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ENZYMOLOGY OF THE REDUCTIVE BIOACTIVATION OF SR 4233

A NOVEL BENZOTRIAZINE DI-*N*-OXIDE HYPOXIC CELL CYTOTOXIN

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Abstract—SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide) is a novel benzotriazine di-*N*-oxide which shows unusually high selective toxicity towards hypoxic cells, probably as a result of reductive bioactivation. Using an HPLC assay for the parent drug and its 2- and 4-electron reduction products (SR 4317 and SR 4330, respectively), we have examined the enzymology of SR 4233 reductive metabolism *in vitro* using a variety of different enzyme preparations. SR 4233 was converted extremely rapidly to SR 4317 under N_2 by mouse liver microsomes, and showed a marked preference for NADPH over NADH as a reduced cofactor. The reaction was inhibited completely in air and boiled preparations. It was also inhibited by 78–86% in carbon monoxide (CO), implicating cytochrome P-450 as the major microsomal SR 4233 reductase. The kinetics of reductive metabolism of SR 4233 to SR 4317 by mouse liver microsomes conformed to Michaelis–Menten kinetics, with a K_m of 1.4 mM and a V_{max} of 950 nmol/min/mg protein. SR 4233 reduction was also catalysed by mouse liver cytosol under N_2. However, rates were markedly slower than for microsomes and showed an equal dependency on NADH and NADPH. The cytosolic enzymes aldehyde oxidase and xanthine oxidase both catalysed SR 4233 reduction to SR 4317 under N_2. Purified buttermilk xanthine oxidase also catalysed this reaction. In contrast to other enzyme preparations, DT-diaphorase from Walker 256 tumour cells reduced SR 4233 predominantly to SR 4330, and this reaction occurred under aerobic conditions. These data illustrate that SR 4233 is reduced rapidly by a wide variety of reductases. We propose that the therapeutic selectivity of SR 4233 will be controlled by the relative expression of reductases in tumour versus normal tissues, and in particular by the differential participation of putative activating versus detoxifying enzymes.

Hypoxic tumor cells continue to represent a problem in cancer therapy and to offer an attractive target for rational and selective drug design [1–3]. The benzotriazine di-*N*-oxide SR 4233 (Fig. 1) is the lead compound in a new class of potent hypoxic cell cytotoxins which are currently being evaluated as potential anticancer drugs [4–6]. A particularly important feature is the unusually high selective toxicity of SR 4233 towards hypoxic cells [4, 6, 7].

In vitro studies have shown that Chinese hamster ovary (CHO) cells reduce SR 4323 to its 2-electron product SR 4317 preferentially under anaerobic conditions, and this is accompanied by selective and extensive cell killing [4, 6, 7]. Rat hepatocytes reduce SR 4233 to SR 4317 more extensively than CHO cells at a rate that is both O_2 and substrate concentration dependent [5, 7]. Both studies showed that the metabolite SR 4317 was relatively non-toxic and that there was no detectable drug binding to DNA [5, 7]. Cytotoxicity is thought to result from bioactivation to a short-lived, oxidizing, one-electron *N*-oxide radical intermediate, in a reaction that is rapidly reversed by molecular oxygen [7–9]. Several reductases are known to catalyse *N*-oxide reduction [10, 11]. Previously it has been shown that purified buttermilk xanthine oxidase can catalyse SR 4233 reduction, apparently causing single and double-strand breaks in plasmid DNA *in vitro* [9]. In a preliminary report we have shown that the microsomal enzymes cytochrome P-450 and NADPH: cytochrome P-450 (cytochrome *c*) reductase can also catalyse reduction of SR 4233 to SR 4317 *in vitro* [12].

In this study we have considerably extended our earlier observations and have characterized the major enzymes responsible for SR 4233 reductive metabolism in mouse liver, as well as providing full kinetics for the major microsomal SR 4233 reductase, identified as cytochrome P-450. In addition we report a novel type of reductive metabolism of SR 4233 by DT-diaphorase from rat Walker 256 tumour cell preparations. These studies represent an example of our enzyme-directed approach to bioreductive drug development, which is based on the proposal that the role and relative participation of particular enzymes in tumour versus normal tissue will determine the therapeutic selectivity of hypoxic cell cytotoxins *in vivo* [13].

MATERIALS AND METHODS

Drugs. SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide; NSC 130181) and SR 4317 (3-amino-1,2,4-benzotriazine-1-oxide) were supplied by Dr Mike Tracy of SRI International (Menlo Park, CA, U.S.A.), and SR 4330 (3-amino-1,2,4-benzotriazine) by Dr Peter Wardman of the CRC Gray Laboratory (Northwood, U.K.) The 2-nitroimidazole internal standard Ro 07-0913 (2-nitroimidazoyl-1-yl)-3-ethoxypropane-2-ol) was supplied by Dr Carey Smithen of Roche Products (Welwyn Garden City,

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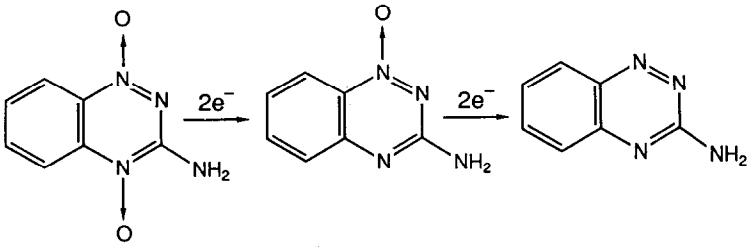

Fig. 1. Reduction of SR 4323 to SR 4317 and SR 4330.

U.K.). The *N*-oxide internal standard 4-nitroquinoline *N*-oxide (NQO), purified buttermilk xanthine oxidase, NADH, NADPH, allopurinol, *N*-methylnicotinamide, menadione, dicoumarol, bovine serum albumin and cytochrome *c* were purchased from the Sigma Chemical Co. (Poole, U.K.). Zero grade nitrogen (<5 ppm O_2) was obtained from BOC (London, U.K.) and Research Grade carbon monoxide (CO, <10 ppm O_2) from Argo International (Barking, U.K.).

HPLC. Concentrations of SR 4233 and its 2- and 4-electron reduction products (SR 4317 and SR 4330, respectively) were determined using isocratic reverse-phase HPLC as described previously [14]. Chromatography was performed using columns and equipment supplied by Waters Associates (Milford, MA, U.S.A.). Separations were carried out on μBondapak phenyl radial compression columns (8 mm × 10 cm; 10 μm beads) fitted in Z-module radial compression units and protected with cyanopropyl (CN) Guard-pak precolumns. Drugs were eluted isocratically at a fixed flow-rate of 3–3.5 mL/min using a mobile-phase consisting of 32% methanol in water. Detection was at 254 nm and drugs and metabolites were identified by chromatographic and spectral properties.

Microsomal preparations. Liver microsomal and cytosolic fractions were prepared from 6–8-week-old male C3H/Km mice which had been fasted overnight [15, 16]. The microsomal pellet was washed once in 20 mM Tris–HCl buffer (pH 7.4) containing 1.15% KCl and resuspended in 100 mM sodium phosphate buffer (pH 7.4) at approximately 10 mg/mL protein. Cytosol was dialysed overnight in 10 L 20 mM Tris–HCl buffer (pH 7.4) containing 1.15% KCl. Microsomes and cytosol were stored at −70° for up to 6 weeks.

Walker 256 tumour cell preparations. The U.K. strain of the Walker 256 tumour cell line was obtained from Dr Richard Knox, Institute of Cancer Research (Sutton, U.K.) and cells were grown in unstirred suspension culture using Dulbecco's modified Eagle's Medium (Gibco, U.K.) supplemented with 1 mM glutamine and 10% horse serum in a humidified 8% CO_2 atmosphere at 37°.

At confluence, cells were harvested, pooled and centrifuged (1000 rpm × 5 min). Pellets were resuspended in a small volume (1–2 mL) of 100 mM sodium phosphate buffer (pH 7.4). A crude preparation of DT-diaphorase was obtained by sonicating this cell suspension on ice using an MSE 150W Ultrasonic disintegrator (4 × 20 sec at 8–10 μm amplitude). The sonicated preparations were centrifuged for 5 min in an Epindorff centrifuge and the supernatants collected and stored at −70°.

Specific enzyme assays. Microsomal cytochrome *c* reductase activity was assayed as previously described using NADPH (0.5 mM) as cofactor [17]. Cytochrome P-450 content was determined from CO binding spectra [18]. DT-diaphorase activity in Walker cell sonicates was assayed at 550 nm using NADH (0.5 mM) as cofactor, menadione (200 μM) as intermediate electron acceptor and cytochrome *c* (77 μM) as terminal acceptor with or without the inhibitor dicoumarol (10 μM) as previously described

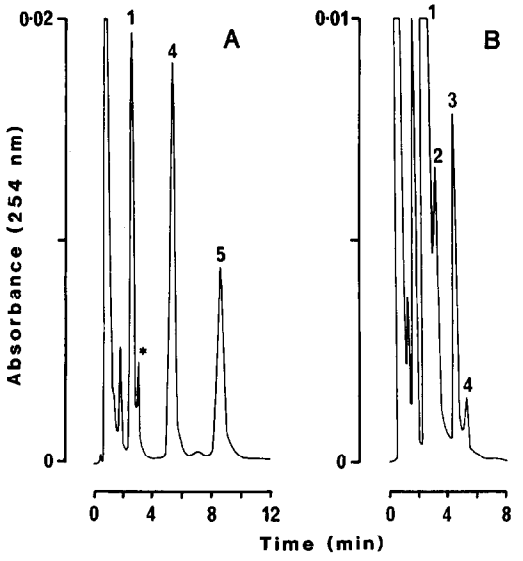

Fig. 2. HPLC chromatograms of an aliquot of a reaction mixture taken from: (A) A 15 min anaerobic mouse liver microsomal incubation (0.5 mM SR 4233) and containing SR 4233 (peak 1; 245 nmoles total), SR 4317 (peak 4; 341 nmoles total) and the internal standard 4-nitroquinoline *N*-oxide (peak 5; 25 mg/L in methanol). An unidentified minor metabolite was also present (*). (B) A 50 min aerobic Walker cell DT-diaphorase incubation (2 mM SR 4233) and containing SR 4233 (peak 1; concentration out of range), SR 4330 (peak 3; 56 nmoles total), SR 4317 (peak 4; 5.6 nmoles total) and internal standard Ro 07-0913 (peak 2; 30 mg/L in methanol).

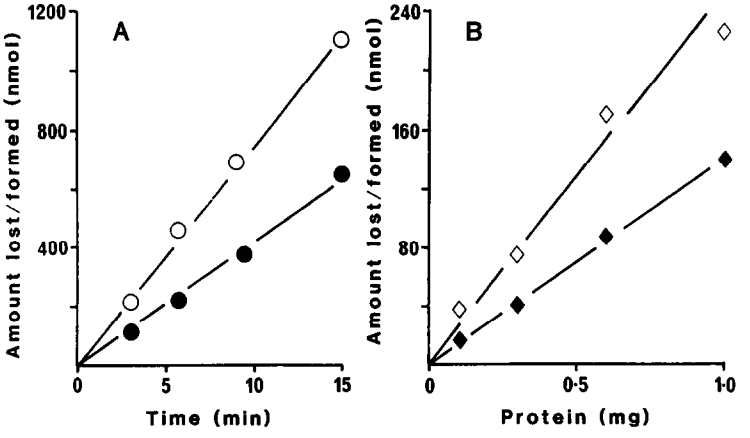

Fig. 3. (A) Typical progress curves for the reductive metabolism of SR 4233 by mouse liver microsomes under nitrogen *in vitro*. Symbols: SR 4233 loss (○) and SR 4317 formation (●). Incubations contained 0.1 mg/mL microsomal protein, 0.9 mM NADH and NADPH and 2 mM SR 4233 in a final volume of 3 ml 0.1 M sodium phosphate buffer (pH 7.4). For other conditions see Materials and Methods. (B) Effects of microsomal protein concentration on the rate of SR 4233 reductive metabolism under nitrogen *in vitro*. Symbols: SR 4233 loss (◇) and SR 4317 formation (◆). Incubations were similar to those in (A) except that total protein concentration was varied from 0.1 to 1.0 mg per assay. Similar results were obtained in repeat experiments.

[19]. Protein concentrations were determined by the method of Lowry *et al.* [20] using bovine serum albumin as a standard.

SR 4233 metabolism. SR 4233 reductive metabolism was carried out under N_2 or CO at 37° in specially modified 25-mL Erhlenmeyer flasks as previously described [21, 22]. Aerobic metabolism was carried out in the above flasks or test-tubes open to air, with vigorous shaking. Standard incubations contained 0.033–0.333 mg/mL microsomal protein, 3.0–16.6 mg/mL cytosolic protein and 0.9 or 3.0 mM NADH and NADPH in a final volume of 1.5 or 3 mL of 100 mM sodium phosphate buffer (pH 7.4). Xanthine oxidase-mediated metabolism in cytosolic preparations (16.7–20 mg/mL) was assayed using the specific substrate *N*-methylnicotinamide (2.5 mM) and inhibitor menadione (10 μM) [15, 23]. Metabolism by DT-diaphorase was assayed in air using 20 units/mL (11.5 units/mg protein), 2 mM NADH as a cofactor and dicoumarol (100 μM) as a specific inhibitor [19] in a final volume of 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4).

Flasks were preincubated for 5–7 min in air, CO or N_2 as appropriate, and the reaction started by the addition of SR 4233 (0.25–10 mM) in DMSO (8–50 μL). Aliquots of the reaction mixture (50–100 μL) were removed at 3–6 consecutive time points, through air-tight septa for anaerobic incubations, and added to 2 volumes of methanol containing internal standard (4-NQO for microsomal and purified xanthine oxidase preparations and Ro 07-0913 for cytosolic and Walker cell enzyme preparations). Samples were centrifuged at 4° (5000 rpm × 5 min) and the supernatants injected into the HPLC system for analysis.

Kinetic analysis. Michaelis–Menten kinetics were established for the microsomal catalysed reaction using the criteria described by Henderson [24]. The apparent K_m and V_{max} values were determined by linear regression analysis of Lineweaver–Burk ($1/v$ versus $1/s$) plots using a statistical package for a desk-top programmable calculator.

RESULTS

Characterization of SR 4233 microsomal reduction

SR 4233 was reduced rapidly by mouse liver microsomes, forming predominantly SR 4317 (Fig. 2A). Small amounts of an unidentified metabolite were also detected (Fig. 2A).

Progress curves for SR 4233 reduction to SR 4317 were linear with time up to at least 15 min (Fig. 3A) and total protein over the range 0.1 to 1.0 mg per assay (Fig. 3B). Initial characterization was carried out at a substrate concentration of 2 mM, for which 0.9 mM cofactor concentrations were non-limiting. Subsequent microsomal enzyme kinetics were carried out at the higher cofactor concentration of 3 mM which was shown to be non-limiting for substrate concentrations ≤10 mM.

Microsomal cytochrome *c* reductase activity in each of the 2 preparations used was 0.46 ± 0.02 and 0.38 ± 0.03 μmol cytochrome *c* reduced/min/mg protein (mean ± 2SE; N = 3) and the cytochrome P-450 content for each was 0.83 ± 0.11 and 0.92 ± 0.13 nmol/mg protein (mean ± 2SE; N = 3).

Table 1 shows the effects of air, CO (a specific inhibitor of cytochrome P-450 [25]) and different cofactors on SR 4233 reductive metabolism to SR 4317 in mouse liver microsomes. It can be seen that SR 4233 loss and SR 4317 formation were affected to similar extents in all cases. The reaction was inhibited almost completely in air. CO inhibited the reaction by 78–86% and there was a minimal activity in the presence of boiled microsomes (≤2% of the control rate). The reduction showed a marked preference for NADPH with only 2–5% of the control activity occurring in the presence of NADH alone.

Table 1. Characterization of SR 4233 reduction by mouse liver microsomal preparations *in vitro*

Conditions	% Rate of reduction in control	
	SR 4233 loss	SR 4317 formation
Control (N_2)*	100	100
CO	22, 18	14, 18
−NADH	100, 106	79,92
−NADPH	≤2, 5.5	≤2, ≤2
+Air	≤2, ≤2	≤2, ≤2
Boiled preparations	≤2, ≤2	≤2, ≤2

The control reaction was carried out under N_2 and contained 0.1 mg/mL microsomal protein, 0.9 mM NADH and NADPH and 2 mM SR 4233 in 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 3 mL. Other conditions as in Materials and Methods. Each value was determined in separate experiments. The lower limit of quantification was ≤2% of the control rate.

* The rate of SR 4233 disappearance and SR 4317 formation in the control was 237 ± 38 and 144 ± 86 nmol/min/mg protein, respectively (mean ± 2SE; N = 3).

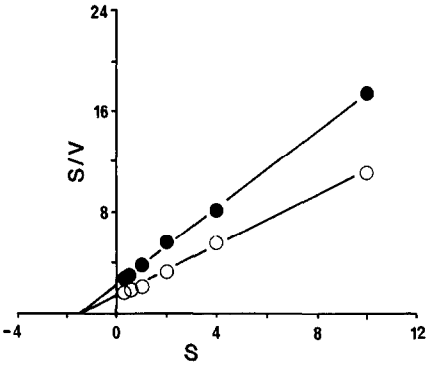

Fig. 4. An s/v versus s plot for the reductive metabolism of SR 4233 by mouse liver microsomes under N_2 *in vitro*. Symbols: SR 4233 loss (○) and SR 4317 formation(●). Data were derived from one experiment with six independent substrate concentrations. Lines were fitted to the data by eye. Similar results were obtained in repeat experiments. For units of s and v, see Table 2. For other conditions see Materials and Methods.

Reaction stoichiometry

Even after prolonged incubations (60 min), SR 4233 was converted predominantly to the 2-electron reduction product SR 4317 by mouse liver microsomes, with minimal (≤4%) formation of the 4-electron product. Consequently SR 4330 formation rates were too low for accurate determination. The average rates of SR 4233 loss and SR 4317 formation were 237 ± 38 and 144 ± 86 nmol/min/mg protein, respectively (mean ± 2SE; N = 3). The stoichiometry of this reaction was therefore 1.8 moles of SR 4233 consumed per mole of SR 4317 formed. The 40–50% of parental material which remains unaccounted for is probably lost during the 2-electron reduction process.

Microsomal reaction kinetics

The conversion of SR 4233 to SR 4317 by mouse liver microsomes was shown to conform to Michaelis–Menten kinetics. Plots of s versus v gave rectangular hyperbolas and plots of s/v versus s [24] were linear (Fig. 4). Table 2 shows that the apparent K_m values for SR 4233 loss and SR 4317 formation were similar at about 1.5 mM. The apparent V_{max} for SR 4233 loss was very high at about 1 μmol/min/mg protein, a value 40% higher than for SR 4317 formation.

Characterization of cytosolic SR 4233 reduction

SR 4317 formation rates were linear with time up to at least 30 min and with total cytosolic protein up to a concentration of 20 mg per assay (data not shown). Mouse liver cytosol was considerably slower at catalysing the reductive metabolism of SR 4233 to SR 4317 as compared to microsomal preparations. For example the average rate for cytosolic SR 4317 formation of 3.5 nmol/min/mg protein is 40-fold less than in microsomes under similar conditions. The cytosolic reaction was equally dependent on NADH and NADPH, and neither cofactor alone could completely support control reduction rates

Table 2. Michaelis–Menten parameters for the reductive metabolism of SR 4233 by anaerobic mouse liver microsomes *in vitro*

Reaction	K_m (mM)	V_{max} (nmol/min/mg protein)
SR 4233 loss	1.7, 1.1	1100, 790
SR 4317 formation	1.5, 1.5	670, 650

Values derived from two independent experiments with six substrate concentrations in each. For other details see Materials and Methods.

Table 3. Characterization of SR 4233 reductases in mouse liver cytosol *in vitro*

Conditions	% Rate of reduction in control	
	SR 4233 loss	SR 4317 formation
Control (N_2)*	100	100
−NADH	45, 41	87, 73
−NADPH	20, 41	52, 62
+Air	≤2, ≤2	≤2, ≤2
Boiled preparations	≤2, ≤2	≤2, ≤2

The control reaction was carried out under N_2 and contained 5 mg/mL cytosolic protein, 0.9 mM NADH and NADPH and 2 mM SR 4233 in 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 3 mL. The lower limit of detection was ≤2% of the control rate. Other conditions as in Materials and Methods. Each value was determined in an independent experiment.

* Control rates of SR 4233 reduction and SR 4317 formation were 5.0 ± 0.7 and 3.5 ± 1.4 nmol/min/mg protein, respectively (mean ± 2SE; N = 3).

(Table 3). The stoichiometry of the conversion of SR 4233 to SR 4317 was similar to that for microsomes with 1.4 moles of SR 4233 consumed per mole of SR 4317 formed. The rate of SR 4330 formation

Table 4. Characterization of the reductive metabolism of SR 4233 by buttermilk xanthine oxidase in mouse liver cytosol *in vitro*

Conditions	Rate of SR 4317 formation (nmol/min/mg protein)
Control (N_2)	3.7, 2.7
+Allopurinol	0.4, 0.5
% Inhibition	88%, 81%

The control incubation was carried out under N_2 and contained 10 mg/mL cytosolic protein, 0.5 mM hypoxanthine and 2 mM SR 4233 in 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 3 mL. For other conditions see Materials and Methods. Each value was determined in an independent experiment.

Table 5 shows that the aldehyde oxidase substrate *N*-methylnicotinamide also supported SR 4233 reduction to its single *N*-oxide SR 4317 in mouse liver cytosol. Menadione inhibited the reaction by 43–65%. Due to the low activity of this enzyme SR 4233 loss could not be accurately measured.

Walker tumour DT-diaphorase

The DT-diaphorase activity of the Walker tumour cell preparation, a rich source of this enzyme [25], was 67 units/mL (12 units/mg protein) of dicoumarol-inhibitable activity. Figure 2B shows that SR 4233 was reduced readily by Walker cell sonicates, forming both the 2- and 4-electron reduction products under air in the presence of NADH. SR 4317

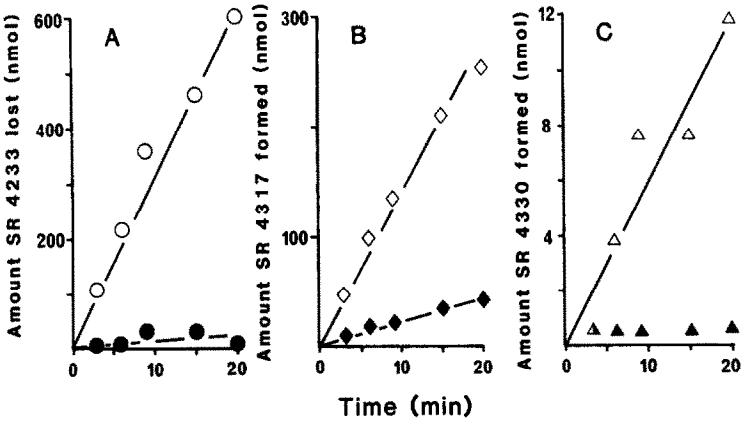

Fig. 5. Typical progress curves for the reductive metabolism of SR 4233 by purified buttermilk xanthine oxidase under N_2 *in vitro*. Open symbols are control rates and closed symbols the rates occurring in the presence of the specific inhibitor allopurinol (0.3 mM). The lower limit of detection was 0.5 nmoles (closed symbols, panel C). Incubations contained 0.5 units buttermilk xanthine oxidase, 1 mM hypoxanthine and 1 mM SR 4233 in 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 3 mL. For other conditions see Materials and Methods. Similar results were obtained in repeat experiments.

in the control was less than 4% of that for SR 4317 formation (data not shown). Minimal SR 4317 formation occurred in air or boiled preparations (≤2% of the control rate).

Involvement of specific cytosolic enzymes

Table 4 shows that cytosolic preparations catalysed SR 4233 conversion to SR 4317 in the presence of the xanthine oxidase substrate hypoxanthine, and that the specific inhibitor allopurinol inhibited the reaction by 81–88%. The role of xanthine oxidase in SR 4233 reductive metabolism was explored further using purified buttermilk xanthine oxidase. Figure 5 shows that SR 4233 was converted predominantly to its 2-electron reduction product by xanthine oxidase. The stoichiometry of this reaction was, however, rather low with approximately 2.4 moles of SR 4233 consumed per mole of SR 4317 formed. The 4-electron reduction product SR 4330 was also formed, but only at the level of 2–6% conversion. Allopurinol inhibited SR 4233 loss and SR 4330 formation completely and SR 4317 formation by 83% (Fig. 5).

Table 5. Characterization of the aldehyde oxidase dependent SR 4233 reduction in mouse liver cytosol *in vitro*

Conditions	Rate of SR 4317 formation (pmol/min/mg protein)
Control (N_2)	430, 610
+Menadione	150, 350
% Inhibition	65%, 43%

The control incubations were carried out under N_2 and contained 16.7 or 20 mg/mL cytosolic protein, 2.5 mM *N*-methylnicotinamide in 0.1 M sodium phosphate buffer (pH 7.4) in a final volume of 1.5 or 3 mL. For other conditions see Materials and Methods. Each value was determined in a separate experiment.

formation was inhibited by 47% and SR 4330 by 82–95% in the presence of 100 μM dicoumarol, a potent and specific inhibitor of DT-diaphorase [19]. The kinetics of this reaction were unusual in that relatively low concentrations of the 2-electron inter-

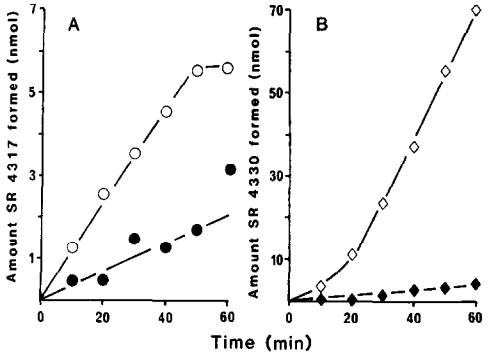

Fig. 6. Representative progress curves for the reductive metabolism of SR 4233 by Walker cell DT-diaphorase preparations under aerobic conditions *in vitro*. Panel (A) SR 4317 formation and (B) SR 4330 formation. Open symbols are control rates and closed symbols the rates in the presence of the specific inhibitor dicoumarol (100 μM). The lower limit of detection was 0.5 nmoles. Incubations contained 10 units of enzyme, 2 mM NADH and 2 mM SR 4233 in a final volume of 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4). For other conditions see Materials and Methods. Similar results were obtained in repeat experiments.

mediate SR 4317 were formed while the terminal 4-electron product SR 4330 accumulated extensively (Figs 2B and 6), the respective formation rates being 0.2 and 1.8 nmol/min/mg protein. Rates of SR 4233 loss were too low to be detected.

DISCUSSION

These results show that in anaerobic mouse liver microsomes the benzotriazine di-*N*-oxide SR 4233 is metabolized very rapidly to the 2-electron product SR 4317 with minimal conversion to the 4-electron product SR 4330. This reaction was inhibited almost completely in air and showed a marked preference for the reduced cofactor NADPH over NADH. The inhibition in air and boiled preparations confirmed that this reaction was catalysed by reductase enzymes, although there was a very small non-enzymatic component (≤2% of control rate). The pronounced dependence on NADPH and 80% inhibition under carbon monoxide implicate cytochrome P-450 as a major microsomal reductase in SR 4233 reductive bioactivation.

Several other *N*-oxides, including the tertiary amine *N*-oxide tiaramide, imipramine and *N,N*-dimethylamine *N*-oxide, have also been shown to undergo reduction by cytochrome P-450 in rat liver microsomes [26–28]. Kato *et al.* [28] have shown that the rate of imipramine *N*-oxide reduction was dependent on the rate of cytochrome P-450 reduction. The reported apparent K_m and V_{max} for these reactions were comparable with each other at between 0.2 and 0.4 mM and 2.5 and 3.3 nmol/min/mg protein, respectively [25]. By contrast SR 4233 reduction to SR 4317 exhibited a higher apparent K_m of 1.0–1.7 mM and a very much greater V_{max} of 800–1100 nmol/min/mg protein. The unusually high V_{max} value may account for the large amounts of reduced metabolites seen in livers of mice administered SR 4233 [29].

In contrast to these results with the tertiary amine *N*-oxides, the antitumour drug indicine *N*-oxide was shown to be reduced equally efficiently by rat liver microsomes in the presence of either NADH or NADPH, though the kinetics were complex with the latter cofactor [30]. Once again the K_m and V_{max} parameters were considerably lower than for SR 4233 metabolism to SR 4317 in mouse liver microsomes. Indicine *N*-oxide reduction was inhibited by CO but stimulated by iron III complexes, and it was concluded that an NADH-dependent cytochrome P-450 might be involved.

The stoichiometry observed in mouse liver microsomes was 1.8 moles of SR 4233 consumed for each mole of SR 4317 formed, leaving some 40% of the starting material unaccounted for. Similar stoichiometries have been reported for this reaction in anoxic CHO cells [7] and in the presence of purified buttermilk xanthine oxidase [9]. Some of the missing material may be accounted for in the unidentified HPLC peak reported here (Fig. 2A) and by other workers [7, 9]. Alternatively, material may be lost at the one-electron reduction level, or conceivably also at the 3-electron level.

Compared to microsomes, cytosolic SR 4233 reductase activity was some 40-fold lower and showed an equal dependence on NADH and NADPH. SR 4233 conversion to SR 4317 was relatively efficient with a stoichiometry comparable to that in microsomes. The major cytosolic enzymes known to catalyse *N*-oxide reduction are aldehyde oxidase [31] and xanthine oxidase [32, 33]. Using the respective aldehyde oxidase-specific substrate and inhibitor, *N*-methylnicotinamide and menadione [23], we have shown that this enzyme can catalyse SR 4233 reduction *in vitro*. Conversion was predominantly to the 2-electron product with no detectable 4-electron product formation. Rates of conversion to SR 4317 were approximately 300 and 10-fold lower than in microsomes and cytosol, respectively, and much lower than the aldehyde oxidase-catalysed *N*-oxide reduction of the tertiary amine *N*-oxides cyclobenzaprine *N*-oxide and imipramine *N*-oxide [31].

Cytosolic xanthine oxidase activity was also demonstrated, with efficient conversion to SR 4317 and, in addition, minor detectable SR 4330 formation. The purified buttermilk enzyme catalysed this reduction predominantly to the 2-electron product. Conversion to the 4-electron product was also detectable, although formation rates were about 20–25-fold lower. These results are in accord with previous observations by Laderoute and Rauth [9] who also showed that these metabolic transformations were accompanied by single and double-strand breaks in plasmid DNA. The mechanism of the reduction by xanthine oxidase is unclear but the pathway for nicotinamide *N*-oxide reduction by milk and pig liver xanthine oxidase under a helium atmosphere has been shown to involve direct transfer of the oxygen molecule from the *N*-oxide to uric acid [32].

All the above enzymes catalyse obligate one-electron reduction and consequently will reduce SR 4233 via a potentially toxic oxidizing radical intermediate, prior to the formation of SR 4317 by transfer of a second electron. The cytotoxic consequences of this

reaction will depend on the half-life and nature of the radicals formed. Under aerobic conditions, oxygen will readily combine with the initial radical forming superoxide and other activated oxygen species. Under anaerobic conditions the more cytotoxic one-electron oxidizing radical species will predominate. By contrast, enzymes catalysing obligate 2-electron donation such as DT-diaphorase, could conceivably by-pass the one-electron intermediate to form SR 4317 directly in a predominantly detoxification pathway. In the case of DT-diaphorase this will occur under aerobic as well as anaerobic conditions. It is therefore of considerable interest that we have demonstrated that DT-diaphorase from rat Walker 256 tumour cells can catalyse SR 4233 reduction to both SR 4317 and predominantly SR 4330 in air. To our knowledge this is the first reported identification of *N*-oxide reduction by this enzyme. DT-diaphorase is known to be present in the brain of rats [34], and this enzyme may be responsible for the intriguing observation of reduced metabolites in brain tissue removed from mice treated with SR 4233 [29].

The predominant formation of the 4-electron product SR 4330 over the 2-electron product SR 4317 by Walker DT-diaphorase preparations was unusual and unexpected. One possible explanation for this peculiar stochiometry is that substrate may remain bound to the enzyme during reduction without releasing the 2-electron intermediate, such that only the terminal product accumulates. Further support for this hypothesis comes from our observation that Walker DT-diaphorase preparations were unable to catalyse SR 4330 formation when the 2-electron intermediate was supplied as substrate at 1.5–2.0 mM under similar conditions to those described here (unpublished data).

In conclusion, we have shown that the benzotriazine di-*N*-oxide SR 4322 is reductively metabolized to SR 4317 by mouse liver microsomes under nitrogen, with a predominant role for cytochrome P-450. The cytosolic enzymes xanthine oxidase and aldehyde oxidase can also catalyse SR 4233 reduction. Most interestingly, the enzyme DT-diaphorase catalysed SR 4233 reduction to SR 4330 in what is presumed to be a detoxication reaction. In view of the potentially important role of bioactivation (1-electron donation) versus detoxification (2- and 4-electron donation) pathways in determining the therapeutic selectivity of SR 4323, studies are underway to examine the principle human enzymes catalysing SR 4233 reductive metabolism.

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