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Characterization of Free Radicals Produced during Oxidation of Etoposide (VP-16) and Its Catechol and Quinone Derivatives. An ESR Study†

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ABSTRACT: Spectroscopic evidence for the radical-mediated metabolism of VP-16, VP-16 catechol, and VP-16 quinone during enzymatic oxidation and autoxidation has been obtained. Autoxidation of the catechol yields the primary semiquinone together with the primary molecular product VP-16 quinone, which subsequently undergoes hydrolytic oxidation to form secondary quinones and semiquinones. Both primary and secondary phenoxyl radicals were detected during peroxidatic oxidation of VP-16. Neither the primary nor the secondary radicals react with DNA at a detectable rate. Evidence for the production of hydroxyl radical during iron-catalyzed oxidation of VP-16 catechol was obtained. These free radical reactions may have implications for the mechanism of antitumor action of VP-16.

VP-16-213 (NSC 141540, **1**) (Scheme I) is an antitumor drug currently in use for the treatment of several forms of cancer (Rozencweig et al., 1977; Issell, 1982; O'Dwyer et al., 1985). Efforts to understand its mechanism of action are in progress (Sinha & Myers, 1984; Sinha et al., 1985; Loike & Horwitz, 1976;; Wozniak & Ross, 1983; Kalwinsky et al., 1983; Van Maanen et al., 1985a). VP-16 induces both single- and double-strand DNA breaks in tumor cells (Loike & Horwitz, 1976; Wozniak & Ross, 1983; Kalwinsky et al., 1983; Long et al., 1984, 1985; Row et al., 1985). These effects are implicated in its cytotoxicity (Wozniak & Ross, 1983; Long et al., 1984; Row et al., 1985). One of the factors responsible for DNA damage has been attributed to the metabolism of

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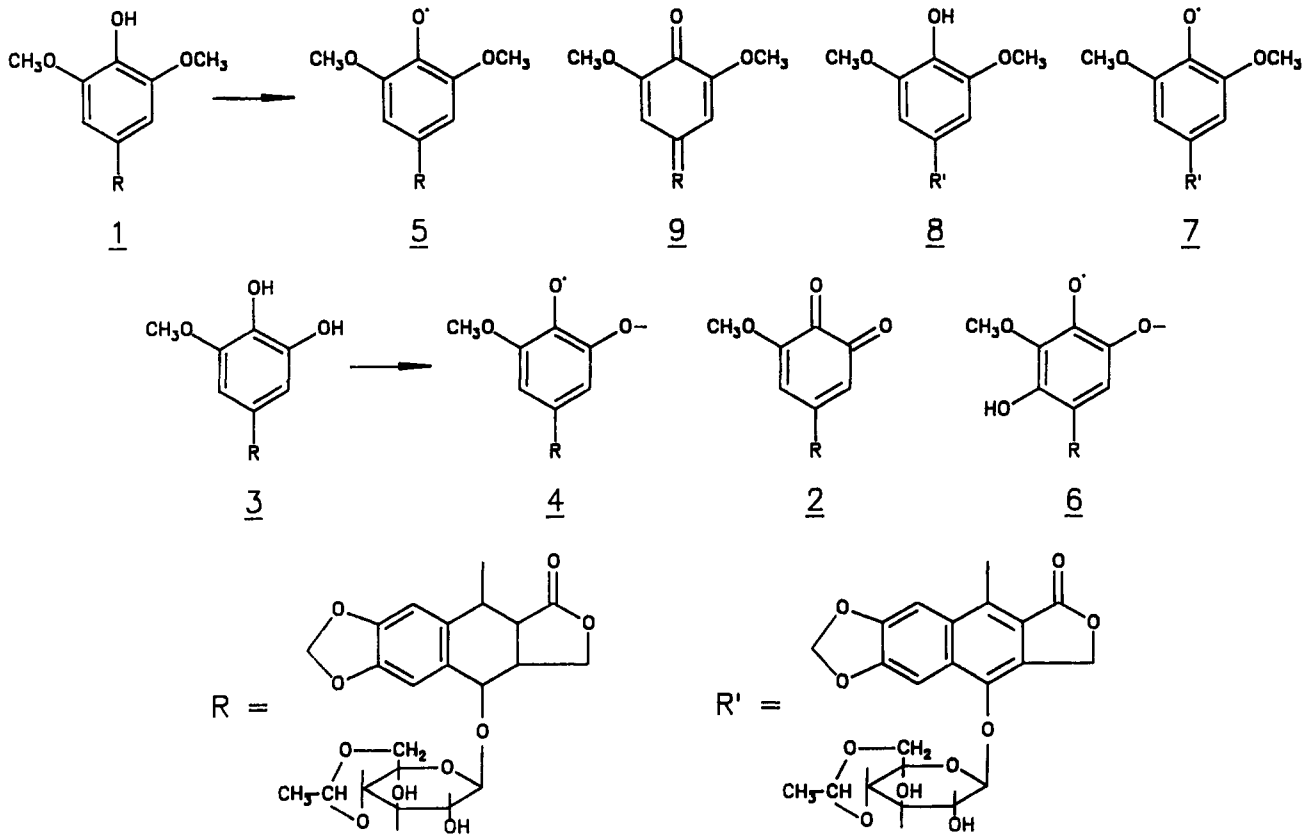
Scheme I



VP-16 to electrophilic intermediates, for example, semiquinones and quinones (Sinha et al., 1983, 1985; Van Maanen et al., 1984, 1985a; Haim et al., 1986, 1987a).

Several enzymatic systems, viz., rat liver microsomes/NADPH (Sinha et al., 1985; Van Maanen et al., 1984, 1985a; Haim et al., 1987a), horseradish peroxidase (HRP)[1]/H_2O_2 (Sinha et al., 1983; Haim et al., 1986), prostaglandin (PG) synthase/arachidonic acid (Haim et al., 1986), and myeloperoxidase/H_2O_2 (Van Maanen et al., 1984), metabolize VP-16 to products capable of irreversible binding to proteins and DNA. The ultimate species responsible for covalent binding has been shown to be the quinone **2** (Van Maanen et al., 1984, 1985a; Haim et al., 1986, 1987a,b) derived from the corresponding catechol **3**. Both semiquinone **4** and phenoxyl radical **5** also have been proposed to be formed as intermediates during metabolism of **1** in the above systems (Van Maanen et al., 1985a; Haim et al., 1986, 1987a). Structures of the precursors and the intermediates are shown in Scheme I.

VP-16 has been shown to cause oxidative stress in vivo (Katki et al., 1987), as evidenced by the depletion of reduced glutathione (GSH) and the formation of oxidized glutathione (GSSG). In vitro the VP-16 phenoxyl radical **5** was shown to react with GSH forming GS$^{\bullet}$ and GSSG (Katki et al., 1987). Taken together, these findings implicate formation of reactive intermediates during the in vivo metabolism of VP-16. More recently, it was shown that the VP-16 catechol–iron complex, in the presence of GSH and hydrogen peroxide, caused extensive nicking of SV40 DNA (Sinha et al., 1988). The DNA damage was related to the production of hydroxyl radicals. The present study was undertaken to further our understanding of the free radical mediated oxidation of VP-16 and its metabolites.

The phenoxyl radical **5** formed during peroxidase-catalyzed oxidation of VP-16 has been previously detected directly by electron spin resonance (ESR) spectroscopy (Sinha et al., 1983; Haim et al., 1986; Van Maanen et al., 1984). Semiquinone **4**, which is likely to be formed via enzymatic or nonenzymatic oxidation of **3**, has not previously been characterized. We now report the detection and characterization of (i) both primary and secondary semiquinones, (ii) primary and secondary phenoxyl radicals, and (iii) hydroxyl radicals formed during enzymatic oxidation and autoxidation of VP-16 and its catechol and quinone oxidation products. Semiquinone radicals **4** and **6** were detected by the ESR–spin stabilization method, as previously described (Kalyanaraman & Sealy, 1982; Kalyanaraman et al., 1984a,c). The diamagnetic divalent cations Zn^{2+} and Mg^{2+} were used to stabilize *o*-semiquinones in acid and neutral solutions, respectively. Phenoxyl radicals **5** and **7** were detected by direct ESR and the hydroxyl radical by ESR–spin trapping. Effects of DNA on the production of semiquinone and phenoxyl radicals in this system are reported and mechanisms for the production of hydroxyl radicals also discussed.

Materials and Methods

VP-16 (NSC 141540) was a gift of Bristol Myers Pharmaceuticals, Syracuse, NY, and the National Cancer Institute, NIH, Bethesda, MD. VP-16 catechol and VP-16 quinone were synthesized from VP-16 by oxidative O-demethylation. The structure and the purity were confirmed by mass spectral analysis and thin-layer chromatography. Calf thymus DNA (highly polymerized, type 1), catalase, HRP (type VI), superoxide dismutase, and *o*-phenylenediamine hydrochloride

[1] Abbreviations: ESR, electron spin resonance; HRP, horseradish peroxidase; DMPO, 5,5-dimethyl-1-pyrroline 1-oxide; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy; EDTA, ethylenediaminetetraacetic acid; SOD, superoxide dismutase; DETAPAC, diethylenetriaminepentaacetic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

were from Sigma Chemical Co., St. Louis, MO. 5,5-Dimethyl-1-pyrroline 1-oxide (DMPO), obtained from Aldrich Chemical Co. (Milwaukee, WI), was redistilled in vacuo prior to use and further purified (if necessary) with activated charcoal (Buettner & Oberley, 1978). Ferric chloride (Sigma Chemical Co.) and 1,10-phenanthroline monohydrate (Aldrich Chemical Co.) were used as received. Chelex 100 (200–400 mesh, sodium form) was from Bio-Rad Laboratories. Chelex 100 treatment for the removal of contaminating metals from buffers was accomplished by using the column method.

The metal ions present in the calf thymus DNA were removed by dialyzing solutions of DNA (1 mg/mL) against 0.1% DETAPAC (18 h at 4 °C), followed by extensive dialysis against the chelexed buffers (48 h at 4 °C). Denatured DNA (1 mg/mL) was prepared by heating to 100 °C for 20 min, rapidly quenching in ice, and then repeating the procedure.

Acetate buffer was prepared by the addition of zinc acetate to 0.2 M acetic acid solution. The buffer was brought to the desired pH by dropwise addition of either 10 M NaOH or glacial acetic acid. Anhydrous magnesium chloride was dissolved in Tris buffer (50 mM, pH 7.5) and the solution filtered to remove insoluble impurities. Experiments in acetate buffer were carried out over a range of pH from 3.0 to 6.0, and those in Tris buffer were carried out over a range of pH from 7.5 to 9.5. At higher pH values, it was necessary to stir the Tris buffer vigorously to dissolve Mg^{2+} ions. Final concentrations of Zn^{2+} and Mg^{2+} in most cases were 0.45 and 0.5 M, respectively.

Enzymatic oxidation of VP-16 was with HRP and H_2O_2 as previously described (Sinha et al., 1983). Autoxidation experiments were done by dissolving VP-16 catechol in various buffers. Semiquinone and phenoxyl radicals were detected in steady-state concentrations in a static system. The oxygen concentrations in incubations for ESR studies were varied by purging with either air, nitrogen, or oxygen for 10 min.

ESR measurements were carried out at ambient temperature on solutions contained in a quartz aqueous flat cell, using a Varian E-109 spectrometer operating at 9.5 GHz and employing 100-kHz field modulation. Magnetic field measurements were made with a Radiopan MJ-110 gaussmeter. For microwave frequency measurements an EiP 200 counter was used. Hyperfine splittings were measured (to 0.05 G) either directly from magnetic field separations or from computer simulations of spectra. g values were obtained from measurements of magnetic field and microwave frequency after correction for the position of the gaussmeter probe using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a standard, taking $g = 2.0037$ for this radical (Weil & Anderson, 1965). Measurements of the g value were to ±0.0001. Radical concentrations were estimated relative to a standard solution of a stable nitroxide radical 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy (CTPO) (Aldrich Chemical Co.), taking into account differences in line width, microwave power saturation, and hyperfine multiplicity between the radical and the nitroxide spectra (Korytowski et al., 1987).

In spin trapping experiments the iron–EDTA complex was added as a preformed complex prepared by adding 1 equiv of Fe^{3+} to 2 equiv of EDTA in acidic media.

Results

Detection and Identification of Primary o-Semiquinones. Addition of catechol **3** to an aerobic buffer containing Mg^{2+} produced a well-resolved ESR spectrum (Figure 1). The intensity of the ESR signal was proportional to the concentration of Mg^{2+}; however, the signal intensity plateaued at the saturating concentration of Mg^{2+}, ca. 400 mM. In the absence of Mg^{2+} no ESR spectrum was detected (Figure 1b). Addition of **3** to an acetate buffer containing Zn^{2+} also gave an intense ESR spectrum (Figure 1c). In the absence of stabilizing metal ions, radicals from **3** were obtained only at high pH (Figure 1d). The uncomplexed radical anions from **3** were not observed at physiological pHs because of their rapid rate of dismutation (Kalyanaraman et al., 1984). The ESR parameters (Table I) were obtained from computer simulations (Figure 2). Comparison with data for related semiquinones (Kalyanaraman et al., 1985, 1986) indicates that species responsible for spectra (Figure 1a,c,d) are **4**–Mg^{2+}, **4**–Zn^{2+}, and **4**, respectively. Radical concentrations were measured to be ca. 0.1–0.5% of the substrate concentrations.

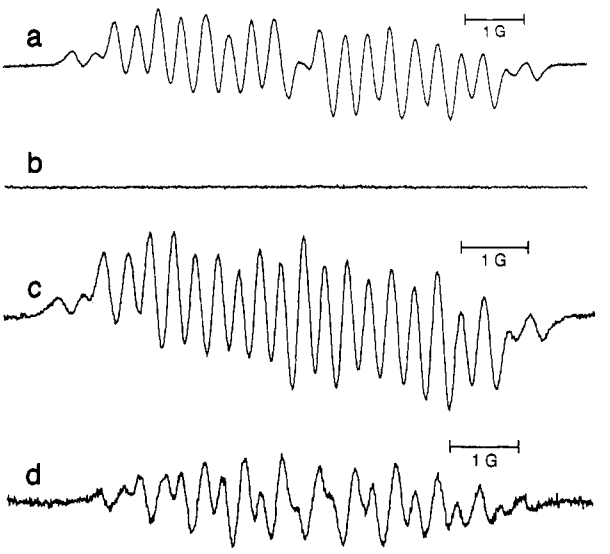


FIGURE 1: ESR spectra of metal-complexed and uncomplexed primary *o*-semiquinones (**4**–Mg^{2+}, **4**–Zn^{2+}, and **4**) obtained from autoxidation of VP-16 catechol (**3**). (a) Spectrum of the **4**–Mg^{2+} complex. The conditions were 0.5 mM substrate and 0.45 M Mg^{2+} in Tris buffer (pH 7.5). (b) Same as (a) but in the absence of Mg^{2+}. (c) Spectrum of the **4**–Zn^{2+} complex. The conditions were 0.5 mM substrate and 0.5 M Zn^{2+} in acetate buffer (pH 5.0). (d) Spectrum of the uncomplexed radical. The condition was 0.5 mM substrate in Tris buffer (pH 9.0). Autoxidation reactions were initiated by adding a DMSO solution of **3** to air-saturated buffers and spectra obtained within 5 min.

Table I: Magnetic Parameters of Radicals

radical	hyperfine couplings (G)				g_{iso}
	$a^H_{OCH_3}$	a^H_{ring}	a^H_β	a^H_γ	
4–Mg^{2+}	0.79 (3)[c]	0.79 (2)	3.6 (1)	0.42 (1)	2.0042
4–Zn^{2+}	0.75 (3)	0.75 (1), 1.0 (1)	3.3 (1)	0.4 (1)	2.0039
4	0.6 (3)	0.6 (1), 1.2 (1)	2.3 (1)	0.35 (1)	2.0045
6–Mg^{2+}		1.8 (1)	3.0 (1)		2.004
5[a]	1.39 (6)	1.39 (2)	4.47 (1)	0.6 (1)	2.0044
5[b]	1.4 (6)	1.40 (2)	4.47 (1)	0.6 (1)	2.0040
7	1.39 (6)	1.39 (2)			2.0040

[a] In methanol/H_2O medium. [b] In DMSO/H_2O medium. [c] Numbers in parentheses denote the number of equivalent protons.

Effects of Scavengers and DNA. Addition of SOD (10 μg/mL) and catalase (100 Sigma units) did not affect significantly the rate of production of **4**–Mg^{2+} (data not shown). This presumably rules out any appreciable contribution from superoxide and hydrogen peroxide to the formation of **4**–Mg^{2+} (cf. Figure 1). However, a dose-dependent decrease in the rate of production of **4**–Mg^{2+} was observed in the presence of *o*-phenylenediamine, which is known to scavenge quinones (Kalyanaraman et al., 1986) (Figure 3).

Addition of DNA to incubations decreased the rate of production of **4**–Mg^{2+}. This effect was greater in the presence

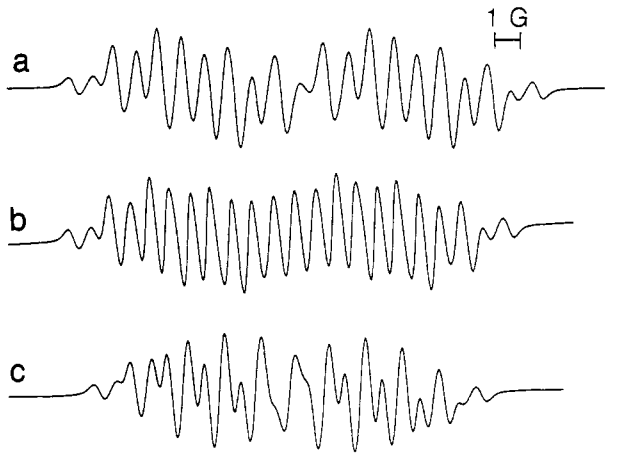


FIGURE 2: Computer simulations of experimental ESR spectra (Figure 1a,c,d) of the metal-complexed and uncomplexed primary semiquinones. Computer simulations were obtained by using a line width of 0.2 G and 50% Lorentzian/50% Gaussian line shapes.

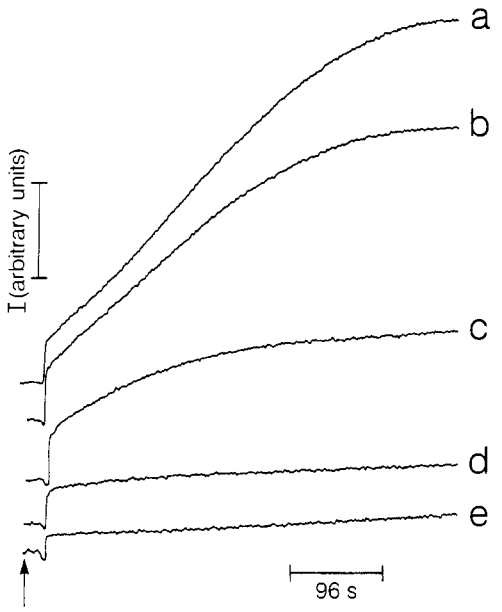


FIGURE 3: Dependence of the rate of production of the magnesium-complexed semiquinone (**4**–Mg^{2+}) on the concentration of *o*-phenylenediamine. The initial rate of production of radicals was monitored following the addition of DMSO solution of **3** to the Tris–$MgCl_2$ buffer (pH 7.5) containing 0.25 mM substrate and *o*-phenylenediamine. Final concentrations of *o*-phenylenediamine were as follows: (a) 0 (control), (b) 0.24 mM, (c) 1.0 mM, (d) 5.0 mM, and (e) 7.6 mM. The final pH was maintained at 7.5 in all cases. At the point marked ↑ reaction was initiated by the addition of substrate. At the high modulation amplitude (~1.25 G) used in this experiment the multiline spectrum shown in Figure 1a collapsed into a single line, and so the rate of production of the semiquinone radical was conveniently measured by sitting on top of this single line and turning the magnetic field off. The lag time represents the time taken to transfer the incubation mixture to the flat cell in the ESR cavity.

of denatured DNA (Figure 4).

Detection and Identification of Secondary Semiquinone. Addition of quinone **2** to a Tris buffer (pH 8.5) containing Mg^{2+} also gave the ESR spectrum of **4**–Mg^{2+} (Figure 5a). With time, however, the above spectrum was gradually replaced (Figure 5) by that of a secondary species, which gave a distinctly different spectrum at high modulation amplitude. The total width of the latter four-line spectrum is less than that of **4**–Mg^{2+}, suggesting that the secondary species has one fewer hyperfine coupling. Since the *g* values of **4**–Mg^{2+} and the secondary species are almost identical, it is very likely that

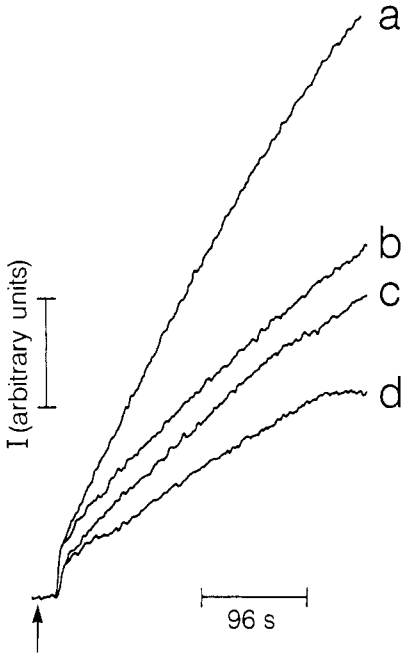


FIGURE 4: Effect of DNA on the rate of production of the magnesium-complexed semiquinone (**4**–Mg^{2+}). The initial rate of radical production was monitored following addition of a DMSO solution of **3** to the Tris–$MgCl_2$ buffer (pH 7.5) containing (a) 0.3 mM substrate, (b) 0.3 mM substrate and DNA (0.5 mg/mL), (c) 0.3 mM substrate and DNA (0.66 mg/mL), and (d) 0.3 mM substrate and denatured DNA (0.66 mg/mL).

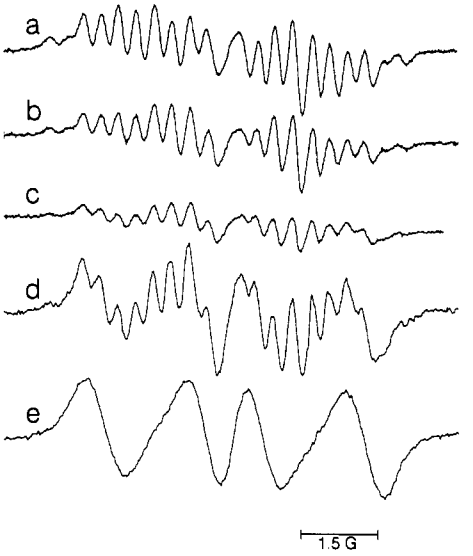


FIGURE 5: ESR spectra of magnesium-complexed primary and secondary *o*-semiquinones (**4**–Mg^{2+} and **6**–Mg^{2+}) obtained from hydrolytic oxidation of VP-16 quinone. (a) Spectrum of the primary semiquinone **4**–Mg^{2+} obtained immediately. Conditions were 0.1 mM substrate and 0.45 M Mg^{2+} in Tris buffer (pH 8.5). (b) Spectrum obtained after 10 min. (c) Spectrum obtained after 20 min. (d) Spectrum obtained after 30 min. (e) Spectrum obtained after 40 min. Note that a higher modulation amplitude (1 G) was used in (e) compared to 0.1 G used in (a–d).

both radicals belong to the *o*-semiquinone type. It is also very likely that endogenous metal ions such as Fe^{2+} catalyzed this reaction. On the basis of ESR parameters (Table I) the four-line spectrum (Figure 5e) was assigned to species **6**–Mg^{2+}. At pH 7.4 the ESR spectral changes occurred at a much slower rate, and as a result the ESR spectrum of the secondary semiquinones was barely detectable (data not shown). Previously, Korytowski et al. have also demonstrated the production of hydroxyl-substituted secondary semiquinones during

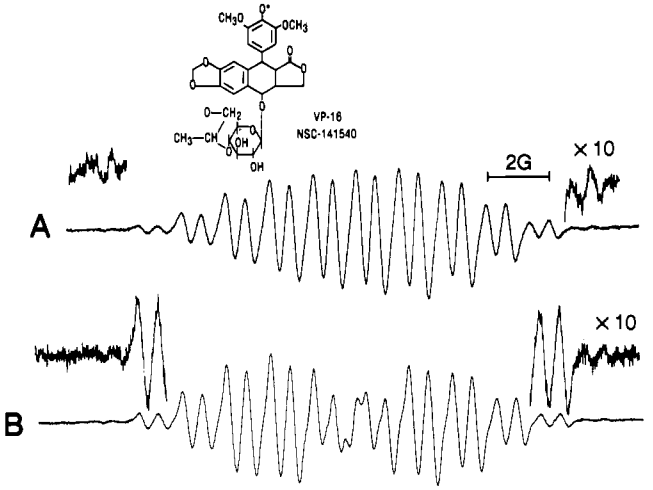


FIGURE 6: ESR spectrum of the primary phenoxyl radical (**5**) obtained during HRP/H_2O_2-catalyzed oxidation of VP-16 (**1**). Conditions were 0.2 mM substrate, 8 μg/mL HRP, and 1.4 mM H_2O_2 in acetate buffer (pH 5.0). (A) In 10% DMSO/H_2O medium and (B) in 10% methanol/H_2O medium. These spectra were obtained with a modulation amplitude of 0.1 G.

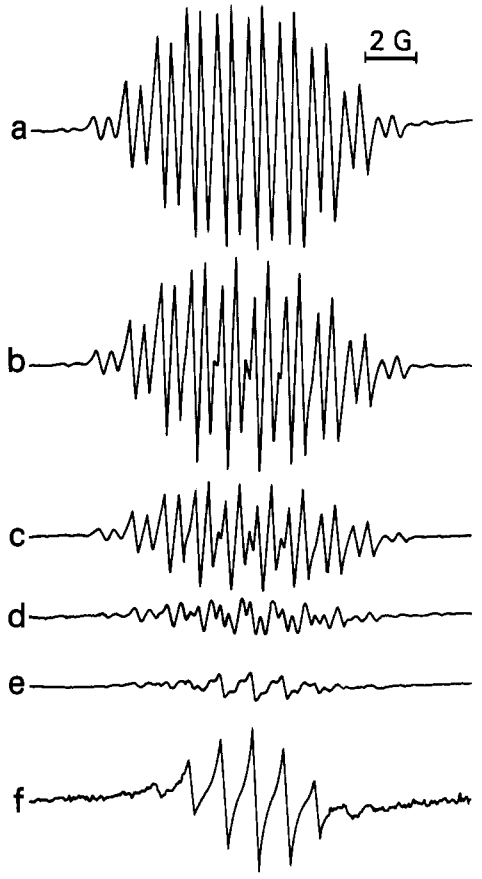


FIGURE 7: ESR spectra of primary and secondary phenoxyl radicals obtained during HRP/H_2O_2-catalyzed oxidation of VP-16. The conditions were 0.14 mM substrate, 1.4 mM H_2O_2, and 5 μg/mL HRP in a carbonate buffer, pH 9.0. Spectra were obtained after (a) 5 min, (b) 10 min, (c) 15 min, (d) 20 min, (e) 25 min, and (f) 30 min.

tyrosinase-catalyzed oxidation of catecholamines (Korytowski et al., 1987).

Detection and Identification of Primary Phenoxyl Radical. Addition of **1** to either acetate, Tris, or phosphate buffer containing HRP and H_2O_2 produced a multiline ESR spectrum (Figure 6). The spectrum was better resolved in a methanol/H_2O mixture than in DMSO/H_2O. Hyperfine parameters (Table I) obtained by computer simulations are

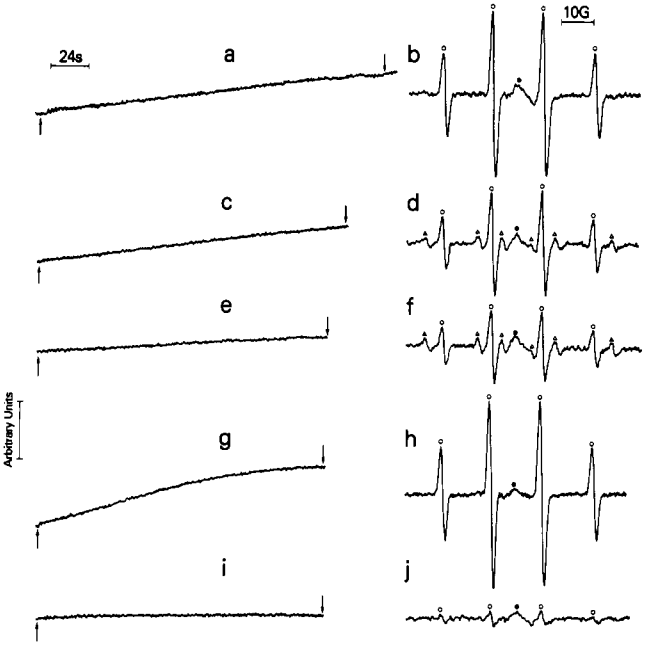


FIGURE 8: Kinetics of the buildup of the DMPO–OH adduct and steady-state ESR spectra obtained during autoxidation of **4**. (a) Rate of production of DMPO–OH. The conditions were 0.1 mM VP-16 catechol, 50 μM Fe^{3+}–EDTA (1:2), and 25 mM DMPO in an air-saturated carbonate buffer (0.1 M, pH 9.0). (b) ESR spectrum obtained after the steady state has been reached in (a). (c) Kinetics of buildup of the DMPO–OH adduct under the same incubation conditions as in (a) but containing ethanol (80 mM). (d) ESR spectrum obtained after the steady state has been reached in (c). (e) Same as in (a) but containing ethanol (160 mM). (f) ESR spectrum obtained after the steady state has been reached in (e). (g) Same as in (a) but containing 10 μg/mL superoxide dismutase. (h) ESR spectrum obtained after the steady state has been reached in (g). (i) Same as in (a) but containing 5 μg/mL catalase. (j) ESR spectrum obtained after the steady state has been reached in (i). Symbols ○, ●, and △ refer to the DMPO–OH, the uncomplexed primary *o*-semiquinone **4**, and DMPO–ethanol adducts, respectively.

consistent with assignment to radical **5**, since the parameters are in close correspondence with those reported for other hindered phenoxyl radicals (Uber & Stegmann, 1979).

Reactions of Phenoxyl Radical. The steady-state concentrations of **5** produced by the HRP/H_2O_2 system remained unchanged in the presence of nitrogen, air, or 100% oxygen, implying that there occurs no reaction between **5** and O_2. Absence of such a reaction has been observed for other hindered phenoxyl radicals (Doba et al., 1984; Caldwell & Steelink, 1969).

The steady-state concentration of **5** showed a square root dependence on enzyme concentration, so that increasing the enzyme concentration by a factor of 4 increases the steady-state radical concentration by a factor of 2, consistent with a bimolecular mechanism of radical decay (Kalyanaraman et al., 1984b). In the presence of DNA (~1 mg/mL) little or no change in the steady-state concentration of **5** was found. Reaction between **5** and the DNA polymer thus does not occur at an appreciable rate under these conditions (cf. Figure 6).

Detection and Identification of Secondary Phenoxyl Radical. The ESR spectrum of the primary phenoxyl radical **5** decayed rapidly at pH 9.0, followed by the appearance of another spectrum (Figure 7). On the basis of ESR parameters (Table I) and previous data on product analysis (Broggini et al., 1985) this spectrum (Figure 7) was assigned to species **7** derived from one-electron oxidation of **8**. Inclusion of DNA (4 mg/mL) did not affect the rate of production of **7**.

Spin Trapping of Hydroxyl Radical. The hydroxyl radical produced during the autoxidation of VP-16 catechol, **3**, was

detected by spin trapping. These experiments were carried out at pH 9 because of increased rate of production of DMPO–OH. Figure 8 shows the kinetics of formation of DMPO–OH during autoxidation of **3** in the presence of Fe^{3+}–EDTA ($d[DMPO–OH]/dt = 0.1\ \mu M/min$). Production of free hydroxyl radicals was confirmed by using ethanol as an $^{\bullet}OH$ radical scavenger (Figure 8). Both the initial rate and the steady-state concentration of DMPO–OH were inhibited by ethanol, and the steady-state ESR spectrum of DMPO–OH was replaced by the ESR spectrum of the DMPO–hydroxyethyl adduct. Inclusion of catalase significantly inhibited the formation of DMPO–OH, suggesting that hydrogen peroxide formed is a precursor to DMPO–OH. In the presence of SOD, we found a small increase (rather than a decrease) in the rate of formation of DMPO–OH (Figure 8). This suggests that superoxide is not the primary reductant of iron chelates in this system.

Discussion

Autoxidation of catechols produces semiquinones together with superoxide and hydrogen peroxide (Fridovich, 1986). It has been proposed that the superoxide radicals subsequently react with most hydroxy-substituted catechols to form H_2O_2 and the semiquinone (Fridovich, 1986). In the presence of the quinone-trapping agent, *o*-phenylenediamine, the concentration of semiquinone **4**–Mg^{2+} was reduced in a dose-dependent manner (Figure 3). Since *o*-semiquinones do not appear to react with *o*-phenylenediamine, this effect may be attributed to a reaction between *o*-quinone and *o*-phenylenediamine (Bolt & Kappus, 1974). This is consistent with semiquinones also being formed in a back-reaction between quinone and the parent compound:

$$\text{VP–catechol} + O_2 \overset{M^{n+}}{\rightleftharpoons} \text{VP-16 semiquinone} + O_2^{\bullet-} \quad (1)$$

$$O_2^{\bullet-} + \text{VP-16 catechol} + 2H^+ \rightleftharpoons \text{VP-16 semiquinone} + H_2O_2 \quad (2)$$

$$2(\text{VP-16 semiquinone}) \rightleftharpoons \text{VP-16 quinone} + \text{VP-16 catechol} \quad (3)$$

$$\xrightarrow{o\text{-phenylenediamine}} \text{scavenged products}$$

In the presence of DNA, semiquinone levels from autoxidation also were reduced. As described before, this effect is probably due to binding of the VP-16 quinone to DNA. We believe that significant binding of semiquinone **4** to DNA is unlikely, as addition of DNA did not significantly affect the rate of formation of VP-16 semiquinones in the HRP/H_2O_2 system. The binding of VP-16 quinone to DNA also has been proposed to occur during microsomal activation of VP-16 (Sinha et al., 1985; Van Maanen et al., 1985a). This would also be consistent with previous data demonstrating inactivation of the bacteriophage DNA by the VP-16 quinone (Van Maanen et al., 1985b). Evidence also exists for inactivation of the DNA polymerase by *o*-quinones (Wick, 1979; Wick & Fitzgerald, 1981). The fact that denatured DNA exhibited a greater effect than nondenatured DNA may be attributed to an increased availability of quinone-binding sites in denatured DNA.

Previous studies have established that oxidation of catechols by horseradish peroxidase/H_2O_2 proceeds primarily via the one-electron oxidation intermediate, the *o*-semiquinone, and that the *o*-semiquinone decays with second-order kinetics, no doubt reflecting disproportionation to the corresponding catechol and quinone (Mason et al., 1961). The linear relationship between steady-state radical concentration and the square root of the enzyme concentration indicates that disappearance of the phenoxyl radical from VP-16 also follows second-order kinetics, i.e.

$$k[E] = 2k_t[\mathbf{5}]_{ss}^2 \quad (4)$$

where $k[E]$ is the rate of radical formation for enzyme concentration, [E], $2k_t$ is the rate constant for radical termination, and $[\mathbf{5}]_{ss}$ is the steady-state concentration of the phenoxyl radical. Thus at physiological pH the one-electron oxidation of VP-16 in tissues containing peroxidases is expected to lead to the accumulation of the corresponding quinone methide via transient phenoxyl radicals (eq 5). On the basis of ESR analysis of the secondary radical and previous data on product analysis (Broggini et al., 1985), formation of the characteristic dimer product is excluded. It is also likely that the stereochemical hindrance would make dimer formation less favorable.

$$\text{VP-16} \xrightarrow{-1e,\ -H^+} \mathbf{5} \quad (5)$$

$$\mathbf{5} + \mathbf{5} \rightarrow \text{VP-16} + \text{VP-16 quinone methide (}\mathbf{9}\text{)} \quad (6)$$

Since the concentration of **5** in our experiments is independent of $[O_2]$, its mechanism of decay should be independent of $[O_2]$. This is consistent with data for the tocopheroxyl radical and other hindered phenoxyl radicals (Doba et al., 1984; Caldwell & Steelink, 1969) but differs from the decay mechanism proposed for phenoxyl radical derived from butylated hydroxytoluene (Thomson & Wand, 1985). The square root relationship between the HRP concentration and **5** also rules out the possibility of oxidation of **5** to **9** by compounds I and II.

Addition of DNA did not affect the steady-state concentrations of phenoxyl radicals (either primary or secondary) produced during oxidation of VP-16 in the HRP/H_2O_2 system. Since phenoxyl radicals are produced as obligate intermediates in the HRP/H_2O_2 system, this lack of an effect implies that VP-16 phenoxyl radicals do not react at an appreciable rate with DNA. This conclusion also was reached by previous investigators (Van Maanen et al., 1985b) who showed that the VP-16 phenoxyl radical did not inactivate the bacteriophage DNA.

One of the products isolated during peroxidatic oxidation of VP-16 contained an aromatized group **8** (R′ in Scheme I). We now propose that the quinone methide of VP-16, **9**, is a precursor to secondary phenoxyl radical **7**. The ESR spectrum of the secondary phenoxyl radical is suggested to result from the one-electron oxidation of **8** (eq 7).

$$\mathbf{9} \rightarrow \rightarrow \mathbf{8} \xrightarrow{HRP/H_2O_2} \mathbf{7} \quad (7)$$

It has been recently suggested that the quinone methide of VP-16 could bind irreversibly to DNA (Haim et al., 1987b). However, if this were a major reaction, one would expect a decrease in the concentration of **7**, since the quinone methide **9** presumably is its precursor. Such a decrease was not found in the present study, indicating a low binding ratio between **9** and DNA.

Free radicals have been implicated in DNA strand breakage in photochemical and enzymatic systems (Decuyper et al., 1984; Brawn & Fridovich, 1981). Free radicals derived from one-electron oxidation of estradiol have been proposed to bind to DNA (Jellinck & Fletcher, 1971), but there exists very little direct spectroscopic evidence for such reactions, although Nagata et al., using ESR, were able to demonstrate the binding of phenoxyl and semiquinone radicals (derived from the metabolites of benzo[*a*]pyrene) to DNA bases (Nagata et al., 1982). In the present work we, in fact, obtained ESR evidence

that neither the semiquinones nor the phenoxyl radicals react at an appreciable rate with DNA. Other studies have shown that nitro anion radicals also do not react with DNA at an appreciable rate (Polnaszek et al., 1984).

It is becoming increasingly clear that reactive intermediates, formed during metabolism of xenobiotics and carcinogens, are less likely to be detected under in vivo conditions. However, their intermediacy could be inferred on the basis of identification of the "marker products". For example, the identification of *cis*-diethylstilbestrol and dienestrol during metabolism of *trans*-diethylstilbestrol, a well-established environmental carcinogen, is diagnostic of formation of the semiquinone and quinone of diethylstilbestrol, respectively (Liehr et al., 1986). Similarly, the detection of 8-hydroxydeoxyguanosine and specific cholesterol hydroperoxides is indicative of the production of hydroxyl radical and singlet oxygen, respectively (Floyd et al., 1986; Kalyanaraman et al., 1987). On the basis of reactions 5–7, it is proposed that species **8** or its derivative could serve as useful in vivo marker products of radical- or quinone-mediated metabolism of VP-16.

Iron-catalyzed production of hydroxyl radical is considered to mediate the antitumor action of several drugs (Dugue & Muenier, 1985; Doroshow, 1983; Lown, 1985; Muindi et al., 1985), and hydroxyl radical scavengers have been shown to decrease the cytotoxicity of VP-16 (Wozniak & Ross, 1983; Wozniak et al., 1984). In the present work we have provided evidence for generation of hydroxyl radical from VP-16 catechol. Although the addition of iron chelates was found to be a prerequisite for generating hydroxyl radical in this system, such reactions may still be biologically relevant due to the presence of "iron pools" in proliferating neoplastic cells (Dugue & Muenier, 1985; Trowbridge & Lopez, 1982). Moreover radical-mediated mobilization of iron from ferritin (which is present in high levels in tumor tissues) has been demonstrated in several systems (Thomas et al., 1985; Thomas & Aust, 1986). Of the several radicals formed during peroxidatic oxidation of VP-16 and VP-16 catechol, species **6** appears to be more reducing than others (Kalyanaraman et al., 1988).

Since the rate of formation of hydroxyl radical is increased in the presence of added SOD, it appears that the iron-catalyzed Haber–Weiss mechanism (in which superoxide is the reductant of Fe^{3+}) is not responsible for the production of hydroxyl radical in our system. By analogy with data for Fe(III)–catechol complexes (Hider et al., 1983), we propose the following reactions for the production of hydroxyl radicals in the system containing VP-16 catechol and Fe^{3+}–EDTA. The reduction of Fe^{3+}–EDTA by **3** presumably occurs either by an outer-sphere electron transfer (Pelizzetti & Mentasti, 1977) or by a redox decomposition of an unstable bidentate complex (Mentasti et al., 1976). These equations, together

$$Fe^{3+}\text{–EDTA} + \mathbf{3} \rightleftharpoons Fe^{3+}\text{–}\mathbf{3} + \text{EDTA} \quad (8)$$

$$Fe^{3+}\text{–}\mathbf{3} \underset{-1e}{\overset{+1e}{\rightleftharpoons}} Fe^{2+}\text{–}\mathbf{3} \quad (9)$$

$$Fe^{2+}\text{–}\mathbf{3} + \text{EDTA} \rightleftharpoons Fe^{2+}\text{–EDTA} + \mathbf{3} \quad (10)$$

with eq 1–3, form the basis of the Fenton reaction (eq 11).

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + {}^{\bullet}OH + OH^- \quad (11)$$

In a recent communication, Chrisey et al. demonstrated that the plant-derived catechols such as epicatechin and procyanidin B_2 cause DNA strand scission in a reaction catalyzed by Cu^{2+} and O_2 (Chrisey et al., 1988). Several other structurally related catechol flavanoids caused less efficient DNA damage under similar conditions (Chrisey et al., 1988). It was, therefore, suggested that the above catechols or their Cu^{2+} complexes associate with DNA prior to the production of oxy radicals. The VP-16 catechol–Fe^{3+} complex might also react in a similar manner (Sinha et al., 1988).

Recently Teicher et al. have shown that the antitumor activity of VP-16 is enhanced following the addition of Fluosol–DA and carbogen breathing (Teicher et al., 1985). This effect was felt to be mediated by oxy radicals. On the basis of the present work it is clear that VP-16 phenoxyl radicals do not react with molecular oxygen at an appreciable rate. Therefore, this reaction is not likely to increase the production of oxy radicals. However, the present data suggest that increased oxygenation leads to an increase in the production of quinones, semiquinones, and hydrogen peroxide (data not shown), which may account for enhanced cytotoxic effects observed during the combined modality (Telcher et al., 1985). Previously, Haim et al. have shown an increase in the metabolism of VP-16 in the presence of oxygen (Haim et al., 1987).

In conclusion we have detected and characterized both primary and secondary radicals from VP-16, VP-16 catechol, and VP-16 quinone. Neither the primary nor the secondary radicals react with molecular oxygen or DNA at an appreciable rate. The hydroxyl radical was detected during the iron-catalyzed oxidation of VP-16 catechol. It is likely that these reactions may lead to the characterization of marker products during metabolism of VP-16 in tumor cells of relevance to an understanding of the antitumor activity of VP-16.

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