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Peroxynitrite-Dependent Aromatic Hydroxylation and Nitration of Salicylate and Phenylalanine. Is Hydroxyl Radical Involved?

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There is considerable dispute about whether the hydroxylating ability of peroxynitrite ($ONOO^-$)-derived species involves hydroxyl radicals ($OH^•$). This was investigated by using salicylate and phenylalanine, attack of $OH^•$ upon which leads to the formation of 2,3- and 2,5-dihydroxybenzoates, and *o*-, *m*- and *p*-tyrosines respectively. On addition of $ONOO^-$ to salicylate, characteristic products of hydroxylation (and nitration) were observed in decreasing amounts with rise in pH, although added products of hydroxylation of salicylate were not recovered quantitatively at pH 8.5, suggesting further oxidation of these products and underestimation of hydroxylation at alkaline pH. Hydroxylation products decreased in the presence of several $OH^•$ scavengers, especially formate, to extents similar to those obtained when hydroxylation was achieved by a mixture of iron salts, H_2O_2 and ascorbate. However, $OH^•$ scavengers also inhibited formation of salicylate nitration products. *Ortho*, *p*- and *m*-tyrosines as well as nitration products were also observed when $ONOO^-$ was added to phenylalanine. The amounts of these products again decreased at high pH and were decreased by addition of $OH^•$ scavengers. We conclude that although comparison with Fenton systems suggests $OH^•$ formation, simple homolytic fission of peroxynitrous acid (ONOOH) to $OH^•$ and $NO_2^•$ would not explain why $OH^•$ scavengers inhibit formation of nitration products.

Keywords: Peroxynitrite, hydroxyl radical, aromatic hydroxylation, phenylalanine, salicylate, nitric oxide

INTRODUCTION

Peroxynitrite ($ONOO^-$) is a cytotoxic species generated by the rapid (rate constant $>10^9$ $M^{-1}s^{-1}$)[1] reaction of superoxide ($O_2^{•-}$) with nitric oxide ($NO^•$)[2]

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$$

Peroxynitrite formation has been implicated as a contributor to tissue injury in several human diseases, including atherosclerosis,[3] rheumatoid arthritis,[4] inflammatory bowel disease,[5] neurodegenerative disorders[6–8] and adult respiratory distress syndrome.[9] Peroxynitrite can be directly damaging to biological molecules e.g. it can oxidise protein thiol groups and methionine residues.[10–12] In addition, at physiological pH, $ONOO^-$ proto-

nates to peroxynitrous acid [ONOOH] which generates a wide range of cytotoxic products, whose chemical identity is not well established. Suggested cytotoxic species include nitrogen dioxide ($NO_2^{\bullet}$), nitronium ion (NO_2^+), a vibrationally-excited state *trans* isomer of peroxynitrous acid and hydroxyl radical ($^{\bullet}OH$).[10–14] Although simple homolytic fission of ONOOH, would be expected[16] to produce $NO_2^{\bullet}$ and $OH^{\bullet}$

$$ONOOH \rightarrow NO_2^{\cdot} + OH^{\cdot}$$

it has been argued that this reaction is thermodynamically unlikely[17] and studies of solvent effects have been used to argue that a "caged radical" mechanism is also unlikely.[12,18] Nevertheless, some evidence has been presented to show that $OH^{\bullet}$ can be produced by decomposition of peroxynitrite at pH 7.4[2,14,15,16] although there is also evidence to the contrary.[17,19]

Two of the most definitive techniques for detecting $OH^{\bullet}$ are ESR-spin trapping and aromatic hydroxylation.[20,21] Attempts to detect $OH^{\bullet}$ from $ONOO^-$ by spin trapping experiments suggested that small amounts[22] or none[19,23] are formed. On the other hand, van der Vliet *et. al.*[14] showed that $ONOO^-$ caused hydroxylation of phenylalanine (Phe), forming *ortho-*, *meta-*, and *para*-tyrosines, (in low yields) at pH 7.4. Formation of these products, which are characteristic of $OH^{\bullet}$ attack upon Phe,[21,24] was partially inhibited by the $OH^{\bullet}$ radical scavenger mannitol. However, species other than $OH^{\bullet}$ can hydroxylate aromatic compounds, e.g. singlet oxygen ($^1O_2$) and cytochromes P450 can convert salicylate to 2,5-dihydroxybenzoate.[21,25] It seems likely that one of the problems in all these studies is that $ONOO^-$ and/or products derived from it can undergo further reactions with the initial products of attack upon detector molecules such as spin traps.[19,22]

In the present paper, we evaluated and compared hydroxlation of aromatics upon $ONOO^-$ addition and by a known $OH^{\bullet}$ generating system[26], a mixture of $FeCl_3$-EDTA, H_2O_2 and ascorbate, at pH 6.0, 7.4 and 8.5. We used two aromatic detector molecules, namely phenylalanine (Phe) and salicylic acid (2-hydroxybenzoate, HB). The characteristic products of hydroxylation of Phe by $OH^{\bullet}$ are *ortho-*, *meta-* and *para*-tyrosines[21,24] while attack of $OH^{\bullet}$ on HB generates 2,3- and 2,5-dihydroxybenzoates.[21,27–29] Detection of these products would be strong circumstancial evidence for $OH^{\bullet}$ formation[21] from $ONOO^-$ breakdown. Although cytochromes P-450 and $^1O_2$ can hydroxylate salicylate, only 2,5-dihydroxybenzoate and not 2,3-dihydroxybenzoate is formed.[21,25,29]

MATERIALS AND METHODS

DL-Phenylalanine (Phe), L-Dopa, tyrosines (*o-*, *m-* and *p*-tyr), 3-nitro-L-tyrosine (NO_2tyr), *p*-nitro-DL-phenylalanine (NO_2Phe), salicylic acid (HB), 2,3- and 2,5-dihydroxybenzoic acids (2,3- and 2,5-DHB), catechol (cat), 5-nitrocatechol (NO_2–cat), 5-nitro-2-hydroxybenzoic acid (NO_2HB), deoxyribose, histidine, mannitol, sodium formate, ascorbate and all other reagents were of the highest purity available from Sigma Chemical Company, UK.

Peroxynitrite was synthesised as previously described.[10] Briefly, an acidic solution (5 ml, 0.6M HCl) of H_2O_2 (0.7M) was mixed with KNO_2 (5 ml, 0.6M) on ice for 1 sec and the reaction quenched with ice cold NaOH (5 ml, 1.2M). This stock was then frozen overnight (–20°C) and the top layer of the solution collected for the experiment. Concentrations of stock $ONOO^-$ were determined before each experiment[10] by measuring the absorbance at 302 nm; ϵ_{302} = 1670 $M^{-1}cm^{-1}$; the usual concentrations achieved were 250–300 mM.

Reaction Conditions

Salicylate or phenylalanine (both 5 mM) in potassium phosphate buffer (100 mM) were prepared at various pH values—6.0, 7.4 and 8.5. The final concentration of peroxynitrite was 1 mM in all

experiments. Final concentrations of Fenton reagents used were Fe(III) = 5 μM, EDTA = 15 μM, ascorbate = 50 μM and H_2O_2 = 50 μM. $FeCl_3$ and EDTA were pre-mixed before addition to the reaction system.

The final incubation volumes were 1 ml and all incubations were carried out at 37°C for 20 min. Peroxynitrite was added to a preincubated (10 min) reaction mixture with rapid vortex-mixing. Hydroxyl radical scavengers sodium formate (100 mM), mannitol (100 mM), deoxyribose (100 mM) or histidine (10 mM and 50 mM) were used at the final concentrations stated.

DETECTION AND ANALYSIS OF PRODUCTS BY HPLC

Columns (25 × 4.6 mm) were from HPLC Technology, Wellington House, Cheshire, UK, except for the Hibar guard with a C_{18} cartridge (BDH, Poole, UK). The pump was from Polymer Laboratories, Essex Road, Church Stretton, Shropshire, UK.

The identity of the peaks detected was confirmed by retention time, spiking with authentic standards and cyotodiode array detection using a Gynkotek diode array detector (model UVD 320) from HPLC Technology. Peak heights were measured and concentrations determined from a standard curve. All separations were at ambient temperature. *Products of hydroxylation and nitration of Phe* were analysed using a Nucleocil 5 μm C_{18} column with guard. The eluent was 500 mM KH_2PO_4-H_3PO_4 (pH 3.0) with methanol (90:10, v/v) at a flow rate of 1 ml min^{-1} and the UV detector set at 274 nm (sensitivity 0.01). *Products of hydroxylation of salicylate* were measured using a Spherisorb 5 μm ODS2 C_{18} column with guard. The eluent was 30 mM sodium citrate/27.7 mM acetate buffer (pH 4.75) with methanol (97:3, v/v) at a flow rate of 0.9 ml min^{-1}. The DHB's were detected with an electrochemical detector (EDT, LCA 15) equipped with a glassy carbon working electrode and a Ag/AgCl reference electrode. The detector potential was set at +0.65 volts. *Nitrocatechol and nitrosalicylic acid* were measured using a Nucleocil 5 μm C_{18} column with guard. The eluent was 500 mM KH_2PO_4-KOH (pH 6.6) with methanol (70:30, v/v) at a flow rate of 1 ml min^{-1}. The UV detector was set at 320 nm.

RESULTS

Peroxynitrite-Dependent Salicylate Hydroxylation and the Effect of Scavengers.

Attack of $^{\bullet}OH$ upon salicylate gives two characteristic products, 2,3- and 2,5-DHB. Addition of $ONOO^-$ or an authentic $^{\bullet}OH$-generating system (iron-EDTA/ascorbate/H_2O_2) to salicylate in buffer (100 mM KH_2PO_4) at pH 6.0, 7.4 and 8.5 resulted in the formation of the two DHB's in approximately equimolar amounts (Fig. 1A and B). The amounts formed decreased sharply with pH in the case of $ONOO^-$, but not as markedly in the Fenton system. The $ONOO^-$-dependent hydroxylation was inhibited by several $OH^{\bullet}$ scavengers in a concentration-dependent manner (data shown for histidine) to similar extents as observed in the Fenton systems (Table 1). Products of nitration of salicylate and catechol were also observed and an HPLC method was developed to separate these (Fig. 2). Their levels were also decreased in the presence of $OH^{\bullet}$ scavengers (Table 2). Generation of products in this and other experiments reported in this paper was not inhibited by treating the solution of $ONOO^-$ with MnO_2 to remove H_2O_2, or by adding catalase (100 enzyme units ml^{-1}). Although high concentrations of $ONOO^-$ can inactivate catalase, inactivation was insignificant under our reaction conditions. The pH of the reaction mixture remained approximately the same after the addition of $ONOO^-$.

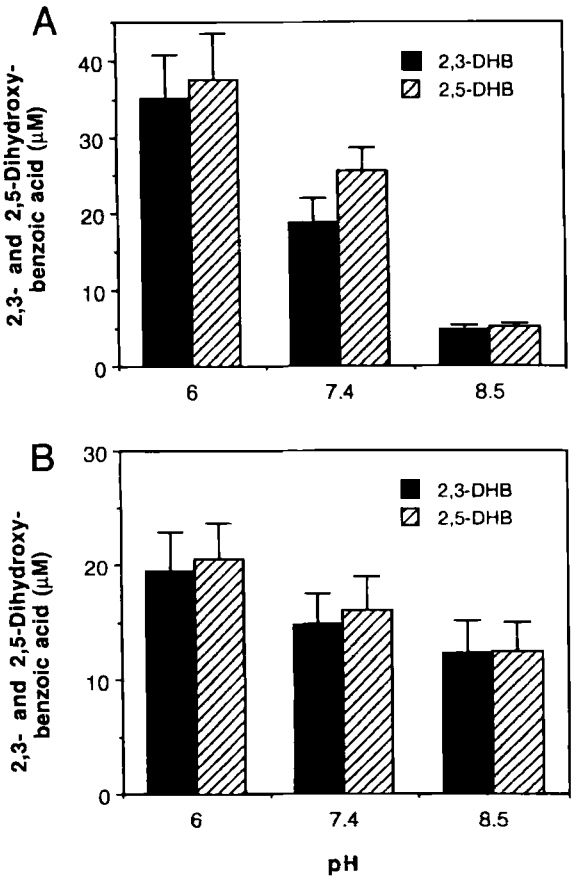

FIGURE 1 **A** Amounts of 2,3- and 2,5-DHB's measured after peroxynitrite (1.0 mM) was added to salicylate (5.0 mM) in KH_2PO_4 buffer (100 mM) at pH 6.0, 7.4 and 8.5. Data are mean ± S.E., n = 4. **B** Amounts of 2,3- and 2,5-DHB's measured after salicylate (5.0 mM) was reacted with Fenton reagents (Fe^{3+} = 5 μM, EDTA = 15 μM, Ascorbate = 50 μM and H_2O_2 = 50 μM) in KH_2PO_4 buffer (100 mM) at pH 6.0, 7.4 and 8.5. Data are mean ± S.E., n = 4.

The Amounts of 2,3- and 2,5-DHB's Measured may be Underestimates of those Actually Formed at pH 8.5

We found that 2,3-DHB and 2,5-DHB are rapidly lost on addition of $ONOO^-$ (data not shown). It was therefore important to examine whether this happens in reaction mixtures containing salicylate, which would result in an underestimate of hydroxylation. Salicylate was mixed with 2,3-DHB and 2,5-DHB (25 μM each) before the addition of $ONOO^-$. Table 3 shows that at pH 6.0 and 7.4 essentially all of the added dihydroxybenzoates were recovered whereas this was not the case at pH 8.5.

Phenylalanine Hydroxylation by Fenton and $ONOO^-$ Systems at 37°C

Addition of $ONOO^-$ or Fenton reagents to Phe in buffer (100 mM KH_2PO_4) at pH 6.0, 7.4 and 8.5 led to the formation of *o*-, *m*- and *p*-tyrosines, consistent with earlier data[14] (Fig. 3). Traces of dihydroxyphenylalanine (dopa) were also observed (Table 4). Peroxynitrite-dependent generation of these products (Fig. 3A) decreased at higher pH values, consistent with the salicylate data (Fig. 3A). Again, pH had little effect in the Fenton system. Hydroxylation of phenylalanine upon addition of $ONOO^-$ was inhibited by $^{\bullet}OH$ scavengers in a similar way to salicylate hydroxylation and to hydroxylation of phenylalanine by the Fenton system (Table 4). Nitrotyrosine and nitrophenylalanine were also detected and the amounts again decreased on addition of $^{\bullet}OH$ scavengers (Table 5).

Effect of Added Hydroxylated Products

If dopa, or tyrosines (*o*-, *m*-, *p*-) or 3-nitrotyrosine (10 μM each) were mixed with Phe before addition of $ONOO^-$, *m*- and *o*-tyrosines were essentially quantitatively recovered at all pH values. There appeared to be some loss of *p*-tyrosine at alkaline pH and considerable loss at all pH values of dopa (Table 6). Interestingly, inclusion of these products did not increase the amounts of nitrophenylanine (NO_2Phe) or nitrotyrosine (NO_2tyr) detected after addition of $ONOO^-$ (Table 6, last 2 columns). Loss of dopa may be due to its facile oxidation/nitration because of the presence of two hydroxyl groups on the benzene ring. This could also explain why some previous researchers have not detected dopa.

TABLE I Products of hydroxylation of salicylic acid (HB) at pH's 6.0, 7.4 and 8.5 with Fenton reagents or with $ONOO^-$. The effect of hydroxyl radical scavengers.

Reaction Conditions		2,3 DHB-μM		2,5 DHB-μM	
		$ONOO^-$	Fenton	$ONOO^-$	Fenton
pH 6.0		30.4	34.8	25.6	28.3
+ Deoxyribose	(100 mM)	23.8 (22%)	18.1 (48%)	23.6 (8%)	20.0 (29%)
+ Mannitol	(100 mM)	11.5 (62%)	6.8 (80%)	9.8 (62%)	7.7 (73%)
+ Formate	(100 mM)	2.0 (93%)	0.7 (98%)	0.1 (99.6%)	0.8 (97%)
+ Histidine	(10 mM)	14.9 (51%)	27.6 (21%)	15.6 (39%)	13.5 (52%)
+ Histidine	(50 mM)	4.0 (87%)	—	4.5 (82%)	—
pH 7.4		22.5	21.3	27.7	29.4
+ Deoxyribose	(100 mM)	15.3 (32%)	20.9 (1.8%)	17.0 (39%)	29.0 (1.4%)
+ Mannitol	(100 mM)	5.5 (76%)	5.9 (72%)	7.1 (74%)	8.5 (71%)
+ Formate	(100 mM)	4.2 (81%)	0.6 (97%)	5.3 (81%)	0.9 (97%)
+ Histidine	(10 mM)	21.8 (3%)	17.4 (18%)	21.6 (22%)	24.5 (16%)
+ Histidine	(50 mM)	5.5 (76%)	—	2.5 (91%)	—
pH 8.5		5.2	18.1	6.1	13.5
+ Deoxyribose	(100 mM)	0.5 (90%)	5.8 (68%)	0.3 (95%)	3.7 (73%)
+ Mannitol	(100 mM)	0.1 (98%)	6.5 (64%)	0.2 (97%)	4.6 (66%)
+ Formate	(100 mM)	0.1 (98%)	0.5 (97%)	0.1 (98%)	0.6 (96%)
+ Histidine	(10 mM)	0.6 (88%)	14.6 (19%)	0.4 (93%)	16.1 (19%)
+ Histidine	(50 mM)	n.d.	—	n.d.	—

The percentage inhibition by scavengers is given in brackets. Results are expressed as a mean of duplicate experiments with reproducibility of 90–100%.
n.d.—not detected. 2,3-DHB 2,3-dihydroxybenzoate; 2,5-DHB 2,5-dihydroxybenzoate.

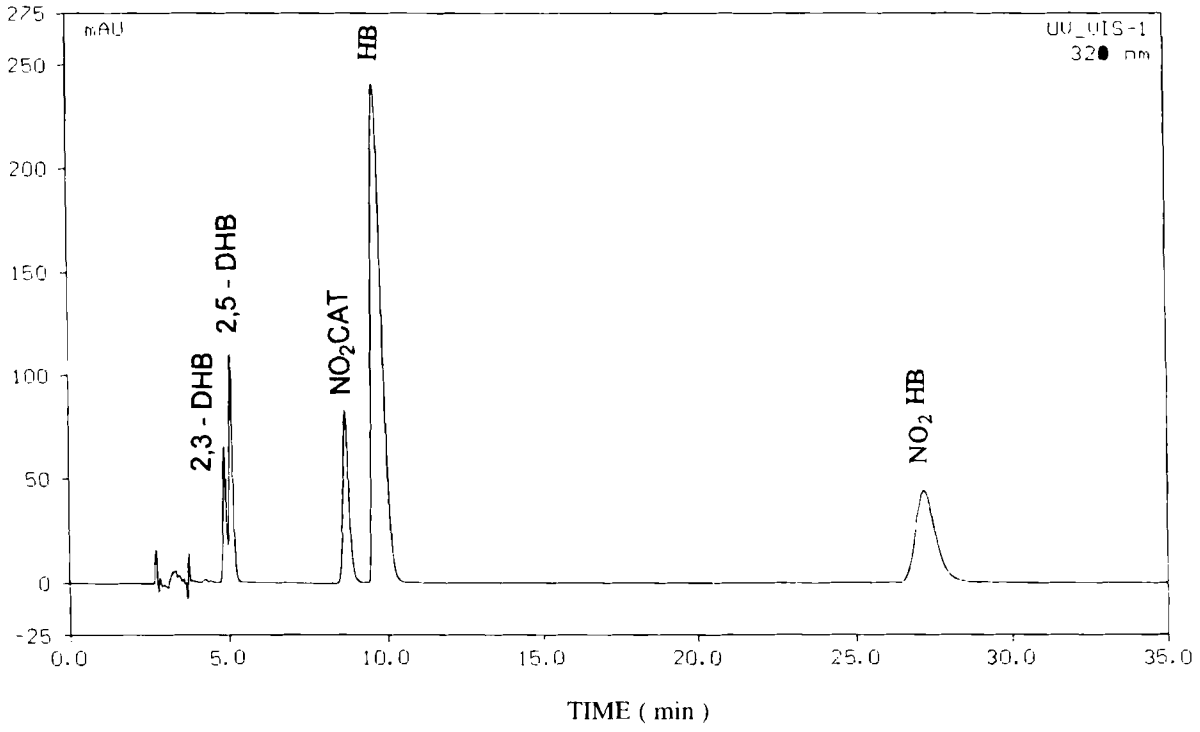

FIGURE 2 HPLC separation of 2,3-, 2,5-DHB (25 μM each), 5-nitrocatechol (25 μM, NO_2CAT), 5-nitrosalicylic acid (25 μM, NO_2HB) and salicylic acid (1.25 mM, HB) using the HPLC conditions outlined in the Materials and Methods section. Detection was at 320 nm.

TABLE II Products of nitration of salicylic acid (HB) at pH's 6.0, 7.4 and 8.5 with/without $OH^{\bullet}$ scavengers.

Reaction Conditions		NO_2cat μM	NO_2HB μM
pH 6.0		1.5	10.7
+ Deoxyribose	(100 mM)	1.0 (33%)	9.5 (11%)
+ Mannitol	(100 mM)	0.5 (67%)	6.5 (39%)
+ Formate	(100 mM)	0.2 (87%)	2.3 (79%)
+ Histidine	(10 mM)	1.0 (33%)	8.5 (21%)
+ Histidine	(50 mM)	0.3 (80%)	2.8 (74%)
pH 7.4		0.5	12.9
+ Deoxyribose	(100 mM)	0.2 (60%)	11.5 (4%)
+ Mannitol	(100 mM)	0.1 (80%)	9.0 (25%)
+ Formate	(100 mM)	0.1 (80%)	1.5 (88%)
+ Histidine	(10 mM)	0.4 (20%)	8.5 (29%)
+ Histidine	(50 mM)	0.1 (80%)	2.8 (77%)
pH 8.5		n.d.	5.6
+ Deoxyribose	(100 mM)	n.d.	3.1 (45%)
+ Mannitol	(100 mM)	n.d.	2.6 (54%)
+ Formate	(100 mM)	n.d.	2.3 (59%)
+ Histidine	(10 mM)	n.d.	4.2 (25%)
+ Histidine	(50 mM)	n.d.	3.6 (36%)

n.d.—not detected. NO_2cat 5-Nitrocatechol; NO_2HB 5-nitrohydroxybenzoate (5-nitrosalicylate). The percentage inhibition by scavengers is given in brackets. The results are expressed as a mean of duplicate experiments with reproducibility of 90–100% and are obtained from the same experiments as shown in Table 1.

DISCUSSION

Koppenol *et. al.*[30] have suggested that the reaction of $ONOO^-$ with aromatic compounds produces products of both hydroxylation and nitration by direct attack of *trans* peroxynitrous acid rather than by $OH^{\bullet}$, although effects of $OH^{\bullet}$ scavengers were not examined. These reactions have the same activation energy as the isomerization to nitrate (NO_3^-), 18 Kcal mol^{-1}, and do not depend on the concentration of the aromatic compound.

We show that addition of $ONOO^-$ to aromatic compounds (salicylate or phenylalanine) at pH's 6.0, 7.4 and 8.5 led to a pattern of hydroxylation characteristic of trapping $OH^{\bullet}$, viz., 2,3-

TABLE III Effect of peroxynitrite upon 5 mM salicylate (HB) in the presence or absence of added 2,3- and 2,5-DHB's (25 μM each). The results are expressed as a mean of duplicate experiments with reproducibility of 90–100%.

Reaction		2,3-DHB-μM	Total amount of the added 2,3-DHB recovered-μM	2,5-DHB-μM	Total amount of the added 2,5-DHB recovered-μM
pH 6.0	HB + $ONOO^-$	30.4	—	25.6	—
	HB + $ONOO^-$ + DHB's	53.2	22.8	49.8	24.2
pH 7.4	HB + $ONOO^-$	22.5	—	27.7	—
	HB + $ONOO^-$ + DHB's	45.8	23.3	51.7	24.0
pH 8.5	HB + $ONOO^-$	16.1	—	17.5	—
	HB + $ONOO^-$ + DHB's	19.2	3.1	19.8	2.3

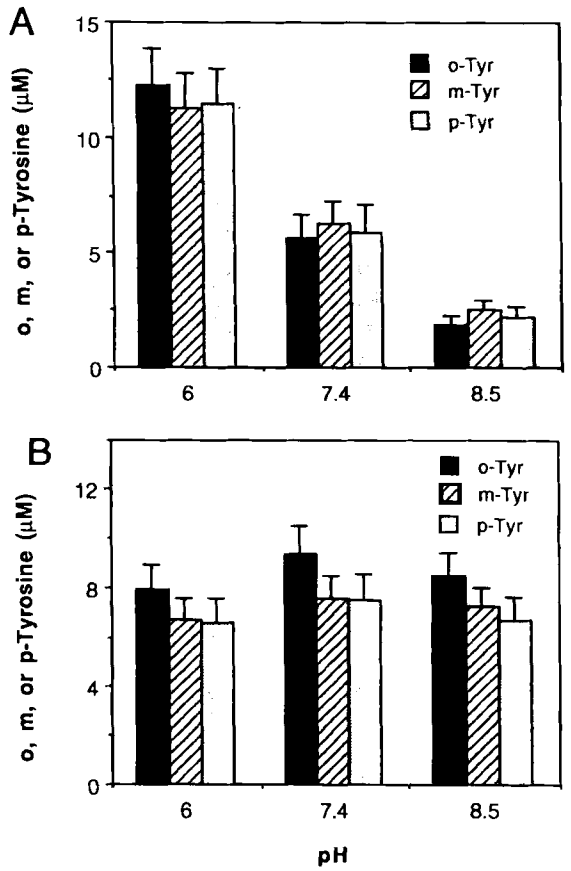

FIGURE 3 **A** Amounts of tyrosines (*o*-, *m*-, *p*-) measured after peroxynitrite (1.0 mM) was added to phenylalanine (5.0 mM) in KH_2PO_4 buffer (100 mM) at pH 6.0, 7.4 and 8.5. Data are mean ± S.E., n = 4. **B** Amounts of tyrosines (*o*-, *m*-, *p*-) measured after phenylalanine (5.0 mM) was reacted with the Fenton reagents (Fe^{3+} = 5 μM, EDTA = 15 μM, Ascorbate = 50 μM and H_2O_2 = 50 μM) in KH_2PO_4 buffer (100 mM) at pH 6.0, 7.4 and 8.5. Data are mean ± S.E., n = 4.

and 2,5-DHB from salicylate, and dopa, *o*-, *m*- and *p*-tyrosines from phenylalanine. Similar products were obtained in parallel experiments with Fenton systems. Other known hydroxylating systems such as enzyme-based oxo-iron species (e.g. in cytochromes P-450) and singlet O_2 give different patterns of hydroxylation from OH•.[25]

Further evidence that OH• is somehow involved is provided by the inhibitory effects of a wide range of OH• scavengers (only mannitol was used in our earlier study[14]). The inhibitions obtained are comparable to those in Fenton systems assayed in parallel. Although there has also been considerable dispute regarding formation of OH• in Fenton systems (reviewed in[31]), the major groups who originally contested this now appear to accept that OH• is formed when iron chelated to EDTA reacts with H_2O_2.[32,33] It seems unlikely that *trans* peroxynitrous acid would react with all the scavengers and hydroxylate two different aromatic compounds in a manner so similar to OH• from an authentic Fenton system.

But is "free" OH• really formed? Attempts to detect it using spin trapping have been equivocal. One problem to be considered is the effect of $ONOO^-$ and products derived from it on the assay methodology, as illustrated by the problems with detecting dopa observed in the present study. Thus inability to detect OH• using the spin trap DMPO in an ESR-spin trapping experiment could be due to destruction of the DMPO-•OH spin adduct by $ONOO^-$ or its decomposition products.[22] The major products of aromatic hydroxylation (2,3 DHB, 2,5 DHB, *o*, *m*, *p*-tyrosines) do not appear to suffer greatly from this problem.

Although our evidence suggests that OH• is somewhow involved, it also seems unlikely that the mechanism involves simple homolytic fission of ONOOH to OH• and reaction of the latter with our aromatic detectors/OH• scavengers. Indeed, formation of *nitration* products was also inhibited by OH• scavengers, in agreement with our earlier finding[34] that OH• scavengers (dimethylsulphoxide, ethanol, mannitol) inhibit $ONOO^-$-induced nitration of tyrosine. All three of these scavengers (at 50 mM) increased the production of nitrite (NO_2^-) when 1 mM $ONOO^-$ was added to 50 mM phosphate buffer at pH 7.4.[34] Further, addition of tyrosines (*o*-, *m*-, *p*-) to Phe did not lead to an increase in the level of 3-nitrotyrosine detected on subsequent addition of $ONOO^-$, suggesting that the nitrotyrosine detected does not simply result from the conversion Phe → tyrosine → nitrotyro-

TABLE IV Products of hydroxylation of phenylalanine with $ONOO^-$ or Fenton reagents at pH's 6.0, 7.4 and 8.5 with/without $OH^\bullet$ scavengers.

Reaction conditions		DOPA-μM		*o*-Tyr-μM		*m*-Tyr-μM		*p*-Tyr-μM	
		$ONOO^-$	Fenton	$ONOO^-$	Fenton	$ONOO^-$	Fenton	$ONOO^-$	Fenton
pH 6.0		3.9	2.3	18.1	7.7	13.0	7.1	12.5	7.3
+ Deoxyribose	(100 mM)	3.7 (5%)	2.2 (4%)	15.8 (13%)	4.6 (40%)	8.0 (38%)	4.4 (38%)	11.3 (10%)	5.1 (30%)
+ Mannitol	(100 mM)	1.0 (74%)	1.0 (57%)	7.6 (58%)	1.9 (75%)	4.5 (65%)	1.5 (79%)	9.2 (26%)	2.2 (70%)
+ Formate	(100 mM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.0 (28%)	3.4 (53%)
+ Histidine	(10 mM)	3.1 (21%)	1.6 (30%)	13.1 (28%)	6.5 (16%)	11.5 (12%)	5.6 (21%)	11.2 (10%)	6.3 (14%)
pH 7.4		2.4	3.8	4.1	14.4	4.5	8.0	10.3	9.6
+ Deoxyribose	(100 mM)	1.9 (21%)	1.9 (37%)	3.3 (20%)	12.5 (13%)	3.5 (22%)	5.5 (31%)	8.2 (20%)	5.8 (40%)
+ Mannitol	(100 mM)	1.6 (33%)	0.7 (82%)	1.9 (54%)	3.1 (78%)	1.8 (60%)	1.5 (81%)	4.6 (55%)	2.2 (77%)
+ Formate	(100 mM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	9.3 (10%)	7.3 (24%)
+ Histidine	(10 mM)	1.5 (38%)	4.6 (21%)	2.8 (32%)	11.3 (22%)	3.5 (22%)	7.5 (6%)	9.5 (10%)	8.8 (8%)
pH 8.5		1.4	3.5	1.8	7.0	2.8	0.6	5.2	0.8
+ Deoxyribose	(100 mM)	1.0 (29%)	2.2 (37%)	1.6 (11%)	4.3 (39%)	1.6 (43%)	0.3 (50%)	4.3 (17%)	0.5 (38%)
+ Mannitol	(100 mM)	0.5 (64%)	1.0 (71%)	0.6 (67%)	1.6 (77%)	0.4 (86%)	0.4 (33%)	1.7 (67%)	0.6 (25%)
+ Formate	(100 mM)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.6(12%)	0.3 (62%)
+ Histidine	(10 mM)	1.0 (29%)	1.3 (37%)	1.7 (5%)	5.9 (16%)	0.8 (71%)	0.2 (66%)	4.3 (17%)	0.5 (38%)

n.d.—not detected. *o*-Tyr, *o*-tyrosine; *m*-tyr, *m*-tyrosine; *p*-tyr, *p*-tyrosine.
The results are a mean of duplicate experiments. The percentage inhibition by scavengers is given in brackets.

TABLE V Products of nitration of phenylalanine with $ONOO^-$ at pH's 6.0, 7.4 and 8.5 with/without $OH^\bullet$ scavengers.

Reaction Conditions		NO_2Tyr-μM	NO_2Phe-μM
pH 6.0		17.9	19.5
+ Deoxyribose	(100 mM)	11.8 (34%)	11.5 (41%)
+ Mannitol	(100 mM)	2.9 (84%)	3.5 (82%)
+ Formate	(100 mM)	n.d.	n.d.
+ Histidine	(10 mM)	15.9 (11%)	10.6 (46%)
pH 7.4		1.25	3.0
+ Deoxyribose	(100 mM)	1.15 (8%)	2.5 (17%)
+ Mannitol	(100 mM)	0.4 (68%)	0.6 (80%)
+ Formate	(100 mM)	n.d.	n.d.
+ Histidine	(10 mM)	0.8 (36%)	2.3 (23%)
pH 8.5		0.8	0.5
+ Deoxyribose	(100 mM)	0.6 (25%)	0.1 (80%)
+ Mannitol	(100 mM)	0.6 (25%)	0.1 (80%)
+ Formate	(100 mM)	n.d.	n.d.
+ Histidine	(10 mM)	0.7 (13%)	0.5

n.d.—not detected. NO_2Tyr, 3-nitrotyrosine; NO_2Phe, *p*-Nitrophenylalanine. The results are a mean of duplicate experiments. The percentage inhibition by scavengers is given in brackets. Data are from the same experiments as in Table 4.

sine but may involve some concerted mechanism. Of course, we must bear in mind the possible formation of other isomers of nitrotyrosine or nitrophenylalanine as well as other products such as dihydroxy, dinitro, nitro-dihydroxy-, dinitrohydroxy-adducts, since there are as yet unidentified peaks on the chromatograms. Another factor we have not considered is the complex reactions between $ONOO^-$ and CO_2/HCO_3^-, which are present in all air-exposed aqueous solutions and at 25mM levels in human blood plasma.[2,36,37]

The simplest explanation of our results would be that decomposition of ONOOH can produce a species with reactivity very similar to $OH^\bullet$. It may be that addition of $OH^\bullet$ scavengers/detectors induces the formation of this species by promoting ONOOH decomposition to a hydroxylating species, without forming a nitrating species. However, the amount of hydroxylation/nitration that takes place is only a small percentage of the total $ONOO^-$ added, suggesting it is not a major reaction pathway. Even if $OH^\bullet$ were formed, the relevance of this *in vivo* is uncertain giving the direct damaging effects of $ONOO^-$/ONOOH themselves and of other products of their decomposition. However, it will be another source of this damaging species in a metal ion independent pathway, arising from elevated levels of $NO^\bullet$ and $O_2^{\bullet-}$.[35] Our development of a method for measuring nitration products of salicylic acid might also be useful in detecting $ONOO^-$ formation in biological systems, especially as salicylate has already been used to detect $OH^\bullet$ in rheumatoid patients[29].

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TABLE VI Effect of adding $ONOO^-$ to phenylalanine (Phe, 5 mM) or to a mixture of Phe with dopa and the three tyrosines (*o*-, *m*-, *p*-) at a final concentration of 10 μM each.

Product measured	DOPA -μM	Total amount of added dopa recovered -μM	*p*-tyr -μM	Total amount of added *p*-tyr recovered -μM	*m*-tyr -μM	Total amount of added *m*-tyr recovered -μM	*o*-tyr -μM	Total amount of added *o*-tyr recovered -μM	NO_2-tyr -μM	NO_2Phe -μM
pH 6.0 Phe + $ONOO^-$	3.9	—	12.5	—	13.0	—	18.1	—	17.9	19.5
" " + dopa + tyr (*o*-, *m*-, *p*-)	5.4	1.5	25.2	13.0	22.4	9.4	29.3	11.2	18.7	21.0
pH 7.4 Phe + $ONOO^-$	2.4	—	10.3	—	4.5	—	4.1	—	1.25	2.5
" " + dopa + tyr (*o*-, *m*-, *p*-)	2.96	0.6	11.9	1.6	13.3	8.8	13.2	9.1	1.65	3.2
pH 8.5 Phe + $ONOO^-$	1.4	—	5.2	—	2.8	—	1.8	—	0.8	0.5
" " + dopa + tyr (*o*-, *m*-, *p*-)	2.4	1.0	13.4	8.2	11.1	8.3	10.7	8.9	2.4	0.9

The results are expressed as a mean of duplicate experiments with reproducibility of 90–100%.

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