ACTIVATION OF BIOREDUCTIVE ANTITUMOUR AGENTS BY DT-DIAPHORASE

By

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Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Medicine, University of Glasgow

October 1989-January 1991
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January 1991-May 1995
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May, 1995

Summary

Bioreductive anticancer agents possess two potential targets for selectivity, hyperexpressed activating enzymes and hypoxia. The subject of this thesis is part of an 'enzyme directed approach' to rational drug development in which compounds are tailored to suit the catalytic preferences of key bioreductive enzymes. Studies have focused on the two-electron reducing flavoenzyme DT-diaphorase due to its increased expression and activity in tumour versus normal tissue and its ability to catalyse metabolism of substrates with structural similarity to principal bioreductive agents. The overall aim of the thesis was to determine the involvement of DT-diaphorase in the activation of the novel indoloquinone EO9, aziridinylbenzoquinone AZQ and selected cyclopropamitosenes. Structure-activity relationships were examined using analogues of these compounds.

Initial experiments to characterise metabolism of EO9 showed it to be efficiently reduced by DT-diaphorase present in sonicates of human HT29 and rat Walker cells as well as by a highly purified form of the rat enzyme. Chemosensitivity assays also demonstrated EO9 to be a highly potent cytotoxin for these cell lines, which express high levels of DT-diaphorase. Study of EO9 analogues showed that structural modification altered both ability to undergo metabolism catalysed by DT-diaphorase and cytotoxicity. A clear correlation was observed between cytotoxicity and metabolism with analogues which were better substrates for DT-diaphorase being more potent cytotoxins than the more poorly reduced derivatives. This supported the importance of DT-diaphorase in activation of the indoloquinones to their cytotoxic species. Inhibitor studies further substantiated this hypothesis but indicated involvement of additional enzymes. The latter possibility was supported by results of electron spin resonance spectroscopy experiments where NADPH: cytochrome P450 reductase was shown to metabolise EO9. The mechanism of reaction has to some extent been elucidated. Both DT-diaphorase and NADPH: cytochrome P450 reductase metabolised EO9 to highly oxygen sensitive metabolites which, in the presence of air, were auto-oxidised to generate highly reactive and potentially damaging oxygen and drug based radicals.

In intact cells EO9 induced DNA damage in the form of strand breaks and interstrand cross-links at pharmacologically relevant concentrations. This damage was more extensive in the high DT-diaphorase expressing cell line HT29 than in the BE cell line which does not express a functional form of the enzyme. Using a cell free system the

importance of DT-diaphorase activation for generation of both strand breaking and interstrand cross-linking species was determined. Monoadducts could be formed with DNA in the absence of activation although the sequence selectivity exhibited by the compound was found to be altered following reduction.

Neither hypoxia or pH altered the initial rate of EO9 reduction catalysed by DT-diaphorase. Hypoxia did however alter the extent of DNA damage and other investigators have shown that both pH and hypoxia can influence cytotoxicity of this compound. Metabolism studies suggested that these parameters will influence the stability of metabolites formed.

AZQ and some cyclopropamitosenes were also reduced by purified rat Walker DT-diaphorase but less efficiently than EO9. In a similar manner to EO9 some correlation between metabolism and cytotoxicity was observed with the aziridinylbenzoquinones although the differences were within a far narrower range than for the indoloquinones.

To conclude, data presented in this thesis has confirmed a role for DT-diaphorase in activation of EO9, AZQ and cyclopropamitosenes. In addition NADPH: cytochrome P450 reductase has also been shown to metabolise EO9. The mechanism of reaction and cytotoxicity has been partially elucidated and structure-activity relationship studies have highlighted features of the drug molecules which are important for both metabolism and cytotoxicity. Such information may be important in the design of improved analogues and for optimisation of clinical protocols employing these novel, bioreductive agents.

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Publications Arising From This Thesis : enclosed in envelope inside rear cover

Bailey, S.M., Suggett, N., Walton, M.I. & Workman, P. (1992) Structure-activity relationships for DT-diaphorase reduction of hypoxic cell directed agents: indoloquinones and diaziridinyl benzoquinones. *International Journal of Radiation Oncology, Biology, Physics*, **22**, 649-653.

Moody, C.J., O'Sullivan, N.O., Stratford, I.J., Stephens, M.J., Workman, P., Bailey, S.M. & Lewis, A. (1994) Cyclopropamitosenes: novel bioreductive anticancer agents-mechanism of action and enzymatic reduction. *Anti-Cancer Drugs*, **5**, 367-372.

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List of Abbreviations

A	adenine
Ah	aromatic hydrocarbon
ARE	antioxidant response element
arg	arginine
AZQ	diaziquone; 3,6-bis(carboxyamino)-2,5-diaziridinyl-1,4-benzoquinone
BSA	bovine serum albumin
C	cytosine
cys	cysteine
DCPIP	2,6-dichlorophenolindophenol
DETEPAC	diethylenetriamine pentaacetic acid
DMF	dose modification factor (IC_{50} drug / IC_{50} drug + inhibitor)
DMPO	5,5-dimethyl-1-pyrroline-1-oxide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
EO9	3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4, 7-dione)prop-β-en-α-ol
EORTC	European Organization for Research and Treatment of Cancer
ESR	electron spin resonance spectroscopy
FAD	flavin adenine dinucleotide
g	acceleration due to gravity
G	guanine
gly	glycine
Gy	Gray
HCR	hypoxic cytotoxicity ratio (IC_{50} in air / IC_{50} hypoxia)
HPLC	high performance liquid chromatography
IC(n) or ID(n)	inhibitory concentration or dose (concentration which inhibits n% of colony formation or in the case of the MTT assay that which results in a n% decrease in absorbance compared with the control)
i.v.	intravenous
Km	concentration of substrate at which the rate of enzyme reduction is half-maximal
lys	lysine
$LD_{(10)}$	concentration which killed 10% of animals

mRNA	messanger RNA
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NCI	National Cancer Institute
MRC	Medical Research Council
PBS	phosphate buffered saline
PCR	polymerase chain reaction
ppm	parts per million
S	substrate concentration
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ser	serine
T	thymine
thr	threonine
tyr	tyrosine
v	initial velocity of enzyme reaction
Vmax	maximum velocity of enzyme reaction when substrate is at saturation
XRE	xenobiotic responsive element

SI units are used throughout the text and, due to their common acceptance are not defined in this abbreviation section

Acknowledgements

I wish to express my sincere thanks and appreciation to my supervisor, Professor Paul Workman for all his guidance, support and continual encouragement throughout my PhD. I would also like to thank everyone in the laboratories in which I have spent time for their friendship. Unfortunately the list is too extensive to acknowledge everyone individually.

I am extremely grateful to the following people for their particular contributions:- MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge - Professor Bleehen for finding funding for the first year of my PhD and for acting as a second supervisor during this period; Jane Donaldson and Drs Mike Walton and Michael Binger for providing valuable advice during my first few months; Nigel Suggett for his contribution towards some of the metabolism studies in Chapter 6. Institute of Cancer Research, Sutton, Surrey - Dr Richard Knox for his general advice and considerable encouragement; Frank Friedlos for assistance with protein purification and alkaline elution; Dr Terry Jenkins for helpful discussion; Dr Steve Hobbs and Richard for proof reading; Professor Tom Connors for support throughout my career and the rest of the "Molecular Pharmacology Group" for welcoming me in to their laboratory. Department of Pharmacy, De Montfort Univerisity, Leicester - Dr Laurence Patterson for use of his ESR equipment and to both Laurence and Dr Geoff Fisher for constructive advice on this subject. Department of Medical Oncology, University College and Middlesex School of Medicine (UCMS), London - Dr John Hartley for allowing me to carry out the cross-linking and *taq* polymerase assays in his lab and to both John and Mike Wyatt for sharing their knowledge with me on these subjects. CRC Department of Medical Oncology, Glasgow - Drs Ian Freshney, Martin Graham, Bob Brown and Rob Riley for helpful advice; Liz the librarian; Drs Jane Plumb and Alex Lewis, for their considerable guidance and encouragement particularly during the preparation of this document and Dr Matt Lohmeyer for his friendship, support and for always being there to help out in a crisis. Professor Ian Harts department St Thomas' Hospital for their patience during my writing up period. I would also like to thank Professors Stan Kaye, Glasgow and Norman Bleehen, Cambridge for permitting me to make use of the laboratory facilities in their departments and to the CRC for funding the final years of my PhD.

Finally, I wish to thank my parents for all their support and encouragement, particularly my father for his extensive help with proof reading of this thesis.

I dedicate this thesis to my family

Declaration

I declare that the work presented in this thesis was solely carried out by myself except where outlined below:-

Some of the initial experiments included in Chapter 6 determining the metabolism of the aziridinylbenzoquinones were carried out by Nigel Suggett, a summer student in our laboratory, under my supervision. I did however confirm a large proportion of the results in subsequent repeat experiments.

The cross-linking and sequence selectivity assays described in Chapter 5 were performed by myself in Dr John Hartleys laboratory at University College and Middlesex School of Medicine (UCMS), London, UK. Due to lack of time, as this work was carried out at the end of my PhD, repeat experiments were performed using the same conditions by Mike Wyatt (UCMS). An autoradiogram from one of these repeat experiments was used in Figure 5.13 as it provided the clearest representation of the work obtained.

Chapter 1

General Introduction

This thesis examines the activation of bioreductive anticancer agents by DT-diaphorase as part of an 'enzyme directed' approach to drug development. The introduction which follows presents the background to this subject area, beginning with an outline of the requirement for innovative, rational drug development. Hypoxia is presented as a problem for conventional chemotherapy and radiotherapy and strategies of circumventing or exploiting hypoxia are discussed. In particular bioreductive anticancer agents and the enzymes involved in their activation are described in detail.

1.1 Cancer Therapy and Drug Development

The term cancer refers to a multitude of diseases which share the common characteristic of disordered growth. In Britain it is estimated that 1 in 3 people will develop cancer and 1 in 4 will die as a result of the disease. Current therapy of cancer includes surgery, radiotherapy and chemotherapy. Surgery is highly effective in localised non-metastatic tumours as well as non-malignant forms of the disease. Radiotherapy is also beneficial for localised treatment in many types of cancer but its use is limited by resistance and damage to normal tissues. Since most patients die as a result of metastatic spread, systemic treatment is essential for the majority of patients. Over the last 50 years several chemotherapeutic agents have been used clinically. Although significant advances have been made towards cure of particular types of cancer such as childhood leukaemia and testicular cancer there has been relatively little success against the major solid tumours and cancer remains the second biggest killer in the UK after coronary heart disease. The problem with established chemotherapy is that, in general, these drugs lack selectivity for tumour versus normal cells and consequently have a low therapeutic index.

Data obtained in 1986 showed that approximately 48% of cancers presented in a localised state and therefore were suitable for treatment with surgery and / or radiotherapy (Souhami and Tobias, 1986). Of these around 60% were curable. In contrast a cure rate of only 5% was achieved with metastatic cancer. Thus there is an

obvious requirement for improvements in cancer therapy, and particularly in systemic treatments effective against disseminated disease.

It may be argued that the lack of effectiveness of many chemotherapeutic agents is a result of the drug discovery methods previously employed. Initially these were highly empirical and involved random screening of a diverse range of compounds for toxicity followed by analogue development. The Chester Beatty Research Institute in London, UK, which carried out much of this work in Britain, adopted a screen which involved testing compounds *in vivo* for activity against a Walker tumour grown in rats (Rosenoer *et al*, 1966). The National Cancer Institute (NCI) in the USA also used rodent models *in vivo* but focused on leukaemias e.g. the L1210 and P388 murine tumours as the preliminary screens and this may explain why agents identified as possessing promising activity were largely effective against leukaemias but were generally much less effective against solid tumours (Schwartsmann and Workman, 1993). Despite leading to the identification of a number of currently used, active drugs such as cisplatin, a criticism of these models has been that they were rodent tumours which may not necessarily be a good indication of therapeutic activity in humans. In 1975 animal solid tumours and human tumour xenografts were also included as secondary *in vivo* model systems at the NCI. However, relatively little success has been achieved and in recent years drug development has been considered to have reached a plateau of effectiveness. A few highly selective compounds were discovered in preclinical screens but were found to be ineffective in humans and in general, the currently employed drugs are what is often described as 'dirty', that is, they have multiple sites of action. More encouragingly exciting breakthroughs have been made in understanding the molecular basis of cancer. This knowledge is now being exploited in current research strategies where both empirical and rational, mechanism-based approaches to drug development are combined. Designing drugs directed against specific molecular targets may overcome the problems of selectivity observed with the currently employed compounds. Cellular targets such as oncogenes, signalling molecules and the cell membrane have been identified in place of DNA and microtubule targets, and a wide range of compounds of both synthetic and natural origin are assayed against for activity against them (Workman, 1992b). Screens are increasingly of high throughput and may involve cell-free studies with purified, often recombinant, target proteins, or alternatively may involve cell lines which naturally or as a result of genetic modification express appropriately high levels of the target molecule. Following detection, lead compounds can be structurally modified to produce analogues with enhanced potency and selectivity.

As an alternative approach to strategies aimed at particular novel molecular targets the US NCI has recently developed a new *in vitro* screen which consists of a large number of cell lines derived from human solid tumours. These have been well characterised and represent a range of tumour types (Grever *et al*, 1992). Agents are identified that have activity against particular disease types and these may be further tested for activity *in vivo* against the same human tumours grown as xenografts in nude mice. Computer programmes are used to give drugs characteristic 'fingerprints' based on their cytotoxicity profiles on the range of cell lines. These profiles can be compared for different drugs. Promising new agents are selected on the basis of disease specificity, potency, a unique structure and antitumour profile which indicates a different mechanism of action from agents previously used.

In addition to these more innovative but potentially high risk approaches, elucidating the molecular mechanism of action of anticancer drugs which have either previously undergone or are presently undergoing clinical trial may permit optimisation of compounds to improve the therapeutic index. This knowledge may also allow the development of strategies to overcome undesirable side effects. The disadvantage with this approach is that dramatic improvements in clinical performance are unlikely.

Aside from novel drug development approaches based on small molecule 'chemical' approaches additional strategies are being examined for the treatment of cancer (Workman *et al*, 1992). These include principally immunotherapy, antisense oligonucleotides, gene therapy, hormone and growth factor treatments.

To conclude the introductory section, although significant advances have been made in the treatment of specific types of cancer, approximately 25% of the population still die of the disease, the large majority as a result of metastatic spread. This highlights the urgent need for major improvements in systemic chemotherapy which may now be possible through innovative approaches to rational drug development.

1.2 Hypoxia and Bioreductive Antitumour Agents

1.2.1 Hypoxia in Tumours

Solid tumours are known to outgrow their vascular supply (Thomlinson and Gray, 1955; Coleman, 1988). This results in a diffusion gradient extending away from the capillaries along which the supply of oxygen and nutrients becomes progressively

decreased. Adjacent to vasculature are well oxygenated viable cells but at a distance of 100-200 μm from the nearest functional capillary is a population of anoxic, necrotic cells which receive insufficient oxygen and nutrients to maintain viability. Between these two extremes are cells which exist at an intermediate oxygen tension between fully aerated and anoxic conditions and which are known as hypoxic cells (Fig 1.1).

Cells which are permanently hypoxic due to the diffusion limitations described above are said to be chronically hypoxic. Areas also exist in experimental model tumours where hypoxia is more transient. This is known as acute hypoxia and results from microregional fluctuations in blood flow (Chaplin *et al*, 1986). The precise reasons for this phenomenon are at present unknown although suggestions include collapse of vessels due to high interstitial pressure in tumours (Sevick and Jain, 1989) and blocking of vessels by circulating blood or tumour cells (Jain, 1988).

Hypoxia is widely believed to be a therapeutic problem in solid tumours where it is thought to limit response to both radio and chemotherapy (Coleman, 1988). Since these therapy-resistant hypoxic cells are capable of repopulating a treated tumour, it is important to develop methods to identify and treat them.

Several methods have been employed for the detection of tumour hypoxia including fluorescent antibodies (Cline *et al*, 1990), labelled nitroimidazole hypoxic cell sensitisers (Urtasun *et al*, 1986; Franko *et al*, 1987), cryospectrophotometry (Mueller-Klieser *et al*, 1981), oxygen electrodes (see below), the comet assay (Olive *et al*, 1993) and magnetic resonance spectroscopy (Rofstad *et al*, 1988) amongst others. These methods actually measure different parameters. The only technique to measure oxygen partial pressure directly is the oxygen electrode. Using this method strong evidence for hypoxia within human solid tumours has been obtained. The median pO_2 in breast tumours was 30 mm Hg compared to 65 mm in normal breast tissue (Vaupel *et al*, 1991). Similarly an increase in the proportion of hypoxic cells has been observed in cervical cancer with a median value of 12 mm Hg in tumours and 48 mm Hg in normal cervix of nulliparous women (Hockel *et al*, 1991). Hypoxic regions have also been detected in brain tumours (Rampling *et al*, 1994). Intra-tumoural heterogeneity of pO_2 levels has been reported for human brain tumours (Rampling *et al*, 1994) as well as in human breast and cervix tumours (Kallinowsky *et al*, 1990). The percentage of cells existing at an oxygen tension of <2.5 mm Hg is however thought to be the value which is of most significance radiobiologically. Pooled data from a study of ten patients with glioblastoma showed that 28% of readings were less

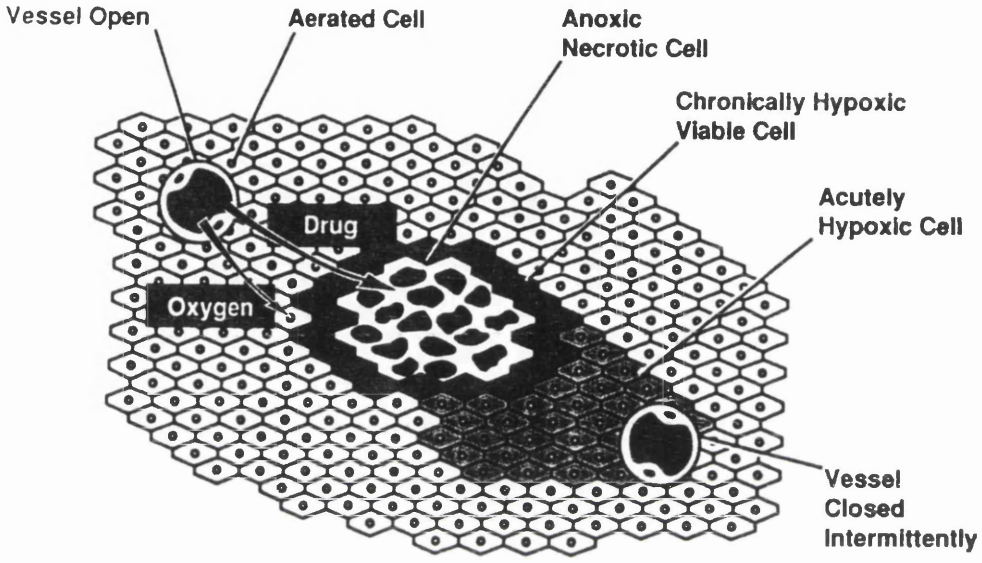


Fig 1.1 Schematic diagram of a solid tumour showing areas of chronic and acute hypoxia. Reprinted with permission from Workman (1993).

than 2.5 mm Hg (Rampling *et al*, 1994). In contrast, during the same study, a low grade tumour showed no readings of pO_2 <2.5 mm Hg.

1.2.2 Hypoxia-induced Radioresistance and Chemoresistance

The correlation between oxygen level and radiosensitivity was first made by Petry in 1923 (see Hall, 1978) although the effect had been observed previously by a number of other workers without realisation of the significance. Oxygen has been shown to sensitise both bacterial systems and mammalian cells to radiation when it is present either during or within a few milliseconds of radiation (see Hall, 1978). The nature of the oxygen effect is not fully understood but can be explained by the oxygen fixation hypothesis. This is based upon the fact that radiation induces formation of free radicals which may cause biological damage. In the presence of oxygen these free radicals can react to form organic peroxides which lead to a more permanent form of damage which can not be so easily repaired, e.g. by thiols (see Hall, 1978).

Studies performed to determine the amount of oxygen required for radiation sensitisation found little difference in radiation response between 12 mm Hg and 600 mm Hg oxygen tension. A rapid decrease in sensitivity occurs below 30 mm Hg. Fully oxygenated cells are approximately 3 times more sensitive to radiation than their anoxic counterparts (see Hall, 1978).

In 1955, whilst examining histological sections of lung carcinoma, Thomlinson and Gray discovered that the thickness of the viable cells in a solid tumour was always between 100-180 μm. At greater distances from the capillary cells were necrotic. This coincided with the calculated diffusion distance of oxygen which was estimated to fall to zero between 150-180 μm from the blood vessel. These findings suggested that hypoxic cells may exist within human tumours and led to a renewed interest in radiation insensitivity and hypoxia. Studies carried out using oxygen electrodes in breast, cervical carcinoma (Hockel *et al*, 1993) and head and neck cancer (Gatenby *et al*, 1988) have shown a good correlation between hypoxia and response to radiotherapy.

In addition to being hypoxic, solid tumours were also thought to be acidic in nature. This theory originally arose from work carried out by Warburg and co-workers in 1930 (see Griffiths, 1991) who reported a preferential conversion of glucose to lactic acid in tumours. Initial studies using pH electrodes confirmed this belief (reviewed in Wike Hooley *et al*, 1984 and Vaupel *et al*, 1989). However due to the relatively large

size of the micro-electrode compared to the cell, such measurements tend to reflect the extracellular pH (Vaupel *et al*, 1989). More recent studies have employed ^{31}P Nuclear Magnetic Resonance Spectroscopy, a non-invasive technique that enables measurement of intracellular pH (Griffiths, 1983). These have revealed that, if anything, the intracellular pH of tumours is neutral to slightly alkaline. It has been suggested that intracellular pH may be regulated by export systems of the cell which would result in a higher acidity of the extracellular fluid whilst still retaining intracellular pH at near neutrality (Griffiths, 1991).

A number of strategies have been developed to circumvent the problem of tumour hypoxia. These include:-

- fractionated radiotherapy
- increasing oxygen supply to the tumour
- radiation sensitisers
- hypoxia-activated cytotoxins

These different approaches will be discussed briefly in turn.

Fractionated radiotherapy works on the principal that oxic cells in the proximity of tumour vasculature will be killed by radiation thus permitting reoxygenation of previously hypoxic cells at a greater distance from the blood supply. Cells in these areas will then be killed by successive cycles of radiation (see Hall *et al*, 1978).

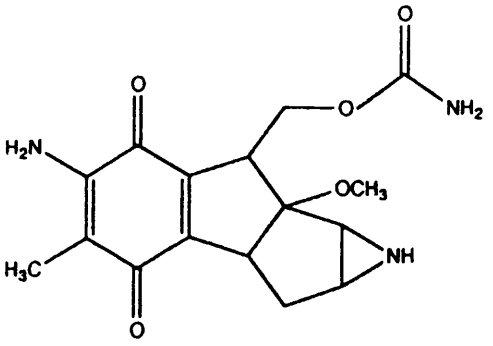
Attempts have been made to confer radiation sensitivity by increasing tumour oxygenation. Techniques employed include oxygen or carbogen breathing under normobaric or hyperbaric conditions (Suit *et al*, 1972), blood flow modifiers, (e.g. Hirst *et al*, 1993), perfluorochemical carriers (Coleman, 1988) and calcium antagonists (Kaelin, 1982). These approaches may have use in chronic hypoxia but are likely to provide only limited benefit with acute hypoxia.

Radiation sensitisers as their name suggests are chemicals which are able to sensitise the tumour cells to radiation therapy. Nitroimidazole compounds have been shown to function as effective radiation sensitisers *in vivo*. One of these agents, metronidazole (1-β-hydroxyethyl-2-methyl-5-nitro-imidazole), a 5-nitroimidazole compound, has been employed clinically as an antiparasitic drug (see Hall *et al*, 1978), This agent entered clinical trial for the treatment of cancer but was subsequently found to be less potent than some 2-nitroimidazoles such as misonidazole [α-(methoxymethyl)-2-nitro-1H-imidazole-1-ethanol] (see Workman and Stratford, 1993). Metronidazole

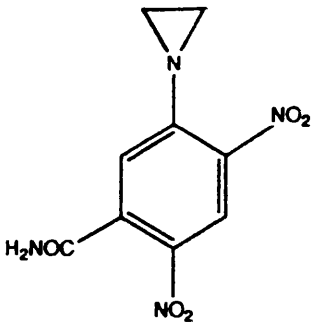
penetrated well into tumours and substantial sensitisation was reported in experimental tumours. Despite some evidence of clinical sensitisation the use of this agent was limited due to the peripheral and central neurotoxicity (Dische, 1985). Analogues of misonidazole, etanidazole [1H-imidazole-1-acetamide, N-(2-hydroxyethyl)-2-nitro , SR 2508, and pimonidazole [Ro 03-8799; 1-(2-nitroimidazole-1-yl)-3-(1-piperidino)-2-propanol] were subsequently developed in attempt to increase sensitisation whilst reducing toxicity. Phase III randomised clinical trials of these compounds are now underway. Etanidazole is more hydrophilic than misonidazole, is absorbed more slowly into the brain and peripheral nerves and thus causes less neurotoxicity (Brown and Workman, 1980). This compound is equally potent to misonidazole as a radiation sensitiser and can be given at three times the dose of misonidazole (Coleman *et al*, 1987). Pimonidazole possesses greater sensitising ability than misonidazole but is lipophilic, basic and readily accumulates in the tumour tissue (Newman *et al*, 1988). In contrast to etanidazole, the dose-limiting toxicity of pimonidazole is an acute CNS syndrome but this agent does not cause peripheral neuropathy (Roberts *et al*, 1980). As a result of their different toxic mechanisms and an additive radiosensitisation of pimonidazole and etanidazole in murine models clinical trials of the two agents in combination chemotherapy were initiated (Bleehen *et al*, 1989).

Other radiation sensitisers include a 5-nitroimidazole, nimorazole [4-[2-(5-nitroimidazole-1-yl) ethyl] morpholine] which has shown some activity in clinical trial (Overgaard *et al*, 1991) and RSU 1069 [(1-2-nitro-1-imidazoyl)-2-(1-aziridinyl)-2-propanol] (Fig 1.2). The latter compound is a mixed function agent containing a nitro group which provides sensitising ability as well as an aziridine moiety which gives it the capacity to act as a bifunctional hypoxic cell cytotoxin following reductive activation (Jenkins *et al*, 1990). Phase I clinical trial of RSU 1069 (Horwich *et al*, 1986) revealed dose-limiting gastrointestinal toxicity. Recently a prodrug of RSU 1069, RB 6145 [1H-imidazole-1-ethanol,α-([2-bromoethyl amino] methyl)-2-nitro, monohydrobromide] has been developed with a bromoethyl side chain (Jenkins *et al*, 1990). Radiosensitisation has been observed with this agent in animal models (Adams *et al*, 1992) and clinical trials are awaited with one of the enantiomers.

These radiation sensitisers were found to have activity dependent on their redox potential or 'electron affinity' and thus were known as electron-affinic radiosensitisers. A comprehensive review by Adams *et al* (1992) summarises the characteristics and mechanisms of action of these agents. In brief, one-electron reduction potential was found to reflect the radiosensitising efficiency of the compounds. It has been



Mitomycin C



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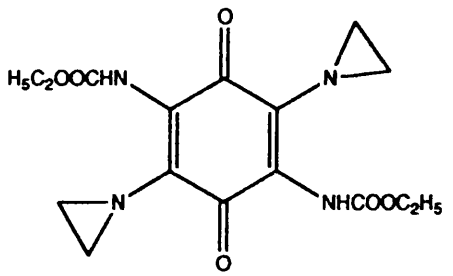
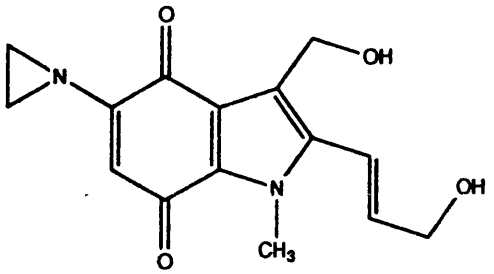
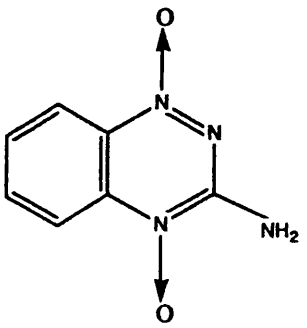


AZQ



EO9



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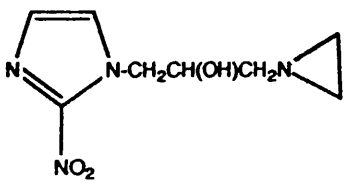


RSU 1069

Fig 1.2 Chemical structures of key bioreductive anticancer agents

proposed that the radiation sensitiser localises close to DNA, traps electrons generated by radiation and thus affects the distribution of free radical centres on DNA. This then leads to DNA damage. The review by Adams also discusses the ability of radiation sensitisers to act as bioreductive anticancer agents. This feature is also related to electron affinity but occurs by different mechanisms to those which cause radiation sensistisation.

1.2.3 Bioreductive Anticancer Agents

Bioreductive anticancer agents, by definition, are compounds which require metabolic reduction to generate their toxic species. Although hypoxia is not actually required for bioreductive activation, preferential metabolism to the cytotoxic species often occurs under low oxygen conditions. This is due to enhanced stability of oxygen sensitive metabolites.

The main bioreductive agents employed in cancer therapy may roughly be divided into three main categories, namely nitro compounds, N-oxides and quinone compounds. In addition various novel agents can be grouped into a final miscellaneous category. The chemical structures of key compounds which fall into these categories are shown in Fig 1.2.

Nitro Compounds

In addition to their activity as radiation sensitisers the nitroimidazoles were also found to exhibit activity as bioreductively activated hypoxic cell cytotoxins. Misonidazole showed an 11 fold increase in toxicity in V79 cells under hypoxic compared with oxic conditions. Metronidazole is less electron affinic with a hypoxic : oxic cytotoxicity ratio (HCR) of 2 whereas that of the highly electron affinic nitrofurazone was 8.5 (Stratford and Stephens, 1989).

As described previously, misonidazole was modified by replacing the methoxy group with an aziridine moiety to generate RSU 1069 (Adams *et al*, 1984) . The aziridine group provides an alkylating moiety and gives improved potency and selectivity as a hypoxic cell cytotoxin (Silver and O'Neill, 1986). The HCR increased to 67 for V79 cells and this was attributed to the bifunctional character of the compound (Stratford and Stephens, 1989). The bromoethyl prodrug of RSU 1069, RB 6145 retains hypoxic cell selectivity whilst showing reduced toxicity in animals (Jenkins *et al*, 1990).

Nitroimidazole activation is believed to be a multistep process involving a variety of enzymes. The cytotoxic metabolites are thought to be the one-electron reduced nitro anion radical, the two-electron reduced nitroso and the four-electron reduced hydroxylamine. The six-electron reduced amine derivative is not considered to be important in the mechanism of cytotoxicity (see Workman, 1992a). Under aerobic conditions free radical generation following redox cycling of the radical anions may predominate. The ultimate DNA alkylating species is undetermined but is believed to be generated from the nitroso or hydroxylamine or their ring-opened cleavage products under hypoxic conditions. In the case of aziridinyl nitroimidazoles e.g. RSU 1069 the possession of two reactive centres, the reduced nitro group and the aziridine, provide sites for alkylation of DNA and thereby DNA cross-linking (Silver and O'Neill, 1986).

Another nitro compound which sensitises hypoxic cells (Stratford *et al*, 1981) is CB 1954 (Fig 1.2; 5-(aziridin-1-yl)-2,4-dinitrobenzamide). In addition to its radiosensitising ability CB 1954 also acts as a hypoxic cell cytotoxin. Although this agent was highly potent and selective in its activity against the rat Walker tumour (Khan and Ross, 1969 / 1970) it did not display any significant effect against other transplantable tumours (Cobb *et al*, 1969; Connors and Melzack, 1971). CB 1954 did however cause only minimal haematological toxicity but at toxic doses damage to the liver and urinary tract was observed (Cobb, 1970).

Despite the lack of antitumour activity displayed against a range of animal and human tumour xenografts (Workman *et al*, 1986), CB 1954 was entered in to clinical trial in the 1970's (see Knox *et al*, 1993) as a result of the extraordinarily high therapeutic index observed in the Walker tumour model. Toxicity was minimal with diarrhoea being the most significant side effect. No bone marrow toxicity or liver dysfunction was noted. However, disappointingly, no tumour regression was observed and CB 1954 was labelled as ' a drug in search of a human tumour to treat' (Workman *et al*, 1986).

N-oxides

The lead compound of the benzotriazine di-N-oxides is SR 4233 (3-amino-1, 2, 4-benzotriazine 1,4-dioxide; WIN 59075) also known as tirapazamine (Fig 1.2). This agent exhibits a high degree of hypoxic cell selectivity *in vitro* (Zeman *et al*, 1986;

Costa *et al*, 1989; Zeman *et al*, 1989) with HCRs as large as 75-200 and 15-50 being reported for rodent and human tumour cells respectively (Zeman *et al*, 1986). Unlike many other hypoxic cell cytotoxins SR 4233 shows an increased cytotoxic potency over a wider range of oxygen concentrations (Koch, 1993). In addition to its activity *in vitro* SR 4233 also appears to act as an effective antitumour agent in experimental tumours *in vivo*. Thus it caused extensive cell death when used in combination with fractionated radiotherapy (Zeman *et al*, 1988; Brown and Lemmon, 1990) or with agents which induce tumour hypoxia (Sun and Brown, 1989). SR 4233 is currently undergoing phase I clinical trial in Glasgow, UK, Stanford and Harvard, USA.

Several enzymes are involved in the bioreductive activation of SR 4233 including cytochromes P450 (Walton and Workman, 1990; Walton *et al*, 1992c), cytochrome P450 reductase (Cahill and White, 1990; Lloyd *et al*, 1991, Walton *et al*, 1992c), xanthine oxidase (Walton and Workman, 1990), aldehyde oxidase (Walton and Workman, 1990) and DT-diaphorase (see Section 1.4). Experiments using mouse liver microsomes indicate that cytochromes P450s, particularly the subfamilies IIB, IIC and IIIA, are the most important enzymes involved in metabolism of SR 4233 (Walton *et al*, 1992c). Other authors have found somewhat different results and it appears that the involvement of particular enzymes in activation of SR 4233 may differ between species and with oxygen status.

HPLC studies have detected the two (SR 4317) and four-electron (SR 4330) reduced compounds as the predominant metabolites (Baker *et al*, 1988; Walton and Workman, 1990). Neither of these species was found to be toxic (Baker *et al*, 1988; Costa *et al*, 1989). It was therefore suggested that SR 4233 is metabolised to form SR 4317 via a radical intermediate (Lloyd *et al*, 1991), which, in the presence of air, is thought to undergo auto-oxidation and redox cycling to generate oxygen radicals. Under hypoxia it has been proposed that the intermediate is able to gain a second electron by extracting hydrogen from DNA and thus forms the stable mono-N-oxide SR 4317 while concomitantly causing DNA double strand breaks (Costa *et al*, 1989). This damage is believed to be responsible for the cytotoxicity observed.

Analogues of SR 4233 are also being investigated. One of these, the 3-desamino analogue is also highly potent but has reduced systemic toxicity (Zeman *et al*, 1989). Further studies are in progress.

Quinones

Quinone compounds are widely distributed in nature and a large number of both naturally occurring and synthetic quinones has been screened for antitumour activity. Several promising compounds were identified and have entered clinical trial including streptonigrin, mitoxantrone, mitomycin C (Fig 1.2), adriamycin and the aziridinyl-benzoquinones.

Many of these quinones are bioreductive alkylating agents, that is they possess groups which, following reduction, are capable of alkylating cellular targets. The prototype bioreductive alkylating agent is the quinone-containing antibiotic mitomycin C. This compound was first isolated in 1958 from *Streptomyces caespitosis* by Wakaki and co-workers at the Kyowa Fermentation Institute, Japan (Wakaki *et al*, 1958). Antitumour activity was noted and in 1960 clinical trials of mitomycin C were carried out in Japan. Myelosuppression and resistance were observed; however, by altering the dose schedule these problems were reduced and mitomycin C was approved for clinical use in the USA by 1974.

The literature contains many reports of clinical trials involving mitomycin C. It is effective against a number of tumours where it is commonly used in combination chemotherapy regimes. Activity has been observed in gastrointestinal tract cancer and particularly in stomach cancer where it is used as part of the FAM regime (5-fluorouracil, adriamycin and mitomycin C). It also has a role in the treatment of both superficial and bladder cancer *in situ* where it is administered by intravesical installation therapy, as second and third line therapy in breast cancer and in non-small cell lung carcinoma (Veeder *et al*, 1992) where it is given in combination with cisplatin and ifosphamide. Some single agent activity has been observed in prostate cancer. The main problem with mitomycin C is a delayed and somewhat unpredictable myelosuppression.

Mitomycin C acts as a potent cell cytotoxin *in vitro* and exhibits selective toxicity towards hypoxic cells (Iyer and Szybalski; 1963; Kennedy *et al*, 1980; Rockwell *et al*, 1982; Rauth *et al*, 1983). The hypoxic : oxic cytotoxicity ratio is however lower than that of many of the other bioreductive anticancer agents and for some cell lines no enhancement in potency was observed (Keyes *et al*, 1984). Some hypoxic cell selectivity has been observed for mitomycin C in experimental tumours *in vivo* (Rockwell and Kennedy, 1979; Rockwell, 1983).

Several enzymes have been identified as being able to catalyse reduction of mitomycin C including NADPH: cytochrome P450 reductase (Bachur *et al*, 1979; Pan *et al*, 1984), xanthine oxidase (Pan *et al*, 1984; Bligh *et al*, 1990), xanthine dehydrogenase (Gustafson and Pritsos, 1992a and b) and DT-diaphorase (see Section 1.4). The precise mode of reduction and the identity of the cytotoxic species are not fully understood but it appears that they may differ under aerobic and anaerobic conditions.

The cytotoxic mechanism of mitomycin C is thought mainly to involve DNA damage via alkylation with oxygen radical damage also being important in air. Following either one or two-electron reduction the C1 position of the aziridine moiety is activated and can form an adduct with guanine at the N-2 position (Iyer and Szybalski, 1963). Loss of the carbamate group generates a second reactive centre at C-10 which can form an adduct with guanine N-2 to generate a cross-link in the minor groove of DNA (Tomasz *et al*, 1987).

Several aziridinylbenzoquinones have undergone development as anticancer agents including trenimon (triaziquone; 2,3,5-tris (aziridinyl)-1,4-benzoquinone), carbaziliquone (carboquone; 2,5-bis(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl-)-6-methyl-1,4 benzoquinone), AZQ (diaziquone; 3,6-bis(carboxyamino)-2,5-diaziridinyl-1,4-benzoquinone) and BZQ (3,6-bis[(2-hydroxyethly)amino]-2,5-diaziridinyl-1,4-benzoquinone). AZQ (Fig 1.2) was synthesised by Driscoll and co-workers in 1976 (Chou *et al*, 1976) at the NCI following a screening programme in which aziridinyl compounds showed activity in a number of murine tumour models. It was designed to have high lipid solubility and low ionisation potential to aid passage across the blood-brain barrier whilst being water soluble to facilitate formulation. The carboethoxyamino groups confer lipid solubility whilst the quinone and aziridine moieties allow reductive activation and alkylation reactions to occur respectively. BZQ was also synthesised by Chou and co-workers (1976). This has greater lipid solubility than AZQ and is more easily synthesised.

In animal models AZQ was active against intraperitoneal L1210 leukaemia, P388 leukaemia and B16 melanoma (O'Brien, 1985) as well as against murine intracranially and intraperitoneally implanted tumours (Driscoll, 1979). Experiments carried out *in vivo* in athymic mice found AZQ to be active against human glioma and medulloblastoma. An increase in growth delay was observed when it was combined with carmustine or procarbazine but only slight enhancement of therapeutic effect was

seen when combined with cyclophosphamide, cisplatin or vincristine (Schold *et al*, 1984).

Human Phase I, II and III clinical trials have been performed. As a single agent AZQ showed activity in brain tumours (Castleberry *et al*, 1990; Berg *et al*, 1992) and in acute nonlymphocytic leukaemia as a second line or consolidation therapy in first remission patients when given as a continuous infusion (Lee *et al*, 1986; Schulman *et al*, 1987). Little effect has been reported against other tumour types. Dose-limiting toxicity consisted of delayed myelosuppression and thrombocytopaenia with few non-haematological side effects (Schilsky *et al*, 1982). The plasma clearance is fairly rapid with a half-life of 30 minutes (Schilsky *et al*, 1982). AZQ has also been investigated as an agent in combination chemotherapy e.g. with 2,2-anhydro-arabinosyl-5-fluorocytosine (Meyers *et al*, 1989) and etoposide (Lee *et al*, 1990).

AZQ, like mitomycin C, possesses the quinone and aziridine moieties, thus suggesting that bioreductive alkylation may be involved in its mechanism of cytotoxicity. It is highly cytotoxic with a large variation in potency being observed between cell lines and exhibits selectivity towards hypoxic cells (for detail see Chapter 7). This was most marked below 0.6 mm Hg oxygen concentration.

Reduction has been suggested to be important for AZQ activation. Evidence for AZQ metabolism within intact cells has been provided by electron spin resonance (ESR) experiments (Gutierrez *et al*, 1985). Further studies have shown that microsomes, rat liver nuclei, purified NADPH: cytochrome P450 reductase (Gutierrez *et al*, 1982; Fisher and Gutierrez, 1991a) and DT-diaphorase (Siegel *et al*, 1990a; Ross *et al*, 1990; Fisher and Gutierrez, 1991a and b) are all able to catalyse reduction of this compound.

The mechanism of cytotoxicity is believed to involve DNA damage. Following treatment of cells with AZQ both DNA strand breaks and interstrand cross-links have been detected (King *et al*, 1984; Szmigiero *et al*, 1984) although cell killing correlates with cross-link formation rather than strand break induction. It has been suggested that cross-link formation may involve alkylation through the aziridine groups with activation to the DNA damaging species occurring in the nucleus (King *et al*, 1984). Indeed in isolated nuclei and in the presence of cofactor AZQ has been shown to induce strand breaks via oxygen radical formation as well as interstrand cross-links (Szmigiero and Kohn, 1984). With isolated DNA, AZQ caused both DNA strand

breaks and DNA interstrand cross-links when chemically reduced (King *et al*, 1984) or enzymatically using DT-diaphorase (Lee *et al*, 1992).

AZQ may induce cytotoxicity via a number of mechanisms including free radical damage and / or alkylation following reductive activation. The enzyme profile of the cell will determine the spectrum of metabolites generated and might therefore influence sensitivity towards this agent.

EO9 [(3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4, 7-dione)prop-β-en-α-ol) ; Fig 1.2] is the lead compound in a series of novel indoloquinone anticancer agents synthesised by Oostveen and Speckamp (1987) at the Free University Hospital in Amsterdam. Its structural similarity to mitomycin C suggests that it may also undergo bioreductive activation to form an alkylating agent. However it exhibits a different antitumour profile from mitomycin C, being generally a more potent cytotoxin with particular preference for solid tumours over leukaemias (Hendriks *et al*, 1993). It also has the advantage of showing an improved hypoxic cell selectivity (for further information see Chapter 4). As discussed previously (Section 1.2) tumour pH may also play a role in antitumour activity. In a similar manner to mitomycin C, decreasing pH has been found to increase the cytotoxic potency of EO9 and to decrease stability of the drug (Phillips *et al*, 1992). It is presently unclear how pH will affect the activity of EO9 *in vivo*.

An indication of possible solid tumour selectivity of EO9 *in vivo* came from studies using the Corbett two tumour assay (Hendriks *et al*, 1993). This involves examining the sensitivity of tumour cell lines derived directly from solid tumours. Further experiments using OC-Tol small cell lung carcinoma cells (Roed *et al*, 1989) have also suggested that the cytotoxicity data obtained *in vitro* correlates well with activity seen *in vivo*.

The activity of EO9 was assessed in a range of animal models. This agent was inactive against the P388 murine leukaemia as well as the ascetic murine tumour MAC 15 and subcutaneous MAC 13. Moderate activity was however detected against the mouse colon carcinomas MAC 16 and MAC 26 (Walton *et al*, 1992a; Hendriks *et al*, 1993).

Antitumour activity was also investigated using human tumour xenografts (Hendriks *et al*, 1993). EO9 was administered to the tumour-bearing mice at a dose of 4-6 mg/kg by intravenous injection at days 0 and 7. No antitumour activity was observed

in the renal RXF 243 xenograft whereas a growth delay was detected in the breast MAXF449 and LXFL 329 tumours and regression was seen in gastric GXF97 and ovarian MRI-H-207 xenografts. Similar experiments carried out with mitomycin C indicated a comparable antitumour profile in the tumour xenografts.

Toxicology and pharmacokinetics of EO9 administered to mice and rats were examined (Hendriks *et al*, 1993). In C3H/HE mice side effects observed following a single i.v. injection of the drug included transient sluggishness with piloerection, pallor and transient irritation at the injection site. Interestingly however, no bone marrow toxicity was detected. The LD_{50} values were 12.99 mg/kg in male mice and 13.95 mg/kg in female mice with death occurring between 3 and 25 days following drug administration. Again where mice were given multiple dose regimes at 1.5 mg/kg no drug related death or bone marrow toxicity was detectable. Side effects included temporary anaemia, decrease in overall body weight as well as changes in the weight of other organs including testes, spleen, kidney and liver, congested blood vessels and haemorrhagic spots on the jejunum. Rats were injected i.v. with a single dose of EO9 corresponding to the LD_{10} obtained in mice (2.7 mg/m^2). This was found to be safe with no display of toxicity. Where multiple injections (2.7 mg/m^2) were given to rats the only detectable problem was inflammation at the injection site. Dose-limiting toxicity in animal studies was gastrointestinal. As a result of these animal studies a starting dose of 2.7 mg/~~kg~~ m^2 was recommended for the human clinical trial.

Preclinical pharmacokinetic studies were also carried out in rats, mice (Binger and Workman, 1990; Workman *et al*, 1992) and finally in a small number of beagle dogs (see Workman *et al*, 1992). Interestingly the half-life of EO9 (1.9 minutes) in mice is far less than that of mitomycin C (16 minutes) and plasma levels achieved are four times higher for mitomycin C. It is possible that the reduced cytotoxicity *in vitro* (Hendriks *et al*, 1993), increased half-life and plasma levels seen with mitomycin C compared with EO9 may be due to a decreased rate of metabolism. In beagle dogs (Workman and Binger, personal communication) a higher plasma concentration was achieved than in mice and also a longer half-life. In addition, the low toxicity observed suggested that doses of EO9 required for activity could be attained.

Following the extensive preclinical evaluation, *in vivo* EO9 was taken into clinical trial. Phase I studies were carried out on patients aged between 45 and 73 bearing solid tumours (Schellens *et al*, 1993, 1994). Most were colo-rectal tumours and patients had received prior chemo or radiotherapy. EO9 was administered as an i.v.

infusion for 5 minutes at 3 weekly intervals. Pharmacokinetic data were obtained. A wide variation between patients was observed with a range of total plasma clearance values of 3.2 to 9.7 l/minute. Elimination from the central compartment was rapid and non-linear with a terminal half-life of 0.8 to 19 minutes, protein binding was 62± 8%. Negligible amounts of EO9 and the hydrolysis product EO5A were found in the urine. Drug levels attained were higher than those achieved in animal models. Dose-limiting toxicity occurred at 27 mg/m^2 and was proteinuria associated with temporary oedema, sodium and water retention and serum creatine increase. Hypertension also occurred in 2 of the patients. Moderate nausea and vomiting but no bone marrow toxicity was observed. Most symptoms were reversible at day 15. Results appeared promising with partial responses being observed. One patient bearing an adenocarcinoma of unknown primary origin showed a 50% reduction in tumour size and a second also with adenocarcinoma of unknown primary origin showed a 50% reduction of lung metastasis and stabilisation in liver metastasis. Finally a third patient with bile duct cancer exhibited a 45% tumour reduction in liver metastasis.

A second phase I clinical trial using a different schedule and phase II trials are now underway .

Novel Compounds

In addition to the bioreductive agents described efforts have been initiated to develop novel types of compounds. Denny's group have examined the possibility of using prodrugs that are activated under hypoxia to deliver diffusible cytotoxins which would kill adjacent cells (Denny and Wilson, 1993). This could overcome the problem of cells which are at an oxygen pressure low enough to be sensitive to radiation but too high to activate the bioreductive drugs. Denny and Wilson (1993) suggested that the compound should be activated to an initial one-electron reduced intermediate that would be sensitive to oxygen and thus selective activation would only occur in hypoxic cells. The ultimate cytotoxic species should however not be affected by oxygen status, pH or cell cycle and thus could diffuse out of the activating hypoxic cell to kill adjacent oxic cells.

Mustard compounds fit the criteria outlined above. The addition of either a nitro group or cobalt (III) to form nitro-deactivated aromatic mustards or cobalt (III) complexed deactivated aliphatic mustards provides compounds which can be activated following reduction. Development of these drugs is presently underway.

Another approach is to use DNA intercalators. Wilson *et al* (1992) described a nitroacridine which although being active *in vitro* was poor *in vivo* . The failure of the nitroacridines is thought to be due to the fact that they are such potent intercalators that their extravascular diffusion to the chronically hypoxic region of the tumour is limited.

Patterson (1993) has examined the potential of developing bioreductive drugs which are active against acute hypoxia. Anthraquinones have been developed to fulfil these requirements, of which the lead compound is the N-oxide prodrug, 1,4-bis{[2-(dimethyl-amino-N-oxide)ethyl]-amino}5,8-dihydroxyanthracene-9,10-dione (AQ4N). Under hypoxic conditions this can be reduced to AQ4 which is stable in air and is DNA-affinic.

1.2.4 Enzymes Involved in Bioreductive Activation of Anticancer Agents

Numerous enzymes can catalyse the reduction of bioreductive agents. The main ones will be discussed in turn, with particular emphasis on DT-diaphorase which is a central focus of this thesis.

Xanthine Oxidase and Dehydrogenase

Xanthine oxidase and dehydrogenase convert xanthine to uric acid. During this process NAD is reduced to NADH by xanthine dehydrogenase and superoxide is produced by xanthine oxidase. Xanthine dehydrogenase (EC 1.1.1.204) is more prevalent in the cell than is xanthine oxidase (Battelli *et al*, 1972; Anderson *et al*, 1989). Xanthine dehydrogenase exists as a dimer with one flavin adenine dinucleotide molecule and two iron-sulphur centres (Amaya *et al*, 1990) and may undergo oxidation or proteolytic cleavage to form xanthine oxidase (Porras *et al*, 1981). This may also occur during purification, thus rendering separation of the two enzymes difficult. The proteolytic conversion is irreversible and it has been suggested that this may involve a Ca^{2+}-activated protease (McCord, 1985) whereas the oxidative conversion is reversible and occurs via reduction of oxidised thiols (Stripe and Della Corte, 1969).

Studies involving purified preparations of xanthine oxidase and xanthine dehydrogenase have found both enzymes to be capable of metabolising mitomycin C (for references see Section 1.2.3).

Doxorubicin *in vivo* increases both xanthine oxidase and xanthine dehydrogenase activities in rat kidney and it appears that this is due to an increase in protein levels (Ghiggeri *et al*, 1990). Cardiac tissue levels of xanthine oxidase in the mouse are also increased following exposure to doxorubicin (Gustafson *et al*, 1991). These authors suggested that this resulted from a conversion of xanthine dehydrogenase to the oxidase form.

Carbonyl Reductase

Carbonyl reductase (EC 1.1.1.184) is a cytosolic, monomeric NADPH-dependent oxidoreductase. It exhibits a broad substrate specificity (Jarabak *et al*, 1991) and metabolises a variety of quinone compounds including menadione (Wermuth *et al*, 1986) A number of K-region and non-K-region polycyclic aromatic hydrocarbon o-quinones and their glutathione adducts (Jarabak *et al*, 1991) as well as benzo and naptho-quinones (Wermuth *et al*, 1986) act as the best substrates. The enzyme has been purified from a number of sources including human, pig and chicken (see Wermuth *et al*, 1986) The carbonyl reductase isolated from human liver and brain has been found to consist of multiple molecular forms and enzyme from both sources were able to metabolise a wide range of quinone substrates with equal efficiency (Wermuth *et al*, 1986). The enzyme is also maintained in permanent cell lines (Gebel and Maser, 1992). Little is as yet known about its physiological role although it has been suggested that it may be involved in quinone metabolism (Wermuth *et al*, 1986). Wermuth and co-workers (1986) have suggested that although DT-diaphorase may be the principal two-electron transferring quinone reductase in rat liver, carbonyl reductase may be more important for this role in human liver. The enzyme is selectively inhibited by rutin (Wermuth, 1981) and quercetin (Felsted and Bachur, 1980) although there are also indications that indomethacin and dicoumarol may inhibit carbonyl reductase (see Gebel and Maser, 1992)

Dihydrodiol Dehydrogenase

Dihydrodiol dehydrogenase (EC 1.3.1.20) is a cytoplasmic enzyme that oxidises aromatic dihydrodiols to catechols. It utilises NADPH as a cofactor (see Klein *et al*, 1992). In addition to metabolism of the dihyrodiols, dihydrodiol dehydrogenase purified from rat liver reduces a range of quinone compounds although menadione itself is not a substrate. Redox cycling with concomitant generation of toxic radicals has also been observed during quinone reduction catalysed by this enzyme (Klein *et al*, 1992)

Cytochrome P450

The cytochromes P450 are haem proteins which absorb at 450 nm on binding of carbon monoxide and show considerable inter- and intraspecies variation. The human cytochrome P450 superfamily is comprised of at least 20 genes, the protein products of which have a wide substrate specificity. These enzymes catalyse a number of reactions including hydroxylations, dehalogenations, dealkylations, deaminations and reductions. Each family member has its own characteristic substrates and regulatory mechanisms although some overlap does exist between enzymes. Regulation may be controlled at the transcriptional level (Gonzalez and Nebert, 1990) by methods involving the aromatic hydrocarbon receptor (Ah), xenobiotic response element (XRE) and steroids or may involve other mechanisms such as substrate-induced protein stabilisation e.g. by ethanol (Gonzalez and Nebert, 1990). Carbon monoxide acts as an inhibitor of cytochromes P450 and therefore of microsomal oxidations. In contrast cyanide inhibits mitochondrial oxidation.

NADPH: Cytochrome P450 Reductase

NADPH: cytochrome P450 reductase (cytochrome c reductase; NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4) is an 80 kD flavoprotein which has activity linked to cytochrome P450 (Haniu *et al*, 1986). The two enzymes usually exist together in the endoplasmic reticulum (Hall *et al*, 1989) although cytochrome P450 reductase can occur and function alone. NADPH: cytochrome P450 reductase supplies electrons to both mitochondrial cytochrome P450 and enzymes involved in fatty acid metabolism (Ilan *et al*, 1981; Dailey and Strittmatter, 1980). It has been reported to exist in a number of tissues including human and rat brain (Ghershi-Egea, 1993). This enzyme has been shown to metabolise a number of bioreductive drugs including SR 4233 (Cahill and White, 1990; Walton *et al*, 1992c), mitomycin C (Bachur *et al*, 1979) and AZQ (Fisher and Gutierrez, 1991a).

NADH-Cytochrome b_5 Reductase

NADH-cytochrome b_5 reductase is an oxidoreductase associated with the haemeprotein cytochrome b_5. It contains one FAD group (Strittmatter, 1965), is located on the outer membrane of mitochondria and utilises external NADH as an electron donor. NADH-cytochrome b_5 reductase can catalyse reduction of ferricyanide and quinones.

Nitric Oxide Synthase

Nitric oxide synthase (EC. 1.14.13.39) consists of a number of isoforms all of which are dimeric proteins with molecular weights ranging from 130 kD - 160 kD. They act to convert L-arginine to nitric oxide, an endogenous vasodilator. Modulation of nitric oxide synthase by drugs e.g. Nitro-L-arginine (Wood *et al*, 1993) may therefore alter tumour sensitivity to both radiation and bioreductive drugs. In addition protein sequencing data has shown significant amino acid homology between the C terminus of the enzyme and that of rat liver NADPH: cytochrome P450 reductase (Bredt *et al*, 1991). Thus it is possible that nitric oxide synthase may be capable of metabolising bioreductive drugs.

1.3 DT-diaphorase (NAD(P)H: (quinone acceptor) oxidoreductase (NQO) (E.C. 1.6.99.2))

1.3.1 Historical Background and Characteristics

DT-diaphorase was first discovered in 1955-6 by Ernster and Navazio whilst studying the intracellular distribution of NAD and NADP-dependent dehydrogenases and was reported by these authors in 1958. The enzyme was found to utilise the cofactors NADH and NADPH with equal efficiency (Ernster and Navazio, 1958), to be present in the cytosol and to be inhibited by a variety of compounds. DT-diaphorase was subsequently purified and characterised (Ernster *et al*, 1962). It was highly sensitive to inhibition by dicoumarol (Ernster *et al*, 1962), a property which was consistent with a previously described enzyme vitamin K reductase (see Ernster, 1987). Despite original differences in other properties described for the two enzymes later reports indicated that the two enzymes were in fact identical.

The enzyme was originally named DT-diaphorase (Ernster *et al*, 1962) due to its ability to utilise both NADH and NADPH, formerly known as DPNH and TPNH, as cofactors. Subsequently it has been referred to by a variety of other names including vitamin K reductase, phylloquinone reductase, menadione reductase, quinone reductase, nicotinamide menadione oxidoreductase (NMOR) and NAD(P)H: (quinone acceptor) oxidoreductase (NQO1) (E.C. 1.6.99.2).

DT-diaphorase is a dimeric flavoenzyme consisting of two equally sized (32 kD) subunits and binding two molecules of FAD (Hojeberg *et al*, 1981). It is capable of

catalysing direct two-electron reduction (Iyanagi and Yamazaki, 1970). Although the mechanism of electron transfer is not yet definitively established it has been suggested that two rapid, successive one-electron reduction reactions may occur while the substrate remains bound to the enzyme (Iyanagi, 1987).

DT-diaphorase is widely distributed in the animal kingdom where both inter and intra-species variation has been observed (see Ernster, 1987; Lind *et al*, 1973).

The physiological role for DT-diaphorase is not clear. It has however been suggested that it may be involved in quinone detoxification as well as in the vitamin K-dependent carboxylation of prothrombin (see Ernster, 1987).

Quinones, as discussed previously, commonly exert their toxicity via the semiquinone radical and / or oxygen radicals formed during redox cycling. Two-electron reduction catalysed by DT-diaphorase would bypass this toxic pathway, thus acting as a detoxification mechanism. In support of this theory is the observation that the toxicity of some quinones e.g. menadione (Roberts *et al*, 1989) and streptonigrin (Hochstein unpublished data in Ernster, 1967) is enhanced by the DT-diaphorase inhibitor dicoumarol. In addition, treatment of rats with compounds such as azo dyes led to an increase in DT-diaphorase levels and subsequently to induced resistance and reduced carcinogenic effects of these compounds (Huggins *et al*, 1965). The initial interest for a role of DT-diaphorase in vitamin K-dependent carboxylation of prothrombin came from the finding that dicoumarol and other oral anticoagulants were able to inhibit DT-diaphorase. This role is as yet unconfirmed.

1.3.2 Purification

The first reports of DT-diaphorase purification date back to the 1960s when the enzyme was isolated from both rat (Ernster *et al*, 1960) and beef liver (see Ernster,1987). Subsequently, however, DT-diaphorase has also been purified from a range of sources and from both induced and uninduced animals. Initially conventional purification methods were employed which involved largely acetone precipitation, fractionation with ammonium sulphate and DEAE-cellulose chromatography (Ernster, 1967). Several modifications were made and alternative procedures have been adopted. The more favoured of these appears to be affinity chromatography, which reduced the number of purification steps and resulted in a more homogenous enzyme preparation with a yield of ~80% (Sharkis and Swenson, 1989)

Purification has also permitted isolation of isoenzymes of DT-diaphorase with varying substrate specificities. A number of different forms of rat liver DT-diaphorase have been detected (Raftell and Bloomberg, 1980; Hojeberg *et al*, 1981). Segura-Aguilar and Lind (1987) showed the presence of three different forms using chromatofocusing techniques. It has been reported that two different isoforms, a hydrophobic and a hydrophilic type, exist in mouse liver (Prochaska and Talalay, 1986).

In humans, genetic evidence indicates the existence of four gene loci encoding diaphorases (oxidoreductases) denoted DIA1-DIA4. DIA4 corresponds to the gene encoding DT-diaphorase (NQO1) and is located on chromosome 16 (Jaiswal *et al*, 1988). A second human diaphorase, designated NQO2, located on chromosome 6 has recently been identified (Jaiswal *et al*, 1990) and has both DNA and protein sequence homology with DT-diaphorase. The substrate specificities of DT-diaphorase and NQO2 protein differ with the NQO2 protein being 50-100 fold less active at reducing DCPIP and menadione. Induction also differs between the two isoforms.

1.3.3 Expression and Induction

DT-diaphorase is expressed in most animal tissues (Edwards *et al*, 1980) but its distribution varies widely. In animals the tissue which expresses the highest levels of DT-diaphorase is the liver although it is also present in the heart, kidney, brain, small intestine, skeletal muscle and mammary glands. High levels of DT-diaphorase have been found in preneoplastic liver nodules of rats treated with chemical carcinogens (Schor *et al*, 1978). In humans, high levels of DT-diaphorase are present in stomach and kidney (Schlager and Powis, 1990). Interestingly, increased DT-diaphorase expression has been reported in tumour tissue such as human colon carcinoma (Schor and Cornelisse, 1983), non-small cell lung carcinoma (Malkinson *et al*, 1992) and hepatocellular carcinoma (Cresteil and Jaiswal, 1991) compared with normal tissue of the same origin (see also Schlager and Powis, 1990). There are however some exceptions. Lower DT-diaphorase activity has been reported in stomach and kidney tumours compared to normal tissues (Schlager and Powis, 1990). An increase in DT-diaphorase mRNA expression has also been observed in normal liver surrounding the tumours of patients with hepatocellular carcinoma compared with that of normal individuals (Cresteil and Jaiswal, 1991).

Smoking and alcohol have been reported to affect DT-diaphorase levels (Shlager and Powis, 1990; Gasdaska *et al*, 1993). Patients smoking at the time of examination had

similar DT-diaphorase expression in tumour tissue to normal tissue whereas non-smokers and past smokers had elevated levels in tumour tissue. Alcohol induced an increase in lung tumour DT-diaphorase but had no effect on normal lung tissue.

At the subcellular level DT-diaphorase is mainly located in the cytosol (>90%) although it is also present in mitochondria and microsomes (Ernster *et al*, 1960; 1962). It is believed to be located in the matrix or inner surface of the inner membrane of mitochondria (Conover and Ernster, 1962) and to exist as a glycoprotein form on the cytosolic surface of the endoplasmic reticulum.

Induction of DT-diaphorase was first reported in the 1960's by Huggins and co-workers (Huggins *et al*, 1965). These authors observed increased levels of DT-diaphorase in the liver and other tissues of rats and mice treated with polycyclic aromatic hydrocarbons and azo dyes. In addition these animals developed resistance to the toxic and carcinogenic effects of the inducers. Since these initial descriptions induction has been widely studied and many compounds have been identified as inducers of DT-diaphorase both *in vitro* and *in vivo*. These include the polycyclic aromatic hydrocarbons such as β-napthoflavone (Kumaki *et al*, 1977), 3-methylcholanthrene (e.g. Lind and Ernster, 1974) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Kumaki *et al*, 1977), azo dyes (Huggins and Pataki, 1978), phenolic antioxidants such as 2(3)-tert-butyl-4-hydroxanisole (BHA) (Benson *et al*, 1980) amongst many others. (For a comprehensive review see Talalay and Prochaska, 1987). Induction has also been reported *in vitro* by a wide variety of compounds e.g. daunorubicin (Galaris and Rydstrom, 1983), tert-butyl hydroquinone, various substituted 1,2- and 1,4-diphenols and aromatic diamines (Prochaska *et al*, 1985).

The enhanced expression of DT-diaphorase in tumour tissue, induction of DT-diaphorase by carcinogens and subsequent protection against some cytotoxic effects of these compounds led to the belief that DT-diaphorase may play a role in cellular defence against environmental and dietary carcinogens. It can also act as a selective target for chemotherapy (Riley and Workman, 1992b)

The increased levels of DT-diaphorase may be related to enhanced synthesis of new protein (Hojeberg *et al*, 1981). Evidence indicates that this may result from increased transcription of the gene (Prochaska and Talalay, 1988) Hypomethylation status has also been correlated with this enhanced expression (Bayney and Pickett, 1987).

Inducers are of two main classes, those which induce only phase II enzymes and those which induce phase I and phase II. Prochaska and Talalay (1988) proposed a model to explain this phenomenon. Monofunctional inducers include compounds such as 2(3)-tert-butyl-4-hydroxy-anisole (BHA) and selectively induce only phase II enzymes (e.g. glutathione-S-transferase (GST) and DT-diaphorase). It is believed that induction by these agents does not involve the Ah (aryl hydrocarbon) receptor but acts via an electrophilic signal. In contrast, bifunctional inducers e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce both phase I and phase II enzymes. Depending on the particular compound they are thought either to bind to the Ah receptor directly or alternatively to be metabolised to electrophilic species by phase I enzymes which can then subsequently act via a similar mechanism to the monofunctional inducers.

The molecular mechanisms involved in induction of DT-diaphorase are more thoroughly discussed in the next section (1.3.4).

1.3.4 Gene and Protein Sequence

Rat DT-diaphorase cDNA was cloned and fully sequenced by Robertson and co-workers in 1986 (Robertson *et al*, 1986). The cDNA is 1,501 base pairs long with an open reading frame extending from bp 75-899 which encodes a 274 residue protein of molecular weight 30,946 D (Robertson *et al*, 1986). In 1988 cDNA of human cytosolic DT-diaphorase was cloned, sequenced and the protein composition elucidated (Jaiswal *et al*, 1988). The human cDNA is 2448 bp long encoding a protein of 274 residues with molecular weight of 30,880 D. Comparisons of the rat and human DT-diaphorase showed an 83% and 85% homology of cDNA and protein respectively (Jaiswal *et al*, 1988).

Subsequently the genomic DNA for both the human (Jaiswal, 1991) and rat (Bayney *et al*, 1989) DT-diaphorase (NQO1) were also cloned and sequenced. The human gene is approximately 20 kb long. Both genes consist of six exons and five introns with exons 2-5 being highly conserved between the two species (Jaiswal, 1991). The sixth exon is 1833 base pairs in length in the human gene and contains four potential polyadenylation signal sequences (AATAAA) and a single copy of the Alu repetitive sequence. In contrast the sixth exon of the rat gene is 907 bp long contains only one polyadenylation sequence and no Alu repetitive sequences. This region would account for the presence of three distinct mRNA species for DT-diaphorase observed in the Hep-G2 (human hepatoblastoma) cell line.

Site directed mutagenesis studies have permitted further characterisation of the DT-diaphorase gene. The residues Gly 150, Ser 151 and Tyr 155 which occur within the single glycine rich region (residues 145 - 162) of rat liver DT-diaphorase and are conserved in the human protein have been implicated in the binding of the cofactors NADH and NADPH (Ma *et al*, 1992b). Residues bearing hydroxyl groups within this glycine rich region have also been suggested to stabilise cofactor binding by hydrogen bonding with the pyrophosphate group of the nucleotide (Ma *et al*, 1992b). Within the same study Ma and co-workers also identified the Tyr 128 residue as an important factor in dicoumarol binding. Further confirmation of this involvement came from photoaffinity probe analysis where Thr 127 and Tyr 128 residues were modified by a photoaffinity analogue of 4-hydroxycoumarin (Myszka and Swenson, 1991). Due to the competitive inhibition of dicoumarol with the cofactors NADH and NADPH suggestions were also made that these residues may define part of the nucleotide binding site (Myszka and Swenson, 1991). Other sites which have been characterised by site directed mutagenesis studies include Lys 76 which appears to be involved in NADPH binding (Ma *et al*, 1992a), Cys 179 which maintains stability of the enzyme (Ma *et al*, 1992a) and Arg 177 which may be important for protein conformation and FAD binding (Chen *et al*, 1992).

Gene sequencing and mutagenesis studies have also led to further elucidation of the molecular mechanisms involved in the regulation of expression and induction of the DT-diaphorase gene.

Within a 1850 base pair 5' flanking region of the human DT-diaphorase gene are a number of elements important in its transcriptional regulation (Jaiswal, 1991). A TATA box has been located running from base pairs -37 to -32 upstream from the CAP site. This is highly conserved between the rat and human and is suggested to play a role in initiation of transcription. A CCAAT sequence exists at nucleotide -649 in the human gene (Jaiswal, 1991) and -129 in the rat (Bayney *et al*, 1989). This has been identified in many eukaryotic promoters and functions to increase the rate of transcription. Also detected are two regions known to be important in induction of transcription, the XRE or xenobiotic response element and the ARE or antioxidant response element. The XRE with a sequence of GCGTG and was present at -740 in the human gene (Jaiswal, 1991) and -393 in the rat gene (Favreau and Pickett, 1991). The involvement of the XRE in induction by chemicals has been extensively studied for the cytochrome P450 1A1 gene (Whitlock, 1987). A sequence known as the Ah locus codes for a 280 kD protein product known as the Ah receptor. This is thought

to bind to chemical inducers and then translocates to the nucleus where it binds to the XRE and initiates transcriptional activation of the gene. This then leads to an increase in the corresponding mRNA and ultimately an increase in the levels of protein. It has been suggested that a similar mechanism may occur in the case of the DT-diaphorase gene. The observations that the DT-diaphorase gene is not induced in cell lines deficient in the cytochrome P450 1A1 gene and that induction of DT-diaphorase appears to have a delayed onset over that of other enzymes has led to the theory that some inducers may be metabolised by enzymes such as cytochrome P450 1A1 and that the metabolites may actually act as the inducers of DT-diaphorase. Both the human and rat genes possess an ARE located between -467 and -447 for the human gene (Jaiswal, 1991) and -434 to -404 for the rat gene (Favreau and Pickett, 1991). Within this region 6 base pairs have been identified as being essential for induction, GTGAC NNNGC (where N = any nucleotide), with TGAC being required for basal expression (Rushmore *et al*, 1991). An AP1 binding site (TGACTC) has also been identified contained within the ARE sequence of the human gene (Jaiswal, 1991); this has 5 of the 7 base pairs conserved in the rat AP1 site (Favreau and Pickett, 1991) and is important for both basal expression and induction of the gene by some compounds. AP1 proteins form homo or heterodimers with other proteins such as Jun B, Jun D and Fos, and create a transcription factor. This is then thought to bind to the AP1 site within the ARE, regulating expression of the gene.

1.3.5 Mechanisms of Reaction

DT-diaphorase is commonly considered to react by a binary complex or 'ping-pong' mechanism with its electron donor and electron acceptor. These two substrates are competitive inhibitors with respect to each other which suggests some relationship between the cofactor and substrate binding sites within the enzyme. It has been suggested that DT-diaphorase is an obligate two-electron transferring enzyme (Iyanagi and Yamazaki, 1970).

As discussed previously, both nicotinamide adenine dinucleotide cofactors NADH and NADPH are able to act as electron donors (Ernster *et al*, 1962). The enzyme also has a wide substrate specificity being reported to metabolise a wide variety of quinones as well as azo-dyes, (Huang *et al*, 1979a and b), nitro compounds (e.g. Sugimura *et al*, 1966) and chromium compounds (De Flora *et al*, 1985). DCPIP, menadione and some other benzo and napthoquinones were the most active substrates (for summary see Ernster *et al*, 1962).

A number of compounds also act as inhibitors of DT-diaphorase, the most potent of which is dicoumarol (Ernster *et al*, 1962). Others include coumarin derivatives e.g. warfarin, indandione derivatives (Lind and Hojeberg, 1981; Hollander and Ernster 1975), Dicoumarol is believed to inhibit the enzyme by competing for the NAD(P)H cofactor binding site (Ernster *et al*, 1962).

1.4 DT-diaphorase in Cancer Chemotherapy

The involvement of DT-diaphorase in cancer chemotherapy has recently been the subject of a number of publications and has been comprehensively reviewed by Riley and Workman (1992b).

CB 1954

The high selectivity of CB 1954 for the rat Walker tumour with lack of effect in human tumours prompted detailed research into the mechanism of activation of the compound. The enzyme responsible for activation of CB 1954 in the rat Walker cells was found to be DT-diaphorase (Knox *et al*, 1988a and b), and its involvement was clarified by inhibitor studies (Roberts *et al*, 1989). DT-diaphorase reduced CB 1954 by 4-electron reduction to generate 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (Knox *et al*, 1988b) which then reacted with acetyl CoA or thioesters to form 4-(N-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide (Knox *et al*, 1991). The latter compound is a difunctional alkylating species and was shown to form interstrand cross-links in Walker cell DNA. Interstrand cross-links are believed to be intrinsically more cytotoxic than single stranded di-adducts or monofunctional adducts (Roberts and Friedlos, 1987). The sensitivity of rat Walker cells to CB 1954, in addition to being a function of activation, may also be enhanced by an inherent sensitivity to these adducts (Knox *et al*, 1991). DNA damage studies have shown that the reaction predominantly occurs with the C8 position of deoxyguanosine and molecular modelling suggests that the second arm of the cross-link will be at the O6 of deoxyguanosine (Knox *et al*, 1993).

The lower sensitivity of human tumours to CB 1954 is thought to result from reduced activation of the compound. DT-diaphorase has been reported to exist in high levels in human tumours and is similar in protein structure to that present in rodents. It appears however, that the human enzyme is less efficient at catalysing metabolism of CB 1954 than is the rat form of the enzyme (Boland *et al*, 1991).

Ongoing studies in Dr Knox's laboratory at the Institute of Cancer Research, Sutton, Surrey, UK (Knox *et al*, 1993) are examining analogues of CB 1954 in attempt to find one that is more efficiently metabolised by human DT-diaphorase. CB 10-200 (5-(aziridin-1-yl)-2,4-dinitro-1-isobutylbenzoate), the analogue in which the carboxamide has been substituted by an isobutylcarboxylate moiety, was reduced 40 times more rapidly by the human enzyme than CB 1954 (Lambert *et al*, 1992). It also retained selectivity for cells expressing DT-diaphorase.

SR 4233

Initial indications of the involvement of DT-diaphorase in metabolism of SR 4233 came from Walton and Workman (1990). Sonicates of rat Walker tumour cells reduced SR 4233 under aerobic conditions to generate the two and four-electron reduced products SR 4317 and SR 4330 respectively. Formation of these metabolites was largely inhibited by dicoumarol (SR 4330 by 95% and SR 4317 by 47%) suggesting the involvement of DT-diaphorase (Walton and Workman, 1990).

Confirmation of the ability of DT-diaphorase to metabolise SR 4233 came from studies carried out by Riley and Workman (1992a) using a highly purified preparation of the rat Walker enzyme. Reduction conformed to Michaelis-Menten kinetics and was not affected by change in pH over the range 5.8 - 8.0. The rate of SR 4233 metabolism was far lower than that observed for menadione. Under aerobic conditions SR 4330 was the major product following prolonged incubation although SR 4317 was also detected at comparable levels for 10-15 minutes. Formation of SR 4330 was almost completely inhibited by dicoumarol. SR 4330 was also found to act as a substrate for DT-diaphorase whereas SR 4317 could not. It is possible that a further metabolite may be the oxygen-sensitive six-electron reduced compound detected during electrochemical and chemical reduction. Cahill and co-workers (1993) published similar findings. In addition these latter investigators examined anaerobic reduction of SR 4233 by purified Walker DT-diaphorase. Metabolism of the drug in the absence of air was found to be approximately three fold higher than in air with differences in metabolites also being observed. SR 4317 was the major product of reduction but approximately 50% of metabolites formed were unidentified.

In the whole cell system SR 4317 was also a major metabolite formed within Walker cells (Cahill *et al*, 1993). Generation of this metabolite was not greatly affected by dicoumarol although it led to partial reduction of DNA damage.

Dicoumarol inhibition studies with sonicates of a human fibrosarcoma cell line (HT 1080) also indicated that DT-diaphorase may play a role in metabolism of SR 4233 (Wang *et al*, 1993).

Thus it appears that SR 4233 is able to undergo metabolism catalysed by DT-diaphorase under both aerobic and hypoxic conditions although the metabolites formed may differ depending on oxygen status. As SR 4317 and SR 4330 are the predominant products detected it has been suggested that DT-diaphorase may play a protective role against SR 4233 toxicity by bypassing the putative damaging radical intermediate (Riley and Workman, 1992a; Walton *et al*, 1992c; Cahill *et al*, 1993). The role played by DT-diaphorase in the toxicity of SR 4233, as with other bioreductive agents will depend on the levels and affinities of other enzymes within the cell as well as the nature of the metabolites formed. In practice, results from cell studies suggest that DT-diaphorase may not be important in the metabolism and cytotoxicity of SR 4233 in intact cells (Plumb *et al*, 1994a and b).

Mitomycin C

Despite numerous studies examining mitomycin C activation the involvement of DT-diaphorase has been highly controversial (Workman and Walton, 1989). Initial indications of a role for DT-diaphorase in bioreductive activation of mitomycin C came from cytotoxicity experiments *in vitro* on a range of tumour cell lines, including initially the effects of dicoumarol on the response of EMT6 cells (Sartorelli, 1988). A murine lymphoblast cell line, L5178Y made resistant to benzoquinone had 20 fold greater DT-diaphorase expression than the parental line and showed an increased sensitivity to mitomycin C (Begleiter *et al*, 1992). The levels of NADPH: cytochrome P450 reductase, also shown to metabolise mitomycin C, were similar in both the parental and resistant lines indicating that DT-diaphorase may be an important factor in activation of the compound. In support of this theory, mitomycin C was also found to be more potent towards other cell lines containing high levels of DT-diaphorase compared with their low / non-expressing counterparts. These include the 3437T and GM38 human fibroblast cells derived from patients from a cancer prone family and healthy donor respectively (Marshall *et al*, 1989, 1991b) and pairs of repair-proficient and deficient Chinese hamster ovary cells (Dulhanty and Whitmore, 1989; 1991). In addition, the DT-diaphorase inhibitor dicoumarol reduced the sensitivity of the high DT-diaphorase expressing lymphoblasts (Begleiter *et al*, 1988, 1992) and fibroblasts (Marshall *et al*, 1989, 1991a and b) to mitomycin C as

well as decreasing interstrand cross-link formation (Begleiter *et al*, 1988, 1992). Interestingly studies with EMT6 mouse mammary tumour cells showed some inhibition of mitomycin C toxicity under aerobic conditions in the presence of dicoumarol but not under hypoxia (Keyes *et al*, 1985).

The evidence obtained in favour of mitomycin C activation by DT-diaphorase was therefore largely indirect and based heavily on inhibitor studies using dicoumarol. Dicoumarol has been shown to exert pleiotropic effects and therefore caution should be taken when interpreting data obtained using this inhibitor (Workman and Walton, 1989). To clarify the involvement of DT-diaphorase in the metabolism of mitomycin C purified enzyme was employed. Schlager and Powis (1988) were unable to detect reduction of mitomycin C catalysed by DT-diaphorase purified from either human kidney or rat liver. Mitomycin C also acted as a poor substrate for DT-diaphorase present in sonicates of rat Walker tumour cells which contain high levels of the enzyme (Walton *et al*, 1992b). Another cell line expressing high levels of DT-diaphorase, the HT29 human colon carcinoma line, was more sensitive to mitomycin C than the BE colon carcinoma line which does not express a functional form of the enzyme (Siegel *et al*, 1990b). However, when DT-diaphorase purified from the HT29 cells was used to examine reduction of mitomycin C at neutral pH no metabolism was detected. Kennedy and co-workers (1985) had previously reported an increased cytotoxicity of mitomycin C at low pH. pH was also subsequently found to influence the metabolism of mitomycin C catalysed by purified DT-diaphorase derived from rat liver and human HT29 cells with reduction being detected as pH was decreased to 5.8 (Siegel *et al*, 1990b). They suggested that the reason for the enhanced metabolism at lower pH values was the formation of the quinone methide metabolite. At high pH (above 7.4) this was proposed to alkylate the enzyme thus inactivating it whereas at lower pH values protonation may occur, converting the quinone methide to 2,7-diaminomitosene which may be released from the DT-diaphorase permitting metabolism to proceed.

The role of DT-diaphorase in the cellular cytotoxicity of mitomycin C remains controversial. Whereas Robertson *et al*, (1994) found no correlation between mitomycin C sensitivity and DT-diaphorase activity in a large panel of cell lines, Fitzsimmons *et al*, (1994) did find a correlation in the NCI human tumour panel.

AZQ

In a similar manner to mitomycin C, AZQ acted as a more potent cytotoxin in HT29 than in BE cells. Dicoumarol inhibited this cytotoxicity as well as DNA damage in HT29 cells, thus suggesting that DT-diaphorase may be responsible for AZQ activation (Siegel *et al*, 1990a). Siegel and co-workers (1990) confirmed that DT-diaphorase present in HT29 cell cytosol as well as highly purified preparations derived from HT29 cells and rat liver could catalyse metabolism of AZQ.

Further evidence of AZQ activation by DT-diaphorase came from spectrophotometric and ESR studies performed by Fisher and Gutierrez (1991b). They found that AZQ was metabolised to give a semiquinone radical by the dicoumarol-inhibitable component of the S9 fraction of MCF-7 human breast carcinoma cells. Similar results were subsequently obtained using a purified preparation of DT-diaphorase. Data from both groups indicated that AZQ was metabolised by DT-diaphorase to an oxygen sensitive hydroquinone which auto-oxidised under aerobic conditions to generate a semiquinone radical. DT-diaphorase was also able to catalyse AZQ reduction to a DNA interstrand cross-linking species at neutral pH (Lee *et al*, 1992).

EO9

At the outset of the work carried out in this thesis few studies had examined the role of DT-diaphorase in metabolism of EO9. Sonicates of rat UK Walker 256 mammary tumour cells had been found to metabolise EO9 (Walton *et al*, 1992b). This activity was largely inhibitable by dicoumarol and was therefore attributed to DT-diaphorase. Preliminary studies using HT29 human colon carcinoma cell sonicates (Walton *et al*, 1991) which also contain high levels of DT-diaphorase similarly exhibited dicoumarol inhibitable metabolism of EO9.

Subsequently a number of investigators have compared sensitivity of a range of tumour cell lines to EO9 with their DT-diaphorase activity. In addition to data presented in this thesis, evidence to support the hypothesis that DT-diaphorase may play a role in determining the sensitivity of tumour cells *in vitro* and *in vivo* was provided by Walton and co-workers (1992a). These authors reported that EO9 exhibited more potent cytotoxicity in HT29 colon carcinoma cells which express high levels of DT-diaphorase compared to BE cells which, due to a mutation in the NQO1 gene, do not express a functional form of the enzyme. In addition, data obtained *in vivo* showed that DT-diaphorase activity was 16 fold higher in the mouse

adenocarcinoma MAC 16 than in the mouse adenocarcinoma MAC 26 which exhibits greater resistance to EO9. Further data which substantiate a role for DT-diaphorase in EO9 cytotoxicity under aerobic conditions have been provided by Robertson and co-workers (1992, 1994), Plumb and co-workers (1994a and b) and Smitskamp and co-workers (1994) in a large range of cell lines. In general, these studies showed a good correlation between levels of DT-diaphorase and EO9 cytotoxicity. Collard and Double (1992) reported that, although cells expressing high levels of DT-diaphorase tended to be more sensitive to EO9, there was no absolute agreement between the two parameters. However, this study examined only a small number of cell lines. In a recent survey of the extensive NCI human tumour cell line panel, Fitzsimmons *et al*, (1994) confirmed the relationship between DT-diaphorase expression and sensitivity to EO9.

Interestingly hypoxia increased the sensitivity to EO9 of cells expressing low levels of DT-diaphorase but had little effect on cells expressing high levels of the enzyme (Plumb and Workman, 1994; Robertson *et al*, 1994; Plumb *et al*, 1994b). In fact an inverse correlation has been suggested for the hypoxic cell cytotoxicity of EO9 and DT-diaphorase activity (Plumb *et al*, 1994b).

1.5 Enzyme Directed Prodrug Therapy (EDPT)

Enzyme-directed prodrug therapy involves the design of prodrugs that are converted to cytotoxic compounds following enzymatic activation (Workman and Walton, 1990; Workman, 1994). Tumour cell selectivity is attained by targeting enzymes which exhibit increased expression or activity in tumour versus normal tissue. The work described in this thesis is part of an 'enzyme directed approach to bioreductive drug development' adopted by our laboratory. It is hoped that this will permit the optimisation of drug structure to suit the catalytic preferences of key bioreductive enzymes.

Further information is required for this approach to be of clinical benefit including:-

(1) Characterisation of other enzymes involved in metabolism of these bioreductive agents.

(2) 'Enzyme profiling' of tumour versus normal tissue - i.e. characterisation of the levels and activity of relevant activating and detoxifying enzymes as well as their affinities for the bioreductive agents.

(3) Definition of resistance mechanisms for particular compounds so that methods of circumventing resistance may be developed.

(4) Determination of tumour hypoxia and pH.

1.6 Aims of This Thesis

As part of the enzyme directed approach to bioreductive drug development outlined above, the overall goal of the work described in this thesis was to determine the mechanism of activation and cytotoxicity of selected bioreductive antitumour agents. An understanding of these features may lead to optimisation of drug structure for optimal antitumour activity. Studies have focused on the novel indoloquinone agent EO9 and related agents but have also examined the structurally similar aziridinylbenzoquinone compound AZQ and its analogues, as well as certain cyclopropamitosenes.

As discussed earlier, the enzyme DT-diaphorase shows increased expression and activity in some solid tumours compared with normal tissue and catalyses metabolism of compounds that are structurally similar to EO9 and AZQ. It was therefore considered likely that this enzyme may be especially important in the activation of EO9 and AZQ. The initial aims were therefore first to determine whether DT-diaphorase was capable of catalysing metabolism of the bioreductive compounds EO9, AZQ and cyclopropamitosenes. Secondly to compare the ability of the agents to undergo reduction catalysed by DT-diaphorase with their cytotoxic potency in order to assess whether this was an activation or detoxification process. Thirdly, to examine DNA damage as a possible mechanism of cytotoxicity and to clarify the involvement of DT-diaphorase in activation of these compounds to DNA damaging species. Finally, by studying structural analogues of these compounds to highlight areas where drug structure could be modified for optimal metabolism and antitumour activity.

Chapter 2

General Materials and Methods

This chapter covers the main materials and methods used throughout the thesis. Individual chapters contain methods particular to those chapters and also include more details of procedures described in this section.

2.1 Chemicals and Biochemicals

I am grateful for the gifts of EO9 and related indoloquinone compounds from the EORTC New Drug Development Office (Amsterdam, Netherlands) and aziridinylbenzoquinone analogues of diaziquone (AZQ) from Dr John Butler (Paterson Institute, Manchester, UK). The purity of EO9 and analogues EO5A and EO12 was determined as >99% by HPLC (Binger and Workman, 1990). Additional confirmation of EO9 purity was provided by Dr. E. Bailey (MRC Toxicology Unit, Carshalton, Surrey, UK, unpublished data) using gas chromatography and mass spectral analysis.

Highly purified (95%) rat Walker UK 256 tumour cell DT-diaphorase (NAD(P)H: quinone acceptor oxidoreductase, EC 1.6.99.2) used in initial experiments was kindly provided by Dr R. Knox (Institute of Cancer Research, Sutton, Surrey, UK) as well as rabbit anti-rat Walker tumour DT-diaphorase antiserum. For later experiments rat Walker DT-diaphorase was prepared as outlined in Section 2.4. Purified rat liver NADPH: cytochrome P450 reductase was a gift from Professor R. Wolf (University of Dundee, Dundee, Scotland, UK).

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK) and all tissue culture media and sera from Gibco Ltd. (Paisley, Scotland, UK) unless otherwise stated in the text.

2.2 Cell Culture

2.2.1 HT29 and BE Cells

HT29 and BE cell lines are derived from human adenocarcinomas of the colon. The HT29 cells were obtained from Dr J. Fogh who originally derived the cell line (Fogh and Trempe, 1975). The BE cells were a gift from Dr N. W Gibson (University of Southern California, Los Angeles, California, USA). Both of these lines, which grow as a monolayer, have similar morphology to their original counterparts *in vivo* (Erickson *et al*, 1978). The HT29 cells grow in an organised fashion whereas the BE cells are relatively disorganised and are interspersed with giant cells.

HT29 tumour cells differ from the BE cells in a number of aspects. Of particular relevance to this thesis is the fact that HT29 cells express high levels of DT-diaphorase (Siegel *et al*, 1990a) whereas BE cells do not express a functional form of the enzyme due to a mutation in the NQO1 gene (Traver *et al*, 1992). In addition BE cells are deficient in the DNA repair enzyme O6 alkylguanine-DNA alkyltransferase and are often described as mer- (Fornace *et al*, 1990). HT29 cells possess this enzyme and are denoted mer+ (Fornace *et al*, 1990). Siegel and co-workers (1990a) compared activities of other enzymes present in sonicates of the two cell lines. They reported little difference in levels of NADPH : cytochrome P450 reductase, xanthine oxidase was not detected in either the HT29 or BE cells, and 4-5 fold lower expression of glutathione and glutathione-S transferase was observed in BE cells compared to HT29.

BE and HT29 cell lines were cultured under identical conditions. Stock cultures of cells were grown in monolayer, seeded at a density of 1 x 10^5 cells / 75 cm^2 plastic tissue culture flask (Falcon Plastics, Crawley, Sussex, UK). They were maintained in Eagle's minimal essential medium supplemented with 10% foetal calf serum (Seralab, Crawley Down, Sussex, UK), 1 mM glutamine and the antibiotics penicillin and streptomycin (100 units / ml and 100 µg / ml respectively). Cells were incubated at 37°C in a humidified incubator with an atmosphere of 8% CO_2 and were subcultured weekly by trypsinisation. This involved removal of medium and washing twice in a mixture of trypsin (0.1%) and EDTA (0.04%) in PBS before incubation at 37°C for 15 minutes. Cells were then resuspended in a small volume of medium, disaggregated using a pipette, and finally counted using a haemocytometer before dilution to the appropriate concentration. Medium was replaced twice weekly between subculturing.

2.2.2 Walker Cells

The Walker tumour from which the cell line was ultimately derived was initially identified in 1928 by Dr G. Walker at the John Hopkins University Medical School. It was observed as a mammary adenocarcinoma present in a pregnant albino rat although subsequently the adenomatous features have disappeared. The tumour has been maintained at the Institute of Cancer Research, London since 1944 and has been used as a screen for antitumour agents (Knox *et al*, 1991). It was noted that the tumour was particularly sensitive to difunctional alkylating agents in 1966 (Rosenoer *et al,* 1966; Everett, 1966). Sensitivity was retained in the rat Walker UK 256 cell line established from this tumour (Knox *et al*, 1991). A subline resistant to chlorambucil was developed in Professor K. Harrap's laboratory (Institute of Cancer Research, London, UK) and this was subsequently found to be cross-resistant to a number of other difunctional alkylating agents (Knox *et al*, 1991). It is however, no longer kept under selective pressure (Knox *et al*, 1991). This subline was designated Walker Resistant (WR) while the parent cell line was denoted Walker Sensitive (WS).

The Walker tumour cell line was, like the HT29 cells, of particular interest for this research due to its expression of the enzyme DT-diaphorase (Boland *et al*, 1991) which confers its high sensitivity to CB 1954 (Knox *et al*, 1988b). Both Walker Sensitive and Resistant lines were a gift from Dr R. Knox.

The two strains of the Walker cells, WR and WS were cultured in an identical manner. Cells were grown as a suspension culture at a density of 1 x 10^5 cells / ml in 75 cm^2 plastic tissue culture flasks and were subcultured 3 times weekly. They were maintained in Dulbecco's minimal essential medium supplemented with 10% horse serum, 1 mM glutamine and antibiotics penicillin and streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 8% CO_2.

All cell stocks were checked for mycoplasma on a frequent basis and were found to be free from infection.

2.3 Preparation of Cell Sonicates

Cell sonicates of both the HT29 and Walker cells were prepared for use in enzyme assays. HT29 cells, harvested at confluence by trypsinisation, and Walker cells taken on reaching a density of approximately 1 x 10^7 cells / ml were pooled and washed

three times in 10 mM sodium phosphate buffer (pH 7.5) by alternate centrifugation (5 minutes at 150 g using a Beckman TJ6 centrifuge) and resuspension. Aprotonin (final concentration 1%) was added to the resultant cell suspension to prevent breaking down of reductive enzymes by proteases. Cells were then disrupted by sonication (MSE Soniprep 150 sonicator, MSE Scientific Instruments, Crawley, Sussex, UK) on ice using 3 x 10 second exposures at maximum power before centrifugation at 15000 g which served to remove cell debris and high molecular weight components. The supernatant was taken and filtersterilised using a Millipore filter (0.2 μm pore size; Millipore UK Ltd.,Watford, Herts, UK). Finally sonicates were aliquotted and frozen at -70°C permitting utilisation without repetitive freeze-thawing procedures which may damage the enzyme.

2.4 Protein Purification

2.4.1 Introduction

A highly purified preparation of DT-diaphorase was derived from rat Walker UK 256 tumour cells. The method was identical to that described by Knox and co-workers (1988). Walker cells were grown as a bulk preparation and sonicated before purification. This involved two cycles of gel filtration chromatography which separated out the proteins on the basis of molecular size. Active fractions were then further separated on the basis of ionic charge by two cycles of anion exchange chromatography. Fractions were assayed for DT-diaphorase activity following each purification stage and were subjected to SDS polyacrylamide gel electrophoresis and Western blot analysis as described in Section 2.5 in order to confirm the identity and purity of the protein.

2.4.2 Preparation of Walker Cell Sonicate

Cells were grown in suspension culture as previously described (Section 2.2.2) in a 175 cm^2 flask for about one week until cells reached a density of approximately 1.5 x 10^6 cells / ml. These were used as to inoculate a 20 l vessel containing 18 l of Dulbecco's minimal essential medium, horse serum (10%) L'-glutamine (1 mM) and antibiotics penicillin and streptomycin. This was connected to a 5% CO_2 supply and maintained as a stirred suspension culture at 37°C until cells had reached an optimum density (2 x 10^6 cells / ml) for harvesting (approximately 7 days).

Cells were then concentrated by centrifugation initially using a Beckman J68 centrifuge (150 g for 10 minutes) and on reaching a volume of less than 1 l cells were further concentrated using a Beckman TJ-6 centrifuge (7 minutes at 150 g). Residual medium was removed by decantation, the resulting cell pellet resuspended in PBS (2.5 ml of cells : 1 ml of PBS) and aprotonin was added to give a final concentration of 1% before disruption by sonication. Three 10 second pulses were given at maximum intensity using a sonicator (MSE Soniprep 150) with medium sized head, cooling the sample on ice between each stage. The resultant sonicate was transferred to Beckman tubes on ice for centrifugation at 4°C, 15000 g for 30 minutes (Beckman L8-55 ultracentrifuge) to remove high molecular weight matter. Finally the supernatant was collected and filtered through Millipore sterile filters to clear particulate matter before gel filtration chromatography.

2.4.3 Gel Filtration Chromatography

Aliquots of the filter sterilised Walker cell sonicate were injected on to a TSK G3000 SWG (21.5 x 600 mm) gel filtration column (Anagel) fitted with a 2 ml injection loop. The sample was then eluted isocratically with 10 mM sodium phosphate buffer pH 7 at a flow rate of 4 ml / minute. Fractions (2 ml) were collected and kept on ice before analysing for DT-diaphorase activity. This was carried out using the cytochrome *c* reduction assay outlined in Section 2.7 of this thesis employing a set concentration of menadione (10 μM). In addition samples were examined for their ability to catalyse metabolism of EO9 (10 μM). In this case 4 ml fractions were collected. Active fractions (numbers 5-20 (Fig 2.2)) were yellow in colour and eluted between 23 and 29 minutes with the peak at ~25 minutes as confirmed by absorbance at 280 nm using a UV detector (Fig 2.1). An increase in absorbance at 280 nm was also observed coincident with sample elution at around 37 minutes and minor peaks at 52-56 and 63 minutes. Only the fractions eluting at 37 minutes did however show activity with menadione and EO9 (Fig 2.2). Fractions containing the peak of activity were pooled and concentrated using an Amicon Centriprep 10 until a final volume of approximately 5 ml remained. This was then again injected on to the gel filtration column for a second elution step carried out as described previously. Fractions were collected, assessed for DT-diaphorase activity and active fractions pooled and concentrated. In all cases activity of protein was found to be inhibited by dicoumarol and no reduction was seen in the absence of either menadione or EO9. Furthermore when analysing fractions NADH was found to function with equal efficiency to NADPH as a cofactor. This is a characteristic of DT-diaphorase and further confirms the identity of the enzyme purified.

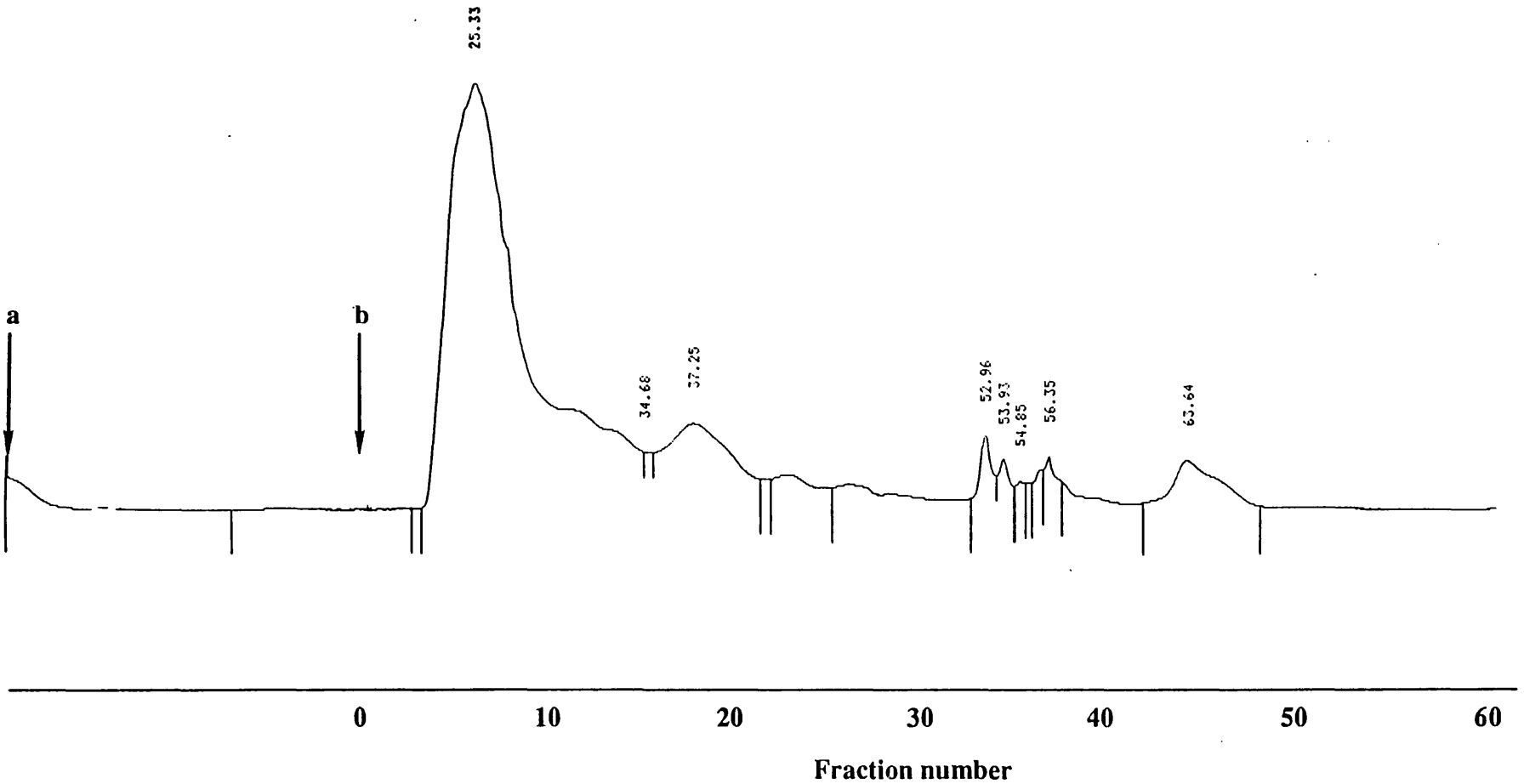


Fig 2.1 Gel filtration chromatography of a sonicate of UK rat Walker 256 tumour cells. Absorption was measured at 280 nm. (a) represents the start of elution and (b) the point at which fraction collection began. Numbers given above the peaks show elution time in minutes. The active fractions 15 - 20 which eluted around 35 - 40 minutes were collected for further purification.

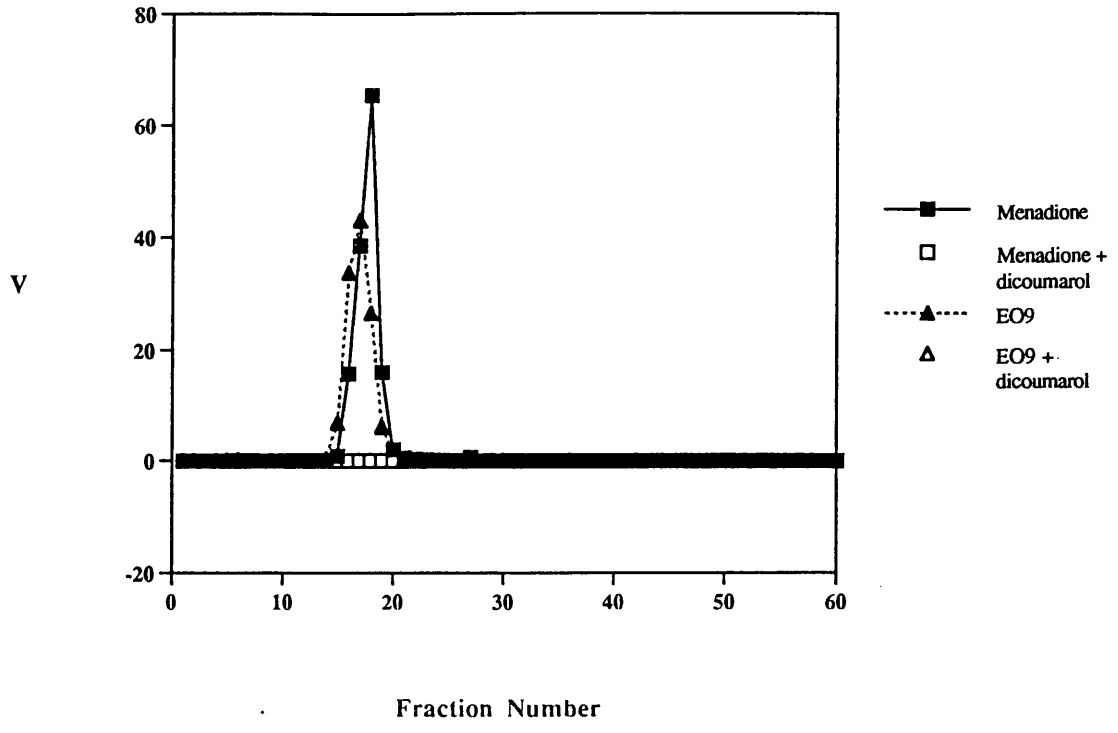


Fig. 2.2 Reduction of menadione and EO9 by fractions eluted from gel filtration chromatography of an extract of rat Walker tumour cells. Experiments were carried out in the presence and absence of the DT-diaphorase inhibitor dicoumarol. Activity (V) for menadione is expressed as the rate of change in absorbance of cytochrome *c* / 1 µl sample measured at 550 nm. In the case of EO9 activity was multiplied by a factor of 10. Data are taken from an individual experiment. Δ indistinguishable from control

2.4.4 Anion Exchange Chromatography

The active, concentrated fractions obtained from gel filtration were injected on to a TSK DEAE-5PW 115 x 10 nm anion exchange column with a 2 ml loop. Samples were eluted at 1 ml / minute using a linear gradient of 0-0.5 M sodium chloride in 10 mM NaH_2PO_4, pH 7.0 over 30 minutes and 0.5 ml fractions were collected. These were assessed for DT-diaphorase activity as described in Section 2.7. Active fractions (numbers 15-33, Fig. 2.4) eluted between 6 and 10 minutes with a peak of activity at 7.68 minutes as detected by spectral changes at 280 nm (Fig. 2.3). These fractions were collected, concentrated and desalted by adding 10 mM sodium phosphate buffer pH 7.0 at an equivalent volume to the concentrate. This sample was then reinjected on to the column and the active fractions were again collected. An approximate sample concentration was determined spectrophotometrically (450 nm) and was found to contain 1 mg / ml of protein. This was diluted to give 0.5 mg / ml with ice cold PBS before filtersterilisation using a Millipore filter (0.2 μm pore size) The purified DT-diaphorase was then aliquotted to avoid necessity for freeze / thawing and was stored at -70°C.

The identity of the purified enzyme obtained was confirmed by SDS-PAGE followed by Coomassie blue staining and western blotting. DT-diaphorase was present as a single band which corresponded to a protein of approximately 33 kD molecular weight (Fig. 2.5 and 2.6) and reacted with rabbit anti-rat DT-diaphorase antiserum (Fig. 2.6). Samples of eluate taken following gel filtration and anion exchange chromatography steps showed two extra bands in addition to that for DT-diaphorase in the western immunoblot (Fig 2.6). These were believed to represent breakdown products of the enzyme.

2.5 Electrophoretic Analysis of Proteins

2.5.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method described by Laemmli (1970) using a Bio-Rad Protean 2 kit (Bio-Rad, Hemel Hempsted, Herts. UK).

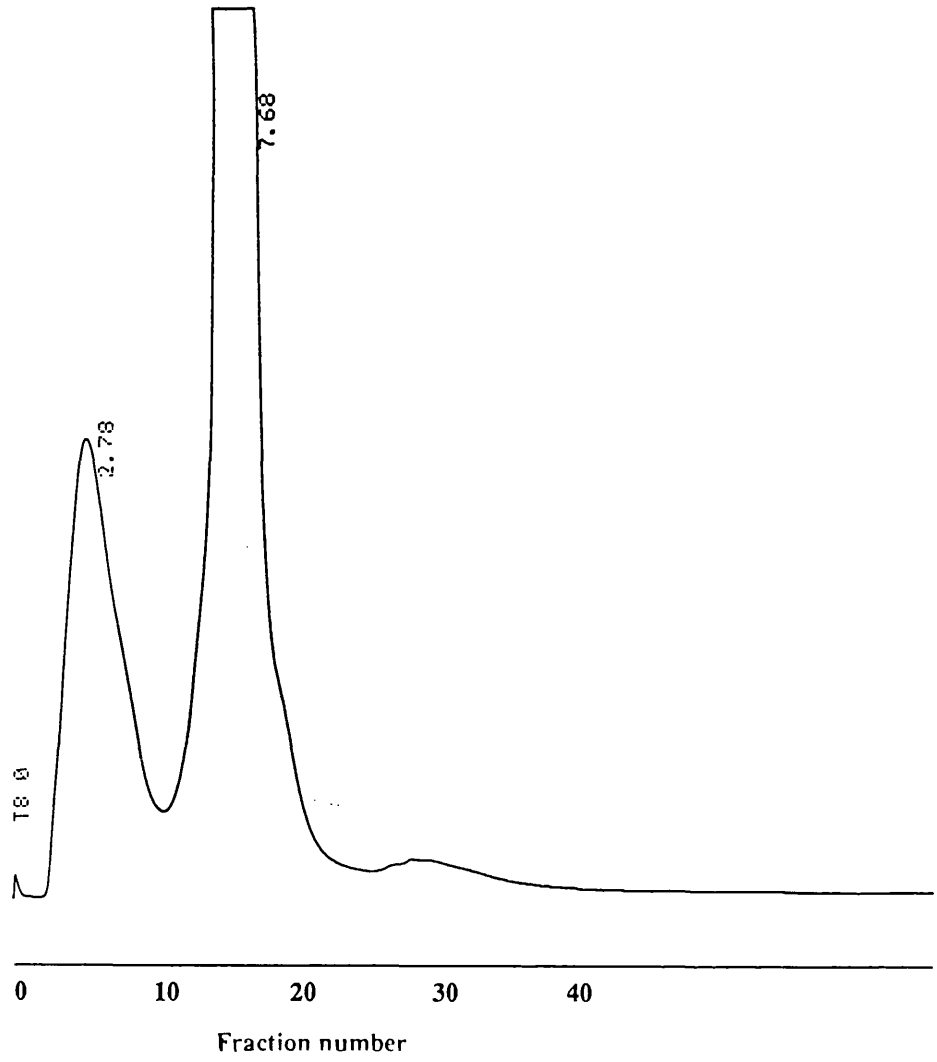


Fig 2.3 Anion exchange chromatography of pooled active fractions collected from gel filtration chromatography. Absorption was measured at 280 nm during sample elution. Fractions were collected at 30 second intervals with a flow rate of 1 ml / minute. Fraction collection was coincident with the initiation of elution. The active fractions (15 -33) eluted between 6 and 10 minutes and constituted purified DT-diaphorase. Elution time in minutes is shown alongside the peaks.

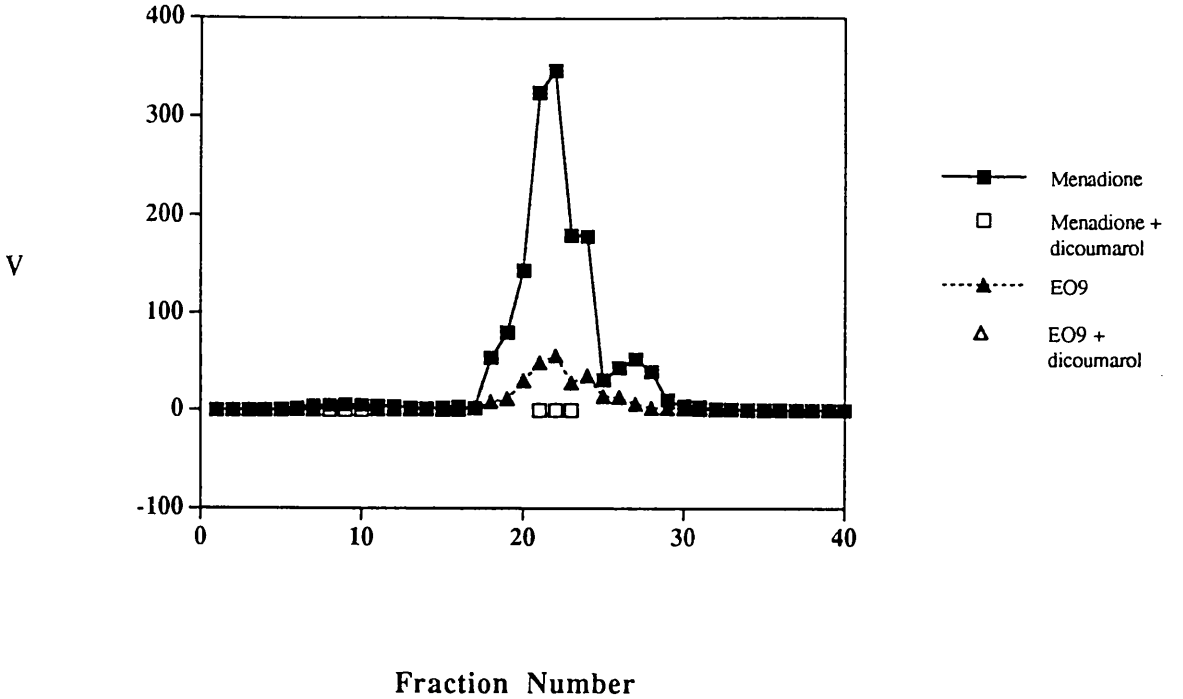


Fig 2.4. Reduction of menadione and EO9 by fractions eluted from anion exchange chromatography of an extract from Walker tumour cells. Activity (V) is expressed as the rate of change in absorbance of cytochrome *c* / 1 µl sample measured at 550 nm. Assays were carried out in the presence and absence of the DT-diaphorase inhibitor dicoumarol. Data are taken from an individual experiment.

Δ indistinguishable from control

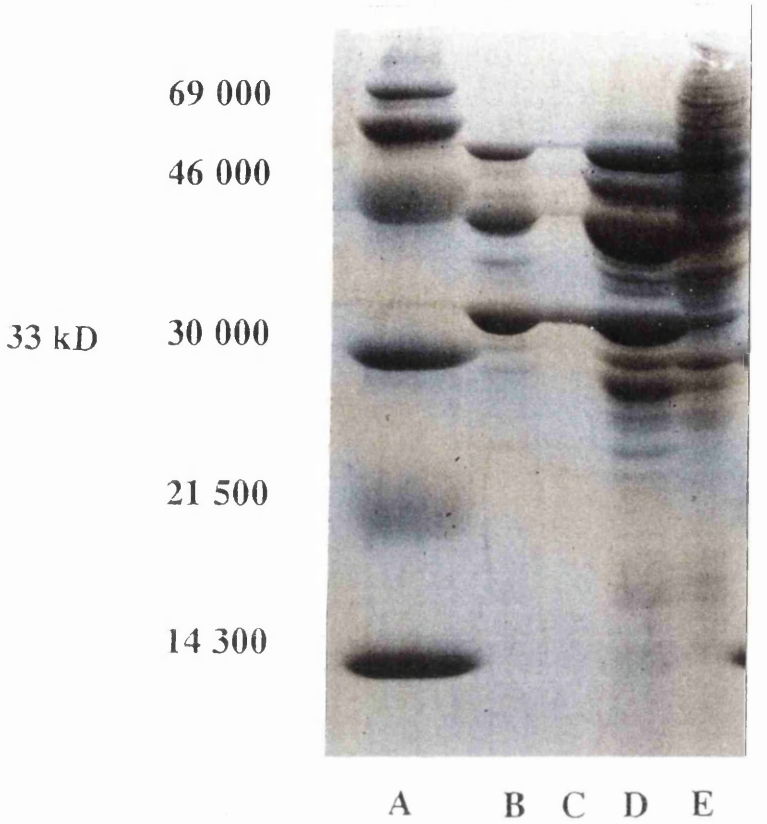


Fig. 2.5 SDS PAGE, Coomassie blue stained gel of samples taken during rat Walker tumour cell DT-diaphorase purification. (A) molecular weight markers. (B, C and D) pooled active fractions eluted from (B) the second cycle of gel filtration chromatography, (C) anion exchange chromatography (purified enzyme), (D) the first cycle of gel filtration chromatography. (E) cell sonicate. Samples were loaded at concentrations of 5-10 µg protein per sample except for the purified enzyme for which 0.5 µg was used. Data are taken from an individual experiment and were confirmed in an independent repeat study as well as in three repeat assays carried out at the same time using different concentrations of enzyme.

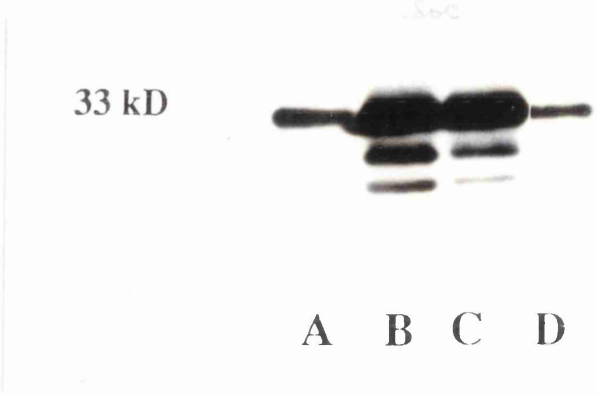


Fig. 2.6 Western Immunoblot analysis of the purification of Walker cell DT-diaphorase. (A) sonicate of Walker cells. (B) pooled active fractions eluted during gel filtration chromatography cycle 1. (C) pooled active fractions eluted from gel filtration chromatography cycle 2. (D) concentrated, pooled active fractions eluted from anion exchange chromatography. Samples were loaded at concentrations of 5-10 μg protein per sample except for the purified enzyme for which 0.5 μg was used. Data are taken from an individual experiment and were confirmed in an independent repeat study as well as in three repeat assays carried out at the same time using different concentrations of enzyme.

Glass plates (12 cm x 16 cm) and spacers were cleaned with alcohol, assembled in casting stands and a 12% polyacrylamide separating gel was poured to a level of 16 cm. This contained 14.8 ml (12% (w/v)) of polyacrylamide (30% w/v acrylamide and 1.57% bisacrylamide, Serum Biotech. Ltd., Kidderminster, UK); 9.25 ml separating buffer (Tris [1.5 M], SDS [0.5%], pH 8); 11 ml distilled deionised water. These constituents were thoroughly mixed before addition of freshly prepared ammonium persulphate (APS; Bio-Rad) 2 ml (1% (w/v)) and N,N,N1,N1-tetramethylethylene diamine (TEMED [20 µl]). The gel was overlayed with distilled water and left to set for 30 minutes. Following polymerization of the gel, water was poured off, a comb (previously cleaned in alcohol) was inserted and the stacking gel poured. The latter was prepared in an identical manner to the separating gel but consisted of 1.5 ml of 30% acrylamide and bisacrylamide (4.5%), 2.5 ml stacking gel buffer (Tris [0.5 M], SDS [0.5%], pH 6.6), 5.7 ml distilled deionised water, 0.3 ml APS (1%), 10 µl TEMED. The glass plates containing the gel were then detached from the casting stand and clipped in to the upper reservoir of the Bio-Rad kit. The latter had been previously moistened to enhance the seal. The assembled plates and upper reservoir were lowered in to the lower reservoir and approximately 1.5 l of electrode buffer (Tris, [0.52 M], glycine [0.53 M], SDS [35 mM], pH 8.3 diluted 1/10 in distilled deionised water) were added. The comb was then gently removed from the glass plates and electrode buffer added to cover the electrodes. Following protein determination the samples were diluted to the required concentration, mixed with an equal volume of boiling mix (SDS [2%], β-mercaptoethanol [5%], glycerol [10%], bromophenol blue [0.005%], stacking gel buffer [10%]) and were boiled for 5 minutes. A 10 µl aliquot of these samples was then loaded on to the gel and electrophoresis was carried out for 1.5 hours at 50 mA to allow migration of the protein through the stacking gel before running overnight at 6 mA.

2.5.2 Coomassie Blue Staining

Total protein content was visualised by staining the gel in Coomassie blue (75 ml / l glacial acetic acid; 45.5 ml / l methanol; 2.5 g / l Coomassie blue in distilled deionised water) for a period of 2-3 hours and destaining (70 ml / l glacial acetic acid; 50 ml / l methanol in distilled deionised water) until ready to photograph.

2.5.3 Western Immunoblotting

Samples separated by SDS-PAGE were analysed for the presence of DT-diaphorase by western blotting. The method was essentially that described by Towbin and co-workers (1979) with modification by Adams and co-workers (1985). The gel was first transferred on to an Immobilon, polyvinylidene difluoride microporous membrane (Millipore, UK Ltd.) by a semi dry method. Six sheets of Whatman filter paper 16 cm x 16 cm were soaked in transfer buffer (Tris [48 mM]; glycine [39 mM]; SDS [0.037% w/v] in distilled deionised water) and were placed centrally in the Milliblot Graphite Electroblotter 1 apparatus (Millipore). A polyvinylidene difluoride microporous membrane (16 cm x 16 cm) which had previously been soaked in methanol, rinsed in water and then soaked in transfer buffer was then added. The stacking gel was removed from the separating gel and the latter was gently lowered on top of this stack before finally covering with a further 6 sheets of filter paper prepared as described above. Where two gels were transferred together a layer of dialysis tubing was placed between the two gels. A pipette was rolled over the top in order to ensure that the gel was completely flat against the membrane. The transfer process was then carried out at 200 mA for 45 minutes.

Following transfer of the proteins on to the membrane the latter was removed, rinsed in TBST (NaCl [165 mM]; Tris base [49.5 mM], pH 7.9; Tween 20 [0.05%]) and left to block overnight in 5% skimmed milk (Marvel™) in TBST. The membrane was then rinsed for 2 x 10 minutes before incubation with rabbit anti-rat Walker DT-diaphorase antiserum (1/500 dilution) in 10% Marvel™ for 1 hour. The antiserum had been reported to be specific for DT-diaphorase and cross-reactive with the human form of the enzyme (Boland *et al* 1991) The membrane was then rinsed again in TBST (4 x 15 minutes) before adding the secondary antibody, protein A horseradish peroxidase linked (1/5000 dilution; Amersham Int. Plc., Little Chalfont, Bucks, UK) in 10% Marvel™ in TBST for 15 minutes. Finally the latter was removed by washing for 30 minutes in TBST changing the buffer frequently during this period. The bands were visualised by staining with ECL (Amersham Int. Plc.) for one minute followed by enhanced chemiluminescence detection. This involved removal of excess buffer by gently blotting the membrane between filter paper, staining in ECL (mixed in equal proportions of reagent 1 and 2 as provided in the kit) for 1 minute, blotting off excess stain and wrapping in Saran Wrap™ before exposure to Polariod film.

2.6 Protein Determination

The protein concentration of various enzyme samples was determined where necessary using one of two commercial kits produced by Pierce or Bio-Rad.

The Bio-Rad method is to some extent based on the Bradford assay (Bradford, 1976) and utilises the fact that a solution of acidic Coomassie Brilliant Blue G-250 changes peak absorbance from 465 nm to 595 nm when it binds to proteins. The dye reagent supplied contains dye, phosphoric acid and methanol.

A standard curve was prepared using known concentrations of bovine serum albumin (BSA) according to the kit instructions. Samples were mixed with the dye reagent at a ratio of 1 part sample to 5 parts reaction mixture. Absorbance was monitored spectrophotometrically using a Uvicon 860 spectrophotometer (Kontron, Zurich, Switzerland) at a wavelength of 595 nm at a time point between 5 - 60 minutes after initiating the reaction.

Protein samples of unknown concentration were assayed in a similar manner and their concentration interpolated from the standard curve.

The Pierce (BCA) assay kit depends on the detection of Cu^{1+} which is formed from Cu^{2+} on its reaction with protein under alkaline conditions and forms a complex with bicinchoninic acid (BCA) to result in a change in absorbance at 562 nm. The absorbance change is monitored spectrophotometrically and protein content of samples determined from comparison with a standard curve of BSA. Briefly, 0.1 ml of protein sample were added to a cuvette and mixed with 2.0 ml of reagent. This was incubated at 37°C for 30 minutes before cooling to room temperature and reading the absorbance.

2.7 Cytochrome *c* Reduction Assay

DT-diaphorase reduction of quinone compounds was monitored spectrophotometrically using a method similar to that described by Ernster (1962). The reaction was followed by detecting a decrease in absorbance of cytochrome *c* at 550 nm using a Uvicon 860 spectrophotometer. Cytochrome *c* acts as a signal molecule accepting electrons from the reduced quinone substrate. Reaction constituents include cytochrome *c* (77 μM), BSA (0.14%), purified rat Walker DT-

diaphorase, quinone dissolved in a mixture of DMSO (final concentration of 1% or 2%) and Tris buffer with BSA (0.14%) in a final volume of 1ml of Tris-Cl buffer pH 7.5. The reaction was started by addition of the electron donor NADH (2 mM) and was monitored at 37^0C for 1-2 minutes. Menadione, the conventional substrate for DT-diaphorase, was included as a standard and as a positive control. Reactions were repeated in the presence and absence of 100 μM dicoumarol, the relatively selective and potent inhibitor of DT-diaphorase. The activity attributed to this enzyme was calculated as that fraction which was inhibited by dicoumarol. Appropriate controls were carried out where each individual reaction component was omitted and reaction conditions were confirmed to be optimum. Within an individual experiment assays were repeated at least twice.

The amount of DMSO used as a solvent for compounds was also found to affect DT-diaphorase activity. A 12-13 % decrease in menadione metabolising activity was observed when increasing DMSO concentration from 1 to 2%. For this reason care was taken to prepare drug dilutions in vehicle to maintain the concentration of DMSO constant. Variation between repeat assays carried out within a day was small, in general being less than 5%.

2.7.1 Calculation of Kinetic Constants

Activity was calculated in nmoles of cytochrome *c* reduced / minute / mg protein using the extinction coefficient of 21.1 mM cm $^{-1}$ for cytochrome *c*. In some cases activity has been expressed in μmoles of cytochrome *c* reduced / minute / mg, due to the rapid rate of metabolism for some substrates by this enzyme. Results are also expressed in units of menadione reductase activity (n or μmoles cytochrome *c* reduced / minute / U). One U is defined as the amount of enzyme which reduces 1 μ mole cytochrome *c* / minute. Kinetic constants were determined from Hanes Woolf plots where substrate concentration (S) / initial velocity (v) is plotted against substrate concentration as this has been reported to be the favourable graphical representation from which to determine such parameter values (Henderson, 1978). Values were obtained using the computer package Enzyme Kinetics (Trinity Software, Campton, NH, USA) and were confirmed by comparison with other graphical methods. Some values of substrate concentration were observed to inhibit the enzyme reaction or to be saturating to a great extent when displayed as a substrate against initial velocity plot. These were omitted, in the conventional way, in calculations of kinetic constants as they would skew the data.

2.8 General Policies

2.8.1 Repeat Experiments

Where n is given in the figure legend it refers to the total number of experiments i.e. the number of independent repeats plus the original experiment.

2.8.2 Referencing

In general the references cited in the text refer to the original work. Where a review is quoted which includes the data described the reference is given as (see author, year).

Chapter 3

Reductive Metabolism of Indoloquinones

3.1 Introduction

As discussed in Chapter 1 quinone compounds may undergo one-electron reduction catalysed by a variety of enzymes including cytochrome P450s and NADPH:cytochrome P450 reductase amongst others to generate semiquinone free radicals. These may be toxic in their own right but under aerobic conditions are generally unstable and undergo auto-oxidation to regenerate the parent quinone. This process of redox cycling concomitantly generates potentially damaging oxygen-based radicals and may lead to oxidative stress. In contrast two-electron reduction catalysed by DT-diaphorase generates the hydroquinone species. This bypasses the toxic radical producing intermediates, at least initially, and is therefore often considered to be a detoxification process. However some compounds such as bioreductive alkylating agents possess groups which are activated upon two-electron reduction and thus in these cases DT-diaphorase catalysed reduction may actually result in activation.

Studies described here have examined the potential of DT-diaphorase and NADPH: cytochrome P450 reductase to catalyse reduction of EO9 because of their broad substrate specificity and in particular because of their ability to catalyse metabolism of structurally similar compounds to EO9, e.g. quinoneimines (Powis *et al*, 1987), 1,4-naphthoquinones (Buffington *et al*, 1989), anthraquinones (Fisher *et al*, 1992), menadione (Ernster *et al*, 1962) and mitomycin C (Bachur *et al*, 1979; Siegel *et al*, 1990b).

Previous experiments carried out by Walton and co-workers (1991, 1992b) have shown that EO9 could undergo metabolism catalysed by rat Walker tumour DT-diaphorase. The aims of experiments described in this chapter were initially to confirm that EO9 could be reduced by DT-diaphorase present in sonicates of rat Walker cells and to fully characterise the kinetic parameters using a highly purified (>95%) form of the rat Walker enzyme. As no purified human DT-diaphorase was made available the reductive ability of the enzyme present in sonicates of HT29 cells was examined. Walton and co-workers had also previously shown that this human

enzyme was able to reduce EO9 (100 μM) although kinetic parameters were not determined.

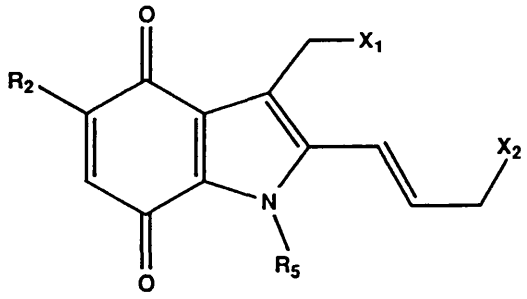
Structural modification has been reported to alter DT-diaphorase catalysed metabolism of a range of compounds. For example, the dinitrophenyl aziridine antitumour agent CB 1954 and its analogues 5-chloro-2,4-dinitrobenzamide and 3,5-dinitrobenzamide were able to readily undergo reduction catalysed by DT-diaphorase derived from rat Walker cells (Knox *et al*, 1988b) whereas 2,4-dinitro-5-(2'-hydroxyethylamino)-benzamide was not (Knox *et al*, 1988b). Similarly the benzotriazine-di-N-oxide SR 4233 acted as a substrate for DT-diaphorase as did the four-electron reduced analogue SR 4330, however the two-electron reduced derivative SR 4317 was not detectably metabolised by this enzyme (Riley and Workman, 1992a). For this reason a selected range of analogues related to EO9 were also studied (Fig 3.1). It was hoped that the information obtained may aid understanding of the structural features preferred for metabolism by this enzyme and facilitate an 'enzyme-directed approach' to the design of improved agents (Workman and Walton, 1990, Workman, 1994). Finally, the effect of hypoxia and pH on DT-diaphorase metabolism have been examined because of their possible physiological relevance in tumour tissue (Chapter 1).

DT-diaphorase-mediated metabolism was monitored directly by UV visible spectrophotometry, HPLC and electron spin resonance spectroscopy (ESR). ESR permits detection of drug-based free radical metabolites in addition to oxygen radicals which may be concomitantly generated during reductive metabolism, as discussed above. Thus ESR method provides an alternative method for detecting reduction and in addition provides valuable information about reactive metabolites which due to their instability may be difficult to detect using other techniques.

3.2 Methods

3.2.1 Spectrophotometric Assay for Metabolism of Quinones - Aerobic Conditions

Indoloquinone metabolism was determined spectrophotometrically using the cytochrome *c* reduction assay described previously (Chapter 2). In this assay, cytochrome *c* acts as the terminal electron acceptor and reporter molecule for quinone reduction. Purified rat Walker DT-diaphorase as well as sonicates of both rat Walker



Indoloquinone core structure

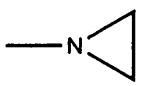
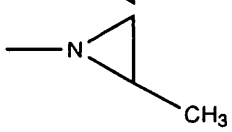
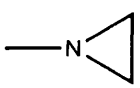
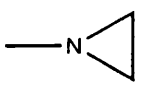
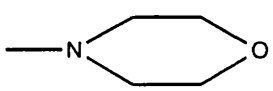
Analogue	R_2	X_1	X_2	R_5
EO9		OH	OH	CH_3
EO8		OH	OH	CH_3
EO7	OCH_3	OH	OH	CH_3
EO88		OH	$OCONH_2$	CH_3
EO5A	$NHCH_2CH_2OH$	OH	OH	CH_3
EO72		$OCOOCH_3$	OH	CH_3
EO56	NHC_2H_5	OH	OH	CH_3
EO16	OCH_3	OH	OH	C_4H_9
EO12		OH	OH	CH_3

Fig. 3.1 Chemical structure of indoloquinones related to EO9

and human HT29 colon carcinoma cells (Chapter 2) were used as sources of the enzyme. Where sonicates were employed potassium cyanide (KCN; 1 mM) was also added to the reaction mixture to inhibit mitochondrial enzymes (Stryer, 1981). Results were calculated in terms of both mg of protein and in units of menadione activity to account for any small between day differences in enzyme activity.

To confirm results obtained using this assay, reduction was also monitored directly in the absence of cytochrome *c* by following change in absorbance of the reaction components over the range 200 nm - 700 nm at 1 minute intervals for a 10 minute period. For these experiments a quartz cuvette was used to permit measurement over the range of wavelengths examined, the cofactor, NADH concentration was reduced to 200 μM and EO9 and purified rat Walker DT-diaphorase were employed at concentrations of 50 μM and 0.25 μg respectively in a total volume of 1 ml.

Additional experiments monitored loss of EO9 absorbance, under aerobic conditions, at 500 nm. Although this was not the λ max of EO9 it was selected because it was a principal wavelength at which EO9 continued to absorb light but which avoided overlap with the absorption peak of the nicotinamide cofactor, NADH. Due to the oxygen sensitivity of EO9 metabolites a small magnetic flea was included in the cuvette (3 ml volume) where possible to ensure complete mixing of the reaction components. In order to accommodate a magnetic stirrer an LKB spectrophotometer (LKB Ultrospec 4050, Pharmacia, Milton Keynes, UK) was employed with a magnetic pad placed beneath it. This replaced the Kontron machine used for all other experiments. Reaction constituents included EO9 (100 μM), NADH (500 μM) and purified rat Walker DT-diaphorase (5 μg) in a total volume of 3 ml Tris-Cl buffer (50 mM; pH 7.5) containing BSA (0.14%)

3.2.2 Effect of Hypoxia on Metabolism of Indoloquinones

The effect of hypoxia on the metabolism of the indoloquinones EO9, EO8, EO7 and EO88 catalysed by purified rat Walker DT-diaphorase was investigated by the cytochrome *c* reduction assay described in Chapter 2. A special apparatus was used for these experiments. This consisted of a quartz cuvette with an outlet tube and Thunberg type bulb attached (Belmont Instruments, Glasgow, Scotland, UK). Plastic cuvettes were not used due to possible absorption of oxygen by the surface. Reaction constituents were identical to those for aerobic studies. The NADH cofactor, used to initiate the reaction was placed in the bulb at the top of the apparatus and was therefore separated from the remaining reaction constituents located in the cuvette.

The latter was attached via a short length of tubing to a glass manifold fitted with a two way tap. This in turn was connected to a vacuum and to a gas cylinder containing nitrogen (black spot grade < 6 parts per million; British Oxygen Company, London, UK). By use of the two way tap the sample was alternately evacuated and gassed at approximately 5 second intervals over a period of 5 minutes. Individual experiments in which the gassing procedure was extended from 5 to 15 minutes, nitrogen was substituted for helium (black spot grade; British Oxygen Company) and an oxygen trap was included, confirmed the gassing procedure was sufficient for hypoxia to be induced. The reaction was initiated by rapid inversion of the cuvette. The effect of hypoxia on DT-diaphorase metabolism was further investigated by HPLC as described below.

3.2.3 Effect of pH on the Metabolism of EO9

The effect of pH on EO9 metabolism was again examined by the cytochrome *c* reduction assay. This was essentially as described in Chapter 2, however Tris-Cl buffer was substituted with sodium phosphate buffer (100 mM) due to its broader buffering capacity. Comparison was made of both buffers at pH 7.4. Metabolism was investigated at pH 7.8, 7.4, 7.0, 6.4 and 5.8. Menadione was included as a standard and mitomycin C as a positive control.

3.2.4 High Performance Liquid Chromatography (HPLC)

Enzyme catalysed reduction of EO9 was carried out essentially as described for the spectrophotometric assay (Chapter 2) except for the exclusion of cytochrome *c*. Reaction constituents included EO9 (100 μM), NADH (500 μM) and 5 ng, 50 ng, 250 ng or 25 μg of purified rat Walker DT-diaphorase in a total volume of 1 ml Tris-Cl buffer pH 7.5 containing BSA (0.14%). These were placed in an Eppindorff tube, mixed, 0.5 ml was transferred to a 0.7 ml HPLC vial and the reaction was kept on ice until being initiated by incubation at 37°C. Reduction was monitored by HPLC by following change in absorbance of EO9, cofactor NADH and metabolites using a Gilson HPLC (Gilson, Anachem, Luton, Beds., UK) with a Partisphere C18 column and a mobile phase of 40% methanol 60% water. Following initiation of EO9 reduction, aliquots of 10 μl were injected on to the HPLC column at 6 minute intervals for analysis. Detection was carried out at 260 nm and 340 nm wavelength and also by monitoring fluorescence using a diode array detector (1000S diode array detector, Applied Biosystems, Warrington, Cheshire, UK). Controls involved omitting individual reaction components i.e. enzyme, NADH and drug.

3.2.5 Electron Spin Resonance Spectroscopy (ESR)

Electron spin resonance spectroscopy was carried out using a Varian E109 Century Series X-band (9.3 GHz) spectrophotometer (Varian Instruments, Palo Alto, CA, USA). ESR parameters were in general: scan range ± 50 G, time constant 1 second, modulation amplitude 1.25 G, receiver gain 1.25 x 10^5 and 1.25 x 10^6, microwave power 5 mW, field set 3394 G, scan time 4 minutes, modulation frequency 100 cps and microwave frequency 9.51 G. All reactions were carried out at room temperature.

Standard reaction constituents included: DT-diaphorase (0.25 μg) or NADPH: cytochrome P450 reductase (1.3 μg), EO9 (800 μM dissolved in DMSO) and NADPH (1 mM) made up to a total volume of 400 μl Tris-Cl buffer pH 7.4. Where DT-diaphorase was used bovine serum albumin (BSA, 0.14%) was also included to stabilise the enzyme. In some experiments the effect of altering DT-diaphorase concentration to 0.007 μg, 2 μg or 5 μg was also investigated. Spin trapping studies were carried out using DMPO (100 mM) and in the presence and absence of superoxide dismutase (400U) and catalase (200U). For inhibition studies dicoumarol (100 μM) or rabbit anti-rat Walker DT-diaphorase antiserum (43 μl) were included. The latter was preincubated for 15 minutes at room temperature with purified DT-diaphorase before addition of other reaction components. Control experiments were carried out where enzyme alone was preincubated for 15 minutes to ensure that no damage was occurring to the DT-diaphorase during this period. Reactions were initiated by addition of cofactor to the reaction mixture and rapidly mixing. NADH (1-2 mM) was supplemented for NADPH in some assays with DT-diaphorase. Constituents were then transferred directly to the flat cell for analysis by ESR.

3.3 Results

3.3.1 DT-diaphorase Catalysed Metabolism of Quinones: Spectrophotometry

Cytochrome c Assay

Menadione

The ability of menadione to act as a substrate for DT-diaphorase is well established (see Chapter 1 for references). The results shown in Fig 3.2 and Table 3.1 using the cytochrome *c* reduction assay have confirmed that menadione reduction is catalysed

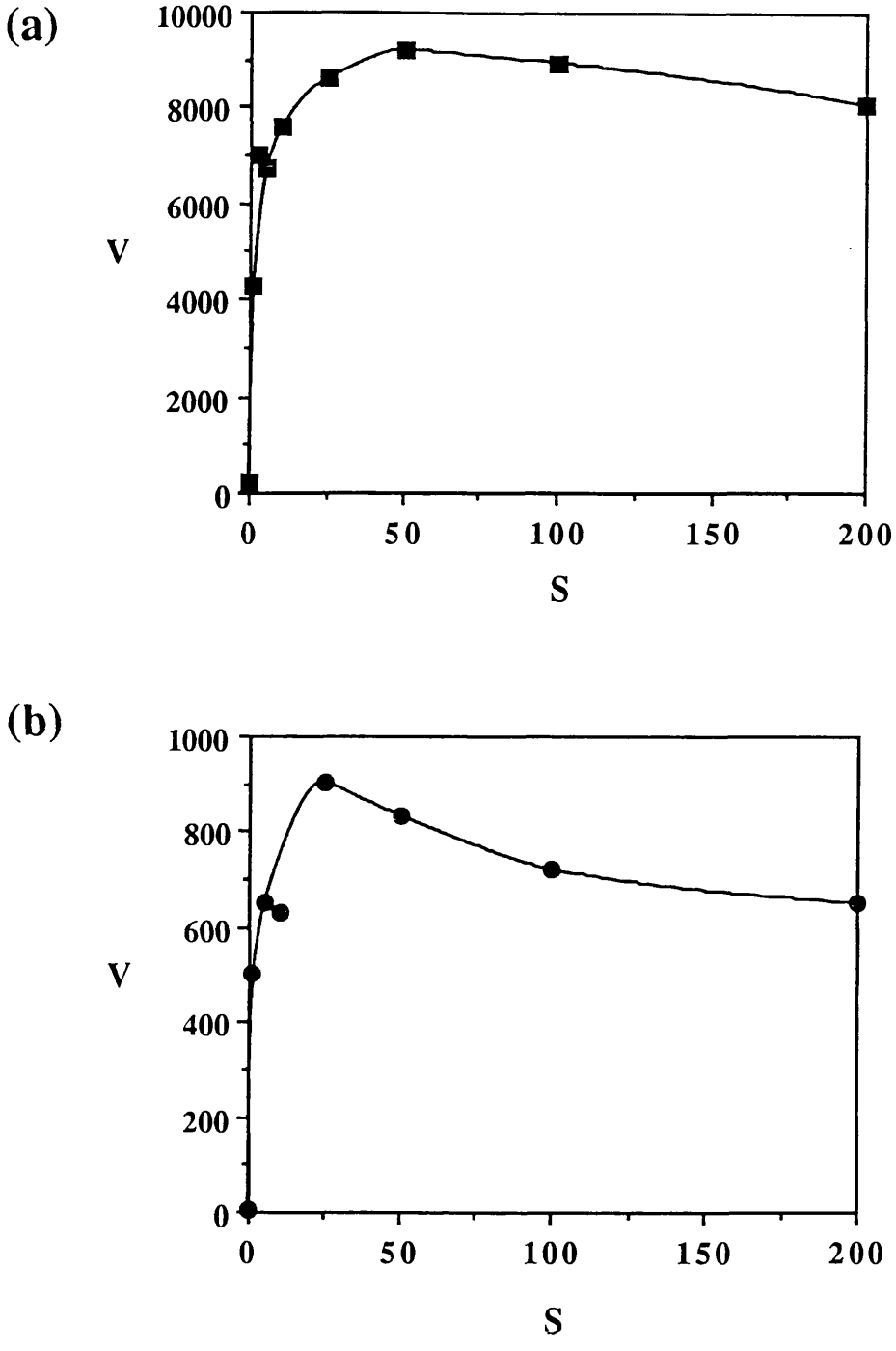


Fig. 3.2 Plot of (v) initial velocity (n moles cytochrome *c* reduced / minute / mg protein) versus (S) menadione concentration (μM) for DT-diaphorase present in (a) Walker cell sonicates and (b) HT29 cell sonicates in the presence of cofactor NADH. Data are taken from an individual experiment but were confirmed in at least two independent repeats.

Compound	Walker		HT-29	
	Km (μM)	Vmax (μmol/min/ mg)	Km (μM)	Vmax (μmol/min/ mg)
Menadione	3.59[a]	13.23[a]	4.27[a] ± 2.82 (n=3)	1.40[a] ± 0.36 (n=3)
	1.69[a]	9.42[a]		
	6.67[b]	11.99[b]	2.73[b]	1.38[b]
EO9	24.29[a]	2.15[a]	17.35[a]	0.074[a]
	7.61[a]	2.52[a]		

Table 3.1 Kinetic parameters for the metabolism of menadione and indoloquinone EO9 catalysed by DT-diaphorase present in sonicates of rat Walker and human HT29 tumour cells. Data are expressed per mg of protein. Where more than two independent repeat experiments were carried out data are stated as a mean ± standard error with the number of individual experiments (n). For studies with less than two repeats results of individual experiments are given. The effect of varying DMSO concentration was examined (a) represents a final DMSO concentration of 2% and (b) of 1% in the reaction buffer.

by DT-diaphorase present in sonicates of both rat Walker and human HT29 tumour cells. Consistent with its role as an inhibitor of DT-diaphorase, dicoumarol (100μM) decreased menadione reductase activity by 96-97% in the case of the Walker extract and 94-95% for the human preparation at saturating substrate concentrations. Progress curves were observed to be linear for, in general, the first 30 seconds of the assay over the range of substrate concentrations examined and at the lower concentrations they remained linear for even longer periods (data not shown). Initial velocity (v) was calculated from the linear portion of these progress curves. When v was plotted against substrate concentration (S) this gave a rectangular hyperbola (Fig 3.2) indicative of an enzyme reaction conforming to Michaelis-Menten kinetics. This was particularly clear for the Walker preparation (Fig 3.2 a). An increase in initial velocity was seen as substrate concentration increased with saturation occurring at around 25 μM menadione for both Walker and HT29 cell sonicates. Activity was shown to be linear with respect to enzyme concentration over the range examined (data not shown). Kinetic parameters Km and Vmax are displayed in Table 3.1. These were calculated from the Hanes Woolf plot for menadione with both Walker and HT29 enzyme preparations, as is shown in Fig 3.3 a. The linearity of these plots provided the evidence of Michaelis-Menten kinetics. The Km values obtained for the rat and human cell DT-diaphorase were similar at around 2-7 μM whereas the maximum reduction rates (Vmax) differed by approximately 8 fold between the two preparations, the Walker extracts being the most active.

EO9

Using the cytochrome *c* reduction assay EO9 was also shown to undergo efficient reduction catalysed by DT-diaphorase present in sonicates of both rat Walker tumour cell and human HT29 colon carcinoma cells in the presence of cofactor NADH (Fig 3.3 b). As for menadione, reaction kinetics were consistent with Michaelis-Menten behaviour. The rate of indoloquinone reduction appeared linear with respect to DT-diaphorase concentration over the range examined (results not shown). Controls where either enzyme or cofactor were omitted resulted in very low levels of background activity (data not shown). In the case of Walker cells both the mean Vmax, of 2.3 μmol cytochrome *c* reduced / minute / mg protein and mean Km, of 16 μM were only ~6 fold lower and higher respectively than the benchmark substrate menadione. In the case of HT29 cell sonicates the Vmax was approximately 16-17 fold lower than that of menadione while the Km value was only four fold higher (Table 3.1). Thus with EO9 as a substrate the rat Walker cells were more active with regard to DT-diaphorase activity than HT29 cells. Inclusion of dicoumarol (100 μM),

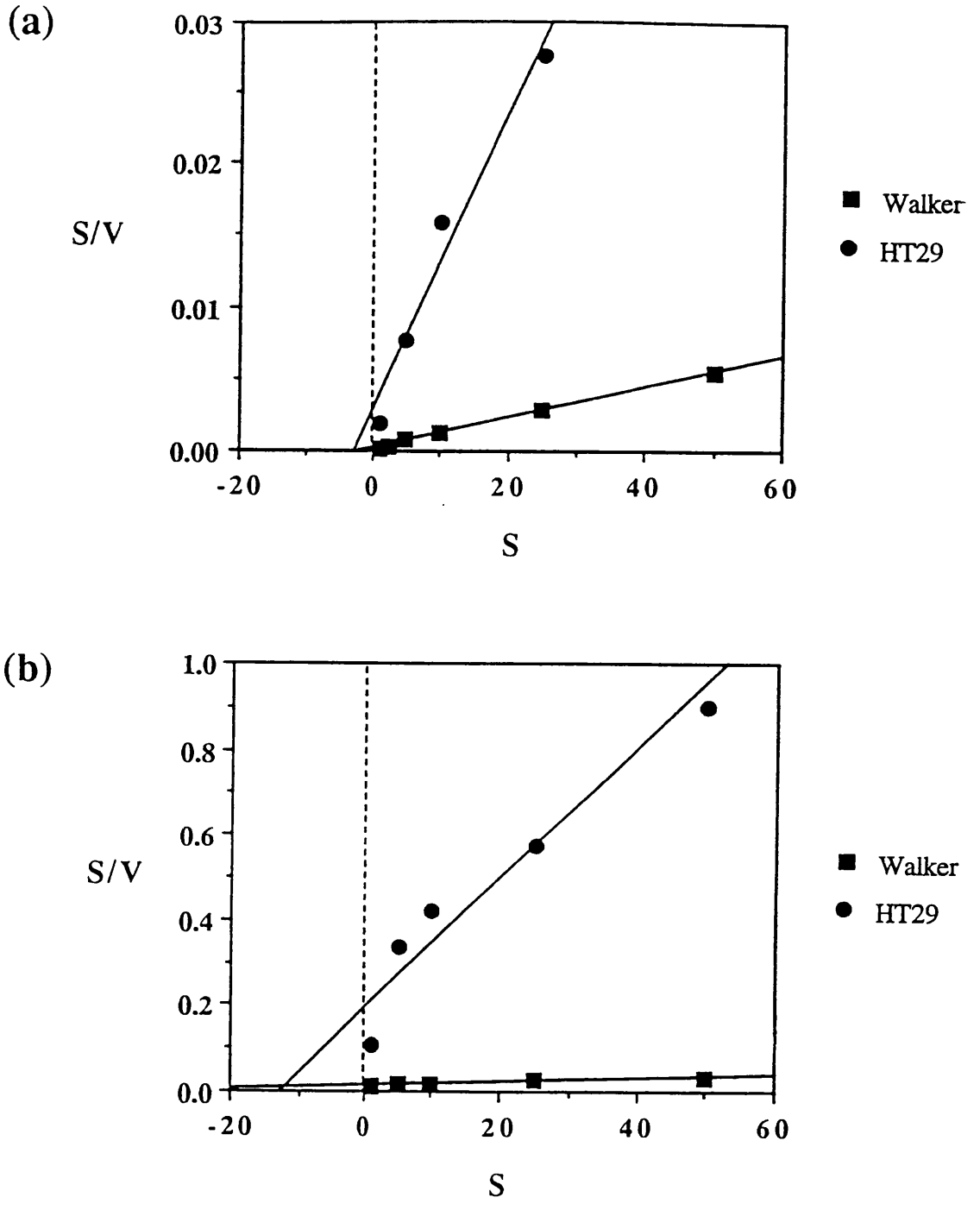


Fig. 3.3 Hanes Woolf plot for the metabolism of (a) menadione and (b) EO9 catalysed by DT-diaphorase present in sonicates of rat Walker and human HT29 cells with the cofactor NADH. v is initial velocity (n moles cytochrome *c* reduced / minute / mg protein) and S is substrate concentration (μM). Data are taken from an individual experiment but were confirmed in at least one independent repeat.

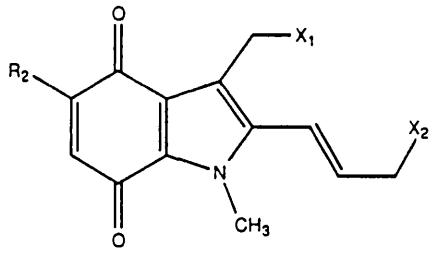
the inhibitor of DT-diaphorase, reduced the activity by ~96-97% at saturating substrate concentrations for the Walker enzyme and by around 65% for the HT29 cell preparation. The dicoumarol inhibitable activity in the sonicates was attributed to metabolism catalysed by DT-diaphorase and the residual assigned to other enzymes.

EO9 Analogues

Reduction of indoloquinone derivatives of EO9 was also found to be catalysed by DT-diaphorase present in sonicates of the rat and human tumour cells with activity varying according to the structural modification made (Table 3.2). As in the case of EO9, reduction of the analogues was linear with increasing enzyme concentration and minimal activity was observed in the absence of cofactor, enzyme or drug (data not shown). For the rat Walker enzyme both the methyl-aziridine compound, EO8 and EO7, the analogue possessing a methoxy substituent in place of the aziridine, exhibited reduced activity compared to EO9 when considering Vmax values (0.138 and 0.993 μmol / minute / mg for EO8 and EO7 respectively compared with a mean of 2.3 μmol / minute / mg for EO9). The Km value for EO7 of 5.44 μM was, however, lower than that of EO9 (16 μM) although that of EO8 was higher being 49.1 μM. In the case of the HT29 cell enzyme both EO7 and EO8 acted as poorer substrates than EO9 in terms of Vmax but exhibited lower Km values of (9.15 μM and 5.78 μM respectively compared with 17.35 μM for EO9). EO7 acted as a better substrate for both enzyme preparations than EO8 in terms of Vmax values. The Km for EO7 was approximately 10 fold lower than that of EO8 for the Walker cell enzyme but was similar in the case of the HT29 enzyme. EO88, the compound possessing a carbamate leaving group in place of the hydroxyl leaving group in EO9 at the X_2 position was however able to undergo five fold more rapid reduction catalysed by rat Walker cell DT-diaphorase than EO9 itself when considering Vmax values. Furthermore the Km value was almost half that of EO9. Interestingly for EO5A, the aziridine ring-opened metabolite, no reduction was observed with either enzyme preparation at the limit of detection of the assay.

In addition to use of sonicates the reduction of EO9 and its analogues was compared using a highly purified preparation of the rat Walker DT-diaphorase. As shown in Fig 3.4 and Tables 3.3 and 3.4 the purified DT-diaphorase was able to catalyse reduction of EO9 and various analogues. Metabolism of the indoloquinone analogues by purified rat Walker DT-diaphorase was of a similar order to that obtained using the sonicates although activity was of course proportionally higher when expressed per mg of protein. For example, EO88 was confirmed as a more efficient substrate with

Compound	Walker			HT-29		
	Km (μM)	Vmax (μmol/min/ mg)	Vmax (μmol/min/ U)	Km (μM)	Vmax (μmol/min/ mg)	Vmax (μmol/min/ U)
EO9	24.29 7.61	2.15 2.5	216.00 87.35	17.35	0.074	63.89
EO7	5.44	0.993	103.08	9.15	0.032	31.32
EO8	49.08	0.138	11.50	5.78	0.0137	12.60
EO88	14.04	10.07	299.97	ND	ND	ND
EO5A	No activity observed at background limit of assay					



Indoloquinone core structure

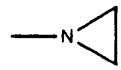
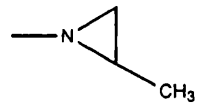
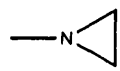
Analogue	R_2	X_1	X_2
EO9		OH	OH
EO8		OH	OH
EO7	OCH_3	OH	OH
EO88		OH	$OCONH_2$
EO5A	$NHCH_2CH_2OH$	OH	OH

Table 3.2 Kinetic parameters for the metabolism of indoloquinones related to EO9 catalysed by DT-diaphorase present in sonicates of rat Walker and human HT29 tumour cells. Data are expressed both per mg of protein and in units of menadione activity. Where more than two repeats were carried out results for both experiments are quoted. ND means not determined.

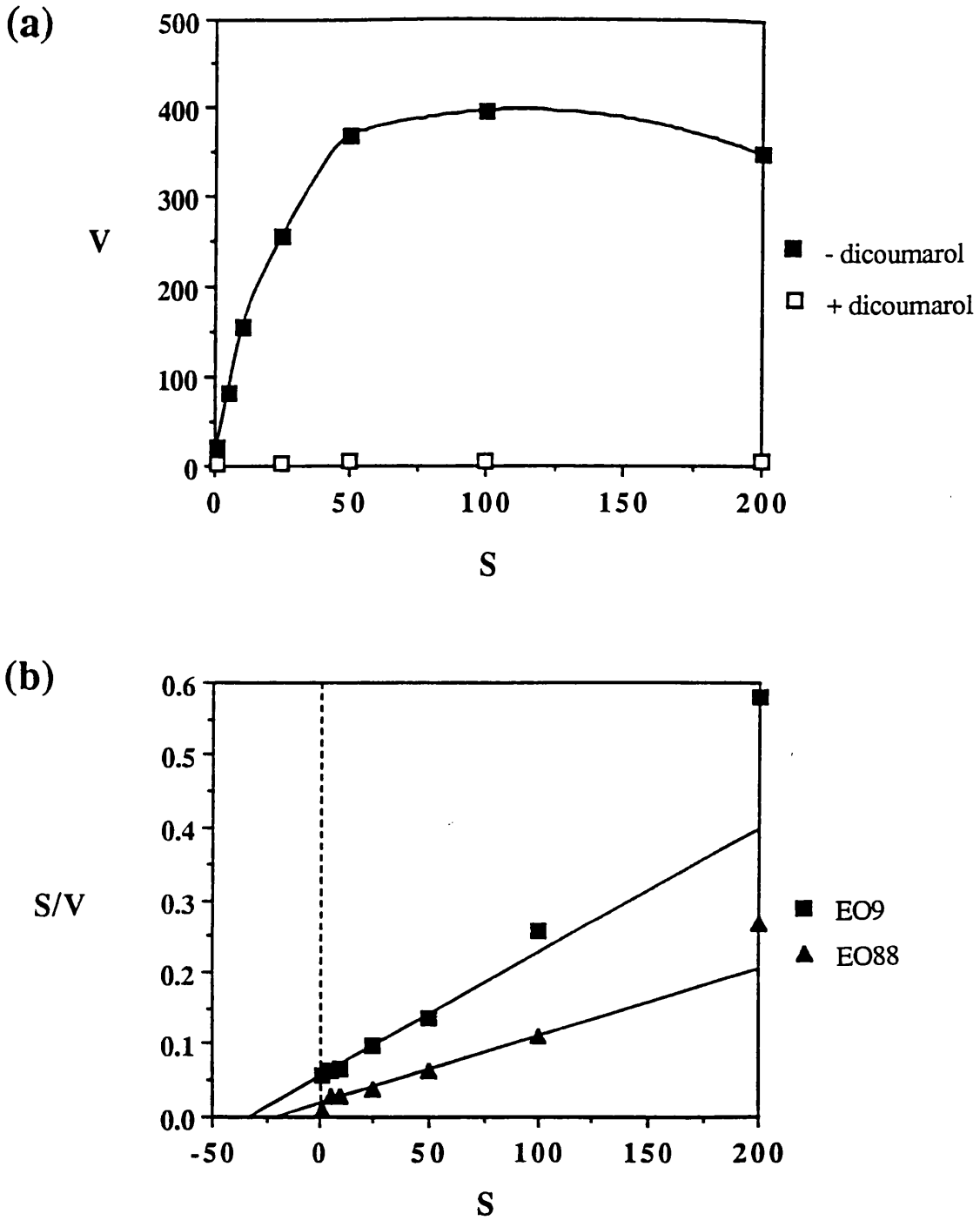


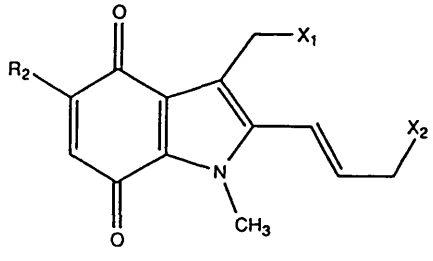
Fig. 3.4 (a) Plot of initial velocity (v, n moles cytochrome *c* reduced / minute / mg protein) versus substrate concentration (S, μM) for the reduction of EO9 catalysed by purified rat Walker DT-diaphorase with increasing drug concentration in the presence and absence of dicoumarol.

(b) Hanes Woolf plot for the metabolism of EO9 and EO88 by purified rat Walker DT-diaphorase. Units are identical to those in (a). Data for both (a) and (b) are taken from an individual experiment and were confirmed in an independent repeat.

Drug	Km (μM)	Vmax (μmoles / min / mg)
Menadione	1.87[a]	1002[a]
	1.65[b]	1198[b]
EO9	31.36[a]	504[a]
	31.41[a]	505[a]

Table 3.3 Kinetic parameters for the metabolism of menadione and indoloquinone EO9 catalysed by purified rat Walker cell DT-diaphorase. Data are quoted for two independent repeat experiments. The effect of varying DMSO concentration was examined: (a) represents a final DMSO concentration of 2% and (b) of 1% in the reaction buffer.

Drug	Km (μM)	Vmax (μmoles / min / mg)	Vmax (nmoles / min / U)
EO9	31.36 31.41	504.15 505.77	166.32 109.05
EO8	16.80 30.58	15.55 61.49	7.22 11.48
EO7	5.55 ± 0.59 (n=3)	264.64 ± 33.96 (n=3)	84.61 ± 13.56 (n=3)
EO88	18.23 ± 2.99 (n=4)	1378.33 ± 165.49 (n=4)	372.93 ± 5.40 (n=4)



Indoloquinone core structure

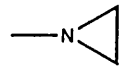
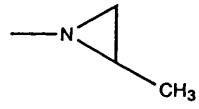
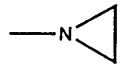
Analogue	R_1	X_1	X_2
EO9		OH	OH
EO8		OH	OH
EO7	OCH_3	OH	OH
EO88		OH	$OCONH_2$

Table 3.4 Kinetic parameters for the metabolism of indoloquinones related to EO9 catalysed by purified rat Walker cell DT-diaphorase. Data are expressed both per mg of protein and in units of menadione activity. Where more than two repeats were carried out results are quoted as a mean ± standard error with the number of independent repeat assays (n). Where only two repeat experiments were carried out the result for each individual experiment is given.

around 3-fold higher Vmax and a 2-fold lower Km than EO9. EO7 exhibited a 2-fold lower Vmax but also a 5-fold lower Km compared to EO9 while EO8 showed a much lower Vmax but a smaller Km. Two additional compounds were screened with the purified enzyme, EO72 which possessed a carboxymethyl leaving groups in place of the hydroxyl leaving group of EO9 (Fig 3.1) and EO16 where the aziridine was substituted with a methoxy group and the R_5 methyl group was replaced by a butyl. Both EO72 and EO16 were poorly metabolised by rat Walker DT-diaphorase (data not shown) with activity being around the background levels of the assay.

Mitomycin C

Reduction of mitomycin C catalysed by purified rat Walker DT-diaphorase was also examined. Experiments were limited to two concentrations of mitomycin C as this compound was an extremely poor substrate for DT-diaphorase and more detailed studies would have necessitated the use of large quantities of enzyme. At a drug concentration of 100 μM, DT-diaphorase catalysed reduction of mitomycin C was approximately 150 fold slower than for EO9 with an activity of 2.84 μmoles cytochrome *c* reduced / minute / mg of enzyme (taken from a mean of 2 repeat assays carried out within the same experiment). Where a concentration of 50 μM mitomycin C was employed the activity was 0.895 ± 0.304 μM cytochrome *c* reduced / minute / mg DT-diaphorase (mean ± SD where n=4) which was around 720 fold lower than EO9.

Direct Spectrophotometry

In order to provide further evidence for EO9 reduction catalysed by DT-diaphorase, changes in the concentrations of reaction constituents were measured directly by spectrophotometry. This was carried out over an absorbance range of 200-700 nm and also by monitoring change in absorbance of EO9 specifically at a fixed absorbance of 500 nm. For these experiments conditions were changed slightly from those described for the cytochrome *c* reduction assay described earlier. The principal difference was that cytochrome *c* was omitted. Other constituents varied in concentration and are described below.

Experiments monitoring change in absorbance between 200-700 nm wavelength during the reduction of EO9 (50 μM) by DT-diaphorase (0.25 μg / ml) showed a decrease in peak height at 340 nm over a 10 minute period consistent with loss of the cofactor NADH (200 μM) (Fig 3.5). Controls where drug was substituted by vehicle

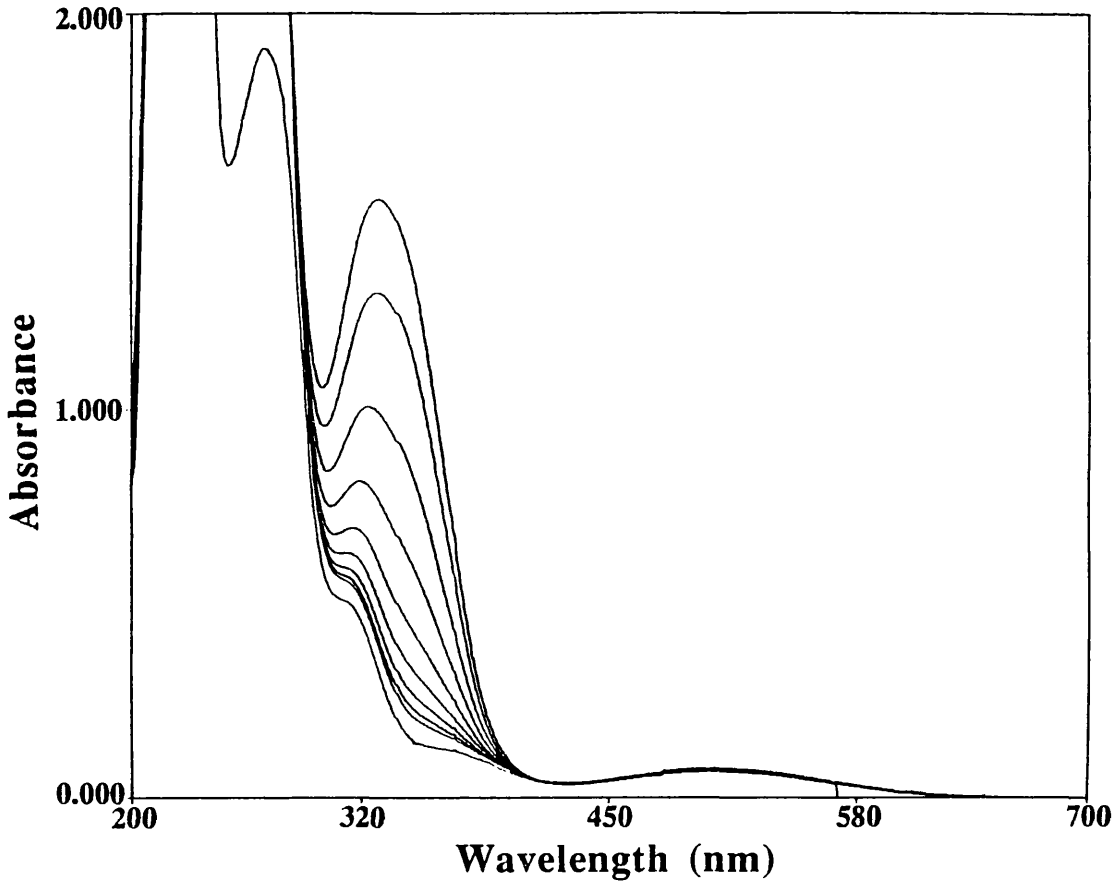


Fig. 3.5 Sequential UV-visible absorbance scans associated with the reduction of EO9 catalysed by DT-diaphorase in the presence of cofactor. The lower curve represents EO9 in buffer in the absence of cofactor and enzyme and the uppermost EO9 plus NADH in the absence of enzyme. Subsequent scans were taken each minute following initiation of reduction by addition of DT-diaphorase. Data are taken from an individual experiment and were confirmed in a single repeat assay.

alone or where enzyme was omitted resulted in no detectable decrease in NADH peak height. Little reduction in absorbance was noted at 450-550 nm wavelength which corresponds to EO9 under these conditions. However, in experiments where EO9 (100 μM) absorbance was monitored continuously at 500 nm wavelength during reduction catalysed by a higher concentration of DT-diaphorase (5 μg / 3 ml) in the presence of an increased concentration of NADH (500 μM) a decrease in the reading was observed (data not shown). The absorbance remained low for ~3 minutes before increasing to near original levels after ~6-7 minutes. The reduction was visible to the naked eye as a change from the purple coloured EO9 to colourless on initiation of reaction, followed by regeneration of purple coloration. The reappearance of colour was initially evident at the reaction mixture / air interface but with the aid of a magnetic flea was made uniform throughout the cuvette.

3.3.2 The Effect of Hypoxia on DT-diaphorase Metabolism of Indoloquinones

The initial velocity of DT-diaphorase metabolism of EO9, EO8 (Fig 3.6 b), EO7 and EO88 (Fig 3.6 a) was found to be almost identical under hypoxic and aerobic conditions as determined by the cytochrome *c* reduction assay.

3.3.3 The Effect of pH on DT-diaphorase Metabolism of EO9

When Tris-Cl buffer and sodium phosphate buffer were compared at pH 7.4 with both the standard substrate menadione (50 μM) and EO9 (50 μM and 10 μM) only slight differences in rate were observed. The effect of changing pH to 5.8 was also examined. The rate of menadione reduction catalysed by purified rat Walker DT-diaphorase decreased by ~30 % with lowering pH whereas that of EO9 showed a 20% decrease at 50 μM but no change at 10 μM. Subsequent experiments were carried out to further investigate the effect of altering pH on EO9 metabolism over a wider range including 7.8, 7.4, 7.0, 6.4, and 5.8. Results indicated that at concentrations of both 50 μM and 10 μM menadione, DT-diaphorase-catalysed metabolism was more rapid at pH 7.4 with activity decreasing both above and below this level (Fig 3.7 a). In contrast, EO9 (50 μM and 10 μM) showed little variation over the range of pH values examined although if anything a slight optimum was seen at pH 7.0 (Fig 3.7 b).

Mitomycin C was also included in these experiments as studies carried out using HPLC analysis by Siegel and co-workers (1990b) had noted a profound effect of pH on reduction by purified rat hepatic DT-diaphorase. Interestingly, using the cytochrome *c* reduction assay, we observed no difference in the initial velocity of

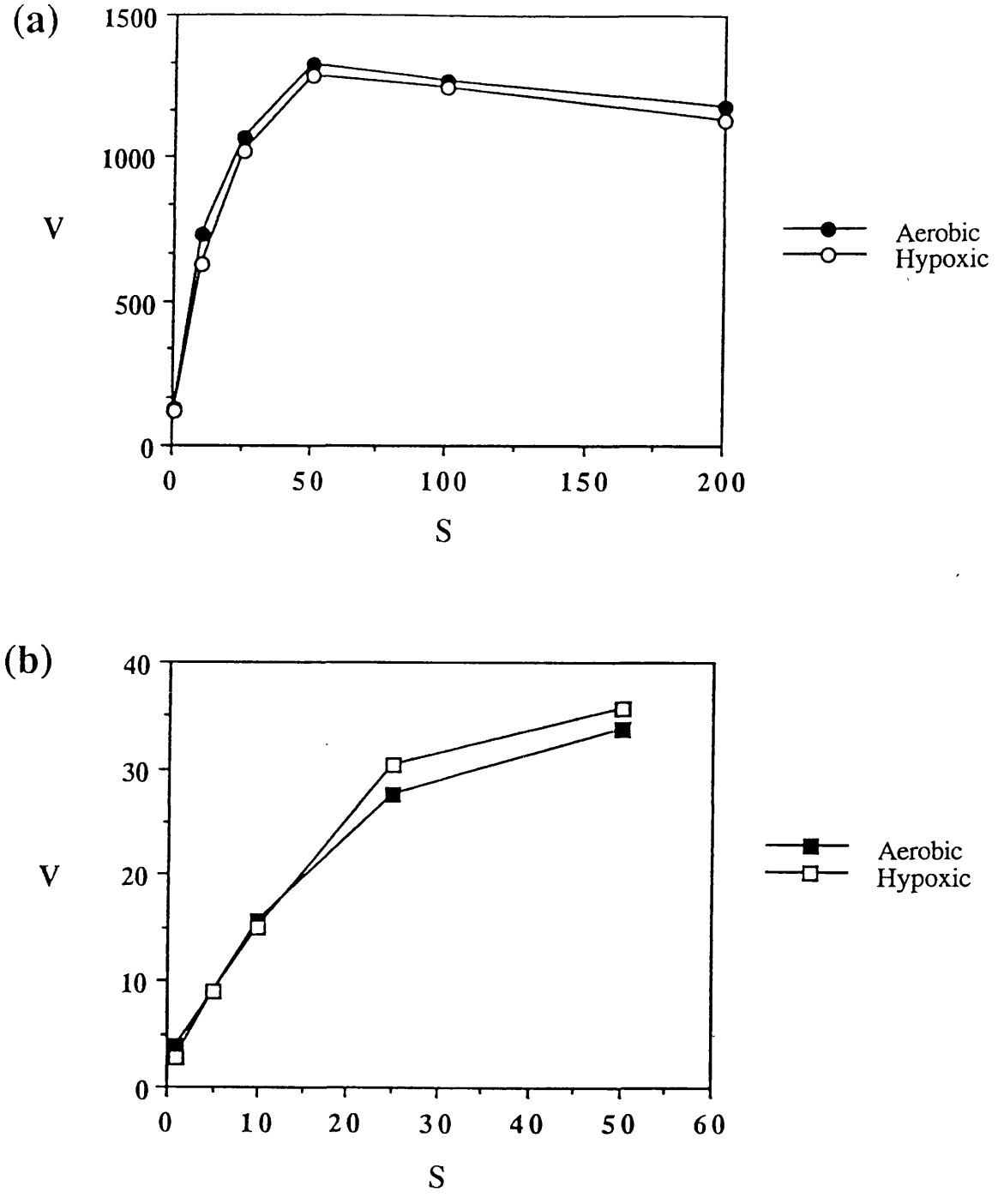


Fig 3.6 Effect of hypoxia on the initial velocity of (a) EO88 and (b) EO8 reduction catalysed by purified rat Walker DT-diaphorase in the presence of cofactor NADH. Units of activity (v) are in n moles of cytochrome c reduced / minute / mg of purified rat Walker DT-diaphorase. S is substrate (μM). Data were obtained from an individual experiment but similar results were obtained in at least two repeat assays which were carried out for most substrate concentrations.

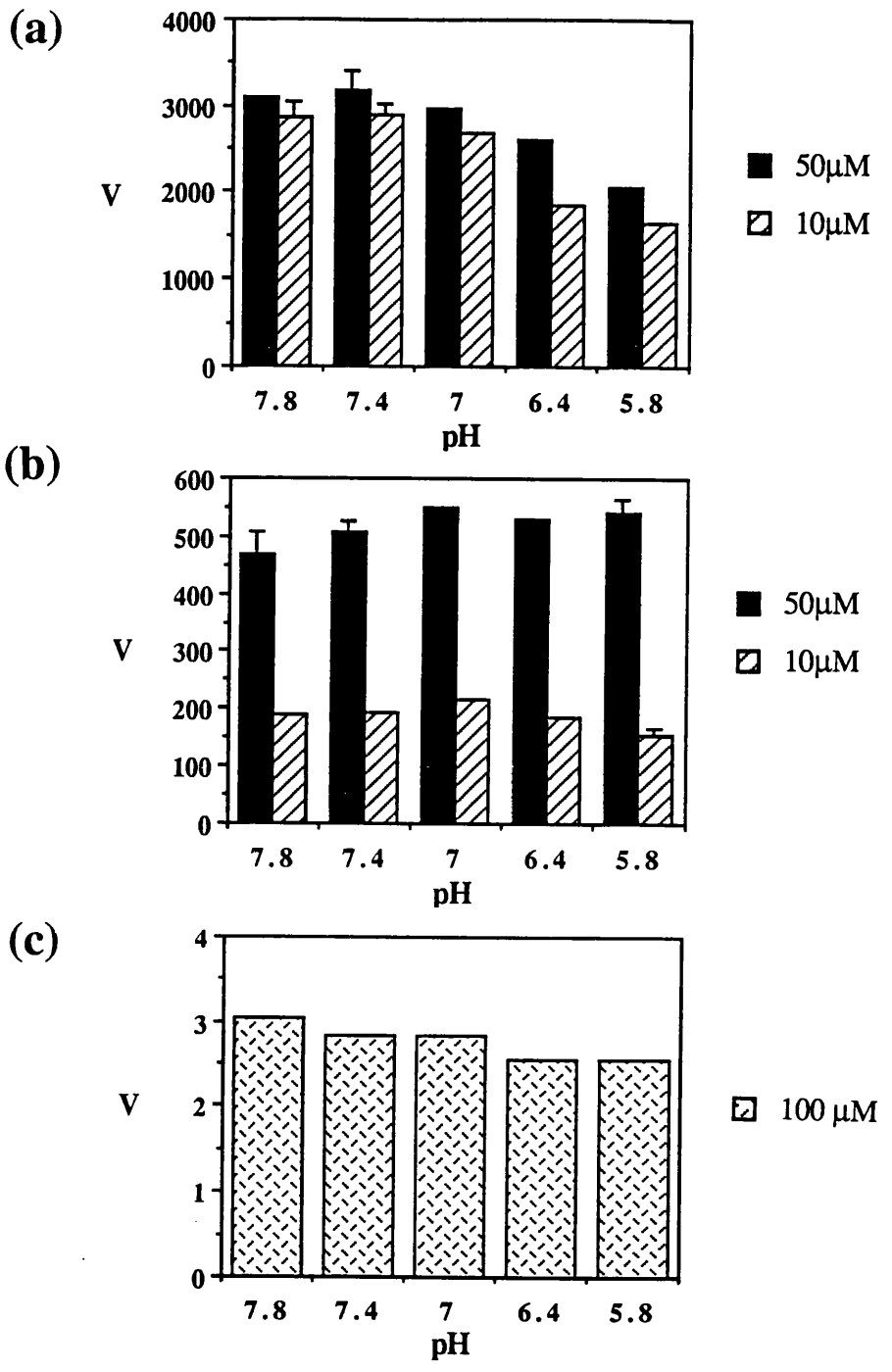


Fig. 3.7 Effect of pH on the metabolism of (a) menadione, (b) EO9 and (c) mitomycin C. Data are taken from an individual experiment and standard deviations where given or illustrated were calculated from at least three repeat assays. Results were confirmed in an independent experiment for pH 5.8 and 7.4. Units of activity (v) are in n moles of cytochrome c reduced / minute / mg of purified rat Walker DT-diaphorase.

mitomycin C reduction catalysed by purified rat Walker DT-diaphorase at various pH values (Fig 3.7 c).

3.3.4 DT-diaphorase Catalysed Reduction of EO9: HPLC

HPLC experiments confirmed that EO9 could undergo reduction catalysed by purified rat Walker cell DT-diaphorase. This was indicated by a decrease in peak height of NADH cofactor which eluted at 0.55-0.6 minutes (Fig 3.8). Interestingly, only a slight decrease in the peak height of EO9 itself was observed. As in the case of spectrophotometric assays, increasing enzyme concentration increased the rate of reduction of EO9 as measured by loss of cofactor (data not shown). Little change was noted for control incubations where either enzyme or drug were omitted.

The effect of hypoxia on DT-diaphorase-catalysed reduction of EO9 was also examined. Following reduction of EO9 results revealed the formation of a colourless metabolite, as visualised by the naked eye. This was presumably the hydroquinone. However, when this sample was injected into the HPLC equipment the chromatogram again showed little or no decrease in EO9 peak height, indicating that reoxidation had occurred during the analyses.

3.3.5 DT-diaphorase and NADPH: Cytochrome P450 Reductase Catalysed Reduction of Quinones: ESR Experiments

Radical Formation Following DT-diaphorase Reduction of EO9

Aerobic incubation of EO9 (800 μM), purified rat Walker DT-diaphorase (0.25 μg) and cofactor NADPH (1 mM) in a total volume of 400 μl buffer (Tris Cl and 0.14% BSA) led to the production of a highly reproducible ESR spectrum. This was apparent 3 minutes after initiation of the reaction (Fig 3.9, spectrum a). Signal intensity increased over time and after 8 minutes it was clearly distinguishable as an 8 line spectrum (Fig 3.9, spectrum b) which was stable over 30 minutes (not shown). The spectrum is characteristic of a drug-based radical, and by analogy with other quinones this almost certainly represents the semiquinone species. Interestingly, either decreasing the DT-diaphorase concentration to 0.007 μg or increasing it to 2 μg or 5 μg resulted in abrogation of the detection of drug-based radicals (data not shown).

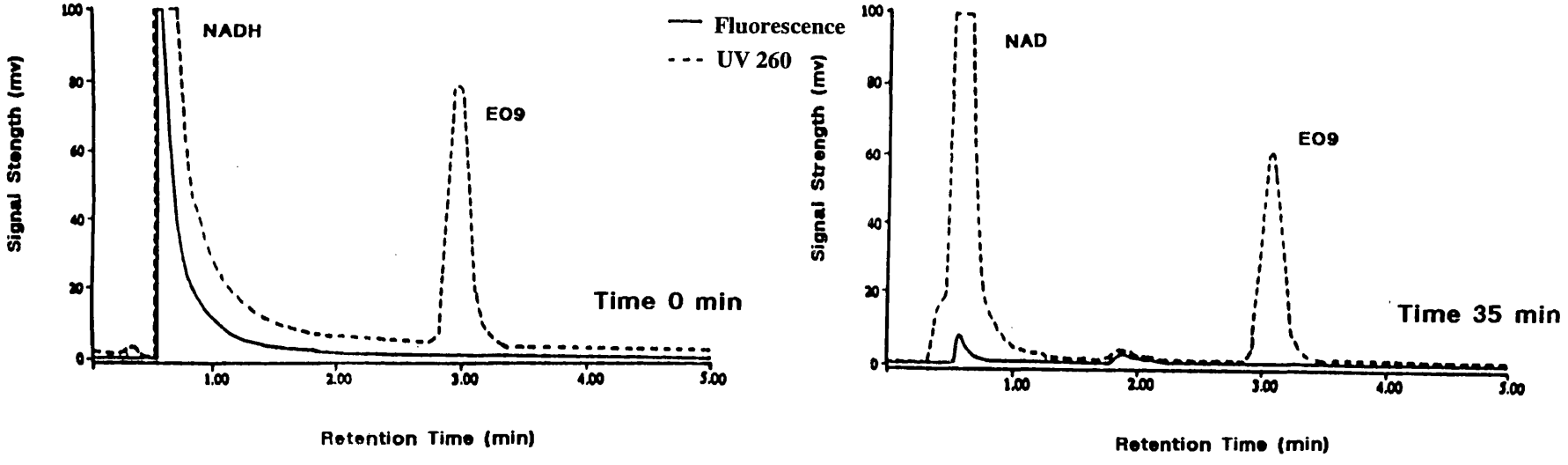
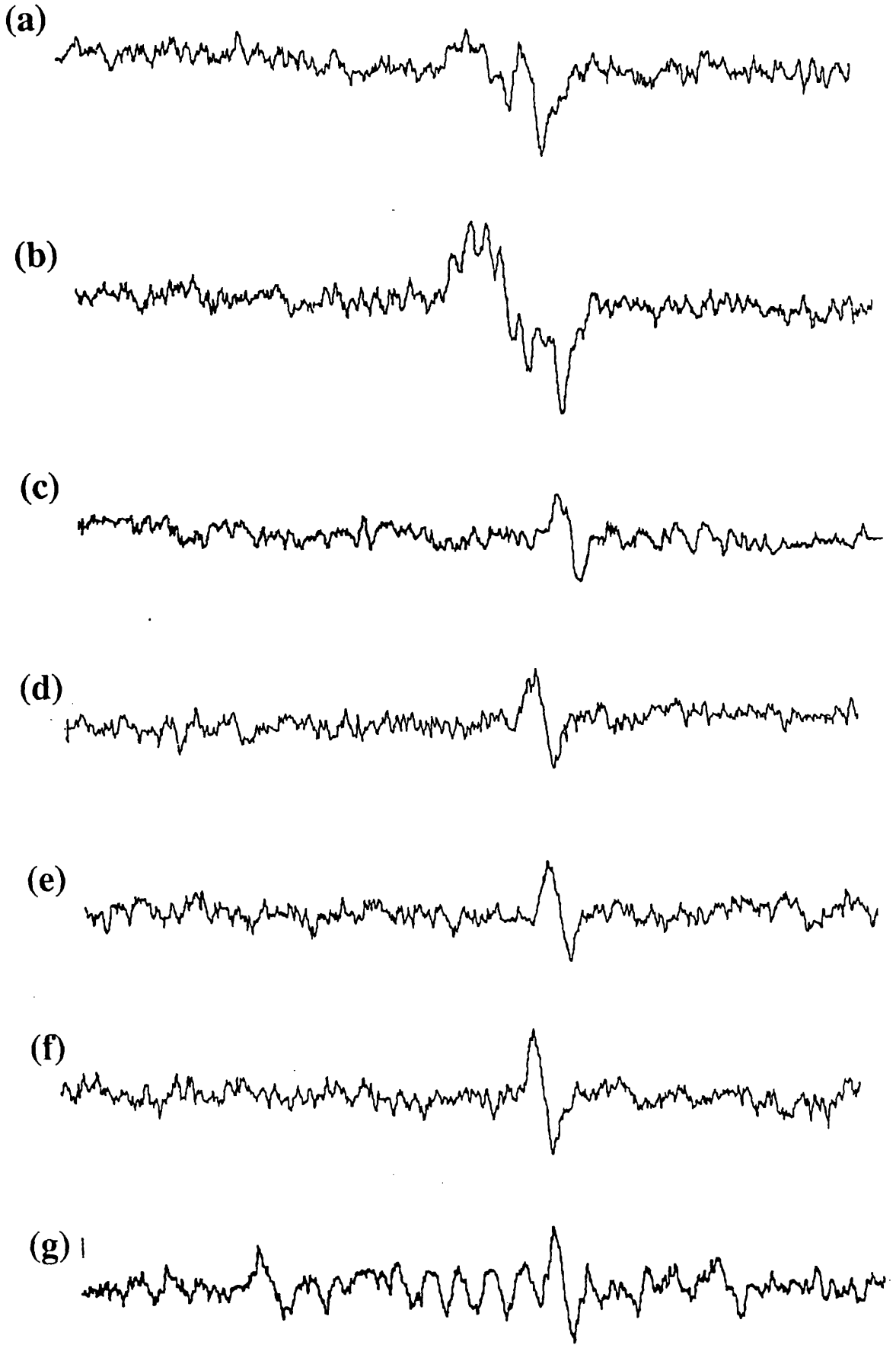


Fig. 3.8 HPLC chromatograms showing change in peak height of EO9 and NADH absorbance at 260 nm and by fluorescence absorbance during DT-diaphorase catalysed metabolism. Reaction constituents included: 50 µl of 1:100 dilution of purified Walker DT-diaphorase, 500 µM NADH and 100 µM EO9 in Tris-Cl buffer pH 7.5. Data are taken from an individual experiment. The same trends were seen in similar experiments where only enzyme concentration was altered.

Fig 3.9 ESR spectra obtained during metabolism of EO9 catalysed by purified rat Walker DT-diaphorase.

Standard reaction conditions included DT-diaphorase (0.25 μg), EO9 (800 μM) and NADPH (1mM) in a volume of 400 μl. (a and b) complete system after (a) 3 minutes and (b) 8 minutes, (c) inclusion of 100 μM dicoumarol, (d) inclusion of 43 μl anti-DT-diaphorase antiserum and (e, f and g) controls where either (e) NADPH, (f) drug or (g) enzyme were omitted. With the exception of (b) all spectra were taken 3 minutes after initiation of reaction. Where possible most of the data were confirmed in an independent repeat experiment.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A series of control experiments were carried out. The generation of a detectable signal was blocked by inclusion of the inhibitor dicoumarol (500 μM) (Fig 3.9, spectrum c), pre-incubation with rabbit anti-rat DT-diaphorase antiserum (43 μl) (Fig 3.9, spectrum d) and omission of the cofactor NADPH (Fig 3.9, spectrum e), EO9 (Fig 3.9, spectrum f) or DT-diaphorase (Fig 3.9, spectrum g).

Replacing the cofactor NADPH (1 mM) with NADH (1 mM) gave almost identical results (Fig 3.10). This Figure also illustrates that an increase in cofactor concentration to 2 mM gave a similar intensity of peak initially but after 9 minutes this began to decrease in a linear fashion, leaving no detectable peak after 24 minutes.

Given that DT-diaphorase is thought to catalyse direct two-electron reduction to the hydroquinone the presence of a drug-based radical here would suggest that auto-oxidation may have occurred. Thus it would be expected that oxygen-based radicals would also have been generated. These radicals such as the superoxide anion are highly unstable and therefore due to their short life span are extremely difficult to detect by conventional ESR. For this reason spin traps are commonly included to permit detection of short lived oxygen-based radicals. The nitrone compound DMPO (5,5-dimethyl-1-pyrroline-1-oxide) has been used successfully in similar experiments involving the aziridinylbenzoquinone bioreductive agent AZQ (Fisher and Gutierrez, 1991a and b) and was therefore selected as the spintrap in these experiments. DMPO reacts with superoxide and hydroxyl radicals to form adducts with a longer lifetime and characteristic spectrum (Finkelstein, 1979). Following a 3 minute reduction of EO9 catalysed by DT-diaphorase a 6 line spectrum was observed (Fig 3.11, spectrum a). This is characteristic of a DMPO-CH_3 radical formed on reaction of DMSO, present as the drug solvent, with the DMPO-OH radical (Pou *et al*, 1989). On theoretical grounds, the methyl-DMPO radical should appear as a 1:1:1:1:1:1 spectrum. However presumably, due to the short life span of the adduct, signal intensity decreased during the initial 3 minute scan resulting in peaks of unequal height. There is also some indication of a further radical present masked by the oxygen-related species. The 8 minute scan (Fig 3.11, spectra b and c), where total loss of oxygen radicals has occurred, revealed this to be the EO9-based radical which then remained stable over 29 minutes (not shown).

Use of superoxide dismutase and catalase enabled more clear identification the source of oxygen radicals produced in the system (Fig 3.11). Inclusion of these two enzymes led to an inability to detect oxygen-based radicals (Fig 3.11, spectra d and e). The quality of the EO9-based radical (Fig 3.11, spectrum e) remained unaffected by

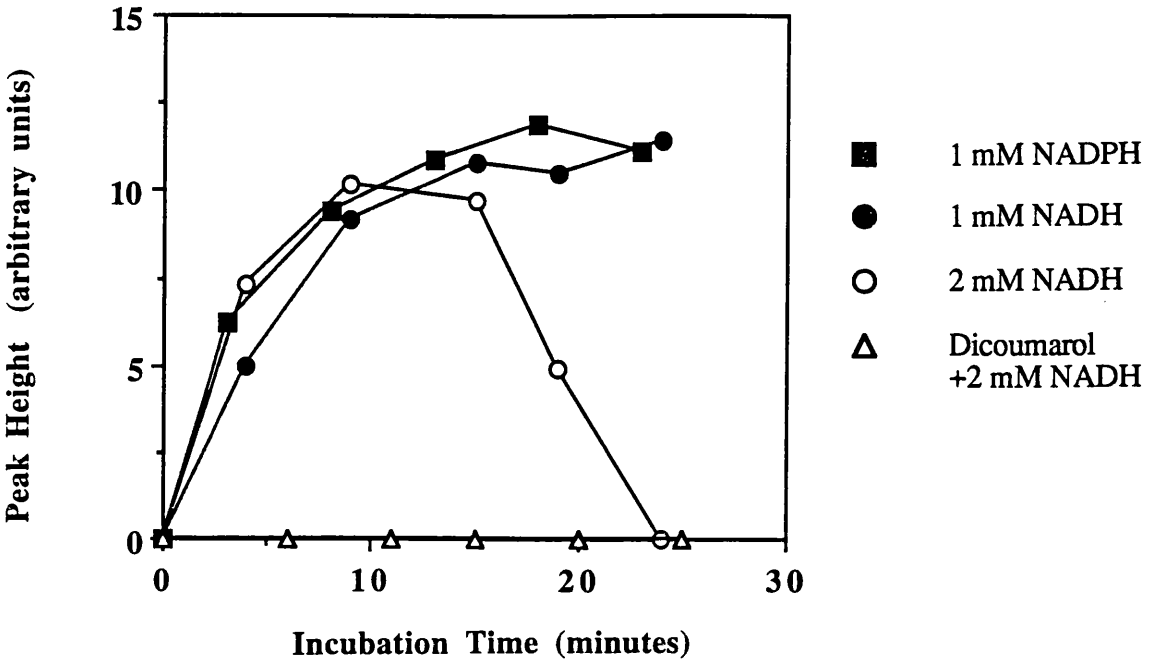
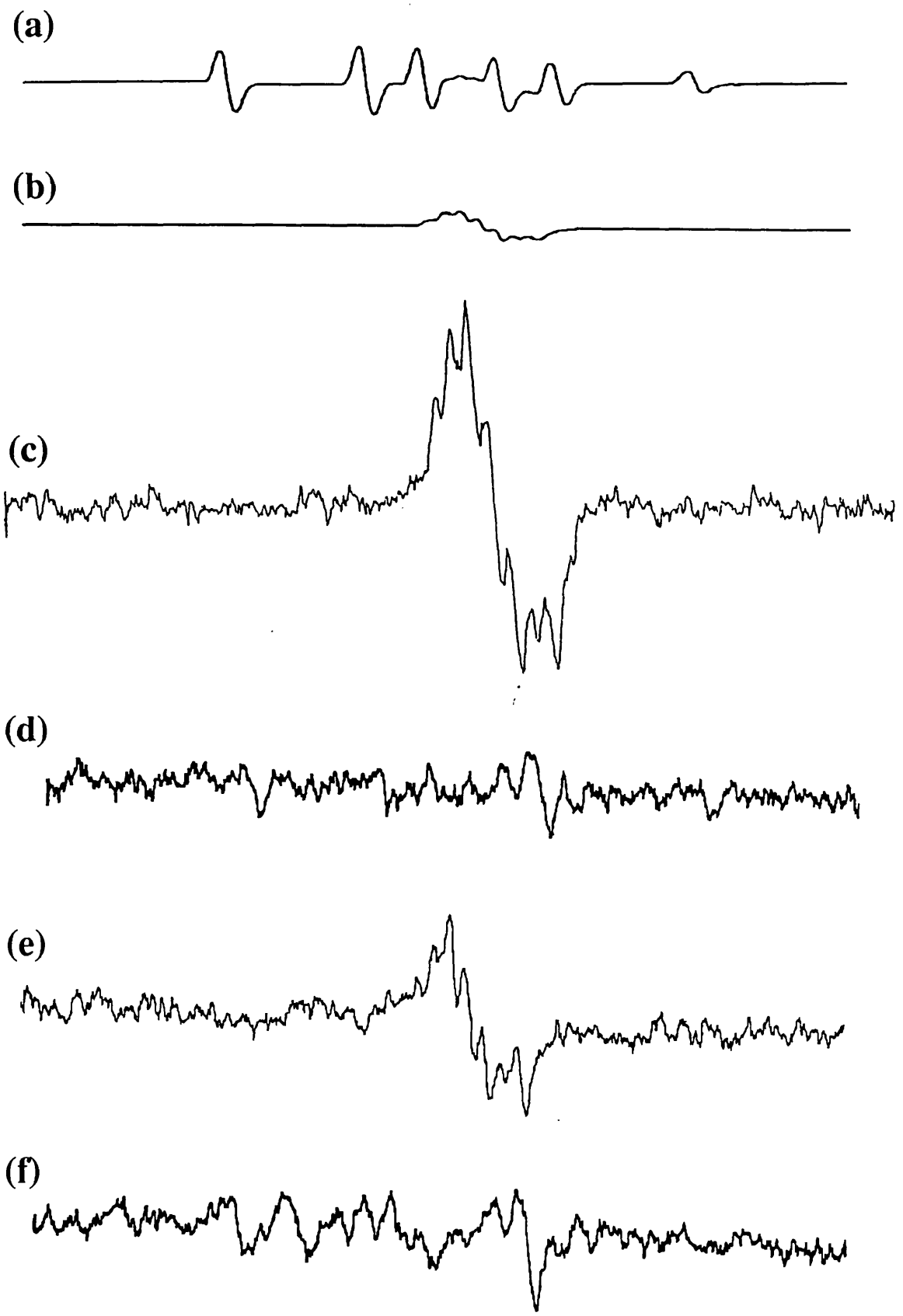


Fig. 3.10 Kinetics of EO9-based radical formation during reduction catalysed by DT-diaphorase in the presence of 1 mM NADH, 1 mM NADPH, 2 mM NADH or 2 mM NADH plus dicoumarol. Data are taken from an individual experiment.

Fig. 3.11 ESR spectra of EO9 and oxygen radicals formed during the reduction of EO9 catalysed by DT-diaphorase in the presence of spin trap DMPO (100 mM).

Standard reaction conditions included DT-diaphorase (0.25 μg), EO9 (800 μM) and NADPH (1mM) in a total volume of 400 μl. (a and b) spectra generated after 3 and 8 minutes following initiation of reaction respectively (c) repeat of (b) with an increased signal intensity, (d and e) complete system plus superoxide dismutase (400 U) and catalase (200U) taken (d) 3 and (e) 8 minutes after the initiation of the reaction, (f and g) complete system plus catalase after (f) 3 minutes and (g) 8 minutes and (h) complete system plus superoxide dismutase (3 minutes), (i) complete system plus 43 μl of anti-DT-diaphorase antiserum (5 minute), (j) complete system plus dicoumarol (100 μM) taken at 5 minutes, (k, l and m) are controls where (k) NADPH, (l) drug or (m) enzyme were omitted. In most cases and where possible, data were confirmed in an independent repeat experiment.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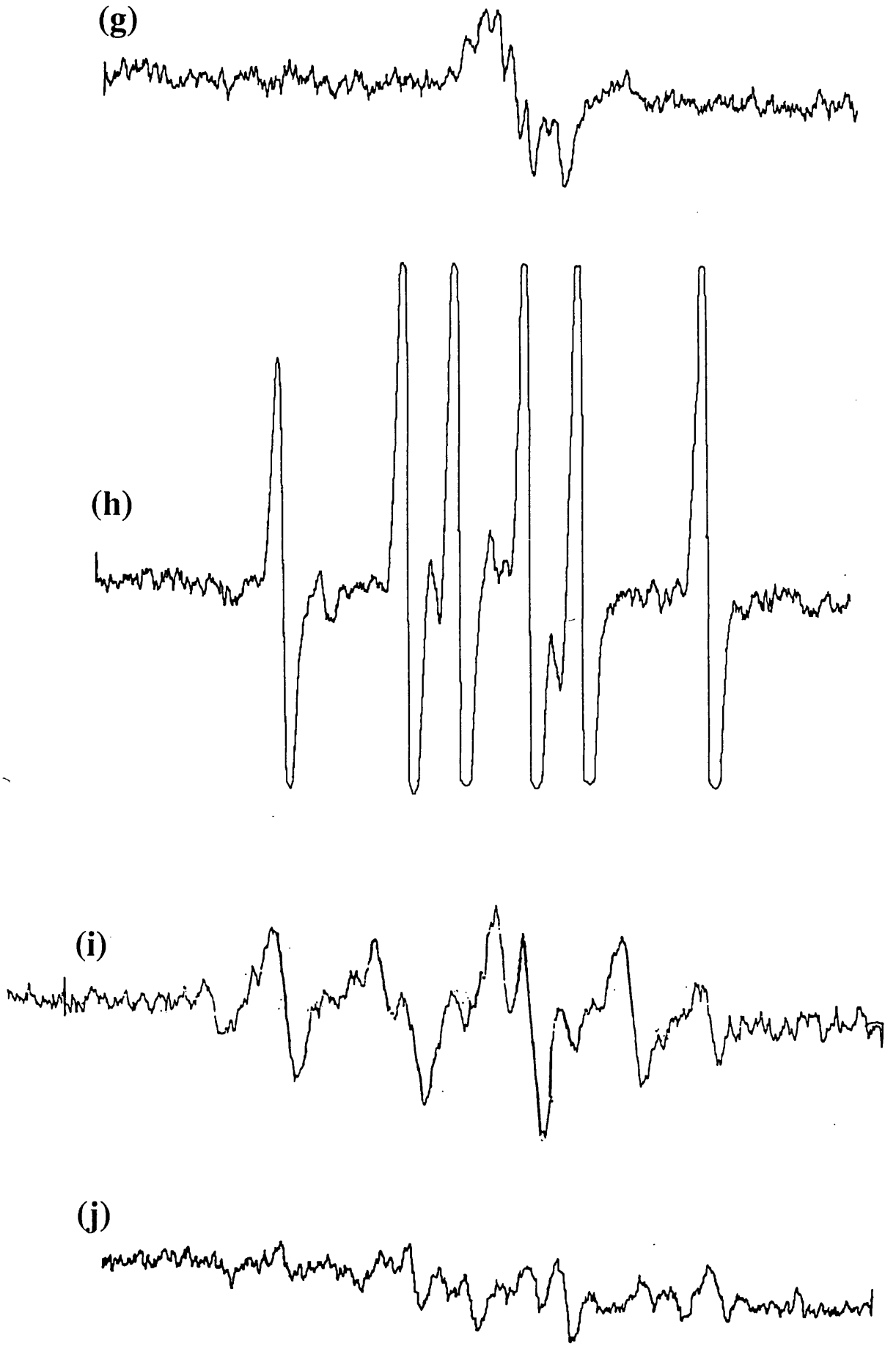
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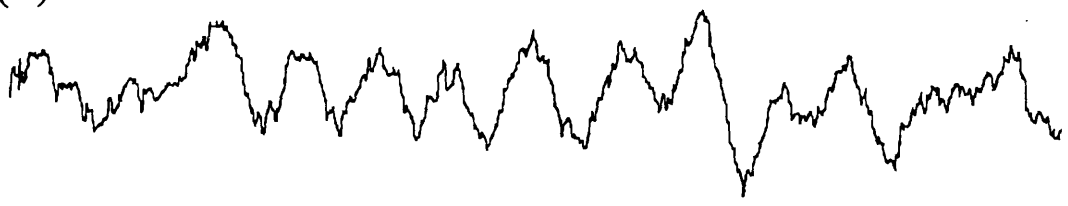

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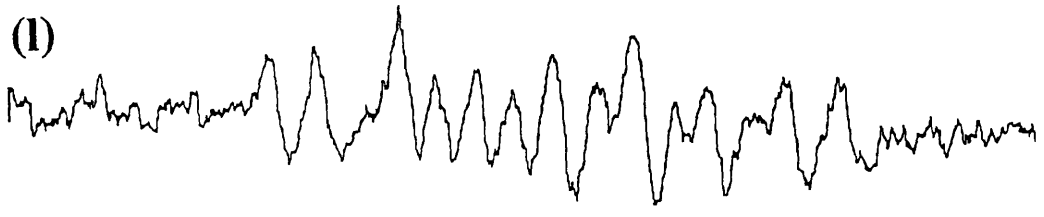

(m)

addition of both superoxide dismutase and catalase but was delayed in appearance (not present at 3 minutes; Fig 3.11, spectrum d) and was diminished in size (Fig 3.11, spectrum e). A similar spectrum was observed when inhibiting with catalase alone (Fig 3.11, spectra f and g). In contrast, superoxide dismutase alone resulted in no inhibition of oxygen radical - DMPO adducts and may have even increased the signal intensity to a small extent (Fig 3.11, spectrum h).

Inhibition of both EO9 and methyl-DMPO radical formation by each of anti-DT-diaphorase antiserum (43 μl) (Fig 3.11, spectrum i) and dicoumarol (100 μM) (Fig 3.11, spectrum j) was noted. Removal of any one of the components, i.e. cofactor (Fig 3.11, spectrum k), EO9 (Fig 3.11, spectrum l) or DT-diaphorase (Fig 3.11, spectrum m), from the system also resulted in loss of signal.

Radical Formation Following NADPH: Cytochrome P450 Reductase Metabolism of EO9

In addition to DT-diaphorase, other enzymes are likely to play a role in metabolism of EO9. NADPH: cytochrome P450 reductase, having been shown to catalyse metabolism of similar compounds was a probable candidate and was therefore examined here by ESR. Incubation of NADPH:cytochrome P450 reductase with EO9 in the presence of cofactor produced a detectable radical 4 minutes after initiation of the reaction (Fig 3.12, spectrum a). As with DT-diaphorase, this was an 8 line spectrum characteristic of a drug-based radical, presumably the semiquinone species. This was persistent over 30 minutes (not shown). Similarly, as in the case of DT-diaphorase, formation of the drug-based radical was dependent on the presence of NADPH (Fig 3.12, spectrum b), EO9 (Fig 3.12, spectrum c) and cytochrome P450 reductase (Fig 3.12, spectrum d) as removal of any one of these components resulted in abolition of the signal.

Spin trap studies with DMPO revealed the presence of oxygen-based radicals following 5 minutes activation of EO9 by purified cytochrome P 450 reductase (Fig 3.13, spectrum a). This was also a 6 line spectrum characteristic of a DMPO-methyl radical. Clearly superimposed upon this is the appearance of a semiquinone radical, more evident after 10 minutes where complete loss of the short lived oxygen-based radicals has occurred (Fig 3.13, spectrum b). This EO9 radical was fairly stable over 30 minutes (not shown). Oxygen-based radical production, like that of the drug, was also dependent on the presence of cofactor (Fig 3.13, spectrum c), drug (Fig 3.13, spectrum d) and NADPH:cytochrome P450 reductase (Fig 3.13, spectrum e).

Fig. 3.12 ESR spectra obtained during the reduction of EO9 catalysed by NADPH: cytochrome P450 reductase.

Standard reaction conditions included NADPH: cytochrome P450 reductase (1.3 μg), EO9 (800 μM) and NADPH (1mM) in a total volume of 400 μl. (a) complete system taken 4 minutes after initiation of reaction, (b, c, d) controls where either (b) NADPH, (c) drug or (d) NADPH were omitted taken 3 minutes after initiation of reaction. In most cases and where possible data were confirmed in an independent repeat experiment.

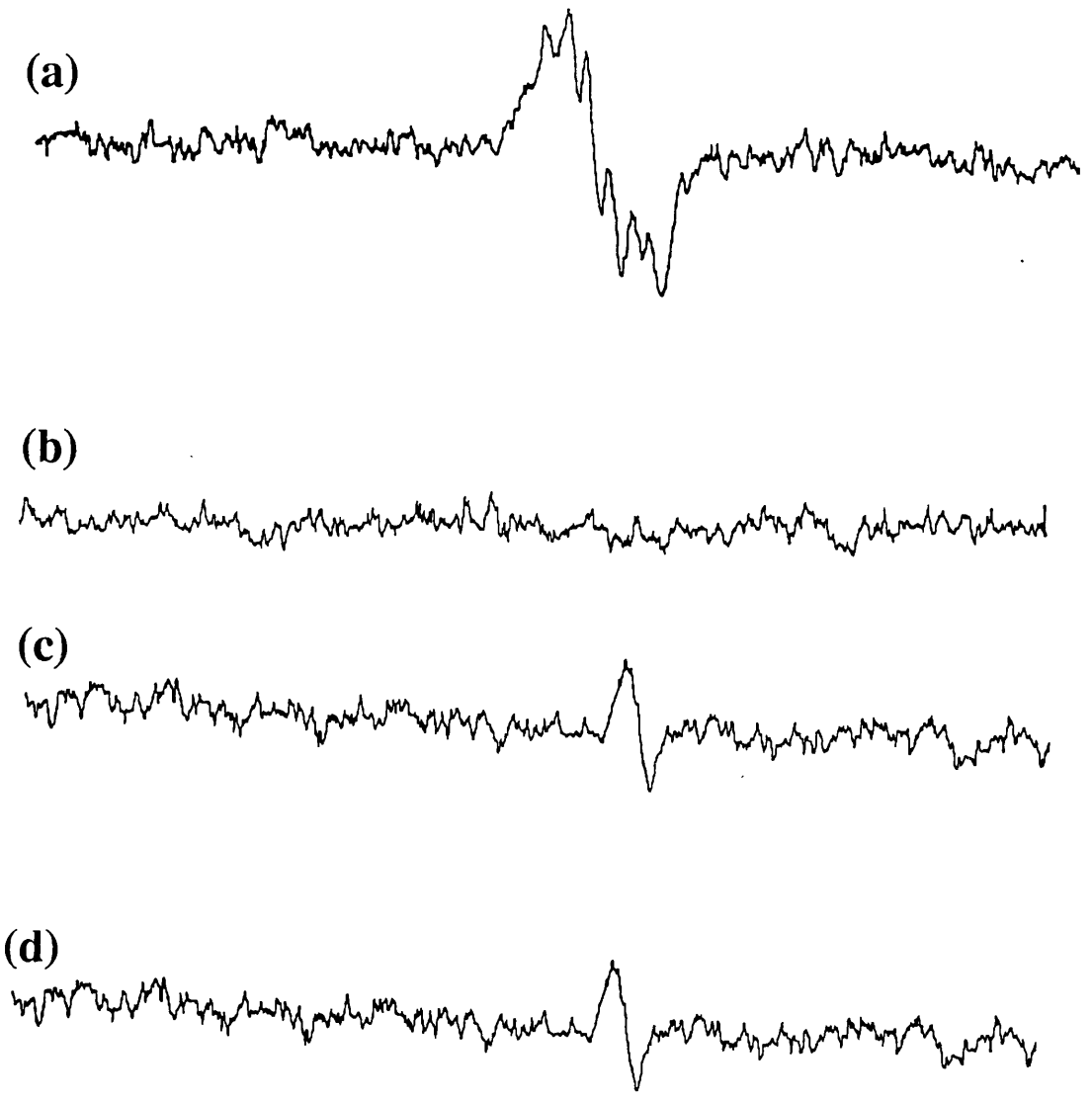
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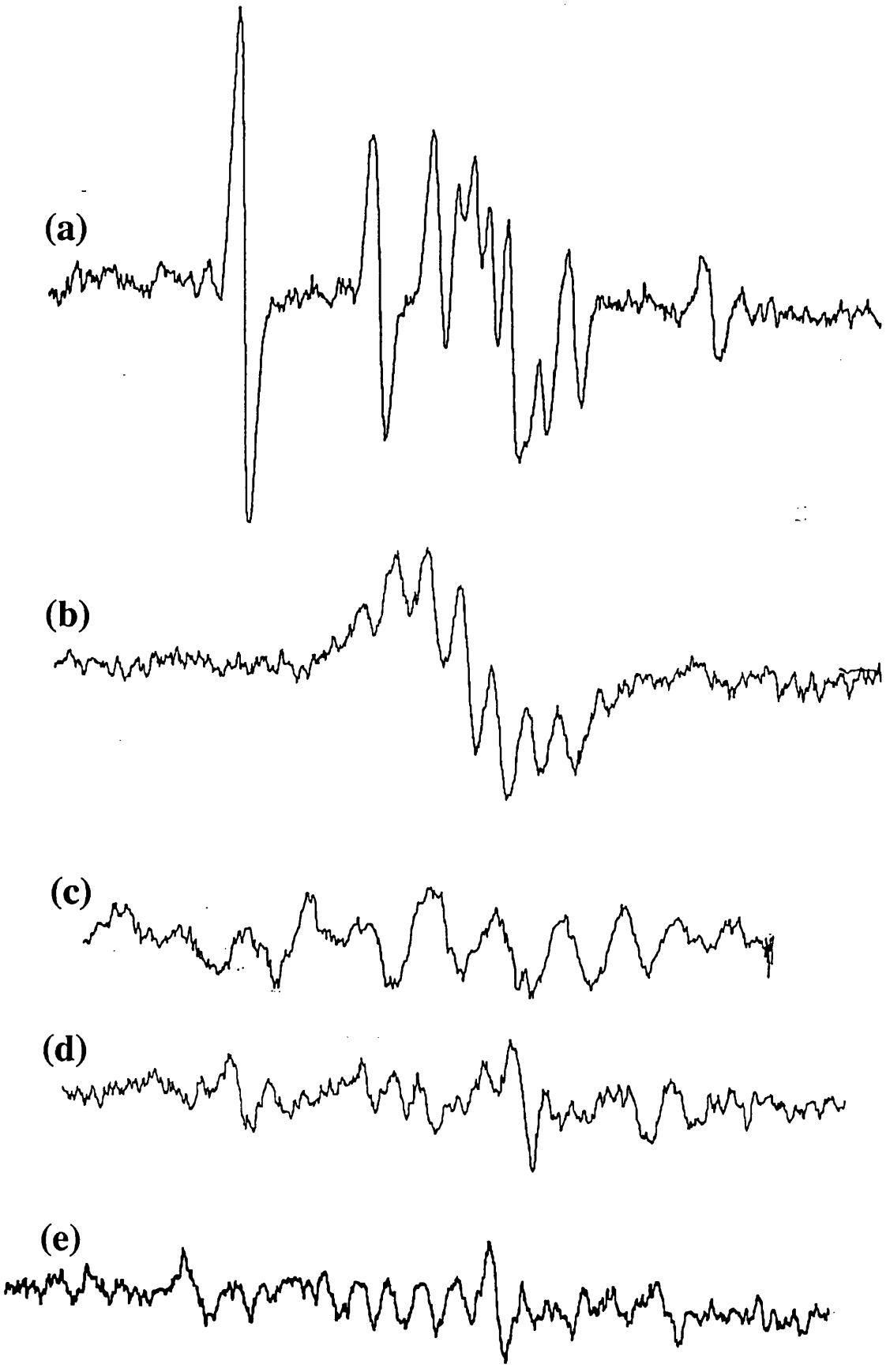
Fig. 3.13 ESR spectra of EO9 and oxygen radicals formed during the reduction of EO9 catalysed by NADPH: cytochrome P450 reductase in the presence of spin trap DMPO (100 mM).

Standard reaction conditions included NADPH: cytochrome P450 reductase (1.3 μg), EO9 (800 μM) and NADPH (1mM) in a total volume of 400 μl. (a and b) spectra generated after 5 and 10 minutes following initiation of reaction respectively, (c, d and e) controls where (c) NADPH, (d) drug and (e) enzyme were omitted, (f and g) complete system plus superoxide dismutase (400 U) and catalase (200 U) taken (f) 3 and (g) 8 minutes after the inititation of the reaction, (h and i) complete system plus superoxide dismutase after (h) 3 minutes and (i) 8 minutes and (j) complete system plus catalase (3 minutes). In most instances and where possible data were confirmed in an independent repeat experiment.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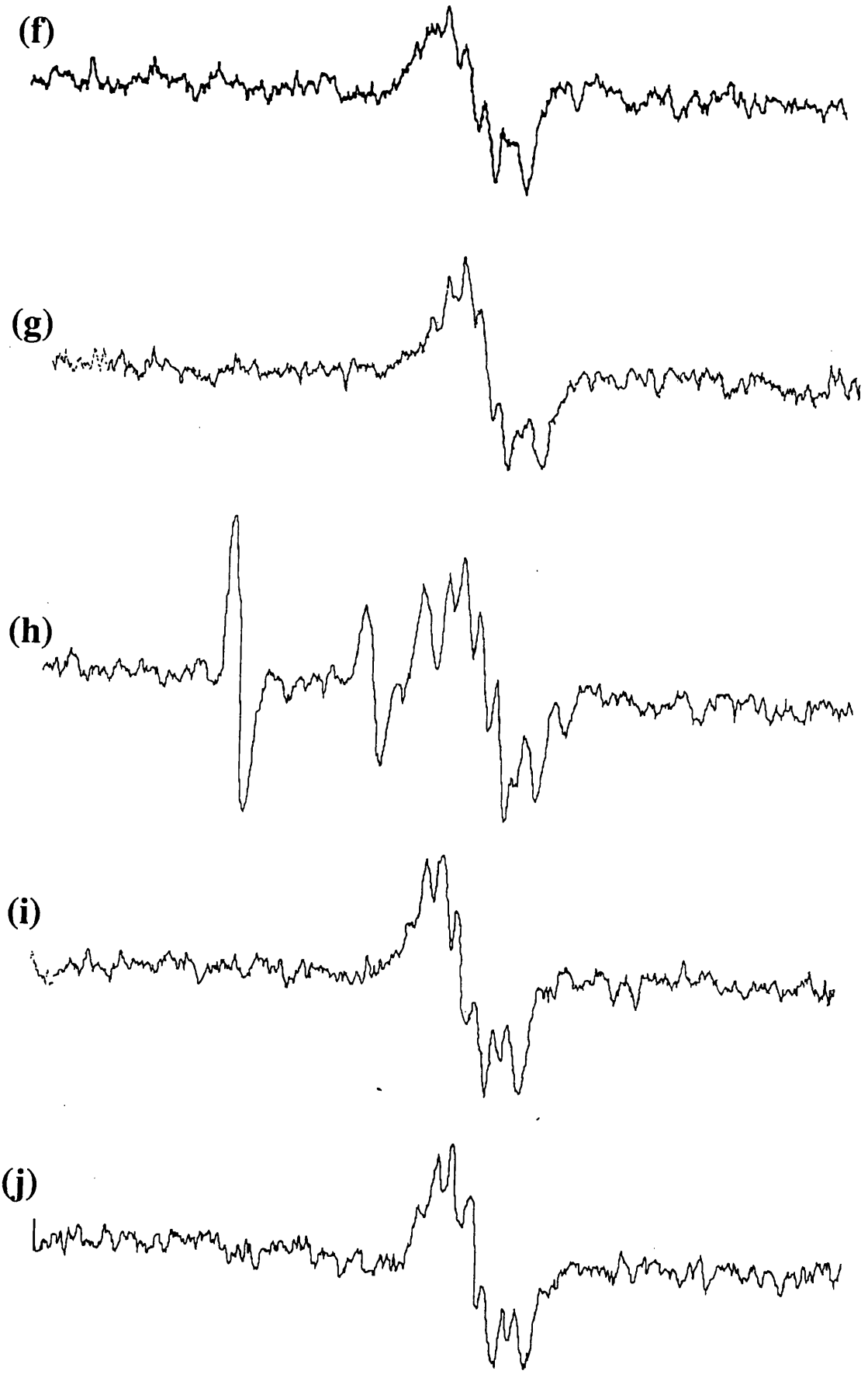
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Addition of either superoxide dismutase and catalase (Fig 3.13, spectra f and g), or catalase alone (Fig 3.13, spectrum j) resulted in inhibition of the oxygen-based radical although the presence of the drug-based radical was maintained. This was therefore similar to the pattern observed with DT-diaphorase. Where superoxide dismutase was included the DMPO- methyl radical was still apparent (Fig 3.13, spectra h and i), but was reduced in intensity.

3.4 Discussion

The structural similarity of EO9 to other quinone substrates of DT-diaphorase suggested that this enzyme may play a role in metabolism of EO9. At the outset of this thesis, previous work in our laboratory had shown that sonicates of rat Walker cells could catalyse reduction of EO9 (Walton *et al*, 1991; 1992b). This activity was inhibitable by dicoumarol, thus indicating the involvement of DT-diaphorase. Preliminary experiments with human HT29 cells had given similar results (Walton *et al*, 1992b). For this reason, the studies described here examined the ability of DT-diaphorase present in sonicates of rat Walker tumour and human HT29 colon carcinoma cells, as well as a purified form of the enzyme, to catalyse reduction of EO9 and related indoloquinone compounds.

A number of methods are available for analysing the reduction of agents by DT-diaphorase. Commonly used techniques include the use of HPLC or direct spectrophotometry to monitor the loss of the agent or NAD(P)H cofactor. Both of these methods have limitations. HPLC is fairly time consuming, particularly when determining kinetic parameters for a large number of substrates and tends to require relatively large quantities of drug and enzyme both of which were in limited supply. It does however have advantages of monitoring unambiguously the individual reaction components and often permits identification of the metabolites. The spectrophotometric method is even more direct but has complications for agents such as the aziridinylbenzoquinones which absorb at around 340 nm, a wavelength which is coincident with that of the cofactors NADH and NADPH. In addition, a large number of the quinone compounds have reduced metabolites which are oxygen sensitive and therefore it is difficult to determine kinetics by following the loss of parent drug unless anaerobic conditions are employed. One method of clarifying the levels of auto-oxidation is by also measuring oxygen consumption. Because of these complications, the cytochrome *c* reduction assay was employed routinely in this thesis. Although this is an indirect method because reduction of the quinone is

monitored via cytochrome *c* as the terminal electron acceptor and reporter molecule, it has the advantages of being rapid and widely acceptable, permitting screening of a large number of analogues, and also of high sensitivity, thus utilising less drug and enzyme.

Results presented in this chapter, using a variety of techniques, have provided strong evidence that DT-diaphorase present in sonicates of rat Walker and human HT29 tumour cells is able to catalyse the metabolism of EO9. Further unambiguous support in the case of the Walker tumour was provided by the observation that the reduction of EO9 and certain analogues was also catalysed by the DT-diaphorase purified from this source.

EO9 acted as an excellent substrate for DT-diaphorase being only 4-6 fold poorer in terms of Vmax than the conventional benchmark substrate menadione in the case of the rat Walker tumour sonicate and 14-16 fold poorer for the human sonicate. The Km values for EO9 with both sonicates were also higher than those obtained with menadione. Similar results were obtained with the purified DT-diaphorase although in this case activity per mg of protein was obviously higher than that exhibited by sonicates. Interestingly this novel anticancer drug was very much more efficiently metabolised by DT-diaphorase at pH 7.4 than was mitomycin C, the prototype bioreductive alkylating agent. Overall these results compare well with those obtained by Walton and co-workers (1992b).

It was observed that rat Walker tumour cells were more active than the human HT29 colon carcinoma cells with respect to DT-diaphorase activity. This may be due to differences in the concentrations of DT-diaphorase present in the two cell lines or alternatively to the enzymes differing in their ability to reduce the indoloquinones on a molecule to molecule basis. Western blotting experiments suggested that Walker cells possessed more DT-diaphorase protein than HT29 cells although this may reflect differences in cross-reactivity of antibody used (data not shown). In order to understand the basis of this difference a purified form of the human enzyme was required and was not available during the time course of this thesis. Subsequent experiments carried out by Dr A. Lewis in our laboratory have further investigated this issue by using recombinant forms of rat and human DT-diaphorase produced in bacteria (Lewis *et al*, 1994, Chen *et al*, in press). The rat recombinant form of the enzyme was shown to compare well with the purified rat Walker DT-diaphorase with regard to its metabolism of EO9. Importantly, the recombinant rat enzyme was able to catalyse reduction of EO9 approximately seven times more efficiently than the

recombinant human enzyme. Providing that the recombinant human enzyme also acts as a good model for the intrinsic human cell DT-diaphorase, these data would support the hypothesis that the rat Walker enzyme was able to catalyse reduction of EO9 more efficiently than the form of the enzyme present in HT29 colon carcinoma cells. A similar difference in the reducing ability of rat Walker cell DT-diaphorase and human Hep-G2 cell DT-diaphorase has also been observed with CB 1954 using purified forms of the enzyme (Boland *et al*, 1991).

It must be emphasised that experiments utilising sonicates of rat Walker and human HT29 cells as a source of enzyme rely on the inclusion of the inhibitor dicoumarol for determination of DT-diaphorase activity. Dicoumarol was originally thought to be a specific inhibitor of DT-diaphorase but more recently has been reported to inhibit a xanthine oxidase and dehydrogenase (Gustafson and Pritsos, 1992b), carbonyl reductase (Wermuth, 1981), NADH cytochrome b_5 reductase (Hodnick and Sartorelli, 1993) and a variety of other enzymes (for detailed references see Murray *et al*, 1982). Despite these concerns the affinity of DT-diaphorase for dicoumarol is however extremely high and it is expected that this would provide a reasonable measure of the role played by DT-diaphorase, particularly in the high expressing cell lines used here. Furthermore the involvement of DT-diaphorase in catalysing reduction of the indoloquinones was demonstrated unambiguously using the highly purified form of the rat Walker enzyme.

Structural modification of compounds has previously been reported to alter their ability to undergo reduction catalysed by DT-diaphorase. An understanding of the key structural features may be important for future drug design to give optimal activity, advocated by the 'enzyme-directed approach' (Workman and Walton, 1990; Workman, 1994). For this reason the DT-diaphorase catalysed reduction of a range of structural analogues of EO9 was examined.

Quite subtle structural modification of EO9 resulted in an altered ability to be metabolised by purified rat Walker DT-diaphorase as well as that present in the rat Walker and human HT29 cell sonicates. Interestingly the 2-hydroxyethylamino derivative EO5A, in which the aziridine ring of EO9 has been opened was extremely poorly metabolised, if at all. A similar phenomenon has been described for the aziridine ring-opened analogue of CB 1954 2,4-dinitro-5-(2'-hydroxyethylamino)-benzamide (Knox *et al*, 1988b). This suggests a preference for an intact aziridine ring for DT-diaphorase-catalysed reduction. However the results obtained with EO7 suggest that this is not a unique structural requirement. EO7 has a methoxy

substituent in place of the aziridine moiety and yet still undergoes efficient metabolism catalysed by both human and rat DT-diaphorase. This conclusion is substantiated by experiments carried out by Knox and co-workers (1988b) which revealed that the analogues of CB 1954, 5-chloro-2,4-dinitrobenzamide and 3,4-dinitrobenzamide, which also lack the aziridine group, are however still reduced by this enzyme. Nevertheless, EO8, the methylaziridine analogue, and EO7 both acted as poorer substrates for rat Walker and human HT29 cell DT-diaphorase than did EO9. Interestingly, EO88, an analogue which retains the aziridine moiety but also exhibits a carbamate leaving group at the X2 position in place of the hydroxyl group of EO9 (Fig 3.1) was much more efficiently metabolised by the enzyme when comparing both Km and Vmax values. This observation highlights an additional area of the molecule where relatively minor structural modification can result in an unexpected enhancement of DT-diaphorase catalysed reduction.

The reduction potential of a compound, which might be expected to determine its ability to undergo enzymatic reduction, will clearly be influenced by the nature of the molecular substituents. In the case of the indoloquinones related to EO9 all the groups at the R_1 and R_2 position of the molecule are electron donating. The greater the capacity of the particular substituent to donate electrons the more negative will be its reduction potential and the more readily it would be predicted to undergo reduction. A limited amount of data are available regarding the half-wave potentials ($E_{1/2}$) of the indoloquinones (on file at the EORTC). These values were determined electrochemically at pH 8 using a Ag/Ag Cl reference electrode. Reduction potentials determined in this way may have some limitations but serve to give a relative order for predicted ease of reduction which was, from most to least electronegative, EO9>EO8>EO7 for the indoloquinones. Although no value was available for EO88 it would be expected to be similar to EO9 as substituents at the leaving groups would be unlikely to have much effect on the redox potential of the quinone. Interestingly, despite only small differences in the $E_{1/2}$ values, large differences were observed in the ability of the indoloquinones to undergo DT-diaphorase-catalysed reduction. Moreover, the order of metabolism does not directly follow that predicted by reduction potential. Thus, redox potentials do not appear to be of great benefit in predicting DT-diaphorase-catalysed reduction of indoloquinone compounds within the range examined. It is likely however, that a threshold level for reduction potential will exist beyond which reduction becomes more difficult. This could be examined using other analogues with far more negative reduction potentials. Such a threshold level has been suggested for quinone epoxides between -500 mV and -600 mV (Brunmark *et al*, 1988). A lack of correlation of redox potential and DT-diaphorase

mediated reduction has been reported for a variety of other compounds such as 1,4-napthoquinones (Buffington *et al*, 1989) and quinone epoxides (Brunmark *et al*, 1988)

In addition to reduction potential, other factors would be expected to influence the ability to undergo reductive metabolism. These include steric considerations, in particular substrate fit in to the active site of the enzyme and lipophilicity. The effect of these parameters could be determined by examination of additional analogues.

DT-diaphorase, being an obligate two-electron reducing enzyme would be expected to convert quinones directly through to hydroquinones, thus avoiding the oxygen sensitive semiquinone radical stage. For this reason some authors have suggested that reduction catalysed by this enzyme should be unaffected by oxygen (see Workman, 1992a). However, depending on the stability of the hydroquinone product, auto-oxidation may or may not occur generating oxygen radicals and the semiquinone radical. In addition, comproportionation-disproportionation reactions may also generate radical products. Results presented in this chapter show that in the case of EO9 and the analogues EO8, EO7 and EO88 there was clearly no difference in the initial rate of DT-diaphorase reduction between aerobic and hypoxic conditions, as determined by the cytochrome *c* reduction assay. A similar lack of differential between aerobic and hypoxic metabolism catalysed by DT-diaphorase has also been reported for other bioreductive compounds including mitomycin C (Siegel *et al*, 1990b), AZQ (Siegel *et al*, 1990a) and CB 1954 (Knox *et al*, 1988b). Hypoxia may however affect the nature and levels of the metabolites formed by affecting their stability. It must be emphasised that the present results were obtained using very brief reaction times.

Controversy exists over the pH status of solid tumours. Current opinion supports the view that the pH of extracellular fluid in tumours is more acidic than normal cells but that intracellular pH is well regulated (see Chapter 1). In light of these reports and the observation that activity of the quinone agent mitomycin C is enhanced under acidic conditions it was decided to investigate the effect of pH on the reduction of EO9. Results obtained using the cytochrome *c* reduction assay showed that the rate of DT-diaphorase-mediated metabolism of EO9 remained relatively constant over the pH range 5.8 - 7.8. These pH values were selected as pH values in human tumours as low as 5.8 have been reported (Vaupel *et al*, 1989). In addition, this was the range used in experiments carried out by Siegel and co-workers (1990b) on mitomycin C. Menadione, the conventional substrate for DT-diaphorase, was also examined as a control. The reduction rate as monitored by the cytochrome *c* reduction assay

decreased only slightly with decreasing pH over the range 7.4 - 5.8. This is consistent with the lack of appreciable decrease in menadione metabolism reported by Siegel and co-workers (1990b) at equivalent pH values. However, in contrast, to results obtained from Siegel and co-workers (1990b) data presented here showed no effect of pH on the DT-diaphorase-catalysed reduction of mitomycin C. A major difference is that Siegel and co-workers (1990b) used HPLC to monitor reduction in mitomycin C concentration after 30 minutes sample incubation. They suggested that the reason for the enhanced metabolism at lower pH values involved the pH dependent formation of the quinone methide metabolite. At high pH (above 7.4) this may alkylate DT-diaphorase thus inactivating it catalytically, whereas at lower pH values protonation of the metabolite may occur, thereby converting the quinone methide to 2,7-diaminomitosene which may be released from the enzyme permitting ongoing metabolism to proceed. In the present studies, the cytochrome *c* reduction assay was carried out over a 1-2 minute period. It is therefore possible that this gave insufficient time for extensive enzyme inactivation to occur, thus resulting in apparent lack of effect of pH on metabolism.

Phillips and co-workers (1992) found that the cytotoxic potency of EO9 increased when extracellular pH was decreased from 7.5 to 5.8 (Phillips *et al*, 1992). In addition, they observed EO9 to be unstable in media at acid pH at 37°C and identified EO5A as a breakdown product. These authors suggested that the increased potency of EO9 under acidic conditions may be due to either enhanced enzymatic activation of the indoloquinone in a similar manner to that reported for mitomycin C, or alternatively to chemical modification of EO9. However, results presented in this chapter suggest that the initial rate of DT-diaphorase-catalysed reduction is not affected within this pH range. In addition, intracellular pH has been reported to be well regulated and thus is unlikely to be affected by the low extracellular pH in tumours. Furthermore, the results discussed in this chapter have shown that the breakdown product EO5A acts as a very poor substrate for DT-diaphorase. One explanation for the increased cytotoxicity observed by Phillips and co-workers (1992) is that a reactive intermediate may have been formed during breakdown of EO9 to EO5A which has not so far been detected. There is, however, no data to support this possibility.

In addition to the spectroscopy studies, HPLC experiments confirmed that EO9 could be metabolised by purified rat Walker cell DT-diaphorase and that all components of the reaction mixture, i.e. enzyme, drug and cofactor, were required for this process to proceed. This evidence relied on a decrease in cofactor concentration. In contrast

relatively little decrease in EO9 concentration was noted. It is likely that this apparent discrepancy was due to formation of a highly oxygen-sensitive metabolised DT-diaphorase, presumably the hydroquinone which, in the presence of molecular oxygen, was auto-oxidised to regenerate the parent compound. This would result in little overall loss in EO9 while supporting a consumption of cofactor. Similar tendencies to undergo rapid auto-oxidation following metabolism by DT-diaphorase have been reported for the aziridinylbenzoquinone AZQ (Siegel *et al*, 1990a) but data reported by Siegel and co-workers (1990b) suggested that, in contrast, mitomycin C metabolites are insensitive to oxygen. In the case of experiments carried out under hypoxic conditions, the metabolite should have been stabilised and therefore have been detectable. Again the lack of decrease in EO9 peak height suggested that auto-oxidation was occurring during sample injection of the reduced metabolite on to the column implying extreme oxygen sensitivity. Spectrophotometric analysis of DT-diaphorase-catalysed reduction of EO9 in the absence of cytochrome *c* revealed a decrease in absorbance of EO9 at 500 nm wavelength immediately after initiation of the reaction. Following a lag period of a couple of minutes reappearance of a peak at 500 nm was observed when air was introduced in to the system indicating formation of the parent compound. This reaction was visible with the naked eye as a conversion of the purple coloured EO9 solution to colourless and then subsequent reappearance of the purple form. It is possible that a metabolite of EO9 could also absorb light at 500 nm however the HPLC data showing little loss of EO9 supports the view that a EO9 is reduced to a highly oxygen sensitive metabolite which is then reoxidised to EO9 itself.

In order to further investigate the mechanism of the DT-diaphorase-catalysed reduction of EO9 and to identify the nature of the metabolites, ESR studies were carried out. These revealed that DT-diaphorase-catalysed reduction of EO9 to generate a drug-based radical. This exhibited an 8 line spectrum and by analogy with other quinones this is likely to be the semiquinone radical. However, without further sophisticated computer analysis, it is not possible to definitively confirm the identity of this species.

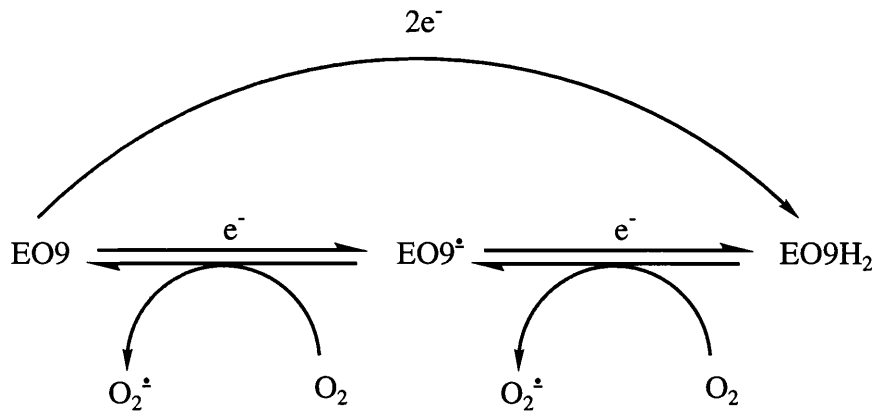
DT-diaphorase, widely considered to be an obligate two-electron reducing flavoenzyme (Iyanagi and Yamazaki, 1970) would be expected to catalyse metabolism of EO9 directly to a hydroquinone. On this simple model one would not have expected to see evidence of a semiquinone radical during this reduction process. However, similar results have been obtained following DT-diaphorase catalysed reduction of AZQ (Fisher and Gutierrez, 1991a). In this case it has been suggested

that the hydroquinone generated auto-oxidises to produce the semiquinone and parent compound. HPLC and spectrophotometric data presented in this chapter indicates a similar oxygen sensitivity of EO9 metabolites and may provide an explanation for the generation of the semiquinone (Fig 3.14; Equation 1). An additional method by which the semiquinone radical could be generated under these conditions are by comproportionation : disproportionation reactions (Fig. 3.14; Equation 6). This may also contribute to the production of the semiquinone radical.

Controls where either drug, enzyme or cofactor were omitted showed no evidence of radical formation. This strongly supports the view that both oxygen radicals and drug-based radicals are generated following reduction of EO9 catalysed by DT-diaphorase using NADPH as an electron donor. In addition, experiments where the DT-diaphorase inhibitor dicoumarol or anti-DT-diaphorase anti-serum were pre-incubated with the enzyme, did not generate an ESR signal. These results further confirm the involvement of DT-diaphorase in reduction of EO9 and radical generation.

It is interesting that in experiments where either the enzyme concentration was lowered to 0.007 μg (in 400 μl reaction volume) or increased to 2 or 5 μg (in 400 μl) the drug-based radical was no longer observed. At lower enzyme concentrations it is likely that the hydroquinone is produced more slowly and therefore auto-oxidation occurs to generate the semiquinone and quinone without permitting sufficient amounts of semiquinone to build up to form a detectable signal. At higher enzyme concentrations, reduction of EO9 would be very rapid and thus may quickly consume the oxygen present in the system causing the sealed flat cell to become hypoxic. Under these conditions the hydroquinone would be stabilised by preventing auto-oxidation and therefore once again reducing the levels of semiquinone. This phenomenon would also suggest that the drug-based radical is predominantly formed by auto-oxidation rather than comproportionation : disproportionation reactions. Visible evidence existed for the occurrence of EO9 metabolism at these higher enzyme concentrations in that the contents of the flat cell were observed to be colourless rather than possessing the purple coloration of the parent compound.

Consistent with the idea that the drug-based radical results from metabolism of EO9 catalysed by DT-diaphorase is the finding that replacing the cofactor NADPH with NADH gave almost identical results. As discussed previously (Chapter 1) a characteristic of this enzyme is its ability to utilise NADH and NADPH with equal efficiency. Increasing NADH concentration to 2 mM resulted in a similar initial peak



[1]

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad [2]$$

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \quad [3]$$

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2 \quad [4]$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad [5]$$

$$2EO9^{\cdot}H \rightleftharpoons EO9 + EO9H_2 \quad [6]$$

$$O_2^{\cdot-} + EO9H_2 \rightleftharpoons EO9^{\cdot-} + H_2O_2 \quad [7]$$

$$DMSO + {}^{\cdot}OH \longrightarrow CH_3 \quad [8]$$

$$DMPO \longrightarrow \downarrow \longrightarrow DMPO\text{-}CH_3$$

Fig 3.14 Proposed reaction mechanism for reduction of EO9. Direct $2e^-$ reduction in equation [1] may be catalysed by DT-diaphorase whereas the slower sequential $1e^-$ reducing steps may involve enzymes such as NADPH: cytochrome P450 reductase.

intensity to that obtained with 1 mM; however, in contrast to the result with 1 mM cofactor the signal began to decrease after 9 minutes giving no detectable peak after 24 minutes (Fig 3.10). Based on the presumption that EO9 is being reduced to the hydroquinone by DT-diaphorase and that auto-oxidation subsequently occurs, the additional amount of reducing equivalent may continue to drive the forward reaction until hypoxia prevails. This would stabilise the hydroquinone and result in negation of the radical species.

If the proposed mechanism (Fig 3.14; Equation 1) for the generation of semiquinone following DT-diaphorase metabolism of EO9 is correct, oxygen-based radicals should also have been generated as a by-product (Fig 3.14; Equation 1). As previously discussed, due to the short life span of these species they are largely undetectable by conventional ESR. For this reason, the spin trap DMPO was employed. As expected, where DMPO was included a 6 line methyl-DMPO radical (Fig 3.14; Equation 8) was observed. This had an extremely short life span and rapidly disappeared being no longer present after 8 minutes. At this stage it was evident that the drug-based free radical was also present. The detection of oxygen radicals therefore confirmed that auto-oxidation of a DT-diaphorase metabolite, presumably the semiquinone, was occurring.

Identification of the oxygen radicals produced was possible by inclusion of superoxide dismutase and catalase. Superoxide is expected to be generated as a by-product during auto-oxidation of the hydroquinone and semiquinone species (Fig 3.14; Equation 1). This spontaneously dismutates to produce hydrogen peroxide and oxygen (Fig 3.14; Equation 2). Superoxide dismutase catalyses this reaction thus removing superoxide from the system. In addition superoxide dismutase should inhibit the reduction of Fe^{3+} which is part of the Haber-Weiss reaction (Fig 3.14; Equation 4 and 5). Catalase is involved in conversion of hydrogen peroxide to water and oxygen (Fig 3.14; Equation 3) and thus removes hydrogen peroxide from the system preventing the Fenton reaction (Fig 3.14; Equation 5). In the experiments described in this chapter superoxide dismutase did not result in inhibition of either the drug radical species or the DMPO-methyl radical. This would suggest that there was already sufficient reduced ferric ions present in the incubation to permit the Haber-Weiss reaction to occur. Inclusion of a metal ion chelator such as diethylenetriamine pentaacetic acid (DETEPAC) could clarify this issue by removing the ferric ions and thus abolish the signal observed. The lack of inhibition of the DMPO-methyl radical signal also indicates that H_2O_2 is not generated significantly by hydroquinone oxidation by superoxide (Fig 3.14; Equation 7). Catalase inhibited the generation of

the DMPO-methyl radical (Fig 3.11), thus suggesting that hydrogen peroxide reacts with Fe^{2+} to generate OH^- and OH·. The hydroxyl radical then reacts with DMSO to produce a methyl radical which reacts with DMPO to generate the DMPO-CH_3 radical observed.

In addition to DT-diaphorase, other enzymes are likely to be involved in the reductive metabolism of EO9. For this reason, and the fact that NADPH: cytochrome P450 reductase has been reported to metabolise structurally similar substrates, this enzyme was investigated here. ESR studies carried out with NADPH: cytochrome P450 reductase showed the generation of a drug-based radical similar to that produced following DT-diaphorase reduction. However, in contrast to DT-diaphorase this one-electron reducing enzyme would however be expected to catalyse the formation of EO9 to the semiquinone radical in a single one-electron step (Fig 3.14, Equation 1). Once at its optimum intensity the radical remained stable over 30 minutes. It is likely that the semiquinone is highly oxygen sensitive and may be auto-oxidised to the parent compound but that an equilibrium situation is achieved thus enabling its visualisation. It is also possible that initial redox cycling of semiquinone could utilise oxygen present in the closed system generating hypoxia and thus stabilising the radical species.

As in the case of DT-diaphorase catalysed activation of EO9, the presence of a radical was dependent on cofactor, EO9 and NADPH: cytochrome P450 reductase. These data have therefore provided the first evidence to show that NADPH : cytochrome P450 reductase can catalyse reduction of EO9.

Experiments where spin trap DMPO was included also resulted in detection of a methyl radical, identical to that observed for DT-diaphorase metabolism of EO9. This suggested the generation of an oxygen sensitive metabolite.

ESR experiments have served to confirm the metabolism of EO9 catalysed by DT-diaphorase as well as providing the first evidence that NADPH: cytochrome P450 reductase may also catalyse reduction of this compound. In addition they have provided valuable insight into the nature of the reaction, detecting and putitatively identifying the radical metabolites which may be involved in the mechanism of EO9 toxicity under aerobic conditions. This will be further discussed in Chapter 5. Some authors have reported ESR studies involving incubation of whole cells with various compounds (Gutierrez *et al,* 1986; Butler *et al*, 1990; Dzielendziak *et al*, 1990). This would be a useful experiment to carry out with EO9 as it would indicate whether

radicals were generated in a whole cell system containing a variety of enzymes as well as mechanisms to protect against free radical damage. Various inhibitors could be used together with cells of differing enzyme content.

To conclude, data presented in this chapter have confirmed that EO9 can undergo reduction catalysed by DT-diaphorase derived from both rat and human tumour cells and has to some extent elucidated the mechanism of reaction. Results indicated that this reduction generates a highly oxygen-sensitive metabolite which, under aerobic conditions, is rapidly auto-oxidised to yield the semiquinone radical and ultimately the parent quinone. The latter is then available for further reduction. This process of redox cycling concomitantly produces potentially damaging oxygen-based radicals. Results of ESR experiments have also provided the first evidence that EO9 can be reduced by NADPH: cytochrome P450 reductase. This also generates semiquinone and oxygen-based radicals similar to those formed by DT-diaphorase reduction although these are likely to result from a direct one-electron reduction process. Hypoxia did not affect the initial rate of reduction of the indoloquinones by DT-diaphorase but it is likely to affect the stability of metabolites so generated as well as of those produced following reduction by other enzymes such as NADPH: cytochrome P450 reductase. The nature of the substituent groups have been reported to affect the auto-oxidation rate of 1,4-napthoquinones (Brunmark *et al*, 1988). It is therefore possible that analogues of EO9 may differ in their stability under oxic conditions. Altering pH also had no effect on the rate of DT-diaphorase reduction of EO9. It may however alter the chemical nature of either EO9 itself or of its metabolites. For example in the case of the quinone compound AZQ, protonation of the aziridine ring may be facilitated upon reduction of the quinone group and acidic conditions may encourage the protonation (Mossaba *et al*, 1985). Thus hypoxic and acidic conditions which have been reported to occur in solid tumours may result in a different spectrum of metabolites to those produced by the same enzyme reactions under aerobic conditions. This may have consequences for the degree of cytotoxicity seen in the different environments.

The limited structure-activity relationship studies also reported in this chapter clearly showed that structural modification of EO9 had major effects on the ability of indoloquinones to be metabolised by DT-diaphorase. The information gained from such studies may lead to the optimisation of drug structure for enhanced or reduced metabolism by DT-diaphorase depending on whether it is found to activate or detoxify the indoloquinone anticancer agents. In this regard it is promising that, within the limited number of analogues studied here, individual compounds were

identified which were either very much poorer or alternatively better substrates for DT-diaphorase than EO9 itself. In order to demonstrate the cellular consequences of a varying ability to act as a substrate for DT-diaphorase it is necessary to investigate also the cytotoxicity of the indoloquinones. These studies are described in the following chapter.

Chapter 4

Cytotoxicity of Indoloquinones

4.1 Introduction

4.1.1 Cytotoxicity of Indoloquinones

At the outset of the work described in this thesis only limited information was available concerning the *in vitro* cytotoxicity of EO9. Subsequently however, this issue has been extensively studied by a number of investigators in a large range of cell lines. The effect of hypoxia, pH, exposure time and cell density amongst other factors have also been characterised.

In initial experiments, the aerobic cytotoxicity of EO9 was determined *in vitro* against cell lines derived from human small cell lung cancer (Roed *et al*, 1989), rat Walker tumour, human colon carcinoma (this thesis published in Bailey *et al*, 1992; Walton *et al*, 1992a) in addition to leukaemia, colon and breast tumours (Collard *et al*, 1992). More extensive analyses followed which documented the sensitivity of larger panels of cell lines to EO9. These included, in particular, studies carried out by Robertson *et al* (1992; 1994) of 23 human tumour cell lines (breast, lung and colon) as well as 8 rodent lines and those of Plumb and co-workers involving 8 human lung cancer cell lines (Plumb *et al*, 1994a) and 23 cell lines derived from breast, glioma, ovary and lung tumours (Plumb *et al*, 1994b). Similar experiments were conducted by Phillips and co-workers (1992) and by Smitskamp-Wilms *et al* (1994). In the largest survey conducted to date, the US National Cancer Institute (NCI) in collaboration with the European Organisation for Research and Treatment of Cancer (EORTC), has determined the *in vitro* aerobic cytotoxicity of EO9 in its panel of 56 human tumour cell lines (reported in Hendriks *et al*, 1993). These included eight different tumour types, namely leukaemic, non-small cell lung cancer, small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer and renal cancer in addition to the MCF-7 human breast adenocarcinoma and P388 murine leukaemia cell lines and their multidrug resistant variants. The cytotoxic potency was obtained using the sulpharhodamine B (SRB) protein assay with a 48 hour continuous drug exposure. In general EO9 was found to be highly cytotoxic with a mean GI_{50} value (the concentration at which 50% growth inhibition occurred) of 17 nM taken from all the

lines in the panel. Colon, melanoma and CNS tumour lines exhibited particular sensitivity to EO9 with GI_{50} values generally being 10 nM or below. Similar levels of response were also observed in individual cell lines derived from renal and non-small cell lung cancer. In this survey (Hendriks *et al*, 1993) and in other studies referred to above it was generally found that cell lines derived from solid tumours tended to be more sensitive to EO9 than leukaemic cell lines . However, Phillips *et al* (1992) did not observe such a preference in their investigation. In support of the general *in vitro* trend, some selectivity for solid tumours compared with leukaemias has also been noted in *in vivo* studies (Hendriks *et al*, 1993; Walton *et al*, 1992a).

Experiments carried out with EO9 on the small cell lung cancer tumours, OC-tol and NCI-N592 (Roed *et al*, 1989) and mouse colon adenocarcinomas MAC 16 and 26 (Walton *et al*, 1992a) *in vivo* correlated well with the sensitivity of the equivalent *in vitro* cell lines. This supports the potential usefulness of these cell lines as an *in vitro* screen for the prediction of antitumour activity.

Under identical conditions to those described for EO9, mitomycin C, the prototype bioreductive alkylating agent, exhibited a totally different antitumour profile to that of EO9 when examined in the NCI panel of cell lines. A similar lack of correlation between the aerobic cytotoxic potency of mitomycin C and EO9 was noted in a range of cell lines by Phillips and co-workers (1992) and by Robertson and co-workers (1992; 1994) again using a 96 hour continuous exposure time. Mitomycin C was in general less potent (mean GI_{50} value 71 nM) in the NCI panel, showed no preference for any particular tumour type with the exception of melanoma, and was not selective for solid tumour versus leukaemic cell lines. In addition, EO9 did not exhibit differential sensitivity between the CHO-K1 cell line and its mitomycin C resistant variant (Stratford and Stephens, 1989). These data would suggest possible differences in the mode of drug activation and / or cytotoxicity between EO9 and mitomycin C.

A number of experiments have examined the effect of oxygen status on the toxicity of EO9 (Adams *et al*, 1992; Hendriks *et al*, 1993; Plumb and Workman, 1994; Plumb *et al*, 1994a and b; Robertson *et al*, 1994). These have revealed an enhancement of toxicity in some cell lines, but not others, under hypoxic conditions *in vitro* with hypoxic cytotoxicity ratios as large as 1700 in the case of BE colon carcinoma cells (Plumb and Workman, 1994). This is much greater than that observed for other bioreductive compounds such as mitomycin C which showed only a 9 fold potentiation of toxicity under hypoxia using the BE cell line. A high degree of EO9 sensitivity towards hypoxic cells has also been observed *in vivo* with the KHT

sarcoma (Adams *et al*, 1992). Interestingly, studies involving the use of the vasoactive agent hydralazine have shown an enhancement in sensitivity of MAC 26 murine colon adenocarcinoma to EO9 *in vivo* (Bibby *et al*, 1993). This may be due to increased levels of hypoxia induced by the modulation of blood flow with hydralazine, although pharmacokinetic effects can not be ruled out.

Data presented in Chapter 3 showed that structural analogues of EO9 varied in their ability to undergo reduction catalysed by DT-diaphorase. No publications were available regarding the cytotoxic potency of these compounds. Thus experiments described in this chapter were designed primarily to investigate whether these analogues also varied in their cytotoxic potency and in particular the extent to which the structure-activity relationships for DT-diaphorase metabolism versus cytotoxicity were related. This in turn allowed initial evaluation of the relationship between indoloquinone sensitivity and metabolism by DT-diaphorase.

4.1.2 Chemosensitivity Assays

A number of methods are available for determining the sensitivity of cell lines to particular agents. These include clonogenic assays, cell counting following trypan blue exclusion, ^{3}H thymidine incorporation, MTT and related tetrazolium dye reduction and protein staining assays such as crystal violet amongst others.

These various methods actually measure different parameters. For example, the clonogenic assay determines the ability of cells to undergo clonogenic proliferation whereas the ^{3}H thymidine incorporation assay measures the number of cells which are actively synthesising DNA. Depending on how the tetrazolium dye reduction assays are carried out this may measure predominantly either cytotoxicity or cytotstasis, or a combination of these. In addition, some compounds, due to their mode of action may not be suited to particular assay types. The choice of assay employed will therefore depend both on the nature of the compounds under examination as well as the type of information required. The chemosensitivity assays employed in this thesis are discussed in more detail below.

MTT Assay

A tetrazolium dye-based microtitration assay was first described by Mossman (1983). This assay is based on the conversion of a yellow, water soluble monotetrazolium salt (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; MTT) to an insoluble

purple formazan when reduced. The mitochondrial dehydrogenases are involved in MTT reduction via electron transport from NAD or NADP diaphorases and therefore active, viable mitochondria are required for this reduction to occur. Thus only living, not dead cells are able to reduce MTT (Mossman, 1983).

The MTT assay employed in this thesis is a rapid, semi-automated method by which a large number of samples can be easily screened. It has been shown to be highly reproducible and in general it correlates well with results obtained by other assays (Alley *et al*, 1988; Carmichael *et al*, 1987). Unlike some methods it does not require the use of radioactivity or expensive equipment. In addition to giving an immediate visual qualitative result, quantitative information may also be obtained. This requires prior determination of the formazan production for a known cell number. Despite the large benefits of the MTT assay there are also a few disadvantages. The procedures employed are often unable to distinguish between cytotoxicity and cytostasis. Caution must also be taken when dealing with compounds which either reduce MTT directly or which may interfere with the reductive systems involved in MTT metabolism. A relevant example of this is dicoumarol which has been reported to act as an uncoupler of mitochondrial enzymes and inhibit a number of reductive enzymes (Wermuth, 1981; Gustafson and Pritsos, 1992b; Hodnick and Sartorelli, 1993; Murray *et al*, 1982). In addition, the quinone compound menadione, following reduction catalysed by DT-diaphorase, generated a metabolite which could directly reduce MTT (Prochaska and Santamara, 1988). It is important to validate the MTT assay for individual cell lines and compounds, and in view of the above comments, this is particularly true in the case of quinone-type agents.

The MTT assay was selected as the principal method for determining cytotoxic potency in this work as it was commonly employed in our laboratory because it provides rapid and easy screening of large numbers of compounds in different cell lines.

Crystal Violet Staining

Crystal violet stains protein and may therefore be used to determine cell number. This may be quantitative if optical absorbance is determined for known cell densities. It must however be noted that if an agent induces an increase in cell size this may be misinterpreted as an increase in cell number (a feature which can also apply to the tetrazolium dye reduction assays). In addition dead cells may to some extent remain

adherent to the substratum leading to further errors. Despite these possible limitations the assay was used here solely to confirm the data obtained by MTT assay.

Clonogenic Assay

The clonogenic assay measures the ability of cells to undergo clonogenic proliferation and is thus able to distinguish between cytotoxic and cytostatic effects. This method is however time consuming and was therefore only employed to provide further data on Walker cell sensitivity to EO9. It would have been extremely difficult to confirm the MTT assay results in the Walker cell line by crystal violet staining due to the fact that the cells are grown in suspension culture.

4.2 Methods

4.2.1 MTT Dye Reduction Assay

The cytotoxicity of a number of the indoloquinones was determined by means of the MTT dye reduction assay. The method employed was based on that described by Mossman (1983). As discussed by Carmichael and co-workers (1987) cells must be in an exponential phase of growth when MTT is added and MTT dye reduction should be linear with respect to cell number. It was therefore important that the optimum cell number and length of assay were determined for each cell line used. As both the HT29 and Walker cell lines were routinely used in the MTT assay in our laboratory optimum conditions had already been determined by J. Donaldson but were reconfirmed in the present work (data not shown). These optimal conditions were employed in the experiments detailed in this chapter.

Walker and HT29 cells cultured routinely as described in Chapter 2 were harvested for assay during log phase of growth. A single cell suspension was prepared and the cell number determined using a haemocytometer. Samples were resuspended in fresh medium to give a density of 1×10^4 cells / ml for HT29 cells and 5×10^3 cells / ml for Walker cells. Aliquots (200 µl) of the cell suspension were placed in to each of 88 wells of a 96 well microtitre plate (Falcon). The initial row of 8 wells was filled with 200 µl of medium alone to act as a blank. Plates were then incubated at 37°C, 8% CO_2 in a humidified atmosphere for 4-6 hours to permit recovery of cells from mechanical disruption and, in the case of HT29 cells, to allow adhesion to the substratum before drug treatment. Compounds were diluted in DMSO (to give a

maximum final concentration of 0.05%) and applied to the cells in 20 µl aliquots to provide the required range of concentrations. Cells were exposed to drug continuously over a nominal period of 72 or 96 hours for Walker and HT29 cells respectively under standard incubation conditions. No particular allowance was made for any decrease in drug concentration during the period of the assay. At the end of the exposure time 20 µl of MTT dye solution (5 mg / ml in sterile PBS) was added to each well and the plates returned to incubate for a further 2 hours. This time had been determined to be within the linear range of the MTT dye reduction curve for each cell line. The medium containing MTT solution was then gently removed from the wells by aspiration leaving the reduced tetrazolium salt present as blue crystals in the cells adherent to the microtitre plate. As Walker cells grow in suspension culture it was necessary to pellet the cells before aspiration. This was carried out by centrifugation of the plates at 200g for a period of 5 minutes at 4°C. The tetrazolium salt was then solubilised by addition of 200 µl of DMSO to each of the 96 wells of the microtitre plate followed by agitation using a plate shaker (10 minutes). Absorbance at 540 nm was determined by use of a Titertek Multiscan MCC ELISA plate reader (Flow laboratories, Helsinki, Finland) with a reference beam of 690 nm. The medium blank was automatically subtracted. Results were expressed in terms of absorbance as a percentage of the vehicle control. Each drug concentration was repeated 4-8 times per experiment and in general 3 between day repeats were carried out. The results of repeat wells within the same experiment were averaged and the data were expressed graphically as log of the drug concentration (x axis) plotted against absorbance as a % of vehicle control (y axis). The IC_{50} (concentration which reduces the absorbance to 50% of the control wells) was then determined by eye.

In order to investigate the involvement of DT-diaphorase in the cytotoxicity of the indoloquinones experiments were carried out where the DT-diaphorase inhibitor dicoumarol was included. In general this was dissolved in DMSO (final concentration of 0.05%) although experiments were also carried out where 0.05 M sodium hydroxide was used as the solvent (giving a final concentration of 227 µM) and this was found to give similar results. Dicoumarol was used at the highest non-toxic concentration which was determined to be in general around 50 µM for HT29 and 10 µM for Walker cells. In occasional experiments some degree of toxicity was apparent at these concentrations and therefore 25 µM dicoumarol for HT29 cells and 5 µM for Walker cells were also employed. Inhibitor was applied 30 minutes prior to addition of the test drug and remained present throughout the exposure period. Due to the complications of dicoumarol in the MTT assay described in the Introduction to

this chapter the cytotoxic levels of dicoumarol were confirmed by the crystal violet assay.

The modulation of cytotoxicity exerted by dicoumarol was expressed as the dose modification factor (DMF) determined by the formula:-

$$DMF = \frac{IC_{50}}{IC_{50} \text{ in the presence of dicoumarol}}$$

Validation of MTT Assay for Drugs Used

Literature precedent had indicated the possibility of both quinone compounds (Prochaska and Santamaria, 1988) and dicoumarol, by virtue of its inhibition of reductive enzymes (Ernster 1987; Wermuth, 1981; Gustafson and Pritsos, 1992b; Hodnick and Sartorelli, 1993), interfering with MTT reduction by cells. Hence experiments were carried out to determine the extent of this effect. Menadione was selected as a representative quinone for these studies.

Quinones

For menadione, HT29 cells were plated out as described for the MTT assay but at densities of 1 x 10^4 and 5 x 10^3 cells / ml. Following 96 hours incubation various concentrations of menadione ranging from 4 μM - 100 μM were applied to each of three identical plates which were then incubated at 37°C for 30 minutes. The plates were subsequently treated individually as described below:-

1. MTT was added immediately to cells.

2. Medium containing drugs was removed from cells, the plate was washed twice to remove residual drug and metabolites and fresh medium plus MTT were added.

3. Cells were treated as for plate 2 except that, following washing, medium containing fresh drug was added.

The plates were then incubated for a period of 2 hours and optical density of the formazan product was determined as described previously.

Dicoumarol

Walker cells were plated out at densities of 5 x 10^3 and 10^3 cells / ml and were incubated with 0.39 μM-100 μM dicoumarol for various lengths of time before adding MTT and determining the optical density of the formazan product. The times were chosen to be insufficient to cause cytotoxicity, thus any effect seen should result from a direct influence on MTT formazan development.

4.2.2 Crystal Violet Staining for Determination of Cytotoxicity

Crystal violet is a protein stain which has been used to determine cell number (Drysdale *et al*, 1986). In order to confirm results obtained by the MTT assay, crystal violet staining was also carried out on HT29 cells which had been treated under identical conditions. Following drug treatment (see Section 4.2.1) medium was decanted and an excess of crystal violet solution was applied (200 μl; 0.2% in 2% ethanol in distilled water). This was left for 10 minutes prior to removal and thorough rinsing in tap water for 2 minutes. Solvent (200 μl ; 0.5% SDS; 50% ethanol in distilled water) was then added to each well and the plates returned to the incubator for 1 hour at 37°C before agitating for 10 minutes using a plate shaker. Absorbance was determined on a plate reader at 610 nm wavelength.

In order to determine whether absorbance of crystal violet remained in the linear range, cells were plated out at various densities, incubated for periods of 24, 48, 72 and 96 hours and the optical density measured with crystal violet as described above.

4.2.3 Clonogenic Assay

Cytotoxicity of the indoloquinones was also determined in both Sensitive and Resistant forms of the rat Walker UK 256 tumour cells by clonogenic assay. Cells which had been cultured routinely (Chapter 2) were harvested during log phase of growth and were diluted to give a cell density of 2 x 10^5 cells / ml. Aliquots (10 ml) were taken and placed in 15 ml tubes (Falcon) for drug treatment. Various concentrations of the test compounds (in DMSO; maximum final concentration of 1%) were applied to the cells and the latter incubated under standard conditions for 2 hours. Control samples were treated with an equivalent amount of solvent. Drug was then removed by centrifugation (7 minutes at 10g in a Beckman centrifuge), washing twice in medium and resuspension in 10 ml of fresh medium. Serial dilutions were made of the cells to give 2 x 10^5, 2 x 10^4, 2 x 10^3, 2 x 10^2 and 2 x 10 cells / ml and 2

ml aliquots of each of these were mixed with 3 ml of semi-solid agar (0.12% in McCoys 5A medium supplemented with glutamine [1 mM] and horse serum [20%]). This was carried out in quadruplet for each treatment. Tubes were then placed in ice water to allow solidification of the agar prior to incubation under standard conditions for a period of ~10 days. On reaching a size of over approximately 50 cells, colonies were counted using a dissecting microscope. Quadruplet values were averaged and results were corrected for the plating efficiency determined from the control. Data were expressed as a % survival and were illustrated graphically by plotting log % survival against drug concentration.

Clonogenic assays were also carried out including the various concentrations of the inhibitor dicoumarol (100 μM - 600 μM) which had previously been determined to be sub-cytotoxic over the two hour exposure period employed. This was solubilised in DMSO (to give an maximum final concentration of 3%; which had also previously been determined to have no effect on the plating efficiency) and was applied 10 minutes prior to addition of the test compound. Two controls were employed, one containing dicoumarol alone and the second vehicle alone.

4.3 Results

4.3.1 Validation and Optimisation of Assay Conditions

Crystal Violet Assay

In addition to reconfirming conditions for the previously optimised MTT assay (Section 4.2.1) it was also necessary to determine an optimum cell density for use in the crystal violet assay. Fig 4.1 illustrates that a fairly linear relationship exists for optical absorbance of crystal violet with increasing cell number over the range 1 x 10^3 to 4 x 10^4 cells / ml. Thus the cell density of 1 x 10^4 cells / ml was selected as optimum for use in cell survival assays.

Effect of Dicoumarol on the MTT Assay

As mentioned in Section 4.1 it was possible that dicoumarol as well as quinone compounds could have an effect on the MTT assay. The results revealed that, where low concentrations of dicoumarol (< 5 μM) were incubated with Walker cells, optical density of the formazan was either reduced to a minimal extent (in general <10%) or

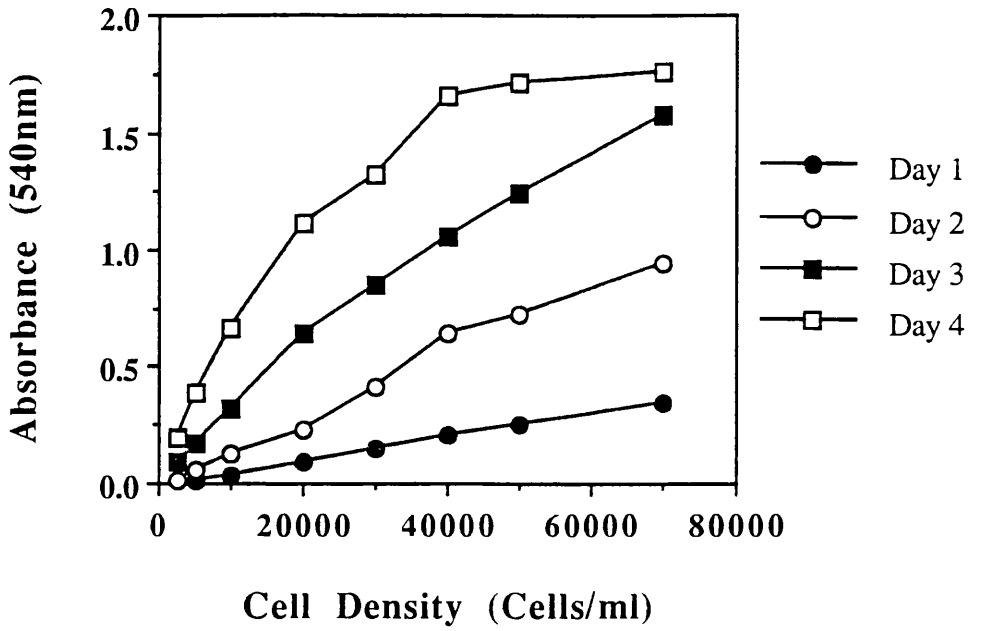


Fig 4.1 Dependence of absorbance on plating density for the crystal violet assay. The level of staining is measured by absorbance at 540 nm for various cell densities 1, 2, 3, or 4 days after plating. Data were taken from an individual experiment and confirmed in an independent repeat.

unchanged compared with the control over the 0-2 hour incubation time. In contrast, with increasing dicoumarol concentration above 5 μM, an increase in optical density corresponding to enhanced formazan production was observed over time. No effect of dicoumarol on the production of formazan was noted in the absence of cells, thus indicating that this observation was not due to any direct effect of the compound on MTT. Both DMSO and sodium hydroxide were employed as solvents in these experiments with little difference being observed between results. In addition controls examining the effect of the appropriate amounts of these vehicles revealed little / no effect on MTT reduction in the absence of dicoumarol. Despite these observed differences in formazan production at higher (>5μM) dicoumarol concentrations, IC_{50} values obtained using HT29 cells were similar for both the MTT dye reduction assay and crystal violet staining (with mean values ± SD of 95 μM ± 13.23 and 76.67 μM ± 2.89 respectively). In the one experiment using the Walker cell line where the MTT assay was compared with direct cell counting, similar results were again obtained (IC_{50} values of 33 μM and 30 μM respectively). This would indicate that although dicoumarol affects MTT dye reduction in the short term it may not significantly alter the IC_{50} values obtained over a longer exposure time.

Effect of Quinone Drugs on the MTT Assay

The effect of the quinone menadione on MTT reduction was also examined. Cells were incubated with menadione for 30 minutes before either analysing directly with MTT or rinsing, replacing with either medium alone or medium containing fresh drug and then adding MTT. Reduction of MTT was found to be highest for cells where drug had not been rinsed off before incubation with the formazan (95-130% of the vehicle control for $2x10^3$ cells) over the range of menadione concentrations examined (4-100 μM). Where cell supernatant had been replaced with fresh medium absorbance was lower (48-90% of vehicle control) whereas an intermediate degree of MTT reduction was observed for cells where the medium had been replaced with medium containing fresh drug (59-119%). Thus, over the 30 minute exposure time employed in these assays, menadione did appear to affect the production of formazan from MTT. However, the cytotoxicity of menadione determined by the MTT dye reduction assay gave almost identical results to those obtained using the crystal violet assay. This suggested that, while menadione metabolites may affect MTT dye reduction in the short incubation periods used in these validation assays, it may not have an important effect over the longer incubation times used for cytotoxicity testing.

Determination of Sub-cytotoxic Concentration of Dicoumarol for Inhibition Assays

In order to use dicoumarol to investigate the effect of DT-diaphorase inhibition on the toxicity of the quinone compounds it was necessary to determine the highest sub-cytotoxic concentration. Concentration-response curves determined by the MTT assay are shown in Fig 4.2 for both the rat Walker and human HT29 cell lines. It is evident that the Walker cells are far more sensitive to dicoumarol than the HT29 cells with mean IC_{50} values of 16.8 μM and 90 μM respectively. Little difference was observed between the results obtained using sodium hydroxide and DMSO as a solvent (data not shown). In general toxic affects were noted in the HT29 cell line at concentrations above 50 μM and in the Walker cell line above 5-10 μM. For this reason 50 μM and 25 μM dicoumarol were selected as inhibitor concentrations for quinone toxicity modulation experiments for HT29 cells and 10 μM and 5 μM for Walker cells.

4.3.2 Toxicity of Quinone Compounds

Both the human HT29 colon carcinoma and rat Walker tumour cells were found to be relatively sensitive to menadione, the conventional substrate for DT-diaphorase, when compared with published data for a number of anticancer agents. Values obtained from MTT assays in HT29 cells compare closely with those obtained by crystal violet staining; for example the mean IC_{50} values for menadione using the MTT and crystal violet assay were 15.8 μM ± 5.15 and 10.2 μM ± 2.91 respectively (mean ± SE of a minimum of four repeat experiments). These results therefore indicated the validity of the MTT method. Interestingly, both cell lines exhibited equal sensitivity to menadione with mean IC_{50} values as determined by MTT assay of 15.8 μM for HT29 cells and 13.7 μM for Walker cells (Table 4.1).

The indoloquinone EO9 was found to be an extremely potent cytotoxin in both the human HT29 and rat Walker tumour cells (Fig 4.3 and Table 4.1). In contrast to menadione a marked differential toxicity was observed between the two cell lines, the Walker cells being more sensitive to EO9 with an IC_{50} of 0.047 nM compared with an IC_{50} of 14.8 nM for HT29 cells. As with menadione, results obtained with the MTT assay for HT29 cells compared well with those obtained using the crystal violet assay (mean IC_{50} values ± SE of 14.8 nM ± 0.35 and 10 nM ± 0.22 respectively)

Despite the fairly modest changes in chemical structure, the 9 analogues of EO9 examined were observed to vary to a considerable extent in their cytotoxic potency to

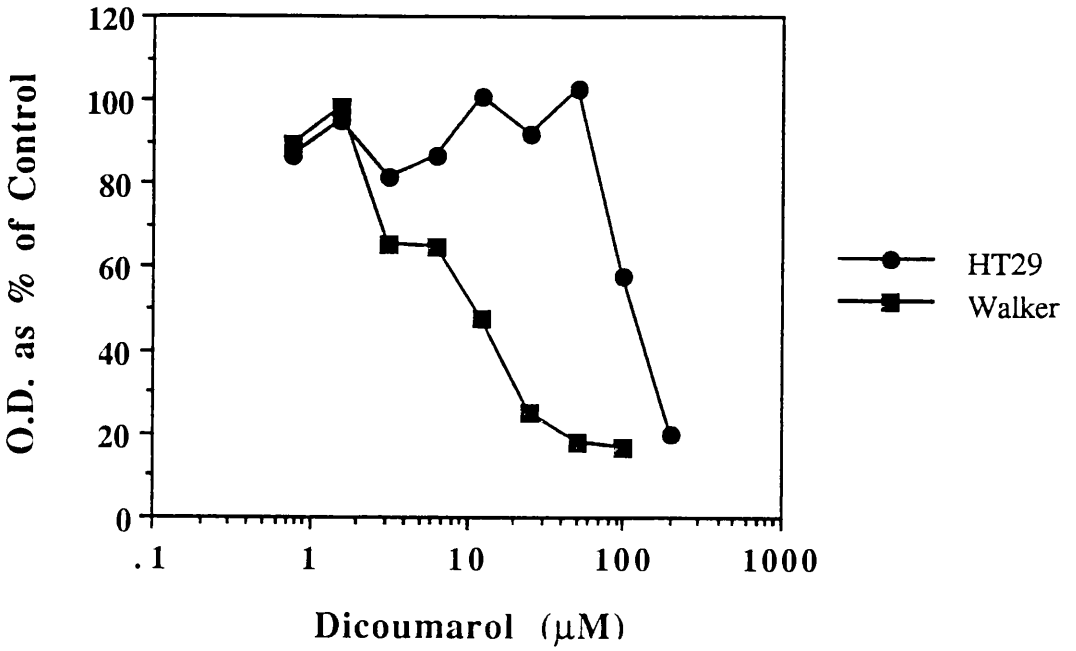


Fig 4.2 Concentration-response curves for dicoumarol against HT29 and Walker Sensitive cells. Data are taken from an individual experiment and are representative of at least three independent repeat assays.

Drug	HT29			Walker Sensitive		
	Mean IC_{50} (μM)	Standard Error	Sample Number	Mean IC_{50} (μM)	Standard Error	Sample Number
Menadione	15.8	5.15	5	13.6	7.89	3
EO9	0.0148	0.0035	5	0.000054 0.000039		2
EO7	1.26	0.086	3	1.43	0.291	3
EO8	0.710	0.143	3	< 0.062 < 0.019		2
EO12	23		1	ND		
EO5A	> 326		1	ND		
EO40	30		1	ND		
EO16	6.70 5.50		2	0.50		1
EO56	> 100		1	ND		
EO72	< 0.39		1	< 0.0035 < 0.19		2
EO88	< 0.239		1	<0.239		1

Table 4.1 Cytotoxicity of menadione and indoloquinone compounds related to EO9 in HT29 and Walker Sensitive cells as determined by the MTT dye reduction assay.

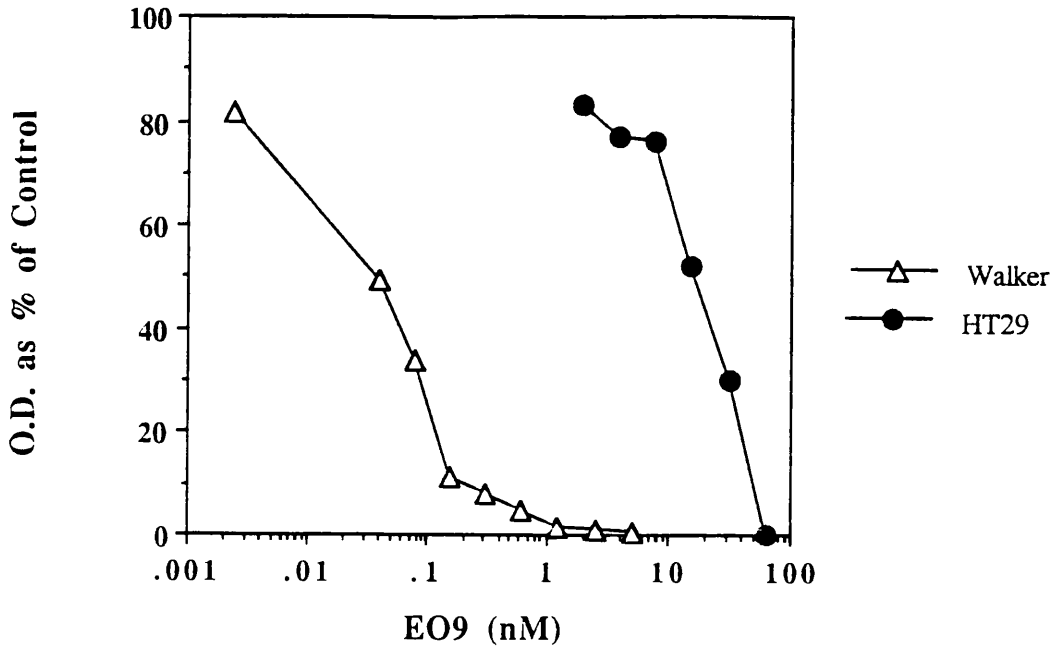


Fig 4.3 Concentration-response curve for HT29 tumour cells and rat Walker Sensitive tumour cells exposed to EO9 continually over 4 or 3 days respectively. Data are from an individual experiment and are representative of 5 repeat assays in the case of HT29 and of 2 repeats for the Walker cell line.

both rat Walker and human HT29 cell lines (Table 4.1). EO9 and also EO88, the compound in which one of the hydroxyl leaving groups (in the X_2 position, Chapter 3, Fig 3.1) of EO9 was substituted with a carbamate group, were the most toxic. In striking contrast HT29 cells were far less sensitive to EO5A, which had a ring-opened aziridine (2-hydroxyethyl amino group) in place of the intact ring, than to all other analogues examined. The IC_{50} value was greater than 327 μM compared with a value of 14.8 nM for EO9. Other analogues with various substituents were intermediate between these two extremes (Table 4.1). In general all compounds which possessed the aziridine moieties including EO9, EO88, EO8 and EO72 exhibited greater cytotoxicity than those lacking this functional group. Similar to menadione, some analogues appeared to exert equal potency towards both rat Walker and human HT29 tumour cells whereas others showed a differential sensitivity between the two cell lines.

Dicoumarol modulation

In general, fairly small differences if any in the toxicity of quinones were seen in the presence of dicoumarol. Inclusion of dicoumarol (50 μM) in the toxicity assay for menadione resulted in a 1.5 fold decrease of the IC_{50} value obtained in HT29 cells (Table 4.2, Fig 4.4 a). Thus dicoumarol potentiated menadione toxicity, consistent with the view that DT-diaphorase functions to detoxify menadione. The results obtained for modulation of indoloquinone cytotoxicity by dicoumarol were fairly variable with some difference being observed between data obtained using the crystal violet assay and those using the MTT assay (Table 4.2). In general however, little alteration in cytotoxicity was observed with EO9 (Fig 4.4 b and Table 4.2) or the analogues examined in the presence of inhibitor. The main exception being EO20 which showed a consistent potentiation of cytotoxicity in the presence of dicoumarol of 1.2-2 fold. The sensitivity of HT29 cells to EO8 was also consistently greater in the presence of dicoumarol (Table 4.2). The concentration-response curve for HT29 cells in the presence of EO9 and modulation by dicoumarol are shown in Fig 4.4 b.

Clonogenic Assay

Clonogenic assays examining the effect of EO9 on Walker Sensitive and Resistant lines confirmed that EO9 was an extremely potent cytotoxin (Fig 4.5 a). The IC_{50} values were however higher than those obtained using the MTT assay for the Walker Sensitive cell line. Preliminary data with EO88 revealed a similar cytotoxic potency to EO9 in both these cell lines (Fig 4.5 a and b).

Drug	DMF Determined by MTT Assay		DMF Determined by Crystal Violet Assay	
	50 μM dicoumarol	25 μM dicoumarol	50 μM dicoumarol	25 μM dicoumarol
Menadione	1.60±0.206 (n=3)	1.38 2.08	1.55 2.2	1.13 1.83
EO9	0.715 ± 0.064 (n=4)	1.10 ± 0.031 (n=4)	1.215 ± 0.265 (n=4)	1.281 ±0.23 (n=3)
EO7	0.52 0.15	0.79 0.62	1.2	1.1
EO8	0.94 0.19	0.91 0.53	0.4	0.86
EO40	0.79	1.25	ND	ND
EO16	1	1.1	ND	ND
EO20	2.07	1.4	1.89	1.23

Table 4.2 Modification of indoloquinone cytotoxicity by 50 μM and 25 μM dicoumarol as determined by the MTT and crystal violet methods. Data from independent repeat experiments are presented as a mean ID_{50} value ± standard error and sample number (n). Where two or less repeats were carried out the individual ID_{50} values are given.

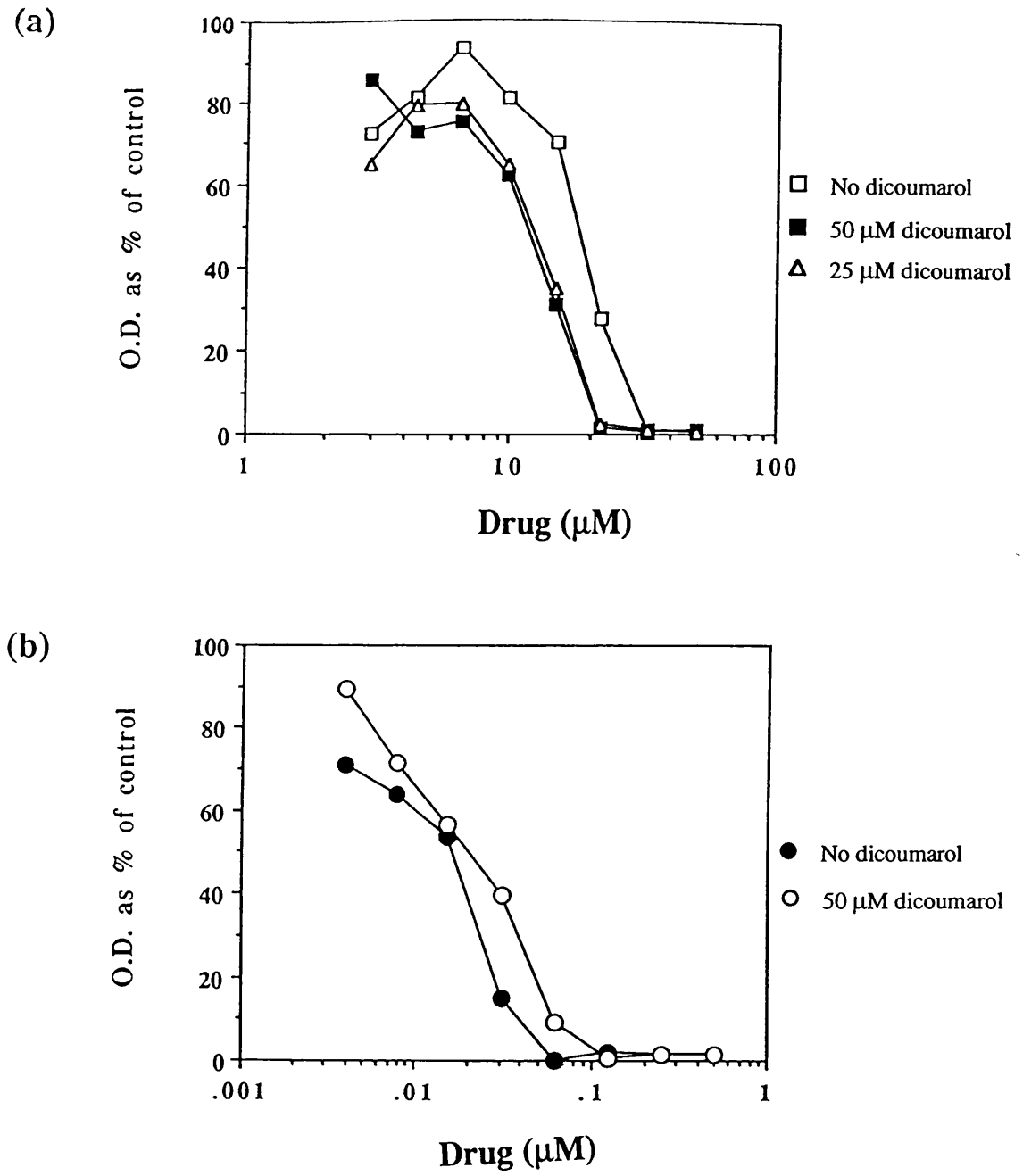


Fig 4.4 Concentration-response curves showing modulation of (a) menadione and (b) EO9 toxicity in the presence and absence of dicoumarol using HT29 cells as determined by the MTT dye reduction assay. Data are taken from individual experiments and are representative of at least 2 independent repeats in the case of menadione and 5 repeat assays for EO9.

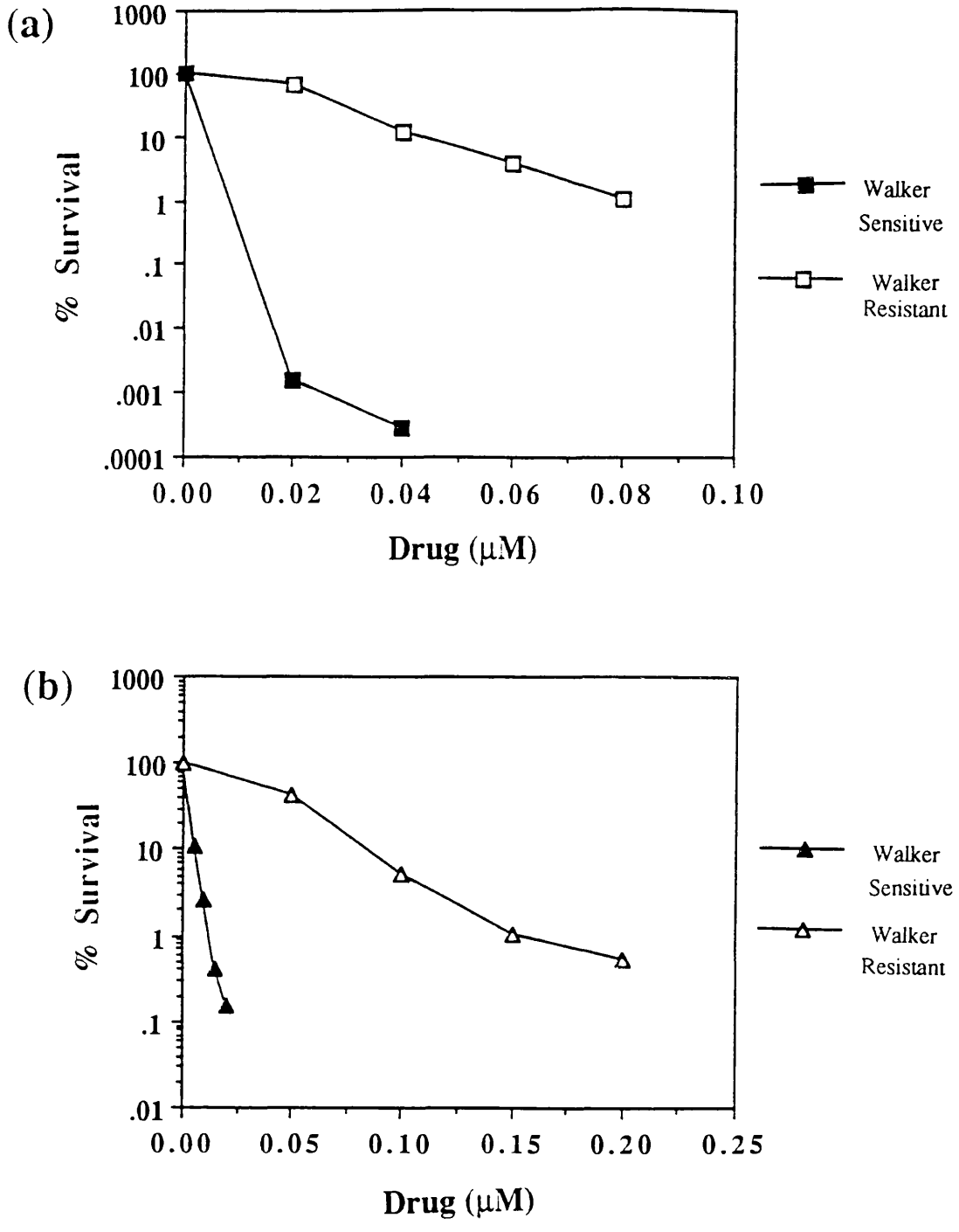


Fig 4.5 The effect of (a) EO9 and (b) EO88 on the colony forming ability of Walker Resistant and Walker Sensitive cells following a two hour exposure. Data are taken from an individual experiment and were confirmed in at least one repeat assay. Each of the data points are an average of four repeat assays carried out within a single expermint.

A differential in toxicity was demonstrated between the Resistant and Sensitive strains of Walker cells in response to EO9 (Fig 4.5 a) and EO88 (Fig 4.5 b) suggesting that these compounds may act as bifunctional alkylating agents. Where dicoumarol was included in the cell survival assays for EO9 (Fig 4.6) and EO88 (data not shown) some protection against cytotoxicity was noted. These results were generally similar to those obtained with HT29 cells by the MTT and crystal violet assay.

4.4 Discussion

Having determined in Chapter 3 that EO9 was able to undergo metabolism catalysed by DT-diaphorase, experiments described in this chapter were designed to elucidate whether this may represent an activation or detoxification process. The cytotoxicity of EO9 was therefore investigated in two cell lines which express high levels of DT-diaphorase, namely HT29 human colon carcinoma and Walker rat tumour cells. In addition the effect of structural modification on cytotoxicity was also examined, since this also represents a means of relating metabolism to cytotoxicity and may potentially lead to further improvements over EO9.

4.4.1 Validation of Assays

Effect of Quinones on the MTT Aassay

Because tetrazolium dye assays are based on metabolic reduction, experiments were carried out to determine the effect of menadione, as a representative quinone, on MTT dye reduction. These revealed some increase in formazan production where menadione was present before and during incubation with MTT, suggesting that metabolites might facilitate reduction of MTT. This finding is consistent with reports that menadione metabolites could directly reduce MTT (Prochaska and Santamaria, 1988). It is possible that over the 72 or 96 hour period of drug exposure employed in the assay such an effect would not occur or would be insignificant. Results obtained using the MTT assay for these quinone compounds agree well with data obtained using the crystal violet assay. This shows that any interference of quinones with MTT dye reduction does not affect the IC_{50} value obtained.

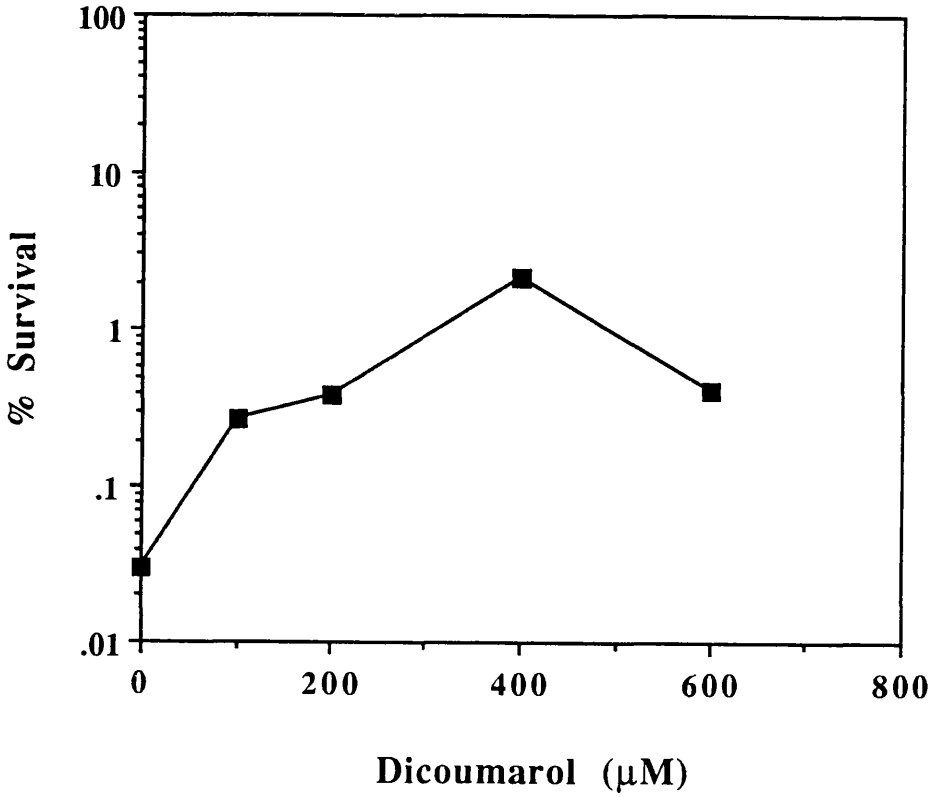


Fig 4.6 Protection conferred by dicoumarol on the cytotoxicity of EO9 (0.015 μM) towards Walker Sensitive cells as determined by clonogenic assay. Data are taken from an individual experiment and were confirmed in at least one independent repeat assay. Each point shown is an average of three or more repeat assays carried out within the same experiment.

Effect of Dicoumarol on the MTT Assay

Where dicoumarol was applied to Walker cells at low concentrations (<5 μM) a decrease in MTT formazan production was observed. This was indicated by a decrease in absorbance of this product at 590 nm ranging from 7 - 40 % depending on the concentration and exposure time. Surprisingly, however, at higher dicoumarol concentrations MTT dye reduction increased both with increasing time of exposure and concentration of compound. This implied that dicoumarol interfered with enzymes involved in MTT dye reduction. Dicoumarol is known to inhibit DT-diaphorase (Ernster, 1987), an enzyme implicated in tetrazolium dye reduction (Romyhr, 1987; Schor and Cornelisse, 1983), in addition to acting as a mitochondrial uncoupler (Murray *et al*, 1982). This would explain the observed decrease in formazan production at low concentrations of dicoumarol. Recent reports by Gustafson and Pritsos (1992b) have suggested that dicoumarol may also inhibit the enzymes xanthine dehydrogenase and xanthine oxidase. This results in an increase in metabolism of mitomycin C. If MTT were able to act as a substrate for xanthine oxidase and xanthine dehydrogenase it is possible that a similar effect may occur at higher concentrations of dicoumarol. These data would indicate that over a short exposure time dicoumarol effects the MTT dye reduction process, presumably by virtue of its inhibition of bioreductive enzymes. Whether dicoumarol would exert any effect on MTT dye reduction following a 72-96 hour incubation period remains unknown. An alternative method for determining cell viability, crystal violet staining, did however result in a similar IC_{50} values. This indicates that any effect is not significant. At lower concentrations of dicoumarol the MTT assay sometimes gave higher values for cell survival than did the crystal violet assay.

One approach that could be employed to reduce the effect of both quinone metabolites and dicoumarol on the MTT assay would be to wash the cells before adding the dye. This would however incur greater variability in the assay, particularly for suspension cultures. In view of the relatively small effects involved it was decided that this precaution was not necessary.

Determination of a Sub-cytotoxic Concentration of Dicoumarol

Sub-cytotoxic concentrations of dicoumarol needed to be established for inhibition assays. A decrease in cell survival following exposure to dicoumarol was in general evident above 50 μM for the HT29 cell line and thus both 50 μM and 25 μM dicoumarol concentrations were selected. Walker cells were far more sensitive to

dicoumarol with an IC_{50} value of around 16 μM and cytotoxic effects occurring at concentrations above 5 μM. The reason for this difference is not known.

4.4.2 Quinone Toxicity

Both the rat Walker and human HT29 tumour cell lines were highly sensitive to EO9 with IC_{50} values of 0.047 nM and 14.8 nM respectively (Table 4.1). These data are consistent with the findings of the NCI *in vitro* screening programme where the compound was found to be extremely potent towards human solid tumour cell lines in general. A GI_{50} value (concentration which inhibits 50% of growth) of 10 nM was quoted for the HT29 cells in the NCI screen which is similar to the value presented in this chapter despite the difference in assay and exposure time. Other authors have reported IC_{50} values of 62.5 nM (Phillips *et al*, 1992), 160 nM (Robertson *et al*, 1994) and 33 nM (Van Ark-otte *et al*, 1994) in this cell line. Extending the exposure time of HT29 cells to EO9 from 24 to 72 hours has however been found to have little effect on cytotoxicity although there was a 10 fold difference in IC_{50} values between a 1 hour and 24 hour incubation period (Van Ark-otte *et al*, 1994). To date there have been no publications reporting the cytotoxicity of EO9 in the rat Walker tumour cells.

Structural modification of EO9 resulted in very significant alterations in the cytotoxic potency (Table 4.1). EO9 and EO88, the analogue in which one of the hydroxyl leaving groups of EO9 has been replaced by a carbamate group, were observed to be the most potent cytotoxins of the 9 analogues investigated in both the HT29 and Walker cell lines. Although an absolute IC_{50} value was not obtained for EO88 using the MTT assay, data obtained using the clonogenic assay revealed that EO88 had a similar cytotoxic potency to EO9 in Walker Sensitive cells. In striking contrast EO5A, the analogue containing a ring-opened and thereby deactivated aziridine group, was at least 22,000 fold less toxic than EO9 (Table 4.1). During the course of this work Phillips and co-workers (1992) reported similar results where EO5A is 84 fold less toxic to DLD-1 human colon adenocarcinoma cells compared to EO9. The only structural difference between EO9 and EO5A involves the aziridine ring. It is possible that this may be responsible for the differences in cytotoxicity observed either by altering the ability of the compound to a) be metabolised and / or b) to cause bifunctional adducts as the activated aziridine is a potent alkylating species. EO9 and EO88 possess an aziridine ring and were both efficiently metabolised by DT-diaphorase (Chapter 3) and acted as potent cytotoxins to HT29 and Walker cells. This may indicate the importance of an aziridine ring in catalysing reduction of the indoloquinones to a cytotoxic species. However, EO7 lacks the aziridine moiety

(Chapter 3 Fig 3.1) and yet is also well metabolised by DT-diaphorase (Chapter 3) and is a potent cytotoxin. These data therefore provided the first indication that DT-diaphorase metabolism may be important for the activation of the indoloquinones. Moreover, the results with EO7 show that the aziridine moiety is not an essential structural requirement for metabolism catalysed by DT-diaphorase. Consistent with the idea that cytotoxic potency may result from ability of the aziridine moiety to confer alkylating ability is the finding that analogues EO8, EO72, EO9 and EO88 all possess the aziridine group and exhibit comparatively low IC_{50} values. In contrast the other 6 analogues examined lacked the aziridine moiety and had higher IC_{50} values of 1-30 μM. Results obtained with EO7 suggest that the possession of the aziridine moiety may not be totally responsible for cytotoxic effects as this compound lacks the aziridine ring but retains a similar potency to the aziridine ring-containing compound EO8. Thus cytotoxic potency appears to be a combination of both ability to be activated by enzymes such as DT-diaphorase and possession of the aziridine ring. The latter most probably acts to alkylate critical molecules within the cell and facilitates DNA cross-linking. The complication in this analysis is that the aziridine will contribute to the ability to act as a substrate for DT-diaphorase and to the ability to cross-link DNA.

Structure-activity relationships have been examined for other series of bioreductive compounds such as the dinitrobenzamide CB 1954. In a similar manner to EO9, Knox and co-workers (1988) observed that the aziridine moiety was not necessary for metabolism of dinitrophenyl aziridine compounds related to CB 1954 by DT-diaphorase. Interestingly the aziridine ring opened derivative of this agent has also been found to be far less potent *in vivo* than the parent compound (Khan and Ross, 1969 / 1970) in addition to acting as a very poor substrate for DT-diaphorase (Knox *et al*, 1988). Intact aziridine rings have similarly been reported to be important for growth inhibition of the quinone agent AZQ in human leukaemic cell lines (Egorin *et al*, 1985).

As discussed above, data presented in this thesis have shown that HT29 and Walker cells which possess high levels of DT-diaphorase are extremely sensitive to EO9. In addition, structure-activity relationship studies found that analogues better metabolised by DT-diaphorase acted as more potent cytotoxins than those which were more poorly reduced. These data provide the first strong evidence for the involvement of DT-diaphorase in the activation of EO9. This hypothesis has been further substantiated in studies carried out by other investigators. In a pair of colon carcinoma cell lines, HT29 cells which express high levels of DT-diaphorase were

found to be 15-30 fold more sensitive to EO9 than the BE cell line (Walton *et al*, 1992a; Plumb and Workman, 1994) which does not express a functional form of the enzyme due to a mutation in the NQO1 gene (Traver *et al*, 1992). In the same study a correlation between DT-diaphorase expression and sensitivity to EO9 was also observed *in vivo* in a pair of mouse colon tumours. Studies on large panels of cell lines which cover a variety of tumour types of both human and rodent origin continued to show a correlation between aerobic sensitivity to EO9 and DT-diaphorase expression (Robertson *et al*, 1992, 1994; Phillips *et al*, 1992; Plumb *et al*, 1994a and b; Smitskamp-Wilms *et al*, 1994; Fitzsimmons *et al*, 1994; Paull *et al*, 1994).

Data presented in this chapter have also given a strong indication that DNA damage may be involved in the cytotoxic mechanism of EO9. A differential sensitivity was observed between rat Walker Sensitive and Resistant cells to both EO9 and EO88, the analogue with a modified pendant leaving group. This is a characteristic feature of difunctional alkylating agents (Knox *et al*, 1991).

It may be that data obtained with other cell lines may also aid elucidation of the mechanism of cytotoxicity exerted by a particular compound. A 315 fold differential in sensitivity was observed with EO9 between the rat Walker and human HT29 cell lines. However, in contrast to EO9, certain quinone compounds such as menadione and the indoloquinone analogue EO7 exhibited equal cytotoxic potency towards both the Walker and HT29 cell lines. It is possible that this observation may result from variation in the activating enzymes between the two cell lines which alter their ability to metabolise some compounds and not others. Alternatively it may reflect a difference in the mechanism of cytotoxicity exerted by the various quinones. Menadione is thought to induce cell death by oxidative stress and DNA damage following redox cycling (Ngo *et al*, 1991). If the two cell lines were to possess similar levels of relevant activating and protective enzymes, or alternatively the same overall balance of these enzymes, a similar sensitivity to menadione would be expected. Alternatively, for compounds such as EO9, the mechanism of toxicity may include alkylation as well as free radical damage and oxidative stress. Walker Sensitive cells are known to be particularly sensitive to damage caused by bifunctional alkylating agents (Knox *et al*, 1991). Results obtained in this chapter revealed a differential in sensitivity between Walker Sensitive and Resistant strains to EO9 suggesting that this drug acted as a bifunctional alkylating agent which induced DNA interstrand cross-links. For a similar reason, compounds which induce toxicity primarily by DNA alkylation may show an enhanced cytotoxic potency in the Walker

cells as compared with HT29. Menadione and EO7 both lack the aziridine moieties which are potent alkylating groups and thus may be less likely to mediate toxicity by bifunctional alkylation than would EO9 and EO8 which possess this group. Interestingly the former two compounds are those which exhibit similar cytotoxic potency in the HT29 and Walker cell lines and the latter those which do not. This would substantiate the theory that where a differential is observed the compound is more likely to act as an alkylating agent. If, upon examination of a wider range of compounds, this phenomenon were to remain consistent comparison of the HT29 and Walker cells in cell survival experiments may provide a useful screen for indication of the mechanisms of toxicity.

Some information on the redox potentials of the indoloquinones ($E_{1/2}$) as determined by polarography was available from the EORTC (unpublished data on file). As reported in Chapter 3, the order of reduction potential from most to least electronegative was EO12, EO8, EO9~EO88, EO7. The order of cytotoxicity was from most to least cytotoxic EO12, EO7, EO8, EO9~EO88. Therefore, although there is some weak relationship between the reduction potential and aerobic cytotoxicity the correlation is not absolute. As discussed in Chapter 3 however the ease of reduction did not correlate well with that predicted by redox potential and the half-wave potential values are within a fairly narrow range such that little effect on metabolism would be expected. A similar lack of correlation between $E_{1/2}$ values and *in vitro* cytotoxicity was observed with mitosene compounds related to mitomycin C (Malipaard *et al*, 1992). Thus one-electron reduction potentials determined by polarography may not be a very useful measure of ability to undergo reduction catalysed by DT-diaphorase or of cytotoxicity. As discussed in a recent review by Workman (1994) a long-standing view was that the redox potential of the bioreductive drug would determine its efficacy as a hypoxic-cell cytotoxin. Although the hypoxic sensitivity of cell lines to EO9 has not been evaluated in the work presented in this thesis, data obtained under aerobic conditions suggests that factors other than redox potential may also be important in determining both ability to be reduced and cytotoxic potency.

In addition to ability to undergo reductive activation, a number of other factors will affect the cytotoxic potency of the indoloquinone compounds. These may include other physicochemical characteristics such as solubility, lipophilicity, cellular uptake and efflux, as well as the levels of and relative affinities for and maximum velocities of different activating and detoxifying enzymes. Depending on the mechanisms of

damage involved, systems for protection against oxidative stress, and various DNA repair enzymes will also play a role in determining cellular sensitivity to these agents.

Inclusion of dicoumarol in MTT viability assays of the effect of menadione, the classical substrate for DT-diaphorase, resulted in ~ 1.5 fold increase in toxicity (Fig 4.4 a). Although the degree of modulation is fairly small these data were reproducible and are in fact consistent with classical results obtained in hepatocytes at the Karolinska laboratory (Thor *et al*, 1982). The protection conferred by dicoumarol would suggest that DT-diaphorase is able to detoxify menadione. The relatively modest degree of protection is likely to be due to the ability of the hydroquinone formed by DT-diaphorase to generate the semiquinone free radical, by reoxidation or comproportionation : disproportionation reactions, before the hydroquinone can be eliminated by glucuronide or sulphate conjugation. It is also possible that a higher degree concentration of dicoumarol, if it had been tolerated, would have given more protection.

In contrast to the result obtained with menadione, EO9 showed a 1.4 fold decrease in toxicity where 50 μM dicoumarol was included, whereas at 25 μM dicoumarol little modification was observed. Repeating these experiments using the crystal violet assay resulted in an apparent increase in toxicity by dicoumarol. Due to the variation between the repeat experiments using crystal violet assays the slightly more consistent results obtained with the MTT assay appear more reliable. EO8 and EO7 also showed an approximate 1.5 fold decrease in toxicity where dicoumarol was included. These were again compounds which were highly cytotoxic to HT29 cells and were well metabolised by DT-diaphorase. Thus although the modification of indoloquinone toxicity observed in the presence of dicoumarol was small it was of a similar range to that observed with menadione.

Clonogenic assays with Walker Sensitive cells confirmed that dicoumarol caused a decrease in the cytotoxicity of EO9 but not complete protection (Fig 4.6). Dicoumarol could bind to serum protein and it is therefore possible that the lack of complete inhibition of cytotoxicity is due to low concentrations of the compound being achieved in cells. As the cytotoxicity of the compound CB 1954 was almost totally alleviated by these concentrations of dicoumarol (data not shown) this would be an unlikely explanation. Thus data obtained using both the MTT assay and clonogenic assay are supportive of a role for DT-diaphorase in activation of EO9, EO8 and EO7 but the lack of complete protection conferred by dicoumarol against their toxicity would suggest the additional involvement of other enzymes. Indeed

data obtained from ESR experiments (see Chapter 3) provided evidence for the metabolism of EO9 by cytochrome P450 reductase to generate potentially toxic drug and oxygen-based radicals. In addition, caution must be taken when interpreting data obtained using dicoumarol due to the pleiotropic effects seen with this compound (see Chapter 3 and Workman *et al*, 1989).

Hypoxia may enhance the selectivity of bioreductive drugs towards hypoxic tumours (Chapter 1). Although the effect of hypoxia and pH on the cytotoxicity of EO9 and hypoxia have not been examined in this chapter they have been examined by other investigators. Cell survival assays have indicated an enhancement in sensitivity of HT29, BE (Plumb and Workman, 1994), V79 (Stratford and Stephens, 1989), KHT (Adams *et al*, 1992), V79 and CHO (Hendriks *et al*, 1993) cells towards EO9 under hypoxia compared with aerobic conditions. The ratio of hypoxic : oxic sensitivity to EO9 does however vary greatly between cell lines. Interestingly, recent data have shown that whereas the aerobic sensitivity of cells to EO9 correlate well with DT-diaphorase activity an inverse correlation has been observed for DT-diaphorase expression and hypoxic sensitivity (Plumb *et al*, 1994b; Robertson *et al*, 1994). Data presented in Chapter 3 of this thesis showed no difference in the rate of DT-diaphorase metabolism between aerobic and hypoxic conditions as determined by the cytochrome *c* reduction assay. In addition, results of ESR experiments revealed that an oxygen-sensitive metabolite is generated following reduction of EO9 catalysed by DT-diaphorase. Thus it is likely that hypoxia influences the stability of the metabolite produced by this reduction process and that this may explain the hypoxic enhancement of EO9 cytotoxicity observed by other investigators. Hypoxia may also affect metabolites generated by enzymes other than DT-diaphorase, particularly as BE cells do not possess a functional form of DT-diaphorase and showed an extremely large hypoxic cytotoxicity ratio (HCR).

To conclude, data presented in this chapter have shown EO9 to act as a highly potent toxin to both the rat Walker and human HT29 cell lines which express high levels of DT-diaphorase. Studies of structural analogues of EO9 found a correlation between cytotoxicity and ability to undergo DT-diaphorase-catalysed reduction. Thus, taken together, these results provide the first strong evidence to suggest the importance of DT-diaphorase in activation of indoloquinones. Further support of this involvement has been provided by dicoumarol inhibition studies and more recently by data from a number of laboratories in which sensitivity of cells have been correlated with their levels of DT-diaphorase activity. The lack of complete inhibition of toxicity observed with dicoumarol suggests the additional involvement of other enzymes in activation

of these compounds. Another good candidate enzyme would be NADPH: cytochrome P450 reductase as data presented in Chapter 3 of this thesis have shown that in air it also can metabolise EO9 to generate similar metabolites to those formed by DT-diaphorase. In addition to this important link between DT-diaphorase and cytotoxicity, structure-activity relationships have revealed other significant findings. They show that small modifications to EO9 can profoundly alter the cytotoxic potency of the compound, thus indicating a potential for new drug development. Areas of the molecule which favour both metabolism and toxicity have been highlighted with the aziridine group being of particular importance although not absolutely essential. Finally, despite the fact that redox potential is often considered to be a determinant of cytotoxicity, these experiments have not shown a direct correlation but have emphasised the necessity to examine other parameters such as enzymatic reduction. The differential toxicity between the rat Walker Sensitive and Resistant cells suggested that the mechanism of EO9-induced cytotoxicity may involve DNA damage. For this reason experiments described in the following chapter examined different forms of DNA damage both in intact cells at pharmacologically relevant concentrations and in cell free systems to enable the role of individual enzymes to be determined.

Chapter 5

DNA Damage

5.1 Introduction

The chemistry of EO9 suggests that its mechanism of cytotoxicity may involve DNA damage. As discussed previously (Chapter 3) reduction of EO9 could generate the semiquinone radical with concomitant formation of oxygen radicals under aerobic conditions. Both these species may be potentially damaging to DNA by inducing DNA strand breaks. In addition, reduction of EO9 may facilitate activation of the aziridine ring and / or one or both of the hydroxyl leaving groups (Fig 5.1) to produce monofunctional or bifunctional alkylating species which could form adducts with DNA. Results of clonogenic assays described in Chapter 4 of this thesis further suggested the ability of EO9 to induce DNA damage. These studies showed a clear differential in cytotoxicity between Walker Sensitive and Resistant cells, a feature which is typical of a large number of known bifunctional alkylating agents. Therefore, the experiments described in this chapter were designed firstly to examine whether DNA damage was evident in cells following exposure to pharmacologically relevant concentrations of EO9 in order to determine whether this could be a possible mechanism of EO9-induced cell kill. A second aim was to use of cell free assays, to investigate the possible involvement of DT-diaphorase and NADPH: cytochrome P450 reductase in induction of this DNA damage. A final objective was to characterise the damage at a more molecular level by examination of the sequence selectivity of EO9 binding to DNA both before and following activation by DT-diaphorase.

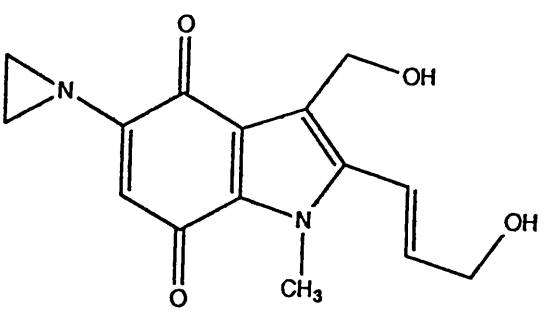


Fig 5.1 Chemical Structure of EO9

DNA damage was assessed in intact cells by the technique of alkaline elution using a method similar to that described by Kohn and co-workers (1981). The rat Walker mammary tumour cell line and two human colon carcinoma cell lines, HT29 and BE, were selected for theses studies because the cytotoxicity of EO9 and DT-diaphorase catalysed metabolism of the drug had been characterised precisely in these cells (Chapters 3 and 4 of this thesis and Plumb and Workman, 1994). Utilisation of irradiated and unirradiated cells permitted both the DNA strand break and interstrand cross-link frequency to be determined. Studies carried out by Plumb and Workman (1994) had found a 2-5 fold increase in the cytotoxic potency of EO9 in HT29 cells and 1000-3000 fold in BE cells under hypoxic compared with oxic conditions. If DNA damage were involved in the cytotoxic mechanism of EO9 the degree of this damage would also be expected to change under hypoxia. For this reason the effect of hypoxia on DNA damage was investigated in the HT29 and BE cells.

Alkaline elution data obtained with the high DT-diaphorase expressing cell line HT29 were compared with those for the BE cell line which does not express a functional form of the enzyme. This gave an indication of the role played by DT-diaphorase in activation of EO9 to a DNA-damaging species. However, in order to clarify the involvement of DT-diaphorase and the one-electron reducing enzyme NADPH: cytochrome P450 reductase in this activation process, cell free assays were also carried out. Two agarose gel electrophoresis methods were employed for this, one to examine DNA strand breaks and the other to measure DNA interstrand cross-links. In addition the selectivity of EO9 binding to DNA was investigated using a *Taq* polymerase gel assay.

The DNA strand break assay was similar to that described by Walton and co-workers (1991) and is based on conformational changes of pBR 322 supercoiled plasmid DNA when it is subject to strand breaks, resulting in an altered electrophoretic mobility. A single strand break converts the supercoiled plasmid to the relaxed, open circular conformation (form II), whereas a double strand break produces the linear form (III) (Fig 5.2). These three species of DNA were resolved by agarose gel electrophoresis. Gels were visualised by staining with ethidium bromide and quantified by densitometry.

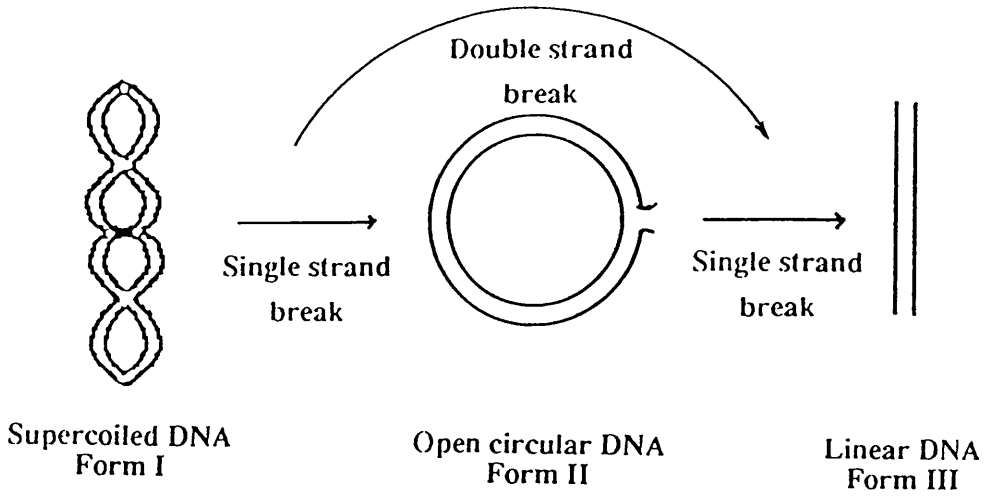


Fig. 5.2 Conformational changes in plasmid DNA following strand breakage

DNA cross-links can be detected by a variety of techniques in a cell free system including alkaline sucrose gradient centrifugation and fluorescence methods amongst others. These techniques do, however, have disadvantages. The agarose gel method described by Hartley and co-workers (1991) has the advantage of being highly sensitive and requires relatively little DNA and drug. Following treatment, DNA is heat denatured at 95°C and cooled on ice to provide the single stranded form. In the presence of a cross-link, however, DNA remains double stranded. The double and single strands of DNA are resolved by agarose gel electrophoresis due to their different electrophoretic mobility. The amount of cross-linking induced can be quantified by densitometry.

Having examined DNA strand breaks and cross-links, DNA damage was examined at a molecular level by determining the sequence selectivity of EO9 binding to DNA in order to more fully understand the mechanism of action of EO9. Selectivity of DNA binding has been reported for a number of antitumour agents (Mattes *et al*, 1986; Hartley *et al*, 1986; Hartley *et al*, 1991b) and it is possible that this preferential binding may be related to their antitumour activity (D'Incalci *et al*, 1992). An understanding of the sequence selectivity may therefore allow design of compounds with enhanced binding to specific DNA sequences important in eliciting an antitumour response. An example of such rational design is clomesome (2-chloroethyl(methylsulphonyl) methanesulphonate) which is currently undergoing clinical trial in the UK. This compound is based on the chloroethylnitrosoureas and possesses chloroethylating potential, which is thought to be related to antitumour activity, but lacks hydroxyethylating or carbamoylating activity which are not believed to be important and may contribute to toxicity (Hartley, 1993). Another point of interest is that many alkylating agents bind to guanines present in GC sequences. The human genome has a rich GC content and these regions exist in some oncogenes as well as being common in genes associated with proliferation (Mattes *et al*, 1988). It is possible that the antitumour activity observed may be related to the sequence-specific binding exhibited by these compounds, but this issue is controversial and remains to be clarified.

The specificity of EO9 binding to DNA was examined using the *Taq* polymerase assay described by Ponti and co-workers (1991). This method is based on the ability of a thermostable DNA polymerase isolated from *Thermus aquaticus* to synthesise new strands of DNA *in vitro*. In the presence of a lesion the polymerase is unable to continue DNA strand elongation and thus the newly synthesised strand of DNA becomes truncated at a site corresponding to the adduct. Products are subject to linear amplification using the technique of polymerase chain reaction (PCR) and are analysed on polyacrylamide sequencing gels. Fragment mobility and band thickness will relate to the site of adduct formation and the frequency of that lesion respectively. The method has been reported to give consistent results to those obtained using other procedures (Ponti *et al*, 1991). In addition it is more sensitive, does not require a strand breakage step and can detect lesions at any base. The assay has also been suggested to have a potential use for examining sequence selectivity of binding in intact cells which have been exposed to drug at pharmacologically relevant concentrations (D'incalci *et al*, 1992)

5.2. Methods

5.2.1 Alkaline Elution

Aerobic DNA Alkaline Filter Elution with Rat Walker UK 256 Tumour Cells

Rat Walker UK 256 tumour cells, cultured as described in Chapter 2, Section 2, were harvested on reaching a log phase of growth and were resuspended in fresh medium to give a density of 3 x 10^5 cells / ml. Flasks containing 50 ml of these cells were then radiolabelled with either ^3H (plus 0.48 μg / ml of cold thymidine) or ^{14}C at a specific activity of 1 μCi / ml. Following a 24 hour incubation period at 37°C, radiolabelled cells were pelleted by centrifugation (Beckman TJ6) at 150 g for 7 minutes in 25 ml Falcon tubes. Medium was decanted and cells were washed twice in PBS before resuspending to a cell density of 2 x 10^5 cells / ml. Aliquots of 10 ml of these Walker cells were then transferred to 15 ml tubes (Falcon) for drug treatment.

Alkaline elution experiments generally involve exposing cells to a single dose of drug for a set period of time. In the case of the Walker cells a range of conditions were investigated before finally optimising the doses to favour detection of cross-links. Drug concentrations which had previously been found to be cytotoxic in clonogenic assay (Chapter 4) were employed. Initially cells were incubated for a period of two hours with EO9 at a final concentration of either 80 nM or 400 nM (dissolved in a maximum final concentration of 0.1% DMSO). Following treatment half of the cells were harvested for immediate examination of DNA damage and the remaining half were resuspended in fresh medium and returned to the incubator for a further 24 hour period to allow DNA repair. In a second experiment a final concentration of 10 nM EO9 was applied to cells for varying continuous exposure times (2, 4, 7.25, 12, 18 and 24 hours) before elution. In order to further optimise conditions the amount of EO9 was increased to 20 nM for continuous exposures of 2, 6 or 20 hours. In a final experiment four repeat doses of either 4 nM or 20 nM (dissolved in a maximum final concentration of 0.02% DMSO) EO9 were applied to cells at four hourly intervals. Following a further 12 hour incubation period drug was removed by centrifugation (7 minutes at 150 g) and cells were washed twice in medium. Half of the cells were then taken, resuspended in 10 ml of medium and returned to the incubator at 37°C for a further 24 hours in order to examine DNA repair. The remaining cells were prepared for elution as described later in this Section. Control incubations involved exposing cells to the solvent DMSO at the same concentration as that applied to treated cells. The dinitrophenyl aziridine compound CB 1954 has been shown to induce a high

frequency of DNA interstrand cross-links in Walker cell DNA using alkaline elution (Knox *et al,* 1991). For this reason CB 1954 treated cells were included in the experiment as positive controls. These had been treated with a single dose of compound at a final concentration of 10 μM for a period of 1.5 hours before elution.

DNA Alkaline Filter Elution with HT29 and BE Tumour Cells

HT29 and BE cells were routinely cultured as described in Chapter 2, Section 2. On reaching log phase of growth these were harvested by trypsinisation, washed, resuspended in medium and seeded at a density of 10^6 cells in 75 cm^2 tissue culture flasks. Cells were left for a period of 6-12 hours to permit adherence to the substratum before addition of radiolabel. Labelling was carried out as described previously in this Section but for a period of 72 hours. Following removal of the radiolabel, cells were resuspended in medium, seeded on to sterile, glass petri dishes (5 cm diameter) at a density of 1 x 10^6 cells / dish and incubated at 37°C for a chase period of 24 hours. This allowed adherence of cells and ensured incorporation of label into high molecular weight DNA. Cultures were then treated with EO9 or vehicle (DMSO in PBS) as a control.

As experiments with Walker cells (detailed above) had been able to detect cross-links following multiple additions of drug at four hourly intervals, initial studies with HT29 and BE cells adopted a similar procedure. Cells were exposed under aerobic conditions, to three repeat aliquots of 20 nM EO9 (dissolved in DMSO to give a final concentration of 0.02%) at four hourly intervals followed by a further incubation period before eluting 24 hours after the first addition of drug. In a second experiment the concentration was further increased to 100 nM but this was applied in a single dose for a 20 hour continuous incubation period. Finally, for experiments where hypoxic conditions were compared with aerobic ones a short incubation period was required. Concentrations of 0.25 μM EO9 and 10 μM EO9 were therefore selected as these had been previously shown to be around the IC_{50} values of EO9 in HT29 and BE cells respectively for a 3 hour exposure under aerobic conditions (Plumb and Workman, 1994). Incubations were carried out within a plastic cake box adapted to include two tubes which acted as an inlet and outlet for the appropriate gas. Before entering the box, gas was passed through a dreshall bottle containing sterile, distilled, deionised water incubated at ~39°C. A pregassing period of 30 minutes allowed the water to become saturated with nitrogen and acted to humidify the gas so reducing evaporation of the incubation medium. Tissue culture medium was removed from the petri dishes and was replaced by 2 ml of sample in PBS which had previously been

gassed for a period of 20 minutes with either nitrogen or air/CO_2 for hypoxic and aerobic experiments respectively. Petri dishes were rapidly placed in the box which was then tightly sealed with tape and connected to the gas supply. For hypoxic incubations nitrogen was used (British Oxygen Company) and for aerobic incubations 5 % CO_2 in air was employed. Incubations were carried out for a period of 2 hours at 37°C in the warm room before rapidly removing medium using a Pasteur pipette. The cells were then trypsinised and treated as described below.

Preparation of Cells for Alkaline Elution Following Treatment

Harvested cells were counted on a haemocytometer and were resuspended in ice cold, sterile PBS to give a final concentration of 1 x 10^6 cells / ml in 15 ml tubes (Falcon). These were kept on ice to prevent DNA repair. Samples were divided to provide cells for irradiation and those to remain unirradiated to allow the examination of both interstrand cross-links and strand breaks. The ^{14}C labelled, treated samples for irradiation were given a dose of 6 Gy. A 0.2 ml aliquot of treated cell suspension was mixed with 10 ml of ice cold PBS in a 15 ml tube to give a final cell concentration of 2 x 10^4 cells / ml. In order to compensate for any difference in elution rate between columns of the apparatus an internal standard of ^3H labelled cells was employed, these were exposed to 1.5 Gy. Where internal standard was included, 0.1 ml of ^3H labelled cells were added to 0.1 ml of ^{14}C labelled cells in 10 ml of ice cold PBS again resulting in a concentration of 2 x 10^4 cells / ml. Similar dilutions were made for unirradiated cells to examine DNA strand break formation. Samples were then maintained in the dark on ice until they were eluted as described below.

Alkaline Elution Procedure

The elution apparatus consisted of a Swinnex type 50 ml syringe barrel and Swinnex filter holder mounted vertically in groups of eight channels per apparatus. DNA was eluted through a polycarbonate filter of 2 μm pore size (25 mm diameter, Nucleopore, High Wycombe, Bucks., UK.), placed on the filter holder. Elution rate was regulated using an 8 channel peristaltic pump (Miniplus 2, Gilson, Anachem, Luton, Beds., UK) fitted with 0.38 mm internal diameter PVC miniplus 3 tubing (Anachem). The latter was attached to the filter holder via a stainless steel needle. Fractions were collected in 6.5 ml scintillation vials (Packard Instrument Co., Gronigen, Netherlands) using an LKB Ultrorac II fraction collector (LKB Instruments Ltd, Selsdon, Surrey, UK).

Before sample elution the polycarbonate filters were moistened with distilled water, placed over the filter holder grid, overlaid with rubber O rings to form a tight seal and holders were screwed into the Swinnex barrels. Correct functioning of the equipment was checked by addition of 5 ml of ice cold PBS to each channel. Cell samples (5 ml) were then gently loaded on to the filters and permitted to flow through by gravity leaving a total of 10^5 cells deposited on each filter. In all cases samples and solutions were added gently in order to avoid introduction of air bubbles and disruption of the DNA. All procedures were carried out in the dark and with the apparatus covered during elution in order to avoid degradation of naked DNA by UV light. Lysis solution (5 ml: glycine [0.2 M; pH 10]; Na_2EDTA [0.05 M; pH 9.7]; SDS [0.07 M]) containing 0.5 mg / ml of proteinase K was added to each channel. The latter constituent digested and removed protein thus allowing measurement of DNA-DNA interstrand cross-links without interference caused by DNA-protein cross-links. This was followed by 5 ml of lysis solution and 5 ml of wash ($Na_2EDTA\ 2H_2O$ [20 mM], tetraethylammoniumhydroxide [0.4 % w/v; pH 9]) which served to remove the high salt concentration and detergent. When all of the wash solution had passed through the columns they were connected to the pump tubing and 10 ml of elution solution ($Na_2EDTA\ 2H_2O$ [20 mM], tetraethylammoniumhydroxide [7.5 % w/v; pH 12]) was added. The high pH of the elution solution disrupts hydrogen bonds between parallel strands of DNA converting them to single strands and permitting them to pass through the filter. Samples were eluted overnight in the dark at room temperature using a peristaltic pump at a flow rate of 1 ml / 90 minutes and 10 x 1 ml fractions were collected over 15 hours.

Having completed the elution procedure the filters were gently removed from their holders using a pair of blunt tweezers and were placed in scintillation vials. The solvent, Soluene 350 (1 ml; Packard Instrument Co.) was added to each of the samples which were then placed in a Grant heater (Grant, Cambridge, UK) at 50°C for 20-30 minutes before vortexing for a short time to ensure complete solubilisation of the filters. To both the eluted fractions and solubilised filters 4 ml of the liquid scintillation cocktail, Hionic-Fluor (Packard Instrument Co.) was added and mixed thoroughly. The radioactivity (^{14}C and ^{3}H content) of the samples was determined using a Wallac 1410 scintillation counter (Pharmacia Ltd., St Albans, Herts., UK).

Calculations and Presentation of Data

The data obtained from alkaline elution experiments are conventionally displayed graphically as an elution profile where the fraction of ^{3}H DNA remaining on the filter

(y-axis) is plotted against the fraction of ^{14}C DNA remaining on the filter (x-axis). These values are obtained by use of the following equation.

$$F(i) = \frac{T - (A(i))}{T}$$

Where F is the fraction of DNA retained on the filter, T is the total number of counts per channel (those eluted plus those remaining on the filter) and A is the sum of the counts eluted from the first fraction up to and including fraction i.

The rate at which DNA is eluted from the filter is represented by the slope of the elution profile. Where DNA strand breaks are present the DNA will elute more rapidly than the control unirradiated DNA whereas the presence of DNA interstrand cross-links will cause a decrease in the rate of elution compared with the control.

The DNA damage induced was quantified with the application of published formulae (O'Connor and Kohn, 1990). This may be expressed as either damage occurring per 10^9 daltons of DNA or in rad equivalents. For compounds which induce both DNA strand breaks and DNA interstrand cross-links the situation becomes more complex as cross-linked DNA which has also received strand breaks will elute more rapidly than in the absence of DNA strand breaks and therefore the extent of DNA cross-linking is masked. Inclusion of unirradiated samples permits calculation of the DNA strand break frequency. This value may be used to correct the calculated DNA cross-link frequencies.

DNA Strand Break Frequency

Calculations to quantify the DNA damage frequencies are carried out using only one, arbitrarily selected, value for the fraction of DNA retained on the filter. The value selected is the retention of the ^{14}C label where that of the internal standard (^3H) is 0.5. This value is known as the "relative retention" and is denoted as Ro and ro for the irradiated and unirradiated controls and R1 and r1 for the irradiated and unirradiated, drug treated samples respectively.

The apparent drug induced probability of a strand break (PBD) is calculated using the following formula.

$$PBD = \frac{PBR \quad (\log (r1 / ro)}{(\log (Ro / ro)}$$

PBR is the probability of a single strand break produced by the X-ray dose alone. This value for 6 Gy is 5.3 (Roberts and Friedlos, 1987).

5.2.2 Plasmid Strand Break Assay

Aerobic Incubations of pBR 322 with Purified DT-diaphorase Activating System

Plasmid pBR 322 DNA (Boeringer Manheim UK Ltd., Lewis, East Sussex, UK) was supplied in the supercoiled form although some batches contained a small percentage of linearised DNA. The extent of contamination was determined by running a control lane of untreated plasmid on each gel. Assay conditions were based on those described by Walton and co-workers (1991) and were similar to those employed in enzymatic assays (Chapter 3, Section 2.1). Reactions were carried out in a 1 ml Ependorff tube and standard conditions included: pBR 322 supercoiled DNA (1.6 μg), EO9 (100 μM), purified rat Walker DT-diaphorase (2 μg) and bovine serum albumin (0.14%) in 100 mM sodium phosphate buffer pH 7.4 to give a total reaction volume of 100 μl. Initial experiments used Tris-Cl (50 mM; pH 7.5) buffer but as a result of the high background damage this was substituted by sodium phosphate. The reaction was initiated by addition of the cofactor NADH (2 mM) and pulse spinning briefly using a microfuge to bring reaction components together. Incubation was carried out in a warm room at 37°C for 30 minutes although initial experiments had a one hour incubation period. The reaction mixture was agitated at frequent intervals during the experiment to ensure complete aeration. In order to stop the reaction 20 μl aliquots were taken and added to 5 μl of stop buffer (SDS [0.5%], EDTA [5 mM], glycerol [60% v/v] and bromophenol blue [0.001%]). Samples were gently mixed, briefly microfuged and kept on ice before loading on to a 1% agarose gel. Assays were also carried out where concentrations of DT-diaphorase (0.002, 0.02, 0.2, 2, 4, 8 and 12 μg), EO9 (0.1, 1, 10, 100 and 200 μM) and incubation time (0, 5, 10, 20, 30, 45 and 60 minutes) were varied. Control incubations involved omitting individual components or adding either prior boiled enzyme or dicoumarol (100 μM in DMSO). In order to identify the species responsible for the DNA damage observed, superoxide dismutase (300U) and catalase (300U) were included in some assays.

Aerobic Incubations of pBR 322 with Purified NADPH:cytochrome P450 Reductase Activating System

NADPH:cytochrome P450 reductase incubations were carried out under almost identical conditions to those described for DT-diaphorase assays previously in this Section. Standard reaction conditions included EO9 (100 μM), pBR 322 (1 μg), purified rat NADPH cytochrome P450 reductase (0.1 μg), NADPH (2 mM) in 100 mM sodium phosphate buffer pH 7.4 made up to a total reaction volume of 60 μl. The effect of varying the concentrations of NADPH:cytochrome P450 reductase (0, 0.025, 0.05, 0.1, 0.2 and 1 μg), EO9 (1, 50, 100, 200 and 400 μM) and incubation time (0, 5, 10, 20, 30, 45, 60, 90 minutes) was also investigated.

Hypoxic Plasmid Strand Break Assays

Hypoxic incubations were again almost identical to those carried out in air. The apparatus employed to obtain hypoxia consisted of a metal 'pig' attached to a nitrogen cylinder (0 grade with < 5 ppm O_2; British Oxygen Co.) via an oxygen trap (Oxytrap). The latter acted to remove any trace contamination of oxygen. Reactions were carried out in sealed 1 ml glass HPLC vials. Plastic was avoided due to possible absorption of oxygen on to the surface. Syringe needles connected the multiple outlets of the pig with the reaction vials. A second syringe needle perforated the seals of each tube to permit removal of gas. Samples were pregassed with nitrogen for a period of 10 minutes before initiation of the reaction by injection of pregassed NADH. In order to confirm that this period of time was sufficient to remove a significant amount of oxygen comparisons were made with samples gassed for 30 minutes. An optimum time for gassing was required which permitted removal of oxygen without significant evaporation occurring. Reactions were terminated by removing 20 μl aliquots of the reaction mixture using a Hamilton syringe and mixing with 5 μl of stop buffer.

Agarose Gel Electrophoresis

Samples were analysed by agarose gel electrophoresis. The three possible forms of plasmid present supercoiled, open circular and linear have different mobilities when electrophoresed and therefore run as distinct bands on an agarose gel. The latter was prepared by making a 1% (w/v) agarose solution in TAE buffer (Tris base [40 mM], glacial acetic acid [20 mM] and EDTA [1 mM]; pH 8.1). This was placed in a microwave oven and heated for 5 minutes on medium power to ensure solubilisation

of the agarose before pouring into the gel tank with a 15 well comb. On solidification of the gel TAE running buffer was added to the tank and the comb removed. Samples and lambda hind III restriction fragments (Gibco BRL Life Sciences, Paisley, Scotland, UK) were mixed with loading buffer and were loaded on to the gel. Electrophoresis was carried out overnight at 40 volts for 14-16 hours.

The gel was examined by staining with ethidium bromide in TAE (2 μg/ml for 1 hour), rinsing in TAE and viewing under a UV transilluminator. A record of the gel was taken in both positive and negative form using polaroid type 55 film and a polaroid camera fitted above the transilluminator. On removal of the film the negative was washed for 5 minutes in a solution of sodium sulphite and rinsed for a further 5 minutes in distilled water before drying. The density of bands was quantified using a laser densitometer. Due to differential binding capacity of ethidium bromide with supercoiled plasmid compared with open circular and linear forms a standard correction factor was applied. This involved multiplying density values for the supercoiled form by 1.22 (Fisher and Gutierrez, 1991a).

5.2.3 Plasmid Cross-linking Assay

Cutting and Labelling Plasmid

Plasmid pBR 322 DNA (20 μg) (Northumbria Biologicals Limited, Cramlington, Northumbria, UK) was linearised by digesting with 3 μl of the restriction endonuclease Bam H1 (Gibco BRL) and 17 μl of 10x reaction buffer 3 (Gibco BRL) made up to 170 μl in distilled deionised water at 37°C for 1 hour. The reaction was terminated by precipitation of DNA by addition of 1/10th volume of sodium acetate (7 M) and 3 volumes of 95% cold ethanol, mixing thoroughly, chilling for 10 minutes in a dry ice / ethanol bath and centrifugation at 150 g for 10 minutes. The supernatant was carefully removed and the DNA pellet dried by lyophilisation (10-15 minutes, Edwards freeze drier). Following resuspension in TE (Triethanolamine [25 mM] and EDTA [1 mM]) buffer, linearised DNA was then dephosphorylated by incubation at 65°C for 1 hour with bacterial alkaline phosphatase (62.5 Units; Gibco BRL) and 20 μl of 5x bacterial alkaline phosphate buffer (to give final concentrations of: Tris-HCl [50 mM; pH 8.0] and NaCl [600 mM]) made up to a total volume of 100 μl in distilled deionised water. A phenol and chloroform wash was used to extract the dephosphorylated DNA. An equal volume of phenol was added, mixed thoroughly and the sample briefly centrifuged. The aqueous layer then removed and added to a mixture of isoamyl alcohol and chloroform, mixed thoroughly, centrifuged and the

water layer again removed. A second chloroform / isoamyl alcohol wash was carried out. The DNA present in the water layer was precipitated with alcohol, pelleted by centrifugation and the supernatant removed. It was then dried by lyophilisation (5-10 minutes). Finally the DNA was resuspended in distilled deionised water to give a final concentration of 0.5 μg/μl. Linearisation was confirmed by agarose gel electrophoresis (Section 5.2.2).

Having been linearised and dephosphorylated the plasmid DNA was 3' end labelled with [γ-^{32}P] ATP (5000 Ci/m mol, Amersham International, Amersham, Bucks, UK). This involved incubation of 5 μg DNA (prepared as described above), 5x forward buffer (to give a final concentration of : Tris-HCl [70 mM; pH 7.6], $MgCl_2$ [10 mM], DTT [5 mM]) supplied in the labelling kit (20 % v/v), [γ-^{32}P] ATP (10 μCi) and T4 polynucleotide kinase (5U units) in distilled deionised water at 37°C for 30 minutes. Unincorporated ATP was them removed by precipitating the DNA with 1/10 th volume of ammonium acetate and 3 volumes of 95% ethanol (as described above), removing the supernatant and drying by lyophilisation for 5 minutes. DNA was resuspended in 50 μl of distilled, deionised water and was precipitated with 1/10th of the volume of sodium acetate and 3 volumes of ethanol as described above. The dried pellet was resuspended in distilled deionised water to give a final concentration of 100 ng/μl and was maintained frozen at -20°C until required.

Aerobic Incubations of Plasmid DNA with Purified DT-diaphorase Activating System.

Approximately 10 ng of end-labelled DNA was incubated with EO9 (0.01, 0.1, 1, 10 and 100 μM final concentration), purified rat Walker cell DT-diaphorase (0.0175, 0.175, 1.75, 17.5 and 175 ng) and 100 μM NADH in TE buffer. Control experiments involved omission of either drug, cofactor or enzyme as well as a sample of untreated DNA. Reactions were carried out for 2 hours at 37°C before termination by addition of an equal volume of stop solution (sodium acetate [0.6 M], EDTA [20 mM], tRNA [100 μg/ml]). Cold ethanol 95% (3 volumes) was then used to precipitate DNA as described above. The DNA pellets obtained were dried by lyophilisation (approximately 15 minutes) and were resuspended in 10 μl of strand separation buffer (DMSO [30%], EDTA [1 mM], bromophenol blue [0.04%], xylene cylanol [0.04%]). Samples were then denatured at 95°C for 2 minutes and chilled immediately in an ice-water bath before loading on to an agarose gel for electrophoresis. One sample, the undenatured control was resuspended in 10 μl loading buffer (sucrose [6%], bromophenol blue [0.04%]) in place of strand separation buffer and was not heated.

Agarose Gel Electrophoresis

Samples were loaded on to a 0.8 % agarose gel and electrophoresed in TAE buffer at 40V for 16 hours. The gel was removed and dried on to a sheet of 3 MM Whattman filter paper covering a layer of DE81 filter paper at 80°C for 2 hours using a gel drier (Bio-rad model 583). Bands were visualised by autoradiography with a hyperfilm MP (Amersham) film, exposed for approximately 4 hours using an intensifying screen (DuPont-Cronex Lightening-plus intensifying screen).

5.2.4 *Taq* DNA Polymerase Assay

Preparation of DNA

pBR322 DNA was digested with the restriction enzyme Bam HI and dephosphorylated as described in Section 5.2.3.

Drug Treatment of DNA

Bam HI digested pBR322 was incubated for 2 hours at 37°C in the presence of EO9 (0.1, 1, 10 and 100 μM in DMSO at 1% final DMSO concentration), 1 mM NADH and purified rat Walker DT-diaphorase (0.175 μg) in TEA buffer pH 7.2 to give a final volume of 50 μl. Control reactions involved omission of one or more of the reaction constituents. In addition a positive control, chlorambucil (100 μM in DMSO 1% final concentration), was included.

Following drug treatment the reaction was terminated by addition of an equal volume (50 μl) of stop buffer (sodium acetate [0.6 M], EDTA [20 mM], tRNA [100 μg/ml]) and DNA was precipitated with three volumes of ethanol (300 μl) as described in Section 5.2.3. The supernatant was then gently removed using a Pasteur pipette and the remaining pellet vacuum dried (at least 10 minutes, Edwards Freeze drier). Finally the pellet was resuspended in 50 μl of TE buffer.

DNA Amplification

A synthetic oligonucleotide primer with the sequence 5' TATGCGACTCCTGCATTAGG 3' was employed. This was selected as it provides a relatively GC rich fragment of desirable strand length for optimal resolution. The primer was 5'- end labelled by incubation for 1 hour at 37°C with 20% (V/V) 5x

forward buffer (to give a final concentration of : Tris-HCl [70 mM; pH 7.6], $MgCl_2$ [10 mM], DTT [5 mM]), [γ-^{32}P] ATP (10 μCi), 4% V/V T4 Kinase in a total volume of 25 μl of distilled water. This was then passed through spin columns to remove unincorporated label. The drug treated DNA samples were mixed with 2.5 mM dNTP mix, 2.5 mM $MgCl_2$, 0.2% gelatin, labelled primer (5%), 1U *Taq* polymerase (all from Advanced Biotechnologies, Leatherhead, Surrey, UK) in buffer and were amplified using a Perkin Elmer Cetus PCR machine (30 cycles consisting of 1 minute denaturation at 94°C, 2 minutes annealing 60°C, 2 minutes chain elongation at 72°C).

Following linear amplification the samples were transferred to a 1.5 ml Eppendorf tube and the DNA precipitated and dried by addition of 1/10 volume of sodium acetate and 3 volumes of ethanol as described previously (Section 5.2.3). Finally the DNA pellet was resuspended in 3 μl formamide loading dye (98% formamide [98%], EDTA [10 mM; pH 8.0], bromophenol blue [0.025%], xylene cyanol [0.025%]), denatured by heating for 2 minutes at 90°C, cooled in an ice water bath and loaded on to a 6% polyacrylamide gel for electrophoresis.

Polyacrylamide Gel Electrophoresis

The principal employed was similar to that described in Chapter 2 for Western blotting. The Bio-rad sequencing gel kit was assembled and 5 ml of the 6% polyacrylamide gel was poured in to the base of the casting tray to seal the apparatus. A 6% gel was then poured and left to set before addition of TBE buffer (Tris base, [90 mM], boric acid [90 mM] and EDTA pH 8.3 [20 mM]) and prewarming at 55°C for 3 hours. Following loading of samples electrophoresis was carried out at 3000V for 3 hours. The gel was dried and an autoradiogram developed as described for agarose gels in Section 5.2.3.

5.3 Results

5.3.1 Alkaline Elution

DNA Damage in Walker Cells

With the purpose of obtaining optimal conditions for the detection of cross-links, a variety of drug concentrations were investigated. Initial experiments utilised 80 nM and 400 nM EO9 applied to cells for a period of 2 hours with elution being carried out

either immediately or after a 24 hour recovery period. Under these particular conditions a significant amount of DNA strand breaks were indicated by the more rapid elution of treated samples as compared to the control. No DNA cross-links were clearly evident although the biphasic appearance of the curve suggested that they may be present but masked by DNA strand breaks. When applied to Walker tumour cells the positive control CB 1954 showed a high DNA interstrand cross-link frequency of approximately 3.18 cross-links / 10^9 daltons of DNA.

In order to optimise conditions for detection of cross-links with EO9, varying exposure regimes were adopted. Cells subjected to continuous exposure of 10 nM EO9 were examined at various incubation times (2-12 hours). DNA cross-links which increased in extent over time were detected in these experiments but their frequency was still fairly low, with a maximum of 0.18 cross-links / 10^9 daltons DNA present at 18 hours drug incubation when compared with that of CB 1954 which gave a cross-link frequency of 2.88 cross-links / 10^9 daltons of DNA. A further change was therefore made where 20 nM EO9 was applied continuously over 2-20 hours. Unirradiated cells were also employed to permit determination of DNA strand break frequency and to correct for this occurrence when calculating DNA cross-link frequency. For the shorter exposure times DNA strand breaks were significant with no cross-links being apparent (Fig 5.3). After 20 hours treatment a small quantity of DNA cross-links were detectable but the amount of DNA strand breaks were less than observed with the short exposure times (Fig 5.3). In a final attempt to detect DNA cross-links either 4 nM or 20 nM EO9 was added to cells on four occasions at four hour intervals. This resulted in an enhancement in the DNA cross-link frequency detected (Fig 5.4). With repeat doses of 20 nM EO9 the cross-link frequency, following correction for DNA strand breaks, was 1.14 links / 10^9 daltons of DNA and for 4 nM EO9 the frequency was 0.658 cross-links / 10^9 daltons of DNA for samples eluted 12 hours after initial addition of drug. Repeat assays carried out within the same day were found to be highly reproducible.

DNA Damage in HT29 Cells

In attempt to optimise the drug exposure conditions for alkaline elution of HT29 cells a variety of drug concentrations were investigated. Initial experiments used 3 repeat doses of 20 nM EO9 at four hourly intervals followed by a further incubation period before harvesting cells 24 hours after the addition of the first aliquot of EO9. This resulted in a DNA cross-link frequency, when corrected for DNA strand breaks, of 0.45 links / 10^9 daltons of DNA. EO9 concentration was then increased to 100 nM

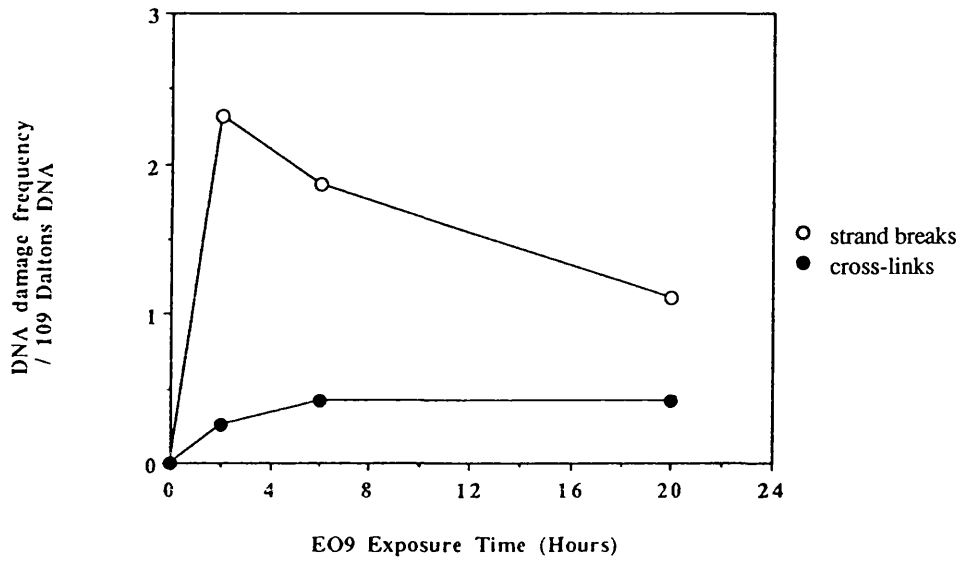


Fig. 5.3 Formation of DNA strand breaks and DNA interstrand cross-links in rat Walker tumour cells over time following continuous incubation with 0.02 μM EO9. Data are taken from a typical experiment.

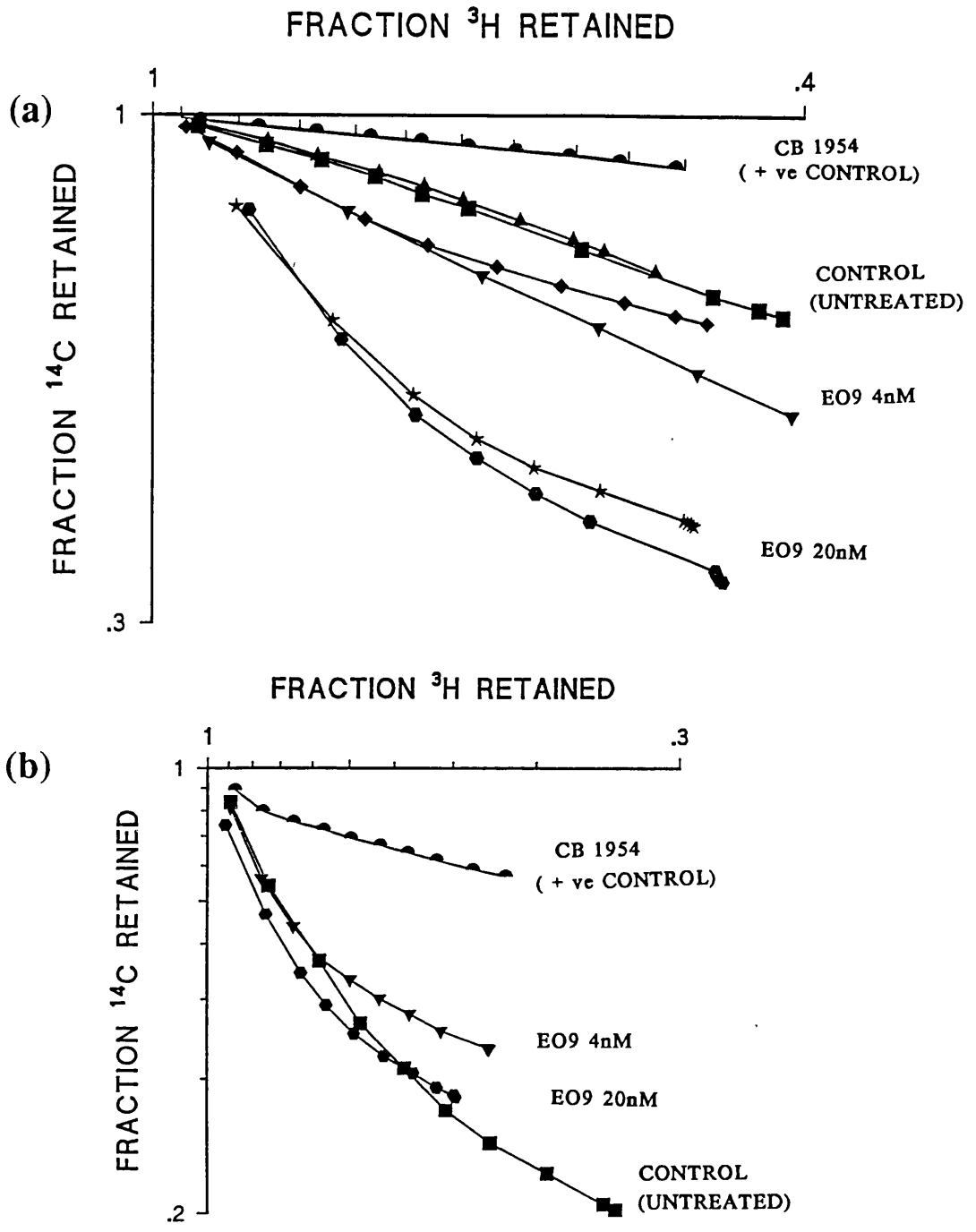


Fig 5.4 Alkaline elution profiles for rat Walker tumour cells exposed to four repeat doses of 4 or 20 nm EO9 given at four hourly intervals under aerobic conditions. (a) shows data obtained using unirradiated cells and (b) results seen with irradiated cells. CB 1954 was also included as a positive control due to its known ability to induce DNA interstrand cross-links in this system. Data were taken from an individual experiment and were typical of the trend seen in other similar experiments during optimisation of the protocol.

given as a single dose over a 20 hour continuous exposure time. This resulted in a slight further increase in DNA interstrand cross-link frequency (data not shown). Finally conditions were optimised to allow DNA cross-links to be visualised following only a 2 hour drug exposure which was necessary to permit comparison of aerobic and hypoxic conditions. In these experiments HT29 cells were exposed, under aerobic conditions to 0.25 μM EO9, a concentration shown to be around the IC_{50} value for HT29 cells by clonogenic assay (Plumb and Workman, 1994). Results presented in Fig 5.5 a show that DNA damage was evident as detected by alkaline filter elution. The unirradiated, treated sample eluted more rapidly than the respective control, as indicated by a steeper elution curve. This is characteristic of the presence of DNA strand breaks. Calculations show that the average DNA strand break frequency taken from a number of experiments is 4.62 DNA strand breaks / 10^9 daltons DNA. A possibility of DNA cross-link formation was also indicated from the elution profile of irradiated samples. However, on calculation of the DNA cross-link frequencies it became evident that none were present. Although no DNA interstrand cross-links were evident from initial calculations some of these lesions were detected when correcting for the DNA strand breaks thus indicating that the extensive DNA strand breaks masked cross-link detection by increasing the flow of linked DNA fragments through the filter. Correcting for this effect revealed a small incidence of cross-links 0.59 lesions / 10^9 daltons DNA.

The amount of EO9 to which HT29 cells were exposed was increased to 10 μM. This concentration was previously found to be around the IC_{50} value for EO9 with BE cells following a three hour exposure under aerobic conditions (Plumb and Workman, 1994). In contrast to results obtained with BE cells (see below) this resulted in little further increase in DNA damage above that induced by 0.25 μM under aerobic conditions (data are not shown).

Finally, experiments were carried out to compare the DNA damage under aerobic and hypoxic conditions. Results (Fig 5.5 a and b) showed a similar degree of DNA strand breaks were induced under hypoxic conditions as in the presence of air. However, in certain experiments a slight increase in DNA interstrand cross-link frequency was observed under hypoxia.

BE Cell DNA Damage

In contrast to HT29 cells, BE cells treated for 2 hours with a concentration of 0.25 μM EO9 under aerobic conditions showed little or no evidence of any DNA damage

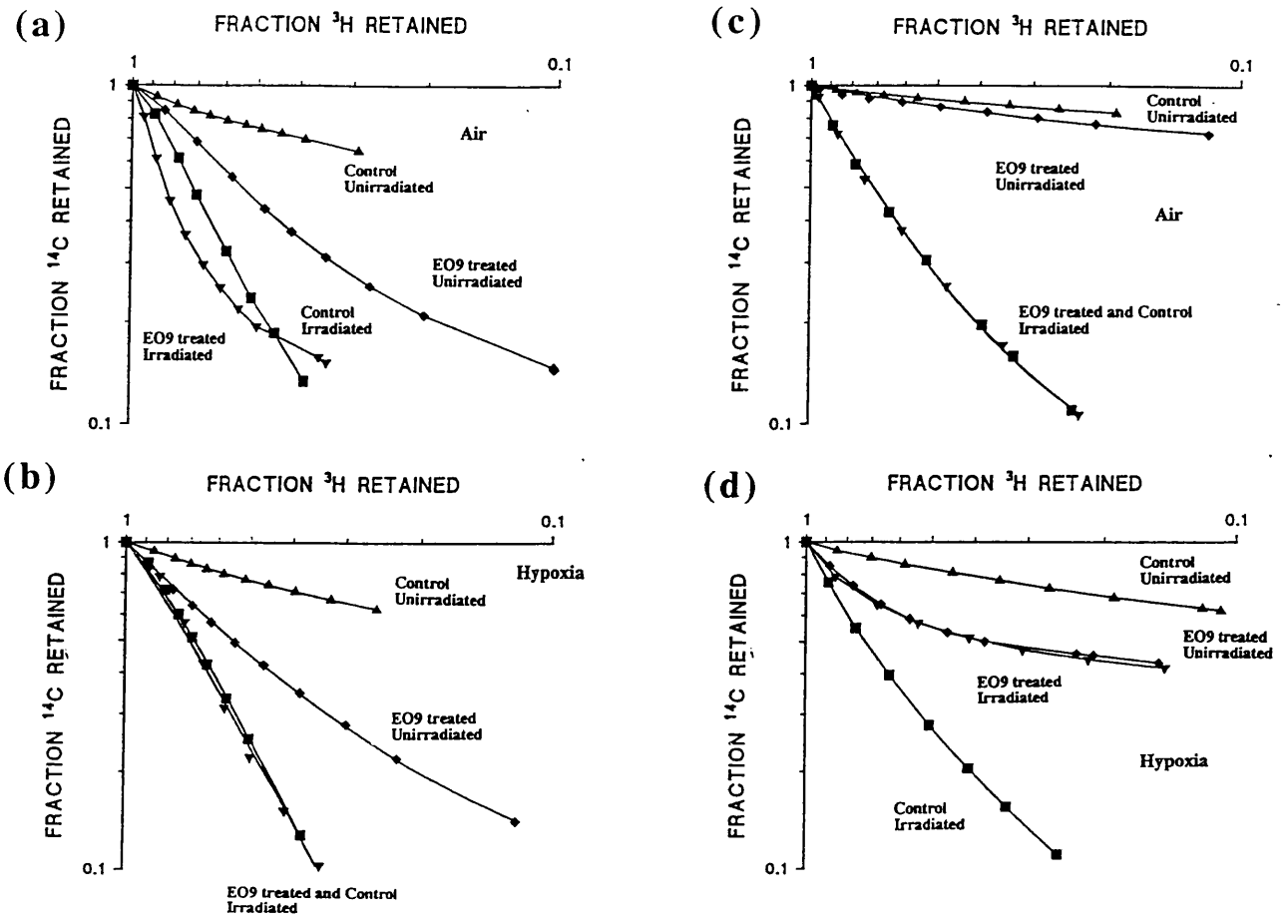


Fig. 5.5 Alkaline elution profiles for human (a and b) HT29 and (c and d) BE cells exposed to 0.25 μM EO9 for a period of 2 hours under (a and c) aerobic or (b and d) hypoxic conditions. Data were taken from an individual experiment although similar trends were seen in repeat experiments carried out under identical conditions.

(Fig 5.5 c). This was indicated in the elution profiles where curves of both irradiated and unirradiated treated samples were almost coincident with those of their respective controls. The frequency of DNA damage was low at 0.147 cross-links / 10^9 daltons of DNA and 0.247 strand breaks / 10^9 daltons of DNA respectively.

Where EO9 concentration was increased to 10 μM, a concentration previously shown to be toxic over a 3 hour exposure under aerobic conditions (Plumb and Workman, 1994), DNA damage was evident (elution profiles not shown). A significant quantity of DNA strand breaks were induced with a frequency of 6.42 strand breaks / 10^9 daltons of DNA. DNA cross-link induction was however still low at 0.37 lesions / 10^9 daltons of DNA

In contrast to results seen with HT29 cells, hypoxia dramatically altered the DNA damage induced by EO9 in BE cells. in terms of both DNA interstrand cross-links and DNA strand breaks. The difference between aerobic and hypoxic DNA damage was greater at the lower concentration of 0.25 μM EO9. Fig 5.5 d illustrates the elution profile for 0.25 μM EO9 exposure to BE cells under hypoxic conditions. The curve for the irradiated treated cell sample now lies well above that of the control, indicating a retarded rate of elution, characteristic of DNA interstrand cross-links. A larger proportion of DNA strand breaks were also apparent as shown by a faster rate of elution of the unirradiated sample as compared to the control. Calculation of the frequencies revealed a strand break frequency of 1.98 lesions / 10^9 daltons DNA and DNA interstrand cross-link frequency of 3.36 lesions / 10^9 daltons DNA.

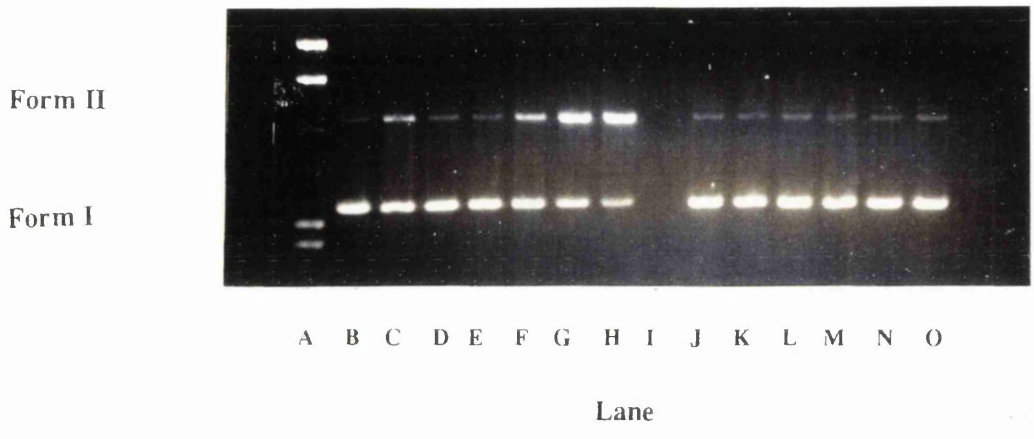
5.3.2 Plasmid Strand Break Assay

Following aerobic incubation of plasmid DNA with EO9, DT-diaphorase and NADH cofactor, DNA strand breaks were detected by agarose gel electrophoresis (Figs 5.6 5.7 and 5.8). Plasmid DNA in the supercoiled form is converted to open circular and finally linear DNA following single or double strand break induction respectively. As a result of their differing conformation these migrate unequally in the gel and are therefore resolvable. The extent of DNA strand breaks was found to increase with increasing drug (Fig 5.6) or enzyme concentration (Fig 5.7) as well as with increasing incubation time (Fig 5.8) up to 90 minutes. Control incubations where either drug, enzyme or cofactor were omitted showed little or no evidence of DNA damage (Figs 5.6, 5.7 and 5.8).

Fig 5.6 The effect of altering drug concentration on EO9 induced plasmid strand break frequency following activation by DT-diaphorase in the presence of cofactor. Standard reaction conditions included pBR 322 (1.0 μg), DT-diaphorase (2 μg) and NADH (2 mM) with varying concentrations of EO9 (0 - 200 μM).

Upper Panel
Agarose gel. Lanes: (A) λ hind III markers; (B) plasmid control; (C) no EO9 control; (D-H) complete system with varying EO9 concentrations (0.1, 1, 10, 100, 200 μM); (I) blank; (J-N) controls where enzyme was omitted for increasing drug concentration (0.1, 1, 10, 100, 200 μM) and (O) no NADH control.

Lower Panel
Quantification of DNA damage by densitometry. Data are from lanes C-H in the upper panel.


Form II
Form I
A B C D E F G H I J K L M N O
Lane

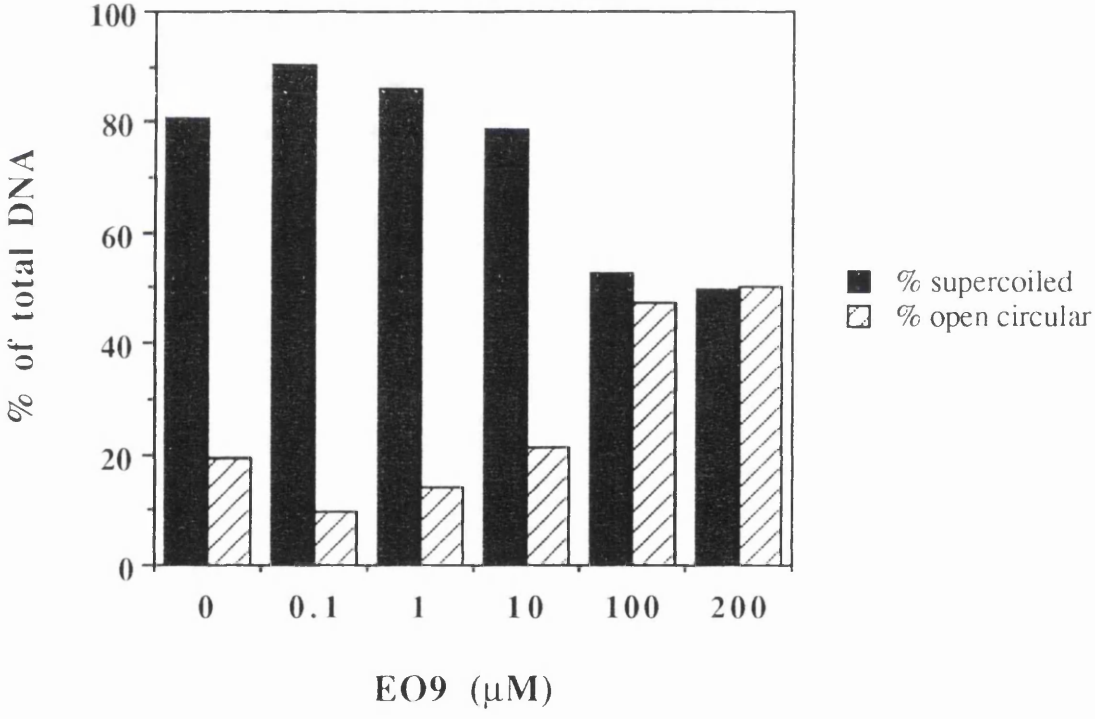
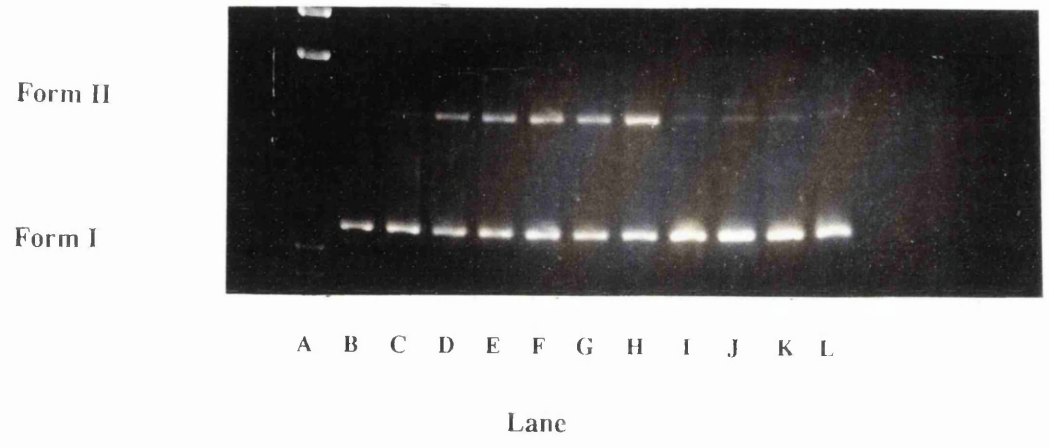

100
80
60
40
20
0
% of total DNA
0 0.1 1 10 100 200
EO9 (μM)
% supercoiled
% open circular

Fig 5.7 The effect of altering enzyme concentration on EO9 induced plasmid strand break frequency following activation by DT-diaphorase in the presence of cofactor. Standard reaction conditions included pBR 322 (1.0 μg), EO9 (100 μM) NADH (2 mM) with varying concentrations of DT-diaphorase (0-8 μg).

Upper Panel
Agarose gel. Lanes: (A) λ hind III markers; (B) plasmid control; (C) no enzyme control; (D-H) complete system with varying enzyme concentrations (0.02, 0.2, 2, 4, 8 μg); (I-M) controls where drug was substituted for vehicle with increasing enzyme concentration (0. 0.02, 0.2, 2, 4 μg).

Lower Panel
Quantification of DNA damage by densitometry. Data are from lanes C-H in the upper panel.


Form II
Form I
A B C D E F G H I J K L
Lane

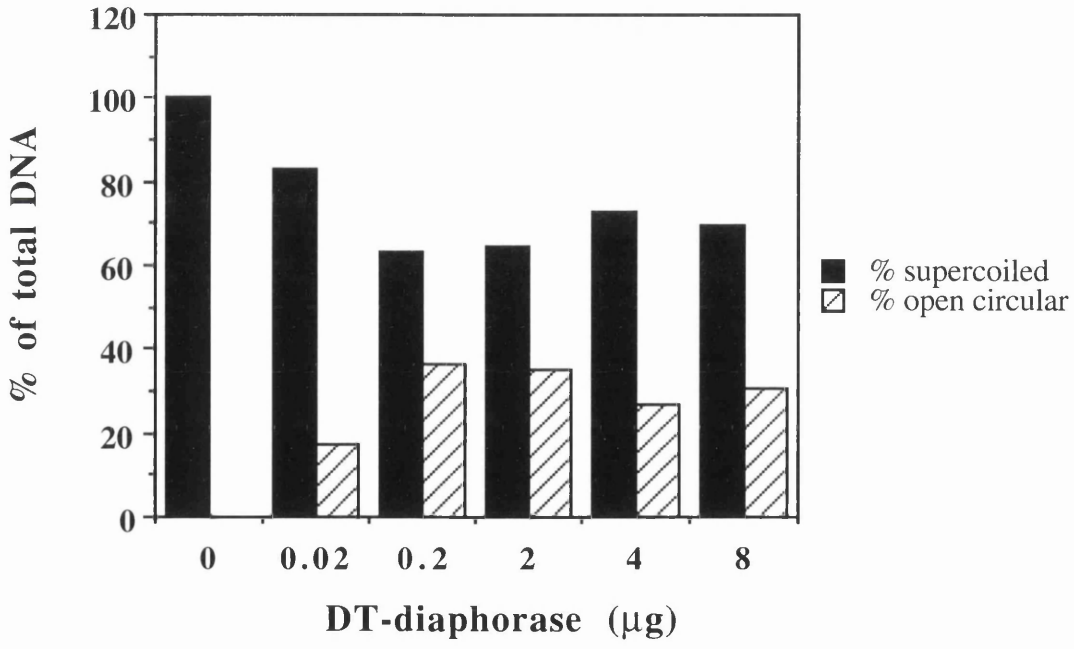

120
100
80
60
40
20
0
% of total DNA
0 0.02 0.2 2 4 8
DT-diaphorase (μg)
% supercoiled
% open circular

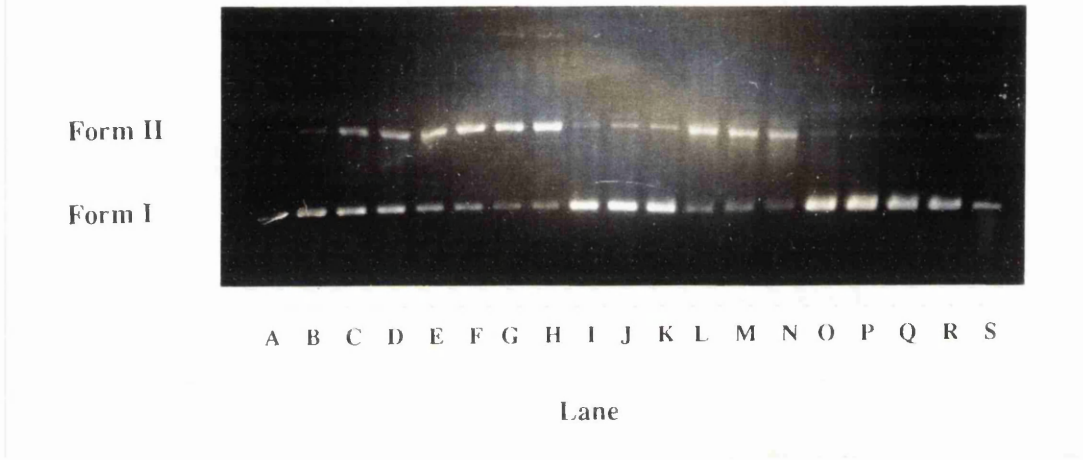
Fig 5.8 The effect of altering incubation time and enzyme concentration on EO9 induced plasmid strand break frequency following activation by DT-diaphorase. Standard reaction conditions included pBR 322 (1.0 μg), EO9 (100 μM), NADH (2 mM) and DT-diaphorase (2 μg).

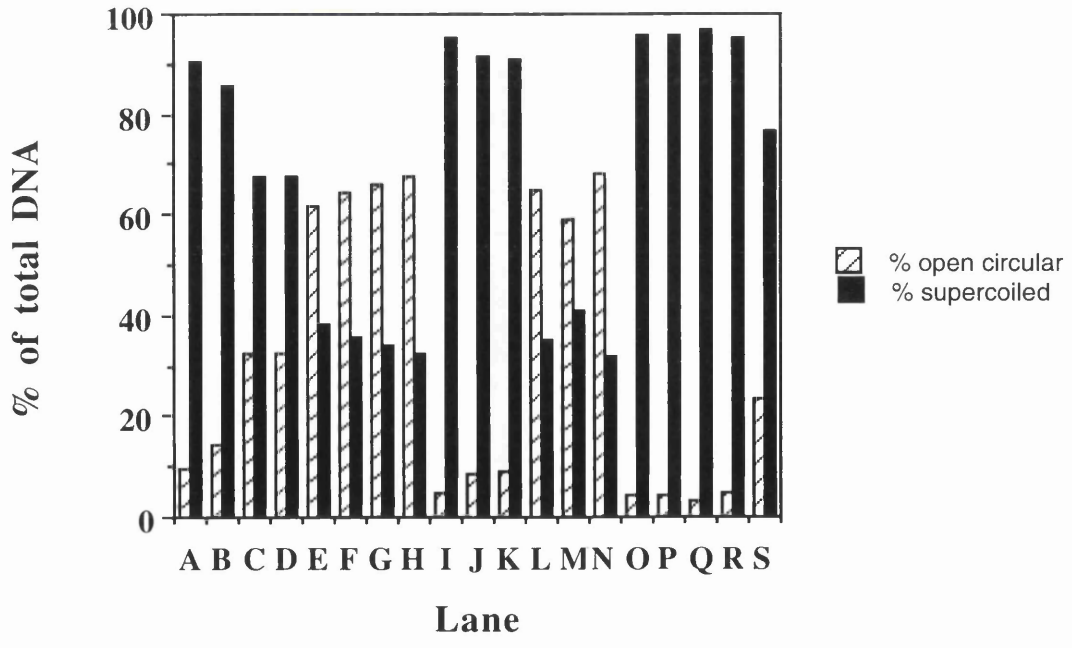
Upper Panel

Agarose gel. Lanes: (A) plasmid control; (B-H) complete system with varying incubation time (0, 5, 10, 20, 30, 45 and 60 minutes); (I) no enzyme control; (J-N) varying enzyme concentration (0.002, 0.02, 0.2, 2, 12 μg); (O and P) no NADH control for complete system with 2 μg and 12 μg DT-diaphorase; (Q and R) no drug control for 2 μg and 12 μg DT-diaphorase and (S) complete system plus 100 μM dicoumarol.

Lower Panel

Quantification of DNA damage by densitometry. Data are from lanes C-I in the upper panel.


Form II
Form I
A B C D E F G H I J K L M N O P Q R S
Lane


% of total DNA
100
80
60
40
20
0
A B C D E F G H I J K L MN O P Q R S
Lane
% open circular
% supercoiled

Inclusion of superoxide dismutase in the reaction mixture resulted in approximately 30-40% decrease in DNA strandbreak formation (Fig 5.9). However, where either catalase was added alone or in combination with superoxide dismutase, DNA strand breaks were completely abolished (Fig 5.9).

When DT-diaphorase was replaced with NAD(P)H: cytochrome P450 reductase similar results were obtained. An increase in the quantity of single DNA strand breaks occurred with increasing drug (Fig 5.10) and enzyme concentration (Fig 5.11) as well as with incubation time (Fig 5.12).

Experiments were also carried out to investigate DNA strand break formation under hypoxic conditions. Results (data not shown) showed that damage was caused to DNA when it was incubated with drug and cofactor in the presence of DT-diaphorase. Surprisingly this was inhibitable by superoxide dismutase and catalase suggesting that damage resulted from oxygen radicals. Some degree of protection against DNA strand break formation was also detected with SR 4233 using the same system. These data would therefore suggest that conditions were not sufficiently anoxic and contained a low percentage of oxygen or alternatively that oxygen could cause DNA damage following termination of the reaction during loading of the gel.

5.3.3 Plasmid Cross-linking Assay

Following incubation of EO9 with DT-diaphorase in the presence of cofactor (NADH) cross-link formation in plasmid pBR 322 DNA was detected using the plasmid cross-link assay (Fig 5.13). This was indicated by conversion of single stranded denatured DNA to the double stranded form. The two bands present in the denatured control correspond to the two single strands of DNA which differ in electrophoretic mobility. Cross-link formation increased with increasing drug concentration from 0-1 μM but then decreased again from 1-100 μM. The extent of DNA-cross-links also increased with increasing enzyme concentration and was not evident in controls where drug, enzyme or cofactor were omitted. Interestingly, EO9 induced a greater extent of cross-links than equivalent concentrations of the established bifunctional alkylating agent chlorambucil.

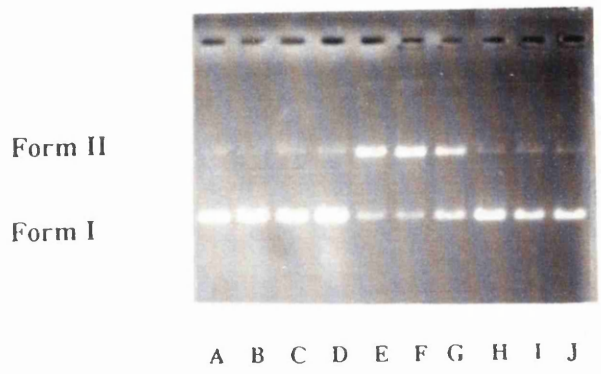
5.3.4 *Taq* Polymerase Assay

The results obtained for the analysis of the sequence selectivity of EO9 are presented in Fig 5.14. In the untreated DNA control the majority of the DNA has undergone

Fig 5.9 The effect of superoxide dismutase and catalase on EO9 induced plasmid strand break frequency following activation by DT-diaphorase in the presence of cofactor, NADH. Standard reaction conditions included pBR 322 (1.0 μg), EO9 (100 μM), NADH (2 mM) and DT-diaphorase (2 μg).

Upper Panel
Agarose gel. Lanes: (A) plasmid control; (B) minus NADH control; (C) no enzyme control; (D) no drug control; (E) complete system; (F) complete system; (G) complete system plus superoxide dismutase (300 U); (H) complete system plus catalase; (I) complete system plus superoxide dismutase (300 U) and catalase (300 U) and (J) complete system minus drug plus superoxide dismutase (300 U) and catalase (300 U).

Lower Panel
Quantification of DNA damage by densitometry. Data are from upper panel.


Form II
Form I
A B C D E F G H I J
Lane

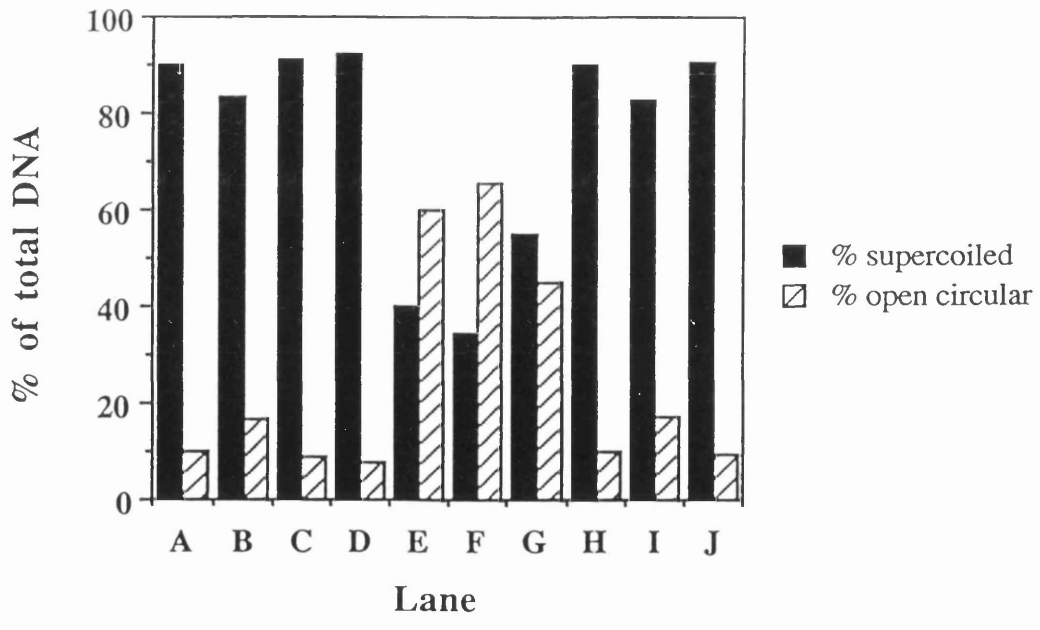

100
80
60
40
20
0
% of total DNA
A B C D E F G H I J
Lane
% supercoiled
% open circular

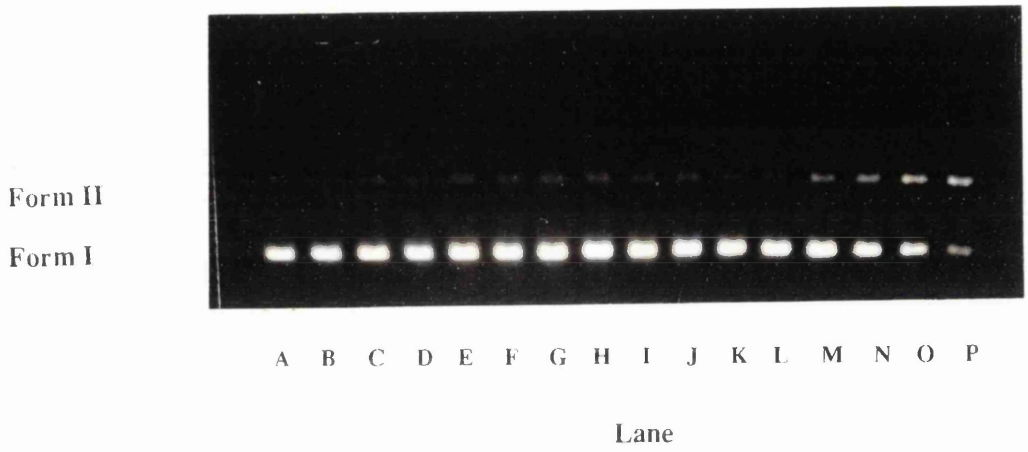
Fig 5.10 The effect of altering drug concentration on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase in the presence of cofactor. Standard reaction conditions included pBR 322 (1.0 μg), NADPH: cytochrome P450 reductase (0.1 μg) and NADPH (2 mM) with varying concentrations of EO9 (0-400 μM).

Upper Panel

Agarose gel. Lanes: (A) plasmid control; (B) plasmid and NADPH: cytochrome P450 reductase (0.1 μg); (C) plasmid plus NADPH; (D) no drug control; (E) control where drug was substituted by vehicle; (F) vehicle control minus NADPH; (G-K) no NADPH control for 1, 50, 100, 200 and 400 μM EO9 concentrations; (L-P) increasing EO9 concentration 1, 50, 100, 200 and 400 μM.

Lower Panel

Quantification of DNA damage by densitometry. Data are from lanes E and L-P in the upper panel.


Form II
Form I
A B C D E F G H I J K L M N O P
Lane

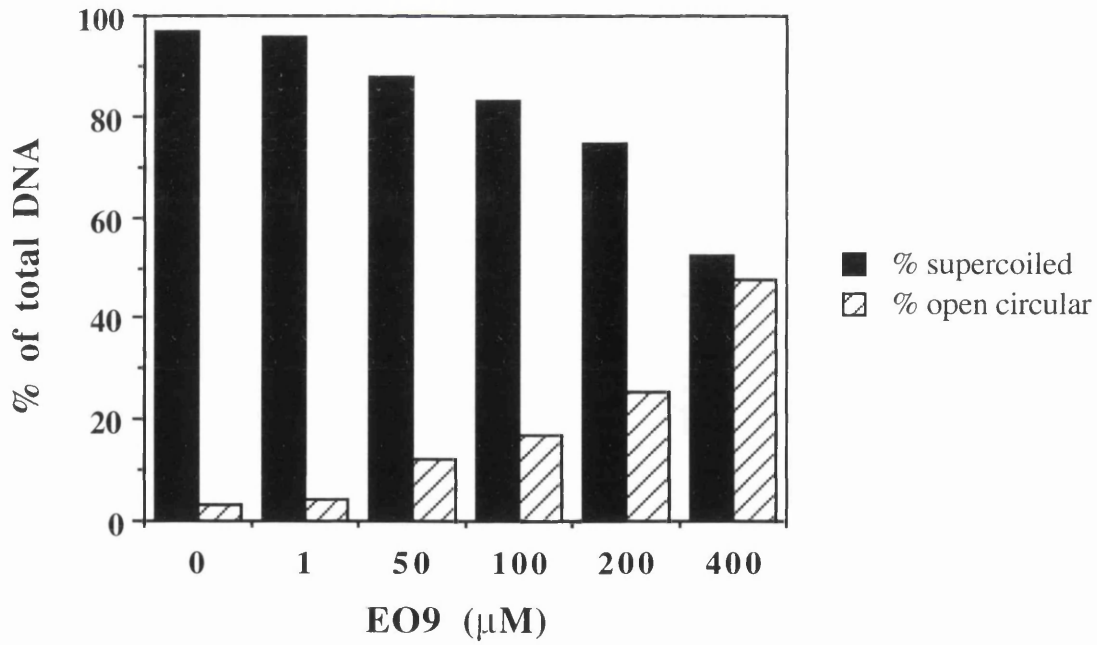

100
80
60
40
20
0
% of total DNA
0 1 50 100 200 400
EO9 (μM)
% supercoiled
% open circular

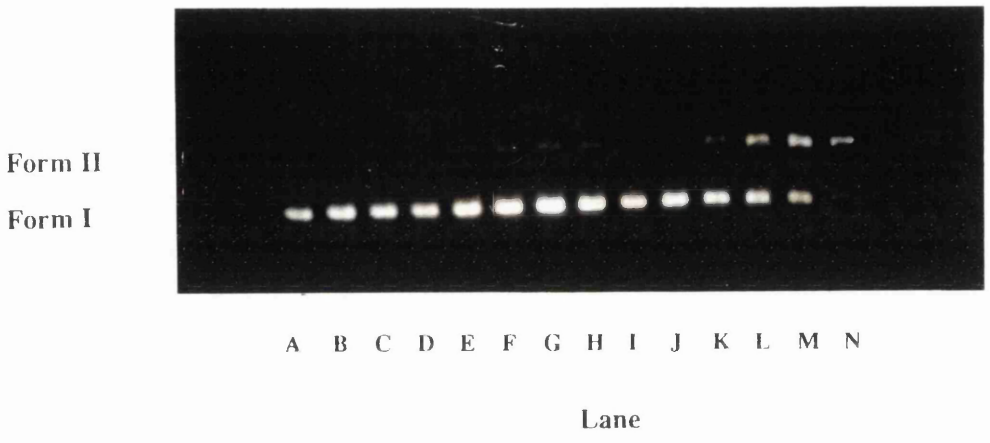
Fig 5.11 The effect of altering enzyme concentration on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase in the presence of cofactor. Standard reaction conditions included pBR 322 (1.0 μg), EO9 (100 μM), NADPH (2 mM) with varying concentrations of NADPH: cytochrome P450 reductase (0-1 μg).

Upper Panel

Agarose gel. Lanes: (A) plasmid control; (B) vehicle control; (C-H) no NADPH control for varying enzyme concentration (0, 0.025, 0.5, 0.1, 0.2, 1 μg); (I-N) increasing enzyme concentration (0, 0.025, 0.5, 0.1, 0.2, 1 μg).

Lower Panel

Quantification of DNA damage by densitometry. Data are from lanes I-N in the upper panel.


Form II
Form I
A B C D E F G H I J K L M N
Lane

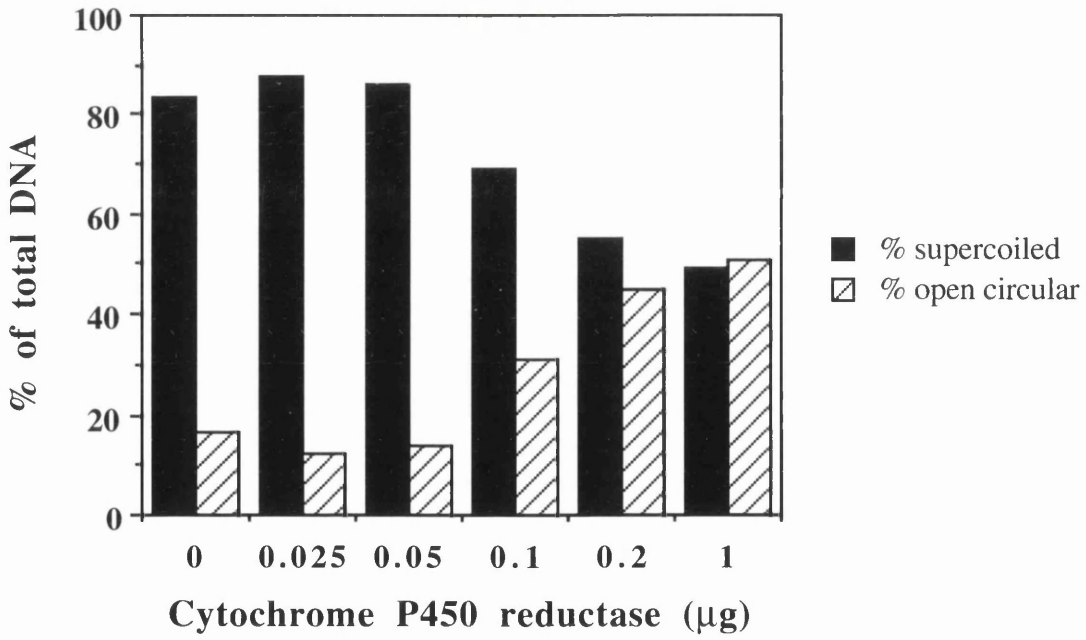

100
80
60
40
20
0
% of total DNA
0 0.025 0.05 0.1 0.2 1
Cytochrome P450 reductase (µg)
% supercoiled
% open circular

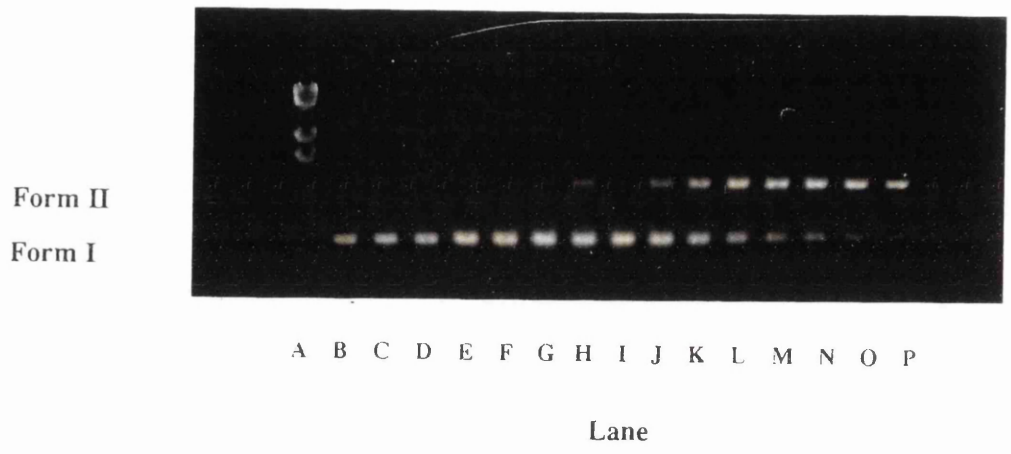
Fig 5.12 The effect of altering incubation time on EO9 induced plasmid strand break frequency following activation by NADPH: cytochrome P450 reductase. Standard reaction conditions included pBR 322 (1.0 μg), EO9 (100 μM), NADPH (2 mM) and NADPH: cytochrome P450 reductase (0.1 μg).

Upper Panel

Agarose gel. Lanes: (A) λ hind III markers; (B) plasmid alone; (C) plasmid and EO9; (D) plasmid and NADPH: cytochrome P450 reductase; (E) plasmid and NADPH; (F) no NADPH control; (G) no enzyme control; (H) no drug control; (I-P) increasing incubation time (0, 5, 10, 20, 30, 45, 60, 90 minutes).

Lower Panel

Quantification of DNA damage by densitometry. Data are from lanes I-P in the upper panel.


Form II
Form I
A B C D E F G H I J K L M N O P
Lane

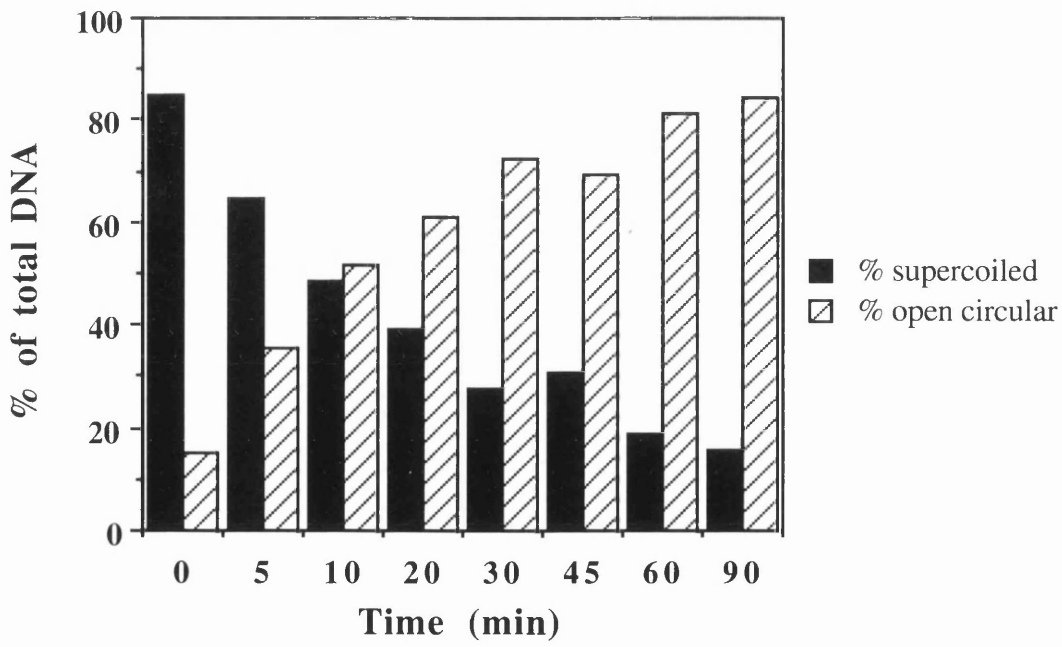
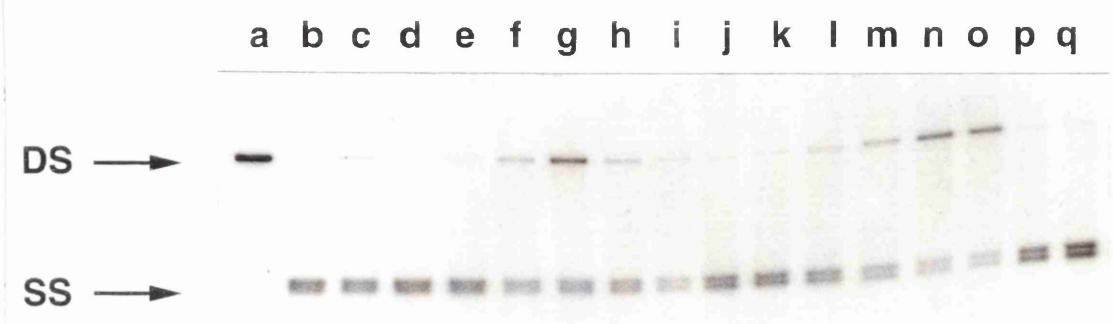

100
80
60
40
20
0
% of total DNA
0 5 10 20 30 45 60 90
Time (min)
% supercoiled
% open circular

Fig 5.13 The effect of altering enzyme and drug concentration on EO9 induced DNA interstrand cross-link frequency following activation by DT-diaphorase in the presence of cofactor, NADH. Standard reaction conditons included pBR 322 (10 ng), EO9 (1 μM), NADH (100 μM) and DT-diaphorase (0.175 μg).

Data are taken from an individual experiment. Initial results were obtained during the course of this thesis. Due to time constraints a repeat assay was carried out by M. Wyatt (UCH, London, UK) using identical conditions, as part of a collaborative project. The latter experiment gave very similar results but a clearer photograph of the gel was obtained. For this reason this photograph is shown here.

Upper Panel
Agarose gel. Lanes: (A) plasmid - undenatured double stranded control; (B) plasmid denatured, untreated control; (C) no NADH control; (D) no drug control; (E-I) increasing EO9 concentration (0.01, 0.1, 1, 10 and 100 μM); (J) no enzyme control; (K-O) increasing DT-diaphorase concentration (0.175, 1.75, 17.5, 175 ng); (P) 1 μM EO9 in the absence of enzyme and cofactor; (Q) 10 μM EO9 in the absence of enzyme and cofactor.

Lower Panel
Quantification of DNA damage by densitometry. Data are from upper panel.


a b c d e f g h i j k l m n o p q
DS
SS

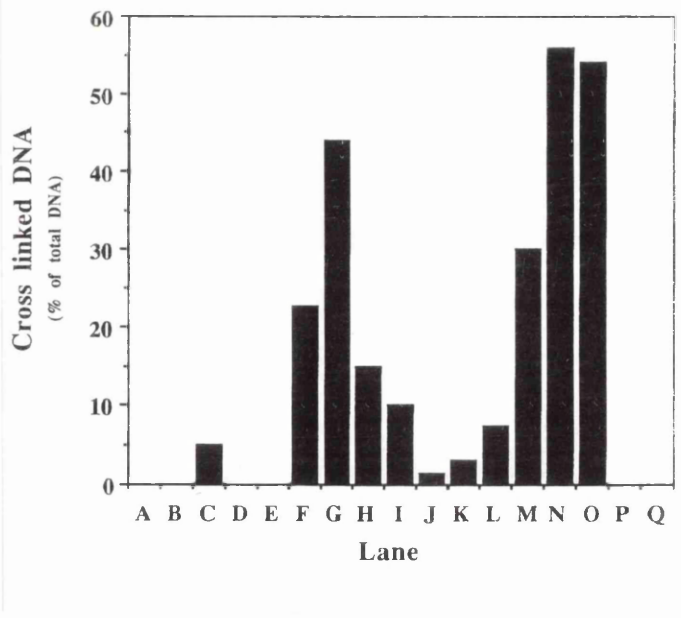

Cross linked DNA
(% of total DNA)
60
50
40
30
20
10
0
A B C D E F G H I J K L M N O P Q
Lane

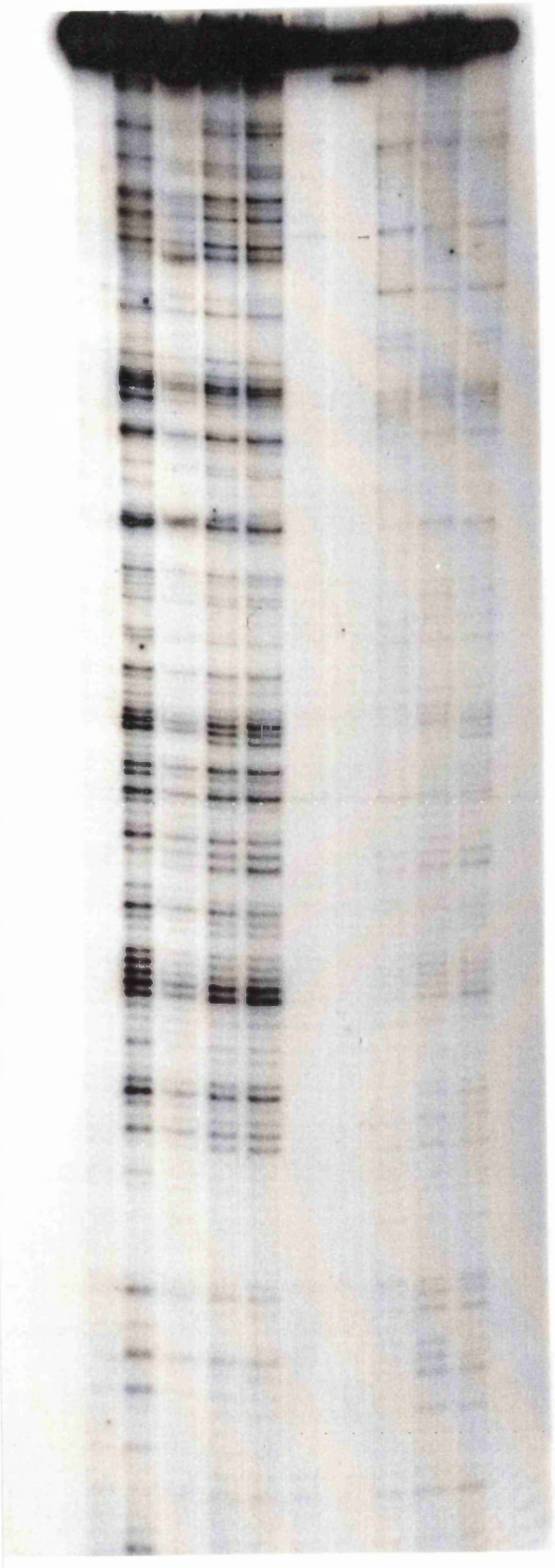
Fig 5.14 (a) Autoradiogram of a polyacrylamide gel showing the sequence selectivity of EO9 adduct formation with DNA determined using the *Taq* polymerase stop assay. Standard reaction conditions included EO9 (100 μM), NADH (1 mM), DT-diaphorase (0.175 μg) and pBR 322 DNA (10 ng). Lanes: (A) untreated DNA control; (B) chlorambucil treated DNA; (C-E) controls in which (C) NADH or (D) enzyme were omitted or (E) where drug was incubated with DNA alone. Finally lanes (F-I) show the effect of increasing drug concentration (0, 0.1, 1, 10 and 100 μM) on DNA adduct formation. The arrows indicate a selection of the bases where the alkylation pattern has been altered with some specific pBR 322 base numbers given for reference.

(b) Differences in sequence selectivity of EO9 compared with chlorambucil and of unactivated EO9 compared with enzymatically activated drug. Data are taken from the autoradiogram in (a) of this figure. The bases where alkylation has occurred are underlined and sequences are given in a 5' to 3' order.

(c) Summary of results displayed in (b) of this figure showing the frequency of sequences which had been alkylated more than once. As in (b), underlined bases represent those which have undergone alkylation.

(d) Part of the pBR 322 gene sequence used in the *taq* polymerase assay.

(a)



(b)

Alteration in sequence selectivity of unactivated EO9 compared with chlorambucil

Highly enhanced or new sequences with unactivated EO9		Sequences where alkylation has been lost with unactivated EO9	
base number	Sequence	base number	Sequence
497	GCGCT	502	TGTTT
500	CTTGT	508	GGCGT
509	GCGTG	510	CGTGG
515	GTATG	519	GGTGG
527	GCCCT	523	GCAGG
544	TGTTG	524	CAGGC
551	CGCCA	529	CCGTG
562	TGCAT	563	GCATG
564	CATGC	567	GCACC
566	TGCAA	570	CCATT
568	CACCA	575	CCTTG
		576	CTTGC

Alteration in sequence selectivity of EO9 following activation

Highly enhanced or new sequences with activated EO9		Sequences where alkylation has been lost with activated EO9	
base number	Sequence	base number	Sequence
491	TCATG	494	TGAGC
505	TTCGG	507	CGGCG
560	CTTGC	514	GGTAT
		518	TGGTG
		525	AGGCC
		531	GTGGC
		550	CTTGT

(c)

Alteration in sequence selectivity of unactivated EO9 compared with chlorambucil

	sequence	frequency
highly enhanced or new sequences	GCG	2
	GCA	2
	CCA	2
sequences where alkylation has been lost	GTGG	2
	GCA	3
	CAT	2

Alteration in sequence selectivity of EO9 following activation

	sequence	frequency
highly enhanced or new sequences	TGC	2
sequences where alkylation has been lost	GGC	2

(d)

371 410
CTGTGGATCCTCTACGCCGGACGCATCGTGGCCGGCATCA

411 450
CCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGA

451 490
CATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTC

491 530
ATGAGCGCTTGTTTTCGGCGTGGGTATGGTGGCAGGCCCGT

531 570
GGCCGGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCA

571 610
TTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTAC

611 650
TGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCG

complete chain elongation and thus a strong band corresponding to a full length fragment of 263 base pairs was visible at the top of the gel with additional feint bands corresponding to a few shorter length fragments arising from random polymerase detachment (Fig 14 (a), Lane A). Chlorambucil, the positive control, exhibited a number of distinct bands along the length of the gel (Fig 14 (a), Lane B) indicating the occurrence of covalent DNA lesions resulting in the synthesis of shortened fragments of DNA. In general, chlorambucil bands correspond to lesions at guanine residues, particularly in runs of consecutive guanines. Where EO9 alone was incubated with the plasmid, DNA modification was again observed (Fig 14 (a), Lane E). Some of the lesions observed for EO9 were co-incident with those of chlorambucil, with EO9 also showing a preference for guanines although fewer were modified. However, the banding pattern was in general, different from that of chlorambucil with EO9 binding more specifically. In particular, a number of bases modified by chlorambucil either were not alkylated or were alkylated to a lesser degree by unactivated EO9 e.g. adenines in the sequence GCA. EO9 did however alkylate some bases more strongly than chlorambucil such as the adenine in the sequence GTATC (base pair number 515 in pBR 322 DNA), two cytosines in the sequence GCA and three cytosines in the sequence pu pu C pu (where pu denotes purine). Where cofactor was included with drug (Fig 14 (a), Lane D), but still in the absence of enzyme an almost identical pattern was observed to that of drug alone. For incubations containing enzyme, either in the presence (Fig 14 (a), Lanes G, H, I and J) or absence of cofactor (Fig 14 (a), Lane C), a reduction in band intensity was observed indicative of fewer lesions. When EO9 was omitted (Fig 14 (a), Lane F) or was at low concentration (Fig 14 (a), Lane G) no damage above the control (Lane A) was observed. Interestingly, where EO9 had been incubated with a complete activating system consisting of cofactor, NADH and DT-diaphorase (Fig 14 (a), Lanes G, H, I and J) a different banding pattern was observed to that of drug alone (Fig 14 (a), Lane E). As can be seen from Fig 5.14 (a) there were distinct areas of difference with binding being more specific following activation. Some bases were modified with activated EO9 that were not alkylated by either chlorambucil or unactivated drug (Fig 14, (a), (b) and (c)). These included principally, four changes in cytosines, two of which were in the sequence TGC (positions 562 and 566) and one adenine in the sequence CAT (position 491). Further distinct differences were visible however, unfortunately these were towards the top of the gel which was less clearly resolvable and therefore difficult to determine the base number.

5.4 Discussion

The chemistry of EO9 suggests that upon reduction it could generate potentially damaging radicals. These may exert a range of cellular effects including the induction of DNA strand breaks. In addition, the presence of the aziridine moiety and hydroxyl leaving groups confer the potential to act as a bifunctional alkylating agent. Thus it was expected that the cytotoxic mechanism of EO9 may involve DNA damage as has been suggested for a number of other related agents such as AZQ (Siegel *et al*, 1990a) and mitomycin C (for review see Powis, 1987). Experiments described in this chapter were therefore designed to investigate whether cytotoxic concentrations of EO9 caused DNA damage. The involvement of individual enzymes in activation of EO9 to a DNA damaging species was also examined using cell free systems.

Alkaline elution experiments investigated DNA strand break and interstrand cross-link induction in DNA following exposure of intact tumour cells to EO9. Initial experiments examined DNA damage in rat Walker tumour cells. This cell line was selected for a two reasons: a) the differential toxicity to EO9 between sensitive and resistant cells seen in cytotoxicity studies which indicate the likely presence of cross-links: b) DT-diaphorase used in enzyme studies had been purified from Walker cells. Following continuous exposure to EO9 a significant amount of strand-breaks were detected but DNA cross-link frequency appeared to be extremely low. This was due to the DNA strand breaks obscuring detection of DNA cross-links. By optimising the dose schedule to 4 nM applied four times at four hourly intervals cross-links were detectable. It is likely that both DNA strand breaks and cross-links are formed following each addition of EO9 but that during the interval between doses DNA repair occurs. This is more efficient for DNA strand breaks than for DNA interstrand cross-links and thus cross-links accumulate with successive doses whilst DNA strand breaks frequency is maintained at a lower level.

It must be noted that this alkaline elution procedure does not distinguish between frank DNA strand breaks and alkali-labile sites. These are areas where a drug-DNA adduct has been formed which renders the DNA sensitive to breakage under the alkaline conditions present during the elution process. These sites may also undergo repair within the cell but the kinetics of repair are likely to differ from those of frank breaks. The data presented in this chapter have shown that a large extent of the EO9-induced strand breaks formed in Walker cell DNA were repaired over a 20 hour

recovery period and it is therefore likely that these were due to frank breaks rather than the presence of alkali labile sites.

Walker cells have large amounts of DT-diaphorase, an enzyme shown to metabolise EO9 (Chapter 3) and are very sensitive to this drug (Chapter 4). It was therefore possible that this enzyme may play a role in activation of EO9 to a DNA damaging species which is responsible for the cytotoxicity observed. The involvement of DT-diaphorase in the cytotoxic mechanism was also indicated from studies using a pair of human colon carcinoma cell lines, HT29 and BE tumour cells. HT29 cells contain a high level of DT-diaphorase and were found to be more sensitive to EO9 under aerobic conditions than are BE cells which do not express a functional form of the enzyme (Walton *et al*, 1992a; Plumb and Workman, 1994). A similar correlation between levels of DT-diaphorase and sensitivity to EO9 has also been reported for a large range of cell lines by a number of other investigators (Robertson *et al*, 1994; Smitskamp-Wilms *et al*, 1994). Interestingly, under hypoxia the BE cells were greatly sensitised to EO9 whereas little effect was observed with the HT29 cells (Plumb and Workman, 1994).

Results from alkaline elution experiments (Section 5.3.1) show that following treatment with 0.25 μM EO9 both DNA strand breaks and DNA interstrand cross-links were formed in HT29 cells but little damage was evident in BE cells. This concentration of drug is around the IC_{50} value for EO9 in HT29 cells with a 3 hour exposure (Plumb and Workman, 1994). Alkaline elution experiments used a 2 hour exposure time although little difference in toxicity would be expected between 2 and 3 hours. Under identical treatment conditions little cell kill was noted in BE cells (Plumb and Workman, 1994), thus the trend in DNA damage correlates well to the cytotoxic potency of EO9. Increasing the concentration of EO9 to 10 μM, which is around the IC_{50} value (3 hour exposure) for BE cells, caused a large amount of strand breaks in their DNA. Hence again a correlation between DNA damage and cytotoxicity was apparent. The small extent of cross-links induced in BE cell DNA at this cytotoxic concentration of EO9 may reflect a different mechanism of cytotoxicity to that occurring with HT29 cells under aerobic conditions. Little further increase in DNA damage of HT29 cells was observed at 10 μM EO9 compared with 0.25 μM.

Incubations carried out under hypoxic conditions showed little or no increase in strand break induction in HT29 cell DNA although a small increase in DNA cross-link frequency was observed in some experiments. This correlates with the small (2.9 fold) increase in cytotoxicity seen under hypoxia in HT29 cells (Plumb and

Workman, 1994). Interestingly, BE cells showed a dramatic 30 fold increase in the extent of cross-links formed when hypoxic conditions prevailed. This may explain the 1000-3000 hypoxic cytotoxicity differential reported by Plumb and Workman (1994).

As well as the difference in DT-diaphorase activity, HT29 cells posses the DNA repair enzyme O6-alkyl guanine alkyl transferase whereas the BE cells do not. It is however unlikely that this enzyme would be involved in the repair of adducts formed by EO9 although it has been implicated in mitomycin C damage repair (Dusre *et al*, 1989). In addition if the alkyl transferase deficiency were important in repair of EO9 induced DNA damage the HT29 cells would be expected to be less sensitive to EO9 than the repair deficient BE cells and in fact the opposite situation is observed under aerobic conditions (Plumb and Workman, 1994).

To summarise, DNA damage was detected at pharmacologically relevant, cytotoxic concentrations in both human and rat tumour cell lines following exposure to EO9 under aerobic conditions. Hypoxia increased the level of DNA damage moderately in HT29 cells but considerably in the BE cells thus the trend in DNA damage followed that of cytotoxicity. This suggested that DNA strand breaks and interstrand cross-links may be involved in the mechanism of EO9 induced cell kill. The greater aerobic cell sensitivity and DNA damage observed in the high DT-diaphorase expressing cell lines, Walker and HT29, as compared to that of the BE cells which does not posses a functional form of the enzyme, supports the involvement of DT-diaphorase in the activation of EO9 to a cytotoxic species. One way of examining this involvement further would be to include dicoumarol, an inhibitor of DT-diaphorase, in the damage assays. The problems involved with using this compound have however been outlined previously (Chapter 3). For this reason the ability of both DT-diaphorase and of NADPH: cytochrome P450 reductase, which has also been shown to metabolise EO9 (Chapter 3) to activate EO9 to a DNA damaging species has been determined in cell free systems. In these cell free studies the concentrations of reaction constituents were optimised to suit the sensitivity of the assay and therefore were not of direct pharmacological relevance.

An agarose gel method employing plasmid DNA was used to examine strand break induction by EO9 following its activation by DT-diaphorase and NADPH: cytochrome P450 reductase. In addition to showing that both these enzymes can metabolise EO9, ESR experiments (Chapter 3) have indicated that the reduction generates both drug and oxygen-based radicals. These are potentially damaging

species and may be responsible for the DNA strand breaks observed in alkaline elution experiments. Results obtained using the plasmid agarose gel method (Section 5.3.2) confirmed the findings of Walton and co-workers (1991) that, in a cell free system, DT-diaphorase is able to activate EO9 to a DNA strand breaking species. The extent of this form of DNA damage was dependent on the concentration of enzyme and EO9 up to saturating levels at which point no further increase was observed. The level of damage also increased with increasing incubation time. Inclusion of both superoxide dismutase and catalase together decreased the proportion of DNA strand breaks to near background levels. These enzymes function to remove superoxide and hydrogen peroxide from the system, thus this would indicate that oxygen radicals, rather than drug-based radical species, were responsible for the damage observed. This is consistent with the finding that superoxide dismutase and catalase abolished the oxygen radical signal but not drug radical signal obtained by ESR (Chapter 3). Walton and co-workers (1991) had not previously investigated the addition of catalase as well as superoxide dismutase to the system. In the experiments described here, inclusion of superoxide dismutase alone in the incubation of DT-diaphorase with EO9 and cofactor led to a small if any reduction in the amount of DNA strand breaks induced. These results are also in good agreement with those obtained by Walton and co-workers (1991).

Interestingly, in preliminary experiments where Tris-Cl buffer was employed in place of phosphate buffer a high level of background DNA damage was observed where DT-diaphorase was incubated with DNA and cofactor alone in the absence of drug. Other investigators have observed the generation of oxygen radicals by ESR following incubation of cofactor with DT-diaphorase in the absence of drug (Knox *et al*, personal communication). It appears that DT-diaphorase, in the presence of cofactor, may be able to reduce molecular oxygen. Due to this activity subsequent experiments utilised phosphate buffer and followed the method described by Walton and co-workers (1991) where no background damage had been reported.

The original intentions of these studies were firstly to confirm the results obtained by Walton and co-workers (1991) and then to further characterise the involvement of oxygen radicals involved in DNA damage by using superoxide dismutase and catalase. A further aim was to examine the effect of hypoxia on DNA strand break induction. In Chapter 3 it was suggested that both oxygen and drug-based radicals were formed following DT-diaphorase reduction of EO9, predominantly by auto-oxidation of the metabolite in the presence of molecular oxygen. Theory would therefore suggest that under hypoxic conditions little or no oxygen radicals should be

generated. The proportion of drug-based radicals should also be reduced although some may be formed by comproportionation : disproportionation reactions. Thus a reduction in DNA damage would be expected under low oxygen conditions. Surprisingly, assays carried out under hypoxia also showed evidence of DNA strand break induction which was inhibitable by superoxide dismutase and catalase. Fewer (1.5 to 2 fold) DNA strand breaks were formed under hypoxia than in air and these were inhibitable by about 50 % with superoxide dismutase and catalase compared with about 70 % inhibition experienced in aerobic incubations. Interestingly the amount of damage remaining in the presence of superoxide dismutase and catalase was similar when incubations were carried out under either air or hypoxia and the levels were only slightly higher than the background of the assay. The hypoxic protection conferred by superoxide dismutase and catalase could be due to the presence of residual oxygen in the system despite the stringent conditions used, although this seems unlikely as increasing the pregassing time from 10 to 30 minutes did not significantly alter damage observed. The extreme oxygen sensitivity of the EO9 metabolite formed by DT-diaphorase reduction has been discussed in Chapter 3 Following termination of the assay by addition of stop buffer the sample is loaded on to the agarose gel for electrophoresis, during which time it inevitably comes in to contact with oxygen. It is possible that the high oxygen sensitivity of the metabolite permits auto-oxidation to occur at this stage generating strand breaking oxygen-based radicals. Experiments carried out by other investigators using the benzotriazine di-N-oxide SR4233 in the same system showed DNA damage following activation by DT-diaphorase under hypoxic but not aerobic conditions. This was partially inhibited by superoxide dismutase and catalase indicating that the a sufficient degree of hypoxia prevailed to stabilise the DNA damaging metabolite to some extent. Thus the assay system may be suitable for some compounds but not others depending upon the oxygen sensitivity of the metabolites generated.

ESR studies (Chapter 3) have shown that in addition to DT-diaphorase, NADPH:cytochrome P450 reductase could activate EO9 to generate drug and oxygen-based radicals in the presence of cofactor. For this reason the involvement of this enzyme in activation of EO9 to a DNA damaging species was also investigated. The results showed that DNA strand breaks were induced where EO9 was incubated under aerobic conditions with NADPH: cytochrome P450 reductase and cofactor. As in the case of DT-diaphorase, controls where drug, cofactor or enzyme were omitted resulted in no or very low level of DNA strand break induction. In addition, the extent of damage was dependent on the concentration of drug and enzyme as well as incubation time. In the case of NADPH: cytochrome P450 reductase the drug radical

should be formed directly by one-electron reduction rather than by auto-oxidation of the metabolites. ESR experiments have shown that the radical, presumably the semiquinone, is also oxygen sensitive and auto-oxidises to generate oxygen radicals. Due to the lack of time and problems encountered with hypoxic DNA strand break assays these were not carried out for cytochrome P450 reductase.

Experiments to investigate the effect of DT-diaphorase and NADPH: cytochrome P450 reductase on activation of AZQ to a DNA strand breaking species were performed by Fisher and Gutierrez (1991a). Similar results were obtained to those presented in this chapter for EO9, i.e. DT-diaphorase and NADPH: cytochrome P450 reductase activation of AZQ led to DNA strand break formation. Protection by superoxide dismutase and catalase along with results of ESR experiments suggested that oxygen radicals generated via auto-oxidation of metabolites were the damaging species.

Alkaline elution experiments have shown DNA cross-link induction following EO9 treatment of cell lines high in DT-diaphorase whereas the BE cells which do not express a functional form of the enzyme showed comparatively low levels of DNA damage. This would indicate the possible involvement of DT-diaphorase in activation of EO9 to a DNA cross-linking species. A cell free, plasmid cross-link assay was employed to investigate this. Despite the fact that, as with the DNA strand break assay, pharmacologically relevant concentrations could not be employed, the experiments provided a model system for determination of the ability of particular enzymes to catalyse metabolism of EO9 to a DNA cross-linking species.

As discussed in Section 5.1. the plasmid cross-link assay is based on the facilitated reannealing of single stranded DNA to double stranded DNA in the presence of cross-links. Where EO9 (100μM) was incubated with DT-diaphorase and cofactor an increase in the percentage of double stranded DNA was observed compared with the control (Fig 5.13) indicating that DNA cross-links had been induced. The level of this damage increased with increasing DT-diaphorase concentration up to certain levels at which complete conversion of single stranded to double stranded DNA was observed. DNA cross-link frequency also increased with drug concentration up to 1 μM. Above this molarity the extent of this DNA damage decreased slightly. In control incubations where either drug or cofactor were omitted no double stranded DNA was evident implying that neither enzyme combined with cofactor nor enzyme combined with drug could cause the DNA interstrand cross-links. However, where drug was incubated in the presence of cofactor but in the absence of enzyme a small

quantity of double stranded form was observed suggesting that cofactor alone can slowly reduce EO9 to a difunctional alkylating species. Following reductive activation, EO9 was found to cross-link plasmid DNA more efficiently than an equivalent concentration of the bifunctional alkylating agent chlorambucil.

Thus these experiments have confirmed that DT-diaphorase reduction of EO9 functions to activate the compound to a difunctional alkylating species capable of cross-linking plasmid DNA. In a similar manner the aziridinylbenzoquinones related to AZQ (Hartley *et al*, 1991a; Lee *et al*, 1992) have also been found to require activation catalysed by DT-diaphorase to induce DNA cross-links at physiological pH (Hartley *et al*, 1991a; Lee *et al*, 1992). Some DNA damage was however observed at pH 7 in the absence of activation by the related compounds BZQ (3,6-Bis[(2-hydroxyethyl)amino]-2,5-diaziridinyl-1,4-benzoquinone and DZQ (2,5-diaziridinyl-1,4-benzoquinone). As in the case of EO9, cross-links were however formed at physiological pH when drugs were incubated with 0.173 μg of DT-diaphorase purified from rat liver (Lee *et al*, 1992). For the methyl AZQ analogue, as with EO9, the frequency of these lesions increased with increasing drug concentration from 2.5 μM to 50 μM (Lee *et al*, 1992). The effect of pH and chemical reduction was also investigated by these authors on the DNA damage caused by the aziridinylbenzoquinones. These parameters were not examined for EO9. When pH was decreased the percentage of cross-links induced by the aziridinylbenzoquinones increased to a small degree (Hartley *et al*, 1991a) whereas chemical reduction with ascorbic acid gave cross-links in the case of AZQ and methyl AZQ only. Where both reducing conditions and low pH prevailed a significant level of cross-linking was observed for all analogues. The extent of this damage, in general, decreased with increasing size of substituent.

The enhanced cross-linking ability of both aziridinylbenzoquinones and indoloquinones following reduction is likely to result from aziridine ring activation. This process is assisted by the change in electron distribution upon quinone reduction to either the semiquinone or hydroquinone species (Butler *et al*, 1989). Low pH may also assist aziridine ring activation by protonation.

As discussed in Section 5.1 the specificity of drug binding to particular sequences in DNA may be relevant to antitumour activity. The *Taq* polymerase assay was employed to examine the sequence selectivity of covalent modification to DNA by EO9. It was hoped that this would further the understanding of EO9 induced DNA

damage at a more molecular level. In addition, it is possible that by understanding this selectivity compounds may be rationally designed to target specific nucleotides.

Untreated DNA ran predominantly as a single band corresponding to a 263 base pair fragment due to the absence of DNA adducts. Additional bands of very low intensity were also observed and may be due to random lesions or the *Taq* falling off the DNA in the absence of lesions.

Chlorambucil is an established alkylating agent which predominantly forms covalent adducts with guanine residues resulting in the characteristic banding pattern seen in Fig 5.14. This result is consistent with that obtained by other investigators (Wyatt *et al*, unpublished data). Lesions were observed to be more common in runs of contiguous guanines than with isolated, bases as has been reported for a number of alkylating agents (Ponti *et al*, 1991)

Surprisingly, perhaps, where EO9 was incubated with DNA in the absence of an activating system DNA modifications did occur. It is likely that these covalent adducts are due to monofunctional alkylation through the aziridine moiety. Aziridines are known to be highly reactive groups which can readily bind to nucleophiles. Results presented in this chapter show that binding predominantly occurs at guanine residues which is consistent with results obtained for other aziridine containing compounds (Mattes *et al*, 1986; Lee *et al*, 1992). This may be explained by the fact that alkylation has been suggested to be related to the electrostatic potential of guanine N-7 (Kohn *et al*, 1988). This differs according to the base adjacent to the molecule with the most negative bases being runs of guanines (Pullman and Pullman, 1981). Interestingly, the banding pattern seen with EO9 is distinct from that of chlorambucil with lesions being more specific. Notably, fewer bases were modified and all of the adenines in the sequence GCA were alkylated more strongly by chlorambucil than by unactivated EO9. This could possibly result from steric hindrance. EO9 was able to alkylate some bases more strongly than chlorambucil including an adenine in the sequence GTATC, two cytosines in the sequence GCA and three cytosines in the sequence pu pu C pu (where pu indicates a purine). Hartley (1993) reported that the non-alkylating portion of the molecule could influence the sequence selectivity of alkylation and thus this may to some extent explain the differences between EO9 and chlorambucil. Where the cofactor, NADH, was also included in the presence of drug a similar pattern of DNA adducts was obtained. This indicated that if any chemical reduction of the compound was occurring by NADH it did not alter the affinity or sequence selectivity of EO9 for

DNA. For incubations containing the enzyme DT-diaphorase and EO9 but in the absence of a complete activating system a similar pattern of DNA modification was once again observed but the intensity of binding was decreased. The phenomenon of reduced intensity remained consistent wherever enzyme was present even in the presence of complete activating system. The reason for the reduction in band intensity is unknown. One possibility is that EO9 may be binding to the active site of the protein and thus the concentration of free drug available for DNA binding is reduced.

The effect of DT-diaphorase activation on the sequence selectivity of DNA binding of EO9 was investigated for various concentrations of drug. The results revealed that, in the presence of the enzyme activating system EO9 was still able to bind covalently to DNA although the amount of adducts appeared to be reduced. Of considerable potential importance is the observation that the DNA sequence specificity was distinctly different to that obtained in controls where components of the activating system had been omitted. In addition to a reduction of band intensity, the compound appeared more selective, binding to fewer and different sequences than when unactivated. In particular, a few bases were alkylated by the activated drug that were not modified by either the unactivated drug or by chlorambucil. These included four cytosines of which two were in the sequence TGC, one adenine in the sequence CAT and two thymines. Additional distinct differences were in regions of the gel which were difficult to interpret as they were less clearly resolved hence the identity of the modified bases remain undetermined. In future experiments, running the gel further would permit resolution of this region. A change in sequence selectivity following activation by DT-diaphorase has been reported during the course of this work for the aziridinylbenzoquinones methyl DZQ and DZQ (Lee *et al*, 1992). A number of theories may advanced to explain these observations. (i) The chemistry of EO9 suggests that upon reduction, activation of one or both of the two leaving groups to an alkylating species may occur. These themselves may be binding to DNA in preference to the aziridine group or alternatively may change the shape or electrostatic potential of the molecule and thus alter its binding capacity. (ii) It is likely that EO9 may be activated to a bifunctional alkylating agent capable of inducing DNA cross-links under these conditions. Indeed the results of the agarose gel cross-link assay (Section 5.3.3 of this chapter) have confirmed that such a reaction occurs. Activation to a bifunctional alkylating agent may result in drug only being able to cause cross-links at certain sequences and thus restricting lesions to fewer but more specific sites. (iii) Activation to a bifunctional alkylating agent which has bound to the protein (enzyme) present in the system to form a DNA-protein cross-

link. A further complication may exist in the fact that these concentrations of reaction constituents would be expected to generate damaging oxygen radical species which have been shown to induce strand breaks in DNA under aerobic conditions (Section 5.3.2). Strand breaks could potentially shorten the fragments of DNA and thus give artifactual results mimicking those of a covalent lesion.

One point of concern, as with all the cell free assays, is whether the sequence specificity obtained with naked DNA is similar to that which occurs in the whole cell system. Experiments carried out by Hartley and co-workers (1992) showed that the sequence selectivity seen with mechlorethamine, uracil and quinacrine mustard using an isolated 340 base pair a sequence of human DNA was maintained when DNA was analysed following extraction from cells treated with these compounds. This would indicate that *in vitro* assays using naked DNA may be useful in predicting the nature of cellular damage.

It has been suggested that chemical substituents may alter the selectivity of binding of compounds to DNA (Hartley, 1990); thus the possibility of modifying an agent to obtain an analogue with enhanced specificity exists. Unfortunately due to time restrictions analogues of EO9 were not examined using this assay. This may have yielded valuable information and it is suggested that these are carried out in the future.

Experiments described in this chapter have confirmed that EO9 is able to cause DNA damage in the form of strand breaks and DNA interstrand cross-links in human and rat cell DNA when applied at cytotoxic doses. The enhanced DNA damage observed with increasing cytotoxicity indicates that this may be one mechanism by which EO9 induces cell kill. Results obtained by comparing the high and low DT-diaphorase expressing cell lines, HT29 and BE respectively implicates this enzyme in reductive activation of EO9 to a DNA damaging and cytotoxic species. Indeed, data presented in previous chapters of this thesis have shown that EO9 is metabolised by DT-diaphorase derived from both rat and human tumour cells and that this leads to the formation of potentially damaging drug and oxygen-based radicals under aerobic conditions. Results from the plasmid DNA strand break and cross-link assays show that purified DT-diaphorase in a cell free system can activate EO9 to a species which can cause both DNA strand breaks and interstrand cross-links. Using superoxide dismutase and catalase, it appears that DNA strand break formation may result from oxygen radicals rather than drug-based radicals. NADPH: cytochrome P450 reductase has also been shown to metabolise EO9 to generate oxygen and drug-based

radicals (Chapter 3). Strand break assays confirmed that this reduction may also activate EO9 to a species capable of inducing strand breaks in plasmid DNA. Due to lack of time the involvement of this enzyme in activation of EO9 to a cross-linking species was not investigated.

The use of these cell free systems has permitted identification of enzymes which can metabolise EO9 to a DNA damaging species. These have also shown that EO9 in the absence of an activating system, at physiological pH, cannot cause significant strand breaks or interstrand cross-links in naked DNA. Monofunctional adducts were however detected. The significance of these activating systems in eliciting cellular DNA damage does however remain to be elucidated. The involvement of a particular enzyme in metabolism of EO9 will depend on the levels of other bioreducing enzymes present as well as their relative affinities for EO9. It must also be noted that the experiments described in this chapter used plasmid DNA which may differ from the reactions experienced with human DNA. DNA repair systems within the cell will also be a determining factor in the level and importance of the various forms of damage induced.

In the case of HT29 and Walker cells both one and two-electron reduction may occur. Under aerobic conditions it is likely that both the semiquinone and hydroquinone products will auto-oxidise to generate the toxic radicals which may cause DNA strand breaks. Strand break experiments suggest that this form of DNA damage is generated by oxygen-based radicals rather than drug-based radicals. In the isolated cell system DNA cross-links were also formed under oxic conditions following aerobic reduction of EO9 by DT-diaphorase. This may indicate that the one-electron reduced metabolite formed by auto-oxidation of the two-electron reduced product is the DNA cross-linking species. Alternatively it is possible that, due to the close proximity of the strong nucleophile, the hydroquinone binds to DNA causing a cross-link before oxidation can occur. Hypoxia should favour formation of the hydroquinone over the semiquinone. Thus if this two-electron reduced metabolite were the DNA damaging species DNA interstrand cross-links should be enhanced in the absence of oxygen. In fact only a small increase in DNA damage was observed in HT29 cells. In contrast BE cells showed a large increase in DNA cross-link formation under hypoxia. The semiquinone formed by one-electron reduction would be stabilised under these conditions which would suggest that this was the product responsible for DNA cross-links. However, by stabilising the semiquinone, further one-electron reduction may be permitted to generate the hydroquinone. Thus the nature of the DNA cross-linking species remains unclear. One point of note is that under low oxygen conditions the

sensitivity of BE cells to EO9 is greatly increased beyond that of HT29 cells. It is possible that although the BE and HT29 cells posses relatively similar levels of NADPH: cytochrome P450 reductase, the lack of DT-diaphorase in the BE cells favours the one-electron reduction pathway to generates more semiquinone than hydroquinone and that it is this metabolite which is the more toxic.

To conclude, results presented in this chapter have shown that DNA damage in the form of both DNA interstrand cross-links and DNA strand breaks are induced in rat Walker tumour cell lines and human HT29 and BE cell lines following treatment with EO9. The occurrence of these lesions at concentrations of EO9 relevant to cytotoxicity indicate that DNA damage may be an important mechanism of EO9 induced cell kill. Results of cell free assays suggest that both DT-diaphorase and NADPH: cytochrome P450 reductase may be involved in activation of EO9 to these cytotoxic, DNA damaging metabolites, the identity of which remains to be clarified.

Chapter 6

Metabolism of Aziridinylbenzoquinone Compounds by DT-diaphorase

6.1 Introduction

The structurally related quinone compound AZQ has the potential to undergo bioreductive activation in a similar manner to that described earlier in this thesis for EO9 (Chapter 3). This may involve either one or two-electron reduction generating the semiquinone or the hydroquinone species respectively. Toxicity may then result from oxidative stress and / or alkylation of critical biological components.

AZQ has been shown to be reduced electrochemically, chemically, by intact cells and by microsomes, as well as by certain isolated enzyme systems (for references see below). Electrochemical reduction experiments (Mossaba *et al*, 1985) have revealed a number of important features of AZQ and its reduced products. In aqueous medium electrochemical reduction leads to the formation of the two-electron reduced dianion which is auto-oxidised to the semiquinone and parent compound. In DMSO one-electron reduction steps are possible; this permitted characterisation of AZQ and the one and two-electron reduced species by UV-visible absorption spectroscopy (Mossaba *et al*, 1985). Further experiments carried out by Mossaba and co-workers (1985) using ESR have characterised the semiquinone formed by electrochemical reduction. These investigators also suggested that the reduction of AZQ to the dihydroquinone occurred more rapidly at lower pH values. Results from cyclic voltammetry experiments indicate that aziridine ring opening is assisted following reduction of the quinone (Mossaba *et al,* 1985).

Reduction of AZQ can also be carried out chemically using sodium borohydride. ESR studies with low concentrations of this reducing agent have detected the yellow AZQ free radical anion which is stable under anaerobic conditions. Higher concentrations of reducing agent yield the hydroquinone which is capable of undergoing auto-oxidation to the free radical form. This may then react further to yield a monoaziridine (Guttierez *et al*, 1987). Studies using either sodium borohydride or NADPH have revealed that reduction of AZQ is required for the induction of DNA interstrand cross-linking (King *et al*, 1984; Szmigiero and Kohn,

1984). These reactions are pH dependent with greater DNA damage being exhibited at low pH.

Evidence of AZQ reduction within intact cells has again been provided by ESR studies which have detected free radicals following exposure of cells to AZQ. The cell lines in which this has been demonstrated include K562 and L1210 (Egorin *et al*, 1985; Lea *et al*, 1988; Butler *et al*, 1990; Dzielendziak *et al*, 1990), Hep-2 (Gutierrez, 1986), HL 60 (Egorin *et al*, 1985) and BSM and RAJI cells (Dzielendziak *et al*, 1990).

The ability of particular subcellular fractions and of certain purified enzymes to catalyse reduction of AZQ has also been determined. Metabolism of AZQ catalysed by rat liver microsomes and rat liver nuclei, as well as by purified NADPH : cytochrome P450 reductase, was indicated by NADPH-dependent oxygen consumption and by production of a free radical intermediate as detected by ESR (Gutierrez *et al*, 1982; Fisher and Gutierrez, 1991b).

Initial studies employing HT29 colon carcinoma cells revealed that AZQ could undergo metabolism and exert cytotoxicity in a fashion which was inhibitable by dicoumarol (Ross *et al*, 1990). This suggested a role for DT-diaphorase in both the reduction and bioactivation of AZQ in this cell line. Further support for the involvement of DT-diaphorase came from experiments in which HT29 cells were compared with the BE colon carcinoma cell line which, due to a mutation in the NQO1 gene encoding DT-diaphorase, does not express a functional form of DT-diaphorase (Traver *et al*, 1992). In addition to cytotoxicity, interstrand cross-linking was reduced by dicoumarol in the HT29 cell line whereas no effect was seen with this inhibitor in the case of the less sensitive BE cells (Siegel *et al*, 1990a). Subsequent experiments involving use of a highly purified form of the HT29 cell human DT-diaphorase confirmed that AZQ could act as a substrate for this enzyme. Further examination of the DT-diaphorase catalysed reduction of AZQ was carried out using a more readily available purified rat hepatic enzyme (Siegel *et al*, 1990a).

Flavin reductases including DT-diaphorase present in the S9 fraction of MCF-7 human breast cancer cells have also been shown to metabolise AZQ under both aerobic and hypoxic conditions (Fisher and Gutierrez, 1991b). DT-diaphorase reduction generated both the drug free radical anion and oxygen radicals. It appears that these are formed from auto-oxidation of the metabolite presumed to be the hydroquinone, as well as from comproportionation reactions.

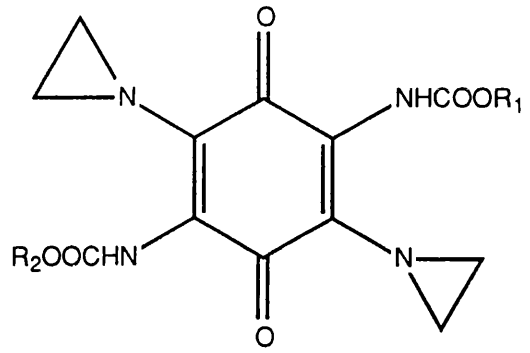
A number of studies carried out on compounds structurally related to AZQ have indicated that modification alters many characteristics including metabolism. One example is BZQ, the analogue in which the carboethoxyamino groups have been substituted with ethanolamine groups. This differed from AZQ in stability, one-electron reduction potential, enzymatic reduction, DNA alkylation, cellular uptake and efflux (Butler *et al*, 1990) as well as in cytotoxicity (Lea *et al*, 1988). For this reason Dr John Butler and co-workers at the Patterson Institute, Manchester, synthesised a set of novel diaziridinylbenzoquinones in which the carboethoxyamino moieties were substituted by a variety of different hydrocarbon groups as illustrated in Fig 6.1 (Dzielendziak and Butler, 1989). Initial experiments carried out on these analogues revealed differences in their cytotoxicity, free radical generation, DNA cross-linking ability, one-electron reduction potential and metabolism by NADPH-requiring enzymes in cell sonicates (Dzielendziak *et al*, 1990).

At the time of carrying out the studies outlined in this chapter there had been no publications reporting the metabolism of these AZQ analogues by DT-diaphorase. We were particularly interested in the relevance of this enzyme due to its ability to metabolise AZQ (Siegel *et al*, 1992) as well as its increased expression and activity in some tumours (see Chapter 1) but most importantly to complement the work carried out on EO9 analogues (Chapter 3). The purpose of the work described in this chapter was therefore to investigate the ability of the AZQ analogues to be metabolised by DT-diaphorase. It was hoped that this may aid elucidation of structural features which may optimise the reductive activation of AZQ by DT-diaphorase.

6.2 Methods

6.2.1 Metabolism of Aziridinylbenzoquinones

The metabolism of AZQ and related analogues was analysed spectrophotometrically using the cytochrome *c* reduction assay. The method was identical to that described in Chapter 2 except that full kinetics were carried out using 0.1 μg of purified rat Walker DT-diaphorase in a total volume of 1 ml of buffer. Linearity of the substrate with respect to enzyme concentration was examined by varying the concentration of DT-diaphorase between 0 and 0.1 μg / ml. The cofactor NADH concentration was also optimised. A slight lag phase occurred during the progress of the reaction and the initial activity was calculated from the linear portion of the curve.



Analogue	R group (R1=R2)
methyl	CH_3
ethyl (AZQ)	$-CH_2-CH_3$
n-propyl	$-CH_2-CH_2-CH_3$
iso-propyl	$-CH(CH_3)-CH_3$
n-butyl	$-CH_2-CH_2-CH_2-CH_3$
sec-butyl	$-CH_2-CH(CH_3)-CH_3$
iso-butyl	$-CH(CH_3)-CH_2-CH_3$

Fig 6.1 Structures of aziridinylbenzoquinones

6.3 Results

6.3.1 General Features of the Cytochrome *c* Reduction Assay

Results obtained using the cytochrome *c* reduction assay demonstrated that AZQ was able to undergo metabolism catalysed by purified rat Walker DT-diaphorase (Fig 6.2 and Table 6.1). Typical progress curves obtained during this reduction process with various concentrations of AZQ are shown in Fig 6.2 a. These illustrate that the rate of reduction was linear throughout the two minute duration of the assay, and this was also true for reactions carried out over 4 minutes (data not shown). Increasing AZQ concentration increased the rate of reaction whilst still retaining a linear progress curve (Fig 6.2 a). Controls carried out where drug was omitted resulted in an extremely low level of activity (0.47 nmoles cytochrome *c* reduced / minute / mg) and this was therefore disregarded for calculation purposes. Exclusion of enzyme did give a fairly high background but for all analogues the activity in the absence of enzyme was similar if not slightly higher than that in the presence of dicoumarol. This non-enzymatic, dicoumarol insensitive activity could therefore be attributable to direct chemical reduction of the quinones by NADH. For the experiments where NADH was omitted no activity was noted.

Structural modification of AZQ resulted in an altered ability to be metabolised by DT-diaphorase (Tables 6.1 and 6.2). It was also observed that for some AZQ analogues, particularly *sec*-butyl AZQ and *iso*-butyl AZQ, a completely linear progress curve was not always obtained but an initial lag phase was present (Fig 6.2). The length of this varied to a small extent between repeat experiments carried out for individual analogues for a given drug concentration. The progress curves for AZQ and methyl AZQ, which in contrast did give a linear progress curve are also illustrated in Fig 6.2. The most striking lag phase was noted with *sec*-butyl and *iso*-butyl AZQ (Fig 6.2) where this persisted for approximately 60 seconds. In standard fashion, all activities were calculated from the linear part of the curve. Experiments where the reaction was initiated by cofactor, enzyme or substrate all showed a similar lag phase. Where dicoumarol was included again a lag phase was observed.

Effect of Altering Cofactor Concentration

The effect of altering the concentration of the NADH cofactor on the rate of reaction was investigated for each analogue in order to confirm that this was optimum. In general increasing NADH concentration did not result in any significant increase in

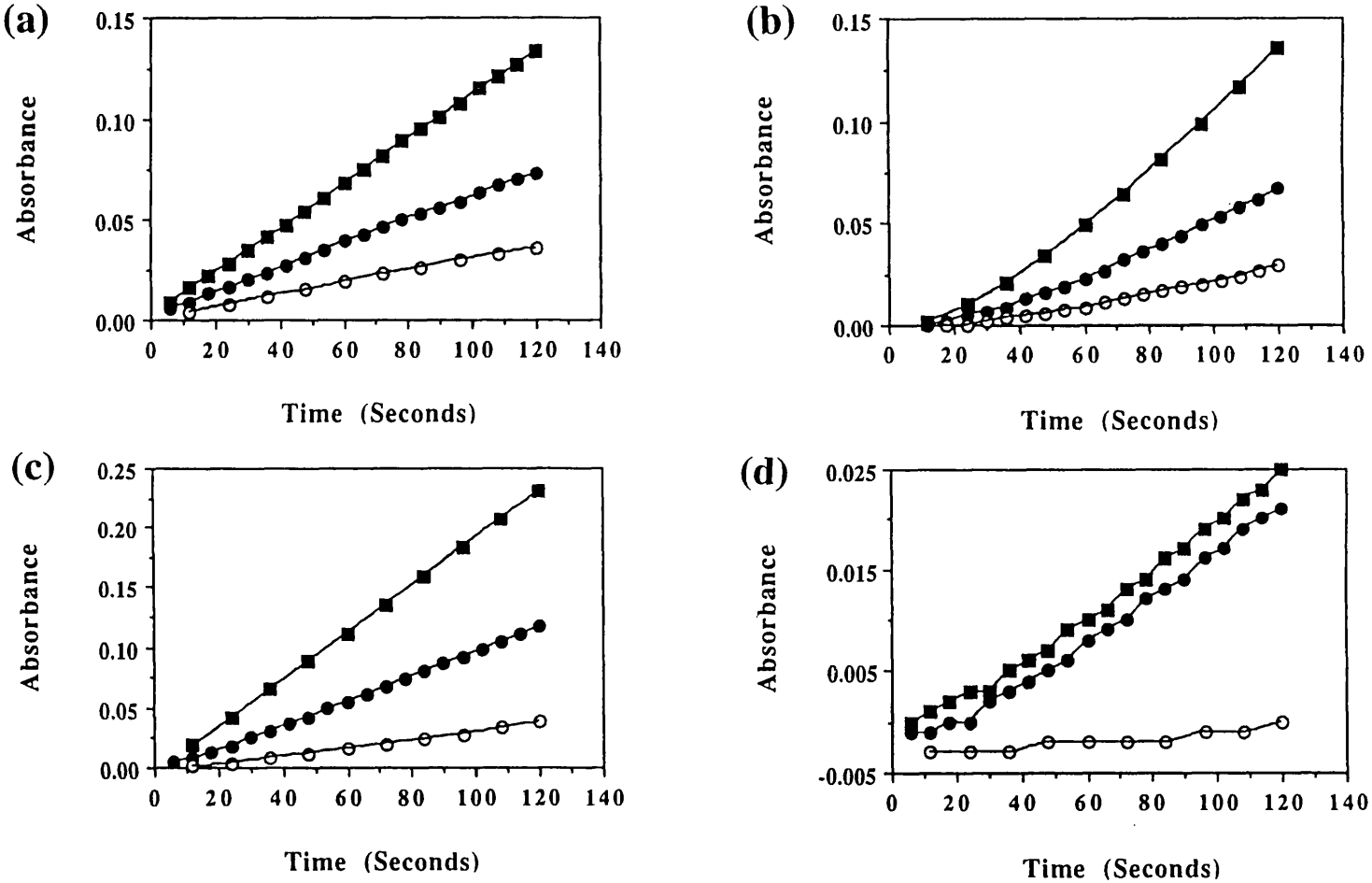


Fig 6.2 Reduction of (a) AZQ, (b) *iso*-butyl AZQ, (c) methyl AZQ, (d) *sec*-butyl AZQ catalysed by purified rat Walker DT-diaphorase over a two minute time period. Data are taken from an individual experiment but the same trends in results were seen in at least one independent repeat experiment as well as in repeats carried out on the same day.

■ 50 μM drug ● 25 μM drug ○ 25 μM drug + dicoumarol

Analogue		Activity (μ moles cytochrome c reduced / minute / mg protein)					
		200 μM drug			400 μM drug		
		mean	SD	n	mean	SD	n
methyl AZQ		86.23*	122.61	3	143.49	42.919	8
AZQ		53.209	20.44	11	73.46	18.87	9
n-propyl AZQ		38.62*	ND	2	81.44	9.37	6
iso-propyl AZQ		11.85*	ND	2	9.42	6.24	8
n-butyl AZQ		72.27*	ND	2	124.29	71.21	8
iso-butyl AZQ		111.56	32.99	5	85.40*	6.32	5
sec-butyl AZQ	a	9.95*	ND	2	13.43	2.43	3
	b	11.21	4.088	3	21.01	8.52	6

Table 6.1 The reduction of aziridinylbenzoquinones catalysed by purified rat Walker DT-diaphorase expressed per mg of protein. Data are from pooled experiments (both within-and between-day repeats) and are expressed as a mean, standard deviation (SD) and sample number (n). * - data taken from only a single experiment due to limited supply of compound. ND - not determined.

Analogue		Activity (U menadione activity)					
		200 μM drug			400 μM drug		
		mean	SD	n	mean	SD	n
methyl AZQ		31.71*	4.50	3	56.32	10.68	8
AZQ		20.01	7.25	11	30.04	4.86	9
n-propyl AZQ		21.46*	ND	2	44.83	5.82	6
iso-propyl AZQ		6.51*	ND	2	5.32	3.36	8
n-butyl AZQ		47.05*	ND	2	77.21	5.60	8
iso-butyl AZQ		54.23	19.32	5	32.60*	2.41	5
sec-butyl AZQ	a	3.36*	ND	2	4.89	0.88	3
	b	3.95	1.27	3	7.99	4.22	6

Table 6.2 The reduction of aziridinylbenzoquinones catalysed by purified rat Walker DT-diaphorase expressed in units of menadione activity (U). Data are from pooled experiments (both within- and between-day repeats) and are expressed as a mean, standard deviation (SD) and sample number (n). * - data taken from only a single experiment due to limited supply of compound. ND - not determined.

the dicoumarol inhibitable activity as exemplified by AZQ in Table 6.3. In most cases the overall activity did rise but the background obtained in the presence of dicoumarol similarly increased, resulting in no overall change. In the case of *iso*-propyl AZQ reducing the concentration of cofactor NADH did however decrease the activity obtained (Table 6.3). The results therefore indicated that 2 mM NADH, in addition to being optimum concentration for menadione metabolism, was also favourable for the majority of the aziridinylbenzoquinones analysed. In the case of *iso*-butyl AZQ, the dicoumarol inhibitable activity obtained on use of 4 mM and 8 mM NADH was actually lower than that at 2 mM, thus suggesting some inhibition due to cofactor. *Sec*-butyl AZQ was exceptional. This analogue continued to show an increase in dicoumarol inhibitable activity up to 10 mM before a slight decrease in activity was again observed at 20 mM, this indicating an optimum of around 10 mM NADH.

Effect of Altering Enzyme Concentration

Selected experiments to examine the effect of altered enzyme concentration were also performed. Due to shortage of compound these were repeated only for the *iso*-propyl and *sec*-butyl analogues but similar results were seen for the other compounds. In general at least three enzyme concentrations were studied in addition to the minus enzyme control. A 400 μM substrate concentration was employed for all compounds with the exception of AZQ for which 200 μM was used. The *n*-butyl and *iso*-butyl analogues showed good linearity with respect to enzyme concentration within the range 0.02-0.1μg / ml, AZQ and the methyl, *n*-propyl, and *sec*-butyl analogues were reasonably linear within this range. Initial studies with the *iso*-propyl analogue showed a deviation from this trend with little increase in activity when enzyme concentration was doubled from 0.05 μg / ml to 0.1 μg / ml. Repeat experiments using 50 μM *iso*-propyl AZQ did reveal linearity within the range 0.0083-0.1 μg / ml. The *sec*-butyl analogue was also re-examined due to the limited number of concentrations used initially. Again a 50 μM concentration was employed which resulted in linearity although the activity obtained was extremely low.

Effect of Altering Substrate Concentration

The effect of increasing the concentration of each compound on the rate of reduction is shown in Fig 6.3. In the case of AZQ, activity increased in a linear fashion with increasing substrate concentration up to 800 μM. A similar type of result was obtained with *n*-propyl AZQ. A linear increase in activity was also seen for *iso*-

Aziridinylbenzoquinone	Activity (μM cytochrome c reduced / minute / mg enzyme)					
	1 mM NADH	2 mM NADH	4 mM NADH	8 mM NADH	10 mM NADH	20 mM NADH
methyl AZQ	ND	163.34	156.40	ND	ND	ND
AZQ	ND	38.23	40.28	37.44	ND	ND
***n*-propyl AZQ**	87.68	97.87	75.35	ND	ND	ND
***iso*-propyl AZQ**	5.69	13.74	7.35	12.32	ND	ND
***n*-butyl AZQ**	ND	126.30	ND	112.32	ND	ND
***iso*-butyl AZQ**	ND	91.62	83.41	61.14	ND	ND
***sec*-butyl AZQ**	ND	13.74	18.48	23.70	30.80	4.74

Table 6.3 Effect of varying cofactor, NADH concentration on aziridinylbenzoquinone reduction catalysed by purified rat Walker DT-diaphorase. In all cases 400 μM drug concentration was employed except for AZQ when 200 μM was used. Data are taken from individual experiments and in general represent an average of two or more within-day repeat assays. ND = not determined.

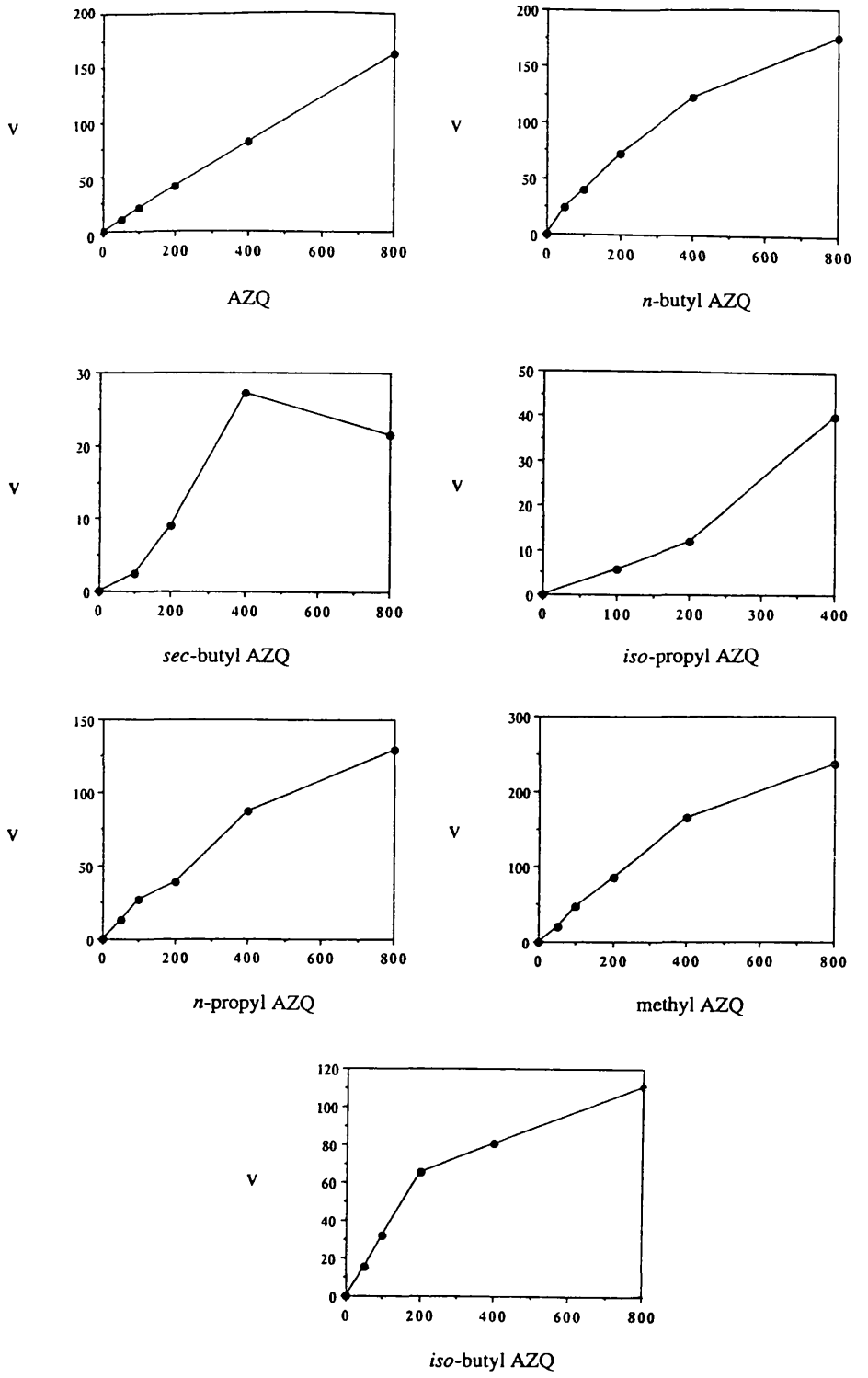


Fig 6.3 Effect of increasing aziridinylbenzoquinone concentration (μM) on the rate of reduction (v, n moles of cytochrome *c* reduced / minute / mg protein) catalysed by rat Walker DT-diaphorase. Data are taken from an individual experiment and in general (except where drug availability was limiting) were confirmed in an independent repeat study. Repeats were however also carried out for several of the data points within the assay.

propyl AZQ up to 200 μM drug concentration, however at 400 μM the profile curved upwards in a more exponential manner. The lack of evidence for substrate saturation observed with AZQ and analogues *n*-propyl and *iso*-propyl AZQ would suggest a high Km value. In contrast, the curves obtained for *n*-butyl, methyl and *iso*-butyl AZQ were linear up to concentrations of 200 - 400 μM after which they began to plateau, indicative of substrate saturation occurring. The Km values for these compounds are likely to be high compared with certain other quinone compounds such as EO9 (see Chapter 3) but lower than for AZQ and its *n*-propyl and *iso*-propyl analogues. *Sec*-butyl AZQ was exceptional in the fact that activity decreased when increasing the substrate concentration above 400 μM. It is however probable that this phenomenon resulted from the limited solubility of the compound.

6.3.2 Enzyme Kinetic Parameters

A number of methods of graphical analysis exist for the determination of enzyme kinetic parameters. Displaying the data as a Lineweaver Burk plot (where 1/v is plotted against 1/S) reveals a linear relationship for AZQ (Fig 6.4 b) and also the *n*-butyl and *iso*-propyl analogues (data not shown). Other structural analogues deviated to a varying degree from linearity (e.g. Fig 6.5 b). As previously discussed (Chapter 2) the Hanes Woolf plot (S/v against S) has been considered the most accurate method for determination of the Km and Vmax values. When the data are displayed in this manner, a linear relationship is again observed for AZQ (Fig 6.4 c) in addition to the *n*-butyl analogue (data not shown). However, the *iso*-butyl compound (Fig 6.5 c) deviates from linearity as do the *sec*-butyl, *iso*-propyl, methyl and *n*-propyl analogues indicating that in general these aziridinylbenzoquinones do not conform to simple Michaelis-Menten kinetics. For this reason Km and Vmax values could not be obtained.

Because of this, in order to compare the relative abilities of the compounds to be reduced by DT-diaphorase, the activities have been reported in Tables 6.1 and 6.2 at two concentrations of drug, that is 200 and 400 μM. Different batches of enzyme may vary slightly in activity for equivalent amounts of protein due to a number of factors including the age of the preparation. In addition, small differences may also be observed in reaction constituents between days leading to a different activity being obtained for the same concentration of enzyme and compound. For this reason the benchmark quinone menadione was included as a positive control in each set of assays carried out. Thus in addition to expressing the activities in mg of protein

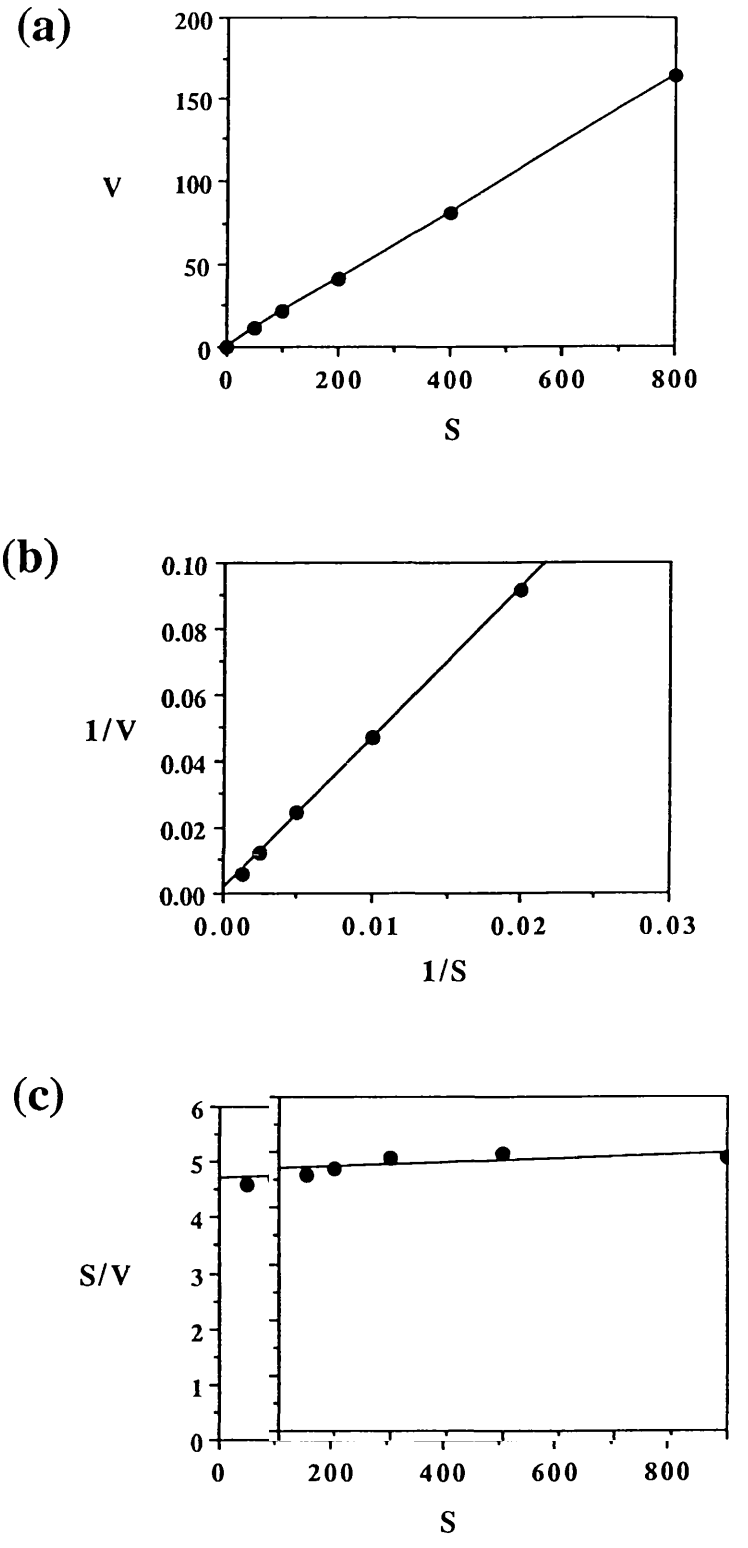


Fig 6.4 Metabolism of AZQ catalysed by purified rat Walker DT-diaphorase displayed as (a) a plot of activity (v, n moles of cytochrome *c* reduced / minute / mg protein) against substrate concentration (S, μM), (b) Lineweaver Burk plot, (c) Hanes Woolf plot. Data are taken from an individual experiment and were confirmed in an independent repeat study. Several within day repeats giving similar results were also carried out for most drug concentrations.

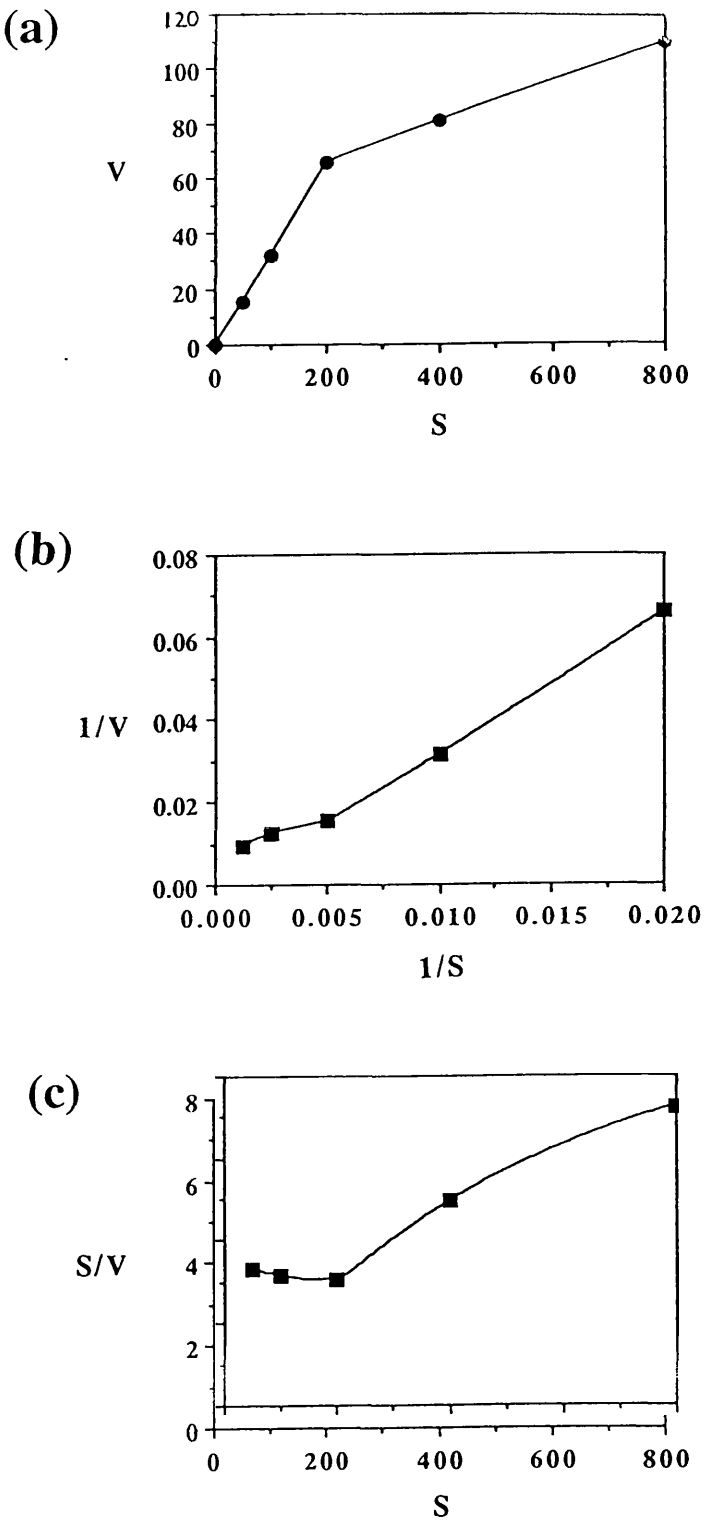


Fig 6.5 Metabolism of *iso*-butyl AZQ catalysed by purified rat Walker DT-diaphorase displayed as (a) a plot of activity (v, n moles of cytochrome *c* reduced / minute / mg protein) against substrate concentration (S, μM), (b) Lineweaver Burk plot, (c) Hanes Woolf plot. Data are taken from an individual experiment and were confirmed in an independent repeat study. Several within day repeats giving similar results were also carried out for most drug concentrations.

(Table 6.1) the results have also been presented in units of menadione activity to compensate for these small between day variations observed (Table 6.2). The results in Tables 6.1 and 6.2 are means of pooled data taken from both within-day and between-day repeats. Due to limited amount of compound, between-day repeats were not always carried out at the listed concentrations.

Results displayed in Table 6.1 show that structural modification of AZQ resulted in an altered rate of DT-diaphorase catalysed metabolism. At a concentration of 400 μM, the methyl analogue was the most rapidly reduced of the 7 compounds examined whereas *iso*-propyl was the most poorly metabolised. The relative order of DT-diaphorase catalysed metabolism was methyl > *n*-butyl > *iso*-butyl > *n*-propyl > ethyl (AZQ) > *sec*-butyl > *iso*-propyl. Only slight variations in order were obtained when expressing data in Units of menadione activity and when comparing values obtained at the 200 μM concentration.

Experiments were carried out to examine the effect of hypoxia on the rate of metabolism of the parent compound AZQ at 50 and 25 μM concentration. These showed no significant difference between aerobic and hypoxic conditions, the activities at 50 μM AZQ being 14 and 16 nmoles cytochrome *c* reduced / minute / mg protein respectively.

6.4 Discussion

The aims of the experiments presented in this chapter were to investigate AZQ metabolism catalysed by highly purified DT-diaphorase isolated from rat Walker tumour cells and to determine the effect of structural modification on this reduction process. The results obtained clearly demonstrate that AZQ acted as a substrate for the Walker cell DT-diaphorase (Fig 6.2, 6.3 and 6.4). At a substrate concentration of 100 μM activity was ~5 fold higher than that achieved with the prototype bioreductive alkylating agent mitomycin C which has been suggested to be activated by DT-diaphorase (Siegel *et al*, 1992). AZQ was however ~30 fold more poorly metabolised than EO9 and ~180 fold more poorly metabolised than the conventional substrate menadione (Chapter 3). As with EO9, structural modification of AZQ was found to significantly alter its ability to act as a substrate for DT-diaphorase (Table 6.1 and 6.2).

As discussed in Chapter 3, of a number of methods available for monitoring DT-diaphorase metabolism of quinone compounds the cytochrome *c* reduction assay was considered preferable for our requirements.

Controls carried out where either drug or cofactor were omitted resulted in an extremely low level of activity. Exclusion of enzyme did however give a fairly high background activity indicating that the compounds could be reduced directly by the NADH cofactor. This is in contrast to results obtained by Siegel and co-workers (1990a) who reported a negligible non-enzymatic loss of quinone under both aerobic and anaerobic conditions. These authors did however use a 10 fold lower concentration of NADH. In addition, the experiments where negligible background was reported were carried out by direct UV spectrophotometry which may not be as sensitive as the cytochrome *c* reduction assay. Assuming that dicoumarol did not interact with the drugs and that it was acting purely as an inhibitor of DT-diaphorase, it would be expected that the activity in the presence of dicoumarol would be identical to that obtained in the absence of enzyme. In fact for all analogues the background in the absence of enzyme was similar if not slightly higher than that in the presence of dicoumarol. This further supported the fact that dicoumarol was totally inhibitory of enzyme activity at the concentration employed (100 μM). Although there are a number of problems associated with using the inhibitor dicoumarol (Chapter 3) these are unlikely to apply here since a highly purified preparation of DT-diaphorase was used.

In contrast to the situation with EO9 and its analogues (Chapter 3), examination of the progress curves (Fig 6.2) revealed a brief but significant initial lag phase in the case of certain analogues. This was particularly noticeable for *sec*-butyl and *iso*-butyl AZQ. There was however no difference observed between reactions initiated by enzyme, drug or NADH. Following the lag phase the reaction proceeded in a linear fashion.

The rate of reduction of the aziridinylbenzoquinones was dependent on enzyme, drug and cofactor concentration. The NADH concentration of 2 mM employed in these experiments was optimum for all substrates with the exception of *sec*-butyl AZQ which showed increasing activity with up to 10-20 mM cofactor. Increasing drug concentration resulted in an enhancement in activity, the only exception again being the *sec*-butyl analogue which exhibited decreased activity above 400 μM drug concentration. It is likely however, that this reduced activity was a result of the limited solubility of the *sec*-butyl analogue. The lack of

substrate saturation observed for most analogues would imply a high Km value. This is consistent with previous findings with AZQ utilising rat hepatic DT-diaphorase (Siegel *et al*, 1990a). These authors have also subsequently examined metabolism of the aziridinylbenzoquinone analogues by the purified rat liver enzyme (Gibson *et al*, 1992). They did not however discuss either the effect of increasing drug concentration on the rate of reaction or the Km values obtained with the analogues. Results presented in this chapter demonstrated a lack of linearity for Hanes Woolf plots suggesting that the aziridinylbenzoquinones do not in general conform to Michaelis-Menten kinetics and thus no Km values could be determined.

Experiments indicated that, in general, the compounds exhibit linearity with respect to enzyme concentration over a range of 0.02-0.1 μg / ml of protein, with increasing concentration leading to enhanced activity. Repeat experiments are however required to confirm this observation. For most assays 0.1 μg / ml of protein was employed as this was the lowest concentration to give a reasonable rate of reduction. It may have been preferable to utilise higher concentrations of enzyme, particularly for the investigation of some of the poorer substrates where activity was very low with 0.1 μg of DT-diaphorase / ml of reaction mixture. However, this would have consumed very large amounts of purified enzyme and the results were considered sufficient for the necessary conclusions to be drawn.

Since the aziridinylbenzoquinones do not tend to conform to Michaelis-Menten kinetics activities were compared at two drug concentrations (200 and 400 μM) and were expressed in both units of menadione activity as well as mg of protein for the reasons previously described (Chapter 3). The order of activity of the analogues was largely similar when 200 μM drug concentration to that obtained with 400 μM . The results obtained for 400 μM aziridinylbenzoquinones will be discussed as these involved a greater number of repeats. The order of activity for this concentration when expressed in mg of protein was, from most to least active, methyl > *n*-butyl > *iso*-butyl > *n*-propyl > ethyl (AZQ) > *sec*-butyl > *iso*-propyl. Converting these results to units of menadione activity merely reversed the order of the methyl and *n*-butyl and *n*-propyl and *iso*-butyl analogues. Similar studies carried out by Gibson and co-workers (1992) who found an order of reduction by rat hepatic DT-diaphorase of methyl > *n*-butyl > ethyl (AZQ) > *iso*-butyl >*n*-propyl >*sec*-butyl >*iso*-propyl. Although this sequence does vary slightly from that obtained from the experiments described in this chapter, the overall trend is very similar. It is possible that the differences may be due to the different source of enzyme employed between the two studies, i.e. rat liver versus rat Walker tumour. The advantage of using the purified

rat Walker tumour enzyme was that a direct comparison was possible between the rate of reduction catalysed by this enzyme and cytotoxicity in the cell line from which the enzyme was derived (see Chapter 7).

The one-electron reduction potentials (E^1_7) determined by pulse radiolysis range from -44 for the methyl analogue to -167 for the *iso*-butyl derivative. The other aziridinylbenzoquinones are intermediate between these two extremes and assume the following order: methyl > AZQ > *n*-butyl > *n*-propyl > *iso*-propyl > *sec*-butyl > *iso*-butyl (Dzielendziak and Butler, 1989). Two-electron reduction potentials are thought to follow a similar trend to the one-electron reduction potentials (Swallow, 1982) and thus the possible ease of reduction catalysed by DT-diaphorase should also be predictable from the one-electron reduction potentials. In fact no direct correlation can be seen between the rate of reduction of the analogues by DT-diaphorase (Table 6.1) and the one-electron reduction potentials. This is consistent with results obtained by Gibson co-workers (1992) with purified rat hepatic DT-diaphorase. Furthermore other investigators have similarly reported a lack of correlation between one-electron reduction potentials and DT-diaphorase metabolism of a range of quinone compounds (Brunmark *et al*, 1988; Buffington *et al*, 1989). In addition to the electron reduction potential other factors will influence the ability of a compound to undergo metabolism catalysed by the enzyme which may explain the lack of correlation observed. Lipophilicity as well as steric factors may be important, in particular the substrate fit in to the active site of DT-diaphorase.

Cytochrome *c* reduction assays were carried out to examine the effect of hypoxia on the reduction of AZQ by DT-diaphorase. No apparent difference was observed between the rates of metabolism obtained under aerobic verses hypoxic conditions. These data compare well with those obtained by Siegel and co-workers (1990a) using purified rat hepatic enzyme. They monitored NADH oxidation spectrophotometrically and found similar initial rates of cofactor utilisation under aerobic and hypoxic conditions, although AZQ removal was more rapid under anaerobic conditions (5.6 and 3.5 nmol / minute / μg for anaerobic and aerobic conditions respectively). This suggested that in the presence of air the hydroquinone formed by reduction was rapidly auto-oxidised to regenerate the quinone. They also found that at pH 7.8 the rate of re-oxidation of $AZQH_2$ was higher.

To conclude, data presented in this chapter provide clear evidence that the aziridinylbenzoquinones related to AZQ are able to undergo metabolism catalysed by rat Walker tumour cell DT-diaphorase. The somewhat unusual enzyme kinetics have

been documented. The rate of reduction varies depending on the structural modification made. This may have implications for the cytotoxicity of the compounds and hence their therapeutic efficacy. This will be discussed in the following chapter.

Chapter 7

Cytotoxicity of Aziridinylbenzoquinones

7.1 Introduction

The cytotoxicity of aziridinylbenzoquinones, particularly of diaziquone or AZQ, *in vitro* has been fairly well documented. A number of these findings have been summarised in Table 7.1. AZQ is highly cytotoxic under aerobic conditions with IC_{50} values as low as 0.1 μM being reported in the K526 leukaemic cell line. It is however difficult to draw detailed quantitative comparisons between the cytotoxicities reported in the numerous cell lines due to variations in exposure times, parameters quoted (i.e. IC_{50} and IC_{90}) and methods employed. What is however evident, is that cytotoxicity varies to quite a large extent between cell lines and depends on the exposure time as well as the technique used.

Structural modification of AZQ has also been found to alter cytotoxicity. For example BZQ (a derivative in which the carboethoxyamino groups of AZQ have been replaced by ethanolamine moieties) has a 43 fold greater toxicity than AZQ in the L1210 murine leukaemic cell line (Lea *et al*, 1988). Also for a series of analogues where the carboethoxyamino group has been replaced by a variety of other moieties a large range of cytotoxicities were observed (De Mol *et al*, 1988).

Several studies have indicated that reduction of AZQ may be necessary for the generation of cytotoxic species. Cytotoxicity of AZQ in the CHO Chinese hamster ovary cell line was increased following incubation with the chemical reducing agent ascorbate (O'Brien *et al*, 1990). An indication of the importance of enzymatic reduction in the cytotoxicity of AZQ came from experiments using the HT29 human colon carcinoma cell line. These studies revealed that dicoumarol, an inhibitor of DT-diaphorase, decreased the cytotoxicity of AZQ and thus implicated this enzyme in the activation of the aziridinylbenzoquinone (Siegel *et al*, 1990a). Further evidence for the involvement of DT-diaphorase in the activation of AZQ was provided by the observation that the HT29 cell line, which expresses high levels of the enzyme, was more sensitive to AZQ than the BE human colon carcinoma line, which has a mutation in the NQO1 gene and as a result does not express a functional form of the enzyme (Siegel *et al*, 1990a).

Cell Line	Tumour type	Species	Cytotoxicity		Time	Method	Reference
			IC50 (μM)	IC90 (μM)			
L1210	Leukaemic	murine	93.6	-	1 hour	cell counting	Lea, J. S., et al. 1988
K526	Leukaemic	human	5.6	-	1 hour	cell counting	Lea, J. S., et al. 1988
CHO AA8-4	Ovarian	hamster	1	-	1 day	clonogenic	O'Brien, P. J., et al. 1990
CHO AA8-4	Ovarian	hamster	0.7	-	7 days	clonogenic	O'Brien, P. J., et al. 1990
HT29	Colon	human	-	18	1 hour	clonogenic	Szmigiero, L and Kohn, K.W., 1984
VA-13	Embryo	human	-	45	1 hour	clonogenic	Szmigiero, L and Kohn, K.W., 1984
IMR	Embryo	human	-	82	1 hour	clonogenic	Szmigiero, L and Kohn, K.W., 1984
L1210	Leukaemic	murine	-	100	1 hour	clonogenic	Szmigiero, L. and Kohn, K.W., 1984

Table 7.1 Sensitivity of a number of cell lines to AZQ. The dash under the cytotoxicity column means not determined.

As previously discussed (Chapter 1), AZQ has the potential to act as a bioreductive alkylating agent and as such may be selectively toxic towards hypoxic cells. In one study a 3-4 fold increase in cell kill was observed under hypoxic verses aerobic conditions for the CHO AA8-4, Chinese hamster ovary cell line (O'Brien *et al*, 1990). In these experiments oxygen concentrations below 0.6 mmHg were more effective in enhancing toxicity than those above 0.6 mmHg. Rauth and Marshall (1990), however, have pointed out that it is between 0.6 mmHg and 6 mmHg oxygen where radiation sensitivity initially falls. Thus from the point of view of complementing radiation therapy, an ideal hypoxic cell cytotoxin would exhibit selective cell kill below 6 mmHg oxygen concentrations. Other investigators claimed no effect of hypoxia on the cytotoxicity of AZQ in the CHO cell line, although in fact a 2.3 fold decrease was observed (Powis *et al*, 1987). In addition, a study in C3H mice has shown no selectivity for hypoxic cells in the 16/C mouse mammary carcinoma *in vivo* (Tannock, 1983).

Results presented earlier in this thesis (Chapter 6) have shown that the series of aziridinylbenzoquinone analogues synthesised by Dr John Butler and co-workers (Patterson Institute, Manchester, UK) differed in their ability to be metabolised by DT-diaphorase (Chapter 6). Given this information and that bioreduction has been implicated in the activation of AZQ, it was of interest to determine whether the cytotoxicity of these analogues towards rat Walker tumour cells was related to their ability to be metabolised by DT-diaphorase purified from the same cell line. It was hoped that this would clarify the role played by DT-diaphorase in the bioactivation of aziridinylbenzoquinones and may elucidate structural features that were important in this process.

7.2 Methods

7.2.1 Cell Culture and Cytotoxicity Testing

Rat Walker UK 256 Sensitive tumour cells were employed to examine the cytotoxicity of the aziridinylbenzoquinones in order to compare results with data obtained from metabolism studies which used enzyme purified from these cells. Walker 256 tumour cells were cultured routinely as described previously (Chapter 2).

Cell viability was assessed by means of the MTT dye reduction assay carried out in the presence and absence of the DT-diaphorase inhibitor dicoumarol. The method was essentially identical to that outlined in detail in Chapter 2. Briefly, Walker tumour cells were seeded in microtitre plates at a density of 5 x 10^3 cells / ml and were treated with varying concentrations of drug. Following a standard 72 hour exposure period plates were analysed by the MTT assay for cell viability. For assays where dicoumarol was included it was desirable to select the highest sub-cytotoxic dose of inhibitor which was in general 5 μM. However in some experiments this was found to have a small cytotoxic effect and therefore 1 μM was also employed. Dicoumarol was added to the cells 30 minutes before drug treatment and was left on during the 72 hour exposure to the aziridinylbenzoquinone. Results were calculated as absorbance as a percentage of the appropriate vehicle control. This was 0.2% DMSO (final concentration) in the case of drug alone and either 5 μM or 1 μM dicoumarol plus 0.7% DMSO (final concentration) for the inhibitor-containing treatments. Initial MTT assays were carried out in Cambridge with some experiments repeated in Sutton. However, the results were generally very similar from both laboratories. Where all analogues were not analysed during the same experiment care was taken to ensure that a standard was included, which was generally AZQ itself. Most of the equipment used in both laboratories was identical as was the original source of the cells. Microtitre plates used in Sutton were obtained from Nunclon (Life Technologies, Paisley, Scotland, UK) rather than Falcon. However the results obtained did not vary to any appreciable extent between experiments carried out in the two laboratories. A limited supply of a number of the analogues permitted only duplication of the dicoumarol inhibition studies.

For dicoumarol inhibition studies the dose modification factors were determined for each individual experiment using the formula:

$$\text{Dose modification factor (DMF)} = \frac{IC_{50}\ \text{Drug + Dicoumarol}}{IC_{50}\ \text{Drug alone}}$$

7.2.2 Clonogenic Assay

A preliminary experiment was carried out on the Walker Sensitive and Walker Resistant cells to determine the cloning efficiency following exposure to AZQ. The method is identical to that previously described in Chapter 4, Section 4.2.

7.3 Results

The aziridinylbenzoquinones related to AZQ exhibited highly potent cytotoxicity when applied to rat Walker UK 256 tumour cells (Table 7.2). The mean IC_{50} values for all analogues examined fell within a relatively narrow range, from 15 to 44 nM. The ranking for cytotoxicity was *n*-propyl AZQ = *iso*-butyl AZQ > *sec*-butyl AZQ = methyl AZQ > *n*-butyl AZQ > AZQ > *iso*-propyl AZQ (in decreasing order of potency). Variation between the repeat IC_{50} values obtained was small with standard errors ranging from 0.004 to 0.015 (Table 7.2). Typical concentration-response curves of the seven aziridinylbenzoquinones are shown in Fig 7.1. The shape and steepness of these curves were very similar for the different analogues.

Dicoumarol was observed to modulate the cytotoxicity of the aziridinylbenzoquinones in a protective fashion (Table 7.3). In most cases the degree of protection against cytotoxicity obtained with 1 μM dicoumarol was less than that obtained with 5 μM dicoumarol, the exception being *iso*-butyl AZQ. At 5 μM concentration the average DMF varied from 1.65 to 3.02 across the range of analogues. Structural modifications did not appear to greatly influence the DMF (Table 7.3). The concentration - response profiles are shown in Fig 7.2. In all cases the curve where dicoumarol is included lies to the right of that of the drug alone indicative of a protective effect of dicoumarol on the toxicity of the aziridinylbenzoquinones.

A preliminary experiment examining the cloning efficiency of Walker Sensitive and Walker Resistant cells following exposure to AZQ revealed a large differential between the two cell lines. Fig 7.3 shows that the Walker Sensitive line is much more sensitive to AZQ than is the Walker Resistant. As previously discussed (Chapter 4, Section 4.3) is indicative of a bifunctional alkylating agent.

7.4 Discussion

The aziridinylbenzoquinones examined were all highly potent towards the rat Walker tumour cells (Table 7.2). Structural modification had only a fairly modest effect on cytotoxicity. The most cytotoxic of the analogues were the *iso*-butyl and *n*-propyl AZQ and the least potent was *iso*-propyl AZQ (Table 7.2, Fig 7.1) with the difference between the two extremes being ~3 fold. During the course of the present work a similar series of compounds was analysed for cytotoxicity in human HT29 colon

Aziridinylbenzoquinone	Mean IC_{50} (μM)	Standard Error	Sample Number (n)
AZQ	0.032	0.0076	10
methyl AZQ	0.020	0.0132	5
iso-butyl AZQ	0.015	0.0040	9
iso-propyl AZQ	0.044	0.0146	4
n-propyl AZQ	0.015	0.0045	4
sec -butyl AZQ	0.020	0.0072	5
n-butyl AZQ	0.025	0.0085	5

Table 7.2 Cytotoxicities of aziridinylbenzoquinones as determined by the MTT assay (Section 7.2). Data are expressed as a mean plus standard error of (n) independent experiments.

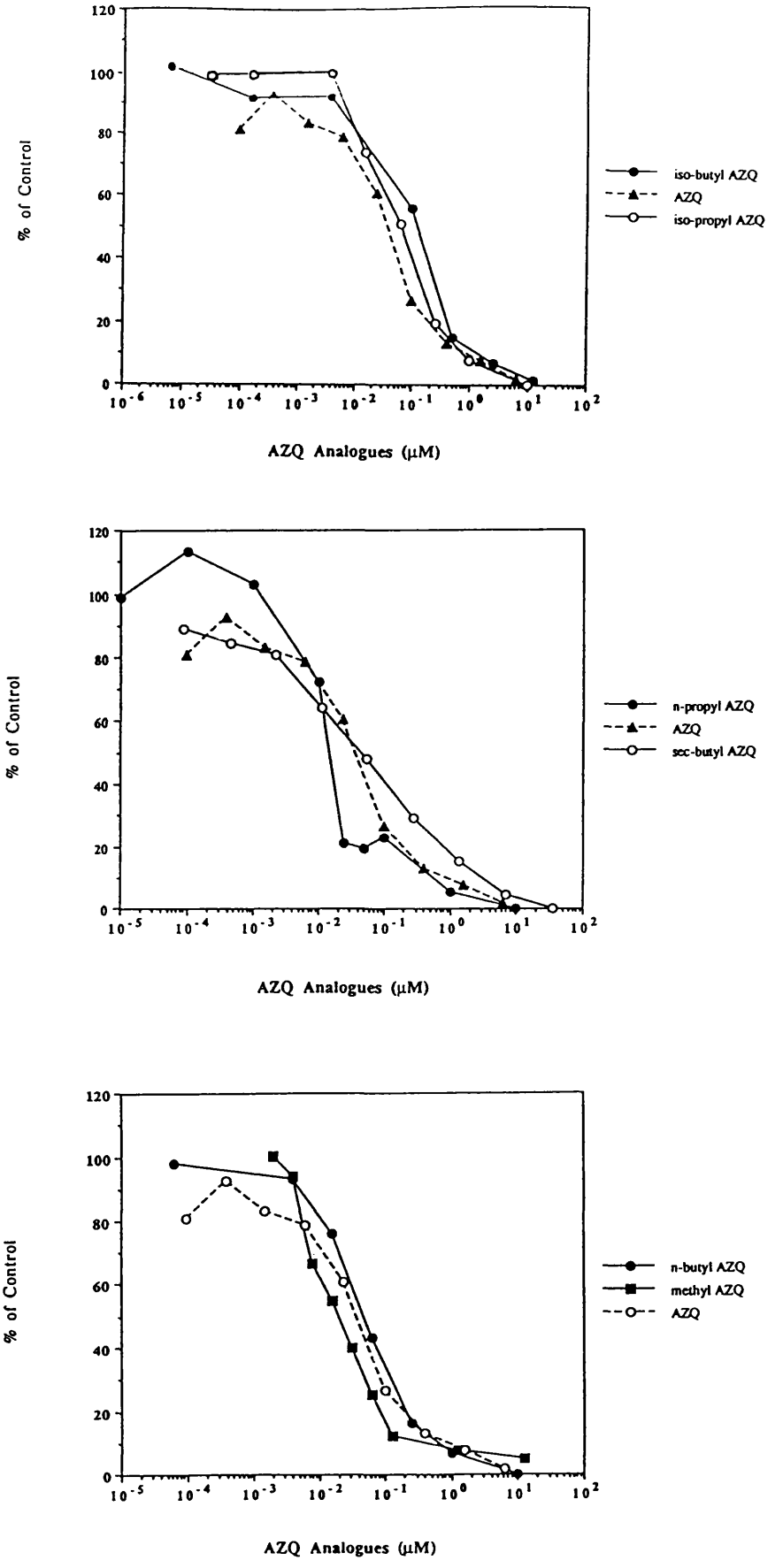


Fig. 7.1 Concentration - response curves of aziridinylbenzoquinones as determined by MTT assay. Data are taken from individual experiments but are representative of 4 - 10 independent repeats (see Table 7.2).

Drug	5μM Dicoumarol			1μM Dicoumarol		
	Mean DMF	Standard Error	Sample No. (n)	Mean DMF	Standard Error	Sample No. (n)
AZQ	1.80	0.314	5	1.76	0.704	3
methyl AZQ	1.65	0.437	3	1.17	*	2
iso-butyl AZQ	1.86	0.406	6	2.49	0.938	5
iso-propyl AZQ	2.32	*	2	0.57	*	1
n-propyl AZQ	2.01	*	2	1.20	*	1
sec-butyl AZQ	3.02	1.071	3	1.00	*	2
n-butyl AZQ	2.99	0.776	4	2.14	0.930	3

Table 7.3 Dicoumarol modulation of aziridinylbenzoquinone cytotoxicity in rat Walker tumour cells. Data are presented as dose modification factor (DMF) with standard error and sample number (n). The symbol * indicates that experimental repeats were curtailed by limited drug supply.

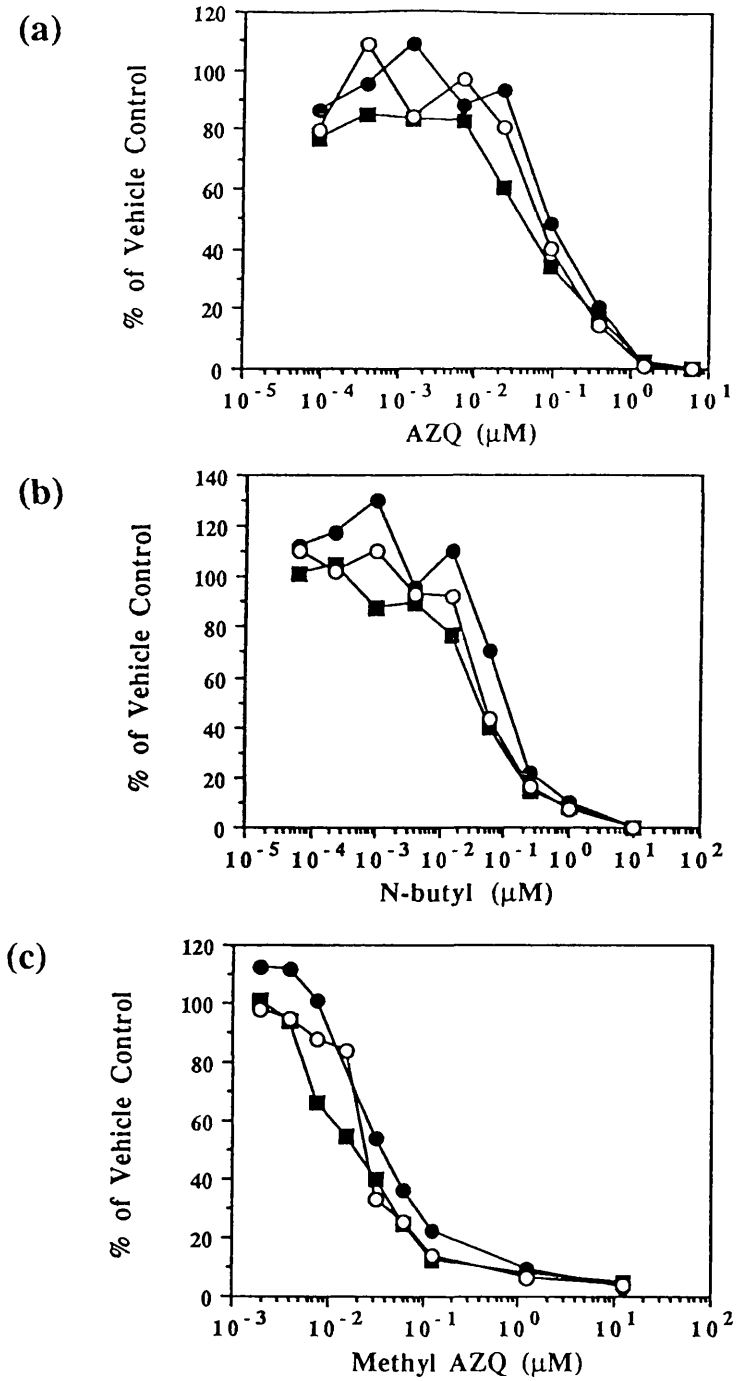


Fig 7.2 Typical concentration - response curves of AZQ analogues (a) AZQ (b) *n*-butyl AZQ and (c) methyl AZQ and their modulation by dicoumarol. Data are taken from an individual experiment and are representative of at least 3 independent repeat experiments with the exception of the curve for 1 μM dicoumarol inhibition of methyl AZQ which was repeated twice.

—■— No Dicoumarol —●— 5 μM Dicoumarol

—○— 1μM Dicoumarol

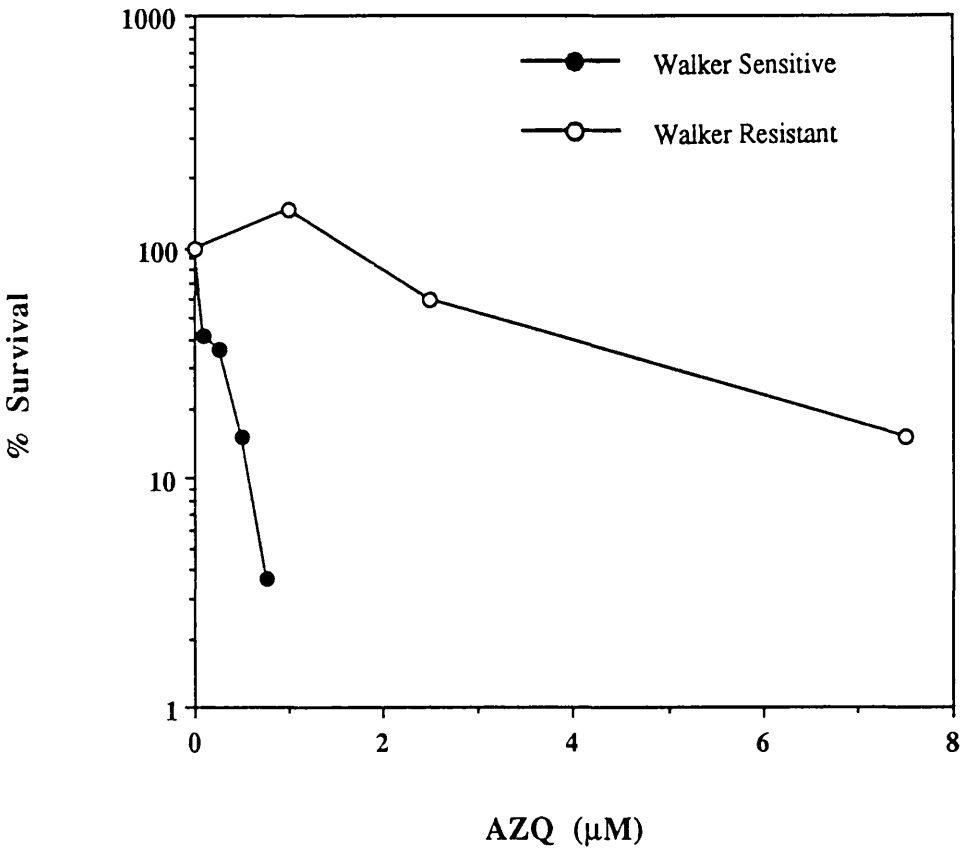


Fig 7.3 Colony forming ability of rat Walker Resistant and Walker Sensitive tumour cells following exposure to AZQ.

carcinoma cells (Gibson *et al*, 1992) and the leukaemic K562, BSM, L1210 and RAJI cell lines (Butler *et al*, 1990). The order of cytotoxicity obtained in these experiments varied to some extent from data presented in this chapter using the Walker cell line. However, broadly consistent with the present data, Gibson and co-workers reported only a small degree of variation in potency between the analogues with *iso*-propyl AZQ being the least toxic. Minor alterations in the order of cytotoxicity are understandable in view of the fact that there were differences in both the species of origin and nature of cell lines used as well as in the experimental conditions employed. Indeed, experiments with EO9 analogues presented earlier in this thesis (Chapter 4) have shown differences in cytotoxicity between human HT29 cells and the rat Walker cell line with the latter being more sensitive. The cytotoxicity experiments reported in this chapter have used the MTT assay following a 72 hour drug exposure and IC_{50} values were used for comparison. Those obtained by Gibson and co-workers (1992) were IC_{90} values calculated from clonogenic assay with a 2 hour exposure. Exposure time has been reported to be an important factor in determining the toxicity of aziridinylbenzoquinones. Around a 50 fold difference was observed in the ID_{50} value for AZQ in the K562 leukaemic cells when drug incubation time increased from 1 hour to 5-7 days (Butler *et al*, 1990). In contrast O'Brien and co-workers (1990) have reported very little increase in toxicity between a 24 hour and 7 day exposure in CHO AA8-4 cells.

Enzymology studies described earlier in this thesis (Chapter 6) revealed that purified rat Walker DT-diaphorase was able to reduce AZQ very efficiently and that structural modification significantly altered the rate of this metabolism. For this reason it was considered important to investigate a potential role for DT-diaphorase mediated metabolism in the activation of the aziridinylbenzoquinones to a cytotoxic species. An initial indication of such an involvement was obtained by comparing the cytotoxicity of the analogues in the rat Walker cell line with their ability to be metabolised by DT-diaphorase purified from the same cell line (Chapter 6). The more toxic *iso*-butyl and methyl analogues were amongst the more favourably metabolised whereas the least toxic compound *iso*-propyl AZQ was the most poorly reduced (when assayed at 400μM concentration). This would support a role for DT-diaphorase in the reductive activation and cytotoxicity of the aziridinylbenzoquinones in the Walker cell line. Other analogues examined exhibited intermediate cytotoxicity and in most cases reduction rates were between these two extremes. Despite the overall correlation, the order does not equate precisely with either their ability to undergo DT-diaphorase metabolism or with their order of reduction as predicted from one-electron reduction potentials (see Chapter 6). The results obtained

are similar to the overall correlation reported during the course of this work by Gibson and co-workers (1992). Although finding a different order of toxicity they also saw no absolutely precise correlation with either DT-diaphorase reduction or one-electron reduction potentials. It should be emphasised that they compared cytotoxicity in human HT29 colon carcinoma cells with reduction by a purified rat hepatic enzyme. Previous studies using CB 1954 (Boland *et al*, 1991) and EO9 (Walton *et al*, 1991 and Chapter 3 of this thesis published in Bailey *et al*, 1992) have reported differences in metabolism by the rat and human enzyme. Within the cell a number of additional factors will influence cytotoxicity including drug uptake and efflux as well as levels of activating and protective enzymes which may vary in their affinities for different substrates. For this reason we would not necessarily expect a perfect correlation between DT-diaphorase reduction and cytotoxicity. Nevertheless, given the relatively narrow range of cytotoxicities and enzyme reduction rates seen with the range of AZQ analogues used, the overall correlation was quite reasonable and supportive of a role for DT-diaphorase in the activation mechanism.

To clarify further the role of DT-diaphorase metabolism in the cytotoxicity of the aziridinylbenzoquinones the DT-diaphorase inhibitor dicoumarol was included in some of the cytotoxicity assays. The data obtained illustrated that dicoumarol, when applied at a sub-cytotoxic concentration, was able to modulate the toxicity of the compounds towards rat Walker cells to a modest extent (up to 3 fold) (Table 7.2). The level of modulation observed was however consistent with that obtained using menadione in hepatocytes (Thor *et al*, 1982) and C3H/10$T_{1/2}$ mouse embryo fibroblast cells (Atallah *et al*, 1987). In general dicoumarol decreased the cytotoxicity exerted by the aziridinylbenzoquinones thus implicating DT-diaphorase in the activation of these compounds (Fig 7.2). Although the protective effect appears quite modest, similar degrees of protection were observed when 20 μM dicoumarol was applied to HT29 cells (Gibson *et al* 1992). An exception to this observation was the *n*-butyl analogue which exhibited a 6 fold protective effect in the HT29 cell line (Gibson *et al*, 1992). Interestingly this analogue also manifested the highest degree (3 fold) of protection in the Walker cell line in the present experiments (Table 7.3). This greater modulation of cytotoxicity in the presence of dicoumarol would suggest that DT-diaphorase activation of *n*-butyl AZQ is more heavily involved in the cytotoxic mechanism of the compound. Consistent with this hypothesis is the observation that *n*-butyl AZQ was the most efficiently metabolised of all the analogues by both the purified rat Walker DT-diaphorase (Chapter 6) and rat hepatic DT-diaphorase (Gibson *et al*, 1992).

As in the case of EO9, the chemistry of the aziridinylbenzoquinones would suggest various possible mechanisms of cytotoxicity including oxidative stress following redox cycling, alkylation and drug free radical damage. The differential toxicity observed between the Walker Resistant and Sensitive cell lines would imply that AZQ could act as a bifunctional alkylating agent (Fig 7.3). Indeed, examination of a number of cell lines following exposure to AZQ has revealed the presence of both DNA interstrand cross-links and strand breaks (Szmigiero *et al,* 1984; King *et al,* 1984) with the nature and extent of these two types of damage varying greatly between different cell lines. Cytotoxicity has been correlated with the formation of interstrand cross-links but not of strand breaks (Szmigiero *et al,* 1984). Evidence of DNA-protein cross-links in CHO-K1 cells treated with AZQ has also been reported (King *et al,* 1984). In addition they found more rapid repair of strand breaks than of cross-links in this cell line.

Experiments using chemical reducing agents have indicated that reduction may enhance cytotoxicity of AZQ in intact cells (Section 7.1). Indeed in cell free systems a number of authors have reported that either chemical reduction (Szmigiero and Kohn, 1984; King *et al* , 1984; Gutierrez *et al,* 1986; Butler *et al,* 1989) or enzymatic reduction (Fisher and Gutierrez, 1991a and b) is required for the induction of DNA damage. A recent publication by Lee and co-workers (1992) has reported an increase of DNA interstrand cross-links induced by these aziridinylbenzoquinones following reduction catalysed by DT-diaphorase. Further evidence has come from the detection of the semiquinone, the one-electron reduced metabolite, in intact cells by ESR as well as following reduction by purified enzymes (Egorin *et al,* 1985; Gutierrez *et al,* 1986; Lea *et al,* 1988; Butler *et al,* 1990; Dzielendziak *et al,* 1990). The semiquinone radical is potentially a DNA damaging species and has been implicated in the toxicity of AZQ (King *et al,* 1984). Some authors report a lack of correlation between semiquinone radical detection and cytotoxicity (Lea *et al,* 1988) although later reports suggest that this may be due to efflux of this metabolite from the cell (Butler *et al,* 1989). The precise nature of the cytotoxic species is as yet unclear and may vary between cells.

It is possible that both hypoxia and pH may influence the cytotoxicity of the aziridinylbenzoquinones. The few publications that have documented the effect of hypoxia on AZQ cytotoxicity have shown a minimal effect *in vitro* and *in vivo* (Powis, 1987; O'Brien *et al,* 1990; Rauth and Marshall, 1990). For this reason and the limited amount of compounds available the effect of oxygen concentration was not examined in this study. Decreasing the pH increases AZQ-induced DNA damage

(e.g. Szmigiero and Kohn, 1984; King *et al*, 1984; De Mol *et al*, 1988) as well as that of the analogues reported in this chapter following reduction by DT-diaphorase (Lee *et al*, 1992). The effect of pH could in theory be due to either an effect on DT-diaphorase or alternatively a direct activation of the aziridines.

To summarise, reductive activation has been implicated in the cytotoxicity of AZQ and the use of cell free systems has permitted identification of enzymes which may be involved in this process. Those enzymes implicated to date include NADPH: cytochrome P450 reductase and DT-diaphorase. The experiments reported in Chapters 6 and 7 of this thesis provide further evidence for the metabolism of aziridinylbenzoquinones by DT-diaphorase. In addition they indicate that this reduction process may be an important factor in determining the cytotoxicity of the compounds. The incomplete protection of toxicity conferred by dicoumarol would suggest additional involvement of other activating enzymes. However the problems of using this inhibitor and the caution required when interpreting results has been discussed in detail in Chapter 3. Structural modification of AZQ clearly plays a role in the ability of the aziridinylbenzoquinones to be metabolised by DT-diaphorase and in their cytotoxicity. The data obtained would suggest a greater importance for DT-diaphorase in the activation of the *n*-butyl analogue but that it may be less relevant for other compounds such as *iso*-propyl and *sec*-butyl AZQ which are poorer substrates.

Despite the relationship between DT-diaphorase metabolism and cytotoxicity the aziridinylbenzoquinones are likely to exert their toxicity through a variety of mechanisms which may involve different metabolites. The particular mechanism of activation and cytotoxicity will depend on the chemistry of the individual analogue as well as the levels of activating and protective enzymes present in the various cell types.

Chapter 8

Discussion

Over the years the field of cancer chemotherapy has seen significant advances in the treatment of specific tumours. Cancer does however remain a major cause of mortality world-wide emphasising a need for innovative new drug development. Traditional, empirical methods of drug discovery are now being superseded by more rational approaches which exploit new, potentially more selective targets. It is hoped that this change of strategy will result in the identification of novel agents which possess an enhanced therapeutic index over those compounds currently available.

Solid tumours are largely incurable by radiotherapy and conventional chemotherapy. This is thought to be at least in part due to the presence of resistant hypoxic cell populations (Coleman, 1988). Hypoxia does however provide a selective target for bioreductive anticancer agents which are prodrugs that undergo metabolism to generate oxygen-sensitive cytotoxic metabolites (Workman and Stratford, 1993). In addition some solid tumours have been shown to express increased levels of certain reducing enzymes involved in activation of these compounds, thus providing a second level of selectivity (Workman and Walton, 1990).

DT-diaphorase has been reported to be capable of both activating and detoxifying quinone compounds and to have increased levels and activity in a number of solid tumours when compared with normal tissue of the same origin (Riley and Workman, 1992b). For these reasons the work in this thesis was designed to investigate a role for DT-diaphorase in the activation and cytotoxicity of bioreductive anticancer agents. The investigation has focused on two classes of compound: the novel indoloquinones related to EO9 (Oostveen and Speckamp, 1987) and aziridinylbenzoquinone analogues of AZQ (Dzielendziak and Butler, 1989; Dzielendziak *et al*, 1990). Results of studies carried out previously had suggested that both EO9 (Walton *et al*, 1991 and 1992) and AZQ (Siegel *et al*, 1990a) could undergo metabolism catalysed by DT-diaphorase although at the outset of this project there had been no publications documenting reduction of analogues.

The initial aims of the work were therefore firstly to confirm that DT-diaphorase could catalyse metabolism of these categories of bioreductive anticancer agents and

secondly to determine their cytotoxic potency. This latter property could then be correlated with the ability to undergo reduction in an attempt to determine whether DT-diaphorase-catalysed metabolism represented a major activation or detoxification process.

Quinones may undergo one-electron reduction catalysed by a variety of enzymes such as cytochrome P450 reductase to generate the corresponding semiquinone radical (see review by Powis, 1989). These radicals are in general highly oxygen sensitive and under aerobic conditions are auto-oxidised to generate the parent quinone. Further rounds of reduction and oxidation then occur in a process known as redox cycling with the concomitant generation of oxygen radicals. These latter species may cause DNA damage in the form of strand breaks, in addition to a multitude of other effects which lead to a potentially lethal condition known as oxidative stress. In the absence of air the semiquinone is likely to be stabilised and further reduced to generate the hydroquinone. For a number of standard quinones (e.g. menadione) the hydroquinone is more readily conjugated (e.g. to the sulphate or glucuronide) and subsequently eliminated and is therefore less toxic than the semiquinone radical (Buffington *et al*, 1985). DT-diaphorase catalyses two-electron reduction of quinones to generate the hydroquinone directly, thus bypassing the toxic radical-producing reactions. For this reason it is often considered to be a detoxification enzyme under aerobic conditions. However, some quinone compounds have two-electron reduced metabolites which are also highly oxygen sensitive and may participate in redox cycling. In addition a number of compounds possess substituents such as the aziridine moiety and / or leaving groups which following reduction to either the semiquinone or the hydroquinone are activated to alkylating species. Thus for these compounds two-electron reduction catalysed by DT-diaphorase as well as one-electron reduction, by for example NADPH: cytochrome P450 reductase, may lead to activation rather than detoxification. Mitomycin C, the prototype bioreductive alkylating agent, is one such compound (Ross *et al*, 1993). It contains an aziridine group which, after reduction, is activated to an alkylating moiety capable of producing an initial monoadduct; following that further rearrangement results in a crosslink in the minor groove of DNA. This is thought to be a major mechanism of mitomycin C induced cytotoxicity. The structure of EO9 is based on that of mitomycin C and it was therefore considered likely that its mechanism of cytotoxicity would also involve DNA damage. The quinone group confers the ability for redox cycling and radical production whereas the aziridine moiety and two leaving groups on the sidechains permit activation to an alkylating agent. An additional aim of this thesis was therefore to investigate DNA damage as a possible mechanism of cell kill of EO9. By using cell free systems the

involvement of particular enzymes such as DT-diaphorase in this process could be elucidated. Equivalent studies to determine the mechanism of AZQ induced cytotoxicity were performed during the course of this work (Siegel *et al*, 1990a).

The ability of DT-diaphorase to metabolise bioreductive agents was determined primarily by the highly sensitive spectrophotometric, cytochrome *c* reduction assay which was selected for the reasons discussed in Chapter 3. HPLC and spectrophotometric monitoring of the direct loss of cofactor were also employed to further confirm findings. Results showed that DT-diaphorase present in sonicates of both rat Walker and human HT29 colon carcinoma cells could catalyse metabolism of EO9 very efficiently. Data compared well with those obtained by Walton and co-workers (1991) using Walker tumour cell preparations and with preliminary findings of these authors using the HT29 cell sonicates (Walton *et al*, 1992b). In order to determine the extent of activity attributable to DT-diaphorase the widely used reagent dicoumarol was included as an inhibitor. It should be noted however that this compound has subsequently been found to also inhibit a number of other enzymes including xanthine oxidase and dehydrogenase (Gustafson and Pritsos, 1992b), carbonyl reductase (Wermuth, 1981), NADH cytochrome b_5 reductase (Hodnick and Sartorelli, 1993) and is therefore less specific than was originally thought. A purified form of rat Walker DT-diaphorase was therefore used to provide unambiguous evidence and to determine kinetic parameters. Studies with sonicates of tumour cells may however provide valuable information not obtainable with a purified enzyme system. They may more closely reflect the natural environment within the cell and allow examination of drug reduction by a particular enzyme in a situation where competing enzymes are also present. For these reasons the metabolism of EO9 was compared with both purified DT-diaphorase and enzyme-rich sonicates.

Data obtained with EO9 using tumour cell sonicates suggested that the rat Walker cells were more efficient than the human HT29 cells in terms of EO9 reduction. This would either indicate that the rodent enzyme was more active than human DT-diaphorase or that DT-diaphorase was present at higher concentrations in the rat cells per mg of protein than in human HT29 cells. As discussed in Chapter 3 further experiments are required to clarify this issue by a direct comparison of rat and human enzyme preparations. This issue is currently being addressed by Dr. A. Lewis in our laboratory using recombinant rat and human DT-diaphorase produced in *E.coli.* Results of these experiments suggest that the rat form of the enzyme is approximately 7 times more active than the human form with regard to EO9 reduction (Lewis *et al*, 1994). Similar quantitative differences in activity between the rat and human forms

of DT-diaphorase have previously been reported for the dinitrophenyl aziridine compound CB 1954 (Boland *et al*, 1990). This has been suggested to be the reason for the high therapeutic index of CB 1954 in rodent tumours in contrast to the apparent lack of activity in a human clinical trial.

Work carried out in this thesis using purified rat Walker enzyme confirmed that both EO9 and AZQ were able to act as excellent substrates for DT-diaphorase. Activity was only 2-5 fold poorer than the conventional substrate menadione in the case of EO9 and ~200 fold poorer for AZQ when considering Vmax values. Both compounds were far more efficiently metabolised than the prototype bioreductive agent mitomycin C. During the course of this work Siegel and co-workers (1990) also reported that purified rat hepatic DT-diaphorase was able to catalyse metabolism of AZQ.

In the case of EO9, HPLC data confirmed that purified rat Walker DT-diaphorase was able to catalyse reduction of the compound and also indicated that a highly oxygen sensitive metabolite was formed. Further evidence for the generation of an oxygen sensitive metabolite came from ESR studies where both oxygen radicals and a drug-based radical, almost certainly the semiquinone, were detected when EO9 was incubated under aerobic conditions with DT-diaphorase in the presence of cofactor. Similar results also from ESR experiments had previously been obtained for AZQ (Siegel *et al*, 1990a; Fisher and Gutierrez, 1991a). Thus it appears that DT-diaphorase may catalyse reduction of both EO9 and AZQ to their corresponding hydroquinone form which is then auto-oxidised in the presence of molecular oxygen to the parent quinone via the semiquinone intermediate. Other possibilities are that DT-diaphorase catalyses two-electron reduction by rapid successive one-electron reduction steps which are nevertheless detected by ESR, or alternatively that comproportionation : disproportionation reactions are occurring.

Bioreductive agents are designed to have specificity for hypoxic tumours. Studies carried out in our own laboratory and by others have shown increases in cytotoxic potency of both EO9 (Hendriks *et al,* 1993; Plumb and Workman, 1994; Robertson *et al*, 1994; Plumb *et al*, 1994a and b) and AZQ (O'Brien *et al*, 1990; Rauth and Marshall, 1990) under hypoxic compared with aerobic conditions. As DT-diaphorase may be involved in the activation of EO9 to a cytotoxic species the influence of hypoxia on the rate of DT-diaphorase-catalysed reduction of EO9 was investigated. Results obtained using purified rat Walker tumour DT-diaphorase showed that the initial velocity for reduction of EO9 occurred at an identical rate in the presence and

absence of air as detected by the cytochrome *c* reduction assay. Assays with AZQ revealed similar findings. Other investigators have reported that the initial reduction of mitomycin C (Siegel *et al*, 1990b), CB 1954 (Knox *et al*, 1988a) and AZQ (Siegel *et al*, 1990a) is similarly unaffected by oxygen. Nevertheless, due to the oxygen sensitivity of the DT-diaphorase metabolites it is likely that the oxygen status of the tumour will influence the cytotoxicity of EO9 by affecting the stability of these species, despite the oxygen-independence of the initial reduction rate.

In addition to being hypoxic, tumours have been considered in the past to be acidic in nature. This has however been open to controversy and current opinion suggests that whereas extracellular pH may be slightly acidic due to increased lactic acid production the intracellular pH of the tumour remains well regulated (Griffiths *et al*, 1991; 1993). Modification of pH has been shown to affect DT-diaphorase-mediated reduction of mitomycin C (Siegel *et al*, 1990b; Keohane *et al*, 1990) and also the cytotoxicity of EO9 (Phillips *et al*, 1992) and mitomycin C (Kennedy *et al*, 1985). It should be noted that the pH may affect the stability of the quinone prodrug before reduction and thus alter its ability to act as a substrate and the nature of the metabolite produced following drug reduction. For example low pH may lead to protonation of the aziridine ring which may enhance its activation to an alkylating moiety and increase its cytotoxic potency, independently of enzyme activation. In addition, the pH of the cell may directly affect the enzyme, thereby modifying its catalytic ability. Due to the possible relevance of pH to drug activation within tumours, the effect of pH on DT-diaphorase metabolism of EO9 was examined. Using the spectrophotometric cytochrome *c* reduction assay no difference in the rate of metabolism was observed over the pH range 5.8 to 7.8 with EO9. Interestingly, no effect was seen in our experiments with mitomycin C using this assay system. Siegel and co-workers (1990b; Ross *et al*, 1993) proposed that the quinone methide is the metabolite formed by DT-diaphorase-catalysed reduction of mitomycin C and that this alkylates the enzyme, thereby inactivating it. Under acidic conditions this may be converted to 2,7-diaminomitosene which is released from the enzyme-substrate complex thus permitting further metabolism. It is likely that the short time course of the spectrophotometric assay does not permit enzyme inactivation to be detected and thus no benefit of decreasing pH was observed. In contrast, Siegel and co-workers (1990b) measured the production of metabolites by HPLC after more prolonged incubation. This difference could be tested by preincubating mitomycin C with NADH and the enzyme before measuring reduction rate in the cytochrome *c* reduction assay.

Structural modification of compounds often leads to alteration of a number of characteristics including their cytotoxicity and ability to be metabolised. It is therefore important to determine the structure-activity relationships of anticancer compounds in order to improve drug design for increased cytotoxicity and selectivity. This may be particularly useful in the so-called 'enzyme-directed' approach to drug development (Workman and Walton, 1990; Workman, 1992a; Workman, 1994) by enabling optimisation of drug structure to suit the catalytic preferences of key bioreductive enzymes. A number of analogues of both EO9 and AZQ were made available to us. For both classes of compound, structural modification was found to alter the rate of DT-diaphorase-catalysed reduction. In the case of the indoloquinone analogues too few compounds were analysed to give any conclusive structure-activity relationships but certain important observations can be made nevertheless. It does for example appear that the aziridine ring-opened form of the compound EO5A is very poorly metabolised by DT-diaphorase. Similar results have been reported for CB 1954 (Knox *et al*, 1988b). This suggests that the aziridine function may undergo a desirable interaction with the active site of DT-diaphorase. EO7, the analogue where the aziridine ring is replaced by a methoxy group was however only 2-fold less active a substrate for DT-diaphorase than was EO9 itself when considering Vmax values. This indicated that possession of an intact aziridine was not a structural requirement for activation catalysed by this enzyme. In contrast, EO8 which possessed a modified aziridine ring in the form of a methyl aziridine was 15-20 fold more poorly metabolised than EO9. Interestingly, altering the hydroxyl leaving group of EO9 to a carbamate in the case of EO88 led to an increase in rate of reduction. As regards the aziridinylbenzoquinones, analogues available for study exhibited modifications of the side chain rather than the aziridine group. Little difference was observed between the ability of these analogues to undergo reduction catalysed by DT-diaphorase and thus no clear structure-activity relationships were evident. The order of activity at a concentration of 400 μM compound was, from most to least active, *n*-butyl > methyl > *iso*-butyl > *n*-propyl > ethyl (AZQ) > *sec*-butyl > *iso*-propyl. This order differed to only a small extent from that obtained during the course of this work with the purified rat hepatic enzyme by Gibson and co-workers (1992).

One of the properties which might potentially affect the rate of reduction of quinones is the redox potential. When comparing the half-wave ($E_{½}$) reduction potentials provided for the indoloquinones with the order of DT-diaphorase-catalysed reduction no direct correlation was observed. Similarly the one-electron reduction potential of the azirdinylbenzoquinones did not directly follow their ability to undergo DT-diaphorase reduction. In the latter case reduction potential was expressed as an E^1_7

thermodynamically precise and therefore.
value determined by pulse radiolysis. This is believed to be a more reliable measure of reductive capacity than $E^1/_2$. It might be argued that it would have been more correct to compare DT-diaphorase catalysed reduction with two-electron reduction potentials. On the other hand, DT-diaphorase catalyses two rapid but sequential one-electron reduction steps, and so it could equally well be argued that the one-electron reduction step is in fact appropriate. In practice the two-electron reduction potentials were not available; moreover, the one and two-electron reduction potentials are thought to follow a similar trend. A lack of correlation with reduction potential has been reported by other authors for aziridinylbenzoquinones (Gibson *et al*, 1992), 1,4-napthoquinones (Buffington, 1989) and quinone epoxides (Brunmark, 1988). It should be pointed out, however, that the reduction potentials of both EO9 and AZQ analogues were within a fairly narrow range ($E^1/_2$ values of -367 to -380 for the indoloquinones and E^1_7 values ranging from -44 to -161 for the aziridinylbenzoquinones) and it is possible that an effect might have been seen had a broader range of values been studied. Nevertheless it does seem likely that the reduction of quinones by DT-diaphorase is much less redox-dependent than more classical one-electron reducing enzymes such as cytochrome P450 reductase (Workman, 1994). In addition other properties are likely to be important such as lipophilicity and steric factors including in particular substrate fit into the active site of the enzyme. Thus we can conclude that in future drug design related to DT-diaphorase, electron reduction potentials may not be an important consideration providing they do not vary beyond the range studied here, while the rate of reduction can still be modified by changing the pendant groups in relatively subtle ways. Thus the 'enzyme-directed' bioreductive drug design approach is particularly suited to DT-diaphorase.

The cytotoxic potency of the indoloquinones and aziridinylbenzoquinones was determined by the MTT dye reduction assay. In the case of the indoloquinones the results were confirmed by other techniques. The human HT29 colon carcinoma and rat Walker mammary tumour cell lines were selected for analysis because of their high expression of DT-diaphorase and the fact that they were the source of enzyme for the metabolism studies described in this thesis. EO9 was found to exhibit highly potent cytotoxicity in both the HT29 and Walker cell lines compared to that shown by a number of other commonly used anticancer drugs in both these and other cell lines. This level of cell kill is consistent with reports of other investigators for EO9 in HT29 cells (Hendriks *et al*, 1993; Phillips *et al*, 1992; Plumb and Workman, 1994; Robertson *et al*, 1994; Van Ark-otte, 1994). Results presented in this thesis also

showed AZQ to act as a highly potent cytotoxin to the Walker cell line although these particular cells were more sensitive to EO9.

As for DT-diaphorase metabolism, modifying the structure of EO9 led to alteration of cytotoxic potency in both HT29 and Walker cells. Structural modification also resulted in a slight alteration in the cytotoxic potency of the azirdinylbenzoquinones in Walker cells. For the indoloquinones the variation between IC_{50} values for the analogues was fairly large. This ranged from 15 nM for EO9 to >326 μM for EO5A (the aziridine ring opened metabolite) in HT29 cells and from 46 pM for EO9 to 1.43 μM in the case of EO7 (the analogue where the aziridine of EO9 has been replaced by a methoxy group) in the Walker cells. In contrast the IC_{50} values of the aziridinylbenzoquinones were more similar to each other with the *iso*-butyl and *n*-propyl analogues being most toxic, having an IC_{50} value of 15 nM, and the *iso*-propyl analogue being least toxic with an IC_{50} value of 44 nM. This finding was consistent with data obtained during the course of this work by Gibson and co-workers (1992) using the HT29 cell line.

Determination of both DT-diaphorase-catalysed reduction rates and cytotoxicity for the same analogues allows the relationship between the two parameters to be assessed. Interestingly, analogues which were more efficiently metabolised by DT-diaphorase were in general the more toxic; for example the indoloquinones EO9 and EO88 (the analogue with a carbamate leaving group in place of the side-chain hydroxyl group of EO9) were the most rapidly metabolised and the most cytotoxic whereas the more poorly metabolised analogue EO5A was the least toxic. Similarly for the aziridinylbenzoquinones methyl and *iso*-butyl AZQ were efficiently metabolised and exhibited greater toxicity whereas the least toxic analogue *iso*-propyl AZQ was amongst the more poorly metabolised. This would support a potentially important role for activation of these agents by DT-diaphorase. These data are again consistent with findings of Gibson and co-workers regarding the aziridinylbenzoquinones (1992). The cytotoxic potency of compounds is however unlikely to be solely a reflection of reductive capacity but will be influenced by the levels of other activating and protective enzymes within the cell as well as by physico-chemical factors such as solubility and lipophilicity.

A property which may well be affected by structural modification is the extent of reaction with DNA, either before or after enzymatic activation. The aziridine moiety is generally considered to confer alkylating ability following activation and therefore to increase cytotoxicity of quinone bioreductive agents. This moiety remained

unmodified in the aziridinylbenzoquinones analysed. In the case of the indoloquinones, analogues possessing this group were in general the more potent whereas EO5A, the aziridine ring-opened compound, was ~ 21,000 times less toxic in the HT29 cell line. This clearly supports the involvement of the aziridine group in the mechanism of toxicity. A few compounds such as EO7 were however exceptions, lacking the aziridine but retaining a considerable degree of potency, but not so great as with EO9 itself. Interestingly a difference in sensitivity to some indoloquinone compounds but not others was observed between the rat Walker and human HT29 cell line. It is possible that this may reflect different mechanisms of cytotoxicity of the various analogues. Walker cells are known to be particularly sensitive to difunctional alkylating agents and thus compounds which have an increased cytotoxic potency in this cell line may cross-link DNA. Alternatively, it may involve interspecies differences in structural preferences of DT-diaphorase. Clearly it is possible that the aziridine group is favoured because it encourages both efficient metabolism and also is involved in DNA interactions. In order to test these hypotheses and to draw further deductions regarding structure-activity relationships additional analogues need to be examined. The results described here do, however, provide a useful lead into this area of investigation.

Results of structure-activity relationship studies presented in this thesis (Bailey *et al*, 1992) provided the first evidence to link cytotoxic potency of the indoloquinones with their ability to undergo reduction catalysed by DT-diaphorase. Further indication of the importance of DT-diaphorase in the activation of both the aziridinylbenzoquinones and indoloquinones came from comparisons of the sensitivity of two human colon carcinoma cell lines to these agents. EO9 was 15 to 30-fold more toxic to HT29 cells which express high levels of DT-diaphorase than to BE cells which do not possess a functional form of this enzyme because of a point mutation in the NQO1 gene (Walton *et al*, 1992a; Plumb and Workman, 1994). Similarly AZQ showed a 2 fold differential between the two cell lines at 80% cell kill (Siegel *et al*, 1990a). HT29 and BE cells exhibit similar levels of other activating enzymes such as cytochrome P450 reductase (Siegel *et al*, 1990a) but differ in their expression of the DNA repair enzyme O6-alkyl guanine alkyl transferase. However, this is unlikely to affect their sensitivity to EO9 as discussed previously in Chapter 4 of this thesis. Subsequently, a number of studies have been carried out by various laboratories on panels of human and rodent cell lines where the levels of DT-diaphorase and other bioreducing enzymes have been correlated with sensitivity to EO9. Robertson and co-workers (1992) found a link between the cytotoxic potency of EO9 and its ability to undergo metabolism catalysed by DT-diaphorase in 15

human tumour cell lines derived from breast, lung and colon. This study was later updated (Robertson *et al*, 1994) to include additional human cell lines as well as 8 rodent cell lines. The correlation was still retained. Experiments carried out by the Bradford (UK) group at around the same time (Collard and Double, 1992; Phillips *et al*, 1992) again indicated a link between DT-diaphorase and activation of EO9, as did those of Plumb and co-workers (1994a; 1994b), Smitskamp-Wilms *et al* (1994) and the NCI-Glasgow collaboration (Hendriks *et al*, 1993; Paull *et al*, 1994; Fitzsimmons *et al*, 1994)

In order to help clarify a possible involvement of DT-diaphorase in the cytotoxic mechanism of indoloquinones and aziridinylbenzoquinones the enzyme inhibitor dicoumarol was included in toxicity assays. Only a small degree of modulation was noted, as observed also by Gibson and co-workers (1992) with the aziridinylbenzoquinone analogues. Interestingly the data presented in Chapter 7 of this thesis and those reported by Gibson and colleagues show a more significant modulation of *n*-butyl AZQ cytotoxicity by dicoumarol than is the case for the other analogues. Notably, this was also the compound most rapidly metabolised by rat Walker DT-diaphorase (Chapter 6) and the rat hepatic enzyme (Gibson *et al*, 1992). This appears to confirm the view that DT-diaphorase may be equally important in activation of the more efficiently metabolised analogues such as *n*-butyl AZQ but is less important for others. There are however a number of problems associated with use of dicoumarol in cytotoxicity studies, as discussed in Chapter 4. Hence data obtained using this inhibitor should not be relied upon too strongly . Another approach could be to carry out cytotoxicity assays on cell lines such as HT29 following addition of various enzyme inducers. It is however likely that this would give co-induction of other enzymes, resulting in difficulty in dissecting out specific changes due to DT-diaphorase. A more favourable alternative approach to answer this question would be to transfect a cell line expressing low levels of DT-diaphorase with the cDNA for the enzyme under the transcriptional control of a specific inducible promoter. Sensitivity of the cell line to the agents could then be examined following selective induction of DT-diaphorase.

The chemistry of EO9 suggests that its mechanism of cytotoxicity may involve DNA damage. Additional support for this hypothesis came from experiments described in this thesis on the cytotoxicity of indoloquinones against the Walker cell lines sensitive and resistant to bifunctional alkylating agents. These cell lines showed a differential toxicity towards EO9 and EO88, a feature which is commonly diagnostic of interstrand cross-links (Knox *et al*, 1991). EO88 differs from EO9 only in the

substitution of one of its hydroxyl leaving groups with a carbamate moiety and thus, like EO9, bears three potential alkylating sites when activated which could in theory form cross-links with DNA. As a result of the indication that these two indoloquinones were able to induce DNA interstrand cross-links, the extent of DNA damage induced by cytotoxic concentrations of EO9 in whole cells was investigated by the technique of alkaline elution. Walker cells which possess high levels of DT-diaphorase were found to contain both DNA strand breaks and interstrand cross-links following treatment with EO9. Visualisation of the cross-links by alkaline elution required dose optimisation. This involved 4 repeat applications of EO9 at 4 hourly intervals followed by a further 12 hour incubation, probably permitting repair of strand breaks and accumulation of less readily repaired interstrand cross-links. This finding may have important clinical implications if DNA damage is confirmed to be responsible for EO9-induced cell kill. DNA interstrand cross-links have often been considered to be a more toxic form of DNA damage than single strand breaks. Thus it is possible that an increase in tumour cell kill may be achieved by administering the drug by continuous infusion or multiple doses rather than by a single high dose regimen. This proposal requires further investigation using *in vivo* tumour models.

The ESR experiments presented in this thesis (Chapter 3) have shown that the reduction of EO9 by DT-diaphorase generated both oxygen radicals and a drug-based radical, both of which have the potential to cause DNA strand breaks. It is therefore likely that the DNA damage detected in DT-diaphorase rich Walker cells treated with EO9 is due to DT-diaphorase activation of this compound. This possibility was further investigated in the present work using the DT-diaphorase proficient and deficient human colon carcinoma cell lines HT29 and BE. Results showed that both DNA strand breaks and interstrand cross-links were induced in HT29 cells at a dose of EO9 which corresponded to the IC_{50} value. Interestingly this concentration was non-toxic to BE cells (Plumb and Workman, 1994) and caused no detectable damage to their DNA (this thesis). Increasing the concentration of EO9 to an equipotent concentration (i.e. the IC_{50} of EO9 in BE cells) resulted in the detection of DNA damage in these cells also. Thus the presence and extent of DNA damage appeared to correlate very well with cytotoxicity of EO9 in the human colon carcinoma cell line pair, consistent with the view that it may be an important mechanism of cell kill. The higher doses required to induce both cytotoxicity and DNA damage in the BE cells were also consistent with the importance of DT-diaphorase in the production of DNA damaging lesions. Because of the large hypoxic differential observed in BE cells (Plumb and Workman, 1994) experiments were also carried out under hypoxic versus aerobic conditions. Under hypoxia levels of DNA damage in both BE and HT29 cells

also showed a correlation with cytotoxicity. It was hypothesised in Chapter 5 that the cytotoxic, DNA damaging species may therefore be either a one-electron reduced metabolite such as the semiquinone stabilised under hypoxia or alternatively the two-electron reduced product. The latter could be formed by either direct two-electron reduction in cells possessing DT-diaphorase or by two sequential one-electron reduction steps in cell lines such as BE which lack a functional form of this enzyme.

DNA damage experiments using whole cells are highly informative as they give data regarding the mechanisms of cytotoxicity in a more physiologically relevant system where a variety of enzymes are present. Inhibitor studies could be employed to investigate the role of a particular enzyme in drug activation to a DNA damaging species. However, in the case of DT-diaphorase this was not considered advisable due to the pleiotropic effects of dicoumarol discussed previously. For this reason cell free assays were used to determine the potential involvement of DT-diaphorase in activation of EO9 to metabolites capable of inducing strand breaks and interstrand cross-links in plasmid DNA.

Data presented in Chapter 5 showed that EO9 was activated by DT-diaphorase under aerobic conditions to a strand breaking species in a dose-dependent manner. These results compared well with those obtained previously by Walton and co-workers (1991) in the same system. Experiments using superoxide dismutase and catalase have also provided evidence implicating oxygen radicals as the causal species of the strand breaks observed under the aerobic conditions used. In addition, preliminary experiments investigated the effect of hypoxia on strand break formation. Results of these studies also showed EO9 to induce strand breaks under hypoxia but rather surprisingly a substantial degree of protection of this damage was observed when superoxide dismutase and catalase were included in the assays. These enzymes should remove oxygen radicals from the system and the results therefore suggest that damage observed was due to either a small amount of residual oxygen within the apparatus or due to auto-oxidation of the metabolites during loading of the reaction mixture on to the gel. Evidence in support of the latter was provided by the observation that the purple colour of the EO9 solution was restored rapidly upon introduction of air into an enzyme system which had previously metabolised EO9 to colourless metabolite(s) under anoxia. Because of the potential problems with reoxidation, these hypoxia experiments were not pursued further. It should be emphasised however that there was a component of the DT-diaphorase-catalysed DNA strand break capablity that was not protected by superoxide dismutase and

catalase. This indicates that DNA damage can be due in part to the release of EO9 reduction products catalysed by DT-diaphorase.

The ability of DT-diaphorase to activate EO9 to a bifunctional alkylating agent capable of inducing interstrand cross-links in DNA was also examined using a cell free plasmid, agarose gel-based assay. Results of these studies confirmed that EO9 could cause cross-links in DNA following aerobic reduction catalysed by DT-diaphorase at physiological pH. In the absence of activation no cross-links were detectable. This supports the view that DT-diaphorase may be one of the enzymes involved in activation of EO9 to a cytotoxic, DNA cross-linking species in the intact cell.

Studies with AZQ described in this thesis and those from other investigators have indicated a similar mechanism of DNA damage to that caused by EO9. AZQ is reduced by DT-diaphorase (Chapter 3 and Siegel *et al*, 1990a). This generates oxygen radicals and a drug-based radical (Fisher and Guttierez, 1991a), presumably by auto-oxidation of a highly oxygen sensitive two-electron reduced metabolite. Following treatment with AZQ, HT29 cells showed evidence of DNA damage in the form of both interstrand cross-links and strand breaks whereas DNA damage was not detected in BE cells (Siegel *et al*, 1990a). This again suggested a role for DT-diaphorase in activation of AZQ to a DNA damaging species. In addition the strong correlation between DNA damage and cytotoxicity supported the view that this may be a mechanism of AZQ-induced cell kill. Experiments involving cell free systems have confirmed that AZQ could be activated by DT-diaphorase and NADPH: cytochrome P450 reductase to species causing strand breaks (Fisher and Guttierez, 1991a).

In addition to strand breaks and interstrand cross-links, other types of DNA damage may be formed by alkylating agents including DNA monofunctional adducts. Reaction with DNA can occur at different bases and can also be DNA sequence-dependent. In order to more fully understand this DNA damage at a molecular level, a highly sophisticated *Taq* polymerase assay was employed. This permitted the sequence selectivity of EO9 binding to DNA to be determined. Results with this assay, presented in Chapter 5, showed that EO9 was able to form covalent DNA adducts in the absence of activation. This was presumably due to the instability of the aziridine ring in the close proximity of strong nucleophilic sites on DNA. Binding was, as for many other alkylating agents, predominantly with guanine residues particularly where they occurred in runs of consecutive guanines. This is believed to be due to the high electronegativity of these sequences (D'Incalci, 1992). However,

the precise sequence dependence was different from that obtained with the conventional nitrogen mustard chlorambucil which was included as a standard. Both the number of nucleotides alkylated and the extent of modification was reduced with binding being more slightly more specific for certain guanines. In particular, it was noted that of the all the adenines in the sequence GCA within the readable part of the sequencing gel were alkylated more strongly by chlorambucil than by unactivated EO9. This suggested that EO9 was, possibly for steric reasons, unable to bind to this particular series of bases. Only one adenine was more strongly alkylated by EO9 than chlorambucil, that being in the sequence GTATC (base pair number 515 in pBR322 DNA). Other bases which were modified by EO9 but not chlorambucil included two cytosines in the sequence GCA and three incidences of cytosine in the sequence pu pu C pu (where pu denotes purine). Interestingly, where DT-diaphorase was included in the reaction mixture the degree of DNA binding was decreased, regardless of whether the NADH cofactor was included. The reasons for this phenomenon are unknown. It is possible that when EO9 is bound to the enzyme this reduces its availability to interact with DNA. This may be to some extent an artefact of the model system used. Of greater potential importance was the observation that, in the presence of both DT-diaphorase and NADH cofactor, the spectrum of EO9 adducts produced was found to differ from those formed in the absence of an activating system. In general, this involved loss or decreased extent of alkylation compared with the unactivated drug, with binding being more specific. Guanines were the most common base for which this occurred. A few bands were however present following activation that were not evident with either unactivated drug or chlorambucil. Four of the changes noted were cytosines with two of these being in the sequence TGC (positions 562 and 566). Only one adenine was noted to have increased alkylation with the sequence CAT (position 491). Two incidences of thymine modification were also detected. Further distinct differences between EO9 and reduced forms were clearly visible in a region of the gel which was poorly resolved for technical reasons and in which it was therefore difficult to determine the sequence. In addition to the possible sequestration of EO9 by the DT-diaphorase molecule mentioned earlier additional suggestions put forward in Chapter 5 to explain the decreased alkylating in the presence of DT-diaphorase included DNA cross-link formation restricting further monofunctional alkylation and strand break induction causing artifactual results. Such altered specificity following enzymatic activation has been observed by other investigators with the bioreductive quinone alkylating agents DZQ (3,6-diaziridinyl-1,4-benzoquinone) and methyl-DZQ (2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone). It is hoped that such sequence-selective DNA interactions could lead to selective antitumour activity (Hartley, 1990).

Studies examining the inhibitory effect of dicoumarol on EO9-induced cytotoxicity suggested a role for DT-diaphorase in EO9 activation but indicated that other enzymes may also be involved. The one-electron reducing enzyme NADPH: cytochrome P450 reductase has been reported to be involved in the activation of a number of bioreductive agents including mitomycin C (Bachur *et al*, 1979; Pan *et al*, 1994) and AZQ (Fisher and Gutierrez, 1991a). For this reason experiments were carried out to determine the possible importance of this enzyme in EO9-induced cytotoxicity. Results from ESR experiments provided the first evidence for the ability of NADPH: cytochrome P450 reductase to metabolise EO9. In addition, they showed that under aerobic conditions this reduction generates both a drug-based radical and oxygen radicals. These were similar in nature to those produced following EO9 reduction by DT-diaphorase. Similarly, DNA damage assays indicated that these oxygen radicals are able to induce DNA strand breaks. Thus it appears that NADPH: cytochrome P450 reductase may also be involved in the activation of EO9 in intact cells. Due to time limitations further investigations with NADPH: cytochrome P450 reductase were not performed, but it would be interesting to evaluate the role of this enzyme in comparison with DT-diaphorase.

In summary, data presented in this thesis have shown that both EO9 and AZQ acted as potent cytotoxins whose mechanism of cell kill was likely to involve DNA damage. The experiments have provided important information to understand the nature of EO9 bioreductive metabolism, particularly with regard to the involvement of DT-diaphorase. From these data a scheme for the mechanism of activation and action of EO9 has been postulated (Fig 8.1). In this scheme DT-diaphorase reduces EO9 to form a colourless metabolite which is relatively stable under hypoxic conditions. This is likely to be the two-electron reduced metabolite, the hydroquinone. In the presence of molecular oxygen this species undergoes auto-oxidation to generate a drug-based radical (presumably the semiquinone) and ultimately the parent quinone. Reactive oxygen radicals are generated concomitantly during this process. NADPH: cytochrome P450 reductase also catalyses reduction of EO9 to generate the same metabolite as that formed by auto-oxidation of the two-electron reduced metabolite. Thus it is likely that under aerobic conditions two-electron reduction of EO9 catalysed by DT-diaphorase and also one-electron reduction by NADPH: cytochrome P450 reductase will both generate a drug-based radical, thought to be the semiquinone, as well as oxygen radicals. In addition the hydroquinone may be generated either by direct by two-electron reduction or two sequential cycles of one-electron reduction. It is likely that in an intact cell, under aerobic conditions, the spectrum of metabolites ultimately formed will depend on the levels of oxygen and proximity of strong

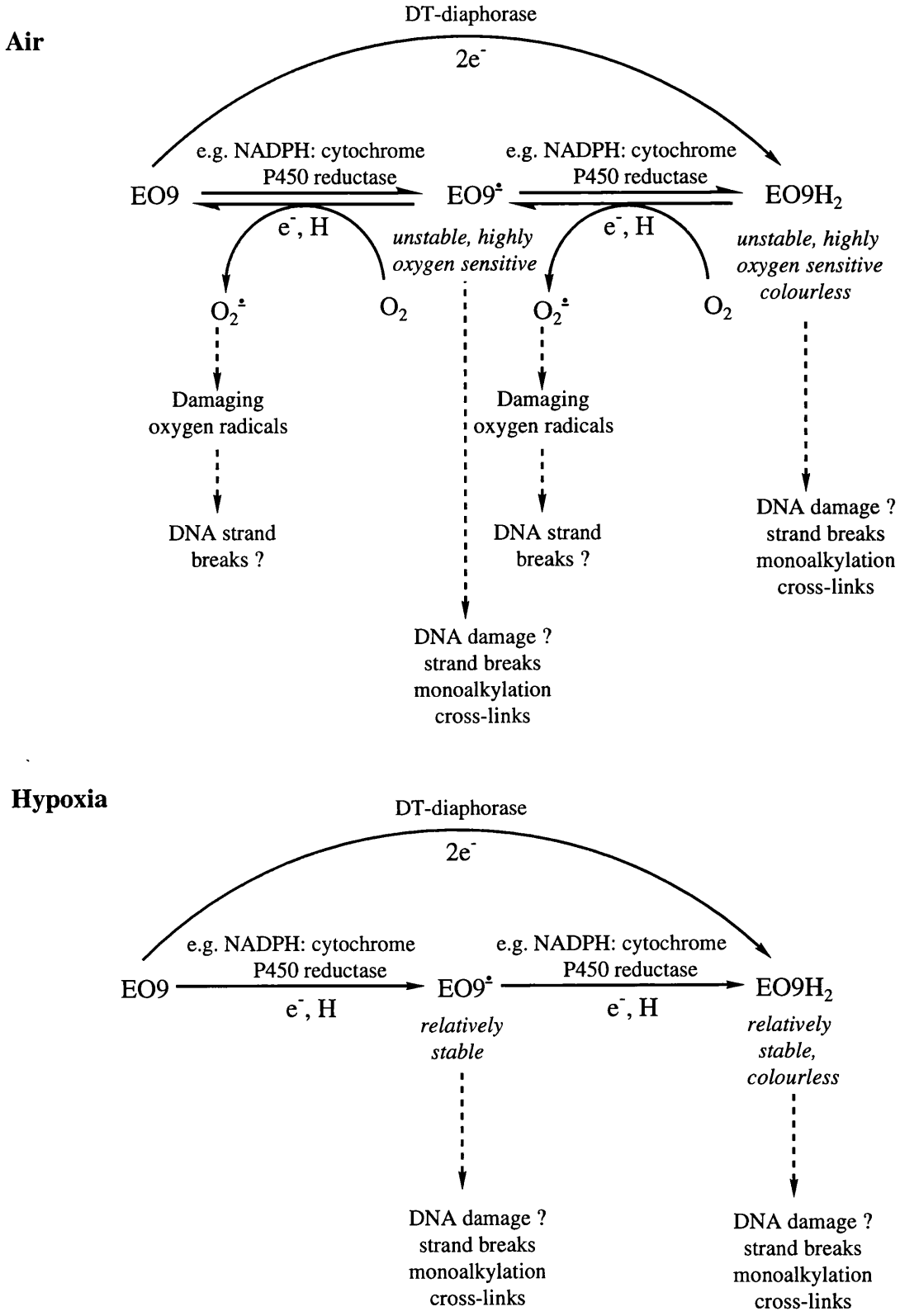


Fig. 8.1 Postulated mechanism of activation and action of EO9

nucleophiles. Under hypoxia the product of DT-diaphorase metabolism appears to be stabilised and the drug and oxygen radicals would be less likely to be formed. Similarly one-electron reduction catalysed by enzymes such as NADPH: cytochrome P450 reductase under hypoxia should produce a stabilised semiquinone and could even undergo further cycles of one-electron reduction to generate the hydroquinone. A further complication is that, in addition, the semiquinone and hydroquinone may both be formed by comproportionation : disproportionation reactions.

DNA damage appears to be a major mechanism by which EO9 causes cytotoxicity. Strand breaks are induced which are likely to be due to oxygen radicals formed during redox cycling. Following reduction the aziridine and leaving groups present on the side chains are likely to undergo activation to form alkylating moieties capable of causing DNA interstrand cross-links. The identity of the reactive metabolite responsible for DNA cross-links is unknown. On chemical grounds cross-links could be formed from either the semiquinone or hydroquinone. Hypoxic DNA damage experiments may clarify this issue.

Concluding Remarks

The initial aims outlined at the beginning of this thesis have largely been fulfilled. Inevitably the results obtained have raised new questions which remain to be answered by further research. The data presented have strongly indicated a role for DT-diaphorase in the bioreductive activation and cytotoxic mechanism of EO9. Experiments have however to some extent relied on use of the DT-diaphorase inhibitor dicoumarol which is now known to exert pleiotropic pharmacological effects. Other authors have warned that caution should be taken when interpreting results obtained using this compound (Workman and Walton, 1989; Ross *et al*, 1993). For this reason the ability of DT-diaphorase to metabolise both the indoloquinones and aziridinylbenzoquinones was confirmed using a purified form of the enzyme derived from rat Walker tumour cells. Experiments have however indicated that the rat and human DT-diaphorase enzymes differ in their ability to metabolise certain compounds such as EO9 and CB 1954 (Chapter 3 and Boland *et al*, 1991). An understanding of these metabolic intricacies provides a basis for rational drug development. Analogues may be identified with enhanced reduction by the human tumour enzymes compared with the rat forms of DT-diaphorase. In the case of the indoloquinones, EO8 acted as a slightly better substrate for DT-diaphorase present in sonicates of human HT29 cells than of rat Walker cells (Chapter 3). An analogue of CB 1954, 5-(aziridin-1-yl)-2,4-dinitro-1-isobutylbenzoate (CB 10-200), also showed

preference for the human enzyme (Lambert *et al*, 1992). This information also highlights the importance of using relevant model systems which will reflect the human *in vivo* situation most closely. It would be favourable, in this instance, to screen compounds for activity using the purified human enzyme. This was however not available and is reported to be difficult to purify in quantity. In terms of the ultimate clinical relevance it would obviously be important to analyse the reduction of EO9 using a purified human enzyme. A good indication of the involvement of DT-diaphorase in the cytotoxicity of EO9 towards human tumour cells has been provided by comparative studies of high and low DT-diaphorase expressing cell lines, such as HT29 and BE colon carcinoma cells (Plumb and Workman, 1994). DNA damage studies on these cell lines reported in this thesis are strongly supportive of the proposed mechanism. In addition, a number of studies have reported a clear correlation between DT-diaphorase expression and the aerobic cytotoxicity of EO9 in larger panels of human tumour cell lines (Phillips *et al*, 1992; Walton *et al*, 1992; Robertson *et al*, 1992; Robertson *et al*, 1994; Plumb *et al*, 1994a and b; Smitskamp-Wilms *et al*, 1994; Fitzsimmons *et al*, 1994). The issue could however be further clarified using cell lines transfected with DT-diaphorase cDNA. Levels of this enzyme could then be regulated under the control of an inducible promoter.

Structural modification of EO9 and AZQ resulted in an altered cytotoxic potency and ability to be reduced by DT-diaphorase. In general, the SARs for cytotoxicity and DT-diaphorase were similar, thus further supporting a causal role for DT-diaphorase in the cytotoxic mechanism. This information highlights the potential for tailoring of analogues to suit metabolism by key bioreductive enzymes as part of enzyme-directed drug development programmes (Workman and Walton, 1990; Workman, 1994). For the optimal structural requirements to be determined a wider range of analogues need to be analysed. Based on present work, such studies should include metabolism, cytotoxicity and DNA damage endpoints. EO9 itself was based on the structure of the established bioreductive alkylating agent mitomycin C. Results presented in this thesis and elsewhere have shown that this novel indoloquinone agent exhibits important differences from mitomycin C, particularly in its increased ability to undergo reduction catalysed by DT-diaphorase, its preference for solid tumours versus leukaemias and importantly the fact that it does not induce dose-limiting myelosuppression.

The lack of complete inhibition of drug metabolism and cytotoxicity in the presence of dicoumarol suggested the involvement of additional enzymes in activation of indoloquinones. Experiments using NADPH: cytochrome P450 reductase have

shown that this enzyme can also catalyse metabolism of EO9 to generate DNA damaging metabolites under aerobic conditions. The role played by any one enzyme in the activation of the indoloquinones or aziridinylbenzoquinones will depend on the levels and affinities of other enzymes present in the cell. For this reason it is important that experiments are carried out to examine the metabolism of these bioreductive agents catalysed by other enzymes such as cytochrome P450, xanthine oxidase and dehydrogenase and carbonyl reductase amongst others. In addition, for an enzyme-directed approach to cancer treatment to be successful the 'enzyme profile' of the tumour needs to be determined for each patient. With such information at hand it may be possible to tailor treatment for the individual by selecting drugs which are activated by enzymes expressed at high levels within a particular tumour. Ideally, such enzyme profiling would be conducted in parallel with measurement of the level of tumour hypoxia (e.g. see Rampling *et al*, 1994)

The importance of understanding the relative contributions of enzyme content and the level of hypoxia in a given tumour is highlighted by the recent observation that the role of DT-diaphorase in activation of EO9 may differ under aerobic and hypoxic conditions (Plumb and Workman, 1994a and b; Robertson *et al*, 1994). These studies have shown that under aerobic conditions tumour lines rich in DT-diaphorase are the most sensitive to EO9 but show very little increase in sensitivity under hypoxia; in contrast, cell lines with low DT-diaphorase activities are relatively resistant to EO9 in air but exhibit a substantial increase in sensitivity under hypoxic conditions. DNA damage studies on HT29 and BE cells described in this thesis are consistent with this pattern. It would be of particular interest to isolate and identify the metabolites formed under both aerobic and hypoxic conditions so that the cytotoxic species may be determined. This may prove difficult due to the apparent high reactivity of metabolites formed. As discussed previously, bioreductive agents are thought to be selective to hypoxic cells and / or those expressing increased levels of bioactivating enzymes. It is important that these agents are active against hypoxic cells which are resistant to radiation. This tends to be observed at an oxygen pressure of below 6 mm Hg. Many bioreductive agents currently available, e.g. mitomycin C, are most effective below 0.6 mm Hg oxygen and hence there are cells which will remain viable after combined therapy with mitomycin C and radiation. More encouragingly, one of the recently developed bioreductive agents, the benzotriazine di-N-oxide SR 4233, shows a more 'suitable' cytotoxicity profile over varying oxygen concentrations (Koch, 1993). The levels of oxygen present in cells should therefore be correlated with cytotoxicity in order to identify compounds effective over the desired range of oxygen tension. Nevertheless it is possible that compounds which do not completely

cover this window of hypoxic selectivity may still be effective due to a phenomenon known as the 'bystander effect'. This is a situation where cytotoxic metabolites generated within a cell are able to diffuse out and kill adjacent cells. However, this would require the metabolites produced on bioreductive activation to display the appropriate level of stability to facilitate sufficient but not excessive, diffusion.

With regard to the relative importance of the DT-diaphorase activities versus the extent of hypoxia in tumours under physiological conditions, it will be important to assess the contribution of these two parameters *in vivo*, for example using human tumour xenografts.

Clinical trials of EO9 are presently underway. As discussed above the ideal situation ultimately would be to tailor drug treatment depending on the characteristics of an individual patients tumour both in terms of enzyme profile and levels of hypoxia. This does however require further elucidation of the enzymology of these bioreductive anticancer agents. In the meantime it may be informative to take biopsies of tumours prior to treatment and carry out enzyme profiling on these specimens in order to determine whether enzyme status relates to response to EO9 therapy in the clinic. Such data may throw light on the involvement of particular enzymes in EO9 activation in humans *in vivo*.

Currently, results obtained by Plumb and co-workers (1994) and Robertson *et al* (1994) would suggest that *in vitro* DT-diaphorase may be important in activation of EO9 under aerobic conditions but that under hypoxia the situation is reversed. If these relationships hold true *in vivo* the patients which also benefit most from EO9 treatment would be those with either hypoxic tumours containing low levels of DT-diaphorase or those with high DT-diaphorase levels and low hypoxia.

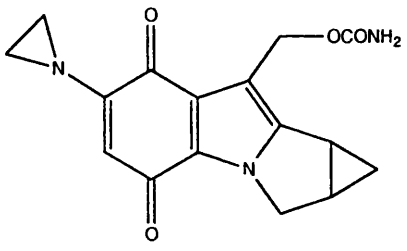
The data presented in this thesis contributes to the 'enzyme directed approach' to bioreductive drug development adopted by our laboratory (Workman and Walton, 1990; Workman, 1994). The results have enhanced our understanding of the mechanisms of both enzymatic activation and DNA damage of EO9, from the whole cell down to molecular level. The importance of DT-diaphorase in the metabolism of both indoloquinones related to EO9 and aziridinylbenzoquinone analogues of AZQ has also been confirmed. Information gained from this study and other similar investigations may be important either for improved analogue development and / or for optimisation of clinical protocols employing bioreductive anticancer agents.

Appendix 1

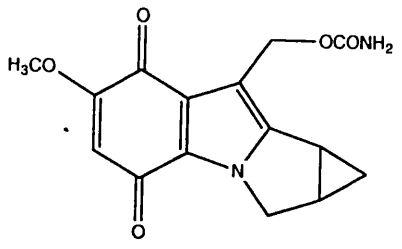
A1.1 Introduction

A series of cyclopropamitosenes (Fig A1.1) have recently been synthesised by Professor C. Moody and Dr N. O'Sullivan at Loughborough University, UK in an attempt to investigate the involvement of the C-10 group in alkylation. The compounds are structurally related to mitomycin C but possess a cyclopropane group in place of the aziridine ring at the C1 position, thus reducing the electrophilicity at this site. In theory one or two-electron reduction of the cyclopropamitosenes followed by elimination of the carbamate moiety could generate a powerful electrophile capable of alkylating DNA. It is also possible that radical-induced opening of the cyclopropane may occur producing a highly reactive radical which may cause strand breaks in DNA by extracting a hydrogen atom.

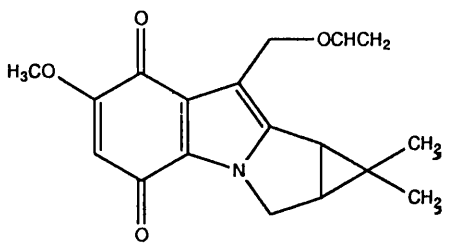
The *in vitro* cytotoxic potency of two of the lead compounds the 7-methoxycyclopropamitosene (RB 91007X) and the 7-aziridinylcyclopropamitosene (RB 91008X) has been determined by Drs I. J. Stratford and M. Stephens at the MRC Radiobiology Unit, Harwell, UK using V79 lung fibroblasts (Moody *et al*, 1994). RB 91008X was found to be highly toxic compared to many other agents against V79 cells, with an IC_{50} value of 3 nM under aerobic conditions. This was 1000 fold more potent than RB 91007X (IC_{50} 3 μM). For comparison, mitomycin C and EO9 were intermediate between these two extremes. The sensitivity of cells to RB 91007X increased 25 fold when experiments were carried out under hypoxia but no effect was observed with RB 91008X. These results suggested that the two compounds may differ in their mechanism of activation, with RB 91007X being metabolised by an oxygen-sensitive process and RB 91008X by an oxygen-insensitive one. Since DT-diaphorase reduction has generally been considered to be independent of oxygen, the involvement of this enzyme in the activation of the two cyclopropamitosenes was investigated by including the DT-diaphorase inhibitor dicoumarol in cytotoxicity assays. The results indicated that DT-diaphorase may be important in the activation of RB 91008X but not of RB 91007X. To complement the cytotoxicity studies and to provide more definitive evidence of the possible involvement of DT-diaphorase, experiments described in this appendix were designed to directly investigate the ability of DT-diaphorase to metabolise the cyclopropamitosenes.



RB 91008X



RB 91007X



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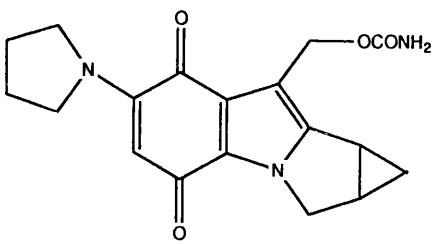

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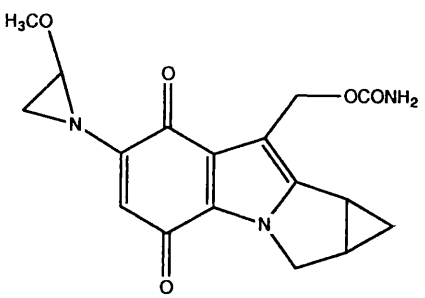

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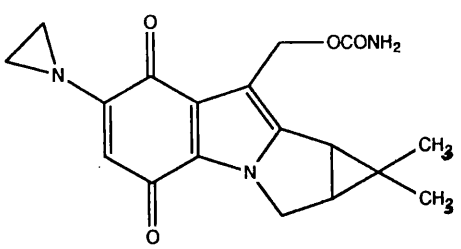

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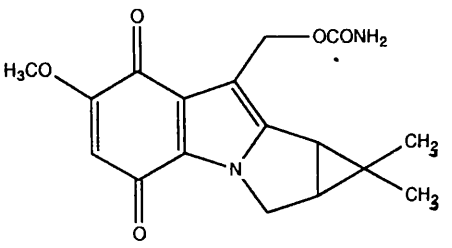
Fig. A1.1 Chemical structures of cyclopropamitosenes



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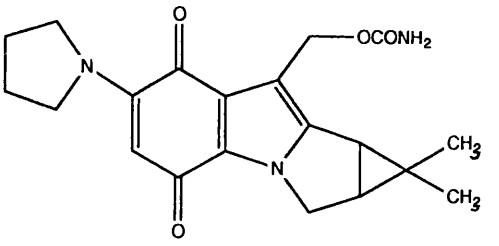

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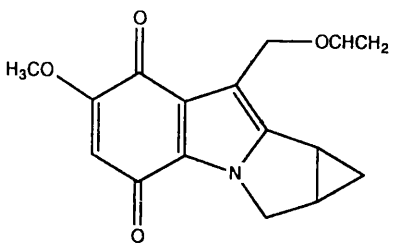

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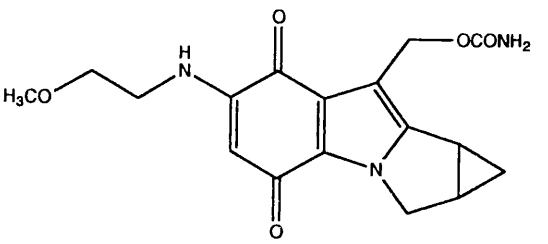

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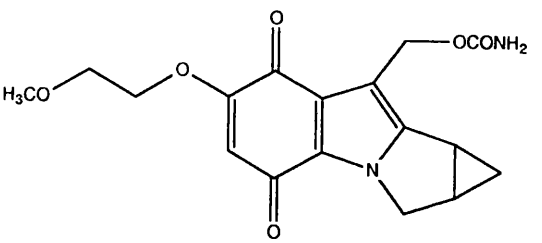

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A1.2 Methods

DT-diaphorase-catalysed metabolism of the two lead cyclopropamitosene compounds RB 91007X and RB 91008X, together with 7 structurally related analogues (Fig A1.1) was determined spectrophotometrically by the cytochrome *c* reduction assay described in Chapter 2. Due to the limited amount of drug and enzyme available the experiments were restricted and between-day repeats were not performed. Activity was in general determined at 50 μM drug concentration using two low dilutions of DT-diaphorase (0.008 μg and 0.05 μg in a total reaction volume of 1 ml) although 10 μM drug was also employed in some cases. Higher concentrations (0.5, 1, 2 and 4 μl / ml of reaction buffer) of enzyme were used only for the lead compounds RB 91007X and RB 91008X. Reduction of RB 91007X and RB 91008X was confirmed directly by monitoring oxidation of cofactor at 340 nm. Controls were carried out where each reaction component was omitted. The restraints on enzyme availability resulted in dicoumarol inhibition studies being performed on all compounds except for the lead analogues RB 91007X and RB 91008X.

The cyclopropamitosenes were kindly donated by Professor C. Moody (Loughborough University). These were diluted in DMSO and were used freshly where possible. Alternatively stocks were maintained frozen at -20°C, aliquotted to avoid freeze thawing.

A1.3 Results

Results obtained using the cytochrome *c* reduction assay showed that the two lead compounds RB 91007X and RB 91008X (Fig A1.1) could undergo metabolism catalysed by purified rat Walker DT-diaphorase in the presence of cofactor (Table A1.1). RB 91008X was metabolised approximately three fold more efficiently than was RB 91007X with activities of 4.64×10^3 and 1.60×10^3 nmoles of cytochrome *c* reduced / minute / mg protein respectively (Table A1.1). Assays where reduction was monitored directly following a change in absorbance at 340 nm confirmed that RB 91007X and RB 91008X could act as substrates for DT-diaphorase, as well as the relative rates of metabolism (Fig A1.2). Using the cytochrome *c* reduction assay incubations containing enzyme and cofactor alone resulted in some reduction. Both cofactor and the cyclopropamitosenes absorb at around 340 nm, thus it is not possible to quantify reduction of either compound by monitoring change in absorbance at this wavelength. Controls where cofactor or enzyme were omitted resulted in either no or

Compound	Rate of Reduction (n moles cytochrome c reduced / minute / mg protein)
Menadione	4.96×10^6
RB 91007X	1.60×10^3
RB 91008X	4.64×10^3
EO9	6.48×10^5
Mitomycin C	8.96×10^2

Table A1.1 Rate of DT-diaphorase - catalysed metabolism of the cyclopropamitosenes RB 91007X and RB 91008X compared with that of the conventional substrate menadione and the bioreductive anticancer drugs EO9 and mitomycin C. Data are taken from an individual experiment but are the means of at least 4 repeat assays.

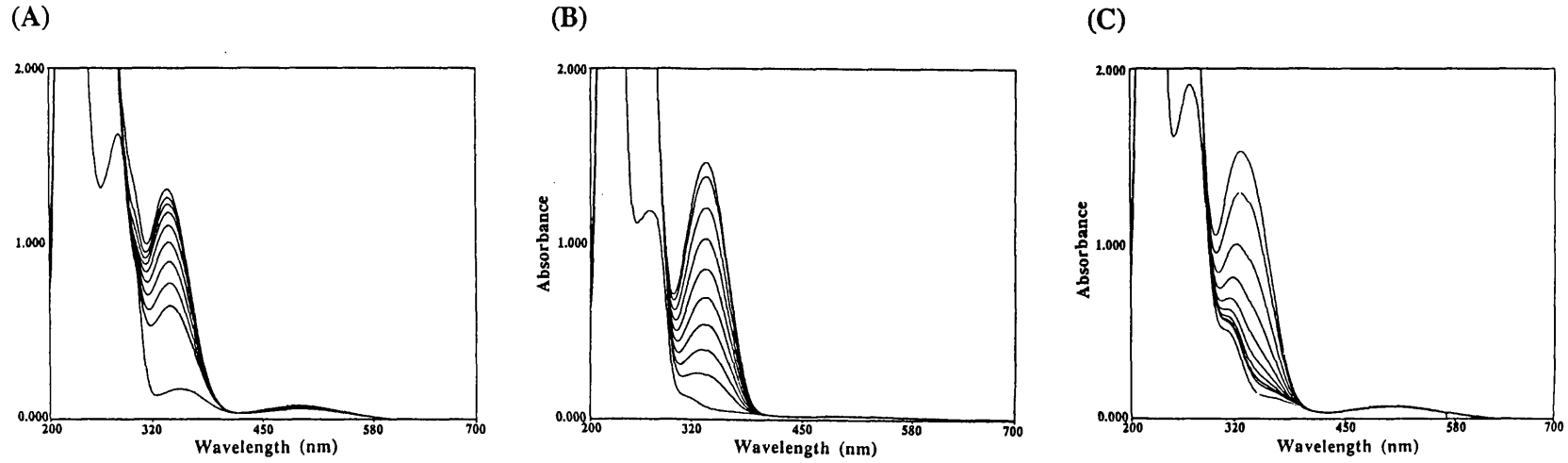

Fig A1.2 Reduction of (a) RB 91007X, (b) RB 91008X and (c) EO9 catalysed by purified rat Walker cell DT-diaphorase. Reduction is monitored by detecting change in absorbance of reaction constituents with time over the wavelength range 200-700 nm. The lower curve represents drug in buffer in the absence of cofactor and enzyme and the uppermost drug plus NADH. Subsequent scans were taken each minute following initiation of reduction by addition of DT-diaphorase. 2.5 μg of DT-diaphorase were employed for all experiments except for those involving EO9 which utilised 0.25 μg in a total volume of 1 ml. Data are taken from an individual experiment and were confirmed in a single repeat assay.

very low levels of activity. RB 91007X showed a non-linear progress curve with an initial sharp increase in absorbance followed by a more linear progress curve when change in absorbance was plotted against time (minutes). Activity was therefore calculated from the linear portion of the curve.

Although with menadione and EO9 as substrates the preparation of DT-diaphorase used had been confirmed to be linear with respect to increasing enzyme concentrations (data not shown) a deviation from linearity was observed with RB 91007X and RB 91008X over the range 1-4 μg of protein.

For the additional analogues examined, DT-diaphorase activity was extremely low nearing the background level of activity for the assay. This was due to the small amount of enzyme which could be employed. It was therefore difficult to determine the efficiency of the cyclopropamitosenes as substrates for DT-diaphorase from the data obtained. Given more enzyme and compound a wider range of drug concentrations and higher enzyme concentrations could have been be used. It was possible however to conclude that none of the compounds were more efficiently metabolised than EO9 which was run in parallel as a standard and positive control. Only two of the analogues (Fig A1.1) 3/28 and 3/42 had any reasonable levels of activity above background. For a DT-diaphorase concentration of 0.05 μg / ml and drug concentration of 10 μM, the activity seen with the analogues was about 5 fold lower than that obtained with EO9.

A1.4 Discussion

DT-diaphorase has been implicated in the activation of both EO9 (Chapters 1, 3 and 4) and mitomycin C (for references see Chapter 1). The cyclopropamitosenes described in this thesis chapter possess a similar chemical structure to these compounds and thus it was possible that they may also be metabolised by DT-diaphorase.

Cytotoxicity experiments carried out in V79 cells with the two lead compounds (Fig A1.1) RB 91007X and RB 91008X suggested that they are highly potent cytotoxins which differ in their modes of activation. In contrast to RB 91008X, RB 91007X appears to be metabolised to oxygen-sensitive metabolites, thus showing enhanced toxicity under hypoxic conditions. Dicoumarol inhibition assays suggested a role for

DT-diaphorase in activation of the aziridinylcyclopropamitosene RB 91008X but not of the methoxycyclopropamitosene RB 91007X.

Experiments described in this appendix have shown that both RB 91007X and RB 91008X are able to act as substrates for DT-diaphorase purified from rat Walker tumour cells. The aziridine analogue RB 91008X was 3 fold more efficiently metabolised than was RB 91007X, fitting in with the protection conferred by dicoumarol against its toxicity. Similar results have been observed with the indoloquinone compounds related to EO9. The analogue EO7 bearing a methoxy group in place of the aziridine ring of EO9 was also able to act as a substrate for DT-diaphorase but was ~2 fold less efficiently reduced than was EO9 itself. This supports the view that aziridine moieties may favour binding to DT-diaphorase (see Chapter 8). The aziridine-containing cyclopropamitosene was also found to be more cytotoxic towards V79 cells than was the methoxy compound (Moody *et al*, 1994). This characteristic also parallels results obtained with EO9 where the parent compound was more cytotoxic than the methoxy analogue. This was considered to be due to either increased activation by DT-diaphorase and / or the fact that aziridines may function as potent alkylating groups (Chapter 4).

As noted in the methods section, dicoumarol was not included in assays performed on the lead compounds due to limited availability of drug and enzyme. In theory, with purified enzyme activity in the presence of dicoumarol the observed activity should be similar to that in the absence of enzyme. Control experiments where either cofactor or enzyme were omitted showed low background levels of activity. Where cofactor and enzyme were incubated alone in the absence of drug some activity was observed. This value was subtracted from the overall activity when calculations were made. The cytochrome *c* reduction detected was concluded to result from reduction of cyclopropamitosenes catalysed by DT-diaphorase in the presence of cofactor NADH.

This particular preparation of rat Walker DT-diaphorase had been confirmed to give a linear increase in reduction rate of menadione. Deviation from linearity was however detected with RB 91007X and RB 91008X. In addition an unusual result was obtained for the change in absorbance over time for RB 91007X. The reaction progress curve showed an initial high rate followed by a levelling off and then a more linear increase. Due to these irregularities, DT-diaphorase reduction of the cyclopropamitosanes was confirmed by directly monitoring the change in absorbance of drug and cofactor NADH during the reaction. As both drug and cofactor were

found to absorb at 340 nm it was not possible to determine kinetics from these experiments. A decrease in absorption was however noted at 340 nm for both RB 91007X and RB 91008X where drug was incubated with cofactor in the presence of enzyme. In the absence of any one reaction constituent no change in absorbance was noted. This indicated that DT-diaphorase was able to metabolise the cyclopropamitosenes in the presence of cofactor. The more rapid decrease in absorbance over time of RB 91008X than RB 91007X confirmed that it was able to act as a more efficient substrate. Both RB 91007X and RB 91008X were however more slowly reduced than EO9.

Several additional cyclopropamitosene analogues (Fig A1.1) were also analysed for their ability to undergo reduction catalysed by DT-diaphorase. Low levels of activity were seen for each of the compounds using the concentrations of DT-diaphorase that were available for the experiments. EO9 which is known to act as a good substrate for DT-diaphorase was employed as a standard as well as the conventional substrate menadione. At the enzyme concentrations used menadione was found to be well metabolised by the purified rat Walker DT-diaphorase. EO9 was far less efficiently reduced but measurable rates were seen. In order to draw firm conclusions regarding the relative ability of the cyclopropamitosenes to act as substrates for DT-diaphorase higher enzyme concentrations would need to be employed. What is clear is that all the compounds examined were poorer substrates than EO9 but that 3/42 and 3/28 were reduced only about 5 fold less efficiently than EO9 using 0.05 μg of DT-diaphorase in a total reaction volume of 1 ml.

To conclude, data presented in this appendix have demonstrated that purified rat Walker DT-diaphorase is able to catalyse metabolism of both cyclopropamitosenes RB 91008X and RB 91007X. The 7-aziridinyl analogue RB 91008X is metabolised 3 times more efficiently than the 7-methoxy analogue RB 91007X. What is particularly striking about these results is that such a modest change in ability to act as a substrate for DT-diaphorase nevertheless produces a major shift in the extent of the enhancement of cytotoxicity by hypoxia and in the modulation of cytotoxicity by dicoumarol.

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