EPR and Spin-Trapping Investigation of Free Radicals from the Reaction of 4-Methoxybenzenediazonium Tetrafluoroborate with Melanin and Melanin Precursors

Krzysztof J. Reszka* and Colin F. Chignell

Contribution from the Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

Received December 1, 1992

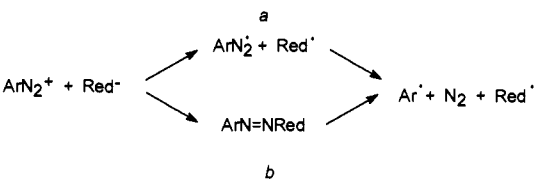
Abstract: The interaction of synthetic DOPA melanin (DM) and its precursors (catechols and phenols) with 4-methoxybenzenediazonium tetrafluoroborate (4-MeO–PhN_2BF_4) has been studied using EPR spectroscopy and the spin-trapping technique. We found that DM, catechol, 3,4-dihydroxybenzoic acid, 3,4-dihydroxyhydrocinnamic acid, 3,4-dihydroxyphenylalanine, and 6-hydroxydopamine all react with 4-MeO–PhN_2^+ through a one-electron-transfer process which gives rise to an aryl radical (4-MeO–Ph$^\bullet$) derived from the diazonium compound and to radicals from melanin and from the catechol(amine)s. The formation of aryl radicals is an autocatalytic process. To explain the autocatalysis, we postulate a mechanism in which the key step is the formation of *o*-quinones. In aerated solutions the aryl radicals react with oxygen, which leads to oxygen consumption. The reaction was found to be order 1, 0.5, and 0.35 with respect to 4-MeO–PhN_2^+, catechol, and oxygen concentration, respectively. Phenol, 4-hydroxyanisole, and tyrosine do not reduce 4-MeO–PhN_2^+ unless they are activated by the enzyme tyrosinase. In the presence of tyrosinase, tyrosine produces the most efficient reducing agent. This indicates that the conversion of phenols to *o*-dihydroxybenzene derivatives by tyrosinase is essential for aryl radical formation from 4-MeO–PhN_2^+. These observations substantiate the ability of hydroquinones and semiquinone radicals to promote the homolysis of diazonium salts to generate aryl radicals. Such reductive activation of diazonium compounds may be pertinent to their biological, mutagenic, and carcinogenic action.

Introduction

Arenediazonium compounds undergo homolytic dediazoniation to produce aryl radicals[1-3] upon reacting with certain electron donors. Two particular mechanisms of this reaction are recognized. One is an outer-sphere mechanism (path *a* in Scheme I) in which direct electron transfer from a reducing agent (Red^-) to ArN_2^+ takes place. The second is an inner-sphere mechanism (path *b* in Scheme I) that involves the formation of an intermediate complex (Ar—N=N—Red) which subsequently decomposes into radicals. If the reaction proceeds according to the former mechanism (*a*), it produces aryldiazenyl radicals (Ar—N=N$^\bullet$) first, which subsequently decompose spontaneously in aqueous solution, liberating N_2 and the corresponding aryl radicals. Evidence for the involvement of free radicals in dediazoniation reactions has been obtained mostly through product analysis and EPR measurements.[4-8]

There have been few studies of the reactions of diazonium ions with dihydroxybenzenes and semiquinone radicals. While the reductive fragmentation of ArN_2^+ has been shown to occur in the presence of catechol, metal ions, such as Cu^{2+}, were always intentionally added to the system.[6] The primary function of catechol was to reduce the metal ion, which then reacted with ArN_2^+ (Sandmeyer reaction). The possibility that catechol can directly reduce ArN_2^+ was not considered.

Scheme I



On the other hand Brown and Doyle[9] have performed a detailed study of the reaction between diazonium ions and *p*-benzohydroquinone (*p*-QH_2). They found that in oxygen-free aqueous buffer, pH 7, the reduction of *p*-nitrobenzenediazonium by *p*-QH_2 results in the formation of *p*-quinone (*p*-Q) and nitrobenzene as the major final products and (*p*-nitrophenyl)-1,2-benzoquinone, formed by arylation of *p*-Q, as a minor product. Kinetic measurements and product analyses indicated that the reaction occurs with the net stoichiometry ArN_2^+/*p*-QH_2 of 2:1, which implicates both the hydroquinone and *p*-semiquinone radical (*p*-QH$^\bullet$ or *p*-$Q^{-\bullet}$) in the reduction of ArN_2^+. The lack of new absorption bands in the UV spectrum of ArN_2^+ in the presence of *p*-QH_2, beyond those attributable to the added reducing agent, suggested that this reaction occurs through an outer-sphere electron-transfer mechanism. Further evidence for this mechanism was provided by showing that the values of the rate constants obtained experimentally could be predicted by Marcus theory of outer-sphere electron transfer processes.

Experimental evidence that *p*-benzosemiquinones reduce ArN_2^+ has been provided by Jirkovsky et al.[10] In their experiments semiquinones were generated by flash photolysis of *p*-Q in the presence of 2,4,6-tri-*tert*-butylphenol as an electron donor. They showed that in this system the rate of the reduction of ArN_2^+ is

* Author to whom correspondence should be addressed: tel no., (919) 541-4751; Fax no., (919) 541-7880; electronic mail, RESZKA@NIEHS.NIH.GOV.

(1) Galli, C. *J. Chem. Soc., Perkin Trans. 2* **1981**, 1459–1461.
(2) Yasui, S.; Nakamura, K.; Ohno, A. *J. Org. Chem.* **1984**, *49*, 878–882.
(3) Galli, C. *Chem. Rev.* **1988**, *88*, 765–792.
(4) Bunnett, J. F.; Takayama, H. *J. Org. Chem.* **1968**, *33*, 1924–1928.
(5) Besse, J.; Zollinger, H. *Helv. Chim. Acta* **1981**, *64*, 529–546.
(6) Hanson, P.; Jones, J. R.; Gilbert, B. C.; Timms, A. W. *J. Chem. Soc., Perkin Trans. 2* **1991**, 1009–1017.
(7) Beckwith, A. L. J.; Norman, R. O. C. *J. Chem. Soc. B* **1969**, 403–412.
(8) Gilbert, B. C.; Hanson, P.; Jones, J. R.; Whitewood, A. C.; Timms, A. W. *J. Chem. Soc., Perkin Trans. 2* **1992**, 629–634.
(9) Brown, K. C.; Doyle, M. P. *J. Org. Chem.* **1988**, *53*, 3255–3261.
(10) Jirkovsky, J.; Fojtik, A.; Becker, H. G. O. *Collect. Czech. Chem. Commun.* **1981**, *46*, 1560–1565.

pH-dependent and decreases on going to acidic pH. This is due to protonation of the radical anion, p-$Q^{-\bullet} + H^+ \Rightarrow p$-$QH^\bullet$ (pK_a = 4.4, ref 10), to give the neutral radical which shows lower reactivity when compared to that of p-$Q^{-\bullet}$. The reduction of p-MeO–PhN_2^+ by p-$Q^{-\bullet}$ at pH 7 proceeds with the rate constant of $k = 1.2 \times 10^6$ M^{-1} s^{-1}.

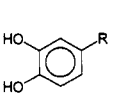
Semiquinone radicals derived from anthraquinone-2-sulphonic acid and anthraquinone-2,6-disulphonic acid also reduce arenediazonium compounds.[11] The anthraquinones were UV irradiated in alcohol/water mixtures, which afforded the semiquinones and alcohol-derived radicals by abstraction of hydrogen atom from alcohol molecules by anthraquinone triplets. In this environment ArN_2^+ was reduced by both the anthrasemiquinones and the alcohol radicals. Thus these experiments indicate that p-hydroquinone, p-semiquinone, and anthrasemiquinone radicals can reduce arenediazonium cations.

Numerous physiologically relevant compounds possess p- and o-hydroquinone groups, and the question can be raised as to whether they also can promote homolytic dediazoniation of ArN_2^+. The question is pertinent, because diazonium cations have been shown to be mutagenic[12] and carcinogenic.[13] It is likely that the biological activity of diazonium compounds might be related to their ability to produce aryl radicals *in vivo*. The reaction might be particularly efficient in those cell regions which possess high concentrations of reducing species.

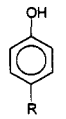
Among the natural substances which possess the o-hydroquinone and o-quinone moieties are eumelanins (referred to as melanins). These are brown-to-black pigments responsible for the coloration of skin and hair in animals and humans. It is believed that the physiological role of melanin is protection against harmful visible and UV radiation.[14] *In vivo* the pigment is synthesized in highly specialized organelles, melanosomes, in melanocytes present in the skin.[15] According to the classical Raper–Mason's model, synthesis of melanin consists of several stages: (1) hydroxylation of tyrosine (a phenol) to DOPA (3,4-dihydroxyphenylalanine); (2) oxidation of DOPA to DOPA quinone; (3) cyclization to leucodopachrome; (4) oxidation to dopachrome; (5) decarboxylation or rearrangement to 5,6-dihydroxyindole or 5,6-dihydroxyindole-2-carboxylic acid; and (6) oxidation to melanin. The two first steps are catalyzed by the enzyme tyrosinase. The current view is that melanin is a heterogeneous polymer containing dihydroxyindole (and its carboxylic acid) and DOPA at various stages of oxidation.[16,17]

Melanin exhibits a persistent endogenous EPR signal originating from the semiquinone radicals trapped in its structure.[16] The intensity of this signal can be increased by one-electron oxidation of hydroquinone or reduction of quinone subunits, or it can be decreased by oxidation or reduction of melanin semiquinones.[18] Changes in the EPR spectra of melanins doped with di- and trivalent metal ions are consistent with complex formation between the metal ion and a radical that has a chelating structure.[19] Thus EPR spectra of metal-complexed radicals in melanins derived from DOPA or catechol indicate that the radicals are o-indolesemiquinones and o-semiquinones.[16,19]

Because the quinol and quinone subunits undergo facile oxidation and reduction, their simultaneous presence in melanin render this pigment highly reactive. Melanin may exchange electrons with various exogenous and endogenous electron donors and acceptors.[18,20-24] For example, melanin can reduce ferricyanide[21,22] and oxidize NAD(P)H[21,22] and ascorbic acid.[20] It is therefore likely that the pigment might be able to reduce arenediazonium cations; however, nothing is known about its reactivity toward this class of compounds. In the present work we examined the reaction between 4-methoxybenzenediazonium tetrafluoroborate (4-MeO–PhN_2F_4B) and synthetic DOPA melanin (DM), a model of the natural eumelanin pigment, and melanin precursors, catechols and phenols (see below). We tested



R	
H	Catechol
COOH	3,4-DHBA
CH_2CH_2COOH	3,4-DHHCA
$CH_2CH(NH_2)COOH$	DOPA
$CH_2CH_2NH_2$	6-OH-dopamine



R	
H	PhOH
OCH_3	4-HA
$CH_2CH(NH_2)COOH$	Tyr

the hypothesis that melanin can induce homolytic dediazoniation of 4-MeO–PhN_2^+, seeking EPR evidence for the formation of the resultant free radicals. Additionally, we examined the ability of selected melanin precursors, catechol(amine)s and phenols to induce similar reactions. We used EPR spectroscopy in conjunction with the spin-trapping technique and found that 4-MeO–PhN_2^+ can be readily reduced by DM and certain catecholic melanin precursors, producing the respective aryl (4-MeO–Ph$^\bullet$) and semiquinone radicals.

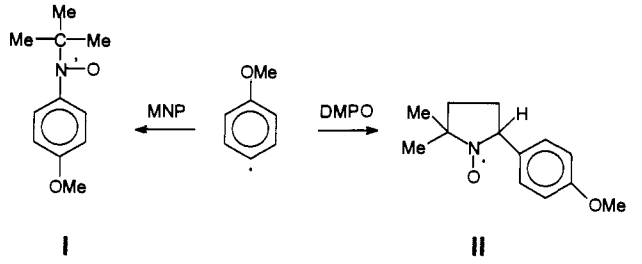
Experimental Section

Materials. 4-MeO–PhN_2BF_4, phenol (PhOH), tyrosine (Tyr), 4-hydroxyanisole (4-HA), catechol, 3,4-dihydroxybenzoic acid (3,4-DHBA), 3,4-dihydroxyhydrocinnamic acid (3,4-DHHCA), 3,4-dihydroxyphenylalanine (DOPA), 6-hydroxydopamine hydrochloride (6-OH-DOPAMINE), zinc acetate, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and 2-methyl-2-nitrosopropane (MNP) were obtained from Aldrich Chemical Company (Milwaukee, WI). Mushroom tyrosinase (EC 1.14.18.1, 3870 units/mg of solid), superoxide dismutase (SOD, EC 1.15.1.1, 2470 units/mg of solid), and catalase (EC 1.11.1.6, 9300 units/mg of solid) were purchased from Sigma Chemical Company (St. Louis, MO). DMPO was purified by distillation under reduced pressure and was stored under nitrogen at −20 °C. The stock solution of zinc acetate (0.47 M) was prepared in acetate buffer to which several drops of glacial acetic acid were added to attain the desired pH value of 4.6. Synthetic DOPA melanin (DM) was a gift from Dr. C. C. Felix (Medical College of Wisconsin, Milwaukee, WI). The method of its preparation has been described.[19]

Methods. EPR measurements were made using a Varian E-Line Century Series EPR spectrometer operating at 9.4 GHz with a 100-kHz modulation frequency and equipped with a TM_{101} cavity. EPR measurements were performed in a flat quartz aqueous cell at room temperature. Hyperfine (hf) splitting constants were evaluated by computer simulation using a program developed by Mr. David R. Duling. EPR kinetic measurements were performed recording the low-field component of a DMPO adduct in time intervals. Oxygen consumption measurements were performed at 25 °C using an oxygraph equipped with a Clark-type oxygen electrode (Yellow Springs, OH). Unless

(11) Fomin, G. V.; Mordvintsev, P. I.; Mkhitarov, R. A.; Gordina, T. A. *Russ. J. Phys. Chem. (Engl. Transl.)* **1980**, *54*, 135–137.
(12) Malaveille, C.; Brun, G.; Kolar, G.; Bartsch, H.; *Cancer Res.* **1982**, *42*, 1446–1453.
(13) Toth, B.; Taylor, J.; Mattson, B.; Gannett, P. *In Vivo* **1989**, *3*, 17–21.
(14) Kollias, N.; Sayre, R. M.; Zeise, L.; Chedekel, M. R. *J. Photochem. Photobiol., B* **1991**, *9*, 135–160.
(15) Jimbow, K.; Fitzpatrick, T. B.; Quevedo, W. C., Jr. In *Biology of the Integument*; Matoltsy, A. G., Ed.; Springer: New York, 1984; Vol. 2, pp 278–292.
(16) Sealy, R. C. In *Free Radicals in Molecular Biology, Aging, and Disease*; Armstrong, D., Sohal, R. S., Cutler, R. G., Slater, T. F., Eds.; Raven Press: New York, 1984; pp 67–76.
(17) Ito, S. *Biochim. Biophys. Acta* **1986**, *883*, 155–161.
(18) Lukiewicz, S.; Reszka, K.; Matuszak, Z. *Bioelectrochem. Bioenerg.* **1980**, *7*, 153–165.
(19) Felix, C. C.; Hyde, J. S.; Sarna, T.; Sealy, R. C. *J. Am. Chem. Soc.* **1978**, *100*, 3922–3926.
(20) Chauffe, L.; Windle, J. J.; Friedman, M. *Biophys. J.* **1975**, *15*, 565–572.
(21) Gan, E. V.; Lam, K. M.; Haberman, H. F.; Menon, I. A. *Br. J. Dermatol.* **1977**, *96*, 25–28.
(22) Gan, E. V.; Haberman, H. F.; Menon, I. A. *Arch. Biochem. Biophys.* **1976**, *173*, 666–672.
(23) Menter, J. M.; Willis, I. *J. Invest. Dermatol.* **1980**, *75*, 257–260.
(24) Menter, J. M.; Townsel, M. E.; Moore, C. L.; Williamson, G. D.; Soteres, B. J.; Fisher, M. S.; Willis, I. *Pigment Cell Research* **1990**, *3*, 90–97.

Scheme II



otherwise stated all experiments were performed in aerated phosphate (50 mM, pH 7.0) or acetate (50 mM, pH 4.6) buffers.

Results

Reductive Fragmentation of Arenediazonium Compounds. In our study we exploited the ability of diazonium compounds to produce aryl radicals upon one-electron reduction (Scheme I). Aryl radicals are short-lived at room temperature and cannot be detected directly by EPR. For this reason we resorted to the method of spin trapping[25] using MNP and DMPO as trapping agents. Aryl radicals add to these spin traps to form more stable adducts (MNP/Ar• and DMPO/Ar•), which are suitable for EPR analysis. The aryl radical derived from 4-MeO–PhN_2^+ adds to MNP and DMPO to form adducts **I** and **II**, respectively (Scheme II). Before we studied the interaction of 4-MeO–PhN_2^+ with melanin, we first characterized its reaction with catecholic and phenolic melanin precursors.

Reaction of 4-MeO–PhN_2^+ with Catechol(amine)s. (A) Spin Trapping. The addition of catechol (1 mM) to 4-MeO–PhN_2^+ (0.1 mM) and MNP in phosphate buffer (pH 7.0) generated the EPR spectrum shown in Figure 1A. The spectrum shows hyperfine (hf) couplings to nitrogen (15.21 G (1N)) and two pairs of equivalent hydrogens (1.81 G (2H) and 0.89 G (2H)), as expected for **I**. Spectra identical to that shown in Figure 1A were observed when DOPA, 6-OH-DOPAMINE, 3,4-DHHCA, or 3,4-DHBA were used as reductants. Control experiments showed that the presence of these agents was necessary to generate these spectra. This confirms that interaction of catechol(amine)s with 4-MeO–PhN_2^+ generates the expected aryl radical (4-MeO–Ph•).

When DMPO (40 mM) was used instead of MNP, the spectrum shown in Figure 1B was observed. Its hf coupling constants (a_N = 16.1 G and a^{β}_H = 25.1 G) indicate formation of a DMPO spin adduct of a carbon-centered radical. Because no other DMPO adducts were observed and because DMPO is very efficient in trapping carbon-centered radicals, the spectrum in Figure 1B was attributed to adduct **II**, formed by trapping of the same aryl radical (4-MeO–Ph•) that was trapped by MNP. The hf couplings of adduct **II** are very close to those found for the same species produced by photolysis of 4-MeO–$PhSO_3Na$ in the presence of DMPO (a_N = 15.92 G and a_H^{β} = 24.95 G).[26]

The amplitude of the EPR signal of **II** changes with reaction time. The reaction proceeds with the fastest rate at higher concentrations of the reducing agent (Figure 1C). At [catechol] = 0.38 and 1 mM, the amplitude of the EPR signal from **II** reached its maximum within 12 min and then decreased (Figure 1C). It is likely that the decrease of the amplitude may be due, at least in part, to reduction of the nitroxide **II** by *o*-semiquinones formed in this system (*vide infra*). The kinetic curves observed at [catechol] = 0.1 and 0.2 mM have an S-shaped profile, which indicates that the reaction is autocatalytic. A similar conclusion has been reached by measuring oxygen consumption (*vide infra*).

Brown and Doyle[9] have reported that the rate of reduction of arenediazonium salts by *p*-hydroquinone is pH-dependent. The rate increases rapidly on going from pH 6 to pH 9, which reflects

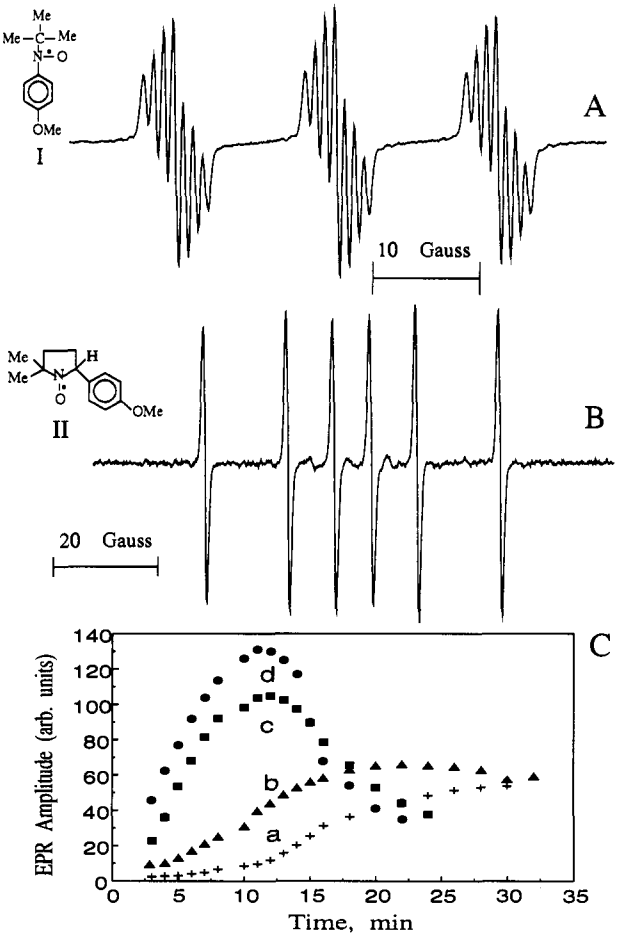


Figure 1. EPR spectra of spin adducts **I** (A) (hf couplings: a_N = 15.21 G, a_H = 1.81 (2H), a_H = 0.89 G (2H)) and **II** (B) (hf couplings: a_N = 16.1 G, a^{β}_H = 25.1 G) generated by the addition of the *p*-MeO–Ph• radical to MNP (A) and DMPO (B). The aryl radicals were generated by the reduction of 4-MeO–PhN_2^+ (0.1 mM) with catechol (1 mM) in the presence of (A) MNP (ca. 14 mM) or (B) DMPO (40 mM) in aerated phosphate buffer (pH 7.0). Instrumental settings: microwave power 10 mW (A), 20 mW (B); modulation amplitude 0.165 G (A), 0.33 G (B); gain 4×10^2 (A), 6.3×10^3 (B); time constant 0.128 s (A), 0.25 s (B); scan rate 16 min (A), 4 min (B). (C) Kinetics of the EPR signal from **II** at various catechol concentrations (mM): a, –0.1; b, –0.2; c, –0.38; d, –1.0.

the higher reactivity of the monobasic form (*p*-QH^-) when compared to the nonionized hydroquinone (*p*-QH_2). We found that when 4-MeO–PhN_2^+ (0.1 mM) reacted with catechol (1 mM) in acetate buffer at pH 4.6, no signal from the adduct **II** was observed. This may indicate that at this pH the reduction of 4-MeO–PhN_2^+ does not occur or is very slow and accordingly the aryl radicals cannot be detected. The pK_a value of catechol is 9.45,[27] which means that at pH 4.6 the concentration of *o*-QH^- is very low ([*o*-QH^-]/[*o*-QH_2] is ca. $10^{-4.85}$). When concentrations of catechol and 4-MeO–PhN_2^+ were increased to 10 and 2 mM, respectively, only a very weak signal from **II** was observed (Figure 2A shows the signal observed during the second scan). However, upon the addition of zinc acetate (50 mM), a significantly stronger signal from the radical **II** was observed at this pH (Figure 2B). Simultaneous with the production of **II** was the formation of semiquinone radicals. The spectrum in Figure 2B shows lines from the *o*-semiquinone/Zn^{2+} complex (*o*-$Q^{-\bullet}/Zn^{2+}$) superimposed on the lines from **II**.

(B) Semiquinone Radicals. The reaction of catechol(amine)s with 4-MeO–PhN_2^+ generates semiquinone radicals. Because these radicals cannot be detected readily in static EPR exper-

(25) Janzen, E. G. *Acc. Chem. Res.* **1971**, *4*, 31–40.
(26) Cholvad, V.; Szabova, K.; Stasko, A.; Nuyken, O.; Voit, B. *Magn. Reson. Chem.* **1991**, *29*, 402–404.
(27) Steenken, S.; Neta, P. *J. Phys. Chem.* **1979**, *83*, 1134–1137.

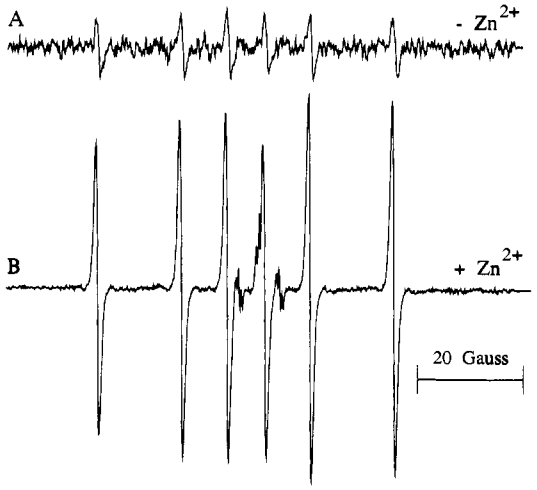


Figure 2. EPR spectra from II observed during interaction of catechol (10 mM) with 4-MeO–PhN_2^+ (2 mM) in acetate buffer, pH 4.6, in the presence of DMPO (40 mM): (A) second scan with Zn^{2+} omitted; (B) in the presence of 50 mM zinc acetate. In the central part there is a signal, triplet of triplets, from the *o*-$Q^{-\bullet}/Zn^{2+}$ complex. Instrumental settings: microwave power 20 mW; modulation amplitude 0.165 G; gain 2.5×10^4 (A), 4×10^3 (B); time constant 0.25 s (A), 0.128 s (B); scan rate 4 min.

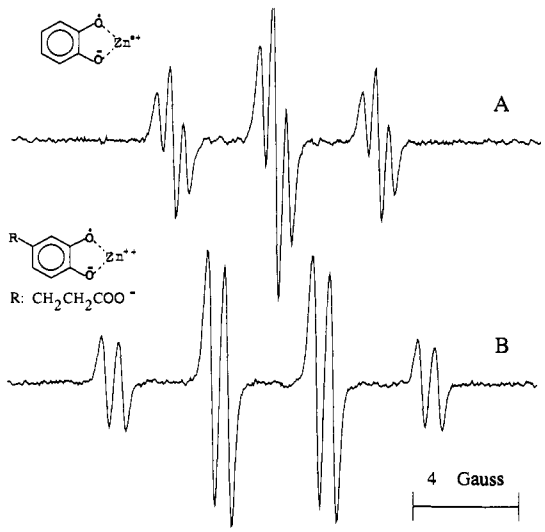


Figure 3. EPR spectra of *o*-semiquinone radical/Zn^{2+} complex from catechol (A) and from 3,4-DHHCA (B). Radicals were generated by the addition of 4-MeO–PhN_2^+ (1.8 mM) to catechol (9 mM) or 3,4-DHHCA (9 mM) in aerated acetate buffer, pH 4.5, containing zinc acetate (80 mM). Hf couplings: 3.94 G (2H) and 0.49 G (2H) for radicals from catechol (A); 4.02 (3H) and 0.62 G (1H) for radicals from 3,4-DHHCA (B). Instrumental settings: microwave power 5 mW; modulation amplitude 0.165 G; gain 10×10^3 (A), 8×10^3(B); time constant 0.25 s; scan rate 8 min.

iments, we used the metal ion-spin stabilization technique.[28] *o*-Semiquinone radicals form complexes with non-paramagnetic divalent metal ions (e.g., Zn^{2+}, Mg^{2+}) that are more stable than metal-free radicals. The addition of 4-MeO–PhN_2^+ (1.8 mM) to catechol (9 mM) and zinc acetate (80 mM) in pH 4.6 acetate buffer produced an EPR spectrum with hf splittings (3.94 G (2H), 0.49 G (2H)) characteristic of the *o*-semiquinone radical (Figure 3A). When catechol was replaced by 3,4-DHHCA (9 mM), an EPR spectrum with hf splittings of 4.02 G (3H) and 0.62 G (H), characteristic of the 3,4-DHHCA-derived semiquinone radical, was observed (Figure 3B). DOPA and 3,4-DHBA also produced semiquinones when added to 4-MeO–PhN_2^+ in the presence of zinc ions (not shown). No signals were observed

(28) Kalyanaraman, B.; Sealy, R. C. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 1119–1125.

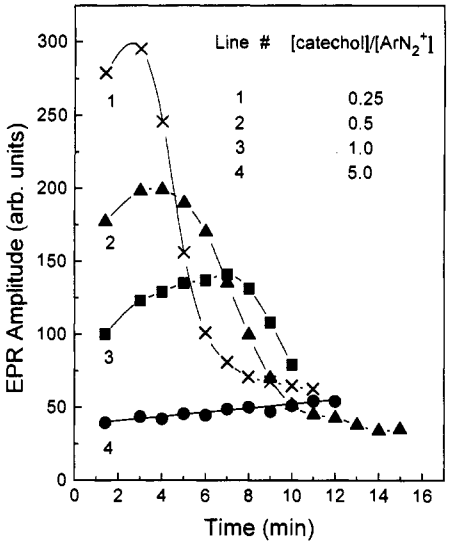


Figure 4. Amplitude of the EPR signal from *o*-semiquinone/Zn^{2+} complex *versus* time for various [catechol]/[ArN_2^+] ratios.

when Zn^{2+} was omitted. The EPR spectra produced by treatment of the catechol(amine)s with 4-MeO–PhN_2^+ are similar to those observed during their UV photolysis or photosensitized oxidation.[29,30]

The intensity of the EPR signal from the *o*-$Q^{-\bullet}/Zn^{2+}$ complex depends on the [catechol]/[4-MeO–PhN_2^+] ratio. This is illustrated in Figure 4, which shows the kinetics of the formation of the EPR signal from this radical in aerated buffer (pH 4.6) in the presence of Zn^{2+} ions at constant catechol concentration (5 mM) and at [catechol]/[4-MeO–PhN_2^+] ratios of 0.25, 0.5, 1.0, and 5. It may be seen that the radicals are generated immediately after addition of 4-MeO–PhN_2^+ (time "zero" in Figure 4). At a 5-fold excess of catechol over 4-MeO–PhN_2^+, the signal was weak and its amplitude remained virtually constant during several minutes of observation. This low concentration of radicals may be due to a rapid exhaustion of the diazonium compound. More intense EPR signals were observed at lower [catechol]/[4-MeO–PhN_2^+] ratios. Under these conditions the amplitude of the EPR signal reached a maximum, after which it decreased. The most intense signals were observed at the lowest [catechol]/[4-MeO–PhN_2^+] ratio (0.25).

We found that the presence of air had a profound effect on the formation of the radicals. In aerated solutions the reaction of catechol with 4-MeO–PhN_2^+ produced an EPR signal from *o*-semiquinone/Zn^{2+} radical that was significantly stronger (ca. 3-fold) compared to the EPR signal observed in a nitrogen-saturated solution (not shown). This observation suggests that the semiquinone radicals are produced more efficiently in aerated solutions.

(C) Oxygen Consumption. EPR measurements showed that oxygen enhances the formation of semiquinone radicals in our systems. Therefore it was of interest to examine whether oxygen is consumed during the reaction of 4-MeO–PhN_2^+ with catechols. Control measurements showed that in pH 7.0 phosphate buffer in the absence of 4-MeO–PhN_2^+ no oxygen uptake occurs in samples containing catechol, 3,4-DHBA, 3,4-DHHCA, or DOPA (0.25 mM each).[31] The addition of 4-MeO–PhN_2^+ to these samples caused either oxygen consumption (catechol, 3,4-DHBA, 3,4-DHHCA, and DOPA) or a marked acceleration of this process

(29) Kalyanaraman, B.; Felix, C. C.; Sealy, R. C. *Environ. Health Perspect.* **1985**, *64*, 185–198.

(30) Reszka, K.; Lown, W. J.; Chignell, C. F. *Photochem. Photobiol.* **1992**, *55*, 359–366.

(31) There was a background oxygen consumption from 6-OH-DOPAMINE even in the absence of the diazonium compound. This catecholamine is known to readily undergo autoxidation even at neutral pH. The process is dependent on the presence of traces of redox-active metal ions, mostly iron.[32]

(32) Gee, P.; Davidson, A. J. *Arch. Biochem. Biophys.* **1984**, *231*, 164–168.

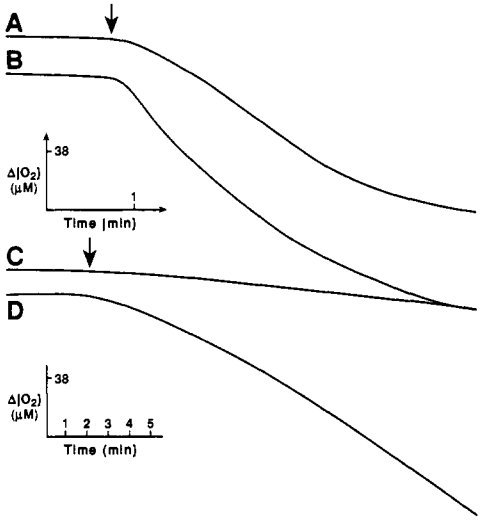


Figure 5. Oxygen consumption during the reduction of 4-MeO–PhN_2^+ in pH 7.0 phosphate buffer, (A) with catechol (0.25 mM) and (B) with DM (ca. 2.75 mg/mL); and in acetate buffer (pH 4.6), (C) with catechol (10 mM), Zn^{2+} omitted, and (D) same as in part C in the presence of Zn^{2+} (47 mM).

Table I. Rate of Oxygen Consumption during the Reaction of 4-MeO–PhN_2^+ (1.25 mM) with Catechol(amine)s (0.25 mM) in pH 7.0 Phosphate Buffer (50 mM)

catechol(amine)	rate, 10^{-3} M min^{-1}
catechol	0.056 ± 0.001
DOPA	0.156 ± 0.004
6-OH-DOPAMINE	0.323 ± 0.026
3,4-DHBA	0.032 ± 0.001

(6-OH-DOPAMINE). The oxygen concentration *versus* time profile has a Z-shape (Figure 5A), which suggests that the reaction is autocatalytic. Consequently, the rate of oxygen consumption varies with reaction time. To characterize the oxygen uptake we used the central, approximately linear sectors of kinetic runs, such as that in Figure 5A. Table I gives such determined rates of oxygen consumption during the interaction of 4-MeO–PhN_2^+ with some selected catechol(amine)s. The addition of SOD (100 units/mL) or catalase (370 units/mL) did not influence the rate of the O_2 uptake, indicating that neither superoxide nor hydrogen peroxide is involved.[33] We conclude that the oxygen uptake must be predominantly due to its reaction with organic radicals, most likely the aryl radicals detected in our spin-trapping experiments (eq 1). This hypothesis is supported by the observation that addition of DMPO (radical scavenger) to the reaction mixture inhibits oxygen consumption (not shown).

$$\text{4-MeO–Ph}^{\bullet} + O_2 \Rightarrow \text{4-MeO–Ph–O–O}^{\bullet} \Rightarrow \Rightarrow \text{products (phenols and hydroquinones)} \quad (1)$$

The rate of oxygen consumption measured at constant catechol concentration was linearly dependent on diazonium concentration (Figure 6A), implying that this reaction is first-order with respect to 4-MeO–PhN_2^+. Kinetic measurements, conducted at two different diazonium concentrations of 0.25 and 1.25 mM, showed that the rate of oxygen consumption is proportional to the square root of the catechol concentration (Figure 6B). This suggests that the reaction is of order 0.5 with respect to catechol. Results from runs at different $[O_2]$, at constant [catechol] and [4-MeO–PhN_2^+], indicated that the reaction is of order 0.35 with respect to oxygen concentration (Figure 6C). Thus the rate of oxygen uptake in the linear region (fast O_2 consumption) can be approximated by eq 2.

$$-d[O_2]/dt = k[\text{4-MeO–PhN}_2^+][\text{catechol}]^{0.5}[O_2]^{0.35} \quad (2)$$

The rate of oxygen consumption is pH-dependent. As Figure 7A shows, no oxygen uptake was observed at pH below 6.5 but above pH 7.0 the rate of this reaction increased significantly. There is a linear relationship between the rate of oxygen uptake and the catecholate anion concentration (Figure 7B). The actual *o*-QH^- concentrations were estimated using eq 3

$$[o\text{-QH}^-] = K[QH_2]_T/([H^+] + K) \quad (3)$$

where $[QH_2]_T$ = 0.25 mM (total catechol concentration) and K(catechol) = $10^{-9.45}$ (ref 27). The lack of oxygen uptake at acidic pH (<6.5) is in agreement with the lower reactivity of *o*-QH_2 when compared to that of *o*-QH^- ions, indicating that radical formation either is very slow or does not occur at all. This idea is supported by the absence of an EPR signal from **II** in this pH range. When the concentrations of catechol and diazonium salt were increased to 10 and 2 mM, respectively (conditions which produced a very weak signal from adduct **II**, Figure 2A), there was some slow oxygen uptake (Figure 5C). When zinc acetate (50 mM) was added, the rate of this process was markedly accelerated (Figure 5D and Table II), and the shape of the $[O_2]$ *versus* time curve indicated that the process is autocatalytic. This is in agreement with the enhanced generation of the radical **II** in the presence of Zn^{2+} observed in our spin-trapping experiments (Figure 2B).

Reaction of 4-MeO–PhN_2^+ with Phenols. Tyrosinase-Catalyzed Dediazoniation. When PhOH, Tyr, or 4-HA (1.0 mM each) were added to a solution containing 4-MeO–PhN_2^+ (0.1 mM) and DMPO (40 mM) at pH 7.0, no radical formation was observed, which indicates that these phenols alone are not capable of reducing the diazonium salt. When similar experiments were performed in the presence of tyrosinase (19.2 μg/mL), the formation of the adduct **II** was observed (Figure 8). The three phenols differ significantly in their ability to induce the formation of **II**. Tyrosine was the most efficient agent because it produced a strong EPR signal from **II** that reached maximum amplitude within ca. 7 min from the start of the reaction (i.e., enzyme addition). On prolonged incubation the signal decreased (Figure 8). PhOH was significantly less efficient, as it produced an EPR signal with an intensity equal to only ca. 20% of that produced by tyrosine (Figure 8). 4-HA was the least efficient in producing **II**, as shown in Figure 8. The low reactivity of 4-HA in this particular assay may be a reflection of the extremely low stability of the 4-HA-derived *o*-hydroquinone and/or the respective *o*-semiquinone radical.[34]

Reaction of 4-MeO–PhN_2^+ with DM. (A) Spin Trapping. The addition of DM to 4-MeO–PhN_2^+ and MNP in aerated phosphate buffer (pH 7.0) generated an EPR spectrum similar to that produced by catechol(amine)s and 4-MeO–PhN_2^+ (Figure 1A). Because hf couplings in both cases were identical, the spectrum produced by the DM + 4-MeO–PhN_2^+ system was also assigned to adduct **I**.

Similarly, the addition of DM to 4-MeO–PhN_2^+ and DMPO in aerated buffer (pH 7.0) produced an EPR spectrum similar to that generated by catechol(amine)s (Figure 1B), and accordingly, it was assigned to adduct **II**. The amplitude of the EPR signal of **II** changed with the time of reaction. For [DM] of 2.6 and 5 mg/mL, the signal reached a maximum after ca. 6 and 4 min from the start of the reaction, respectively, and then began to decrease (Figure 9). The decrease of the EPR signal may result from reaction of **II** with melanin radicals or with the melanin oxidizing groups. Because interaction of the pigment with 4-MeO–PhN_2^+ increases the concentration of melanin radicals and melanin quinone groups (*vide infra*), this explanation for the decay of **II** is plausible.

(33) Some catalase-dependent O_2 recovery was observed from the 6-OH-DOPAMINE sample. In this case the formation of H_2O_2 may be due to autoxidation of that catecholamine and is not related to the reaction with the diazonium compound.

(34) Nilges, M. J.; Swartz, H. M.; Riley, P. A. *J. Biol. Chem.* **1984**, *259*, 2446–2451.

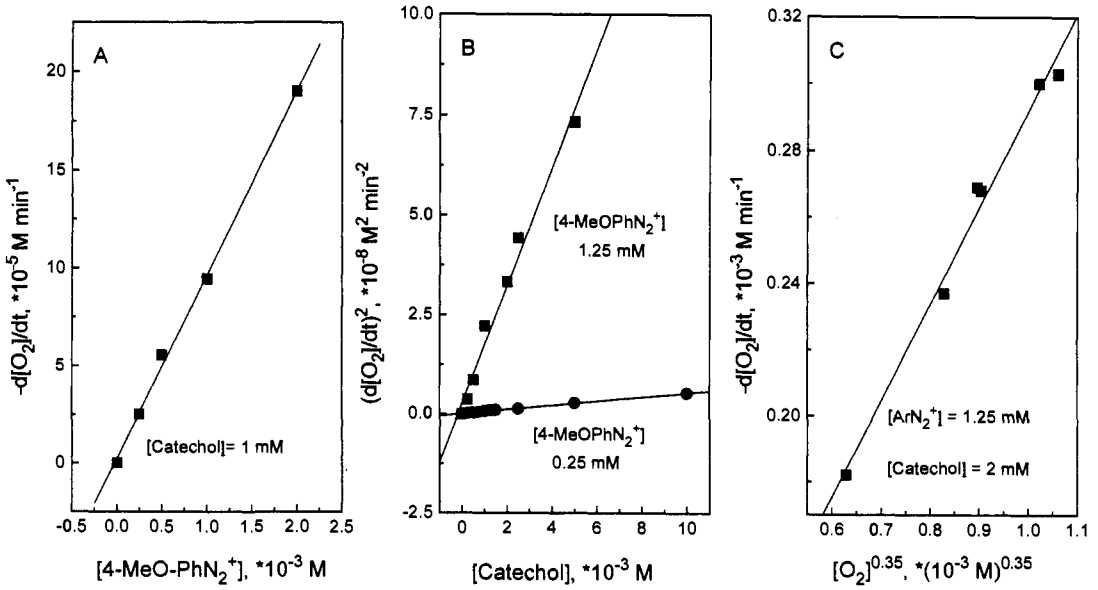


Figure 6. Rate of oxygen consumption during reduction of 4-MeO–PhN_2^+: (A) *versus* [4-MeO–PhN_2^+] at constant [catechol] of 1.0 mM; (B) *versus* [catechol] at constant [4-MeO–PhN_2^+] of 0.25 mM (●) and 1.25 mM (■); (C) *versus* $[O_2]$ at constant [catechol] of 2.0 mM and [4-MeO–PhN_2^+] of 1.25 mM.

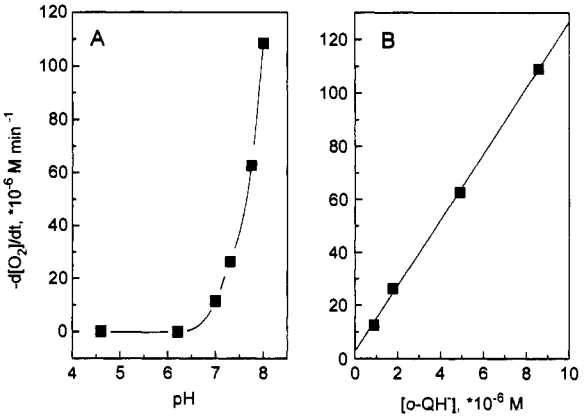


Figure 7. Dependence of the rate of oxygen consumption during reduction of 4-MeO–PhN_2^+ by catechol ploted *versus* pH (A) and *versus* catecholate anion concentration (B). [4-MeO–PhN_2^+] = 0.25 mM, $[catechol]_T$ = 0.25 mM.

Table II. Amount of Oxygen Consumed during the Reaction of Catechol (10 mM) with 4-MeO–PhN_2^+ (2 mM) at pH 4.6: Effect of Zinc Ions

	$-\Delta[O_2]$, 10^{-6} M	
	after 5 min	after 10 min
control	3.5	11
$+Zn^{2+}$ (50 mM)	26.3	66.6

(B) Melanin Radicals. Melanin possesses a natural EPR signal originating from semiquinone radicals present in its structure. It is believed that these radicals exist in equilibrium with nonradical groups (quinones and hydroquinones) as described by eq 4.

$$Q + QH_2 \rightleftarrows 2Q^{\cdot-} + 2H^+ \quad (4)$$

We examined the effect of 4-MeO–PhN_2^+ on the intrinsic melanin EPR signal. After the addition of the arenediazonium compound to DM in buffer (pH 7.0), the EPR signal of the pigment increased. The rate of this process was relatively slow, and the maximum amplitude was reached after several minutes of incubation. Figure 10 shows the signal of the melanin pigment recorded before (A) and after (B) 4-MeO–PhN_2^+ addition. The signal amplitude increases as [4-MeO–PhN_2^+] increases, but then for [4-MeO–PhN_2^+] > 2 mM ([DM] = 2.75 mg/mL), the signal reached a plateau (Figure 10). Comparison of the second integrals of the

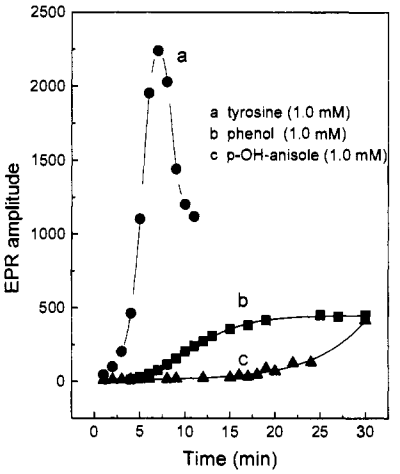


Figure 8. Formation of adduct II in systems containing tyrosine (●), 4-HA (▲), phenol (■) (1 mM), DMPO (40 mM), and mushroom tyrosinase (19.2 μg/mL) in aerated phosphate buffer, pH 7, at 25 °C.

melanin signals, recorded before and after 4-MeO–PhN_2^+ addition, indicated that the increase in signal intensity is real (and is not due to the oxygen-broadening effect), which implies the formation of new melanin radicals. At pH 7 and for [DM] = 2.75 mg/mL, the maximum increase in the radical concentration was ca. 4-fold.

(C) Oxygen Consumption. We found that oxygen was consumed when DM reacted with 4-MeO–PhN_2^+. However, in contrast to the catechol(amine)s, the reaction with DM is not autocatalytic (Figure 5B). This indicates that the reduction of 4-MeO–PhN_2^+ occurs predominantly *via* reaction with only one type of electron donor. Figure 11 shows that the initial rate of oxygen uptake is linearly dependent on [DM] and it can be described by eq 5. Melanins are known to reduce oxygen to

$$-d[O_2]/dt = (1.13 + 24.0[DM, mg/mL]) \times 10^{-6}\ M\ min^{-1} \quad (5)$$

superoxide ion slowly.[35] However, the level of O_2 in phosphate buffer (pH 7.0) containing DM was constant prior to 4-MeO–PhN_2^+ addition. The addition of SOD (100 units/mL) or catalase (370 units/mL) had no effect on the rates of oxygen consumption,

(35) Korytowski, W.; Hintz, P.; Sealy, R. C.; Kalyanaraman, B. *Biochem. Biophys. Res. Commun.* **1985**, *131*, 659–665.

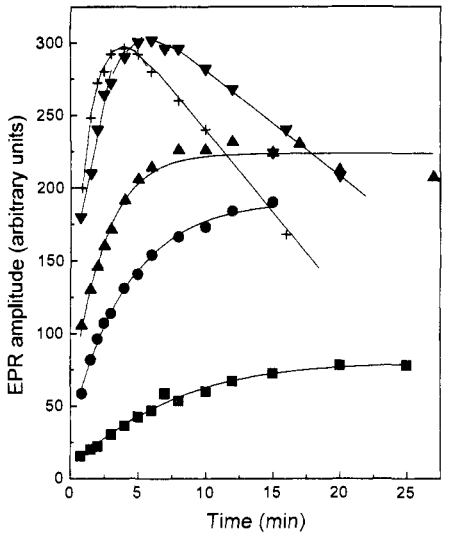


Figure 9. Kinetics of the generation of the EPR signal from II during the reaction of 4-MeO–PhN_2^+ (0.1 mM) with DM in the presence of DMPO (40 mM) in phosphate buffer (pH 7.0). Melanin concentration (mg/mL): (■) 0.1; (●) 0.5; (▲) 1.1; (▼) 2.6; (+) 5.0.

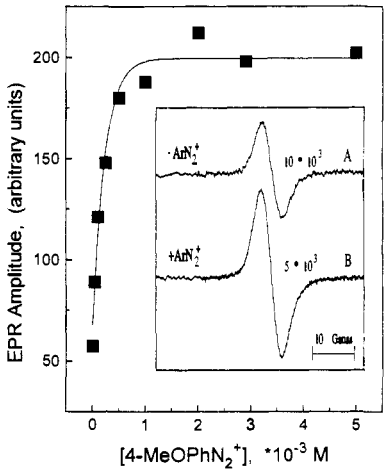


Figure 10. Amplitude of the EPR signal from melanin *versus* [4-MeO–PhN_2^+]. Inset: Melanin EPR signals observed before (A) and after (B) addition of 4-MeO–PhN_2^+. Experimental conditions: [DM] = 2.75 mg/mL, [4-MeO–PhN_2^+] = 0 (A), [4-MeO–PhN_2^+] = 3 mM (B). Instrumental settings: microwave power 1 mW; modulation amplitude 3.3 G; gain 10×10^3 (A), 5×10^3 (B); time constant 0.25 s; scan rate 4 min.

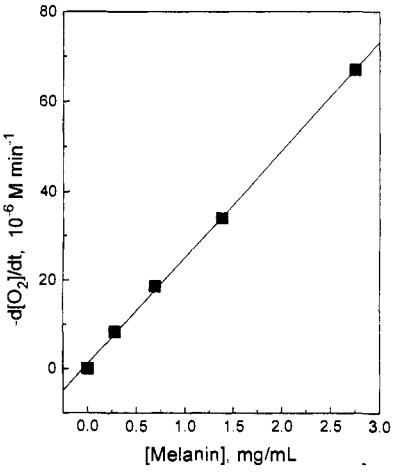


Figure 11. Initial rates of oxygen consumption *versus* [DM] in phosphate buffer (50 mM, pH 7.0) at 25 °C. [4-MeO–PhN_2^+] = 1.25 mM.

which suggests that neither the superoxide radical nor hydrogen peroxide is involved in the oxygen consumption. This implies that the removal of oxygen occurs *via* the reaction of O_2 with the product of the reaction of melanin with 4-MeO–PhN_2^+, most likely with the 4-methoxyphenyl radical (eq 1).

Discussion

In this work we have demonstrated that synthetic DOPA melanin and catechol(amine) melanin precursors reduce 4-MeO–PhN_2^+ to form free radicals derived from both the reducing agents and the diazonium salt. PhOH, 4-HA, and Tyr do not reduce 4-MeO–PhN_2^+, unless activated by tyrosinase. Identification of the aryl radical has been achieved using EPR spectroscopy and the spin-trapping technique. Using the EPR approach we also observed the formation of semiquinone radicals from the catechol(amine)s and from the melanin pigment. Detection of the aryl and the semiquinone radicals is consistent with the homolytic mechanism of fragmentation of this diazonium compound although it may not be immediately obvious whether this occurs *via* the outer- or the inner-sphere electron-transfer process (Scheme I). Brown and Doyle have proposed that the reduction of arenediazonium cations by *p*-hydroquinone may be described adequately by the outer-sphere mechanism,[9] and it is likely that catechols reduce ArN_2^+ through the same pathway. This is because the likelihood of direct electron transfer to ArN_2^+ is dependent on redox potentials of electron donors, and *o*-hydroquinones are almost as good reducing agents as *p*-hydroquinones.

Our results are consistent with the reduction of 4-MeO–PhN_2^+ by the monobasic and semiquinone forms of catechol(amine)s. It has been reported that *p*-QH_2, and its ionized form (*p*-QH^-), differ in their capacities to reduce diazonium salts.[9] While *p*-QH_2 is virtually unreactive toward 4-MeO–PhN_2^+, its monobasic form reacts with a rate constant of 4.47×10^3 M^{-1} s^{-1} (ref 9). Because the one-electron oxidation potentials of *p*- and *o*-hydroquinones do not differ markedly (459, 460, and 530 mV, respectively, for the $Q^{-\bullet}/QH^-$ couples at pH 7 from *p*-hydroquinone,[36] DOPA,[37] and catechol[27]), the rate constants for the reduction of 4-MeO–PhN_2^+ by the respective monobasic forms of DOPA and catechol should be in the same range.[38]

As illustrated in Scheme I, the diazonium group functions as a one-electron-oxidizing agent, and therefore in reactions with catechol(amine)s, semiquinone radicals are the obligatory intermediate products (eq 6a). Following reaction 6a, two situations

$$QH^- + ArN_2^+ \rightleftharpoons Q^{-\bullet} + H^+ + Ar^\bullet + N_2 \qquad (6a)$$

$$Q^{-\bullet} + ArN_2^+ \rightleftharpoons Q + Ar^\bullet + N_2 \qquad (6b)$$

$$2Q^{-\bullet} + H^+ \rightleftharpoons QH^- + Q \qquad (6c)$$

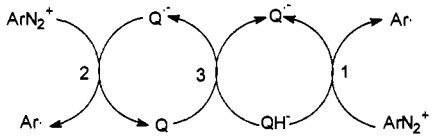
are possible. Semiquinone radicals may continue the reduction process reacting with other molecules of diazonium cations to produce new $Ar^\bullet$ radicals and *o*-quinones (eq 6b), or alternatively they may disproportionate to hydroquinones and quinones (eq 6c). Jirkovsky et al.[10] have found that the rate constant for the reduction of our diazonium compound by *p*-$Q^{-\bullet}$ is 1.2×10^6 M^{-1}

(36) Ilan, Y. A.; Czapski, G.; Meisel, D. *Biochim. Biophys. Acta* **1976**, *430*, 209–224.

(37) Jovanovic, S. L.; Simic, M. G. In *Oxygen Radicals in Biology and Medicine*; Simic, M. G., Taylor, K. A., Ward, J. F., von Sonntag, C., Eds.; Plenum Press: New York, 1987; pp 115–122.

(38) As suggested by one of the reviewers, good reducing agents might be formed by arylation of catechols. In fact, Brown and Doyle (ref 9) identified products arising from addition of $Ar^\bullet$ to *p*-quinones (minor products), which implies the existence of transient aryl radical–(hydro)quinone conjugates. This reaction is more likely to occur in systems without externally added radical scavengers (spin traps DMPO and MNP) which may compete with the hydroquinones for $Ar^\bullet$. This is because the traps efficiently scavenge $Ar^\bullet$ radicals (k is ca. 10^7 M^{-1} s^{-1}) and because they are usually present at concentrations much higher than that of hydroquinones. Conditions which might favor the formation of such radical conjugates exist only in samples used to measure oxygen consumption (spin traps omitted). It is possible that the complex rate law for oxygen consumption (eq 2) is the result of intermediacy of several different types of reducing agents appearing during the course of the reaction.

Scheme III



s^{-1}. This is 3 orders of magnitude higher than the rate constant for the reduction by a monobasic hydroquinone[9] and reflects the superior reducing power of the semiquinone radicals when compared to that of hydroquinones. It is likely that *o*-semiquinone radicals will also be more reactive than the respective catecholate anions (reduction potential for the *o*-Q/*o*-Q$^{-\bullet}$ couple is 210 mV at pH 7.0[39] while that for *o*-Q$^{-\bullet}$/*o*-QH$^-$ is 530 mV[27]). Because *o*-Q$^{-\bullet}$ is a weaker electron donor than *p*-Q$^{-\bullet}$, the anticipated rate constant for reaction 6b (for *o*-Q$^{-\bullet}$ and 4-MeO–PhN$_2^+$) should be close to, but less than, 10^6 M^{-1} s^{-1}.

Semiquinone radicals formed in reaction 6a may also disproportionate to quinones and hydroquinones (eq 6c). At pH 7.0 semiquinones disproportionate with a rate constant on the order of 10^7–10^8 M^{-1} s^{-1} (refs 29 and 40). Consequently, under conditions where reaction 6b predominates over reaction 6c (this is more likely to happen at low [catechol] and high [4-MeO–PhN$_2^+$]), *o*-quinones are produced at a fast rate. Then, through comproportionation (reverse of eq 6c) they produce two semiquinone radicals which may enter reaction 6b recovering the quinone, and then the cycle is repeated. The overall effect of such a cycle is acceleration of the generation of aryl radicals (autocatalytic effect), preceded by a slow induction period during which the quinones are formed. This was observed in our EPR/spin-trapping (Figure 1C, lines a and b) and oxygen consumption experiments (Figure 5A,D).[41] If, on the other hand, disproportionation of *o*-Q$^{-\bullet}$ (eq 6c) prevails over reduction of 4-MeO–PhN$_2^+$ (eq 6b), reaction 6b may be masked by reaction 6a and no autocatalytic effect can be observed (Figure 1, lines c and d). Thus, depending on which particular mechanism, 6a or 6b, dominates under given experimental conditions, the formation of aryl radicals may show either autocatalytic or noncatalytic character.

We postulate that the formation of quinones is the critical step which determines efficacy of the overall dediazoniation process. The importance of this step relies on the fact that quinones and catechols comproportionate to semiquinone radicals (eq 6c), which are better electron donors than the starting catechols. The cyclic and self-stimulating (quinone-driven) nature of this process is depicted in Scheme III, in which the following three steps can be distinguished: reaction 1, which is the slow initiation process (corresponding to eq 6a); reaction 2, which produces aryl radicals and quinones (corresponding to eq 6b); reaction 3, which produces semiquinone radicals which again enter reaction 2. The cycle will repeat although, as one or both reactants (catechol, ArN$_2^+$) are exhausted, the rate of this process will decrease.[42]

The role of oxygen in the dediazoniation process in aqueous solutions is known to be complex. Product analysis has shown that oxygen influences both the type and yields of the material formed and also the kinetics of arenediazonium decomposition.[2,4,5,7] In particular, it has been observed that in aqueous alkaline solutions (pH 9–10) decomposition of 4-chlorobenzenediazonium tetrafluoroborate is slow in thoroughly deoxygenated solutions, but it is markedly faster in the presence of oxygen, even at low oxygen concentration. The kinetic profiles of the decomposition reaction exhibited the characteristic Z-shape in the presence of oxygen, suggesting that this is an autocatalytic process. It has been postulated that the catalytic role of oxygen may be indicative of a radical chain mechanism of dediazoniation.[5,11] In the presence of oxygen, both phenols and hydroquinones derived from arenediazonium compounds are formed.[5,7] They may arise from aryl peroxyl radicals, which are the primary products of reaction 1, through a series of transformations, as proposed in earlier work.[4,5,7,43–45] Because phenolate ions and hydroquinones are good electron donors, the accelerated decomposition of arenediazonium compounds in aerated solutions has been linked to the accumulation of these products during the induction period followed by their rapid oxidation by ArN$_2^+$.[5] In fact, in the example given above, 4-chlorophenol has been identified as the most important catalyst; the addition of 4-chlorophenol to the reaction mixture eliminated the induction period, indicating that the reaction was no longer autocatalytic.[5]

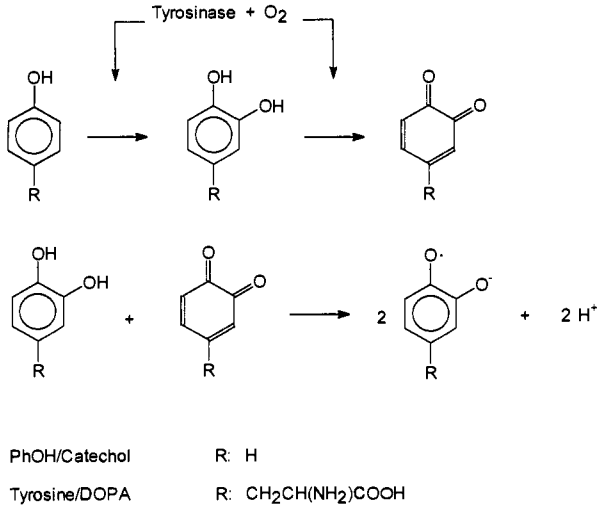
We believe, however, that the autocatalysis observed in our experiments cannot be explained by the formation of a phenol from 4-MeO–PhN$_2^+$. One reason is that, as we have shown, phenols, including 4-HA (4-MeO–PhOH), do not reduce 4-MeO–PhN$_2^+$ at pH 7. Another reason is that catechols, which are better reducing agents then phenols, were intentionally added to our samples so they did not have to be produced *in situ*. A more plausible mechanism of the autocatalysis involves the formation of *o*-quinones (*vide supra*, Scheme III).

We have found that in acidic solutions both adduct **II** formation and oxygen consumption are significantly slowed down in comparison to these processes in neutral and alkaline solutions. This may be due to a less efficient generation of the aryl radicals at acidic pH. There are three major contributing factors for this effect. The first is the lower concentration of the initiating agent (i.e. the monobasic form of catechol) as already mentioned above. The second is that at low pH a significant fraction of the semiquinone radicals exists in the nonionized state (QH$^\bullet$) and the neutral radicals dismutate with a rate constant that is $\geq$1 order of magnitude higher than that for radical anions (Q$^{-\bullet}$). Because the pK_a value for the semiquinone radical from catechol is 5 (ref 27), at pH 4.6 the neutral form will dominate, and the momentary concentration of QH$^\bullet$ and Q$^{-\bullet}$ radicals might be too low for an efficient reaction with the diazonium compound. The third is that the reducing capacity of the neutral radical is low in comparison to its anionic counterpart.[10]

We have shown that the low reactivity of catechol in acidic solutions can be circumvented by the addition of Zn^{2+} ions. This observation can be rationalized assuming that at low pH oxidation of *o*-QH$_2$ by 4-MeO–PhN$_2^+$ to *o*-QH$^\bullet$ and *o*-Q$^{-\bullet}$, although nonmeasurably slow, nevertheless may occur. Then, in the presence of Zn^{2+} the *o*-semiquinone radicals can be stabilized in the form of *o*-Q$^{-\bullet}$/Zn^{2+} complexes (Figure 3). Because the rate constant for disproportionation of the *o*-Q$^{-\bullet}$/Zn^{2+} complexes is ca. 400 times lower than that of the uncomplexed radical, the semiquinones may accumulate in higher steady-state concentrations.[28,29] Zinc forms such complexes only with radical anions. Therefore in the presence of Zn^{2+} the momentary equilibrium QH$^\bullet$ $\rightleftarrows$ Q$^{-\bullet}$ + H$^+$ is shifted to the right, thereby increasing the effective concentration of the radical anions (most of which exist as *o*-Q$^{-\bullet}$/Zn^{2+} species). Then, *o*-Q$^{-\bullet}$ and perhaps mostly *o*-Q$^{-\bullet}$/Zn^{2+} will react according to eq 6b. Here, zinc ions function

(39) Swallow, A. J. In *Function of Quinones in Energy Conserving Systems*; Trumpower, B. L., Ed.; Academic Press: New York, 1982; pp 59–72.
(40) Rao, P. S.; Hayon, E. *J. Phys. Chem.* **1973**, *77*, 2274–2276.
(41) Note that for autocatalytic reactions the kinetic runs, in which the accumulation of a product is monitored, assume S-shaped profiles, while those in which consumption of a substrate is monitored are Z-shaped.
(42) The role of quinones in the autocatalysis was confirmed in an independent experiment in which EPR signal amplitudes of DMPO/*p*-MeO–Ph$^\bullet$ from samples containing catechol (0.1 mM) and 4-MeO–PhN$_2^+$ (0.1 mM) were compared with signals from samples to which *p*-benzoquinone (*p*-BQ) (1 mM) was added. The signal from the sample containing *p*-BQ attained maximum intensity in significantly shorter time, 4 min, than the control signal, which continued to increase even after 30 min of incubation; signal intensity was approximately 28-fold greater in the presence of *p*-BQ than in its absence. Similar effects were observed on oxygen consumption: in the presence of *p*-BQ the induction period was shortened or even eliminated, depending on the type of reducing agent used (manuscript in preparation).
(43) Russell, G. A.; Bridger, R. F. *J. Am. Chem. Soc.* **1963**, *85*, 3765–3766.
(44) Li, A. S. W.; Chignell, C. F. *Photochem. Photobiol.* **1987**, *46*, 445–452.
(45) Lipczynska-Kochany, E.; Kochany, J.; Bolton, J. R. *J. Photochem. Photobiol., A* **1991**, *62*, 229–240.

Scheme IV



as a sort of catalyst by prolonging the effective lifetime of the *o*-semiquinone radicals. This renders the homolysis of 4-MeO–PhN_2^+ more efficient even at low pH.[46,47]

The interaction of 4-MeO–PhN_2^+ with Tyr, PhOH, or 4-HA does not generate free radicals unless tyrosinase is present. It is known that tyrosinase hydroxylates phenols into *o*-dihydroxybenzene derivatives.[15,16] This is illustrated in Scheme IV for PhOH and Tyr, which are converted into catechol and DOPA, respectively. The oxidation potentials of phenols are significantly higher (>800, 940, and 600 mV at pH 7 for PhOH,[48] tyrosine,[49] and 4-HA,[48] respectively) than the oxidation potentials of catechol(amine)s (*vide supra*) and the redox potential of 4-MeO–PhN_2^+ (481 mV).[50] Thus the finding that phenols induce dediazoniation only in the presence of tyrosinase may be rationalized in terms of the formation of metabolites (catechol(amine)s) which are better reducing agents than their phenolic precursors. An additional factor which may influence efficacy of dediazoniation is that, while the first step of the enzymic activation (hydroxylation of phenols) is slow, the second step (oxidation of catechol(amine)s to quinones) is very fast (Scheme IV). Therefore, production of semiquinones through comproportionation of the *in situ*-produced *o*-hydroquinones and *o*-quinones is expected to occur (Scheme IV, eq 6c). Thus, the reduction of the diazonium compound may be accomplished both by catechol(amine)s and the respective semiquinones. The efficacy of the tyrosinase-catalyzed formation of adduct **II** in the presence of tyrosine and phenol is in qualitative agreement with the oxygen consumption data, which show a higher rate of oxygen uptake in the presence of DOPA (metabolite of tyrosine) than in the presence of catechol (metabolite of phenol) (Table II).

Oxidation of melanin by 4-MeO–PhN_2^+ generates new radicals in the pigment. Melanin contains hydroquinone groups from DOPA[16] and from 5,6-dihydroxyindole derivatives,[16,17] which exist in equilibrium as described by eq 4. The formation of new melanin radicals can be rationalized by a mechanism analogous to that proposed for the formation of radicals from catechol(amine)s, i.e. a one-electron oxidation of the melanin hydroquinone groups to semiquinone radicals (eq 6a). It is reasonable to expect that 4-MeO–PhN_2^+ will react exclusively with those QH^- groups which are located on the polymer surface, as they are more easily accessible to the diazonium compound. It is also highly probable that melanin semiquinones may react with 4-MeO–PhN_2^+ to give oxidized melanin (melanin quinones) and aryl radicals. It is therefore unusual that the reaction of DM with 4-MeO–PhN_2^+ increases the EPR signal of the pigment. The observation that the concentration of melanin radicals reached a saturation level at high [4-MeO–PhN_2^+] (Figure 10) may indicate exhaustion of all superficially located hydroquinone and semiquinone groups in the polymer due to their reaction with the diazonium compound. Then, the only radicals which remained would be those located inside the melanin polymer. We found that in this state melanin contains a higher concentration of free radicals, which may be indicative of strong interaction between the "outer" and "inner" redox groups in the polymer matrix, which leads, under these particular oxidative conditions, to a new equilibrium (eq 4) characterized by a higher content of the free radicals. Thus, the use of arenediazonium compounds allows us to modify the redox status of the melanin pigment.

From pulse radiolysis studies[51] it is known that aryl radicals add to aromatic compounds at the rate of ca. 7.6×10^6 M^{-1} s^{-1}. It was therefore anticipated that melanin may be a target for these radicals. Our EPR/spin-trapping experiments have shown that, in the presence of DM, 4-MeO–Ph$^\bullet$ radicals can be readily trapped with DMPO, even at increasing DM concentrations (Figure 9). This suggests that DMPO competes efficiently with DM for these radicals. This may be due to the high value of the rate constant for addition of 4-MeO–Ph$^\bullet$ to DMPO and high [DMPO]. For a phenyl radical reacting with DMPO in water, the rate constant has been reported[52] to be 7×10^7 M^{-1} s^{-1}.

The interaction of melanin with diazonium compounds induces oxygen consumption, which, in contrast to the situation with catechol(amine)s, is a noncatalytic process (Figure 5B). This is presumably due to the simultaneous presence of the endogenous semiquinones, hydroquinones, and quinones in the pigment (eq 4). The first species initiate, from the very beginning, the fast reduction process. This situation is maintained by a continuous and rapid adjustment of equilibrium 4 to produce new semiquinone radicals. It is likely that the oxygen consumption observed during the reaction of DM with 4-MeO–PhN_2^+ may be due to the reaction of these aryl radicals which are not trapped by the pigment.

Conclusions

Melanin and catechol melanin precursors induce the homolytic dediazoniation of 4-methoxybenzenediazonium cation *via* a one-electron-transfer mechanism. This reaction is the source of free radicals derived from melanin and the catechol(amine)s and of the aryl radical from the diazonium compound. In aerated solutions the reductive activation of the diazonium cation leads to oxygen consumption, presumably due to the reaction of oxygen with aryl radicals. Both oxygen consumption and radical formation processes are autocatalytic. We postulate that the catalysis involves the formation of semiquinone radicals through comproportionation of catechols with the *in situ*-produced quinones. The accelerated production of aryl radicals is due to interaction of 4-MeO–PhN_2^+ with semiquinones, which are better reducing agents than catechols and catecholate anions. Phenol, tyrosine, and 4-hydroxyanisole do not initiate the radical formation unless they are activated by tyrosinase. It is likely that the formation of aryl radicals, stimulated by chemical and/or metabolic reduction, might be involved in the biological action of arenediazonium salts.

(46) That it is the complexation with zinc ions that is required to stimulate oxygen consumption was confirmed by the use of *p*-QH_2. The *p*-$Q^{-\bullet}$ radical does not chelate Zn^{2+}, and the rate of oxygen consumption at pH 4.6 was the same both in the presence and in the absence of zinc ions.

(47) The absorption spectrum of catechol at pH 4.6 does not change upon addition of Zn^{2+}, which suggests that formation of *o*-QH_2/Zn^{2+} or deprotonation of phenolic groups does not occur. Therefore the increased reactivity of the system in the presence of Zn^{2+} cannot be attributed to the reduction of 4-MeO–PhN_2^+ by catecholate anion.

(48) Scurlock, R.; Rougee, M.; Bensasson, R. V. *Free Rad. Res. Commun.* **1990**, *8*, 251–258.

(49) DeFelippis, M. R.; Murthy, C. P.; Faraggi, M.; Klapper, M. H. *Biochemistry* **1989**, *28*, 4847–4853.

(50) Doyle, M. P.; Guy, J. K.; Brown, K. C.; Mahapatro, S. N.; VanZyl, C. M.; Pladziewicz, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1536–1540.

(51) Madhavan, V.; Schuler, R. H.; Fessenden, R. W. *J. Am. Chem. Soc.* **1978**, *100*, 888–893.

(52) Kalyanaraman, B. In *Reviews in Biochemical Toxicology;* Hodgson, E., Bend, J. R., Philpot, R. M., Eds.; Elsevier Biomedical: New York, 1982; Vol. 4, pp 73–139.