Biochemical Pharmacology, Vol. 39, No. 12, pp. 2051–2055, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

REACTIONS OF A FREE RADICAL INTERMEDIATE IN THE OXIDATION OF AMODIAQUINE

ROGER H. BISBY
Department of Biological Sciences, University of Salford, Salford M5 4WT, U.K.

(*Received* 15 *January* 1990: *accepted* 19 *February* 1990)

Abstract—One electron oxidation of the antimalarial drug amodiaquine by inorganic radicals was investigated by pulse radiolysis. A transient species was observed and identified as the semiiminoquinone radical, which has recently been implicated in the toxicity of amodiaquine. Pulse radiolysis was used to determine the reactivity of this radical. In the absence of other solutes it decays rapidly in a second order process. No reaction between the semiiminoquinone radical and oxygen could be observed. In the presence of ascorbate or a phenolic antioxidant (Trolox C) the semiiminoquinone radical was rapidly repaired. Similar reductants have been reported (Maggs JL *et al.*, *Biochem Pharmacol* 37: 303–311, 1988) to inhibit irreverisble protein binding during amodiaquine autoxidation, and the present results support the involvement of the radical during these reactions.

The antimalarial drug amodiaquine may cause serious myelotoxicity and hepatotoxicity in man [1, 2]. These effects have been postulated to be due to oxidation of the 4-aminophenol moiety of amodiaquine (AQ*) [3, 4], which leads through consecutive one-electron oxidations of the parent molecule to the semiiminoquinone free radical and to the iminoquinone (Fig. 1). It is not known which of these species is the cause of host toxicity, although data on the inhibition of protein binding during autoxidation of amodiaquine suggest the involvement of both species [4]. Similar reactions have been more extensively studied with acetaminophen [5, 6], which is also hepatotoxic in large doses [7]. The formation of free radicals from acetaminophen by enzymatic reactions both *in vitro* [8] and *in vivo* [9] has been reported.

The technique of pulse radiolysis [10, 11] is ideally suited to the investigation of drug free radicals in solution, and has previously been used to determine the properties of the radicals formed by one-electron oxidation of acetaminophen [12, 13]. Radiolysis of a dilute aqueous solution with a submicrosecond pulse of energetic electrons creates oxidizing (hydroxyl, OH) and reducing radicals (the hydrated electron, e^-_{aq} and hydrogen atom, H·):

$$H_2O \rightsquigarrow {}^{\cdot}OH, H^{\cdot}, e^-_{aq}. \quad (1)$$

Saturation of the solution with nitrous oxide converts the hydrated electron to hydroxyl radicals, which then represent over 90% of the radical yield:

$$N_2O + e^-_{aq} \rightarrow {}^{\cdot}OH + OH^- + N_2. \quad (2)$$

The hydroxyl radical may be used as the primary oxidizing radical, or may be converted to one of several less reactive but more specific oxidizing inorganic radicals ($X^{\cdot}$ or $X_2^{\cdot-}$) by addition of an inorganic scavenging anion ($X^- = Br^-$, SCN^-, N_3^- etc) to the solution:

$$^{\cdot}OH + X^- \rightarrow OH^- + X^{\cdot}, \quad (3)$$

$$X^{\cdot} + X^- \rightleftharpoons X_2^{\cdot-}. \quad (4)$$

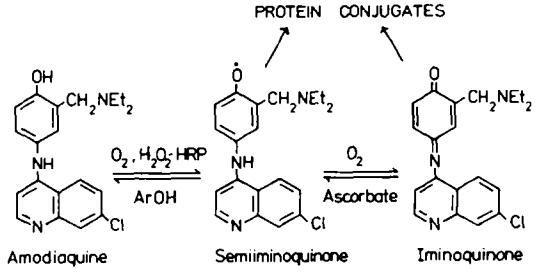


Fig. 1. Scheme proposed for the oxidation of amodiaquine (AQ) to reactive semiiminoquinone and iminoquinone intermediates, after Maggs *et al.* [3, 4].

* Abbreviation: AQ, amodiaquine.

MATERIALS AND METHODS

Amodiaquine (AQ, camoquine, 4-[(7-chloro-4-quinolinyl)amino] - 2 - [(diethylamino)methyl - phenol]) was a gift from Warner-Lambert (U.K.) Ltd. Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was a gift from Hoffman-LaRoche. Other reagents were AnalaR grade where available. Water was obtained from a Milli-Q system (Millipore Ltd) and solutions were prepared in baked glassware.

The pulse radiolysis facility at the University of Salford, based on a Febetron 705B electron accelerator, was used. Solutions were irradiated with electron pulses (approx. 1.5 MeV, 50 nsec duration) in a silica cell of 2 cm optical pathlength. Transient absorptions were recorded using a Datalab 905 digitizer and transferred to an Apple II microcomputer system for storage and analysis. The dose per pulse was between approximately 1 and 5 Gy for most experiments. Spectra are plotted as the product of extinction coefficient (ϵ, units m^2/mol) and radiation

BP 39:12-0

Table 1. Second order rate constants for reactions of free radicals with amodiaquine

Radical	pH	Second order rate constant ($M^{-1}\,sec^{-1}$)
$^{\cdot}OH$*	6.8	$(6.0 \pm 0.5) \times 10^9$
$N_3^{\cdot}$†	6.8	$(1.20 \pm 0.05) \times 10^9$
$Br_2^{\cdot-}$†	6.8	$(2.06 \pm 0.06) \times 10^8$
e_{aq}^-‡	7.1	$(3.9 \pm 0.1) \times 10^{10}$

* Measured by competition with formate, taking $k(^{\cdot}OH + HCO_2^-) = 3.2 \times 10\,M^{-1}\,sec^{-1}$.

† Measured from formation of the amodiaquine radical absorption at 460–470 nm.

‡ Measured from the decay of the hydrated electron radical absorption at 700 nm.

chemical yield (G, units mol/J). Dosimetry was performed with aerated solutions of KSCN (10 mM), taking $G\varepsilon$ (480 nm) $= 2.47 \times 10^{-4}\,m^2/J$ [10].

RESULTS AND DISCUSSION

Reactions of free radicals with amodiaquine

The reactions of some free radicals with AQ were studied by pulse radiolysis and the second order rate constants for their reactions are summarized in Table 1. Experiments were restricted to neutral and slightly acidic solutions due to the insolubility of AQ in alkaline solutions, most probably due to deprotonation of the quinoline ring. From the effect of pH on solubility and on the absorption spectrum, this deprotonation appears to occur with a pK of 7–8. One electron oxidation of amodiaquine was studied using $^{\cdot}OH$, $N_3^{\cdot}$ and $Br_2^{\cdot-}$ as the oxidant radicals, generated as described in the introduction. Typical changes in optical absorption as a function of time in pulse irradiated N_2O saturated solutions of sodium azide and AQ are shown in Fig. 1. At the concentrations of azide employed, the azide radical ($N_3^{\cdot}$) is formed from reaction with $^{\cdot}OH$ within the duration of the pulse (<50 nsec) and the increase in absorbance at 460 nm shown in Fig. 2A is due to formation of an amodiaquine free radical from the reaction of $N_3^{\cdot}$ with AQ. Under the experimental conditions this reaction occurs with first order kinetics. A plot of the observed first order rate constant versus AQ concentration, as in the inset to Fig. 3A, gives a second order rate constant for the reaction of $N_3^{\cdot}$ with AQ of $(1.20 \pm 0.05) \times 10^9\,M^{-1}\,sec^{-1}$ at pH 6.8. The non-zero intercept in this and similar plots is due to the competing second order decay of the radicals. Observation of the amodiaquine radical over a longer timescale, as in Fig. 2B, shows that the radical is unstable and decays over a period of milliseconds.

The spectrum of the transient radical formed in the reaction of $N_3^{\cdot}$ with AQ was constructed from transient absorption profiles, such as those in Fig. 2, recorded at regular wavelength intervals in the visible spectral region. At wavelengths below 400 nm measurements are hindered by the strong ground state absorbance of AQ. The transient spectrum, obtained by pulse radiolysis, from reaction of $N_3^{\cdot}$ with AQ obtained at pH 6.8 (Fig. 3A) has a pronounced peak at 450–460 nm and a shoulder at longer

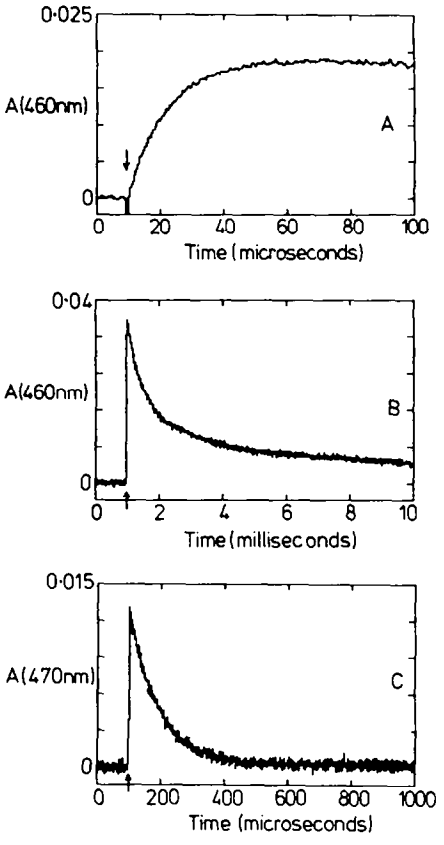


Fig. 2. Transient absorptions at 460–470 nm obtained on pulse radiolysis of N_2O saturated AQ solutions. The time of the pulse is indicated by the arrow. (A) Formation of the AQ radical absorption at 460 nm on reaction of the $N_3^{\cdot}$ radical in solutions containing AQ (52 μM), NaN_3 (0.1 M) and phosphate buffer (10 mM) at pH 6.80. Dose = 1.5 Gy/pulse. (B) Second order decay of the AQ radical absorption at 460 nm in solution containing AQ (0.5 mM), NaN_3 (0.05 M) and phosphate buffer (20 mM) at pH 6.75. Dose = 2.0 Gy/pulse. (C) First order decay of the AQ radical absorption at 470 nm on reaction with ascorbate in solution containing AQ (2.5 mM), ascorbate (0.28 mM), NaN_3 (0.1 M), EDTA (50 μM) and phosphate buffer (50 mM) at pH 6.62. Dose = 1.4 Gy/pulse.

wavelengths. The dotted line in the spectrum in Fig. 3A shows the corrected spectrum, adjusted for ground state bleaching in the region 400–450 nm. The best estimate for the extinction coefficient for the radical absorption at 460-nm is $1400 \pm 100\,m^2/mol$, assuming a yield (G) of 0.62 μmol/J for the $N_3^{\cdot}$ radical. A prominent absorption maximum in the region of 400–500 nm is characteristic of semiquinone and semiiminoquinone radicals [13–15] and indicates that the observed spectrum may be that of the semiiminoquinone radical obtained by one-electron oxidation of the 4-aminophenol group of amodiaquine.

The $Br_2^{\cdot-}$ radical was also found to react rapidly with AQ with a second order rate at pH 6.8 of $(2.06 \pm 0.06) \times 10^8\,M^{-1}\,sec^{-1}$ ($I = 0.12$), obtained from the plot in the inset to Fig. 3B. The transient spectrum of the amodiaquine radical formed on reaction of $Br_2^{\cdot-}$, shown in Fig. 3B, is virtually identical

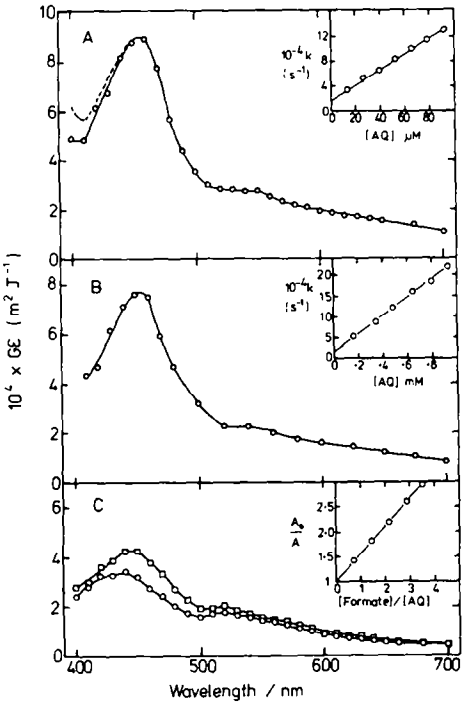


Fig. 3. Transient absorption spectra from pulse radiolysis of N_2O saturated AQ solutions. (A) From reaction of $N_3^\cdot$ radicals in a solution containing AQ (0.2 mM), NaN_3 (20 mM) and phosphate buffer (5 mM) at pH 6.9. Measured 15 μsec after a pulse of 2.9 Gy. The dashed line shows the radical spectrum, corrected for bleaching of the ground state absorption between 400–450 nm. Inset: effect of AQ concentration of the first order rate (k) of formation of the AQ radical absorption at 460 nm on reaction of $N_3^\cdot$. (B) From reaction of $Br_2^{\cdot-}$ radicals in solution containing AQ (0.8 mM), KBr (0.1 M) and phosphate buffer (10 mM) at pH 6.8. Measured 20 μsec after a pulse of 2.9 Gy. Inset: effect of AQ concentration of the first order rate (k) of formation of the AQ radical absorption at 460 nm on reaction of $Br_2^{\cdot-}$. (C) From reaction of ·OH radicals in a solution containing AQ (0.2 mM) and phosphate buffer (5 mM) at pH 7.0. Measured 2 μsec (○) and 15 μsec (□) after a pulse of 5.5 Gy. Inset: effect of formate concentration on the AQ tion of $Br_2^{\cdot-}$. (C) From reaction of ·OH radicals in a solution

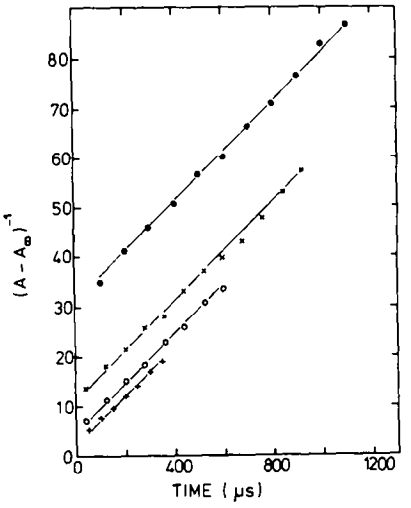
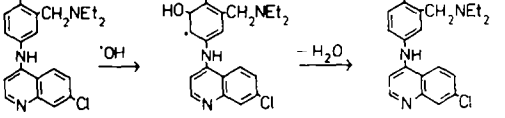


Fig. 4. Second order plots for the decay of the amodiaquine radical absorption (A) corrected for the permanent residual absorption (A_∞) in N_2O-saturated solutions containing AQ (0.5 mM), sodium azide (50 mM) and phosphate buffer (20 mM) at pH 6.75. Measured after pulses of 2.0 Gy (●), 5.1 Gy (×), 14.1 Gy (○) and 29.2 Gy (+).

to that obtained on reaction of $N_3^\cdot$. This provides strong support for the conclusion that the amodiaquine free radical observed in these experiments is a simple one-electron oxidation product, rather than a radical addition product.

The competition method was used to obtain the rate constant of $(6.0 \pm 0.5) \times 10^9\ M^{-1}\ sec^{-1}$ for reaction of the hydroxyl radical with AQ from the data in the inset to Fig. 3C, using formate as the reference solute and taking $k\ (OH^\cdot + HCO_2^-) = 3.2 \times 10^9\ M^{-1}\ sec^{-1}$ [16]. The transient product radical spectra measured on reaction of ·OH with AQ are shown in Fig. 3C. At the concentration of AQ used (0.2 mM) the half-life for reaction of ·OH with AQ is 0.6 μsec, calculated from the rate constant given above. The initial transient spectrum measured 2 μsec after the pulse is considerably less intense than that measured after oxidation with $N_3^\cdot$ or $Br_2^{\cdot-}$, and was observed to continue to increase in intensity for approximately 10–15 μsec after the pulse. As is shown in Fig. 2C, this effect is especially pronounced within the region of the main absorption peak (440–500 nm) of the other spectra in Fig. 2. The low intensity of the spectra from reaction of ·OH suggests that the hydroxyl radical may react with sites (for example the quinoline ring) other than the 4-aminophenol moiety in AQ. Hydroxyl radicals often react with aromatic systems by addition, and the increase in absorbance after hydroxyl radical reaction indicates, by analogy with similar reactions [13, 17], dehydation of an initial dihydroxycyclohexadienyl radical to give the semiiminoquinone radical (Eqn 5).



(5)

The observation that this leads to an increase in absorbance within the region of maximum absorption in spectra obtained from reaction of $N_3^\cdot$ and $Br_2^{\cdot-}$ strengthens the conclusion that these inorganic radicals react to produce the semiiminoquinone radical of AQ.

The rate of reaction of the hydrated electron with AQ was also measured, since it is necessary information for the design of other pulse radiolysis experiments. The second order rate constant was obtained from measurements of the first order rate of decay of the hydrated electron absorption at 700 nm in solutions at pH 7.1 ± 0.1 containing AQ (0–11 μM), giving a value of $(3.9 \pm 0.1) \times 10^{10}\ M^{-1}\ sec^{-1}$.

Reactions of the amodiaquine radical

The semiiminoquinone radical of amodiaquine is unstable, as noted above and demonstrated in Fig. 2B. At the initial radical concentrations provided by pulse radiolysis in these experiments, the decay occurs over a timescale of milliseconds and results in a small permanent residual absorption at 460 nm with a $G\varepsilon$ of approximately 7×10^{-5} m^2/J. When corrected for the absorbance of this permanent product (A_∞) the decay follows second order kinetics, as shown by the linear plots of $(A - A_\infty)^{-1}$ versus time in Fig. 4 at several different initial radical concentrations. The slope of these plots is numerically equal to $2k/\varepsilon l$, where l is the optical pathlength of the sample cell and $2k$ is the rate constant for the second order decay of the radicals. Using the value of the extinction coefficient of the radical obtained above (1.4×10^3 m^2/mol), the data in Fig. 4 give $2k = (1.4 \pm 0.3) \times 10^9$ M^{-1} sec^{-1}. Such a second order decay may indicate either a radical dimerization or a disproportionation reaction. If the latter is the case, then the residual absorption at 460 nm may be due to the amodiaquine iminoquinone.

The decay of the amodiaquine semiiminoquinone radical was also observed in a solution saturated with a N_2O/O_2 (100:20) mixture, using a pulse of 1.1 Gy. The decay was indistnguishable from one obtained under saturation with N_2O alone, with a first half-life for decay of approximately 1 msec. This suggests that the rate constant for reaction of the amodiaquine semiiminoquinone radical with oxygen is less than 2×10^6 M^{-1} sec^{-1}. This is consistent with previous reports that no reaction could be detected between the acetaminophen semiiminoquinone radical and oxygen, either by electron spin resonance spectroscopy [8] or by pulse radiolysis [13].

Maggs *et al.* [3, 4] showed that the irreversible binding of AQ to protein during oxidation of the drug in aqueous solution at physiological pH was inhibited by a number of reducing agents, including NADPH, ascorbate, *N*-acetylcysteine, reduced glutathione and butylated hydroxytoluene (BHT). Whilst two-electron reduction by ascorbate and thiols would implicate the iminoquinone as the reactive product of autoxidation, the observation that BHT reduced protein binding suggested a role for the semiiminoquinone radical, since BHT is a typical phenolic antioxidant capable of "repairing" organic free radicals [18]. Pulse radiolysis has therefore been used to directly measure the reactivity of the amodiaquine semiiminoquinone free radical with some of these reducing agents.

The decay of the amodiaquine semiiminoquinone radical was observed at pH 6.65 in solutions containing increasing concentrations of ascorbate. As shown in Fig. 2C, the decay is considerably faster in the presence of 0.28 mM ascorbate. A plot of the observed first order rate constant for amodiaquine radical decay against ascorbate concentration (Fig. 5) was used to obtain a rate constant of $(3.3 \pm 0.1) \times 10^7$ M^{-1} sec^{-1} for the reaction of ascorbate with the radical. Trolox C is a water soluble analogue of vitamin E and acts as an effective phenolic antioxidant in the same way as BHT [18, 19]. It has been demonstrated to repair a number of different types of organic radicals, including those derived from fatty acids [19], acetaminophen [12] and amino acids [20]. Increasing concentrations of Trolox C in solution also caused an increase in the decay of the amodiaquine semiiminoquinone radical. From the data in Fig. 5 a second order rate constant of $(1.00 \pm 0.05) \times 10^7$ M^{-1} sec^{-1} was determined for the repair of the amodiaquine semiiminoquinone radical by Trolox C at pH 6.7.

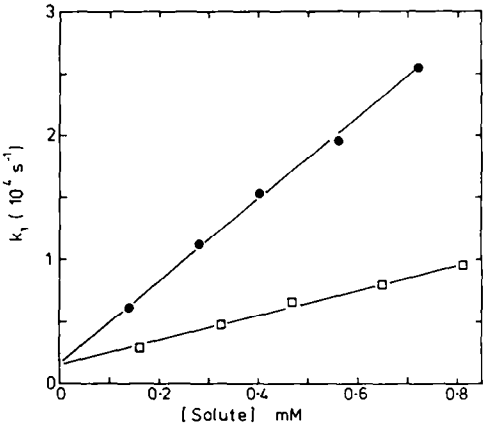


Fig. 5. Effect of the concentration of ascorbate (●) or Trolox C (□) on the first order rate of decay of the AQ radical absorption at 460–470 nm in solutions containing AQ (2.5 mM), NaN_3 (0.1 M) and phosphate buffer (50 mM) at pH 6.7 ± 0.1. In the solutions containing ascorbate, EDTA (50 μM) was also added. Measured using a dose of 1.4 Gy/pulse.

The data obtained here therefore shows that the inhibition by both BHT and ascorbate of amodiaquine binding to proteins during autoxidation may be due to the very rapid reactions of these reductants with the amodiaquine semiiminoquinone radical. Such reactions are consistent with those in the scheme (Fig. 1) proposed by Maggs *et al.* [3, 4]. However, the postulated reaction of oxygen with the semiiminoquinone radical, in which the superoxide radical would be one product, could not be detected by pulse radiolysis. Further studies of the properties and reactivity of the amodiaquine radical using pulse radiolysis, for example determination of its one electron reduction potential and rate of reaction with thiols, are presently limited by the low solubility of AQ in even slightly alkaline solutions. Further experiments are being undertaken to overcome this obstacle.

REFERENCES

1. Hatton CSR, Bunch C, Peto TEA, Pasvol G, Russell SJ, Singer CRJ, Edwards G and Winstanley P, Frequency of severe neutropenia associated with amodiaquine prophylaxis against malaria. *Lancet* i: 411–413, 1986.
2. Neftel KA, Woodtly W, Schmid M, Frick PG and Fehr J, Amodiaquine induced agranulocytosis and liver damage. *Br Med J* **292**: 721–723, 1986.
3. Maggs JL, Kitteringham NR, Breckenridge AM and Park BK, Autoxidative formation of a chemically reactive intermediate from amodiaquine, a myelotoxin and

hepatotoxin in man. *Biochem Pharmacol* **36**: 2061–2062, 1987.
4. Maggs JL, Tingle MD, Kitteringham NR and Park BK, Drug–protein conjugates—XIV. Mechanisms of formation of protein-arylating intermediates from amodiaquine, a myelotoxin and hepatotoxin in man. *Biochem Pharmacol* **37**: 303–311, 1988.
5. Birge RB, Bartolone JB, Nishanian EV, Bruno MK, Mangold JB, Cohen SD and Khairallah EA, Dissociation of covalent binding from oxidative effects of acetaminophen: studies using dimethylated acetaminophen derivatives. *Biochem Pharmacol* **37**: 3383–3393, 1988.
6. Bartolone JB, Birge RB, Sparks K, Cohen SD and Khairallah EA, Immunochemical analysis of acetaminophen covalent binding to proteins: partial characterization of the major acetaminophen binding liver proteins. *Biochem Pharmacol* **37**: 4763–4774, 1988.
7. Savides MC and Oehme FW, Acetaminophen and its toxicity. *J Appl Toxicol* **3**: 96–111, 1983.
8. Fischer V, West PR, Harman LS and Mason RP, Free-radical metabolites of acetaminophen and a dimethylated derivative. *Environ Health Perspect* **64**: 127–137, 1985.
9. Rosen GM, Singletary Jr WV, Rauckman EJ and Killenburg PG, Acetaminophen hepatotoxicity: an alternative mechanism. *Biochem Pharmacol* **32**: 2053–2059, 1983.
10. Baxendale JH and Busi F, *The Study of Fast Processes and Transient Species by Electron Pulse radiolysis*. Reidel, Dordrecht, 1982.
11. Bensasson RV, Land EJ and Truscott TG, *Flash Photolysis and Pulse Radiolysis*. Pergamon Press, Oxford, 1983.
12. Bisby RH, Cundall RB and Tabassum N, Formation and reactivity of free radicals of acetaminophen formed by one electron oxidation. *Life Chem Rep* **3**: 29–34, 1985.
13. Bisby RH and Tabassum N, Properties of the radicals formed by one-electron oxidation of acetaminophen—a pulse radiolysis study. *Biochem Pharmacol* **37**: 2731–2738, 1988.
14. Swallow AJ, Physical chemistry of semiquinones. In: *Functions of Quinones in Energy Conserving Systems* (Ed. Trumpower BL), pp. 59–72. Academic Press, New York, 1982.
15. Neta P, Radiation chemistry of quinoid compounds. In: *The Chemistry of Quinoid Compounds* (Eds. Patai S and Rappoport Z), Vol II, pp. 879–898. Wiley, Chichester, 1988.
16. Buxton GV, Greenstock CL, Helman WP and Ross AB, Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. *J Phys Chem Ref Data* **17**: 513–886, 1988.
17. Land EJ and Swallow AJ, Pulse radiolysis studies of aqueous phenol. *Trans Faraday Soc* **63**: 1181–1190, 1967.
18. Burton GW and Ingold KU, Vitamin E: application of the principles of physical organic chemistry to the exploration of its structure and function. *Acc Chem Res* **19**: 194–201, 1986.
19. Pryor WA, Strickland T and Church DF, Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous sodium dodecyl sulfate micelle solutions. *J Am Chem Soc* **110**: 2224–2229, 1988.
20. Bisby RH, Ahmed S and Cundall RB, Repair of amino acid radicals by a vitamin E analogue. *Biochem Biophys Res Commun* **119**: 245–251, 1984.