medium after 24 hours. Cell lines 93-8 and 93-17 were established in the laboratory and were never frozen. They were on passages 10-14 and 2-4 respectively at the time of assay. Cell line 93-15, obtained from NIA Cell Repository, was on passage 10-12 and had been frozen for more than 10 years. [a]Significantly different from corresponding control $P<0.001$; two-tailed t-test.

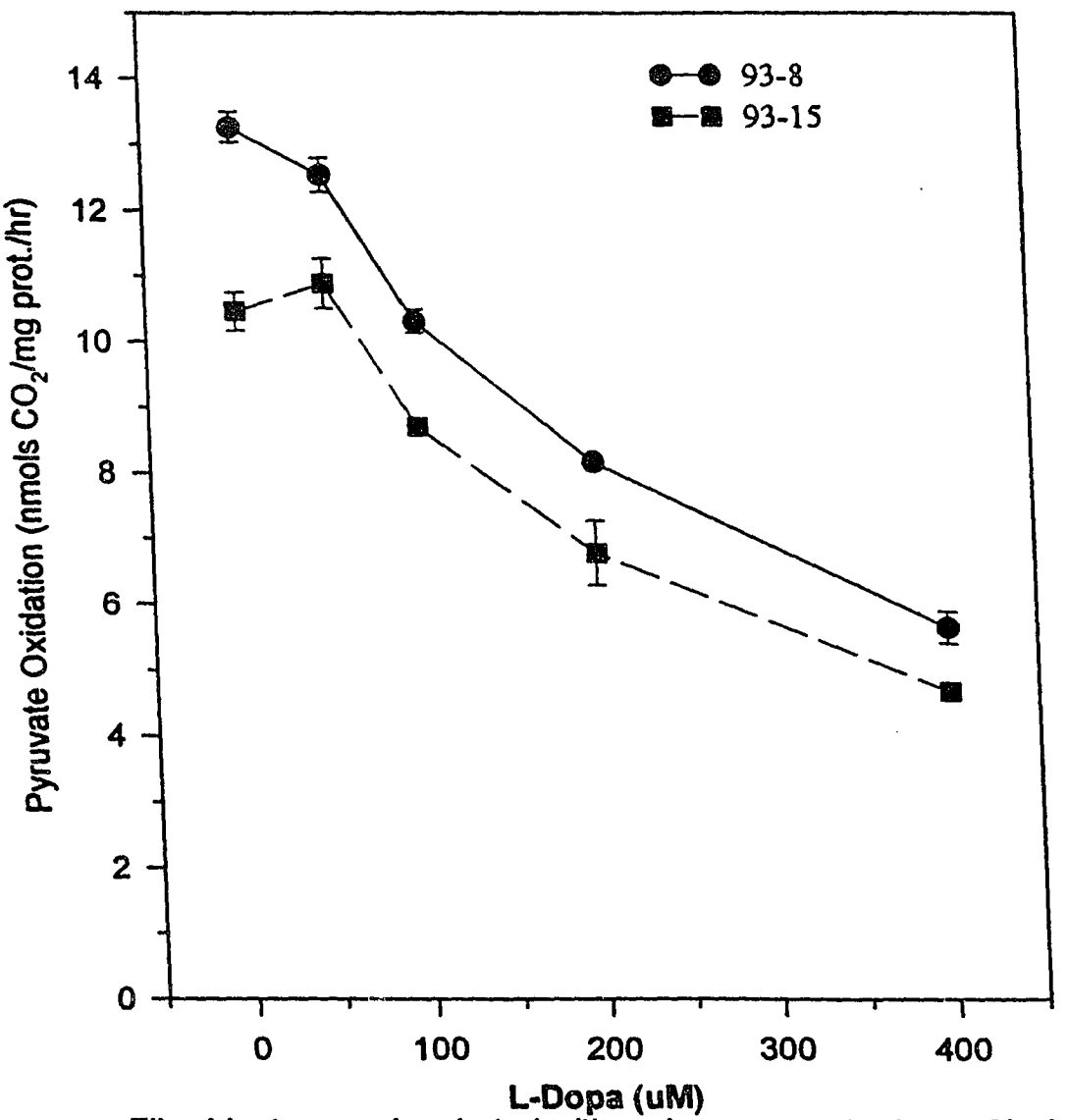
5.1.5: L-dopa dose-response

The concentration dependence of the effect of L-dopa on cellular respiration was also studied in two fibroblast cell lines. At concentrations between 100 and 400 μM, L-dopa treatment caused a dose-dependent reduction in pyruvate oxidation (**Figure 13**). The effect was similar in both cell lines. At these concentrations, the autoxidation of L-dopa was visible from the darkening of the feeding medium.

Concentrations of 50 μM L-dopa or lower failed to show an effect on pyruvate oxidation. To determine whether low concentrations of L-dopa could be effective if applied over a longer period of time, fibroblasts (93-8) were incubated for one week (7 day) with lower concentrations of L-dopa, with daily changes of the medium. Even with prolonged treatment, L-dopa, given at 10, 25 and 50 μM, did not reduce cellular respiration measured as pyruvate oxidation. The respiration (as percent of control ± SEM) found was 107 ± 1% (10 uM) 105 ± 3 % (25 μM) and 118 ± 3 % (50 μM) of control, respectively.

FIGURE 13

Effect of L-Dopa Concentration on Pyruvate Oxidation in 2 fibroblast cell lines



Fibroblasts were incubated with various concentrations of L-dopa for 48 h. and assayed (pyruvate oxidation). Shown are means and S.E.M. $p < 0.005$ compared to control for 100, 200 and 400 uM

5.1.6: Washout experiments

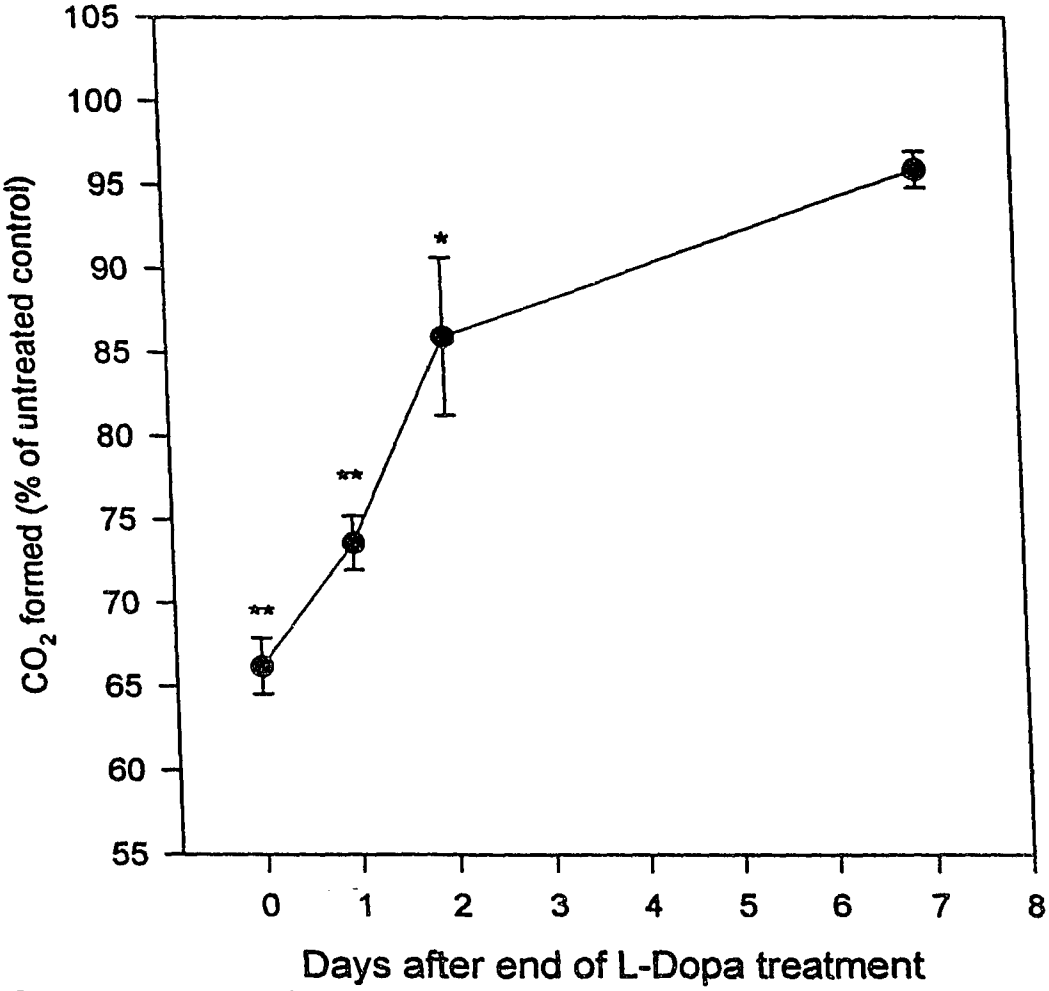
To investigate whether the effect of L-dopa on cellular respiration is reversible, the rate of pyruvate oxidation in fibroblasts was assayed immediately and 1, 2 or 7 days after 48 hour treatment with 200 μM L-dopa (**Figure 14**). After removal of L-dopa, pyruvate oxidation gradually returned to control values over the course of 7 days. However, at 24 and 48 hours following L-dopa removal the decarboxylation of pyruvate remained significantly lower, at 73.6% and 86.0% of control, respectively.

The next series of experiments addressed a number of questions that arose from the results listed above. Oxidative processes were seemingly involved in the inhibition of pyruvate oxidation by L-dopa, since the presence of the antioxidant ascorbic acid was able to fully prevent this effect. As mentioned in the introduction, several possible pathways exist to inflict an oxidative stress on cells using L-dopa. It could autoxidize, and thus generate reactive oxygen species such as $\cdot O_2^-$ and H_2O_2.

On the other hand, it could be metabolized to DA, and then exert its damaging action via the MAO dependent oxidative deamination of DA, its main metabolite. Therefore, experiments were conducted that addressed these two possibilities.

FIGURE 14

Recovery of respiration in human fibroblasts (93-8) following 48 h exposure to 200 uM L-Dopa



Shown are mean +/- S.E.M. of 5 experiments conducted in quadruplicate. Fibroblasts (93-8) were incubated for 48 h with or w/o 200 uM L-dopa, rinsed and refed with medium w/o L-dopa and assayed for pyruvate oxidation at the time points indicated. * $p < 0.02$ and ** $p < 0.001$ compared to corresponding control; two-tailed t-test.

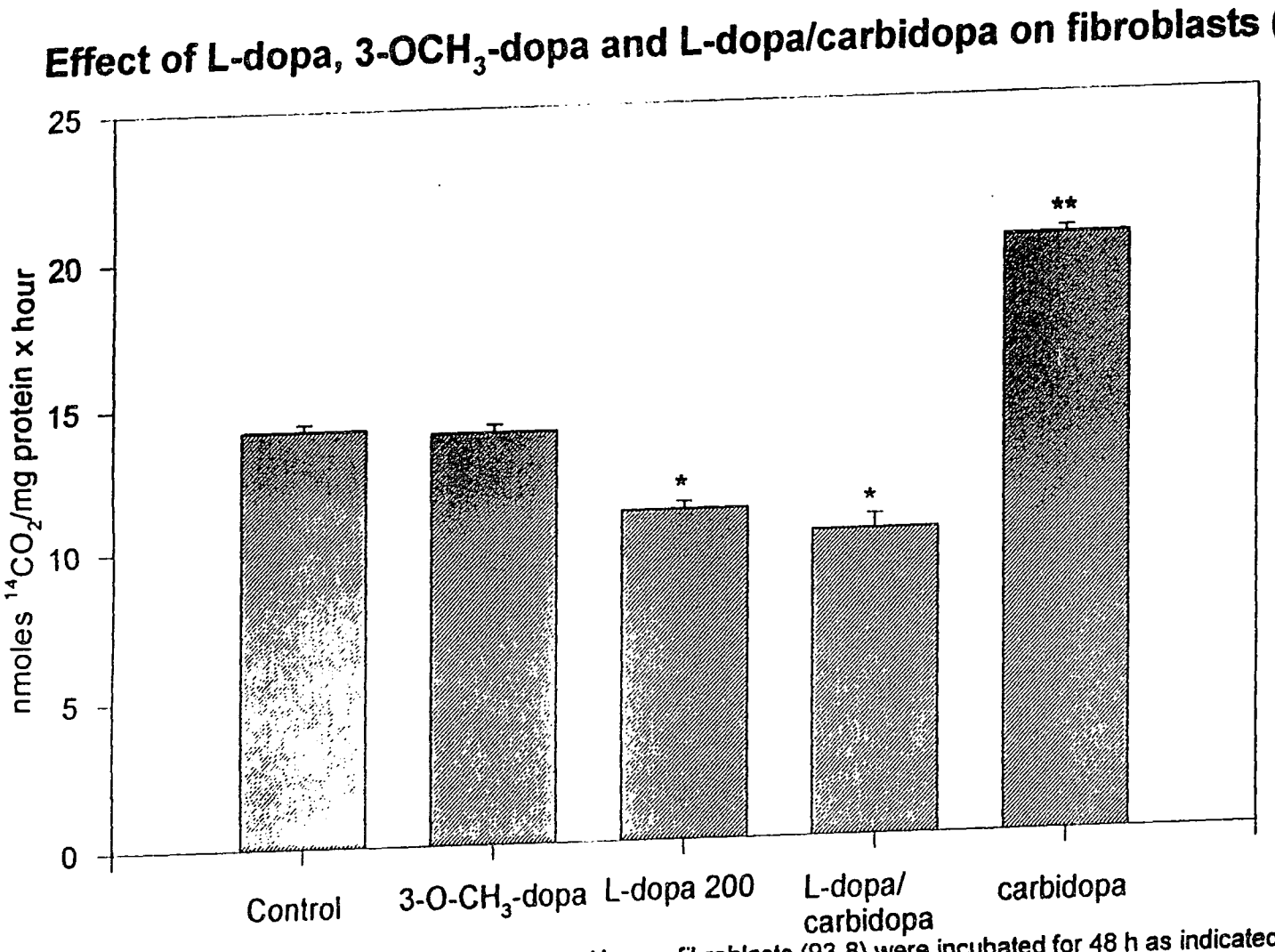
5.1.7: Experiments with 3-O-methyl-dopa and carbidopa

Fibroblasts (93-8) were incubated for 48 h in the presence or absence of 200 μM 3-O-methyl-dopa. This compound, a non-autoxidizable derivative of dopa, addresses the question whether the autoxidation of the catechol moiety is necessary for the effect on cellular respiration. In addition, another group of cells was treated again with 200 μM L-dopa, but now in the presence or absence of 50 μM of the aromatic amino acid decarboxylase (AADC) inhibitor carbidopa. Carbidopa, a suicide substrate for AADC, is used clinically to prevent peripheral conversion of L-dopa to DA. In these experiments, it was added to suppress the conversion of L-dopa to DA, thus ultimately preventing H_2O_2 production by MAO, since no substrate for it would be present.

5.1.7a: 3-O-Methyldopa

As in the initial experiments, L-dopa at 200 μM caused a significant decrease in pyruvate oxidation, while 3-Omethyldopa was unchanged from control, with 98.7 ± 1.7% of mean control activity (**Figure 15**). L-dopa was significantly different from both control and 3-Omethyl-dopa ($p < 0.001$). The lack of effect of 3-Omethyl-dopa strongly suggests that the presence of an unprotected, autoxidizable catechol function is required for the inhibitory action of L-dopa. However, the conversion of L-dopa to DA with subsequent

FIGURE 15



Shown are two experiments in quadruplicate. Human fibroblasts (93-8) were incubated for 48 h as indicated. * significantly differerent from control and 3-OCH_3-dopa ($p < 0.01$);** significantly different from all other conditions, $p < 0.001$. 3-OCH_3-dopa was not significantly different from control. L-Dopa/carbidopa was not significantly different from L-dopa alone. MANOVA followed by Tukey post-hoc.

generation of H_2O_2 by MAO was still a possible mechanism for the inhibition of cellular respiration.

5.1.7b: Pargyline

In another experiment, a subset of samples was treated with 10 μM pargyline, which inhibits both MAO-A and MAO-B. Treatment with pargyline did not modify the inhibitory effect of L-dopa on pyruvate oxidation (expressed as % of control: L-dopa 45 ± 1; L-dopa + pargyline 42 ± 1). Pargyline alone had no effect on pyruvate oxidation. These data indicated that DA turnover by MAO may not be involved in the inhibitory action of L-dopa on pyruvate oxidation, and the experiments with carbidopa were to further probe this assumption. Enforcing the data obtained with pargyline are the results of experiments where carbidopa was given together with L-dopa.

5.1.7c: Effect of carbidopa

Addition of carbidopa to the L-dopa treatment did not change the inhibition observed with L-dopa alone. Pyruvate oxidation was reduced as with L-dopa alone (**Figure 15**). This indicates that conversion of L-dopa to DA is not required to inhibit cellular respiration. The argument could be raised that carbidopa by itself may inhibit mitochondrial activity, thus

obscuring any beneficial effects of inhibiting DA generation by the AADC. However, 50 μM carbidopa by itself actually stimulated cellular respiration significantly (157 ± 1.7 % of control, $p < 0.001$), creating a puzzling observation, since similar concentrations of L-dopa had failed to have any significant effect on pyruvate oxidation.

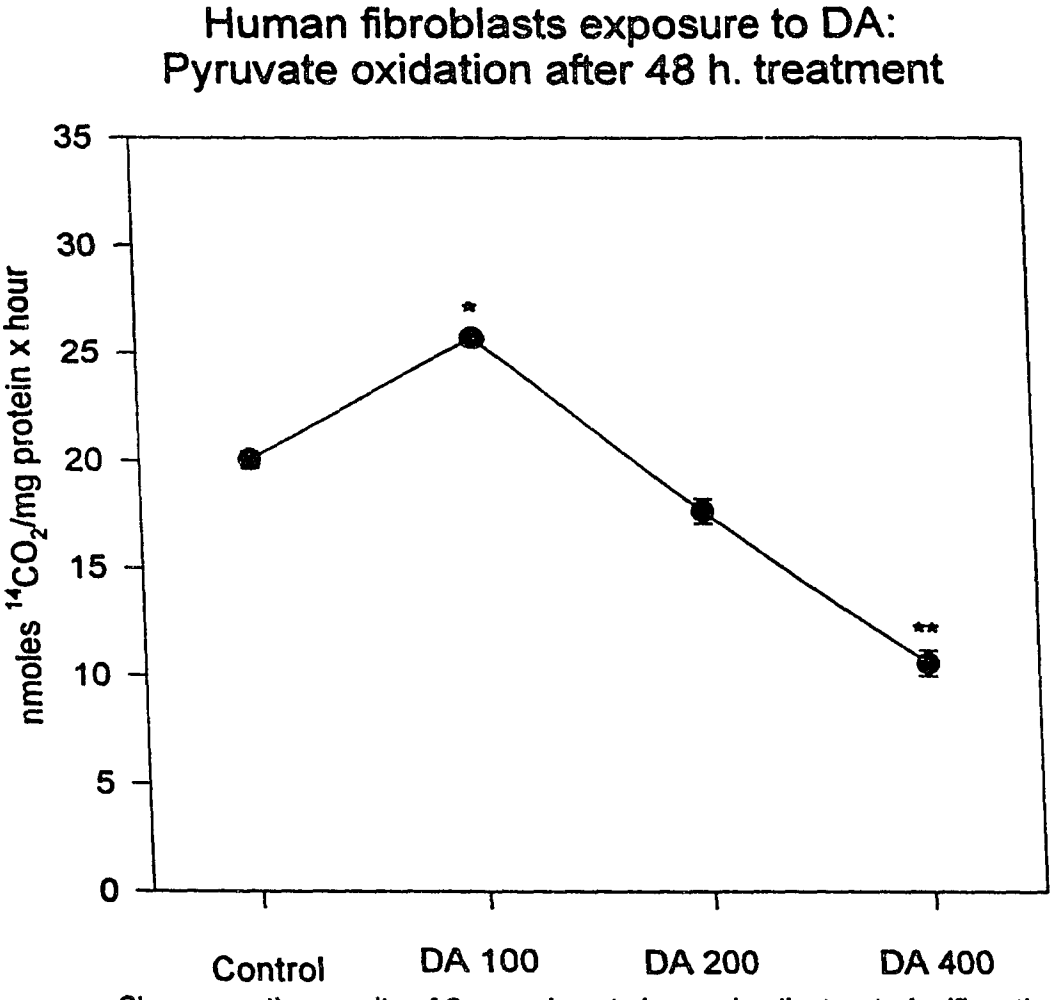
5.1.8: Experiments with DA

In separate set of experiments, dopamine was employed, to investigate the effect of a catechol compound that is not an amino acid on pyruvate oxidation. DA was also chosen because it is the main natural metabolite of L-dopa. Fibroblasts (93-8) were exposed to DA at concentrations ranging from 100 - 400 μM for 48 h, with one medium change and then analyzed for pyruvate oxidation using 2-^{14}C-pyruvate (**Figure 16**). The response of cellular respiration to DA-exposure was concentration dependent, and, in this respect, different from L-dopa.

Whereas incubation of fibroblasts with 100 μM L-dopa had resulted in a significant reduction of $^{14}CO_2$ release, treatment with 100 μM DA led to a significant and reproducible increase of cellular respiration compared to control (128 ± 0.8 %, $p < 0.001$). When the concentration of DA was raised to 200 μM, no significant difference to control was observed (88 ± 3% of

control, $p > 0.05$), whereas DA given at 400 μM resulted in a sizable and significant decrease in pyruvate oxidation (- 47.3 ± 3 %, $p < 0.001$) (**Figure 16**).

FIGURE 16



Shown are the results of 2 experiments in quadruplicate. * significantly different from control, 200 and 400 uM, $p < 0.01$. ** significantly different from control, 100 and 200 uM , $p < 0.001$; 200 uM was not significantly different from control. MANOVA followed by Tukey-Kramer post-hoc test.

5.1.8a: Experiments with DA: FCCP

Since the increase in pyruvate oxidation seen at 100 μM DA was puzzling, FCCP, an uncoupler of mitochondrial respiration, was used to investigate whether this increase reflected increased capacity or increased basic consumption, which could be due to e.g. a "leaky" electron transport chain. Following 48 h treatment with or without 100 μM DA the stimulation of mitochondrial respiration was assayed in the presence of 0.1 μM FCCP. The uncoupler increased pyruvate oxidation by 166.8 ± 5.7 % for FCCP-control vs. unstimulated control, which is in keeping with previous data obtained when mitochondrial respiration was uncoupler-stimulated by FCCP. Fibroblasts treated with 100 μM DA for 48 h showed similar and significant increases in respiratory activity when compared to FCCP-control (129.4 ± 4.7 %, $p<0.001$) (**Table 7**), a similar relative increase as observed in unstimulated fibroblasts (128 ± 0.8 %, $p< 0.001$, see above). It seems therefore that exposure of fibroblasts to lower levels of DA, and possibly also carbidopa, can lead to an increase in respiratory capacity in these cells. The reason for this effect remains elusive.

TABLE 7

The effect of treatment with 100 μM DA for 48 h on the respiration in human embryonic fibroblasts (93-8), stimulated by FCCP

	CONTROL	DA 100 μM
nmoles $^{14}CO_2$/mg protein hour (± SEM)	53.52 ± 1.145	69.242 ± 2.521[a]

Shown are the means and S.E.M. of 2 experiments conducted in quadruplicate. Fibroblasts (93-8) were treated with DA (100 μM) for 48 h and the rate of pyruvate oxidation determined. During the assay, the respiration was stimulated by the presence of 0.1 μM FCCP.

[a] significantly different from control $p < 0.001$ (MANOVA followed by Tukey post-hoc test).

5.1.9: Experiments with catalase and SOD

Since the protective action of ascorbate against the effect of L-dopa (**Table 4**) suggested that oxidative events underlie the inhibition of pyruvate metabolism by L-dopa, experiments were conducted to test whether removal of extracellular H_2O_2 and superoxide by the addition to the fibroblast cultures of catalase (10 µg/mL) and superoxide dismutase (5 µg/mL) could protect. No protection was observed by this treatment (expressed as % of control: L-dopa 51 ± 6; L-dopa + SOD + catalase 55 ± 1).

5.2: DISCUSSION

Exposure of human fibroblasts to L-dopa results in an inhibition of the oxidation of pyruvate, but not succinate. Some of the observations with L-dopa and human fibroblasts have been published (Werner et al., 1994). The assay used employed the release of $^{14}CO_2$ from 2-^{14}C -pyruvate, which reflects Krebs cycle activity, and from [1,4]-$^{14}CO_2$-succinate, which measures complex II/III activity. Inhibition of pyruvate oxidation is independent of the origin of the cells, since reduction in pyruvate oxidation was reproduced in three different fibroblast cell lines. In control fibroblasts, the oxidation of pyruvate was inhibited more than 95% by the complex I inhibitor rotenone, and could be stimulated strongly by FCCP, an uncoupler of mitochondrial respiratory control. Both observations indicate that the reduction equivalents supplied by pyruvate do indeed pass through complexes I-IV of the respiratory chain. Pyruvate oxidation has been successfully used by others to determine respiratory chain activity in fibroblasts (Robinson et al., 1987; Slipetz et al., 1991). Therefore, L-dopa is likely to cause inhibition at the level of complex I of the respiratory chain or upstream of it. The fact that the oxidation of succinate remained unchanged in our experiments precludes an involvement of enzymes downstream of complex I. The observations with cultures of human cells (fibroblasts) is supported by the

experimental observations of others, who found decreased complex I activity in dopamine-rich regions of rat brain after long-term exposure (30-days) to high doses of L-dopa (Przedborski et al., 1993).

The inhibition of mitochondrial respiration was dose-dependent. The minimum concentration of L-dopa necessary to achieve significant inhibition of pyruvate concentration was 100 μM. This lower limit could not be overcome by prolonged exposure. Exposure to lower concentrations of L-dopa (10-50 μM) for 7 days did not significantly alter the rate of pyruvate oxidation.

One possible site of the inhibitory action is the uptake of pyruvate into the cell. Pyruvate is a good substrate for the monocarboxylic acid transporter (Poole and Halestrap, 1993). Therefore, interference with the uptake sites by L-dopa (e.g. by binding of the quinone to the transporter) would certainly be expected to affect pyruvate oxidation. However, when cells were homogenized after pretreatment with L-dopa in order eliminate cell membrane transport, pyruvate oxidation was still markedly reduced. Therefore, pyruvate transport is not involved in the inhibition of cellular respiration by L-dopa.

The inhibition of pyruvate oxidation by L-dopa was reversible: withdrawal of the drug following 48 h exposure to 200 μM L-dopa resulted in a slow return to normal levels of respiration (Figure 12). However, pyruvate oxidation was still significantly lower then control at 48 h after washout. Pyruvate oxidation returned to normal at 7 days. One possible mechanism for the slow return of pyruvate oxidation capacity is a replacement of damaged proteins via new protein synthesis. Inactivation of enzymes by binding of quinones to cysteine groups is well established (e.g. Waring et al., 1986). Binding of L-dopa quinone to sulfhydryl groups in complex I is a potential mechanism for inhibition of pyruvate metabolism.

The beneficial effect of the antioxidant ascorbic acid suggests the involvement of oxidative events in the inhibitory action of L-dopa. Since ascorbic acid prevented the visible autoxidation of L-dopa (formation of melanin), quinone intermediates of melanin formation are likely candidates for mediators of the toxic effect on pyruvate oxidation. This hypothesis was tested by employing the non-autoxidizable analogue 3-O-methyldopa. Because 3-O-methyldopa does not autoxidize, it should not suppress complex I. Treatment of fibroblasts with 200 μM 3-O-methyldopa did not

reduce pyruvate oxidation (Figure 13), in keeping with its non-autoxidizable character.

Subsequent experiments eliminated H_2O_2 production by mitochondrial MAO activity as a cause of the observed inhibition of pyruvate oxidation. L-Dopa is not a substrate for MAO, but after decarboxylation (by the aromatic amino acid decarboxylase), the DA formed from L-dopa can serve as a substrate for MAO. Carbidopa inhibits the decarboxylation of L-dopa to DA, thereby preventing the formation of the MAO substrate. When carbidopa was combined with L-dopa, inhibition of pyruvate oxidation was still observed, even though carbidopa alone had a stimulatory effect on pyruvate oxidation. These observations indicate that conversion to DA is not required for the inhibitory action of L-dopa, leaving the autoxidation of L-dopa as the most likely explanation. This was also confirmed by the ineffectiveness of the MAO inhibitor pargyline in preventing the damaging effect of L-dopa exposure on cellular respiration. Pargyline is non-selective and inhibits both MAO-A and MAO-B at the concentrations used.

It is known that L-dopa, which is one of the drugs most commonly used in the treatment of Parkinson's disease, can cause a variety of side effects during the treatment of Parkinson patients. This may be related to an oxidative stress, which could be brought about by two mechanisms, namely, (1) chemically via autoxidation and (2) biochemically via the H_2O_2 generated during the catabolism its main metabolite, the neurotransmitter dopamine. Evidence for both pathways exist. L-dopa added to synaptosome preparations increases the GSSG level; this increase is sensitive to the inhibition of MAO (Spina and Cohen, 1988), indicating biochemical formation of H_2O_2 via MAO activity. In the experiments presented in this thesis, treatment of human fibroblasts with L-dopa reduced cellular respiration, an effect that could not be blocked by pargyline (MAO inhibitor), but was blocked by the antioxidant ascorbic acid.

Experiments were also conducted with DA as an alternative, autoxidizable, catechol compound. These experiments revealed a surprising

dose-dependent biphasic effect on respiratory activity. DA at 100 μM actually increased cellular respiration by approximately 30 %; this was also true when mitochondrial respiration was stimulated with FCCP. Higher concentrations of DA however led to a decrease in pyruvate oxidation: treatment of fibroblasts with 400 μM DA for 48 h reduced cellular respiration by 47%. The nature of this biphasic effect remains elusive. One possibility is that cells may respond to a low level autoxidative stress with increased biosynthetic activity, as was recently shown for upregulation of glutathione in cell cultures (Mytilineou et al., 1993), an effect which could require increased ATP production. On the other hand, such responses could be rendered ineffective due to cellular damage at a higher oxidant stress level.

6: SUMMARY and CONCLUSIONS

The work presented in this thesis investigates how chemical and biochemical features of DA and L-dopa may lead to oxidative events that can affect the mitochondrion and compromise cellular respiration. These two compounds are of particular interest due to their prominent role in Parkinson's disease, a chronic neurodegenerative disorder affecting mainly the dopaminergic neurons of the basal ganglia (Ehrlich and Hornykiewicz, 1960). Both L-dopa and DA are capable of producing reactive oxygen species, L-dopa via autoxidation, and DA via autoxidation and also by its oxidative deamination by monoamine oxidase (MAO), producing H_2O_2 (reviewed by Cohen, 1983). The selective loss of neurons that use DA as a neurotransmitter has been attributed to the potential hazards involved with these pathways, and it has been suggested that DA can act as an "endogenous neurotoxin" (Fahn, 1989).

In the experiments with isolated mitochondria, the enzymatic pathway of DA metabolism via MAO was investigated. To this end, glutathione disulfide (GSSG), a marker of H_2O_2 detoxification, and protein-mixed disulfides (PrSSG), another marker of oxidative stress, were measured in isolated mitochondria challenged with MAO substrates.

Increases were observed for both GSSG and PrSSG. For example, when brain mitochondria were challenged with 100 μM DA for 10 min. showed an 11-fold increase in the level of GSSG, and a 3.9-fold increase in the level of protein-mixed disulfides. Mitochondria are not only the site of the enzyme (MAO) that catalyzes the deamination of DA leading to H_2O_2 production, but also one of the potential targets for H_2O_2 , as shown by the build-up of both GSSG and PrSSG in mitochondria upon MAO activity. This oxidative stress has functional consequences, as was shown by the partial inhibition of aldehyde dehydrogenase, which was examined in both rat liver and brain mitochondria. It is remarkable that the reduction in aldehyde dehydrogenase activity was more pronounced in brain mitochondria

compared to liver mitochondria; incubation with 1 mM DA resulted in twice the loss of activity in brain mitochondria (ca. 40%) then in liver mitochondria (ca. 20%). Turan et al. (1989a) also reported the inactivation of aldehyde dehydrogenase in rat liver mitochondria following the incubation with 1 mM DA for 90 min.; however, as mentioned previously, the inactivation of aldehyde dehydrogenase by oxidation of the DA catechol was probably enzyme catalyzed (Turan et al., 1989a) and sex-specific, namely observed only in female rat liver mitochondria. Turan et al. (1989) reported no detectable coloration (i.e. catechol oxidation) even after 90 min. incubation time when male rat liver mitochondria were used.

In the second part of this thesis work, the effect of L-dopa was studied on intact cells, namely, human skin fibroblasts. L-Dopa is an example of an autoxidizable compound. L-dopa (a catechol) autoxidizes, producing $\cdot O_2^-$, H_2O_2 , and dopaquinone in the process, as discussed in the introduction. Oxidative stress has been implicated as a cause of mitochondrial malfunction, and quinoidal compounds are potent inhibitors of oxidative

phosphorylation (e.g., Bironaite et al., 1992). Since defects in mitochondrial respiration have been implicated in Parkinson's disease (Schapira et al. 1989, 1990; Walker et al. 1990), the effect of L-dopa on cellular respiration in human cells was investigated.

The treatment of fibroblasts with 100 μM or higher concentrations of L-dopa for a minimum of 48 h caused a reduction in pyruvate, but not succinate oxidation. This indicates a detrimental effect of L-dopa on mitochondrial respiration upstream of complex II, which is in agreement with results by Przedborski et al. (1993), who observed complex I inhibition in rat brain after long-term treatment of the animals with high doses of L-dopa. The autoxidation of L-dopa, which could be followed visually by the darkening of the medium, was slow; even high doses (e.g., 400 μM), darkening of the medium required a minimum of 12 h. This is in keeping with our observation that short-term incubations of cells with L-dopa did not lead to any measurable changes in mitochondrial activity. The long lag period is also consistent with the observation, cited above, that darkening of

the medium was not observed over a 20-minute time span in experiments conducted with isolated mitochondria.

The effect of L-dopa on fibroblast cultures was dose-dependent and slowly reversible over time. The involvement of autoxidation of L-dopa was verified using the O-methylated dopa-derivative, 3-O-methyldopa, which cannot autoxidize. 3-O-Methyldopa had no effect on cellular respiration. This observation is consistent with autoxidation as a requirement for the inhibitory action of L-dopa.

A surprising observation was made when ascorbate was added as an antioxidant in experiments with L-dopa autoxidation: with or without added L-dopa, ascorbate consistently boosted mitochondrial respiration, almost doubling pyruvate oxidation rates. Succinate oxidation, on the other hand, was not increased. These findings imply a beneficial effect of ascorbate for mitochondrial function. In this regard, it should be noted that recent findings indicate decreased mitochondrial respiratory chain capacity, namely reduced

complex I activity, in Parkinson's disease (Schapira et al., 1989, 1990; Parker et al., 1989; Walker et al. 1990; Shoffner et al., 1991). In addition, other investigators have shown increased markers of oxidative stress in affected brain regions of patients with Parkinson's disease (e.g. Dexter et al., 1986; Sian et al., 1994; Fahn and Cohen, 1992). Oxidative stress appears also to be involved in the neurotoxicity of MPP^+, the toxic metabolite of MPTP. It is known that MPTP poisoning results in loss of complex I activity of mitochondria (Nicklas et al., 1985). It was later shown that oxygen was required for damage, and that antioxidants, such as ascorbic acid, were highly protective (Cleeter et al., 1993). This indicates a particular vulnerability of complex I to damage by oxidative stress.

The impairment of complex I in Parkinson's disease may, therefore, be due, in part, to the chemical and biochemical reactivity of the neurotransmitter DA, and its metabolic precursor, L-dopa. Increased DA turnover by MAO has been shown to increase GSSG both in vitro and in vivo (e.g. Spina and Cohen, 1989; Spina and Cohen, 1990). My own results with

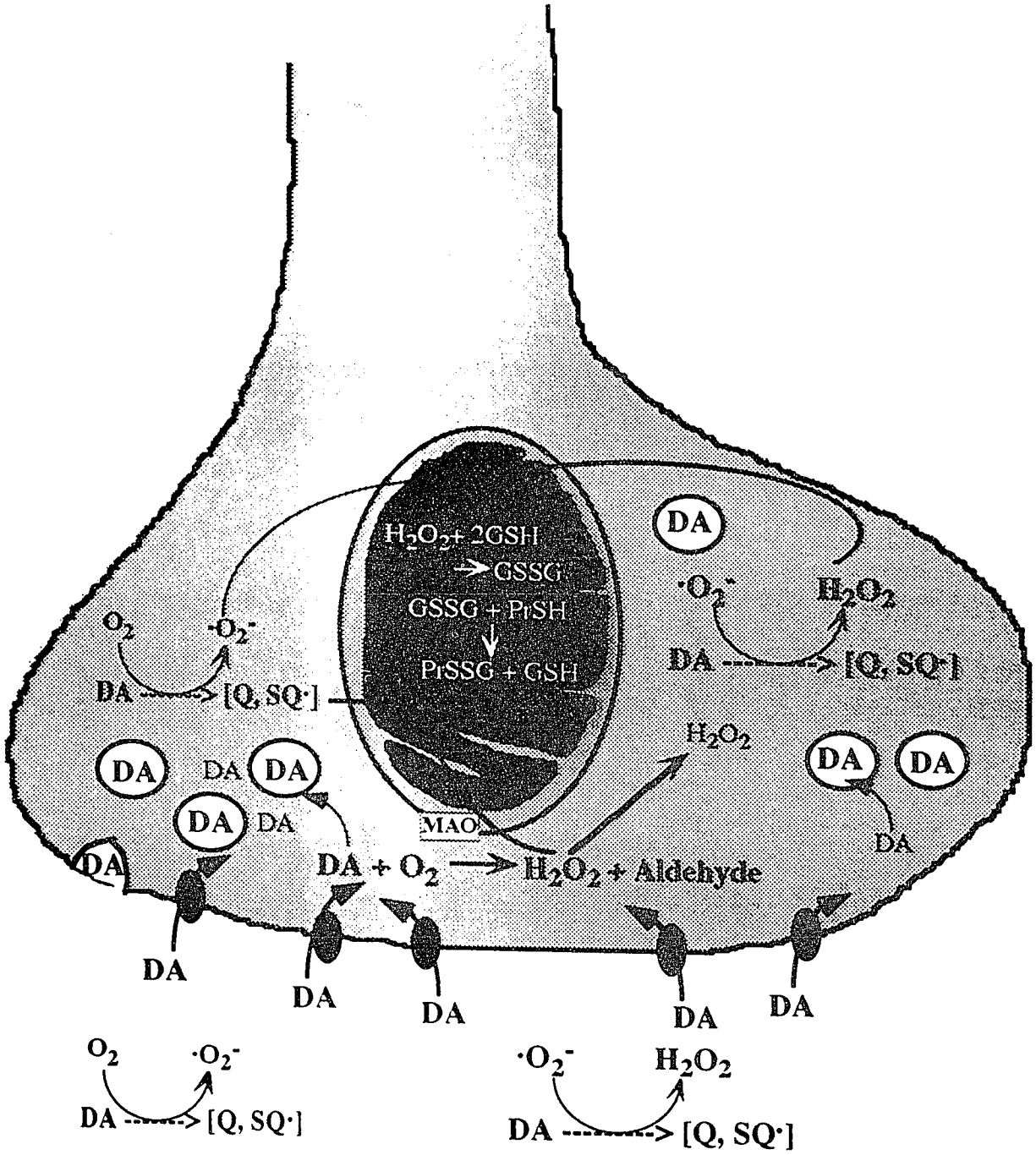
mitochondria show that the turnover of DA by monoamine oxidase results in a significant oxidative stress in the form of GSSG and PrSSG accumulation, which reduces the activity of a representative enzyme (aldehyde dehydrogenase) of the mitochondrial matrix. Therefore, increased DA turnover by MAO, possible combined with autoxidation of catecholamines or administered L-dopa, may be one of the reasons for the impairment of mitochondrial function in Parkinson's disease. Since DA turnover occurs wherever MAO is localized, these phenomena would not be restricted to DA neurons. They may even be an explanation for an apparent contradiction: After the loss of 80% or more of the DA neurons in Parkinson's disease, the remaining DA neurons account for only a small part of the total cell population in the substantia nigra, yet the complex I inhibition observed in Parkinson's disease is usually in the range of 30-40%.

It should be noted that evidence for an increased turnover of DA exists: The ratio of DA to MAO-derived metabolites is shifted towards the metabolites in Parkinson's disease (Hornykiewicz and Kish, 1986), indicating

an increased flux of H_2O_2 in the environment of the remaining DA neurons. An oxidative stress imposed on the mitochondria of DA neurons and neighboring cells, including glia, may be one of the factors compromising the general mitochondrial function in the substantia nigra.

Figure 17 shows schematically in a simplified DA terminal the two different mechanisms dealt with in this thesis that may contribute to the demise of DA neurons in Parkinson's disease. As shown in the first part of this thesis, the mitochondrion can be subject to oxidative stress evoked by MAO activity on DA (or any other MAO substrate), with H_2O_2 reaching the inner compartments of the mitochondrion. Following the detoxification of H_2O_2 by glutathione peroxidase (GPx), GSSG is formed. If GSSG is allowed to build up, it can undergo a disulfide-exchange with protein-thiol groups (PrSH) and form mixed disulfides (PrSSG), altering enzyme function if the protein is an enzyme. Evidence for increased MAO activity with its potential to increase the oxidative load in Parkinson's disease exists. DA turnover is increased in Parkinson's disease (Hornykiewicz and Kish, 1986),

FIGURE 17


H_2O_2+ 2GSH
GSSG
GSSG + PrSH
PrSSG + GSH
O_2
$\cdot O_2^-$
DA
[Q, SQ·]
H_2O_2
MAO
DA + O_2 → H_2O_2 + Aldehyde

and experimental stimulation of DA turnover in vivo has been shown to cause increased H_2O_2 production (Spina and Cohen, 1989). These insults, which presumably result from increased free, non-vesicle bound DA being oxidatively deaminated by MAO may be exacerbated by increased autoxidation of L-dopa, DA and its metabolites, which results in the production of reactive oxygen species and the binding of autoxidation products to thiol-bearing molecules in the cell. The detrimental effect of catechol autoxidation on cellular respiration was shown in this thesis using the DA precursor L-dopa, which reduced cellular respiratory activity in human fibroblasts (Werner et al., 1994). The stress exerted by MAO generated H_2O_2 may thus be exacerbated by the detrimental effects of catechol-autoxidation of L-dopa, DA and its metabolites. Fornstedt et al. (1989) showed an increase in catechol-autoxidation rate in dopamine-rich areas of brains that showed a degeneration of the basal ganglia by measuring the 5-S-cysteinyl adducts of DA, DOPAC and Dopa, respectively. Recent in-vitro data indicate that these adducts may be breakdown products

of cysteine-containing proteins, to which oxidized catechol had bound (Hastings and Zigmond, 1994).

Mitochondria are susceptible to oxidative stress (e.g. Boveris, 1994), and show functional and structural alterations under such conditions. For example, mitochondria change their volume under oxidative stress. It is of interest to note that, historically, mitochondrial glutathione peroxidase was identified as a contraction factor (Neubert et al., 1962). The complexes I, II and IV of the mitochondrial respiratory chain are vulnerable to oxidative stress (e.g. Benzi et al., 1991). Beneficial effects of supplying antioxidants on mitochondrial structure and function have been reported. Ascorbate was found to have a protective effect on mitochondrial function in ischemia-reperfusion injury (Nishinaka et al., 1992). The importance of glutathione for mitochondrial structure and function and the interdependence of mitochondrial glutathione and ascorbate may be one of the possible explanations (Meister, 1995).

In summary, oxidative stress elicited by both the chemical and biochemical properties of DA and L-dopa was capable of affecting mitochondrial functions, albeit over different pathways. The two mechanisms, autoxidation and H_2O_2 production via MAO, provide the potential for a two-pronged assault on mitochondria in Parkinson's disease. The apparent reduction in antioxidant defenses such as GSH (Sian et al., 1994) may be an indication for an impairment of the cells capacity to respond to an oxidative challenge. Under such a paradigm, an oxidative stress which could otherwise be handled may become a lethal challenge for the cells of the substantia nigra by strangling oxidative phosphorylation in those cells. In addition, recent evidence suggests a crucial role of oxidative stress on mitochondria in apoptosis (programmed cell death), especially with the recognition of the oncogene Bcl 2, which protects against oxidative stress induced cell death (e.g. Korsmeyer et al., 1995). With oxidative stress being more of a trigger then the final cause of cell death, even a lower level oxidative stress, which targets the mitochondrion, may push a cell into a downward spiral leading to its demise. Oxidative stress in mitochondria, brought about by the turnover of DA as well as by the autoxidation of L-dopa, DA and DOPAC, may be a double-pronged mechanism which could bring about such a scenario.

7: ADDENDUM

In addition to the work directly related to my thesis, I was also involved in two additional projects, the results of which are published. The projects are listed under the titles of the publications, both of which are included in the appendix.

"Impaired oxidative decarboxylation of pyruvate in fibroblasts from patients with Parkinson's disease" C. Mytilineou, P. Werner, S. Molinary, A. DiRocco, G. Cohen and M.D. Yahr. J. Neural. Transm. [P-D Sect], (1994) 8: 223-228

The first project dealt with the assay of the respiratory activity of skin fibroblasts obtained from patients suffering from Parkinson's disease. This project was to establish whether or not skin fibroblasts, which can be generated from a benign and almost painless biopsy, display the respiratory defect seen in PD (Schapira et al., 1989, 1990, Parker 1989, Shoffner et al., 1990). While the reduction of complex I activity in the affected brain regions of PD patients is generally accepted, a controversy over findings in non-CNS tissue exists (see DiMauro, 1993, for review). I was involved in the establishment of the assay used in this study, as well as in carrying out the experiments. The method used is identical to the method described in specific aims 2 of this thesis.

Our results indicated that the defect is indeed displayed in PD fibroblasts, compared to age- and sex-matched controls. Since fibroblasts can be propagated in culture for a prolonged time, they are an ideal model system to study biochemical and genetic defects in primary, genetically unaltered cells.

"Lipid peroxidation in brain: Interaction of l-dopa/dopamine with ascorbate and iron." C. L. Li, P. Werner and G. Cohen. Neurodegeneration 4: 147-153

The second project dealt with the interaction of L-dopa and ascorbic acid with lipid peroxidation in a rat brain homogenate model. Throughout the experiments, I had the pleasure of working with Chun-Lun Li, who carried most of the project while being a summer student in Dr. Cohen's laboratory. I was involved in setting up the analytical procedure and aided in the design of the experiments. The assays were conducted using the TBARS (thiobarbituric acid reactive substances) method, which indicates the presence of degradation products of lipid peroxides, especially malondialdehyde.

Our results indicated a dualistic role of L-dopa in lipid peroxidation. With only the ascorbate endogenous to the tissue present in the homogenate, L-dopa was partially protective against lipid peroxidation. This effect was reversed when ascorbate was added at concentrations of 0.5-2.0 mM.

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