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DRUG–PROTEIN CONJUGATES—XIV

MECHANISMS OF FORMATION OF PROTEIN-ARYLATING INTERMEDIATES FROM AMODIAQUINE, A MYELOTOXIN AND HEPATOTOXIN IN MAN*

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Abstract—The enzymic and non-enzymic formation of protein-arylating intermediates from amodiaquine (AQ,7-chloro-4-(3′-diethylamino-4′-hydroxyanilino) quinoline), an anti-malarial associated with agranulocytosis and liver damage in man, was studied *in vitro*. [^{14}C]AQ in phosphate buffer, pH 7.4, under air was autoxidized to a reactive derivative(s) which possessed characteristics indicative of a semiquinone/quinone imine: reduction by NADPH and ascorbic acid, conjugation with thiols and irreversible binding to microsomal and soluble proteins. Cysteinyl SH groups were major sites of arylation. Radiolabelled material irreversibly bound to HSA after 24 hr and to human liver microsomes after 4 hr represented 26.5 ± 1.8% and 31.4 ± 0.6% ($\bar{x}$ ± SD, N = 3) of incubated [^{14}C]AQ (10 μM), respectively. The quinone imine of AQ(AQQI) was synthesized, and displayed the same oxidative and electrophilic reactions as the product(s) of AQ's autoxidation. A water-soluble product formed in buffered solutions of AQ and *N*-acetylcysteine was identified as an AQ mercapturate by comparison with an adduct prepared from synthetic AQQI. Irreversible binding of [^{14}C]AQ was inhibited by a radical scavenger; this indicated that the semiquinone imine contributed to the binding. Although AQ was extensively de-ethylated by human liver microsomes, oxidation by cytochrome P-450 did not appear to be principally responsible for its activation and irreversible binding in microsomal incubations. AQ was oxidized to protein-arylating intermediates by horseradish peroxidase. It also formed reactive derivatives, possibly *N*-chloro compounds, in chlorine solutions. These findings indicated that AQ can give rise to chemically reactive species by at least three distinct mechanisms, viz. autoxidation in neutral solution under air, peroxidase-catalyzed oxidation and *N*-chlorination. Formation of such species in liver and myeloid cells might be responsible for the adverse reactions associated with AQ.

The toxicity of many stable compounds is attributable to their oxidative metabolism to chemically reactive intermediates [1–4]. The disposition and reactivity of an intermediate will largely determine the expression of the tissue injury. However, the primary reactions may be blocked by protective mechanisms [5, 6] and reparative processes may intervene to prevent subsequent biochemical responses leading to irreversible cellular damage [7, 8].

Although several enzyme types have been implicated in the metabolic activation of drugs and other xenobiotics [2–4], the cytochrome P-450 family of mono-oxygenases is considered to be responsible for the oxidation of a great variety of compounds to reactive electrophiles. Amongst the intermediates generated by cytochrome P-450 the *p*-quinone imine of paracetamol has been the focus of particular attention. It is thought to be the ultimate reactive and toxic intermediate of the drug [9–11] and to be primarily responsible for paracetamol-induced hepatic necrosis. The biochemical and cellular mechanisms of its toxicity remain to be clarified [7, 12–14] but arylation of vital intracellular proteins is generally viewed as a critical initiating event [1–3, 15, 16].

Clearly, any compound containing the p-hydroxyanilino moiety of paracetamol may undergo cytochrome P-450-mediated one-/two-electron oxidation to a semiquinone/quinone imine. One such drug is the 4-aminoquinoline anti-malarial amodiaquine (AQ; Camoquin; 7-chloro-4-(3′-diethylamino-4′-hydroxyanilino) quinoline; Fig. 7) [17], the subject of recent clinical concern because of serious dose-related toxicity [18]. It has been associated with occasionally fatal agranulocytosis [18–20] and liver damage [19, 21]; the latter varies from mild hepatitis [21] to sub-massive necrosis [19]. AQ is rapidly and extensively metabolized following absorption in man [22–24], de-ethylation and C-2 hydroxylation to desethylamodiaquine, bidesethylamodiaquine and 2-hydroxydesethylamodiaquine being the principal routes of metabolism [22, 23].

In view of the above considerations, the metabolism of AQ *in vitro* was studied with reference to the formation of electrophilic intermediates which reacted irreversibly with protein. However, it became apparent that AQ was readily *autoxidized* to such intermediates in neutral solution under air. Further experiments revealed a number of mechanisms by which they could be formed and provided details of the species' identities and reactivity. This

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enabled a comparison with drugs which yield protein-arylating derivatives only via metabolism. Aspects of this work have been published in abstract form [25, 26].

MATERIALS AND METHODS

Chemicals, proteins and enzymes. [Quinoline-2-^{14}C]AQ dihydrochloride monohydrate (3.2 mCi/mmol) was synthesized by Amersham International plc (Bucks, U.K.) [27]. It was repurified to 97–99% radiochemical purity by TLC; purity was determined by TLC on silica plates (developed with triethylamine–methanol, 1:1, v/v) and HPLC on a phenyl column [23]. [^{14}C]AQ (9–10 mg; radiochemical purity 86%) in ethanol was chromatographed on two silica gel 60 plates (20 × 20 × 0.2 cm) developed with triethylamine–methanol (1:1,v/v), located under UV light (254 nm; R_f 0.68) and eluted with ethanol (gel corresponding to a band of [^{14}C]AQ extracted 8 ml × 3). Silica gel was removed by centrifugation and HCl (11.45 M; 42.36 μmol/10 mg [^{14}C]AQ) added to the ethanol solution to produce a solution of the hydrochloride; this was stored at −30°. Unlabelled AQ dihydrochloride dihydrate (Camoquin), desethylamodiaquine (DEAQ) and bidesethylamodiaquine (biDEAQ) were obtained from Parke-Davis plc (Pontypool, Wales, U.K.). NADPH (tetrasodium salt) was purchased from Boehringer Mannheim GmbH, Biochemica (Mannheim, F.R.G.). Butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (Poole, Dorset, U.K.). HPLC-grade solvents were products of Fisons plc (Loughborough, Leics, U.K.). Scintillation fluid (Aqua Luma Plus) was from Lumac/3M B.V. (Schaesberg, Netherlands). Human serum albumin (HSA, Fraction V; 96–99% pure), bovine serum albumin (BSA, Fraction V), bovine α-casein (Bα-c, *ca.* 70% pure, balance primarily β-casein), peroxidase (Type VI from horseradish; 300 Units/mg) and catalase (purified powder from bovine liver; 2750 Units/mg) were obtained from Sigma.

Syntheses. The putative quinone imine of AQ(AQQI) was synthesized by the method of Dahlin and Nelson [28] for preparing *N*-acetyl-*p*-benzoquinone imine. Unlabelled AQ (0.4 g, 0.86 mmol) in chloroform (40 ml) over anhydrous Na_2SO_4 (*ca.* 0.2 g) was stirred with added silver oxide (0.5 g, 2.15 mmol) at room temperature for 30 min. The mixture was filtered and then passed through a column of hexane-washed chromatography-grade silica gel (20 cm × 20 cm; 100–200 mesh; Sigma) which was eluted with methanol–chloroform (2:98,v/v). The eluate was filtered through paper and evaporated to dryness *in vacuo* at 30°. An orange product (0.24 g, 0.68 mmol; 79% yield) was obtained: λ_{max} (*n*-hexane) 271 nm ($\varepsilon 0.23 \times 10^4\ M^{-1}\ cm^{-1}$); IR (Nujol) 3400 (medium, OH) and 3140 (weak, NH) of AQ absent, 1615 (strong, quinone imine). Characteristically [29, 30], as revealed by rapid discharge of its dark red colour in biphasic chloroform–water systems, the product was reduced by ascorbate and reacted with thiols to form water-soluble compounds.

[^{14}C]AQ mercapturates were prepared by reacting *N*-acetylcysteine (0.815 g, 5 mmol) in water with [^{14}C]AQQI (4 μCi/mmol) prepared as described above. The two-phase reaction proceeded rapidly at room temperature; the quinone imine's colour disappeared and yellow products partitioned into the aqueous phase. The reaction mixture was centrifuged to separate the aqueous and chloroform phases. Aliquots (20 μl × 30) of the former were chromatographed on a μBondapak C_{18} column (30 cm × 0.39 cm i.d., 10 μm; Waters Associates, Hartford, Cheshire, U.K.) using a gradient of acetonitrile (20–25% at 0.2%/min) in $NH_4H_2PO_4$ (10 mM, pH 4.6) containing octane sulphonate (5 mM). The flow rate was 1.5 ml/min. An LKB 2150 HPLC pump and 2152 HPLC controller (LKB, Bromma, Sweden) were used. Eluate was monitored at 254 nm with an LKB 2151 UV monitor. Fractions (0.5 ml) corresponding to what were presumed to be the three regio-isomeric (2′-, 5′- and 6′-) mercapturates of [^{14}C]AQ (UV peaks designated I, II and III, R_t 9 min, 11 min and 17 min, respectively; formed in the approximate proportions 1:0.3:1.3) were pooled. Acetonitrile was removed *in vacuo* at *ca.* 40° and the aqueous residues passed through C_{18} cartridges (Sep-Pak, Waters Associates). Unbound material was eluted with water (5 ml × 5) and the radiolabelled bound material with methanol (1.5 ml). Aliquots of the methanolic eluates were taken for UV-visible spectroscopy (spectra recorded with a Cecil CE 599 instrument). UV λ_{max} (methanol): AQ, 257 nm (aromatic), 344 (quinolyl); I, 254,338; II, 255,339; III, 254, 344. These λ_{max} are indicative of an intact (4′-hydroxyanilino)quinolyl moiety [31]. The remaining eluates were evaporated to dryness under N_2 at 20° and the residues analysed by fast-atom (FAB) mass spectrometry. Low-resolution positive-ion FAB spectra were obtained with either a Kratos MS-50 (samples in *m*-nitrobenzyl alcohol) or a V.G. Micromass 70-70F (samples in glycerol) instrument. Negative-ion spectra were also obtained with the latter. Positive-ion FAB spectra (samples in *m*-nitrobenzyl alcohol) contained a peak at m/z 517 ($[M + 1]^+$; relative intensity (RI): I, 22%, II, 13%; III, 61%); the spectrum of adduct III also contained diagnostically useful ions at m/z 444 ($[M\text{-}N.(C_2H_5)_2]^+$; RI 42%) and m/z 315 ($[m/z\ 444\text{-}129]^+$; loss of *N*-acetylcysteine fragment $HO_2.C(CH_2).NH.COCH_3$; RI 37%). Qualitatively identical spectra were obtained when the samples were dissolved in glycerol. The weaker negative-ion spectra all contained a peak at m/z 515 ($[M - 1]^-$), and variously possessed fragment ions at m/z 387 ($[M - 129]^-$), m/z 443 ($[M - 1 - N.(C_2H_5)_2]^-$) and m/z 314 ($[m/z\ 443 - 129]^-$).

Histologically normal livers were obtained from seven kidney transplant donors (4 males, 3 females; aged 4–60 years). Ethical approval was granted and consent to removal of the liver samples obtained from the donors' relatives. Washed microsomes were prepared as described previously [32] and used immediately. Their cytochrome P-450 contents were 0.15–0.80 nmol/mg protein (mean, 0.32 nmol/mg). [^{14}C]AQ (10 μM; 0.13 μCi) was incubated with microsomes (8 mg protein) in 0.1 M sodium phosphate buffer (pH 7.4; 4 ml) at 37° for up to 4 hr. Triplicate incubations were performed in 25 ml

Ehrlenmeyer flasks which were shaken in a water bath under air. They were stopped by cooling on ice, and unreacted [^{14}C]AQ was extracted into Aristar ether (peroxide-free; BDH Ltd., Poole, Dorset, U.K.; 5 ml × 2). Radiolabelled material irreversibly bound to microsomal protein (*ca.* 500–2000 dpm/mg after 30 min) was assayed by exhaustive extraction following precipitation with acetone (1:1, v/v) at 5° over *ca.* 15 hr. The microsomal pellets were extracted according to a procedure employed in previous studies of the irreversible binding of reactive intermediates [32, 33]. Successive extracts were assayed for radioactivity to ensure that the removal of reversibly bound material was complete. The final pellets were dissolved in 2 ml 1 M NaOH by heating at 60° for *ca.* 2 hr. Aliquots (50 μl) were taken for protein determination by the method of Lowry *et al.* [34], using BSA as the standard, and for liquid scintillation counting (500 μl aliquots mixed with 12 ml of scintillant). Autoxidation of [^{14}C]AQ to chemically reactive species under air was assessed by measuring the radiolabelled material irreversibly bound to microsomal suspensions purged with N_2 prior to addition of the [^{14}C]AQ and incubated under N_2 thereafter. The identities of the species were investigated by co-incubating nucleophiles (reduced glutathione, *N*-acetylcysteine and N^2-acetyl-lysine; 1 mM), reductants (ascorbate, 0.05–1.0 mM; NADPH, 1.0 mM) and the radical scavenger BHT (0.1–1.0 mM).

Incubation of [^{14}C]*AQ with soluble proteins.* HSA, BSA and Bα-C (2 mg/ml) were incubated with [^{14}C]AQ (10 μM; 0.13 μCi) under the same conditions as the human liver microsomes for up to 24 hr. The binding to HSA after 24 hr was also determined using a range of protein concentrations (0.25–3.0 mg/ml). Evaporation over 16–24 hr was prevented by placing loose aluminium foil caps on the flasks. The incubations were stopped and extracted and the protein precipitated as described above. Protein pellets were exhaustively extracted with methanol (5 ml × 3–6) for determination of irreversible binding. They were solubilized in the same way as microsomal pellets; 50–200 μl aliquots of the solubilized pellets were taken for protein assay and 500 μl aliquots for determination of radioactivity.

Incubation of [^{14}C]*AQ with horseradish peroxidase and HSA.* [^{14}C]AQ (10 μM or 100 μM, 0.13 μCi) was incubated with HSA (2 mg/ml), H_2O_2 (1 mM; from 30%, v/v, solution; BDH Ltd) and horseradish peroxidase (HRP; 0.5 Units, 1.7 μg protein) in 4 ml 0.1 M sodium phosphate buffer, pH 7.4, at 20° for 30 min. H_2O_2 and HRP were omitted from control incubations. Reactions were performed in capped test tubes and initiated by addition of the HRP in phosphate buffer (25 μl). They were stopped with catalase (300 Units in 50 μl phosphate buffer). The incubations were extracted with ether (5 ml × 2) and the irreversibly bound radiolabelled material measured as described above.

Generation of reactive N-chloro derivatives of [^{14}C]*AQ.* A number of compounds become irreversibly bound to protein in the presence of chlorine (unpublished data). Binding is thought to proceed via reactive *N*-chloro derivatives [35]. *N*-Chlorination of [^{14}C]AQ (633 nmol; 0.13 μCi) was attempted by incubating drug with chlorine solutions (0–1.41 mM; quadruplicate incubations) in 2.45 ml 0.2 M sodium acetate buffer, pH 6.5, in the dark at room temperature for 5 min; the stock solution of chlorine was prepared by bubbling gas into distilled water and adjusting its pH to 7.0 with NaOH. After 5 min, HSA (5 mg, 73.5 nmol, in 50 μl acetate buffer) was added and the incubation continued for 20 min. Thereafter, incubations were extracted with ether (5 ml × 2) and the protein precipitated with dimethylformamide (5 ml) at 5° overnight. The protein pellets were extracted with methanol (5 ml × 2) and 70% (v/v) methanol (5 ml), and finally dissolved in 3 ml 0.5 M NaOH. Aliquots were removed for determination of radioactivity (1 ml) and protein (200 μl). In order to exclude the possibility that the weak oxidizing action of chlorine in aqueous solution was responsible for the observed formation of protein-arylating [^{14}C]AQ derivatives, the above experiments were repeated using equimolar concentrations (0.73 mM and 7.3 mM) of chlorine and H_2O_2.

Analysis of microsomal metabolites. Unchanged [^{14}C]AQ and metabolite extracted from 30-min incubations containing microsomes and NADPH (1 mM) were analysed by reversed-phase HPLC. The method of Winstanley *et al.* [23] was used. Combined ether extracts were evaporated to dryness under N_2 at 30° and reconstituted in 300 μl of methanol. Aliquots (25–50 μl; 12–22 × 10^3 dpm) were chromatographed on a Rad-Pak μBondapak phenyl column (10 cm × 8 cm i.d., 10 μm; Waters Associates, U.K.) using an eluent consisting of water–triethylamine–methanol (79:1:21, v/v) adjusted to pH 2.75 with H_3PO_4. The flow rate was 3.5 ml/min. Eluate was monitored at 340 nm, collected in 0.3 min (1.05 ml) fractions and dissolved in scintillant (4 ml per fraction) for measurement of radioactivity. The peaks of radioactivity were chromatographically identified by comparing their retention times with those of co-injected authentic unlabelled compounds (AQ,R_t 5 min; DEAQ, R_t 3.5 min; biDEAQ, R_t 3.0 min). Recoveries of chromatographed radioactivity were 85–90%.

Analysis of water-soluble products of [^{14}C]*AQ autoxidation and HRP-catalyzed oxidation.* Radiolabelled water-soluble materials formed when [^{14}C]AQ (10 μM) was incubated with either HSA (2 mg/ml) and *N*-acetylcysteine (1 mM) for 24 hr or HSA, H_2O_2, HRP and glutathione (1 mM) for 30 min were analysed by HPLC.

Ten 4 ml incubations containing HSA and *N*-acetylcysteine were extracted with ether and the residual ether evaporated under N_2. The aqueous phases were combined and passed through a C_{18} cartridge. Aliquots (20 μl) of the methanolic eluate (1.5 ml) were chromatographed on a C_{18} column as described above. Several radiolabelled compounds were resolved (Fig. 5); the compound of R_t 17 min co-chromatographed with adduct III formed by the reaction between synthetic AQQI and *N*-acetylcysteine, which had been identified as a mercapturate of AQ. Eluate fractions containing putative adduct III were combined and concentrated, and the material (*ca.* 0.5 μg) isolated in the same manner as the synthetic adduct. It was analysed by FAB mass spectrometry.

Three HSA–H_2O_2–HRP–glutathione incubations

Table 1. Irreversible binding of [^{14}C]AQ to microsomes from human livers

Liver	Irreversible binding (% incubated radioactivity)	
	No NADPH	NADPH
1	5.7 ± 0.7	2.1 ± 0.1**
2	5.4 ± 0.1	3.4 ± 0.3**
3	5.2 ± 0.5	3.2 ± 0.3*
4	4.1 ± 0.5	1.9 ± 0.2*
5	2.5 ± 0.1	1.3 ± 0.1**
6	2.3 ± 0.1	1.4 ± 0.0**
7	1.5 ± 0.1	1.5 ± 0.1

Microsomes were incubated with 10 μM [^{14}C]AQ in air at 37° for 30 min. Irreversibly bound radiolabelled material was determined by exhaustive extraction of precipitated microsomes. Data are $\bar{x}$% ± SD (triplicate determinations). Except for Liver 7, binding was significantly lower in presence of NADPH (1 mM); * $P < 0.005$, ** $P < 0.001$ (Student's non-paired t-test).

were extracted with ether and the HSA precipitated with acetone. The buffer–acetone supernatants were combined, evaporated to dryness *in vacuo* at *ca.* 40° and reconstituted in 600 μl of water for HPLC analysis on the C_{18} column.

RESULTS

Irreversible binding and metabolism of [^{14}C]*AQ in microsomal incubations*

[^{14}C]AQ incubated with microsomes from seven human livers in air at 37° for 30 min underwent irreversible binding. Except with microsomes from one liver, the binding was significantly lower in the presence of 1 mM NADPH (Table 1). The time course of binding to microsomes from one human liver in the absence of NADPH is shown in Fig. 1. After 4 hr, 31.4 ± 0.6% ($\bar{x}$ ± SD, N = 3), equivalent to 1.97 ± 0.04 nmol AQ/mg protein, was bound to these microsomes. Of the balance of incubated radioactivity, 64.5 ± 1.9% was recovered in the ether extracts and 3.0 ± 0.1% in the buffer–acetone supernatant obtained upon precipitation of the microsomes. The irreversible binding to pooled

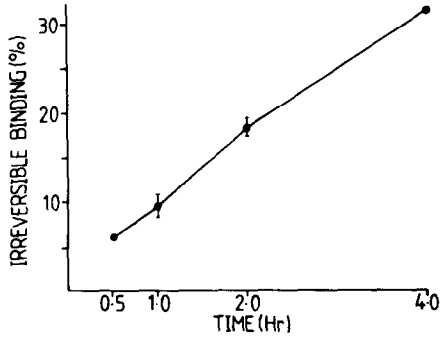

Fig. 1. Time course of irreversible binding of [^{14}C]AQ(10 μM) to human liver microsomes incubated in absence of NADPH at 37°. Points are means of triplicate determinations; bars are SD (omitted when ≤1.0%).

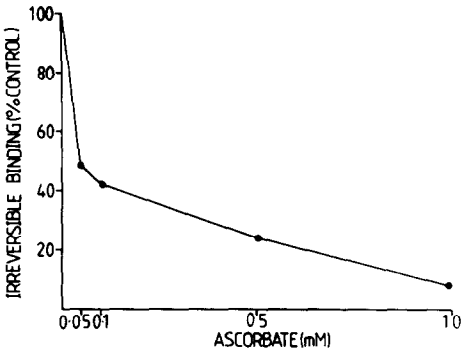

Fig. 2. Inhibition of the irreversible binding of [^{14}C]AQ(10 μM) to human liver microsomes by ascorbate in the absence of NADPH. Incubations performed at 37° over 30 min. Points derived from means of triplicate determinations.

microsomes from three livers when NADPH was omitted (3.7 ± 0.2% over 30 min) was considerably decreased under a N_2 atmosphere (to 0.7 ± 0.2%). It was also inhibited by sulphur nucleophiles, ascorbic acid (Fig. 2) and BHT (Table 2) but not by the nitrogen nucleophile N^2-acetyl-lysine. Inhibition by thiols but not ascorbic acid was associated with increased formation of radiolabelled water-soluble products: from 2.3 ± 0.3% (control) to 5.3 ± 0.3% (glutathione) and 4.0 ± 0.3% (*N*-acetylcysteine).

[^{14}C]AQ (81.5 ± 2.1% incubated radioactivity, N = 7 livers) was recovered unchanged, as determined by HPLC analysis of ether extracts, from 30-min incubations performed without NADPH. In the presence of co-factor it was solely and extensively metabolized to DEAQ, identified by co-chromatography, by microsomes from all seven livers. The fraction of [^{14}C]AQ metabolized to DEAQ, as estimated by radiometric HPLC, was *ca.* 75–100%. Formation of DEAQ was partially inhibited by the cytochrome P-450 inhibitor SKF 525A at 100 μM (unpublished observation).

Table 2. Inhibition of the irreversible binding of [^{14}C]AQ to human liver microsomes and HSA

Inhibitor	Irreversible binding (% control)	
	Microsomes	HSA
Ascorbate	10	3
Glutathione	18	14
N-Acetylcysteine	18	3
NADPH	46	13
BHT	67	44

Microsomes (pooled from three livers) and HSA were incubated with 10 μM [^{14}C]AQ in air at 37° for 30 min and 2 hr, respectively. Inhibitors co-incubated at 1 mM except for BHT (0.5 mM). Data derived from means of triplicate determinations of binding in presence (test) and absence (control) of inhibitor. Maximum inhibition of the binding to microsomes by BHT (used at 0.1–1.0 mM) achieved at inhibitor concentration of 0.5 mM. Test significantly different from control in every case: $P < 0.001$ (Student's non-paired t-test) except for BHT in microsomal incubations when $P < 0.005$.

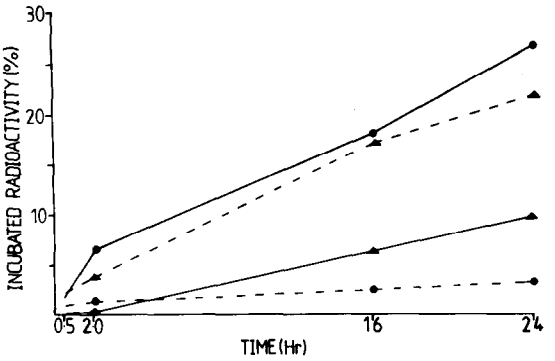

Fig. 3. Irreversible binding of [^{14}C]AQ to human serum albumin (——) and formation of water-soluble products (– – –) in the presence (▲) and absence (●) of reduced glutathione (1 mM). Incubations performed at 37°. HSA concentration, 2 mg/ml. Points are means of triplicate determinations.

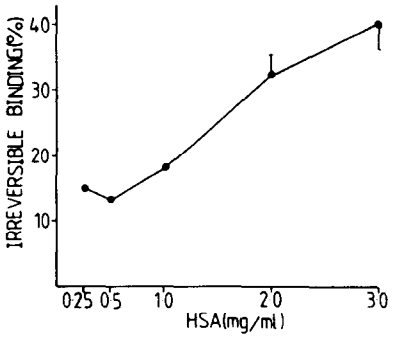

Fig. 4. Irreversible binding of [^{14}C]AQ to human serum albumin over 24 hr at increasing protein concentrations. Incubations performed at 37°. Points are means of triplicate determinations. Bars are SD.

Non-enzymic irreversible binding of [^{14}C]*AQ to soluble proteins*

[^{14}C]AQ in phosphate buffer, pH 7.4, reacted spontaneously and irreversibly with two soluble proteins which possess a sulphydryl group, HSA and BSA, and one, Bα-C, which does not (Table 3, Fig. 3). After 24 hr, the radiolabelled material bound to HSA, which represented 26.2 ± 1.8% of incubated radioactivity, was equivalent to 1.30 ± 0.09 nmol AQ/mg; for a molecular weight of 68,000 this equalled 88 mmol/mol. The fraction of [^{14}C]AQ bound to HSA at 24 hr increased with protein concentration (Fig. 4). It was much lower in 0.1 M phosphate buffer, pH 5.2, (1.7 ± 0.8%). Binding at pH 7.4 over 2 hr was inhibited by thiols, ascorbate, NADPH and BHT (Table 2) but not N^2-acetyllysine. As with microsomal incubations, the effects of the thiols were combined with the formation of polar derivatives: water-soluble radiolabelled material increased from 1.4 ± 0.2% (control) to 3.8 ± 0.2% (glutathione) and 2.0 ± 0.0% (*N*-acetylcysteine). Over 24 hr, considerable fractions of [^{14}C]AQ were converted to such derivatives (Fig. 3): in HSA incubations, water-soluble material rose from 3.2 ± 0.1% (control) to 21.8 ± 0.7% in the presence of 1 mM glutathione and from 4.2 ± 0.8% (control) to 21.5 ± 0.3% with 1 mM *N*-acetylcysteine. The irreversible binding to Bα-C was inhibited in the same manner as the binding to the thiol proteins, viz. by *N*-acetylcysteine (23% inhibition) and BHT (37%) but not by N^2-acetyl-lysine.

Peroxidase-dependent irreversible binding of [^{14}C]*AQ to soluble proteins*

Hydrogen peroxide (1 mM) oxidized [^{14}C]AQ (10 μM) to derivatives that reacted irreversibly with HSA, and HRP catalyzed more extensive binding (Table 4). In the latter case the binding was decreased to less than the control value by ascorbate; whereas glutathione only completely abolished the peroxidase-dependent binding. HRP-H_2O_2 also caused the formation of substantial amounts of water-soluble products in the presence of glutathione: the water-soluble radioactivity was increased from 0.6 ± 0.0% added radioactivity in control incubations (10 μM [^{14}C]AQ) to 0.9 ± 0.1% in those containing H_2O_2 and 15.9 ± 1.4% in the additional presence of HRP and glutathione (1 mM).

Formation of thioether adducts of [^{14}C]*AQ via autoxidation and HRP-catalyzed oxidation*

The radiolabelled water-soluble products of reactions between autoxidized [^{14}C]AQ and *N*-acetylcysteine over 24 hr were resolved into four main components (Fig. 5). Of these, only one, that of R_t 17 min, co-chromatographed with one of the synthetic mercapturates of [^{14}C]AQ prepared via [^{14}C]AQQI (adduct III). Its positive-ion FAB spectrum (sample in glycerol) contained the same diag-

Table 3. Irreversible binding of [^{14}C]AQ to soluble proteins

Protein	Irreversible binding (% incubated radioactivity)	
	2 hr	24 hr
Human serum albumin	5.8 ± 0.6	26.5 ± 1.8
HSA + glutathione	0.3 ± 0.1	9.7 ± 2.0
Bovine serum albumin	4.4 ± 1.1	23.6 ± 0.9
Bovine α-casein	3.1 ± 0.2	12.3 ± 1.5

Proteins (2 mg/ml, 4 ml) were incubated with [^{14}C]AQ(10 μM) under air in 0.1 M sodium phosphate buffer, pH 7.4, at 37°. Irreversibly bound radiolabelled material was determined by exhaustive extraction of precipitated protein. Data are $\bar{x}$% ± SD (triplicate determinations).

Table 4. Oxidation of [^{14}C]AQ to protein-arylating intermediates by horseradish peroxidase

Incubation	Irreversible binding (% incubated radioactivity)
10 μM [^{14}C]AQ	0.3 ± 0.1
100 μM [^{14}C]AQ	0.1 ± 0.0
10μM [^{14}C]AQ + H_2O_2	2.5 ± 0.1
10 μM [^{14}C]AQ + H_2O_2 + HRP (complete)	8.2 ± 0.5
100 μM [^{14}C]AQ + H_2O_2 + HRP	5.3 ± 0.4
Complete + ascorbate (1 mM)	0.1 ± 0.0
Complete + glutathione (1 mM)	1.5 ± 0.3

[^{14}C]AQ was incubated with HSA (2 mg/ml), H_2O_2 (1 mM) and HRP (0.5 Units) at 20° for 30 min. Data are $\bar{x}$% ± SD (triplicate determinations).

nostically significant ions as that of adduct III: m/z 517([M + 1]$^+$; RI: adduct, 28%; product, 5%), m/z 444 ([M-N. $(C_2H_5)_2$]$^+$; RI: adduct, 26%; product, 3%) and m/z 315 ([m/z 444-129]$^+$; RI: adduct, 34%; product, 6%). The products of R_t 5 min and R_t 8 min eluted before the other synthetic mercapturates. HPLC resolved the water-soluble products of HRP-H_2O_2-mediated oxidation of [^{14}C]AQ in the presence of glutathione into three putative glutathione adducts: R_t 2.5 min (51% eluate radioactivity), R_t 6 min (7%) and R_t 9 min (31%).

The effect of chlorine on the irreversible binding of [^{14}C]AQ

The irreversible binding of [^{14}C]AQ to HSA was dependent on the concentration of chlorine with which [^{14}C]AQ was incubated prior to addition of protein (Fig. 6). With 100 ppm (1.41 mM) chlorine, 6.8 ± 2.0% (586.2 ± 170.5 mmol equivalents/mole protein) ($\bar{x}$ ± SD, N = 4) became bound. Under identical experimental conditions but in the presence of either 0.73 mM chlorine or 0.73 mM H_2O_2 the irreversibly bound [^{14}C]AQ equalled 0.9 ± 0.2% ($\bar{x}$ ± SD, N = 3) and 0.1 ± 0.0% of the incubated drug, respectively; the binding in absence of either oxidizing agent was 0.1 ± 0.0%. The corresponding data for 7.3 mM chlorine and 7.3 mM H_2O_2 were 4.4 ± 1.5% and 0.14 ± 0.1%, respectively. Thus irreversible binding of [^{14}C]AQ was significantly elevated above control values ($P < 0.005$ and $P < 0.01$ for 0.73 mM and 7.3 mM chlorine, respectively; Student's non-paired t-test) only in the presence of the weaker oxidizing agent, chlorine.

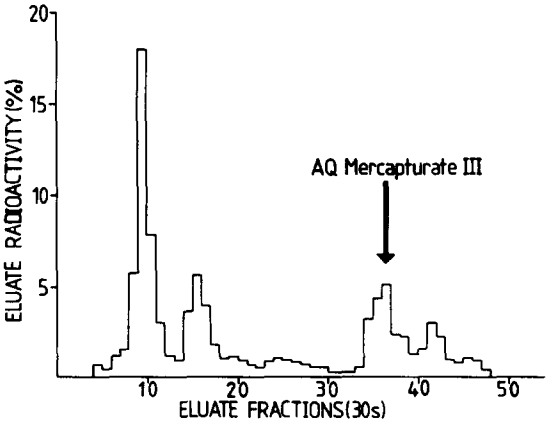

Fig. 5. Chromatogram of water-soluble compounds formed in incubations containing HSA (2 mg/ml), [^{14}C]AQ(10 μM) and N-acetylcysteine (1 mM). Incubations performed at 37° for 24 hr. Compounds were analysed on a 10-μm C18 column using a gradient of acetonitrile (20% to 25% over 25 min) in $NH_4H_2PO_4$ (10 mM, pH 4.6) containing octane sulphonate (5 mM). The component of R_t 17 min corresponded to the principal N-acetylcysteine adduct (III) of AQ.

DISCUSSION

Chemically reactive and hence potentially toxic species may occur within animals as consequences of numerous mechanisms. In the simplest instance, an exogenous compound is intrinsically reactive; thus 2,4-dinitrofluorobenzene [36] and a variety of alkylating agents [37] modify proteins *in vivo*. A more familiar mechanism, and the one most frequently encountered with drugs, is the oxidative metabolism of stable compounds to reactive intermediates [2–4]. Paracetamol is a paradigm of drugs undergoing such activation [9–16]. Although enzymic oxidations by cytochrome P-450 and peroxidases have received the greatest attention, it is possible that non-enzymic processes may also contribute to the activation of some compounds, e.g. p-phenetidine [30]. In this

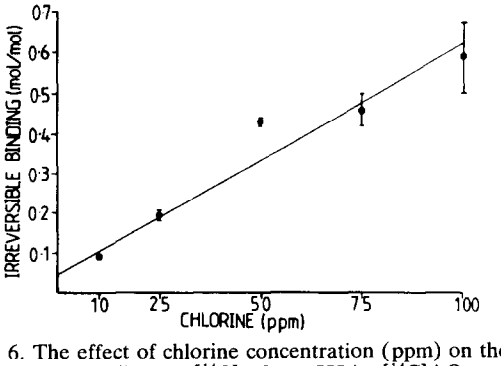

Fig. 6. The effect of chlorine concentration (ppm) on the irreversible binding of [^{14}C]AQ to HSA. [^{14}C]AQ was incubated with chlorine for 5 min, HSA added and incubations continued for 20 min. Points represent means of four determinations; bars are SEM.

study we have found that several diverse mechanisms may be involved in the formation of reactive intermediates from a drug. Therefore the ability of AQ to damage both liver and bone marrow might be a consequence of multiple pathways of activation.

An unexpected finding was the notable susceptibility of AQ to autoxidation in air at physiological pH; the much lower irreversible binding to HSA at pH 5.2 was a reflection of the increased stability of a base at acid pH. Moreover, and contrary to the usual observation of NADPH-dependent activation of drugs to reactive metabolites, AQ's binding was lower in the presence of NADPH. In marked contrast, paracetamol demonstrated 2.5 ± 1.1% ($\bar{x}$ ± SD, N = 8 livers) and 1.3 ± 1.1% irreversible binding to human liver microsomes over 30 min in the presence and absence of NADPH, respectively, when incubated under the same conditions as AQ [38]. Reduction and hence deactivation of the arylating derivative is clearly the favoured reaction with AQ. The presence of an aromatic *N*-substituent is presumably the major determinant of the susceptibility of AQ's *p*-hydroxyanilino moiety to oxidation; a presumption indirectly supported by the rapid autoxidation of *N*-(4-ethoxyphenyl)-*p*-hydroxyaniline to the corresponding benzoquinone imine [30]. DEAQ, the only metabolite of AQ recovered from microsomal incubations and a major metabolite in man [22, 23], is also likely to undergo rapid autoxidation in neutral solution under air.

Taken collectively, the data indicated that the protein-arylating products of autoxidation of AQ in neutral solution under air, in both microsomal and protein incubations, were a mixture of semiquinone imine and quinone imine derivatives. Thus the inhibition of arylation displayed characteristics known, largely from studies of paracetamol and *p*-phenetidine, to be typical of such derivatives. Both types of electrophile form thioether adducts [10, 16, 29, 39, 40] and *N*-acetyl-*p*-benzoquinone imine (NAPQI) is reduced by ascorbate [29] and NADPH [40]. Additionally, as noted with NAPQI and paracetamol [41], binding of AQ was not inhibited by a nitrogen nucleophile. Glutathione can act as both a nucleophile and reductant [40] but the conversion of large fractions of AQ to water-soluble products suggests predominance of the nucleophilic action. A specific contribution of the semiquinone imine to protein binding of AQ was tentatively indicated by the inhibitory effect of the radical scavenger BHT. This represents a difference between AQ and paracetamol since free radicals are thought not to play a significant role in the binding of paracetamol to protein *in vitro* [41]. Further evidence of the formation of AQQI was obtained by trapping the product(s) of autoxidation as a stable *N*-acetylcysteine adduct. However, amongst a mixture of water-soluble compounds formed in the presence of *N*-acetylcysteine only one was identified, by co-chromatography and mass spectrometry, as an AQ mercapturate (adduct III). The remaining compounds were not identified but it seems possible, from a study of the reactions of NAPQI in phosphate buffer [40], that they were polymers of AQ and AQ mercapturate derived from free radicals. It is evident that quinone imines in aqueous solutions undergo reactions leading to additional electrophilic species [40]; one of these reactions, comproportionation between the quinone imine and parent compound to yield a semiquinone imine, is a potential source of AQ semiquinone imine.

Although the cytochrome P-450 mono-oxygenases appear to be principally responsible for the enzymic formation of reactive intermediates in liver [2, 3, 42], alternative oxidative reactions catalysed by peroxidases and hydroperoxidases may be predominant in other tissues [3, 4, 42]. For example, the hydroperoxidase activity of prostaglandin synthetase (PS) [4] catalyses the one-electron oxidation of paracetamol; both HRP- and PS-generated paracetamol

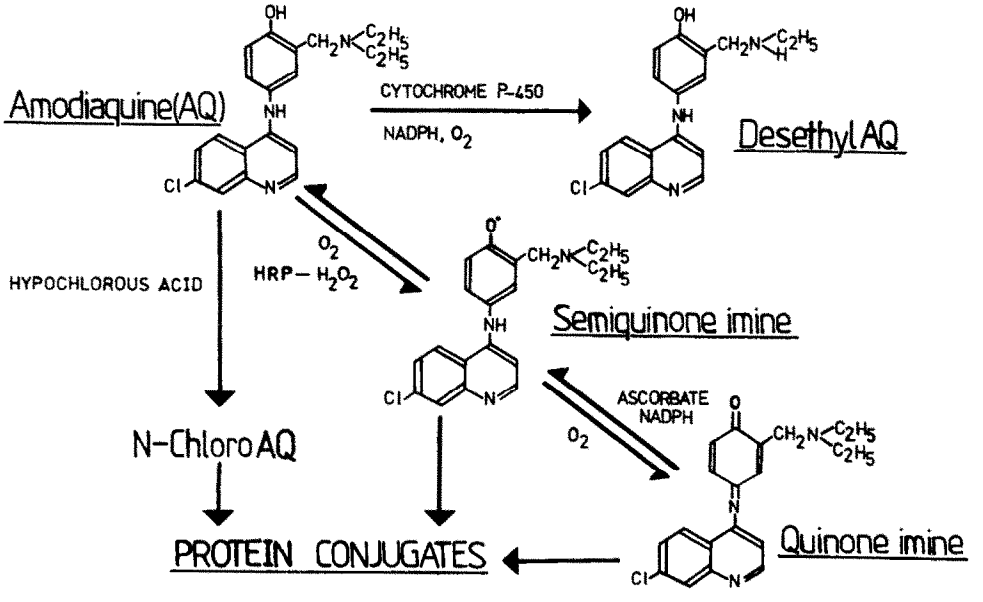

Fig. 7. Proposed mechanisms for the formation of chemically reactive intermediates from [^{14}C]AQ *in vitro*.

metabolites arylate proteins and form glutathione adducts [4, 39]. In view of the myelotoxicity of AQ, HRP-catalyzed formation of reactive AQ derivatives is noteworthy since bone marrow contains appreciable amounts of myeloperoxidase (MPO) [44]. MPO has been implicated in the myelotoxicity of benzene and oxidizes phenol to protein-binding species *in vitro* [43]. As well as catalyzing H_2O_2-dependent oxidations of organic substrates [43, 45], MPO oxidizes chloride to hypochlorous acid; this, in turn, reacts with nitrogen compounds to yield *N*-chloro derivatives [45, 46] which are long-lived hydrophilic oxidants [46]. Those of the anti-microbial agent chlorhexidine become irreversibly bound to protein *in vitro* [35]. The present finding that protein-reactive AQ derivatives are generated in chlorine solutions under conditions which do not favour the much more powerful oxidizing action of H_2O_2 suggests that they may be products of *N*-chlorination [35] rather than simple oxidation. AQ could be exposed to MPO-generated hypochlorous acid in bone marrow and to exogenous hypochlorous acid in areas where drinking water is heavily chlorinated. Finally, recent studies of a novel cellular mechanism for generating reactive drug metabolites have shown that stimulated human neutrophils, which release powerful oxidants and MPO, activate AQ *in vitro* [47].

Cysteinyl sulphydryl groups were shown to be major sites at which AQ arylates soluble proteins. Over 24 hr, irreversible binding of [^{14}C]AQ to Bα-C, a protein which lacks cysteine residues, was 52% of the binding to BSA, which contains a single free sulphydryl group but otherwise has a similar amino acid composition [41]. Thus the drug's binding characteristics are similar to those of paracetamol *in vivo* and *in vitro* [48] and of NAPQI *in vitro* [41]. Clearly, other residues are arylated by AQ's intermediates; lysyl N^6-amino groups are possible sites since electrophiles such as 2,4-dinitrofluorobenzene [36] and a reactive metabolite of chloramphenicol [49] react with them.

In conclusion, it is evident that reactive intermediates of AQ are generated by a number of mechanisms (Fig. 7) which may operate *in vivo*. If an intermediate such as AQQI, which is toxic towards human lymphocytes and myelocytes *in vitro* [50], were to be formed in liver and bone marrow cells, it might initiate the cellular damage associated with AQ.

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