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METABOLISM OF 3-AMINO-1,2,4-BENZOTRIAZINE-1,4-DIOXIDE (SR 4233) BY PURIFIED DT-DIAPHORASE UNDER AEROBIC AND ANAEROBIC CONDITIONS

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Abstract—Purified DT-diaphorase [NAD(P)H (quinone acceptor) oxidoreductase (EC.1.6.99.2)] from Walker cells was used to investigate the reductive metabolism of 3-amino-1,2,4-benzotriazine-1,4-dioxide (SR 4233) under aerobic and anaerobic conditions. In the presence of NADPH, under aerobic conditions, HPLC analysis showed the four-electron reduction product 3-amino-1,2,4-benzotriazine (SR 4330) was the major reaction product. In contrast, anaerobically, the 2-electron reduction product 3-amino-1,2,4-benzotriazine-1-oxide (SR 4317) was the predominant metabolite. Anaerobic reduction of SR 4233 to the known metabolites SR 4317 and SR 4330, catalysed by DT-diaphorase, was 3-fold higher than reduction under aerobic conditions. Anaerobically, approximately half of the substrate utilized could not be accounted for by the formation of known products. Aerobically, the majority of the SR 4233 lost could be accounted for by its conversion to SR 4317 and SR 4330. In Walker cells incubated with SR 4233 anaerobically, SR 4317 was the major metabolite formed. Dicoumarol (100 μM) had little effect on the rate of formation of this metabolite in this cell line or in a rat liver epithelial derived (JBJ) cell line. Dicoumarol did however partially reduce the induction of unscheduled DNA synthesis caused by SR 4233 in Walker cells but not in JB1 cells, suggesting the action of dicoumarol may be specific to Walker cells. It is concluded that DT-diaphorase plays only a minor role in the overall reduction of SR 4233 in the two cell lines studied.

DT-Diaphorase [NAD(P)H (quinone acceptor) oxidoreductase (EC 1.6.99.2)] is an obligate two electron-reducing [1] dimeric flavoenzyme [2] of approximate M_r = 55,000. It contains two molecules of FAD [3] and is located primarily in the cytosol [1] although activity has been detected in mitochondria [3], microsomes [4] and the Golgi apparatus [5]. Distribution of this enzyme is widespread [6, 7] with especially high levels of activity in the liver, kidney and gastrointestinal tract.

Recently, DT-diaphorase has received increased attention, mainly due to its role in the toxification and detoxification of potential anti-tumour agents [8]. Evidence has shown that the activity of this enzyme is elevated in tumour cells when compared to normal cells [9]. Thus, increased levels of activity have been reported in some rat, mouse and human hepatomas [10–12]. Very high levels have also been observed in human colonic carcinomas [13] and some human breast carcinomas [14]. Recently DT-diaphorase has been shown to metabolize a wide range of currently topical anticancer drugs. These include indoloquinones (EO9) [15], benzotriazine-di-*N*-oxides (SR 4233) [16, 17], quinonoid bioreductive alkylating agents (mitomycin C) [18], aziridinyl benzoquinones (AZQ§) [19] and the aziridinyl nitrobenzamide compound CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzanide) [20].

SR 4233 (3-amino-1,2,4-benzotriazine-1,4-dioxide, Fig. 1) demonstrates a high selective cytotoxicity towards hypoxic cells [21, 22]. These cells, present in solid tumours, are believed to be one of the most important contributory factors in the resistance of neoplasms to both chemo- and radiotherapy [23]. Investigations into the metabolism of SR 4233 by DT-diaphorase have demonstrated [17] predominantly four-electron reduction to the triazine base SR 4330 (3-amino-1,2,4-benzotriazine) with minor formation of the two-electron reduction product SR 4317 (3-amino-1,2,4-benzotriazine-1-oxide) (see Fig. 1). This is in contrast to cytochrome P450 reductase [NADPH-ferrihaemoprotein reductase (EC.1.6.2.4)] [24, 25] which, under anaerobic conditions, metabolizes SR 4233 predominantly to the two-electron reduction product SR 4317. Previous work on the DT-diaphorase-catalysed metabolism of SR 4233 has been performed under aerobic conditions [17], a situation not necessarily mimicked in hypoxic cells.

This paper reveals the differences in metabolism of SR 4233 by purified DT-diaphorase under either

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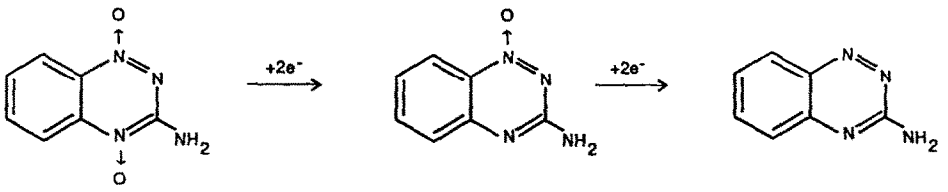
§ Abbreviations: SR 4233, 3-amino-1,2,4-benzotriazine-1,4-dioxide; SR 4317, 3-amino-1,2,4-benzotriazine-1-oxide; SR 4330, 3-amino-1,2,4-benzotriazine; AZQ, diaziquone; CB 1954 5-(aziridin-1-yl)-2,4-dinitrobenzamide; UDS, unscheduled DNA synthesis; MMS, methyl methanesulphonate; DMSO, dimethyl sulphoxide.



SR 4233 SR 4317 SR 4330

Fig. 1. Chemical structures of SR 4233 and its reductive metabolites.

aerobic or anaerobic conditions and investigates this enzyme's role in the metabolism and genotoxicity of SR 4233 in the Walker and JB1 carcinoma cell lines under hypoxic conditions.

MATERIALS AND METHODS

Chemicals. SR 4233, SR 4317 and SRF 4330 (of >98% purity as judged by HPLC) were generously supplied by Dr G. M. Blackburn (Dept of Chemistry, University of Sheffield, U.K.) and by Stirling Drug Inc. (New York, NY, U.S.A.). Walker cell DT-diaphorase, judged pure by the presence of a single component following SDS–gel electrophoresis and staining with Coumassie blue, was a kind gift from Dr R. J. Knox (Institute of Cancer Research, Sutton, Surrey). Common laboratory chemicals used throughout this work were of ANALAR grade or above and were purchased from BDH Ltd (Dagenham, U.K.) or Fisons Laboratory Supplies (Loughborough, U.K.). Deionized water was used throughout. Bovine serum albumin (type V), calf thymus DNA, cytochrome *c*, dicoumarol, dimethyl sulphoxide (DMSO), hydroxyurea and NADPH were purchased from the Sigma Chemical Co. (Poole, U.K.). Foetal calf serum, gentamicin, Liebovitz L_{15} medium, L-glutamine and trypsin were purchased from Gibco (Uxbridge, U.K.). Other chemicals were obtained from the following: phosphate-buffered saline from Unipath Ltd (Basingstoke, U.K.), and methyl-[^{3}H]thymidine (sp. act. 79 Ci/mmol); >98% radiochemical purity from Amersham International plc (Amersham, U.K.).

Cell lines and culture conditions. Walker 256 rat carcinoma cells (a kind gift from the late Prof J. J. Roberts, Institute of Cancer Research, Sutton, Surrey) were cultured in Nunclon 260 mL tissue culture flasks (Gibco) in complete William's media E [William's E supplemented with 5% (v/v) foetal bovine serum, 1% (w/v) L-glutamine and 50 μg/mL gentamycin] under 5% carbon dioxide in air at 37°.

Preparation of cytosolic fraction from Walker cells. Walker cells (1×10^9) in 0.14 M NaCl, 0.05 M Tris–HCl, pH 7.4 at 4°, were disrupted using 3×20 sec bursts from a Ystral X-1020 homogenizer (Ystral Dottinson, Germany) and centrifuged at 10,000 *g* for 20 min. The supernatant was decanted and centrifuged at 105,000 *g* for 60 min. The cytosolic fraction obtained was immediately frozen in liquid nitrogen and stored at −70° until use. No significant loss of DT-diaphorase activity using menadione substrate could be detected between the freshly prepared cytosolic fraction and samples stored up to 4 weeks at −70°.

DT-diaphorase activity. The DT-diaphorase activity of both the purified enzyme and the Walker cytosolic fraction was determined spectrophotometrically with NADPH (0.5 mM) at 37° using a modification of the method of Ernster [26] with menadione (10 μM) as the substrate and cytochrome *c* (70 μM) as the terminal electron acceptor. Protein content was assayed using the method of Lowry *et al.* [27] using bovine serum albumin as standard.

SR 4233 metabolism by DT-diaphorase. Metabolism was assayed aerobically (open to the air) and anaerobically (<1% v/v oxygen). A 1 mL reaction mixture containing KCl (0.15 M), $MgCl_2$ (1 mM), EDTA (5 mM) and phosphate buffer (0.1 M) pH 7.4 was made hypoxic by passing humidified oxygen-free nitrogen 500 mL/min) over the surface. Oxygen concentrations in the reaction mixtures were measured using an oxygen electrode (Jenway Ltd, Felsted, U.K.). NADPH (2 mM) was added, followed by purified enzyme. Reactions were initiated by the addition of SR 4233 (1 mM final concentration dissolved in 10 μL DMSO) and stopped after various times as indicated with ice-cold CH_3CN (1 mL). This was mixed and centrifuged (10,000 *g* for 2 min in an Eppendorf centrifuge), and 10 μL aliquots of the supernatant were analysed directly by HPLC.

Determination of NADPH concentrations in reaction mixtures. Using incubation mixtures as described above, aliquots (10 μL) were removed every minute for up to 15 min and added to ice-cold CH_3CN/H_2O (1:1 v/v, 0.99 mL). The concentration of NADPH was determined spectrophotometrically at 340 nm using an extinction coefficient of 6200 L/mol/cm for NADPH [28].

SR 4233 metabolism by Walker and JB1 cells. Walker or JB1 cells (1×10^7) were incubated as suspensions in supplemented Liebovitz L_{15} medium (10 mL) together with SR 4233 (1×10^{-4}M, dissolved in DMSO) under N_2 at 37° for up to 2 hr. Aliquots (0.2 mL) were removed at the indicated time points and added to an equal volume of ice-cold CH_3CN in an Eppendorf centrifuge tube. Samples were

mixed, centrifuged (10,000 *g* for 2 min) and analysed using HPLC as described below.

HPLC analysis of SR 4233 and its major metabolites. SR 4233, 4317 and 4330 were assayed by reverse-phase chromatography using a Millipore-Waters system (Millipore, Watford, U.K.) comprised of a WISP 710 autoinjector, pumps (model 510A) and solvent programmer (model 680). Separations were achieved using a 125 × 4 mm LiChrospher 100 RP-18 5μM endcapped column with a 4 × 4 mm precolumn of the same material (E. Merck, Darmstadt, Germany). Optical absorbance of the eluate was determined at 268 nm using a Kontron 432 UV detector (Kontron Instruments, Watford, U.K.). Peak areas were integrated using a Kontron PC integration pack. The initial mobile phase consisted of CH_3CN/water (1:10 v/v). A linear gradient to CH_3CN/water (1:1 v/v) was run over 10 min. The flow rate was 1.2 mL/min.

In some instances, the UV detector was replaced by a 1000S diode array detector (Applied Biosystems, Warrington, U.K.) and absorbance spectra (200–400 nm) were acquired when the signal in the detection channel (254 nm) exceeded the threshold of the background noise.

Unscheduled DNA synthesis studies. Walker cells (1×10^7) were incubated in the presence of hydroxyurea (10 mM), [^{3}H]thymidine (10 μCi) and the test compound in Liebovitz L_{15} medium (10 mL) for 2 hr at 37° under N_2. At the end of this time, cells were harvested and the DNA extracted as described previously [24]. DNA concentrations were measured by the method of Burton [29].

RESULTS

Aerobic versus anaerobic metabolism of SR 4223 catalysed by DT-diaphorase

Two distinct batches of DT-diaphorase were purified from Walker rat mammary tumour cells. Using menadione as the substrate, the activities of the two enzyme preparations were 8223 and 1800 nmol of cytochrome *c* reduced/min/μg protein. Addition of dicoumarol (100 μM) reduced the enzyme activities to 226 ± 21 nmol/min/μg protein (mean ± SE for four experiments) and zero, respectively. Both sources produced similar metabolic profiles of SR 4233 reduction when compared under aerobic or anaerobic conditions, as monitored by HPLC. Figure 2 shows typical HPLC chromatograms from experiments carried out under aerobic and anaerobic conditions. Under aerobic conditions the four-electron reduction product (SR 4330) predominates, accounting for approximately 80% of the known metabolites formed. Anaerobically, however, metabolism of SR 4233 differs markedly, with the two-electron reduction product (SR 4317) predominating. Aerobic metabolism of SR 4233 by DT-diaphorase was completely inhibited when dicoumarol (100 μM) was added to the reaction mixture (Table 1). Under hypoxic conditions, formation of the two-electron and four-electron reduction products could only account for 50% of total SR 4233 loss (Table 1). Similar results have been obtained for cytochrome P450 reductase and mouse liver cytochrome P450 [24], indicating the possibility of unknown metabolite formation or substrate degradation. Addition of dicoumarol to the reaction mixture caused approximately 80% inhibition of metabolism to the known metabolites. Figure 3 shows a typical product formation time course from SR 4233 reduction by DT-diaphorase under both aerobic and anaerobic conditions. It shows that under hypoxic conditions, SR 4317 predominates, with linear formation over the first 15 min. In contrast, under aerobic conditions the four-electron reduction product (SR 4330) is the major metabolite. Formation of SR 4330 appears to accelerate after 5 min.

We wished to examine whether the differences in metabolism of SR 4233 aerobically and anaerobically were due to the presence of oxygen and if the effects were reversible. SR 4233 was incubated for 5 min in the presence of purified enzyme under aerobic conditions. At the end of this incubation, the reaction was made anaerobic and finally stopped after 30 min. At the same time a reaction was carried out anaerobically for 5 min then aerobically for a further 25 min. Aliquots were removed from both sets of experiments after 5 and 30 min and subjected to HPLC analysis. Table 2 shows after 5 min, under aerobic conditions, the major metabolite formed was SR 4330. On switching to anaerobic conditions (a process taking approximately 10 min), SR 4330 formation continues but mono-*N*-oxide formation becomes predominant. The reverse can be seen when the reaction is begun under hypoxic conditions. SR 4317 formation predominates initially but when the reaction mixture is made aerobic, SR 4317 formation ceases and the triazine base becomes the major metabolite. The concentration of SR 4317 formed anaerobically after 5 min decreased by approximately 30% during 25 min incubation. This may be due to its conversion to the fully reduced triazine base. However, SR 4317 was not found to be a substrate for DT-diaphorase metabolism under aerobic or anaerobic conditions, confirming the observations of Riley and Workman [17].

Stoichiometry of NADPH utilization and SR 4233 metabolism

The cofactor requirements of DT-diaphorase under aerobic and anaerobic conditions were investigated by incubating SR 4233 with purified enzyme and NADPH (1 mM) and analysing the loss of NADPH spectrophotometrically at 340 nm. NADPH content decreased in a linear fashion over the 15 min time period. Results from this experiment showed that after 5 min incubations, under anaerobic conditions, 317 ± 28 nmol (mean ± SE for four experiments) of NADPH were utilized and this was associated with the disappearance of 182 ± 16 nmol of SR 4233. Aerobically, however, 290 ± 37 nmol of cofactor were accompanied by the loss of 61 ± 8 nmol of di-*N*-oxide. When SR 4233 was incubated with cofactor in the absence of enzyme, cofactor loss was negligible. This disparity between anaerobic and aerobic stoichiometry may be due to the four-electron reduction metabolite SR 4330 which is formed predominantly under aerobic conditions (*cf* Fig. 2).

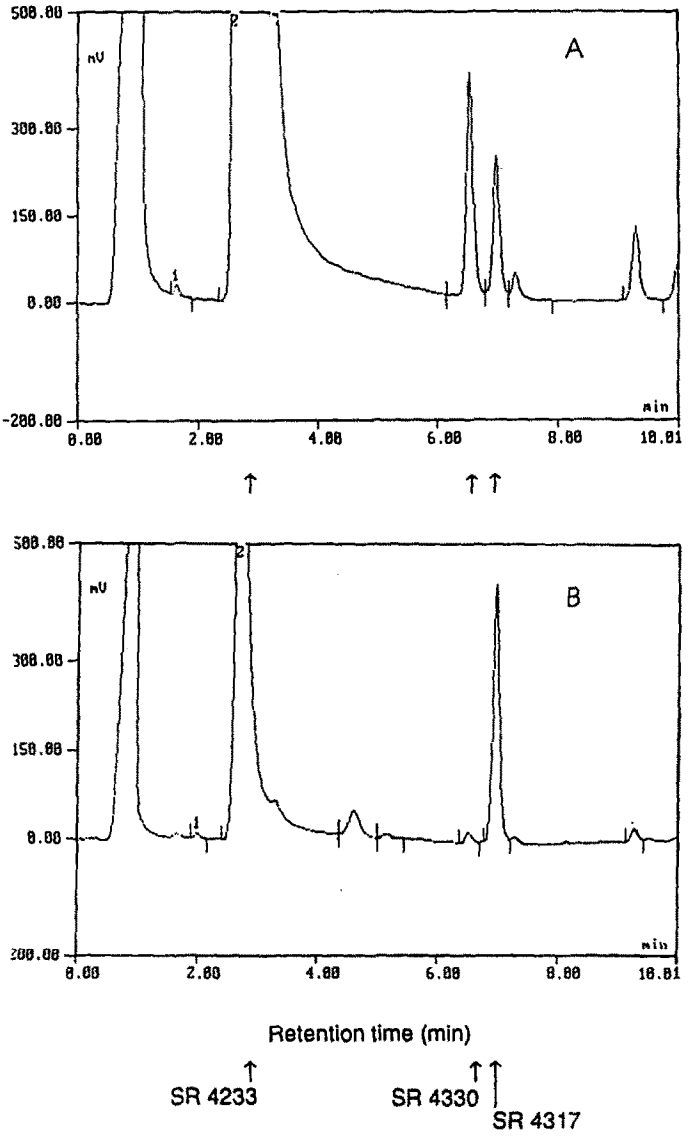


Fig. 2. HPLC chromatogram for the reduction of SR 4233 (1 mM) under aerobic (A) and anaerobic (B) conditions using purified DT-diaphorase (25 μg protein). Reactions were performed over 15 min in the presence of NADPH (2 mM). DT-diaphorase activity was 8223 nmol/min/μg using menadione as the substrate and cytochrome *c* as the terminal acceptor. Ordinates represent relative absorbance at 268 nm.

Effects of pH values on reaction rates

The effect of pH on SR 4233 disappearance and mono-*N*-oxide formation under hypoxic conditions was investigated using Walker cell cytosol and purified DT-diaphorase. In both systems SR 4233 disappearance showed no pH dependence over the range pH 6.0 to 7.4 (data not shown). In this respect, results are similar to that seen for DT-diaphorase aerobically [17]. Anaerobically, the formation of mono-*N*-oxide, however, shows an apparent pH dependence. Using Walker cell cytosol there was a significant increase in SR 4317 formation as the pH was progressively lowered (Table 3). A similar pH dependence was found for SR 4317 formation when the enzyme was partially inhibited by dicoumarol (20 μM). In the case of the purified enzyme, some increase in SR 4317 formation was also observed as the pH was lowered, while the SR 4233 disappearance showed again no pH dependence.

Metabolism of SR 4233 by Walker and JB1 cell lines: effects of dicoumarol

An attempt was made to relate the purified enzyme findings under anaerobic conditions to that found in whole cells. Two cell lines with differing levels of DT-diaphorase (Table 4) were used. In both cases, following incubation with SR 4233, the mono-*N*-oxide was the major metabolite formed. Figure 4

Table 1. Metabolism of SR 4233 to SR 4317 and SR 4330 by purified DT-diaphorase under aerobic and anaerobic conditions

	Rate of loss or formation (nmol/min/mg protein)		
	SR 4233	SR 4317	SR 4330
Aerobic	179 ± 91	15 ± 6	93 ± 32
Aerobic plus dicoumarol (100 μM)	ND	ND	ND
Anaerobic	780 ± 108	318 ± 70	47 ± 4
Anaerobic plus dicoumarol (100 μM)	125 ± 29	57 ± 3	ND

Incubations were as described in Materials and Methods.
Purified DT-diaphorase activity was 1800 nmol/min/μg protein using menadione as the substrate and cytochrome *c* as the terminal electron acceptor.
Results represent the mean ± SE of three experiments.
ND, not detected.

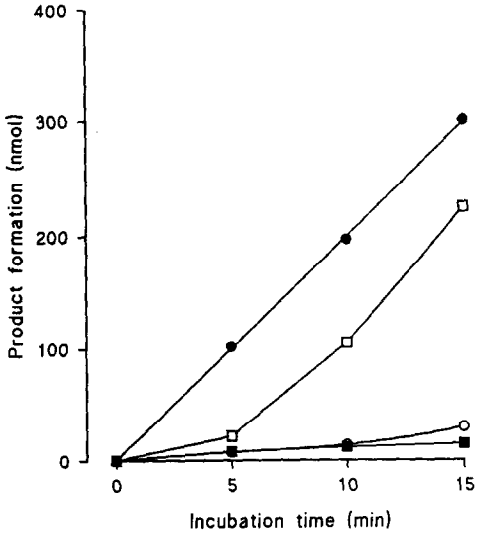


Fig. 3. Time dependence formation of SR 4317 and SR 4330 from SR 4233, catalysed by DT-diaphorase (25 μg protein) under aerobic or anaerobic conditions. DT-diaphorase activity was 8223 nmol/min/μg protein using menadione and cytochrome *c* as described in Materials and Methods. The formation of SR 4317 (○, ●) and of SR 4330 (□, ■) under aerobic (open symbols) or anaerobic (closed symbols) conditions is the mean of duplicate experiments.

Table 2. Effect of changes in oxygen tension on the metabolism of SR 4233 by purified DT-diaphorase

	Metabolite formation (nmol/mL reaction mixture)	
Conditions	SR 4317	SR 4330
Aerobic 5 min	4 ± 1	20 ± 5
Aerobic made anaerobic, 30 min	102 ± 9	66 ± 7
Anaerobic 5 min	90 ± 4	10 ± 1
Anaerobic made aerobic, 30 min	65 ± 10	49 ± 8

Incubation conditions were as described in Materials and Methods. Purified DT-diaphorase activity was 1800 nmol/min/μg. Reaction mixtures contained 50 μg/mL DT-diaphorase protein.
Results represent the mean ± SE of three experiments.

shows SR 4317 product formation over a 2 hr time period in both cell lines in the presence and absence of dicoumarol (100 μM). In neither case did dicoumarol cause a reduction in the rate of SR 4317 formation, despite observation that such inhibition occurred in cytosolic preparations (Table 3). The rate of conversion of SR 4233 to SR 4317 was much lower in Walker cells compared to JB1 cells despite the considerably higher levels of DT-diaphorase in cytosolic fractions of Walker cells (Table 4). This suggests that, in the cell lines studied, DT-diaphorase plays a minor role in the metabolism of SR 4233 under hypoxic conditions. It is more likely that cytochrome P450 reductase, which displays greater activity in JB1 cells than in Walker cells [23], play the predominant role in SR 4233 metabolism. Both cell lines used possess no detectable levels of cytochrome P450 [29].

Induction of unscheduled DNA synthesis by SR 4233: effects of dicoumarol

The effect of DT-diaphorase activity on the potential DNA damaging capability of SR 4233 under hypoxic conditions was investigated using unscheduled DNA synthesis (UDS) as an index of genotoxicity. Table 5 shows the effects of dicoumarol on the induction of UDS by SR 4233 in both Walker and JB1 cell lines. Increasing concentrations of dicoumarol up to 100 μM results in a corresponding decrease in UDS. With 50 and 100 μM dicoumarol

Table 3. Effect of pH on the formation of SR 4317 by Walker cell cytosol and purified Walker DT-diaphorase under anaerobic conditions

pH	SR 4317 formation (nmol/min/mg protein)	
	Walker cytosol	Walker cytosol plus dicoumarol (20 μM)
6.00	9.41 ± 1.08*	3.65 ± 1.10*
6.50	8.45 ± 0.68*	2.51 ± 1.16*
6.87	6.23 ± 0.72	1.64 ± 1.11
7.41	4.38 ± 1.30	0.25 ± 0.19
	Purified DT-diaphorase	DT-diaphorase plus dicoumarol (20 μM)
6.00	220 ± 10	50 ± 15
6.50	184 ± 5	41 ± 2
6.87	175 ± 5	42 ± 12
7.41	152 ± 17	15 ± 1

Incubation were performed in MOPSO [3-(*N*-morpholino)-2-hydroxypropanesulphonic acid] buffer for 5 min at 37° under N_2 at the pH values indicated. SR 4317 was assayed as described in Materials and Methods. Purified DT-diaphorase activity was 1800 nmol/min/μg protein.

Results represent the mean ± SED of three experiments.

Significance of difference in SR 4317 formation between test pH value and incubation carried out at pH 7.41, *$P < 0.05$.

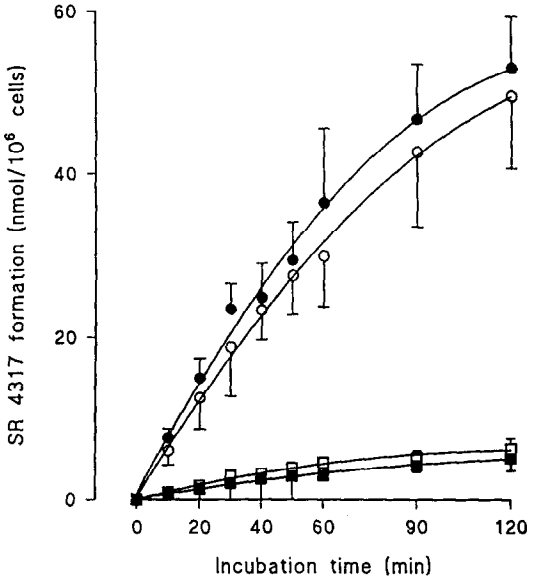


Fig. 4. Time dependence for the metabolism of SR 4233 (1×10^{-4}M) to SR 4317 and SR 4330 by Walker and JB1 cells under anaerobic conditions. Cells (1×10^7) were incubated in Liebovitz L_{15} medium (10 mL) under anaerobic conditions in the presence (open symbols) or absence (closed symbols) of dicoumarol (100 μM). Either Walker (□, ■) or JB1 (○, ●) cells were used. Results represent the mean ± SE for four experiments.

Table 4. Comparison of DT-diaphorase activities in cytosolic preparations from JB1 and Walker cells

Cell line	DT-diaphorase (μmol/min/mg protein)	DT-diaphorase plus 50 μM dicoumarol (μmol/min/mg protein)
JB1	5.48 ± 1.1	0.80 ± 0.27
Walker	15.89 ± 0.41	2.89 ± 0.34

DT-diaphorase activities were assayed spectrophotometrically using menadione, cytochrome *c* and NADPH as a cofactor, as described in Materials and Methods.

Results represent the mean ± SE of four experiments.

this decrease is significant. In contrast, dicoumarol has no inhibitory effect on the induction of UDS by SR 4233 in JB1 cells; in fact it stimulated significantly the UDS. It may be that the effect of dicoumarol is specific for Walker cells. In order to investigate if dicoumarol has an effect on DNA repair enzyme systems, the effect of methyl methanesulphonate (MMS, 1×10^{-4}M) on the induction of UDS in Walker cells was also studied. MMS is a direct-acting genotoxic agent and requires no enzyme activation [9]. Table 5 shows that dicoumarol (100 μM) has no inhibitory effect on UDS caused by MMS. Increasing concentrations of dicoumarol had no inhibitory action on UDS caused by SR 4233 in JB1 cells. The effect of dicoumarol on UDS induced by MMS was not therefore determined in this cell line. Dicoumarol alone had no effect on UDS in Walker or JB1 cells (Table 5).

DISCUSSION

DT-diaphorase plays a significant role in the toxification and detoxification of many potential anti-tumour agents [8] and it has been shown that the activity of the enzyme is elevated in many tumour cell lines [9]. Evidence suggests that, in the rat, the major bioreductive activating enzyme is cytochrome P450 reductase [24, 31], whereas in mouse liver cytochrome P450 has been implicated [32]. The aim of potential bioreductive anti-cancer drugs is to act in hypoxic environments. It is therefore necessary to investigate toxifying and detoxifying enzyme systems under both aerobic and hypoxic conditions.

Under aerobic conditions, DT-diaphorase converts SR 4233 directly via two-electron reduction to both the mono-*N*-oxide and predominantly, via four-electron reduction, to the fully reduced triazine SR 4330. Mechanistically, this would be expected to constitute a detoxification reaction, not involving a nitroxide radical intermediate. This finding is in agreement with the results of Riley and Workman [18]. The present results show unexpectedly that under hypoxic conditions, SR 4233 is reduced by purified DT-diaphorase primarily to the mono-*N*-oxide, SR 4317. Further reduction to the triazine base, SR 4330, is blocked by the *absence* of oxygen. This effect is reversible by the admission of O_2 to the reaction mixture (Table 2). The mechanism of

Table 5. Effects of dicoumarol on unscheduled DNA synthesis caused by SR 4233 (10^{-4} M) or MMS (1×10^{-4}M) following 2 hr exposure under anaerobic conditions

	Dicoumarol concn (μM)	Unscheduled DNA synthesis (dpm/μg DNA) Walker cells	JB1 cells
Controls	0	97 ± 16	41 ± 1
	100	98 ± 10	45 ± 3
SR 4233 (1×10^{-4}M)	0	2159 ± 201	1232 ± 40
	20	1636 ± 199 NS	1623 ± 68†
	50	1464 ± 61*	2212 ± 354*
	100	798 ± 28‡	1625 ± 40*
MMS	0	296 ± 10	ND
(1×10^{-4}M)	100	348 ± 16	ND

Cells (10^7) were incubated in the presence of hydroxyurea (10 mM), [^{3}H]thymidine (10 μCi) and the test compound in Liebovitz L_{15} medium (10 mL) for 2 hr at 37° under N_2. At the end cells were harvested and the DNA extracted and assayed for radioactivity, as described in Materials and Methods.

Results represent the mean ± SE for at least four experiments.

Probability of significance of difference between cells incubated in the presence and absence of dicoumarol: *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$. NS, not significant. ND, not determined.

action of oxygen, on the enzyme protein or a metabolic intermediate, is unclear. Similar effects were observed using crude Walker cell sonicates (Table 3). Stoichiometric evidence shows that during anaerobic metabolism of 1 mole of SR 4233 by purified DT-diaphorase, 2 moles of NADPH are utilized, SR 4317 being the major $2e^-$ reduction product (Fig. 1b, Table 1). Aerobically, 5 moles of NADPH are utilized. This is compatible with the further $4e^-$ reduction of SR 4233 to SR 4330 under aerobic conditions.

The reason for the lower overall rate of metabolism of SR 4233 under aerobic conditions, catalysed by DT-diaphorase (Table 1) is not clear. In the case of $1e^-$ reduction of this compound by cytochrome P450 reductase, lower rates of aerobic metabolism have been attributed to oxidation of the nitrogen oxide radical anion intermediate by O_2 to cause redox cycling [21, 31]. With DT-diaphorase, which is an obligate 2^- reducing enzyme [1], although the addition of each electron is likely to occur sequentially, it is improbable that redox cycling is involved. Radiolabelled SR 4233 was not available, so the precise fate of all the substrate metabolized cannot be established unequivocally. It is possible that the apparent increase in metabolism seen with SR 4233 under hypoxic conditions is due to the formation of an unstable six-electron reduced product that has been described electrochemically [33]. This is oxygen sensitive and may auto-oxidize in air back to SR 4330. The differential profile of metabolism seen under air and nitrogen may also be a consequence of an apparent general increase in metabolism resulting from an increased stability of the reaction products [17]. Such a scenario would be similar to that described for AZQ [34]. The different stoichiometrics of cofactor consumption to stable metabolite production would be consistent with this hypothesis.

It is generally thought that hypoxic cells have internal environments with acidic pH values [35]. The anaerobic metabolism of SR 4233, as judged by loss of this substrate, was largely independent of pH, over the range 6.0 to 7.4 (data not shown) in a similar fashion to that observed aerobically, confirming earlier observations [17]. The formation of mono-*N*-oxide, however, does show some pH dependence (Table 3). The effect of pH on the formation of unknown, possibly toxic, metabolites has yet to be investigated.

The role of DT-diaphorase in SR 4233 genotoxicity was investigated in both Walker and JB1 cells under hypoxic conditions (Table 5). In Walker cells, SR 4233 causes induction of UDS which is inhibited in a dose-dependent fashion by dicoumarol. This suggests that DT-diaphorase might be playing a role in the activation of SR 4233. Dicoumarol itself had no effect on the induction of UDS at the concentrations used. However, when the experiment was repeated using JB1 cells, no such inhibitory effect of dicoumarol was observed (Table 5), suggesting that the action of dicoumarol may be specific only to Walker cells. In addition to inhibiting DT-diaphorase, at the highest concentrations used in these experiments, dicoumarol may inhibit mitochondrial oxidative phosphorylation [36] thus reducing cellular ATP concentrations. Previous studies have suggested caution is required in the interpretation of results regarding the use of dicoumarol as a specific inhibitor of DT-diaphorase in cell systems *in vitro* [37, 38]. Walker cells have significantly higher DT-diaphorase activities than JB1 cells (Table 4). It is not clear why dicoumarol inhibited UDS in Walker cells yet apparently did not inhibit the conversion of SR 4233 to SR 4317 (Fig. 4).

Conclusions

In this paper we demonstrate, using purified Walker cell DT-diaphorase, that the metabolic

pathway of SR 4233 differs under aerobic and anaerobic conditions. Aerobically the triazine base SR 4330 is the major metabolite. Anaerobically the mono-*N*-oxide, SR 4317, is the major metabolite but the fate of almost 50% of the substrate utilized cannot be traced. In Walker and JB1 cell lines, results suggest that relative to cytochrome P450 reductase, DT-diaphorase plays only a minor role in the overall metabolism of SR 4233.

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