

The Hydroxylation of Phenylalanine and Tyrosine: A Comparison with Salicylate and Tryptophan¹

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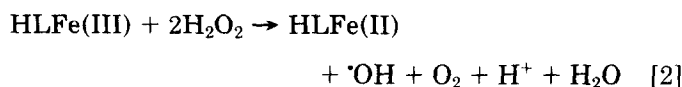
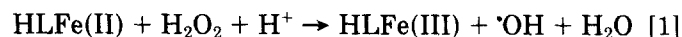
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The hydroxylation of phenylalanine by the Fenton reaction and γ -radiolysis yields 2-hydroxy-, 3-hydroxy-, and 4-hydroxyphenylalanine (tyrosine), while the hydroxylation of tyrosine results in 2,3- and 3,4-dihydroxyphenylalanine (dopa). Yields are determined as a function of pH and the presence or absence of oxidants. During γ -radiolysis and the Fenton reaction the same hydroxylated products are formed. The final product distribution depends on the rate of the oxidation of the hydroxyl radical adducts (hydroxycyclohexadiene radicals) relative to the competing dimerization reactions. The pH profiles for the hydroxylations of phenylalanine and tyrosine show a maximum at pH 5.5 and a minimum around pH 8. The lack of hydroxylated products around near pH 8 is due to the rapid oxidation of dopa to melanin. The relative abilities of iron chelates (HLFe(II) and HLFe(III)) to promote hydroxyl radical formation from hydrogen peroxide are nitrilotriacetate (nta) > ethylenediaminediacetate (edda) \gg hydroxyethylenediaminetriacetate > citrate > ethylenediaminetetraacetate > diethylenetriaminepentaacetate > adenosine 5'-triphosphate > pyrophosphate > adenosine 5'-diphosphate > adenosine 5'-monophosphate. The high activity of iron-nta and -edda chelates is explained by postulating the formation of a ternary Fe(III)-L-dopa complex in which dopa reduces Fe(III). The hydroxylations of phenylalanine and tyrosine are similar to that of salicylate (Z. Maskos, J. D. Rush, and W. H. Koppenol, 1990, *Free Radical Biol. Med.* 8, 153-162) and tryptophan (preceding paper) in that oxidants augment the formation of hydroxylated products by catalyzing the dismutation of hydroxyl radical adducts to the parent compound and a stable hydroxylated product. A comparison of salicylate and the amino acids tryptophan, phenylalanine, and tyrosine clearly shows that salicylate is the best indicator of hydroxyl radical production.

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We recently investigated the mechanism and products of the hydroxylations of salicylate (1) and tryptophan (preceding paper) by radiation and Fenton chemistry. Tryptophan gives a variety of products and requires the presence of a metal to obtain measurable amounts. Salicylate gives two products and is often used as a scavenger of the hydroxyl radicals (2-6). However, it has been suggested that phenylalanine and tyrosine also can be used to detect hydroxyl radicals (7), although neither the yield of hydroxylated products nor the reaction mechanism has been determined. The suitability of these amino acids to detect hydroxyl radicals is examined in this paper. Hydroxyl radicals result from the one-electron reduction of hydrogen peroxide by ferrous complexes as in Reaction [1]. Ferric complexes can be reduced by hydrogen peroxide as in Reaction [2] (8), a reaction which also produces hydroxyl radicals and is unfavorable by only a few kcal/mol. The Gibbs energy change of the reduction of a ferric complex ($E^{\circ} \sim 0.1$ V) by a single hydrogen peroxide is +14 kcal/mol (8) and is not considered here.



We studied the yield of the hydroxylated products of phenylalanine and tyrosine in both systems to determine the abilities of various ferrous and ferric chelates to generate hydroxyl radicals. The chelating agents selected for investigation included those which have biological importance such as citrate, pyrophosphate, and adenine-containing nucleotides, as well as aminopolycarboxylates

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TABLE I

Hydroxylation of 5.0 mM Phenylalanine by γ -Radiolysis and the Fenton Reaction in 10 mM Acetate Buffer, pH 5.5

Reaction and conditions	Total yield ^a (%)	Product distribution				
		Hydroxyphenylalanine (μ M)			Dopa (μ M)	
		4-	3-	2-	2,3-	3,4-
γ -Radiolysis (dose: 570 Gy)						
N ₂ O	(24)	12	12	10	0.4	2
N ₂ O, 0.50 mM Fe(III)-edta	(44)	22	20	21	2	3
Air	(65)	33	31	38	1	1
Fenton reaction						
N ₂ , 1.0 mM Fe(II)-edta, 1.0 mM H ₂ O ₂	(24)	32	31	28	9	4

^a The percentage total yield is relative to the theoretical yield, which is taken to be one product per two hydroxyl radicals. In the calculation of the total yield the amounts of 2,3- and 3,4-dopa were left out.

such as dtpa,³ edta, hedta, edda, and nta which have been used widely in model studies (9).

EXPERIMENTAL PROCEDURES

γ -Radiolysis experiments were carried out at a dose rate of 19 Gy/min with the ⁶⁰Co source of the Nuclear Science Center of Louisiana State University, as described previously (1) and in the preceding paper. Reaction products were separated by high-pressure liquid chromatography; see Ref. (1) and the preceding paper. Products were eluted at a flow rate of 0.85 ml/min from a 250 × 4.6-mm Microsphere 300 C₁₈ 5- μ m reverse phase column (Alltech). The mobile phase consisted of 30 mM citric acid and 27 mM acetic acid. The retention times, relative to phenylalanine, of 3,4-dihydroxyphenylalanine (3,4-dopa), 2,3-dihydroxyphenylalanine (2,3-dopa), tyrosine, 3-hydroxyphenylalanine, and 2-hydroxyphenylalanine relative to phenylalanine were 0.54, 0.63, 0.67, 0.80, and 0.92, respectively. Concentrations of products were determined from calibration with a standard solution containing phenylalanine, 3,4-dihydroxyphenylalanine, tyrosine, 3-hydroxyphenylalanine, and 2-hydroxyphenylalanine or calculated from the intensity of the electrochemical signal generated from 2,3-dihydroxyphenylalanine.

Fenton reactions were carried out anaerobically (nitrogen) or aerobically (air) in the presence of 5.0 mM phenylalanine. The scavengers 3-hydroxyphenylalanine, 2-hydroxyphenylalanine, and tyrosine were used at a concentration of 1.0 mM, due to their limited solubility in water. The reactions were initiated by mixing equal concentrations of the ferrous complex with hydrogen peroxide in 10 mM buffer solution. The final concentrations of ferrous complexes were 1.0, 0.20, or 0.10 mM, depending on the experiment. Generally, the ligand concentration was sufficient to ensure at least 99% complexation of ferrous ions, but the molar ratio of ligand to ferrous ion, [L]/[Fe(II)], did not exceed 10.

The decomposition of hydrogen peroxide by Fe(III)L was studied in air-saturated solutions. The reactions were initiated by addition of hydrogen peroxide to a solution containing 5.0 mM phenylalanine or 1.0 mM tyrosine and 0.10 mM of Fe(III)L. The concentration of hydrogen peroxide before reaction was 0.40 mM. The solutions were buffered with 10 mM acetate, pH 5.5. The [L]/[Fe(III)] ratio in all these experiments

was 2. The concentrations of hydroxylated products were measured every 2–4 min. The decomposition of hydrogen peroxide by Fe(II)L was carried out under the same conditions.

The oxidation of 3,4-dopa was carried out in an air-saturated solution and in the presence of 10 mM buffer which was acetate, phosphate, or borate depending on the pH. The yields of oxidation products—dopachrome (5,6-indolinedione-2-carboxylate) and melanin—were observed spectrophotometrically as a function of pH. The concentration of dopachrome was confirmed by HPLC. The retention time of dopachrome relative to phenylalanine is 0.65. Dopachrome was prepared as described by Palumbo *et al.* (10). Preparation of synthetic melanin was carried out according to Felix *et al.* (11). L-Phenylalanine, L-tyrosine, DL-3-hydroxyphenylalanine, DL-2-hydroxyphenylalanine, and DL-3,4-dihydroxyphenylalanine were purchased from Sigma. Water was deionized and purified by reversed osmosis (Marcor Osmonics).

RESULTS

Phenylalanine

The reaction of the hydroxyl radical with phenylalanine leads to the formation of three isomeric tyrosines: 2-hydroxyphenylalanine, 3-hydroxyphenylalanine, and tyrosine (Table I). A secondary hydroxylation is also observed and yields some 2,3-dopa and 3,4-dopa. Isomeric hydroxyphenylalanines were formed in equal amounts independent of hydroxylating system or condition, in agreement with other studies (12–15). These data suggest that during γ -radiolysis and Fenton reactions the same hydroxycyclohexadienyl radicals (or hydroxyl radical adducts) are formed.

In the absence of any oxidant, the yield of hydroxylated products at pH 5.5 in γ -radiolysis experiments did not exceed 24% of the theoretical yield. A theoretical yield of 100% amounts to one hydroxylated product per two hydroxyl radicals, $G(\text{product}) = 0.5 G(^{\bullet}\text{OH})$. The yield of hydroxylated products increases when Fe(III)-edta or dioxygen is present, as reported for salicylate (1). The combined yield of monohydroxylated products reaches a plateau of 44% for a sample containing 0.20 mM of Fe(III)-edta, as indicated by the dashed line in Fig. 1. This yield corresponds to G values of 0.37, 0.33, and 0.35 for 4-, 3-,

³ Abbreviations used: adp, adenosine 5'-diphosphate; amp, adenosine 5'-monophosphate; atp, adenosine 5'-triphosphate; dopa, 3,4-dihydroxyphenylalanine; dtpa, diethylenetriaminepentaacetate; edda, ethylenediaminediacetate; edta, ethylenediaminetetraacetate; hedta, hydroxyethylethylenediaminetriacetate; nta, nitrilotriacetate.

⁴ Dr. A. Sygula, personal communication.

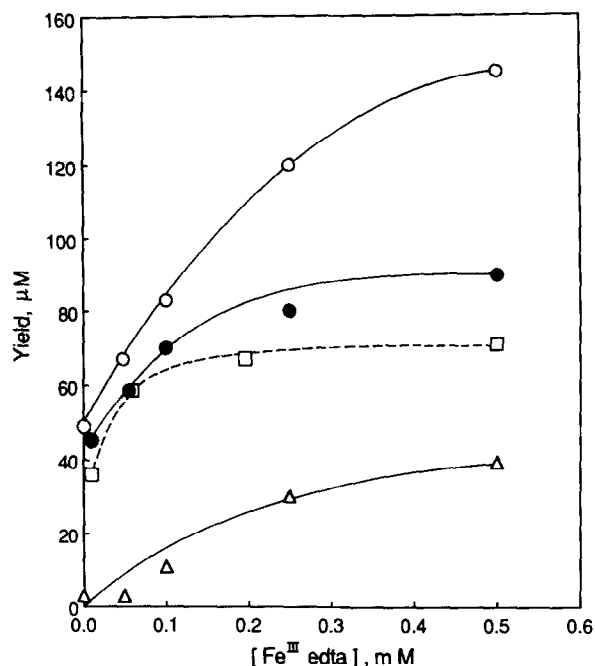


FIG. 1. Yields of hydroxylated products as a function of Fe(III)-edta concentration in irradiated, N_2O -saturated solutions. Dose: 570 Gy; pH 5.5. Symbols: (□) amount of 2-, 3-, and 4-hydroxyphenylalanine (tyrosine) from 5.0 mM phenylalanine; (Δ) 2,3-dopa from 1.0 mM 2-hydroxyphenylalanine; (●) 3,4-dopa from 1.0 mM tyrosine; (○) 2,3- + 3,4-dopa from 1.0 mM 3-hydroxyphenylalanine.

and 2-hydroxyphenylalanine, respectively, which are similar to those reported by those reported by Simic *et al.* (16) in the absence of metal chelates or oxygen.

Tyrosine

A comparison of the yields of dihydroxyphenylalanines from 2-, 3-, and 4-hydroxyphenylalanine, shown in Fig. 1 and Table II, shows that hydroxylation of 2-hydroxyphenylalanine gives exclusively 2,3-dopa, 4-hydroxy-

phenylalanine gives 2,4-dopa, and 3-hydroxyphenylalanine results in a mixture of 2,3- and 3,4-dopa. These observations are in agreement with the notion that the hydroxyl radical is an electrophile: the sites of reaction are those with the highest electron densities, as calculated according to the AM1 method with the MOPAC program (17) by Dr. A. Sygula.

pH Dependences

The effect of pH was studied on the yields of the hydroxylation of phenylalanine and hydroxyphenylalanines during γ -radiolysis and the Fenton reaction. The results are shown in Figs. 2A and 2B. The yields of hydroxylated products of phenylalanine decrease sharply above pH 7, reach a minimum at pH 8, and increase at higher pH. Similarly, yields of the dihydroxyphenylalanines show a maximum at pH 5.5 and decrease below pH 8, but do not show an increase. Both pH profiles show a lack of hydroxylated products at pH values near 8. Monohydroxyphenylalanines are stable and the observation that they are not formed at pH 8 might indicate that dimerization occurs. Solutions of hydroxyphenylalanines that were exposed to hydroxyl radicals at pH 8 became yellow and a black precipitate, probably melanin, formed. This phenomenon did not occur above pH 9.

We briefly investigated the effect of pH on the conversion of 3,4-dopa to melanin. This reaction occurs via several oxidation steps involving intermediates such as semidopaquinone, dopaquinone, or dopachrome (18). Figure 3 shows the optical spectra of an aerated 3,4-dopa solutions after γ -radiolysis. The absorbances at 475 and 600 nm were taken as a measure of dopachrome and melanin concentration, respectively. As shown in Fig. 4, the concentration of dopachrome increases with pH and then declines sharply above pH 7. However, at pH 8 the concentration of black melanin increases and reaches a maximum. The fast rate of 3,4-dopa oxidation and its con-

TABLE II

Hydroxylation of 1.0 mM Hydroxyphenylalanines by γ -Radiolysis and the Fenton Reaction in 10 mM Acetate Buffer, pH 5.5

Reaction and conditions	Yields of dopa from hydroxyphenylalanines, μM (%)		
	2-OH	4-OH	3-OH
	2,3-Dopa ^a	3,4-Dopa	2,3-Dopa/3,4-dopa
γ -Radiolysis (dose: 570 Gy)			
N_2O	3 (3) ^b	48 (29)	15/34 (30)
N_2O , 0.50 mM Fe(III)-edta	40 (25)	93 (57)	74/72 (90)
Air	64 (40)	93 (57)	77/63 (86)
Fenton reaction			
N_2 , 1.0 mM Fe(II)-edta, 1.0 mM H_2O_2	57 (11)	92 (18)	143/150 (59)

^a The percentage yield is given in parentheses and is related to a theoretical yield of one product per two hydroxyl radicals.

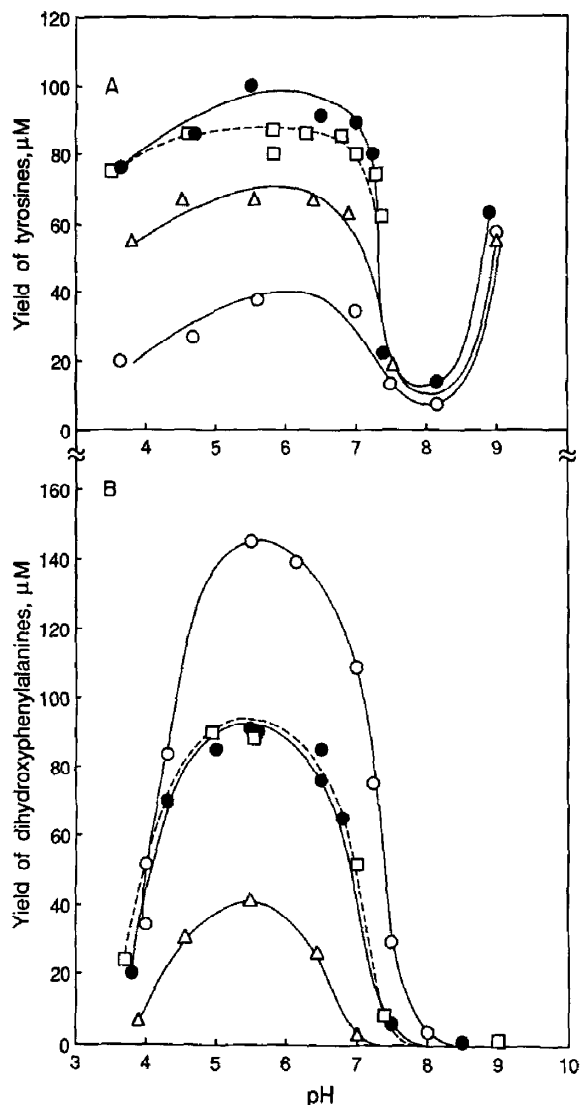


FIG. 2. (A) pH dependence of yields of 2-, 3-, and 4-hydroxyphenylalanine (tyrosine) from irradiated (570 Gy) 5.0 mM phenylalanine solutions. Symbols: (○) N_2O -saturated solution; (△) N_2O -saturated solution containing 0.50 mM Fe(III)-edta; (●) air-saturated solution. (B) pH dependence of yields of dihydroxyphenylalanines from irradiated N_2O -saturated solutions (same dose) containing 0.50 mM Fe(III)-edta. Symbols: (△) 2,3-dopa from 2-hydroxyphenylalanine; (●) 3,4-dopa from tyrosine; (○) 2,3- + 3,4-dopa from 3-hydroxyphenylalanine. The dashed lines and the open squares in A represent the combined yield of 2-, 3-, and 4-hydroxyphenylalanine from the Fenton reaction of 1.0 mM Fe(III)-edta with 1.0 mM hydrogen peroxide. In B the dashed line and open squares indicate the yield of hydroxylated tyrosine (3,4-dopa) from the Fenton reaction under the same conditions.

version to melanin at pH 8 may explain the absence of hydroxylated products at that pH.

Effect of Chelators on the Yield of the Fenton Reaction

Shown in Table III are the effects of different iron chelates on the yields of hydroxyphenylalanines and 3,4-dopa from phenylalanine and tyrosine, respectively, in nitro-

gen- and air-saturated solutions. The yields of hydroxylated products in nitrogen-saturated phenylalanine solutions are small and range between not detectable and $14 \mu M$. The majority of hydroxyl radical adducts dimerize and form diphenylalanine after loss of water (16). Different results are obtained for nitrogen-saturated tyrosine solutions. The yield of 3,4-dopa varied from $5 \mu M$ in the absence of ligand to $84 \mu M$ in the presence of nta. Under the conditions of the experiment a cumulative concentration of $200 \mu M$ hydroxyl is formed, which—based on two hydroxyl radicals per stable product—would give $100 \mu M$ of monohydroxyphenylalanine or dopa. After the oxidation by hydrogen peroxide iron is present in the ferric form, which may catalyze the disproportionation of the hydroxyl radical adduct as described in the preceding paper (Reaction [4]), in competition with dimerization (Reaction [3]). Given the observed yields, ferric ions coordinated to nta, edda, hedta, or citrate are able to catalyze the disproportionation of the hydroxyl radical adduct of tyrosine with some efficiency, but not the disproportionation of the phenylalanine-hydroxyl radical adduct. Dioxygen (0.25 mM at $25^\circ C$ and 50 mM ionic strength (8)) generally improves the yield of hydroxylated products, especially that of the monohydroxyphenylalanines. Since oxygen alone is not very effective in some phenylalanine experiments—it does not improve the yield of experiments

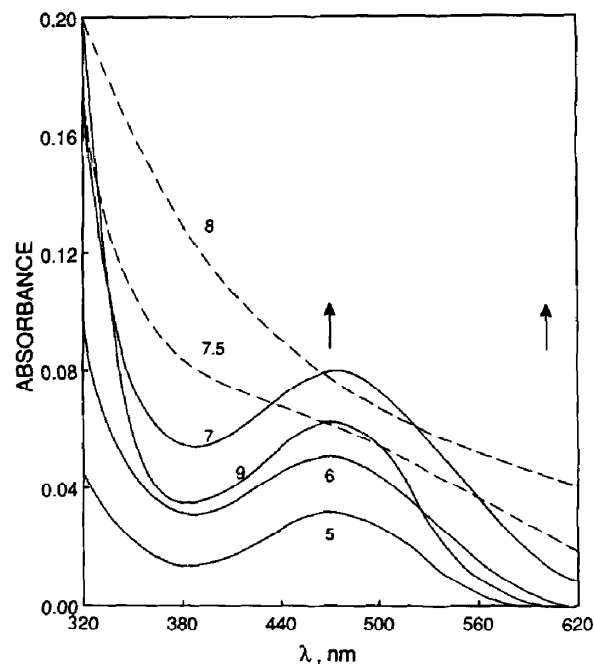


FIG. 3. Optical spectra of aerated 3,4-dihydroxyphenylalanine solutions after γ -radiolysis (570 Gy) in the range of pH 5–9. Optical densities at 475 nm (dopachrome) and 600 nm (melanin) were used to determine the relative amounts of the indicated species. pH values are indicated in the figure. The spectra of the solutions at pH 7.5 and 8.0 are dashed to indicate that the absorbances of these solutions were not stable due to the formation of melanin.

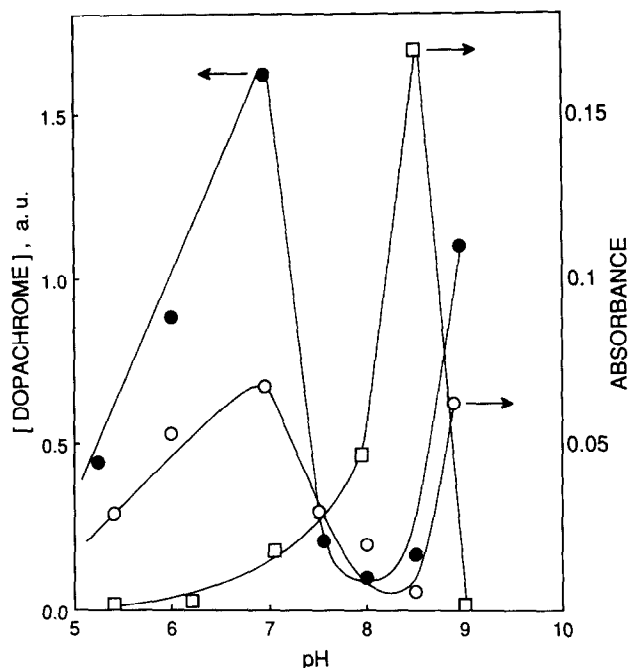


FIG. 4. pH dependence of the yields of oxidation products from irradiated (570 Gy) air-saturated 3,4-dihydroxyphenylalanine solutions. Symbols: (O) absorbance at 475 nm as a measure of the dopachrome concentration; (□) absorbance at 600 nm as a measure of melanin concentration; (●) detection of dopachrome by HPLC (arbitrary units). The solution at pH 7 contains both dopachrome and melanin. We corrected for the presence of the latter by subtracting the absorbance at 600 nm from that at 475 nm.

in which edta, dtpa, or the more biologically relevant chelating agents are present—it is possible that aminopolycarboxylate complexes with solvent accessible coordination sites are reduced by the phenylalanine hydroxyl radical adduct and subsequently oxidized by oxygen. The superoxide so formed may then reduce another phenylalanine hydroxyl radical adduct to phenylalanine and water, thereby completing the catalytic cycle.

The effect of varying the ligand concentration on the formation of 3,4-dihydroxyphenylalanine at a fixed concentration of 0.20 mM iron(II) is shown in Fig. 5. In Table III chelators are listed in order of diminishing stability constants. The efficiency of biological chelators is lower than that of aminopolycarboxylates and decreases as follows: nta > edda > hedta > edta > dtpa. This shows that increased coordination by the aminopolycarboxylate chelator decreases the yield of the hydroxylated products. The biologically relevant chelators, because of their low effective stability constants, were used in higher concentrations ($[L]/[Fe(II)] > 4$). Since the molar ratio of ligand to iron did not exceed 10, a significant part of the ferrous ions was not complexed. The presence of atp or adp at a ligand to iron ratio greater than 3 decreases the yield of hydroxylated products (Fig. 5, lines 9 and 10), probably because the ligands now compete with the scavenger for hydroxyl radicals. No such decrease is observed for edta,

dtpa, hedta, citrate, or pyrophosphate chelates, which generally react slower with the hydroxyl radical. For nta and edda chelates maxima at $[L]/[Fe(II)]$ molar ratio of approximately 2 were observed. It seems likely that the decrease at higher ligand to metal ratios is due not only to scavenging of the hydroxyl radical by the ligand, but also to the formation of an ineffective 2:1 ligand to metal complexes.

Figure 6 presents the yield of hydroxylated products formed in a solution of tyrosine as a function of time. The hydroxylation reactions were carried out in air-saturated solutions to maximize the production of stable hydroxylated products. Hydroxyl radicals were generated in two Fenton-type systems. In the first system, $Fe(II)L + H_2O_2$, the decomposition of hydrogen peroxide was catalyzed by ferrous chelates. In the second system, $Fe(III)L + H_2O_2$, the reaction was induced by ferric chelates. Lines 1 and 1A in Fig. 6 show an increase of the yield of the hydroxylated product of tyrosine with time for both the $Fe(II)$ -nta + H_2O_2 and the $Fe(III)$ -nta + H_2O_2 systems. The formation of 3,4-dopa in both systems takes place in two stages. In the first few seconds of the reaction the yield of 3,4-dopa rapidly increases. The amount of product formed in this stage is proportional to the initial concentration of ferrous nta. A further increase of the amounts of 3,4-dopa takes place over a period exceeding 10 min. A comparison of the ligands edda, hedta, citrate, and edta shows that nta is the most efficient in producing 3,4-dopa.

TABLE III

The Effect of Iron Chelators on the Yields of Hydroxyphenylalanines and 3,4-Dihydroxyphenylalanine in Aerated and Nitrogen-Saturated Solutions

Chelator ^a	Hydroxyphenylalanines from phenylalanine, μM		3,4-Dopa from tyrosine, μM	
	Nitrogen	Air	Nitrogen	Air
dtpa	11	14	6	42
edta	13	14	9	42
hedta	13	40	55	65
edda	11	54	74	74
nta	11	70	84	83
Citrate	— ^b	— ^b	15	54
atp	— ^b	— ^b	6	35
Pyrophosphate	— ^b	— ^b	9	32
adp	— ^b	— ^b	7	12
amp	— ^b	— ^b	8	15
None	— ^b	— ^b	5	9

Note. Reactions were carried out for 10.0 min (phenylalanine) or 5.0 min (tyrosine) in acetate buffer at pH 5.5. $[Phe] = 5.0$ mM, $[Tyr] = 1.0$ mM, $[Fe(II)] = [H_2O_2] = 0.20$ mM.

^a Equilibrium constants for the reaction $Fe(II) + HL \rightarrow Fe(II)L + H^+$ (calculated from Ref. (43)) decrease in the order given. Chelators were present in excess, as given under Experimental Procedures.

^b Amounts of products were small and difficult to determine.

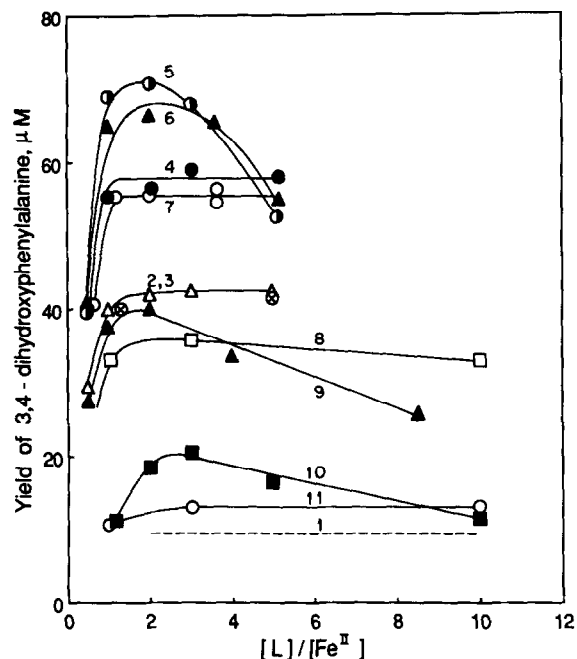


FIG. 5. Yield of 3,4-dihydroxyphenylalanine from an air-saturated tyrosine solution as a function of the chelator to iron ratio in the Fenton reaction. Conditions: $[\text{Fe(II)L}] = 0.20 \text{ mM}$, $[\text{H}_2\text{O}_2] = 0.20 \text{ mM}$, $[\text{tyrosine}] = 1.0 \text{ mM}$, 5.0 mM acetate buffer, pH 5.5; Reaction time $< 30 \text{ s}$. 1, no ligand; 2, edta; 3, dtpa; 4, hedta; 5, edda; 6, nta; 7, citrate; 8, pyrophosphate; 9, atp; 10, adp; 11, amp.

The ability of ferric edda to catalyze the decomposition of hydrogen peroxide was slightly lower than that of ferric nta. No biphasic behavior as described above for nta and edda is observed with hedta, citrate, and edta. The ability of the $\text{Fe(III)-nta}/\text{H}_2\text{O}_2$ system to generate hydroxyl radicals was confirmed by using phenylalanine as a scavenger and monitoring the formation of hydroxyphenylalanines (not shown).

The unusually high activity of iron-nta and -edda chelates is related to the formation of a blue compound in tyrosine solution. In the case of nta a solution of tyrosine immediately turned blue after mixing with the Fe(II) complex and hydrogen peroxide. When Fe(III)-nta was used, the formation of blue color took approximately 10 min. The absorbance spectrum of the blue compound shows a broad peak centered on 620 nm, a smaller peak at 400 nm, and an approximately five times more absorbing peak at 300 nm. These observations indicate that a blue ternary complex is formed among Fe(III) , nta (or edda), and 3,4-dopa.

DISCUSSION

A comparison of hydroxylated products from the reaction of ferrous or ferric chelates with hydrogen peroxide and from radiation experiments suggests that identical hydroxyl radical adducts are formed. The yield of the hydroxylated products strongly depends on pH, oxidant, and

reaction time. The ability of dioxygen to produce hydroxylated products was higher than that of Fe(III)-edta , as shown in Table I. This effect might be due to a more efficient disproportionation of the phenylalanine-hydroxyl radical adduct, thereby diminishing product formation via the dimerization pathway. Our studies show that under optimal conditions it is possible to use phenylalanine and tyrosine for the detection and quantification of hydroxyl radicals.

In a pulse radiolysis study of the reaction of hydroxyl radicals with phenylalanine the distribution of the three isomers of the phenylalanine-OH adduct is reported as 50% *ortho*, 30% *para*, and 14% *meta* (19). The distribution of these transients is determined not only by the electrophilic character of hydroxyl radical and its preferential attack at the least electron deficient ring position, but also by the energetic stabilities of the transients. The final distribution of hydroxylated products differs from that of the hydroxyl radical adducts found in the pulse radiolysis study due to the relative rates of competing side reactions such as dimerization, elimination of water in acid-base-catalyzed reactions, or recombination of hydroxyl radical adducts with hydrogen atom radical adducts. Furthermore, the presence of oxidants influence the final product distribution. A case in point is the hydroxylation of salicylate: equal amounts of the 3- and 5-hydroxycyclohexadiene radical are formed, but the ratio of 2,3- to 2,5-

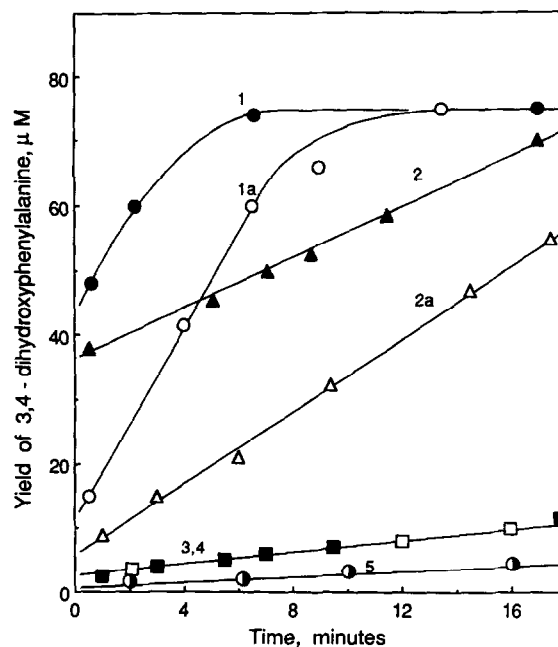


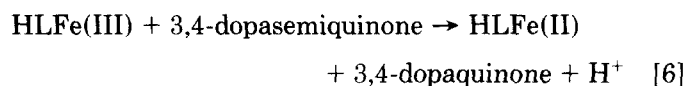
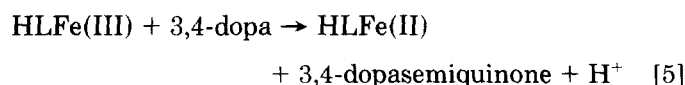
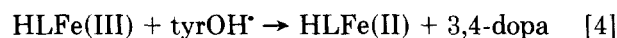
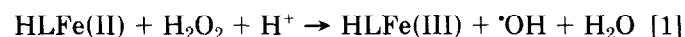
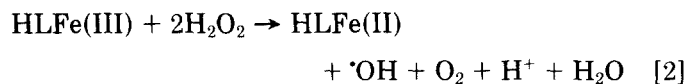
FIG. 6. Time dependence of the yield of 3,4-dihydroxyphenylalanine from an air-saturated tyrosine solution exposed to hydrogen peroxide and 1, Fe(III)-nta ; 1a, Fe(III)-nta ; 2, Fe(II)-edda ; 2a, Fe(III)-edda ; 3, Fe(III)-hedta ; 4, Fe(III)-citrate ; 5, Fe(III)-edta . Reaction conditions: $[\text{HLFe}] = 0.10 \text{ mM}$; $[\text{HL}]/[\text{Fe}] = 2$; $[\text{H}_2\text{O}_2] = 0.40 \text{ mM}$; 5.0 mM acetate buffer, pH 5.5.

dihydroxybenzoate is 5 to 1 and changes to nearly 1 to 1 when oxidants are present.

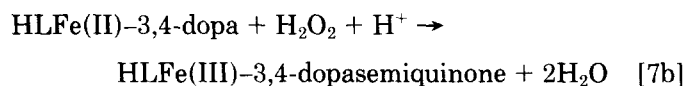
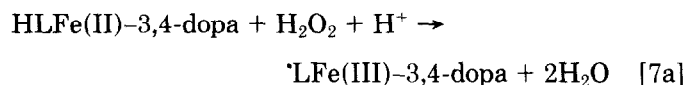
According to a pulse radiolysis study of the reaction of hydroxyl radical with tyrosine (20), 2-OH and 3-OH radical adducts are formed with yields of 50 and 35%, respectively. In our experiments, the hydroxylation of tyrosine led only to the formation of 3,4-dopa. The lack of 2,4-dihydroxyphenylalanine may be due to the fast dimerization reaction of the intermediate hydroxyl radical adduct (20).

The rather rapid formation of hydroxylated products from the reaction of Fe(III)-nta or -edda and hydrogen peroxide as shown in Fig. 6 is somewhat unexpected. Rate constants for Reaction [1], the Fenton reaction, range between $0.80 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ with dtpa as a ligand and $1.0 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ with pyrophosphate (9, 21). At this point no further hydroxyl radical production is expected, because the one electron reduction of a metal complex such as Fe(III)-edta by hydrogen peroxide is thermodynamically unfavorable (8). Less unfavorable is the reaction of one Fe(III) complex with two hydrogen peroxides, as shown in Eq. [2]. The rate of reduction of the ferric complex might well be the limiting factor in this process and could conceivably depend on the reduction potential of the Fe(III)/Fe(II) complex. However, no significant differences are observed: for example, the reduction potential of Fe(III)-/Fe(II)-edta is 0.12 V (22), whereas that of the nta-chelated iron is 0.15 V (23). Thus, the high reactivity of ferric nta in the decomposition of hydrogen peroxide in comparison with that of ferric edda cannot be explained in terms of reduction potentials. The following sequence was established for the ability of different chelating agents to generate hydroxyl radicals: nta > edda > hedta > citrate > edta > dtpa > atp > pyrophosphate > adp > amp. In a study of chelates that catalyze the formation of hydroxyl radicals from superoxide at pH 4.8 similar results were obtained (24). It is possible that the reduction proceeds faster in the case of chelates with water ligands in the coordination sphere. Such an "open" coordination allows the formation of ternary complexes with the hydroxylated product or the hydroxyl radical adduct. In acidic media dopa appears to form several complexes with iron(III) (25, 26) and copper(II) (27). At neutral pH the formation of a ternary complex, Cu(II)-dopa-threonine, was observed (28). Our preliminary spectrophotometric study at pH 5.5 of the blue complex, presumably a ternary complex of Fe(III)-nta(or edda)-dopa, shows maxima at 400 and 615 nm. A similar spectrum for the Fe(III)-2,3-dihydroxybenzoic acid complex was obtained in acidic solution (29, 30). A mechanism for the oxidation of benzene-1,2-diols by ferric ion has been proposed and involves bidentate complex formation, followed by intramolecular and intermolecular electron transfer with ferrous ion and the corresponding *o*-benzoquinone as products (29-32). Analogous to such reactions dopaquinone and ferrous nta (or edda) can be formed as a result of Fe(III)-nta-dopa

complex formation, followed by intramolecular and intermolecular electron transfer.



Ternary complex formation may affect the detection of hydroxyl radicals if these react with the ligand or dopa:



Chain terminations are caused by the disproportionation of the semiquinone radical, the dimerization of tyrosine radicals, and the generation of superoxide from the dopasemiquinone radical, followed by disproportionation. The maximum observed at 400 nm may be due to dopaquinone (33). Reactions [1]-[6] would explain the high yield of hydroxylated products of tyrosine in a system of hydrogen peroxide and ferric complexes of nta, edda, hedta, or citrate.

Ferric ions complexed to nta and edda show the highest catalytic reactivity to produce hydroxyl radicals from hydrogen peroxide. These radicals might be responsible for the toxic effect of nta and edda *in vivo*. Our data support the results of others (34, 35) which show that the reaction of ferric nta with hydrogen peroxide causes damage to the bases of DNA.

It is worth noting that Fe(III)-citrate, a complex that might be of physiological relevance (36), promotes a small but significant increase in the yield of 3,4-dihydroxyphenylalanine. It would seem that this complex can undergo redox cycling, which may be the explanation for the toxicity of iron in a reducing intracellular environment.

In vivo and often *in vitro* it is not possible to scavenge all hydroxyl radicals. This is easily demonstrated by constructing a table of all molecules and ions present, their concentrations, their second-order rate constants with the hydroxyl radical, and the product of the last two quantities (37). This product is the first-order rate of disappearance of the hydroxyl radical due to reaction with a particular

TABLE IV
Comparison of Hydroxyl Radical Scavengers

Scavenger	Rate constant with $\cdot\text{OH}$, $\text{M}^{-1} \text{s}^{-1}$ ^a	Selected hydroxylation product	Yield	
			% ^b	G ^c
Salicylate ^d	1.2×10^{10}	2,3-Dihydroxybenzoate	58	1.6
Tryptophan ^e	1.3×10^{10}	7-Hydroxytryptophan	14	0.38
Indole ^e	3.2×10^{10}	6-Hydroxyindole	6	0.16
Tyrosine ^f	1.3×10^{10}	3,4-Dihydroxyphenylalanine	57	1.5
Phenylalanine ^g	6.5×10^9	2-Hydroxyphenylalanine	24	0.63

^a From Ref. (44).

^b Yield based on a maximum yield of one product per two hydroxyl radicals. The salicylate value is from Ref. (1), and the tryptophan and indole values are from the preceding paper.

^c The G value is the number of molecules formed per 100 eV absorbed. The G value of the hydroxyl radical in a N₂O-saturated solution is 6.0.

^d Yield constant from pH 5 to 9; unlike 2,5-dihydroxybenzoate, the yield of 2,3-dihydroxybenzoate is not dependent on the presence of metals or oxygen.

^e N₂O-saturated solution, 0.40 mM Fe(III)-edta present.

^f Metabolite; N₂O-saturated solution, 0.50 mM Fe(III)-edta present; pH 5.5.

^g Air saturated.

solute. For a high sensitivity a scavenger should react at a diffusion-controlled rate with the hydroxyl radical and give a small number of hydroxyl radical adducts. Furthermore, the hydroxyl radical adduct must disproportionate rather than form dimers, and the final product should be stable and conveniently detected. In Table IV we compare five hydroxyl radical scavengers. The yields of hydroxylated products apply to γ -radiolysis experiments, and those in the Fenton experiments are generally lower. Clearly, the best scavenger is salicylate. Phenylalanine can be used to detect hydroxyl radicals, but its rate constant for reaction with the hydroxyl radical is half as large as that of the salicylate and tyrosine. Tyrosine is useful at pH 5.5, but less suitable at higher pH, because its hydroxylation product, dopa, readily undergoes oxidation near pH 8. The yield of 3,4-dopa from tyrosine is only 18% in the Fenton reaction. In this system 3-hydroxyphenylalanine may be more useful, as equal amounts of 2,3- and 3,4-dopa are formed with a combined yield of 59%. Tryptophan gives low yields for two reasons: there are five major products and redox-active metal complexes do not catalyze the hydroxyl radical adducts efficiently. The yields of the various hydroxylated products depends on the presence of oxygen or metal complexes, as is the case for tryptophan (see preceding paper), phenylalanine, tyrosine, and salicylate (1). In this respect the scheme for the hydroxylation of salicylate presented by Halliwell and co-workers (38), which was recently republished (39), is an oversimplification. The use of aromatic hydroxyl radical scavenger *in vitro* or *in vivo* can be complicated further by specific enzymic hydroxylations (40). We have drawn

attention to the fact that the ratio of 2,3- to 2,5-dihydroxybenzoate can vary from 5 to 1.2 in radiolysis and Fenton studies (1), but that this ratio is about 1 or smaller in two *in vivo* studies (38, 41). The specific hydroxylation of salicylate to 2,5-dihydroxybenzoate by cytochrome P450 (39, 42) provides an explanation for that observation.

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