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PROPERTIES OF THE RADICALS FORMED BY ONE-ELECTRON OXIDATION OF ACETAMINOPHEN—A PULSE RADIOLYSIS STUDY

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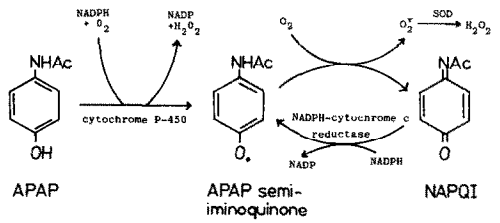
Abstract—The semi-iminoquinone radical of acetaminophen, which has previously been proposed as a possible hepatotoxic intermediate in the cytochrome P-450 catalysed oxidation of acetaminophen, has been generated and studied by pulse radiolysis. In the absence of other reactive solutes, the radical decays rapidly by second order kinetics with a rate constant ($2k_2$) of $(2.2 \pm 0.4) \times 10^9\,M^{-1}\,sec^{-1}$. In alkaline solutions the radical deprotonates with a pK of 11.1 ± 0.1 to form a radical-anion, as confirmed by the effect of ionic strength on the rate of radical decay. The acetaminophen radical-anion reacts with resorcinol at high pH values, leading to the formation of a transient equilibrium from which the one-electron reduction potential of the semi-iminoquinone radical of actaminophen is estimated to be $+0.707 \pm 0.01$ V at pH 7. This value predicts that acetaminophen should be oxidised by thiyl radicals. This was confirmed by pulse radiolysis experiments for reaction of the cysteinyl radical, for which rate constants of $7 \times 10^6\,M^{-1}\,sec^{-1}$ at pH 7 and $2.7 \times 10^8\,M^{-1}\,sec^{-1}$ at pH 11.3 were obtained. The reaction of O_2 with the acetaminophen semi-iminoquinone radical could not be detected by pulse radiolysis, and alternative mechanisms for superoxide radical formation are discussed.

The widely used analgesic drug acetaminophen (APAP, paracetamol)† causes severe hepatic toxicity when ingested in large amounts [1]. De Vries [2] has suggested that the hepatotoxicity of APAP might be due to cytochrome P-450 mediated one-electron oxidation to the semi-iminoquinone radical. This radical species might then redox cycle through *N*-acetyl-*p*-benzoquinone imine (NAPQI) leading to the formation of reactive oxygen radicals (Scheme 1). In model experiments, oxidation of APAP by horseradish peroxidase (HRP) led to the formation of a reactive intermediate which could become covalently bound to proteins [3]. In the same study, a free radical species was detected by electron spin resonance (ESR) spectroscopy. Covalent binding of APAP metabolite(s) to proteins was also detected by Dahlin *et al.* [4] in mouse liver microsomes. In this case the reactive species were concluded to be



Scheme 1. Proposed mechanism for acetaminophen oxidation and generation of active oxygen species through redox cycling between the APAP semiiminoquinone radical and NAPQI. After references 2 and 6.

NAPQI and hydroquinone. Kinetic studies of the decomposition of NAPQI in aqueous solutions of APAP [5] indicated a comproportionation reaction leading to semi-iminoquinone radical formation. Rauckman and Rosen [6] have also postulated a role for the semi-iminoquinone radical in APAP-induced hepatotoxicity. Rosen *et al.* [7] reported that superoxide radical, detected by spin-trapping, was formed during comproportionation of NAPQI and APAP in the presence of oxygen. Evidence was also obtained for H_2O_2 formation, through superoxide disproportionation, on incubation of APAP in hamster hepatic microsomes and an ESR signal indicative of free radical formation was detected. The ESR signal observed in microsomal incubations with APAP [7] has been shown subsequently not to be that of the semi-iminoquinone radical, but to be that of an APAP-derived melanin-like polymer. The semi-iminoquinone radical of APAP could only be detected on the millisecond time scale, employing fast-flow ESR spectroscopy [8] and HRP/H_2O_2 or lactoperoxidase/H_2O_2 as the oxidants. Static ESR spectroscopy of incubations of NAPQI with purified NADPH-cytochrome P-450 reductase revealed only the melanin-like free radical, although analogous experiments with *N*-acetyl-3,5-dimethyl-*p*-benzoquinone imine showed the formation of the corresponding semi-iminoquinone radical [9] which had previously been demonstrated to be relatively stable [10]. In contrast to previous results [11], Fischer *et al.* [9] were unable to detect the formation of superoxide from the semi-iminoquinone radical of APAP, using DMPO as a spin trap. Additionally, the intensity of the semi-iminoquinone radical ESR signal was independent of oxygen concentration [12], showing the lack of reactivity with oxygen. The results of Mason's group using ESR spectroscopy for

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† Abbreviations used: APAP, acetaminophen; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; HRP, horseradish peroxidase: NAPQI, *N*-acetyl-*p*-benzoquinone imine; SOD, superoxide dismutase.

the study of APAP oxidation have recently been summarized [13], and it is suggested that enzymic deacylation of APAP and its metabolites, leading to *p*-aminophenoxyl radicals and *p*-benzoquinone imine may be a critical factor in accounting for the hepatotoxicity of APAP.

In a preliminary publication [14], we have shown that the APAP semi-iminoquinone radical may be generated and studied by pulse radiolysis. The radical was rapidly repaired by both ascorbate and a vitamin E analogue. We now report a more extensive pulse radiolysis study of the APAP semi-iminoquinone radical.

MATERIALS AND METHODS

Acetaminophen was obtained from Sigma (St Louis, MO). All other chemicals were AnalaR grade when available. Solutions were prepared in baked glassware, using water from a Millipore Milli Q system.

Pulse radiolysis experiments were undertaken using the Febetron 705B electron accelerator at the University of Salford as described previously [14]. A 2 cm optical pathlength cell was used for all the experiments. Dosimetry was performed using air saturated solutions of KSCN (10 mM), taking $G((SCN)_2^-) = 0.29\ \mu M/Gy$ and $\varepsilon((SCN)_2^-)$ at 480 nm to be $7600\ M^{-1}\ cm^{-1}$. Transient spectra of radical intermediates are presented as plots of extinction coefficient ($M^{-1}\ cm^{-1}$) versus wavelength, using an assumed *G*-value for the oxidising radicals of $0.62\ \mu M/Gy$ in N_2O-saturated solution. The pH of the solutions was adjusted by addition of NaOH. All experiments were performed at room temperature.

Prior to irradiation, solutions were saturated with N_2O in a syringe system [15], in order that radiolytically produced hydrated electrons be converted to hydroxyl radicals:

$$H_2O \rightsquigarrow {}^{\cdot}OH, {}^{\cdot}H, e_{aq}^-, H_2O_2, H_2 \qquad (1)$$

$$e_{aq}^- + N_2O \rightarrow {}^{\cdot}OH + OH^- + N_2 \qquad (2)$$

In N_2O-saturated solutions the resulting radical species produced following irradiation are the ·OH radical ($G = 0.62\ \mu M/Gy$) and ·H ($G = 0.057\ \mu M/Gy$) [16]. For experiments at high pH values, the solutions were initially deoxygenated by bubbling with N_2O before addition of alkaline buffer.

RESULTS AND DISCUSSION

(1) *Spectra of radicals formed by one-electron oxidation of acetaminophen*

Transient spectra of the radical products formed on pulse radiolysis of N_2O-saturated solutions of acetaminophen solutions are shown in Fig. 1. In N_2O-saturated solutions containing acetaminophen alone (Fig. 1A), the hydroxyl radical is the predominant (>90%) species reacting with the acetaminophen. Reaction of ·OH leads to an initial product observed immediately (1 μsec) after the pulse with a single absorption maximum at 330 nm. The spectrum measured 20 μsec after the pulse shows that an additional peak of 450 nm has appeared. The rate of formation of the species with an absorption

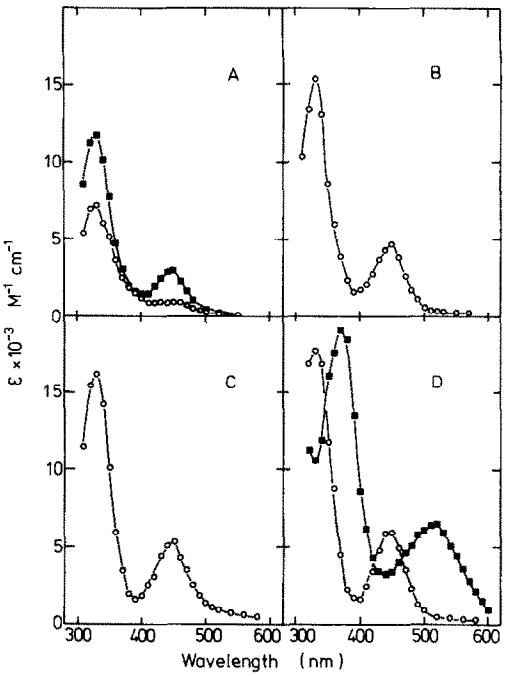
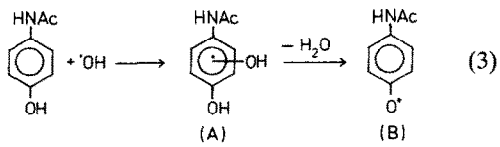


Fig. 1. Transient absorption spectra from one-electron oxidation of acetaminophen by inorganic radicals, measured by pulse radiolysis in N_2O-saturated solutions. A *G*-value of 0.62 μM/Gy was assumed for each oxidising radical. (A) Oxidation by ·OH radicals in a solution containing APAP (0.5 mM) and phosphate (0.5 mM, pH 7.7). Spectra measured 1 μsec (○) and 20 μsec (■) after the pulse. (B) Oxidation by Br_2^- radicals in a solution containing APAP (5 mM), phosphate (10 mM, pH 8.1) and KBr (0.5 M). Measured 15 μsec after the pulse. (C) Oxidation by $(SCN)_2^-$ radicals in a solution containing APAP (5 mM), phosphate (10 mM, pH 8.2) and KSCN (0.5 M). Measured 15 μsec after the pulse. (D) Oxidation by $N_3^\cdot$ radicals in solutions containing APAP (4 mM), phosphate (10 mM) and NaN_3 (0.2 M). Measured 2 μsec after the pulse at pH 8.5 (○) and pH 12.1 (■).

at 450 nm was found to be increased by both increasing the pH and phosphate buffer concentration in the solution, as shown in Fig. 2. It is apparent from comparison of these results with previous investigations of the reaction of ·OH with phenols [17], that the initial product is a dihydroxycyclohexadienyl radical (A), which subsequently dehydrates to a semi-iminoquinone radical (B) (equation (3)) in a reaction that is base catalysed.



(3)

Spectra almost identical to that of the final product in this reaction were also obtained on pulse radiolysis of N_2O-saturated solutions containing either Br^-, SCN^- or N_3^- in neutral or slightly alkaline solution (Fig. 1 B–D). Under these conditions the acetaminophen is oxidised by Br_2^-, $(SCN)_2^-$ or $N_3^\cdot$ respectively.

Reactions of these radicals appear to lead directly to formation of the semi-iminoquinone radical. At

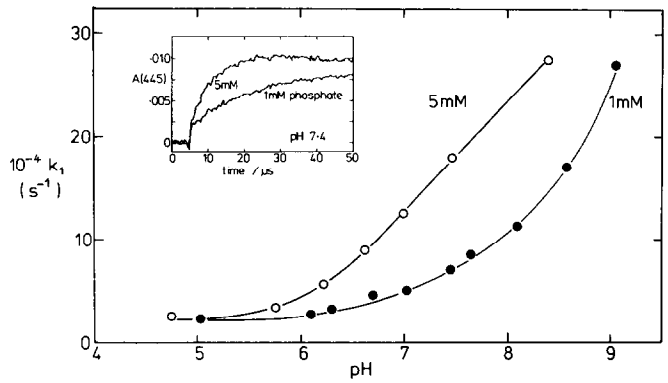


Fig. 2. Effect of pH and phosphate concentration (○, 5 mM and ●, 1 mM) on the first order rate constant for dehydration of the APAP-·OH adduct (equation (3)), measured by formation of the transient absorption at 445 nm. Inset: oscillograms showing the formation of the 445 nm transient absorption after pulse radiolysis of N_2O-saturated solutions containing APAP (0.5 mM) and phosphate buffer (pH 7.4, 1 mM and 5 mM as indicated). Dose = 2.7 Gy/pulse.

neutral pH the rates of oxidation of APAP by these inorganic radicals (Table 1) follow the order expected from previous studies [18, 19]. In alkaline solutions the rates of reaction of these radicals with acetaminophen increase due to ionization of the phenolic group. We have previously shown [14] that the increase in reactivity of Br_2^- with acetaminophen with increasing pH corresponds directly to this ionization with $pK = 9.9 \pm 0.1$, corresponding to the published value of $pK = 9.82$ for deprotonation of the phenolic group of APAP [20]. On one-electron oxidation of acetaminophen with $N_3^\cdot$ at pH 12.1 the transient absorption spectrum of the resulting radical is displaced to longer wavelengths (Fig. 1D) corresponding to deprotonation of the semi-iminoquinone radical for which a pK of 11.1 ± 0.1 has been determined [14]. The transient spectrum from pulse radiolysis of acetaminophen solutions containing sodium carbonate at pH 12.6, where the CO_3^- radical is the oxidising species, also corresponds to that of the acetaminophen radical-anion (Fig. 5A, see below).

(2) *Decay of the semi-iminoquinone radical*

The semi-iminoquinone radical of acetaminophen is unstable. Under the conditions of the pulse radiolysis experiments, the radical decays over a period of several hundred microseconds. At pH 8.3 the decay of the APAP semi-iminoquinone radical follows second order kinetics. The value of the second order rate constant ($2k_2$) for the decay of the radical, defined according to equation (4), is $(2.2 \pm 0.4) \times 10^9\ M^{-1}\ sec^{-1}$. A value of $(5.9 \pm 0.5) \times 10^3\ M^{-1}\ cm^{-1}$ was obtained for the extinction coefficient of the semi-iminoquinone radical at 445 nm under optimal conditions, which is slightly higher than that previously reported [14].

$$\frac{d[R]}{dt} = -2k_2[R]^2 \qquad (4)$$

As the pH of the solution is increased, the rate of decay decreases (Fig. 3), the pH region of the decrease corresponding approximately to the pK of the radical. Full analysis of the effect of pH on the radical decay the neutral semi-iminoquinone radical and the radical anion in terms of the three possible reactions:

(5)

Table 1. Second order rate constants for reaction of inorganic radicals with acetaminophen

Radical	pH	Second order rate constant ($M^{-1}\ sec^{-1}$)
·OH	7.0	$(9.8 \pm 0.4) \times 10^9$
$N_3^\cdot$	7.1	$(3.8 \pm 0.2) \times 10^9$
	11.1	$(5.8 \pm 0.3) \times 10^9$
Br_2^-	8.3	$(2.5 \pm 0.1) \times 10^7$
	11.7	$(5.7 \pm 0.2) \times 10^8$
$(SCN)_2^-$	6.9	$(7.5 \pm 0.2) \times 10^6$
	11.0	$(5.1 \pm 0.3) \times 10^8$
CO_3^-	12.7	$(1.9 \pm 0.2) \times 10^9$

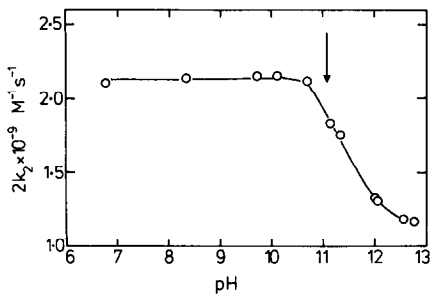
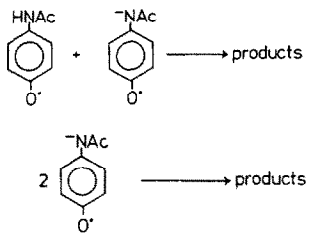


Fig. 3. Effect of pH on the second order rate of decay ($2k_2$) of the APAP semi-iminoquinone radical. Transient decays were recorded at 462 nm (the isobestic point of the neutral- and anion-radical spectra, assuming an extinction coefficient of $4.33 \times 10^3\ M^{-1}\ cm^{-1}$) on pulse radiolysis (17 Gy/pulse) of N_2O-saturated solutions of APAP (0.2 mM), phosphate (10 mM) and NaN_3 (0.2 M). The arrow indicates the pK_a of the semi-iminoquinone radical.



is prevented by ionic strength effects at the higher pH values (see below). However, the results appear to show that the rates of reactions (5) and (6) are similar and both rather higher than for reaction (7). Reaction (7) would be expected to be slower because only in this case are there two negatively charged species reacting together. The kinetic results fail to distinguish between dimerisation and disproportionation as possible alternatives for reactions (5)–(7). In the HRP/H_2O_2 catalysed oxidation of APAP, Potter *et al.* [21, 22] observed the formation of six acetaminophen polymers which were proposed to be formed by reactions of the semi-iminoquinone radical.

The effect of ionic strength on the decay of the radical species at both pH 8.3 and pH 12.0 was further investigated to confirm the radical charge under these conditions. The acetaminophen radicals were generated by oxidation with N_3^- in pulse radiolysis experiments. The ionic strength was changed by addition of increasing concentrations of sodium azide. Due to requirement for $^{\cdot}OH$ radicals to be scavenged by N_3^- the lowest concentration of NaN_3 used was 2 mM. The results of these experiments are shown in Fig. 4, plotting the logarithm of the slope of second order plots versus $\sqrt{I}(\sqrt{I}+1)$ according to the Debye–Huckel equation [23]. At pH 8.3 there is little, if any, effect of ionic strength on the rate of decay of the acetaminophen radical. This indicates that at this pH the radical has zero charge and corresponds to the assignment of the species as a neutral radical at this pH. At pH 12.0, the rate of decay of the radical increases with increasing ionic strength and the plot gives a maximum slope at the lower ionic strengths of 0.7. The primary salt effect predicts that reaction of species with unit charge of the same sign should give a plot with unit positive slope. Therefore the results indicate that at pH 12.0 the radicals have unit charge, and since they are derived from a neutral radical by deprotonation they must be radical anions. That the slope of the plot at pH 12 is less than the value of unity may be due to the experiment being carried out at a pH only 0.9 units higher than the radical pK, and the relatively high ionic strengths required for the experiment. Measurements at higher pH would have further restricted the ionic strength range available.

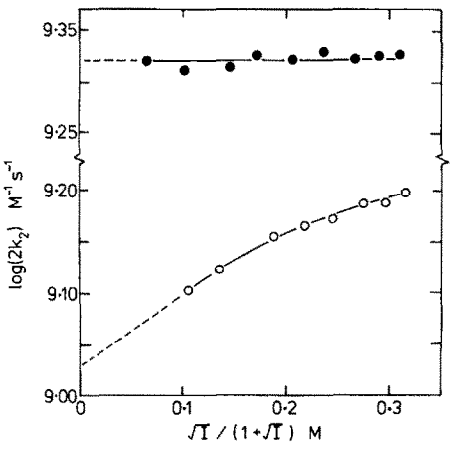


Fig. 4. Effect of ionic strength on the second order rate ($2k_2$) for decay of acetaminophen radicals formed on oxidation with N_3^- radicals at pH 8.3 ± 0.1 (●, measured at 445 nm, taking an extinction coefficient of $5.9 \times 10^3\ M^{-1}\ cm^{-1}$) and at pH 12.0 ± 0.1 (○, measured at 520 nm, taking an extinction coefficient of $6.7 \times 10^3\ M^{-1}\ cm^{-1}$). The ionic strength was varied by addition of increasing concentrations of NaN_3 (2–200 mM) to solutions of APAP (0.2 mM).

(3) *Reaction of the hydrated electron with acetaminophen*

The second order rate constant for reaction of the hydrated electron with APAP was measured from the rate of decay of the e_{aq}^- absorption at 700 nm, following pulse radiolysis of N_2-purged solutions containing APAP (100–500 μM) buffered to pH 7.0 with phosphate (2 mM). A second order rate constant of $(2.5 \pm 0.3) \times 10^8\ M^{-1}\ sec^{-1}$ was obtained. This result shows that APAP concentrations of up to 40 mM may be used in pulse radiolysis of N_2O-saturated solutions with insignificant (>5%) reduction in the yield of $^{\cdot}OH$ radicals from competition between APAP and N_2O for scavenging of e_{aq}^-.

(4) *Determination of the one-electron reduction potential of the acetaminophen semi-iminoquine radical*

Our previous results have shown that the semi-iminoquinone radical of acetaminophen is rapidly repaired by ascorbate and by Trolox C (a vitamin E analogue) at neutral pH. The radical-anion of acetaminophen was also found to be repairable by hydroquinone at pH 12.6 with a second order rate constant of $(1.4 \pm 0.1) \times 10^8\ M^{-1}\ sec^{-1}$. In these reactions there was no indication of reversibility. This implies that the one-electron reduction potential of the APAP radical is substantially (>100 mV) higher than that of Trolox C ($E_7 = 480$ mV) [24]. Further experiments to measure the repair of the acetaminophen radical anion by resorcinol (RES) at pH >12 showed that a transient equilibrium (equation (8)) was established in the reaction from which a one electron reduction potential (E^1) for the acetaminophen semi-iminoquinone radical could be estimated

$$APAP^{\cdot} + RES \rightleftharpoons APAP + RES \qquad (8)$$

Figure 5A shows the transient spectra recorded from pulse radiolysis of separate N_2O-saturated solutions of acetaminophen and of resorcinol, both containing sodium carbonate (0.1 M, pH 12.6). The spectrum of the radical formed by one-electron oxidation of resorcinol under these conditions is similar to that previously reported [24], with a peak at approximately 450 nm. Figure 5B shows the transient

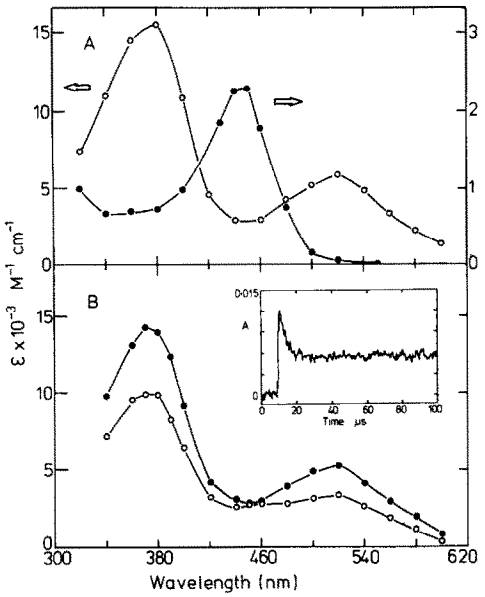


Fig. 5. Demonstration of a transient equilibrium between the APAP radical-anion and resorcinol at high pH. A G-value for the radical species of 0.62 μM/Gy was assumed. (A) Transient absorption spectra from pulse radiolysis of N_2O-saturated solutions containing APAP (18 mM, ○) or recorcinol (2 mM, ●) and Na_2CO_3 (0.1 M) at pH 12.6. Both spectra measured 20 μsec after the pulse. Note the difference in absorbance (ε) scale for the two spectra. (B) Transient absorption spectra measured after 2 μsec (●) and 30 μsec (○) following pulse radiolysis of an N_2O-saturated solution containing APAP (37 mM) and resorcinol (3.7 mM) and Na_2CO_3 (0.1 M) at pH 12.4. Inset: oscillogram showing the decay of the APAP radical-anion absorption at 380 nm in a solution of APAP (37 mM), resorcinol (11.1 mM) and Na_2CO_3 (0.1 M) at pH 12.4. Dose = 1.6 Gy/pulse.

absorption spectra recorded 2 μsec and 30 μsec after pulse radiolysis of an N_2O-saturated solution containing APAP (37 mm), recorcinol (3.7 mM) and sodium carbonate (0.1 M) at pH 12.4. The spectrum observed 2 μsec after the pulse is clearly that of the acetaminophen radical-anion, which at 30 μsec after the pulse has partially decayed, except in the region of the resorcinol radical absorption between 440 and 460 nm. The inset to Fig. 5B shows a typical decay at 380 nm of the acetaminophen radical-anion in a similar solution containing both acetaminophen and resorcinol. This illustrates the initial decay of the acetaminophen radical-anion followed by the establishment of a transient equilibrium.

The equilibrium constant (K_{eq}) for the transient equilibrium was determined by two methods. Initially measurements of the APAP radical anion concentration at the equilibrium were made from the transient absorption values, from which K_{eq} was determined according to equation (10) [24, 25],

$$K_{eq} = ([APAP] \cdot [RES^{\cdot}])/([APAP^{\cdot}] \cdot [RES]) \quad (9)$$

$$= \frac{[APAP]\,(A_{obs} - A_{APAP^{\cdot}})}{[RES]\,(A_{RES^{\cdot}} - A_{obs})} \quad (10)$$

where A_{obs} is the observed absorbance at equilibrium in solutions containing both APAP and RES, and $A_{RES^{\cdot}}$ and $A_{APAP^{\cdot}}$ are the absorbance values in solutions of only RES or APAP respectively. Values of $(A_{obs} - A_{APAP^{\cdot}})/(A_{RES^{\cdot}} - A_{obs})$ were measured at 520 nm for ratios of [RES]/[APAP] of up to 0.3 at pH 12.8, giving $K_{eq} = 8.13 \pm 0.2$.

An alternative method of determination of K_{eq} is to measure the first order rate of approach to the transient equilibrium (K_{obs}) [25], for which

$$k_{obs} = k_8\,[RES] + k_{-8}\,[APAP] \quad (11)$$

and

$$K_{eq} = k_8/k_{-8}. \quad (12)$$

Measurement of k_{obs} in N_2O-saturated solutions of APAP (37 mM), sodium carbonate (0.2 M) and resorcinol (1.85–11.1 mM) at pH 12.4 led to the results in Fig. 6. Values of $k_8 = (1.7 \pm 0.12) \times 10^7\ M^{-1}\,sec^{-1}$ and $k_{-8} = (2.3 \pm 0.22) \times 10^6\ M^{-1}\,sec^{-1}$ were obtained, giving $K_{eq} = 7.4 \pm 0.7$.

Taking the more accurate value of $K_{eq} = 8.13 \pm 0.2$ from the transient absorbance measurements, the difference in one-electron reduction potential (ΔE^1) between the APAP and resorcinol radicals was calculated from $\Delta E^1 = 0.059 \log(K_{eq})$, giving $\Delta E^1 = 53.7 \pm 3$ mV. The one-electron reduction potential (E^1) of the resorcinol radical has been measured to be +385 mV at pH 13.5 [24]. Using the ground state and radical pK values for resorcinol quoted by Steenken and Neta [26], $E^1_{12.8}$ is calculated to be virtually unchanged at +386 mV. Hence at pH 12.8 ± 0.1 the one electron reduction potential for the APAP radical-anion is +440 ± 10 mV, uncorrected for the effect of ionic strength. E^1 at other pH values may be calculated from equation (13) [26, 27].

$$E^1_{pH} = E^1_o + 0.059 \log (\{K_1K_2 + K_1[H^+] + [H^+]^2\}/\{K_r + [H^+]\}) \quad (13)$$

where K_1 and K_2 are the first and second acid dissociation constants for APAP, and K_r is the acid dissociation constant of the APAP semi-iminoquinone radical (pK_r = 11.1 [14]). pK_1 is given in the literature as 9.82 [20], and we estimate p$K_2 \geq 15$ from the lack of any change in the absorption spec-

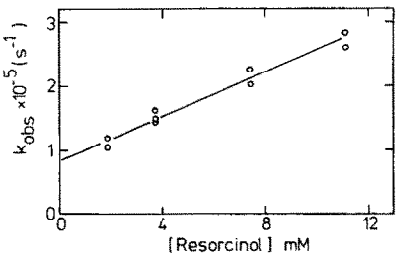


Fig. 6. Determination of the equilibrium constant for the transient equilibrium between the APAP radical-anion and resorcinol at pH 12.4 ± 0.1, using the kinetics of the approach to equilibrium (equation (11)). The observed first order decay (k_{obs}) of the APAP radical-anion at 380 nm and 520 nm was measured by pulse radiolysis of N_2O-saturated solutions containing APAP (37 mM), resorcinol (1.85–11.1 mM) and Na_2CO_3 (0.1 M).

trum of APAP on increasing pH from 12 to 14. The value of the one-electron reduction potential at pH 7.0 (E_7^1) is calculated to be $+707 \pm 10$ mV, and E_0^1 to be $+1.12 \pm 0.01$ V. Using a value for the Hammett constant (σ_p^+) of -0.6 for the CH_3CONH– group and a comparison with published potentials and Hammett substituent constants for other phenols, a value for $E_{13.5}^1$ of +0.43 V is predicted for the acetaminophen radical-anion (P. Wardman, personal communication). This is in reasonable agreement with the value obtained in the present work.

(5) *Reaction of acetaminophen radicals with sulphydryl compounds*

The hepatotoxicity of acetaminophen is only expressed when intracellular levels of glutathione have been depressed [1]. Administration of thiol compounds and their precursors, especially methionine, has been proposed as a treatment for acetaminophen poisoning [28–31]. It is therefore important to consider the reactivity of thiol compounds, and glutathione in particular, with the acetaminophen semi-iminoquinone radical. The one-electron reduction potential of the sulphur radical, RS$^\cdot$, derived from the one-electron reduction of the peptide $(Cys\text{-}Gly)_2$ has recently been estimated at approximately +0.73 V at pH 13 [32]. Using a pK value of 8.3 for the thiol group of cysteine [33], a reduction potential of +0.81 V at pH 7 is calculated. This is approximately 100 mV greater than that for the APAP semi-iminoquinone radical at pH 7. Assuming this value, then reaction (14).

$$RSH + APAP^\cdot \rightleftharpoons RS^\cdot + APAP \qquad (14)$$

has an equilibrium lying to the left-hand side with an equilibrium constant of $\sim 2 \times 10^{-2}$. This accounts for our observation that the decay of the APAP semi-iminoquinone radical appears to be unaffected in pulse radiolysis experiments by the presence of thiols such as cysteine. We have attempted to study the reverse of reaction (14) by pulse radiolysis of deaerated solvents of cystine (RSSR) with t-butanol as an $^\cdot OH$ radical scavenger, in which the cysteinyl radical is generated [34]:

$$RSSR + e_{aq}^- \rightarrow RSSR^{\cdot -} \qquad (15)$$

$$RSSR^{\cdot -} \rightarrow RS^\cdot + RS^- \qquad (16)$$

Under these conditions, thiol (other than the approximately micromolar amount produced by reaction (16) is absent) and the reverse reaction (14) is expected to be observed.

The inset to Fig. 7 shows an oscillogram recorded at 520 nm from pulse radiolysis of a deaerated solution of cystine (CysSSCys, 2 mM), t-butanol (0.85 M) and APAP (0.2 mM) at pH 11.3. The initial very rapid formation and decay is due to the $CysSSCys^{\cdot -}$ radical-anion which has a broad absorption centered at 410 nm, and which decomposes into CysS$^\cdot$ (transparent at 520 nm) and $CysS^-$ with a first order rate of $\geqslant 3 \times 10^5$ sec^{-1} [34]. The subsequent increase in absorbance represents the formation of the APAP radical-anion, as confirmed by the transient spectrum (data not shown). The pseudo-first order rate of growth of the absorbance was proportional to APAP concentration (Fig. 7), giving a second order rate

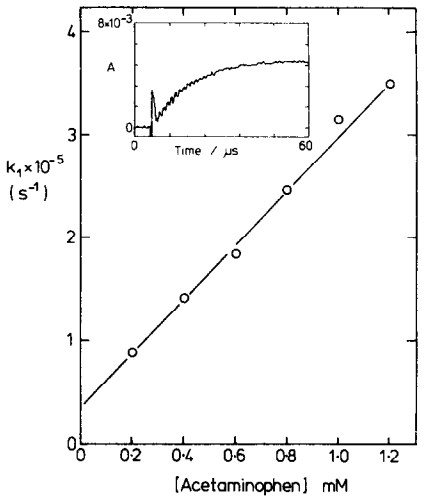


Fig. 7. Effect of acetaminophen concentration on the first order rate constant for reaction of the cysteinyl radical (CysS$^\cdot$), measured by the rate of formation of the acetaminophen radical absorption at 450 or 520 nm on pulse radiolysis of deaerated (N_2-purged) solutions containing cystine (2 mM), APAP (0.2–1.2 mM), t-butanol (0.85 M) and phosphate (10 mM) at pH 11.3 ± 0.1. Inset: oscillogram showing the transient absorption changes at 520 nm on pulse radiolysis of a deaerated solution containing cystine (2 mM), APAP (0.2 mM), t-butanol (0.85 M) and phosphate (10 mM) at pH 11.3. Dose = 3.1 Gy/pulse.

constant (k_{-14}) of $(2.7 \pm 0.1) \times 10^8$ M^{-1} sec^{-1} at pH 11.3. Similar pulse radiolysis experiments at pH 10.5 showed that the intensity of the transient absorption due to the APAP radicals corresponded to quantitative oxidation of APAP by CysS$^\cdot$. The value of k_{-14} was also measured at pH 7.0 and was found to be $(7 \pm 1) \times 10^6$ M^{-1} sec^{-1}. A lower rate at smaller pH values is expected from analogous results on the oxidation of tyrosine by RS$^\cdot$ [32] and is due to protonation reactions [35] accompanying electron transfer at pH 7. At pH 11.3 the reaction is expected to proceed purely by electron transfer. An increased rate for k_{-14} at high pH values is also expected from the increase in ΔE^1 between the cysteinyl radical and the acetaminophen radical due to the combined effects of radical and ground state pKs. Further work is in progress to investigate whether this value of ΔE^1 may be determined by pulse radiolysis methods.

Experiments such as those described here allow the reaction of RSH/RS$^\cdot$ with APAP to be studied only under pulse radiolysis conditions. It has been suggested [35, 36] that under physiological conditions thermodynamically unfavourable reactions such as reaction (14) may still proceed in the forward direction if RS$^\cdot$ is removed by a series of coupled reactions such as

$$RS^\cdot + RS^- \rightleftharpoons RSSR^{\cdot -} \qquad (17)$$

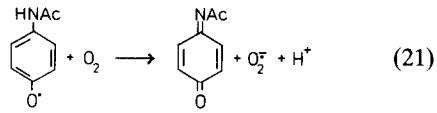
$$RSSR^{\cdot -} + O_2 \rightarrow RSSR + O_2^{\cdot -} \qquad (18)$$

$$2O_2^{\cdot -} \xrightarrow{SOD/2H^+} H_2O_2 \qquad (19)$$

$$RS^\cdot + O_2 \rightarrow RSO_2^\cdot \qquad (20)$$

At present we have not been able to investigate the role of these additional reactions, due to the rapid decay of the APAP semi-iminoquinone radical at the relatively high initial radical concentrations (~1–5 μM) generated in pulse radiolysis experiments.

Using pulse radiolysis we have not been able to observe the reaction between O_2 and the APAP semi-iminoquinone radical (equation (21)), as originally proposed for a mechanism of superoxide generation [2, 6].



(21)

Our experiments now place an upper limit of $\sim 10^5\ M^{-1}\ sec^{-1}$ for the rate of this reaction. This agrees well with the measurements of Mason and coworkers [9, 12, 13] who were also unable to detect this reaction using ESR spectroscopy. Coupled reactions such as those represented by equations (17)–(20) may provide a mechanism for O_2^- and H_2O_2 formation for which evidence has been claimed in microsomal incubations with APAP [7].

REFERENCES

1. Savides MC and Oehme FW, Acetaminophen and its toxicity. *J Appl Toxic* **3**: 96–111, 1983.
2. De Vries J, Hepatotoxic metabolic activation of paracetamol and its derivatives phenacetin and benorilate: oxygenation or electron transfer? *Biochem Pharmacol* **30**: 399–402, 1981.
3. Nelson SD, Dahlin DC, Rauckman EJ and Rosen GM, Peroxidase-mediated formation of reactive metabolites of acetaminophen. *Mol Pharmacol* **20**: 195–199, 1984.
4. Dahlin DC, Miwa GT, Lu AYH and Nelson SD, *N*-Acetyl-*p*-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci USA* **81**: 1327–1331, 1984.
5. Dahlin DC and Nelson SD, Synthesis, decomposition kinetics, and preliminary toxicological studies of pure *N*-acetyl-*p*-benzoquinone imine, a proposed toxic metabolite of acetaminophen. *J Med Chem* **25**: 885–886, 1982.
6. Rauckman EJ and Rosen GM, Intermediacy of intracellular superoxide in drug mediated hepatotoxicity. In: *Oxy radicals and their Scavenger Systems*, Vol. II: *Cellular and Medical Aspects* (Eds. Greenwald RA and Cohen G), pp. 193–198. Elsevier Biomedical, New York (1983).
7. Rosen GM, Singletary WV, Rauckman EJ and Killenberg PG, Acetaminophen hepatotoxicity: an alternative mechanism. *Biochem Pharmacol* **32**: 2053–2059, 1983.
8. West PR, Harman LS, Josephy PD and Mason RP, Acetaminophen: enzymic formation of a transient phenoxyl free radical. *Biochem Pharmacol* **33**: 2933–2936, 1984.
9. Fischer V, West PR, Nelson SD, Harvison PJ and Mason RP, Formation of 4-aminophenoxyl free radical from the acetaminophen metabolite N-acetyl-p-benzoquinone imine. *J Biol Chem* **260**: 11446–11450, 1985.
10. Fischer V and Mason RP, Stable free radical and benzoquinone metabolites of an acetaminophen analogue. *J Biol Chem* **259**: 10284–10288, 1984.
11. Rosen GM, Rauckman EJ, Ellington SP, Dahlin DC, Christie JL and Nelson SD, Reduction and glutathione conjugation reactions of *N*-acetyl-*p*-benzoquinone imine and two dimethylated analogues. *Mol Pharmacol* **25**: 151–157, 1984.
12. Fischer V, West PR, Harman LS and Mason RP, Free radical metabolites of acetaminophen and a dimethyl derivative. *Envir Health Perspect* **64**: 127–137, 1985.
13. Mason RP and Fischer V, Free radicals of acetaminophen: their subsequent reactions and toxicological significance. *Fed Proc* **45**: 2493–2499, 1986.
14. Bisby RH, Cundall RB and Tabassum N, Formation and reactivity of free radicals of acetaminophen formed by one electron oxidation. *Life Chemistry Reports* **3**: 29–34, 1985.
15. Willson RL, The reactions of oxygen with radiation-induced free radicals in DNA and related compounds. *Int J Radiat Biol* **17**: 349–358, 1970.
16. Swallow AJ, Applications of pulse radiolysis to the study of aqueous organic systems. In: *The Study of Fast Processes and Transient Species by Electron Pulse Radiolysis* (Eds. J. H. Baxendale and F. Busi), pp. 289–315. Reidel, Dordrecht (1982).
17. Land EJ and Ebert M, Pulse radiolysis studies of aqueous phenol. *Trans Faraday Soc* **63**: 1181–1190, 1967.
18. Adams GE, Aldrich JE, Bisby RH, Cundall RB, Redpath JL and Willson RL, Selective free radical reactions with proteins and enzymes: reactions of inorganic radical anions with amino acids. *Radiat Res* **49**: 278–289, 1972.
19. Land EJ and Prutz WA, Reactions of azide radicals with amino acids and proteins. *Int J Radiat. Biol.* **36**: 75–83, 1979.
20. Legler G, Sinnott ML and Withers S, Catalysis by glucosidase A3 of *Aspergillus wentii*. *J Chem Soc Perkin Transactions* **II**: 1376–1383, 1980.
21. Potter DW, Miller DW and Hinson JA, Identification of acetaminophen polymerization products catalysed by horseradish peroxidase. *J Biol Chem* **260**: 12174–12180, 1985.
22. Potter DW, Miller DW and Hinson JA, Horseradish peroxidase-catalysed oxidation of acetaminophen to intermediates that form polymers or conjugate with glutathione. *Mol. Pharmacol* **29**, 155–162, 1986.
23. Atkins PW, *Physical Chemistry*, 2nd Edn., p. 327. Oxford University Press, Oxford, 1983.
24. Steenken S and Neta P, One-electron redox potentials of phenols. Hydroxy- and aminophenols and related compounds of biological interest. *J. Phys Chem* **86**: 3661–3667, 1982.
25. Wardman P and Clarke ED, One-electron reduction potentials of substituted nitroimidazoles measured by pulse radiolysis. *J Chem Soc, Faraday Trans I* **72**: 1377–1390, 1976.
26. Steenken S and Neta P, Electron transfer rates and equilibria between substituted phenoxide ions and phenoxy radicals. *J Phys Chem* **83**: 1134–1137, 1979.
27. Clark WM, *Oxidation–Reduction Potentials of Organic Systems*. Williams & Wilkins, Baltimore, 1960.
28. Chiu S and Bhakthan NMG, Experimental acetaminophen-induced hepatic necrosis. *Lab Invest* **39**: 193–203, 1978.
29. Vale JA, Meredith TJ and Goulding R, Treatment of acetaminophen poisoning. *Arch Int Med* **141**: 394–396, 1981.
30. Skoglund LA and Skjelbred P, Comparison of a traditional paracetamol medication and a new paracetamol/paracetamol-methionine combination. *Eur J Clin Pharmacol* **26**: 573–577, 1984.
31. Nagasawa HT, Goon DJW, Muldoon WP and Zera RT, 2-Substituted thiazolidine-4(*R*)-carboxylic acids as prodrugs of L-cysteine. *J Med Chem* **27**, 591–596, 1984.
32. Prutz WA, Butler J, Land EJ and Swallow AJ, Unpaired electron migration between aromatic and sulphur peptide units. *Free Radical Res Commun* **2**: 69–75, 1986.

33. Serjeant EP and Dempsey B, Ionisation constants of organic acids in aqueous solution. *IUPAC Chemical Data Series–No. 23*. Pergamon Press, Oxford, 1979.
34. Hoffman MZ and Hayon E, One-electron reduction of the disulfide linkage in aqueous solution. *J Am Chem Soc* **94**: 7950–7957, 1972.
35. Wardman P and Wilson I, Control of the generation and reactions of free radicals in biological systems by kinetic and thermodynamic factors. *Free Radical Res Commun* **2**: 225–232, 1987.
36. Wilson I, Wardman P, Cohen GM and D'Arcy Doherty M, Reductive role of glutathione in the redox cycling of oxidizable drugs. *Biochem Pharmacol* **35**: 21–22, 1986.