A STUDY ON THE MECHANISMS OF ACROLEIN AND DIAZIQUONE-INDUCED CYTOTOXICITY IN ISOLATED HEPATOCYTES

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JOSE MANUEL LADEIRA E SILVA

A STUDY ON THE MECHANISMS OF ACROLEIN AND DIAZIQUONE-INDUCED CYTOTOXICITY IN ISOLATED HEPATOCYTES

BY

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A Thesis submitted in conformity with the requirements
for the degree of Master of Science
in the
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St. John's, NFLD., Canada
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ABSTRACT

Acrolein may be a causative agent of hepatic necrosis during the biotransformation of allyl alcohol. It has also been implicated in hemorhagic cystitis of the bladder induced by the antitumor agent cyclophosphamide. The mechanism of cytotoxicity is believed to involve DNA and protein alkylation. AZQ, 2,5-diazirinyl-3,6-bis(carboethoxyamine)-1,4-benzoquinone, is a lipid soluble antitumor agent that has successfully completed Phase II drug trials. The mechanism of cytotoxicity is believed to involve DNA cross-linking. However, the following evidence using isolated hepatocytes suggests that the cytotoxicity may involve oxidative stress. Isolated rat hepatocytes were prepared and incubated with acrolein or AZQ.

A) Acrolein

1. Hepatocyte cytotoxicity induced by acrolein ensued following depletion of cellular GSH. No GSSG was formed.

2. The addition of the reducing agent DTT to hepatocytes preincubated with acrolein prevented cytotoxicity.

3. Malondialdehyde, a lipid peroxidation metabolite, was formed during either acrolein or allyl alcohol incubation with hepatocytes.

4. Although malondialdehyde formation could be prevented by the presence of antioxidants and desferrioxamine in the hepatocyte incubate, cytotoxicity was only delayed.

5. The xanthine oxidase inhibitor, allopurinol, did not inhibit acrolein induced lipid peroxidation or protect against cytotoxicity.

6. Acrolein readily induced Ca^{2+} release by isolated energized mitochondria. Intramitochondrial NAD(P)H was not affected indicating that oxidative stress was not involved. The reducing agent DTT could prevent the

release. A reversible alkylation of proteins involved in Ca^{2+} release could be the mechanism involved.

These results suggest two mechanisms of cytotoxicity induced by acrolein. One involving lipid peroxidation and another slower mechanism involving alkylation.

B) AZQ

1. Hepatocyte cytotoxicity induced by AZQ ensued following depletion of GSH.

2. Incubation of AZQ with isolated rat hepatocytes stimulated cyanide-resistant respiration and stoichiometrically oxidized GSH to GSSG. The GSSG levels remained high, as GSSG was not reduced back to GSH. This was found to be the result of reversible inactivation of GSSG reductase.

3. No malondialdehyde was formed.

4. If the hepatocytes were compromised with azide to inhibit catalase, cytotoxicity was increased 10-fold.

5. AZQ readily induced Ca^{2+} release by isolated energized mitochondria. Ascorbate markedly enhanced the effectiveness of AZQ and catalase delayed the release. H_2O_2 formed by redox cycling may therefore cause mitochondrial Ca^{2+} release.

These results suggest that under aerobic conditions AZQ participates in futile redox cycling and oxygen activation. The H_2O_2 formed may mediate cell death in compromised cells.

TABLE OF CONTENTS

Page

Abstract ii
List of Tables v
List of Abbreviations ix
List of Figures xi
Acknowledgements xiii

1. INTRODUCTION

1. 1 Xenobiotic Metabolism

1. 1. 1 Introduction 1
1. 1. 2 Covalent binding theory 2
1. 1. 3 Oxidative stress theory 4

1. 2 Rat Hepatocyte Toxicology

1. 2. 1 Introduction 7

1. 2. 2 Cellular defense
1. 2. 2. 1 Glutathione 8
1. 2. 2. 2 Superoxide dismutase 11
1. 2. 2. 3 Catalase 11
1. 2. 2. 4 Glutathione peroxidase 12
1. 2. 2. 5 Vitamins A and E 13

1. 3 Role of Cellular Ca^{2+} Homeostasis 14

1. 4 Aims 16

2. MATERIALS AND METHODS

2. 1 Materials

2. 1. 1 Chemicals 18
2. 1. 2 Gifts 18

2. 2 Methods Related to Hepatocyte Studies

2. 2. 1 Preparation of isolated rat hepatocytes

2. 2. 1. 1 Isolation of hepatocytes 19
2. 2. 1. 2 Incubation of hepatocytes 21
2. 2. 1. 3 Determination of viability 22

2. 2. 2 Determination of glutathione (GSH) and oxidized glutathione (GSSG) in hepatocytes 22

2. 2. 3 Determination of malondialdehyde (MDA) in hepatocytes 23

2. 2. 4 Preparation of subcellular fractions

2. 2. 4. 1 Preparation of liver microsomes, cytosolic fraction and S9 fraction from rats 24

2. 2. 5 Determination of oxygen consumption by hepatocytes and microsomes 25

2. 2. 6 Enzyme assays

2. 2. 6. 1 Determination of alcohol dehydrogenase activity 25
2. 2. 6. 2 Determination of aldehyde dehydrogenase activity 26
2. 2. 6. 3 Determination of glutathione reductase activity 26

2. 3 Methods related to mitochondria Ca^{2+} homeostasis

2. 3. 1 Preparation of rat liver mitochondria
- 2. 3. 1. 1 Isolation of mitochondria 27
- 2. 3. 1. 2 Incubation of mitochondria 27

2. 3. 2 Determination of Ca^{2+} fluxes from mitochondria 28

2. 3. 3 Determination of transmembrane potential of mitochondria 28

2. 3. 4 Determination of redox status of pyridine nucleotides [NAD(P)H, NAD(P)] in isolated mitochondria 29

2. 4 Statistical analysis 29

3. ACROLEIN STUDY

3. 1 Introduction 30

3. 2 Results 33

3. 2. 1 Hepatocyte studies
- 3. 2. 1. 1 Effects of acrolein on the viability of isolated rat hepatocytes 33
- 3. 2. 1. 2 Effects of acrolein on cellular GSH levels 36
- 3. 2. 1. 3 Effects of thiol agents on acrolein-induced cytotoxicity 36

3. 2. 1. 4 Effects of antioxidants and desferrioxamine on allyl alcohol and acrolein-induced cytotoxicity in isolated hepatocytes 40
3. 2. 1. 5 Effects of allopurinol on hepatocyte cytotoxicity induced by allyl alcohol or acrolein 43

3. 2. 2 Mitochondrial Ca^{2+} study
3. 2. 2. 1 Effect of acrolein on Ca^{2+} homeostasis of isolated rat liver mitochondria 46
3. 2. 2. 2 The effect of thiol agents on mitochondrial Ca^{2+} homeostasis 50

3. 3 Discussion 52

4. AZQ STUDY

4. 1 Introduction 59

4. 2 Results 63

4. 2. 1 Hepatocyte studies
4. 2. 1. 1 Effects of AZQ on the viability of isolated rat hepatocytes 63
4. 2. 1. 2 Induction of oxygen uptake by AZQ 63
4. 2. 1. 3 Effects of AZQ on GSH/GSSG levels in isolated rat hepatocytes 66
4. 2. 1. 4 Effect of catalase inhibition of AZQ-induced hepatocyte cytotoxicity 66
4. 2. 1. 5 Effect of AZQ on GSH recovery after hepatocyte GSH oxidation by H_2O_2 71

4. 2. 2 Mitochondrial study
    4. 2. 2. 1 Effect of AZQ on isolated rat liver mitochondrial Ca^{2+} homeostasis 76

4. 3 Discussion 80

5. Summary and conclusions 84

6. References 88

7. Publications arising from this work 96

LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
AU	absorbance units
AZQ	2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone
BHA	butylated hydroxyanisole
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid disodium salt
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
GSH	glutathione
GSSG	glutathione disulfide
HPLC	high pressure liquid chromatography
Lazaroid U-74500A	21-[4-(3,6-bis(diethylamino)-2-pyridinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)triene-3,20-dione hydrocholoride
MDA	malondialdehyde
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OD	optical density

LIST OF TABLES

Table 1.1 Characteristics of freshly, isolated hepatocytes

Table 3.1 The effect of cyanamide, disulfiram and ethacrynic acid on acrolein-induced cytotoxicity in isolated rat hepatocytes

Table 3.2 Protection of acrolein and allyl alcohol-induced hepatocytes cytotoxicity by desferrioxamine, BHA and Lazaroid V-745000A

Table 3.3 Protection of acrolein and allyl alcohol-induced hepatocyte cytotoxicity by DMSO and allopurinol

Table 4.1 AZQ cytotoxicity in isolated rat hepatocytes

Table 4.2 AZQ-mediated oxygen activation with liver microsomes, isolated hepatocytes and ascorbate

Table 4.3 Cytotoxicity induced by AZQ in catalase inactivated isolated hepatocytes

Table 4.4 Effect of AZQ on glutathione-reductase activity in rat liver cytosol

Table 4.5 H_2O_2-induced cytotoxicity in AZQ treated isolated hepatocytes

LIST OF FIGURES

Figure 1.1 One electron reduction of 1,4-benzoquinone

Figure 1.2 Mechanisms of regulation of cytosolic Ca^{2+} concentration in hepatocytes

Figure 1.3 Structures of diaziquone and acrolein

Figure 2.1 Perfusion apparatus

Figure 3.1 Proposed mechanism of acrolein cytotoxicity

Figure 3.2 The effect of acrolein on the viability of isolated rat hepatocytes

Figure 3.3 GSH depletion induced by acrolein in isolated hepatocytes

Figure 3.4 Enhancement of allyl alcohol-induced hepatocyte cytotoxicity by cyanamide

Figure 3.5 Potentiation of allyl alcohol-induced GSH depletion by cyanamide in isolated rat hepatocytes

Figure 3.6 Protection of acrolein-induced hepatocyte toxicity by thiol reagents

Figure 3.7 Sequence of events of mitochondrial Ca^{2+} release (A), transmembrane potential (B) and NAD(P)H oxidation (C)

Figure 3.8 Potentiation of acrolein-induced mitochondrial Ca^{2+} release by ethacrynic acid and cyanamide

Figure 3.9 Effect of dithiothreitol (DTT), glutathione (GSH) and N-acetylcysteine on Ca^{2+} uptake and retention in acrolein treated mitochondria

Figure 3.10 Formation of acrolein-DNA adduct

Figure 4.1 Possible mechanisms of AZQ induced cytotoxicity

Figure 4.2 GSH depletion (A) and GSSG formation (B) induced by AZQ in isolated hepatocytes

Figure 4.3 H_2O_2-induced changes in hepatocyte GSH/GSSG levels

Figure 4.4 AZQ prevents GSH recovery after H_2O_2-induced GSH depletion

Figure 4.5 Sequence of events of mitochondrial Ca^{2+} release (A) and transmembrane potential (B)

Figure 4.6 Potentiation of AZQ-induced mitochondrial Ca^{2+} release by ascorbate

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CHAPTER 1

Introduction

1. 1 Xenobiotic Metabolism

1. 1. 1 Introduction

Over the last two centuries, mankind has spent an increasing amount of resources in the development of chemicals in order to benefit his existence. These products range from industrial chemicals to pharmaceuticals to cosmetics. Because of the widespread use of chemicals in our environment, the human body is continuously exposed to a wide range of substances which are not natural constituents of the body, collectively termed xenobiotics or foreign compounds (Mason et al., 1985). As a result, the body has developed mechanisms to excrete these foreign compounds in order to prevent their toxic effect. The major routes for excretion is into the urine and bile; the lungs, salivary glands, nails and hair are other minor excretory routes (Briggs and Briggs, 1974).

Since excretion requires transportation via body fluids, the compounds being excreted have to be water soluble. Because the majority of chemicals that get access to the body are nonpolar lipid-soluble substances, the body has developed the capability to chemically transform most xenobiotics into different molecular structures. These are usually more polar water-soluble derivatives of which rapid excretion is favored. This concept of xenobiotic metabolism has led to the now well established theory that chemicals are generally metabolized in two phases, namely "Phase I metabolism" in which new functional groups are introduced into the lipophilic chemicals and "Phase II metabolism" or conjugation. Phase I metabolism comprises oxidations, reductions and hydrolysis which generally results in the introduction of small functional groups such as hydroxyl, carboxyl, amino or thiol into the parent compound (Parke, 1978). This results in a slightly more water-soluble metabolite than the parent lipophilic compound. Phase II metabolism increases still further the polarity of the metabolite. The latter phase occurs by the enzyme catalysed addition of small endogenous molecules including glucuronic acid or sulfate and glycine or glutathione, to the Phase I metabolites making the molecule less lipophilic, more polar and hence more readily excreted from the cell (Parke 1978).

In some instances, the xenobiotic burden may be as such that it overwhelms the cells defense capabilities or in other cases the metabolic transformation may produce an increase in toxic metabolites resulting in chemical-induced tissue injury. Two working hypotheses for chemical induced tissue injury have been suggested to include the covalent binding theory and the oxidative stress theory.

1. 1. 2 Covalent binding theory

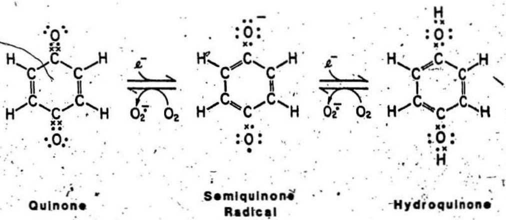
It has been known for a long time that certain chemicals, after administration to experimental animals, can react with cellular macromolecules. However, it was not until 1947, when Miller and Miller demonstrated covalent binding between the hepatocarcinogen N,N-dimethyl-4-aminoazobenzene and liver proteins, that the covalent binding theory was established (Miller and Miller, 1947). The logic behind this theory revolves around the observation that binding is related to toxicity. This theory suggests that carcinogenesis is initiated when electrophilic metabolites interact with nucleophilic groups of macromolecules. Evidence for this general mechanism is based on the correlation between the amount of covalent binding of a toxicant to proteins and the extent of toxicity. Therefore, if the formation of the toxic electrophilic metabolite is prevented or if it is removed before it can bind to a target macromolecule, toxicity is prevented. An example of this is in the now classic studies involving the toxicity of the analgesic drug acetaminophen, which produces centrilobular liver necrosis in man and other susceptible species (Davis et al., 1974). Acetaminophen is thought to be metabolized by the cytochrome P-450 system to a highly reactive electrophilic metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). If large doses of acetominophen are given to experimental animals the detoxifying pathways may become saturated allowing the reactive metabolite to covalently bind to nucleophilic sites of macromolecules and cause necrosis (Moldeus et al., 1978). Potter et al., (1974) demonstrated that acetaminophen-induced hepatic necrosis in hamsters could be prevented by pretreating the animals with piperonyl butoxide, an inhibitor of the cytochrome P-450 system, or increase toxicity by 3-methylcholanthrene, an inducer of the cytochrome P-450 metabolizing system. Indeed, N-acetylcysteine, a sulfhydryl agent known to bind to NAPQI, has been found to be beneficial in the treatment of acetaminophen toxicity in humans (Piperno and Bressenbrugge, 1976). In general, the covalent binding theory has become widely accepted as a mechanism for cellular toxicity.

1. 1. 3 Oxidative stress theory

Oxidative stress has been referred to as toxicity resulting from intracellular oxidant formation which results in oxidative changes to essential macromolecules (Rossi et al., 1986). This was first hypothesized to explain drug-induced hemolysis in humans with deficient erythrocyte levels of glucose-6-phosphate dehydrogenase. Antimalarial drugs such as primaquine, capable of generating active oxygen species, were developed to combat malarial infection when it was found that the malarial parasite showed a surprisingly high susceptibility to oxygen toxicity (Beutler, 1959). Glutathione has a direct role in protecting red blood cells from oxidative damage, but it must be in its reduced form (GSH). Normally, GSSG is prevented from accumulating by reduction with NADPH to GSH, catalyzed by glutathione reductase. Red cells depend on the pentose phosphate shunt to provide NADPH, and since the pathway is defective in glucose-6-phosphate dehydrogenase deficient erythrocytes, these cells are prone to toxicity by the antimalarial compounds.

The most important example of compounds which are able to form active oxygen species are redox cyclers, such as quinones. In the presence of molecular oxygen, reduced quinones are capable of generating superoxide radicals (O_2^-) via redox cycling between quinone, semiquinone radical and hydroquinone. Figure 1.1 exemplifies this one-electron reduction of a quinone (1,4-benzoquinone) to its semiquinone free radical and hydroquinone with molecular oxygen as the oxidizing agent. Since most semiquinone radicals react rapidly with molecular oxygen to form superoxide (Patel and Wilson, 1973), the process of autoxidation of the semiquinone to the quinone can yield

Figure 1.1 One electron reduction-of 1,4 benzoquinone


e⁻
O₂⁻
O₂
e⁻
O₂⁻
O₂
Quinone
Semiquinone Radical
Hydroquinone

large quantities of O_2^- (Kappus and Sies, 1981). Hence, no stable reduction products are formed except under anaerobic conditions. Quinone redox cycling can be catalyzed by a variety of flavoenzymes including NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and NADH:ubiquinone oxidoreductase (Iyanagi and Yamazaki, 1970) or directly by intracellular electron carriers, such as ascorbate or glutathione (Wefers and Sies, 1983).

The enzymatic or spontaneous dismutation of O_2^- yields H_2O_2. O_2^- can then react with H_2O_2 in a process catalyzed by trace metals (M) to form hydroxyl radicals (HO·) [eq. 4] and singlet oxygen ($^1O_2$) [eq. 5], (Beauchamp and Fridovich, 1970). Those reactions forming (HO·) and ($^1O_2$) are illustrated below:

$$O_2^- + M^{n+1} \longrightarrow M^{n+} + O_2 \quad \text{[eq. 1}$$

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \text{[eq. 2]}$$

$$M^{n+} + H_2O_2 + H^+ \longrightarrow M^{n+1} + HO\cdot + H_2 \quad \text{[eq.3]}$$

$$O_2^- + H_2O_2 + H^+ \xrightarrow{\text{met. cat.}} HO\cdot + O_2 + H_2O \quad \text{[eq. 4]}$$

$$O_2^- + H_2O_2 \xrightarrow{M^{n+}} OH^- + HO\cdot + {}^1O_2 \quad \text{[eq. 5]}$$

Since both (HO·) and ($^1O_2$) are strong oxidizing agents, they are probably the chemical species responsible for oxidative-stress induced enzyme inactivation, lipid peroxidation and DNA strand breakage (Halliwell and Gutteridge, 1986).

1. 2 Rat Hepatocyte Toxicology

1. 2. 1 Introduction

The liver plays a major role in xenobiotic metabolsim mainly because of two characteristics. One, because of its blood supply, the liver tends to receive higher concentrations of xenobiotics than do most other organs and secondly it is the major site of drug-metabolizing enzymes. Earlier studies showed that the events of xenobiotic-induced toxicity in isolated hepatocytes tend to occur in a similar sequence to those observed during the development of hepatotoxicity *in vivo*, suggesting that isolated hepatocytes can as a useful model for the study of *in vivo* hepatotoxicity (Fry and Bridges, 1979). As a result, hepatocytes both in suspension and in culture have recently been extensively used for xenobiotic metabolism studies.

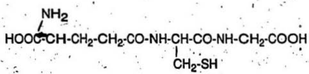
Early attempts to isolate hepatocytes witnessed the use of mechanical force and subsequently, perfusion of the liver with Ca^{2+} and K^+ chelators. These methods however, were unsuccessful in obtaining viable cells in high yields (Jacob and Bharava, 1962). Robbell in 1964 then demonstrated the isolation of fat cells by digesting adipose tissue with a collagenase/hyaluronidase mixture. Following this, Howard and Pesch (1968), described the isolation of viable adult rat hepatocytes by digestion of liver slices with collagenase/hyaluronidase. Berry and Friend (1969) improved this method by introducing a liver perfusion technique using this enzyme mixture. The perfusion method has been modified by various goups to suit specific goals. The technique used in the present study was introduced by Moldeus (1978), and comprises of a two-step perfusion method involving perfusion of the liver with a Ca^{2+}-chelating solution first, followed by a collagenase mixture containing Ca^{2+}. The result is a homogeneous suspension of parenchymal cells with little contamination of other cell types. The characteristics of the freshly-isolated cells are described in table 1.1.

Freshly isolated hepatocytes in suspension while remaining viable for only approximately 10 hours have the advantage of retaining their physiological characteristics (Moldeus et al., 1978). Cultures of viable hepatocytes tend to lose certain specialized functions as the culture time increases. The possibility of transformation after division may also make cultured hepatocytes unsuitable as a model for in vivo hepatotoxicity studies.

1. 2. 1 Cellular Defense System

1. 2. 2. 1 Glutathione

The hepatocyte has several mechanisms to rid the cell of toxicants and of these, conjugation with GSH is probably the most important. GSH is a tripeptide of glutamic acid, cysteine and glycine with the following structure:



$$\mathrm{HOO\overset{NH_2}{C}\text{-}CH\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}NH\text{-}\underset{CH_2\text{-}SH}{CH}\text{-}CO\text{-}NH\text{-}CH_2\text{-}COOH}$$

γ-glutamylcysteinylglycine

This nucleophilic tripeptide found in all tissues may be conjugated with many types of xenobiotics. Substrates for this reaction must possess an

TABLE 1.1 Characteristics of freshly, isolated hepatocytes.

Recovery	~ 2 - 4x10^7 cells/g liver
Trypan Blue exclusion	~ 90%
Glutathione	~ 50 nmol/10^6 cells
NADPH/(NADP$^+$ + NADPH)	~ 0.8
NADH/(NAD$^+$ + NADH)	~ 0.2
ATP	~ 20 nmol/10^6 cells
O_2 consumption	~ 12 nmol/10^6 cells per min
Cytochrome P-450	~ 0.25 nmol/10^6 cells
Cells with blebs	~ < 5%

Moldeus et al.,1978

electrophilic centre which binds covalently with the nucleophilic sulfur of the cysteinyl group of the the GSH to yield a conjugate. The formation of glutathione S-conjugates can occur either directly or catalyzed by glutathione S-transferases, a group of cytosolic enzymes with broad substrate specificities (Jakoby and Habig, 1980). The conjugate then undergoes removal of glutamate and glycine followed by acetylation to yield mercapturic acid. The ability of GSH to directly reduce free radicals with the concomitant formation of thiyl radicals and GSSG has been speculated, however, the extent to which this reaction occurs *in vivo* is still unknown.

Along with conjugation with many toxic molecules or metabolites produced as a result of Phase I drug metabolism, GSH is also involved in the detoxification of hydroperoxides (O'Brien, 1988). Hydroperoxides are powerful oxidizing agents, and can oxidize compounds containing SH groups, as can the free radicals formed during hydroperoxide degradation (discussed later). GSH has been shown to play an important role in the protection of cells from these peroxides by acting as the cofactor for the enzyme glutathione peroxidase. This results in the conversion of the peroxide to H_2O or alcohol and of GSH to GSSG. Since accumulation of GSSG is cytotoxic, cells have developed a mechanism to extrude the disulfide from the cell and/or to reduce it back to GSH with the aid of the enzyme, glutathione reductase and NADPH.

Because of the efflux of GSSG and GS-conjugate during oxidative stress and covalent binding, the ability of the cell to resynthesize GSH may be compromised by a lack of substrate. As a result, toxicity is found to ensue after GSH depletion by xenobiotics. Since the supply of cysteine appears to be the rate-limiting step in hepatic glutathione synthesis, administration of cysteine precursors like methionine or N-acetylcysteine may protect cells from oxidative stress and be of benefit in the treatment of acetaminophen toxicity (Piperno and Bressenbruegge, 1976). Hence GSH serves a protective function against both oxidative stess and electrophilic attack by reactive intermediates during xenobiotic metabolism.

1. 2. 2. 2 Superoxide dismutase

Superoxide dismutase (SOD) is the first line of defense against superoxide radicals ($O_2^{\cdot-}$). SOD catalyzes the disproportionation or dismutation of superoxide by the following equation:

$$2\,O_2^{\cdot-} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

In hepatocytes, there are two types of SOD: the Cu/Zn-containing enzyme located in the cytosol, and the Mn-containing enzyme found mostly in the mitochondria (Weisiger and Fridovich, 1972).

1. 2. 2. 3 Catalase

Catalase is present in almost all mammalian cells and is mostly compartmentalized in peroxisomes. Its main function is the removal of H_2O_2 from the cell via the following reaction:

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

Catalase is a conjugated protein with protohaematin as its prosthetic group. An unusual characteristic unique of catalase which differs from other haematin dervatives is that it cannot be reduced even by such powerful reducing agents as sodium hyposulfite. Catalytic decomposition of hydrogen peroxide occurs when iron is in the ferric form. Hepatocytes deficient in catalase activity may be obtained by treating the cells with sodium azide. This results in the formation of an azide-catalase complex which can be reduced to the ferrous form by H_2O_2, rendering the catalase inactive (Keilin and Hartree, 1945).

1. 2. 2. 4 Glutathione peroxidase

Glutathione peroxidase (GSH-Px) catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides (R-OOH) by the following equations (Little and O'Brien, 1968):

$$\mathrm{HOOH} + 2\,\mathrm{GSH} \xrightarrow{\text{GSH-Px}} \mathrm{GSSG} + 2\,H_2O$$

$$\mathrm{ROOH} + 2\,\mathrm{GSH} \xrightarrow{\text{GSH-Px}} \mathrm{GSSG} + H_2O + \mathrm{ROH}$$

Essential to the action of GSH-Px on hydroperoxides is the level of GSH which is maintained by *de novo* synthesis of GSH as well as by the reduction of GSSG to GSH with NADPH. The latter reduction is catalysed by glutathione reductase, an enzyme found in the cytosol and mitochondria. NADP+ is maintained in its reduced form by glucose-6-phosphate dehydrogenase of the pentose phosphate pathway, isocitrate dehydrogenase and other enzymes of the Krebs cycle (Reed, 1986; Eggleston and Krebs, 1974). The catalytic site of GSH-Px contains selenocysteine (Forstrom et al., 1978) and its activity can vary with the amount of selenium in the diet (Coombs and Coombs, 1984). GSH-Px acts in concert with catalase to remove H_2O_2, but has a much lower Km for

H_2O_2 than catalase. Therefore, at high GSH and relatively low peroxide levels, H_2O_2 is decomposed by GSH-Px faster than by catalase (Jones et al., 1981).

1. 2. 2. 5 Vitamins E and A

Vitamin E is a one-electron donor. The hydroxyl group of the benzene ring of vitamin E (vit-E-OH) acts as a reductant, upon oxidised with free radicals, and generates a vitamin E free radical (vit-E-O.):

$$ROO\cdot + \text{vit-E-OH} \longrightarrow \text{R-OOH} + \text{vit-E-O}\cdot$$

The resulting vitamin E radical may then be reduced back to vitamin E by polar donors such as ascorbic acid (AH_2) (Packer et al., 1979) and/or GSH:

$$\text{vit-E-O}\cdot + AH_2 \longrightarrow AH\cdot + \text{vit-E-OH}$$
$$\text{vit-E-O}\cdot + \text{GSH} \longrightarrow \text{GS}\cdot + \text{vit-E-OH}$$

The resulting vitamin C radical is in turn enzymatically reduced back to vitamin C by NADH-dependent systems, while the glutathione free radicals form GSSG which can then be reduced back to GSH by glutathione reductase.

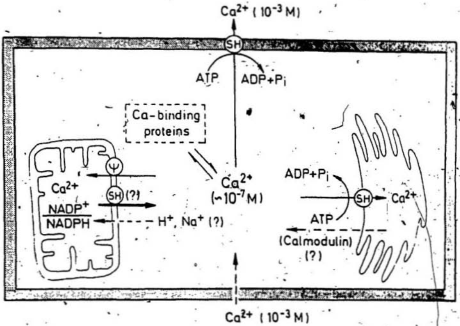
The antioxidant activity shown by carotenoids (vitamin A) has been related to their capacity to quench oxidant species such as singlet molecular oxygen (Foote and Denny, 1968). Apparently, cis-carotene reacts with $^1O_2$, yielding an excited carotenoid which subsequently disperses its energy by reversible isomerization to trans-carotene. This characteristic inherent of -carotene is the basis for its protective role against damage initiated by visible light, as well as its possible use in the treatment of certain photosensitive diseases (Cadenas, 1985). It has been sugggested that dietary -carotene could be protective against cancer development (Cadenas, 1985).

1. 3 Role of cellular Ca^{2+} homeostasis in toxicology

The intracellular concentration of Ca^{2+} plays a major role in the regulation of intermediary as well as motile and secretory cell functions (Duncan, 1976). As illustrated in fig. 1.2, its concentration in the cytosol is rigorously controlled by active compartmentation into the endoplasmic reticulum and mitochondria, extrusion by the plasma membrane and by calcium binding to specific proteins including calmodulin (Carafoli, 1987). Mitochondrial Ca^{2+} homeostasis is regulated by a cyclic mechanism involving Ca^{2+} uptake by an electrogenic ruthenium-red sensitive uniporter and Ca^{2+} release, which is probably mediated by a Ca^{2+}/H^+ antiporter. The latter appears to be regulated by the redox level of intramitochondrial pyridine nucleotides (Lehninger et al., 1978), although a recent study has shown that thiols may also be important in modulating mitochondrial Ca^{2+} transport (DiMonte et al., 1984). In addition, the existence of a Ca^{2+}/Na^+ antiporter may also contribute to Ca^{2+} efflux in liver mitochondria. The active transport of calcium ions through the endoplasmic reticulum and plasma membrane is mediated by Ca^{2+}-stimulated Mg^{2+}-dependent ATPases which both appear to depend on free sulfhydryl groups for activity (Moore et al., 1975).

Recently, a role for cytosolic ionized calcium as a mediator in cell injury has been suggested. Drug-induced hepatocyte toxicity has been associated with an increase in cytosolic Ca^{2+} concentration, which appears to involve the mobilization of intracellular Ca^{2+} stores as well as influx of extracellular Ca^{2+} (Bellomo and Orrenius, 1985). The mechanism(s) by which cytosolic Ca^{2+}

Figure 1.2 Regulation of intracellular calcium


Ca^{2+} (10^{-3} M)
SH
ATP
ADP+Pi
Ca-binding proteins
Ψ
Ca^{2+}
SH (?)
$NADP^+$ / NADPH
H^+, Na^+ (?)
Ca^{2+} (~10^{-7} M)
ADP+Pi
SH
Ca^{2+}
ATP
(Calmodulin) (?)
Ca^{2+} (10^{-3} M)

Nicotera, P. and Orrenius, S. (1987)

mediates toxic cell injury may involve interactions with calmodulin, cytoskeletal components such as actin and tubulin and activation of hydrolytic enzymes such as phospholipases and proteases (Nicotera and Orrenius, 1987). Hence, these events may represent an early and common step in drug-induced toxicity.

2.4 Aims

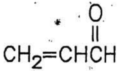
The major objective of this thesis is to determine the importance of alkylation and oxidative stress in the toxicity of xenobiotics using isolated rat hepatocytes both as the metabolizing system and target cells.

Chemical-induced tissue injury has been suggested to occur through alkylation and oxidative stress. However, the relative contributions of each of these mechanisms in inducing cytotoxicity are still being debated. The major part of this study is to characterize the biochemical mechanisms leading to toxicity when isolated hepatocytes are exposed to compounds which theoretically are good candidates for iether alkylation or oxidative stress.

In the present study, the effects of diaziquone and acrolein exposure to isolated rat hepatocytes will be presented in order to illustrate the parallels between the effects of these two agents in the development of cytotoxicity (figure 1.3 shows their structure) . Diziquone is a benzoquinone which theoretically can undergo redox cycling via the activated semiquinone, and acrolein is the reported reactive intermediate in the metabolism of allyl alcohol.

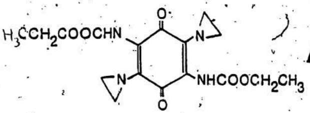
Alterations in Ca^{2+} homeostasis have been suggested to be critical in the initiation of irreversible chemical-induced injury. The other part of this thesis focuses on the ability of the two model compounds to disrupt cellular Ca^{2+} homeostasis. To accomplish this, isolated rat liver mitochondria were used to monitor Ca^{2+} fluxes upon exposure to the chemical agent.

Figure: 1.3



$$CH_2{=}CH\overset{\overset{O}{\|}}{C}H$$

Acrolein



AZQ

CHAPTER 2

MATERIALS and METHODS

2. 1 Materials

2. 1. 1 Chemicals

Sodium azide, hydrogen peroxide, 2,4-dinitrofluorobenzene, dimethylsulfoxide, glutathione, oxidized glutathione, potassium cyanide, cyanamide, ascorbic acid, rotenone, succinic acid, trichloroacetic acid, thiobarbituric acid, allopurinol, pyrazole, disulfiram and butylated hydroxyanisole were purchased from Sigma Chemical Co. , ST. Louis, Missouri .

Catalase, albumin, collagenase, NADH, NADPH and hepes were obtained from Boehringer Mannheim, Dorval, Quebec .

Allyl alcohol, metaphosphoric acid, safranin O and arsenazo III, were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin .

Acrolein was obtained from BDH Chemicals, Toronto. HPLC solvents were obtained from Caledon, Georgetown, Ontario.

2. 1. 2 Gifts

AZQ was provided by the National Cancer Institute Bethesda, Maryland.

Desferrioxamine was provided by Ciba-Geigy Canada LTD., Mississauga, Ontario.

Lazaroid U-74500A was provided by UpJohn Company, Toronto Ontario.

2. 2 Methods related to hepatocyte studies

2. 2. 1 Preparation of rat hepatocytes

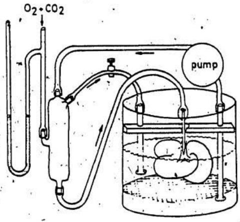
2. 2. 1. 1 Isolation of rat hepatocytes

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing 200-225 g were used for the preparation of hepatocytes. They were given food and water *ad libitum*.. Isolated rat hepatocytes were prepared according to the method described by Moldeus et al., 1978. Four different buffers were used. Buffer A (200 ml) was a modified Hanks buffer, pH 7.4 [8.0 g NaCl; 0.4 g KCl; 0.2 g $MgSO_4\cdot 7H_2O$; 0.06 g $Na_2HPO_4.2H_2O$; 0.06 g KH_2PO_4; 2.19 g $NaHCO_3$, in a volume of 1 litre] containing 0.5 mM ethanedioxybis(ethylamine)-tetracetate (EDTA), 2% albumin and 12.5 mM Hepes. Buffer B (100 ml) was the same modified Hanks buffer containing 0.075% collagenase, 4 mM $CaCl_2$ and 12.5 mM Hepes. Buffer C (200 ml) was a Krebs-Henseleit buffer, pH 7.4, [6.9 g NaCl; 0.36 g KCl; 0.13 g KH_2PO_4; 0.295 g $MgSO_4\cdot 7H_2O$; 0.374 g $CaCl_2\cdot H_2O$; 0.2 g $NaHCO_3$, in a volume of 1 litre] containing 2% albumin and 12.5 mM Hepes. Buffer D (400 ml) was the same Krebs-Henseleit buffer containing 12.5 mM Hepes. All solutions were equilibrated with carbogen gas (95% O_2, 5% CO_2) and heated to 37°C prior to use.

The apparatus used for all perfusions was setup as shown in fig. 2.1. It consisted of an oxygenator connected with Teflon tubing to a steel cannula, a plastic rack containing two vertical tubes with a horizontal bar at the top and a plastic screen at the bottom placed in a beaker, and a roller pump. The flow through the cannula was controlled by changing the gas pressure.

The rats were anaesthetized with ether, and the peritoneal cavity was

Figure 2.1 Rat liver perfusion apparatus


$O_2 \cdot CO_2$
pump

opened by a midventral incision. Heparin (500 units in 0.1 ml) was injected in the caval vein. A ligature was applied around the portal vein, an incision was made and the cannula was immediately inserted allowing the liver to be perfused with buffer A. The inferior vena cava was then cut to allow for the outflow of the perfusate to waste and the liver was excised from the body. After 4 min of perfusion with buffer A, the plastic rack was placed in another beaker containing buffer B. Buffer B was recirculated for approximately 6 min while the pressure was kept constant (10-15 cm H_2O). At the end of the perfusion, the liver was removed form the cannula and immersed in buffer C. The capsule was then cut open, and the cells were dispersed with a pair of tweezers and gentle swirling, followed by filtration through cotton gauze. The cell suspension was then distributed into plastic centrifuge tubes and centrifuged (two times) at 50 x g for 40 seconds. The supernatant (containing cell debris and non-parenchymal cells) was discarded and the loose cell pellet was re- suspended in buffer D and counted in a Improved Neubauer counting chamber (Hauksley, London, England) using a low power light microscope (40 x).

2. 2. 1. 2 Incubation of hepatocytes

After isolation of the hepatocytes the sedimented cells were diluted in buffer D to a final concentration of 1 x 10^6 cells/ml. The cells were pipetted (20 ml) into round-bottom flasks fitted on a standard-taper distillation adapter for five flasks which was rotated (30 rpm) on a rotary evaporator with the flasks dipping down in the thermostated water (37°C). Carbogen gas was continuously applied to the surface of the incubation medium through the central vacuum exit of the evaporator. Cells were preincubated for 30 minutes before the start of each experiment at which time, recovery from the shock of the isolation procedure was reached. Water insoluble agents were dissolved in dimethyl-sulfoxide (DMSO) and added to the hepatocyte incubation in a volume of 50 µl or less.

2. 2. 1. 3 Determination of viability

Cell viability was determined by trypan blue penetration. This procedure monitors the integrity of the plasma membrane and is both sensitive and easy to perform. Aliquots of cell suspension (100µl) were added to an equal volume of 0.5% trypan blue (w/v; dissolved in buffer D) and examined using a low power light microscope (40 x). The percentage of cells taking up the stain were classified as the percentage of nonviable cells. Experiments were performed when a successful hepatocyte isolation procedure obtained a yield of cells with 85% or greater viability.

2. 2. 1. 4 Determination of glutathione (GSH) and oxidized glutathione (GSSG) in hepatocytes

Determination of reduced and oxidized glutathione was carried out by high-performance liquid chromatography as described by Reed et al., (1980). An aliquot of 0.8 ml incubation mixture was mixed vigorously with 0.2 ml solution of 25% w/v metaphosphoric acid. After centrifugation, (5 min, 600 x g, room temperature) to precipitate the protein, 0.5 ml of supernatant was added to test tubes containing approximately 20 mg of sodium bicarbonate, followed immediately with the addition of 50 µl of iodoacetic acid solution (15 mg/ml H_2O) and left for 60 minutes in the dark. A 0.5 ml solution of 1-fluoro-2,4-dinitrobenzene (DNP) (1.5% v/v) in absolute ethanol was added and allowed to react for 4 hours at room temperature in the dark to form N-DNP derivatives. N-DNP derivatives of standard GSH and GSSG were also formed in a similar way as described above.

Separation of DNP-derivatives were performed on a Waters Bondapak amine liquid chromatography column. Samples (50 µl) were injected with a Wisp 710 A automatic injector.. The solvents were delivered with a 660 solvent programmer solvent delivery system. The column was washed with 80% methanol and then equilibrated for 5 minutes with a solvent system containing 640 ml; methanol, 160 ml water and 200 ml from a solution containing 272 g sodium acetate trihydrate, 122 ml water and 378 ml galcial acetic acid. The elution of DNP derivatives of GSH and GSSG was carried out using the same solvent system as described, for 15 minutes at a flow rate of 1.5 ml per minute. The eluted compounds were detected at 365 nm using a Waters Model 440 absorbance detector. The signals from the detector were integrated and recorded on a Waters Data Module. After elution, the column was washed for 5 minutes with 80% methanol before analyzing the next sample. The concentrations of GSH and GSSG in the samples were calculated from the respective standard curves and are expressed as nmoles GSH/10^6 cells, ± SE of at least 3 separate experiments.

2. 2. 3 Determination of malondialdehyde (MDA) in hepatocytes

Lipid peroxidation was assessed by periodically removing 1 ml aliquots from the hepatocyte incubation mixture and measuring thiobarbituric-acid reacting substances (MDA) by the method of Ottolenghi, (1958). Aliquots of 1 ml incubation mixture were diluted to 2 ml with deionized water. 1 ml of 35% trichloroacetic acid (TCA) was then added followed by 2 ml of 0.75% aqueous thiobarbituric acid and the contents mixed. The tubes were then placed in a boiling water bath for 15 minutes. After cooling, evaporation losses were replaced by addition of water followed by 2 ml of 70% TCA. After a 20 minute period tp allow for complete extraction of the colour adsorbed on to the protein pellets, the tubes were centrifuged for 15 mintues. The abrorbance of the supernatants were read in a double beam DU-7 Beckman spectrophotometer at 535 nm. Blanks containing the incubation medium plus the chemical agent being studied but without cells, were also processed and their absorbances subtracted from those of the complete incubations. Results are expressed as the change in absorbance or optical density (OD) per 10^6 cells.

2. 2. 4 Preparation of subcellular fractions

2. 2. 4. 1 Preparation of liver microsomes, cytosolic fraction and S9 fraction from rats

Microsomes were prepared from male Sprague-Dawley rats (200-250 g), as described by Ernster et al., (1962) with the exception that the food and water was given *ad libitum*. Animals were killed by cervical dislocation followed by perfusion of the liver through the portal vein with ice-cold 0.9% saline to remove blood. The liver was excised from the body, blotted dry and weighed. All steps were carried at 0-4°C. The liver was then immersed in 3 volumes (w/v) of 0.1M potassium phosphate buffer (pH 7.4), minced into fine pieces with scissors and homogenized by a hand-operated Potter-Elvehjem homogenizer. The homogenate was then transferred to centrifuge tubes and centrifuged at 9000 x g for 10 minutes in a Sorval RC-2B centrifuge using a SS-34 rotor. The supernatant (designated the S9 fraction) was decanted, filtered through cheesecloth, and centrifuged at 105,000 x g for 75 minutes in a Model L5-65B Beckman ultracentrifuge, equiped with a 50 Ti rotor. This supernatant was designated the cytosolic fraction and frozen at -80°C. The microsomal pellet was resuspended in 0.1M phosphate buffer pH 7.4 by gentle homogenization with a hand homogenizer and centrifugated at 105,000 x g for 60 minutes. The pellet was resuspended by homogenization in phosphate buffer in a volume equal to the initial weight of the liver and frozen in suitable aliquots at -80°C. Protein determination was performed as described by Bradford, (1972) using bovine serum albumin as a standard.

2. 2. 5 Determination of oxygen consumption by hepatocytes and microsomes

Oxygen consumption was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co. Inc. , Model 5300) in a 2 ml chamber, maintained at 37°C. Before use, hepatocytes, at a concentration of 1 x 10^6/cells, were kept at 37°C in Krebs-Henseleit buffer containing 12.5 mM Hepes (pH 7.4) and allowed to equilibrate with 95% air and 5% CO_2. KCN (1 mM neutrilized with HCl) was added to inhibit mitochondrial respiration.

Oxygen consumption by microsomes was conducted in the same manner described above except that the buffer used was 0.1 M Tris-HCl (pH 7.4) and NADPH was added to supply reducing equivalents for the cytochrome P-450 reductase system. Results are expressed as nmoles $O_2/10^6$ cells/min for hepatocytes, and nmoles O_2/mg protein/min for microsomes ± standard error of at least three separate experiments.

2. 2. 6 Enzyme assays

2. 2. 6. 1 Determination of alcohol dehydrogenase activity

Alcohol dehydrogenase (ADH) activity of rat liver cytosol was measured at 25°C as described by Rikans and Moore, (1987). The reaction mixture was comprised of 90mM potassium-phosphate (pH7.4), 40mM KCl, 0.5 mM NAD+, the alcohol substrate as indicated and rat liver cytosol in a final volume of 1ml.

ADH activity was followed by monitoring NADH production as the increase in absorbance at 340nm in a Beckman DU-7 Spectrophotometer. Values were expressed as nmol NAD+ reduced/min/g liver ± standard error of at least three separate experiments.

2. 2. 6. 2 Determination of aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity of rat liver S9 fraction was determined spectrophotometrically by measuring the production of NADH at 340 nm, as described by Mitchell and Peterson (1988). The S9 ALDH activity was assayed, at 23°C, in a 1.0 ml reaction mixture containing 100 mM sodium phosphate buffer (pH 7.4), 1.0 mM NAD+, 2.0 mM pyrazole and rat liver S9 protein. The reaction was started by the addition of the aldehyde substrate. Values were expressed as nmol NAD+ reduced/min/mg protein ± standard error of at least three separate experi- ments.

2. 2. 6. 3 Determination of glutathione reductase activity

Glutathione reductase activity was measured as described by Carlberg and Mannervik (1985). The reaction was carried out in a 1 ml cuvette and contained 0.5 ml of 0.2 M potassium phosphate buffer (pH 7.0) containing 2 mM EDTA, 50 µl of 2 mM NADPH in 10 mM Tris-HCl (pH 7.0), 50 µl of 20 mM GSSG and deionized water to give a final total volume of 1 ml. The reaction was initiated by the addition of rat liver cytosol to the cuvette. Glutathione reductase activity was followed by monitoring the oxidation of NADPH in a DU-7 Beckman double beam spectrophotmeter at 340 nm at room temperature. Dicoumarol (100 µM), was included in the reaction mixture to inhibit NADPH oxidation due to DT-diaphorase activity. DT-diaphorase catalyses the reduction of quinones to hydroquinones by NAD(P)H (Iyanagi and Yamasaki, 1970). Values were expressed as nmol NADPH oxidized/min/mg protein ± standard error of at least three separate experiments.

2. 3 Methods related to mitochondrial Ca^{2+} homeostasis

2. 3. 1 Preparation of rat liver mitochondria

2. 3. 1. 1 Isolation of rat liver mitochondria

Mitochondria were prepared from male Sprague-Dawley rats (225-250 g, fed *ad libitum*) as described by Bellomo et al., 1984. The rats were killed by decapitation and the abdominal cavity was opened. The liver was immediately perfused with cold 0.9% NaCl solution and placed in a homogenization medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Hepes, (pH 7.4) (MSH buffer) and 1 mM EDTA. Liver tissue was minced with scissors and homogenized with a Potter (glass teflon) homogenizer in a total volume of 80-100 ml (1:10 homogenate). The homogenate was then centrifuged for 10 min at 4°C at 400 x g in a Sorvall RC-2B centrifuge. The supernatant was then centrifuged at 9000 x g for 10 min. The resulting pellet was washed by resuspension in EDTA-free MSH buffer followed by centrifugation at 9000 x g for 10 min. The final pellet was resuspended in a small volume of EDTA-free MSH buffer. Protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin used as a standard. The mitochondrial suspension was kept on ice and used immediately.

2. 3. 1. 2 Incubation of mitochondria

Mitochondria were incubated (1 mg/ml) at room temperature in MSH buffer containing 3 μM rotenone and 5 mM succinate. Rotenone was included in order to maintain mitochondrial pyridine nucleotides in a reduced state and to induce the release of endogenous Ca^{2+} (Frei et al., 1985). Succinate was used as the energy supply for mitochondria. $CaCl_2$ at a concentration of 50 nmol/mg of mitochondrial protein was then added to the mitochondria and allowed to incubate for approximately 5 min to allow for the Ca^{2+} to be taken up by the mitochondria.

2. 3. 2 Determinations of Ca^{2+} fluxes from rat liver mitochondria

Ca^{2+} movements across the inner mitochondrial membrane were followed at 25°C by dual wavelength spectrophotometry using the metallochromic dye Arsenazo III [40 µM, 2,2'-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-diazo)-bis- (benzene arsenic acid)] (Kendrick et al., 1977). Arsenazo III was added to a 3 ml cuvette containing the mitochondrial incubation mixture. Upon addition of Ca^{2+}, a blue colour appeared instantly as a result of an interaction between the dye and the added Ca^{2+}. This blue color gradually returned to pink as the Ca^{2+} dissociated from the dye complex and was taken up by the mitochondria. Absorbance changes were monitored continuously in an Aminco DW2 dual-wavelength spectrophotometer operating with the wavelength pair 654-685 nm. A recorded increase in absorbance signified a release of Ca^{2+} from mitochondria.

2. 3. 3 Determination of transmembrane potential of rat liver mitochondria

Mitochondrial transmembrane potential was measured using the dye safrarin O (10 µM) as described by Akerman and Wikstrom, (1976) at 25°C. Safrarin O was added to a 3 ml cuvette containing the mitochondrial incubation mixture. Upon addtition of Ca^{2+}, the absorbance decreased as the Ca^{2+} was taken up into the mitochondria and thereby signifying a drop in the transmembrane potential. Absorbance returned to original levels thereafter indicating a restoration of the transmembrane potential and a complete uptake of the added Ca^{2+}. Absorbance changes were monitored continuously in an Aminco DW2 dual wavelength spectrophotometer with the wavelength pair 533-511 nm. A decrease in absorbance signified a drop in the mitochondrial transmembrane potential.

2. 3. 4 Determination of redox status of pyridine nucleotide [NAD(P)H/NAD(P)] in isolated mitochondria

Mitochondrial pyridine nucleotides were monitored at 25°C in a DW2 dual wavelength spectrophotometer with the wavelength pair 340-370 nm (Lehninger et al., 1978). Absorbance changes were monitored continuously after the addition of Ca^{2+} to the mitochondrial incubation mixture. Compounds being tested were added thereafter as indicated in the figures.

2. 4. Statistical Analyses

Statistical significance of differences between treatment groups in these studies was determined by the Student's t-test. The minimal level of significance chosen was $p<0.05$.

CHAPTER 3

Acrolein Study

3. 1 Introduction

Acrolein is a highly reactive molecule having two reactive centers consisting of a carbon-carbon double bond and an aldehydic group. Its main use is in the chemical industry as a synthetic intermediate (Patel et al., 1980). Acrolein is a by-product of overheated frying oils (Zitting and Heinonen, 1980), a component of tobacco smoke (Izard and Liberman, 1978) and automobile exhaust (Lipari and Sivarin, 1982). It is also a metabolite of the anticancer drug cyclophosphamide (Ohno and Ormstead, 1985).

Because of its importance, acrolein has been widely studied and its toxicities have been categorized. Hales (1982) reported acrolein to be teratogenic in pregnant Sprague-Dawley rats. Malformations produced by acrolein included edema, hydrocephaly, open eyes, cleft palate, micrognathia, omphalocele, bent tail and forelimb and hindlimb defects. Acrolein was also found to be extremely bacteriotoxic (Hales, 1982) but lacked any mutagenic activity using the Salmonella typhimurium TA 1535 test system (Hales, 1982; Ellenberger and Mohn, 1977). However, in some test systems acrolein causes chromosomal damage (Au et al., 1980). Inhibition of mitochondrial respiration by acrolein was reported by Zollner (1973), while Patel et al., (1984) showed that addition of acrolein to lung and liver microsomal suspension resulted in total inhibition of NADPH-cytochrome c reductase.

Reid (1972), showed that metabolism of allyl alcohol to acrolein in rats resulted in hepatic periportal necrosis. This necrosis as well as the binding of ^{14}C-allyl alcohol to periportal hepatocytes was found to have been markedly reduced by pretreating the animals with pyrazole, therefore inhibiting the bioactivation of allyl alcohol to acrolein (Reid, 1972). This provided ~~Hence~~ providing the first direct evidence *in vivo* that allyl alcohol causes periportal necrosis through the covalent binding of the metabolite acrolein to periportal hepatocytes. Detoxification of acrolein was then suggested to occur via conjugation with GSH and oxidation to acrylic acid by aldehyde dehydrogenase (ALDH) (Ohno et al., 1985) (fig. 3.1). In support of this mechanism, Jaeschke et al. (1987) showed that allyl alcohol-induced necrosis increased if ALDH was inhibited with disulfiram or cyanamide.

The mechanism of acrolein-induced cytotoxicity is still poorly understood. Acrolein-induced death of isolated rat hepatocytes or liver damage following perfusion is reported to be preceded by GSH depletion and to be associated with extensive lipid peroxidation (Zitting and Heinonen, 1980). GSH depletion and modification of protein thiols can result in perturbation of intracellular Ca^{2+} homeostasis, alteration of cellular morphology and cell death (Nicotera and Orrenius, 1987). Whether acrolein induced hepatotoxicity specifically occurs as a result of lipid peroxidation catalyzed by production of superoxide anions from the xanthine oxidase system as suggested by Badr et al. (1986), or whether acrolein simply alkylates vital thiol groups and macromolecules resulting in cell death is still being debated.

The aim of this study is to investigate the relationship between GSH depletion, lipid peroxidation and cytotoxicity when isolated rat hepatocytes are treated with acrolein.

Figure 3.1 Proposed mechanism of acrolein metabolism in the hepatocyte

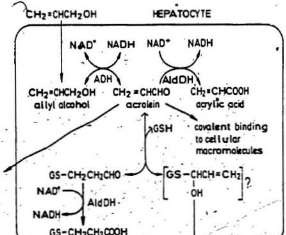
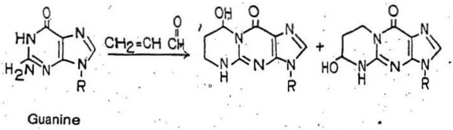

CH2=CHCH2OH
HEPATOCYTE
NAD+
NADH
NAD+
NADH
ADH
AldDH
CH2=CHCH2OH
allyl alcohol
CH2=CHCHO
acrolein
CH2=CHCOOH
acrylic acid
GSH
covalent binding to cellular macromolecules
GS-CH2CH2CHO
[GS-CHCH=CH2 | OH]?
NAD+
AldDH
NADH
GS-CH2CH2COOH

Figure 3.10 Formation of acrolein-DNA adduct


CH2=CH CH
O
Guanine

R = deoxyribose

3. 2 RESULTS

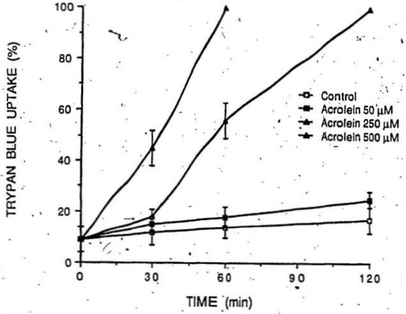
3. 2. 1 Hepatocyte Studies

3. 2. 1. 1 Effects of Acrolein on the Viability of Isolated Rat Hepatocytes

Addition of acrolein at a concentration of 250 and 500 µM to isolated rat hepatocytes resulted in the appearance of surface blebs followed by cell death at 2 hours and 1 hour respectively, as assessed by trypan blue penetration (figure 3.2). A dose of 50 µM acrolein was found not to significantly affect hepatoyte viability within 120 minutes ($p>0.1$) while higher doses caused cell death in a time-dose dependent manner.

Both cyanamide and disulfiram are reported to be potent inhibitors of aldehyde dehydrogenase (Marchner and Tottmar, 1978; Vallari and Pietrunzko, 1982), the enzyme reported to be responsible for metabolizing acrolein to the more hydrophilic and less reactive acrylic acid (Rikans and Moore,1987). Table 3.1 demonstrates that pretreatment of hepatocytes with either cyanamide or disulfiram increased the susceptibility of hepatocytes to acrolein-induced cytotoxicity. Concentrations of a previously subtoxic dose of 50 µM acrolein caused 100% cell death within 2 hours of incubation. Hepatocyte viability was not significantly altered from that of untreated cells when either disulfiram or cyanamide were incubated alone for a 2 hour incubation period ($p>0.1$). Depletion of GSH by preincubating hepatocytes with ethacrynic acid also significantly increased acrolein-induced cytotoxicity ($p<0.001$) (table 3.1).

Figure 3. 2 The effect of acrolein on the viability of isolated rat hepatocytes.



Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4 containing Hepes (12.5 mM), with varying concentrations of acrolein. Cell viability at 30, 60 and 120 minutes was determined as described in Methods (2. 2. 1. 3). Three separate experiments were carried out. Values are means ± standard error.

Table 3. 1 The effect of cyanamide, disulfiram and ethacrynic acid on acrolein-induced cytotoxicity in isolated rat hepatocytes

Treatment (μM)	Cytotoxicity (% trypan blue uptake) min: 30	60	120
Control	12±2	14±2	17±3
Acrolein (50)	13±2	16±3	20±3
+ cyanamide (200)	22±3	53±4	100
+ disulfiram (150)	18±3	45±4	100
+ ethacrynic acid (500)	20±3	52±4	100
Cyanamide (200)	13±2	15±2	19±3
Disulfiram (150)	12±2	15±2	18±3
Ethacrynic acid (500)	14±2	16±2	20±3

Hepatocytes (10^6 cells/ml) were preincubated at 37°C for 5 minutes in Krebs-Henseleit buffer, pH 7.4,, with either cyanamide, ethacrynic acid or disulfiram. Where indicated, acrolein was then added to the incubation mixture. Cell viability at 30, 60 and 120 minutes was determined as described in Methods (2. 2. 1. 3). Three separate experiments were carried out. Values are means ± standard error.

Recently, Mitchell and Peterson (1988) reported that acrolein was not a substrate for either mitochondrial or cytosolic ALDH but was instead a potent inhibitor. Our results with rat liver S9 confirm this since oxidation of acrolein could not be measured spectrophotometric by the production of NADH. However the addition of 1.0 mM GSH to the ALDH assay medium containing 1.0 mM acrolein and 1.0 mM NAD^+ resulted in an ALDH activity of 7.2 ± 1.1 nmol of NADH formed/ min/mg of protein. Therefore, in the presence of GSH, acrolein was found to be a good substrate for ALDH. Presumably either the GSH - acrolein conjugate was a substrate for ALDH or GSH protected ALDH from inactivation by acrolein.

3. 2. 1. 2 Effects of Acrolein on Cellular GSH Levels

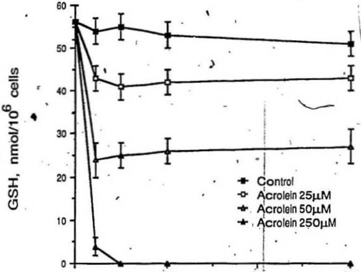
Hepatocyte GSH was rapidly depleted (< 1 min) without subsequent recovery when toxic doses of acrolein (250 μM) were incubated with the cells (fig. 3.3). At a subtoxic dose of 50 μM acrolein however, GSH depletion was immediate, but did not go to completion. No GSSG formation occurred.

Since GSH depletion upon addition of acrolein was very rapid, the effect of allyl alcohol on GSH levels was also monitored as GSH depletion is slower with allyl alcohol because acrolein availability is less (Ohno et al.,1985). Figure 3.4 confirms this report and shows that when the ALDH inhibitor, cyanamide, is included in the cell incubate, allyl alcohol-induced GSH depletion (fig. 3.5) as well as cytotoxicity (fig. 3.4) is increased.

3. 2. 1. 3 Effects of Thiol Agents on Acrolein-Induced Cytotoxicity

Various thiol agents were compared for their effectiveness in preventing

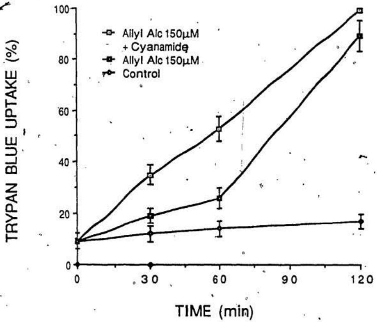
Figure 3. 3 GSH depletion induced by acrolein in isolated hepatocytes.



TIME, (min)

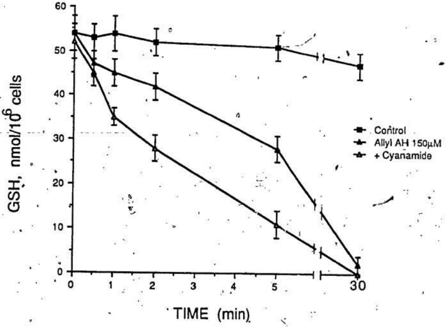
Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), and varying concentrations of acrolein. At times indicated 0.8 ml aliquots of cell suspensions were removed and total GSH levels were determined by HPLC analysis as described in Methods (2. 2. 2). Three separate experiments were carried out. Values are means ± standard error.

Figure 3. 4. Enhancement of allyl alcohol-induced hepatocyte cytotoxicity by cyanamide.



Hepatocytes (10^6 cells/ml) were preincubated at 37°C for 5 minutes in Krebs-Henseleit buffer, containing Hepes (12.5 mM), pH 7.4, ± cyanamide (200 µM). Allyl alcohol (150 µM) was then added where indicated and cell viability at 30, 60 and 120 minutes was determined as described by Methods (2. 2. 1. 3). Control represents cells ± cyanamide. Three separate experiments were carried out. Values are means ± standard error.

Figure 3. 5. Potentiation of allyl alcohol-induced GSH depletion by cyanamide in isolated rat hepatocytes.



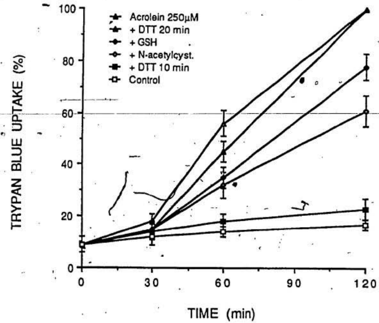
Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), ± cyanamide (200 µM). Allyl alcohol (150 µM) was then added as indicated in the figure. At the times indicated, 0.8 ml aliquots of cell suspensions were removed and total GSH levels were determined by HPLC analysis as described in Methods (2. 2. 2). Control represents samples ± cyanamide. Three separate experiments wre carried out. Values are means ± standard error.

subsequent cytotoxicity initiated by acrolein in isolated hepatocytes (fig. 3.6). Cells were first preincubated for 10 minutes with 250 µM acrolein, followed by washing (gentle centrifugation 50 x g and resuspension in fresh Krebs-Henseleit buffer, containing 12.5 mM Hepes) prior to treatment with various thiol reagents. As shown in fig 3.6, addition of 2 mM dithiothreitol, completely protected ($p<0.001$) the cells from 250 µM acrolein since no loss of cellular viability was observed within the 120 minute incubation period. However, if the introduction of dithiothreitol was delayed for 20 minutes, no significant protection of acrolein-induced cytotoxicity was found ($p>0.1$). Addition of 2mM GSH or N-acetylcysteine caused a significant delay ($p< 0.01$) of the cytotoxicity in 250 µM acrolein-treated hepatocytes but was less effective than dithiothreitol. N-Acetylcysteine was more effective than GSH (fig. 3.6).

3. 2. 1. 4 Effects of antioxidants and desferrioxamine on allyl alcohol and acrolein-induced cytotoxicity in isolated rat hepatocytes

Allyl alcohol-induced necrosis of perfused rat liver has been reported to be preceded by the formation of lipid peroxidation (Badr et al., 1986), as determined by malondialdehyde (MDA) formation. Table 3.2 shows that the addition of a toxic dose of allyl alcohol (250 µM) to hepatocytes also induced the production of MDA. Pretreating hepatocytes with low concentrations of the antioxidant butylated hydroxyanisole (BHA) or Lazaroid U-74500 (Braughler et el., 1987) significantly delayed ($p<0.005$) cell death. MDA formation due to allyl alcohol was totally inhibited when these antioxidants were included in the incubation (table 3.2). Desferrioxamine, an iron trapping agent, was also found to inhibit lipid peroxidation and allyl alcohol-induced hepatocyte cytotoxicity

Figure 3. 6 Protection of acrolein-induced hepatocyte toxicity by thiol reagents.



Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), for 10 minutes with 250 µM acrolein. Either 2 mM dithiothreitol (DTT), 2 mM glutathione (GSH) or 2mM N-acetylcysteine was then added and the viability was assessed at 30, 60 and 120 minutes as described in Methods (2. 2. 1. 3). Three separate experiments were carried out. Values are means ± standard error.

Table 3. 2 Protection of acrolein and allyl alcohol-induced hepatocyte cytotoxicity by desferrioxamine, BHA and Lazaroid U-74500A

	Cytotoxicity (% trypan blue uptake)			MDA (OD 535 nm /10^6 cells)
Treatment	min: 30	60	120	120
Control	12±2	14±2	17±3	0.05±0.01
Allyl alcohol	21±3	62±5	100	0.52±0.08
+ Desferrioxamine	15±2	22±3	25±3	0.08±0.02
+ BHA	19±3	27±3	55±5	0.07±0.02
+ Lazaroid U-74500A	16±3	20±3	26±4	0.07±0.02
Acrolein	18±3	56±5	100	0.48±0.07
+ Desferrioxamine	17±3	27±4	72±5	0.08±0.02
+ BHA	18±3	35±4	88±6	0.06±0.01
+ Lazaroid U-74500A	17±3	31±3	82±6	0.05±0.01

Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), for 5 minutes with either desferrioxamine (100 µM), BHA (100 µM) or lazaroid U-74500A (100 µM). Acrolein (150 µM) or allyl alcohol (250 µM) was then added to the mixture as indicated and viability was assessed at 30, 60 and 120 minutes as described in Methods (2. 2. 1. 3). At the end of each experiment (120 minutes) a 1 ml aliquot of cell suspension was taken to determine formation of malondialdehyde (MDA) as described in Methods (2. 2. 3). Three separate experiments were carried out. Values are means ± standard error.

was significantly (p<0.001) prevented.

Addition of a toxic dose of acrolein (150 µM) to hepatocytes caused the formation of MDA which could be prevented if either of the antioxidants BHA or lazaroid U-74500A or the iron chelator desferrioxamine was also included in the incubation medium (table3.2). However, unlike allyl alcohol, cytotoxicity was delayed to a relatively lesser extent.

3. 2. 1. 5 Effect of allopurinol on hepatocyte cytotoxicity induced by allyl alcohol or acrolein

Allopurinol, an inhibitor of xanthine oxidase, protected hepatocytes against allyl alcohol cytotoxicity and prevented MDA formation (table 3.3). However, pretreatment of the cells with allopurinol (1 mM) had no significant effect on acrolein-induced cytotoxicity (p>0.1) or on the formation of MDA (p>0.1). Because of this difference in allyl alcohol and acrolein toxicity, the effects of allopurinol on alcohol dehydrogenase activity in the hepatocytes were investigated. Alcohol dehydrogenase activity of rat liver cytosol was found surprisingly to be markedly inhibited by dimethylsulfoxide (DMSO), the solvent normally used to dissolve allopurinol. Table 3.4 shows that when allyl alcohol or ethanol concentrations were added to rat liver cytosol preincubated with 1mM DMSO, ADH activity was significantly inhibited (p<0.05). DMSO also protected against allyl alcohol-induced hepatocyte cytotoxicity (table 3.3).

Table 3. 3 Protection of acrolein and allyl alcohol-induced hepatocyte cytotoxicity by DMSO and allopurinol

	Cytotoxicity (% trypan blue uptake)			MDA (OD 535 nm /10^6 cells)
Treatment min:	30	60	120	120
None	12±2	14±2	17±3	0.05±0.01
Allyl alcohol	21±3	62±5	100	0.52±0.08
+ Allopurinol	15±3	32±4	35±5	0.12±0.04
+ DMSO	14±3	23±3	26±4	0.09±0.03
Acrolein	18±3	56±5	100	0.48±0.08
+ Allopurinol	17±3	53±6	100	0.50±0.09

Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), for 5 minutes with either dimethylsulfoxide (DMSO) (1 mM) or allopurinol (1 mM) dissolved in DMSO (1mM). Acrolein (150 µM) or allyl alcohol (250 µM) was then added to the incubation mixture as indicated and viability was assessed at 30, 60 and 120 minutes as described in Methods (2. 2. 1. 3). At the end of each experiment (120 minutes) a 1 ml aliquot of cell suspension was taken to determine formation of malondialdehyde (MDA) as described in Methods (2. 2. 3). Three separate experiments were carried out. Values are means ± standard error.

Table 3. 4 Inhibition of rat liver alcohol dehydrogenase by allopurinol

Treatment	ALCOHOL DEHYDROGENASE (μmol NAD$^+$ reduced /min/g liver)	
	ETHANOL 10 mM	ALLYL ALCOHOL 1 mM
NAD$^+$ + cytosol	0.62±0.06	0.45±0.04
NAD$^+$ + cytosol + pyrazole	0.23±0.06	0.12±0.05
NAD$^+$ + cytosol + allopurinol [a]	0.12±0.06	0.12±0.06
NAD$^+$ + cytosol + DMSO	0.22±0.06	0.15±0.06
NAD$^+$ + cytosol + allopurinol [b]	0.60±0.04	0.41±0.03

Rat liver cytosol was incubated in 90 mM potassium phosphate buffer, pH 7.3, and 40 mM KCl containing 0.5 mM NAD$^+$ and 10 mM ethanol or 1 mM allyl alcohol. Pyrazole (0.5 mM), allopurinol (1.0 mM), and dimethylsulfoxide (DMSO) were added as indicated. Alcohol dehydrogenase activity was measured as described in Methods (2. 2. 6. 1) and expressed as, μmol NAD$^+$ reduced/min/g liver. Three separate experiments were carried out. Values are means ± standard error.

Abreviations:

[a] dissolved in DMSO

[b] dissolved in acetonitrile

3. 2. 2 Mitochondrial Ca^{2+} Studies

3. 2. 2. 1 Effect of acrolein on Ca^{2+} homeostasis of isolated rat liver mitochondria

As illustrated in fig. 3.7, respiring mitochondria were able to uptake and retain added Ca^{2+} (50 nmol/mg of mitochondrial protein) for at least 15 minutes. The addition of acrolein at 250μM or higher to Ca^{2+} loaded mitochondria caused a complete collapse of the transmembrane potential, followed by Ca^{2+} release. The amount of time between acrolein addition and Ca^{2+} release was dose-dependent. A dose of 50μM acrolein did not effect the transmembrane potential nor the ability of the mitochondria to retain Ca^{2+}. Addition of EGTA, a Ca^{2+} chelator, to 250μM acrolein treated mitochondria inhibited Ca^{2+} cycling and restored the transmembrane potential (fig. 3.7). This indicates that the inner mitochondrial membrane was not irreversibly depolarized (Masini et al., 1986), hence the acrolein-induced collapse of the membrane potential is not due to membrane damage of the mitochondria but rather to a continuous and energy-draining Ca^{2+} cycling. The NAD(P)H /NAD(P) redox status of the mitochondria remained unchanged after acrolein treatment.

Ethacrynic acid (500 μM) addition to energized mitochondria results in the depletion of mitochondrial GSH (Meredith and Reed,1982), but does not effect Ca^{2+} retention by mitochondria within an incubation period of 10 minutes (fig. 3.8). However, 50 μM acrolein addition to Ca^{2+} loaded mitochondria preincubated with ethacrynic acid caused Ca^{2+} to be released (fig.3.8).

Figure 3. 7 Sequence of events of mitochondrial Ca^{2+} release (A), transmembrane potential (B) and NAD(P)H oxidation (C).

Liver mitochondria (1 mg/ml) were incubated in MSH buffer pH7.4 at 25°C containing rotenone (3 µM) and succinate (5 mM). Ca^{2+} (50 nmol/mg) was then added to mitochondrial suspension and allowed to equilibrate for approximately 5 minutes followed by the addition of acrolein. Concentrations of acrolein added were (a) 50 µM; (b) 250 µM); (c) 500 µM and (d) none. Trace (x) represents NAD(P)H oxidation upon addition of 0.5 mM oxaloacetate. EGTA (1mM) was added where indicated. Ca^{2+} release, transmembrane potential and NADPH oxidation was monitored as described in Methods (2. 3. 2, 2. 3. 3, and 2. 3. 4, respectively).

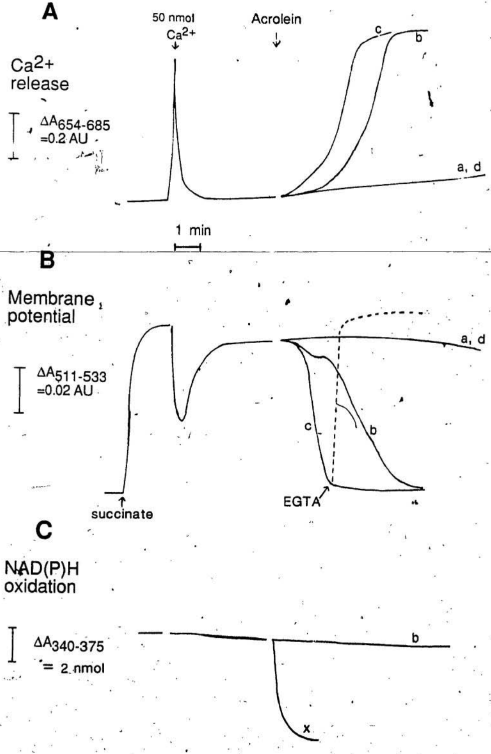
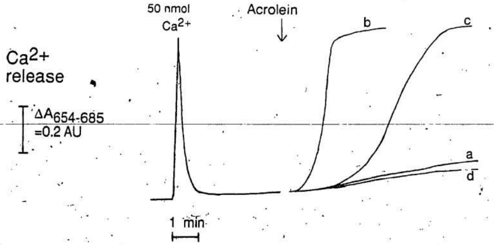

A
50 nmol Ca2+
Acrolein
Ca2+ release
ΔA654-685 =0.2 AU
c
b
a, d
1 min
B
Membrane potential
ΔA511-533 =0.02 AU
a, d
c
b
succinate
EGTA
C
NAD(P)H oxidation
ΔA340-375 = 2 nmol
b
x

Figure 3.8 Potentiation of acrolein-induced mitochondrial Ca^{2+} release by ethacrynic acid and cyanamide.



Liver mitochondria (1mg/ml) were incubated in MSH buffer pH 7.4 containing rotenone (3 µM) and succinate (5 mM). Ca^{2+} (50 nmol/mg) was then added to the mitochondrial suspension and allowed to equilibrate for approximately 5 minutes. Additions were made as follows (a) acrolein (50 µM); (b) ethacrynic acid (500 µM) + acrolein (50 µM); (c) cyanamide (200 µM) + (acrolein 50 µM); (d) ethacrynic acid (500 µM) or cyanamide (200 µM) or none. Ca^{2+} release was monitored as described in Methods (2. 3. 2).

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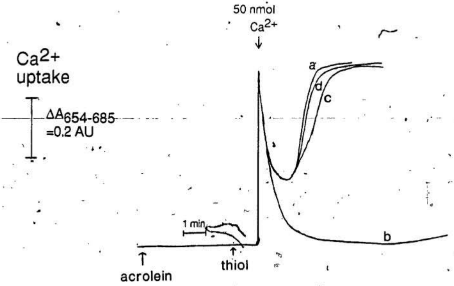
Figure 3. 9 Effect of dithiothreitol (DTT), glutathione (GSH) and N-acetylcysteine on Ca^{2+} uptake and retention in acrolein treated mitochondria.



Liver mitochondria (1 mg/ml) were preincubated in MSH buffer, pH 7.4 at 25°C, containing rotenone (3 µM) and succinate (5 mM) with 250 µM acrolein for 4 minutes. Additions of thiols were made as follows (a) none; (b) DTT (1mM); (c) GSH (1mM); and (d) N-acetylcysteine (1mM). Ca^{2+} (50 nmol/mg) was then added 1 minute after the thiol reagent as indicated in the figure. Ca^{2+} release was monitored as described in Methods (2. 3. 2).

3. 3 Discussion

The periportal necrosis of the liver induced by acrolein has been attributed to the depletion of cellular non-protein and protein sulfhydryl groups (Badr et al.,1986), leading to the modification of cellular proteins and membrane lipid peroxidation by an indirect iron-dependent mechanism. Our study with isolated rat hepatocytes confirms these results as acrolein at concentrations of 250 µM and higher caused an immediate depletion of GSH, malondialdehyde formation and eventual cell death. Previous work by Patel et al., (1980) and Rikans, (1984) suggested that acrolein was oxidatively metabolized to acrylic acid by NAD+ and aldehyde dehydrogenase in both Holtzman and Fischer male rats. This is believed to be a detoxification pathway as acrylic acid is not toxic to perfused rat liver (Belinsky et al., 1986). In further support of this theory, administration of either cyanamide or disulfiram, two ALDH inhibitors, prior to acrolein treatment was found to increase hepatocyte cytotoxicity. A recent study by Mitchell and Peterson (1988) however, reported that acrolein is a potent inhibitor of the high affinity ALDH isoenzymes in both the mitochondria and cytosolic subcellular fractions *in vitro*. They suggested that the ALDH activity reported by Rikans, (1984), in the presence of acrolein was misleading since ALDH activity was assayed in the presence of B-mercaptoethanol. The scenario of a B-mercaptoethanol-acrolein adduct being a substrate for ALDH was therefore suggested.

The formation of an acrolein-GSH adduct *in vitro* was first suggested by DeMasters et al. (1983). Ohno et al. (1985) further suggested without any evidence that the acrolein-GSH adduct was metabolized to the corresponding acid by ALDH present in hepatocytes. Our results with rat liver S9 showed that acrolein inhibited ALDH activity. However, acrolein was found to be a good substrate for ALDH when added to the S9 fraction if GSH was also added. Therefore it is possible that in the cell, acrolein reacts rapidly with GSH forming an acrolein-GSH adduct which is then further oxidized to the non-toxic acrylic acid form by ALDH and NAD+. Inhibition of ALDH by cyanamide and disulfiram would prevent the oxidation of the acrolein-GSH adduct which may be toxic to the cell. However, it is more likely that GSH acts by protecting ALDH from inactivation by acrolein. Inhibition of ALDH by cyanamide and disulfiram would then increase acrolein toxicity by inhibiting its removal by ALDH.

Earlier studies by Badr et al., (1986) showed that toxic doses of allyl alcohol administered to perfused rat liver cause lipid peroxidation and liver necrosis which could be prevented if the liver was treated with either antioxidants or the iron-trapping agent desferrioxamine prior to allyl alcohol infusion. Lipid peroxidation was therefore suggested to contribute to the allyl alcohol-induced necrosis. Results from this study with isolated hepatocytes found allyl alcohol toxicity to be mostly prevented by the antioxidants BHA and lazaroid U-74500A or the iron chelator desferrioxamine. Lipid peroxidation under these conditions was totally inhibited. Incubation of hepatocytes with acrolein instead of allyl alcohol also caused lipid peroxidation that was inhibited if either antioxidants or desferrioxamine was included in the incubate. Cytotoxicity in this case however, was only delayed.

The xanthine oxidase inhibitor allopurinol prevented lipid peroxidation and liver necrosis in livers perfused with allyl alcohol (Badr et al., 1986). As a result, it was concluded that lipid peroxidation may have been initiated by superoxide anion radicals generated by xanthine oxidase. The latter may be formed from xanthine dehydrogenase as a result of increased cytosolic Ca^{2+} activating a Ca^{2+} dependent protease (McCord, 1985). The addition of allopurinol to hepatocytes treated with allyl alcohol also inhibited lipid peroxidaiton and prevented or delayed toxicity. However, acrolein induced lipid peroxidation and cytotoxicity in hepatocytes was not affected by allopurinol. This suggests that xanthine oxidase is not responsible for the lipid peroxidation. Further investigation revealed that DMSO (the solvent normally used to dissolve allopurinol) readily inactivates alcohol dehydrogenase. The alcohol dehydrogenase inhibitor 4-methylpyrazole also prevented allyl alcohol-induced hepatocyte cytotoxicity (Ohno et al., 1985). Therefore, the protective effect of allopurinol against hepatotoxicity induced by allyl alcohol could be due to an inhibition of ADH activity by the DMSO used to dissolve allopurinol. The fact that DMSO, a hydroxyl radical scavenger, did not prevent acrolein induced cytotoxicity and lipid peroxidation in hepatocytes also suggests that hydroxyl radicals are not involved in mediating acrolein catalysed lipid peroxidation.

Incubation of isolated hepatocytes with acrolein (250 µM) for 10 minutes followed by washing and resuspendion of the cells in fresh Krebs-Henseleit buffer did not affect the onset of cell death as compared to acrolein-treated unwashed cells. However, dithiothreitol (2 mM) added after the hepatocytes had been washed, prevented cell death. This suggests that dithiothreitol penetrated the hepatocyte membranes and reversed acrolein-induced damage. That other sulfhydryl agents were unsuccessful at preventing cytotoxicity may be explained by their lower lipid solubility and much lower ability to penetrate the cell membrane.

Incubation of hepatocytes with a variety of toxic agents produces a sustained increase in cytosolic Ca^{2+} concentration (Orrenius and Nicotera, 1987). This event preceds the appearance of surface blebs and the loss of cell viability and seems to be a critical event in the development of toxicity. Amongst several mechanisms in maintaining Ca^{2+} homeostasis in the cell (see chapter 1, 1.3), mitochondria appear to play a major role in regulating cellular

Ca^{2+} levels, since they possess specific routes for both uptake from and release into the cytosol (Carafoli, 1967; Schraer et al., 1973).

Acrolein addition to isolated rat liver mitochondria resulted in the collapse of the transmembrane potential and an immediate release of Ca^{2+} from the mitochondria into the surrounding medium. Previously, mitochondrial Ca^{2+} release induced by various xenobiotics has been linked to the oxidation and hydrolysis of intramitochondrial NAD(P)H with resultant ADP ribosylation of the inner membrane (Frei [a] et al., 1985). However, mitochondrial Ca^{2+} release by acrolein is unusual in that it did not cause oxidation or depletion of NAD(P)H in the mitochondria. Depleting the mitochondrial GSH with ethacrynic acid or inactivation of ALDH with cyanamide also decreased the retention time of the sequestered Ca^{2+} in acrolein treated mitochondria.

Addition of dithiothreitol to the mitochondria seems to reverse the damage caused by acrolein without restoring GSH levels even when added after a 5 minute period to maximize the acrolein modification of the mitochondria. This period of time was sufficient for acrolein to cause disruption in the regulation of mitochondrial Ca^{2+} fluxes as Ca^{2+} release occurred in less than 1 minute upon addition of 250 μM acrolein to Ca^{2+} loaded mitochondria. GSH and N-acetylcysteine were unable to reverse acrolein-induced damage to mitochondria, presumably because of their lower reactivity and inability to penetrate the inner membrane. Allyl alcohol did not release Ca^{2+} from mitochondria presumably because of alcohol dehydrogenase activity is absent. ADH has been shown to be located in the cytosolic fraction (Cornell, 1983).

In conclusion, acrolein is a highly reactive molecule which depletes GSH and protein thiols without GSSG formation in isolated hepatocytes. Depletion of GSH by other toxins has been associated with the elevation of cytosolic Ca^{2+} (Bellomo and Orrenius, 1985) and the activation of intracellular proteases and phospholipases associated with cell death. Acrolein was also found to impair the ability of the mitochondria to retain Ca^{2+} and could, therefore, disrupt cellular calcium homeostasis. An increase in cytosolic free Ca^{2+} levels has been associated with the conversion of phosphorylase b to phosphorylase a which catalyses the breakdown of glycogen into glucose (Exton, 1982). Increase in phosphorylase a activity and glycogen breakdown have been reported in acrolein treated hepatocytes (Ku and Bennings, 1986) and in allyl alcohol perfused liver (Belinksy et al., 1984) respectively.

The reversal of acrolein induced mitochondrial damage by DTT may be due to restoration of vital sulfhydryl groups on the Ca^{2+} transporting mechanism which enables the mitochondria to regulate cellular Ca^{2+} homeostasis. Acrolein treatment of hepatocytes may also inactivate thiol groups on both the plasma and endoplasmic reticular Ca^{2+}-ATPase pumps. Thiol groups in both these pumps may be essential for their activity. Dithiothreitol also protected against acrolein induced cytotoxicity in hepatocytes after preincubating the cells with acrolein. Acrolein has also been shown to cause cytochrome P-450 destruction and its conversion to cytochrome P-420 (Patel et al., 1980). Release of iron from intracellular hemoproteins as a result of modification by acrolein could initiate lipid peroxidation e. g. through a Haber-Weiss type of reaction (Haber and Weiss, 1934) and could explain the inhibition of lipid peroxidation and the delay in acrolein induced hepatocyte cytotoxicity by antioxidants and desferrioxamine. Lipid peroxidation can also result form the autooxidation of acrolein. A carbonyl peroxy radical formed during the autoxidation has been suggested to initiate lipid peroxidation. (O'Brien, et al.,1988). Hence lipid peroxidation may play a role in acrolein-induced cytotoxicity. This role seems just one of several toxic pathways since the inhibition of acrolein-induced lipid peroxidation still results in cell death.

Therefore these results suggest that there are two mechanisms of cytotoxicity induced by acrolein. One involves lipid peroxidation and the other is a slower mechanism involving alkylation of essential thiols in enzymes and membrane proteins.

CHAPTER 4

AZQ Study

4. 1 INTRODUCTION

Quinone-containing antitumor agents have been shown to be activated to their free radical species by a number of processes including microsomes and NADPH-cytochrome P-450 reductase (Bachur et al., 1978), rat liver nuclei (Bachur et al., 1982), xanthine oxidase (Pan et al., 1980) and Erlich ascites cells (Sato et al., 1977). The formation of free radical species may be deleterious to the cell. One possible mechanism would be via direct binding of that particular free radical intermediate to vital macromolecules (Tomaz et al., 1974). Another possible mechanism includes the generation of reactive oxygen species. Reduced oxygen derivatives such as superoxide anion ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2) have been implicated in cell toxicity. Strake and Farber (1985) showed that H_2O_2 can cause irreversible cell injury in cultured rat hepatocytes. Other investigators, using freshly isolated rat hepatocytes, have demonstrated that redox cycling compounds such as menadione give rise to large amounts of $O_2^{\dot{-}}$ resulting in GSH depletion, increase in cytosolic Ca^{2+} and cell death (Bellomo and Orrenius, 1985).

In the present study, the ability of the quinone anticancer drug diaziquone (AZQ) to undergo intracellular redox cycling thereby generating reactive oxygen species has been investigated, using isolated rat hepatocytes as the target system.

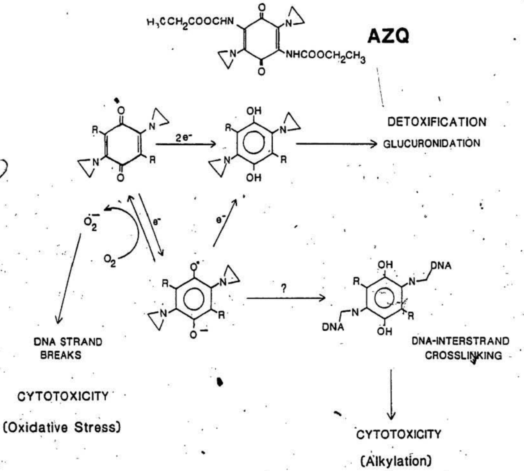
AZQ, [2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone] is a synthetic lipid soluble antitumor drug which was rationally designed to cross the blood-brain barrier for the treatment of the central nervous system tumors (Schold et al., 1984). The two aziridine groups provide bifunctional alkylation while the carboethoxy-amino groups are extremely lipophilic allowing the compound to cross the blood-brain barrier. Phase II trials show that AZQ has promising clinical activity against brain tumors (Curt et al., 1983) and lymphomas (Case et al., 1973) and is the only effective agent against L1210 murine leukemia cells (Driscoll et al., 1979). Preclinical toxicologic studies in mice, dogs and monkeys have reported the major toxicity of AZQ to be in the gastrointestinal , hematopoetic and lymphatic systems . Milder toxicity was also noted in the respiratory, hepatic, nervous and renal systems. Phase I trials revealed bone marrow toxicity and patients developed severe leukopenia and thrombocytopenia (Hacker et al., 1982)

The mechanism by which AZQ acts is still unclear. AZQ has been shown to be reduced to its free semiquinone radical anion by several cell lines (Gutierrez et al., 1985), by rat liver microsomes and NADPH-cytochrome c reductase (Gutierrez et al., 1982). Under aerobic conditions the semiquinone radical could theoretically autooxidize to the parent compound forming $O_2^{\cdot-}$ which can lead to cell toxicity (Fridovich, 1976). In support of free radical formation, Gutierrez et al. (1986) showed that AZQ under aerobic conditions did cause DNA single strand breaks. On the other hand, AZQ has also been suggested to act as a bifunctional reductive alkylating agent, (Massoba et al., 1985). These authors proposed that hydrogen bonding of the hydroquinone hydroxy group to the adjacent aziridine nitrogen may facilitate the opening of the aziridine ring leading to the formation of the aziridinium ion, a carbonium ion and an alkylating species. AZQ was reported to induce DNA interstrand cross-linking in nuclei and cytotoxicity has been correlated with the degree of

DNA cross-linking (Szmigiero et al., 1984). Figure 4.1 illustrates these two possible mechanisms.

In the following studies, the effects of AZQ on cellular GSH levels, mitochondrial Ca^{2+} homeostasis and the involvement of H_2O_2 in AZQ-induced toxicity was investigated in isolated rat hepatocytes.

Figure 4.1 Possible mechanisms of AZQ induced cytotoxicity


H$_3$CCH$_2$COOCHN
NHCOOCH$_2$CH$_3$
AZQ
2e$^-$
DETOXIFICATION
GLUCURONIDATION
O$_2^-$
O$_2$
e$^-$
e$^-$
?
DNA
DNA
DNA STRAND BREAKS
DNA-INTERSTRAND CROSSLINKING
CYTOTOXICITY
(Oxidative Stress)
CYTOTOXICITY
(Alkylation)

4. 2 RESULTS

4. 2. 1 Hepatocyte Study

4. 2. 1. 1 Effects of AZQ on the viability of isolated rat hepatocytes

Incubation of isolated hepatocytes with 500 µM AZQ resulted in the appearance of surface blebs within 30 minutes and loss of cell viability at the end of 4 hour incubation period as assessed by trypan blue penetration (table 4.1). The decrease in cell viability obtained with 500 µM AZQ was gradual with 46% cell death occurring at 2 hours of incubation. A lower dose of 200 µM AZQ did not cause a significant ($p>0.1$) increase in hepatocyte cytotoxicity (table 4.1). Addition of the sulfhydryl agent dithiothreitol (DTT) at 1 hour after incubation with 500 µM AZQ treated hepatocytes resulted in a delay of cytotoxicity at the 2 hour period of incubation (table 4.1). However, if the addition of DTT was delayed for 90 minutes, hepatocyte cytotoxicity caused by 500 µM AZQ was unaltered.

4. 2. 1. 2 Induction of oxygen uptake by AZQ

The addition of AZQ at concentrations as low as 10 µM to isolated rat liver microsomal preparations stimulated O_2 consumption in the presence of NADPH (table 4.2). Increasing the AZQ concentration to 100 µM reached a saturation point in terms of O_2 consumption. As shown in table 4.2, AZQ was also very effective at stimulating respiration of intact hepatocytes in the presence of cyanide, which was added to inhibit O_2 uptake due to mitochondrial respiration.

Table 4.1 AZQ cytotoxicity in isolated rat hepatocytes

	Cytotoxicity (% trypan blue uptake)		
Treament (time)	60'	120'	240'
Control cells	13±2	16±3	20±3
+ AZQ (500µM)	20±3	46±6	100
+ AZQ (200µM)	15±3	19±3	24±4
+ AZQ (100µM)	14±2	18±3	22±4
+ AZQ (500µM) + DTT 60 min	19±3	28±4	100
+ AZQ (500µM) + DTT 90 min	19±3	50±5	100

Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), with varying concentrations of AZQ. DTT (1mM) was added at 60 and 90 minutes after addition of 500µM AZQ, where indicated. Cell viability at 60, 120 and 240 minutes was determined as described in Methods. Three experiments were carried out. Values are means ± standard error.

Table 4.2 AZQ-mediated oxygen activation with liver microsomes, isolated hepatocytes and ascorbate.

Addition	Buffer O_2 uptake (nmol/min)	Microsomal O_2 Uptake (nmol/min/mg)	Hepatocyte O_2 Uptake (nmol/min/10^6 cells)
None	0.03 ± 0.01	8.17±1.24	1.33 ± 0.48
AZQ (5μM)	0.03 ± 0.01	13.32 ± 1.35	5.16 ± 0.92
AZQ (10μM)	0.03 ± 0.01	17.71 ± 1.92	9.12 ± 1.50
AZQ (50μM)	0.03 ± 0.01	28.63 ± 3.49	27.24 ± 3.15
AZQ (100μM)	0.03 ± 0.01	27.34 ± 3.52	84.22 ± 7.54
AZQ (5μM) + ascorbate	58.5±7.55	n.d.	77.53±7.31
AZQ (10μM) + ascorbate	102±13.45	n.d.	142.12±15.32
Ascorbate	0.05±0.01	n.d.	0.07±0.02

Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM) with varying concentrations of AZQ ± 2.5 mM ascorbate as indicated. Microsomes isolated from rat liver were incubated (1 mg/ml) in 0.1 mM Tris-HCl, pH 7.4, 50 mM KCl buffer containing 0.2 mM NAD(P)H with varying concentrations of AZQ ± 1.0 mM ascorbate. Oxygen uptake was measured using a Clarke electrode as described in Methods. Three separate experiments were carried out. Values are means ± standard error.

The rate of O_2 uptake increased in a dose-dependent manner. Introduction of 2.5 mM ascorbate, a reducing agent, to AZQ in buffer caused extensive consumption of O_2 presumably as a result of a one electron reduction of AZQ to an autooxidizable semiquinone (table 4.2). Addition of catalase near the end of the reaction released stoichiometric amounts of oxygen. This suggests that AZQ undergoes futile redox cycling producing $O_2^{\cdot -}$ and resulting in H_2O_2 formation. Control incubations, which received only the DMSO vehicle did not show any increase in oxygen uptake.

4. 2. 1. 3 Effects of AZQ on GSH/GSSG levels in isolated rat hepatocytes

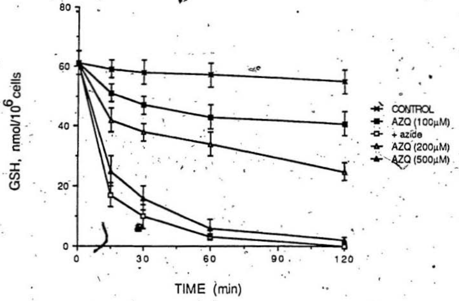
As shown in figure 4.2, (A), the onset of cytotoxicity induced by AZQ was preceeded by a dose-dependent decrease in soluble thiols. Total depletion of GSH occurred only with a toxic dose of AZQ (500 μM) after approximately 90 minutes of incubation. Lower nontoxic concentrations of AZQ also caused a gradual disappearance of GSH but at a much slower rate and never to completion. A corresponding and nearly stoichiometric increase in GSSG was also observed with all AZQ concentrations (fig 4.2, B), thus explaining the disappearance of GSH as an oxidation of GSH.

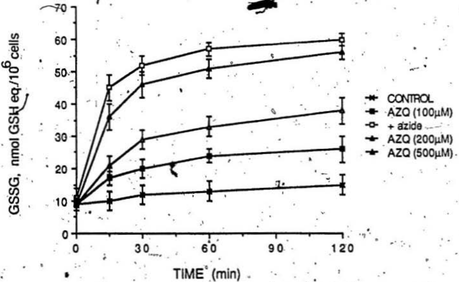
4. 2. 1. 4 Effect of catalase inhibiton on AZQ-induced hepatocyte cytotoxicity

H_2O_2 is thought to be toxic (Fridovitch, 1976) and is converted to H_2O either by catalase, associated with the peroxisomes, or by glutathione

Figure 4. 2 GSH depletion (A) and GSSG formation (B) induced by AZQ in isolated hepatocytes.

Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), with varying concentrations of AZQ. Where indicated, hepatocytes were preincubated with sodium azide (4 mM) for 5 minutes prior to AZQ addition. At various times, 0.8 ml aliquots of cell suspensions were removed and total GSH and GSSG levels were determined by HPLC analysis, as described under Methods (2. 2. 2). Three separate experiments were carried out. Values are means ± standard errors.


GSH, nmol/10^6 cells
80
60
40
20
0
0
30
60
90
120
TIME (min)
CONTROL
AZQ (100μM)
+ azide
AZQ (200μM)
AZQ (500μM)


GSSG, nmol GSH eq./10^6 cells
70
60
50
40
30
20
10
0
0
30
60
90
120
TIME (min)
CONTROL
AZQ (100μM)
+ azide
AZQ (200μM)
AZQ (500μM)

peroxidase present in both the mitochondrial matrix and the cytosol. Addition of 4 mM azide to hepatocytes for 5 minutes results in an almost complete inhibiton of catalase activity without any decrease in cell viability (Rossi et al., 1988). Incubation of 100 μM AZQ with hepatocytes pretreated with 4 mM azide, caused a complete depletion of GSH with a consequent formation of GSSG (figs. 4.2, A) and 4.2, B), followed by cell death within 4 hours of incubation (table 4.3).

In order to investigate if the increase in oxygen consumption by the interaction of AZQ with ascorbate resulted in potentiation of toxicity as a result of an increase in the production of H_2O_2, hepatocytes pretreated with azide were incubated with AZQ and ascorbate. As illustrated in table 4.3, ascorbate greatly enhanced the susceptibility of azide treated hepatocytes to AZQ. Concentrations of AZQ as low as 5 μM proved to be cytotoxic to the cells if ascorbate was present (table 4.3). Both azide and ascorbate were not cytotoxic to the hepatocytes within 4 hours of incubation.

Another method for obtaining hepatocytes with lower catalase activity is by pretreating rats with cyanamide (13 mg/kg i.p.) 1 hour prior to hepatocyte isolation (DeMaster, 1986). Catalase in these hepatocytes was found to have approximately 18% of the activity present in hepatocytes isolated from untreated rats. Hepatocytes from cyanamide treated animals were much more susceptible to AZQ induced cell death (table 4.3). A concentration of 100 μM AZQ caused 100% cell death within a 4 hour incubation period. Addition of ascorbate (2.5 mM) again markedly increased the cells susceptibility to AZQ-induced cytotoxicity. In this case, 10 μM AZQ was cytotoxic to the cells (table 4.3). However, addition of catalase (6400 U) to these catalase-deficient hepatocytes protected them against the cytotoxic effects of AZQ (10 μM) and ascorbate (2.5 mM) (table 4.3).

Table 4.3 Potentiation of AZQ-induced cytotoxicity in isolated hepatocytes by catalase inhibitors

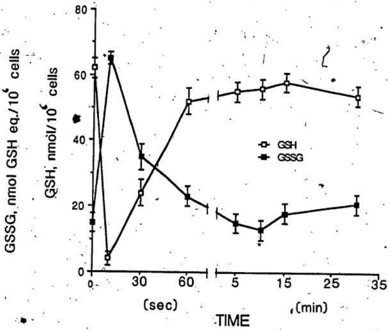
	Cytotoxicity (% trypan blue uptake)			
Treatment	(time)	60'	120'	240'
Control cells		13±2	16±3	20±3
+ AZQ (100µM)		14±2	18±3	22±3
+ AZQ (100µM) + azide		18±3	38±4	100
+ AZQ (5µM) + azide + ascorbate		22±3	78±6	100
+ azide		14±2	18±3	22±4
+ azide + ascorbate		15±3	19±3	22±3
Control cells *(cyanamide treated animals)*		14±2	18±3	22±3
+ AZQ (100µM)		17±2	25±3	100
+ AZQ (10µM)		14±2	20±3	23±4
+ AZQ (10µM) + ascorbate		16±3	32±4	100
+ AZQ (10µM) + ascorbate + catalase		16±3	19±3	24±3
+ ascorbate		15±2	18±3	23±3

Hepatocytes (10^6 cells/ml) from untreated rats and from rats treated with 13 mg/kg cyanamide, injected i.p. 1 hour prior to isolation, were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), with varying concentrations of AZQ. Azide (4 mM), ascorbate (2.5 mM) or catalase (6400 U), were preincubated for 5 minutes prior to AZQ addition, where indicated. Cell viability at 60, 120 and 240 minutes was determined as described in Methods. Three separate experiments were carried out. Values are means ± standard error.

4. 2. 1. 5 Effect of AZQ on GSH recovery after hepatocyte GSH oxidation by H_2O_2

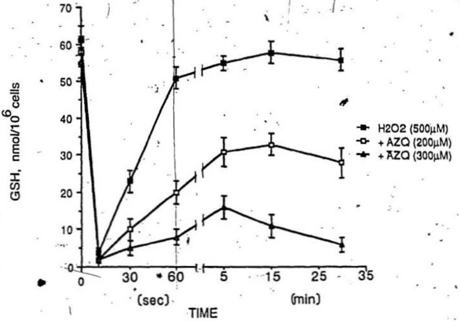
Adding H_2O_2 (500 µM) directly to hepatocytes results in the rapid oxidation of cellular GSH to GSSG and reduction back te original GSH levels within 2 minutes (fig. 4.3), without any detrimental effect on cellular viability. This phenomena demonstrates the active role of glutathione peroxidase and glutathione reductase in the catabolism of H_2O_2 and maintenance of GSH levels in the cell, respectively. However, if H_2O_2 (500 µM) was added to AZQ (200 µM) treated hepatocytes, GSH recovery after the initial depletion was much slower (fig. 4.4). Furthermore, GSH was only restored to a level of about 30 nmol/10^6 cells, representing a loss of approximately 50% GSH in 15 minutes. H_2O_2 (500 µM) addition to 300 µM AZQ treated cells further impeded the recovery of GSH (fig. 4.4). This suggests that glutathione reductase in the presence of AZQ is less efficient in reducing GSSG to GSH. A further investigation showed that AZQ significantly ($p<0.01$) inhibited rat liver cytosolic glutathione reductase activity (table 4.4). Removal of AZQ from the cytosolic fraction by extraction with ethyl acetate however restored the glutathione reductase activity. This suggests that the inhibition of the reductase by AZQ is reversible. AZQ compromised hepatocytes were also found to be more susceptible to H_2O_2 (table 4.5). Thus, although hepatocyte cytotoxicity did not occur with 4 mM H_2O_2 or 200 µM AZQ alone, hepatocyte cytotoxicity was induced with 200 µM AZQ and 500 µM H_2O_2 together.

Figure 4.3 H_2O_2-induced changes in hepatocyte GSH/GSSG levels.



Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM). H_2O_2 (500 µM) was added directly to the cell suspension and total GSH (open squares) and GSSG (close squares) levels were determined by HPLC analysis on 0.8 ml aliquots removed at various times as described in Methods (2. 2. 2). Three separate experiments were carried out. Values are means ± standard error.

Figure 4. 4 AZQ prevents GSH recovery after H_2O_2-induced GSH depletion.



Hepatocytes (10^6 cells/ml) were incubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM). H_2O_2 (500 μM) was added directly to the cell suspension after preincubation with or without AZQ for 1 minute as indicated. At various times after H_2O_2 addition, 0.8 ml aliquots of cell suspension were removed to determine total GSH levels by HPLC analysis as described in Methods (2. 2. 2). Three separate experiments were carried out. Values are means ± standard error.

Table 4.4 Effect of AZQ on glutathione-reductase activity of rat liver cytosol

Treatment	Glutathione Reductase nmol NADPH oxidised/min/mg
NADPH + cytosol	76.7±5.2
NADPH + cytosol + AZQ (100µM)	48.9±4.1
NADPH + cytosol + AZQ(200µM)	33.5±3.7
NADPH + cytosol*	73.4±6.1
NADPH + cytosol* + AZQ(100µM)	70.1±5.8

Rat liver cytosol was incubated in a 1ml cuvette containing 0.1 M potassium phosphate buffer (pH 7.0), 1 mM EDTA , 0.1 mM dicoumarol, 0.1 mM NADPH and 1 mM GSSG at room temperature. Glutathione-reductase activity was assayed at 340 nm as described in Methods and expressed as nmol NADPH oxidised/min/mg protein. Three separate experiments were carried out. Values are means ± standard error.

* Cytosol was extracted 3x with equal volumes of ethyl acetate to remove AZQ before addition of NAD(P)H and GSSG to assay reductase.

Table 4.5 H_2O_2-induced cytotoxicity in AZQ treated isolated hepatocytes

	Cytotoxicity (% trypan blue uptake)		
Treatment (time)	60'	120'	240'
Control cells	13±2	16±3	20±3
+ AZQ (200μM)	15±3	19±3	24±4
+ AZQ (200μM) + H_2O_2 (500μM)	16±3	31±3	71±5
+ H_2O_2 (4mM)	15±3	17±3	22±3

Hepatocytes (10^6 cells/ml) were preincubated at 37°C in Krebs-Henseleit buffer pH 7.4, containing Hepes (12.5 mM), with AZQ (200 μM) ± H_2O_2 (500 μM) or H_2O_2 (4 mM) alone. Cell viability at 60, 120 and 240 minutes was determined as described in Methods. Three separate experiments were carried out. Values are means ± standard error.

4. 2. 2 Effect of AZQ on isolated rat liver mitochondrial Ca^{2+} homeostasis

AZQ was very effective at disrupting the ability of isolated mitochondria to retain added Ca^{2+}. Addition of 70 μM AZQ to mitochondria resulted in the release of Ca^{2+} and the collapse of the transmembrane potential which could be restored by adding 1 mM EGTA (fig. 4.5). Higher concentrations of AZQ were found to be more potent at releasing added Ca^{2+} from mitochondria and the decrease in Ca^{2+} retention times was found to be dose dependent (fig. 4.5).

Since ascorbate was found to increase both oxygen uptake and cytotoxicity in AZQ treated hepatocytes, its effect on Ca^{2+} homeostasis in AZQ treated mitochondria was investigated. As shown in fig. 4.6, ascorbate (1 mM) markedly decreased the retention time of Ca^{2+} in mitochondria compared to AZQ alone. Addition of exogenous catalase (3200 units) to both AZQ and ascorbate treated mitochondria delayed Ca^{2+} release. Neither ascorbate nor catalase added to Ca^{2+} loaded mitochondria caused Ca^{2+} to be released within a 15 minute incubation period.

Figure 4. 5 Sequence of events of mitochondrial Ca^{2+} release (A) and transmembrane potential (B)

Liver mitochondria (1 mg/ml) were incubated at 25°C in MSH buffer pH 7.4 containing rotenone (3µM) and succinate (5 mM). Ca^{2+} (50 nmol/mg) was then added to the mitochondrial suspension and allowed to equilibrate for approximately 5 minutes followed by addition of AZQ. Concentrations of AZQ added were (a) 250 µM; (b) 100µM; (c) 70µM; and (d) none. EGTA (1mM) was added where indicated. Ca^{2+} release and transmembrane potential were monitored as described in Methods (2. 3. 2., and 2. 3. 3 , respectively).

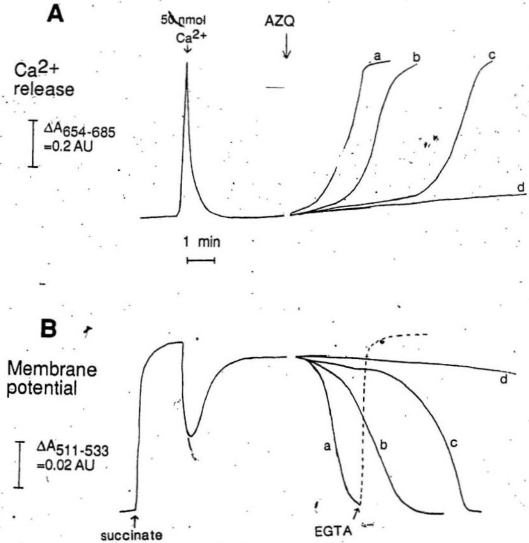
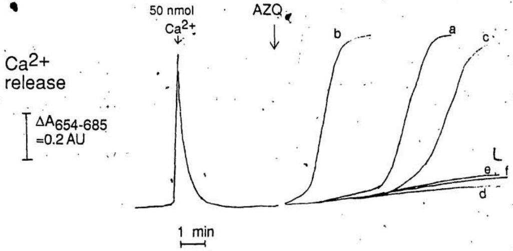

A
50 nmol Ca2+
AZQ
a
b
c
d
Ca2+ release
ΔA654-685 =0.2 AU
1 min
B
Membrane potential
ΔA511-533 =0.02 AU
succinate
EGTA
a
b
c
d

Figure 4. 6 Potentiation of AZQ-induced mitochondrial Ca^{2+} release by ascorbate



Liver mitochondria (1 mg/ml) were incubated at 25°C in MSH buffer, pH 7.4, containing rotenone (3 µM) and succinate (5 mM). Ca^{2+} (50 nmol/mg) was then allowed to equilibrate for approximately 5 minutes. Additions made were, (a) 70 µM AZQ; (b) ascorbate (1 mM) + AZQ (70 µM); (c) catalase (3200 units) + ascorbate (1 mM) + AZQ (70 µM); (e) ascorbate (1mM); (f) catalase (3200 units); and (d) none. Ca^{2+} release was monitored as described in Methods (2.3.2).

4. 3 DISCUSSION

AZQ has been shown to be reduced to an autoxidizable free radical anion by NADPH and rat liver microsomes (Szmigiero et al., 1984). This may occur in the hepatocyte as the addition of AZQ to hepatocytes induced cyanide resistant respiration. This suggests a one-electron reduction of AZQ to the active semiquinone, which then interacts directly with molecular oxygen, regenerating the parent quinone. This futile redox cycling results in the continous generation of superoxide radicals, as a result of the interactions of the semiquinone radical with O_2 (Patel and Wilson, 1973). The superoxide radicals formed would then spontaneously or enzymatically dismutate to H_2O_2.

The hepatocyte is well equipped with enzymatic mechanisms against toxic oxygen species. Superoxide dismutase catalyzes the reduction of O_2^- to H_2O_2 both in the cytosol and mitochondrial compartments (Fridovich, 1976). H_2O_2 is then catabolized to H_2O either by catalase or by glutathione peroxidase. The reduction of H_2O_2 by the latter enzyme uses GSH as a cofactor; thus metabolism of H_2O_2 as a result of redox cycling can lead to GSH depletion and consequently GSSG formation. GSSG accumulation results in cytotoxic protein mixed disulfide formation and hence hepatocytes have developed an active mechanism to reduce GSSG back to GSH by the enzyme glutathione reductase at the expense of NAD(P)H and/or to extrude GSSG from the cell.

A toxic dose of AZQ caused complete GSH depletion in hepatocytes which could be all accounted for by the formation of GSSG. Subtoxic doses of AZQ also resulted in a gradual disappearance of GSH but at a slower rate and never to completion. A slight protection afforded by DTT against AZQ induced hepatocyte cytotoxicity, can be explained by its ability to penetrate the cell and rapidly reduce GSSG to GSH. On the other hand, it may also speed up redox cycling of AZQ thereby increasing H_2O_2 formation which may cancel its protective effect.

In order to investigate the role of H_2O_2 in AZQ induced cell toxicity and to estimate the contribution of catalase to H_2O_2 metabolism, experiments were carried out using two catalase inhibitors, azide and cyanamide. Hepatocytes in which catalase had been inhibited were found to be more susceptible to AZQ induced cytotoxicity. GSH was also found to be depleted at a faster rate and to completion upon the addition of AZQ.

Extensive oxygen uptake resulting in H_2O_2 formation occurred when ascorbate was added to AZQ in the absence of hepatocytes. In the presence of hepatocytes, the marked increase in oxygen uptake by ascorbate was cyanide resistant and did not occur in the absence of AZQ. Addition of catalase when the reaction was nearly complete released oxygen indicating that H_2O_2 was the product. Ascorbate was also found to greatly increase the effectiveness of AZQ at inducing cytotoxicity in catalase deficient hepatocytes. Under these conditions, cytotoxicity occurred at a 100-fold lower concentration of AZQ. Furthermore, AZQ-induced cytotoxicity in these catalase-deficient cells could be prevented by adding catalase to the incubation medium. This suggests that ascorbate and AZQ interact in the medium to generate cytotoxic concentrations of H_2O_2.

Mitochondria have been reported to represent a quantitatively important cellular Ca^{2+} store (Somylo et al., 1985). Disruption of mitochondrial Ca^{2+} homeostasis may be detrimental to the cell (see Chapter I). Continuous redox cycling of quinones resulting in cellular oxidative stress by the formation $O_2^{\cdot}$

and other potentially damaging active oxygen species, have been reported to cause Ca^{2+} release from mitochondria (Moore et al., 1986). This may be of importance for the cytotoxic effects of these compounds in intact cells. AZQ was also found to induce Ca^{2+} release from isolated rat liver mitochondria. Addition of ascorbate increased the efficiency of AZQ to cause Ca^{2+} release from mitochondria while further addition of exogenous catalase delayed Ca^{2+} release. This evidence provides further support to the theory that H_2O_2 generated by the futile cycling of AZQ can induce mitochondrial damage. However, NAD(P)H oxidation directly coupled to AZQ reduction may also occur and this could lead to mitochondrial damage as a result of NAD^+ hydrolysis and consequent ADP ribosylation of mitochondrial proteins (Bellomo et al., 1982).

Hepatocytes are particularly resistant to oxidative stress, hence compounds capable of generating intracellular H_2O_2 are usually not cytotoxic because of the catalase and gluathione peroxidase-reductase defense system (see Chapter I). Our results showed that addition of H_2O_2 to the hepatocytes caused an immediate oxidation of GSH to GSSG which was reduced back to GSH within a 2 minute period. However, when AZQ was also present in the incubation medium, GSH recovery following GSSG formation (as a result of H_2O_2 addition) was impaired. This could be explained by our finding that AZQ reversibly inactivates glutathione reductase. Futhermore, hepatocytes treated with a subtoxic dose of AZQ were much more sensitive to H_2O_2 mediated cytotoxicity. Thus, although hepatocyte cytotoxicity did not occur with 4 mM H_2O_2 or 0.2 mM AZQ alone, a loss in viability did result when 0.5 mM H_2O_2 and 0.2 mM AZQ were incubated with hepatocytes together.

In conclusion, in isolated rat hepatocytes, AZQ may undergo a one-electron reduction to the semiquinone which is immediately oxidized back to the parent quinone by molecular oxygen. This results in a futile redox cycle with a constant production of cytotoxic H_2O_2. Since AZQ is also found to compromise the cellular defense system by reversibly inactivating glutathione reductase, the cells ability to catabolize H_2O_2 is impaired. This may explain the susceptibility of hepatocytes to AZQ-mediated oxidative stress.

CHAPTER 5

SUMMARY and CONCLUSIONS

Several groups have recently carried out studies to establish the relative importance of oxidative stress versus alkylation of intracellular nucleophiles, particularly in the mechanism of quinone toxicity. Rossi et al. (1986) compared a series of methyl substituted benzoquinone derivatives differing in their ability to redox cycle and arylate. They found that unsubstituted benzoquinones depleted GSH by arylation whereas the fully methylated benzoquinone (duroquinone) caused GSH oxidation as a result of a futile reductase catalysed redox cycling resulting in oxygen activation. Whilst the unsubstituted benzoquinones were very toxic, duroquinone was only toxic if the hepatocyte defense system was compromised by inhibiting GSH-reductase, with BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea], or by inactivating catalase with azide. However, the potential role of oxidative stress in hepatocytes with an uncompromised defense system remained unanswered because the fully substituted benzoquinone was found to be less efficient at redox cycling compared to unsubstituted benzoquinones which could also potentially arylate cellular nucleophiles.

In another related study by Miller et al. (1986) the mechanisms by which naphthoquinone derivatives exert their cytotoxicity in isolated hepatocytes were investigated. The toxicity produced by the different naphthoquinone derivatives could be correlated with the degree of GSH depletion but no conclusions could be made about the relative contribution of arylation and oxidative stress.

In an attempt to obtain a better understanding of the relative importance of oxidative stress versus alkylation we studied the cytotoxic mechanisms of acrolein and AZQ. The choice of these compounds was based on their chemical structure and reactivity.

Acrolein is a highly reactive electrophile which has been reported to covalently bind to the thiols and amine groups of liver macromolecules and cause necrosis. The second compound, AZQ, is a fully substituted benzoquinone which has been reported to be able to be reduced to the semiquinone radical and hydroquinone by various cellular systems. This leads to alkylation as a result of the opening of the aziridine ring. Cytotoxicity has been attributed to DNA alkylation and strand breakage. However, the semiquinone radical also autoxidises so that oxidative stress is theoretically possible with AZQ but not with acrolein. A comparison of the effectiveness of acrolein and AZQ at causing hepatocyte toxicity, showed that acrolein was the more potent of the two agents, causing rapid GSH depletion and cytotoxicity even at low levels. In contrast, AZQ did not cause an immediate GSH depletion but rather a gradual oxidation of GSH to GSSG in a time and dose-dependent manner. Hepatocyte GSH oxidation and cytotoxicity induced by AZQ were markedly enhanced by inactivation of catalase. This suggests that both were were caused by H_2O_2 formation produced from redox cycling. The ability of AZQ to redox cycle was confirmed by showing that AZQ induced cyanide resistant respiration in isolated hepatocytes and in microsomes with NADPH.

Hepatocytes were found to be particularly resistant to oxidative stress because of their enzymatic cellular defense system which can neutralize reactive oxygen species. Thus, the addition of H_2O_2 directly to the cells did not result in cytotoxicity as determined by the trypan blue exclusion test. The ability of AZQ to induce cytotoxicity in hepatocytes as a result of oxidative stress could therefore be explained by the finding that AZQ also reversibly inactivates

GSH-reductase, thereby compromising the cell in its ability to protect itself from the toxic effects of oxidant species. This was demonstrated by a direct approach where the enzyme activity was measured in the presence or absence of AZQ and also by a newly rationalized indirect approach where the recovery of cellular GSH in isolated hepatocytes after H_2O_2 treatment was measured. Addition of H_2O_2 to hepatocytes resulted in the immediate oxidation of GSH to GSSG followed by a rapid reduction of GSSG back to GSH within two minutes as a result of glutathione-reductase action. The recovery of GSH should therefore be dramatically affected if the compound in question inactivates glutathione-reductase. This was found to be the case with AZQ .

From the results obtained in this study, oxidative stress-induced toxicity was found to be dependent on two factors: (i) the efficiency of the compound to redox cycle and hence produce H_2O_2 and (ii) the ability of the compound to compromise the cellular defense system. In the case of AZQ, hepatocyte cytotoxicity could be increased twenty fold by compromising the cell with azide thereby inactivating catalase or could be increased 100 fold by further addition of ascorbate, increasing redox cycling.

In contrast, the mechanism of acrolein-induced cytotoxicity was found to be dependent on GSH depletion. Thus cytotoxicity ensued once GSH was depleted. Cytotoxicity has largely been attributed to alkylation. However, two laboratories have attributed allyl alcohol induced liver necrosis *in vivo* and in perfused rat liver to acrolein mediated lipid peroxidation. One of the pathways proposed for this propagation of lipid peroxidation is the interaction of Fe^{2+}, released as a result of covalent binding of acrolein to heme proteins, with reactive oxygen species produced by the induction of xanthine oxidase. Evidence for this hypothesis stems from the protection of allyl alcohol-induced liver damage by allopurinol, desferrioxamine and antioxidants. Results from this study also confirm that lipid peroxidation occurs in acrolein treated hepatocytes. However, lipid peroxidation was concluded to be only one of several pathways leading to toxicity following the addition of acrolein to hepatocytes since both antioxidants and desferrioxamine could successfully eliminate lipid peroxidation and delay cytotoxicity but cell death eventually occurred. Xanthine oxidase was also found not to contribute to the induction of lipid peroxidation since allopurinol did not inhibit acrolein induced lipid peroxidation. Results of other investigators showing that allopurinol protected allyl alcohol-induced lipid peroxidation and cytotoxicity was attributed to the ability of DMSO, the solvent used to dissolve allopurinol, to inactivate alcohol dehydrogenase.

Despite the difference in the mechanism of toxicity between these two agents, they were both able to disrupt mitochondrial Ca^{2+} homeostasis. Whilst AZQ releases Ca^{2+} from mitochondria as a result of H_2O_2 formation in the hepatocyte, Ca^{2+} disruption by acrolein was unusual because Ca^{2+} release occurred without subsequent NADPH oxidation. These results support suggestions made by earlier investigators that both alkylation and oxidative stress-induced cytotoxicity may be mediated through a common pathway involving the disruption of Ca^{2+} homeostasis.

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LATE ADDITIONS

Miller, M. G., Rodgers, A. and Cohen, G. M. Mecanisms of toxicity of naphthoquinones to isolated hepatocytes. Biochem. Pharmacol., 1986, 35 : 1177-1185

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Publications Arising From This Work

Abstracts

1. Silva, J. M. and O'Brien, P. J. (1987). Acrolein induces calcium release from rat liver mitochondria without NAD(P)H depletion. Abstracts of Cell Calcium Metabolism, no:147, Wasington, D. C.

2. Silva, J. M., Drolet, D. L. and O'Brien P. J. (1988). Molecular mechanisms of allyl alcohol induced cytotoxicity in isolated hepatocytes. Abstracts of APS/ASPET '88, Montréal

Full papers

1. O'Brien, P. J., Kaul, H., McGirr, L. and Silva, J. M. (1988) Molecular mechanisms for the involvement of the aldehydic metabolites of lipid peroxides in cytotoxicity and carcinogenesis in "Pharmacological effects of lipids, III" ed. Jon J. Kabara, The American Oil Chemists Society Champaign, Illinois (in press).

2. Silva, J. M. and O'Brien, P. J. (1988) AZQ-induced toxicity in isolated rat hepatocytes is mediated by H_2O_2. Submitted for publication.

3. Silva, J. M. and O'Brien, P. J. (1988) Mechanism of acrolein-induced–cytotoxicity in isolated rat hepatocytes. Submitted for publication.