

One-electron oxidation of 5,6-dihydroxy-2,3-dihydroindole: the influence of Zn^{2+}

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One electron oxidation of 5,6-dihydroxy-2,3-dihydroindole (DDI) and its *N*-ethyl analogue by the azide radical, $N_3^{\cdot-}$, at pH 5 and 9 was studied using pulse radiolysis to further the understanding of chemical pathways leading to melanin formation. One electron oxidation of DDI yields the benzosemiquinone radical with a pK_a value of 5.3. With the *N*-ethyl analogue, the corresponding benzosemiquinone radical is formed, consistent with one of the hydroxy groups being the preferred site for deprotonation of the initially formed radical cation. The benzosemiquinone radicals of DDI disproportionate to yield a stable dopachrome-like product. In the presence of Zn^{2+} at pH 5.0, the benzosemiquinone radicals of DDI react to form, by an alternative route, a Zn ion complex of the *o*-semiquinone radical with a rate constant of $3.0 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. This Zn ion complex decays by second order kinetics to yield a Zn^{2+} -quinone complex which has a lifetime of 3–4 ms. Auto-oxidation of DDI is suggested to lead to the formation of a dopachrome-like intermediate. From these studies, it is concluded that Zn^{2+} significantly influences the reactions involving the semiquinone radical of DDI and these alternative reaction pathways may help to clarify the initial biochemical stage of the free-radical pathway(s) leading to melanin formation.

Introduction

In the last decade, attention has been directed to the elucidation of the pathway(s) of melanin formation, in view of the increase in the incident of malignant melanoma.^{1–3} The scheme of Raper⁴ and Mason *et al.*⁵ was proposed to explain the mechanism of melanin formation involving, among other species, 5,6-dihydroxyindole (DHI). One-electron oxidation of reactive melanin precursors such as DHI, 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and 3,4-dihydroxyphenylalanine together with their analogues have been investigated previously.^{6–12} This has considerably improved the understanding of the chemical pathways involved in melanin formation. One electron oxidation of these compounds leads to the corresponding indolosemiquinone radicals which decay to yield indoloquinones and/or quinone methide and quinoneimine, which have been implicated as intermediates in the melanin pathway. These intermediates are susceptible to interactions with nucleophiles.¹³

According to the Raper–Mason scheme, the leuko compound formed by cyclisation of dopaquinone¹⁴ undergoes oxidation to form a dopachrome compound which then undergoes an intramolecular rearrangement to DHICA which may decarboxylate to DHI.^{15,16} Recently it has been suggested that the conversion of dopachrome to DHI is a key reaction in the melanin biosynthetic pathway and that this conversion may be under the control of various metal ions or a melanocyte-specific enzyme, such as a dopachrome conversion factor (DCF) or dopachrome tautomerase, and tyrosinase-related proteins (TRP1 and TRP2).^{17–22}

Additionally, metal ions including Zn^{2+} interact^{23,24} with catechols and their semiquinone radicals to produce metal–ion complexes. With DHI, the lifetime of its semiquinone radical is increased upon complexation²³ with Zn^{2+} .

The aim of the present study was to investigate one electron oxidation of the substituted indole, 5,6-dihydroxy-2,3-dihydroindole (5,6-dihydroxyindoline or DDI), to further the understanding of free radical species which may be involved in the

pathways leading to melanin formation. DDI represents a very appropriate model for leuco compounds, such as 5,6-dihydroxy-2,3-dihydroindole-2-carboxylic acid, which is thought to be a precursor to DHI in the melanin pathway. Consequently, the question arises as to whether one-electron oxidation of DDI may lead to DHI *via* a dopachrome-like intermediate. The role of Zn^{2+} in the free radical chemistry of DDI was investigated because of the reported¹⁹ significance of metal ions in their interaction with dopachrome.

Experimental

5,6-Dihydroxy-2,3-dihydroindole (DDI) and *N*-ethyl-5,6-dihydroxy-2,3-dihydroindole (E-DDI) were supplied by L'Oreal and used without further purification. Sodium azide, zinc sulfate and all other reagents were of AnalaR grade from BDH and used as received. All solutions were prepared using water which had been purified using a Multi-Q system and deoxygenated stringently (saturated with N_2) in order to minimise auto-oxidation of the indolines, which are especially susceptible to oxidation as observed from the colouration of the solutions in the presence of trace amounts of oxygen especially at pH > 7. Therefore the majority of the experiments were restricted to investigations at pH 5.0. Experiments were only performed using clear solutions. The pH of the solution was adjusted using either $HClO_4$ or NaOH.

The solutions in general contained 0.1 mol dm^{-3} sodium azide and $10^{-4} \text{ mol dm}^{-3}$ DDI (or E-DDI). Solutions were saturated with N_2O (BOC, zero grade) prior to irradiation in order to convert e_{aq}^- into $\cdot OH$ radicals so that the major water-derived radical present is the $\cdot OH$ radical. This concentration of azide ensured that virtually all of the $\cdot OH$ radicals reacted with N_3^- to form $N_3^{\cdot-}$ within < 1 μs , in competition with the reaction of $\cdot OH$ with the indolic compounds.²⁵ Since the azide radical is transparent at $\lambda > 300 \text{ nm}$, the one-electron oxidised transient species may be readily monitored spectrophotometrically.

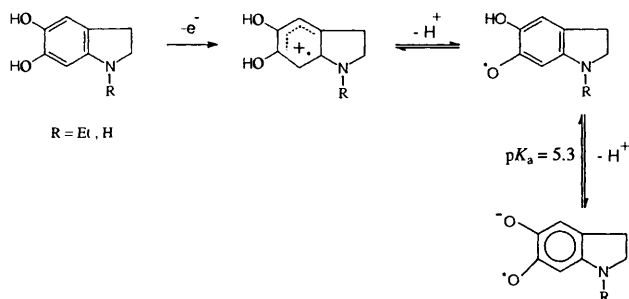
Pulse radiolysis experiments were performed with a 4.3 MeV linear accelerator as previously described.²⁶ The optical

detection system and the data handling processes have been described previously.²⁶ Solutions were irradiated in a quartz cell of 0.2 dm path length at 296 ± 3 K with electron pulses of 1.6 μ s duration. Optical filters were used to minimise photochemical effects. Radiation doses (1–15 Gy/pulse) were determined using KSCN as dosimeter at 480 nm assuming $G = 0.3 \mu\text{mol J}^{-1}$ and $\epsilon = 710 \text{ m}^2 \text{ mol}^{-1}$. The transient optical absorption spectra measured have not been corrected for depletion of the substrates following pulse irradiation since the 'one-pulse one-sample' procedure was used during the generation of the transient optical absorption spectra. The errors in the values of the optical absorbance (A) are $\pm 10\%$.

In steady state experiments, the change in absorbance with time following auto-oxidation of DDI and E-DDI was monitored spectrophotometrically (using a diode array Beckman DU series 7000 spectrophotometer).

Results and discussion

The optical absorption spectra obtained 10 μ s after pulse irradiation of N_2O -saturated solution containing $10^{-1} \text{ mol dm}^{-3}$ azide and $100 \mu\text{mol dm}^{-3}$ DDI at pH 5 and 9 are shown in Fig. 1. It is assumed that N_3^* oxidises DDI by electron transfer^{7,25} to yield the benzosemiquinone radical, the optical absorption of which is pH dependent. From the dependence of the optical absorbance at 475 nm on pH, as shown in the inset of Fig. 1, the $\text{p}K_a$ of the *o*-semiquinone radical of DDI was determined to be 5.3. A rapid deprotonation step, similar to that previously reported upon one-electron oxidation of DHI when $\text{pH} > \text{p}K_a$ of the indolo-quinone radical,⁷ is apparent at pH 9.0 (Scheme 1). The rate of deprotonation of the *o*-semiquinone radical of DDI must be $> 10^5 \text{ s}^{-1}$.



The following experiments were carried out to verify the preferred site of deprotonation of the radical cation produced upon one-electron oxidation of DDI by using E-DDI, in which the N-site cannot undergo deprotonation. The optical absorption spectra measured 10 μ s after pulse irradiation of N_2O -saturated aqueous solution containing $10^{-1} \text{ mol dm}^{-3}$ azide and $100 \mu\text{mol dm}^{-3}$ E-DDI at pH 5.0 and 9.0 are shown in Fig. 2. The optical absorption spectra of the one-electron oxidised species of DDI and E-DDI are similar and show similar pH dependences. From these similarities it is concluded that deprotonation of the radical cation of DDI occurs at one of the hydroxy groups to yield the benzosemiquinone radical and not at nitrogen since this latter deprotonation is prevented in E-DDI by *N*-ethyl substitution. Based upon consideration of the acidity of the amino and hydroxy groups, it is predicted that deprotonation should occur preferentially at the hydroxy group. Scheme 1 summarises the intermediates produced upon one electron oxidation of DDI and E-DDI where the resulting radical cation deprotonates at the hydroxy group.

The semiquinone radical of DDI decays by second order kinetics (see Table 1) to a 'stable' product, the optical absorption spectrum of which is shown in Fig. 3. The product is stable in aqueous solution for at least 10 s, the maximum observation time monitored after the pulse. It is well established

Table 1 Rate constants for second-order decay of the semiquinone radicals produced upon one-electron oxidation of DDI and E-DDI in the presence and absence of Zn^{2+} at pH 5.0.

Compound	λ/nm	$\epsilon/\text{m}^2 \text{ mol}^{-1}$	$k_2/10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$
DDI	475	437	1.6
E-DDI	475	201	1.6
DDI- $2 \text{ mmol dm}^{-3} \text{ Zn}^{2+}$	480		1.6

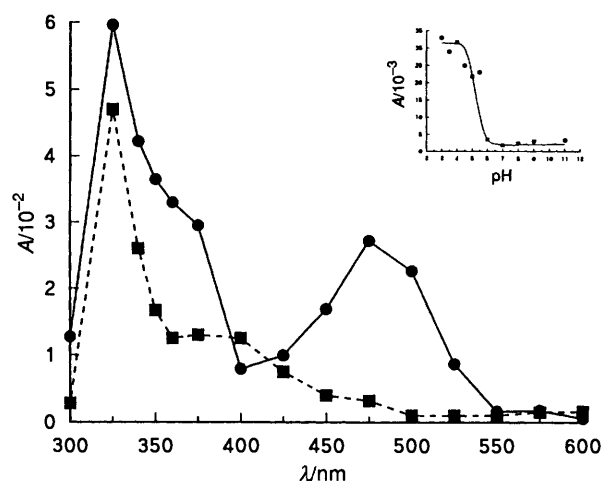


Fig. 1. Optical absorption spectra determined 10 μ s after pulse irradiation of N_2O -saturated solution containing $10^{-1} \text{ mol dm}^{-3}$ azide and $100 \mu\text{mol dm}^{-3}$ DDI at pH 5 (●) and 9 (■). Inset shows the pH dependence of the absorbance change at 475 nm.

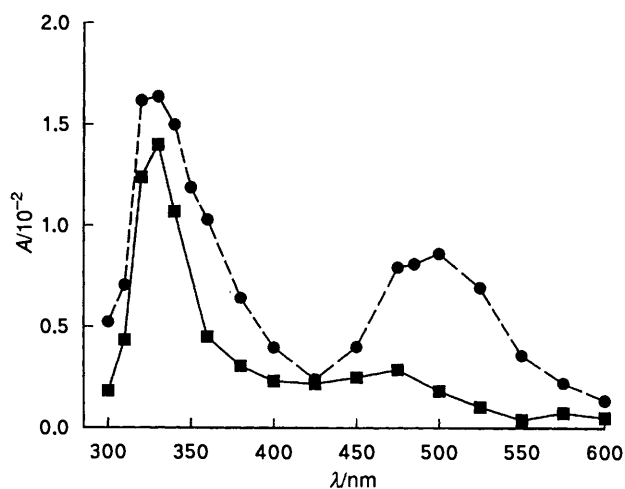


Fig. 2. Optical absorption spectra determined 10 μ s after pulse irradiation of N_2O -saturated solution containing $10^{-1} \text{ mol dm}^{-3}$ azide and $100 \mu\text{mol dm}^{-3}$ E-DDI at pH 5 (●) and 9 (■).

that the bimolecular interaction of *o*-benzosemiquinone radicals occurs preferentially by disproportionation to yield the corresponding benzoquinone.²⁷ Therefore it is proposed that the 'stable' species results from disproportionation and is tentatively assigned to the related dopachrome-like product (Scheme 2) following tautomerisation of the quinone. The chemistry following one-electron oxidation of DDI is therefore similar to that proposed for oxidation of DHI⁹ and occurs in the dihydroxybenzene ring.

N-Ethyl substitution of DDI does not significantly influence the disproportionation reactions of the *o*-benzosemiquinone radical since the rate of loss of the *o*-benzosemiquinone radical (Table 1) and the optical absorption spectrum of the resulting stable product is similar for both DDI and E-DDI under the same conditions.

Under physiological conditions, dopachrome is thought to be stable and requires metal ions or an enzyme with distinct

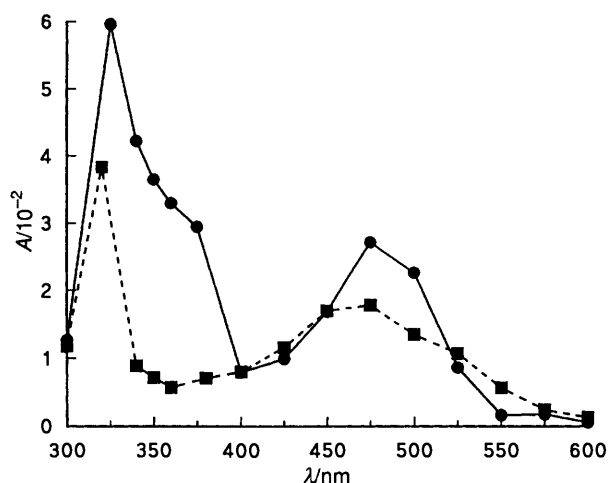
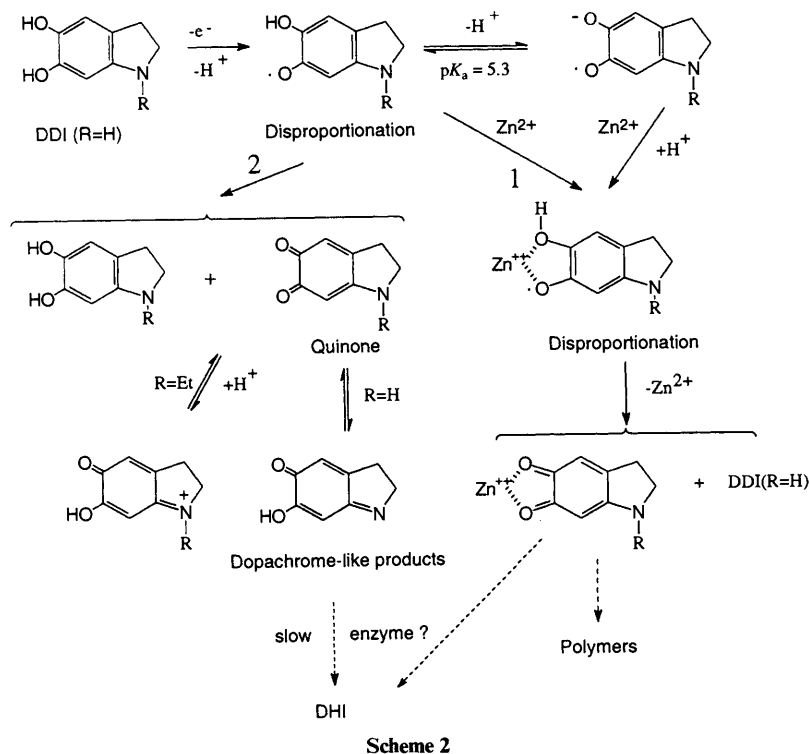


Fig. 3 Optical absorption spectra determined 10 μs (●) and 3 ms (■) after pulse irradiation of N_2O -saturated solution containing $10^{-1} \text{ mol dm}^{-3}$ azide and $100 \mu\text{mol dm}^{-3}$ DDI at pH 5

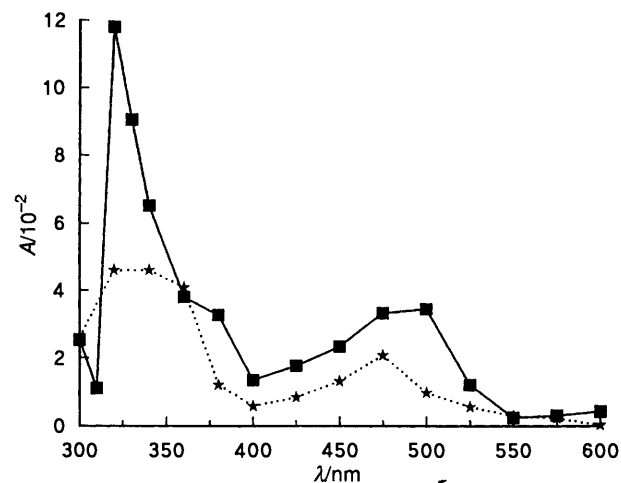


Fig. 4 Optical absorption spectra determined 10 (■) and 180 μs (*) after pulse irradiation of N_2O -saturated solution containing $10^{-1} \text{ mol dm}^{-3}$ azide, $100 \mu\text{mol dm}^{-3}$ DDI and $4 \text{ mmol dm}^{-3} \text{ Zn}^{2+}$ at pH 5.0

melanogenic functions such as DCF and TRP1/2 to convert it into melanotic products.^{19,21} Since metal ions may form metal ion complexes with *o*-semiquinone radicals²³ and modify reactions of dopachrome,^{16,18,19} the influence of the metal ion, Zn^{2+} , upon the stability of the semiquinone radicals of DDI and the dopachrome-like products formed following one-electron oxidation of DDI has been investigated. The optical absorption spectra determined 10 and 180 μs following pulse irradiation of N_2O -saturated aqueous solution containing $10^{-1} \text{ mol dm}^{-3}$ azide, $100 \mu\text{mol dm}^{-3}$ DDI and $4 \text{ mmol dm}^{-3} \text{ Zn}^{2+}$ with a dose of 13 Gy/pulse at pH 5.0 are shown in Fig. 4. One-electron oxidation of DDI is initiated by N_3^{\cdot} radicals and zinc sulfate is radiolytically inert under these conditions. The initial species formed upon one-electron oxidation of DDI, the semiquinone radical, decays in the presence of Zn^{2+} to yield a different species, as depicted by the spectrum at 180 μs . The resulting species has an optical absorption spectrum centred around 450 nm and in the presence of Zn^{2+} it absorbs less strongly at $\lambda < 350 \text{ nm}$ when compared with the absorbance of

the semiquinone radical of DDI (see Fig. 4). It is suggested that a zinc ion complex of the benzosemiquinone radical of DDI is formed as shown in Scheme 2. At pH 5.0, the predominant radical present is the neutral benzosemiquinone radical. It is therefore assumed that this neutral species complexes with Zn^{2+} . Some support for this assumption arises from EPR studies²³ showing that complexation of Zn^{2+} by semiquinone radicals occurs when the semiquinone radical anion is expected to be protonated. The findings presented here do not rule out the possibility of complexation of Zn^{2+} by the semiquinone radical anion of DDI.

The first order rate constant for complexation of the benzosemiquinone radical of DDI determined at 500 nm is dependent upon the concentration of Zn^{2+} ($0.5\text{--}4.0 \text{ mmol dm}^{-3}$) as shown in Fig. 5. From this dependence, the second order rate constant for interaction of Zn^{2+} with the semiquinone radical was determined to be $3 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$. The absorbance at 350 and 500 nm of the zinc complex, following completion of the reaction of the benzosemiquinone

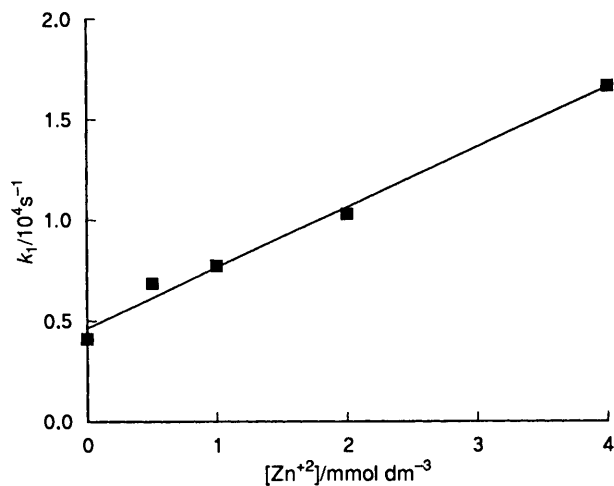


Fig. 5 Dependence of the first-order rate constant for loss of the semiquinone radical of DDI at pH 5.0 on Zn^{2+} concentration determined at 500 nm

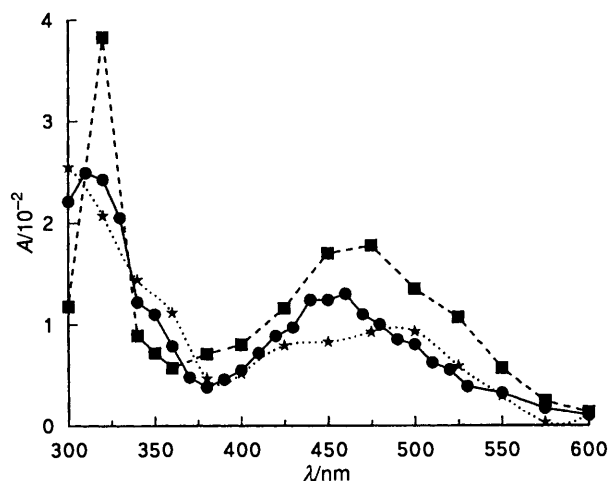


Fig. 6 Optical absorption spectra determined 5 ms after pulse irradiation of N_2O -saturated solution containing $100 \mu\text{mol dm}^{-3}$ DDI in the absence (■) and presence of 2 mmol dm^{-3} (●) and 4 mmol dm^{-3} (*) Zn^{2+}

radical of DDI with Zn^{2+} , is independent of the concentration of Zn^{2+} ($0.5\text{--}4.0 \text{ mmol dm}^{-1}$). Under these conditions it is therefore inferred that if an equilibrium exists between the semiquinone radical of DDI and its corresponding zinc complex, it is predominately in favour of the complex. At lower concentrations of Zn^{2+} , the bimolecular decay of the semiquinone radicals of DDI competes favourably with its reaction with Zn^{2+} . The *o*-semiquinone–zinc complex following its formation in the presence of Zn^{2+} decays by second order kinetics (see Table 1). The optical absorption spectrum of the resulting product at 5 ms is shown in Fig. 6. Presumably a zinc complex of the *o*-benzoquinone is produced as shown in Scheme 2. It has an optical absorption spectrum which is different to that of the dopachrome-like product, produced in the absence of Zn^{2+} , and also shown in Fig. 6 for comparison. In contrast to the stability of the dopachrome-like product formed in the absence of Zn^{2+} , the zinc–benzoquinone complex decays by first order kinetics, as determined at 475 nm, with a rate constant of $300 (\pm 50) \text{ s}^{-1}$ (see Fig. 7).

To gain some qualitative insight into the longer term stability of the dopachrome-like products, auto-oxidation of DDI and the *N*-substituted analogue (E-DDI) was studied in aqueous solutions containing $100 \mu\text{mol dm}^{-3}$ DDI (or E-DDI) and $10^{-1} \text{ mol dm}^{-3}$ azide at pH 7.0 under aerobic conditions at room temperature. These solutions contained azide so that they are similar to those used in the pulse radiolysis studies.

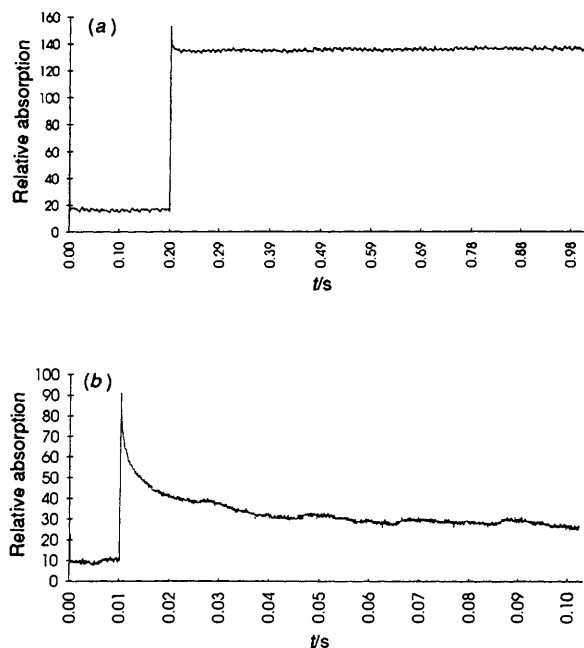


Fig. 7 Time-profile for the change of absorbance at 475 nm on pulse irradiation of DDI ($100 \mu\text{mol dm}^{-3}$) in the absence (a) and presence (b) of 2 mmol dm^{-3} Zn^{2+}

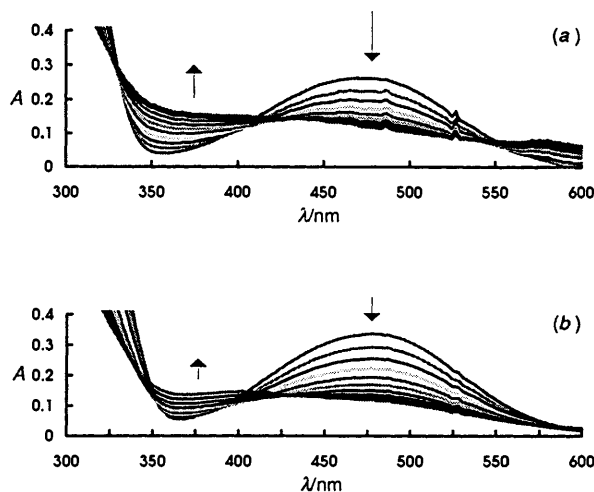


Fig. 8 Optical absorption spectra monitored for 7 h following initiation of aerobic oxidation of $100 \mu\text{mol dm}^{-3}$ (a) DDI and (b) E-DDI at pH 7.0 in the presence of $10^{-1} \text{ mol dm}^{-3}$ azide. The spectra were determined following admittance of oxygen into the cuvette.

Auto-oxidation leads to formation of a 'dopachrome-like intermediate' *i.e.* a stable product centred at 475 nm, within 5 min as shown in Fig. 8. This spectrum is comparable to that produced upon one-electron oxidation of DDI (Fig. 3). The time course of the visible spectral changes associated with decay of the 'stable' products at 475 nm and the formation of persistent products at 365 nm was monitored spectrophotometrically for 7 h with both DDI and E-DDI under these conditions. The dopachrome-like intermediate undergoes further reaction to form a final product(s), the optical absorption spectrum of which is similar to that produced upon auto-oxidation of DHI under similar conditions. *N*-Ethyl-substitution of DDI does not critically influence this transformation reaction within 7 h as shown from the spectral changes with DDI and E-DDI [shown in Figs. 8(a) and (b)]. From the kinetic analyses of the optical changes in Fig. 8, the dopachrome-like intermediate decays by first order kinetics as determined at 475 nm with a rate constant of $2 \times 10^{-4} \text{ s}^{-1}$. The presence of 2 mmol dm^{-3} Zn^{2+} has an effect upon the auto-oxidation of DDI. At pH 7.0

in the presence of Zn^{2+} and aerobic conditions, the final product is formed within 10 mins and has a similar optical absorption spectrum to that shown in Fig. 8(a) determined after 7 h in the absence of Zn^{2+} .

The reaction scheme (Scheme 2) summarises the findings from these pulse radiolysis studies with DDI. One-electron oxidation of DDI results in the formation of its benzo-semiquinone radical which disproportionates resulting in the formation of the corresponding benzoquinone (route 2). This quinone may undergo tautomerisation to the corresponding dopachrome-like product, which is a semi-stable product. From the auto-oxidation studies, its first half-life is estimated to be ca. 1 h. In the presence of Zn^{2+} , the semiquinone radical of DDI chelates Zn^{2+} (route 1) to yield a Zn^{2+} -semiquinone complex which decays to a Zn^{2+} -quinone complex of DDI. The presence of Zn^{2+} significantly modifies the reaction pathways of the radicals following one electron oxidation of DDI. The metal ion benzoquinone complex may be a precursor to subsequent formation of high molecular weight products. Whether this proceeds *via* DHI or by alternative pathways is as yet not known. From the auto-oxidation investigations it is shown that Zn^{2+} acts as a catalysis and may circumvent formation of the dopachrome-like product. Zn^{2+} may be chelated by the *o*-benzoquinone product, making it more susceptible to further reactions. In fact, the final product following auto-oxidation of DDI in the presence of Zn^{2+} is formed within a few minutes.

Conclusions

Zn^{2+} provides an alternative reaction pathway for decay of the benzosemiquinone radical of DHI without leading to a dopachrome-like product. It is considered that Zn^{2+} is able to catalyse the conversion of dopachrome into DHI, however it is proposed that Zn^{2+} may provide alternative pathways for converting DDI into DHI without the intermediate formation of a dopachrome-like product so that subsequent polymerisation leading to melanin may occur.

Acknowledgements

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