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Evaluation of The Pro-oxidant and Antioxidant Actions of L-DOPA and Dopamine in Vitro: Implications For Parkinson's Disease

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The antioxidant and pro-oxidant properties of L-DOPA and dopamine were investigated *in vitro*. Both compounds inhibited the peroxidation of ox-brain phospholipids, with IC_{50} values of 8.5 μM for dopamine and 450 μM for L-DOPA. Dopamine and L-DOPA reacted with trichloromethyl peroxyl radicals ($CCl_3O_2^{\bullet}$) with rate constants of $2.1 \times 10^7 M^{-1}s^{-1}$ and $1.3 \times 10^7 M^{-1}s^{-1}$ respectively. The effects of dopamine and L-DOPA on iron ion- dependent hydroxyl radical generation from H_2O_2 were complex. In general, low concentrations stimulated $OH^{\bullet}$ formation in the presence of ferric-EDTA and, in the case of L-DOPA, ferric-ADP and ferric citrate chelates. Both compounds also reacted with superoxide radical and hypochlorous acid. The products of the reaction with HOCl could still inhibit α_1-antiproteinase and appear to be 'long lived' chloramine-type oxidizing species. Our results suggest that L-DOPA and dopamine might have a complex mixture of pro- and anti- oxidant effects, which could contribute to tissue damage due to oxidative stress in Parkinson's disease and other neurological disorders.

Key Words: Dopamine, L-DOPA, Free Radicals, Antioxidants, Peroxyl Radicals

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INTRODUCTION

Oxidative damage has often been proposed to play an important role in the progression of cell death in Parkinson's disease, other neurodegenerative diseases, and after ischaemic or traumatic brain injury.[1-13] Indeed, free radicals and other reactive oxygen species (ROS) are constantly formed in the human body. Among the commonly encountered ROS are the radicals hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{\bullet -}$) peroxyl ($RO_2^{\bullet}$) and nitric oxide ($NO^{\bullet}$), and the non-radicals hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$). Generation of the highly- reactive $OH^{\bullet}$ is often achieved by reaction of H_2O_2 and $O_2^{\bullet -}$ with 'catalytic' metal ions, especially those of iron and copper. Superoxide radical and H_2O_2 are known to be generated in the brain and nervous system *in vivo*,[1,10] and several

areas of the human brain are rich in iron[14–19] which appears to be easily mobilizable in a form that can stimulate free radical reactions such as $OH^{\bullet}$ formation and lipid peroxidation.[2,10,14] Excess iron deposition in the substantia nigra may contribute to the pathology of Parkinson's disease.[15,17,18]

L-DOPA (L-3,4-dihydroxyphenylalanine) is the immediate biological precursor of dopamine and is commonly used in the treatment of Parkinson's disease.[20] In theory, L-DOPA, dopamine and related compounds could be cytotoxic in man since free radical and quinone metabolites of L-DOPA are toxic to cell lines *in vitro*,[21,22] including genotoxicity,[23] and H_2O_2 is a product of dopamine metabolism by monoamine oxidase.[1] Brain levels of dopamine autoxidation products have been shown to increase with age in small animals.[24] There is evidence of increased lipid peroxidation, oxidative DNA damage and impairment of certain antioxidant defences in Parkinson's disease, as well as an accumulation of iron (possibly catalytic for free radical reactions) in substantia nigra.[3,4,7,14–18,25] Indeed, there has been considerable debate as to whether or not the clinical use of L-DOPA alters the natural course of Parkinson's disease. Chronic treatment with L-DOPA was reported to decrease lipid peroxidation in the cerebral cortex of healthy mice, but to worsen peroxidation in mice with neuronal injury.[26] Thus L-DOPA appears to have a mixture of pro- and anti-oxidant effects *in vivo*, and it could be harmful to tissues damaged by neurodegenerative disease, i.e. it could alleviate the symptoms of the disease whilst worsening the underlying tissue degeneration.

Perhaps the first step in assessing the pro- and anti-oxidant abilities of a compound *in vivo* is to see what it is actually capable of doing, in chemical terms.[27] In order to clarify the possible ability of L-DOPA and its metabolites to affect oxidative damage, we undertook a detailed characterization of its anti-oxidant and pro-oxidant ability *in vitro*. We report here the ability of dopamine and L-DOPA to react with $O_2^{\bullet -}$, $OH^{\bullet}$, and HOCl and to affect metal ion-dependent $OH^{\bullet}$ generation and lipid peroxidation.

EXPERIMENTAL PROCEDURES

Chemicals were of the highest quality available from Sigma Chemical Co. (Poole, Dorset, UK) or from BDH Chemical Company (Gillingham, Dorset, UK). Solutions of L-DOPA and its metabolites were prepared fresh in buffer at pH 7.4 when required.

Peroxidation of Phosopholipid Liposomes

The ability of compounds to inhibit lipid peroxidation at pH 7.4 was tested using ox-brain phospholipid liposomes as described in[28] except that 0.1 ml of 2% (w/v) BHT was added just before the TBA reagents.

Reactions with Trichloromethylperoxyl Radicals

Reaction with trichloromethylperoxyl radical was conducted as described in.[29,30]

Reaction with Superoxide Radical

Superoxide was generated by the hypoxanthine-xanthine oxidase system.[31] Reaction mixtures contained, in a final volume of 3 ml, 0.1 ml 30 mM hypoxanthine (dissolved in minimum KOH solution), 0.1 ml 0.3 mM EDTA, either 0.1 ml of 3 mM cytochrome c or 0.1 ml of 3 mM nitroblue tetrazolium (NBT) and 88 mM (final concentration) KH_2PO_4-KOH buffer (pH 7.4). In each case, the reaction was started by adding 0.3 ml of xanthine oxidase (Sigma type X1875, freshly diluted in the above phosphate buffer to a reasonable rate of absorbance change) and the rate of cytochrome c or NBT reduction measured at 550 and 560 nm, respectively, at 25°C. Any direct effects of the compounds on cytochrome c or NBT were tested in the absence of xanthine oxidase.

Reactions with Hydroxyl Radical: Deoxyribose Assay

The deoxyribose assay was conducted essentially as described in.[32]

Reactions with Hypochlorous Acid

Reaction with hypochlorous acid (HOCl) was studied using the elastase/α_1-antiproteinase (α_1-AP) assay.[33] Control experiments were also carried out to test for any direct effects of the compounds on α_1-antiproteinase and elastase. Reaction of the compounds with HOCl was also studied by examining their ability to protect the enzyme catalase against HOCl-mediated damage.[34]

RESULTS

Inhibition of Liposomal Lipid Peroxidation

Dopamine proved to be a powerful inhibitor of iron ion/ascorbate-dependent lipid peroxidation in ox-brain phospholipid liposomes with an IC_{50} of 8.5 μM (Figure 1). By contrast, L-DOPA had a much weaker effect (IC_{50} 450 μM). Neither of the compounds used interfered with the assay of lipid peroxidation: they had no effect when added with the TBA reagents instead of being included during the assay itself.

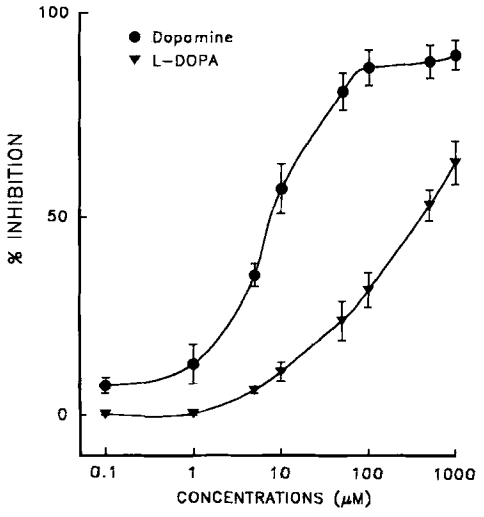


FIGURE 1 Effect of dopamine and L-DOPA on the peroxidation of phospholipid liposomes. Experiments were conducted essentially as described in the Materials and Methods section. Results are mean ± SD from 3 separate experiments; 0% inhibition corresponded to mean absorbances of 1.68 at 532 nm.

Scavenging of Peroxyl Radicals

Peroxyl radical scavenging was examined by generating trichloromethylperoxyl radical ($CCl_3O_2^{\bullet}$) by radiolysis of a mixture of CCl_4, propan-2-ol and buffer[29,30] (equations 1–3).

$$e^-_{aq} + CCl_4 \rightarrow CCl^{\bullet}_3 + Cl^- \quad (1)$$

$$OH^{\bullet} + CH_3CHOHCH_3 \rightarrow H_2O + CH_3\dot{C}OHCH_3 \quad (2)$$

$$CH_3\dot{C}OHCH_3 + CCl_4 \rightarrow CH_3COCH_3 + CCl^{\bullet}_3 + H^+ + Cl^- \quad (3)$$

$$CCl^{\bullet}_3 + O_2 \rightarrow CCl_3O_2^{\bullet} \quad (4)$$

Dopamine and L-DOPA reacted rapidly with $CCl_3O_2^{\bullet}$ with rate constants of $(2.1 \pm 0.23) \times 10^7\ M^{-1}s^{-1}$ and $(1.3 \pm 0.16) \times 10^7\ M^{-1}s^{-1}$ respectively (mean ± SD, n = 3, Figure 2 shows representative data). The rate constants compare well with those for established antioxidants (Table 1). The spectra of the radicals produced from dopamine and L-DOPA after reaction with $CCl_3O_2^{\bullet}$ were very similar (Figure 3).

Effects on Hydroxyl Radical Production

Hydroxyl radicals were generated in a reaction mixture containing ascorbate, H_2O_2 and Fe^{3+}-EDTA at pH 7.4 (equations 5 and 6) and measured by their ability to degrade the sugar deoxyribose.[32] The ascorbic acid increases the rate of $OH^{\bullet}$ generation by reducing iron and maintaining a supply of Fe^{2+}-EDTA (equation 5). Addition of L-DOPA or dopamine to reaction mixtures not containing ascorbate caused a marked stimulation of $OH^{\bullet}$ generation (Figure 4A). Control experiments showed that none of these compounds interfered with the assay used to measure deoxyribose degradation (no effect when added with the TBA reagents), nor did they generate a chromogen in the assay (control with deoxyribose omitted).

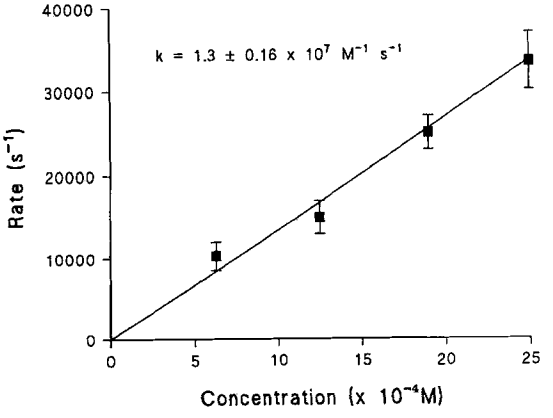


FIGURE 2 Reaction of $CCl_3O_2^{\bullet}$ with L-DOPA to show concentration dependence. The reaction mixtures were as described in the Materials and Methods section. Dopamine was similarly tested.

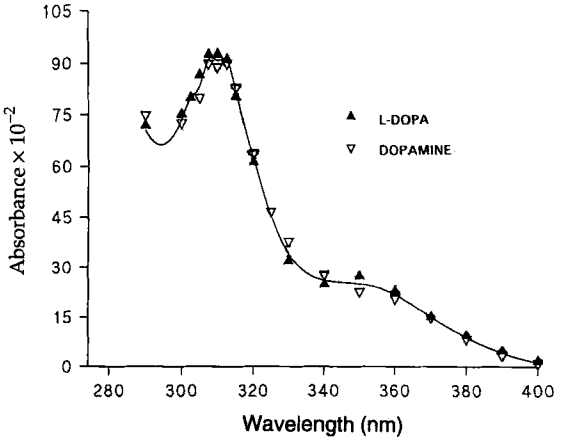


FIGURE 3 Radical absorption spectra of L-DOPA and dopamine after reaction with CCl_3O_2. The data are presented as absorbance per 10 Gy pulse in a 2.5 cm optical cell.

The stimulation of $OH^{\bullet}$ generation tended to be most marked at concentrations below 100 μM and was often less marked at 1 mM. Even when ascorbate was present, stimulation was observed, again becoming less marked at higher concentrations (Figure 4B).

$$Fe^{3+}\text{-EDTA} + \text{Ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{Oxidized Ascorbate} \quad (5)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + OH^{\bullet} + OH^{-} \quad (6)$$

When iron-EDTA was replaced by Fe^{3+}-ADP a pro-oxidant effect of L-DOPA was still observed, but dopamine had much less striking effects (Figure 4C). When another physiological iron complex, Fe^{3+}-citrate, was used L-DOPA again had a significant pro-oxidant effect whereas dopamine did not (Figure 4D).

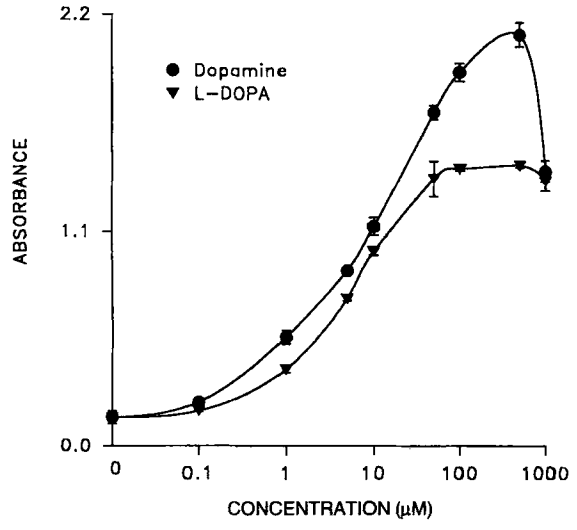
TABLE 1 Second-order rate constants for reaction of DOPA and dopamine with trichloromethylperoxyl radicals. Some established antioxidants are included for comparison.

Compound	Rate constant $M^{-1}s^{-1}$ (CCl_3O_2 + Compound)
L-DOPA	$(1.3 \pm 0.14) \times 10^7$
Dopamine	$(2.1 \pm 0.23) \times 10^7$
Propyl gallate*	$(1.67 \pm 0.08) \times 10^7$
Trolox C*	$(1.6 \pm 0.8) \times 10^8$
Ascorbic acid*	$(1.21 \pm 0.06) \times 10^8$
dl-α-tocopherol*	$(4.89 \pm 0.24) \times 10^8$
α-Lipoic acid*	$(1.8 \pm 0.90) \times 10^8$
Dihydrolipoic acid*	$(2.3 \pm 0.12) \times 10^7$

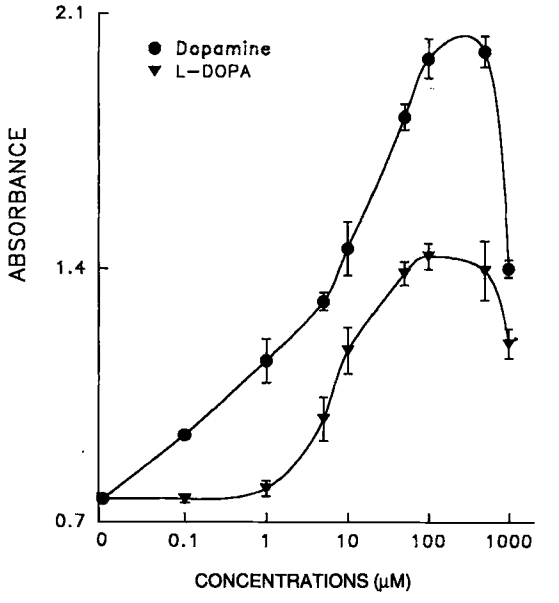
*Values abstracted from the literature[35,36]

Reaction with Superoxide

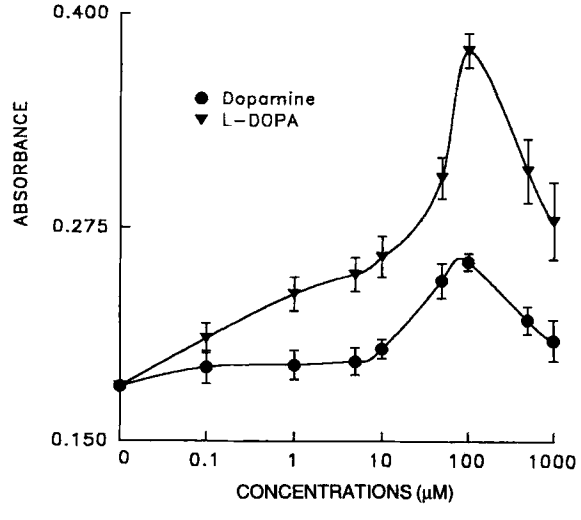
A mixture of hypoxanthine and xanthine oxidase at pH 7.4 generates $O_2^{\bullet -}$ which can be measured by its ability to reduce ferricytochrome c or nitro-blue tetrazolium.[31,32] Dopamine and L-DOPA[31,32] reduced cytochrome c directly and could not be tested in this assay. However, they did not reduce NBT directly and significantly inhibited NBT reduction by $O_2^{\bullet -}$ with IC_{50} values of 8 μM and 4 μM for DOPA and dopamine respectively (data not shown). The approximate rate constants for reaction with $O_2^{\bullet -}$ were calculated[37] to be 1.5×10^5 $M^{-1}s^{-1}$ (dopamine) and 7.7×10^4 $M^{-1}s^{-1}$ (L-DOPA), assuming that NBT reacts with $O_2^{\bullet -}$ with a rate constant[37] of 6×10^4 $M^{-1}s^{-1}$. Neither of these compounds inhibited xanthine oxidase activity, as measured by formation of uric acid (followed at 290 nm in reaction mixtures without NBT).



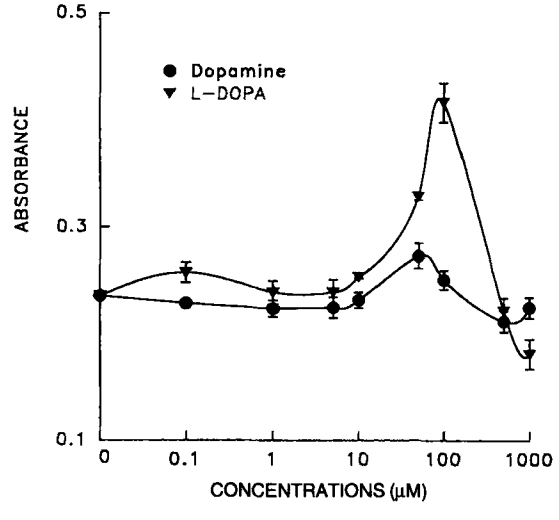
Graph A: Effect on deoxyribose damage by H_2O_2/Fe^{3+}-EDTA in the absence of ascorbate.



Graph B: Effect on deoxyribose damage by H_2O_2/Fe^{3+}-EDTA in the presence of ascorbate.



Graph C: Effect on deoxyribose damage by H_2O_2/ADP-Fe^{3+} in the absence of ascorbate.



Graph D: Effect on deoxyribose damage by H_2O_2/citrate-Fe^{3+} in the absence of ascorbate.

FIGURE 4 Effect of dopamine and L-DOPA on hydroxyl radical generation. Results are mean ± SD from 3 separate experiments.

TABLE 2 Action of dopamine and L-DOPA on inactivation of the elastase-inhibitory capacity of α_1-antiproteinase by hypochlorous acid

Additions to reaction mixture/ μM		Elastase Activity/ ΔA_{410} min^{-1}	α_1-Antiproteinase activity (100 minus % elastase activity)
Elastase only		0.842	0
+ α_1-AP		0.045	95.7
+ α_1-AP and HOCl		0.795	5.6
*α_1-AP, HOCl, and dopamine			
	10.0	0.121	85.6
	25.0	0.621	26.3
	50.0	0.635	24.6
	75.0	0.649	22.9
	100.0	0.664	21.1
	500.0	0.708	15.9
	1000.0	0.710	15.7
L-DOPA	10.0	0.140	83.4
	25.0	0.510	39.4
	50.0	0.549	34.8
	75.0	0.611	27.4
	100.0	0.643	23.6
	500.0	0.643	23.6
	1000.0	0.640	24.0

Experiments were conducted essentially as described in the Materials and Methods section. *Dopamine or L-DOPA at the concentrations stated were incubated with HOCl before addition of α_1-AP. The residual activity of α_1-AP was then assessed by adding elastase and measuring the elastase-inhibitory capacity. Results are means of 3 separate experiments whose results varied by < 5%.

Reaction with Hypochlorous Acid

HOCl scavenging was assessed by measuring the ability of L-DOPA and dopamine to protect α_1-antiproteinase against inactivation by HOCl[33,39] (Table 2).

Low concentrations of L-DOPA and dopamine protected α_1AP against inactivation by HOCl, but the protection diminished at higher concentrations, especially for dopamine (control experiments showed that neither compound directly affected elastase or α_1AP). It may be that these compounds react with HOCl to form products that can themselves inactivate α_1AP (e.g. 'long-lived oxidants' such as chloramines[39]). Evidence consistent with this was obtained by observing spectral changes when HOCl was added to L-DOPA or dopamine (Figure 5). There was a rise in absorbance around 280 nm, consistent with chloramine formation.

Further evidence for reaction of L-DOPA and dopamine with HOCl was obtained by choosing a different biological target, the enzyme catalase. Addition of HOCl to catalase produces marked changes in the enzyme absorbance spectrum.[34] Figure 6 shows that L-DOPA and dopamine could decrease these changes.

DISCUSSION

There has been continuing concern that L-DOPA may exert pro-oxidant actions that could accelerate the degradation of nigral dopamine cells during normal ageing[24] and in Parkinson's disease. Consequently, we have evaluated the ability of L-DOPA and dopamine to scavenge or promote the formation of reactive oxygen species. In general, they were found to exert a mixture of pro- and anti-oxidant abilities. For example, the

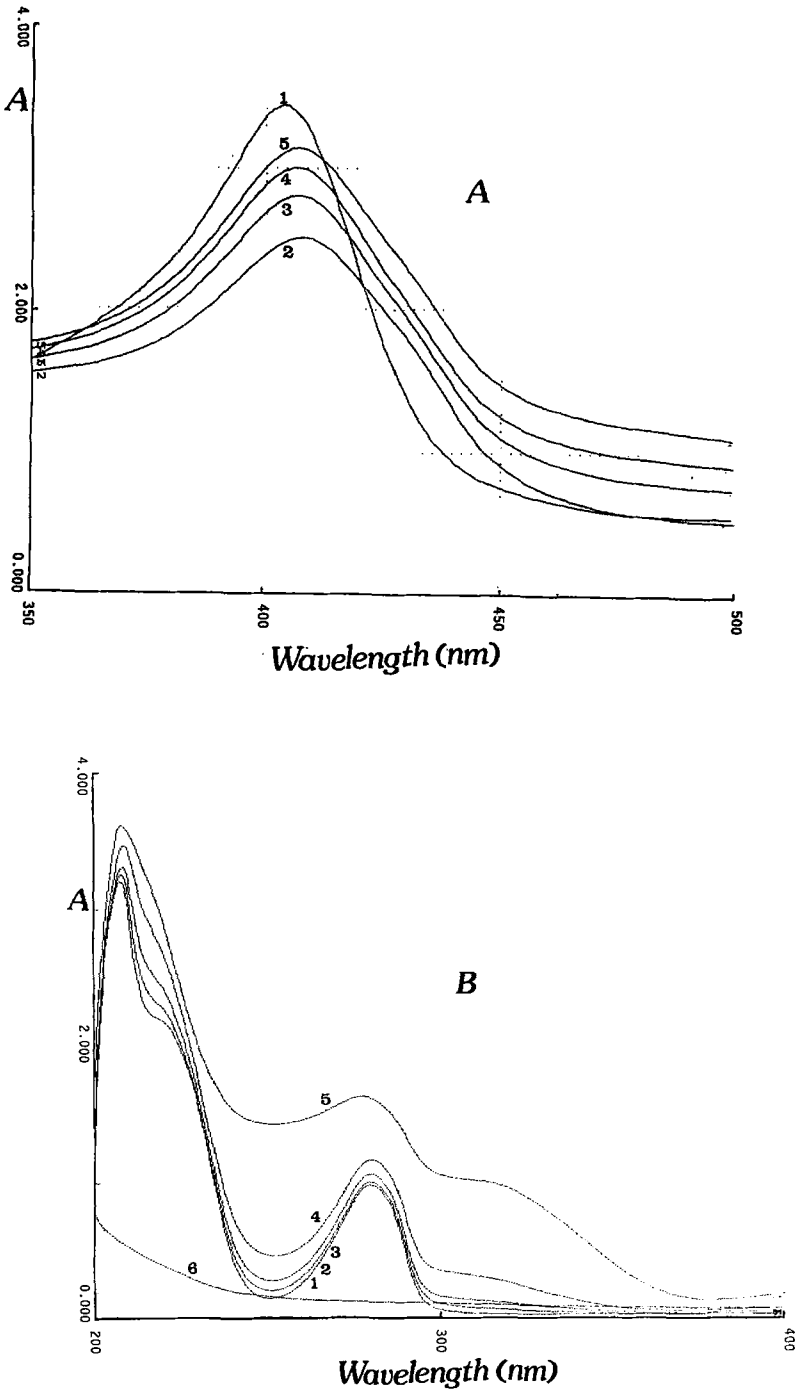


FIGURE 5 Spectral changes upon reaction of hypochlorous acid with L-DOPA and dopamine. The assay was conducted in a final volume of 3 ml. Each cuvette contained an appropriate volume of physiological saline buffer (10 mM Na_2HPO_4-NaH_2PO_4, 0.15 M NaCl) pH 7.4, 330 μM of either dopamine or L-DOPA and varying concentrations of HOCl. Spectra were obtained using a Kontron UVIKON 941 Plus spectrophotometer. Plot A: dopamine, Plot B: L-DOPA; line 1: compound alone; line 2: plus 97 μM HOCl; line 3: plus 194 μM HOCl; line 4: plus 388 μM HOCl; line 5: plus 1000 μM HOCl; line 6: 1000 μM HOCl alone (on plot B).

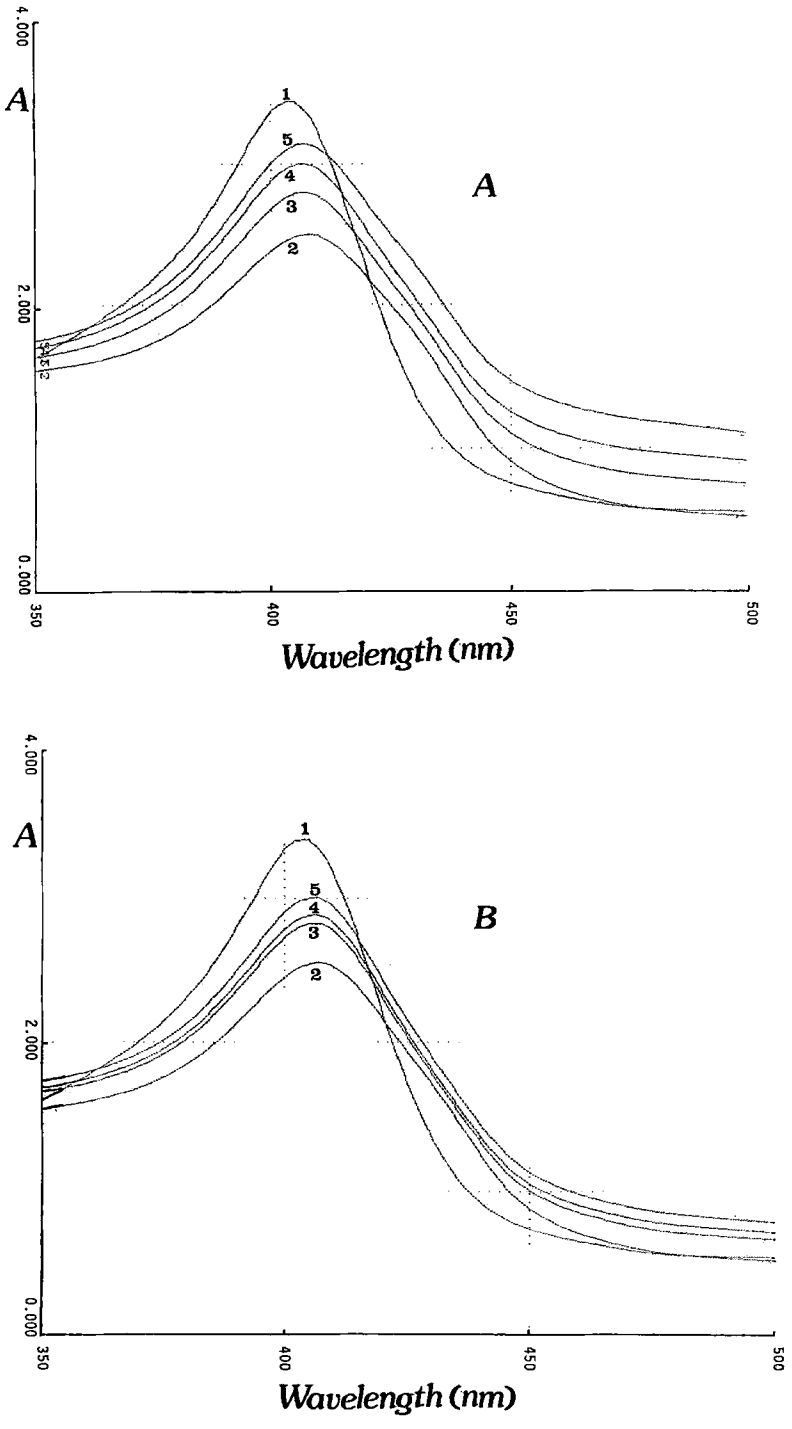
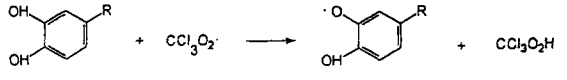


FIGURE 6 Reaction of HOCl with catalase and protection by dopamine and L-DOPA. Panel A: effect of dopamine; B: effect of L-DOPA; Line 1 Spectrum of catalase (18.75 μM); Line 2 catalase plus 1.5 mM HOCl; Line 3 as 2 plus 100 μM dopamine; Line 4 as 2 plus 200 μM dopamine; Line 5 as 2 plus 500 μM dopamine. The lines in panel B were similar, obtained using equivalent concentrations of L-DOPA. All concentrations are the final concentrations in the reaction mixture.

potential of the compounds to inhibit iron ion-dependent lipid peroxidation was demonstrated by the decrease in the level of brain phospholipid liposome peroxidation and their ability to react with the model peroxyl radical $CCl_3O_2^{\bullet}$.[29,30] Dopamine, the more potent scavenger of $CCl_3O_2^{\bullet}$, was also the more effective inhibitor of lipid peroxidation. However, other factors (such as lipophilicity and interaction with iron ions) must also be important since L-DOPA, a much weaker inhibitor of lipid peroxidation, still reacted with $CCl_3O_2^{\bullet}$ at a high rate (Table 1). We used liposomes rather than biological membrane systems (e.g. microsomes) or brain homogenates in our experiments, to avoid problems caused by interactions of added compounds with endogenous membrane antioxidants, such as vitamin E.

L-DOPA and dopamine also reacted with $O_2^{\bullet -}$: the rate constants measured in the biologically-relevant $O_2^{\bullet -}$-generating system used here are comparable to that determined for reaction of $O_2^{\bullet -}$ with adrenalin ($4.0 - 5.4 \times 10^4\ M^{-1}s^{-1}$).[38] Of course, the semiquinone radicals that are probably produced (Scheme 1) by reaction of L-DOPA and dopamine with $O_2^{\bullet -}$ and peroxyl radicals could be cytotoxic themselves by several mechanisms,[22] e.g. they could react with and deplete GSH[24,51] and/or inactivate glutathione-dependent enzymes.[40] Hence the ability of L-DOPA and its derivatives to scavenge $O_2^{\bullet -}$ and peroxyl radicals, measured as antioxidant activities *in vitro*, might ironically, lead to secondary pro-oxidant effects *in vivo*.[51]

In a similarly-equivocal way, L-DOPA and its derivatives reacted with hypochlorous acid, HOCl, which is produced by the action of the neutrophil enzyme myeloperoxidase.[39] They could protect catalase against damage by HOCl as followed by spectral changes (Figure 6), and could protect α_1-antiproteinase at low concentrations. However reaction of L-DOPA and dopamine with HOCl appeared to produce a stable 'long-lived' oxidant, of the type previously described for reaction of HOCl with taurine.[39] These effects might become significant when neutrophils enter the CSF (e.g. in inflammatory diseases such as meningitis) and also in considering the effects of L-DOPA and its metabolites outside the nervous system in patients being given large doses of L-DOPA (e.g. for treatment of Parkinson's disease).



SCHEME 1

The effects of L-DOPA and its metabolites on H_2O_2-dependent $OH^{\bullet}$ generation are complex even *in vitro* and we must not forget the added complexity *in vivo* that oxidation by monoamine oxidase is a source of H_2O_2.[1,7] Low concentrations of L-DOPA could stimulate iron ion-dependent $OH^{\bullet}$ generation from H_2O_2 in the presence of physiological iron chelates such as iron-ADP[41] and iron citrate.[42] Higher concentrations tended to inhibit. All aromatic compounds react very quickly with $OH^{\bullet}$ and L-DOPA and its metabolites will be no exception.[43] Their net effect *in vitro* is probably a balance between stimulation of $OH^{\bullet}$ generation (presumably by reducing iron ions) and direct scavenging of $OH^{\bullet}$,[43] the latter effect probably becoming predominant at higher concentrations. Again, reaction of L-DOPA and dopamine with $OH^{\bullet}$ could generate reactive semiquinones, perhaps able to deplete GSH.[43,51] Given that iron accumulates in Parkinsonian substantia nigra,[14,15,17,18] these interactions could well be physiologically relevant. Indeed, the salicylate hydroxylation technique has been used to detect formation of $OH^{\bullet}$ during dopamine oxidation in the caudate nucleus of living rats.[44]

An important question is whether the concentrations of L-DOPA and dopamine that we have used to achieve the above effects are physiologically relevant. Spina and Cohen[45] reported a peak value of 0.05 mM L-DOPA in the striatum of rats

$$\text{Dopamine} + O_2 + H_2O \rightarrow \text{3,4 dihydroxyphenylacetaldehyde} + NH_3 + H_2O_2 \quad (7)$$

after administration of 100 mg/kg L-DOPA. L-DOPA is quickly decarboxylated to dopamine by the enzyme L- aromatic amino acid decarboxylase. Anden *et al.*[46] estimated a concentration of dopamine of 50 mM within striatal nerve terminals. Hence our studies have used concentrations that are relevant to events *in vivo*.

If L-DOPA metabolites inhibit lipid peroxidation but can stimulate $OH^{\bullet}$ generation (as well as themselves react with $OH^{\bullet}$) under certain circumstances, what could be the consequences of this? GSH depletion could be one, as discussed above. Hydroxyl radicals can damage proteins and DNA.[9,10,13,47,48,49] Indeed, evidence for increased oxidative protein damage has been reported in several neurodegenerative diseases[13] and elevated levels of 8-hydroxy-deoxyguanosine have been reported in parkinsonian substantia nigra.[25] Our data show that one must consider the possibility that these effects are not due to Parkinson's disease itself, but could be consequences of treatment of parkinsonian patients with L-DOPA. Our data *in vitro* show that it would be quite feasible for L-DOPA and dopamine to exert both pro- and anti-oxidant effects,[27,30,36,50] against different biological targets, *in vivo*, as is suggested by animal experimentation.[26,44] We now need *in vivo* experiments to assess the physiological relevance of these effects.

Acknowledgements

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