J. CHEM. SOC. FARADAY TRANS., 1991, 87(18), 2939–2942

Role of Azide Concentration in Pulse Radiolysis Studies of Oxidation: 3,4-Dihydroxyphenylalanine

Chris Lambert and T. George Truscott
Department of Chemistry, Keele University, Keele, Staffordshire ST5 5BG, UK
Edward J. Land*
C.R.C. Department of Biophysical Chemistry, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, UK
Patrick A. Riley
Department of Chemical Pathology, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1P 6DB, UK

The radiolysis of aqueous solutions of 3,4-dihydroxyphenylalanine (dopa) using $N_3^{\bullet}$ as a one-electron oxidant leads to the formation of dopasemiquinone and, successively, dopaquinone and dopachrome. It is now shown that under pH conditions where significant protonation of N_3^- to HN_3 occurs, dopachrome formation is suppressed, probably due predominantly to addition of HN_3 to dopaquinone. The possibility of such nucleophilic reactions occurring needs to be considered in studies of quinone intermediates generated using $N_3^{\bullet}$ as oxidant.

In this paper we consider the effect of azide concentration upon the oxidative processes initiated by reaction of $N_3^{\bullet}$ with dopa, and discuss its importance with respect to the use of $N_3^{\bullet}$ as a general-purpose oxidant in pulse radiolysis.

The use of the azide radical ($N_3^{\bullet}$) as an oxidant in pulse radiolysis is well established.[1,2] The species has been used to investigate the one-electron oxidation of many systems of biological interest.[3–9] The $N_3^{\bullet}$ radical has three useful characteristics[2] in that (i) it does not absorb significantly above 300 nm, (ii) it reacts primarily by an electron-transfer mechanism so does not form adducts and (iii) it is uncharged and thus its reactivity is unperturbed by electrostatic effects.

In such studies, $N_3^{\bullet}$ is often generated by irradiating N_2O-saturated aqueous solutions containing a high concentration of N_3^-. In aqueous solution, the radiolytically induced primary radicals are the hydroxyl radical ($OH^{\bullet}$), the hydrogen atom ($H^{\bullet}$) and the solvated electron (e_{aq}^-). In N_2O-saturated solution the e_{aq}^- rapidly reacts to yield more $OH^{\bullet}$. The subsequent reactions of $OH^{\bullet}$ and $H^{\bullet}$ determine the yield of $N_3^{\bullet}$. These reactions have been extensively studied by Schuler and co-workers.[10,11] Briefly, the azide ion (N_3^-) reacts both with $OH^{\bullet}$ and $H^{\bullet}$:

$$OH^{\bullet} + N_3^- \rightarrow OH^- + N_3^{\bullet}$$

$$H^{\bullet} + N_3^- \rightarrow HN_3^{\bullet -}$$

and under acidic conditions, or in the presence of high concentrations of proton donors, such as buffer, the $HN_3^{\bullet -}$ decays relatively slowly to give more $N_3^{\bullet}$:

$$HN_3^{\bullet -} + H^+ \rightarrow N_3^{\bullet} + H_2$$

In most of the work to date, it has been the radicals formed by oxidation of a substrate that have been of primary interest. Several of our studies,[8,12–16] however, have also used the azide system to initiate a reaction sequence with a view to studying unstable intermediates formed by decay of the initial oxidised radicals. One recent example of this is the conversion of the amino acid dopa to dopachrome.[14,15] The conditions of a typical experiment are arranged so that very rapidly after the electron pulse, virtually the only reactive species in the medium is the $N_3^{\bullet}$ radical. In this system the $N_3^{\bullet}$ reacts efficiently with dopa to give the corresponding oxidised semiquinone, which decays to dopaquinone (λ_{max} = 380 nm) and subsequently dopachrome (λ_{max} = 480 nm).

As stated above, the primary step in the reaction sequence is the conversion of all the radical species produced by the pulse to azide radicals. For this reaction to proceed efficiently, and without interference from reactions of $OH^{\bullet}$ with the substrate, it is advantageous to have a high concentration of azide, typically 5×10^{-2}–10^{-1} mol dm^{-3}.

It has previously been reported[14] that, following the oxidation of dopa by $N_3^{\bullet}$, the *o*-quinone decay and concomitant growth of dopachrome are strongly pH dependent. We have shown that the rate constant for this process increases by a factor of 100 in going from pH 5.6 to 8.6. We have now found that as the pH value is lowered the yield of dopachrome becomes increasingly dependent upon the concentration of azide.† For example, at pH 6 a much smaller amount of dopachrome is formed using 10^{-1} mol dm^3 azide than using 10^{-3} mol dm^{-3} azide, whereas at pH 7 equivalent and larger amounts of dopachrome are formed using both these azide concentrations. It would appear therefore that, under certain conditions, high concentrations of azide may lead to competing side reactions with dopaquinone. Indeed, addition of azide to dopaquinone formed in the enzymatic oxidation of dopa has recently been proposed.[17]

Experimental

3,4-Dihydroxyphenylalanine (dopa) was supplied by Sigma and used as received. All other materials were of AnalaR grade from BDH and were also used as received. The pulse radiolysis experiments were performed with a 9–12 MeV Vickers linear accelerator as previously described,[18,19] using 50–200 ns pulses. Solutions were studied using quartz flow-through cells, optical pathlength 2.5 cm, of either 0.7 or 3 cm^3 internal volume.

Generation of the oxidising species $N_3^{\bullet}$ was achieved by irradiating N_2O-saturated solutions containing 1–30 mmol dm^{-3} NaN_3. At the lowest concentration of azide used, it was estimated that a maximum of 5% of the $OH^{\bullet}$ radical may react with dopa. For some experiments the $Br_2^{\bullet -}$ radical was used as oxidant instead of $N_3^{\bullet}$, and in this case the NaN_3 was replaced by KBr.

† In ref. 14 the concentration of azide was given in error as 5×10^{-2} mol dm^{-3} whereas in fact it was 10^{-3} mol dm^{-3}.

Results and Discussion

The absorption spectrum and decay kinetics of the radical produced on oxidation of dopa were essentially identical whether it was produced by reaction with $Br_2^{\bullet-}$ or $N_3^{\bullet}$. The initial radical spectrum obtained using $N_3^{\bullet}$ oxidation is shown in Fig. 1(*a*). This spectrum is similar to that previously reported,[15] but is given here as the data have been extended to 1000 nm to show the presence of a weak absorption band centred around 700 nm. The radical spectrum was unchanged on increasing the pH from 6 to 7.

The spectra given in Fig. 1(*b*) show that at high concentrations of azide (0.1 mol dm^{-3}) at pH 6, the final absorption band centred around 480 nm,[14] due to dopachrome, which is prominent at low azide concentrations [Fig. 1(*c*)], is absent. This strong absorption at 480 nm is still present if KBr, 5 mmol dm^{-3} azide at pH 6 [Fig. 1(*c*)], or even 0.1 mol dm^{-3} azide at pH 7, are used as the source of oxidants. This phenomenon is examined in more detail in Table 1 in which the first-order constants of the dopaquinone decay and the final absorption due to dopachrome are given as functions of pH and azide concentration. These data demonstrate that, for example, at pH 6.0 an increase in azide concentration from 1 to 30 mmol dm^{-3} causes an increase in the rate of decay of the quinone and a concomitant reduction of the dopachrome absorption. No change in the rate of dopaquinone decay and final dopachrome absorption intensity was noted on varying the concentration of buffer between 10^{-3} and 10^{-1} mol dm^{-3} at pH 5 or 7.

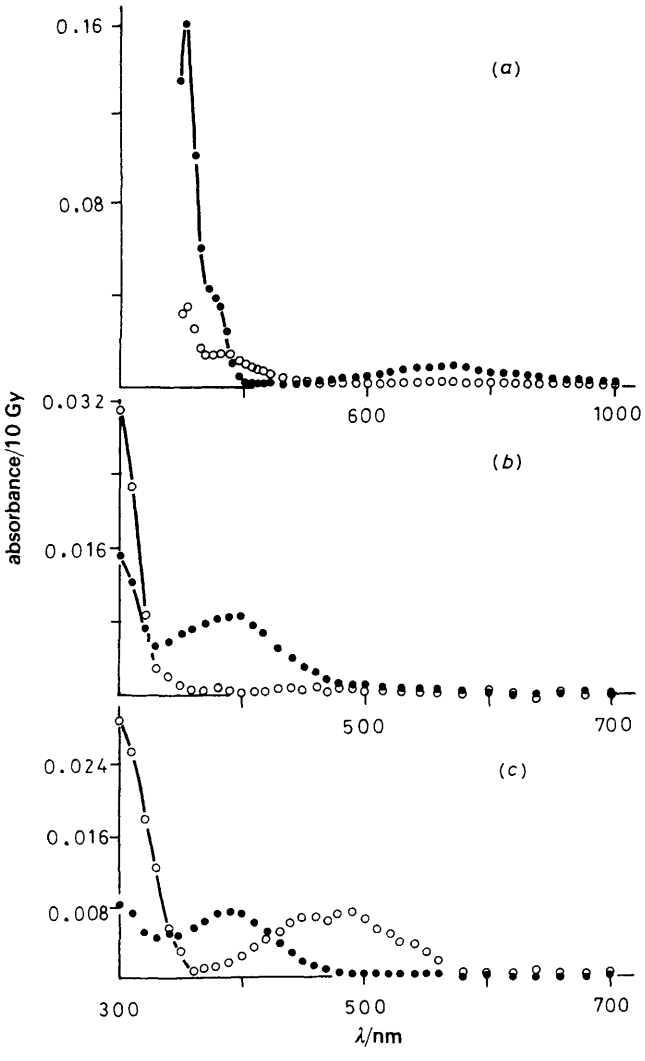


Fig. 1 Absorbance changes at various times after pulse radiolysis of N_2O-saturated aqueous solutions of dopa. (*a*) 9.84 × 10^{-4} mol dm^{-3} dopa + 10^{-1} mol dm^{-3} NaN_3 + 10^{-2} mol dm^{-3} phosphate buffer, pH 6.0: ●, 18 μs; ○, 2.2 ms; dose ≈ 23 Gy. (*b*) 9.94 × 10^{-4} mol dm^{-3} dopa + 10^{-1} mol dm^{-3} NaN_3 + 10^{-2} mol dm^{-3} phosphate buffer, pH 6.0: ●, 28 ms; ○, 720 ms; dose ≈ 14 Gy. (*c*) 2.02 × 10^{-4} mol dm^{-3} dopa + 5 × 10^{-3} mol dm^{-3} NaN_3 + 10^{-2} mol dm^{-3} phosphate buffer, pH 6.0: ●, 40 ms; ○, 8040 ms; dose ≈ 26 Gy

Table 1 Variation in final dopachrome absorbance at 480 nm ($/10^{-2}$) and dopaquinone decay rate constant (s^{-1}, in parentheses) with pH and azide concentration

	azide concentration/mmol dm^{-3}				
pH	1	3	7	15	30
5.2	0.28 (0.2)	0.51 (0.4)	0.27 (0.9)	0.21(1.5)	0.08 (2.8)
5.6	1.19 (0.4)	0.62 (0.6)	0.49 (0.8)	0.48 (1.8)	0.35 (2.5)
6.0	1.10 (0.4)	0.87 (0.6)	0.82 (1.0)	0.67 (1.4)	0.41 (2.1)
6.5	1.15 (1.3)	1.20 (1.2)	1.05 (1.2)	0.94 (1.4)	0.83 (1.8)
7.1	1.29 (3.5)	1.36 (3.5)	1.29 (3.4)	1.25 (3.8)	1.21 (3.5)

The mechanism previously proposed[14] for the formation of the dopachrome involved a cross-reaction between dopaquinone and leucodopachrome. The results indicate that as the concentrations of hydrogen ion and azide increase, the amount of dopachrome formation decreases. The alternative product of the quinone decay does not absorb appreciably in the visible region of the spectrum.

The mechanism by which the azide inhibits the formation of dopachrome may be described by Scheme 1. It is well documented[20,21] that NaN_3 and/or its conjugate acid, hydrazoic acid, can react with quinones. However, these reactions normally involve relatively severe conditions, *i.e.* the presence of acetic acid or even concentrated sulphuric acid. As shown in the scheme, addition of HN_3 can also be important under conditions where the competing reactions of the quinones are relatively slow.

The rate constant for reaction of HN_3 with dopaquinone was estimated by plotting the measured quinone first-order decay rate constants obtained at pH 5.2 (see Table 1), where cyclisation is least important, against the concentration of HN_3, assuming a pK_a for the equilibrium, $HN_3 \rightleftharpoons N_3^- + H^+$, of 4.7.[22] A rate constant (k_1) of 3.6 × 10^2 dm^3 mol^{-1} s^{-1} was thus obtained.

It will be assumed that, by analogy with dopaminequinone,[23] cyclisation of dopaquinone occurs only *via* the form in which its amino group is deprotonated (DQ), and that the pK_a for the NH_3^+/NH_2 equilibrium is close to that of dopa itself, *i.e. ca.* 9. For the pH range studied therefore, *i.e.* 5.2–7.1, dopaquinone will be predominantly in its protonated form (DQH^+) so that the observed rate of quinone decay is described by:

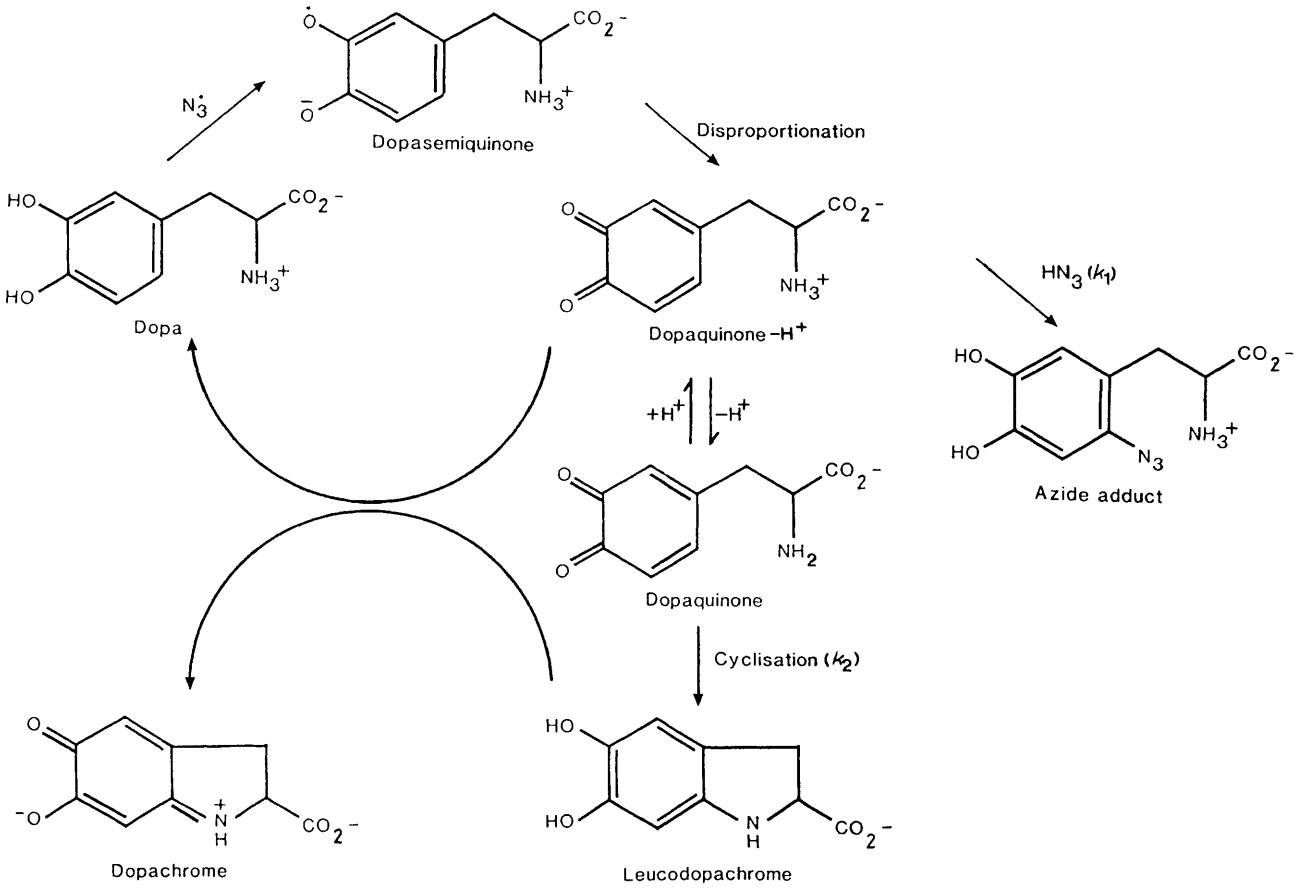
$$\frac{-d(DQH^+)}{dt} = 2k_2[DQ]$$

$$= \frac{2k_2K[DQH^+]}{[H^+]}; \quad \text{where } K = \frac{[DQ][H^+]}{[DQH^+]}$$

$$= k_{obs}[DQH^+]$$

Using the data given in Table 1 for the lowest concentration of azide (10^{-3} mol dm^{-3}), *i.e.* the least affected by HN_3, a plot of k_{obs} against $1/[H^+]$ gives a straight line of slope 2.54 × 10^{-7} = $2k_2K$. Assuming $K = 10^{-9}$, $k_2 = 127$ s^{-1}.

Based on the reaction sequence given in the scheme, it should be possible to predict the proportional yield of dopachrome under all the conditions employed. For the calculation, it is assumed that the only route to dopachrome formation is *via* a rate-determining cyclisation of DQ, the



Scheme 1

other reaction accounting for the decay of dopaquinone being the reaction of DQH^+ with HN_3. The proportion (P) of dopaquinone leading to dopachrome is then given by the fraction

$$P = \frac{\text{routes of decay of dopaquinone that lead to dopachrome}}{\text{all routes of decay of dopaquinone}}$$

$$= \frac{2k_2[DQ]}{4k_2[DQ] + k_1[HN_3][DQH^+]}$$

and substituting for [DQ]

$$= \frac{2k_2 K}{4k_2 K + k_1[HN_3][H^+]}$$

In Fig. 2, the observed final absorbances at 480 nm were plotted against those calculated using the above estimates for $2k_2K$ and k_1, together with the equation for P, on the assumption that the maximum yield of dopachrome under the conditions chosen is represented by an absorbance at 480 nm of 1.28×10^{-2} (see Table 1).

Although the amount of dopachrome produced clearly falls with increasing HN_3 concentration, the plot obtained (Fig. 2) shows considerable curvature, implying that the above scheme is not entirely adequate.

There are several sources of error in the experiments that could lead to scattering of the data. The decay of the dopaquinone in even slightly acidic media takes several seconds for completion and, over this time, there is some analysing light instability. The use of 0.1 mol dm^{-3} azide and 0.01 mol dm^{-3} buffer carries the possibility of impurities, *e.g.* metal ions, being introduced at levels sufficient to interact with the long-lived quinone. However, several batches of both azide and buffer exhibited no difference in behaviour. No attempt has been made to correct for any variation in the pK_a value

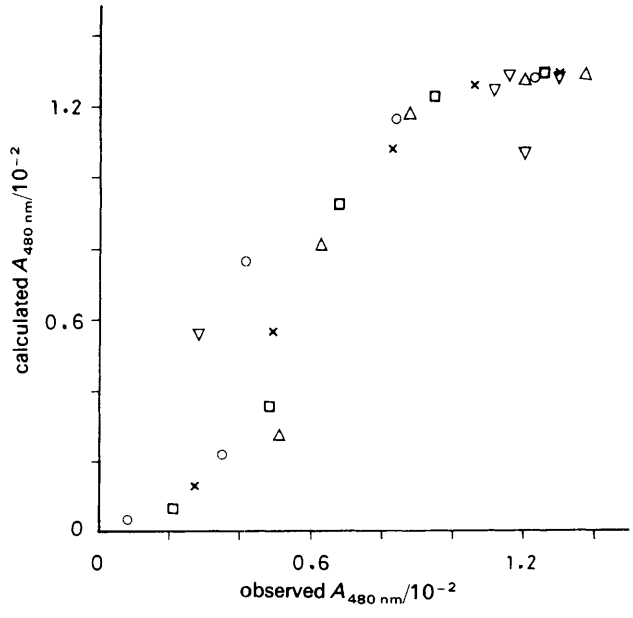


Fig. 2 Absorbance at 480 nm due to dopachrome, calculated from equation P together with the estimates for k_1 and $2k_2K$ (see text), against observed absorbance at 480 nm (see Table 1). Azide concentrations: ○, 30 mmol dm^{-3}; □, 15 mmol dm^{-3}; ×, 7 mmol dm^{-3}; △, 3 mmol dm^{-3}; ▽, 1 mmol dm^{-3}

used to calculate the concentration of HN_3. Debye–Hückel theory predicts that at an ionic strength of 0.1 the pK_a value of HN_3 may be lowered by 0.11 pH units. None of these expected errors would seem to be sufficiently important however to explain the marked deviation from a straight line in Fig. 2, so further work is necessary.

It should be recalled that complex behaviour, only partially explained, in the pH dependence of the dopaquinone–dopachrome transition was also noted earlier.[14] From the biological point of view, this transition is involved in melanin formation resulting from the tyrosinase-mediated oxidation of dopa, which occurs in melanosomes. Any changes of pH in the melanosome may thus have very important consequences with regard to the rate of cyclization, and therefore may be important in controlling the proportion of dopaquinone that undergoes other types of addition reaction, such as with thiols.

The marked effect of azide concentration at low pH values on the lifetime of dopaquinone in the present experiments is quite clear. Previous studies that have used azide as a source of oxidant to generate unstable quinones *via* disproportionation of semiquinones, may or may not be similarly influenced by the presence of high concentrations of azide. 3,4-Mandeloquinone, produced by the oxidation of 3,4-dihydroxymandelic acid, has a half-life of *ca.* 0.25 s when formed in the presence of 5×10^{-2} mol dm^{-3} NaN_3, and this half-life was unaffected on going from pH 5.9 to 7.8.[16] Furthermore, the same half-life was also found using 10^{-1} mol dm^{-3} KBr as the source of oxidant.[24] 4-Methoxy-*o*-benzoquinone, which slowly polymerises in aqueous solution, is stable for at least several seconds in the presence of 5×10^{-2} mol dm^{-3} azide at pH 7.0.[8,13] It thus appears that neither of these *o*-quinones are sensitive to high azide concentrations under the conditions chosen.

However, in a recent study[25] of the oxidation of methoxylated metabolites of indolic melanin precursors, it was noted that the decay of the product initially formed from the decay of the semiquinone of 5-hydroxy-6-methoxyindole, assigned to a quinonoid cation, was dependent upon the concentration of azide. Thus, at pH 7.3, on reducing the azide concentration from 10^{-1} mol dm^{-3} to 5×10^{-3} mol dm^{-3}, the first-order constant for decay of this quinonoid cation decreased from 11.2 to 2.7 s^{-1}, this being attributed to possible proton exchange catalysis. In a related study[12] of the oxidation of several indolic melanin precursors, complex decays of relatively short-lived transient intermediates, assigned to indolequinones, were obtained, which may well be influenced by the high concentration of azide used (10^{-1} mol dm^{-3}). In the light of the present results with dopaquinone, the influence of azide concentration on these systems should be further considered.

We thank the Cancer Research Campaign and the Medical Research Council for financial support.

References

1 E. J. Land and W. A. Prütz, *Int. J. Radiat. Biol.*, 1979, **36**, 75.
2 Z. B. Alfassi and R. H. Schuler, *J. Phys. Chem.*, 1985, **89**, 3359.
3 W. A. Prütz, E. J. Land and R. W. Sloper, *J. Chem. Soc., Faraday Trans. 1*, 1981, **77**, 281.
4 R. Bonnett, R. J. Ridge, E. J. Land, R. S. Sinclair, D. Tait and T. G. Truscott, *J. Chem. Soc., Faraday Trans. 1*, 1982, **78**, 127.
5 S. V. Jovanovic and M. G. Simic, *Life Chem. Rep.*, 1985, **3**, 124.
6 J. Butler, E. J. Land, W. A. Prütz, and A. J. Swallow, *J. Chem. Soc., Chem. Commun.*, 1986, 348.
7 X. Shen, J. Lind and G. Merenyi, *J. Phys. Chem.*, 1987, **91**, 4403.
8 C. J. Cooksey, E. J. Land, P. A. Riley, T. Sarna and T. G. Truscott, *Free Rad. Res. Commun.*, 1987, **3**, 131.
9 T. Mukherjee, E. J. Land, A. J. Swallow, J. M. Bruce, P. C. Beaumont and B. J. Parsons, *J. Chem. Soc., Faraday Trans. 1*, 1988, **84**, 3423.
10 R. H. Schuler, A. L. Hartzell and B. Behar, *J. Phys. Chem.*, 1982, **85**, 192.
11 Z. B. Alfassi, W. A. Prütz and R. H. Schuler, *J. Phys. Chem.*, 1986, **90**, 1198.
12 C. Lambert, J. N. Chacon, M. R. Chedekel, E. J. Land, P. A. Riley, A. Thompson and T. G. Truscott, *Biochim. Biophys. Acta*, 1989, **993**, 12.
13 E. J. Land, C. J. Cooksey and P. A. Riley, *Biochem. Pharmacol.*, 1990, **39**, 1133.
14 M. R. Chedekel, E. J. Land, A. Thompson and T. G. Truscott, *J. Chem. Soc., Chem. Commun.*, 1984, 1170.
15 A. Thompson, E. J. Land, M. R. Chedekel, K. V. Subbarao and T. G. Truscott, *Biochim. Biophys. Acta*, 1985, **843**, 49.
16 M. Bouheroum, J. M. Bruce and E. J. Land, *Biochim. Biophys. Acta*, 1989, **998**, 57.
17 B. Kalyanaraman and T. Sarna, *Pigment Cell Res.*, 1989, **2**, 149.
18 J. P. Keene, *J. Sci. Instrum.*, 1964, **41**, 493.
19 J. Butler, B. W. Hodgson, B. M. Hoey, E. J. Land, J. S. Lea, E. J. Lindley, F. A. P. Rushton and A. J. Swallow, *Radiat. Phys. Chem.*, 1989, **34**, 633.
20 L. F. Fieser and M. Fieser, *Reagents for Organic Synthesis*, Wiley, New York, 1967, p. 447.
21 K. T. Finley, in *The Chemistry of the Quinonoid Compounds*, ed. S. Patai, Wiley, New York, 1974, part 2, p. 877.
22 F. A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry*, Wiley-Interscience, New York, 2nd edn., 1966, p. 339.
23 D. C. S. Tse, R. L. McCreery and R. N. Adams, *J. Med. Chem.*, 1976, **19**, 37.
24 E. J. Land, unpublished results.
25 C. Lambert, E. J. Land, P. A. Riley and T. G. Truscott, *Biochim. Biophys. Acta*, 1990, **1035**, 319.

Paper 1/02060I; *Received* 1st *May*, 1991