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FREE RADICAL FORMATION FROM THE ANTINEOPLASTIC AGENT VP 16-213

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Free radical formation from VP 16-213 was studied by ESR spectroscopy. Incubation of VP 16-213 with the one-electron oxidators persulphate-ferrous, myeloperoxidase (MPO)/hydrogen peroxide and horseradish peroxidase (HRP)/hydrogen peroxide readily led to the formation of a free radical. The ESR spectra obtained in the last two cases, were in perfect accord with that of a product obtained by electrochemical oxidation of VP 16-213 at +550 mV. The half-life of the free radical in 1 mM Tris (pH 7.4), 0.1 M NaCl at 20°C, was 257 ± 4 s. The signal recorded on incubation with HRP/H_2O_2 or MPO/H_2O_2 did not disappear on addition of 0.3 – 1.2 mg/ml microsomal protein. From incubations with rat liver microsomes in the presence of NADPH, no ESR signals were obtained.

Key words: Free Radicals, Antineoplastic Agent, Electron Spin Resonance.

INTRODUCTION

VP 16-213 [4′-demethylepipodophyllotoxin-9-(4,6-O-ethylidene-β-D-glucopyranoside), NSC 141540 (Figure 1)] is an important chemotherapeutic agent used in the treatment of a variety of tumors[1] and is currently undergoing extensive clinical evaluation. Studies on the bioactivation of VP 16-213 have yet failed to identify active metabolites of the drug. Studies of Loike and Horwitz[2] and more recently Wozniak and Ross[3] have suggested that activation of the drug is required for its effect on DNA. Regarding the structure of VP 16-213, formation of a toxic free radical species in the dimethoxyphenol ring (the E-ring) should be considered. Sinha *et al.*[4] reported the generation of a free radical on oxidation by HRP and proposed that this radical was oxygen-centered. In a preliminary study[5], we also observed the formation of a VP 16-213 free radical.

The aim of the present study was to investigate in more detail the properties of the VP 16-213 free radical.

MATERIALS AND METHODS

VP 16-213 was a generous gift from the Bristol Myers Company (Syracuse, N.Y., U.S.A). NADPH was obtained from Boehringer Mannheim GmbH, GFR. MPO, isolated from human granulocytes[6], was a gift from Dr. R. Wever from the University of Amsterdam, The Netherlands. HRP type II (specific activity 165 units/mg) was purchased from Sigma Chemical Company, St. Louis, M.O., U.S.A. All other chemicals used were reagent grade and obtained from J.T. Baker Chemicals B.V., Deventer, The Netherlands.

Male albino Wistar rats (175–200 g) were used for preparation of liver microsomes. Livers of rats treated with 1 g/l sodium phenobarbital in their drinking water for 10 days, were homogenized in 1.15% potassium chloride- 0.01 M potassium phosphate pH 7.4, (4 ml/g liver). Microsomes were prepared by centrifugation of the homogenate at 10,000 g for 20 min and recentrifugation of the supernatant at 100,000 g for 1 hr.

Chemical oxidation of VP 16-213 was carried out using persulphate-ferrous ($S_2O_8^{2-}/Fe^{2+}$). The reaction mixture contained 0.5 ml aqueous 140 mM $K_2S_2O_8$, 0.5 ml aqueous 70 mM $FeSO_4.7H_2O$ and 50 μl 20 mM VP 16-213 in DMSO. ESR spectra were recorded approximately 30 s after mixing. For indirect ESR measurements, the reaction mixture was extracted with 1 ml cold chloroform, the organic layer was evaporated to dryness under a stream of nitrogen and the residue dissolved in 0.3 ml nitrogen-gassed chloroform. Enzymatic oxidation was performed using MPO/H_2O_2, HRP/H_2O_2 or rat liver microsomes in 0.1 M phosphate buffer (pH 7.4). In the MPO system, 1 ml 1 μM MPO, 10 μl 170 mM VP 16-213 in DMSO and 200 μl aqueous 4 mM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.4), in a total volume of 2 ml, were incubated at room temperature for 3 min prior to ESR measurement. In the HRP system, 5–50 μl HRP solution in water (165 units/ml), 1–2 μl 170 mM VP 16-213 in DMSO and 10 μl aqueous 100 mM H_2O_2 in 1 mM Tris HCl buffer (pH 7.4) were incubated in a total volume of 0.5 ml at room temperature for 1 min prior to ESR measurement. The microsomal system consisted of microsomal protein (0.5–3 mg/ml), NADPH 1 mM and VP 16-213 1 mM (added in DMSO) in 100 mM potassium phosphate buffer (pH 7.4). For ESR measurements with an aqueous flat cell, 1 ml microsomal reaction mixture was incubated at 37°C for 30 s. For indirect measurements, 10 ml microsomal suspension was incubated at 37°C for 10 min and thereafter extracted with 10 ml chloroform. The organic layer was evaporated and treated as described above. Electrochemical oxidation of VP 16-213 was carried out at +550 mV using Pt as working and auxiliary electrode and Ag/AgCl as reference electrode. The potentiostate was a G.B.E. model 68 FR 0.5. VP 16-213 was dissolved in methanol/0.1 M phosphate buffer (50:50 v/v), pH 7.4. KNO_3 was added as electrolyte (final concentration 1 M). The VP 16-213 free radical was extracted with chloroform, the organic layer treated as described above and analysed by ESR.

In order to determine the rate of radical generation and the chemical half-life of the radical, on-line coupling of electrochemical oxidation and ESR was used (Figure 2). By fast repetitive scanning at high modulation amplitude, the chemical half-life of the radical was determined in 1 mM Tris (pH 7.4), 0.1 M NaCl and in 1 mM Tris (pH 7.4), 0.1 M NaCl containing 20% methanol (v/v), at 20°C. The concentration of the free radical in the ESR flat cell was calculated from the half-life data, the flow and the current used, as shown in scheme 1.

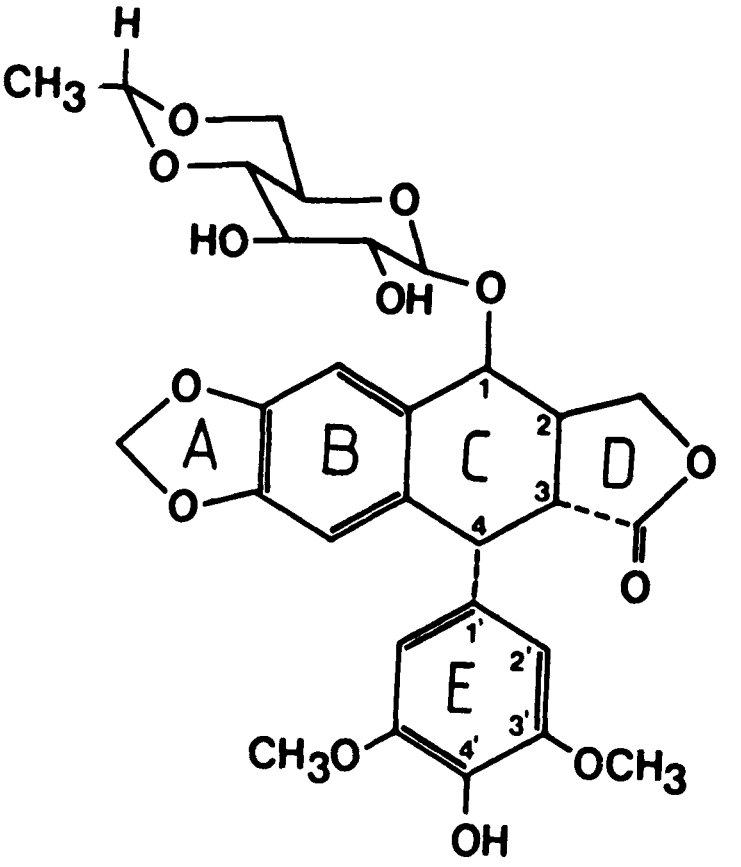

FIGURE 1 Structure of VP 16-213.

ESR spectra were recorded at room temperature on a Varian E-3 spectrometer equipped with an E-4531 multipurpose cavity. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions are described in legends to figures.

RESULTS

In order to determine if VP 16-213 would be oxidized by one-electron transfer, the potent chemical one-electron oxidant $S_2O_8^{2-}/Fe^{2+}$ was used[7]. The results of experiments on VP 16-213 radical generation in the oxidation by $S_2O_8^{2-}/Fe^{2+}$ are shown in Figure 3a–3d. A radical could be detected in the aqueous reaction mixture for 2 min after addition of $S_2O_8^{2-}/Fe^{2+}$ (Figure 3a). After extraction with chloroform, a stable radical was detected (Figure 3c), even after 100 hours at room temperature. No radical formation was observed if VP 16-213 was omitted (Figures 3b and 3d). Free radical formation also occurred on incubation with the enzymatic one-electron oxidants

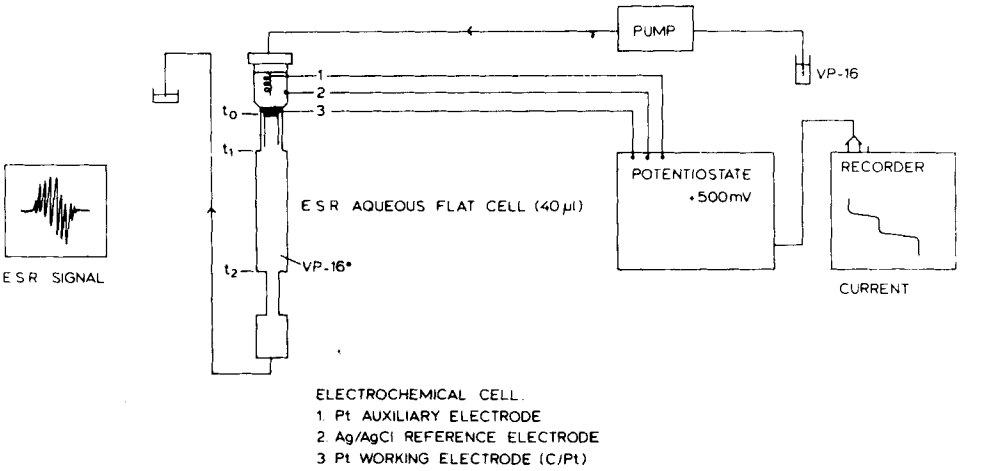

FIGURE 2 On-line coupling of electrochemical oxidation and ESR.

MPO/H_2O_2 and HRP/H_2O_2 (Figure 4a). The g-value and hyperfine splittings of the ESR spectra were in perfect accord with the ESR spectrum of a product obtained on electrochemical oxidation of VP 16-213 at +550 mV (Figures 4a and 4b).

Incubations with microsomes did not give rise to ESR signals, neither directly in an aqueous flat cell, nor after extraction with chloroform. The signal recorded on incubation with the enzymatic one-electron oxidants did not disappear on addition of 0.3–1.2 mg/ml microsomal protein.

Figure 5 shows the ESR spectra of the electrochemically generated VP 16-213 free radical, obtained by fast repetitive scanning at high modulation amplitude. The data from these spectra were used to calculate the half-life of the radical as shown in Figure 6. The half-lives of the free radical in 1 mM Tris (pH 7.4), 0.1 M NaCl and in 1 mM Tris (pH 7.4), 0.1 M NaCl containing 20% methanol (v/v) at room temperature were found to be 257 ± 4 s (n = 3) and 361 ± 13 s (n = 3), respectively. The data used to calculate the concentration of the free radical ($\bar{C}$) by on-line coupling of electrochemical oxidation and ESR, are listed in Table I. The following calibration curve was calculated from the data of the free radical concentration: ESR signal peak height = 1.73 $\bar{C}$ + 3.55; r = 0.92 (Figure 7). The VP 16-213 radical concentration values during enzymatic one-electron oxidation by HRP/H_2O_2 were obtained from the calibration curve and plotted against time (Figure 8).

DISCUSSION

The present study clearly shows that VP 16-213 can be oxidized by one-electron transfer, nonenzymatically as well as enzymatically. From the total linewidth of the spectra, i.e. less than 25 Gauss, we conclude that a radical is formed, which is delocalised in a π-system, suggesting that the B- or E-ring of VP 16-213 is involved in radical formation (Figure 1). The ESR spectra obtained with the enzymatic one-electron oxidants were identical to the ESR spectrum of a product generated by electrochemical oxidation of VP 16-213 (Figure 4). Since the E-ring of VP 16-213 is

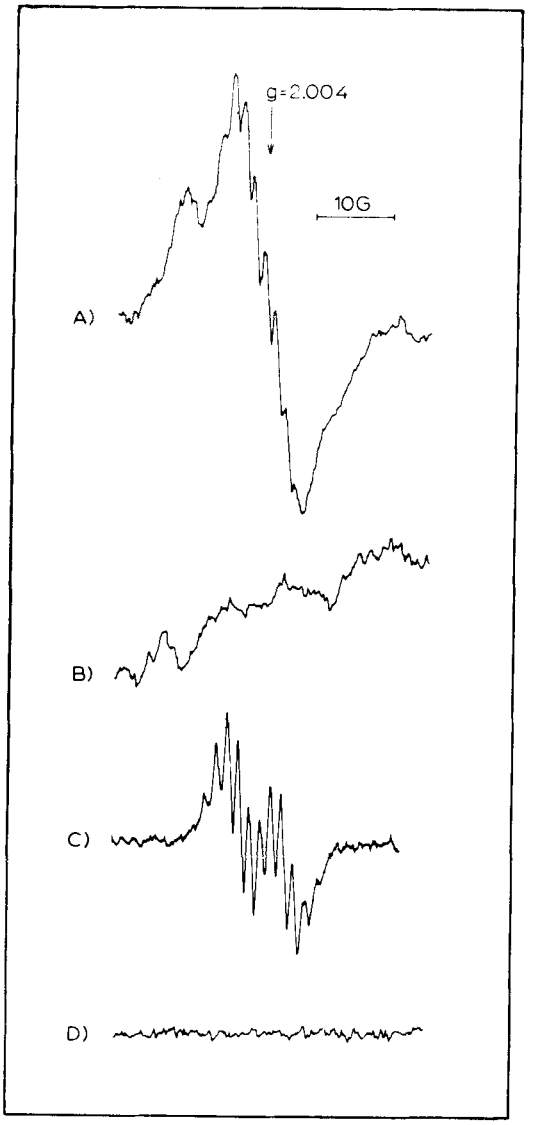

FIGURE 3 Chemical oxidation of VP 16-213 by $S_2O_8^{2-}/Fe^{2+}$: ESR spectra.

A) In aqueous flat cell, 30 s after addition of $S_2O_8^{2-}/Fe^{2+}$. Instrumental conditions were as follows: magnetic field, 3375 G; scan range, 100 G; modulation amplitude, 1.6 G; gain, 2.10^6; power, 32 mW; scan time, 4 min; time constant, 3 s.

B) Conditions as described in A), with the exception that VP 16-213 was omitted.

C) In a standard quartz tube, chloroform extract. Instrumental conditions were as follows: magnetic field, 3375 G; scan range, 100 G; modulation amplitude, 0.5 G; gain, $1.25.10^6$; power, 100 mW; scan time, 60 min; time constant, 10 s.

D) Conditions as described in C), with the exception that VP 16-213 was omitted.

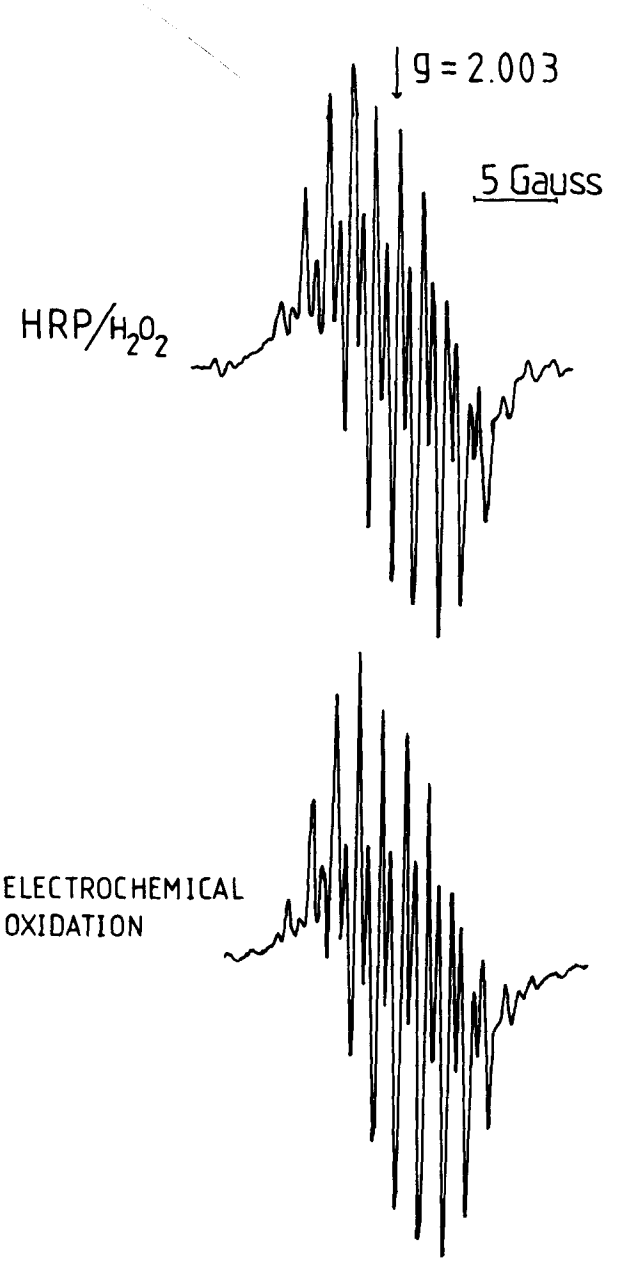

FIGURE 4 Formation of VP 16-213 free radical by horseradish peroxidase and electrochemical oxidation: ESR spectra.

Instrumental conditions were as follows: magnetic field, 3375 G; scan range, 100 G; modulation amplitude, 0.4 G; gain, 2.10^5; power, 100 mW; scan time, 8 min; time constant, 1 s.

TABLE I
Data used to calculate the calibration curve of the VP-16 free radical.

VP-16 conc.[a] (μg/ml)	flow (10^{-3}ml/s)	current A (μA)	ESR-signal peak height (mm)	C_0 (10^{-5} M)	$\bar{C}$ (10^{-5} M)
146	0.92	5.6	146	6.3	5.3
146	1.80	7.4	134	4.3	3.9
323	1.80	22.0	308	12.7	11.6
646	0.92	17.5	250	19.6	16.4
646	1.80	21.5	204	12.4	11.4
840	0.92	19.0	248	21.3	17.8
840	1.80	57.0	624	32.8	30.1

[a] VP-16 in 1 mM Tris (pH 7.4) 0.1 M NaCl, containing 20% v/v methanol.
Current A = current through electrochemical cell.
C_0 = radical concentration in electrochemical cell.
$\bar{C}$ = radical concentration in ESR cell.
ESR spectra were recorded at 20°C. The ESR cell volume was 40 μl. Instrumental conditions were: magnetic field, 3375 G; scan range, 100 G; modulation amplitude, 0.4 G; gain, 5.10^5; power, 100 mW; scan time, 8 min; time constant, 1 s.

$$A = C_0 \text{ (mol/ml)}. \text{ flow (ml/s)}. F \text{ (C/mol)}$$

$$C_t = C_0 e^{-0.693\, t/t_{\frac{1}{2}}} = C_0 e^{-kt}$$

$$\bar{C} = \frac{\int_{t_1}^{t_2} C_0 e^{-kt}\, dt}{t_2 - t_1} = \frac{C_0}{t_2 - t_1} \cdot - \frac{1}{k} \left(e^{-kt_2} - e^{-kt_1}\right)$$

SCHEME I.
Calculation of VP 16-213 free radical concentration ($\bar{C}$) in an ESR cell.

A = current trough electrochemical cell.
C_0 = radical concentration at electrode.
F = faraday constant.
$t_{\frac{1}{2}}$ = half life of the free radical.
t_1 = flow time from electrochemical cell to upper end of the ESR cell.
t_2 = flow time from electrochemical cell to lower end of the ESR cell.

oxidized by two one-electron steps during electrochemical oxidation at +450 mV and +800 mV[8], the ESR data presented here is the first direct evidence for the formation of a VP 16-213 radical by enzymatic oxidation of the phenolic hydroxyl group of the E-ring of the drug.

At room temperature, the VP 16-213 free radical is stable in aqueous solutions and in particular in chloroform. Further, the signal recorded on incubation with HRP or MPO did not disappear on addition of hepatic microsomes, indicating that the radical is not effectively scavenged by microsomal proteins. Moreover, microsomal incubations of VP 16-213, in the presence of NADPH, did not give rise to any detectable ESR signal, neither measured directly in an aqueous flat cell, nor after extraction with chloroform. These findings provide strong evidence that a VP 16-213 free radical is not formed in microsomal incubations in the presence of NADPH, suggesting that the formation of other reactive metabolites can also play a role in the cytotoxicity of VP 16-213. Recently, we observed that incubation of VP 16-213 with purified cytochrome P-450, cytochrome P-450 reductase and NADPH or with cytochrome P-450 and cumene hydroperoxide, resulted in the formation of the ortho-dihydroxy derivative of VP 16-213[9]. This metabolite can be oxidized to the ortho-quinone, a structure

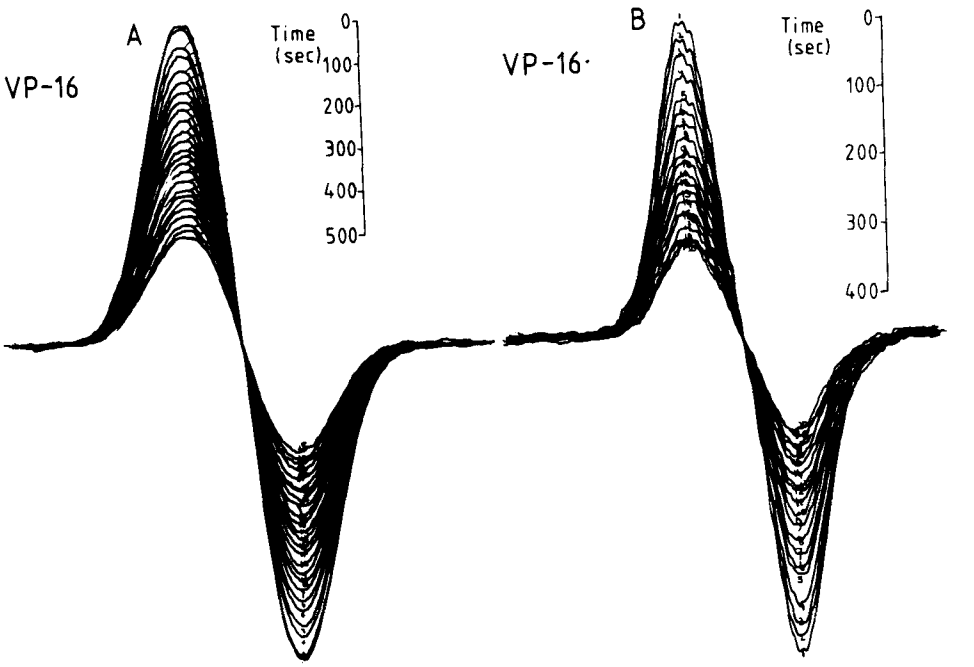

FIGURE 5 Decay of the VP 16-213 free radical.
A) In 1 mM Tris (pH 7.4) 0.1 M NaCl, containing 20% v/v methanol.
B) In 1 mM Tris (pH 7.4) 0.1 M NaCl.

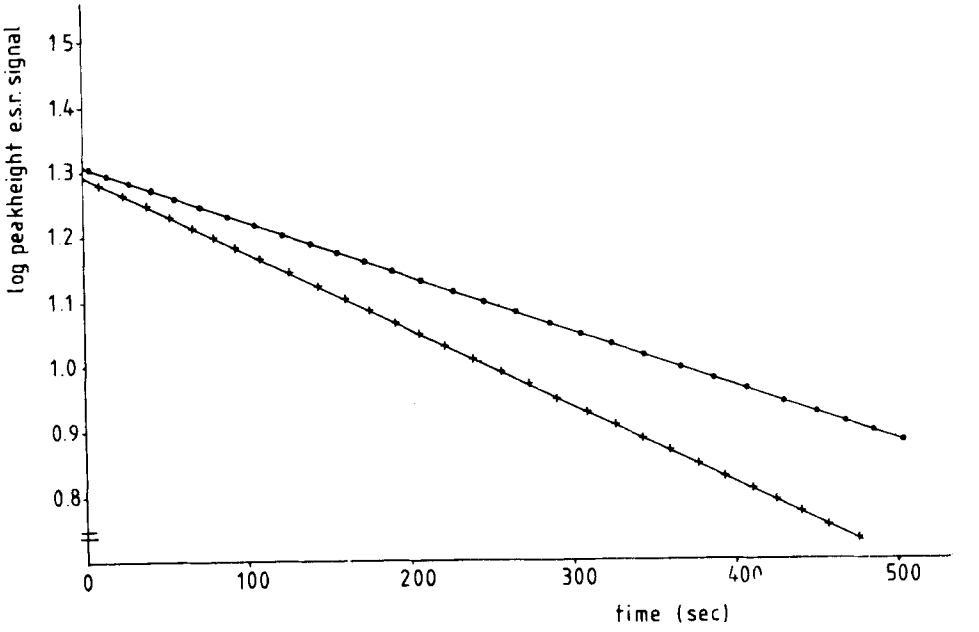

FIGURE 6 Determination of the chemical half-life of the VP 16-213 free radical.
..... In 1 mM Tris (pH 7.4) 0.1 M NaCl, containing 20% v/v methanol; $t_{\frac{1}{2}} = 361 \pm 13$ s (n = 3).
+++++ In 1 mM Tris (pH 7.4) 0.1 M NaCl; $t_{\frac{1}{2}} = 257 \pm 4$ s (n = 3).

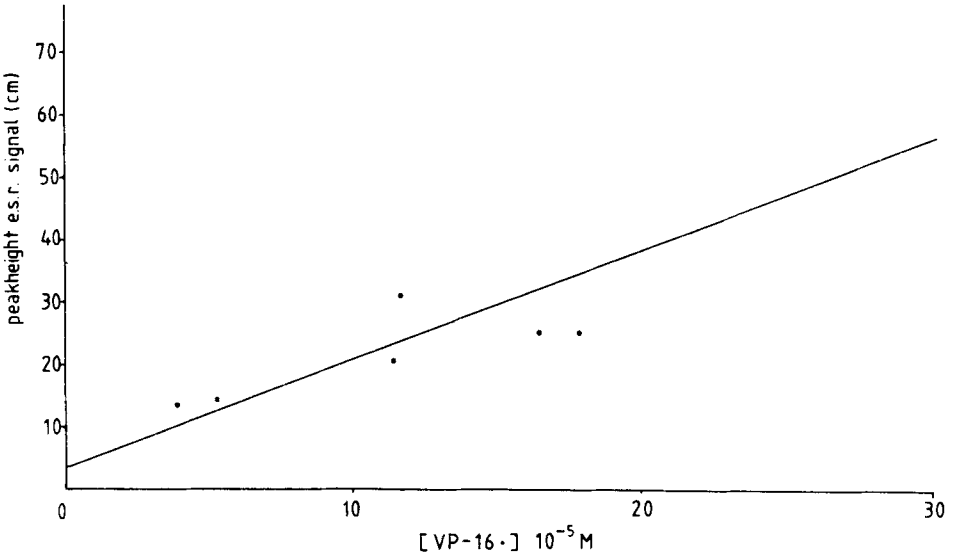

FIGURE 7 Calibration curve of the VP 16-213 free radical: y = 1.73 x + 3.55, r = 0.92.

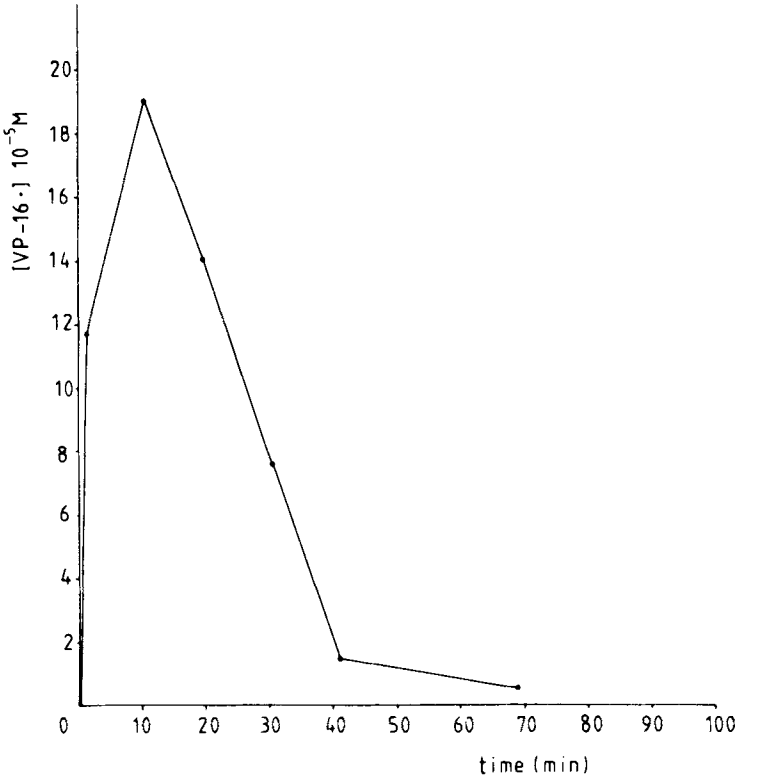

FIGURE 8 Formation of VP 16-213 free radical by horseradish peroxidase: Concentration versus time curve.

Incubation at 20°C of 680 μM VP 16-213, 1.65 unit HRP and 1 mM H_2O_2 in 0.5 ml 1 mM Tris (pH 7.4).

capable of redox cycling. The aim of further studies is to provide structural evidence for the formation of these metabolites and evaluation of their possible interaction with DNA.

Acknowledgements

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