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ON THE INTERACTION OF ANISYL-3,4-SEMIQUINONE WITH OXYGEN

CHRISTOPHER J. COOKSEY[1], EDWARD J. LAND[2‡], PATRICK A. RILEY[3], TADEUSZ SARNA[4] and T. GEORGE TRUSCOTT[5]

[1]*Department of Chemistry, University College, Gordon St., London WC1, U.K.*
[2]*Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, U.K.*
[3]*Department of Chemical Pathology, University College and Middlesex School of Medicine, Windeyer Building, Cleveland St., London W1P 6DB, U.K.*
[4]*Department of Biophysics, Jagiellonian University, 31–120 Krakow, Poland*
[5]*Department of Chemistry, Paisley College of Technology, Paisley PA1 2BE, U.K.*

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Pulse radiolysis studies of anisyl-3,4-semiquinone, formed in the metabolic activation of 4-hydroxyanisole, a possible melanocytotoxic drug under current assessment as a treatment for malignant melanoma, have shown this semiquinone to be unreactive towards oxygen ($k \leqslant 10^5 M^{-1}s^{-1}$), although the reverse reaction of $O_2^{\cdot-}$ with anisyl-3,4-quinone is very rapid ($k = 8.7 \times 10^8 M^{-1}s^{-1}$). Since 1,4 benzoquinone is also unreactive towards anisyl-3,4-semiquinone ($k \leqslant 10^5 M^{-1}s^{-1}$), the one-electron reduction potential, E_7^1 (anisyl-3,4-quinone/anisyl-3,4-semiquinone), is likely to be considerably more positive than 0.1 V. This suggests that the cytotoxicity mechanism does not involve the generation of $O_2^{\cdot-}$ and possible subsequent production of H_2O_2 and/or $OH\cdot$, leading to lipid peroxidation, as previously proposed, but rather involves as yet unknown reactions of anisyl-3,4-quinone. This quinone is unstable in water and its absorption spectrum was measured immediately (< 0.1 s) following disproportionation of anisyl-3,4-semiquinone, before significant decay of the quinone had occurred.

KEY WORDS: Semiquinone, oxygen radicals, cytotoxicity, melanoma, tyrosinase, 4-hydroxyanisole, pulse radiolysis, one-electron reduction potential.

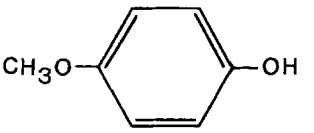
INTRODUCTION

Malignant melanoma, a tumour of melanocytes, is a highly aggressive and increasingly prevalent form of cancer. Many malignant melanomas generate large quantities of melanin and it has therefore been the aim of rational chemotherapy for these tumours to use the melanogenic pathway as a means of targeting cytotoxic therapy.

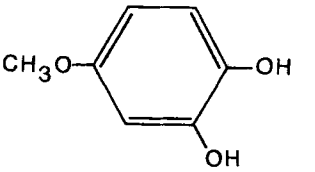
Tyrosinase is present only in melanocytes where it catalyses the oxidation of tyrosine to quinones which polymerise ultimately giving rise to melanin. Certain analogues of tyrosine can be oxidised by tyrosinase to reactive orthoquinones, and 4-hydroxyanisole is currently being investigated[1] as such a possible melanocytotoxic precursor.

In vitro experiments have shown 4-hydroxyanisole to be readily oxidised by mushroom tyrosinase to the 3,4-orthoquinone of anisole, and ESR evidence[2] shows that

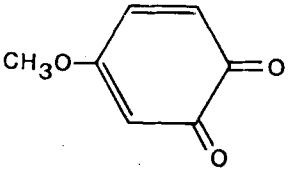
‡Correspondence to: E.J. Land



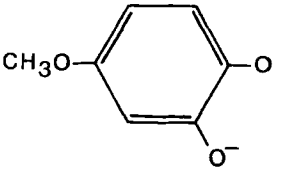
4-hydroxyanisole



anisyl-3,4-hydroquinone



anisyl-3,4-quinone



anisyl-3,4-semiquinone

under certain conditions the corresponding anisyl-3,4-semiquinone radical anion is also formed. It is not known if cytotoxicity is due to the formation of this radical, possibly leading to lipid peroxidation[3] via superoxide formation, or to further reactions of the quinone, for example, addition reactions with –SH compounds.[4]

Nilges and Swartz[5] have demonstrated that *in vitro* the semiquinone radical ESR signal is inversely related to the oxygen concentration and interpret this finding in terms of a cycle of reduction of anisyl-3,4-quinone to the corresponding hydroquinone with enzymatic reoxidation of the hydroquinone back to the quinone, rather than reaction of the semiquinone with oxygen. Assuming that the semiquinone is formed by reverse dismutation of the anisyl-3,4-quinone and the corresponding hydroquinone, the steady-state concentration of semiquinone will remain low while oxygen is available because the hydroquinone concentration will be small.

The influence of tyrosinase on the disappearance of the unstable anisyl-3,4-quinone has however also been examined.[6] While the rate of removal of the quinone is significantly diminished in the presence of tyrosinase, this effect cannot be due to reoxidation of the corresponding hydroquinone because it was also observed in a nitrogen atmosphere. Thus the role of oxygen as a regulator of the semiquinone is somewhat obscure.

We have now generated the semiquinone of anisyl-3,4-quinone using pulse radiolysis in order to provide further kinetic evidence regarding its reaction with oxygen and the reverse reaction of the superoxide anion radical with anisyl-3,4-quinone.

MATERIALS AND METHODS

Preparation of anisyl-3,4-hydroquinone

4-hydroxyanisole was oxidised to anisyl-3,4-quinone with potassium nitrosodisulphonate and the quinone immediately reduced to the hydroquinone with sodium dithionite as follows:

A solution of 4-hydroxyanisole (1.0 g in ether, 10 ml) was added to a solution of potassium nitrosodisulphonate (6 g) dissolved in a solution made by adding potassium dihydrogen phosphate (10 g) to water (200 ml) and ice (200 g), and the mixture was magnetically stirred for 45 minutes. The ether was then removed by rotoevaporation at 20°C and the resulting mixture extracted with chloroform (6 × 25 ml). The first extract was deep orange-red and the last pale yellow.

The combined chloroform extracts of the anisyl-3,4-quinone were shaken with a solution of sodium dithionite (8 g) and disodium hydrogen phosphate (2.2 g) in water (100 ml), cooling in ice. The solution immediately became yellow. The chloroform layer was separated, the aqueous layer then saturated with magnesium sulphate and extracted with more chloroform (200 ml). The combined extracts were dried ($MgSO_4$) and evaporated to give a black oil.

The crude anisyl-3,4-hydroquinone was acetylated to give 1,2-diacetoxy-4-methoxybenzene which was stable and easily purified. The black oil was thus dissolved in pyridine (5 ml) and acetic anhydride (5 ml) added. After 24 hr, water (2 ml) was added to hydrolyse excess acetic anhydride and after a further 4 hr more water (25 ml) was added and the mixture extracted with chloroform (3 × 50 ml). Evaporation of the chloroform gave a dark red pyridine solution which was diluted with ether (50 ml) and washed with portions of 2 M HCl (total 100 ml), then saturated NaCl solution (50 ml) and evaporated to give a dark brown oil. This product was flash chromatographed on 20 g of silica, eluting with 1:1 ethyl acetate-petroleum ether 60/80 giving a pale red viscous oil which solidified on standing. The yield was 0.95 g (53%).

The ^1H NMR spectrum ($CDCl_3$, 400 MHz) showed two acetate singlets (δ2.267 and 2.283), a methoxy singlet (δ3.781) and 3 aromatic ring protons, δ6.725 d (2.96 Hz), 6.775 dd (2.96, 8.96) and 7.077 d (8.96). A nuclear Overhauser experiment, irradiating the methoxy group, showed the expected enhancement of the 6.72 and 6.77 protons. The mass spectrum showed peaks at 224, 182 and 140 (the base peak): the accurate mass was 224.0673, the calculated value for $C_{11}H_{12}O_5$ being 224.0684.

In order to prepare pure anisyl-3,4-hydroquinone from the purified 1,2-diacetoxy-4-methoxybenzene, the acetate (0.94 g) was heated on an oil bath under N_2 with HCl-H_2O (1:1, 10 ml). After 0.5 hr, ethanol (3 ml) was added and after a further 3 hr the solution was filtered with charcoal and celite to give a nearly colourless solution which was evaporated and dried at 45°C and 0.01 mm. ^1H NMR (60 MHz, $CDCl_3$) of the resulting product showed peaks at δ3.77 s, 4.5 broad s, 6.30 dd (7.2), 6.48 d (2) and 6.75 d (7). The mass spectrum showed the molecular ion at m/e 140 with a prominent peak at m/e 125 ($M^+ - CH_3$). The accurate mass was 140.0480 (calculated for $C_7H_8O_3$, mass = 140.0486). The analytical data are thus consistent with the product being anisyl-3,4-hydroquinone. The UV spectrum in water showed maxima at 292 nm (ε = 1880 $M^{-1}cm^{-1}$) and 230 nm (ε = 1620 $M^{-1}cm^{-1}$).

Pulse radiolysis

The pulse radiolysis experiments were performed with a 9–12 MeV Vickers linear accelerator as previously described[7] using 10–50 ns pulses with doses up to 32 Gy and quartz capillary cells of optical path 2.5 cm. Absorbed doses were determined from transient $(SCN)_2^-$ yield of 0.30 μM/Gy and $\varepsilon_{500\,nm}$ = 7100 $M^{-1}cm^{-1}$.[8] Generation of the oxidising species $N_3^\cdot$ was achieved by irradiating N_2O-saturated aqueous solutions of NaN_3 and generation of reducing O_2^- by irradiating O_2-saturated aqueous solutions of HCOONa.

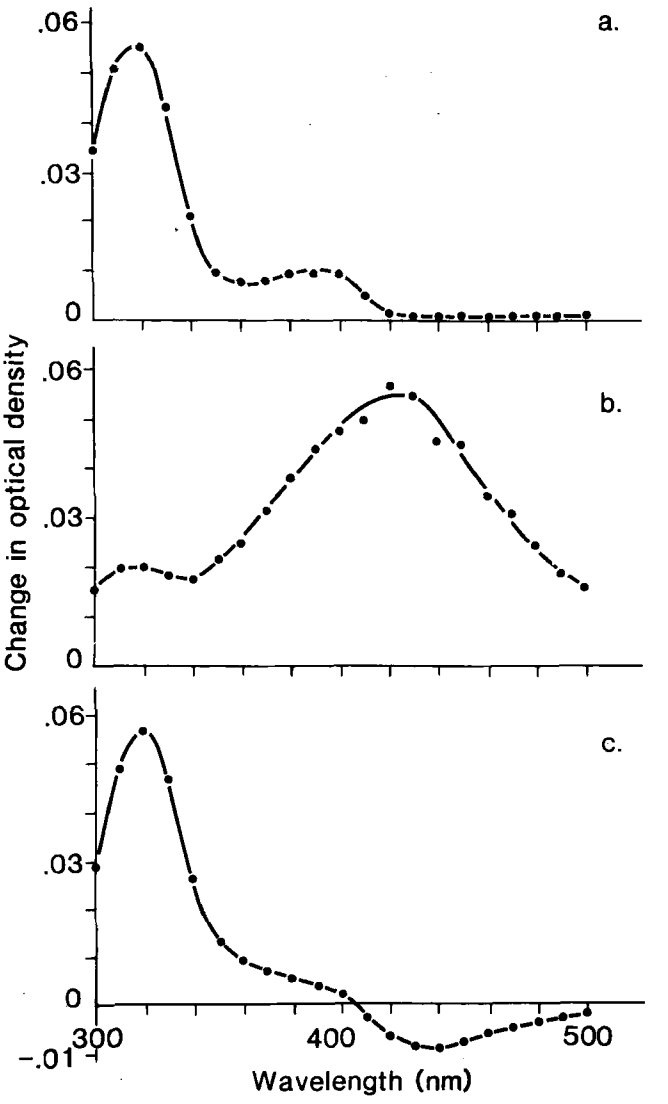
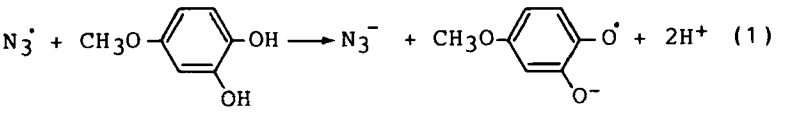


FIGURE 1 Transient change in spectrum (a) 160 μs after pulse radiolysis of 0.93×10^{-4} M anisyl-3,4-hydroquinone in nitrous oxide saturated 5×10^{-2} M NaN_3 containing 10^{-1} M phosphate buffer, pH 7.0. (b) 23 ms after pulse radiolysis of 1.2×10^{-3} M anisyl-3,4-hydroquinone in nitrous oxide saturated 5×10^{-2} M NaN_3 containing 10^{-1} M phosphate buffer, pH 7.0. (c) 260 μs after pulse radiolysis of 1.5×10^{-5} M anisyl-3,4-quinone in oxygen-saturated 10^{-1} M HCOONa + 10^{-1} M phosphate buffer, pH 7.0. This solution also contained 5.2×10^{-5} M anisyl-3,4-hydroquinone (see text).

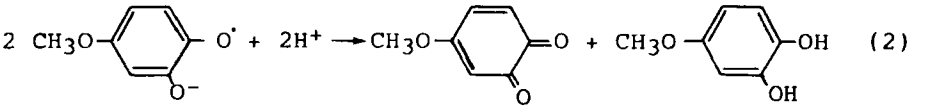
RESULTS AND DISCUSSION

In view of the limited stability of anisyl-3,4-quinone, where possible it was considered most convenient to prepare anisyl-3,4-semiquinone by one-electron oxidation of anisyl-3,4-hydroquinone rather than one-electron reduction of the quinone.

One-electron oxidation of anisyl-3,4-hydroquinone was carried out by pulsed irradiation of a N_2O-saturated 0.93×10^{-4} M solution of the hydroquinone containing 5×10^{-2} M NaN_3, buffered to pH 7.0 with 10^{-1} M phosphate. The transient change in spectrum obtained 160 μs after the pulse is shown in Figure 1(a). This spectrum is assigned to the anisyl-3,4-semiquinone anion radical formed via the following reaction:



This identification with the deprotonated form of the semiquinone is based on ESR evidence obtained at a similar pH[5]. From studies of the pseudo first-order rate of formation of semiquinone absorption at 320 nm, a second-order rate constant of 4.7×10^9 $M^{-1}s^{-1}$ was obtained for reaction (1). Based on a yield for $N_3^{\cdot}$ radicals of 0.62 μM/Gy,[9] after correction for anisyl-3,4-hydroquinone depletion, an extinction coefficient of 10,700 $M^{-1}cm^{-1}$ was found for the semiquinone at 320 nm (Figure 2(a)). The decay of the semiquinone followed second-order kinetics and use of the appropriate extinction coefficient led to a rate constant of $2k = 2.5 \times 10^8$ $M^{-1}s^{-1}$ for this disproportionation reaction:



This reaction provides a convenient and rapid means of measuring the absorption spectrum and extinction coefficients of anisyl-3,4-quinone in water before polymerisation of the quinone begins to occur. Figure 1(b) thus shows the change in spectrum 23 ms after pulse radiolysis of 1.2×10^{-3} M anisyl-3,4-hydroquinone containing 5×10^{-2} M NaN_3 buffered to pH 7.0 with 10^{-1} M phosphate. At this time the disproportionation reaction is complete. On the assumption that two $N_3^{\cdot}$ radicals lead to one quinone molecule, the absolute spectrum of the quinone given in Figure 2(b) was obtained from the data in Figure 1(b), after correction for anisyl-3,4-hydroquinone depletion.

The kinetics of the reaction of oxygen with the semiquinone was sought by addition of oxygen to solutions similar to the above. Since prolonged bubbling with oxygen resulted in the conversion of anisyl-3,4-hydroquinone into the quinone, oxygen was added rapidly by mixing one volume of oxygen-saturated 5×10^{-2} M NaN_3 + 10^{-1} M phosphate buffer with an equal volume of N_2O-saturated 5×10^{-2} M NaN_3 + 10^{-1} M phosphate buffer containing 1.4×10^{-3} M anisyl-3,4-hydroquinone. Using doses of 3.2 Gy per pulse, i.e. as low as possible commensurate with a steady base line over milliseconds, the second order decay of the semiquinone

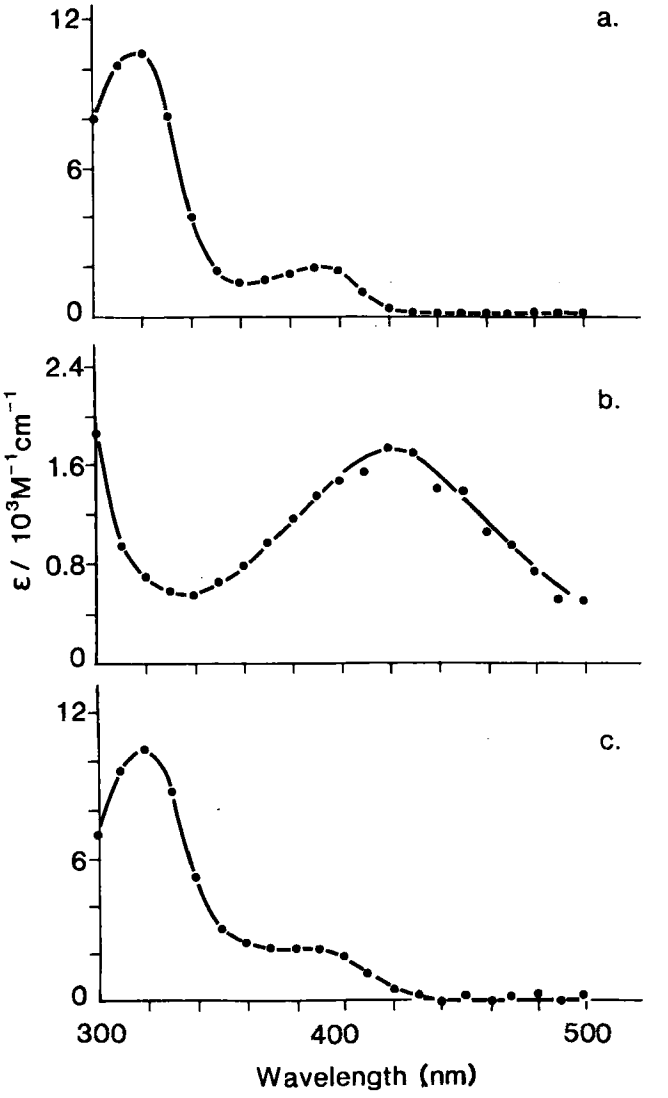
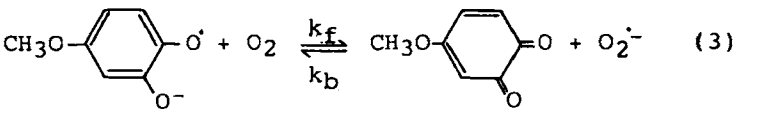


FIGURE 2 Absolute absorption spectra in water of (a) anisyl-3,4-semiquinone formed by one-electron oxidation of anisyl-3,4-hydroquinone (b) anisyl-3,4-quinone formed by disproportionation of anisyl-3,4-semiquinone (c) anisyl-3,4-semiquinone formed by one-electron reduction of anisyl-3,4-quinone.

was found to be, within experimental error, unaffected by the addition of oxygen, consistent with a rate constant (k_f) for the forward reaction (3) of $\leqslant 10^5 M^{-1} s^{-1}$,



(3)

The reverse of reaction (3) was investigated by studying solutions of anisyl-3,4-

quinone in oxygen-saturated 10^{-1} M HCOONa + 10^{-1} M phosphate buffer, pH 7.0. Solutions containing anisyl-3,4-quinone were obtained by bubbling oxygen through solutions of anisyl-3,4-hydroquinone for short periods of time. Under the conditions chosen, starting with a solution of 6.7×10^{-5} M hydroquinone, about 20% conversion to the quinone was obtained on bubbling with oxygen for about 20 mins. The quinone concentration was estimated from its absorption at 420 nm using an extinction coefficient of 1720 $M^{-1}cm^{-1}$. Pulse radiolysis of this oxygen-bubbled solution resulted in the transient difference spectrum shown in Figure 1(c). On the assumption that all $O_2^{\cdot-}$ radicals (yield 0.67 μM/Gy[10]) are scavenged by the quinone, the spectrum of the semiquinone corrected for quinone depletion was obtained, as shown in Figure 2(c). Since this spectrum is almost identical to that shown in Figure 2(a), it seems likely that $O_2^{\cdot-}$ reacts exclusively with the quinone component of the solution to yield the semiquinone. The small difference between Figure 2(a) and (c) may be due to slight polymerisation of anisyl-3,4-quinone in the solution from which the spectrum of Figure 2(c) was obtained. From the pseudo first order growth of semiquinone observed at 320 nm, and depletion of quinone observed at 420 nm, a second-order rate constant (k_b) of 8.7×10^8 $M^{-1}s^{-1}$ was obtained for the reverse reaction (3).

The rapid reaction of $O_2^{\cdot-}$ with quinone is consistent with the couple E_7^1 (anisyl-3,4-quinone/anisyl-3,4-semiquinone) being much more positive than the couple $O_2/O_2^{\cdot-}$ ($E_7^1 = -155$ mV, where the oxygen concentration is expressed as molar). The estimates of both the forward and backward rate constants of reaction (3) may be used to calculate an equilibrium constant limit and hence a one-electron reduction potential limit for anisyl-3,4-quinone. Thus:

$$K_3 = \frac{k_f}{k_b} = \frac{\leqslant 10^5}{8.7 \times 10^8}, \quad \text{and}$$

$$E_7^1(\text{Anisyl-3,4-quinone/anisyl-3,4-semiquinone}) \geqslant -155 + 59 \log (8.7 \times 10^8/10^5)\text{mV}$$

$$\text{i.e.} \geqslant +77\,\text{mV}$$

In order to obtain further information on the one-electron reduction potential of the anisyl quinone/semiquinone couple, the decay of the semiquinone was studied using N_2O-saturated 1.0×10^{-3} M anisyl-3,4-hydroquinone + 5×10^{-2} M NaN_3 + 10^{-1} M phosphate buffer pH 7.0 in the presence and absence of 1.0×10^{-4} M, 1,4 benzoquinone ($E_7^1 = +99$ mV).[11] Since the 1,4 benzoquinone was found to have no detectable effect on the decay of the anisyl-3,4-semiquinone ($k \leqslant 10^5 M^{-1}s^{-1}$), it seems likely that the corresponding E_7^1 for anisyl-3,4-quinone is considerably more positive than 0.1 V. It may be noted that the E_7^1 for the structurally related but unstable 1,2 benzoquinone has been estimated to be +210 mV.[11]

Our studies thus support the conclusion that the cytotoxicity mechanism associated with the administration of 4-hydroxyanisole in the treatment of malignant melanoma does not involve the generation of $O_2^{\cdot-}$, and possible subsequent production of H_2O_2 and/or $OH^{\cdot}$, leading to lipid peroxidation. It seems more likely therefore that the mechanism of action involves reactions of anisyl-3,4-quinone rather than anisyl-3,4-semiquinone. Since pulse radiolysis has already been demonstrated[12] to be a useful technique for the rapid generation and studies of the reactions of unstable quinones, this method may well assist in the further elucidation of the reactions of anisyl-3,4-quinone.

ACKNOWLEDGEMENTS

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