INTERACTION BETWEEN ARYLATING QUINONES AND THIOLS - A PROPOSED MECHANISM OF QUINONE TOXICITY

by

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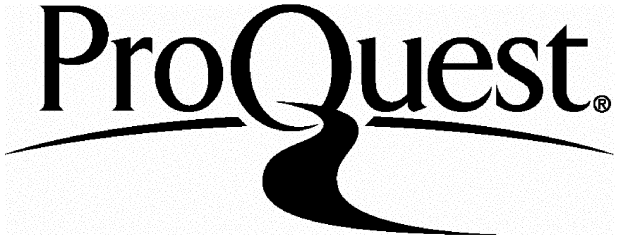

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ABBREVIATIONS

AH	Ascorbate
AMT	Aminotriazole
BQ	1,4-Benzoquinone (*para*-benzoquinone)
BSA	Bovine serum albumin
Cys	Cysteine
DEM	Diethyl maleate
DETAPAC	Diethylenetriamine pentaacetic acid
DiMeBQ	2,6-Dimethyl-*p*-benzoquinone
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EI	Desorption electron impact (mass spectrometry)
FAB	Fast atom bombardment (mass spectrometry)
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
HQ	Hydroquinone (1,4-benzenediol)
HQ-SG	Glutathion-S-yl hydroquinone (also refered to in the text as: GSH-hydroquinone conjugate, hydroquinone-thioether conjugate, GSH-hydroquinone adduct)
LDH	Lactate dehydrogenase
MD	Menadione
MonoMeBQ	Monomethyl-*p*-benzoquinone
NAC	N-acetyl-cysteine
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide-phosphate (reduced form)
NAPQI	N-acetyl-*para*-benzoquinone imine
NEM	N-ethymaleimide
NQ	1,4-Naphthoquinone
$O_2\cdot^-$	Oxygen superoxide
·OH	Hydroxyl radical
OPT	*o*-Phthalaldehyde
PBS	Phosphate-buffer saline
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TetraMeBQ	Tetramethyl-*p*-benzoquinone

ABSTRACT

Interaction between arylating quinones and thiols

- A proposed mechanism of quinone toxicity

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It is a matter of controversy to attribute quinone toxicity to either covalent binding or redox cycling activity.

Paracetamol with its putative reactive metabolite *N*-acetyl-p-benzoquinoneimine (NAPQI) is a classical example of this debate. It has been chosen together with para-benzoquinone (BQ), another possible reactive metabolite of paracetamol and a more stable quinone than NAPQI, to examine interaction of quinones with their main target, thiols.

The initial step in the study has been to assess paracetamol toxicity in a rat liver slice system. The ability of different protecting agents to prevent paracetamol cytotoxicity was investigated and the relation between thiol (glutathione and thiol-protein) status and tissue injury were assessed. Depletion of glutathione (GSH) and oxidative processes but not quantitative loss of protein-SH seemed to relate to the injury.

The second step of investigation was based on the idea that formation of quinone adducts to protein-SH could be necessary but not sufficient to lethally damage the cell. They could, however, if still redox active, trigger a long-lived generation of harmful radical species in the very vicinity of key proteins. *p*-Benzoquinone (BQ) was then shown, in a non-living system, to react with GSH to form HPLC-detectable conjugates which autoxidised to H_2O_2 at a rate which was more rapid than hydroquinone

autoxidation. Different methyl-substituted BQs as well as 1,4-naphthoquinone and menadione reacted with GSH to form H_2O_2 to different extents, depending on their ability to deplete GSH. Synthesised NAPQI was shown to bind GSH and to form some H_2O_2 upon reaction with GSH. Glutathione, and other thiols, in large excess, were able to prevent or delay the autoxidation of hydroquinones.

Similar reactions occurred when BQ was incubated with lens crystallins, the thiol-rich proteins of the lens. In this case, H_2O_2 production was associated with crystallin fragmentation, which seemed to be "site-specific" in terms of the specificity of protein fragment and susceptibility to inhibition by exogenous antioxidants.

We propose that arylation and redox cycling could act together to form a mechanism responsible for cytotoxicity of quinones.

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I. INTRODUCTION

1. Chemically-induced cell injury

1.1. Events observed during cell injury

Over the past two decades cell injury has frequently been studied using hepatocyte-based models and using paracetamol, bromobenzene and menadione as model toxins for the conceptual aspects of their toxicity: metabolism to a reactive metabolite, detoxification by glutathione, covalent binding to critical protein or oxidative stress (Hinson and Roberts, 1992). Injury to cells by chemicals can lead to a complex sequence of events culminating in cell death. There are at least two types of cell death, on one hand necrosis resulting from the failure of endogenous systems to compensate for injury and characterized by gross swelling of the cells, on the other hand programmed cell death (often associated with apoptosis (Martin *et al.*1994)) where cells on the contrary shrink before being rapidly eliminated by phagocytes (Wyllie *et al.*1980; Wyllie and Duvall, 1992). Whilst chemical-induced cell damage often results in necrosis, it can also end in the appearance of apoptotic morphological and biochemical features (Boobis *et al.*1989). We will consider here the events leading to necrosis.

The general pattern of biochemical changes observed in many cell types injured by a variety of agents is very similar (Boobis *et al.*1992; Wyllie and Duvall, 1992). There are early reversible changes like decrease in glutathione (GSH) level, blebbing, or entry of sodium into the cell accompanied by loss of potassium from the cell resulting in the accumulation of water in the cell, manifest as acute cell swelling. There are also later changes like depletion of pyridine nucleotides (NADH and NADPH), decrease in ATP level, modification of protein-thiol, lipid peroxidation and disturbance of calcium homeostasis. Activation of several types of enzymes like proteases, phospholipases, endonucleases (Ray *et al.*1993) as well as alterations in the cytoskeleton are related to rises

in free cytosolic calcium (Boobis *et al.*1992; Nicotera *et al.*1992) or to modification of crucial thiol groups (Torchinsky, 1981; Uchida and Stadtman, 1992).
A scheme representing the relevant biochemical changes observed following cell injury is described in Figure I.1..

1.2. Cause or result

There is often an excellent correlation between a cellular event and detection of tissue injury. However, some events appear to be a consequence rather than a cause of cell death. It is then important to distinguish between correlation and causation, between the marker of cell injury and the agent responsible for the damage.

1.3. Point of biochemical convergence

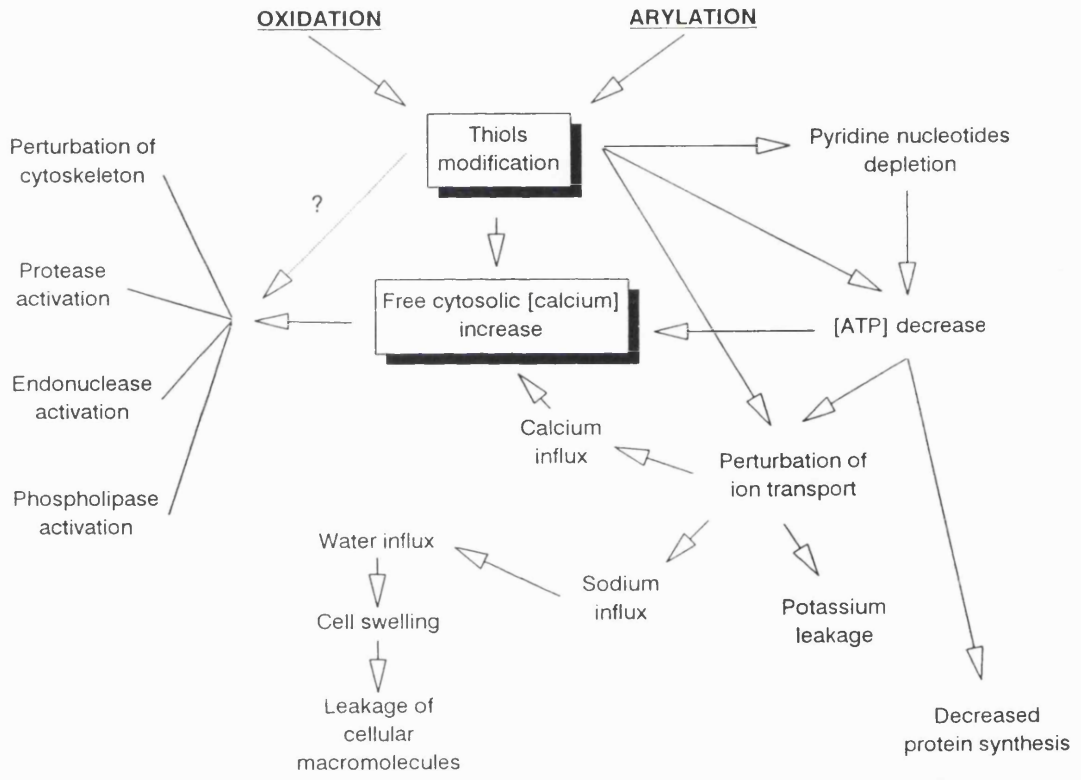
After an extensive insult, there is a point of irreversible damage from which recovery is impossible. This point is defined as "cell death" and is characterized in general by loss of plasma membrane integrity and collapse of cell morphology with leakage of cellular contents.
It is believed that, regardless of the toxic initiation, there may be a point along the pathway towards injury which, once reached, will trigger a cell via a single common pathway towards cell death. This central factor that converts reversible injuries to irreversible injury is not known, though increase in free cytosolic Ca^{2+} has been postulated.

2. Quinones

2.1. Source and nature

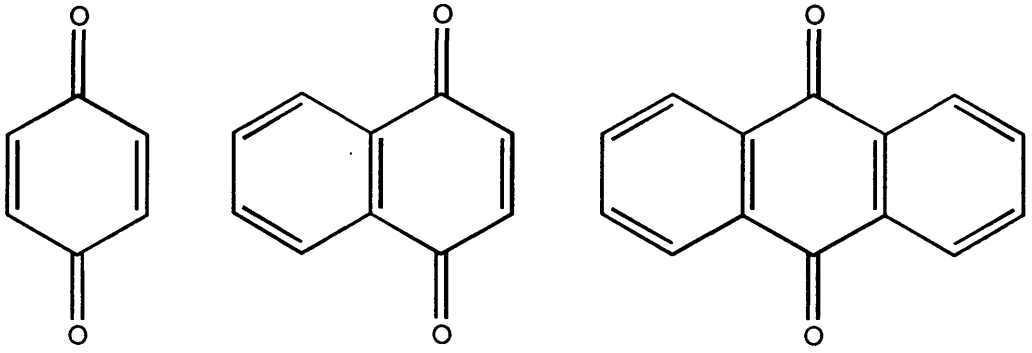
Quinones are widely distributed in nature as components of several functional biological systems (e.g. ubiquinone). Furthermore, the human is exposed to exogenous quinones clinically (i.e. doxorubicin), via diet (e.g. vitamin K_1) or via airborne pollutants (e.g. phenanthrenquinone) (Nohl *et al.*1986; O'Brien, 1991; Monks and Lau, 1992). Their

Figure I.1.: Relevant biochemical changes observed following cell injury



ubiquity and their toxicity have led them to be widely studied as a subject of mechanism of action leading to injury.

Quinones are diketones with the structure $O=C-(C=C-)_nC=O$ derived from aromatic compounds. Quinones, occurring as benzo-, naphtho- and anthraquinones, represent the largest class of quinoid compounds.



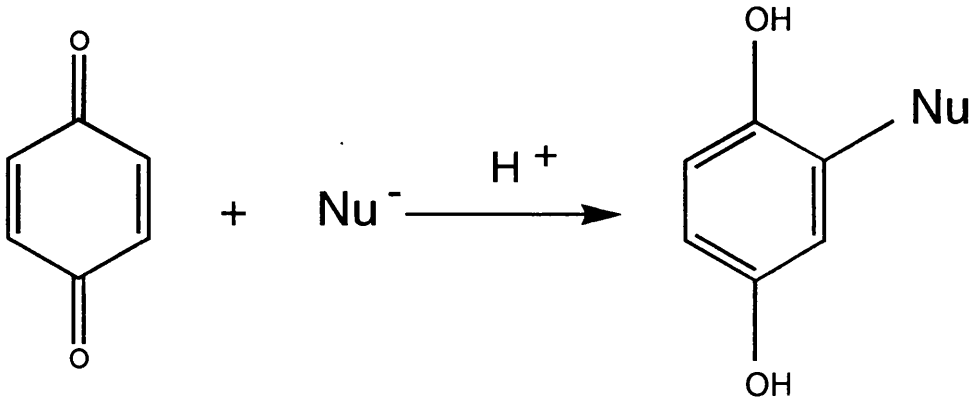
benzoquinone naphthoquinone anthraquinone

There are several types of quinoids depending on the nature of the exocyclic moiety: oxygen, quinones; nitrogen, quinone diimines; both oxygen and nitrogen, quinone imines; sulphur, dithio-quinones; and carbon, quinone methides (Patai and Rappoport, 1988; Brunmark and Cadenas, 1989). We will consider here benzo- and naphthoquinones as well as quinone imines. The chemistry of quinone imines is in many aspects similar to quinones including their ability to undergo redox reactions and addition reactions with nucleophiles. At variance with quinones, quinone imines occur rarely in nature.

Although broadly similar to that of α-β unsaturated ketones, every quinone has a chemistry of its own (Brunmark and Cadenas, 1989) and generalisation regarding their biochemical reactivity should be made with care. The prevalence of a biochemical pathway over another will be determined by, on one hand, the inborn chemical features of the quinone, mainly its reduction potential and, on the other hand, solvent-related factors such as pH and solvation property as well as the availability of electron donors or acceptors.

2.2. Arylation

The polarized carbon-carbon double bond of the quinone is responsible of the nucleophilic addition reaction occurring with cellular nucleophiles such as protein and non-protein sulphydryl. The vast majority of those reactions can be characterized as 1,4-reductive additions of the Michael type (Finley, 1974).



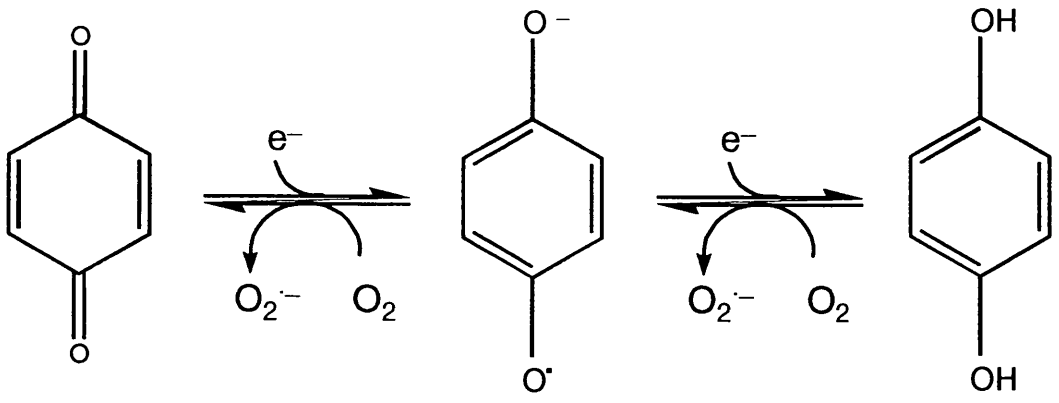
Nu^-: nucleophile

Electron donating substituents decrease the rate of nucleophilic addition to the quinone. The chemistry of nucleophilic addition to 1,4-naphthoquinones is different from that to p-benzoquinones because the adjacent benzene ring in the former limits the addition to one side of the quinoid ring (Brunmark and Cadenas, 1989).

2.3. Oxidoreduction

2.3.1. Quinones are redox cyclers

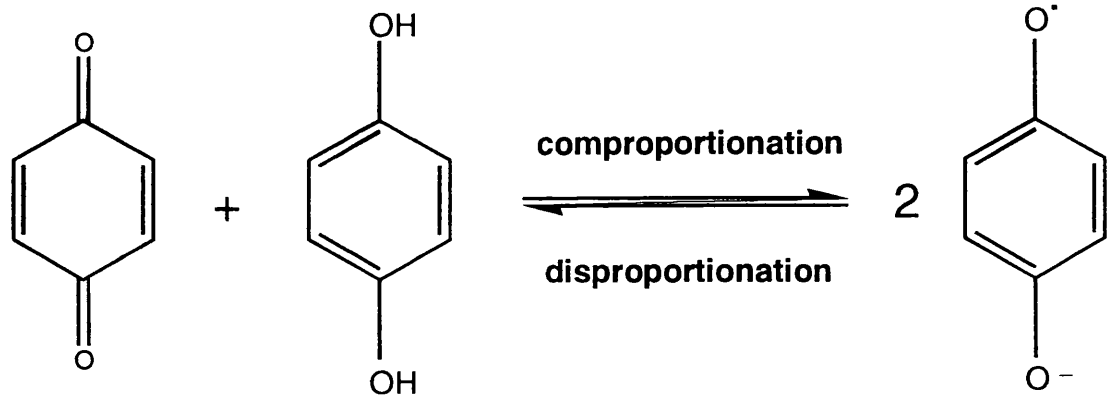
Quinones can undergo one- or two-electron reductions.



quinone semiquinone hydroquinone

Their cellular reduction proceeds either via one-electron transfer mechanism, catalyzed by NADH-cytochrome b_5 reductase and NADPH-cytochrome P450 reductase, or via two-electron transfer process, catalyzed by DT-diaphorase (NAD(P)H:quinone reductase) (Brunmark and Cadenas, 1989). All these enzymes are flavoproteins with relatively low substrate selectivity (Kappus, 1986). Quinones may also be reduced directly by intracellular electron carriers such as ascorbate (one-electron reduction) or GSH (two-electron reduction by two GSH) (Pethig *et al.*1983; Brunmark and Cadenas, 1989; O'Brien, 1991).

These two reduced forms, semiquinone radical and hydroquinone, can react with dioxygen to form the superoxide anion radical, $O_2^{\cdot -}$. This electron transfer to O_2 is non-enzymic and called autoxidation. Most hydroquinones, however, tend to be more stable than their semiquinone (Smith *et al.*1985). H_2O_2 can also be formed during this process if the lifetime of the collisional complex (hydroquinone and O_2) is long enough to allow the second electron transfer or during the oxidation of hydroquinone by $O_2^{\cdot -}$ or lastly due to spontaneous dismutation of $O_2^{\cdot -}$ (Brunmark and Cadenas, 1989; O'Brien, 1991). Another way of transferring electron between different oxidation states of quinones is via comproportionation or disproportionation reactions.



This often happens with great facility enabling semiquinones and quinones to be "redox facilitator" (James and Weissberger, 1938; Monks *et al.*1992).

Quinones can also be reduced enzymically or non-enzymically, before they in turn get autoxidized regenerating the parent quinone, available for rereduction and hence undergo a futile redox cycling. They are therefore called redox cyclers which are defined as NADPH-consuming compounds (Kappus and Sies, 1981), as explained in § I.2.5.4..

2.3.2. Reduction potential

The reduction potential of a quinone is of value in predicting the direction or feasibility, and in some instances the rate constants, of redox reactions with other electron carriers, like oxygen. It depends on the nature of the solvent and on temperature but also on pH and substituents of the quinone (Wardman, 1989).

2.3.3. pH effect

The redox chemistry of quinones is intimately coupled to their acid-base chemistry. For 1,4-benzoquinone (BQ), the quinone and its one-electron reduced form are not protonated at physiological pH, having pK_a of ~ 1 and 4 respectively. Because of charge repulsion, the semiquinone anion radical is a much weaker oxidant than its parent quinone and than the protonated semiquinone radical. The two-electron reduced form of the quinone is protonated at pH 7.4 having both its $pK_a > 10$. This hinders its oxidation which is much easier after deprotonation to the monoanion, partly explaining the so-called stability of hydroquinones (Brunmark and Cadenas, 1989; Monks *et al.* 1992).

2.3.4. Substituent effect

Besides pH effect, the reduction potential of a quinone can be strongly influenced by the nature, number and position of substituents (Chambers, 1974). Electron-donating substituents decrease the reduction potential of quinones, make their hydroquinone form more easily oxidized and raise the pK_a values, while electron-withdrawing substituents have the opposite effect.

2.4. Quinone-thioether reactivity

As seen in § I.2.2., it is now recognised that the nucleophilic attack of thiols on quinones is an addition, reductive in nature, involving a two-electron transfer process and producing a thiol-hydroquinone conjugate, also referred to as quinone-thioether and more precisely named, in the case of GSH, glutathion-S-yl hydroquinone (Finley, 1974;

Wilson *et al.*1987; Wardman, 1990). In the case of GSH, the resulting substituted hydroquinone is not redox inert (Wefers and Sies, 1983a; Ross *et al.*1985; Gant *et al.*1986; Wolff and Spector, 1987; Rao *et al.*1988; Lau *et al.*1988; Brunmark and Cadenas, 1988; Anusevicius and Cenas, 1993) and only minor changes in the reduction potential of the quinones are expected. In fact the thioether substituents often enhance the rate of autoxidation of the hydroquinone (Brunmark and Cadenas, 1988; O'Brien, 1991), in agreement with the relatively weak electron-withdrawing properties of thioether substituents (Brunmark and Cadenas, 1989).
Two different diglutathione conjugates and a triglutathione compound were detected in the bile of rats given HQ (Hill *et al.*1993).

It is important to notice that though, at first glance, oxidive and electrophilic reactivities of quinones seem unrelated, they are instead quite closely related. Indeed, nucleophilic addition to a quinone constitutes a formal two-electron reduction.

2.5. Quinone toxicity

2.5.1. Cellular targets

Addition reactions take place with cellular nucleophiles such as GSH, DNA, RNA, and proteins (Corcoran and Ray, 1992). Covalent binding with nucleic acids is usually accompanied by mutagenic and carcinogenic effects. Binding to proteins has been used by many investigators to explain the mechanism of cytotoxicity leading to liver cell necrosis. The latter will be considered in this work.

2.5.2. Thiol, main target

According to the HSAB (hard and soft acids and bases) principle, addition reactions occur most readily between electrophiles and nucleophiles of similar hardness, chemical hardness and softness being functions of the polarizability of the electrophilic/nucleophilic centre. Therefore thiols being "soft" nucleophiles, compared with "hard" ones like amines and alcohols, are more prompt to nucleophilic addition to quinones which are "soft" electrophiles (Coles, 1984).

The alteration of the intracellular thiol balance during quinone biotransformation results from quinone's two main properties, electrophile and oxidant, and therefore comprises two main processes: thiol arylation and thiol oxidation respectively.

2.5.3. Thiol arylation

Quinones bind readily cellular non-protein and protein thiols either spontaneously or catalyzed by the glutathione S-transferase(s) (Boyland and Chasseaud, 1967), leading to the formation of GSH- and protein-quinone adducts. The sequence of molecular events believed to conduct to cell injury is as follows. Quinones arylate intracellular GSH leading to its depletion. The GSH conjugates are transported out of the cell and further metabolized by the kidney to form urinary mercapturic acids. There is thus a net loss of GSH, which would have to be replenished by a slow synthesis from fresh supply of cysteine. GSH depletion allows quinones to bind protein-thiol covalently thereby causing irreversible changes on postulated key proteins, and resulting in cell death (Brodie *et al.*1971).

2.5.4. Thiol oxidation

Thiol exposure to quinones also results in thiol oxidation which can be described as formation of GSSG (oxidized form of GSH), protein disulphides and glutathione-protein mixed disulphides. The direct action of quinones in the generation of the two first mentioned products is not clear and H_2O_2 resulting from quinone redox cycling could well be the sole cause (Ross *et al.*1985). Glutathione-protein mixed disulphides are the result of the direct or enzymic interaction between GSSG and protein sulphydryl groups (GSSG + P-SH → GSH + P-S-SG) (Brigelius, 1985; Ziegler, 1985; Thomas *et al.*1990).

The mechanism for quinone-mediated cellular injury via thiol oxidation is believed to follow a sequential process. It is proposed that H_2O_2, generated from redox cycling quinones, is reduced by the glutathione peroxidase and results in GSH oxidation (this happens also spontaneously although at much slower rate). GSSG is subsequently re-reduced to the thiol by glutathione reductase at expense of NADPH. However, when NADPH concentration is not maintained at the normal intracellular level, following

ATP depletion, or when the glutathione reductase is inhibited, the intracellular GSSG concentration rises. GSSG accumulation is potentially damaging to the cell owing to its impact on the integrity of many key thiol-proteins (Bellomo *et al.*1983; Brigelius, 1985). Hence, there is a GSSG extrusion system to the extracellular space by a process coupled to a specific ATPase (Akerboom and Sies, 1994), thus also dependant on ATP level. This latter system results in a net loss of total glutathione for the cell.
Oxidizing species will then react with susceptible thiols of proteins and oxidize them, with the same damaging consequence on their functions as seen for arylation. This reaction, unlike protein arylation, is at least initially readily reversible by both exogenous and endogenous thiol-reducing agents (Boobis *et al.*1992).

2.5.5. Arylation *versus* oxidation

There is an excellent correlation between covalent binding and tissue necrosis (Brodie *et al.*1971). However we have to keep in mind that the same reactive species may bind to macromolecules and cause necrosis, without any causal association between the two events. Thus quinone-induced cytotoxicity can be prevented by antioxidants without affecting binding (Boobis *et al.*1992).
It now becomes a major challenge to elucidate the relative contribution to quinone toxicity arising from redox cycling activity versus electrophilic arylating activity. The two hypothesis are often discussed as though they were mutually exclusive (Mitchell *et al.*1981; Gant *et al.*1988; Thor *et al.*1988; Farber *et al.*1988; Hinson and Roberts, 1992). For this purpose different approaches have been used: prevention of quinone injury by antioxidants without affecting binding (Tee *et al.*1986), comparison between changes arising upon exposure to solely oxidizing quinones and mainly arylating ones (Bellomo *et al.*1990), lastly comparison of cytotoxicities due to a substituted quinone where one substitution prevents arylation and another diminishes redox cycling ability (Birge *et al.*1988).
Under both mechanisms, arylation and oxidation, protein-thiol modification seems to be closely related to the occurrence of irreversible cell damage (Bellomo *et al.*1990). Out of sometimes conflicting data (Fernando *et al.*1980; van de Straat *et al.*1986; Birge *et al.*1989), covalent binding appears to be a necessary but not a sufficient factor for the set up of irreversible injury. Concerning oxidation, it seems that, unless the cell is

pretreated with inhibitors of glutathione reductase or catalase, cell machinery such as is found in hepatocyte is adequate to cope with marked oxidative attacks (Rossi *et al.*1986). However for other cell types like cardiac myocyte, their inability to cope with oxidative insult may be a major factor in the predisposition of the heart to the cardiotoxicity of adriamycin (doxorubicin) for instance (Powis, 1989).

2.6. Quinone-thioether toxicity

Compounds that are conjugated with GSH are supposed to be detoxified and are usually excreted in urine as their corresponding cysteine conjugates and/or as mercapturic acids (i.e., S-conjugates of N-acetyl-L-cysteine). However, there is evidence which indicates that a variety of quinone-thioethers derived from GSH possess biological activity. As early as in 1948, GSH conjugate of menadione was shown to retain the antimitotic properties of the unsubstituted quinone (Friedmann *et al.*1948). Eyer and Kiese (1976), in work on the biotransformation of 4-dimethylaminophenol, first noted that S-conjugates could be more toxic than the parent compound. Since then, several halogenated quinones have been identified as potentially irreversible inhibitors of the glutathione-S-transferases (van Ommen *et al.*1988) which are part of the detoxification machinery of the cell against a large number of electrophiles. The N-acetylcysteine conjugate of menadione has been found cytotoxic for rat renal epithelial cells (Brown *et al.*1991). Furthermore, GSH conjugates of 1,4-benzoquinone, in particular 2,3,5-tri(glutathion-S-yl)hydroquinone, cause severe renal proximal tubular necrosis when administered to the rat (Lau *et al.*1988). The selective nephrotoxicity of hydroquinone-linked GSH conjugates is probably a consequence of the localization of the γ-glutamyl transpeptidase on the site of toxicity. This enzyme appears to be essential for this nephrotoxicity (Lau *et al.*1988). Indeed, γ-glutamyl transpeptidase and cysteinyl glycine dipeptidase, which activities are higher in the kidney than in the liver, metabolize glutathione conjugates into the corresponding cysteine conjugates. Those latter may be nephrotoxins either directly, as reactive episulphonium ions, or via the thiomethyl shunt, which directs the metabolism of cysteine conjugates away from mercapturic acid synthesis towards bioactivation by cysteine conjugate β-lyase to form reactive sulphur-containing moieties that are then methylated (Elfarra and Anders, 1984; Jakoby and Stevens, 1984; Monks *et al.*1985).

Thus, conjugation of glutathione with quinones does not necessarily result in detoxification.

2.7. Paracetamol injury

Paracetamol (*N*-acetyl-*p*-aminophenol, also known as acetaminophen) is an over-the-counter analgesic and antipyretic which, in overdose, can cause delayed death due to severe hepatotoxicity with centrilobular necrosis and sometimes associated with acute renal tubular necrosis in the kidney cortex (O'Brien, 1991). Adverse effects due to paracetamol taken at recommended therapeutic doses are rare. Nevertheless, over the past decades paracetamol has been frequently used in suicide attempts (Prescott, 1983). It has also been used over the same period as a model compound to investigate the mechanisms of cell injury (Hinson and Roberts, 1992).

2.7.1. Biotransformation and bioactivation routes

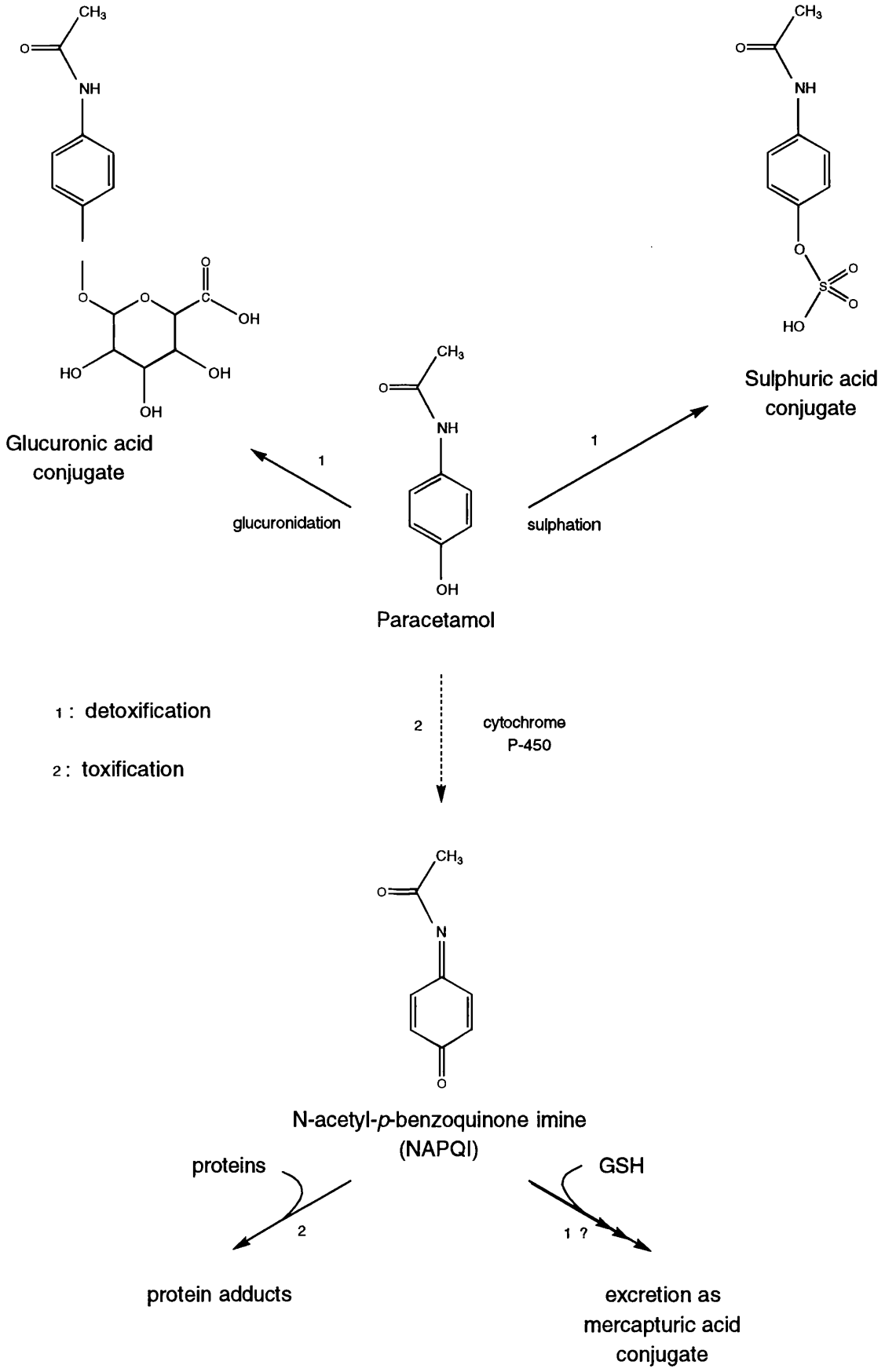
Liver cell injury does not result from paracetamol itself, but from a reactive intermediate formed in a minor metabolic pathway involving the cytochrome P-450 containing mixed-function oxidase system. At normal dose levels, paracetamol is shown to undergo mainly detoxicating metabolism *via* glucuronidation and sulphation in the liver. Only a small part of the administered dose is found to be bioactivated in the liver to such a reactive metabolite, which, being electrophilic, is shown to be detoxified by conjugation with hepatic GSH. The conjugate, upon degradation in the gut and the kidney, is excreted as mercapturic acid in the urine (Vermeulen *et al.*1992).
The overall scheme of biotransformation routes is described in Figure I.2..

2.7.2. Mechanism of toxicity

2.7.2.1. Accepted mechanism

After overdosage, the conjugation system to glucuronides and sulphates being not sufficent to eliminate paracetamol, a more substantial bioactivation of paracetamol proceeds resulting in an extensive depletion of hepatic GSH. Subsequently, covalent

Figure I.2.: Bioactivation and detoxification of paracetamol



binding of the reactive intermediate to cellular thiol-macromolecules increasingly takes place. This last event was first proposed as the mechanism responsible of paracetamol-induced hepatocellular injury (Jollow *et al.*1973; Prescott, 1988).

2.7.2.2. Controversy

Several specific proteins have been identified as being bound by a paracetamol metabolite (Bartolone *et al.*1987; Birge *et al.*1990a; Birge *et al.*1990b; Birge *et al.*1991b; Pumford *et al.*1992; Bartolone *et al.*1992) and the latter to bind with a high degree of selectivity to cysteinyl thiol groups on proteins (Streeter *et al.*1984; Hoffmann *et al.*1985a; Hoffmann *et al.*1985b). Covalent binding to protein has also been proposed as a lesion capable of disrupting Ca^{2+} homeostasis, causing cell death (Corcoran *et al.*1987).

However, dissociation of the covalent binding process from cell death induced by paracetamol has been argued (Labadarios *et al.*1977; Devalia *et al.*1982; Devalia and McLean, 1983) and a major controversy has arisen since as regarding the attribution induced cell death to a covalent binding process or to an oxidative stress related one (Tee *et al.*1986; van de Straat *et al.*1987a; Beutler, 1983; Birge *et al.*1988; Nelson *et al.*1990; Birge *et al.*1991a).

Different events have been observed during the injury process like loss of protein thiol (Moore *et al.*1985; Nelson *et al.*1990; Adamson and Harman, 1993), lipid peroxidation (Albano *et al.*1983; Rosen *et al.*1983; van de Straat *et al.*1988), formation of peroxide in general (Adamson and Harman, 1989), or changes in intracellular Ca^{2+} homeostasis (Landon *et al.*1986; Tirmenstein and Nelson, 1989; Boobis *et al.*1990; Ray *et al.*1993). All could be contributors to the toxicity. However the appearance of those events is often the subject of disagreements among researchers. So, loss of protein thiol, often ascribed to covalent binding, is sometimes mainly attributed to oxidation (Nelson *et al.*1990). Formation of GSSG is detected in some reports (van de Straat *et al.*1986; Jaeschke, 1990) and not found in other studies (Högberg and Kristoferson, 1977). Alteration in Ca^{2+} homeostasis has been itself associated with Ca^{2+}-endonuclease fragmentation of DNA (Ray *et al.*1990a; Ray *et al.*1990b; Ray *et al.*1993). However some investigators indicate that the rise in Ca^{2+} accompanies or follows rather than

precedes cell death (Harman *et al.*1992; Hardwick *et al.*1992). Some studies suggest that lipid peroxidation does not occur in paracetamol-induced hepatotoxicity (Kamiyama *et al.*1993) and rather describe paracetamol as an antioxidant (Albano *et al.*1983; van de Straat *et al.*1988).

2.7.2.3. Model

Other mechanisms of action of hepatotoxins have been found to share similar features, supporting the idea of a common pathway leading to cell injury. Paracetamol has therefore become more or less a model compound for mechanistic studies on toxicity, as well as a research tool for the investigation in the effectiveness of preventive agents against hepatotoxicity (Prescott, 1988; Vermeulen *et al.*1992).

2.7.3. Protecting agents

Paracetamol-mediated cell injury can be prevented by a wide panel of "protecting agents". There are agents related to Ca^{2+} homeostasis like the calcium channel blocking agents nifedipine, chlorpromazine (Landon *et al.*1986) and diltiazem (Deakin *et al.*1991), or like chlorpromazine again, known to inhibit the ability of calcium to activate calmodulin and to inhibit the activation of phospholipase C and protein kinase C (Saville *et al.*1988) or like inhibitors of phospholipase A_2, cyclooxygenase and thromboxane synthetase (Horton and Wood, 1989), or even like aurintricarboxylic acid, a general Ca^{2+}-endonuclease inhibitor (Shen *et al.*1992). There are thiol compounds like diethyldithiocarbamate (Lauriault and O'Brien, 1990), α-mercaptopropionylglycine (Labadarios *et al.*1977), N-acetylcysteine (NAC) (Bruno *et al.*1988; Nasseri-Sina *et al.*1992) and dithiothreitol (DTT) (Albano *et al.*1985; Tee *et al.*1986; Rafeiro *et al.*1994). There are also various compounds with antioxidant activity like dichloro phenol, indophenol, ethanol (Mourelle *et al.*1990), allopurinol, a xanthine oxidase inhibitor (Jaeschke, 1990), α-tocopherol, diphenylenediamine (DPPD) (Albano *et al.*1983; Harman, 1985), or promethazine (Devalia *et al.*1982; Harman, 1985). And finally there are other compounds of less defined modes of action like fructose (Mourelle *et al.*1991) or iloprost, a prostacyclin analogue (Nasseri-Sina *et al.*1992). Two kinds of protection have to be separated however, as far as the protein-arylation role

in cell death hypothesis is concerned: those applied together with paracetamol and which prevent protein arylation (Labadarios *et al.*1977) and those applied at a time point at which covalent binding has already taken place (Devalia *et al.*1982; Harman, 1985; Tee *et al.*1986; Mourelle *et al.*1991). It should also be mentioned that a real protective effect has to be differentiated from just a temporally delayed cell death (Devalia *et al.*1982; Nasseri-Sina *et al.*1992). Not one of the protecting agents listed above is in clinical use, except NAC and methionine (Prescott, 1984).

2.7.4. NAPQI

N-acetyl-p-benzoquinone imine (NAPQI) has been proposed as the ultimate toxic metabolite of paracetamol (Corcoran *et al.*1980) and considered as such by the majority of investigators.

NAPQI is a quinone imine. Since nitrogen electronegativity is less than oxygen, the reduction potential decreases in the same way, however the N-acetyl group should offset this effect at least partly. NAPQI is thus an arylating agent as well as a strong oxidant (Monks *et al.*1992). NAPQI undergoes non-enzymatic reduction by ascorbate (Blair *et al.*1980), NADPH, NADH (Dahlin *et al.*1984) and GSH (Rosen *et al.*1984; Albano *et al.*1985; Coles *et al.*1988) as well as conjugation at the C-3 position with thiols (Hoffmann *et al.*1985a). It is also reduced by NADPH-cytochrome P-450 reductase and by partially purified DT-diaphorase (Dahlin *et al.*1984). Studies both in a chemically defined system (Streeter *et al.*1986) and with hepatocytes (Albano *et al.*1985) indicate that NAPQI binds to protein, with cysteine residues as major sites of arylation (Streeter *et al.*1984). NAPQI-mediated modification of protein-thiol seems to be linked to enzyme inhibition and alteration of intracellular Ca^{2+} homeostasis (Nicotera *et al.*1989). Introduction of methyl substituents at the C-2 and C-6 positions of NAPQI blocks the reduction by GSH but does not block conjugation, whereas introduction of methyl substituents at C-3 and C-5 has the opposite effect (Rosen *et al.*1984). NAPQI and its two dimethylated analogues have been compared on the ground of their different reactivity vis à vis oxidation and arylation (van de Straat *et al.*1987b; Rundgren *et al.*1988; Nicotera *et al.*1990; Weis *et al.*1992). They manifest different levels of toxicity to rat hepatocytes: 3,5-dimethyl-NAPQI < NAPQI < 2,6-dimethyl-NAPQI. Nevertheless, significant amount of GSSG was found with 2,6-dimethyl-NAPQI

(Rundgren *et al.*1988) and the relative toxicity between 3,5-dimethyl-NAPQI and NAPQI was questioned (Weis *et al.*1992).

2.7.5. Is NAPQI the ultimate metabolite

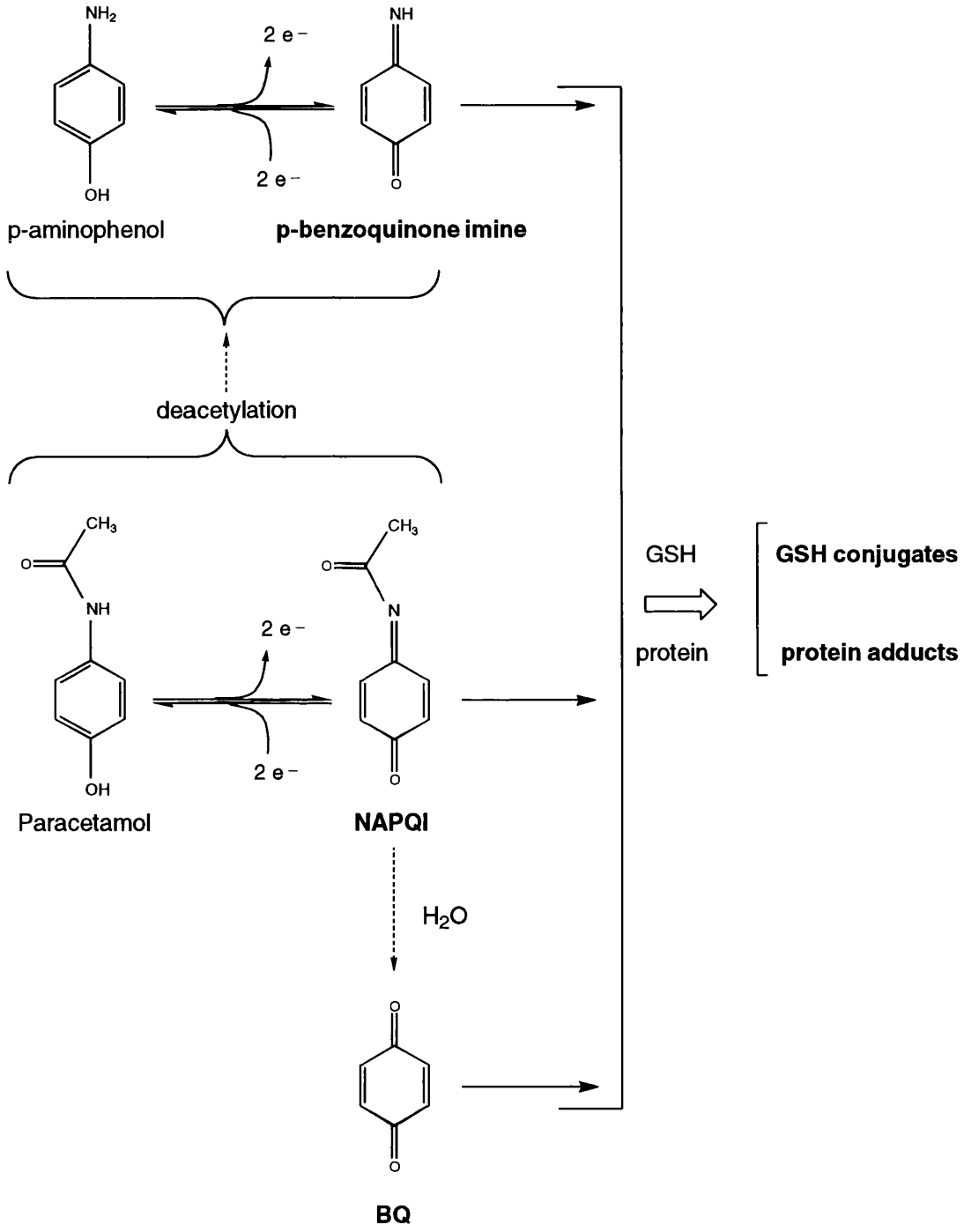
It has to be noted that the killing of cultured hepatocytes by NAPQI occurs by a mechanism different from that of paracetamol (Harman *et al.*1991). Naturally, the mechanisms of toxicity probably differ when the reactive metabolite is added exogenously compared with when it is generated *in situ*. Nevertheless, it is not known wether NAPQI is the only toxic metabolite formed from paracetamol. Other paracetamol metabolites like p-benzoquinone (BQ) and p-aminoquinone imine could also be candidates (see Figure I.3.). NAPQI undergoes hydrolysis to (BQ) (Dahlin and Nelson, 1982), an equally known toxic molecule (Pascoe *et al.*1988) (BQ is the subject of a separate section). Studies of covalent binding to protein by radiolabelled analogues of both NAPQI and paracetamol show that although the majority of covalent binding of paracetamol to proteins is mediated by NAPQI, ~20% were due to BQ (Dahlin *et al.*1984; Axworthy *et al.*1988). Aminophenol and hydroquinone conjugates with cysteine residues in haemoglobin have also been detected after administration of NAPQI *in vivo* (Pascoe *et al.*1988).

2.8. *para*-Benzoquinone (BQ)

Besides its presence in paracetamol metabolism (Pascoe *et al.*1988), BQ is also believed to be an active metabolite of benzene bioactivation (Lunte and Kissinger, 1983; McDonald *et al.*1993a). Benzene is an ubiquitous compound issued from both natural and industrial processes. Chronic exposure to it is associated with various sorts of myelotoxicity. Briefly, benzene is metabolized primarily in the liver via mixed function oxidase enzymes to phenol, hydroquinone and catechol. It is suggested that secondary metabolism within the bone marrow is necessary to elicit toxicity (Thomas *et al.*1990). BQ also appears to be a degradation product of aminophenol. Aminophenols are constituent of a variety of drugs; their biotransformation is found to be accompanied by the formation of deaminated thioadduct (Eckert, 1988).

Furthermore, the stability of BQ and of its thioether conjugates, compared with the

Figure I.3.: Potential active metabolites of paracetamol



susceptibility to rapid hydrolysis of quinoneimines, make BQ an interesting model quinone to study quinone/thiol interaction.

BQ binds GSH to form a sulphydryl conjugate, in a process which seems to be predominantly non-enzymatic (Lunte and Kissinger, 1983). It also binds proteins at the cysteinyl thiol group (McDonald *et al.*1993b). Rat hepatocytes exposed to BQ experience a GSH loss, which is not associated with GSSG formation, and a protein-thiol loss, mainly cytoskeletal, which is not associated with mixed disulphides formation (Bellomo *et al.*1990). The chemical reaction of BQ with GSH results in the formation of poly-glutathione substituents (Lau *et al.*1988). The first conjugation of BQ with GSH rather decreases the reduction potential of the quinone (Lau *et al.*1988; Eckert *et al.*1990a), enhancing its oxidizability. However, subsequent glutathione additions increase the reduction potential of the compound (Lau *et al.*1988). Except for the mono- and tetraglutathion-S-yl conjugates, all the diglutathion-S-yl compounds and especially the triglutathion-S-yl conjugate were nephrotoxic (Lau *et al.*1988).

2.9. 1,4-Naphthoquinone (NQ) and menadione (MD)

MD (2-methyl-1,4-naphthoquinone) is a synthetic derivative of phylloquinone (vitamin K_1). It has found clinical use for its anticancer activity (Powis, 1989; Nutter *et al.*1991), although its research interest has made it a model redox-cycling quinone in rat hepatocytes, used to investigate the mechanisms and consequences of oxidative stress in mammalian cells (Thor *et al.*1982; Di Monte *et al.*1984b).

NQ and MD are closely related quinones and have about equal potentials to induce redox cycling. However, NQ has a higher potential to arylate and is more toxic than menadione (Miller *et al.*1986).

Glutathionyl conjugates of NQ and MD are able to act as substrates for DT-diaphorase. After their reduction by this oxidoreductase they both autoxidize at rates higher than those of the unsubstituted parent compounds (Buffinton *et al.*1989). When presented to rat hepatocytes, MD induces a decrease in both GSH level and in protein sulphydryl groups. This is mainly due to oxidation, although arylation accounts for a small fraction of those losses (Di Monte *et al.*1984b). Rat hepatocytes treated with MD present several other effects: increase in consumption of molecular oxygen and generation of $O_2\cdot^-$

(Thor *et al.*1982; Gant *et al.*1988), alterations in pyridine nucleotide pools (Bellomo *et al.*1982; Miller *et al.*1986; Stubberfield and Cohen, 1989), disturbance of Ca^{2+} homeostasis (Thor *et al.*1982; Bellomo *et al.*1982; Di Monte *et al.*1984a), and DNA damage (Morrison *et al.*1984).

3. Thiols

3.1. Thiol chemistry

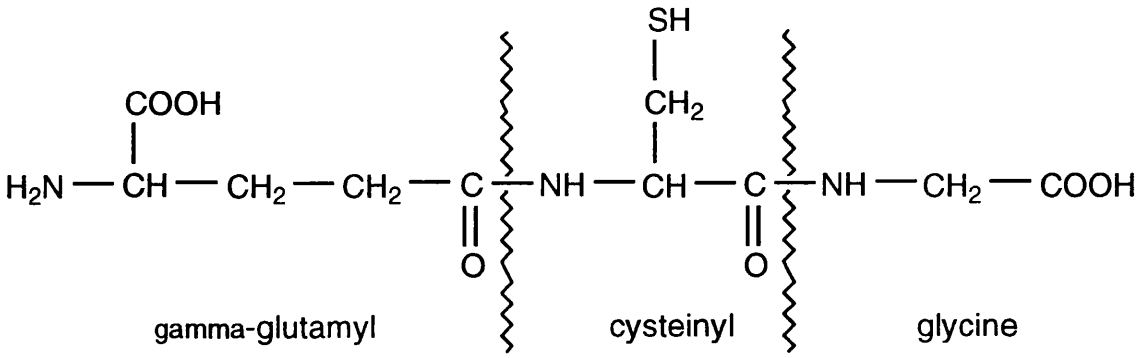
Thiols are reductants, nucleophiles, radical scavengers and metal chelators. They take part in most reactions as thiolate anions (RS^-) (Torchinsky, 1981; Wardman, 1988). Therefore, the reactivity of SH groups depends on their pK_a and of the pH of the solution in which they bathe. Electrostatic influence of neighbouring charged groups and, in the case of protein, spatial arrangement within the molecule clearly influence ionization of thiol groups, thereby thiol reactivity. Thiol groups in native proteins are either fully exposed, partially buried (masked) or buried, consequently their reactivity varies within wide limits (Torchinsky, 1981).

Thiols autoxidize readily in presence of catalytic quantities of metals, especially iron and copper which form complexes with the thiols being oxidized. The removing of metal traces by chelators sharply lowers the rate of thiol oxidation (Torchinsky, 1981).

3.2. Glutathione (GSH)

3.2.1. Crucial role

GSH is the major non-protein low molecular weight sulphydryl in cell (at least 90% of the total non-protein sulphydryl content) (Reed and Fariss, 1984). It exists under two forms, either reduced (GSH) or oxidised (GSSG, considered in a separate section). It is a tripeptide (γ-glutamylcysteinyl glycine), the thiol group of the cysteine being responsible for most of the chemical properties of GSH.



GSH is synthesized at the expense of two ATP molecules and in two sequential reactions. The first reaction is the rate-limiting step and is feedback inhibited by GSH (Meister and Anderson, 1983).

1: gamma-glutamylcysteine synthetase

L-cysteine + L-glutamate + ATP ⟶ gamma-glutamylcysteine + ADP + Pi

2: GSH synthetase

gamma-glutamylcysteine + L-glycine + ATP ⟶ GSH + ADP + Pi

The supply of cysteine can also be a limiting factor in GSH production. It is obtained directly from diet or indirectly from methionine through a pathway involving five enzymes and consuming one ATP molecule (Jocelyn, 1972).

GSH plays an essential role as a nucleophile and as a reductant in the cellular defence system against toxins. As a nucleophile, it intervenes in phase II of the detoxification pathway of xenobiotics (Phase I is the activation of xenobiotics by mixed function oxidases to electrophilic intermediates). This action consists of conjugating electrophiles, directly or via glutathione-S-transferases, to form a more water soluble and frequently less toxic (see § I.2.6.) thioether which is then transported out of the cell and further metabolized by the kidney to form urinary mercapturic acids (O'Brien, 1991). As reductant, via glutathione peroxidases or directly, it prevents tissue damage

mediated by peroxides. The latter reaction results in oxidation of GSH to GSSG which is subsequently reduced back to GSH by glutathione reductase, but at the expense of NADPH. The role of GSH in detoxication is also as a radical scavenger (Wilson *et al.* 1986a; Halliwell, 1990). For instance, the radical-cation of aminopyrine ($AP^{\cdot +}$) formed by prostaglandin H synthase is subsequently reduced by GSH via the reaction: $AP^{\cdot +} + GSH \rightleftarrows AP + GS^{\cdot} + (H^{+})$ (Ishii and Fridovich, 1990).

Beside this protective role, GSH is also involved in the regulation of a number of endogenous functions. For instance it has been shown to be a reductant for heme-containing proteins like myoglobin (Giulivi and Cadenas, 1994) a cofactor necessary in the formation of leukotrienes (Chang *et al.* 1982) and to be crucial for the regulation of Ca^{2+} compart mentation in the hepatocyte (Orrenius *et al.* 1983).

3.2.2. GSH depletion

GSH depletion seems to always precede quinone-induced cell death (Orrenius, 1985; Boobis *et al.* 1989). Although depletion of intracellular GSH levels below the threshold of 20% of physiological level has been considered to be detrimental to the cell (Anundi *et al.* 1979; Younes and Siegers, 1980), it is not *per se* responsible of the injury since GSH can be depleted chemically without further toxicity (Reed and Fariss, 1984; Pascoe and Reed, 1987). It is therefore believed that, following GSH depletion, electrophiles and oxidants react with macromolecules thereby modifying them in a way which can result in cytotoxicity (O'Brien, 1991). Exposure of hepatocytes with lowered levels of intracellular GSH to quinones results in a more rapid loss of protein thiols (Di Monte *et al.* 1984a) and in accelerated cell damage as compared to cells with normal GSH concentrations (Reed and Fariss, 1984).

Some agents prevent quinone-induced cell death without diminishing GSH depletion (Ray *et al.* 1993).

3.2.3. Is GSH a cell protecting agent ?

3.2.3.1. Possible adverse effects of GSH

Non-enzymic reactions of GSH with $O_2^{\cdot -}$ and H_2O_2 are slow but can produce sulphur-

containing radicals more reactive than $O_2^{\cdot -}$ and H_2O_2 themselves (Wefers and Sies, 1983b; Schöneich *et al.*1989). Reaction of ·OH with GSH can also produce thiyl radicals (GS·) that may subsequently react with oxygen to give reactive oxysulphur radicals such as GSO· and GSO_2· (thiyl peroxyl) (Wardman, 1988). In the presence of ferrous iron, GSH will act as a chelator and the $Fe^{II}(GS)_2$ complex being a more powerful reductant than either ferrous salt or GS^-, and activation of oxygen is more likely (Wardman, 1988).

3.2.3.2. GSSG

The autoxidation of GSH is very slow at physiological pH in metal-free solution (Misra, 1974). It can however be oxidized by xenobiotic oxidants directly or via H_2O_2 formation. The oxidation of GSH by H_2O_2 is catalysed by glutathione peroxidase,

$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$.

GSSG can affect protein activity and is therefore potentially toxic for the cell (Brigelius, 1985; Thomas *et al.*1990). Therefore GSSG is kept at low level concentrations (less than 5% of total glutathione (Reed and Fariss, 1984)) by the action of the GSSG reductase and transport of GSSG across the plasma membrane into the bile by an ATP-dependent process, probably common with glutathione S-conjugate transport (Nicotera *et al.*1985; Ishikawa *et al.*1989; Akerboom *et al.*1991). However, if its level rises high enough, protein-SH/GSH mixed disulphides can be produced either spontaneously or *via* a thiol transferase (Brigelius *et al.*1982; Brigelius *et al.*1983) and changes in protein activity can be observed (Yoshida *et al.*1992). This thiolation of crucial sites on protein is believed to alter the activity of key enzymes and, if not rapidly reversed, to mediate toxicity.

3.2.3.3. Thioether reactivity

Quinone S-conjugate reactivity has already been mentioned above (see § I.2.4. & I.2.6.). Reactive glutathione S-conjugates are also formed with other types of compounds such as leukotriene C_4, dibromo- and dichloroethane (Sies, 1988) and a variety of other chemicals (Monks *et al.*1990; Monks and Lau, 1992).

3.3. Protein-SH

3.3.1. Key-proteins, key sites

Many cellular proteins contain highly reactive sulphydryls that are especially prone to modification during alkylation processes or oxidative stress. The concentration of these reactive sites is impressive, reaching or exceeding the concentration of glutathione in many cells. Those proteins often possess key function in the cell (Ziegler, 1985; Brigelius, 1985).

Thiols are essential for many enzymes either in the catalysis or in the binding of cofactors or in maintaining the active conformation of proteins (Torchinsky, 1981; Grässer *et al.*1992).

3.3.2. Protein modification

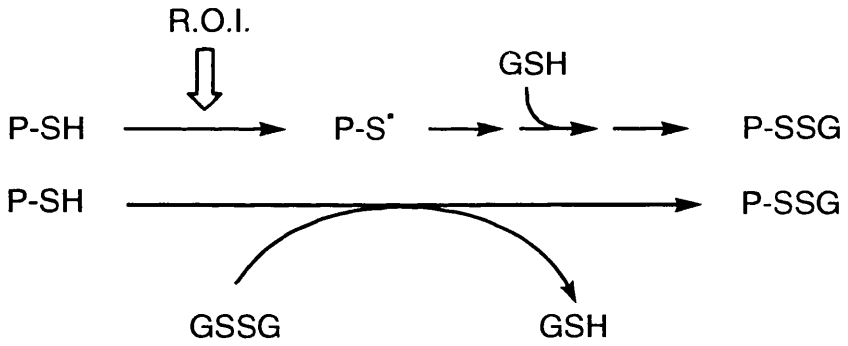
3.3.2.1. Alkylation (or arylation)

It is a common feature of a large number of enzymes to be irreversibly inactivated by thiol alkylating reagents such as N-ethylmaleimide (Thor *et al.*1985). ATP-dependent Ca^{2+} transporter in the hepatocytes plasma membrane has a sulphydryl-dependent functional moiety. Its activity decreases after exposure to paracetamol (Tsokos-Kuhn *et al.*1985), most likely due to covalent binding (Tsokos-Kuhn *et al.*1988). Similarly, the ATP-dependent Ca^{2+} sequestration by rat liver microsomes was inhibited by reagents that cause alkylation of protein sulphydryl groups (Thor *et al.*1985). IP_3-stimulated release of calcium from intracellular pools in hepatocytes was inhibited after treatment with menadione, p-benzoquinone and N-ethylmaleimide (Pruijn *et al.*1991). The kinase and phosphatase activities of rat liver 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase were selectively inhibited or stimulated depending on the treatment with different alkylating agents (El-Maghrabi *et al.*1984). Rabbit skeletal muscle protein phosphatases 1 and 2A are inactivated by a variety of thiol group reagents consisting of alkylating and mercaptide-forming compounds (Nemani and Lee, 1993).

3.3.2.2. Oxidation

3.3.2.2.1. Mechanism

There are different thiol oxidation states: disulphide (RSSR: 2 × $-1e^-$), sulphenic acid (RSOH: $-2e^-$), sulphinic acid (RSO_2H: $-4e^-$) and sulphonic acid (RSO_3H: $-6e^-$) (Jocelyn, 1972; Radi *et al.*1991). Two kind of disulphides are described: intra- or inter-protein disulphide bonds and mixed-disulphides between a protein sulphydryl and a low molecular weight cellular thiol such as GSH, a protein modification termed S-thiolation (Ziegler, 1985) or S-glutathiolation if GSH is involved in the modification. S-glutathiolation can occur enzymatically (catalysed by a thiol transferase (Brigelius *et al.*1982)) or spontaneously caused either by reactive oxygen intermediates (R.O.I.) and without appreciable amounts of GSSG being formed (Park and Thomas, 1988), or by a high ratio of GSSG/GSH. Two different mechanisms are then implied:



Except sulphonic acid, the other oxidation states of thiols can be reduced back to the -SH state (Abate *et al.*1990). Disulphide net reduction can be catalysed by lipoamide dehydrogenase and especially by glutathione reductase and thioredoxin reductase (Ziegler, 1985).

GSH can also reduce a wide variety of disulphides *via* transhydrogenation (Kosower and Kosower, 1983):

$2GSH + R_1SSR_2 \rightleftarrows GSSG + R_1SH + R_2SH.$

These reactions are catalyzed by cytosolic thioltransferases (Axelsson *et al.*1978), glutaredoxin (Luthman and Holmgren, 1982), and by membrane-bound thiol:disulphide oxidoreductases (protein-disulphide isomerases) (Morin *et al.*1983; Morin and Dixon,

1985). However, transhydrogenation reactions do not change the ratio of thiol to disulphide and the reductive capacity of GSH-dependant transhydrogenations depends ultimately on the reduction of GSSG by NADPH-dependant glutathione reductase. NADPH is also needed for thioredoxin reductase (Ziegler, 1985) and appears then to be essential in the overall thiol reduction process.

3.3.2.2.2. Protein biological activity

It has now become clear that the activity of certain enzymes can be modulated by a thiol-disulphide exchange induced by several naturally occurring disulphides (Federici *et al.*1978). In contrast with alkylating processes, this modification is metabolically labile and the rapid "dethiolation" of protein by several reductive processes (Park and Thomas, 1989) prevents lasting damage to the protein. These observations have led to the proposal of a hypothetical "third messenger" function of biological disulphides (Gilbert, 1982).

Many enzymes have been shown to contain accessible cysteinyl residues which can be modified (inactivation or activation) by thiol:disulphide exchange (for review: Ziegler, 1985; Brigelius, 1985). Prominent examples can be listed as fellows: glycogen phosphorylase phosphatase, glycogen synthase, phosphofructokinase (Gilbert, 1982; El-Maghrabi *et al.*1984), hexokinase, pyruvate kinase (Axelsson and Mannervik, 1983), 3-hydroxy-3-methylglutaryl-CoA reductase, serotonin N-acetyltransferase, guanylate cyclase, and glyceraldehyde-3-phosphate dehydrogenase (Brodie and Reed, 1990) all are inactivated by the oxidation process. Conversely, fructose 1,6-biphosphatase, possibly glucose-6-phosphate dehydrogenase, acetyl-CoA hydrolase, probably a protein kinase associated with inhibition of protein synthesis, and leucocyte collagenase (Tschesche and Macartney, 1981) are activated by such processes. Protein kinase C activity is modulated by oxidative processes in a paradoxical manner, activated under moderate oxidation and inhibited under higher degrees of oxidation (Kass *et al.*1989). Those enzymes belong to many different biological function types or pathways like carbohydrate metabolism, fatty acid synthesis, hormone regulation, protein synthesis, signal transduction and proteolysis.

3.3.3. Implications for quinone toxicity

3.3.3.1. Protein-SH depletion

A good correlation has been observed between depletion of protein sulphydryls and loss of cell viability, with loss of protein thiols preceding cell death, (Di Monte *et al.*1984a; Pascoe and Reed, 1989) and attempts have been made to identify distinct pools of protein thiols whose integrity is essential for the maintenance of cell viability.
Protein mixed disulphides (Di Monte *et al.*1984b; Brigelius *et al.*1982) and intermolecular disulphide links (Kosower *et al.*1981) are formed during lethal oxidative stress suggesting that they may have toxicological relevance.

3.3.3.2. Enzyme activation or inactivation

Protein thiol modification is likely to be of importance in expression of toxicity by perturbation of critical cellular functions.

As seen in § I.3.2.2.2.2., thiol redox status of proteins can reversibly affect many metabolic processes, for some in a regulatory manner. However, if oxidative conditions become chronic, disulphide formation may overcome the counteracting systems and direct cellular metabolism into pathways not strictly necessary for cell metabolism but merely imposed by oxidative stress. This process may be deleterious to the cell. The same mechanism of prolonged protein activity change can be conceived to take place when irreversible thiol modifications are imposed by alkylating agents as seen in § I.3.2.2.1..

Furthermore the modification of the biological activity of some proteins may directly contribute to the toxic effects of thiol-modifying toxins.
The ATP-dependent sequestration of Ca^{2+} by the plasma membrane fraction from rat liver is inhibited by the GSH oxidising agents diamide and t-butylhydroperoxide (Bellomo *et al.*1983). The ATP-dependent Ca^{2+} uptake by rat liver microsomes was inhibited by t-butylhydroperoxide (Jones *et al.*1983). The simultaneous inhibition of both transport systems, plasma membrane Ca^{2+}-ATPase and microsome Ca^{2+}-ATPase,

would be expected to result in a disruption of intracellular Ca^{2+} homeostasis. The latter process is a candidate in responsibility for cell killing.

Glutathione S-transferase P-form, considered as a detoxifying enzyme, is inactivated by hydrogen peroxide probably due to the formation of disulphide bonds resulting in steric hindrance of an active site (Shen *et al.* 1993).

Changes in cell morphology and in the cytoskeleton can be observed upon oxidant injury and could be associated with loss of sulphydryl groups from cytoskeletal proteins like actin (Hinshaw *et al.* 1986; Mirabelli *et al.* 1988a; Mirabelli *et al.* 1988b; Moore *et al.* 1992).

Finally, xanthine oxidase has its physiological dehydrogenase activity converted into that of an oxidase by GSSG exposure (Battelli and Lorenzoni, 1982). The production of oxygen superoxide which follows may contribute to cell damage.

3.3.4. Case of the lens

3.3.4.1. Crystallin

Crystallins (for review: Harding and Crabbe, 1984) are the soluble proteins from the lens. The lens is an avascular tissue (instead bathed in aqueous humour) packed with protein (the protein concentrations within the lens are in the ranges found in protein crystals). They provide the high refractive index necessary to focus light on the retina. Although their structure is identical or evolutionarily related to stress proteins, glutathione S-reductase (Wistow, 1993) or even NADPH:quinone reductase (Rao and Zigler, 1992), they have no known function in the lens other than structural. They are exceptionally stable proteins, with only low levels of proteases in lens and virtually no turnover in mature cells (Augusteyn, 1994). The major groups of crystallins are α-, β- and γ-crystallins; only γ-crystallins are monomeric proteins. Crystallin thiol content is high, especially for β and γ. Considering that GSH concentration in the lens of vertebrates is usually considerably higher than those found in other tissues (human lens GSH content is in excess of 10 μmol/g wet weight (Pau *et al.* 1990)), the total sulphydryl content of the lens is extremely elevated.

3.3.4.2. Toxic cataracts

Cataract (for review: Harding and Crabbe, 1984) is the term used to described opacities seen in the lens which could eventually impair vision. Crystallin integrity is believed to be essential to avoid any light scattering. Formation of disulphide-bonded aggregates as well as other alterations of protein conformation are therefore supposed to decrease the transparency. Oxidative stress in general, and H_2O_2 in particular, are suspected to play a role in the formation of many cataracts. Thus, as the human cataract progresses, the SS/SH ratio increases in crystallins and a high level of H_2O_2 is found in the aqueous humour of certain patients (Jahngen-Hodge *et al.*1994).

There is a great variety of insults that can lead to cataract, among them, exposure to toxic chemicals. Quinoids are part of those compounds which have been reported to cause cataract. For instance, oxidation of tyrosine to dopa (3,4-dihydroxyphenylalanine) and thus to dopaquinone results in cataract formation in the rabbit lens accompanied by a loss of both GSH and protein thiol, a rise in GSSG, and some inhibition of glucose 6-phosphate dehydrogenase and lactic dehydrogenase (Srivastava and Beutler, 1969). Naphthalene cataract too, which is well described in the rabbit lens (van Heyningen, 1979), appears to have a quinone for ultimate metabolite. Naphthalene is probably converted by the enzyme catechol reductase to 1,2-dihydroxynaphthalene. This latter intermediate is autoxidized, with production of H_2O_2, to 1,2-naphthoquinone which in turn can react with thiol groups, amino groups, and ascorbate. A redox cycle has been described in the aqueous humour between 1,2-dihydroxynaphthalene and the naphthoquinone. This cycle oxidizes much of the ascorbic acid and generates a large amount of H_2O_2. Paracetamol also has been reported to cause cataract in mice *in vivo* (Shichi *et al.*1978). Finally, opacities develop in embryonic chicken lens in medium containing 1 mM concentration of 1,2-naphthoquinone, 1,4-naphthoquinone, menadione, and hydroquinone (Edwards *et al.*1973).

4. Hydrogen peroxide (H_2O_2)

Due to an efficient H_2O_2-removal mechanism inside the cell, mainly catalase and glutathione peroxidase (Eklöw *et al.*1984), the steady-state H_2O_2 concentration is kept

low (10^{-7}-10^{-9} mol l^{-1} for the rat liver and 10-25 μmol l^{-1} for the lens of the human eye) (Halliwell and Gutteridge, 1989). However, exposure of intact red blood cells to H_2O_2 results in an irreversible inactivation of endogenous superoxide dismutase (Salo *et al.*1990). Furthermore, H_2O_2 alone is likely to be the major cause of cell death due to leukocyte-produced oxygen species as demonstrated by *in vitro* studies with fibroblasts, endothelial cells and other cell types. It can react with virtually all cellular constituents to cause lipid peroxidation, denaturation of proteins and alteration of nucleic acid (Rubin and Farber, 1984).

4.1. Reactive Oxygen Intermediates (R.O.I.)

The term R.O.I., also called reactive oxygen species, is a collective one that includes not only oxygen-centered radicals such as oxygen superoxide ($O_2\cdot^-$) and hydroxyl ($\cdot$OH) but also some non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2) , singlet oxygen ($^1\Delta g$) and hypochlorous acid (HOCl) (Deby and Pincemail, 1986; Halliwell *et al.*1993).

H_2O_2 is formed in vivo by two-electron reduction of O_2 or via enzymic (superoxide dismutase, SOD) and spontaneous dismutation of $O_2\cdot^-$. The latter originates in vivo (Fisher, 1987; Halliwell and Gutteridge, 1992; Nordmann, 1993; Michiels *et al.*1994) either accidentally, by "leakage" from electron transport chains, or intentionally, by activated phagocytic cells and possibly lymphocytes, endothelial cells, and fibroblasts, or finally, again accidentally, through xenobiotic detoxication (Hildebrandt *et al.*1975). Unlike $O_2\cdot^-$, which is mostly reductive, H_2O_2 is an oxidizing agent and is able to cross all biological membranes (Halliwell, 1990). Both of them are poorly reactive and are readily removed from the cell by SOD, catalase and glutathione peroxidase. Yet, they are both potentially toxic due to their conversion into the much more reactive $\cdot$OH (Halliwell and Gutteridge, 1992). Outside the recent mechanism involving $O_2\cdot^-$ interaction with nitric oxide (NO$\cdot$) (Radi *et al.*1991), the mechanism proposed to explain $\cdot$OH formation from $O_2\cdot^-$ and H_2O_2 is the metal-catalysed Haber-Weiss reaction (see following section). $\cdot$OH, reacts at a diffusion-controlled rate with almost all biological molecules. Hence its site of formation will determine its target.

4.2. Fenton and Haber-Weiss reactions

Although the ferryl ion (FeO^{2+}) production associated with haem proteins has been proposed, Fenton chemistry (also referred to as metal-catalysed Haber-Weiss reaction) is the most likely mechanism for producing a highly oxidizing species in biology, i.e. ·OH (Halliwell and Gutteridge, 1992). The Fenton reaction is mainly described as the reaction between H_2O_2 and iron leading to the formation of ·OH (Halliwell and Gutteridge, 1989):

$H_2O_2 + Fe^{2+} \rightarrow {\cdot}OH + OH^- + Fe^{3+}$.

Fe^{3+} is reduced slowly by H_2O_2 but readily by $O_2{\cdot}^-$. The overall process of the metal-catalysed decomposition of H_2O_2 into ·OH and the return of the metal to the reduced state by $O_2{\cdot}^-$ constitutes the Haber-Weiss reaction (Nordmann, 1993):

$H_2O_2 + Fe^{2+} \rightarrow {\cdot}OH + OH^- + Fe^{3+}$

$O_2{\cdot}^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$.

The oxidation reaction is sustained by the presence of metal reductants, such as ascorbate and thiols. Although mainly described for iron, copper appears to react similarly. Reaction of hypochlorite with $O_2{\cdot}^-$ may also be a source of ·OH *in vivo* (Baynes, 1991):

$HOCl + O_2{\cdot}^- \rightarrow O_2 + Cl^- + {\cdot}OH$.

4.3. Source of metals

The question of the Fenton-reactive iron (or copper) availability in vivo is a crucial one to support the toxicity of partially reduced oxygen species. Clearly the organism takes great care to minimize this availability by using transport proteins such as transferrin and storage proteins such as ferritin and haemosiderin for iron, and caeruloplasmin for copper. There is however evidence for metal-dependent free radical reactions in vivo (Starke and Farber, 1985; Halliwell and Gutteridge, 1989). Except in the special case of iron overload, loosely-bound iron concentrations measured in the body fluids by the bleomycin assay are rarely greater than 1-2 μM and are zero in plasma from healthy humans (Halliwell and Gutteridge, 1992). This catalytically active metal ion pool, mainly iron, could come either from a low molecular weight chelator system needed to transfer

metal from storage proteins to biosynthetic pathways (Aust, 1987), or iron release from haemoglobin upon action of H_2O_2 (Gutteridge, 1986), or even iron release from ferritin by $O_2\cdot^-$, organic radicals, or redox cycling chemicals (Aust, 1987). Good evidence suggests that GSH is the intracellular chelator of copper used as a transfer system (Freedman *et al.*1989).

Concerning experimental biology, buffers, such as phosphate and Tris, ligate metals and present metal contamination. Phosphate catalyses ferrous oxidation and is then likely to produce some $O_2\cdot^-$ (Miller *et al.*1990).
Concerning redox cycling quinones which produce H_2O_2, those which are metal-chelators (Weiner, 1994) may be more effective ·OH generators than their non-chelating analogues.

4.4. Antioxidants

An antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1989). Antioxidant compounds can exert their action at different levels of a deleterious oxidative process: by inhibiting the formation of oxygen radicals (e.g. metal chelators), by scavenging oxygen radicals once they are formed (e.g. superoxide dismutase, catalase, glutathione peroxidase, thiols, ascorbate, etc.), or by inhibiting the pathophysiological effects of the oxidized moiety (e.g. vitamin E). In the two last cases, a good antioxidant should have its oxidised form (often a radical itself) poorly reactive and easily restored to its original antioxidant status (Daugherty, 1991).
Molecular oxygen reacts sluggishly with many non-radical molecules due to spin exclusion (Halliwell and Gutteridge, 1989). Catalysts like transition metals (radicals since containing unpaired electrons) are thus needed for such "autoxidation" reactions to take place. Metal chelators belong to a large family of antioxidants. They act by providing a ligand for each of the coordination sites on the metal ion, thus excluding oxygen or by shifting the redox potential of the metal ion so that it is not spontaneously reactive with oxygen. Yet, there is no chelator that binds transition metals in such a way as to render the metals redox inert (Miller *et al.*1990), even reputed good chelators

like DETAPAC and deferrioxamine (Halliwell and Gutteridge, 1984; Borg and Schaich, 1987). Many iron chelates can still react with H_2O_2 in Fenton chemistry (e.g. the EDTA/ferric iron complex is more reactive than ferric salt, but DETAPAC /ferric and ferrous chelates are less reactive than their respective salts) and thereby provide highly soluble redox active forms of iron (the solubility of iron and copper at physiological pH is very low) (Gutteridge, 1990).

5. Protein damage

Outside arylation and oxidation of thiol groups mentioned above, other damage to protein can be observed upon toxic insults.

As seen in § I.2.5.2., quinone attack occurs most readily at the thiol group of cysteinyl residues in protein, being "soft" nucleophiles. Yet sulphur atoms of methionyl residues as well as primary amino groups (arginine and lysine) and secondary amino groups (histidine), being "harder" nucleophiles, are still expected to be attacked by quinones, though at a lesser extent (Coles, 1984).

Exposure of proteins to oxygen radicals *in vitro* leads to modification of amino acids. Histidine, proline, methionine, cysteine and tryptophan are all sensitive to oxidation. Sulphur-containing amino acids are especially sensitive to oxidation, leading to oxidation of cysteine residues to form intra- and inter-molecular disulphide crosslinks in proteins and formation of methionine sulphoxide. Some histidine residues in proteins are even unusually sensitive to oxidation, probably because of their association with metal ions or haem binding sites. As a result of ·OH reactions with amino side groups of protein and phenylalanine there is formation of carbonyl groups (aldehydes and ketones) and hydroxyphenylalanine respectively (Davies, 1987; Davies *et al.*1987a). Protein hydroperoxides have even been identified on free-radical-damaged proteins (Simpson *et al.*1992). Like hydroperoxides, they are relatively stable and can react with transition metals generating protein radicals. Such modifications of amino acids result in gross distortions of secondary and tertiary structure (Davies and Delsignore, 1987). ·OH and possibly intermediates of lipid peroxidation can fragment and crosslink proteins (Wolff and Dean, 1986). H_2O_2, in presence of transition metals, is expected to do the same (Hunt *et al.*1988). Thus, copper ions attached to albumin and

to amino acids can still interact with $O_2^{\cdot -}$ and with H_2O_2 to form ·OH. The reactive species appears to attack the ligand to which the copper is bound and is not released into solution (Gutteridge and Halliwell, 1984).

Crosslinking:

Disulphide bond formation as mentioned above generates intra- and intermolecular crosslinks. Besides those reducible crosslinks, ·OH also induces nonreducible ones such as dityrosine (Halliwell and Gutteridge, 1989).

Random and site-specific fragmentations:

Since ·OH reacts at a diffusion-controlled rate with almost any functional group, the site of the protein damage is expected to be the nearest possible from the site of ·OH formation. As seen in § I.4.1., the most likely mechanism of formation of ·OH is the metal-catalysed Haber-Weiss reaction. Thus the site of ·OH formation depends upon catalytic active metal location. Due to their lack of solubility, iron or copper ions cannot exist free in solution; instead they must bind to molecules.If the molecule is a low molecular weight chelator, a random ·OH attack, dependent on the statistical position of this complex towards the protein, will be observed. However, protein fragmentation by ·OH generates fragments of defined rather than random length. One mechanism could involve a site-specific ·OH attack. Indeed, If the chelating molecule is the protein itself and provided the metal-complex is still Fenton-reactive, ·OH is expected to attack the protein at or near the metal binding site. It is extremely difficult to scavenge ·OH responsible of such a metal-dependent site-specific damage. The success of such a scavenger may depend on the relative metal-binding affinities of the scavenger and the protein (Gutteridge and Halliwell, 1984; Stadtman, 1991). Another mechanism could be that following a random ·OH attack, a rapid electron transfer would direct the damage to a more cleavage-sensitive amino acid residue. For instance, there is evidence that fragmentation occurs by oxidation and subsequent hydrolysis of proline residues (Wolff *et al.*1986).

Oxidatively damaged proteins are rapidly removed by proteases and are then difficult

to detect *in vivo* (Davies and Goldberg, 1987; Davies *et al.*1987b; Davies and Lin, 1988; Marcillat *et al.*1988; Dean *et al.*1993). This is why lens proteins have been a useful model system for studies on protein oxidation because of their negligible turnover with age (Baynes, 1991).

6. Aim

The aim of this study is to obtain further insights into the mechanism of cell injury caused by arylating quinones. More precisely, the interaction between quinones and thiols is considered and the controversial question of recognizing the relative importance of covalent binding and oxidation in the mechanism of cytotoxicity is addressed.

II. MATERIALS AND METHODS

1. Chemicals

N-acetyl-*para*-benzoquinone imine was synthesized as described in § II.8.. *o*-Phthalaldehyde was purchased from Koch-Light Laboratories Ltd. [^{14}C]Formaldehyde (43.6 mCi/mmol, 13.2 mCi/mL), *para*-benzoquinone, monomethyl-*para*-benzoquinone, 2,6-dimethyl-*para*-benzoquinone, tetramethyl-*para*-benzoquinone, 1,4-naphthoquinone, menadione, paracetamol, NADPH, NADH, GSH, GSSG, glutathione reductase, superoxide dismutase, catalase, ammonium ferrous sulphate, xylenol orange, sorbitol, H_2O_2, diethylenetriaminepentaacetic acid, N-ethylmaleimide and other reagents were all obtained from Aldrich, Sigma or BDH.

2. Lens crystallins preparation

The crystallins can be separated from one another by gel chromatography (Harding and Crabbe, 1984). γ-Crystallin was chosen as the main model protein for its monomeric characteristic (Harding and Crabbe, 1984) which renders SDS-PAGE study more convenient. It was prepared from rabbit lens homogenate as in Hunt *et al.* (1992). Homogenisation of decapsulated lenses was carried out in 10 mM potassium phosphate buffer pH 7.0 containing 1 mM DETAPAC, to hinder transition metal-catalysed oxidation during preparation, followed by centrifugation of the resulting crude homogenate at 15,000 g for 15 minutes to yield a clear supernatant. Gel-chromatographic separation of crystallins from this clear supernatant was then performed as described in § II.7.2..

3. Quinone-thiol incubation conditions

Unless otherwise stated, 200 μM thiol was incubated with different concentrations of quinone in 100 mM potassium phosphate buffer pH 7.4, containing 10 μM DETAPAC to avoid metal-catalysed autoxidation of thiols, so that the eventual depletion of thiols would be entirely attributable to quinone effects. Incubations were carried out in small volumes (2 mL) in 10 mL stoppered plastic tubes at 37°C in the dark in a shaking bath ensuring good oxygenation.

4. Liver slices

4.1. Method

Description as well as the pros and cons of the liver slice technique have been well presented (Peters, 1983). Briefly, a drawback is the presence of dying or inactive cells at the centre and on the surface of the slice, the advantages are its simplicity, its rapidity and its maintenance of the organ structure.

An *in vitro* model system developed by McLean *et al.* (1978) was used. This system was elaborated to separate the first phase of exposure to paracetamol, activation of the compound and attachment of the metabolite to cell molecules, from the second phase of cell injury, during which the cell runs down as a consequence of the primary attack. It happens to be a useful tool to assess the progression of events during cell injury and its eventual prevention at a time where the cell seems destined towards irreversible cell damage.

4.2. Protocol

Male albino Wistar rats (OLAC, Bicester, U.K.) weighing 150-200 g were fed stock pellets (SDS, Witham, U.K.) and given a solution containing 1mg of sodium phenobarbitone/mL as the sole source of drinking water to sensitize the liver to paracetamol (McLean and Nuttall, 1978). Phenobarbitone treatment was given for at least 5 days. Vitamin E (5 mg α-tocopherol acetate in two drops of olive oil) was given

by mouth 12-24 hours before rats were killed. Rats were killed by exsanguination under anaesthesia using fentanyl citrate (Janssen, Wantage, U.K.; 0.01 mg/kg, i.m.) and loprazolam (Roussel Ltd, Uxbridge, U.K.; 0.04 mg/kg, i.m.). The liver was rapidly removed and liver slices of 0.3 mm thickness or less were cut by hand on a Stadie-Riggs stage with a long razor blade (A.H. Thomas Co., Philadelphia, PA, U.S.A.). A slice and a lump were kept aside to check the P-450 level of the liver. Slices weighing about 100 mg were put into 25 mL Erlenmeyer flasks (one slice per flask) containing 5 mL of Ringer solution with the following composition: NaCl 125 mM, KCl 6 mM, $MgSO_4$ 1.2 mM, NaH_2PO_4 1 mM, $CaCl_2$ 1 mM, glucose 10 mM, and Hepes 15 mM pH 7.4. Duplicate slices were put into the Ringer solution (with or without other components as indicated) at room temperature and the experiment started by placing the flasks into an incubator bath at 37°C under oxygen with shaking (90 strokes/min). After 2 hours, the slices were taken out of the first flasks and reincubated in 5 mL fresh Ringer solution (with or without other components as indicated) for a further 4 hours as previously described by McLean *et al.* (1978).

At the end of the incubation, slices were transferred into tubes containing 2.9 mL of 5% (w/v) trichloroacetic acid (TCA) with 100 μM DETAPAC and kept on ice to prevent any enzymatic reaction and limit thiol autoxidation. Homogenization of the slices was then rapidly carried out for 10 s using an ultra-turrax homogeniser at full speed to produce a homogenate containing about 30 mg liver/mL. 100 μL of homogenate was kept on ice until assessed for protein content; the remaining part of the homogenate was spun down at 830*g* for 10 minutes. Supernatants were kept on ice until used for measurement of GSH and potassium content; pellets were kept under the same conditions while awaiting measurement of protein-thiol content .

5. Biochemical Analyses

5.1. Measurement of hydrogen peroxide

5.1.1. Method

The concentration of hydrogen peroxide was measured using xylenol orange to detect

H_2O_2-generated Fe^{3+}, according to the method of Michaels and Hunt (1978) as modified by Jiang *et al.* (Jiang *et al.*1990; Wolff, 1994). Ferrous ion (Fe^{2+}), relatively stable to autoxidation in dilute H_2SO_4, can be oxidised by H_2O_2 to yield ferric ion (Fe^{3+}). Xylenol orange binds the latter forming a complex with a maximum absorbance at 560 nm, the colour yield being highly enhanced by sorbitol.

5.1.2. In chemically defined studies

Briefly, 50 μL samples were added to 950 μL xylenol orange reagent (consisting of 250 μM ammonium ferrous sulphate, 100 μM xylenol orange, and 100 mM sorbitol in 25 mM H_2SO_4) and incubated for 30 minutes at room temperature. Absorbance at 560 nm of the Fe^{3+}-xylenol orange complex was calibrated against standard concentrations of H_2O_2. The standard H_2O_2 solution was calibrated using ϵ = 39.4 $M^{-1}cm^{-1}$ at 240 nm (Nelson and Kiesow, 1972). Under the conditions of quinone-thiol incubation used, the measurement of H_2O_2 formed, using the xylenol orange assay, compared closely with those of O_2 uptake as measured using an oxygen electrode.

5.1.3. In liver slices

5.1.3.1. Principle

Since H_2O_2 does not accumulate within slices to an extent to permit direct isolation and characterisation, generation of H_2O_2 in liver slices was detected by measuring irreversible inhibition of endogenous catalase catalytic activity by 3-amino-1,2,4-triazole (AMT) (Cohen and Hochstein, 1964). Briefly, catalase catalytic activity ($2H_2O_2 \rightarrow 2H_2O + O_2$) results from the formation of an active catalase-H_2O_2 complex I and proceeds by the reduction of complex I by a second molecule of H_2O_2 (Aebi, 1984). To irreversibly inhibit this activity, AMT must react with complex I thus requiring the presence of H_2O_2 (Margoliash and Novogrodsky, 1958). This mechanism serves as a detection system for H_2O_2: when irreversible inhibition of catalase by AMT is observed, it may be inferred that H_2O_2 had been present in the test system. Inhibition of catalase was measured by a discontinuous method using the xylenol orange assay described in § II.5.1.2., as in Ou and Wolff (1993).

5.1.3.2. Measurement

Slices were prepared as described in § II.4.2., put into the Ringer solution (other components were added or not as indicated) at room temperature and the experiment started by placing the flasks into an incubator bath at 37°C under 95% oxygen with shaking (90 strokes/min). After 2 hours, the slices were taken out of the first flask and reincubated in 5 mL fresh Ringer solution (other components were added or not as indicated) for a further 2 hours. When catalase activity was measured at 2 hr, 50 mM AMT was added in the first incubation medium; when it was measured at 4 hr, 50 mM AMT was added in the second incubation medium only. At the end of the incubation, slices were washed in phosphate-buffer saline (PBS) to remove any traces of AMT prior to transfer into tubes containing 2 mL ice-cold PBS with 100 μM DETAPAC and homogenized for 10 s using an ultra-turrax homogeniser at full speed. Homogenates were then diluted to 200 μg liver (wet weight)/mL with PBS containing 150 μM H_2O_2, mixed and incubated for 10 minutes at room temperature. Catalase activity was determined by reference to the concentration of H_2O_2 remaining at the end of the incubation period. 50 μL aliquot of H_2O_2-exposed homogenate was added to 950 μL of the xylenol orange reagent (which also halted H_2O_2 consumption by catalase) and the assay carried out as described in § II.5.1.2.. Vials were centrifuged (12,000 g $\times$ 3 min) to remove flocculated material before reading the supernatant at 560 nm. Catalase activity was calculated in terms of H_2O_2 consumed per minute. Under the conditions used, all incubations contained residual H_2O_2 and H_2O_2 consumption was approximately linear over the 10 minutes observed.

5.2. Measurement of GSH

5.2.1. Method

The concentration of GSH was measured using o-phthalaldehyde (OPT) (Cohn and Lyle, 1966; Hissin and Hilf, 1976). GSH was shown by these authors to react specifically with OPT at pH 8.0, by reference to the reaction of OPT with other low molecular weight thiols and non-thiol compounds. This was confirmed in our hands, with cysteine and NAC (data not shown). However, as with other non-enzymatic assay

systems, a high specificity is not guaranteed for biological samples. Indeed, some other biological thiols have been reported to fluoresce when reacting with OPT (Jocelyn and Kamminga A., 1970; Brigelius *et al.*1983). Yet GSH being the most abundant non-protein thiol in the liver, the OPT method is here a useful analytical tool for GSH.

5.2.2. In chemically defined studies

10 μL sample aliquots were added to 1.90 mL 100 mM sodium phosphate buffer pH 8.0 containing 100 μM DETAPAC, to avoid metal-catalysed autoxidation. 100 μL of o-phthalaldehyde (1 mg/ mL in methanol) were then added and rapidly mixed. Fluorescence was measured (Ex, 340 nm; Em, 420 nm) after incubation for 30 minutes at room temperature in the dark, with reference to a GSH standard.

5.2.3. In liver slices

Samples were kept on ice as much as possible to hinder metal-catalysed autoxidation of thiols until reaction with OPT. Aliquots of 5% TCA slice-homogenate supernatants (see § II.4.2.) were diluted 30 times in 100 mM sodium phosphate pH 8.0 containing 100 μM DETAPAC (to avoid metal-catalysed autoxidation). 100 μL of those diluted aliquots were added to 1.8 mL 100 mM sodium phosphate buffer pH 8.0 containing 100 μM DETAPAC. 100 μL of o-phthalaldehyde (1 mg/ mL in methanol) were then added. Fluorescence was measured (Ex, 340 nm; Em, 420 nm) after incubation for 30 minutes at room temperature in the dark, with reference to a GSH internal standard.
When combined studies of catalase activity (§ II.5.1.3.) and GSH content were carried out, 1 mL of slice homogenate in PBS/DETAPAC (see § II.5.1.3.2.) was vortex-mixed with 110 μL 50% TCA containing 100 μM DETAPAC, centrifuged at 830 g for 10 minutes and the supernatant assessed for its GSH content as described above.

5.3. Measurement of GSSG

The concentration of GSSG in quinone-thiol incubation mixtures was monitored by measuring the oxidation of NADPH by glutathione reductase at 340 nm (Akerboom and Sies, 1981),

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$.

Briefly, 5 μL of 40 mM NADPH were added to 1 mL quinone-thiol incubation samples, comprising 100 mM potassium phosphate buffer pH 7.4, 10 μM DETAPAC and various concentrations of GSH, GSSG and quinones. After 2 minutes of stabilisation at 37°C in the spectrophotometer, 5 μL of 200 U/mL glutathione reductase was added with rapid stirring. The rapid decrease in absorbance at 340 nm was calibrated by reference to a GSSG standard. Quinones may confer an optical density at 340 nm, the spectrophotometer should then be zeroed prior to the addition of NADPH. Quinones may also directly oxidize NADPH, however at a clearly lesser rate than glutathione reductase; only the decrease in absorbance due to the fast enzymic reaction should then be considered. A good recovery of a known amount of added GSSG was tested after each measurement.

5.4. Measurement of protein thiols

The concentration of protein thiols was measured using DTNB (5'5'-dithiobis-(2-nitrobenzoic acid), Ellman's reagent) as by Di Monte *et al.* (1984b), with some modifications.

5.4.1. In biochemical studies

500 μL sample aliquots were added to 500 μL 7 M guanidine hydrochloride containing 100 μM DETAPAC and left for 30 minutes at room temperature (to permit complete protein denaturation) prior to the addition of 2 mL of 0.5 M Tris-HCL pH 7.5, containing 100 μM DTNB. Absorbance was measured at 412 nm after 20 minutes' incubation at room temperature and calibrated by reference to a GSH standard curve. Because quinone adducts to protein could contribute to the optical density monitored at 412 nm, a subsequent measurement was carried out after preciptation and removal of proteins and attached quinones (the chromophoric product resulting from reaction of DTNB with thiols is the acid-soluble 2-nitro-5-thiobenzoic acid (Tietze, 1969)). 110 μL 50% trichloracetic acid was added to 1 mL of the measured samples, the mixtures vortex-mixed and allowed to stand 5' on ice prior to be centrifuged (12,000 g $\times$ 5 min). 0.9 mL of the supernatants were collected and the yellow color of 2-nitro-5-

thiobenzoic acid recovered (it disappeared in acidic conditions) by addition of 70 μL 4 M NaOH. GSH standard was subjected to the same treatment and absorbance was measured at 412 nm. In those conditions, good recovery of the optical density was obtained.

5.4.2. In liver slices

TCA-precipitated slice-homogenate pellets (see § II.4.2.) were dissolved in 1 mL 6 M guanidine hydrochloride containing 100 μM DETAPAC and left for 30 minutes at room temperature (to permit complete protein denaturation) prior to the addition of 2 mL of 0.5 M Tris-HCL pH 7.5, containing 100 μM DTNB. Absorbance was measured at 412 nm after 20 minutes' incubation at room temperature and calibrated by reference to a GSH standard curve.

5.5. Measurement of cytochrome P-450

Cytochrome P-450 activity was estimated as in McLean and Day (1974). In the following procedure, the liver homogenates were kept on ice at all times. Briefly, a 2% (wt/vol) homogenates of freshly cut slice and lump were made in KCl /Tris pH 7.0 buffer (0.15 M/25 mM) using an ultra-turrax homogenizer at full speed for 10 s. These homogenates were diluted in 0.5 M potassium phosphate buffer pH 7.5 to give a final concentration of 1% (wt/vol) and then bubbled gently for 20 s with carbon monoxide using a pasteur pipette. Each gassed homogenate was equally divided between two glass cuvettes and the cuvettes scanned between 390-500 nm using a double-beam Pye Unicam SP1750 spectrophotometer. The contents of the tested cuvette were then reduced by adding a few crystals (approximately 1 mg) of sodium dithionite and mixing whilst the control cuvette was oxygenated by a few bubbles of air from a pasteur pipette. After 1 minute, the cuvettes are scanned between 390-500 nm as previously (a second scan was carried out 1 minute later to ensure that the maximum absorbance was observed). The P-450 content was calculated of absorbance from the peak at 450 nm to the trough between 450-500 nm, using the millimolar extinction coefficient of 91.

Satisfactory measurement of cytochrome P-450 can be made using homogenates with optical density *vs* water at 450 nm lesser than 1.0 and with a levelled baseline

(homogenate *vs* homogenate) (McLean and Day, 1974).

Typically, P-450 values were found to range between 60-100 nmol/g liver for phenobarbitone-pretreated rats (compared to 30-50 nmol/g liver without phenobarbitone treatment).

5.6. Measurement of protein concentration

5.6.1. Crystallins

Protein concentration was estimated by absorbance at 260 nm and 280 nm (Dawson *et al.*1986), using the formula:

Protein concentration (mg/mL) = A_{280} × Factor

where Factor is determined from the ratio A_{280} /A_{260}.

Factor was found to be ~1; protein was then estimated by monitoring absorbance at 280 nm assuming ϵ_{280} = 1 mg/mL as in Hunt *et al.* (1992).

5.6.2. Liver slices

Protein content of liver slices was measured using the Lowry-Folin method (Lowry *et al.*1951). Briefly, 100 μL of 5% TCA slice homogenate (see § II.4.2.) was spun down at 830 g for 10 minutes, the supernatant discarded and the pellet allowed to dissolve in 130 μL 1 M NaOH for 30 minutes at 37°C. 10 μL aliquot of the dissolved pellets was added to 0.5 mL 0.5 M NaOH containing 75 mM NaCl followed by the addition of 2.5 mL solution C (see below). Reaction mixtures were well mixed and allowed to stand for 10 minutes prior to addition of 250 μL solution D (see below) and immediately mixed. Absorbance was measured at 740 nm after 30 minutes' incubation at room temperature and calibrated by reference to a bovine serum albumin standard curve.

Solution A: 2% sodium carbonate in distilled water

Solution B: 0.5% cupric sulphate pentahydrate and 1% potassium sodium (+)-tartrate in distilled water

Solution C: 100 (solution A)/ 2 (solution B) (volume/volume)

Solution D: 2.8× dilution in distilled water of Folin & Ciocalteu'phenol reagent

5.7. Measurement of lactate dehydrogenase (LDH) activity

Leakage of lactate dehydrogenase from the slice into the medium was used as a severe irreversible damage indicator of cell injury (Klaassen and Stacey, 1982).

LDH activity in the medium or in homogenates of liver slices was monitored by measuring the oxidation of NADH by LDH, in the presence of pyruvate, at 340 nm as in Amador *et al.*(1963) and Mourelle *et al.*(1990);

Pyruvate + NADH + H^+ → L-Lactate + NAD^+.

Briefly, 20 μL of 11 mM NADH (freshly made) was added to 0.93 mL of 50 mM sodium phosphate buffer pH 7.5 containing 785 μM pyruvate. After 2 minutes of stabilisation at 30°C in the spectrophotometer, 50 μL of medium or of adequately diluted homogenate was added with rapid stirring. Activity was calculated from the slope of the decrease in absorbance at 340 nm recorded over 3 minutes, using ϵ_{340} = 6.22×10^3 $M^{-1}cm^{-1}$ for NADH. LDH released into the medium was expressed as a percentage of the amount of enzyme activity originally added to the flask, based on LDH assays on homogenates of liver slices sampled before incubation.

$$Leakage\ \% = \frac{LDH\ activity\ within\ the\ 5\ mL\ medium \times 100}{LDH\ activity/mg\ of\ liver\ slice \times wt\ of\ slices\ used\ for\ the\ incubation}$$

5.8. Quantification of fragmentation

5.8.1. Radiomethylation of protein

Protein was labelled with [^{14}C]formaldehyde by reductive methylation using sodium cyanoborohydride as described by Jentoft and Dearborn (1979). In this method the only groups labelled are ϵ-amino groups of lysyl residues and the α-amino terminal. The overall reaction sequence is summarized below:

$RNH_2 + CH_2{=}O \rightleftarrows RNH\text{-}CH_2OH \rightleftarrows RN{=}CH_2$

$RN{=}CH_2 + NaCNBH_3 \rightarrow RNH\text{-}CH_3$.

$NaCNBH_3$, at neutral pH, readily reduces Schiff bases (the last step of the above reaction

sequence) but does not reduce aldehydes or ketones. Neither reduction of disulphides by $NaCNBH_3$ nor cross-linking by formaldehyde seem to occur .

5 mg/mL Bovine serum albumin, BSA, (freeze-dried fraction V fatty acid free, Boehringer Mannheim) was incubated with 1 mM DTT, 1mM $NaCNBH_3$ and 24 μM ^{14}C-enriched CH_2OH [taken from a working solution of 14 mM ^{14}C-enriched CH_2OH, 1.16×10^8 dpm/mL, adjusted with cold formaldehyde from an original lot of 43.6 mCi/mmol, 13.2 mCi/mL solution (Sigma)] in 50 mM potassium phosphate buffer pH 7.5 containing 10 μM DETAPAC for 2 hrs 30 min at 37°C in a shaking bath and then dialysed extensively for 14 hours at 4°C against the same buffer.
With this procedure, not more than 2% of reactive amino groups were expected to be methylated. Actual radioactivity of the labelled-BSA solution, after dialysis, was about 30,000 dpm/mL.

5.8.2. Measurement of fragmentation

Fragmentation of proteins was determined by preparing a 5% TCA pellet from the treated protein and measuring the ^{14}C content as in Hunt *et al.*(1988).
0.5 mL labelled-BSA (5 mg/mL, 30,000 dpm/mL) in 50 mM potassium phosphate buffer pH 7.5 containing 10 μM DETAPAC was incubated with different concentrations of BQ in a shaking bath at 37°C in the dark. After 3 hours incubation, 55 μL 50% TCA was added, reaction mixtures were vortex-mixed and allowed to stand 15 minutes on ice prior to centrifugation (12,000 g $\times$ 5 min). Supernatants were discarded and pellets dissolved in 50 μL 1 M NaOH. The dissolved pellets were then diluted 10 times in distilled water and duplicate aliquots (200 μL) were mixed in 5 mL Liquiscint scintillation fluid (National Diagnostics USA) to be counted using a Packard Tri-Carb 460C liquid scintillation system (Packard Instruments Co. Pangbourne, U.K.). A blank containing scintillation fluid and 200 μL 0.1 M NaOH was counted first so that the background value was automatically subtracted from the sample counts. The counts per minute were automatically converted to disintegrations per minute using the quench curve parameter (QIP) for each sample and a standard quench curve, obtained by plotting the efficiency of counting against QIP value for the isotope under the conditions of counting used.

Fragmentation was calculated as the difference of the insoluble pellet before and after the reaction and expressed as a percentage of the insoluble pellet at the beginning of the reaction.

6. SDS-PAGE

The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system developed by Laemmli (1970) was used, prepared as described by Garfin (1990). All the SDS-PAGE presented here were performed on 12.5% crosslinked gel and revealed either by Coomassie Brilliant Blue R-250 or silver staining (Garfin, 1990) as mentioned in legends.

At the end of incubations, a portion of reaction mixtures were included in sample buffer containing 2% (wt/vol) SDS, 0.1 M DTT (for gels run under reducing condition) and 20 mM EDTA and denatured by heating at 100°C for 3 minutes. To optimize the detection of any fragments formed during exposure of protein to quinones, lanes were heavily loaded (10 μg protein/lane).

7. Analytical studies

7.1. Measurement of oxygen consumption

O_2 was measured with a Clark (type YSI model 53) biological oxygen monitor. The sample chamber, kept under constant stirring and at temperature of 37°C, was filled with 10 mL of 100 mM potassium phosphate buffer pH 7.4 containing or not (as indicated) 200 μM GSH and 10 μM DETAPAC. Small volumes (generally 100 μL) of concentrated solutions of biochemicals under study were injected into the sample chamber with a syringe, after thermal equilibration. Starting O_2 concentration was assumed to be 200 μM (Dawson *et al.*1986).

7.2. Gel filtration chromatography

Gel-chromatographic separation of crystallins was performed using an XK-column (Pharmacia) (i.d., 26 mm; length, 100 cm) packed with Sephacryl S200HP (Pharmacia) and a mobile phase of 50 mM potassium phosphate/100 mM potassium chloride pH 7.0 (containing 100 μM DETAPAC). Flow rate was maintained at 1.5 ml/min by a Micropuls 3 Gilson pump and fractions were collected by a Gilson FC203 fraction collector. Protein content of fractions was estimated by absorbance at 280 nm.
An elution profile of rabbit lens crystallins is shown in Figure II.1a.. With this technique, α-,β- and γ-crystallins were resolved (Figures II.1a. and II.1b.).
(The assistance of Dr. J. J. Harding in identifying the different crystallin fractions is gratefully acknowledged)

7.3. High Performance Liquid Chromatography

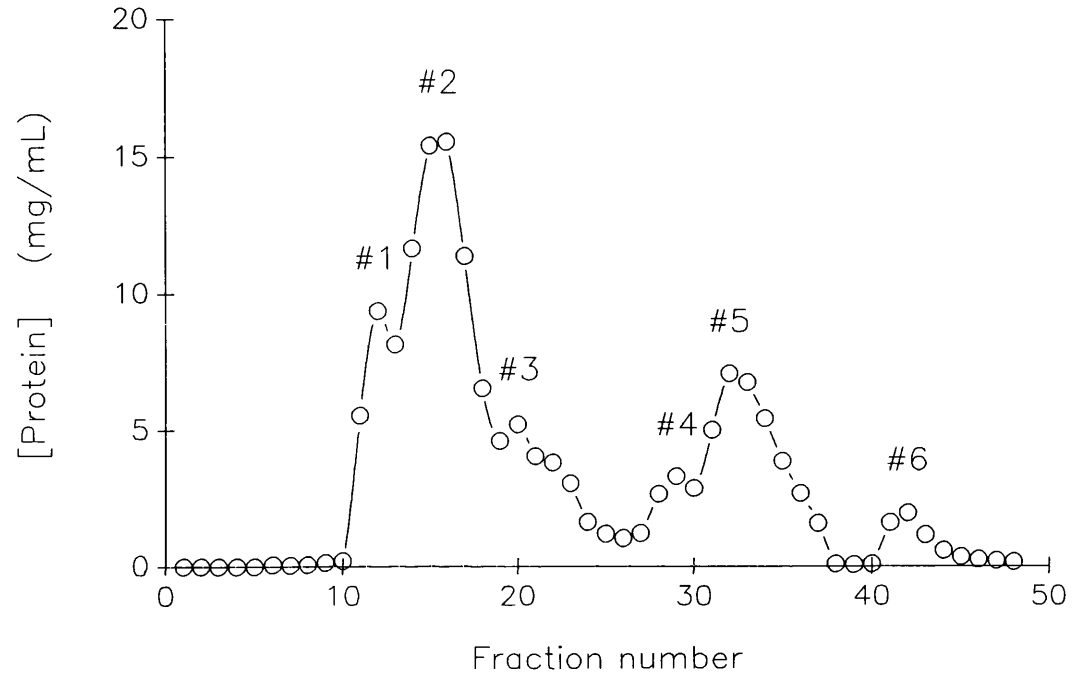
7.3.1. Semipreparative

A 1 mL loop was connected to a μBondapak™ C-18 reverse phase semipreparative column and to a Waters Associates pump. Volumes of 50-200 μL were injected onto the column. Sample were eluted with a linear gradient of acetonitrile/water/trifluoroacetic acid (0/99.9/0.1 to 30/69.9/0.1) at a flow rate of 2 mL/min and monitored with a double detector at 280 nm and 254 nm.

7.3.2. Analytical

20 μL aliquots of the reaction mixture were injected onto a Chrompack spherisorb ODS reverse-phase column connected to a Gilson piston pump model 305. Samples were eluted with an isocratic system of methanol/water/acetic acid (10/89/1) at a flow rate of 0.8 mL/min and monitored at 280 nm.
The mobile phase used in the semipreparative conditions was not found satisfactory in this system and *vice versa*.

Figure II.1a.: Fractionation of rabbit lens crystallins by gel filtration chromatography and thiol content of the resolved fractions

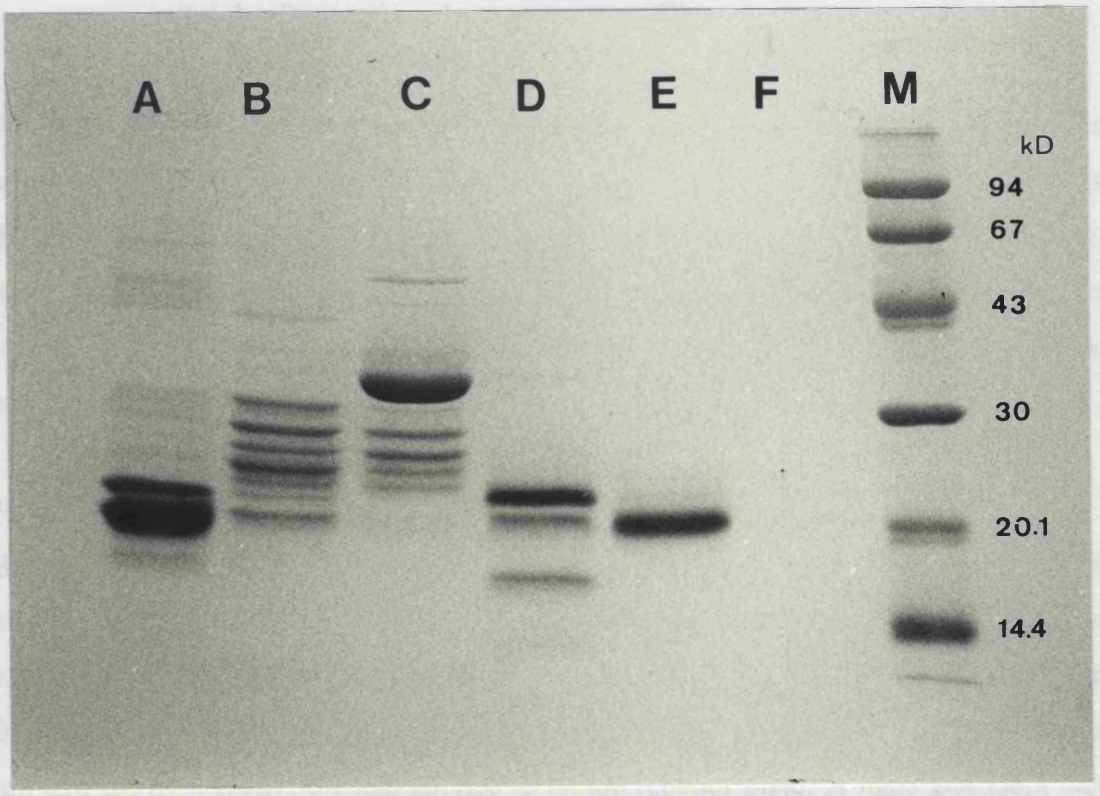


Thiols (nmol/mg protein)					
#1	#2	#3	#4	#5	#6
54.6 ± 0	79.7 ± 0.4	197 ± 2	190.2 ± 0.7	247 ± 3	51 ± 1

The separation was performed using a mobile phase of 50 mM potassium phosphate/100 mM potassium chloride pH 7.0 containing 100 μM DETAPAC over Sephacryl S200HP. Protein content of fractions was estimated by monitoring absorbance at 280 nm assuming ϵ_{280} = 1 mg/mL.

The attached table presents the thiol content of the separated fractions, measured using the Ellman's reagent on denaturated proteins. Data shown are the means ± half the range of duplicate measurements.

Figure II.1b.: SDS-PAGE of fractionated rabbit lens crystallins



SDS PAGE was performed on 12.5% crosslinked gel under reducing conditions and revealed by Coomassie blue. Crystallin fractions are those obtained from the gel chromatography of which the chromatogram is shown Figure II.1a..

Lane A, #1, α-crystallin; Lane B, #2, β-crystallin (+ α-crystallin); Lane C, #3, β-crystallin; Lane D, #4, γ_S-crystallin + some γ-crystallin + fragment; Lane E, #5, γ-crystallin; Lane F, #6, not determined; Lane M, molecular weight standards.

7.4. Mass spectrometry

The assistance of Dr. G.W. Taylor for the mass spectrometry analysis is gratefully acknowledged.

7.4.1. Desorption electron impact (EI)

A Finnigan 4500 was fitted with a Finnigan 'direct exposure probe' utilising a temperature-programmed loop of rhenium wire inserted close to the electron beam from the ion source filament. Samples were desorbed from the loop by applying a current, which was raised from zero to 1.35 A at 10 mA s^{-1}. EI mass spectra (70 eV) were recorded using a mass range of *m/z* 70-500 scanned every 1 s, data being acquired and processed by an Incos series 2000 data system.

Principle:

Briefly, sample vapour at a reduced pressure flows through a region traversed by a 70 eV electron beam. Gas molecules interact with the electrons. Some of these molecules lose an electron to become positively ionised and often subsequently undergo fragmentation. Radical cations formed in the ionising volume are extracted through an ion exit slit by a small potential applied to an ion repeller plate. A mass analysers then separates ions according to their mass-to-charge ratio.

7.4.2. Fast atom bombardment (FAB)

Fast atom bombardment mass spectrometry was carried out using a Finnigan 4500 instrument fitted with an M-Scan FAB gun. Xenon (10 kV) was used as the primary ionising source. Samples were loaded onto the glycerol matrix for analysis. FAB spectra were recorded and processed as for EI.

Principle:

The application of EI ionisation technique is limited by two factors: thermal decomposition of involatile or thermally unstable materials and sometimes extensive fragmentation leading to undetectable molecular ions. A number of new techniques have

then been developed, among them FAB spectrometry.

Briefly, a beam of fast moving ions (xenon), directed to strike the surface of the glycerol matrix (containing the dissolved sample), produces an intense thermal spike whose energy is then dissipated through the matrix and the outer layer of the sample lattice. Molecules are detached from these surface layers as a dense gas containing positive and negative ions. A mass analyser then separates ions according to their mass-to-charge ratio.

The FAB method produces intact molecular ions from very large molecules. Its spectra contain even-electron ions such as $(M + H^+)$ and $(M - H)^-$ rather than $M^{\cdot +}$ or $M^{\cdot -}$. In general terms, the FAB method is better suited to compounds that have relatively acidic or basic centres. Additives such as acids and bases can promote the formation of ions and will result in correspondingly more intense spectra. Addition of a salts such as sodium or potassium can promote cationisation of a compound and is also used to confirm the identification of a protonated molecular ion.

7.5. Spectrophotometry

A single-beam Pye Unicam Series 8700 Spectrophotometer was used for all measurements except for P-450 measurement where a double-beam Pye Unicam SP1750 spectrophotometer was used instead.

7.6. Fluorimetry

A Perkin Elmer LS~5 fluorescence spectrometer was used.

7.7. Measurement of potassium

Loss of potassium from the slices was used to assess injury, indicating more subtle changes than LDH leakage. Potassium changes are reversible (Klaassen and Stacey, 1982).

Potassium content of the slices was measured using a Pye Unicam atomic absorption spectrophotometer SP9 with acetylene gas as fuel (McLean and McLean, 1966; McLean and Nuttall, 1978).

200 μL of the supernatant from the 5% TCA slice homogenates (see § II.4.2.) was added to an acid washed glass vial containing 4.8 mL of 1 M HCl. Absorbance was measured at 766.5 nm using the appropriate hollow cathode lamp and calibrated by reference to a potassium standard curve. The standard potassium solutions were prepared with the same concentrations of TCA and DETAPAC as for the samples.

8. Synthesis and identification of NAPQI

8.1. Synthesis

NAPQI was synthesised in chloroform solution by the oxidation of paracetamol using freshly prepared silver oxide (Ag_2O) (Hugget and Blair, 1983).

Silver oxide preparation:

Silver oxide was freshly prepared by the slow addition, under active stirring, of 100 mL 2.5 M NaOH to 10 mL 1.7 M silver nitrate ($AgNO_3$):

$2AgNO_3 + 2NaOH \rightarrow Ag_2O + 2NaNO_3 + H_2O$.

After stirring for 10 minutes at room temperature in the dark, the mixture was filtered through a glass sinter filter to produce a brown residue of silver oxide. This was washed with distilled water (50 mL), acetone (50 mL) and diethyl ether (50 mL) and left to dry overnight under vacuum at room temperature (Fieser and Fieser 1967).

NAPQI synthesis:

NAPQI is probably very sensitive to air and water, the following procedures were therefore carried out in glasswares pretreated with water repellent (2% dimethyldichlorosilane in 1,1,1-trichloroethane, BDH) and in an atmosphere composed principally of argon (i.e. in flasks or vials, previously filled with argon), for incubations and storage, or within container filled with argon for filtrations and other transfer operations. Freshly prepared Ag_2O (5.2 mmoles) was rapidly added to a conical flask containing paracetamol (0.2 mmoles) and chloroform (10 mL) under constant stirring. The flask was immediately restoppered and the contents were stirred at room temperature in the dark for 40 minutes. The reaction mixture was then filtered through a filter paper

(Watman, grade 1) to retain bulk of insoluble paracetamol and the filtrate was reincubated in the same conditions in a new flask containing 0.35 mmoles Ag_2O for a further 10 minutes to oxidise the remaining soluble paracetamol. This final reaction mixture was filtered as mentioned above to remove silver oxide, aliquoted into small glass vials so that minimum free volume was left and stored, protected from the light, at -20°C. With this method, half life is greater than 6 months (Hugget and Blair, 1983). Typically, NAPQI was used within 2 months following its synthesis.

8.2. Identification

The crude chloroform solution, a clear yellow solution, was analyzed by desorption electron-impact mass spectroscopy and NAPQI identified (Figure II.2.). A good yield of NAPQI was revealed by monitoring the ion currents at m/z 149 (NAPQI) and 151 (paracetamol); less than 5% of paracetamol was present.

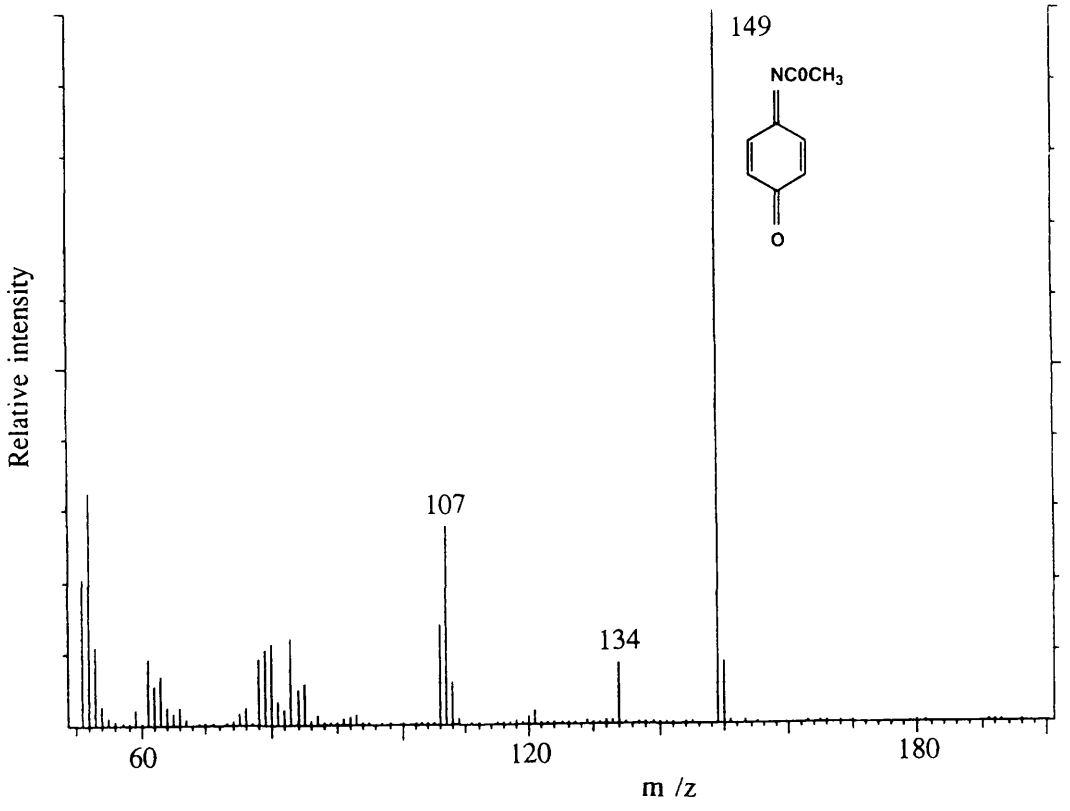
UV max was found to be 263 nm in n-hexane. NAPQI content of the solution was estimated by measuring the absorbance of a 220 times diluted solution in n-hexane at 263 nm (ϵ_{263} = 3.3×10^4 $M^{-1}cm^{-1}$ in n-hexane (Merck Index)) assuming that contaminating paracetamol or other unidentified compounds only negligably participate in the optical density of the solution (paracetamol, λ_{max} = 250 nm, ϵ_{250} = 1.38×10^4 $M^{-1}cm^{-1}$ (Merck Index); BQ, λ_{max} = 248 nm, ϵ_{248} = 18.2 $mM^{-1}cm^{-1}$ (Brunmark and Cadenas, 1988)).

(The assistance of Dr. G.W. Taylor in the identification of NAPQI is gratefully acknowledged)

9. Presentation of data and statistics

Data are presented either as mean ± half the range from duplicate assays or as mean ± standard deviation from quadruplicate assays, as indicated in legends, and are representative of at least two experiments (bars which are not visible on graphs do not exceed the size of the symbol). Comparison of the means of two samples (test of the null hypothesis that the mean is the same for the two samples and the significance of the observed difference) were performed using the Student's t-test. In case of multiple

Figure II.2.: Desorption electron-impact mass spectrum of freshly synthesised NAPQI



Desorption electron-impact mass spectrum of the chloroform solution of freshly synthesised NAPQI.
m/z 149, NAPQI ($M^{\cdot+}$); *m/z* 134, NAPQI - methyl ($M^{\cdot+}$ - $\cdot CH_3$); *m/z* 107, NAPQI - ketene ($M^{\cdot+}$ - CH_2:CO).

analyses, analysis of variance was used followed by the Tukey-Kramer multiple comparisons test. Tests were accomplished using Statgraphics (STSC, Inc.) software or SAS statistical package (SAS Institute, Inc.).

III. RESULTS

The interaction between arylating quinones and thiols was investigated using two model systems: a non-living system where GSH or a thiol-rich protein were directly exposed to quinones and a system where liver slices from phenobarbitone-treated rat were exposed to paracetamol. The fate of the thiol and the quinone were studied and the products of the reaction analysed. In particular H_2O_2 formation was followed as an indicator of O_2 reduction, because of its relative stability in aqueous solution. The two systems were then compared.

1. Chemically defined study with GSH

1.1. GSH exposure to BQ

1.1.1. Conjugation rather than oxidation

Disappearance of SH groups:

In Figure III.1., 0, 100, 150 or 200 μM *p*-benzoquinone (BQ) were incubated for 3 hours with a fixed (200 μM) concentration of GSH in the presence of DETAPAC. The thiol status was followed throughout the incubation time. In the absence of BQ (Figure III.1.a) the level of GSH declined only very slowly. Conversely, there was a rapid loss of titratable thiol groups following the addition of BQ. The initial phase of the reaction appears to involve a 1:1 stoichiometric reaction between GSH and BQ since the addition of 100, 150 or 200 μM BQ to 200 μM GSH led to the rapid loss of approximately and respectively 100, 150 and 200 μM GSH.

This stoichiometric reaction suggests the arylation of GSH rather than simple oxidation of GSSG.

In a second phase of the reaction, clearly shown in Figure III.1.c, the rapid drop in

GSH level which succeeded the addition of 150 μM BQ was followed by a more gradual consumption of GSH.

This slower disappearance of GSH is likely to be due to the formation of polysubstituted hydroquinones as indicated below.

Identification of products:

The loss of GSH was not associated with GSH oxidation to GSSG since only 5 μM GSSG were detected at the end of the incubation of 200 μM GSH with 200 μM BQ in the same conditions as above (Figure III.15). It was rather associated with the electrophilic addition of BQ to GSH as shown by the presence of the monoglutathion-S-yl hydroquinone conjugate (HQ-SG) detected by HPLC-FAB mass spectrometry in a similar equimolar reaction (Figure III.2.a&b). Furthermore, when 200 μM BQ was added to 300 μM GSH, an immediate and complete disappearance of the BQ peak was observed from analytical-HPLC elution profiles of the reaction (Figure III.4.; see BQ alone and zero time with GSH).

Two different diglutathion-S-yl hydroquinone conjugates were also detected in the equimolar reaction mixture by HPLC-FAB mass spectrometry (Figure III.2.a&b).

These two diglutathionyl conjugates were identified at *m/z* 720. The protonated molecular ion would have been expected at *m/z* 721 since the molecular weight of diglutathionyl hydroquinone is 720 and FAB mass spectrometry usually generates protonated molecular ion species ($M + H^+$). This shift to an unprotonated molecular ion ($M^{\cdot +}$ *m/z* 720) can be related to the published work on the complex thermospray mass spectrometric behaviour of the similar compound pyocyanin (Watson *et al.*1986). This chemical, also a redox compound, had its molecular ion species shifted to ($M + H_2^+$) in presence of trifluoroacetic acid, also present in the HPLC mobile phase used in our study. The authors suspected an interaction of this zwitterionic species, analyzed at the oxidised state, with itself or counter ions. Identification of the mono- and the two di-glutahionyl conjugates were confirmed by nuclear magnetic resonance (NMR) analysis carried out by the Imperial College (London).

Presence of triglutathion-S-yl hydroquinone and tetraglutathion-S-yl hydroquinone, not detected here due to the limitation of the Finnigan 4500 instrument used (mass range does not exceed *m/z* 1000), has been identified by others (Lau *et al.*1988).

1.1.2. HQ-SG oxidation, O_2 consumption, $O_2^{\cdot -}$ and H_2O_2 formation

H_2O_2 formation:

The Figure III.1., already referred to above, presents the thiol status and H_2O_2 production during the incubation of 0, 100, 150 or 200 μM BQ with a fixed (200 μM) concentration of GSH in the presence of DETAPAC. Regarding H_2O_2, its formation was only minimal in the absence of BQ or at 100 μM BQ (Figure III.1.a&b). However, when greater concentrationsof BQ were reacted with GSH, a sustained formation of H_2O_2 was revealed (Figure III.1.c&d).

To further examine the formation of reduced oxygen species in this model system, oxygen consumption was monitored during incubation of GSH and BQ (Figure III.5.). Oxygen uptake was immediate upon the addition of BQ to an equimolar amount of GSH, as was also observed for H_2O_2 formation under these circumstances (Figure III.1.d). Supplementation by superoxide dismutase (SOD), 5 minutes after the addition of BQ, greatly accelerated the consumption of O_2 (Figure III.5.a) whereas supplementation by catalase, at the same time, returned some O_2 to the medium and decreased total O_2 uptake by 50 percent (Figure III.5.b).

The catalytic effect of SOD suggests that $O_2^{\cdot -}$ is an intermediate in the oxidation of HQ-SG; on the other hand, the generation of O_2 upon catalase exposure confirms the early formation of H_2O_2 (catalase activity: $2H_2O_2 \rightarrow 2H_2O + O_2$) observed in Figure III.1.d.

Oxidation of HQ-SG:

The presence of H_2O_2 and of diglutathionyl-hydroquinone conjugates (§ III.1.1.1.) suggests that the monoglutathionyl-hydroquinone conjugate, first formed in the reaction, is oxidisable. The mechanism of its oxidation was examined.

The HPLC elution profile of the product of the reaction, at zero time, between 200 μM BQ and 100, 200 or 300 μM GSH (Figure III.3.) revealed that when free BQ remained in excess over HQ-SG (incubation of 200 μM BQ with 100 μM GSH), a significant amount of HQ was formed probably resulting from the oxidation of HQ-SG by BQ. A small amount of HQ was also visible when the reaction occurred with [GSH] $\geq$ [BQ]. However, in the latter case, the presence of HQ was probably due to impurities in the BQ solution, as is shown by the elution profile of BQ alone (Figure III.4.). A large

excess of GSH (800 μM) over BQ (200 μM) also seemed to generate substantial amount of reduced quinone, as indicated Figure III.28. (elution profile at 30 min).

Figure III.4. displays the HPLC elution profile of the product of the reaction of 200 μM BQ with 300 μM GSH at different time points of their incubation. It is not possible to draw a parallel between the semipreparative (Figure III.2.a) and the analytical (Figure III.4.) HPLC elution profiles (mobile phases and columns were different in the two procedures) and thus not possible to assert which peak represents HQ-SG in Figure III.4.. However, it is possible to infer that the peak with retention time of 2:36 (min:s) is likely to be HQ-SG. Indeed, it was the first main new peak appearing upon BQ exposure to GSH and its height decreased progressively with time parallel to increase in the height of other peaks (e.g. the peak with retention time of 6:06 (min:s)). This would suggest that HQ-SG, first formed in the reaction, became gradually oxidised and bound new GSH molecules responsible of the rise of new peaks (corresponding to BQ-SG and HQ-SG_2 for instance). HQ-SG_2 would, in turn, be oxidised, and so forth, resulting in the upsurge of even more peaks (Figure III.4., at 2hr 20min).

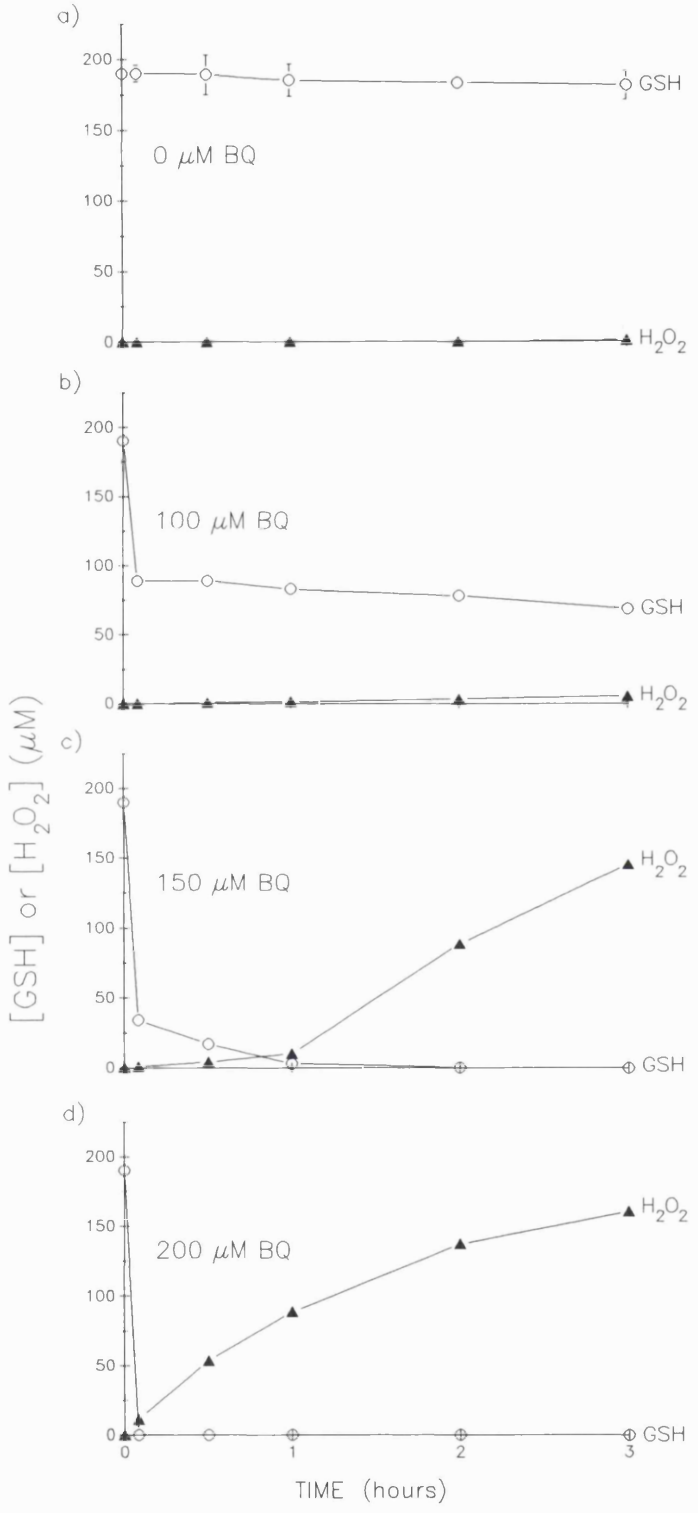
1.1.3. GSH/BQ ratio influence on H_2O_2 formation

The Figure III.1., already referred to above, presents the thiol status and H_2O_2 production during the incubation of 0, 100, 150 or 200 μM BQ with a fixed (200 μM) concentration of GSH. Significant amounts of H_2O_2 formation was observed only after complete exhaustion of titratable thiol groups (Figure III.1.c&d).

In order to study the influence of excess amounts of BQ or GSH on the reaction of the quinone with GSH to yield H_2O_2, three parallel experiments were set up (Figure III.6.a, b & c). First, Figure III.6.a, increasing concentrations of BQ were incubated with a fixed amount of GSH; second, Figure III.6.b, increasing concentrations of GSH were incubated with a fixed amount of BQ; finally, Figure III.6c., equimolar amounts of GSH and BQ were incubated in increasing concentrations.

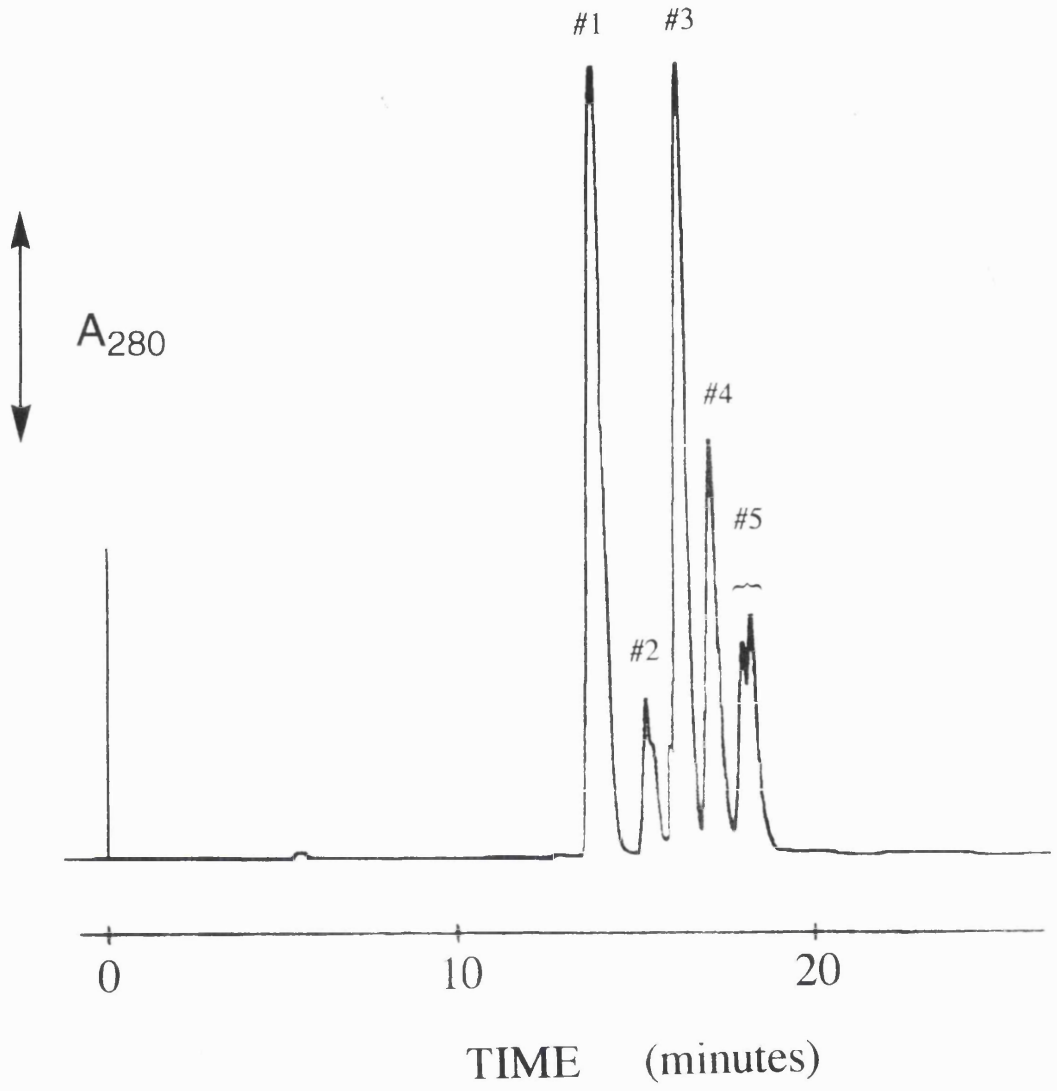
after 3 hours' incubation. In the first two cases (a & b), the yield of H_2O_2 was not linear with concentration of added reactant, unlike the situation found on addition of equimolar amounts to the incubation (c). It rather presented a bell-shaped curve with a maximum around the equimolarity between BQ and GSH and an inflexion point about 100 μM BQ for (a) and about 500 μM GSH for (b).

Figure III.1.: GSH status and H_2O_2 production during GSH-BQ interaction - time course



200 μM GSH was incubated at 37°C for up to 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with (a) 0 μM BQ, (b) 100 μM BQ, (c) 150 μM BQ, and (d) 200 μM BQ. Data shown are the means $\pm$ half the range of duplicate measurements.

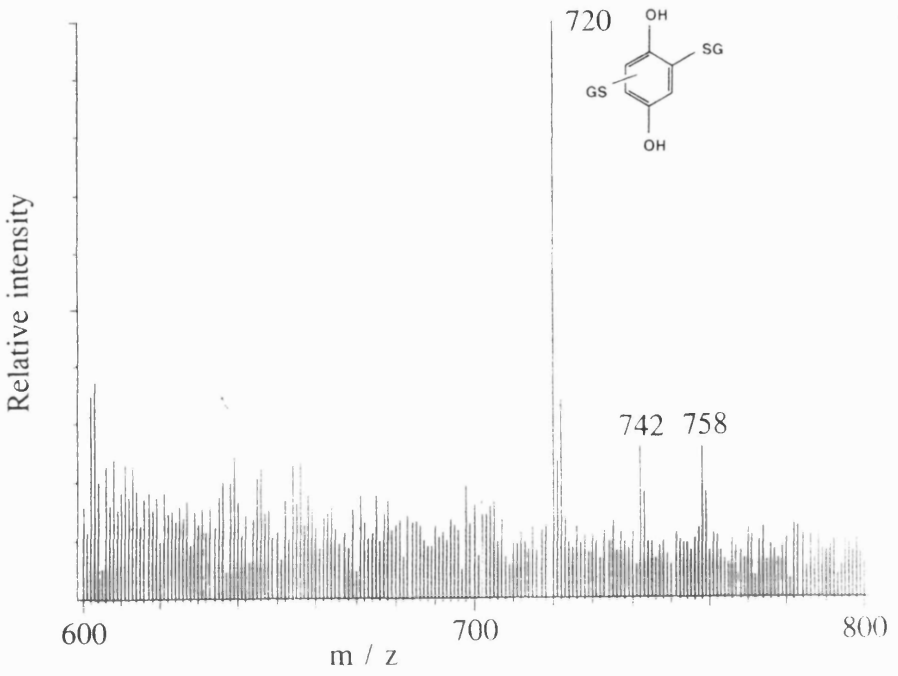
Figure III.2a.: GSH interaction with BQ - formation of mono- and diglutathionyl conjugates - HPLC elution profile



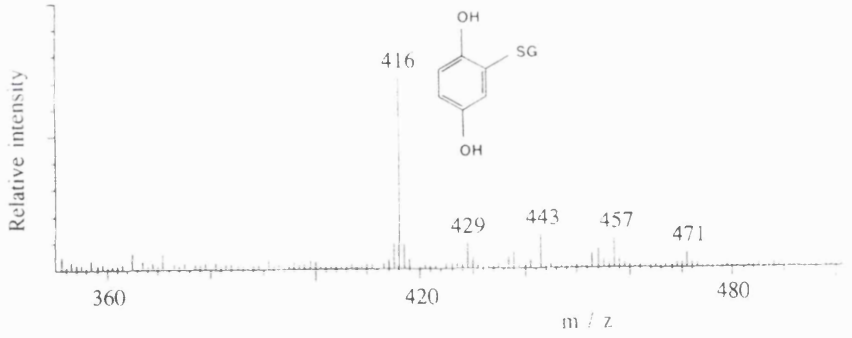
42 mM GSH was incubated with 42 mM BQ in distilled water at room temperature in the dark. After three hours' incubation, the reaction mixture was injected onto a semipreparative HPLC-column. The eluate was monitored in the UV at 280 nm and by fast atom bombardment (FAB) mass spectrometry. Fractions #2, #4 and #5 were identified.
The HPLC elution profile is shown here. The FAB mass spectra of the three identified fractions are presented on Figure III.2b..

Figure III.2b.: GSH interaction with BQ - formation of mono- and diglutathionyl conjugates - FAB mass spectra

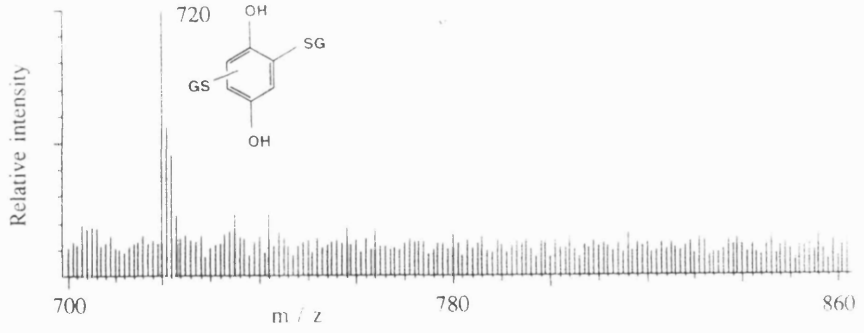
Fraction # 2:



Fraction # 4:

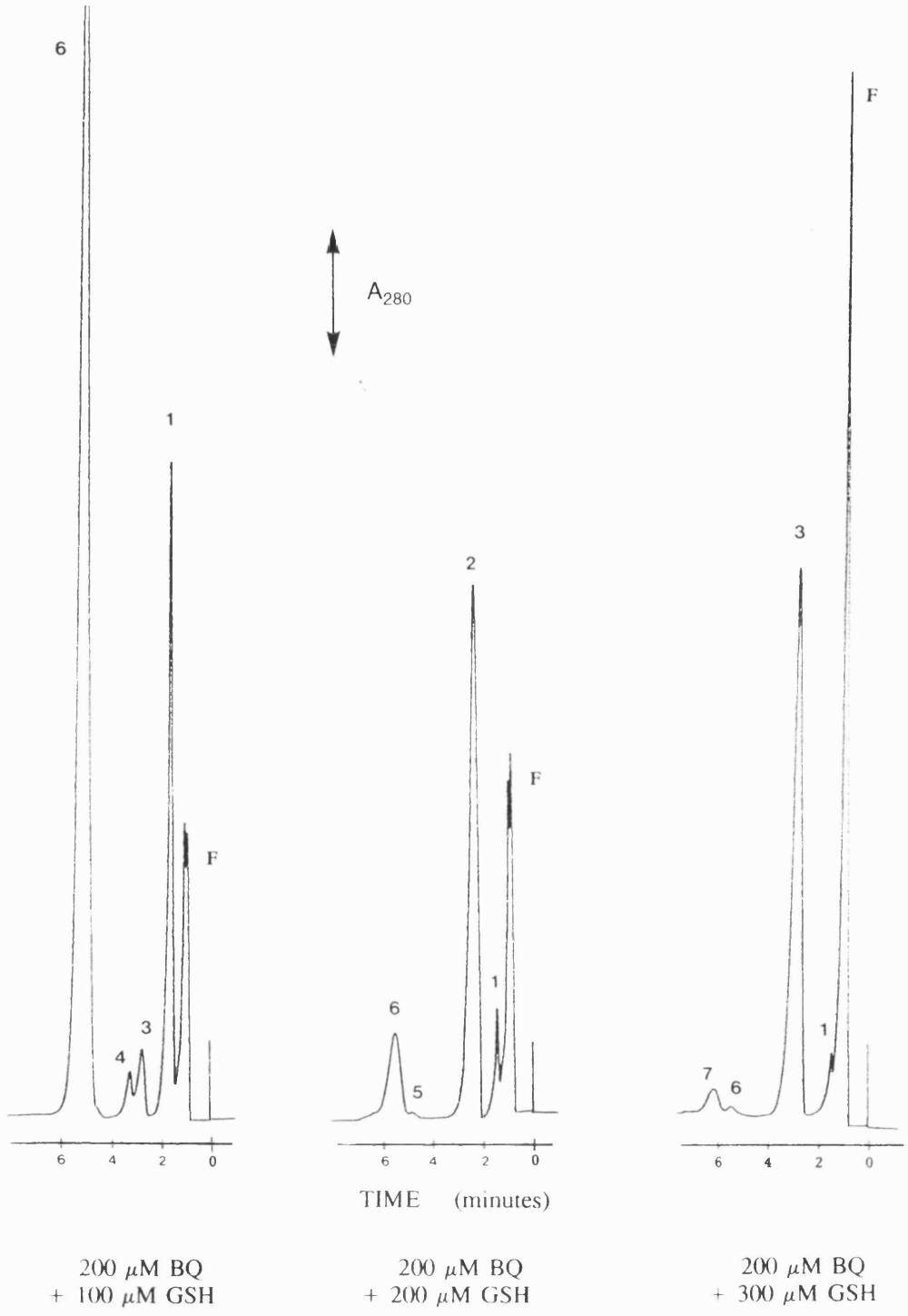


Fraction # 5:



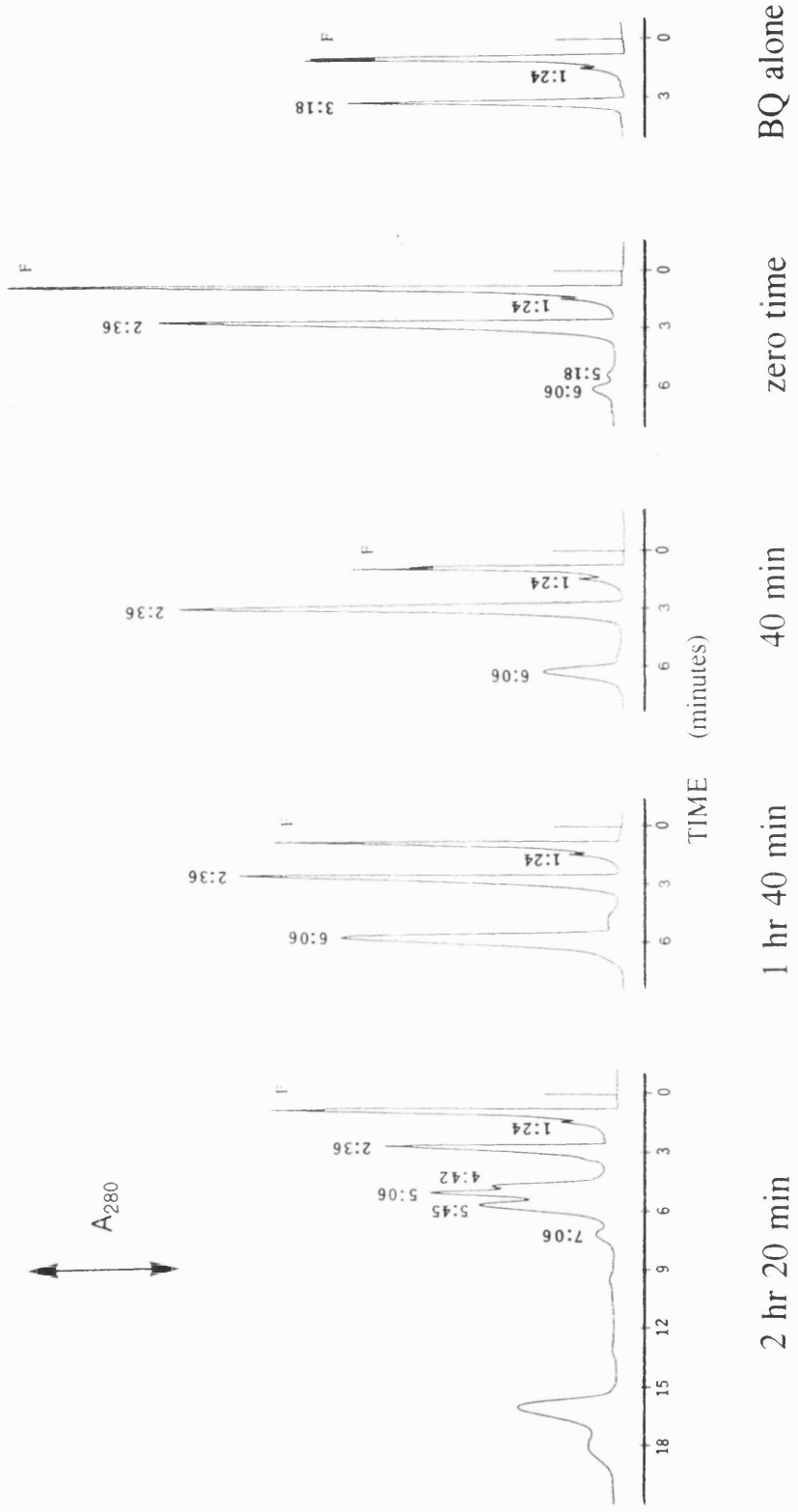
FAB mass spectra of fractions #2, #4 and #5 (see Figure III.2a. for details).
Fraction #2: An unprotonated molecular ion of one of the diglutathion-S-yl hydroquinone (M^+ *m/z* 720) is observed as well as ($M + Na_2^+$ *m/z* 742) and ($M + K_2^+$ *m/z* 758). Fraction #4: A protonated molecular ion of the monoglutathion-S-yl hydroquinone ($M + H^+$ *m/z* 416) is observed as well as ions, 14 units apart (*m/z* 429, 433, 457, and 471), which are probably lipid impurities. Fraction #5: an unprotonated molecular ion of one of the diglutathione-S-yl hydroquinones (M^+ *m/z* 720) is observed.

Figure III.3.: HPLC elution profiles of the products of the reaction of BQ with GSH - dose response



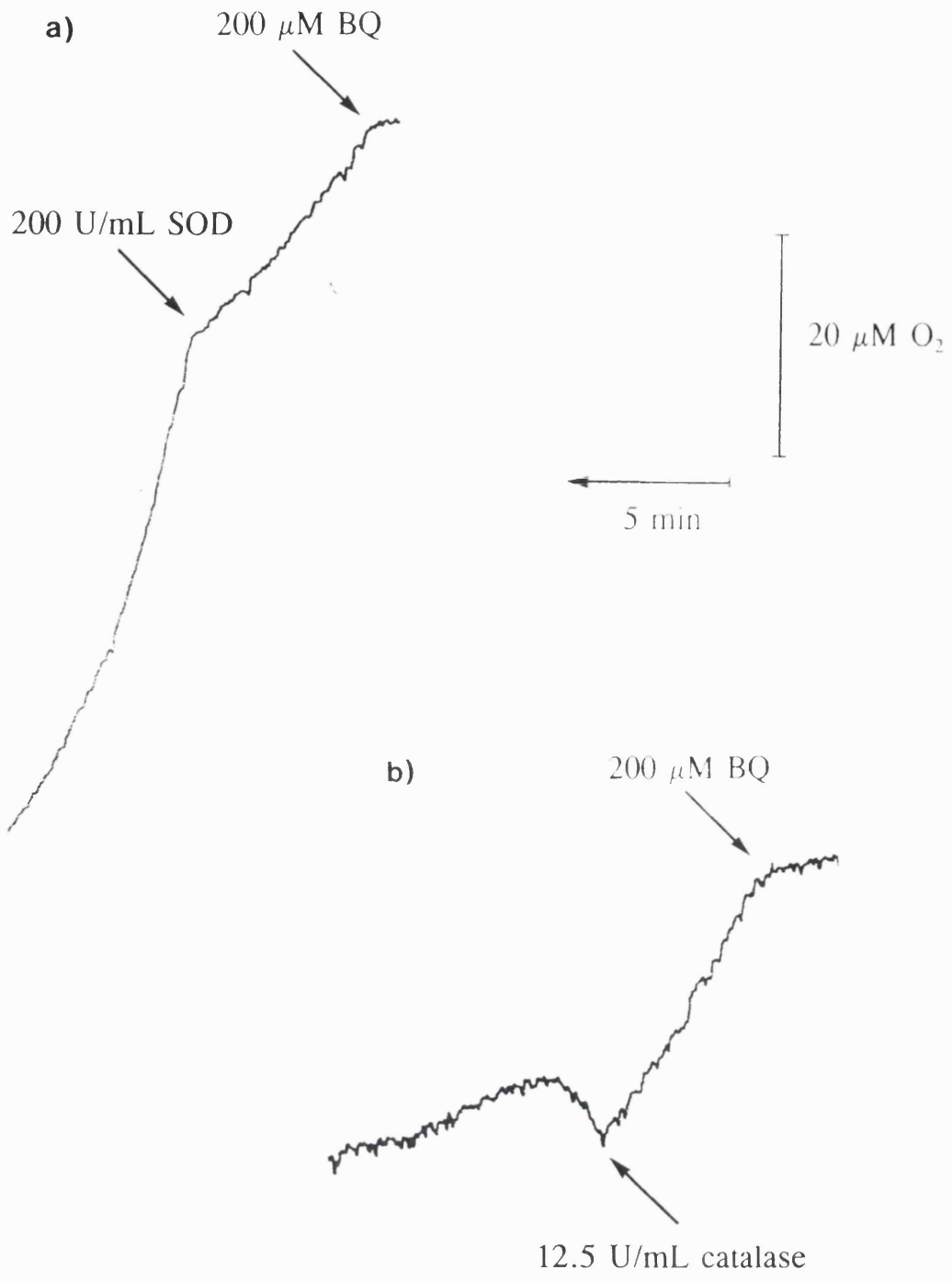
200 μM BQ was incubated at 37°C in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 100, 200 or 300 μM GSH.
Analytical-HPLC elution-profiles of aliquots of the reaction mixtures are presented at zero time. The time parameter for elution progresses from right to left.
F, solvent front; peak 1, 1:25 (min:s), hydroquinone; peak 2, 2:15 (min:s), supposedly same as peak 3; peak 3, 2:40 (min:s), supposedly monoglutathion-S-yl hydroquinone; peak 4, 3:20 (min:s), BQ; peak 5, 4:50 (min:s); peak 6, 5:20 (min:s); peak 7, 6:06 (min:s).

Figure III.4.: HPLC elution profiles of the products of the reaction of BQ with GSH - time course



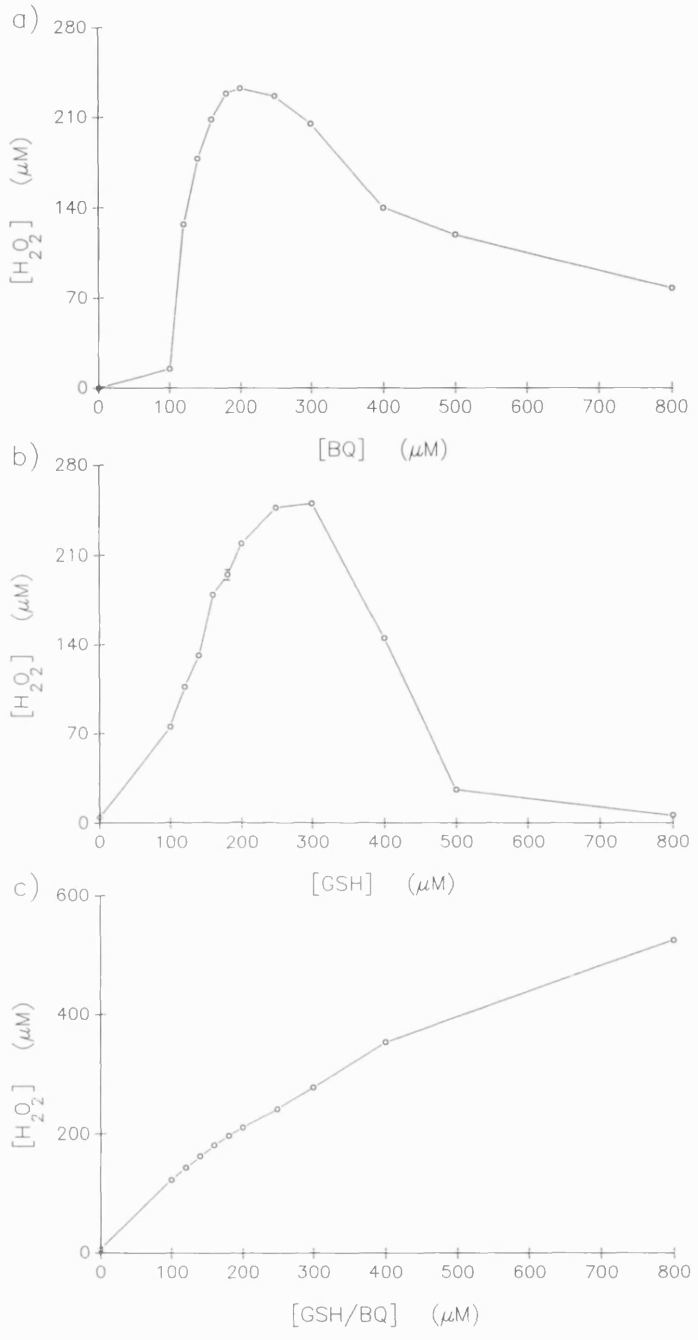
200 μM BQ was incubated at 37°C in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 300 μM GSH. At zero time and after 40 minutes', 1 hour 40 minutes' and 2 hours 20 minutes' incubation, aliquots of the reaction mixture were analysed by HPLC. Elution profile of 200 μM BQ alone in buffer is shown for reference. Time parameters for both elution and incubation progress from right to left. F, solvent front; peak 1:24 (min:s), hydroquinone; peak 2:36 (min:s), supposedly monoglutathion-S-yl hydroquinone; peak 3:18 (min:s), BQ.

Figure III.5.: BQ-GSH interaction - O_2 uptake, $O_2^{\cdot-}$ and H_2O_2 formation



Oxygen consumption during exposure of GSH to BQ.
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ and other components at the points indicated.

Figure III.6.: H_2O_2 production upon GSH-BQ interaction - dose response and GSH/BQ ratio effect



a) 200 μM GSH was incubated at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with increasing concentrations of BQ.
b) 200 μM BQ was incubated at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with increasing concentrations of GSH.
c) Equimolar amounts of GSH and BQ were incubated in increasing concentrations at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC .
Data shown are the means $\pm$ half the range of duplicate measurements.

A significant excess of GSH or BQ over HQ-SG appears thus to restrict the amount of H_2O_2 obtained at the end of the incubation.

This aspect will be further considered in section § III.3.&4..

1.1.4. Temperature, pH, metals and O_2 effects on H_2O_2 formation

Temperature and pH:

Temperature and pH had a dramatic effect on the reaction of GSH with BQ in terms of H_2O_2 formation. The change from room temperature to 37°C enhanced the amount of H_2O_2 found at the end of the three-hour incubation by more than 20 fold (Figure III.7.b). The change of 1 pH unit, from pH 7.0 to pH 8.0, magnified it by more than 7 fold (Figure III.7.a, plot BQ/GSH).

Moreover, this pH effect may account for the variable concentrations of H_2O_2 obtained at the end of the same type of incubation from one experiment to another (*e.g.* Figures III.5.a and III.1.d); a slight imprecision of $\pm$ 0.1 pH unit between two different pH 7.4-buffer batches would be a likely cause.

The pH could intervene at two levels in the process: the pKa of the sulphydryl group - alkaline conditions favour thiolate ion formation thus covalent binding (see § I.3.1.) - or the pKa of the hydroquinone moiety of the conjugate - alkaline conditions favour hydroquinone autoxidation (see § I.2.3.2.). The latter is more likely since, at pH 7.4, covalent binding took place in less than 5 minutes (see § III.1.1.1.) and shortening this step could not explain the increase in H_2O_2 production observed at pH 8.

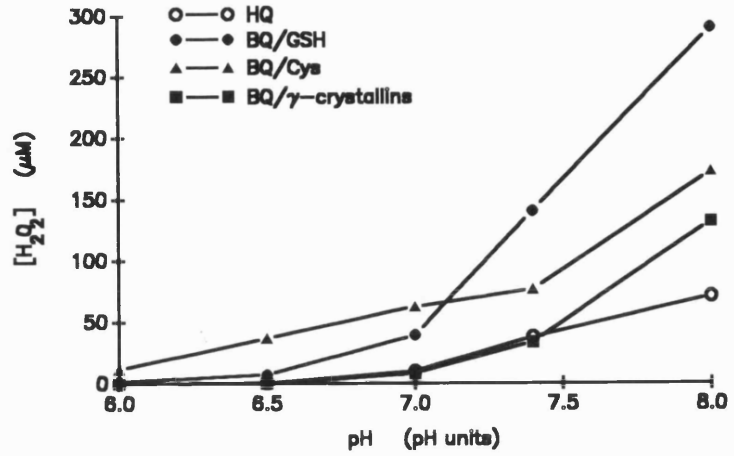
Metals and O_2 concentration:

The concentration of O_2 dissolved in buffer ($\sim$200 μM in potassium phosphate buffer pH 7.4 at 37°C) also influenced the rate of HQ-SG autoxidation, although to a much lesser extent than did temperature and pH. After bubbling the reaction mixture containing equimolar amount of GSH and BQ for 3 minutes with N_2, there was still substantial amount of residual O_2 being reduced to H_2O_2 (Figure III.8.).

The transition metal copper (at concentration above binding capacity of DETAPAC), although catalysing HQ oxidation (data not shown), was ineffective in affecting the rate of oxidation of the glutathione-hydroquinone conjugate as measured by oxygen consumption (Figure III.7.c). The metal chelator desferrioxamine, added under the

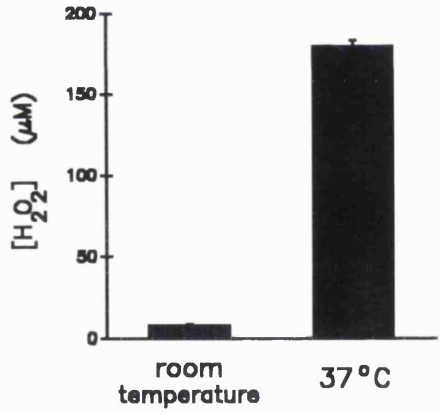
Figure III.7.: H_2O_2 production upon thiol-BQ interaction - pH effect, temperature effect and metal effect

a)



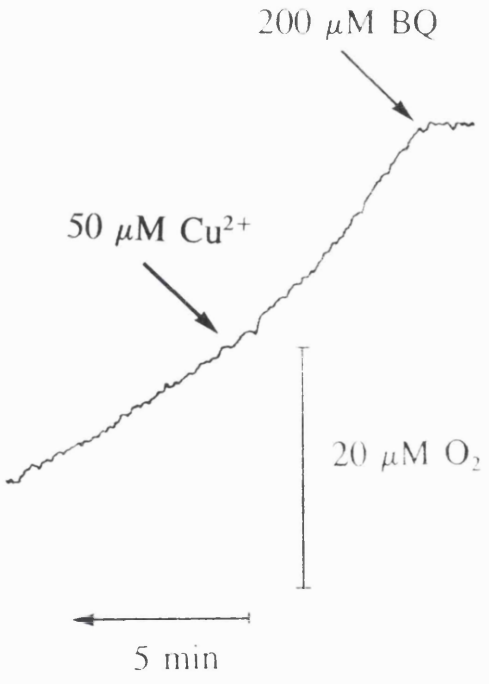
200 μM thiol of GSH, cysteine (Cys) or γ-crystallins were incubated at 37°C for 3 hours with 200 μM BQ in 100 mM potassium phosphate buffer of different pH values, containing 10 μM DETAPAC. H_2O_2 production by 200 μM HQ incubated in the same conditionsis also presented for comparison. Data shown are the means ± half the range of duplicate measurements.

b)



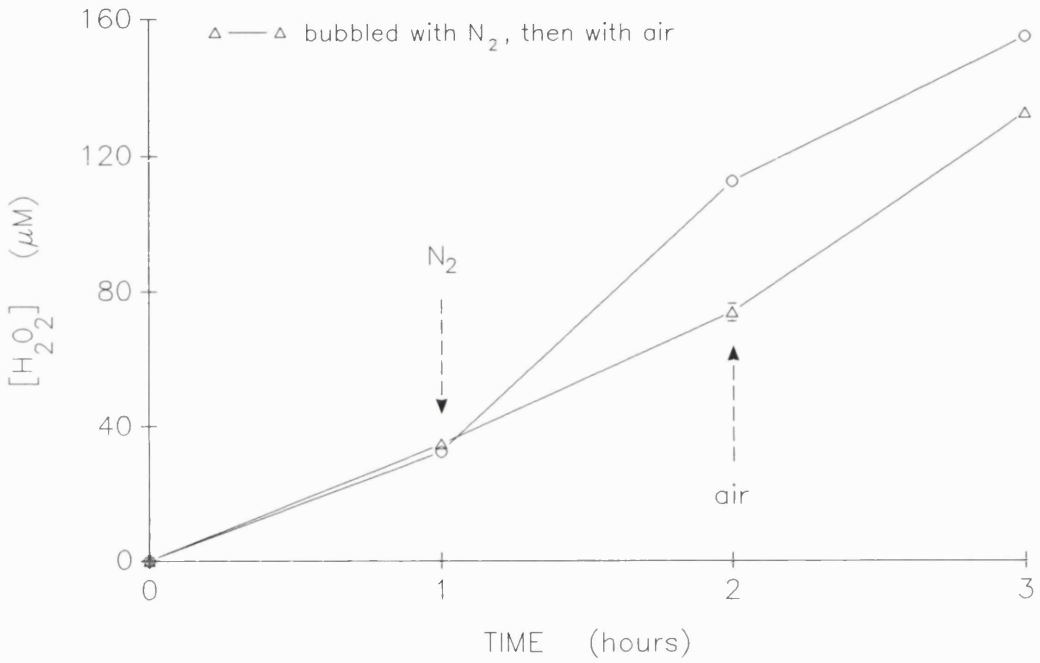
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 200 μM BQ, for 3 hours either at room temperature or at 37°C. Data shown are the means ± half the range of duplicate measurements.

c)



Oxygen consumption during exposure of GSH to BQ.
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ and Cu^{2+} at the points indicated.

Figure III.8.: H_2O_2 production upon GSH-BQ interaction - oxygen effect



200 μM GSH was incubated at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 200 μM BQ. The incubation was carried out either under standard air conditions (2 mL reaction mixture in 10 mL stoppered tube in a shaking bath) (empty circles) or under variable air conditions (empty triangles). In the latter case, after 1 hour's incubation under standard conditions, the reaction mixture was bubbled with nitrogen for 3 minutes and restoppered. Later on, after 2 hours' incubation, the same sample was bubbled with air for 3 minutes and restoppered. Data shown are the means $\pm$ half the range of duplicate measurements.

same conditions, also had no effect on the conjugate autoxidation. It has to be remembered here that the addition of DETAPAC in most reaction mixtures was to prevent artificial metal-catalysed autoxidation of thiols and had no effect on the reaction between quinone and thiol-compound.

1.1.5. HQ and HQ-SG autoxidation - effect of glutathionyl substituent

HQ has been shown to be present in the reaction mixture, though as a minor by-product or impurity (§ III.1.1.2.). The problem was therefore to determine if H_2O_2 observed during BQ and GSH interaction was merely due to HQ autoxidation and in what manner the glutathionyl substituent affects the autoxidisability of hydroquinone.

In Figure III.9., equimolar concentrations of GSH and BQ (a) or HQ alone (b) were incubated in increasing amounts and H_2O_2 formation during the time course of the reaction was measured. H_2O_2 observed in the latter case after three hours' incubation was less than 25 percent of H_2O_2 detected in (a) at the same time. Since the amount of HQ present in such equimolar reaction mixture was negligible as already mentioned, HQ was clearly an unimportant intermediate in H_2O_2 production in solutions of GSH and BQ under these conditions.

The influence of different sulphydryl substituents on hydroquinone autoxidisability was tested by incubating HQ alone or equimolar concentrations of BQ and thiols (GSH, cysteine or γ-crystallins) in buffer of different pH and measuring H_2O_2 accumulation at the end of three hours' incubation (Figures III.7.a). The glutathionyl substituent as well as the cysteinyl one both intensified, though in different manners, the autoxidation ability of hydroquinone especially at higher pH in the case of the glutathionyl residue. γ-Crystallin-cysteinyl substituent (see § III.2.1.1.) increased the autoxidisability of hydroquinone but only when the pH reached 8.0.

Sulphydryl substituents in general and glutathionyl in particular appears then to enhance autoxidation of the corresponding hydroquinone.

1.1.6. GSH exposure to BQ or methyl substituted BQ - role of arylation in H_2O_2 formation

When GSH was incubated with different methylated BQ (Figure III.10.), it was observed that the three non-fully substituted benzoquinones (BQ, monomethylbenzoquinone and dimethylbenzoquinone) all entirely depleted GSH and caused formation of H_2O_2. In contrast, duroquinone (tetramethylbenzoquinone), which cannot bind thiols, neither consumed GSH nor generated H_2O_2. The latter data indicate that H_2O_2 production was dependent upon conjugate formation rather than reduction of the quinone by GSH.

The reaction of the quinones with GSH to yield H_2O_2 decreased in the order BQ> monoMeBQ> diMeBQ. This however did not reflect their respective autoxidisability since the time course of the reaction (data not shown) indicates that H_2O_2 concentrations significantly diminished over a certain stage of the incubation with the methylated quinones, probably due to formation of hydroxyquinones by interaction between quinones and H_2O_2 (James *et al.*1938). Moreover, H_2O_2 levels after the first hour of incubation indicated a decrease in the order monoMeBQ> diMeBQ> BQ.

1.1.7. BQ exposure to thiol and non-thiol molecules

The reaction between BQ and thiol-compounds was further probed (Figure III.11.) by incubating a variety of sulphur- and non-sulphur-containing compounds with BQ. There was only a feeble formation of H_2O_2 when BQ was incubated with compounds where sulphur is involved in C-S-C or C-S-S-C bonds like S-methylglutathione, methionine and GSSG (approximately equal to that produced with the non-sulphur-containing residue glycine) but a far greater extent of H_2O_2 formation when BQ was incubated with thiols (C-S-H) like dithiothreitol (DTT), N-acetylcysteine (NAC), cysteine (Cys), γ-crystallin or dihydrolipoic acid. Instead of the amino group or other functional groups, free sulphydryl seems to account for the reaction of the quinone with GSH which yields H_2O_2. Proline, tyrosine and tryptophan, amino acids usually considered to be sensitive to oxidation, did not exhibit any sign of such a reaction, not even to the slight extent in which glycine did. The nature of the thiol seems to be of importance to the rate with which H_2O_2 was formed. If it is supposed that all the thiols are arylated by BQ, as is

the case for GSH, then the different rate of H_2O_2 production could be related to the diverse influence of these substituents on the corresponding hydroquinone autoxidation as stated in § III.1.1.5..

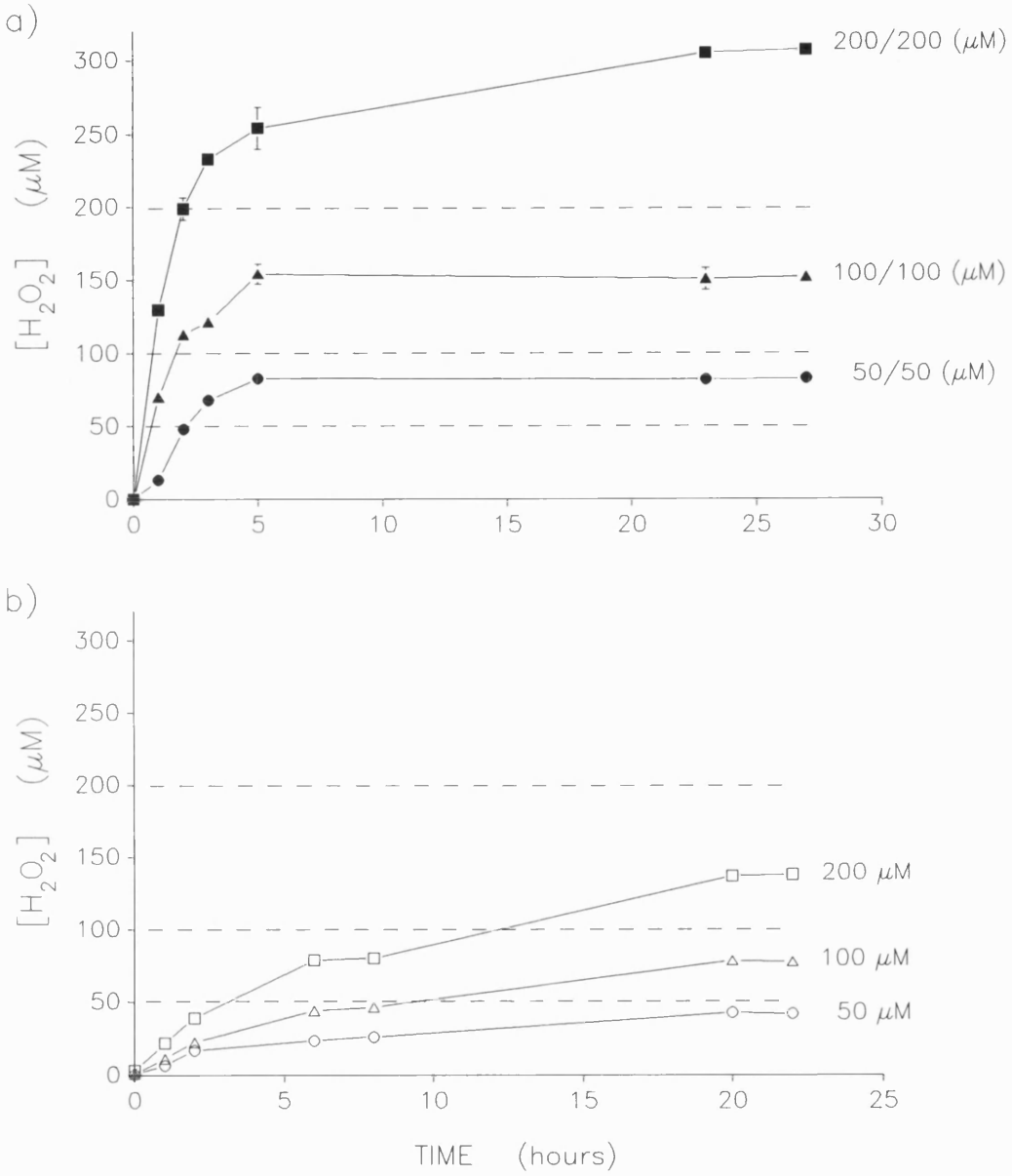
1.1.8. Slower reaction with amino groups

In most experiments carried out in three hours' incubation the final amount of H_2O_2 produced appeared to be broadly stoichiometric with the GSH consumed. In Figure III.9., equimolar concentrations of GSH and BQ (a) or HQ alone (b) were incubated for more than 20 hours in increasing amounts and H_2O_2 formation during the time course of the reaction was measured. Longer incubation times for up to 27 hours (Figure III.9.a) indicated that the H_2O_2 level reached at 3 hours was not the maximum one, the plateau being attained circa 20 hours with a concentration of H_2O_2 generated far beyond the initial concentration of GSH.

GSH understandably effects a two-electron reduction on BQ by its nucleophilic attack (see § I.2.2. and I.2.4.) and H_2O_2 represents a two-electron reduction of O_2; if then all HQ-SG had to be oxidised, a maximum of $[H_2O_2] \leq [HQ\text{-}SG]$ should be expected as observed in Figure III.9.b where $[H_2O_2] \leq [HQ]$.

A reducing moiety, other than the cysteinyl one, must participate in the reaction of the quinone with GSH which yields H_2O_2. Amino group of γ-glutamyl could be this moiety as suggested in Figure III.12.. In this study, 200 μM glycine, 200 μM NAC or 200 μM NAC + 200 μM glycine were incubated with 200 μM BQ for up to 19 hours and H_2O_2 was measured during the incubation time. The different kinetics of the reactions between glycine and BQ on one hand and NAC and BQ on the other hand are clearly visible. Glycine reacted much more slowly with the quinone than NAC did but after a prolonged incubation, it reached a comparable extent of H_2O_2 yield. With NAC, which has its amino group capped by an acetyl, the H_2O_2 production, upon exposure to BQ, was enhanced in the presence of glycine.

Figure III.9.: Comparison between HQ and HQ-SG autoxidation

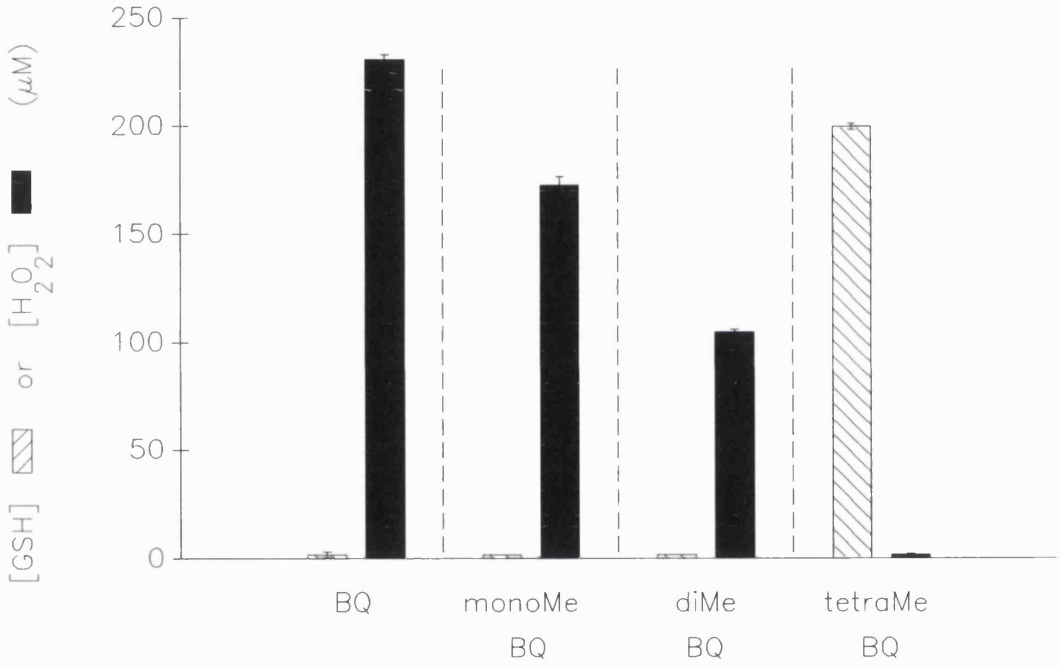


a) Time course for up to 27 hours: 50 μM GSH/50 μM BQ, 100 μM GSH/100 μM BQ or 200 μM GSH/200 μM BQ were incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C.

b) Time course for up to 22 hours: 50 μM HQ, 100 μM HQ or 200 μM HQ were incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C.

Data shown are the means $\pm$ half the range of duplicate measurements.

Figure III.10.: GSH status and H_2O_2 production after incubation of GSH with BQ and methylated BQ



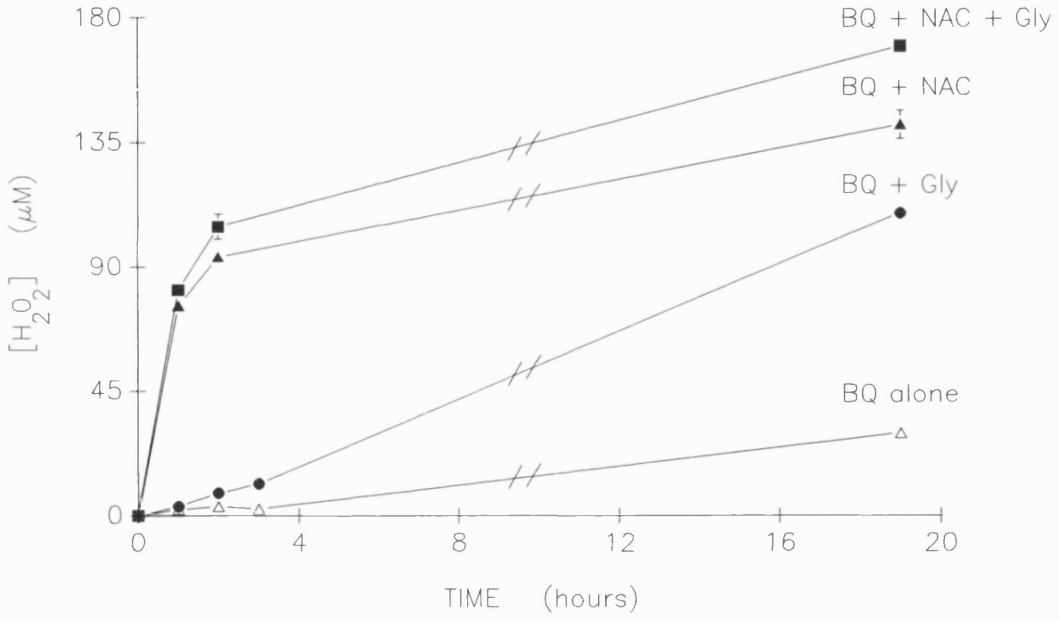
200 μM GSH was incubated, at 37°C for 3 hours, in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 200 μM of BQ, monoMeBQ, diMeBQ or tertraMeBQ. Data shown are the means $\pm$ half the range of duplicate measurements.

Figure III.11.: H_2O_2 production upon exposure of thiol and non-thiol compounds to BQ - comparison

Thiol and non-thiol compounds	H_2O_2 production (μM)
GSH	167.2 ± 0.7
dithiothreitol (DTT)	215 ± 7
dihydrolipoic acid (DHLA)	49.5 ± 0.5
N-acetylcysteine (NAC)	112 ± 1
cysteine	96.8 ± 0.1
γ-crystallin	70 ± 3
S-methylglutathione	9.1 ± 0.1
methionine	14.5 ± 0.3
GSSG	15.2 ± 0.3
glycine	12.2 ± 0.1
proline	6.3 ± 0.1
tyrosine	5.6 ± 0.1
tryptophan	5.8 ± 0.2
BQ	5.9 ± 0.3

200 μM thiol or non-thiol compounds were incubated for three hours with 200 μM BQ. Incubations took place in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C. H_2O_2 production after a three-hour incubation of 200 μM BQ alone in buffer is also presented as a control. Data shown are the means ± half the range of duplicate measurements.

Figure III.12.: Amino groups versus thiol groups in long run incubation with BQ



200 μM glycine (Gly), 200 μM N-acetylcysteine (NAC) or 200 μM NAC + 200 μM Gly were incubated at 37°C in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 200 μM of BQ for up to 19 hours. H_2O_2 production by 200 μM BQ, alone in buffer, during the same period of time is presented for comparison. Data shown are the means $\pm$ half the range of duplicate measurements.

1.2. GSH exposure to NAPQI

N-acetyl-p-benzoquinone imine (NAPQI) wasprepared as described in § II.8..

1.2.1. Conjugation

In Figure III.14., the results from incubation of 0, 100, 150 and 200 μM NAPQI for 3 hours with a fixed concentration of GSH (200 μM) are shown. GSH status was followed throughout the incubation time (a) and GSSG formation was measured at the end of the incubation (b). Figure III.14.a_1 shows that NAPQI, when added to GSH, provoked a rapid loss of titratable thiol groups. The stoichiometry involved, for the doses 50 and 100 μM NAPQI, appeared to be 1 NAPQI for 2 GSH which would rather support the oxidation of GSH to GSSG as confirmed in Figure III.14.b. When NAPQI and GSH were brought to react at same concentration, GSH was totally depleted with half of it oxidised to GSSG. This could imply, assuming no higher oxidised states of GSH had been formed, that the other half was arylated by the quinoneimine.

FAB mass spectrometry investigation of the product of the reaction between NAPQI and GSH (Figure III.13.) confirmed the involvement of both oxidation of GSH and reduction of NAPQI, by detection of paracetamol (*m/z* 152) and GSSG (*m/z* 613), and also arylation, by identification of glutathion-S-yl paracetamol (*m/z* 457). Weak evidence suggested the existence of the diglutathionyl conjugate at *m/z* 761 (data not shown). The formation of an ($M + H^+ - H$, *m/z* 761) species is similar to that observed for the diglutathionyl-hydroquinone conjugates (§ III.1.1.1.) and arises through its redox properties. Paracetamol dimer (*m/z* 301) and trimer (*m/z* 450) were also detected.

It has to be remembered that, in FAB spectrometry, intensity may not reflect relative amount of the different species.

1.2.2. H_2O_2 formation

Figure III.14.a_2 shows H_2O_2 formation during the incubation of 0, 100, 150 and 200 μM NAPQI with a fixed concentration of GSH (200 μM) for 3 hours. The reaction of NAPQI with GSH generated very little H_2O_2. The formation of GSSG itself was not

accompanied by H_2O_2 formation, as indicated at 50 μM NAPQI where 50 μM GSSG was generated without any H_2O_2 detection.

An oxygen electrode study, a less sensitive technique, was not able to reveal any oxygen uptake. Unlike *para*-benzohydroquinone, 200 μM paracetamol incubated in potassium phosphate buffer pH 7.4 at 37°C did not present any sign of autoxidation, as measured by oxygen consumption or H_2O_2 production. The glutathionyl substituent did not seem to markedly increase the autoxidisability of paracetamol; when incubated at same concentration, conditions in which ~90 μM glutathionyl-paracetamol could be expected (see last section), NAPQI and GSH interaction generated 8 μM H_2O_2 after three hours' incubation (Figure III.14.a_2).

1.3. GSH exposure to BQ, NQ, MD and NAPQI - comparison

In Figure III.15., 200 μM GSH was incubated with 200 μM BQ, 1,4-naphthoquinone (NQ), menadione (MD) and NAPQI. After 3 hours' incubation, H_2O_2, GSH and GSSG were measured. The reaction of GSH with different quinones to yield H_2O_2 decreased in the order BQ > NQ > MD > NAPQI. The less GSSG formed during the incubation, the more H_2O_2 was generated. Assuming no higher oxidised states of GSH had been formed, the reaction of the quinones with GSH to form conjugates could be seen as decreasing in the order BQ > NQ > MD $\simeq$ NAPQI and a parallel could be drawn, as in § III.1.1.6., between arylation of GSH and H_2O_2 production. Neither NQ nor MD succeeded in entirely consuming the available GSH.

2. Biochemical study with thiol-proteins

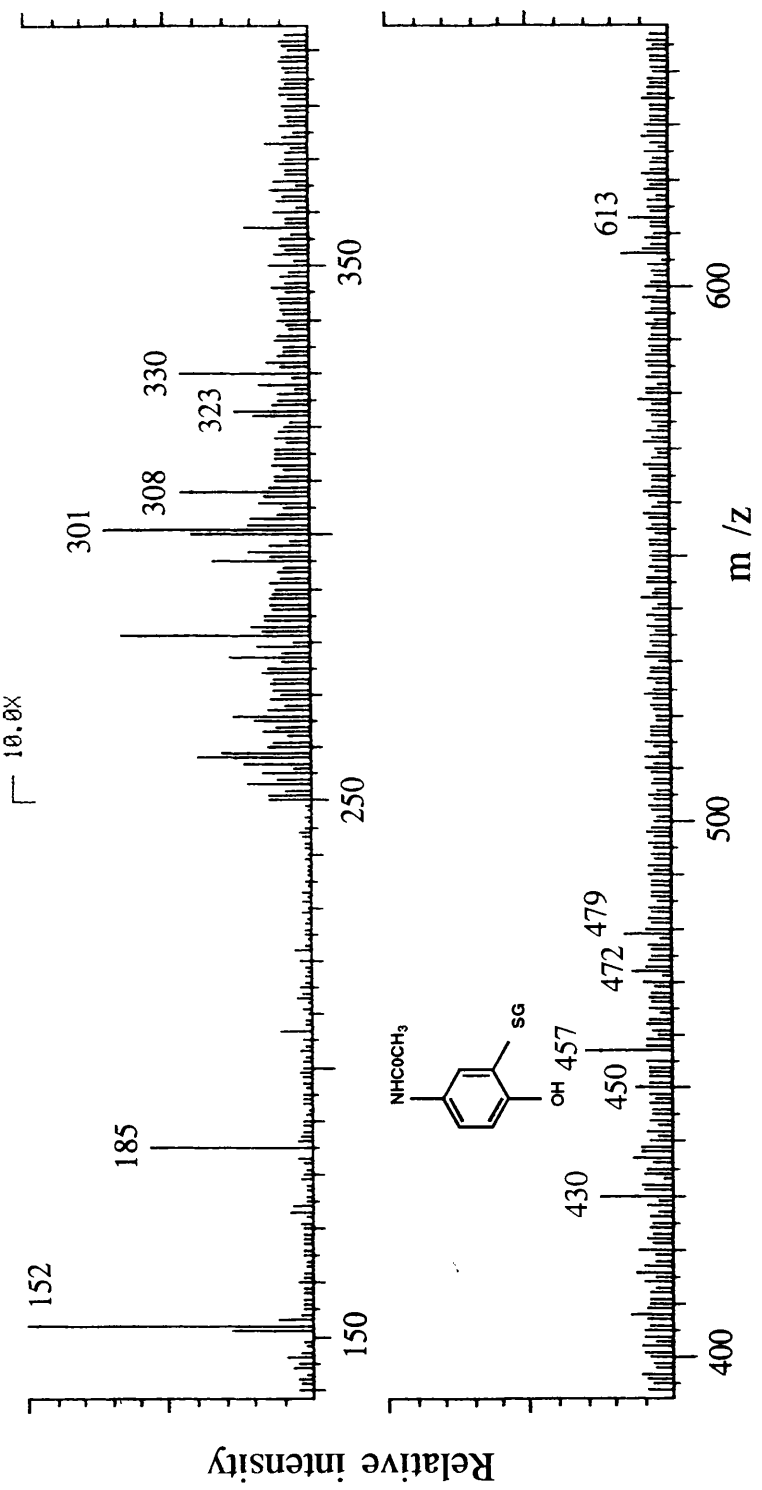
2.1. γ-Crystallin exposure to BQ

2.1.1. Conjugation

As has been shown in § III.1.1.1., BQ readily reacts with GSH and a covalently bound conjugate is formed.

Exposure of BQ to the thiol-rich γ-crystallin also appears to lead to arylation of the

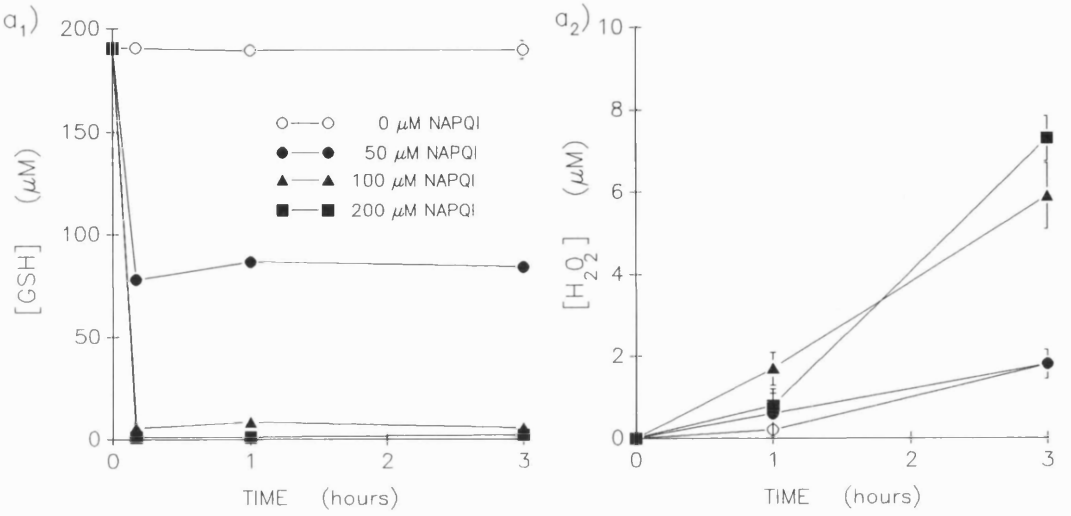
Figure III.13.: Arylation of GSH by NAPQI - FAB mass spectrum



0.5 mL of 20 mM GSH (in distilled water) was added to 1 mL of 8 mM NAPQI (in chloroform). The reaction mixture was immediately vortex-mixed for 5 minutes. The two phases were then separated and concentrated under nitrogen. Each phase was then analyzed by fast atom bombardment (FAB) mass spectrometry. Paracetamol and GSH were detected in the aqueous phase. The FAB mass spectrum of the organic phase is shown above.
m/z 152, protonated molecular ion of paracetamol ($M + H^+$); *m/z* 185, glycerol dimer (from the matrix) ($M + H^+$); *m/z* 301, paracetamol dimer ($M + H^+$); *m/z* 308, glutathione ($M + H^+$); *m/z* 323, paracetamol dimer + sodium ($M + Na_2^+$); *m/z* 330, glutathione + sodium ($M + Na_2^+$); *m/z* 430, possibly paracetamol trimer - ketene + sodium ($M - CH_2{:}CO + Na_2^+$); *m/z* 450, paracetamol trimer ($M + H^+$); *m/z* 457, glutathion-S-yl paracetamol ($M + H^+$); *m/z* 472, possibly paracetamol trimer + sodium ($M + Na_2^+$); *m/z* 479, glutathion-S-yl paracetamol + sodium ($M + Na_2^+$); *m/z* 613, GSSG ($M + H^+$).

Figure III.14.: NAPQI interaction with GSH: GSH status, GSSG formation and H_2O_2 production

a)



200 μM GSH was incubated at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with 0 μM NAPQI, 50 μM NAPQI, 100 μM NAPQI or 200 μM NAPQI.
GSH status (a_1) and H_2O_2 production (a_2) during the three-hour incubation are presented. Data shown are the means ± half the range of duplicate measurements.

b)

	0 μM NAPQI	50 μM NAPQI	100 μM NAPQI	200 μM NAPQI
H_2O_2 (μM)	1.8 ± 0.1	1.8 ± 0.4	5.9 ± 0.8	7.3 ± 0.1
GSH (μM)	190 ± 4	84 ± 0	6 ± 2	1 ± 1
GSSG (μM)	1 ± 0.1	52 ± 0.5	89 ± 0.4	53 ± 0.7

H_2O_2 production, GSH loss and GSSG formation upon GSH exposure to NAPQI.
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C for 3 hours with 0 μM NAPQI, 50 μM NAPQI, 100 μM NAPQI, and 200 μM NAPQI. Data shown are the means ± half the range of duplicate measurements.

Figure III.15.: H_2O_2 production, GSH loss and GSSG formation upon GSH exposure to different quinones - comparison

	BQ	NQ	MD	NAPQI
H_2O_2 (μM)	191.4 ± 0.7	99.4 ± 0.1	10.4 ± 0	7.3 ± 0.1
GSH (μM)	0.2 ± 0.4	8.3 ± 0	30.5 ± 0	1 ± 1
GSSG (μM)	5 ± 0.5	24 ± 0.2	37.2 ± 0.5	53 ± 0.7

200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C for 3 hours with 200 μM BQ, NQ, MD or NAPQI. Data shown are the means ± half the range of duplicate measurements.

protein (Figure III.16.). In this experiment, 200 μM thiol of γ-crystallin was briefly exposed to 0, 100 and 300 μM BQ. The absorption spectral changes of BQ upon exposure to γ-crystallin were observed. Brief exposure of 200 μM thiol of γ-crystallin to 100 μM BQ resulted in the total disappearance of the 253 nm peak, characterizing BQ, in the acid soluble fraction (a_1) and in the rise of a new peak at 263 nm as well as a broad absorbance area around 330 nm in the dissolved pellet (b_1), presumedly due to the quinone-protein adduct. When the crystallin was submitted to an amount of BQ greater than the thiol content of the protein (300 μM BQ for 200 μM thiol), the level of BQ in the acid-soluble fraction fell to just below 100 μM and a peak at 287 nm, characteristic of HQ, started to take shape (a_2). In parallel, in the alkali-dissolved pellet (b_2), the same absorbance figure was obtained as in b_1 with a 50% increase in optical density.

optical density.

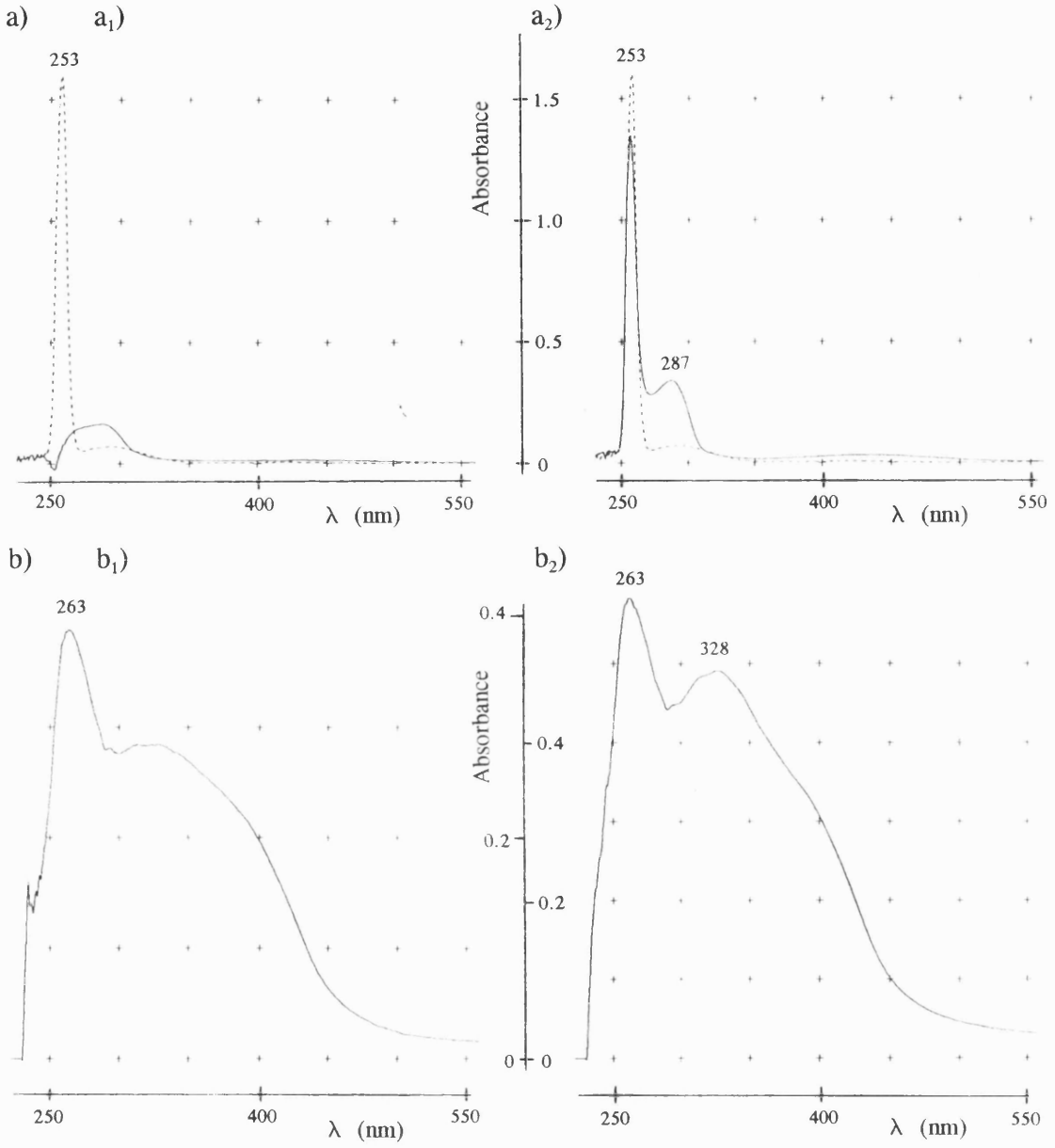
Although the use of radiolabelled BQ would have been a more direct way to demonstrate it (Labelled BQ is not commercially available as a standard product), these data strongly suggest the binding of the quinone to the protein. It is of interest that, as with incubation with GSH (§ III.1.1.2.), an excess of BQ over thiol resulted in the BQ reduction, probably *via* the hydroquinone-thioether conjugate.

The main target of the electrophilic attack is probably sulphydryl group as implied by the close relationship between free quinone left over after the short exposure to the protein and the thiol content of the native protein (Figure III.16.a_1&a_2).

Furthermore, when BQ was added at varying concentrations to a fixed (170 μM) concentration of protein-thiol, there was a rapid loss of titratable thiol groups (Figure III.17.a). There was no stable stoichiometry in the reaction between BQ and protein-SH; 2 thiol groups were consumed per 1 quinone at doses 50 and 100 μM BQ whilst the ratio became less than 1:1 at 200 μM BQ.

The latter ratio possibly finds its basis in the existence of buried sulphydryl groups not directly accessible to the quinone. In that aspect, it is worth noticing the delayed depletion of those thiols presumedly successive to conformational change of the protein imposed by the earlier formed adducts.

Figure III.16.: Arylation of γ-crystallin by BQ

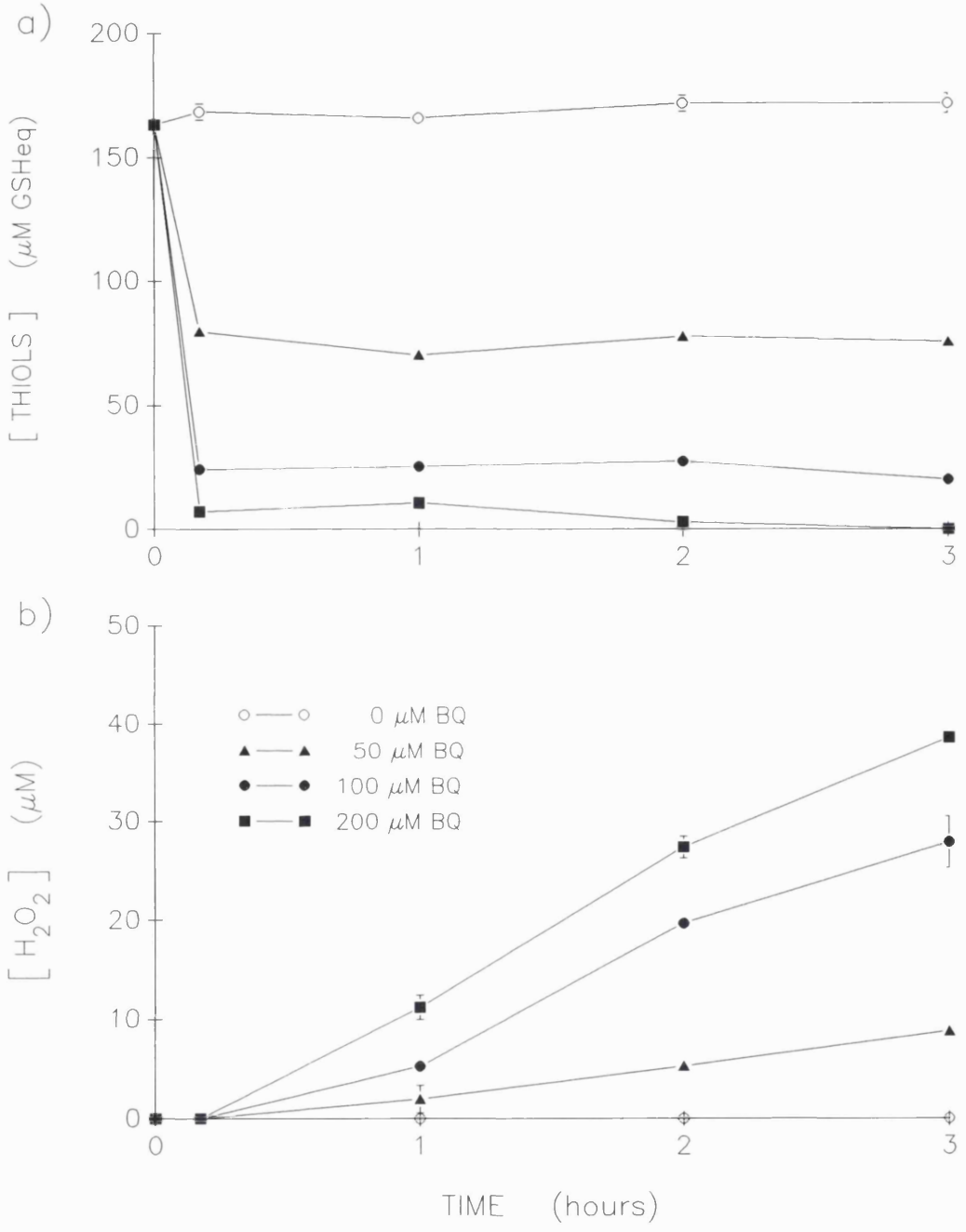


200 μM thiol of γ-crystallin was incubated for 15 min, at 37°C, in 100 mM potassium phosphate buffer pH 7.4, with 0 μM BQ, 100 μM BQ or 300 μM BQ. 1 mL of each of those reaction mixtures was then adjusted, with a 50% TCA solution, to a final 5% TCA concentration. Samples were then spun down for 5 min at 14,000 g.

a) The supernatants were recovered and scanned from 200 to 600 nm (baseline determined with a 5% TCA solution). The spectrograms show the (mathematical) differences between the treated samples and the untreated one (0 μM BQ): a_1) sample treated with 100 μM BQ, a_2) sample treated with 300 μM BQ. The dotted line represents 100 μM BQ alone in 5% TCA as a reference.

b) The pellets were washed once with 5% TCA and then dissolved in 100 μL of 1 M NaOH. Once dissolved the proteins were diluted 10 fold in water and scanned from 200 to 600 nm (baseline determined with a 0.1 M NaOH solution). The spectrograms show the (mathematical) differences between the treated samples and the untreated one (0 μM BQ): b_1) sample treated with 100 μM BQ, b_2) sample treated with 300 μM BQ.

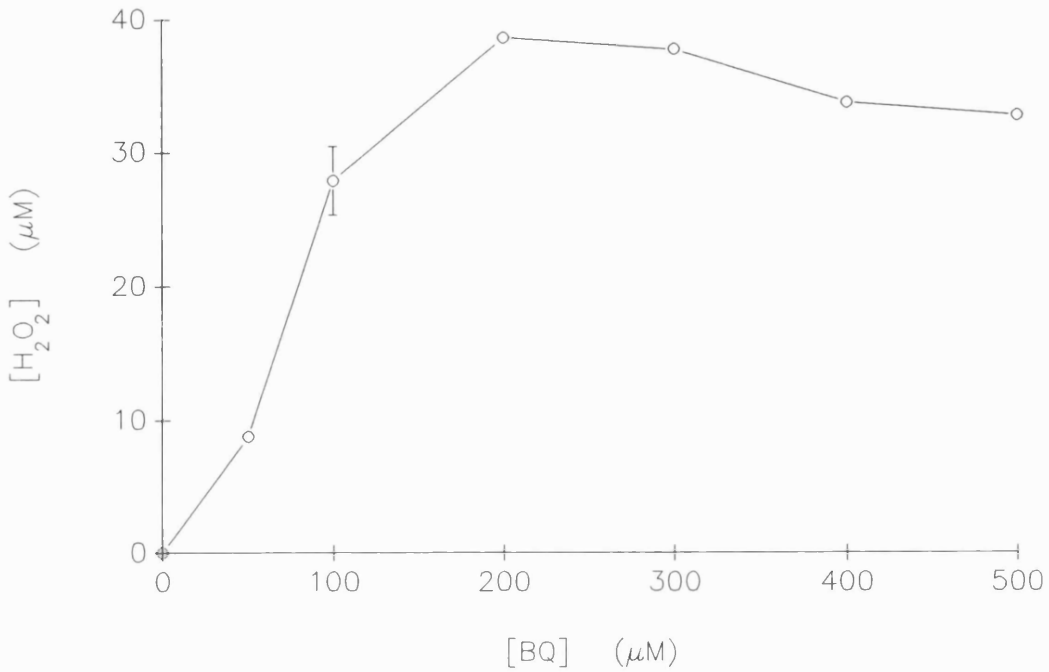
Figure III.17.: γ-Crystallin interaction with BQ: thiol status and H_2O_2 production



170 μM thiol of γ-crystallin was incubated with various concentrations of BQ at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC.
Thiol status during the three-hour incubation is presented in (a); H_2O_2 production during the same incubation is displayed in b). Data shown are the means $\pm$ half the range of duplicate measurements.

Figure III.18.: H_2O_2 production upon γ-crystallin exposure to BQ - dose response curve and role of the adduct

a)



170 μM thiol of γ-crystallin was incubated with various concentration of BQ at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC. Data shown are the means ± half the range of duplicate measurements.

b)

H_2O_2 production (μM)	
without BQ	with BQ
0 ± 0	18.5 ± 0.1

H_2O_2 production from the dialysed product of γ-crystallin exposure to BQ

200 μM thiol of γ-crystallin was first exposed briefly in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C to 0 μM BQ or 300 μM BQ and then dialysed extensively for 14 hours at 4°C against the same buffer. Subsequently, the product of the dialysis was incubated at 37°C and H_2O_2 measured after three hours. Data shown are the means ± half the range of duplicate measurements.

2.1.2. H_2O_2 formation

When increasing concentrations of BQ were incubated to a fixed amount of protein-thiol, there was for each dose of BQ a progressive rise in yield of H_2O_2 (Figure III.17.b). This H_2O_2 production was less pronounced than the one derived from BQ interaction with GSH (see § III.1.1.). H_2O_2 formation did not seem to depend on the remaining unreacted thiols as observed with GSH probably due to the rigidity of the common support of protein thiols.
Similarly to GSH, H_2O_2 formation became restricted at quinone concentrations above the thiol content of the proteins (Figure III.18.a).

Covalent binding precedes H_2O_2 formation:
Formation of H_2O_2 was due to the quinone adduct since the presence of HQ, a plausible byproduct of the reaction, was negligible at least at the dose of 100 μM BQ (§ III.2.1.1.).
Covalent reaction of BQ prior to H_2O_2 formation was further demonstrated by the observation that γ-crystallin, exposed briefly to BQ and subsequently dialysed extensively still generated substantial amounts of H_2O_2 after incubation at 37°C for 3 hours. Under the same conditions crystallin which was not pre-incubated with quinone generated negligible H_2O_2 (Figure III.18.b).

2.1.3. Protein damage

2.1.3.1. Characterisation of the protein damage

Reaction of γ-crystallins with BQ led not only to H_2O_2 formation but also to limited protein fragmentation and formation of high molecular weight, non-reducible aggregates (Figure III.19.). In this figure, γ-crystallin (200 μM thiol equivalents) was incubated with BQ (200 μM) for up to 3 hours. At different time points of the reaction, H_2O_2 was measured (attached Table) and aliquots of the reaction mixture were analysed by SDS-PAGE. Lanes A-E show the depletion of the major 20 kDa peptide and the generation of a major fragment (approximately 18 kDa). There was also some evidence for the production of higher molecular weight material which was not DTT-reducible at 100°C.

In addition, fragmentation of the parent crystallin associated with H_2O_2 formation was efficient. At 30 minutes' incubation more than 50% of the parent crystallin had been fragmented under circumstances in which only 11.7 μM H_2O_2 had accumulated.

In Figure III.20., 200 μM thiol equivalents of γ-crystallin were incubated with 100 μM BQ plus or minus various agents (Lanes B-F) for 2 hours (crystallin incubation alone in buffer is presented Lane A). At the end of the reaction, H_2O_2 was measured (attached Table) and aliquots of the reaction mixture were analysed by SDS-PAGE. Gross fragmentation of the parent crystallin induced by BQ was inhibited by catalase and the hydroxyl radical scavenger sorbitol but both these agents failed to reduce formation of the peptide apparently formed by 2 kDa truncation of the parent crystallin and of high molecular weight aggregates (Lanes A-D). The very similar modified SDS-PAGE pattern obtained with catalase and sorbitol may suggest that H_2O_2 role in this fragmentation process is *via* degradation to ·OH.

Crystallin incubated with copper and H_2O_2 lead to the production of high molecular weight materials and gross fragmentation (Lane G) but not the size-specific fragmentation observed with BQ.

(Lanes E & F will be referred to in § III.3.3. & 4.1.)

2.1.3.2. Comparison with other crystallins and with BSA

Incubation of a fixed (200 μM) thiol concentration of different crystallin fractions, containing either α-, β-, γ_S-crystallins or mixture of them (see Figure II.1.b for content of each fraction), with (Lanes A'-D') or without (Lanes A-D) 100 μM BQ for three hours (Figure III.21.) led to a variable accumulation of H_2O_2 (Table) and different extent of fragmentation and crosslinks (SDS-PAGE). β-crystallin (#3) appeared to be the fraction which, upon exposure to the quinone, experienced the most marked reaction yielding H_2O_2. Formation of high-molecular-weight non-reducible aggregates was detected for all fractions (Lanes A'-D'), yet some bands were more affected than others by the process. For instance, the two main bands in fraction 1 (Lanes A&A') were essentially unchanged upon exposure to the quinone whilst the main band in fraction 3 (Lane C) was almost entirely depleted after exposure to the quinone (Lane C').

When labelled bovine serum albumin (BSA), which contains one free sulphydryl group per molecule (Streeter *et al.*1984), was incubated with varying doses of BQ for three

hours, substantial dose-dependant amounts of trichloroacetic acid-soluble ^{14}C-labelled fragments were observed (Figure III.22.b). This paralleled accumulation of H_2O_2 in the reaction mixture, although not in a close proportional manner (Figure III.22.a). SDS-PAGE of the reaction mixture under similar conditions (Figure III.22.c) confirmed fragmentation of BSA. Depletion of both large aggregates, present in the commercial sample, and BSA monomer (at 68 kDa, intentionally overloaded) were clearly apparent on this gel, which was electrophoresed in non-reducing conditions. Even though the sensitive $AgNO_3$ stain was used for this SDS-PAGE, fragments of specific sizes were not detected suggesting that, in this case, fragmentation related to a rather random event.

2.2. γ-Crystallin exposure to BQ, diMeBQ, NQ, MD and NAPQI - comparison

H_2O_2 formation:

Exposure of γ-crystallin to BQ, diMeBQ, NQ and MD for three hours resulted in H_2O_2 accumulation (Figure III.23., Table). The relative extent of H_2O_2 formation did not reflect that found when the same quinones were exposed to GSH (see § III.1.1.6. & 1.3.). In the latter case, it had been observed that reaction of the quinones with GSH to yield H_2O_2 decreased in the order BQ > NQ > diMeBQ > MD, with γ-crystallin the reaction decreased in the order NQ > diMeBQ > BQ > MD. Reaction of MD with γ-crystallin generated a greater amount of H_2O_2 than with GSH. Conversely, NAPQI, which reacted with GSH with a weak production of H_2O_2, virtually did not form any H_2O_2 in presence of γ-crystallin.

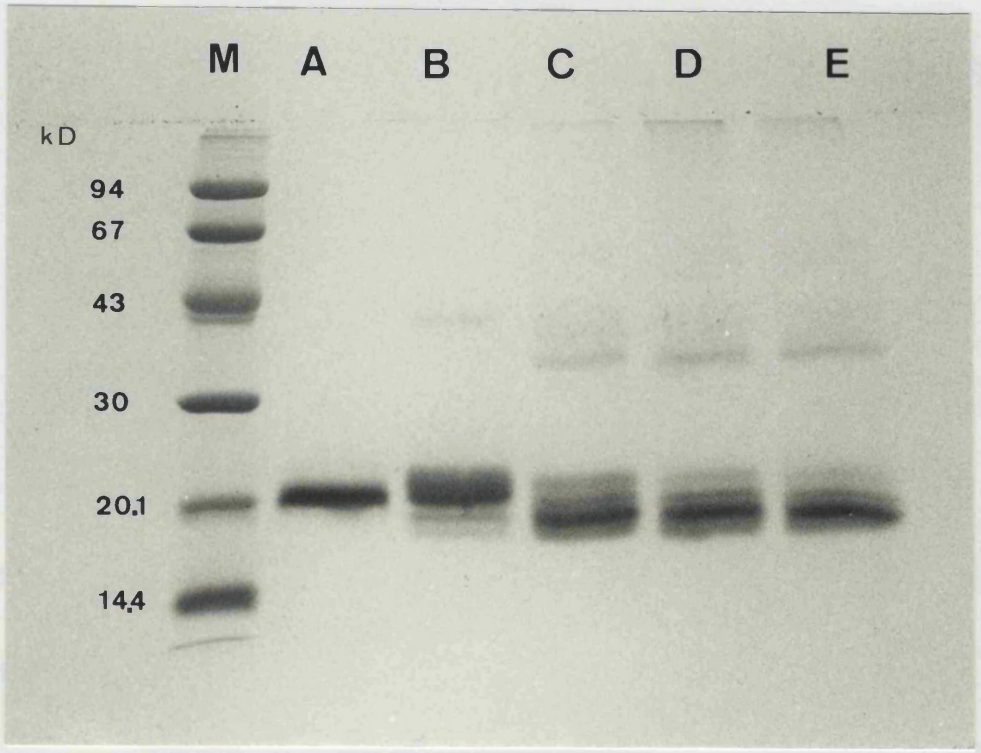
Protein damage:

Regarding protein damage (Figure III.23., SDS-PAGE), all five quinones led to some changes in the pattern of the protein bands. The BQ effect has already been described in § III.2.1.3.1. and was seen to generate overall gross fragmentation, one fragment of specific size and high-molecular-weight non-reducible aggregates. The methylated BQ (Lane C) led to less gross fragmentation (depletion of the parent crystallin was less drastic); however, in addition to the fragment found with BQ, a new fragment probably formed by less than 1 kDa truncation of the parent crystallin was observed. High molecular weight aggregates were also detected.

NQ, MD and NAPQI (Lane D-F) presented similar gel pattern to the diMeBQ, however

Figure III.19.: Protein fragmentation and crosslinking parallel to H_2O_2 production induced by BQ - time course

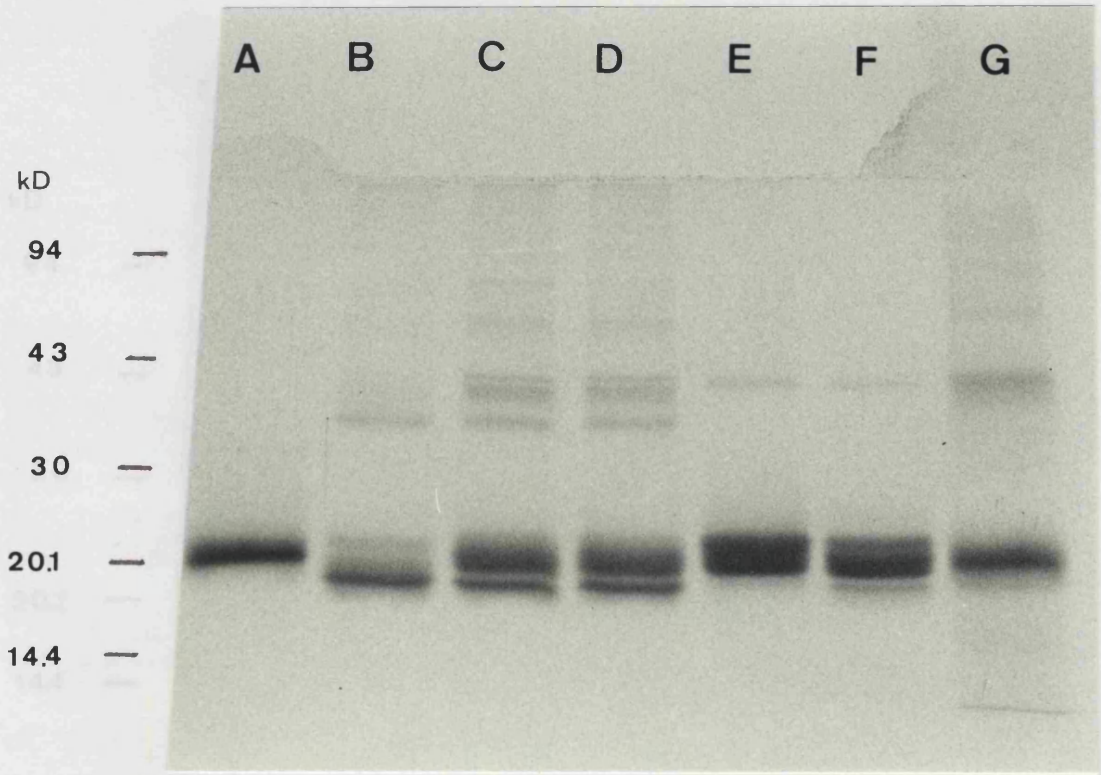
H_2O_2 production (μM)				
Lane A	Lane B	Lane C	Lane D	Lane E
2.4 ± 0.1	2.4 ± 0.1	11.7 ± 0.2	46 ± 1	53 ± 2



SDS PAGE was performed on 12.5% crosslinked gel under reducing conditions and revealed by Coomassie blue.
200 μM thiol of γ-crystallin was incubated with 200 μM BQ, at 37°C, in 100 mM sodium phosphate buffer pH 7.4 containing 15 μM DETAPAC.
Lane M, molecular weight standards; Lane A, native crystallin; Lane B, plus BQ at zero time; Lane C, plus BQ after 30 minutes' incubation; Lane D, plus BQ after 2 hours' incubation; Lane E, plus BQ after 3 hours' incubation.
The attached table gives the corresponding H_2O_2 concentration in the protein incubation mixtures. Data shown are the means ± half the range of duplicate measurements.

Figure III.20.: Protein fragmentation and crosslinking parallel to H_2O_2 production induced by BQ - effect of catalase, sorbitol, GSH and ascorbate

H_2O_2 production (μM)					
Lane A (-BQ)	Lane B (+BQ)	Lane C (+BQ, +catalase)	Lane D (+BQ, +sorbitol)	Lane E (+BQ, +GSH)	Lane F (+ BQ, + AH)
0 ± 0	30.8 ± 0.1	0 ± 0	23.4 ± 0.4	0 ± 0	109 ± 1



SDS PAGE was performed on 12.5% crosslinked gel under reducing conditions and revealed by Coomassie blue.

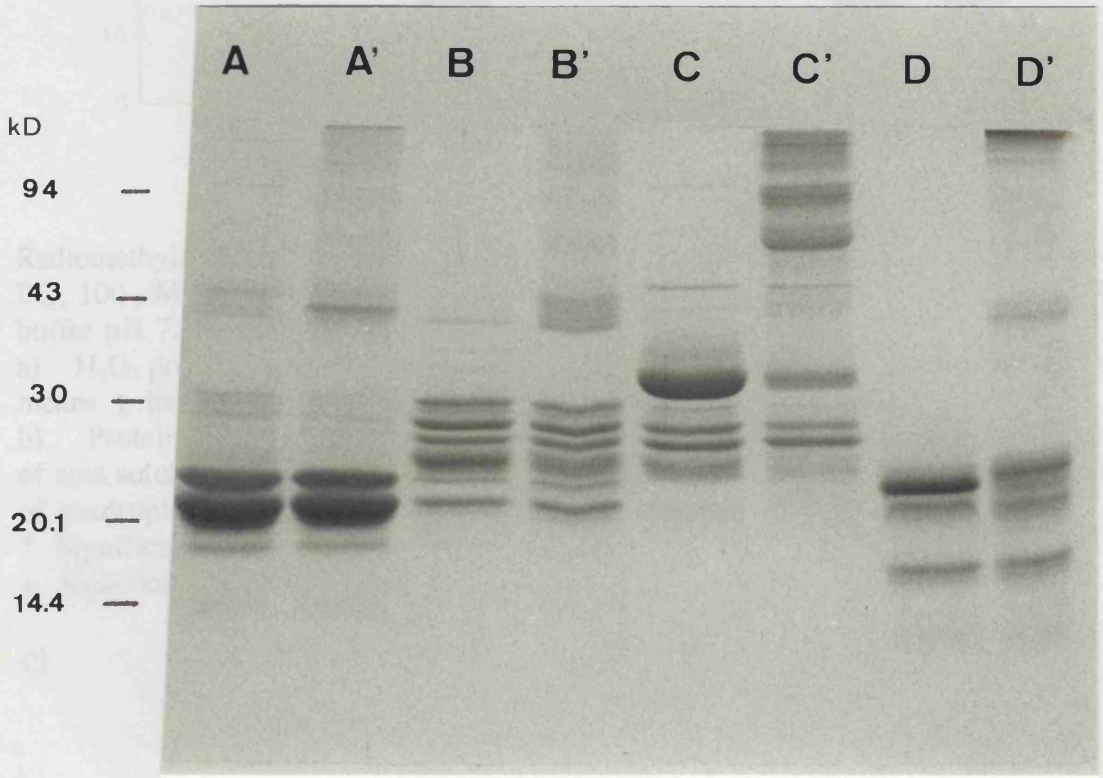
200 μM thiol of γ-crystallin was incubated with or without 100 μM BQ and different agents, at 37°C for 2 hours, in 100 mM sodium phosphate buffer pH 7.4 containing 15 μM DETAPAC.

Lane A, without BQ; Lane B, with BQ alone; Lane C, with BQ + 12.5 U/mL catalase; Lane D, with BQ + 20 mM sorbitol; Lane E, with BQ + 600 μM GSH; Lane F, with BQ + 600 μM ascorbate (AH); Lane G, 100 μM H_2O_2 + 100 μM Cu^{2+} (without BQ). GSH and AH were added 10 minutes after protein exposure to BQ.

The attached table gives the corresponding H_2O_2 concentrations in the protein incubation mixtures. Data shown are the means ± half the range of duplicate measurements.

Figure III.21.: Protein fragmentation and crosslinking parallel to H_2O_2 production induced by BQ - comparison between different crystallins

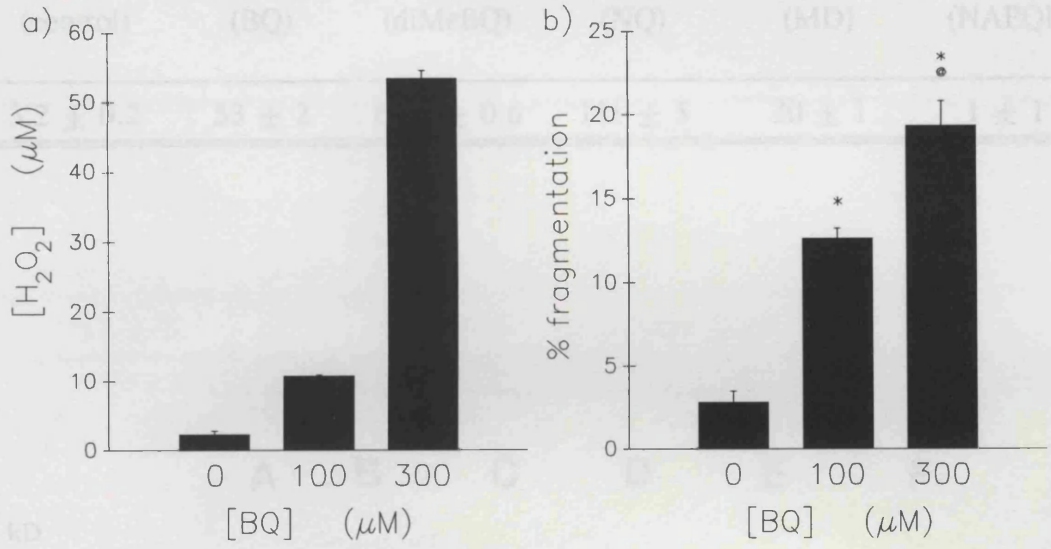
H_2O_2 production (μM)							
# 1		# 2		# 3		# 4	
A (-)	A' (+)	B (-)	B' (+)	C (-)	C' (+)	D (-)	D' (+)
1.1 ± 0.7	13 ± 0.4	0.1 ± 0.1	1.9 ± 0.4	0 ± 0	40 ± 1	0.7 ± 0.7	13.9 ± 0.3



SDS PAGE was performed on 12.5% crosslinked gel under reducing conditions and revealed by Coomassie blue.
200 μM thiol of different crystallins were incubated with or without 100 μM BQ, at 37°C for 2 hours, in 100 mM sodium phosphate buffer pH 7.4 containing 15 μM DETAPAC and 10 mM aminotriazole (to inhibit any catalase activity).
Lane A, #1 without BQ; Lane A', #1 with BQ; Lane B, #2 without BQ; Lane B', #2 with BQ; Lane C, #3 without BQ; Lane C', #3 with BQ; Lane D, #4 without BQ; Lane D', #4 with BQ.
(see Figure II.1b. for the description of the contents of each fraction)
The attached table gives the corresponding H_2O_2 concentrations in the protein incubation mixtures. Data shown are the means ± half the range of duplicate measurements.

Figure III.22.: H_2O_2 production and protein damage induced by BQ - case of bovine serum albumin

a) b)



Radiomethylated bovine serum albumin (BSA) (5mg/mL) was incubated with 0 μM BQ, 100 μM BQ or 300 μM BQ, at 37°C for 3 hours, in 50 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC.

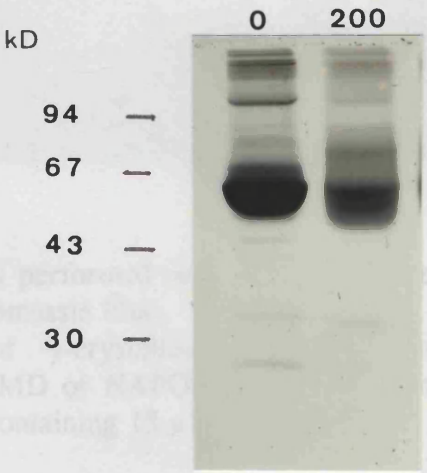
a) H_2O_2 production after the three-hour incubation was measured. Data shown are the means $\pm$ half the range of duplicate measurements.

b) Protein fragmentation after the three-hour incubation was measured as formation of acid soluble radiolabelled material. Data shown are the means $\pm$ standard deviation of quadruplet measurements.

* Significantly different from control (0 μM), $P<0.001$.

@ Significantly different from dose 100 μM BQ, $P<0.001$.

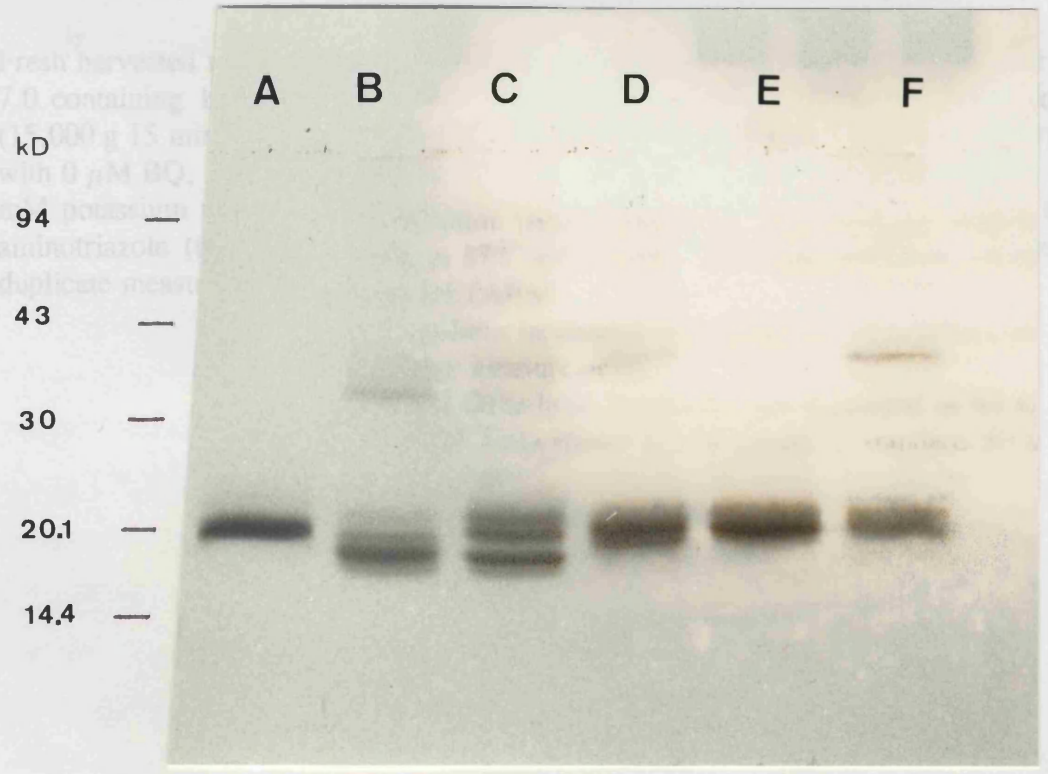
c)



SDS PAGE was performed on 12.5% crosslinked gel and revealed by silver staining. BSA (0.64 mg/mL) was incubated with 0 μM BQ or 200 μM BQ at 37°C for 3 hours in 100 mM sodium phosphate buffer pH 7.4 containing 10 μM DETAPAC.

Figure III.23.: Protein fragmentation and crosslinking parallel to H_2O_2 production induced by different quinones

H_2O_2 formation (μM)					
Lane A (control)	Lane B (BQ)	Lane C (diMeBQ)	Lane D (NQ)	Lane E (MD)	Lane F (NAPQI)
3.2 ± 0.2	53 ± 2	67.5 ± 0.6	111 ± 5	30 ± 1	1 ± 1



SDS PAGE was performed on 12.5% crosslinked gel under reducing conditions and revealed by Coomassie blue.
200 μM thiol of γ-crystallin was incubated in buffer alone or with 200 μM BQ, diMeBQ, NQ, MD or NAPQI, at 37°C for 3 hours, in 100 mM sodium phosphate buffer pH 7.4 containing 15 μM DETAPAC.
Lane A, γ-crystallin in buffer alone; Lane B, with 200 μM BQ; Lane C, with 200 μM diMeBQ; Lane D, with 200 μM NQ; Lane E, with 200 μM MD; Lane F, with 200 μM NAPQI.
The attached table gives the corresponding H_2O_2 concentrations in the protein incubation mixtures. Data shown are the means ± half the range of duplicate measurements.

Figure III.24.: H_2O_2 production upon rat liver homogenate exposure to BQ

H_2O_2 formation (μM)			
0 μM BQ	100 μM BQ	200 μM BQ	500 μM BQ
18 $\pm$ 6	118.7 $\pm$ 0.8	489 $\pm$ 12	653 $\pm$ 33

Freshly harvested rat liver was homogenised in 50 mM potassium phosphate buffer pH 7.0 containing 100 μM DETAPAC. Membranes and other debris were spun down (15,000 g 15 min). 1 mM DTNB accessible thiol of the supernatant was then incubated with 0 μM BQ, 100 μM BQ, 200 μM BQ or 500 μM BQ, at 37°C for 3 hours, in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC and 10 mM aminotriazole (to inhibit catalase). Data shown are the means $\pm$ half the range of duplicate measurements.

without the ~18 kDa fragment, with less gross fragmentation for NQ and MD, virtually no large aggregates for MD but greater amount of high molecular material for NAPQI.

2.3. Rat liver homogenate exposure to BQ

H_2O_2 formation:

In Figure III.24., rat liver homogenate (1 mM DTNB accessible thiol) was incubated for 3 hours with different concentrations of BQ; H_2O_2 was measured at the end of the incubation. BQ was found to interact with the thiols (low molecular weight substrates as well as macromolecules) of hepatocyte cytosol in a similar way to its reaction with GSH or isolated proteins like crystallins or BSA. Large amounts of H_2O_2 were detected in the reaction mixture, always greater than the amounts BQ added.

3. Thiols prevent hydroquinone autoxidation

3.1. GSH effect on HQ

p-Benzohydroquinone (HQ) incubated alone in 100 mM potassium phosphate buffer pH 7.4 at 37°C has been shown to autoxidize, generating H_2O_2 (see § III.1.1.5.). Excess amounts of GSH over HQ-SG have been shown to diminish the accumulation of H_2O_2 at the end of similar incubations (see § III.1.1.3.). The effect of GSH on HQ autoxidation was thus further examined by coincubating HQ with or without GSH in buffer and analysing by HPLC the products of the reaction at zero time and after three hours' incubation (Figure III.25.). Elution profiles attested that the presence of GSH inhibits HQ degradation to BQ (and other products). The table inset in Figure III.25. is evidence of the efficiency of GSH in preventing the concomitant accumulation of H_2O_2. Only 25 μM GSH was able to stop the accumulation of 52 μM H_2O_2 during a three-hour incubation of 200 μM HQ. The ability of GSH to prevent double its amount of H_2O_2 from being formed excludes any mechanisms where GSH reduces H_2O_2 as it is formed (H_2O_2 + 2GSH → GSSG +2H_2O).

3.2. Thiols effect on HQ-SG

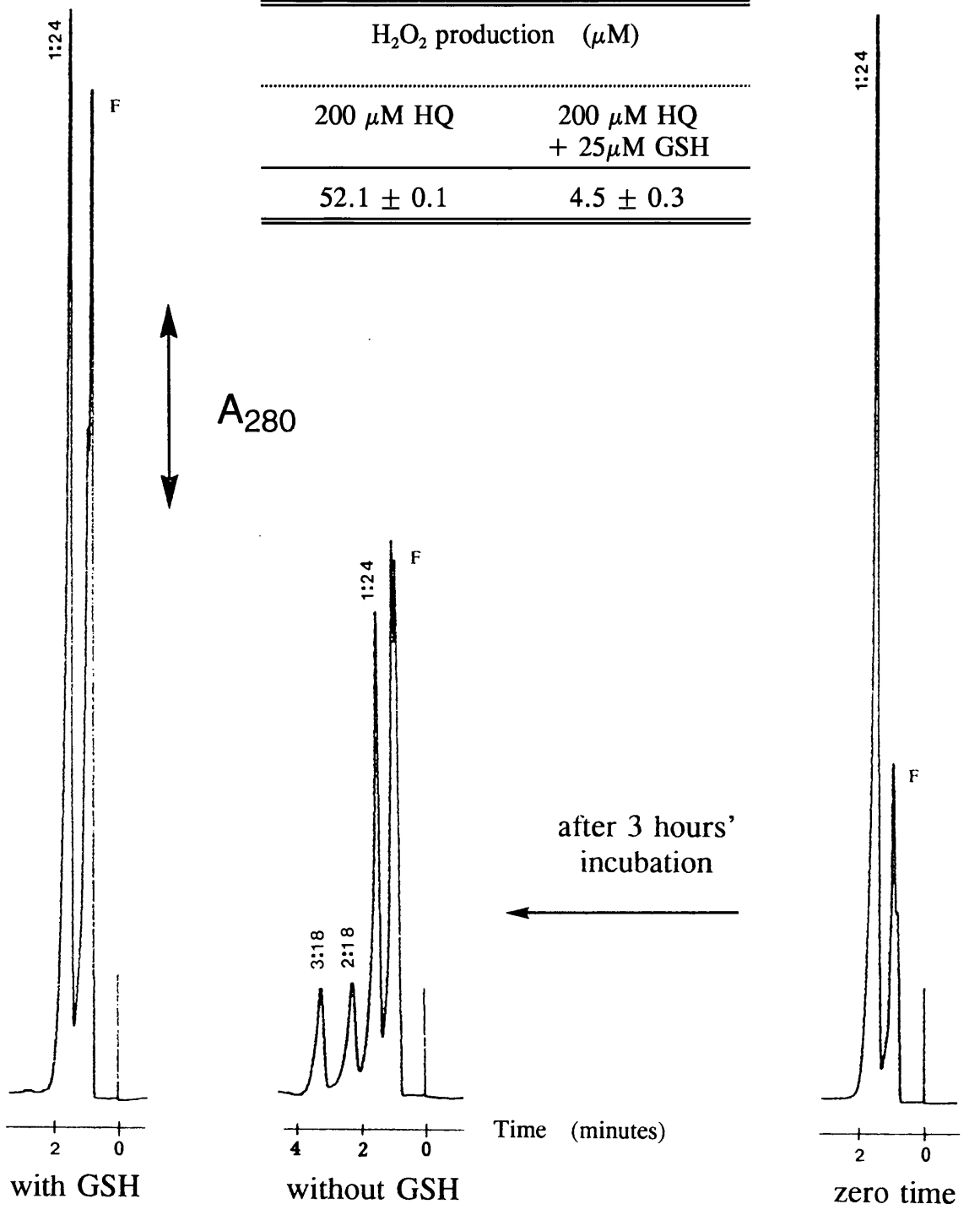
As seen Figure III.6.b, where GSH was added at increasing concentrations to a fixed amount of BQ (200 μM) and H_2O_2 formation measured after 3 hours' incubation, ~600 μM GSH was needed to prevent H_2O_2 accumulation associated with 200 μM HQ-SG oxidation. To make sure that the large excess of GSH added to HQ-SG was not hampering H_2O_2 accumulation by chiefly consuming H_2O_2 before it accumulated, oxygen uptake was monitored during incubation of GSH and BQ at equimolar concentration and subsequent addition of excess GSH or other thiols (Figure III.26.). This figure's charts show that immediate oxygen consumption followed the addition of BQ and that GSH, N-acetylcysteine (NAC), cysteine and dithiothreitol (DTT) were all able to nearly restore the slope of oxygen consumption to its level of before the addition of BQ. NAC was clearly the most effective in this aspect and DTT the less capable. Glycine, used here as reference compound containing amino group but no thiol moiety, failed to significantly alter the slope of the chart of oxygen content of the medium against time.

Figure III.27. further exemplifies the role played by the sulphydryl moiety of GSH in the blocking of HQ-SG autoxidation. In this experiment, BQ and GSH were incubated together, in either equimolar concentration or excess-thiol condition. The excess GSH delayed H_2O_2 formation. When the sulphydryl alkylator N-ethylmaleimide (NEM) was added after the initial 5 minutes where arylation was completed, the blocking effect of GSH on H_2O_2 formation was removed. The two plots with NEM and the one in equimolar condition, without NEM, all followed roughly the same progression of H_2O_2 yield, whereas the plot in excess-thiol condition, without NEM, (empty squares) kept a separate sequence with a very slow rate of H_2O_2 formation though, by the late stage, it finally reached the same level of H_2O_2 accumulation as in the other cases. This last aspect confirms that the low level of H_2O_2 observed up to 8 hr is not the result of the reduction by GSH of newly formed H_2O_2, otherwise a lower level of H_2O_2 would still be expected at 21 hr. The late "catching up" episode is supposedly a consequence of an earlier depletion of the remaining 150 μM GSH.

This hypothesis was investigated (Figure III.28.) for its relevance to the sequence of events observed during quinone-mediated cell injury, where GSH depletion always precedes cell damage (see § I.3.2.2.). 800 μM GSH was incubated in buffer pH 7.4

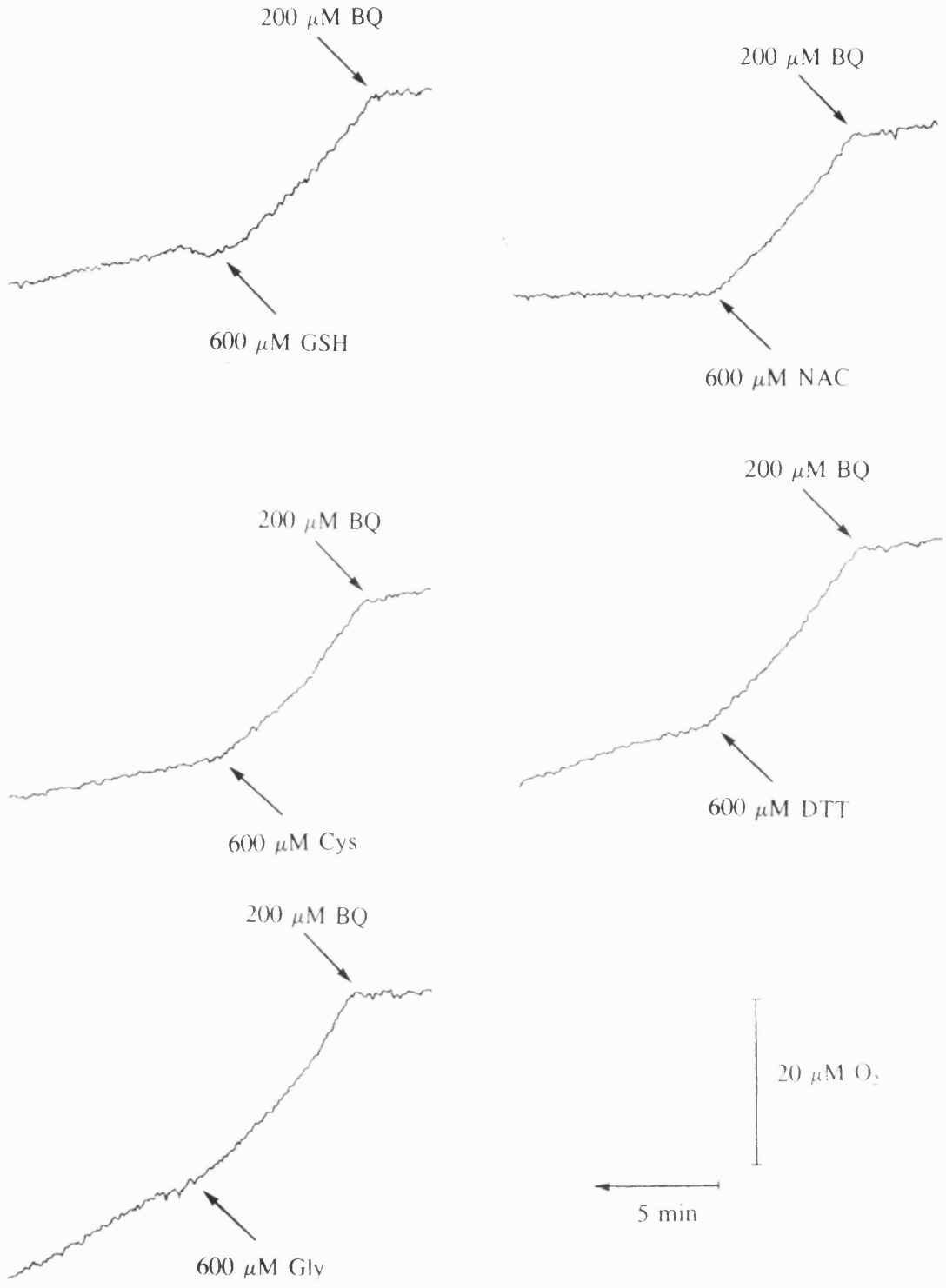
Figure III.25.: GSH effect on HQ autoxidation

H_2O_2 production (μM)	
200 μM HQ	200 μM HQ + 25μM GSH
52.1 ± 0.1	4.5 ± 0.3



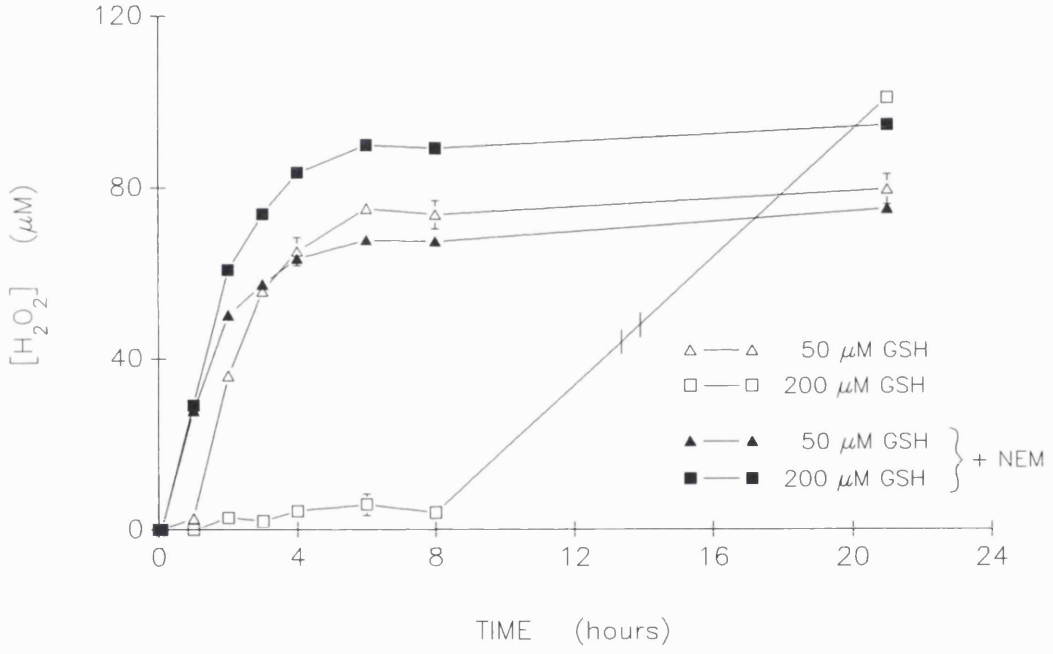
100 μM HQ was incubated at 37°C for 3 hours in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC with or without 100 μM GSH. HPLC elution profiles of the reaction mixtures are presented at zero time (elution profiles were the same with or without GSH) and after three hours' incubation. The time parameter progresses from right to left.
F, solvent front; peak 1:24 (min:s), HQ; peak 3:18 (min:s) BQ.
The attached table gives the H_2O_2 production after a three-hour incubation of 200 μM HQ with or without 25 μM GSH. Data shown are the means ± half the range of duplicate measurements.

Figure III.26.: Thiol effect on HQ-SG autoxidation



Oxygen consumption during exposure of GSH to BQ.
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ and other components at the points indicated.

Figure III.27.: N-ethylmaleimide prevents GSH effect on HQ-SG autoxidation



50 μM GSH or 200 μM GSH were incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C with 50 μM BQ. After five minutes' incubation to allow the arylation reaction to take place, 300 μM N-ethylmaleimide (NEM) was added (or not) to the reaction mixtures and the incubation continued for up to 21 hours. Data shown are the means $\pm$ half the range of duplicate measurements.

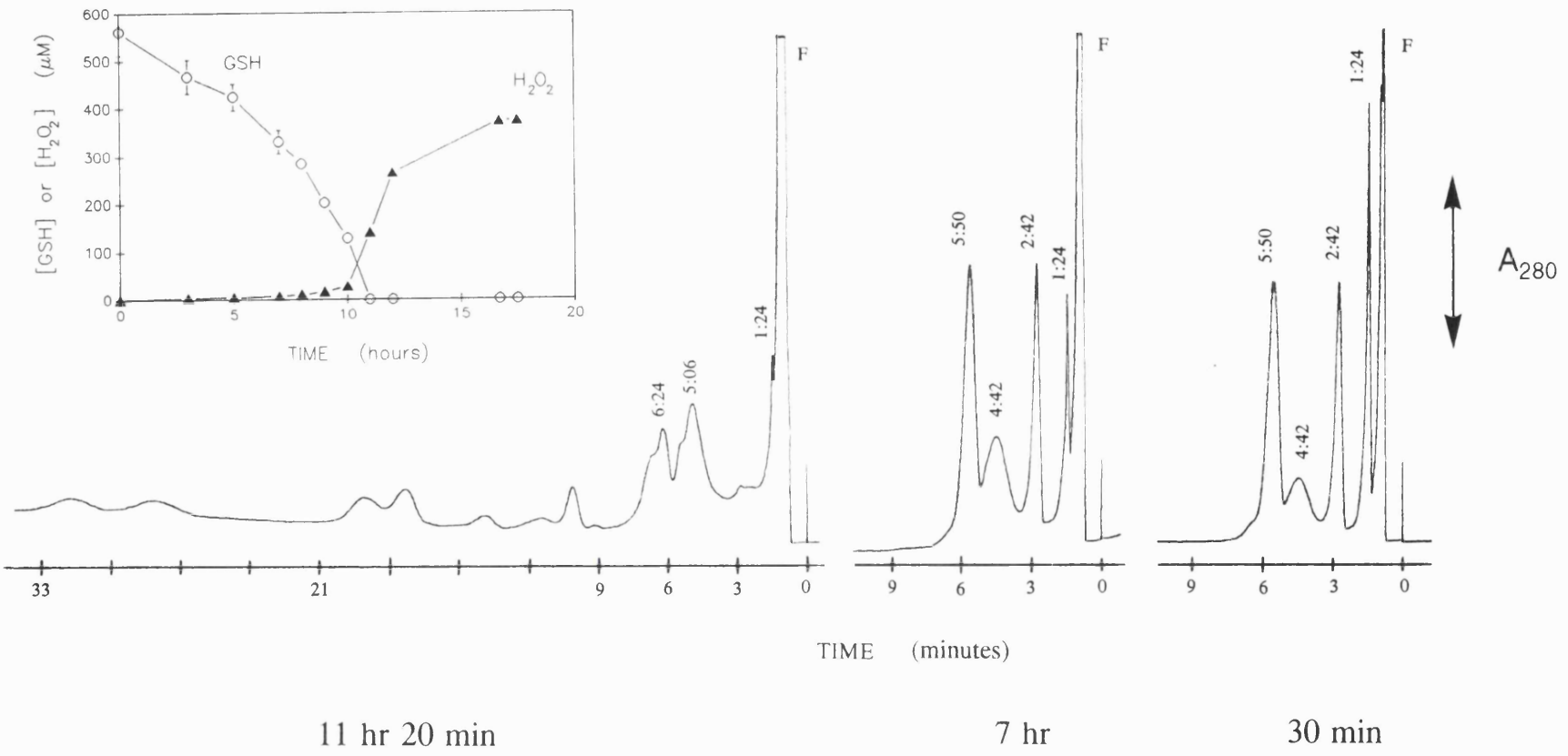


Figure III.28.: Effect of the depletion of excess GSH on HQ-SG autoxidation

800 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C for up to 18 hours with 200 μM BQ. GSH and H_2O_2 levels in the incubation mixture during this time course are presented in the inset (data shown are the means $\pm$ half the range of quadruplet measurements). HPLC elution profiles of the reaction mixture are displayed at several time points of the incubation (time parameters for both elution and incubation progress from right to left).
F, solvent front; peak 1:24 (min:s), hydroquinone; peaks 2:42 (min:s), supposedly monoglutathion-S-yl hydroquinone (see Figure III.4.); other peaks, undetermined (see also Figure III.4.).

containing DETAPAC at 37°C for up to 18 hours during which GSH consumption and H_2O_2 formation were both measured (figure inset III.28.) parallel to a follow through the HPLC elution profiles of the reaction mixture. Three chromatograms were selected at 30 min, 7 hr and 11 hr 30 min to portray the quinone-adduct evolution throughout the course of the incubation and in relation to GSH and H_2O_2 levels. The figure inset shows that the immediate loss of ~200 μM GSH, succeeding addition of 200 μM BQ, was followed by a further sustained consumption of GSH until reaching total depletion at 11 hr. During the same time hardly any H_2O_2 was generated. Once GSH had been entirely depleted, a drastic burst in H_2O_2 production was observed. The quinone adducts represented on the chromatograms looked almost unchanged until at least 7 hr, as referred to the 3 peaks with retention times 2:42, 4:42 and 5:50 (min:s), with only a moderate increase in the height of the centre one (HQ, also formed in the reaction, exhibited a clear decrease in its concentration). Following GSH depletion and accompanying the burst in H_2O_2 production, the three HPLC peaks above mentioned, previously relatively stable, vanished and a plethora of new peaks surged. The new peaks are characterised by higher retention times suggesting a greater lipophil icity and thus perhaps higher oxidised states.

Low molecular weight thiols in general and GSH in particular seem therefore to inhibit hydroquinone autoxidation, the latter occurring overtly after GSH depletion.

3.3. GSH effect on HQ-S-Protein

The inhibiting effect of GSH on hydroquinone autoxidation was further tested on the protein-quinone adduct and the impact on otherwise concomitant protein damage was examined (Figure III.20., Lane E&B). 200 μM thiol of γ-crystallin was briefly exposed to 100 μM BQ, to allow formation of the quinone adduct, and was further incubated in buffer pH 7.4 containing DETAPAC at 37°C with or without 600 μM GSH. After two hours' incubation, H_2O_2 accumulation was measured and an aliquot of the reaction mixture was analysed by SDS-PAGE. No H_2O_2 was detected in the medium at the end of the incubation with GSH, whereas 31 μM H_2O_2 was formed in the absence of GSH. In the electrophoretic study, the presence of GSH (Lane E) not only markedly diminished the loss of the parent crystallin but also totally prevented the formation of the major 18 kDa fragment while both events were induced by BQ without GSH (Lanes A&B and §

III.2.1.3.1.). A high-molecular-weight non-reducible aggregate, apparently a dimer of the parent crystallin, was also visible. It has to be pointed out that the crystallin had to be exposed to BQ for 10 min prior to the addition of GSH and, as seen Figure III.19., where the time course of such γ-crystallin-BQ interaction is followed through by SDS-PAGE, fragmentation and crosslinks appear quickly after the outset of the incubation (Figure III.19 Lane B and § III.2.1.3.1.). Some protein alterations revealed on Figure III.20. Lane E could thus have taken place before GSH was added.

However, GSH appeared to be much more effective than catalase (Lane C) or sorbitol (Lane D) in preventing the quinone damaging effects on γ-crystallin (see also § III.2.1.3.1.).

4. Ascorbate, BQ and diamide effects on the quinone-thioether conjugate

4.1. Ascorbate enhances H_2O_2 yield

Effect on HQ-SG:

Ascorbate (AH) was found to interact with HQ-SG in a complete opposite way to low molecular weight thiols discussed in § III.3.2.. In the experiments summarised in Figure III.29., AH was incubated with BQ alone (a) or with HQ-SG (b) in buffer containing DETAPAC and the oxygen consumption was recorded. Figure III.29.a shows that AH, alone in buffer containing DETAPAC, did not exhibit significant autoxidation. When BQ was added to the oxygen-electrode chamber, an immediate and substantial oxygen uptake followed. In Figure III.29.b, 200 μM GSH was incubated alone in buffer prior to addition at first of 200 μM BQ and subsequently, either 1 min later (Trace 1) or 5 min later (Trace 2), of 600 μM AH. A rapid oxygen uptake followed to the addition of BQ as already described in § III.1.1.2.. This oxygen consumption exhibited an increase after AH supplementation, indicating the presence in solution of a powerful pro-oxidant. AH generated a far lower oxygen uptake with BQ in absence of GSH (Figure III.29.a) suggesting that the powerful pro-oxidant was an oxidized intermediate of HQ-SG. This was indeed illustrated (Figure III.29.b) by the less rapid oxygen consumption observed when AH was added 1 min later than BQ (Trace 1), instead of 5 min (Trace 2), at a time when fewer oxidant molecules had been generated.

Effect on BQ-induced protein damage:

In Figure III.20.Lanes F&B, 200 μM thiol of γ-crystallin was briefly exposed to 100 μM BQ, to allow formation of the quinone adduct, and was further incubated in buffer pH 7.4 containing DETAPAC at 37°C with or without 600 μM AH. After 2 hours' incubation H_2O_2 accumulation was measured and an aliquot of the reaction mixture was analysed by SDS-PAGE. Nearly 4 times more H_2O_2 was detected in the medium at the end of the incubation with AH than without. However, surprisingly, AH diminished not only the loss of the parent crystallin but also the formation of a major 18 kDa fragment (Lane F), both events induced by BQ (Lanes A&B and § III.2.1.3.1.). A high-molecular-weight non-reducible aggregate, apparently a dimer of the parent crystallin, was also visible.

AH, though associated with a greater yield of H_2O_2, appeared to be more effective than catalase or sorbitol but less than GSH (Figure III.20.Lanes C, D and E respectively) in preventing the quinone damaging effects on γ-crystallin.

4.2. BQ and diamide hinder HQ-SG autoxidation

BQ effect on HQ-SG autoxidation:

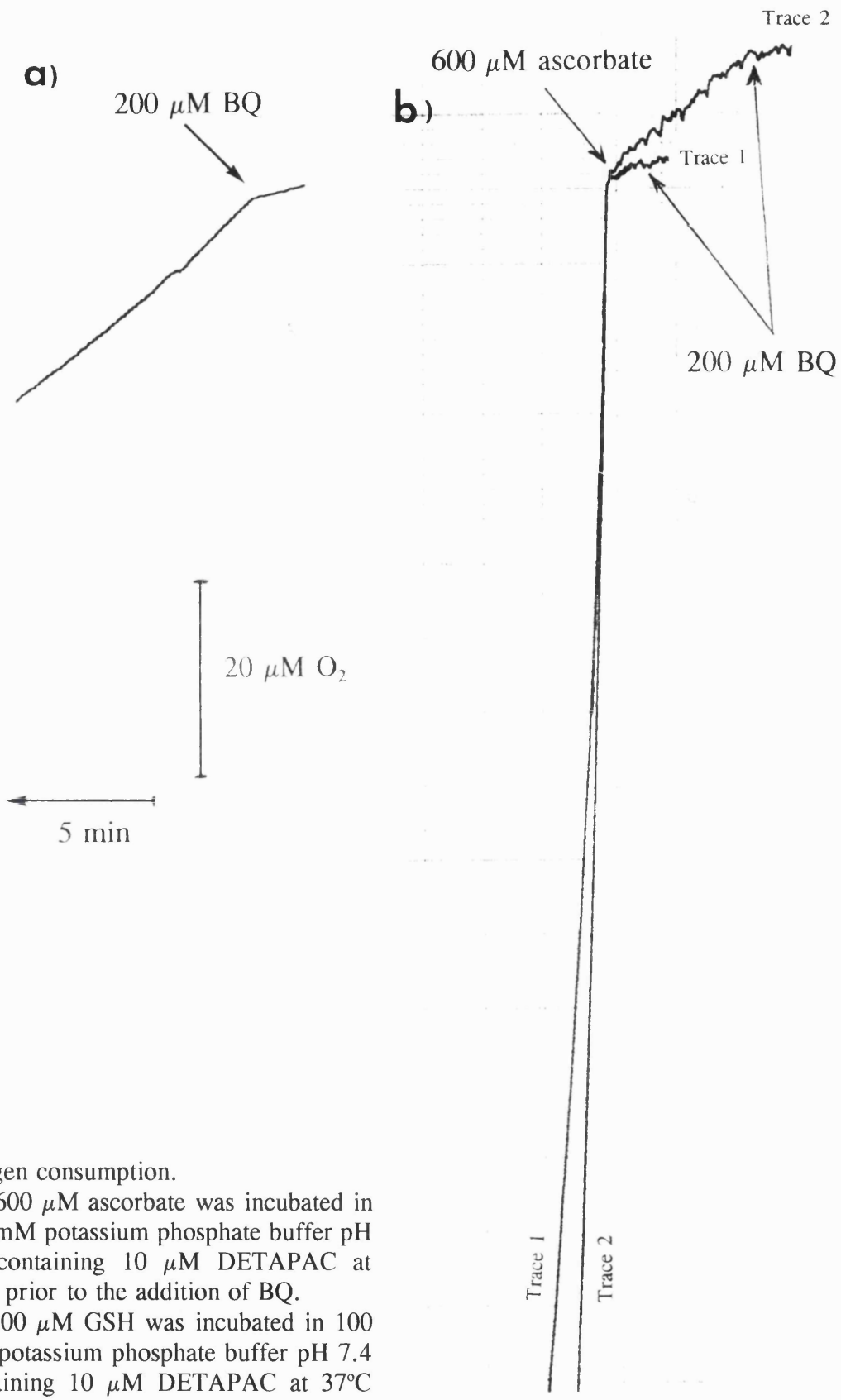
As seen in § III.1.1.3., an excess of BQ over HQ-SG restricted the amount of H_2O_2 otherwise observed at the end of equimolar incubation of GSH and BQ. In the same condition of excess BQ, some HQ was formed and was attributed to the oxidation of HQ-SG by BQ (see § III.1.1.2.). To further understand the action of BQ on the quinone-thioether conjugate, 200 μM GSH was incubated in buffer pH 7.4 at 37°C and its exposure to equimolar and excess amounts of BQ was examined by monitoring oxygen consumption in the solution (Figure III.30.a). Addition, at first, of 200 μM BQ resulted in immediate oxygen consumption, as seen earlier. However, subsequent addition, 5 min later, of 600 μM BQ totally blocked any further oxygen uptake.

Excess amount of BQ over HQ-SG appears then to prevent the conjugate oxidation by oxygen possibly due to its preferential oxidation by BQ itself.

Diamide effect on HQ-SG autoxidation:

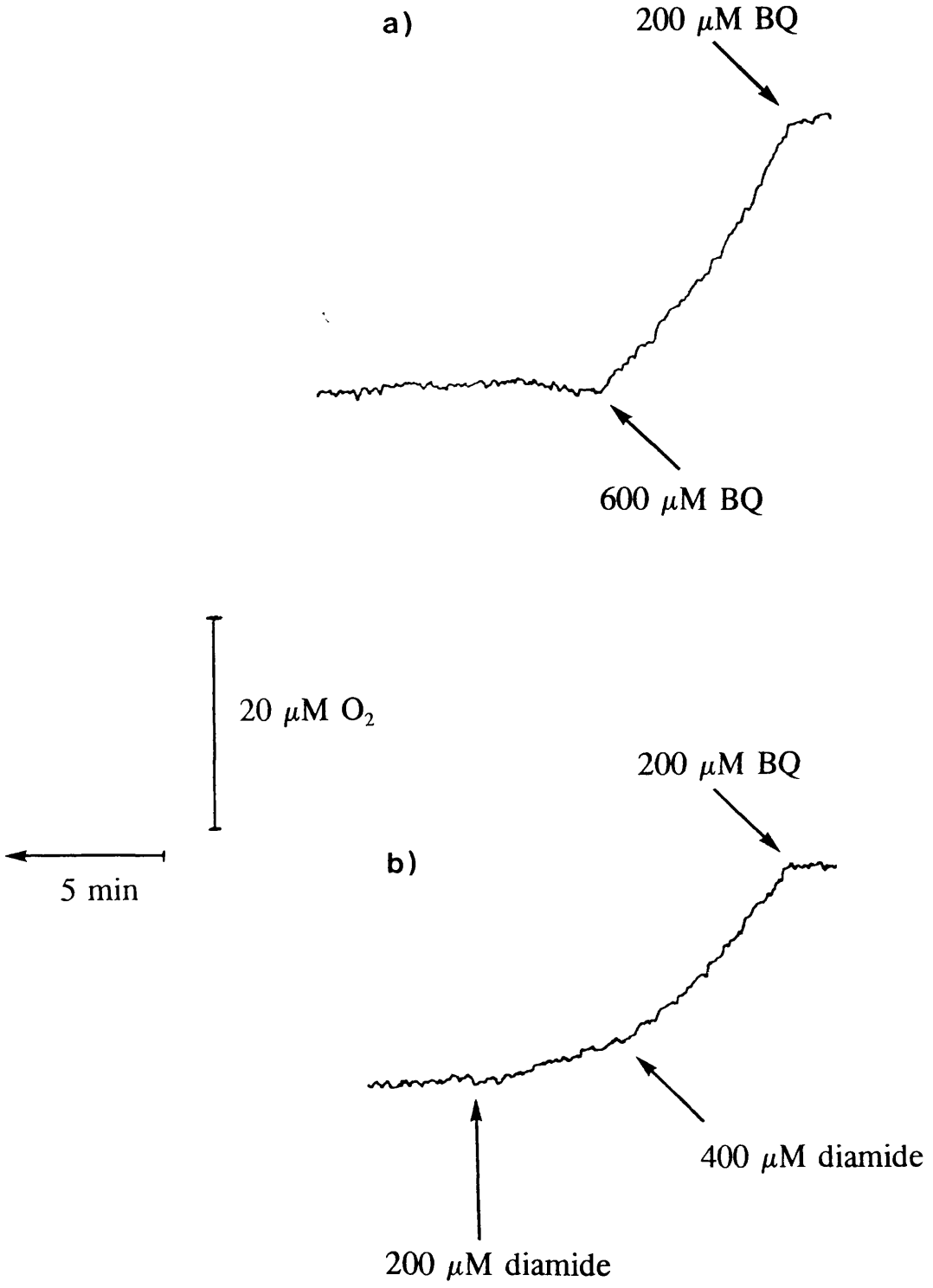
Diamide is known as a thiol-oxidising agent; however, it has also been shown to oxidise a variety of electron donors (O'Brien *et al.*1970). To further test the effect of oxidant

Figure III.29.: BQ and HQ-SG effects on ascorbate



Oxygen consumption.
a) 600 μM ascorbate was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ.
b) 200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ and ascorbate at the points indicated.

Figure III.30.: BQ and diamide effect on HQ-SG autoxidation



Oxygen consumption.
200 μM GSH was incubated in 100 mM potassium phosphate buffer pH 7.4 containing 10 μM DETAPAC at 37°C prior to the addition of BQ and other components at the points indicated.

on HQ-SG autoxidation, diamide was added 5 min after the formation of HQ-SG had started to increase the slope of oxygen utilization (Figure III.30b.), in a similar experiment to the one described above. A first addition of 400 μM diamide diminished the rate of oxygen utilization and an additional 200 μM diamide restored it to its level prior to BQ addition. These data suggest that certain oxidants such as diamide or BQ, at greater concentration than oxygen ($\sim$200 μM dissolved oxygen initially in buffer), are able to oxidise HQ-SG more readily than oxygen does.

5. Liver slice study with paracetamol

5.1. Paracetamol toxicity - quantitative loss of protein-thiol as a poor indicator of irreversible injury

In order to investigate the role of thiols in paracetamol toxicity, the liver-slice model system introduced in § II.4. was used with a toxic dose of paracetamol and both GSH and protein-SH levels were followed throughout the incubation time, in parallel to potassium and lactate dehydrogenase (LDH) leakage. Potassium leakage was used as a marker of "reversible" injury and LDH leakage as a marker of irreversible injury (see § II.7.7. & II.5.7.). As a comparison with the effect of paracetamol on cellular thiols, a non-toxic dose of the thiol-oxidising agent diamide was also applied to liver slices in a parallel experiment. Both sets of data are presented Figure III.31. and described below. Paracetamol (P) has been found to be consistently toxic at the dose of 10 mM in the *in vitro* model system used, where liver slices were obtained from phenobarbital pretreated rats (McLean and Nuttall, 1978; Mourelle *et al.*1991). Liver slices were incubated in Ringer solution alone (control) or with 10 mM paracetamol for a first incubation period of 2 hr (0-2 hr) and then in fresh Ringer solution for a second incubation period of 4 hr (2-6 hr). The control group shows that each of the four parameters measured was relatively stable except that the GSH level diminished and stabilized to half its zero time value by the end of the first incubation period and potassium content also lessened though transiently. This was consistently observed from one experiment to another and is probably due to the stress occasioned to the slices by their preparation. In the paracetamol treated group, GSH was depleted and settled to approximately 10% of

its original level by the end of the first incubation period whereas protein-SH values started to decline only in the second incubation period where paracetamol was no longer present. Potassium content exhibited a sustained decrease from beginning to end while LDH leakage still comparable to control at 3 hr reached about 20% at 6 hr.
When diamide was incubated in the same conditions as paracetamol, but at a dose limit just below toxicity (5 mM), GSH reached the same level as in the paracetamol group at 2 hr but nearly recovered to the control value by the end of the six-hour incubation, whilst protein-SH content declined from the first incubation period, tended to recover at the beginning of the second incubation period but eventually stabilised to reach the paracetamol-treated-group level at 6 hr. Potassium content of diamide treated slices followed the same pattern of change as protein-SH. Its value at 6 hr, however, was much closer to the control than in the paracetamol-treated slices. No LDH leakage was observed at the end of the incubation.
The same quantity of protein-SH loss at 6 hr was associated with tissue damage, in the case of paracetamol treatment, but with tissue survival, in the case of diamide treatment. However, the quantitative loss of protein-SH was apparently associated with cellular dysfunction, as illustrated by its parallel association with potassium release (see diamide plots), and the partial GSH recovery might have prevented the slices from deteriorating.

In Figure III.32., the specific thiol-alkylator N-ethylmaleimide (NEM), the GSH-oxidising agent diamide or the specific thiol oxidant GSSG were added to the first two hours liver-slice-incubation period in the same model system used above. LDH leakage was measured at the end of the six-hour incubation. It appears that these various direct sulphydryl-modifying agents can all provoke severe cell damage, though at lower dose for NEM. Paracetamol, presented here for comparison, is both a thiol-alkylator and -oxidant though acting indirectly following a bioactivation phase.

5.2. Protection against paracetamol-mediated injury

In Figure III.33., the same liver-slice model system was used to assess the ability of a variety of agents to protect cell against paracetamol-mediated injury. Liver slices were incubated with 10 mM paracetamol for the first incubation period of 2 hr and then in

fresh Ringer solution, containing a chemical tested for its protective effect, for a second period of 4 hr. At the end of the six-hour incubation, LDH leakage was measured and comparison was made with liver slices treated with paracetamol only.

Such disparate compounds as N-acetylcysteine (a thiol and precursor of GSH), desferrioxamine (metal chelator), ethanol, N,N'-diphenyl-p-phenylenediamine (lipid-soluble antioxidant), and fructose were found to exhibit some degree of protection in the *in vitro* model system. Although disparate, all these compounds possess, in a wide sense, antioxidant activities. Methionine (a non-thiol sulphur-containing compound and precursor of GSH) failed to significantly prevent lactate dehydrogenase (LDH) leakage probably owing to its lack of free thiol group and more complex path to GSH synthesis in comparison with N-acetylcysteine. The latter aspect would suggest a crucial role for the time parameter in the sequence of events leading to cell damage.

5.3. H_2O_2 formation during paracetamol injury

In order to investigate the participation of oxidative processes in paracetamol-induced cell injury and in an attempt to parallel the reaction of quinones with thiol to yield H_2O_2, observed in non-living system, H_2O_2 formation in liver slices was examined during paracetamol injury (Figure III.34.). The same *in vitro* model system was used as above and H_2O_2 was detected by measuring inhibition of endogenous catalase activity in presence of aminotriazole (AMT) (see § II.5.1.3.). Liver slices were incubated in Ringer solution alone (control) or with 5 or 10 mM paracetamol (non-toxic and toxic doses respectively) for a first incubation period of 2 hr (0-2 hr) and then in fresh Ringer solution for a second incubation period of 2 hr (2-4 hr). Catalase activity and GSH content were measured at 2 hr and 4 hr (Figure III.34.a & b respectively). After the first incubation period (a), a significant decrease in catalase activity was observed for both doses of paracetamol, concomitant with a fall in GSH level.

To make sure that the H_2O_2 detected did not simply ensue from the reduction of GSH content, thus limiting glutathione peroxidase activity, a parallel experiment was set up where the non-redox thiol alkylator diethyl maleate (DEM) was coincubated with or without 5 mM paracetamol. In these conditions, although treatment with DEM alone exhibited some degree of catalase inhibition, exposure to both DEM and paracetamol resulted in a greater inhibition. In both DEM treatment cases, GSH levels matched each

other at values markedly lower than those obtained in the initial experiment (without DEM). GSH depletion, though presenting a slight influence on catalase activity, could not on its own explain the extent of catalase inhibition observed in paracetamol treated groups.

It is conclude that some H_2O_2 was formed during this first incubation period in presence of paracetamol.

After the second incubation period (b), no significant decrease in catalase activity was observed at a dose of 5 mM paracetamol but a pronounced inhibition was shown at the toxic dose of 10 mM paracetamol. At this dose GSH depletion did not exceed the loss produced by DEM exposure.

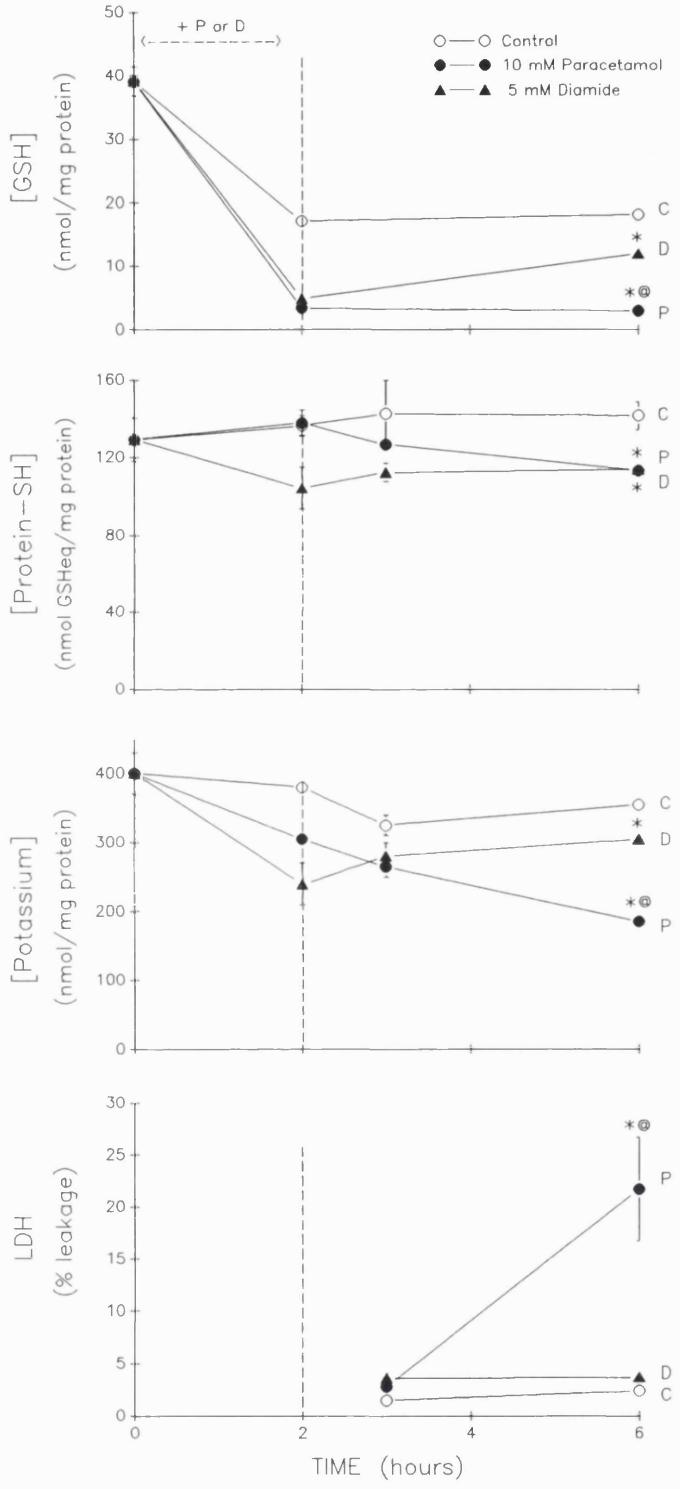
H_2O_2 formation thus was associated with paracetamol-induced cell injury when virtually no free paracetamol existed any more (i.e. independently of paracetamol oxidation to NAPQI through P-450 activity) and when the GSH level had reached its nadir.

Effect of protecting agents on paracetamol-mediated H_2O_2 formation:

N-acetylcysteine (NAC) and fructose (frct) have been shown (§ III.5.2.) to protect liver slices against paracetamol-mediated injury. Figure III.35. shows the effect of these two protecting agents on the H_2O_2 formation observed concomitantly with paracetamol-induced cell injury.

Liver slices were incubated in Ringer solution alone (control) or with 10 mM paracetamol for the first incubation period of 2 hr and then in fresh Ringer solution for a second incubation period of 2 hr with or without NAC or fructose. Catalase activity and GSH content were measured at 4 hr. NAC succeeded in preventing catalase inhibition together with supplying the slices with extra amount of low molecular weight thiols (the contribution of NAC or other byproducts of its metabolism to OPT fluorescence cannot be excluded) whereas fructose failed to curb the decrease in both catalase activity and GSH level, as compared to the paracetamol (only) treated group. This could suggest that a protective dose of low molecular weight thiol is able to prevent paracetamol-mediated H_2O_2 formation in liver slices. However, this H_2O_2 formation, if necessary, does not appear to be sufficient to induce cell injury.

Figure III.31.: Liver slices exposed to paracetamol or diamide - comparison



Liver slices were incubated in Ringer solution alone, with 10 mM paracetamol (P) or with 5 mM diamide (D) for a first incubation period of 2 hours (0-2 hr) and then in fresh Ringer solution for a second incubation period of 4 hours (2-6 hr). Data shown are the means ± standard deviation of quadruplet measurements and significance of differences between groups at 6 hr are indicated.

* Significantly different from control group, $P<0.05$.

@ Significantly different from diamide treated group, $P<0.05$.

Figure III.32.: LDH leakage from liver slices following exposure to paracetamol and other thiol-modifying agents

% LDH leakage (control: 6.2 ± 1.6)			
10 mM paracetamol	250 μM NEM	6 mM diamide	5 mM GSSG
36 ± 1	24 ± 4	30 ± 6	23 ± 1

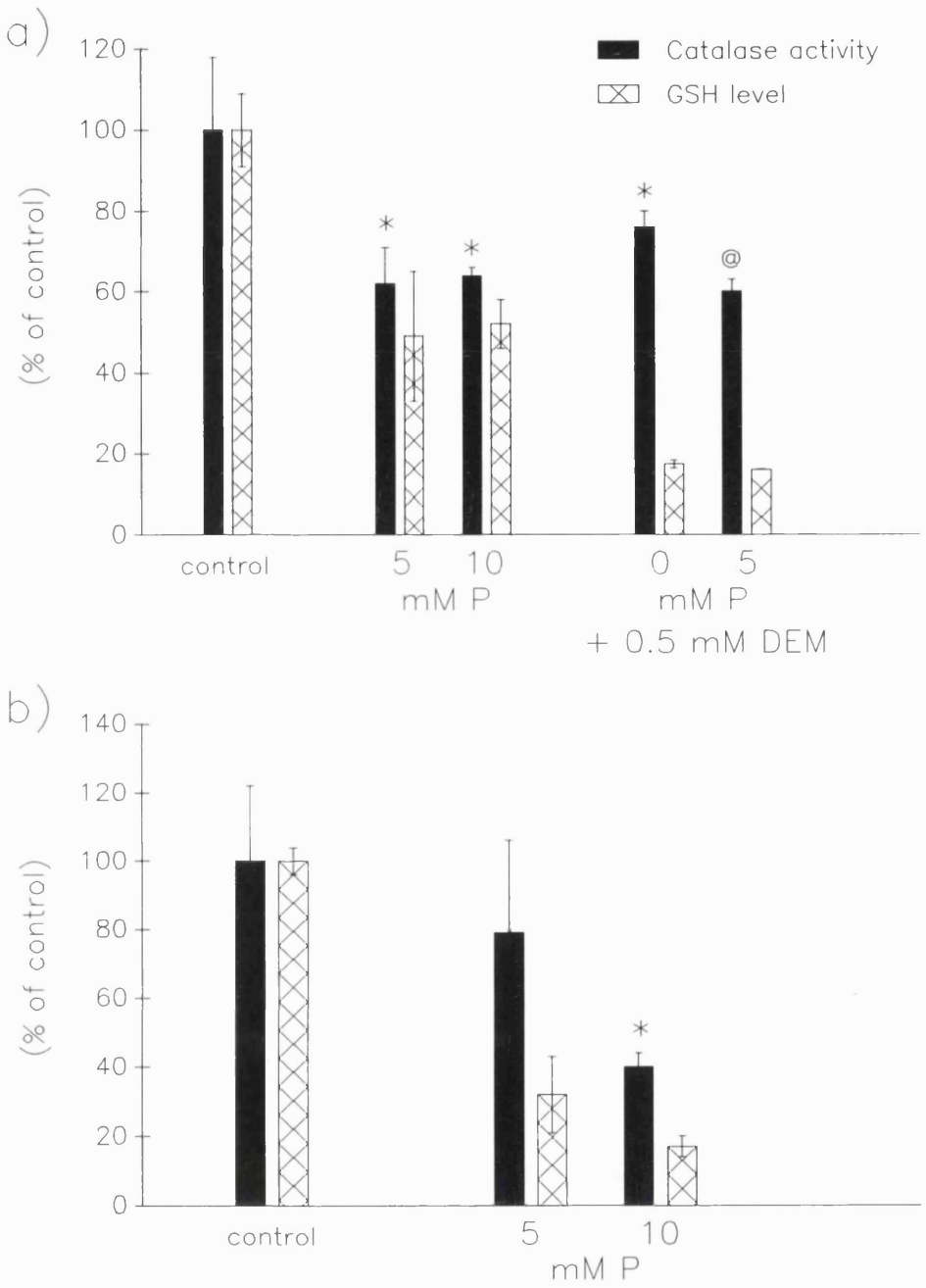
Liver slices were incubated in Ringer solution with 10 mM paracetamol, 250 μM NEM, 6 mM diamide or 5 mM GSSG for a first incubation period of 2 hours (0-2 hr) and then in fresh Ringer solution for a second incubation period of 4 hours (2-6 hr). LDH leakage is expressed as percentages of enzyme activity found in the medium in comparison with the amount originally present in the slices. Data shown are the means ± half the range of duplicate measurements.

Figure III.33.: Prevention of LDH leakage from liver slices following successive exposure to paracetamol and different protecting agents

Components present in the second incubation period	% prevention of LDH leakage
4 mM methionine	6 ± 6
4 mM N-acetylcysteine	56 ± 4
20 mM desferrioxamine	28 ± 2
2 mM ethanol	76 ± 2
38 mM ethanol	94.4 ± 0.8
4 μM DPPD (in 38 mM ethanol)	95.4 ± 0.3
4 μM DPPD (suspension in water)	49 ± 7
20 mM fructose	82 ± 17

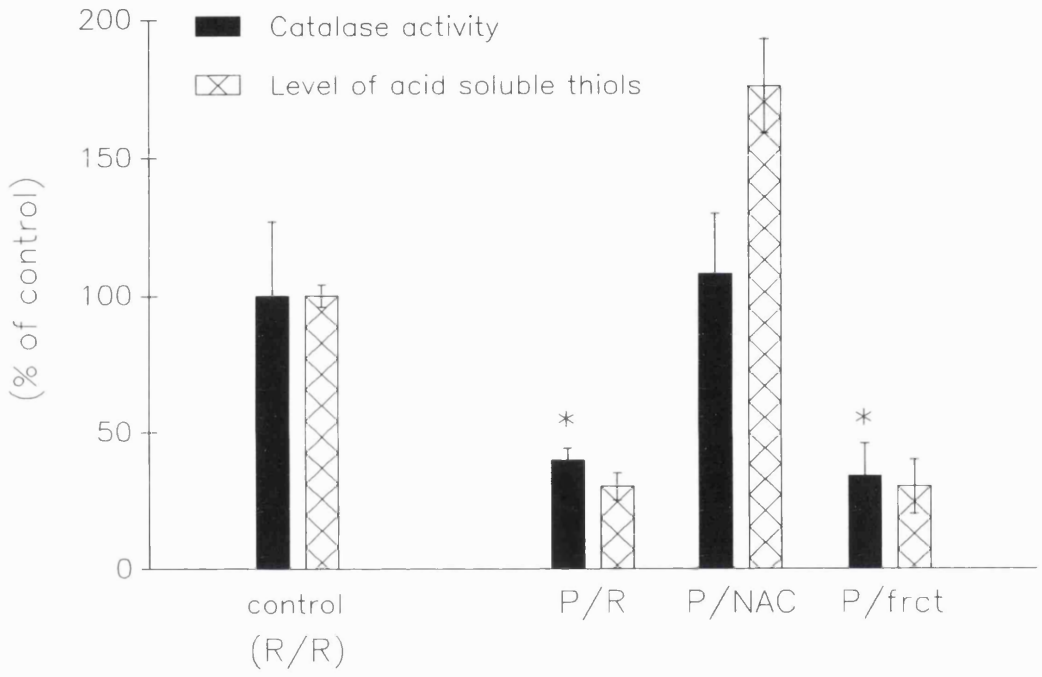
Liver slices were incubated in Ringer solution with 10 mM paracetamol for a first incubation period of 2 hours (0-2 hr) and then in fresh Ringer solution with methionine, N-acetylcysteine, desferrioxamine, ethanol, N,N'-diphenyl-p-phenylenediamine(DPPD) or fructose for a second incubation period of 4 hours (2-6 hr). Prevention of LDH leakage is expressed as percentages of the drop in LDH release, based on the amount of LDH leakage observed in slices solely treated with paracetamol. Data shown are the means ± half the range of duplicate measurements.

Figure III.34.: Generation of H_2O_2 during liver slices exposure to paracetamol



a) Liver slices were incubated for 2 hr in Ringer solution containing 50 mM AMT with either 0 (control), 5 and 10 mM paracetamol (P) or 0.5 mM DEM (diethyl maleate) plus 0 and 5 mM paracetamol.
b) Liver slices were incubated in Ringer solution with 0 (control), 5 and 10 mM paracetamol for a first incubation period of 2 hours (0-2 hr) and then in fresh Ringer solution containing 50 mM AMT for a second incubation period of 2 hours (2-4 hr).
Catalase activity was measured as an indicator of H_2O_2 generation. Data shown are the means $\pm$ standard deviation of quadruplet measurements and significance of differences from control groups, in the case of catalase activity, are indicated.
* Significantly different from control group, $P<0.05$.
@ Significantly different from the group treated with 0.5 mM DEM/0 mM P, $P<0.05$.

Figure III.35.: Generation of H_2O_2 in liver slices upon exposure first to paracetamol then to NAC or fructose



Liver slices were incubated in Ringer solution alone (control) or with 10 mM paracetamol (P) for a first incubation period of 2 hours (0-2 hr) and then in fresh Ringer solution containing 50 mM AMT alone (P/R) or with 4 mM NAC (P/NAC) or 20 mM fructose (P/frct) for a second incubation period of 2 hours (2-4 hr).
Catalase activity was measured as an indicator of H_2O_2 generation. Data shown are the means $\pm$ standard deviation of quadruplet measurements and significance of differences from control group, in the case of catalase activity, are indicated.
* Significantly different from control group, $P < 0.05$.

IV. DISCUSSION

1. Reaction of BQ and other quinones with GSH

1.1. Conjugation versus oxidation

Quinones are known to react with GSH and other thiols by two distinct and competitive reaction mechanisms: firstly by an electrophilic addition and secondly by a redox reaction to the oxidised disulphide and the reduced form of the quinone. BQ presents 4 unsaturated ketone Michael-acceptor moieties (positions 2,3,5 and 6), thereby 4 possible binding sites for GSH. The chemistry of GSH addition to 1,4-naphthoquinones (NQ) is simpler because the adjacent benzene ring limits the nucleophilic addition to one side of the quinone ring (see formulae § I.2.1.). As for NAPQI, it can form GSH conjugates at the C_3 and C_5 positions only (Rosen *et al.*1984). Oxidation of thiol by quinone is less clear, in relation to whether it occurs directly or *via* H_2O_2 generated by quinone redox cycling. Ross *et al.* (1985), analysing the menadione/GSH reaction, found an inhibitory effect of catalase on GSSG formation. Yet, substantial oxidation of GSH was observed even where catalase was present, so indicating the presence of oxidants other than H_2O_2. The electron-transfer from the thiolate anion to the quinone,

$Q + RS^- \rightleftarrows Q^{\cdot -} + RS^{\cdot}$,

is thermodynamically unfavourable (e.g., at pH 7, $E(RSSR^{\cdot -}/2RS^-)$ for cysteine is 650 mV and $E(Q/Q^{\cdot -})$ for BQ, NQ and MD are respectively 78, -140 and -203 mV (Wardman, 1989)). However the removal of the thiyl radical from the system,

$GS^{\cdot} + GS^{\cdot} \rightarrow GSSG$

$GS^{\cdot} + GS^- \rightleftarrows GSSG^{\cdot -}$ & $GSSG^{\cdot -} + Q$ (or O_2) $\rightarrow GSSG + Q^{\cdot -}$ (or $O_2^{\cdot -}$)

$GS^{\cdot} + O_2 \rightarrow GSOO^{\cdot}$,

can easily drive the reaction over to the right (Wilson *et al.*1986a; Wardman, 1990). The results of the chemical reaction of BQ with GSH indicate the prominence of GSH

conjugation over its oxidation. NQ, MD and NAPQI reveal a more balanced reaction between the two processes (§ III.1.3.). The relative importance of each of the two reaction mechanisms is sometimes the subject of conflicting data. The reaction products of quinone-thiol reactions are dependent on various factors such as nature of the solvent, pH, presence or absence of oxygen, and the thiol to quinone ratio (Monks *et al.*1992). For instance, the products of the spontaneous reaction of NAPQI with GSH are reported to be conjugate, paracetamol and GSSG. The latter is formed in amounts stoichiometric with paracetamol, however the ratios found between amounts of conjugate and paracetamol greatly vary among reports (Rosen *et al.*1984; Albano *et al.*1985; Coles *et al.*1988).

1.2. Conjugation coupled with hydroquinone autoxidation

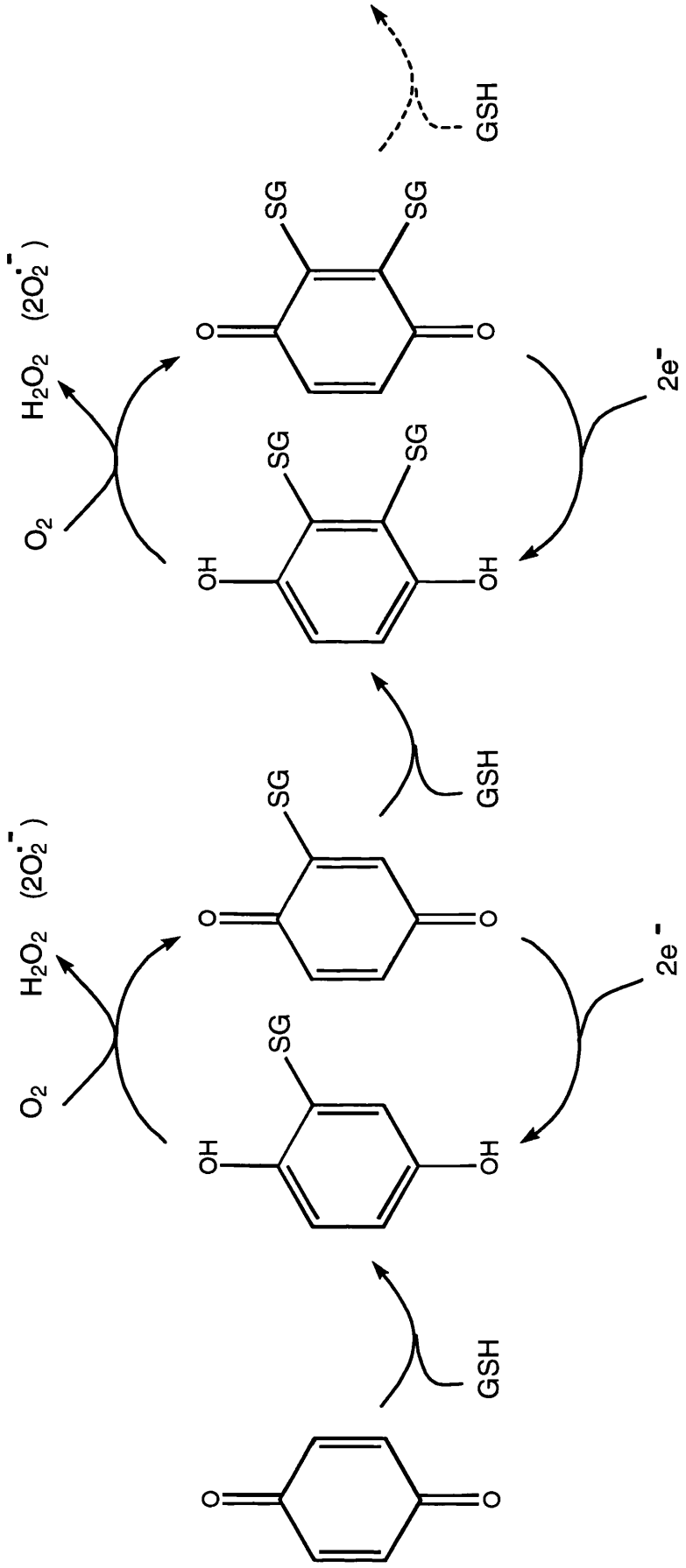
1.2.1. Conjugation as part of a redox cycle

Like their parent hydroquinones, some hydroquinone-thioether conjugates of GSH are subject to autoxidation. Formation of semiquinone free radicals and activated oxygen species can thus occur following electrophilic addition of quinones to thiols. This is in addition to the more generally accepted mechanism of low molecular electron-carrier-driven or enzyme-catalysed one-electron reduction of quinones and one-electron autoxidation of hydroquinones. Considering that the glutathionyl substitute of some quinones enhances the rate of electron transfer to O_2 with ultimate formation of H_2O_2 (§ III.1.1.5.), quinone-thioethers could undergo an even more extreme redox cycle than unsubstituted quinones if they were to be easily reduced.

Now that polyglutathion-S-yl hydroquinones have been identified (Lau *et al.*1988) (see also § III.1.1.1.), a limited redox cycling is attested, where GSH, oxygen and the quinone with increasing degrees of glutathione substitution are the participants. Furthermore it has been proposed that DT-diaphorase, an enzyme catalysing two-electron reduction processes, is able to reduce not only 1,4-naphthoquinones but their glutathionyl conjugates too (Brunmark and Cadenas, 1989; Buffinton *et al.*1989).

Reductive addition of quinone, followed by what could be termed "conjugative oxidation", has then the potential to generate large amounts of reactive oxygen species. The overall scheme of postulated events is described in Figure IV.1..

Figure VI.1.: Scheme representing the succession of reductive addition on quinone and conjugative oxidation as part of a redox cycle



1.2.2. Roles of reduction potential, pK_a and metals

Hydroquinone autoxidation is understood to be a chain reaction (Bandy *et al.*1990) proceeding *via* an initiation reaction,

(1) $H^+ + QH^- + O_2 \rightleftarrows Q^{\cdot -} + 2H^+ + O_2^{\cdot -}$,

followed by propagation reactions,

(2) $H^+ + QH^- + O_2^{\cdot -} \rightleftarrows Q^{\cdot -} + 2H_2O_2$

(3) $Q^{\cdot -} + O_2 \rightleftarrows Q + O_2^{\cdot -}$

(4) $Q + QH^- + H^+ \rightleftarrows 2Q^{\cdot -} + 2H^+$

(5) $H^+ + QH^- + H_2O_2 \rightleftarrows Q^{\cdot -} + {\cdot}OH + OH^- + 2H^+$

(6) $Q^{\cdot -} + H_2O_2 \rightleftarrows Q + {\cdot}OH + OH^-$,

and eventually ending by termination reactions,

(4') $Q^{\cdot -} + Q^{\cdot -} + 2H^+ \rightleftarrows Q + QH^- + H^+$

(7) $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightleftarrows H_2O_2 + O_2$

(8) $Q^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightleftarrows Q + H_2O_2$

(Ishii and Fridovich, 1990; O'Brien, 1991; Dikalov *et al.*1992).

Reduction potential & pK_a:

The reduction of oxygen species by hydroquinone or semiquinone radical implies that the reduction potentials of the quinone forms are lower than those of dioxygen (at pH 7, $E(O_2/O_2^{\cdot -}) = -330$ mV, $E(O_2^{\cdot -}, 2H^+/H_2O_2) = 940$ mV (Wardman, 1989)). However, even quinones with a reduction potential higher than O_2 (e.g., at pH 7, BQ: $E(Q^{\cdot -} + H^+/HQ^-) = 459$ mV and $E(Q/Q^{\cdot -}) = 78$ mV (Wardman, 1989)) still transfer an electron to oxygen to form $O_2^{\cdot -}$(Powis, 1989) probably because of the rapid removal of $O_2^{\cdot -}$ by its spontaneous dismutation to H_2O_2. The ease with which hydroquinones autoxidize therefore reflects only partly their redox potential.

The pK_a of hydroquinones is also of consequence to the rate of their autoxidation, as seen in § I.2.3.2. and III.1.1.4.. For instance trimethylhydroquinone with a reduction potential superior to the one of tetramethylhydroquinone, $E(Q^{\cdot -} + H^+/HQ^-)$ of 385 mV and 350 mV respectively at pH 7, but a lower pK_a, 10.8 versus 11.2, has a higher

autoxidation rate (3.9 nmol/min) than tetramethylhydroquinone (2.7 nmol/min) (O'Brien, 1991). The ease with which hydroquinones autoxidise appears to be a compromise reflecting both the reduction potential and the pK_a of these hydroquinones. The hydroquinones of lowest reduction potential and pK_a values oxidise the fastest.

Transition metals:

Transition metals are potential catalysts of hydroquinone autoxidation (James and Weissberger, 1938; Bandy *et al.*1990). Autoxidation of ferrous (Fe^{2+}) or cuprous (Cu^{+}) ions generate $O_2^{\cdot -}$ (Baynes, 1991) which is a major propagator of the chain reaction (reaction 2). On the other hand, ferric (Fe^{3+}) or cupric (Cu^{2+}) ions probably oxidise the semiquinone radical (Powis, 1989; Dikalov *et al.*1992) regenerating the reduced ions, $Q^{\cdot -} + Fe(III) \rightarrow Q + Fe(II)$.

The involvement of metals in quinone redox activity is supported by the property of some quinones to chelate metal. Quinone-iron complexes have thus been shown to redox cycle more actively with ascorbate or GSH than non-complexed quinones (Dikalov *et al.*1993). This is generally attributed to the ability of ascorbate and GSH to reduce the chelated Fe(III) which in turn facilitates the formation of the semiquinone via intramolecular electron transfer:

$Fe(III)\text{-}Q + AH \text{ (or GSH)} \rightarrow Fe(II)\text{-}Q + AH^{\cdot} \text{ (or } GS^{\cdot}) + H^{+}$

$Fe(II)\text{-}Q \Leftrightarrow Fe(III)\text{-}Q^{\cdot -}$.

The apparent lack of effect of copper on the glutathionyl-hydroquinone conjugate (§ III.1.1.4.) is probably due to the presence of other catalyst(s) such as quinone or semiquinone moieties overpowering the metal action. Metal chelators like DETAPAC (used in most chemically defined studies in § III.) have been reported to have no effect on hydroquinone autoxidation (O'Brien, 1991).

1.2.3. Substituent effect

Alteration of the reduction potential of the quinone by the glutathionyl substituent is not enough to explain the enhanced autoxidisability of the conjugated hydroquinone examined in § III.1.1.5.. It has been previously shown that the one-electron potential ($E(Q/Q^{\cdot -})$) of menadione and its GSH conjugate do not differ significantly (-203 mV

and -192 mV respectively at pH 7 (Wilson *et al.*1986b)), although the rate of autoxidation of the latter was found to be higher (Buffinton *et al.*1989).
As measured by H_2O_2 accumulation after one hour's incubation (see § III.1.1.6.) and in agreement with previous reports (Brunmark and Cadenas, 1988), the autoxidation of the glutathionyl conjugate of the three quinones ranked in decreasing order: 2-methyl-BQ > 2,6-dimethyl-BQ > BQ. This sequence does not only relate to the reduction potentials or the pK_a of the parent hydroquinones since those ($E(Q/Q^{\cdot -})$, $E(HQ^{\cdot -}/HQ^-)$ and pK_a of QH_2) all order as follows: BQ > 2-methyl-BQ > 2,6-dimethyl-BQ (Wardman, 1989; O'Brien, 1991). Thus the glutathionyl substituent probably does not always influence the reduction potential and the pK_a of the hydroquinone conjugates in the same direction and its position on the aromatic ring may be important. Several substituents may also interfere with one other, interfering with the one single substituent influence on the hydroquinone. Indeed the reduction potential of the different glutathionyl p-benzohydroquinones identified by Lau *et al.* (1988) was not found to relate proportionally to their degree of glutathionyl substitution. Furthermore the reduction potential of the different diglutathionyl-hydroquinone conjugates differs depending on the position of the glutathionyl substituents on the ring.

1.2.4. Antioxidant can stimulate quinone autoxidation

One might have anticipated that ascorbate and superoxide dismutase (SOD) would inhibit quinone autoxidation by acting as "antioxidants", yet they were found to markedly enhance quinone autoxidation (§ III.4.1. and III.1.1.2.).

1.2.4.1. Ascorbate

Ascorbate is a powerful reductant. However its action as a prooxidant is well known (Wayner *et al.*1986) but less well understood. Several hypothetical mechanisms may be involved in the action of ascorbate. Its prooxidant activity is usually described as a result of its ability to reduce metals to forms that react with O_2. For instance, the synergistic prooxidant effect of ascorbate on hydroperoxide-dependent lipid peroxidation has been shown (Laudicina and Marnett, 1990). Yet, intracellular reductants such as ascorbate are also able to reduce quinones (Thor *et al.*1982). Analysing the ascorbate-

quinone interactions on Ehrlich ascites tumour cells *in vivo*, a direct correlation between the cytotoxicity of the quinone/ascorbate preparation and the generation of long lived semiquinone free radicals was apparent (Pethig *et al.*1983). A synergism of action between ascorbate and menadione on tumour cell growth *in vitro* seems to be mediated by H_2O_2 generation since the effect is completely abolished by the presence of catalase (Noto *et al.*1989).

One could then speculate that, in our system (§ III.4.1.), ascorbate reduces the newly formed glutathionyl-quinone conjugate by a one-electron reduction to the semiquinone form which in turn could readily react with oxygen and thereby accelerate oxygen uptake (reaction 3 § IV.1.2.2.). Ascorbate could also stimulate oxygen consumption through its property as $O_2\cdot^-$ scavenger (reduction of $O_2\cdot^-$) (Baynes, 1991). In this respect, the mechanism would be similar to the SOD example explained below.

If oxidative reactions between BQ and GSH are of any physiological importance then it is important to recognise that H_2O_2 will be produced not purely as a result of glutathionyl-hydroquinone autoxidation, but also as a result of accumulation of its oxidation products, presumably a powerful catalysis of ascorbate oxidation.

1.2.4.2. Superoxide dismutase

Superoxide dismutase (SOD) has been reported to either inhibit or stimulate autoxidation of different hydroquinones (Ishii and Fridovich, 1990; Bandy *et al.*1990). There are several explanations for the diverse actions of SOD; all reflect the diversity of roles played by $O_2\cdot^-$.

The inhibiting action of SOD is believed to result from both metal chelation by the SOD apoprotein and chain reaction termination (reaction 7 § IV.1.2.2.) by the superoxide dismutase activity. It has even been suggested that SOD could have a superoxide:semiquinone oxidoreductase activity (Cadenas *et al.*1988; Bandy *et al.*1990) which would thus participate in the termination of the chain reaction (reaction 8 § IV.1.2.2.).

On the other hand, stimulatory effects of SOD on autoxidation of hydroquinone (§ III.1.1.2.) are believed to result from the absence of termination reaction (reaction 8 § IV.1.2.2.), because of the removal of one of the reactants ($O_2\cdot^-$), and to occur in

reactions where SOD-generated H_2O_2 is a better oxidant than O_2 (reactions 5 & 6 § IV.1.2.2.), or in which semiquinone or quinone formation is limited by the accumulation of $O_2^{\cdot -}$ (reaction 1 or 3 § IV.1.2.2.). In the last case, SOD removes accumulating $O_2^{\cdot -}$ thus displacing equilibria in favour of the forward reactions, and thereby autoxidation.

1.3. Primary, not solely, role of thiols

As seen in § I.2.5.2., thiols are the main targets of quinone electrophilic attack. However the ability of compounds containing basic nitrogen to undergo Michael addition with a variety of quinones has been well documented (Finley, 1974). Furthermore, Gant and coworkers (Gant *et al.*1986), observed the formation of free radicals following the interaction of BQ and NQ with the amino group of glycine and other amino acids; however this occurred much less readily compared with thiol compounds.

The reaction of BQ with the amino group to yield H_2O_2, suggested in § III.1.1.8., may be of importance in relation to the fate of the quinone adduct on a cysteinyl residue of a protein. In the absence of neighbouring thiol groups or low-molecular-weight reducing agents, the quinone moiety would still be able to react with amino groups from the same protein or from adjacent ones. This would result in the formation of more reactive oxygen species upon further oxidation as well as intra- or intermolecular protein cross-links.

1.4. Different reactivity of thiols

Regarding the different rates of H_2O_2 production observed upon quinone exposure to thiols of different nature (see § III.1.1.7. & III.2.1.3.2.), several explanation are possible. The following discussion concerns both non-protein and protein thiols. Explanations can be found in the differential reactivity of SH groups. This has been well reviewed by Torchinsky (Torchinsky, 1981) and can be listed as follows. Firstly, concerning protein-SH only, the accessibility of the thiol has an obvious influence on its further reactivity. On that subject, it has to be noted that after the binding of a quinone molecule to the first SH group, a conformational change can follow which, in turn, can

increase the accessibility, thereby reactivity, of formerly buried thiol groups. A similar "zipper" mechanism has been observed during the interaction between glyceraldehyde 3-phosphate dehydrogenase and the thiol-modifying agent *p*-mercuribenzoate (Smith and Schachman, 1971). Secondly, the pK_a of the various sulphydryls determine the thiol reactivity since, as seen in § I.3.1., it is the thiolate anion which take part in most reactions. The lower the pK_a is, the faster the reaction will take place. pK_a values of the SH groups of proteins vary within wide limits under the influence of a variety of factors: spatial arrangement within the protein (SH groups buried in a hydrophobic environment have higher pK_a values than SH groups on the surface) and electrostatic influence of neighbouring charged groups (Snyder *et al.*1981) (positively charged groups lower thiol pK_a, whereas negative ones raise it). Finally, functional groups located close to SH groups can facilitate the approach of thiol-reagents by advantageously orientating the reagent vis à vis the SH group or, on the contrary, hamper their approach by repelling them by charge exclusion.

Another explanation for the various intensities observed from the reactions of BQ with several thiols to yield H_2O_2 is the possible different substituent effect exerted by the sulphydryl compounds on the reduction potential and pK_a of the hydroquinone moiety (see above sections).

1.5. Conjugation *versus* oxidation in cytotoxicity - extrapolation to the cell

1.5.1. GSH depletion, ROI generation and synergistic action of GSH conjugate and GSSG

When quinone oxidises GSH, it results in the consumption of two molecules of GSH and in the formation of one molecule GSSG together with one molecule of reduced quinone. The latter compound can then autoxidise, generating H_2O_2, which, with glutathione peroxidase, would lead to even more GSH consumption and GSSG formation.

When quinone binds GSH, an hydroquinone-thioether conjugate of GSH is formed. The autoxidisability of this compound is often greater than the unsubstituted hydroquinone, so more H_2O_2 could be generated and thus, with glutathione peroxidase present, oxidised glutathione will be formed as well as arylated glutathione.

Naturally, a biologically useful aspect of conjugate formation resides in the facilitation of biliary elimination from the liver. Moreover, the maintenance of a low intracellular steady state concentration of both S-conjugate and GSSG is crucial since each of them has a potentially harmful effect (see § I.2.4. & I.3.2.3.2.). Nevertheless the two compounds probably share the same transport system (see § I.3.2.3.2.) and competition between the transport of GSSG and that of glutathione S-conjugates from perfused rat liver into the bile has been demonstrated (Akerboom *et al.*1982; Sies, 1988). The simultaneous presence of both glutathionyl-hydroquinone conjugates and GSSG could then result in the retention of redox-active glutathionyl-hydroquinone conjugates inside the cell. The latter, in turn, may inhibit glutathione reductase (Bilzer *et al.*1984) with the consequence of increasing the intracellular concentration of GSSG even further. Glutathionyl-hydroquinone conjugates and GSSG both formed simultaneously could thus conspire in a synergistic action accelerating the process of cell defence exhaustion and thereby cell death.

In hepatocytes, 2,3-dimethoxy-1,4-naphthoquinone, which does not undergo the arylation reaction, redox cycles as well as menadione, however it is significantly less cytotoxic than menadione to hepatocytes, suggesting that arylation of nucleophiles may be a more selective mechanism for causing cytotoxicity than redox cycling alone (Gant *et al.*1988).

1.5.2. Enlarged target field

The role of GSH in conjugating potentially harmful lipophilic electrophiles is to generate S-conjugates that are frequently less toxic and always more water soluble thereby eliminable via the bile, if the reaction occurs in the liver. However, evidence indicatesthat a variety of quinone-thioethers derived from GSH keep their reactivity (see § I.2.4.), suggesting that GSH-quinone conjugates may provide a "transport" form of a reactive intermediate which will exert its toxic action at sites distant from its formation. Indeed, poly-substituted thioethers of 4-dimethylaminophenol consisting of GSH and cysteine residues of haemoglobin were detected in erythrocytes (Eckert *et al.*1990b).

The bifunctional nature of glutathionyl-hydroquinone conjugates confers on them the potential to interact with enzymes that possess a GSH binding site besides those that contain reactive cysteine residues. There are thus further possible toxicological

consequences. Affinities of the GSH conjugates of menadione and toluquinone for NADP-linked 15-hydroxyprostaglandin dehydrogenases were actually 2- and 10-fold higher, respectively, than the quinones themselves. Both of these quinone-thioethers were also inhibitors of prostaglandin B_1 oxidation (Chung *et al.*1987). Furthermore, GSH conjugates of several halogenated quinones have been synthesized as potentially specific irreversible inhibitors of the GSH S-transferases, enzyme utilizing GSH for the detoxication of a large number of electrophiles. 2,6-Dichloro-3-(glutathion-S-yl)-1,4-benzoquinone succeeded in a 41-fold increase in the rate of enzyme inhibition when compared to the parent quinone (van Ommen *et al.*1988; van Ommen *et al.*1991).

2. Interaction of BQ and other quinones with protein-SH

2.1. Conjugation coupled with hydroquinone autoxidation

BQ was found to interact with thiol-proteins in a way similar to that observed with low molecular weight thiols. Indeed, reductive addition was followed by "conjugative oxidation" with the associated generation of large amounts of reactive oxygen species (§ III.2.1.1 & 2.1.2.).

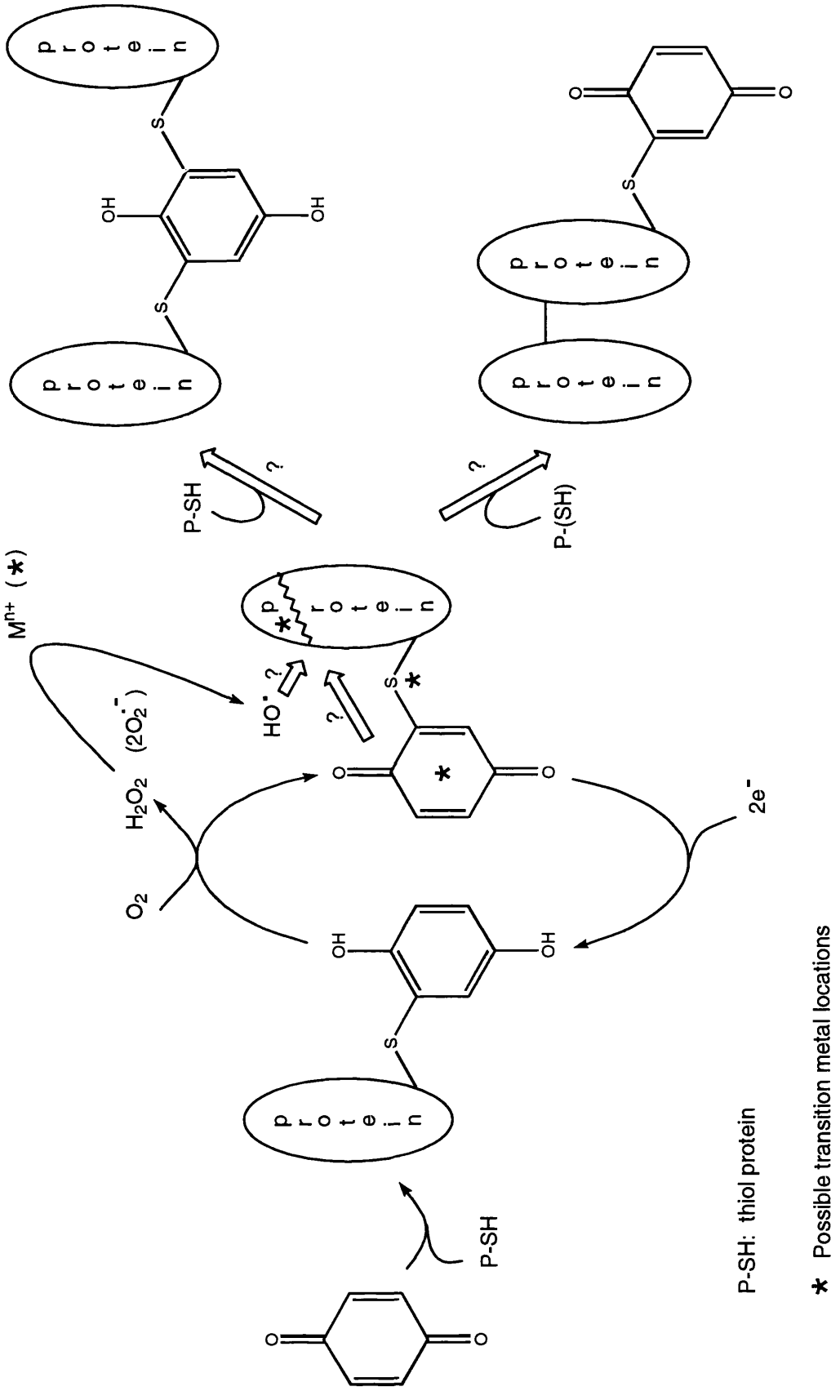
There is however a major difference between the fate of GSH-quinone adducts and protein-quinone adducts. The former are normally rapidly excreted out of the cell, whereas the latter could result in the formation of long-lived prooxidant compounds contributing to constant fluxes of reactive oxygen species over extended periods of time, as well as macromolecular damage (see following section). The overall scheme of postulated events is described in Figure IV.2..

2.2. Protein damage

2.2.1. Fragmentation and cross-linking

Besides arylation of SH groups and probable radical-mediated (reactive oxygen species or semiquinone) intra- and intermolecular disulphide crosslinks in proteins, fragmentation and non DTT-reducible agglomerates were also observed when proteins

Figure VI.2.: Scheme representing the fate of quinone adduct on thiol-protein



were incubated with BQ (§ III.2.1.3.1.).

The last-named damage can be interpreted as either the result of dityrosine bonds induced by ·OH for instance (Halliwell and Gutteridge, 1989) or the product of a protein-quinone adduct electrophilic attack on a second protein molecule, following the same process responsible for the formation of polyglutathionyl-hydroquinones (§ III.1.1.2.).

As to the fragmentation associated with quinone exposure, though few cases have been documented with proteins, fragmentation has been well characterised for DNA (Eliot *et al.*1984; Weiner, 1994; Zhang *et al.*1994). It is known that H_2O_2 can be converted in the presence of transition metals into ·OH, which in turn can cause cleavage of nucleic acid and proteins (§ I.4.1. & I.5.); iron has therefore naturally been suspected as being a necessary factor for the DNA cleavage activity of quinones. As seen in § IV.1.2.2., some quinones can chelate metals. Anthracyclines such as daunorubicin and adriamycin (doxorubicin) form even very strong complexes with metals (Powis, 1989). Adriamycin forms a complex with Fe(III) in which three molecules of adriamycin are bound to one Fe(III) with a stability constant of the complex which is among the highest reported for any Fe(III) chelate. Adriamycin is even able to extract Fe(III) from ferritin, a reaction that is facilitated by the reduction of Fe(III) to Fe(II) by the semiquinone radical or $O_2{\cdot}^-$ (Powis, 1989). Adriamycin-iron complex, bound to DNA, keeps the same capacity to redox cycle with oxygen and thiols that the free complex has and, consequently, causes substantial H_2O_2 yield, DNA aggregates and extensive DNA cleavage (Eliot *et al.*1984). In fact, quinones in their iron-complex stimulate the formation of ·OH even though non-chelating analogs also generate some (Dikalov *et al.*1992).

The various sensitivities of the different crystallins toward BQ-induced fragmentation and crosslinking (§ III.2.1.3.2.) can probably be attributed to the different reactivity of thiol groups of the proteins as explained in § IV.1.4. and in the next section.

2.2.2. Site specific damage

Selective fragmentation of γ-crystallin was observed following its incubation with BQ (§ III.2.1.3.1.). This could be caused by BQ covalently attached to a site on the protein, such as a thiol group, critical for integrity. Similar observations have been made

with DNA. The DNA cleavage induced by the DNA-binding adriamycin-iron complex mentioned above (Eliot *et al.*1984) is not random but gives rise to a fragment of defined size. Similar observation has also been reported with proteins after co-incubation of isolated reaction centres of the photosystem II issue from thylakoids and a quinone, under UV-B irradiation (Friso *et al.*1994). In that case, a fragment of 22 kDa containing the N-terminal moiety of one of the proteins is generated, which seems to be associated with the binding of the quinone to a site that made up an hydrophilic loop. Neither oxygen nor protease activity are involved. The authors then speculate, in view of the relatively high optical absorption properties of the quinone in the UV-B region and of the high reactivity of the semiquinone radical, that the excited semiquinone form of the adduct is the promoter of the peptide bond cleavage.

Two hypotheses may be put forward to explain the BQ-mediated site-specific crystallin cleavage. First, the site could be defined by the presence, next to a reactive SH group, of a cleavage-sensitive amino acid residue like proline (see § I.5.). The semiquinone-form of the hydroquinone-adduct, generated by the adduct's autoxidation, may exert the oxidative attack. Second, the site could be an SH group involved in a Fenton-reactive transition metal complex. The attack would then be brought about by H_2O_2 and its conversion into $^{\cdot}OH$ by the chelated metal.

In both instances, catalase and the hydroxyl radical scavenger sorbitol are unlikely to prevent this site-specific fragmentation because of the absence of oxygen radical in the former case and owing to the vicinity of the reactants (H_2O_2, metal and protein) in the latter. GSH and to a lesser extent ascorbate are able to prevent this fragmentation (§ III.2.1.3.1., 3.3 & 4.1). In the case of GSH, this probably result of its preventing effect on the adduct autoxidation (further discussed in § IV.3.). As to ascorbate, this is paradoxical since ascorbate promotes H_2O_2 formation. The large excess of ascorbate over the protein-quinone adduct used in this experiment (Figure III.20.), if not entirely consumed at the end of the incubation, may explain this observation. The radical scavenging property of ascorbate could have prevail over its prooxidant activity and thereby protected the protein (Halliwell and Gutteridge, 1989).

However this latter result questionsthe role of H_2O_2 in relation to protein damage.

2.2.3. Link with H_2O_2

The adriamycin-iron complex-mediated DNA fragmentation mentioned above (Eliot *et al.*1984) is prevented by addition of catalase, underlying the key role played by H_2O_2. However DNA fragmentation happens at peroxide concentrations more than 3 orders of magnitude below the point where peroxide alone damages DNA. Effectively, the DNA-bound adriamycin-iron complex needs only trace amounts of peroxide to catalyse the cleavage of DNA (Eliot *et al.*1984). Although the ability of quinones to generate oxygen radicals correlates with the capability of cleaving DNA, as far as catalase action is concern, H_2O_2 could be a necessary but not sufficient condition. Indeed adriamycin, which effectively generates H_2O_2 in the presence of DNA and RNA, leads to degradation of double- and single-strand DNAs but does not affect RNA. Now adriamycin can form a complex with DNA, whereas it remains nearly inert toward RNA (Weiner, 1994). Thus, the formation of an adriamycin-nucleic acid complex appears to be necessary for the oxygen radical-mediated cleavage of nucleic acid.
Two types of fragmentation are observed (§ III.2.1.3.1.), random and size-specific. The random one is likely to relate to a substantial yield of H_2O_2 which would react with free metal chelates still fenton-reactive in solution. This would be consistent with the decrease in gross fragmentation observed when the reaction mixture is supplemented with catalase or sorbitol. Conversely, the size-specific fragmentation, occurring *via* one of the two mechanisms described in the last section, would be either H_2O_2-independent or would require only trace amounts of H_2O_2.

2.3. Possible implications in cataract

As described in § I.3.4., transparency of the lens depends on the maintenance of the crystallin structure as well as the short-range order between crystallins. Steady formation of H_2O_2, associated with disulphide formation, as well as fragmentation and non-reducible crosslinks of crystallins (§ III.2.1.) are all to alter crystallin integrity and thus to be cataractogenic.
It is worth emphasizing the dramatic consequence that redox-active crystallin-quinone conjugates can have on cataractogenicity. Lens crystallins with a high content of reducing agents and virtually no turnover in mature cells should be especially sensitive to the

redox activity of the quinone moiety and concomitant protein damage. Indeed, reducing agents such as ascorbate could sustain the redox cycle and produce persistent fluxes of H_2O_2. Furthermore, damaged proteins could accumulate through lack of removal by proteases (§ I.3.4.).

3. Importance of free thiols in preventing hydroquinone autoxidation - paradoxical role of GSH

3.1. Thiol compounds prevent hydroquinone autoxidation

Transition metals are potential catalysts of hydroquinone autoxidation (see § IV.1.2.2.) by their action of increasing the ability of quinones to gain or lose electrons. Protection by GSH against hydroquinone autoxidation (§ III.3.) could thus be compared with the protection by GSH against ascorbic acid autoxidation (Winkler, 1987) which is believed to happen through the formation of complexes between GSH and copper, the catalyst of the autoxidation reaction. Yet, although copper catalyses p-benzohydroquinone autoxidation, it does not significantly affect its glutathionyl-conjugate autoxidation (§ III.1.1.4.). Furthermore, buffers supplemented with DETAPAC or desferrioxamine do not prevent autoxidation. Though GSH could complex metals in a more redox-inert state than DETAPAC and desferrioxamine, metals do not seem to be the important catalyst here.

The work of James and Weissberger (1938) is of great interest. The authors showed that durohydroquinone autoxidises after an induction period and that this induction period is eliminated if some duroquinone is added. They therefore concluded that the quinone formed during the hydroquinone autoxidation acts as a catalyst for the autoxidation, probably via comproportionation. The delaying effect of GSH and other thiols on the autoxidation of the hydroquinone conjugate may thus reflect the removal of the quinone and/or the semiquinone required for the initiation and propagation of autoxidation. Supporting this idea, the same authors demonstrated (James *et al.* 1938) that sodium sulphite (Na_2SO_3) inhibits the autoxidation of hydroquinone and its methylated homologs, mono- di- and tri-methylhydroquinones, with the exception of durohydroquinone (tetramethylbenzoquinone cannot bind nucleophiles); that is to say,

Na_2SO_3 acts in those cases only where it can form hydroquinone sulphonates (HQ-SO_3Na) with the quinone.

3.2. GSH depletion and cell injury

As seen in § I.3.2.2., GSH depletion always precedes cell damage during quinone-mediated cell injury. Nevertheless, the exact relationship between GSH and cell death has not been clarified (Reed and Fariss, 1984; Brunmark and Cadenas, 1989). The prevention of hydroquinone autoxidation by thiol compounds and the correlation between GSH depletion and the onset of hydroquinone autoxidation (§ III.3.), if extrapolated to the cell level, could suggest a possible cause-effect relation between GSH depletion and arylating quinone-induced cell injury. Indeed, a cell exposed to an arylating quinone should experience, before complete depletion of GSH, a globally protective action from the latter. In those conditions, GSH would reduce eventually formed H_2O_2 and prevent macromolecules from arylation. It would also impede both hydroquinone and glutathionyl-hydroquinone autoxidation. However, once depleted, GSH could instead have the potential to actively contribute to quinone-induced toxicity by taking part (through its redox-active quinone-conjugate) in a sudden burst of reactive oxygen species.

It is important to note here that, at the stage following GSH depletion, covalent binding and redox cycling could both occur, even if the quinone exposure was ceased. This is because the GSH-quinone conjugate, whose excretion could be delayed by competitive inhibition with GSSG (see § IV.1.5.1.), would react both as a redox cycler and as an arylating agent much like its parent quinone.

In addition, the protein-quinone adduct, already formed to some extent (covalent binding is detected with sub-hepatotoxic doses of arylating agent like paracetamol and before total depletion of hepatic GSH (Roberts *et al.*1991)), would be freed to redox cycle and thus to generate destructive radical species in the vicinity of the bound protein.

4. Paracetamol-mediated cell injury

4.1. Protein-SH and cell injury - qualitative change *versus* quantitative change

It is well established that cysteine is the main molecular target for the covalent and oxidative modifications of proteins by paracetamol and NAPQI (Streeter *et al.*1984; Hoffmann *et al.*1985a; Hoffmann *et al.*1985b). As seen in § I.3.3., protein-SH comprises key-proteins whose modifications and consequent inactivity are suspected as being crucial for causing cell damage, leading to cell death.

A number of investigators have observed a significant depletion of cellular protein thiol groups in paracetamol- or NAPQI-treated rat hepatocytes (Albano *et al.*1985; Moore *et al.*1985; van de Straat *et al.*1987a). However, similar levels of covalent binding occur after *in vivo* treatment of mice with paracetamol and with a non-hepatotoxic regioisomer. The adduct distribution among the different subcellular compartments of hepatocytes is different and the ability to inhibit key proteins related to calcium homeostasis is opposed (Tirmenstein and Nelson, 1989). The majority of the covalent binding after a cytotoxic dose of paracetamol to hepatocytes occurs on proteins of 44 and 58 kDa (Bartolone *et al.*1987), and data suggest that both the specificity of covalent binding and the extent of binding to the major targets may play an important role in the ensuing toxicity (Birge *et al.*1989). Another report shows that one of the major target protein-thiols of hepatocytes treated with NAPQI is a 17 kDa microsomal protein that was identified as a microsomal glutathione S-transferase (Weis *et al.*1992). Furthermore, protection against paracetamol hepatotoxicity in mice *in vivo* is found to be associated with changes in selective but not total covalent binding (Brady *et al.*1990).

Measurements of the total cellular protein thiol content appear to be a rather insensitive approach to estimating cytotoxicity (§ III.5.1.). This is able not surprising in view of the heterogeneity in the subcellular distribution of cysteine-containing proteins, the differences in their accessibility to thiol-modifying agents, and their different reactivity as discussed in § IV.1.4.. Chemical modification of cysteine residues is not necessarily accompanied by functional changes in the respective protein (Dékány and

Vas, 1984) and the functional changes of some proteins may not lead to cytotoxicity. The 30-kDa SH-protein, carbonic anhydrase III, amounts to approximately 5% of the total cytosolic proteins and is supposed to be a major participant in the liver's response to oxidative stress (Chai *et al.*1991). These authors estimated that if this enzyme were to be completely S-thiolated (following an oxidative stress), approximately 10% of total liver GSH could become bound to it. It is therefore to be expected that a relatively limited number of critical sites exist in the cell, whose activity is essential for cell viability. Protein-thiols represent very substantial pools of reactive sulphydryls that can act to sequester reactive oxygen intermediates as well as alkylating agents. Thus, one can suggest that these major thiol-proteins may provide a significant reservoir of protection against thiol-modifying agents. It could then be hypothesized that toxicity arises from selective alteration of a limited number of critical protein-SH. This selectivity could be directed by the subcellular distribution of the protein in relation to the site of formation of the reactive quinone for instance, or by the matching reactivity of the quinone and the SH group.

4.2. Both arylation and oxidation are lethal

Quinoid compounds like paracetamol are believed to lead to toxicity *via* redox cycling or *via* covalent attachment to macromolecules (Di Monte *et al.*1984a). These mechanisms are considered to be alternative pathways, if not necessarily mutually exclusive (see § I.2.5.5.).

NEM (N-ethylmaleimide) is an alkylating agent specific to thiol groups although reactions with the imidazole group of histidine and the α-amino groups of amino acids and peptides have been reported (Jocelyn, 1972; Torchinsky, 1981). It binds thiol spontaneously through an α,β-unsaturated carbonyl grouping (-C=C-C=O). Unlike quinone, NEM is not a redox cycler. Diamide (diazenedicarboxylic acid bis(N,N-dimethylamide)) is a very efficient GSH oxidising agent, whose reduction product is relatively non-toxic (Reed, 1986), although nonspecific reactions occur (O'Brien *et al.*1970). Among oxidants of SH groups, disulphides like GSSG occupy a special position, their reactions with thiols being absolutely specific (Torchinsky, 1981). As seen in § III.5.1., NEM, diamide or GSSG can all be toxic for liver slices. However, the alkylator NEM exerts its toxicity at a lower dose than oxidants like diamide and

GSSG. This has to be related to the fact that cell machinery as found in hepatocyte is adequate to cope with marked oxidative attacks (Rossi *et al.*1986) and that, provided this machinery is not overpowered by large and long-lasting attack, thiol modification should remain reversible. Instead, alkylation of thiol groups is irreversible. In the case of paracetamol, alkylation has been clearly demonstrated, although dissociated from cell death (see § I.2.7.2.2.). Further, the prevention of paracetamol-mediated cell injury by a wide range of protective agents (see § I.2.7.3. & III.5.2.), many of them acting as antioxidants in one way or another, suggests a role for oxidative processes in the whole mechanism leading to cell death.

In fact, a synergism of action between alkylation and oxidative processes on cell injury could exist. Alkylation, by depleting GSH, could act as a sensitiser for the cell, allowing oxidative processes to arise in an environment impoverished in its defence capability. Indeed, this combined mechanism would be much the same as the one used by researchers intending to prepare cells with increased susceptibility to oxidizing compounds, who pre-treat those cells with such GSH-depleting agents as the thiol-alkylator diethylmaleate, the inhibitor of glutathione reductase BCNU (N,N-bis(2-chloroethyl)-N-nitrosourea), or the inhibitor of GSH synthesis BSO (buthionine sulfoximine) (Meister and Anderson, 1983; Reed, 1986).

4.3. H_2O_2 formation associated with cell injury

NAPQI, the oxidized form of paracetamol and proposed ultimate metabolite of the latter, did manifest a certain H_2O_2 yield in its chemically defined reaction with GSH (§ III.1.2. & 1.3.), though not to the same extent as observed with BQ, another possible paracetamol metabolite. H_2O_2 is also found to be associated with paracetamol-mediated liver slice injury (§ III.5.3.). It is worth noticing the formation of H_2O_2 at a time where paracetamol has been removed from the medium and therefore where paracetamol is virtually absent in its free form, and is present instead attached to GSH or protein-SH as paracetamol adduct or possibly hydroquinone adduct. It can then be speculated that this H_2O_2 production is associated with quinone-thioether redox cycling in a process similar to the one described above where quinones were incubated in chemically defined reactions with GSH or protein-SH.

The proposed mechanism regarding the role of GSH and low molecular-weight thiols

in preventing hydroquinone autoxidation (see § IV.3.2.) would then be supported by the preventive effect of the low molecular weight thiol and GSH precursor, N-acetylcysteine (NAC), on H_2O_2 formation in liver slices, associated with paracetamol injury (§ III.5.3.). This mechanism may furnish a new possible explanation for the protecting effect of such thiol compounds like NAC or dithiothreitol (DTT) against paracetamol-mediated cell injury, other than the commonly advanced regeneration of protein thiols (Albano *et al.*1985). Indeed NAC or DTT could, by restricting hydroquinone-adduct autoxidation, provide to the cell the necessary time for achieving the elimination of the GSH S-conjugates and the turn-over of damaged proteins.

The inability of fructose in preventing H_2O_2 formation, despite its efficiency in protecting liver slices from paracetamol toxicity, dissociates H_2O_2 formation from cell death.

All the same, just as dissociation of covalent binding from cell death does not disprove the role of arylation in the sequence of events leading toward cell killing, the lack of fructose effect could simply underline the cascade system possibly occurring in the process of cell injury. The action of a protective agent at a later stage in the sequence of events leading to cell death can halt (delay or repair) the damaging effects provoked by an earlier episode. Putative causes of injury may thus present the peculiarity of being necessary but rarely sufficient. H_2O_2 is unlikely directly associated with cell damage, instead it is believed to act *via* the formation of GSSG and parallel alteration of protein-thiol homeostasis, as well as *via* its degradation into the highly reactive ·OH. The mechanism of fructose action, as a protective agent, is not known. Nevertheless it is understood to involve multiple pathways; one of which could be to scavenge the ·OH (Silva *et al.*1991).

5. Proposed mechanism of arylating-quinone toxicity

Quinones are believed to lead to toxicity via redox cycling or via covalent binding to macromolecules. These two mechanisms are often considered to be alternative pathways, if not necessarily mutually exclusive. The proposed mechanism of arylating-quinone toxicity which follows is based on the H_2O_2-producing reactions of quinone-

thioethers and the general importance this might have in relation to long-term tissue damage by xenobiotics. It attempts to furnish an explanation for the difficulty often encountered in elucidating the relative contribution to quinone toxicity arising from redox cycling activity *versus* electrophilic arylation. The mechanism put forward consists of sequential, as well as simultaneous events which, if completed, could exhaust cell defences and culminate in the production of severe damage or perhaps even cell death. Those events can be separated into two stages: before and after GSH depletion.

In the first stage, cells exposed to an arylating-quinone experience GSH consumption *via* formation of glutathion-S-yl hydroquinone conjugates. The latter, together with its parent quinone, redox cycle with intracellular electron carriers (enzymic or otherwise) and oxygen leading to H_2O_2 generation. The pace of those redox cycles is restricted by GSH till the GSH level reaches its nadir. Meanwhile a significant amount of GSSG is formed as a result of the glutathione peroxidase removal of H_2O_2 (the extent of GSSG generated could be even greater if the GSH-quinone conjugate inhibits the glutathione reductase). The simultaneous presence of GSH S-conjugate and GSSG could lead to a mutually competitive inhibition of their common excretion system and thereby to their accumulation and spread within the cell. Protein-SH levels decrease slightly under oxidation by GSSG and arylation by the unreacted quinone, at a time when the GSH level, already diminished, loses its buffering role.

Once GSH has been entirely depleted, an acceleration of the events leading to cell injury occurs, in which GSH paradoxically participates. The GSH-quinone and thiol-protein-quinone conjugates are freed to redox cycle and a burst in H_2O_2 yield occurs, accompanied by the consumption of even more of the reducing capacity of the cells (e.g. ascorbate). The cell's overall antioxidant defence-system, already weakened by the GSH depletion, further shrinks. The long-lived semiquinone radical from protein adducts or the H_2O_2 degradation into $\cdot OH$ *via* local metal chelates could then irreversibly damage some key proteins, leading to crucial cellular dysfunction.

The cell's survival would then very much depend o its ability to resynthesise the damaged key-protein(s). Cell protection against such injury could be expected by agents capable of facilitating this resynthesis or able to compensate for the dysfunction of the damaged key-protein(s).

REFERENCES

Abate, C., Patel, L., Rauscher III, F.J. and Curran, T. Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 249:1157-1161, 1990.

Adamson, G.M. and Harman, A.W. A role for glutathione peroxidase/reductase enzyme system in the protection from paracetamol toxicity in isolated mouse hepatocytes. *Biochem.Pharmacol.* 38:3323-3330, 1989.

Adamson, G.M. and Harman, A.W. Oxidative stress in culturated hepatocytes exposed to acetaminophen. *Biochem.Pharmacol.* 45:2289-2294, 1993.

Aebi, H. Catalase *in vitro*. *Methods Enzymol.* 105:121-126, 1984.

Akerboom, T.P.M., Bilzer, M. and Sies, H. Competition between transport of glutathione disulfide (GSSG) and glutathione S-conjugates from perfused rat liver into bile. *FEBS Lett.* 140No1:73-76, 1982.

Akerboom, T.P.M., Narayamaswami, V., Kunst, M. and Sies, H. ATP-dependent S-(2,4-dinitrophenyl)glutathione transport in canalicular plasma membrane vesicules from rat liver. *J.Biol.Chem.* 266:13147-13152, 1991.

Akerboom, T.P.M. and Sies, H. Assay of glutathione , glutathione disulfide , and glutathione mixed disulfides in biological samples. *Methods Enzymol.* 77:373-383, 1981.

Akerboom, T.P.M. and Sies, H. Transport of glutathione disulfide and glutathione S-conjugates in hepatocyte plasma membrane vesicles. *Methods Enzymol.* 233:416-425, 1994.

Albano, E., Poli, G., Chiarpotto, E., Biasi, F. and Dianzani, M.U. Paracetamol-stimulated lipid peroxidation in isolated rat and mouse hepatocytes. *Chem.Biol.Interact.* 47:249-263, 1983.

Albano, E., Rundgren, M., harvison, P.J., Nelson, S.D. and Moldéus, P. Mechanisms of *N*-acetyl-*p*-benzoquinone imine cytotoxicity. *Mol.Pharmacol.* 28:306-311, 1985.

Amador,E., Dorfman,L.E. and Wacker,W.E.C. Serum lactic dehydrogenase activity: an analytical assessment of current assays. *Clin.Chem.* 9: 391-399, 1963.

Anundi, I., Högberg, J. and Stead, A.H. Glutathione depletion in isolated hepatocytes: Its relation to lipid peroxidation and cell damage. *Acta Pharmacol. et Toxicol.* 45:45-51, 1979.

Anusevicius, Z.J. and Cenas, N.K. Dihydrolipoamide-mediated redox cycling of quinones. *Arch.Biochem.Biophys.* 302:420-424, 1993.

Augusteyn, R.C. On the intracellular stability of lens crystallins. *Exp.Eye Res.* 58:127-128, 1994.

Aust, S.D. Sources of iron for lipid peroxidation in biological systems. In: *Oxygen radicals and tissue injury - Procedings of a Brook Lodge Symposium Augusta,Michigan,USA April 27 to 29 1987*, edited by Halliwell, B. Bethesda,Maryland: Upjohn, 1987, p. 27-33.

Axelsson, K., Eriksson, S. and Mannervik, B. Purification and characterization of cytoplasmic thioltransferase (glutathione:disulfide oxidoreductase) from rat liver. *Biochemistry* 17:2978-2984, 1978.

Axelsson, K. and Mannervik, B. An essential role of cytosolic thioltransferase in protection of pyruvate kinase from rabbit liver against oxidative inactivation. *FEBS Lett.* 152:114-118, 1983.

Axworthy, D.B., Hoffmann, K.-J., Streeter, A.J., Calleman, C.J., Pascoe, G.A. and Baillie, T.A. Covalent binding of acetaminophen to mouse hemoglobin. Identification of major and minor adducts formed in vivo and implications for the nature of arylating

metabolites. *Chem.Biol.Interact.* 68:99-116, 1988.

Bandy, B., Moon, J. and Davison, A.J. Multiple actions of superoxide dismutase: why can it both inhibit and stimulate reduction of oxygen by hydroquinones? *Free Radic.Biol.Med.* 9:143-148, 1990.

Bartolone, J.B., Sparks, K., Cohen, S.D. and Khairallah, E.A. Immunochemical detection of acetaminophen-bound liver proteins. *Biochem.Pharmacol.* 36:1193-1196, 1987.

Bartolone, J.B., Birge, R.B., Bulera, S.J., et al. Purification, antibody production, and partial amino acid sequence of 58-kDa acetaminophen-binding liver proteins. *Toxicol.Appl.Pharmacol.* 113:19-29, 1992.

Battelli, M.G. and Lorenzoni, E. Purification and properties of a new glutathione-dependent thiol:disulfide oxidoreductase from rat liver. *Biochem.J.* 207:133-138, 1982.

Baynes, J.W. Oxidation and the role of antioxidants. In: *Radical therapeutics: the role of free radical scavengers and antioxidants beyond 2000*, edited by Daugherty, A. and Baynes, J.W. St.Louis: MedStrategy, 1991, p. 1-36.

Bellomo, G., Jewell, S.A. and Orrenius, S. The metabolism of menadione impairs the ability of rat liver mitochondria to take up and retain calcium. *J.Biol.Chem.* 11558:11562, 1982.

Bellomo, G., Mirabelli, F., Richelmi, P. and Orrenius, S. Critical role of sulfhydryl group(s) in ATP-dependent Ca^{2+} sequestration by the plasma membrane fraction from rat liver. *FEBS Lett.* 163:136-139, 1983.

Bellomo, G., Thor, H. and Orrenius, S. Modulation of cellular glutathione and protein thiol status during quinone metabolism. *Methods Enzymol.* 186:627-635, 1990.

Beutler, E. Active Transport of glutathione disulfide from erythrocytes. *Function of glutathione* 65-75, 1983.

Bilzer, M., Krauth-Siegel, R.L., Schirmer, R.H., Akerboom, T.P.M., Sies, H. and Schulz, G.E. Interaction of a glutathione S-conjugate with glutathione reductase - Kinetic and X-ray crystallographic studies. *Eur.J.Biochem.* 138:373-378, 1984.

Birge, R.B., Bartolone, J.B., Nishanian, E.V., et al. Dissociation of covalent binding from the oxidative effects of acetaminophen - studies using dimethylated acetaminophen derivatives. *Biochem.Pharmacol.* 37:3383-3393, 1988.

Birge, R.B., Bartolone, J.B., McCann, D.J., Mangold, J.B., Cohen, S.D. and Khairallah, E.A. Selective protein arylation by acetaminophen and 2,6-dimethylacetaminophen in cultured hepatocytes from phenobarbital-induced and uninduced mice. *Biochem.Pharmacol.* 38:4429-4438, 1989.

Birge, R.B., Bartolone, J.B., Hart, S.G.E., et al. Acetaminophen hepatotoxicity: correspondence of selective protein arylation in human and mouse liver *in vitro*, in culture, and *in vivo*. *Toxicol.Appl.Pharmacol.* 105:472-482, 1990a.

Birge, R.B., Bartolone, J.B., Tyson, C.A., Hart, S.G.E., Cohen, S.D. and Khairallah, E.A. Selective binding of acetaminophen (APAP) to liver proteins in mice and men. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: Plenum Press, 1990b, p. 685-688.

Birge, R.B., Bartolone, J.B., Cohen, S.D., Khairallah, E.A. and Smolin, L.A. A comparison of proteins S-thiolated by glutathione to those arylated by acetaminophen. *Biochem.Pharmacol.* 42:S197-S207, 1991a.

Birge, R.B., Bulera, S.J., Bartolone, J.B., Ginsberg, G.L., Cohen, S.D. and Khairallah, E.A. The arylation of microsomal membrane protein by acetaminophen is associated with the release of 44 kDa acetaminophen-binding mouse liver protein complex into the cytosol. *Toxicol.Appl.Pharmacol.* 109:443-454, 1991b.

Blair, I.A., Boobis, A.R., Davies, D.S. and Cresp, T.M. Paracetamol oxidation: synthesis and reactivity of N-acetyl-p-benzoquinoneimine. *Tetrahedron Letters* 21:4947-4950, 1980.

Boobis, A.R., Fawthrop, D.J. and Davies, D.S. Mechanisms of cell death. *Trends Pharmacol.Sci.* 10:275-280, 1989.

Boobis, A.R., Seddon, C.E., Nasseri-Sina, P. and Davies, D.S. Evidence for direct role of intracellular calcium in paracetamol toxicity. *Biochem.Pharmacol.* 39:1277-1281, 1990.

Boobis, A.R., Fawthrop, D.J. and Davies, D.S. Mechanisms of cell injury. In: *Oxford textbook of pathology - Vol 1*, edited by McGee, J.O'D., Isaacson, P.G. and Wright, N.A. Oxford: Oxford University Press, 1992, p. 181-193.

Borg, D.C. and Schaich, K.M. Iron and iron-derived radicals. In: *Oxygen radicals and tissue injury - Proceedings of a Brook Lodge Symposium Augusta,Michigan,USA April 27 to 29 1987*, edited by Halliwell, B. Bethesda,Maryland: Upjohn, 1987, p. 20-26.

Boyland, E. and Chasseaud, L.F. Enzyme-catalysed conjugations of glutathione with unsaturated compounds. *Biochem.J.* 104:95-102, 1967.

Brady, J.T., Birge, R.B., Khairallah, E.A. and Cohen, S.D. Post-treatement protection with piperonyl butoxide against acetaminophen hepatotoxicity is associated with changes in selective but not total covalent binding. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: Plenum Press, 1990, p. 689-692.

Brigelius, R., Lenzen, R. and Sies, H. Increase in hepatic mixed disulphide and glutathione disulfide levels elicited by paraquat. *Biochem.Pharmacol.* 31No8:1637-1641, 1982.

Brigelius, R., Muckel, C., Akerboom, T.P.M. and Sies, H. Identification and

quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem.Pharmacol.* 32no17:2529-2534, 1983.

Brigelius, R. Mixed disulfides : biological functions and increase in oxidative stress. *Oxidative stress* 243-273, 1985.

Brodie, A.E. and Reed, D.J. Cellular recovery of glyceraldehyde-3-phosphate dehydrogenase activity and thiol status after exposure to hydroperoxides. *Arch.Biochem.Biophys.* 276:212-218, 1990.

Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G. and Gillette, J.R. Possible mechanism of liver necrosis caused by aromatic organic compounds. *Proc.Natl.Acad.Sci.U.S.A.* 68No1:160-164, 1971.

Brown, P.C., Dulik, D.M. and Jones, T.W. The toxicity of menadione (2-methyl-1,4-naphthoquinone) and two thioether conjugates studied with isolated renal epithelial cells. *Arch.Biochem.Biophys.* 285:187-196, 1991.

Brunmark, A. and Cadenas, E. Reductive addition of glutathione to p-benzoquinone, 2-hydroxy-p-benzoquinone, and p-benzoquinone epoxides. Effect of the hydroxy- and glutathionyl substituents on p-benzohydroquinone autoxidation. *Chem.Biol.Interact.* 68:273-298, 1988.

Brunmark, A. and Cadenas, E. Redox and addition chemistry of quinoid compounds and its biological implications. *Free Radic.Biol.Med.* 7:435-477, 1989.

Bruno, M.K., Cohen, S.D. and Khairallah, E.A. Antidotal effectiveness of N-acetylcysteine in reversing acetaminophen-induced hepatotoxicity - enhancement of the proteolisys of arylated proteins. *Biochem.Pharmacol.* 37:4319-4325, 1988.

Buffinton, G.D., Ollinger, K., Brunmark, A. and Cadenas, E. DT-diaphorase-catalysed reduction of 1,4-naphtoquinone derivatives and glutathionyl-quinone conjugates - effect of substituents on autoxidation rates. *Biochem.J.* 257:561-571, 1989.

Cadenas, E., Mira, D., Brunmark, A., Lind, C., Segura-Aguilar, J. and Ernster, L. Effect of superoxide dismutase on the autoxidation of various hydroquinones - A possible role of superoxide dismutase as a superoxide:semiquinone oxidoreductase. *Free Radic.Biol.Med.* 5:71-79, 1988.

Chai, Y.-C., Jung, C.-H., Lii, C.-K., et al. Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III; characterisation of S-thiolation and dethiolation rections. *Arch.Biochem.Biophys.* 284:270-278, 1991.

Chambers, J.Q. Electrochemistry of quinones. In: *The chemistry of the quinoid compounds - Part 2*, edited by Patai, S. London: John Wiley & Sons, 1974, p. 737-791.

Chang, W.-C., Nakao, J., Orimo, H. and Murota, S.-I. Effects of reduced glutathione on the 12-lipoxygenase pathways in rat platelets. *Biochem.J.* 202:771-776, 1982.

Chung, H., Harvey, R.G., Armstrong, R.N. and Jarabak, J. Polycyclic aromatic hydrocarbon quinones and glutathione thioethers as substrates and inhibitors of the human placental NADP-linked 15-hydroxyprostaglandin dehydrogenase. *J.Biol.Chem.* 262:12448-12451, 1987.

Cohen, G. and Hochstein, P. Generation of hydrogen peroxide in erythrocytes by hemolytic Agents. *Biochemistry* 3:895-900, 1964.

Cohn, V.H. and Lyle, J. A fluorimetric assay for glutathione. *Anal.Biochem.* 14:433-440, 1966.

Coles, B. Effects of modifying structre on electrophilic reactions with biological nucleophiles. *Drug.Metab.Rev.* 15:1307-1334, 1984.

Coles, B., Wilson, I., Wardman, P., Hinson, J.A., Nelson, S.D. and Ketterer, B. The spontaneous and enzymatic reaction of *N*-acetyl-*p*-benzoquinoneimine with glutathione:

a stopped-flow kinetic study. *Arch.Biochem.Biophys.* 264:253-260, 1988.

Corcoran, G.B., Mitchell, J.R., Vaishnav, Y,N. and Horning, E.C. Evidence that acetaminophen and N-hydroxyacetaminophen form a common arylating intermediate, N-acetyl-p-Benzoquinonimine. *Mol.Pharmacol.* 18:536-542, 1980.

Corcoran, G.B., Wong, B.K. and Neese, B.L. Early sustained rise in total liver calcium during acetaminophen hepatotoxicity in mice. *Res.Commun.Chem.Pathol.Pharmacol.* 58:291-305, 1987.

Corcoran, G.B. and Ray, S.D. Contemporary issues in toxicology (the role of nucleus and other compartments in toxic cell death produced by alkylating hepatotoxicants). *Toxicol.Appl.Pharmacol.* 113:167-183, 1992.

Dahlin, D.C., Miwa, g.T., Lu, A.Y.H. and Nelson, S.D. N-acetyl-*p*-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc.Natl.Acad.Sci.U.S.A.* 81:1327-1331, 1984.

Dahlin, D.C. and Nelson, S.D. Synthesis, decomposition kinetics, and preliminary toxicological studies of pure N-acetyl-*p*-benzoquinone imine, a proposed toxic metabolite of acetaminophen. *J.Med.Chem.* 25:885-886, 1982.

Daugherty, A. Antioxidant compounds. In: *Radical therapeutics: the role of free radical scavengers and antioxidants beyond 2000*, edited by Daugherty, A. and Baynes, J.W. St.Louis: MedStrategy, 1991, p. 273-319.

Davies, K.J.A. Protein damage and degradation by oxygen radicals - I. General aspects. *J.Biol.Chem.* 262:9895-9901, 1987.

Davies, K.J.A., Delsignore, M.E. and Lin, S.W. Protein damage and degradation by oxygen radicals -II.Modification of amino acids. *J.Biol.Chem.* 262:9902-9907, 1987a.

Davies, K.J.A., Lin, S.W. and Pacifici, R.E. Protein damage and degradation by

oxygen radicals -IV.Degradation of denatured protein. *J.Biol.Chem.* 262:9914-9920, 1987b.

Davies, K.J.A. and Delsignore, M.E. Protein damage and degradation by oxygen radicals -III.Modification of secondary and tertiary structure. *J.Biol.Chem.* 262:9908-9913, 1987.

Davies, K.J.A. and Goldberg, A.L. Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *J.Biol.Chem.* 262:8227-8234, 1987.

Davies, K.J.A. and Lin, S.W. Oxidatively denatured proteins are degraded by an ATP-independent proteolytic pathway in Escherichia Coli. *Free Radic.Biol.Med.* 5:225-236, 1988.

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. *Data for biochemical research*, Oxford:Clarendon Press, 1986. Ed. 3

Deakin, C.D., Gove, C.D., Fagan, E.A., Tredger, J.M. and Williams, R. Delayed calcium channel blockade with diltiazem reduces paracetamol hepatotoxicity in mice. *Hum.Exp.Toxicol.* 10:119-123, 1991.

Dean, R.T., Gieseg, S. and Davies, M.J. Reactive species and their accumulation on radical-damaged proteins. *Trends Biochem. Sci.* 18:437-441, 1993.

Deby, C. and Pincemail, J. Toxicité de l'oxygène, radicaux libres et moyens de défense. *Presse Méd.* 15:1468-1474, 1986.

Devalia, J.L., Ogilvie, R.C. and McLean, A.E.M. Dissociation of cell death from covalent binding of paracetamol by flavones in a hepatocyte system. *Biochem.Pharmacol.* 31:3745-3749, 1982.

Devalia, J.L. and McLean, A.E.M. Covalent binding and the mechanism of paracetamol toxicity. *Biochem.Pharmacol.* 32:2602-2603, 1983.

Dékány, K. and Vas, M. Inactivation of pig muscle 3-phosphoglycerate kinase by thiol modification depends on reagent size. *Eur.J.Biochem.* 139:125-130, 1984.

Di Monte, D., Bellomo, G., Thor, H., Nicotera, P. and Orrenius, S. Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intracellular Ca^{2+} homeostasis. *Arch.Biochem.Biophys.* 235:343-350, 1984a.

Di Monte, D., Ross, D., Bellomo, G., Eklöw, L. and Orrenius, S. Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. *Arch.Biochem.Biophys.* 235:334-342, 1984b.

Dikalov, S., Alov, P. and Rangelova, D. Role of iron chelation by quinones in their reduction, OH-radical generation and lipid peroxidation. *Biochem.Biophys.Res.Comm.* 195:113-119, 1993.

Dikalov, S.I., Rumyantseva, G.V., Piskunov, A.V. and Weiner, L.M. Role of quinone-iron(III) interaction in NADPH-dependent enzymatic generation of hydroxyl radicals. *Biochemistry* 31:8947-8953, 1992.

Eckert, K.-G. The metabolism of aminophenols in erythrocytes. *Xenobiotica* 18:1319-1326, 1988.

Eckert, K.-G., Eyer, P., Sonnenbichler, J. and Zetl, I. Activation and detoxication of aminophenols. III. Synthesis and structural elucidatuon of various glutathione addition products to 1,4-benzoquinone. *Xenobiotica* 20:351-361, 1990a.

Eckert, K.-G., Eyer, P., Sonnenbichler, J. and Zetl, I. Activation and detoxication of aminophenols. II. Synthesis and structural elucidation of various thiol addition products of 1,4-benzoquinoneimine and N-acetyl-1,4-benzoquinoneimine. *Xenobiotica* 20:333-350, 1990b.

Edwards, A., Gupta, J.D. and Harley, J.D. Photomicrographic evaluation of

drug-induced cataracts in cultured embryonic chick lens. *Exp.Eye Res.* 15:495-498, 1973.

Eklöw, L., Moldéus, P. and Orrenius, S. Oxidation of glutathione during hydroperoxide metabolism. *Eur.J.Biochem.* 138:459-463, 1984.

El-Maghrabi, M.R., Pate, T.M., Pilkis, J. and Pilkis, S.J. Effect of sulfhydryl modification on the activities of rat liver 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase. *J.Biol.Chem.* 259:13104-13110, 1984.

Elfarra, A.A. and Anders, M.W. Renal processing of glutathione conjugates - role in nephrotoxicity. *Biochem.Pharmacol.* 33:3792-3732, 1984.

Eliot, H., Gianni, L. and Myers, C. Oxidative destruction of DNA by adriamycin-iron complex. *Biochemistry* 23:928-936, 1984.

Eyer, P. and Kiese, M. Biotransformation of 4-dimethylaminophenol: reaction with glutathione, and some properties of the reaction products. *Chem.Biol.Interact.* 14:165-178, 1976.

Farber, J.L., Leonard, T.B., Kyle, M.E., Nakae, D., Serroni, A. and Rogers, S.A. Peroxidation-dependent and peroxidation-independent mechanisms by wich acetaminophen kills cultured rat hepatocytes. *Arch.Biochem.Biophys.* 267:640-650, 1988.

Federici, G., Di Cola, D., Sacchetta, P., Di Ilio, C., Del Boccio, G. and Polidoro, G. Reversible inactivation of tyrosine aminotransferase from guinea pig liver by thiol and disulfide compounds. *Biochem.Biophys.Res.Comm.* 81:650-655, 1978.

Fernando, C.R., Calder, I.A. and Ham, K.N. Studies on the mechanism of toxicity of acetaminophen. synthesis and reactions of N-acetyl-2,6-dimethyl- and N-acetyl-3,5-dimethyl-*p*-benzoquinone imines. *J.Med.Chem.* 23:1153-1158, 1980.

Fieser,M. and Fieser,L.F. Reagents for organic Synthesis, *Vol.1* Wiley Interscience, New York, 1967. p.101.

Finley, K.T. The addition and substitution chemistry of quinones. In: *The chemistry of the quinoid compounds - Part 2*, edited by Patai, S. London: John Wiley & sons, 1974, p. 877-1144.

Fisher, A.B. Intracellular production of oxygen-derived free radicals. In: *Oxygen radicals and tissue injury - Procedings of a Brook Lodge Symposium Augusta,Michigan,USA April 27 to 29 1987*, edited by Halliwell, B. Bethesda,Maryland: Upjohn, 1987, p. 34-39.

Freedman, J.H., Ciriolo, M.R. and Peisach, J. The role of glutathione in copper metabolism and toxicity. *J.Biol.Chem.* 264:5598-5605, 1989.

Friedmann, E., Marrian, D.H. and Simon-Reuss, I. Sulphydryl addition compounds of some quinones and related substances in their action on the growth of normal cells. *Br.J.Pharmacol.* 3:335-340, 1948.

Friso, G., Barbato, R., Giacometti, G.M. and Barber, J. Degradation of D2 protein due to UV-B irradiation of the reaction centre of photosystem II. *FEBS Lett.* 339:217-221, 1994.

Gant, T.W., d'Arcy Doherty, M., Odowole, D., Sales, K.D. and Cohen, G.M. Semiquinone anion radicals formed by the reaction of quinones with glutathione or amino acids. *FEBS Lett.* 201:296-300, 1986.

Gant, T.W., Rao, D.N.R., Mason, R.P. and Cohen, G.M. Redox cycling and sulfhydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem.Biol.Interact.* 65:157-173, 1988.

Garfin, D.E. One-dimensional gel electrophoresis. *Methods Enzymol.* 182:425-441, 1990.

Gilbert, H.F. Biological disulfides: the third messenger? *J.Biol.Chem.* 257:12086-12091, 1982.

Giulivi, C. and Cadenas, E. Ferrylmyoglobin: formation and chemical reactivity toward electron-donating compounds. *Methods Enzymol.* 233:189-202, 1994.

Grässer, F.A., LaMontagne, K., Whittaker, L., Stohr, S. and Lipsick, J.S. A highly conserved cysteine in the v-myb DNA-binding domain is essential for transformation and transcriptional trans-activation. *Oncogene* 7:1005-1009, 1992.

Gutteridge, J.M.C. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett.* 201:291-295, 1986.

Gutteridge, J.M.C. superoxide-dependent formation of hydroxyl radicals from ferric-complexes and hydrogen peroxide: an evaluation of fourteen iron chelators. *Free Radic.Res.Commun.* 9:119-125, 1990.

Gutteridge, J.M.C. and Halliwell, B. Iron toxicity and oxygen radicals. *Baillière's haematology - International practice and research - Iron chelating therapy* 2/2:195-256, 1984.

Halliwell, B. How to characterize a biological antioxidant. *Free Radic.Res.Commun.* 9:1-32, 1990.

Halliwell, B., Gutteridge, J.M.C. and Cross, C.E. Free radicals, antioxidants, and human disease: where are we now? *J.Lab.Clin.Med.* 119:598-620, 1993.

Halliwell, B. and Gutteridge, J.M.C. Role of iron in oxygen radical reactions. *Methods Enzymol.* 105:47-56, 1984.

Halliwell, B. and Gutteridge, J.M.C. *Free radicals in biology and medicine*, Oxford:Clarendon Press, 1989. Ed. 2

Halliwell, B. and Gutteridge, J.M.C. Biologically relevant metal ion-dependent hydroxyl radical generation. *FEBS Lett.* 307:108-112, 1992.

Harding, J.J. and Crabbe, M.J.C. *The Eye - Vol.1b*, Orlando:Academic Press, 1984. Ed. 3 pp. 206-492.

Hardwick, S.J., Wilson, J.W., Fawthrop, D.J., Boobis, A.R. and Davies, D.S. paracetamol toxicity in hamster isolated hepatocytes: the increase of cytosolic calcium accompanies, rather than precedes, loss of viability. *Arch.Toxicol.* 66:408-412, 1992.

Harman, A.W. The effectiveness of antioxidants in reducing paracetamol-induced damage subsequent to paracetamol activation. *Res.Commun.Chem.Pathol.Pharmacol.* 49:215-228, 1985.

Harman, A.W., Kyle, M.E., Serroni, A. and Farber, J.L. The killing of cultured hepatocytes by N-acetyl-*p*-benzoquinone imine (NAPQI) as a model of the cytotoxicity of acetaminophen. *Biochem.Pharmacol.* 41:1111-1117, 1991.

Harman, A.W., Mahar, S.O., Burcham, P.C. and Madsen, B.W. Level of cytosolic free calcium during acetaminophen toxicity in mouse hepatocytes. *Mol.Pharmacol.* 41:665-670, 1992.

Hildebrandt, A.E., Tjoe, M. and Roots, I. Mono-oxygenase-linked hydrogen peroxide production and degradation in liver microsomal fractions. *Biochem.Soc.Trans.* 3:807-811, 1975.

Hill, B.A., Kleiner, H.E., Ryan, E.A., Dulik, D.M., Monks, T.J. and Lau, S.S. Identification of multi-S-substituted conjugates of hydroquinone by HPLC-coulorimetric electrode array analysis and mass spectroscopy. *Chem.Res.Toxicol.* 6:459-469, 1993.

Hinshaw, D.B., Sklar, L.A., Bohl, B., et al. Cytoskeletal and morphologic impact of cellular oxidant injury. *Am.J.Pathol.* 123:454-464, 1986.

Hinson, J.A. and Roberts, D.W. Role of covalent and noncovalent interactions in cell toxicity: effects of proteins. *Ann.Rev.Pharmacol.Toxicol.* 32:471-510, 1992.

Hissin, P.J. and Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal.Biochem.* 74:214-226, 1976.

Hoffmann, K.-J., Streeter, A.J., Axworthy, D.B. and Baillie, T.A. Structural characterization of the major covalent adduct formed in vitro between acetaminophen and bovin serum albumin. *Chem.Biol.Interact.* 53:155-172, 1985a.

Hoffmann, K.-J., Streeter, A.J., Axworthy, D.B. and Baillie, T.A. Identification of the major covalent adduct formed *in vitro* and *in vivo* between acetaminophen and mouse liver proteins. *Mol.Pharmacol.* 27:566-573, 1985b.

Horton, A.A. and Wood, J.M. Effects of inhibitors of phospholipase A_2 cyclooxygenase and thromboxane synthetase on paracetamol hepatotxicity in rat. *Eicosanoids* 2:123-129, 1989.

Högberg, J. and Kristoferson, A. A correlation between glutathione levels and cellular damage in isolated hepatocytes. *Eur.J.Biochem.* 74:77-82, 1977.

Hugget, A. and Blair, I.A. Chromatographic analysis of synthetic N-acetyl-*p*-benzoquinoneimine: the putative reactive metabolite of paracetamol. *J.Chromatogr.Sci.* 21:254-258, 1983.

Hunt, J.V., Simpson, J.A. and Dean, R.T. Hydroperoxide-mediated fragmentation of proteins. *Biochem.J.* 250:87-93, 1988.

Hunt, J.V., Jiang, Z.Y. and Wolff, S.P. Formation of hydrogen peroxide by lens proteins: protein-derived hydrogen peroxide as a potential mechanism of oxidative insult to the lens. *Free Radic.Biol.Med.* 13:319-323, 1992.

Ishii, T. and Fridovich, I. Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Radic.Biol.Med.* 8:21-24, 1990.

Ishikawa, T., Kobayashi, K., Sogame, Y. and Hayashi, K. Evidence for leukotriene C_4 transport mediated by ATP-dependent glutathion S-conjugate carrier in rat and liver plasma membranes. *FEBS Lett.* 259:95-98, 1989.

Jaeschke, H. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice *in vivo*: the protective effect of allopurinol. *J.Pharmacol.Exp.Ther.* 255:935-941, 1990.

Jahngen-Hodge, J., Taylor, A., Shang, F., Huang, L.L. and Mura, C. Oxidative stress to lens crystallins. *Methods Enzymol.* 233:512-522, 1994.

Jakoby, W.B. and Stevens, J. Cysteine conjugate beta-lyase and the thiomethyl shunt. *Biochem.Soc.Trans.* 12:33-35, 1984.

James, T.H., Snell, J.M. and Weissberger, A. Oxidation processes. XII. The autoxidation of hydroquinone and of the mono-, di-, and trimethylhydroquinones. *J.Am.Chem.Soc.* 60:2084-2093, 1938.

James, T.H. and Weissberger, A. Oxidation processes. XI. The autoxidation of durohydroquinone. *J.Am.Chem.Soc.* 60:98-104, 1938.

Jentoft, N. and Dearborn, D.G. Labeling of proteins by reductive methylation using sodium cyanoborohydride. *J.Biol.Chem.* 254:4359-4365, 1979.

Jiang, Z.Y., Woolard, A.C.S. and Wolff, S.P. Hydrogen peroxide production during experimental protein glycation. *FEBS Lett.* 268:69-71, 1990.

Jocelyn, P.C. *Biochemistry of the SH group*, London:Academic Press, 1972.

Jocelyn, P.C. and Kamminga A., Development of fluorescence between

o-phthaldialdehyde and thiols. *Anal.Biochem.* 37:417-421, 1970.

Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R. and Brodie, B.B. Acetaminophen-induced hepatic necrosis. II. Role of covalent binding *in vivo*. *J.Pharmacol.Exp.Ther.* 187:195-202, 1973.

Jones, D.P., Thor, H., Smith, M.T., Jewell, S.A. and Orrenius, S. Inhibition of ATP-dependent microsomal Ca2+ sequestration during oxidative stress and its prevention by glutathione. *J.Biol.Chem.* 258:6390-6393, 1983.

Kamiyama, T., Sato, C., Liu, J., Tajiri, K., Miyakawa, H. and Marumo, F. Role of lipid peroxidation in acetaminophen-induced hepatotoxicity: comparison with carbon tetrachloride. *Toxicol.Lett.* 66:7-12, 1993.

Kappus, H. Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. *Biochem.Pharmacol.* 35:1-6, 1986.

Kappus, H. and Sies, H. Toxic drug effects associated with oxygen metabolism: redox cycling an lipid peroxidation. *Experientia* 37:1233-1258, 1981.

Kass, G.E.N., Duddy, S.K. and Orrenius, S. Activation of hepatocyte protein kinase C by redox-cycling quinones. *Biochem.J.* 260:499-507, 1989.

Klaassen, C.D. and Stacey, N.H. Use of isolated hepatocytes in toxicity assessment. In: *Toxicology of the liver*, edited by Plaa, G.L. and Hewitt, W.R. New York: Raven Press, 1982, p. 147-180.

Kosower, N.S., Kosower, E.M., Zipser, Y., Faltin, Z. and Shomrat, R. Dynamic changes of red cell membrane thiol groups followed by bimane fluorescent labeling. *Biochim.Biophys.Acta* 640:748-759, 1981.

Kosower, N.S. and Kosower, E.M. Glutathione and cell membrane thiol status. In: *Fuction of glutathione: biochemical, physiological, toxicological, and clinical aspects*,

edited by Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B. New York: Raven Press, 1983, p. 307-315.

Labadarios, D., Davis, M., Portmann, B. and Williams, R. Paracetamol-induced hepatic necrosis in the mouse-relationship between covalent binding, hepatic glutathione depletion and the protective effect of α-mercaptopropionylglycine. *Biochem.Pharmacol.* 26:31-35, 1977.

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970.

Landon, E.J., Naukam, R.J. and Sastry, B.V.R. Effects of calcium channel blocking agents on calcium and centrolobular necrosis in the liver of rats treated with hepatotoxic agents. *Biochem.Pharmacol.* 35:697-705, 1986.

Lau, S.S., Hill, B.A., Highet, R.J. and Monks, T.J. Sequential oxidation and glutathione addition to 1,4-benzoquinone: correlation of toxicity with increased glutathione substitution. *Mol.Pharmacol.* 34:829-836, 1988.

Laudicina, D.C. and Marnett, L.J. Enhancement of hydroperoxide-dependent lipid peroxidation in rat liver microsomes by ascorbic acid. *Arch.Biochem.Biophys.* 278:73-80, 1990.

Lauriault, V.V.M. and O'Brien, P.J. The antidotal activity of the thiol drug diethyldithiocarbamate against N-acetyl-p-benzoquinone imine in isolated hepatocytes. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: Plenum Press, 1990, p. 311-314.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the folin phenol reagent. *J.Biol.Chem.* 193:265-275, 1951.

Lunte, S.M. and Kissinger, P.T. Detection and identification of sulfhydryl conjugates of p-benzoquinone in microsomal incubations of benzene and phenol.

Chem.Biol.Interact. 47:195-212, 1983.

Luthman, M. and Holmgren, A. Glutaredoxin from calf thymus - purification to homogeneity. *J.Biol.Chem.* 257:6686-6690, 1982.

Marcillat, O., Zhang, Y., Lin, S.W. and Davies, K.J.A. Mitochondria contain a proteolytic system which can recognize and degrade oxidatively-denaturated proteins. *Biochem.J.* 254:677-683, 1988.

Margoliash, E. and Novogrodsky, A. A study of the inhibition of catalase by 3-amino-1:2:4-triazole. *Biochem.J.* 68:468-475, 1958.

Martin, S.J., Green, D.R. and Cotter, T.G. Dicing with death: dissecting the componements of the apoptosis machinery. *Trends Biochem. Sci.* 19:26-30, 1994.

McDonald, T.A., Waidyanatha, S. and Rappaport, S.M. Production of benzoquinone adducts with hemoglobin and bone-marrow proteins following administration of [13C6]benzene to rats. *Carcinogenesis* 14:1921-1925, 1993a.

McDonald, T.A., Waidyanatha, S. and Rappaport, S.M. Measurement of adducts of benzoquinone with hemoglobin and albumin. *Carcinogenesis* 14:1927-1932, 1993b.

McLean, A.E.M. and Day, P.A. The use of new methods to measure: the effect of diet and inducers of microsomal enzyme synthesis on cytochrome P-450 in liver homogenates, and on metabolism of dimethyl nitrosamine. *Biochem.Pharmacol.* 23:1173-1180, 1974.

McLean, A.E.M. and McLean, E.K. The effect of diet and 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane (DDT) on microsomal hydroxylating enzymes and on sensitivity of rats to carbon tetrachloride poisoning. *Biochem.J.* 100:564-571, 1966.

McLean, A.E.M. and Nuttall, L. An in vitro model of liver injury using paracetamol

treatment of liver slices and prevention of injury by some antioxidants. *Biochem.Pharmacol.* 27:425-430, 1978.

Meister, A. and Anderson, M.E. Glutathione. *Ann.Rev.Biochem.* 52:711-760, 1983.

Michaels, H.B. and Hunt, J.W. Determination of peroxides and hydroperoxides in irradiated solutions of nucleic acid constituents and DNA. *Anal.Biochem.* 87:135-140, 1978.

Michiels, C., Raes, M., Toussaint, O. and Remacle, J. Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic.Biol.Med.* 17:235-248, 1994.

Miller, D.M., Buettner, G.R. and Aust, S.D. Transition metals as catalysts of "autoxidation" reactions. *Free Radic.Biol.Med.* 8:95-108, 1990.

Miller, M.G., Rodgers, A. and Cohen, G.M. Mechanisms of toxicity of naphthoquinones to isolated hepatocyes. *Biochem.Pharmacol.* 35:1177-1184, 1986.

Mirabelli, F., Salis, A., Marinoni, V., et al. Menadione-induced bleb formation in hepatocytes is associated with the oxidation of thiol groups in actin. *Arch.Biochem.Biophys.* 264:261-269, 1988a.

Mirabelli, F., Salis, A., Perotti, M., Taddel, F., Bellomo, G. and Orrenius, S. Alterations of surface morphology caused by the metabolism of menadione in mammalian cells are associated with the oxidation of critical sulfhydryl groups in cytoskeletal proteins. *Biochem.Pharmacol.* 37:3423-3427, 1988b.

Misra, H.P. Generation of superoxide free radical during the autoxidation of thiols. *J.Biol.Chem.* 249:2151-2155, 1974.

Mitchell, J.R., Smith, C.V., Hughes, H., Lauterburg, B.H. and Horning, M.G. Overview of alkylation and peroxidation mechanisms in acute lethal hepatocellular

injury by chemically reactive metabolites. *Semin.Liver Dis.* 1:143-150, 1981.

Monks, T.J., Lau, S.S., Highet, R.J. and Gillette, J.R. Glutathione conjugates of 2-Bromohydroquinone are nephrotoxic. *Drug Metab.Dispos.* 13:553-559, 1985.

Monks, T.J., Anders, M.W., Dekant, W., Stevens, J.L., Lau, S.S. and van Bladeren, P.J. Glutathione conjugate mediated toxicities. *Toxicol.Appl.Pharmacol.* 106:1-19, 1990.

Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D. and Graham, D.G. Quinone chemistry and toxicity. *Toxicol.Appl.Pharmacol.* 112:2-16, 1992.

Monks, T.J. and Lau, S.S. Toxicology of quinone-thioethers. *Crit.Rev.Toxicol.* 22(5/6):243-270, 1992.

Moore, M., Thor, H., Moore, G., Nelson, S., Moldéus, P. and Orrenius, S. The toxicity of acetaminophen and *N*-acetyl-*p*-benzoquinone imine in isolated hepatocytes is associated with thiol depletion and increased cytosolic Ca^{2+}. *J.Biol.Chem.* 260:13035-13040, 1985.

Moore, R.B., Hulgan, T.M., Green, J.W. and Jenkins, L.D. Increased susceptibility of the sickle cell membrane Ca^{2+} + Mg^{2+}-ATPase to t-butylhydroperoxide: protective effects of ascorbate and desferal. *Blood* 79:1334-1341, 1992.

Morin, J.E., Axelsson, K. and Dixon, J.E. Purification of thiol:protein disulfide oxidoreductases and possible biological functions. In: *FUNCTION OF GLUTATHIONE biochemical, physiological, toxicological, and clinical aspects*, edited by Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B. New York: Raven Press, 1983, p. 285-295.

Morin, J.E. and Dixon, J.E. Thiol: proteine disulfide exchange enzymes. *Methods Enzymol.* 113:541-547, 1985.

Morrison, H., Jernström, B., Nordenskjöld, M., Thor, H. and Orrenius, S. Induction of DNA damage by menadione (2-methyl-1,4-naphtoquinone) in primary cultures of rat hepatocytes. *Biochem.Pharmacol.* 33:1763-1769, 1984.

Mourelle, M., Beals, D. and McLean, A.E.M. Electron transport and protection of liver slices in the late stage of paracetamol injury. *Biochem.Pharmacol.* 40:2023-2028, 1990.

Mourelle, M., Beals, D. and McLean, A.E.M. Prevention of paracetamol-induced injury by fructose. *Biochem.Pharmacol.* 41:1831-1837, 1991.

Nasseri-Sina, P., Fawthrop, D.J., Wilson, J., Boobis, A.R. and Davies, D.S. Cytoprotection by iloprost against paracetamol-induced toxicity in hamster isolated hepatocytes. *Br.J.Pharmacol.* 105:417-423, 1992.

Nelson, D.P. and Kiesow, L.A. Enthalpy of decomposition of hydrogen peroxide by catalase at 25^{0}C (with molar extinction coefficients of H_2O_2 solutions in the UV). *Anal.Biochem.* 49:474-478, 1972.

Nelson, S.D., Tirmenstein, M.A., Rashed, M.S. and Myers, T.G. Acetaminophen and protein thiol modification. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: Plenum Press, 1990, p. 579-588.

Nemani, R. and Lee, E.Y.C. Reactivity of sulfhydryl groups of the catalytic subunits of rabbit skeletal muscle protein phosphatases 1 and 2A. *Arch.Biochem.Biophys.* 300:24-29, 1993.

Nicotera, P., Moore, M., Bellomo, G., Mirabelli, F. and Orrenius, S. Demonstration and partial characterization of glutathione disulfide-stimulated ATPase activity in plasma membrane fraction from rat hepatocytes. *J.Biol.Chem.* 260:1999-2002, 1985.

Nicotera, P., Rundgren, M., Porubek, D.J., et al. On the role of Ca^{2+} in toxicity of alkylating and oxidizing quinone imines in isolated hepatocytes. *Chem.Res.Toxicol.*

2:46-50, 1989.

Nicotera, P., Hinds, T.R., Nelson, S.D. and Vincenzi, F.F. Differential effects of arylating and oxidazing analogs of N-acetyl-*p*-benzoquinoneimine on red blood cell membrane proteins. *Arch.Biochem.Biophys.* 283:200-205, 1990.

Nicotera, P., Bellomo, G. and Orrenius, S. Calcium-mediated mechanisms in chemically induced cell death. *Ann.Rev.Pharmacol.Toxicol.* 32:449-470, 1992.

Nohl, H., Jordan, W. and Youngman, R.J. quinones in biology: functions in electron transfer and oxygen activation. *Adv.Free Radic.Biol.Med.* 2:211-279, 1986.

Nordmann, R. Radicaux libres, stress oxydatif et vitamines antioxydantes. *C.R.Soc.Biol.* 187:277-285, 1993.

Noto, V., Taper, H.S., Yi-Hua, J., Janssens, J., Bonte, J. and de Loecker, W. Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K_3) treatement on human tumor cell growth *in vitro* - I. Synergism of combined vitamin C and K_3 action. *Cancer* 63:901-906, 1989.

Nutter, L.M., Cheng, A.-L., Hung, H.-L., Hsieh, R-K., Ngo, E.O. and Liu, T.W. Menadione: spectrum of anticancer activity and effects on nucleotide metabolism in human neoplastic cell lines. *Biochem.Pharmacol.* 41:1283-1292, 1991.

O'Brien, P.J. Molecular mechanisms of quinone cytotoxicity. *Chem.Biol.Interact.* 80:1-41, 1991.

O'Brien, R.W., Weitzman, P.D.J. and Morris, J.G. Oxidation of a variety of natural electron donors by the thiol-oxidising agent, diamide. *FEBS Lett.* 10:343-345, 1970.

Orrenius, S., Jewell, S.A., Bellomo, G., Thor, H., Jones, D.P. and Smith, M.T. Regulation of calcium compart mentation in the hepatocyte - a critical role of glutathione. In: *FUNCTIONS OF GLUTATHIONE biochemical, physiological,*

toxicological, and clinical aspects, edited by Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B. New York: Raven Press, 1983, p. 261-271.

Orrenius, S. biochemical mechanisms of cytotoxicity. *Trends Pharmacol.Sci.* FEST supplement:S15-S20, 1985.

Ou, P. and Wolff, S.P. Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and hydrogen peroxide in vitro. *Biochem.Pharmacol.* 46:1139-1144, 1993.

Park, E-M. and Thomas, J.A. S-thiolation of creatine kinase and glycogen phosphorylase *b* initiated by partially reduced oxygen species. *Biochim.Biophys.Acta* 964:151-160, 1988.

Park, E-M. and Thomas, J.A. The mechanisms of reduction of protein mixed disulfides (dethiolation) in cardiac tissue. *Arch.Biochem.Biophys.* 274:47-54, 1989.

Pascoe, G.A., Calleman, C.J. and Baillie, T.A. Identification of *s*-(2,5-dihydroxyphenyl)-cysteine and *s*-(2,5-dihydroxyphenyl)-*N*-acetyl-cysteine as urinary metabolites of acetaminophen in the mouse. Evidence for *p*-benzoquinone as a reactive intermediate in acetaminophen metabolism. *Chem.Biol.Interact.* 68:85-98, 1988.

Pascoe, G.A. and Reed, D.J. Vitamin E protection against chemical-induced cell injury II. Evidence for a threshold effect of cellular α-tocopherol in prevention of adriamycin toxicity. *Arch.Biochem.Biophys.* 256:159-166, 1987.

Pascoe, G.A. and Reed, D.J. Cell calcium, vitamin E, and the thiol redox system in cytotoxicity. *Free Radic.Biol.Med.* 6:209-224, 1989.

Patai, S. and Rappoport, Z. *The chemistry of quinoid compounds, Vol.II*, John Wiley &Sons Ltd, 1988.

Pau, H., Graf, P. and Sies, H. Glutathione levels in human lens: regional distribution in different forms of catarat. *Exp.Eye Res.* 50:17-20, 1990.

Peters, J.T. Methods of studying secretion *in vivo* and in liver slices. *Plasm.Prot.Secret.by the liver* 1983.

Pethig, R., Gascoyne, P.R.C., McLaughlin, J.A. and Szent-Gyorgyi, A. Ascorbate-quinone interactions: electrochemical, free radical, and cytotoxic properties. *Proc.Natl.Acad.Sci.U.S.A.* 80:129-132, 1983.

Powis, G. Free radical formation by antitumor quinones. *Free Radic.Biol.Med.* 6:63-101, 1989.

Prescott, L.F. Paracetamol overdosage - pharmacological considerations and clinical management. *Drugs* 25:290-314, 1983.

Prescott, L.F. Clinical Features and management of analgesic poisoning. *Hum.Toxicol.* 3:75S-84S, 1984.

Prescott, L.F. Paracetamol: a model for toxic injury mediated by the formation of active metabolites. *Liver Cells and Drugs* 164:153-160, 1988.

Pruijn, F.B., Sibeijn, J.-P. and Bast, A. Menadione inhibits the α_1-adrenergic receptor-mediated increase in cytosolic free calcium concentration in hepatocytes by inhibiting inositol 1,4,5-triphosphate-dependent release of calcium from intracellular stores. *Biochem.Pharmacol.* 42:1977-1986, 1991.

Pumford, N.R., Martin, B.M. and Hinson, J.A. A metabolite of acetaminophen covalently binds to the 56 kDa selenium binding protein. *Biochem.Biophys.Res.Comm.* 182:1348-1355, 1992.

Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. Peroxynitrite oxidation of sulfhydryls - the cytotoxic potential of superoxide and nitric oxide. *J.Biol.Chem.*

266:4244-4250, 1991.

Rafeiro, E., Barr, S.G., Harrison, J.J. and Racz, W.J. Effects of N-acetylcysteine and dithiothreitol on glutathione and protein thiol replenishment during acetaminophen-induced toxicity in isolated mouse hepatocytes. *Toxicol.* 93:209-224, 1994.

Rao, D.N.R., Takahashi, N. and Mason, R.P. Characterization of a glutathione conjugate of 1,4-benzosemiquinone-free radical formed in rat hepatocytes. *J.Biol.Chem.* 263:17981-17986, 1988.

Rao, P.V. and Zigler, J.S.,Jr. Purification and characterization of e-crystallin/quinone reductase from guinea pig liver. *Biochim.Biophys.Acta* 1117:315-320, 1992.

Ray, S.D., Sorge, C.L., Raucy, J.L. and Corcoran, G.B. Early loss of large genomic DNA *in vivo* with accumulation of Ca^{2+} in the nucleus during acetaminophen-induced liver injury. *Toxicol.Appl.Pharmacol.* 106:346-351, 1990a.

Ray, S.D., Sorge, C.L., Tavacoli, A., Raucy, J.L. and Corcoran, G.B. Extensive alteration of genomic DNA rise in nuclear Ca^{2+} *in vivo* early after hepatoxic acetaminophen overdose in mice. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: Plenum Press, 1990b, p. 699-705.

Ray, S.D., Kamendulis, L.M., Gurule, M.W., Yorkin, R.D. and Corcoran, G.B. Ca^{2+} antagonists inhibit DNA fragmentation and toxic cell death induced by acetaminophen. *FASEB J.* 7:453-463, 1993.

Reed, D.J. Regulation of reductive processes by glutathione. *Biochem.Pharmacol.* 35:7-13, 1986.

Reed, D.J. and Fariss, M.W. Glutathione depletion and susceptibility. *Pharmacol.Rev.* 36:25S-33S, 1984.

Roberts, D.W., Bucci, T.J., Benson, R,W., et al. Immunohistochemical localization and quantification of the 3-(cystein-S-yl)-acetaminophen protein adduct in acetaminophen hepatotoxicity. *Am.J.Pathol.* 138:359-371, 1991.

Rosen, G.M., Singlrtary, W.V.,Jr, Rauckman, E.J. and Killenberg, P.G. Acetaminophen hepatotoxicity (an alternative mechanism). *Biochem.Pharmacol.* 32:2053-2059, 1983.

Rosen, G.M., Rauckman, E.J., Ellington, S.P., Dahlin, D.C., Christie, J.L. and Nelson, S.D. Reduction and glutathione conjugation reactions of *N*-acetyl-*p*-benzoquinone imine and two dimethylated analogues. *Mol.Pharmacol.* 25:151-157, 1984.

Ross, D., Thor, H., Orrenius, S. and Moldéus, P. Interaction of menadione (2-methyl-1,4-naphtoquinone) with glutathione. *Chem.Biol.Interact.* 55:177-184, 1985.

Rossi, L., Moore, G.A., Orrenius, S. and O'Brien, P.J. Quinone toxicity in hepatocytes Without oxidative stress. *Arch.Biochem.Biophys.* 251:25-35, 1986.

Rubin, R. and Farber, J.L. Mechanisms of the killing of cultured hepatocytes by hydrogen peroxide. *Arch.Biochem.Biophys.* 228:450-459, 1984.

Rundgren, M., Porubek, D.J., harvison, P.J., Cotgreave, I.A., Moldéus, P. and Nelson, S.D. Comparative cytotoxic effects of N-acetyl-p-benzoquinone imine and two dimethylated analogues. *Mol.Pharmacol.* 34:566-572, 1988.

Salo, D.C., Pacifici, R.E., Lin, S.W., Giulivi, C. and Davies, K.J.A. Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. *J.Biol.Chem.* 265:11919-11927, 1990.

Saville, J.G., Davidson, C.P., d'Adrea, G.H., Born, C.K. and Hamrick, M.E. Inhibition of acetaminophen hepatotoxicity by chlorpromazine in fed and fasted mice. *Biochem.Pharmacol.* 37:2467-2471, 1988.

Schöneich, C., Asmus, K.D., Dillinger, U. and Bruchhausen, F.V. Thiyl radical attack on polyunsaturated fatty acids: a possible route to lipid peroxidation. *Biochem.Biophys.Res.Comm.* 161:113-120, 1989.

Shen, H., Tsuchida, S., Tamai, K. and Sato, K. Identification of cysteine residues involved in disulfide formation in the inactivation of glutathione transferase P-form by hydrogen peroxide. *Arch.Biochem.Biophys.* 300:137-151, 1993.

Shen, W., Kamendulis, L.M., Ray, S.D. and Corcoran, G.B. Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: effects of Ca^{2+}-endonuclease, DNA repair, and glutathione depletion inhibitors on DNA fragmentation and cell death. *Toxicol.Appl.Pharmacol.* 112:32-40, 1992.

Shichi, H., Gaasterland, D.W., Jensen, N.M. and Nebert, D.W. Ah locus: gentic differences in susceptibility to cataracts induced by acetaminophen. *Science* 200:539-541, 1978.

Sies, H. Intracellular effects of glutathione conjugates and their transport from the cell. In: *Glutathione conjugation - mechanisms and biological significance*, edited by Sies, H. and Ketterer, B. Lodon: Academic Press, 1988, p. 175-192.

Silva, J.M., McGirr, L. and O'Brien, P.J. Prevention of nitrofurantoin-induced cytotoxicity in isolated hepatocytes by fructose. *Arch.Biochem.Biophys.* 289:313-318, 1991.

Simpson, J.A., Narita, S., Gieseg, S., Gebicki, S., Gebicki, M. and Dean, R.T. Long-lived reactive species on free-radical-damaged proteins. *Biochem.J.* 282:621-624, 1992.

Smith, G.D. and Schachman, H.K. A disproportionation mechanism for the all-or none dissociation of mercurial-treated glyceraldehyde phosphate dehydrognase. *Biochemistry* 10:4576-4588, 1971.

Smith, M.T., Evans, C.G., Thor, H. and Orrenius, S. Quinone-induced oxidative injury to cells and tissues. In: *Oxidative stress*, edited by Sies, H. London: Academic Press, 1985, p. 91-113.

Snyder, G.H., Cennerazzo, M.J., Karalis, A.J. and Field, D. Electrostatic influence of local cysteine environments on disufide exchange kinetics. *Biochemistry* 20:6509-6518, 1981.

Srivastava, S.K. and Beutler, E. Cataract produced by tyrosinase and tyrosine systems in rabbit lens in vitro. *Biochem.J.* 112:421-425, 1969.

Stadtman, E.R Ascorbic acid and oxidative inactivation of proteins. *Am.J.Clin.Nutr.* 54:1125S-1128S, 1991.

Starke, P.E. and Farber, J.L. Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide - evidence for the participation of hydroxyl radicals formed by an iron-catalyzed harber-weiss reaction. *J.Biol.Chem.* 260:10099-10104, 1985.

Streeter, A.J., Dahlin, D.C., Nelson, S.D. and Baillie, T.A. The covalent binding of acetaminophen to protein. evidence for cysteine residues as major sites of arylation in vitro. *Chem.Biol.Interact.* 48:349-366, 1984.

Streeter, A.J., harvison, P.J., Nelson, S.D. and Baillie, T.A. Cross-link of protein molecules by the reactive metabolite of acetaminophen, N-actyl-p-benzoquinone imine, and related quinoid compounds. *Adv.Exp.Med.Biol.* 197:727-737, 1986.

Stubberfield, C.R. and Cohen, G.M. Iterconversion of NAD(H) to NADP(H) - a cellular response to quinone-induced oxidative stress in isolated hepatocytes. *Biochem.Pharmacol.* 38:2631-2637, 1989.

Tee, L.B.G., Boobis, A.R., Huggett, A.C. and Davies, D.S. Reversal of

acetaminophen toxicity in isolated hamster hepatocytes by dithiothreitol. *Toxicol.Appl.Pharmacol.* 83:294-314, 1986.

Thomas, D.J., Sadler, A., Subrahmanyam, V.V., et al. Bone marrow stromal cell biactivation and detoxification of the benzene metabolite hydroquinone: comparison of macrophages and fibroblastoid cells. *Mol.Pharmacol.* 37:255-262, 1990.

Thomas, J.A., Park, E-M., Chai, Y.-C., Brooks, R., Rokutan, K. and Johnston, R.B.,Jr. S-thiolation of protein sulfhydryls. In: *Biological reactive intermediates IV*, edited by Witmer, C.M. New York: plenum Press, 1990, p. 95-103.

Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphtoquinone) by isolated hepatocytes. *J.Biol.Chem.* 257:12419-12425, 1982.

Thor, H., Hartzell, P., Svensson, S.-Å, et al. On the role of thiol groups in the inhibition of liver microsomal Ca^{2+} sequestration by toxic agents. *Biochem.Pharmacol.* 34:3717-3723, 1985.

Thor, H., Mirabelli, F., Salis, A., Cohen, G.M., Bellomo, G. and Orrenius, S. Alterations in hepatocytes cytoskeleton caused by redox cycling and alkylating quinones. *Arch.Biochem.Biophys.* 266:397-407, 1988.

Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione : Applications to mammalian blood and other tissues. *Anal.Biochem.* 27:502-522, 1969.

Tirmenstein, M.A. and Nelson, S.D. Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. *J.Biol.Chem.* 264:9814-9819, 1989.

Torchinsky, Y.M. *Sulfur in proteins*, New York:Pergamon Press, 1981.

Tschesche, H. and Macartney, H.W. A new principle of regulation of enzymic activity (activation and regulation of human polymorphonuclear leukocyte collagenase via disulfide-thiol exchange as catalysed by glutathione cycle in peroxidase-coupled reaction to glucose metabolism). *Eur.J.Biochem.* 120:183-190, 1981.

Tsokos-Kuhn, J.O., Todd, .E.L., McMillin-Wood, J.B. and Mitchell, J.R. ATP-dependent calcium uptake by rat liver plasma membrane vesicles - effect of alkylating hepatotoxins *in vivo* -. *Mol.Pharmacol.* 28:56-61, 1985.

Tsokos-Kuhn, J.O., Hughes, H., Smith, C.V. and Mitchell, J.R. Alkylation of the liver plasma membrane and inhibition of the Ca^{2+} ATPase by acetaminophen. *Biochem.Pharmacol.* 37:2125-2131, 1988.

Uchida, K. and Stadtman, E.R Selective cleavage of thioeher linkage in proteins modified with 4-hydroxynonenal. *Proc.Natl.Acad.Sci.U.S.A.* 89:5611-5615, 1992.

van de Straat, R., de Vries, J., Kulkens, T., Debets, A.J.J. and Vermeulen, N.P.E. Paracetamol, 3-monoalkyl- and 3,5-dialkyl derivatives -comparison of their microsomal cytochrome P-450 dependent oxidation and toxicity in freshly isolated hepatocytes -. *Biochem.Pharmacol.* 35:3693-3699, 1986.

van de Straat, R., de Vries, J., Debets, A.J.J. and Vermeulen, N.P.E. The mechanism of prevention of paracetamol-induced hepatotoxicity by 3,5-dialkyl substitution - the roles of glutathione depletion and oxidative stress -. *Biochem.Pharmacol.* 36:2065-2070, 1987a.

van de Straat, R., de Vries, J. and Vermeulen, N.P.E. Role of hepatic microsomal and purified cytochrome P-450 in one-electron reduction of two quinone imines and concomitant reduction of molecular oxygen. *Biochem.Pharmacol.* 36:613-619, 1987b.

van de Straat, R., Bijloo, G.J. and Vermeulen, N.P.E. Paracetamol, 3-monoalkyl- and 3,5-dialkyl-substituted derivatives - antioxidant activity and relationship between lipid peroxidation and toxicity -. *Biochem.Pharmacol.* 37:3473-3476, 1988.

van Heyningen, R. Naphthalene cataract in rats and rabbits: a resumé. *Exp.Eye Res.* 28:435-439, 1979.

van Ommen, B., den Besten, C., Rutten, A.L.M., et al. Active site-directed irreversible inhibition of glutathione S-transferases by glutathione conjugate of tetrachloro-1,4-benzoquinone. *J.Biol.Chem.* 263:12939-12942, 1988.

van Ommen, B., Ploemen, J.H.T.M., Bogaards, J.J.P., Monks, T.J., Gau, S.S. and van Bladeren, P.J. Irreversible inhibition of rat glutathione S-transferase 1-1 by quinones and their glutathione conjugates. *Biochem.J.* 276:661-666, 1991.

Vermeulen, N.P.E., Bessems, J.G.M. and van de Straat, R. Molecular aspects of paracetamol-induced hepatotoxicity and its mechanism-based prevention. *Drug Metab.Dispos.* 24:367-407, 1992.

Wardman, P. Conjugation and oxidation of glutathione via thiyl free radicals. In: *Glutathione conjugation - mechanisms and biological significance*, edited by Sies, H. and Ketterer, B. London: Academic Press , 1988, p. 43-72.

Wardman, P. Reduction potentials of one-electron couples involving free radicals in aqueous solution. *J.Phys.Chem.Ref.Data* 18:1637-1755, 1989.

Wardman, P. Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Radic.Res.Commun.* 8:219-229, 1990.

Watson, D., MacDermot, J., Wilson, R., Cole, P.J. and Taylor, G.W. Purification and structural analysis of pyocyanin and 1-hydroxyphenazine. *Eur.J.Biochem.* 159:309-313, 1986.

Wayner, D.D.M., Burton, G.W. and Ingold, K.U. The antioxidant efficiency of vitamin C is concentration-dependent. *Biochim.Biophys.Acta* 884:119-123, 1986.

Wefers, H. and Sies, H. Hepatic low-level chemiluminescence during redox cycling of menadione-glutathione conjugate: relation to glutathione and NAD(P)H:quinone reductase (DT-diaphorase) activity. *Arch.Biochem.Biophys.* 224:568-578, 1983a.

Wefers, H. and Sies, H. Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen. *Eur.J.Biochem.* 137:29-36, 1983b.

Weiner, L.M. Oxygen Radicals generation and DNA scission by anticancer and synthetic quinones. *Methods Enzymol.* 233:92-105, 1994.

Weis, M., Morgenstern, R., Cotgreave, I.A., Nelson, S.D. and Moldéus, P. *N*-acetyl-*p*-benzoquinone imine-induced protein thiol modification in isolated rat hepatocytes. *Biochem.Pharmacol.* 43:1493-1505, 1992.

Wilson, I., Wardman, P., Cohen, G.M. and d'Arcy Doherty, M. Reductive role of glutathione in the redox cycling of oxidizable drugs. *Biochem.Pharmacol.* 35:21-22, 1986a.

Wilson, I., Wardman, P., Lin, T.-S. and Sartorelli, A.C. One-electron reduction of 2- and 6-methyl-1,4-naphtoquinone bioreductive alkylating agents. *J.Med.Chem.* 29:1381-1384, 1986b.

Wilson, I., Wardman, P., Lin, T.-S. and Sartorelli, A.C. Reactivity of thiols towards derivatives of 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents. *Chem.Biol.Interact.* 61:229-240, 1987.

Winkler, B.S. In vitro oxidation of ascorbic acid and its prevention by GSH. *Biochim.Biophys.Acta* 925:258-264, 1987.

Wistow, G. Lens crystallins: gene recruitement and evolutionary dynamism. *Trends Biochem. Sci.* 18:301-306, 1993.

Wolff, S.P., Garner, A. and Dean, R.T. Free radicals, lipids and protein degradation.

Trends Biochem. Sci. 11:27-31, 1986.

Wolff, S.P. Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* 233:182-189, 1994.

Wolff, S.P. and Dean, R.T. fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis. *Biochem.J.* 234:399-403, 1986.

Wolff, S.P. and Spector, A. Pro-oxidant activation of ocular reductants. 2. Lens epithelial cell toxicity of a dietary quinone is associated with a stable free radical formed with glutathione in vitro. *Exp.Eye Res.* 45:791-803, 1987.

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. Cell death: the significance of apoptosis. *Int.rev.Cytol.* 68:251-303, 1980.

Wyllie, A.H. and Duvall, E. Cell death. In: *Oxford textbook of pathology - Vol 1*, edited by McGee, J.O'D., Isaacson, P.G. and Wright, N.A. Oxford: Oxford University Press, 1992, p. 141-157.

Yoshida, H., Iijima, M., Kuronuma, Y. and Harada, T. Inhibition of biliary excretion of indocyanine green by the thiol-oxidizing agent, diazenedicarboxylic acid bis[N,N'-dimethylamide]. *Biochem.Pharmacol.* 43:477-482, 1992.

Younes, M. and Siegers, C.-P. Lipid peroxidation as a consequence of glutathione depletion in rat and mouse liver. *Res.Commun.Chem.Pathol.Pharmacol.* 27:119-128, 1980.

Zhang, R., Hirsh, O., Mohsen, M. and Samuni, A. Effects of nitroxide stable radicals on jugulone cytotoxicity. *Arch.Biochem.Biophys.* 312:385-391, 1994.

Ziegler, D.M. Role of reversible oxidation-reduction of enzyme thiols-dissulfides in metabolic regulation. *Ann.Rev.Biochem.* 54:305-329, 1985.