NOVEL ESTERS OF CYSTEINE AS POTENTIAL CHEMOPROTECTANTS

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ABSTRACT

Novel esters of cysteine have been synthesized to evaluate their potential as chemoprotectants. The increased lipohilicity of the esters relative to cysteine should facilitate their entry into cells. Following hydrolysis, the esters should provide an intracellular source of cysteine, to be utilized either for the synthesis of glutathione (GSH) or to act as a direct nucleophilic chemoprotectant. Several esters of cysteine have previously been show to protect against the acute pulmonary oedema induced by perfluoroisobutene, an electrophilic pyrolysis product of Teflon.

Cysteine *iso*propylester (CIPE) produced a rapid but transient elevation of the levels of non-protein sulphydryls in several mouse organs, which was particularly marked in the lung. In laboratory animals with induced hepatic cytochrome P450 activity, CIPE protected against paracetamol and bromobenzene-induced toxicity. CIPE ameliorated the toxicity of paracetamol as effectively as *N*-acetylcysteine. However, CIPE did not prevent naphthalene-induced damage to the lung. Following exposure of mice to diethyl maleate, CIPE did not support the replenishment of hepatic GSH, in contrast to *N*-acetylcysteine.

CIPE appeared to offer best protection against electrophilic attack of short duration, coinciding with elevated levels of tissue cysteine.

High doses of CIPE alone were toxic when administered to mice induced with benzo(a)pyrene but not to control or mice induced with phenobarbitone.

In a rat lung slice model, the esters of cysteine, CIPE and cysteine cyclohexylester, produced considerable rises in intracellular cysteine when compared to other potential cysteine delivery systems, cysteine, *N*-acetylcysteine or L,2-oxothiazolidine-4-carboxylic acid. All these cysteine delivery systems were capable of replenishing GSH in slices previously

depleted of their GSH by diethyl maleate.

Cellular esterases played a key role in the metabolism of cysteine esters by isolated cells. Inhibiting esterase activity, while increasing the extracellular half life of the cysteine esters did not greatly increase the pool of unmetabolised ester detected within cells. A novel role for esterase, mediating the rise in cellular cysteine by the esters, is hypothesised.

Paraquat is a herbicide whose pulmonary toxicity is associated with its selective accumulation into the lungs via a polyamine uptake system. The iron chelators, desferrioxamine, CP51 and 2,3-dihydroxybenzoic acid, partially ameliorated the toxicity of paraquat to rat lung slices. Desferrioxamine was the most effective because it also blocked accumulation of paraquat. Exogenous GSH moderately alleviated the toxicity of paraquat but raising the pulmonary content of GSH with phorone pretreatment did not.

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Abbreviations

ATP - adenosine triphosphate
BCNU - 1,2-bis(2-chloroethyl)-1-nitrosourea
CCHE - cysteine cyclohexylester
CIPE - cysteine isopropylester
CP51 - 1-(2-methoxyethyl)-2-methyl-3-hydroxypyridin-4-one
DMSO - dimethyl sulphoxide
GSH - glutathione (reduced)
GSSG - glutathione disulphide
HBSS - Hanks balanced salt solution
i.p. - intraperitoneal
KRP - Krebs Ringer phosphate buffer
NABQI - N-acetyl-p-benzoquinone imine
NADPH - nicotinamide adenine dinucleotide phosphate (reduced)
NPSH - non-protein sulphydryl
OTZ - L,2-oxothiazolidine-4-carboxylic acid
RibCys - 2-D-*ribo*-(1'.2'.3'.4'.-tetrahydroxybutyl)-thiazolidine-4-carboxylate
SEM - standard error of the mean
SGOT - serum glutamate-oxaloacetate transaminase
SGPT - serum glutamate-pyruvate transaminase

Chapter 1 - INTRODUCTION

1.1 Introduction

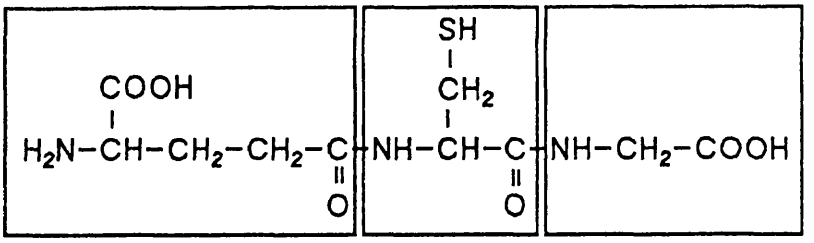
Many drugs and xenobiotics are metabolised to reactive electrophilic intermediates which are detoxified by glutathione (GSH). The supply of the amino acid cysteine can limit the rate of GSH biosynthesis. In this thesis the ability of cysteine esters to supplement intracellular concentrations of cysteine to either support the synthesis of GSH or to act as a direct chemoprotectant, is considered. An initial explanation of the biological and toxicological significance of GSH and how cysteine may influence GSH-mediated and other physiological processes is necessary.

1.2 Functions of glutathione

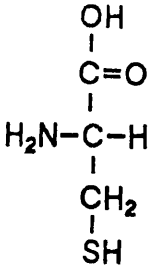
GSH (fig 1.1) is the major non-protein sulphydryl in a wide variety of cells types. Tissue levels of GSH are high, in the millimolar concentration range (0.5 -10mM) and the liver is an especially abundant source [1]. GSH is a tripeptide (γ-glutamylcysteinyl glycine) and the thiol group of cysteine is responsible for many of the chemical properties of GSH while the γ-glutamyl moiety imparts stability to the molecule (fig 1.1). Glutathione exists in two forms, either a reduced state (GSH) or oxidised as a disulphide (GSSG). Much of the intracellular pool of glutathione exists in the reduced form. In addition GSH may form mixed disulphides with the SH groups of proteins and enzymes, or other low molecular weight sulphydryl compounds [1]. Several roles of physiological and toxicological importance have been proposed for GSH, including cellular defence, modulation of enzymic activity [2, 3], the transport of amino acids [4], production of proteins and DNA, immune modulation [5], maintenance of antioxidants, such as ascorbate, in their reduced state [6] and as a reservoir of the amino acid cysteine [7].

As a protectant, GSH may react with electrophiles [8]. The biotransformation of numerous xenobiotics by the cytochrome P450 system is often associated with the formation of toxic electrophiles (fig 1.2). The liver is the major site of

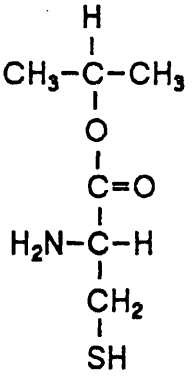
Figure 1.1 - Structures of cysteine, glutathione and cysteine isopropyl-, cyclohexyl- and t-butylester



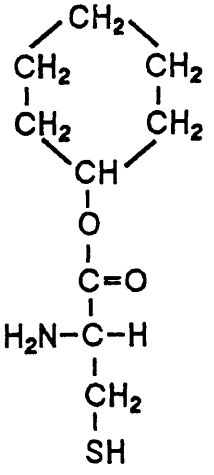
GLUTATHIONE



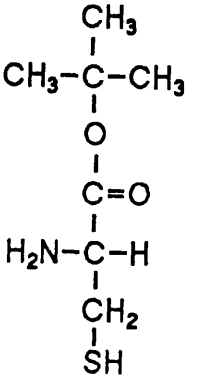
CYSTEINE



CYSTEINE ISOPROPYLESTER



CYSTEINE CYCLOHEXYLESTER



CYSTEINE t-BUTYLESTER

metabolism of xenobiotics but extrahepatic tissues, such as the lung, also have metabolizing capacity. GSH has an important protective function, conjugating electrophiles to form compounds with less biological activity, consequently sparing critical nucleophilic macromolecules from damage and facilitate excretion of the electrophilic xenobiotics [9]. Conjugation may occur directly or via the glutathione-S-transferases [10]. In general, the specificity of GSH-S-transferases for GSH is high but the specificities for the electrophilic substrates are broad and partially overlapping [11]. A diverse group of compounds, some of which are presented in table 1.I are metabolised to electrophilic intermediates, often epoxides, which react with GSH. Increasing or decreasing the expression of cytochrome P450 activity can greatly influence the metabolism and toxicity of these chemicals [12]. Prior depletion of hepatic GSH by diethyl maleate also enhances the toxicity of several compounds, including paracetamol, bromobenzene and carbon tetrachloride [12]. GSH conjugates are usually further metabolised to their corresponding mercapturic acids and excreted into urine. However it is possible that GSH-conjugates may themselves be nephrotoxic [21].

GSH also prevents tissue damage mediated by agents such as H_2O_2, organic hydroperoxides or lipid hydroperoxides by being a substrate for GSH peroxidase [22]. GSH is oxidised to GSSG by GSH peroxidase, with concomitant detoxification of the hydroperoxides. GSH reductase subsequently reduces GSSG back to GSH at the expense of NADPH. NADPH is generated by the pentose-phosphate shunt. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) which inhibits GSH reductase activity and GSH regeneration [23], will potentiate the toxicity of redox cycling compounds such as paraquat [24] and adriamycin [25].

1.3 Synthesis and degradation of GSH

A. Synthesis

GSH is generated from its constitutive amino acids by the sequential action of two enzymes and at the expense of 2 ATP

Figure 1.2 - Detoxification of electrophiles by conjugation with GSH

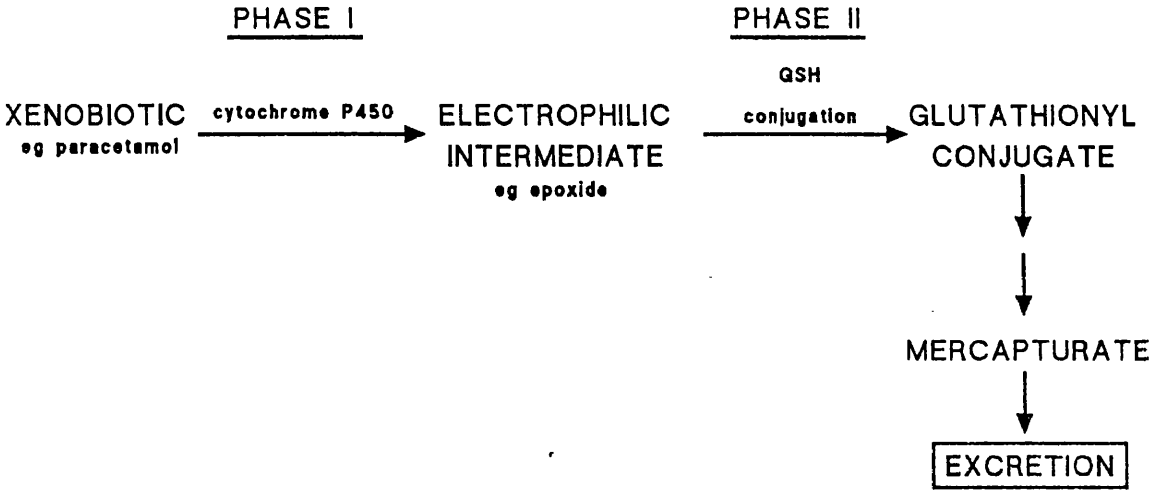


Table 1.I - Compounds whose metabolic intermediates are conjugated by GSH

Compound	Source	Target organ	Reference
paracetamol	analgesic	liver	[13]
bromobenzene	industrial solvent	liver, kidney, lung	[14]
allyl alcohol	synthetic intermediate	liver	[15]
carbon tetrachloride	organic solvent	liver and lung	[12,16]
1,1-dichloro-ethylene		liver and lung	[17,18]
naphthalene	moth balls	liver and lung	[19]
4-ipomeanol	mouldy sweet potatoes	liver and lung	[20]

All the compounds are metabolised by cytochrome P450, except allyl alcohol, which is metabolised by alcohol dehydrogenase to acrolein. The pulmonary toxins mainly effect Clara cells

molecules. A γ-glutamyl link between L-glutamate and L-cysteine is mediated by cytoplasmic γ-glutamylcysteine synthetase [26].

L-cysteine + L-glutamate + ATP
---> γ-glutamylcysteine + ADP + P

This step is rate limiting in the generation of GSH. Physiological levels of GSH compete with L-glutamate for the γ-glutamyl site on γ-glutamylcysteine synthetase, inhibiting the enzyme [27]. Buthionine sulphoximine, by reducing the activity of the enzyme, depletes tissue levels of GSH [28] and potentiates the toxicity of several xenobiotics.

The second step of GSH biosynthesis, catalysed by GSH synthetase, forms a peptide linkage between γ-glutamylcysteine and L-glycine. This step is not feedback inhibited by GSH [26].

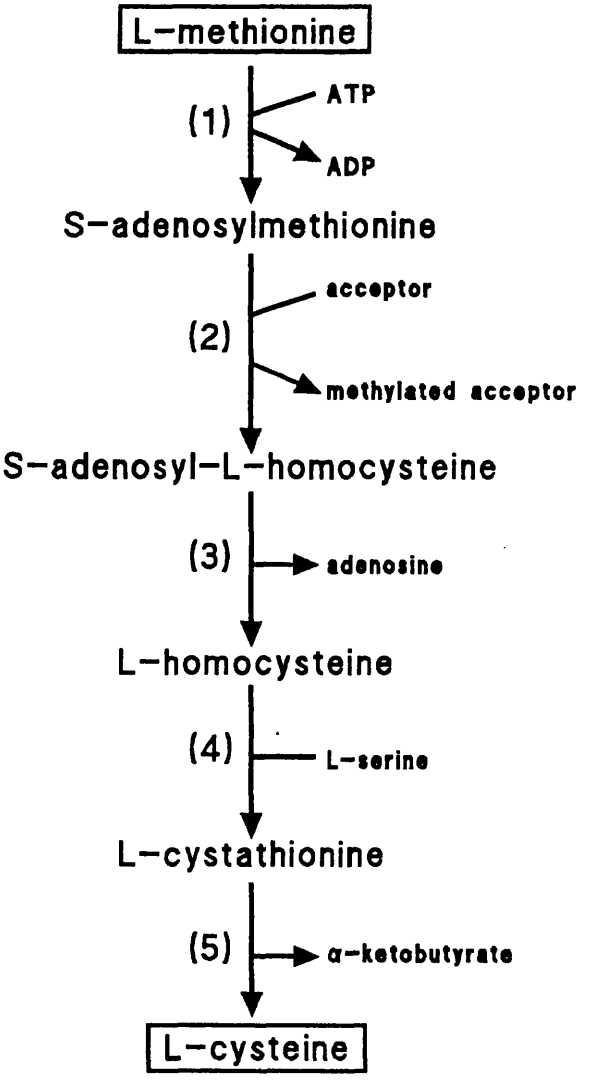
γ-glutamylcysteine + L-glycine + ATP
---> GSH + ADP + P

In addition to feed back inhibition by GSH, the supply of cysteine can also be a limiting factor in GSH production. Normal levels of cellular cysteine are in the range 10-100μM [29], but the K_M of γ-glutamylcysteine synthetase for cysteine is 300μM [30], consequently cysteine availability influences the generation of GSH. Depletion of GSH by conjugation increases the rate of hepatic GSH synthesis (to 2-3 μmol/hr/g wet weight) such that the pool of hepatic cysteine (0.2μmol/g) is rapidly consumed ($t_{½}$ = 2-3 min) [12].

B. Sources of cysteine

Cysteine is usually obtained directly from dietary protein or indirectly from the conversion of methionine. In starved rats, the hepatic levels of both GSH and cysteine decline. When refed, the rate of hepatic GSH generation is proportional to

Figure 1.3 - Cystathionine pathway



(1) S-adenosylmethionine synthetase
(2) methyl-transferases
(3) S-adenosyl-homocysteine hydrolase
(4) cystathionine synthetase
(5) γ-cystathionase

[Adapted from ref. 33]

the cysteine concentration in the diet [7, 31].

The conversion of methionine to cysteine via the cystathionine pathway, is the main supply of cysteine to the liver [32]. The pathway involves five enzymes (fig 1.3). Methionine adenosyltransferase transfers an adenosyl moiety from ATP to methionine forming S-adenosylmethionine (AdoMet). The S-methyl group of AdoMet is transferred to an acceptor molecule, such as glycine or a xenobiotic, yielding S-adenosylhomocysteine, which is in equilibrium with homocysteine. Homocysteine may be methylated, regenerating methionine or alternatively be converted to cysteine by the sequential action of cystathionine ß-synthetase and cystathionine γ-lyase.

C. Interorgan circulation of GSH

The liver has two distinguishing properties giving it a unique position in GSH homeostasis; it is the organ best able to utilise methionine for cysteine generation [33] and exports GSH into plasma and bile [34]. Consequently, the liver influences the level of GSH in extrahepatic tissues, regulated by the γ-glutamyl cycle [35].

The release of GSH from hepatocytes is a major component of GSH hepatic turnover. In perfused liver the rate of GSH efflux is 12-18 nmol/min/g liver weight [36] and while accounting for 90-95% of the rate of GSH depletion, the rate of GSH biosynthesis usually equals the rate of loss by efflux or conjugation [1]. Kaplowitz *et al* [1] suggest the sinusoidal transport of GSH from the hepatocytes to plasma is a carrier mediated process. Damaging the microvilli of the sinusoidal membranes experimentally with thioacetamide or through abuse of alcohol, alters the transmembrane transport of GSH [37], lowering plasma levels of GSH without reducing hepatic GSH content.

GSH exported from the liver can increase the cellular GSH concentration in extrahepatic tissues in two ways. As part of the γ-glutamyl cycle, following extracellular degradation of GSH exported from the liver, cellular GSH could be synthesised

from its constitutive amino acids. Alternatively, GSH may be taken up intact by certain epithelia cells such as type II alveolar epithelia cells [38]. However in general mammalian cells do not accumulate intact GSH.

D. Degradation of GSH

The degradation of GSH is mediated at the cell surface by γ-glutamyl transpeptidase, a membrane bound enzyme (fig 1.4). γ-Glutamyl transpeptidase has a wide tissue distribution and has been found in both the kidney [39] and lung [40], in addition a number of tumours contain high levels of activity.

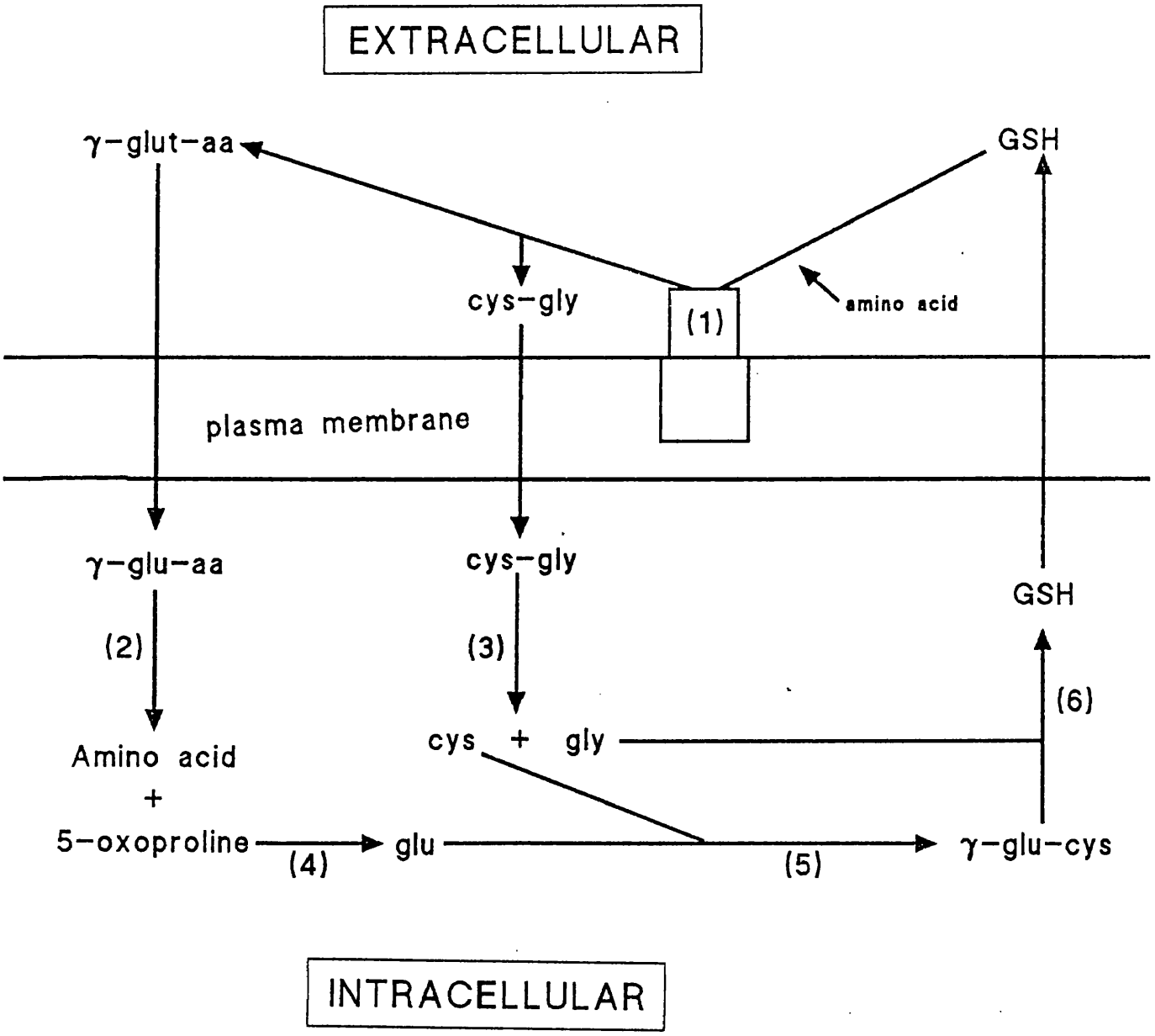
γ-Glutamyl transpeptidase transfers the γ-glutamyl residue of GSH to suitable acceptor substrates, such as L-amino acids or cystine [11], via an enzyme bound γ-glutamyl intermediate, releasing a γ-glutamyl-amino acid complex and cysteinylglycine (fig 1.4). The γ-glutamylamino acid-complex can enter cells, where γ-glutamylcyclotransferase converts it to 5-oxoproline and the corresponding amino acid. Cysteinylglycine can also be carried into cells and is cleaved to cysteine and glycine by dipeptidases. Administration of γ-glutamyl transpeptidase enzyme activity inhibitors, such as AT125 [L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-oxazoleacetic acid], increases the extracelluar levels of GSH [41] and produces glutathionuria.

1.4 Modulation of tissue levels of glutathione

GSH has a pivotal position in cellular defense and modulation of tissue GSH levels may greatly influence the susceptibility of various organs to toxic chemicals, oxidative damage or radiation. Prior depletion of hepatic GSH with diethyl maleate, which conjugates GSH in a GSH-S-transferase catalysed reaction, potentiates the toxicity of paracetamol to of mice [13]. Buthionine sulphoximine, an irreversible inhibitor of γ-glutamylcysteine synthetase, depletes the levels of GSH in human lymphoid cells and enhances the loss of cell viability when irradiated [42].

A variety of different methods have been utilised to

Figure 1.4 - The γ-glutamyl cycle



Enzyme key: (1) γ-glutamyltranspeptidase, (2) γ-glutamylcyclotransferase, (3) dipeptidase, (4) 5-oxoprolinase, (5) γ-glutamylcysteine synthetase and (6) GSH synthetase.

elevate intracellular GSH. In general exogenous GSH is unable to cross cell membranes as the intact tripeptide and performs poorly when administered as a protective agent. However high doses of GSH are tolerated by man [43] and animals [44, 45]. It also protects laboratory animals against the nephrotoxicity of cisplatinum and the urotoxicity of cyclophosphamide [43,44,45]. But liposomally entrapped GSH was much more effective in protecting against paracetamol toxicity than exogenous GSH [46].

In order to facilitate the membrane transport of GSH, a number of hydrophobic esters of GSH have been synthesised, which following hydrolysis elevate intracellular GSH *in vivo* and *in vitro* (table 1.II). GSH monoethylester enhances intracellular GSH in cultured endothelial cells and protects against H_2O_2-induced injury [47]. GSH isopropylester elevates GSH in several mouse organs [48], prevents skeletal muscle degeneration associated with prolonged exposure to buthionine sulphoximine [51] and maintains levels of GSH in ischaemic rat brain [50]. GSH isopropylester also protects against the toxicity of mercuric chloride and the anticancer drug cisplatin [51, 52], and both the methyl and the isopropylesters protect against the hepatotoxicity of paracetamol [48,53]. Prior treatment of a human lymphoid cell line with GSH monoethylester protected against the effects of radiation [54].

1.5 The transport of cysteine in mammalian cells

Cysteine, unlike GSH, is readily transported into cells. The uptake is achieved by the systems present for the transport neutral (dipolar) amino acids (fig 1.5). These systems designated as A, ASC and L were first described in Ehrlich ascites cells [55]. These uptake systems are described as ubiquitous [56] and have been characterised in several isolated and cultured cell systems (table 1.III).

The A and ASC systems are Na^+-dependent, while the L system is Na^+-independent. Being Na^+-dependent gives the A and ASC systems access to the energy stores associated with inorganic ion gradients. The A system transports most dipolar

Table 1.II - Uses of glutathione esters

ESTER	PROTECTION AGAINST	REFERENCE
GSH methylester	paracetamol	[53]
GSH monoethylester	H_2O_2	[47]
	radiation	[54]
	suppressed expression of HIV	[86]
GSH isopropylester	paracetamol	[48]
	mercuric chloride	[51]
	cisplatin	[52]
	BSO-induced muscle degeneration	[49]
	ischaemic brain injury	[50]

Table 1.III - Cells and tissues with cysteine uptake systems

CELL/TISSUE	UPTAKE SYSTEM	REFERENCE
Ehrlich ascites cells	A, ASC, L	55,50
human fibroblasts	A, ASC, L	57,58
Chinese hamster ovary cells	A, ASC, L	59
isolated rat hepatocytes	ASC	62
red blood cells - human	ASC, L	62
- pigeon	ASC	60,62
intestine		
- brush border membrane	ASC	61,62
- basolateral membrane	L	
kidney		
- brush border membrane	ASC	62

amino acids [56] but its activity is usually repressed. Hormones or starving cells of amino acids induces the activity of the A system [63]. System A is subject to *trans*-inhibition by dipolar amino acids and can work against a concentration gradient. 2-(Methylamino)isobutyric acid blocks the uptake of amino acids via the A system [65] and is the tool used to characterise the system.

The component of the Na^+-dependent uptake of neutral amino acids not inhibited by 2-(methylamino)isobutyric acid is classified as the ASC system. This system was first characterised in Ehrlich ascites cells, which were still able to accumulate alanine, serine and cysteine in the presence of 2-(methylamino)isobutyric acid [61], hence ASC system. However this system has a broad specificity for several neutral amino acids in other types of cells [62].

The L system is classified by its inhibition by 2-amino-[2,2,1]-heptane-2-carboxylic acid in Na^+-free medium. In common with the ASC system, it can undergo *trans*-stimulation by which uptake of amino acids by cells can be stimulated by prior loading with the amino acid or an analogue; or alternatively the presence of ASC or L amino acids in the external environment will accelerate the export of amino acids previously accumulated [56, 62].

The uptake of cystine may alter the cellular concentration of cysteine. Cystine can be accumulated by a specific Na^+-independent system, X_c^-, into several cells. The X_c^- system exchanges anionic cystine for intracellular anionic glutamate [66] (fig 1.5). The activity of the transport system has recently been expressed in the membrane of *Xenopus laevis* oocyctes injected with mRNA for the X_c^- system obtained from mouse macrophages exposed to diethyl maleate [67]. A number of reagents and conditions may modulate the uptake of cystine. Following depletion of GSH by diethyl maleate, the uptake of cystine by culture human fibroblasts increases [66]. Inhibition of GSH reductase by BCNU also stimulated the uptake of cystine and glutamate by the X_c^- system of endothelial cells [68]. Imposing oxidative stress on cultured endothelial cells

by addition of either H_2O_2 or polymorphonuclear leukocytes, also enhanced the rate of cystine uptake [69]. Buthionine sulphoximine dramatically inhibits uptake of cystine by the human carcinoma cell line, A549 [70] and by Chinese hamster ovary cells co-incubated with *N*-acetylcysteine or cysteamine [71]. Intracellular cystine levels are kept low by the reducing environment of the cell, generating cysteine which may be used for GSH biosynthesis [66,68,70,71].

Cysteine levels as previously discussed may also be modulated by the activity of the γ-glutamyl cycle.

1.6 Cysteine delivery systems

The supply of cysteine is rate limiting in the biosynthesis of GSH. As the cystathionine pathway is restricted to the liver [32], extrahepatic organs cannot utilise methionine and are largely dependent on preformed L-cysteine. Compounds capable of enhancing cellular levels of cysteine may provide an alternative method to promote GSH. However if tissue levels of GSH are normal, γ-glutamylcysteine synthetase will be inhibited and there will be no net increase in GSH due to excess cysteine. Several methods have been used to elevate cellular cysteine. Methionine supports synthesis of GSH in isolated rat hepatocytes, to prevent the toxicity of bromobenzene [72].

A. *N*-Acetylcysteine

N-Acetylcysteine, is perhaps the best known cysteine prodrug and is used clinically as an antidote to paracetamol intoxication [73] and to alleviate pulmonary congestion [74].

After deacetylation, N-acetylcysteine, generates cysteine. capable of supporting GSH synthesis [75]. When administered after paracetamol intoxication, *N*-acetylcysteine increases the generation of GSH, reverses the paracetamol-induced decline in hepatic GSH and increases the formation of paracetamol-GSH adduct passed into the plasma and bile [73,76,77,78]. The possibility also exists for *N*-acetylcysteine to react with or prevent the generation of the reactive metabolite of

paracetamol by other routes; being a nucleophile it may directly conjugate toxic electrophiles, or enhance sulphation of paracetamol, or inhibit cytochrome P450 activity or delay absorption of paracetamol [73]. However two facts demonstrate the main mechanism of hepatoprotection by *N*-acetylcysteine is due to increased availability of GSH. The unnatural D-isomer of *N*-acetylcysteine which has the potential to conjugate the toxic electrophile directly but not to sustain GSH biosynthesis, did not prevent paracetamol toxicity [79]. Secondly, inhibition of γ-glutamylcysteine synthetase with buthionine sulphoximine, preventing GSH biosynthesis, abrogated the protective effect of *N*-acetylcysteine against the toxicity of paracetamol [80].

N-Acetylcysteine has a wide variety of other effects *in vivo* and *in vitro*. *N*-Acetylcysteine is proposed to act as an antioxidant [81] and is a powerful scavenger of highly reactive hydroxyl radical (·OH) and the myeloperoxidase-derived oxidant hypochlorous acid [82]. Spontaneous and chemically-induced mutations in bacteria are prevented by *N*-acetylcysteine [83] and *in vivo* the thiol enhances the detoxification of carcinogens [84]. Both *N*-acetylcysteine and cysteine influence the activity of lymphocytes by modulating the levels of GSH [5]. The expression of human immunodeficency virus (HIV) in infected cultured cells caused by low levels of GSH, can be suppressed by *N*-acetylcysteine [85,86]. By maintaining levels of GSH, *N*-acetylcysteine prevents the expression of certain transcription factors that stimulate replication of both viral and human genes involved in the disease [5].

B. Thiazolidine derivatives

An alternative method of elevating cellular cysteine concentrations is to use either 5-oxoproline analogues, such as L-2-oxothiazolidine-4-carboxylic acid (OTZ) or the 2-(polyhydroxyalkyl)thiazolidine-4-carboxylic acids (TCA) (fig 1.6), of these OTZ is the best known and studied. OTZ is effectively transported into cells [87] and together with 5-oxoproline, has a high affinity for the cytosolic enzyme 5-

oxoprolinase [88]. 5-Oxoprolinase has a wide tissue distribution and normally cleaves 5-oxoproline to L-glutamate (fig 1.4) [88]. However when OTZ is a substrate, intracellular cysteine is generated (fig 1.6).

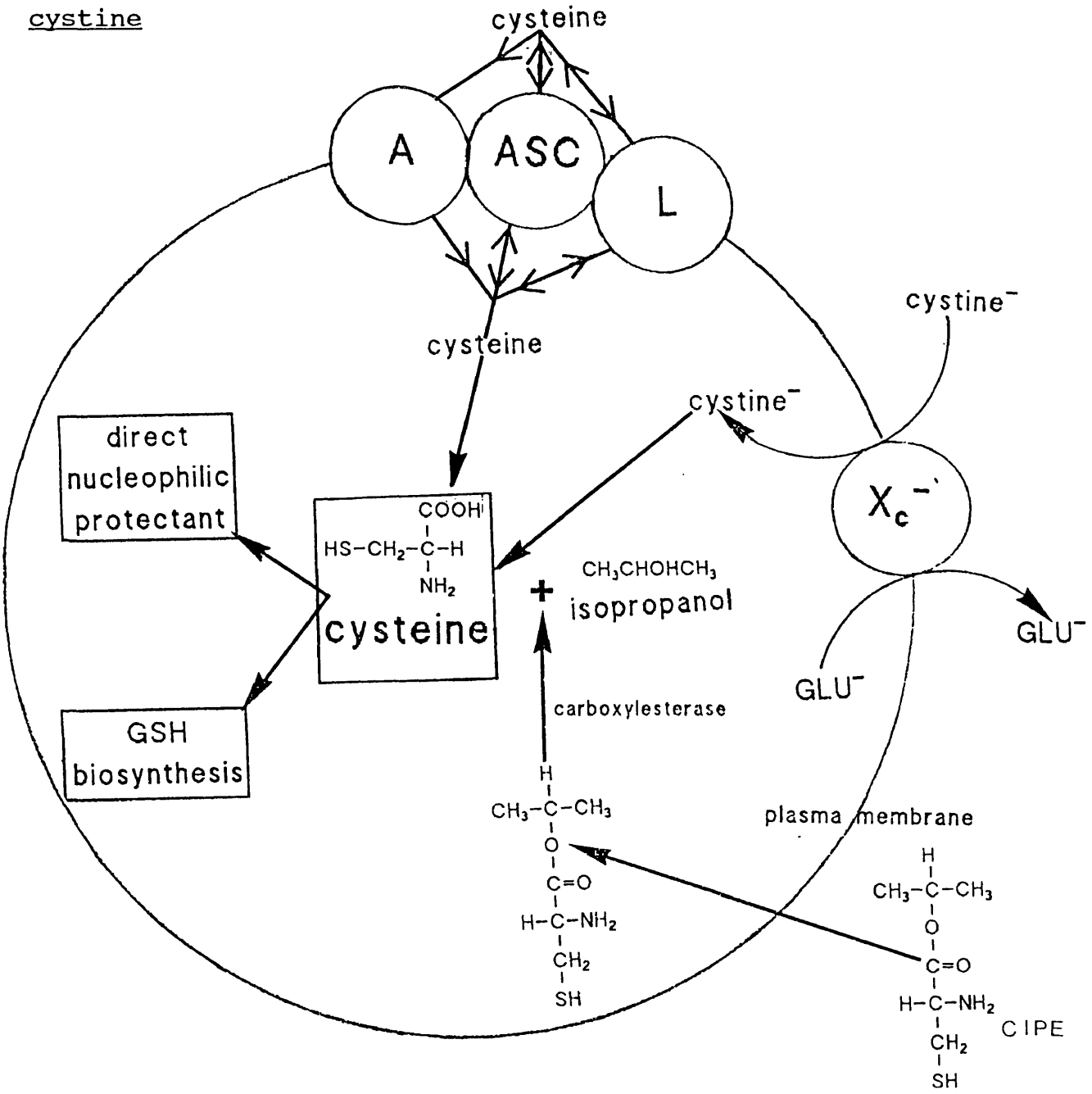
The TCA compounds are generated by condensing cysteine with naturally occurring aldose monosaccharides. 2-D-*Ribo*-(1'.2'.3',4'.-tetrahydroxybutyl)-thiazolidine-4-carboxylic acid (RibCys) (fig 1.6) is the product of condsening cysteine with D-ribose [89] and the TCA with the most promising biological activity. RibCys liberates cysteine following nonenzymic cleavage and hydrolysis (fig 1.6).

Both OTZ and RibCys promote intracellular levels of cysteine and stimulate the formation of GSH in fasted mice [29, 90]. They are capable of protecting against paracetamol intoxication [87, 89]. In the presence of buthionine sulphoximine, a specific inhibitor of γ-glutamylcysteine synthetase, no protection is observed with OTZ. Therefore OTZ protects by increasing GSH availability. Buthionine sulphoximine also inhibits the replenishment of GSH by RibCys in isolated hepatocytes [89]. RibCys protected against the urotoxicity of cyclophosphamide [91]. Recently the efficacy of OTZ as a protective agent has been questioned [48], as intravenous administration of OTZ was not protective against paracetamol-induced liver injury.

C. Esters of cysteine

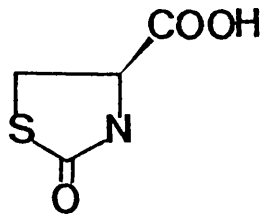
An alternative method to increase cellular cysteine is the use of cysteine esters. A series of esters of cysteine has been synthesized by the Chemical and Biological Defense Establishment, initially with a view to protect against short lived, inhaled, pulmonary electrophiles. The increased lipophilicity of the esters relative to cysteine should ease their passage across cell membranes and following hydrolysis by cytosolic esterases, increase intracellular cysteine (fig 1.6), analogous to the increase in GSH following exposure to esters of GSH. Increased cellular levels of cysteine may act as a direct chemoprotectant or support the biosynthesis of GSH.

Figure 1.5 - Esters of cysteine as intracellular delivery compounds for cysteine and the uptake systems for cysteine and cystine



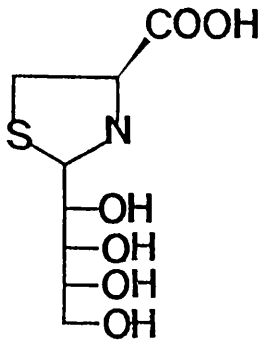
CIPE has been used to illustrate the hypothesis of uptake of esters. CIPE should diffuse across the plasma membrane due to the hydrophobicity of the isopropyl moiety. Cytosolic esterases should hydrolyse the ester to liberate cysteine and isopropanol. The cysteine may then act as a direct chemoprotectant or be used to generate GSH. The uptake of extracellular cysteine is restricted to the neutral amino acid uptake systems (A, ASC and L). Note the unidirectional flow of cysteine by system A, while systems ASC and L allow for both cysteine uptake and efflux. The X_c- systems exchanges anionic cystine for anionic glutamate.

Figure 1.6 - Thiazolidine derivatives as cysteine prodrug



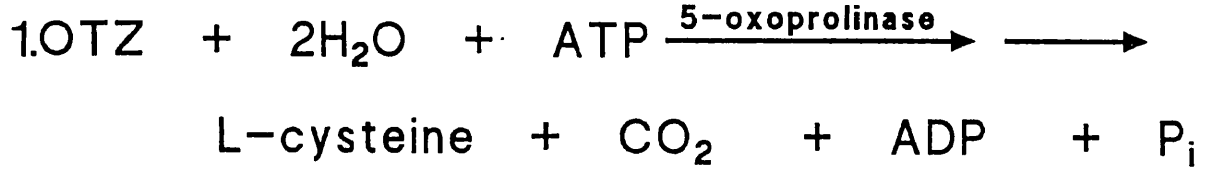
OTZ

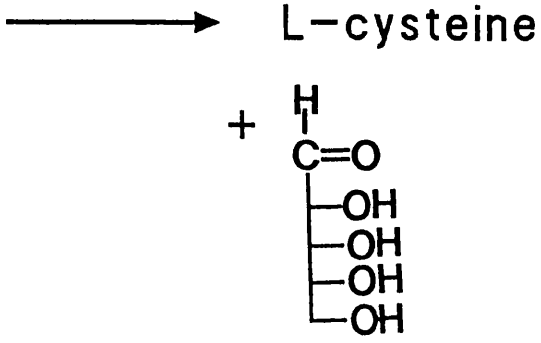
L-2-oxothiazolidine
-4-carboxylate



RibCys

2-D-*ribo*-(1'.2'.3'.4'.
-tetrahydroxybutyl)
-thiazolidine-4-
carboxylate





At physiological pH, the degree of ionisation of the thiol group of cysteine esters is greater than that for either cysteine or GSH (respectively 16%, 6% and 1%) [92]. Therefore a larger portion of cysteine or esters of cysteine would be available to react directly with toxic electrophiles than GSH.

The moderate increase in the tissue content of cysteine following administration of GSH or buthionine sulphoximine *in vivo* is postulated to be responsible for protection respectively against the urotoxicity of cyclophosphamide and cisplatin [44,45] and the hepatotoxicity of 2-methylfuran [93].

Several esters of cysteine were preferentially distributed to the lungs when given to rats and enhanced pulmonary levels of cysteine without increasing the pulmonary content of GSH [94]. The elevation of pulmonary sulphydryls was sufficient to protect against the inhaled electrophile, perfluoroisobutene [94]. ^{35}S-Cysteine ethyl ester produced a considerable but short lived labelling of the lung in contrast to radiolabelled cysteine [95]. The octyl and methyl esters of cysteine, while increasing cellular concentrations of cysteine, were ultimately cytotoxic to cultured human melanoma cells [96].

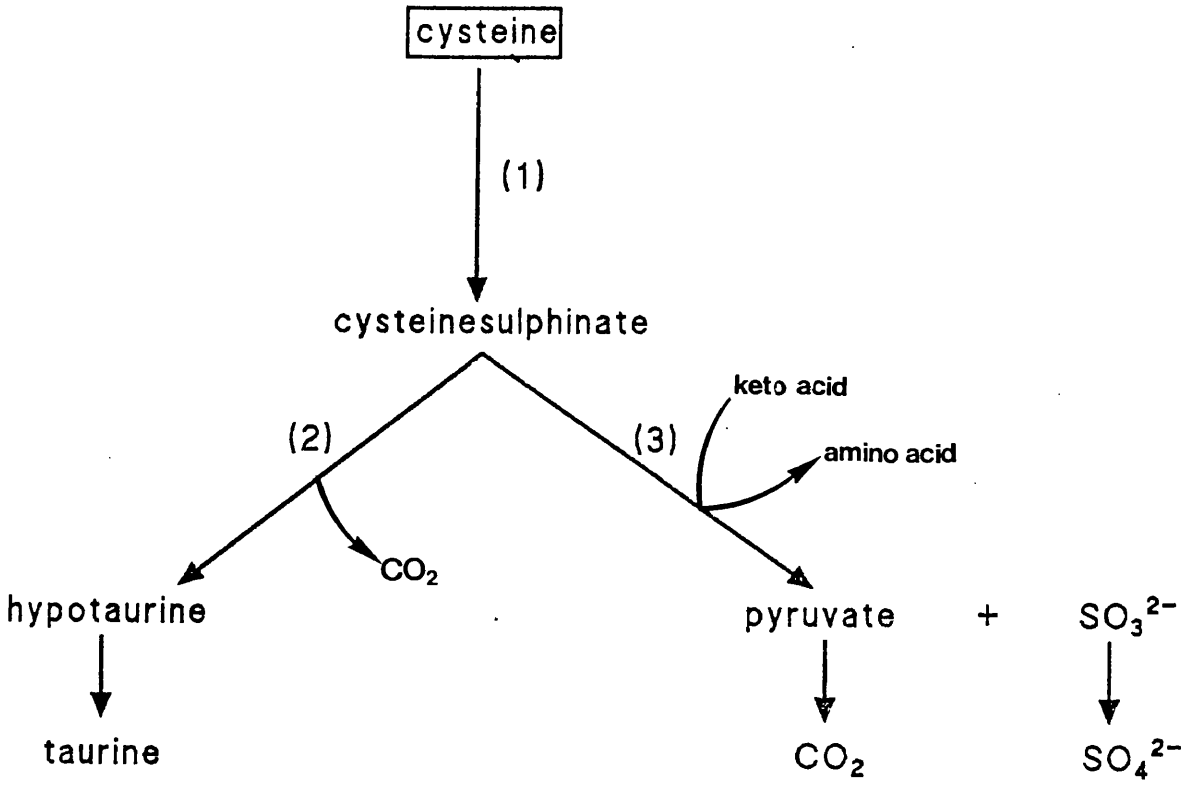
1.7 The metabolism of cysteine

Cysteine metabolism is complex and several pathways of cysteine metabolism have been demonstrated [35, 97, 98] but ultimately, catabolism of cysteine yields taurine and CO_2 or sulphate, urea and CO_2.

The cysteinesulphinate pathway (fig 1.7) oxidises cysteine via the enzyme, cysteine dioxygenase to cysteinesulphinate [99]. Cysteinesulphinate can be decarboxylated to hypotaurine which is readily oxidised to taurine. Alternatively cysteinesulphinate is a substrate for glutamate-oxaloacetate transaminase forming ß-sulphinylpyruvate which spontaneously decomposes to pyruvate and sulphate. In rats, mice and isolated rat hepatocytes ~70-80% of cysteinesulphinate is metabolised to taurine and ~20-30% is transaminated to pyruvate and sulphate [100, 101, 102]

However <10% of the total cysteine catabolism is accounted

Figure 1.7 - Cysteinesulphinate dependent metabolism of cysteine



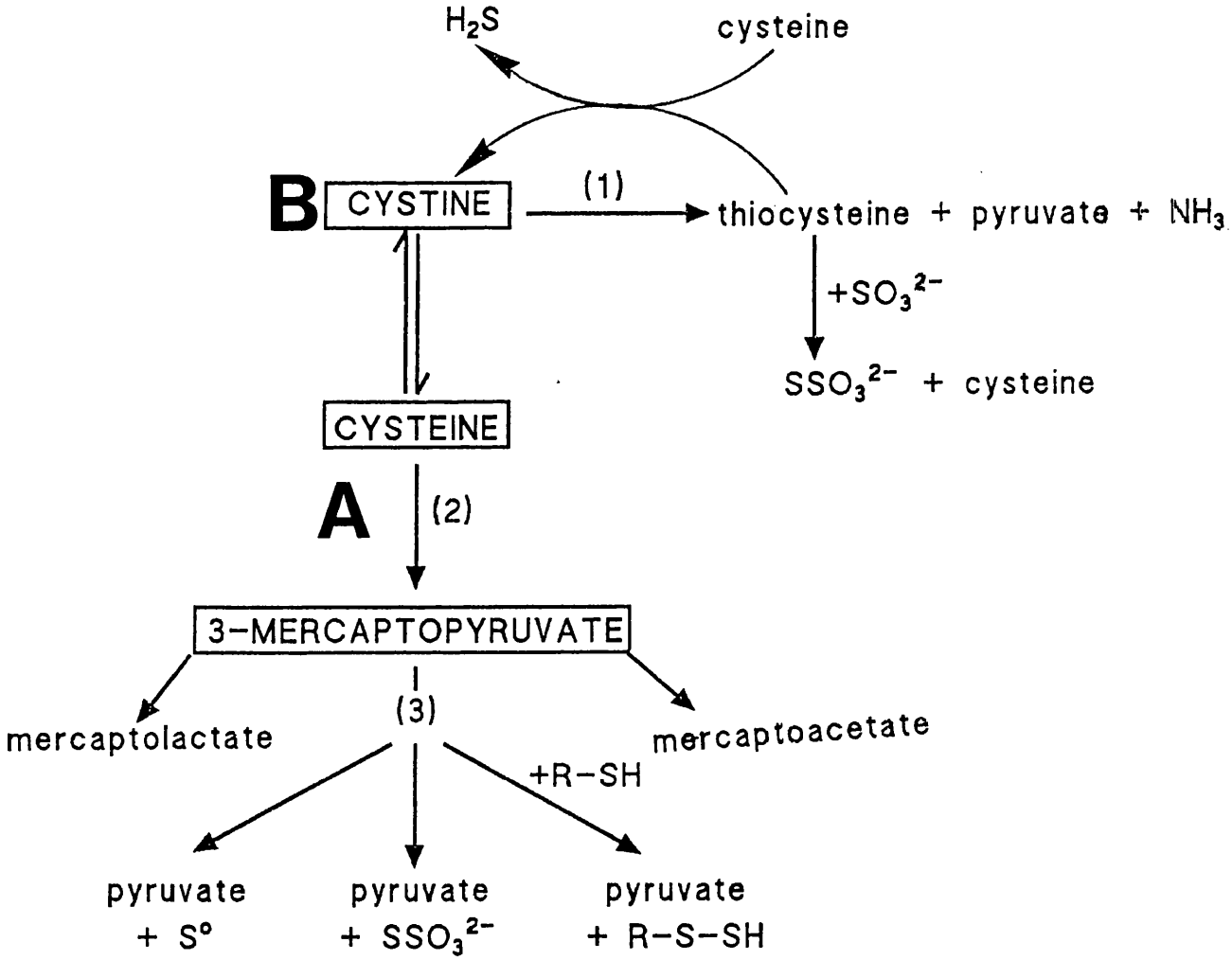
Enzyme Key: (1) - cysteine dioxygenase, (2) - cysteinesulphinate decarboxylase, (3) - glutamate-oxaloacetate transaminase.

[Adapted from ref. 33,97,98]

Figure 1.8 - Cysteinesulphinate independent metabolism of cysteine

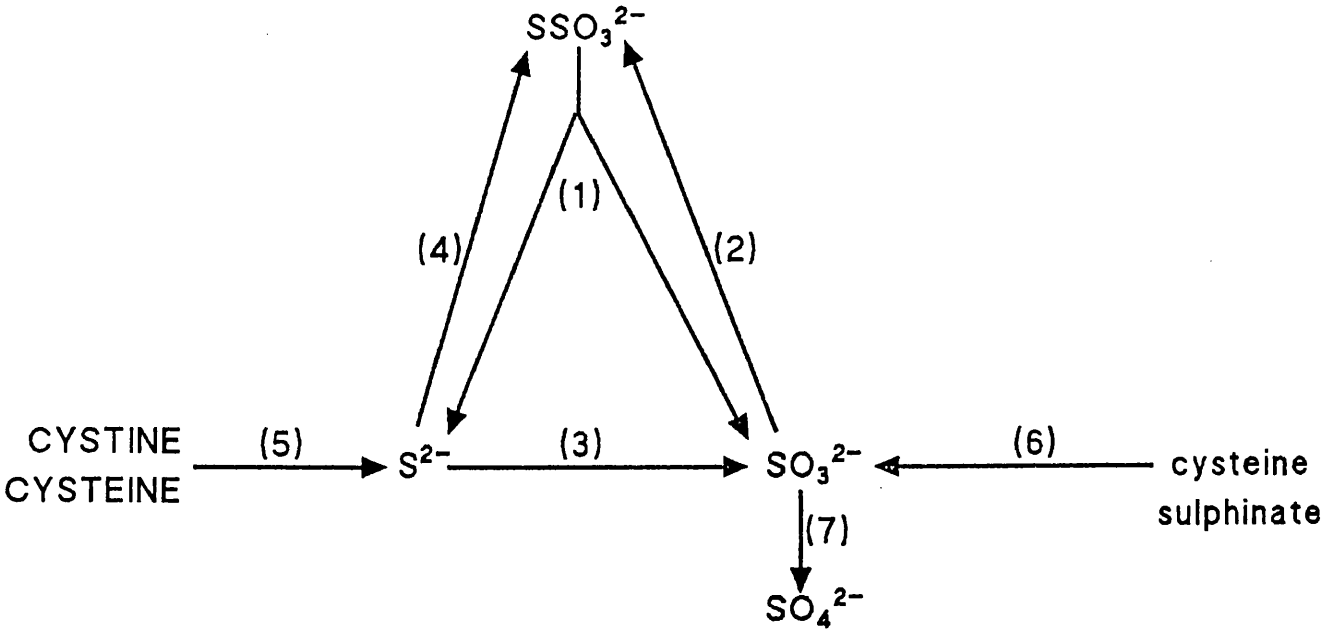
ENZYME KEY

(1) γ-cystathionase
(2) cysteine aminotransferase
(3) 3-mercaptopyruvate sulphurtransferase



[Adapted from ref 33,97,98]

Figure 1.9 - The thiosulphate cycle



Enzyme Key: (1) - rhodanese or thiosulphate reductase, (2) - rhodanese, (3) - nonenzymic, (4) - nonenzymic, (5) S^0 production via γ-cystathionase, (6) - glutamate-oxaloacetate transaminase, (7) - SSO_3^{2-} production from 3-mercaptopyruvate, (8) - sulphite oxidase.

[Adapted from ref 98]

for as taurine and hypotaurine [101] and the formation of sulphate is the major fate for cysteine [99, 103, 104]. Cysteine metabolism to sulphate and CO_2 can be achieved by several pathways independent of cysteinesulphinate formation [101, 105, 106]. Alternative pathways involve the transamination of cysteine with a keto acid to ß-mercaptopyruvate (scheme A, fig 1.8) or cleavage of cysteine or cystine by cystathionase with the release of ammonia (scheme B, fig 1.8) [98, 107]. Propargylglycine, as inhibitor of cystathionase activity, reduced the generation of CO_2 and ammonia from cysteine without affecting levels of cysteinesulphinate, indicative that a large portion of cysteine is metabolised by cysteinesulphinate-independent routes [101]. These pathways generate low molecular weight sulphur-containing compounds, sulphide (S^{2-}, S^{o}), sulphite (SO_3^{2-}) and thiosulphate (SSO_3^{2-}). The thiosulphate cycle in mammalian tissue allows this sulphur to be readily incorporated into sulphate (fig 1.9)[98].

Stipanuk et al [108] demonstrated the pathway which the metabolism of cysteine takes is dictated by the intracellular concentration of cysteine in isolated rat hepatocytes. When availability of cysteine is low, as is seen when hepatocytes are incubated with methionine or OTZ, GSH formation is favoured. However as levels of intracellular cysteine increase, a larger percentage of the cysteine is directed towards the formation of taurine and sulphate [108].

1.8 Cysteine toxicity

Cysteine residues are important in protein and polypeptides, helping to maintain their structure and modulating enzymatic activity. Compounds derived from cysteine such as GSH, coenzyme A and lipoic acid also have roles in controlling the cellular environment. Yet among the amino acids, cysteine is often labelled as toxic , the majority of them having low toxicity.

Administration of cysteine in the diet produced 100% mortality to weanling rats [109]. Diets with a high content

of D,L-methionine or L-cystine, both precursors of cysteine, fed to weanling rats depressed the rate of growth and caused fatalities [110]. A single intraperitoneal administration of cysteine (10mmol/kg) resulted in lethargy and convulsions in rats [29]. Cysteine also damages cultured cells when it is a component in culture media [111]. Leaving media to stand, allowing cysteine to become oxidised, or incorporating pyruvate into the media reduced the cytotoxicity of cysteine.

Cysteine produces acute, diffuse, neuronal degeneration when administered to neonatal rats (12mmol/kg, i.p.) [112]. The damage may be induced by the conversion of cysteine to acidic, neuroexcitatory metabolites such as cysteine sulphinate or cysteic acid [112] or alternatively accumulation of excess cysteine [113]. Concentrations of cysteine >0.6μmol/g wet brain wet damaged the brain [113]. Low doses of OTZ given to fasted mice appreciably elevated the levels of brain cysteine. However the conversion of OTZ by 5-oxoprolinase becomes saturated, so the toxicity seen with high doses of cysteine, due to excessive levels of brain cysteine, were not seen with OTZ [114].

The mechanism by which cysteine exerts its toxicity has not be satisfactorily explained. Oxidation of cysteine generates free radicals [115, 116]. The cysteine thiyl radical (CyS·) has been captured by ESR [115] and several reactive oxygen species ($O_2^{-\cdot}$, H_2O_2 and OH·) are proposed to be generated by oxidation of sulphydryl compounds [116]. Some of the routes for the generation of reactive oxygen species from cysteine are shown below.

(1) $CyS^- + M^{n+} \longrightarrow CyS\cdot + M^{(n-1)+}$

(2) $M^{(n-1)+} + O_2 \longrightarrow M^{n+} + O_2^-$

(3) $CyS^- + CyS\cdot \longrightarrow CyS\dot{S}yC$

(4) $CyS\dot{S}yC + O_2 \longrightarrow CySSyC + O_2^-$

(5) $CySH + O_2^{-\cdot} + H^+ \longrightarrow CyS\cdot + H_2O_2$

Thiolate anion (CyS^-), in a transition metal ion catalysed reaction **(1)**, is converted to the thiyl radical (CyS·).

Reduction of the metal ion at the expense of molecular oxygen liberates the superoxide anion **(2)**. The disulphide radical anion can autooxidise forming cystine and superoxide **(4)**. Superoxide may begin a chain reaction of further cysteine oxidation **(5)**. Cysteine may also form mixed disulphides with both low molecular weight sulphydryls, such as GSH, or with essential SH groups of proteins possibly modifying their activity. Cysteine may also form thiazolidine and hemithioketal derivatives with carbonyl containing compounds [29]. A number of pyridoxal 5'phosphate enzymes can be inhibited by forming stable thiazolidine compounds with cysteine [29].

Cysteine conjugates derived from the GSH-conjugates of a number of xenobiotics, such as halogenated alkanes and alkenes, hydroquinones and quinones are responsible for their nephrotoxicity [117, 118]. The high concentration of enzymes in the kidney such as γ-glutamyltranspeptidase, deacetylase and ß-lyase, responsible for the metabolism of GSH- or mercapturic-conjugates of xenobiotics, are proposed to be responsible for the selective generation of intermediates that damage renal tubules [21, 117, 118].

Several GSH conjugates of hydroquinones are toxic. The GSH conjugate of menadione (2-methyl-3-*S*-glutathionyl-1,4-naphthoquinone) redox cycles [119]. The di- and trisubstituted GSH conjugates of 1,4-benzoquinone are nephrotoxic [120]. AT125, an inhibitor of γ-glutamyltranspeptidase activity, prevents the generation of the cysteine conjugate and averts the nephrotoxicity of 1,4-benzoquinone GSH-conjugates. Whether the cytotoxicity is mediated by alkylation or oxidative stress remains to be elucidated [118]. Renal ß-lyase activity may activate cysteine conjugates to alkylating agents:-

(CYS)-SR ---> NH_3 + pyruvate + $[RSH]^{d+}$ ---> alkylation

Inhibiting ß-lyase activity with aminooxy acetic acid abolished the toxicity of *S*-(1,2-dichlorovinyl)-L-cysteine to isolated rat kidney proximal tubular cells [121]

1.9 Cytotoxic agents

Three compounds; paracetamol, bromobenzene and naphthalene, are known to be metabolised to electrophilic intermediates, which are conjugated by GSH. These compounds have been used to characterise cysteine isopropylester as a chemoprotectant in this thesis.

1.10 Paracetamol

A. Hepatotoxicity of paracetamol

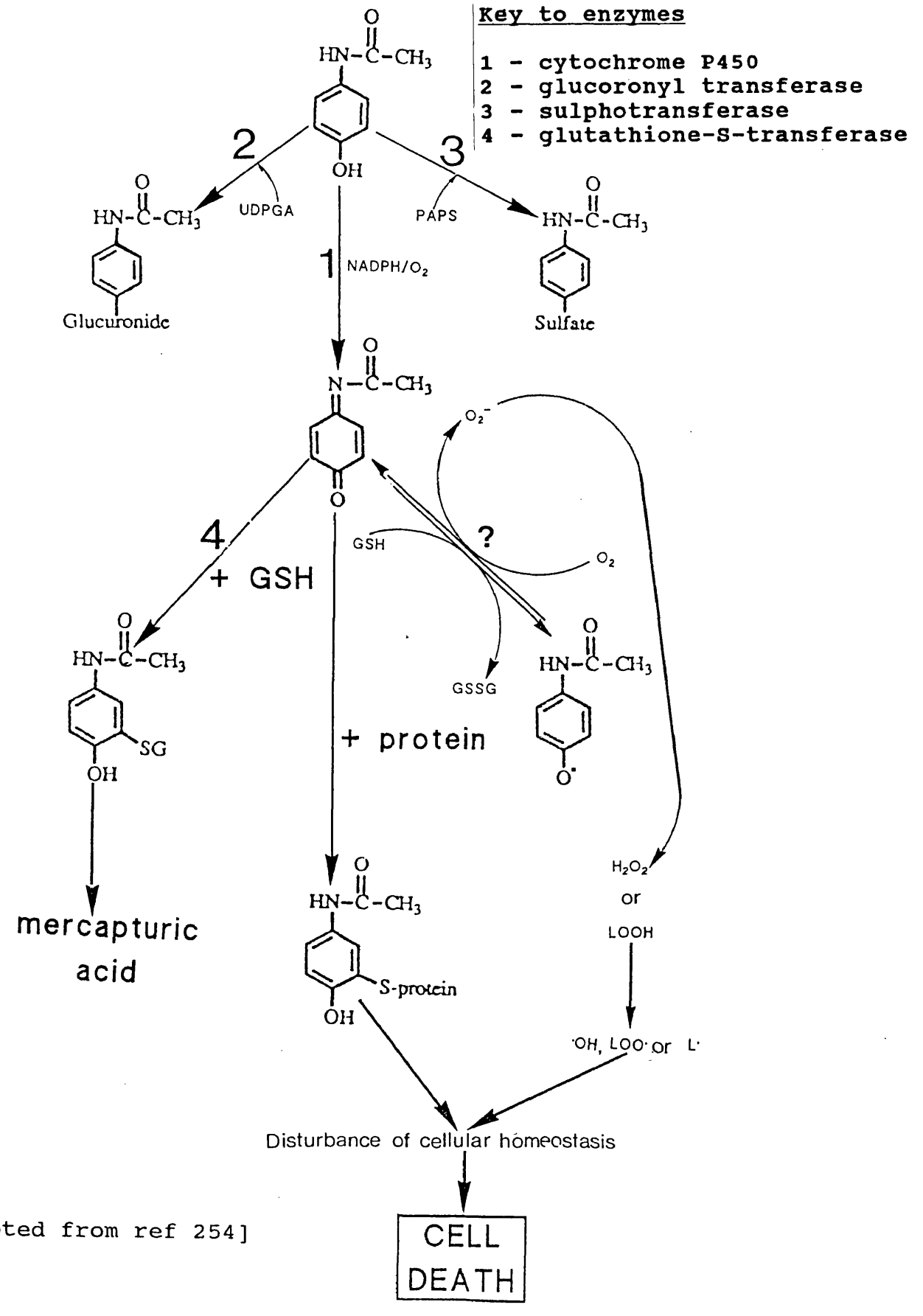
Paracetamol is a commonly used drug for the treatment of pain and fever. Excessive intake of the drug leads to acute hepatic necrosis in both humans and experimental animals [122, 123]. Therapeutic doses of paracetamol are usually metabolised through phase II conjugation with glucuronic acid and sulphate, forming safe excretable products (fig 1.10).

The oxidation of paracetamol by cytochrome P450 enzyme activity generates N-acetyl-p-benzoquinone imine (NABQI), a toxic electrophile. NABQI is usually inactivated by conjugation with GSH. Following an overdose, the supply of GSH becomes limited, resulting in binding of NABQI to alternative nucleophilic macromolecules within the cell to produce hepatotoxicity.

Paracetamol specifically damages the centrilobular region of the classical hepatic lobule (zone 3 of the acinar lobule). The distribution of cytochrome P450 activity and GSH is heterogeneous across the hepatic lobule, the centrilobular region is rich in cytochrome P450 activity but low in GSH, making it susceptible to damage by paracetamol. The hepatotoxicity of paracetamol can be markedly increased by inducers of microsomal enzyme activity such as phenobarbitone or benzo(a)pyrene, conversely inhibitors reduce paracetamol-induced hepatotoxicity in experimental animals [48, 125].

The toxicity of paracetamol can also be affected by the diurnal fluctuation in hepatic GSH [124], toxicity to mice

Figure 1.10 - The metabolism of paracetamol



[Adapted from ref 254]

being greatest when hepatic GSH is at a minimum.

Paracetamol can also be metabolised by prostaglandin synthetase to a reactive metabolite capable of depleting GSH and binding to proteins [126].

The underlying mechanism of paracetamol toxicity to cells following depletion of GSH has not been satisfactorily established. Both covalent and noncovalent interactions may lead to acute cell injury and death [127]. Several mechanisms have been postulated; binding to critical hepatic proteins, oxidation of protein or enzyme sulphydryls, effects on mitochondrial function [128, 129] loss of plasma membrane Ca^{2+}-ATPase activity and impairing Ca^{2+} sequestration, transient generation of reactive oxygen species [130].

A widely accepted hypothesis is binding of paracetamol metabolites to certain critical proteins as the key event leading to cell death. Following depletion of cytosolic and /or mitochondrial GSH, the possibility exists for NABQI to arylate cytosolic and mitochondrial proteins [131]. There is a good correlation between the amount of covalent binding of NABQI and the degree of cell death. However 3'-hydroxyacetanilide, a regioisomer of paracetamol, covalently binds to hepatic proteins in a manner similar to paracetamol, but without any hepatotoxicity. This suggests covalent binding of paracetamol to hepatic proteins may not be an important event in paracetamol toxicity. However, only paracetamol arylates mitochondrial protein, depletes mitochondrial GSH and inhibits plasma membrane Ca^{2+}-ATPase activity [131]. However non-covalent interactions following paracetamol intoxication may also have deleterious effects on cell viability. NABQI, is a chemical oxidant [132] and will oxidise intracellular sulphydryls. Inhibition of GSH reductase activity by BCNU potentiates paracetamol toxicity [133]. Paracetamol also inhibits GSH reductase activity [131] so may render hepatocytes susceptible damage from reactive oxygen metabolites normally generated. Additionally, NABQI may redox cycle, generating superoxide anions, but the generation is unlikely due to the relatively high potential of the semiquinone imine radical

[132]. The GSH conjugate of paracetamol, 3-(glutathion-S-yl) paracetamol is readily oxidised to a free radical by both horseradish peroxidase and NABQI [134]

Nelson and Pearson [127] suggest that until the proteins which are altered by paracetamol are identified, it will be difficult to establish whether binding or oxidant stress produced by NABQI is the event leading to cell death. Hoffman *et al* [135] suggest NABQI will especially arylate hepatic proteins rich in cysteine residues. Using immunochemical techniques, paracetamol appears to selectively bind hepatic proteins of 44 and 58 kDa [136, 137, 138, 139] in mice. When paracetamol is given to mice or following overdosage in humans, the covalent binding to the 58 kDa hepatic protein correlated to the degree of cellular injury [138, 140, 141]. The 58 kDa protein which selectively binds paracetamol was shown to have a large degree of sequence homology with a selenium binding protein (56 kDa) which was not especially rich in cysteine residues [142, 143].

B. Antidotes to paracetamol poisoning

The toxicity of paracetamol has been described in *in vitro* models as a two stage event; the primary stages involve the generation of NABQI, depletion of GSH; while the later stage is degenerative leading to cell death. Clinically, *N*-acetylcysteine and methionine are most effective in the early treatment of patients after large overdoses of paracetamol. However as time progresses the efficacy of sulphur-containing compounds is lowered but still of some therapeutic benefit [144, 145]. Hence the search for alternative therapeutic strategies which may bring about a reversal of the late events in paracetamol toxicity. Fructose [146] and the electron acceptor dichloro phenol and ethanol [129] appeared to delay the degenerative stage of paracetamol toxicity.

Several techniques have been utilised to prevent the toxicity of paracetamol in man, laboratory animals or in *in vitro* models. Treatment with N-acetylcysteine is a clinically proven method to ameliorate paracetamol toxicity [74], with

very few adverse side effects. It alleviates paracetamol toxicity by replenishing hepatic levels of GSH following infusion, as previously discussed. Alternatively *N*-acetylcysteine may be protective in the second, degenerative stage of paracetamol toxicity, by restoring the activity of proteolytic systems to remove proteins arylated by paracetamol [147].

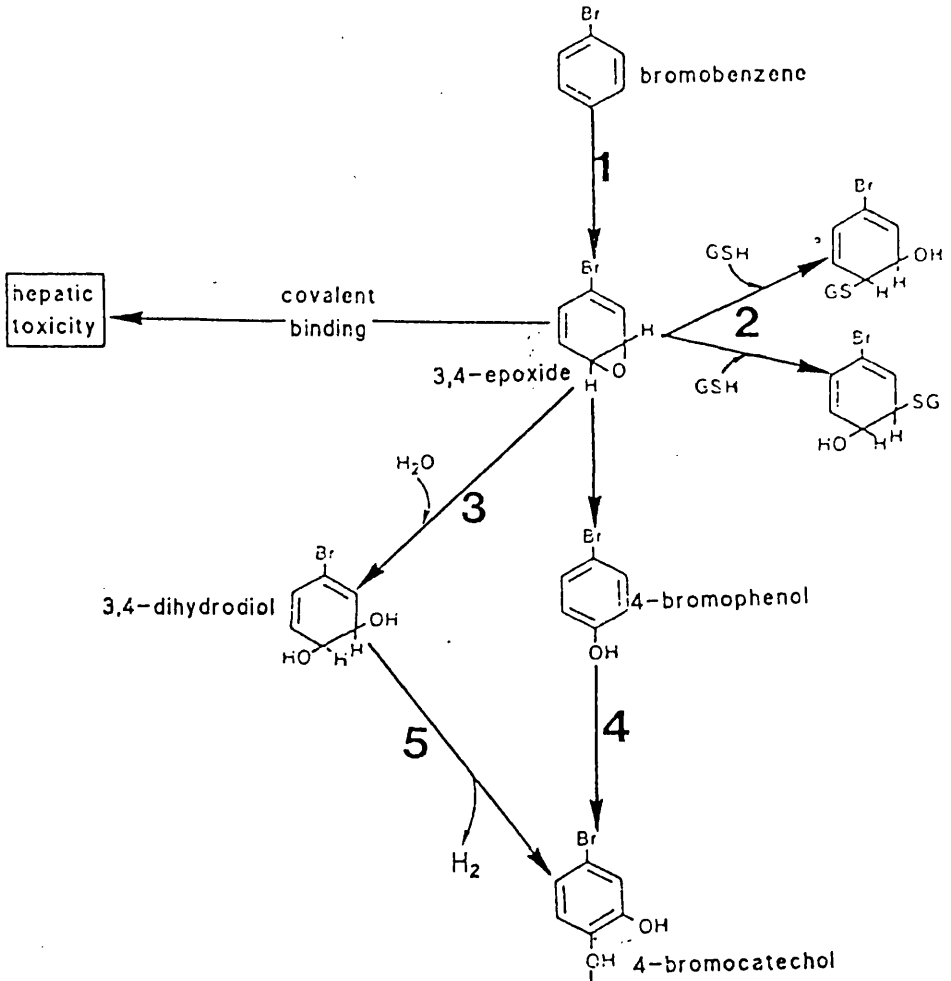
Other sulphur containing compounds have been used to continue the excretion of NABQI. Cysteamine effectively reduced the toxicity of paracetamol when given up to 10 hr postingestion. However it produced side effects; nausea, vomiting and cardiotoxicity [148]. In combination with *N*-acetylcysteine, it was effective against the toxicity of paracetamol at doses that are not protective when given alone to mice [149]. D,L-Methionine is used in the treatment of paracetamol poisoning in humans [150] and probably acts by replenishing cellular levels of GSH [80], following its conversion to cysteine via the cystathionine pathway. S-Adenosylmethionine, an intermediate of this pathway, also protects against the hepatotoxicity of paracetamol to mice [151].

Other attempts have been made to ameliorate toxicity. Acetylsalyclic acid protects possibly by modulation of the lethal events occurring after metabolic activation of paracetamol and depletion of GSH [152]. Ascorbate had no protective effect on the toxicity of paracetamol *in vivo* [153, 154]. Lipopolysaccharide, an immune stimulant, is proposed to protect against paracetamol-induced mortality in mice by depression of cytochrome P450, so reducing generation of NABQI [155].

1.11 Bromobenzene

Bromobenzene is an industrial solvent which is hepatotoxic, nephrotoxic and induces bronchiolar necrosis. The metabolism of bromobenzene to several possibly toxic metabolites is complex (fig.1.11 and 1.12).

Figure 1.11 - The metabolism of bromobenzene to hepatotoxic metabolites



Key to enzymes

1 - cytochrome P450
2 - glutathione-S-transferase
3 - epoxide hydrolase
4 - cytochrome P450
5 - dihydrodiol dehydrogenase

[Adapted from ref. 14]

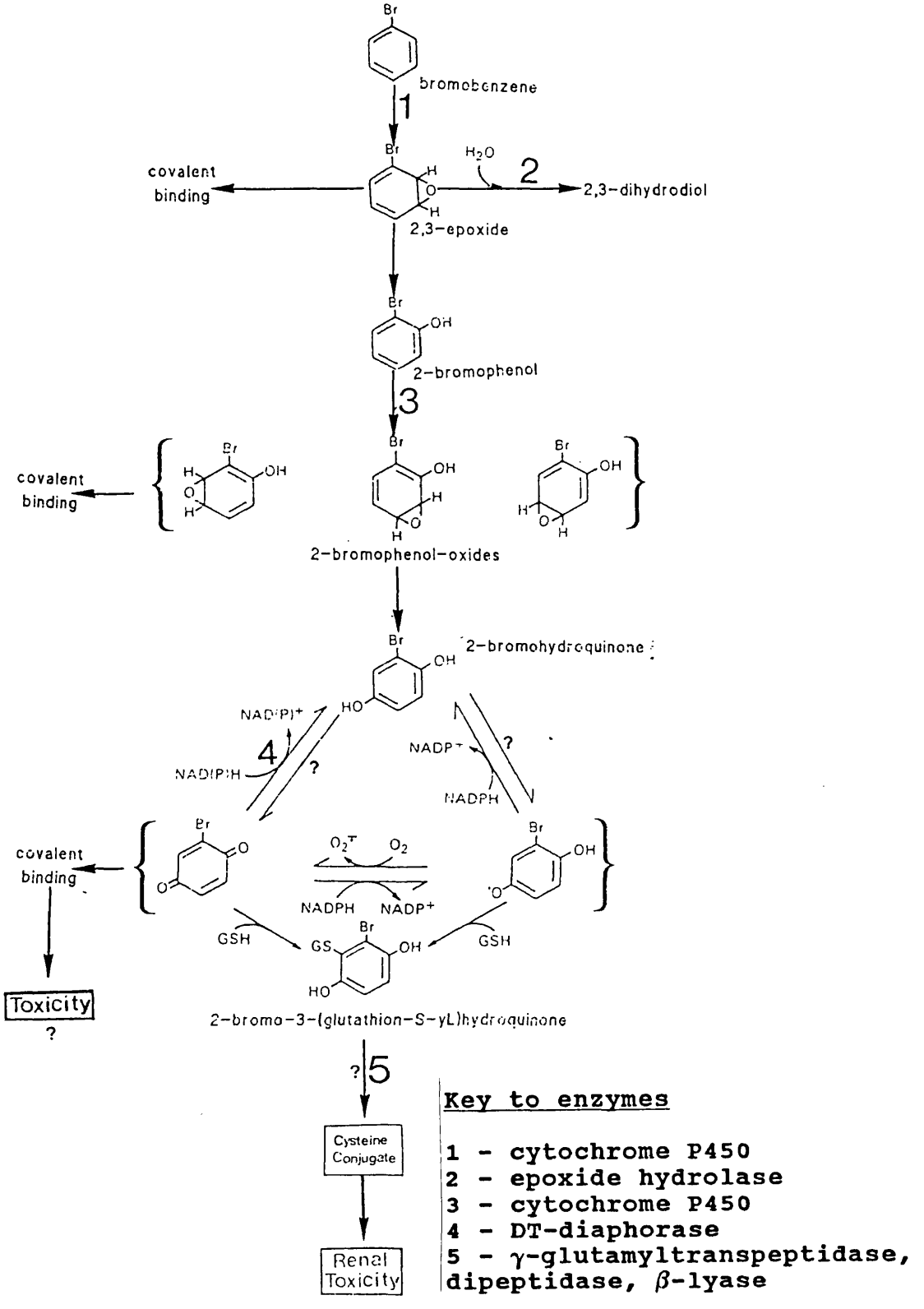
A. Hepatotoxicity of bromobenzene

The postulated mechanism of bromobenzene hepatotoxicity is analogous to that of paracetamol. Bromobenzene is metabolised by cytochrome P450 to generate an epoxide which may conjugate with GSH [156] (fig 1.11). When the supply of GSH becomes limiting the reactive epoxide may arylate alternative nucleophilic sites, possibly critical hepatic proteins [127], leading to cell death. Bromobenzene can form two epoxides, bromobenzene-2,3-epoxide and bromobenzene-3,4-epoxide (fig 1.11 and 1.12), each generated by a different and specific form of cytochrome P450 [14]. The 3,4-epoxide is responsible for the hepatic centrilobular necrosis observed after administration of bromobenzene [157, 158]. Further metabolism of bromobenzene-3,4-epoxide produces 4-bromophenol. Hydration of the epoxide results in a dihydrodiol. Induction of cytochrome P450 activity with phenobarbitone, increases bromobenzene hepatotoxicity via the 3,4-epoxide, the amount of 4-bromophenol excreted in the urine [156] and the formation of GSH conjugates of the 3,4-epoxide [159]. 3-Methylcholanthrene induction of cytochrome P450 protects against the toxicity of bromobenzene by reducing the amount of 3,4-epoxide formed but increasing the formation of the 2,3-epoxide and enhances the toxicity of bromobenzene to the kidney [14].

B. Nephrotoxicity of bromobenzene

The renal toxicity of bromobenzene is mediated by a metabolite formed in the liver and transported by the blood to the renal tubules [161]. The 2,3 epoxide rearranges to yield 2-bromophenol (fig 1.12) which can be further oxidised to 2-bromohydroquinone but only by hepatic microsomes [14]. Administration of chemically synthesized disubstituted GSH conjugates of 2-bromohydroquinone (2-bromo(diglutathion-S-yl)hydroquinone) produced nephrotoxicity similar to that seen with bromobenzene , 2-bromophenol or 2-bromohydroquinone [162]. Several GSH conjugates were detected in the urine of rats given 2-bromo-[^{14}C]-hydroquinone including the disubstituted 2-bromohydroquinone [163]. 2-Bromohydroquinone is proposed to

Figure 1.12 - The metabolism of bromobenzene to nephrotoxic metabolites



[Adapted from ref. 14]

be selectively nephrotoxic due to the action of γ-glutamyltranspeptidase, allowing preferential accumulation of the GSH conjugates into the renal proximal tubules and the subsequent generation of alkylating species by ß-lyase. Pretreatment with AT125, which will inhibit γ-glutamyl transpeptidase activity, protects against the nephrotoxicity of 2-bromohydroquinone [164] and 2-bromo(diglutathion-S-yl) hydroquinone [162].

1.12 Naphthalene

Naphthalene is an environmental contaminant, used in industry and is found in cigarette smoke. Exposure to high doses of naphthalene results in necrosis of the nonciliated bronchiolar epithelial cell (Clara cell) of the mouse lung [165] but not rat lung [166,167].

Naphthalene-induced damage to Clara cells *in vivo* is proposed to be mediated by cytochrome P450 metabolism of naphthalene to reactive intermediates which deplete GSH and can become covalently bound to tissue macromolecules in the lung [20]. The toxic intermediate is a naphthalene epoxide ((1*R*,2*S*)-naphthalene-1,2-epoxide) [168]. The P450 isozyme responsible for naphthalene metabolism to (1*R*,2*S*)-naphthalene-1,2-epoxide has been isolated with sequence homology to the IIF subfamily of P450 genes [169]. However whether this metabolite was generated *in situ* in pulmonary tissue or transported to the lung after generation in the liver, remains a matter of debate. In isolated perfused mouse lung, naphthalene can directly damage Clara cells, deplete pulmonary GSH and covalently bind, demonstrating naphthalene can mediate damage to the lung independent of hepatic metabolism [170]. However when isolated lungs are perfused with naphthalene oxides, the damage to Clara cells is greater [168].

Naphthalene binds covalently to the liver and kidney without toxicity to these non-target organs [20,164]. The specific toxicity to the lung may be explained by stereoselective epoxidation of naphthalene by the P450 isozyme in Clara cells or a rapid pulmonary rate of generation of the

toxic epoxide in comparison to nontarget organs. Lung microsomes from mice preferentially metabolise naphthalene to (1*R*,2*S*)-naphthalene oxide [171] whereas hepatic and renal microsomes are not stereoselective in their metabolism of naphthalene. Pulmonary microsomes derived from rat, hamster and monkey did not appear to preferentially form the (1*R*,2*S*)-naphthalene oxide [171].

1.12 Paraquat

Paraquat is a widely used herbicide, often abused with suicidal intention. Fatalities are usually associated with the selective accumulation of paraquat into pulmonary type I and II alveolar cells. Paraquat is capable of redox cycling leading to cell death.

A. Paraquat accumulation

In vivo and *in vitro*, the lung selectively accumulates paraquat [172,173] by an energy dependent process. Paraquat accumulation by type I and II cells is by the polyamine uptake system [174]. Putrescine and other endogenous and synthetic polyamines can inhibit the pulmonary accumulation of paraquat [175,176]. In autoradiographic studies, paraquat together with the epithelial damaging agent O,S,S trimethyl phosphorodithioate were able to reduce the labelling of type II and Clara cells by tritiated putrescine [177]. Certain structural similarities between paraquat and the oligoamines allow each to interfere with the uptake of the other [178] (fig 1.13). Each contains two quaternary nitrogen atoms separated by similar distances, equivalent to four methylene groups. Diquat, a herbicide structurally similar to paraquat is a potent redox cycling compound [179] but causes little pulmonary injury [180]. The distance between the two nitrogen atoms of diquat precludes it as a substrate for pulmonary polyamine uptake system.

Paraquat-mediated damage to the lungs reduces the pulmonary capacity to accumulate polyamines [181]. Consequently the accumulation of ^{14}C-putrescine has been used

as a functional marker of type I and II cell damage after rat lung slices have been exposed to paraquat in the studies presented.

B Mechanism of paraquat toxicity

The proposed mechanism of paraquat toxicity is illustrated in figure 1.13. After accumulation paraquat has been shown to undergo a one electron reduction under the influence of NADPH-dependent reductases [174]. This generates the reduced paraquat radical ($PQ^{\cdot +}$). $PQ^{\cdot +}$ reacts with molecular oxygen reforming the paraquat cation and producing superoxide anion. Dismutation of superoxide anion **(1)**, either spontaneously or mediated by superoxide dismutase (SOD), produces hydrogen peroxide (H_2O_2).

(1) $2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$

Catalase transforms H_2O_2 into water and oxygen **(2)**.

(2) $2H_2O_2 \longrightarrow 2H_2O + O_2$

Selenium-dependent glutathione peroxidase also reduces both H_2O_2 and organic hydroperoxides **(3)**.

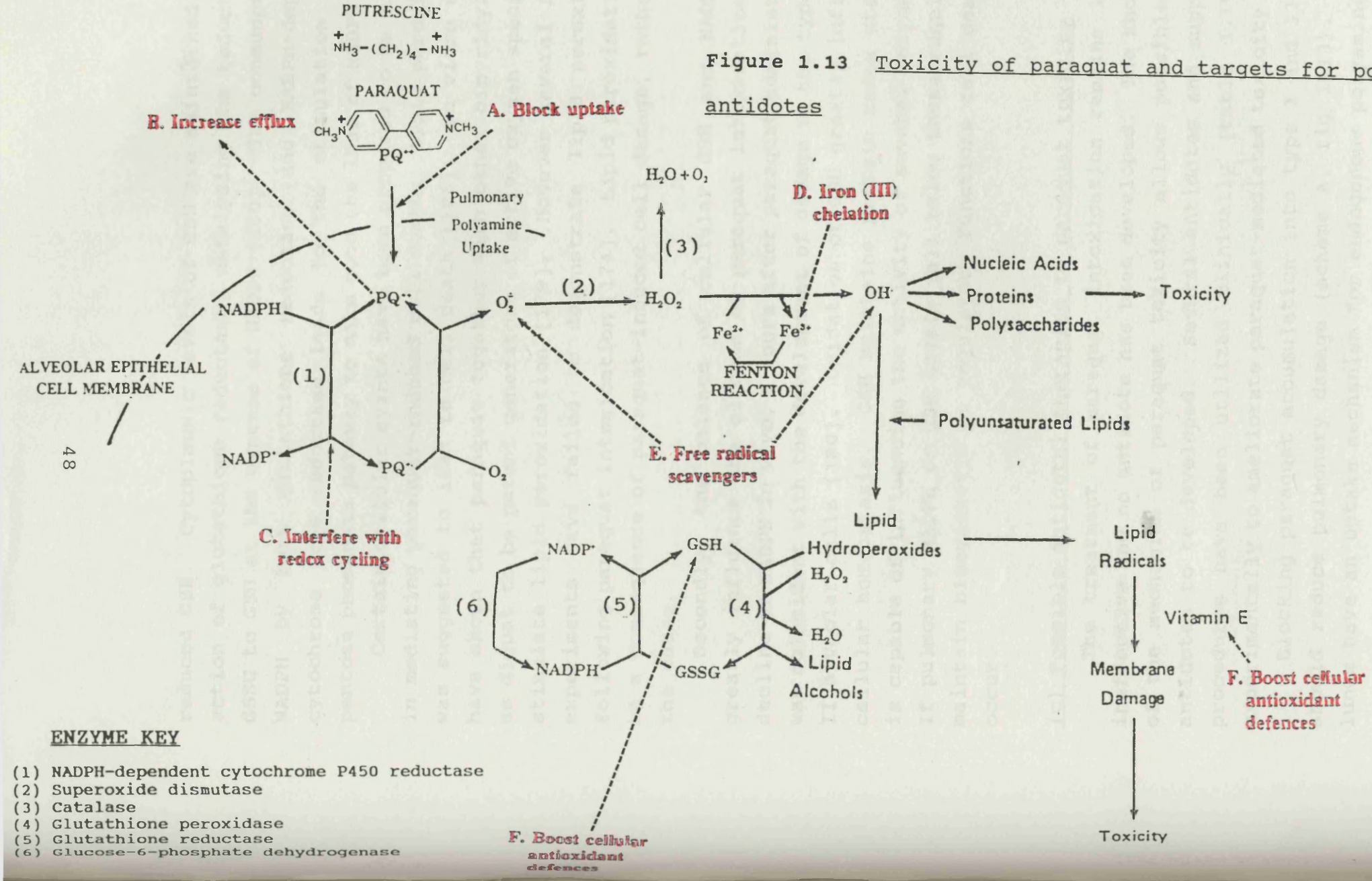
(3) $ROOH + 2GSH \longrightarrow ROH + H_2O + GSSG$

Alternatively, H_2O_2 may be converted to the highly reactive hydroxyl radical ($OH^{\cdot}$) by traces of iron or copper **(4)**.

(4) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$

The damage produced by $OH^{\cdot}$ is dependent on its site of generation. In cells, iron is tightly stored as ferritin [182], preventing its participation in the Fenton reaction. Cellular acidosis as experienced by hypoxic cells or a reductive environment, caused for example by $O_2^{-\cdot}$, ascorbic acid or peroxides cause iron to dissociate from ferritin [182]. The $OH^{\cdot}$ radical has the capacity to initiate lipid peroxidation leading to the production of lipid hydroperoxides (LOOH). LOOH can be converted to the relatively inert lipid alcohol by the action of glutathione peroxidase with the consumption of

Figure 1.13 Toxicity of paraquat and targets for possil antidotes



ENZYME KEY

(1) NADPH-dependent cytochrome P450 reductase
(2) Superoxide dismutase
(3) Catalase
(4) Glutathione peroxidase
(5) Glutathione reductase
(6) Glucose-6-phosphate dehydrogenase

reduced GSH. Cytoplasmic levels of GSH are maintained by the action of glutathione reductase, catalysing the reduction of GSSG to GSH at the expense of NADPH [180]. The consumption of NADPH by both glutathione reductase and NADPH-dependent cytochrome P450 reductase leads to the stimulation of the pentose phosphate pathway to make good the loss of NADPH [183].

Certain cellular events have been proposed to be critical in mediating paraquat-induced cell death. Lipid peroxidation was suggested to lead to cell death [186]. *In vitro* studies have shown that paraquat together with other bipyridyls such as diquat to be potent generators of active oxygen species and stimulate lipid peroxidation [179]. However several *in vivo* experiments have failed to demonstrate lipid peroxidation following paraquat intoxication [174]. Lipid peroxidation may be a consequence of paraquat-induced cell damage, rather than the cause.

Secondly, the balance of cellular GSH and NADPH may greatly influence the outcome of paraquat intoxication. The decline of NADPH *in vivo*, 8 hours after paraquat administration was coincident with the development of damage to the type I and II alveolar cells [180]. Oxidation of GSH greatly influences cellular homeostasis. GSH maintains protein redox state and is capable of influencing the activity of several enzymes [3]. If pulmonary NADPH or GSH levels fall below those required to maintain biosynthetic or regulatory functions cell death may occur.

(c) Possible antidotal therapies to paraquat toxicity

The treatment of paraquat intoxication remains largely ineffective and no antidote has been developed. The knowledge of the mechanism of paraquat toxicity allows possible novel antidotes to be developed. Several antidotes and supportive procedures have been utilized clinically (table 1.IV) and experimentally to ameliorate paraquat-mediated toxicity [185].

Blocking paraquat accumulation into type I and II cells should reduce pulmonary damage (scheme A, fig 1.13). Human lungs have an uptake mechanism for endogenous polyamines and

Table 1.IV Clinical procedures used in the treatment of paraquat intoxication

(1) Prevention of absorption of paraquat from the gut:-

- gastric or whole gut lavage
- induced emesis
- administration absorbent materials such as
  Fuller's earth
  activated charcoal
  ion exchange resins

(2) Removal of paraquat from blood

- haemoperfusion
- haemodialysis

Table 1.V Modulation of enzymes involved in the toxicity of paraquat to reduce or enhance damage

Enzyme	Strategy	Result	Ref
SOD	1.*in vivo* administration	-	[191]
	2.cell line expressing human SOD	-	[192]
NADPH-dependent cytochrome P450 reductase	1.reduced expression in HL60 cell line	+	[193]
GSH peroxidase	1.increased expression in Hela cells	+	[194]
GSH reductase	1.inhibition with BCNU	↑	[24]
	2.increased expression in *Escherichia Coli*	+	[195]

Key to symbols:- + = protection against paraquat toxicity
- = no protection
↑ = enhanced toxicity

paraquat similar to the one described for rat lung [186]. A number of compounds have the capacity to reduce paraquat accumulation *in vitro*, including the polyamines [175], several endogenous amines and exogenous compounds [187]. However putrescine failed to avert paraquat toxicity *in vivo* [188] and the anti-cancer agent methyl glyoxal bis(guanyl hydrazone) (MGBG) while effectively decreasing the accumulation of paraquat *in vitro* [189], shifted the major target of toxicity from the lungs to the kidney *in vivo* [190].

Following paraquat accumulation into type I and II cells the opportunity to interfere with paraquat redox cycling and the generation of reactive oxygen species exists. The outcome of paraquat intoxication depends upon the balance between toxic species generated and endogenous defenses and antioxidants. Several enzymes have been proposed to be involved in the mechanism of paraquat toxicity and modulating their activity could influence the outcome of paraquat intoxication (table 1.V). Removal of transition metal ions (Fe^{3+}, Fe^{2+} and Cu^{2+}) may reduce the generation of $OH^{\cdot}$ from H_2O_2 (scheme D, fig 1.13). This will be discussed in detail in chapter 4.

The balance of antioxidants such as vitamin E, GSH and ascorbate and the rate of reactive oxygen metabolite generation can determine the outcome of a xenobiotic insult to cells [187] (fig 1.13). Ascorbate (vitamin C) is a water soluble antioxidant but also has pro-oxidant activity [22]. Vitamin E is a membrane bound antioxidant which acts as a free radical quencher [22], breaking the chain reaction of lipid peroxidation.

$$ROO^{\cdot} + VitE\text{-}OH \longrightarrow ROOH + VitE\text{-}O^{\cdot}$$

Yasakara *et al* [196] tried to increase the survival rate of humans after paraquat intoxication by administering varying doses of vitamin E but with little success.

Maintaining the levels of NADPH may preserve cell viability. Administration of niacin offered a degree of protection in paraquat-poisoned rats [197] possibly by increasing NADPH synthesis [185].

Materials and Methods

Materials

2.1 Animals

Male BALBc mice (17-25 g) and male Wistar rats (180-250 g) were obtained from Bantin and Kingman (Hull, UK) or were taken from the breeding colonies at the MRC Toxicology Laboratories. Animals supplied by Bantin and Kingman were housed on a 12 hour light/dark cycle with ready access to food and water at the School of Pharmacy animal holding facility for at least 7 days prior to each experiment.

2.2 Chemicals

The radiolabels [1,4-^{14}C]-Putrescine dihydrochloride (111mCi/mmol, 97% pure)and [methyl-^{14}C]-paraquat (111mCi/mmol, 98% pure), were supplied by Amersham International plc (Amersham, UK).

1,3-bis(2-chloroethyl)-1-nitrosourea (Carmustine) (BCNU) obtained from Bristol-Meyer Oncology (Uxbridge) and was used to inhibit GSH reductase activity.

General laboratory reagents and the following chemicals were obtained from Sigma Chemical Co. (Poole, UK); 4-acetamidophenol (paracetamol), N-acetyl-L-cysteine, 2-amino-bicyclo-(2,2,1)heptane-2-carboxylic acid, L-alanine, D,L-aspartic, bromobenzene, choline chloride, L-cysteine hydrochloride monohydrate, 2,3-dihydroxybenzoic acid, diethyl maleate, 2,4-dinitrophenyl hydrazine, 5,5'dithiobis-(2-nitrobenzoic acid), glutathione (reduced), L-methionine, L,2-oxothiazolidine-4-carboxylic acid, phenobarbitone, phytic acid, putrescine dihydrochloride and sulphosalicylic acid.

Benzo(a)pyrene, bis(4-nitrophenyl)phosphate, 4-ethylmorpholine (>99% purity), methane sulphonic acid (99% pure) and phorone (as a 95% solution) were obtained from Aldrich Chemical Co. (Gillingham, UK). Acetic acid (HPLC grade, 99.8% pure) was supplied by BDH (Poole, UK). Halothane was acquired from the Vetinary Drug Co. (Slough, UK).

HPLC grade acetonitrile, dichloromethane, ethanol and

methanol were acquired from Rathburn Chemical Co. (Peebleshire, Scotland).

L-Cysteine isopropyl ester, L-cysteine cyclohexylester and L-cysteine t-butylester were kindly provided by Dr DG Upshall (CBDE, Porton Down,Wiltshire, UK). Paraquat dichloride (>98% pure) and the iron chelator CP51 were kindly donated by Mr I. Wyatt (Central Toxicology Laboratories, ICI plc, Alderley Park, Macclesfield, Cheshire). Desferrioxamine was a gift of the Giba-Giegy Corporation. Pyridoxal isonicotinyl hydrazone was a generous gift of Dr P. Ponka, McGill University, Canada. Paraoxon was kindly provided by Dr MK Johnson, MRC Toxicology Unit, Surrey, UK.

Methods

2.3 Animal dosing

The table below summarises the route, dose range and vehicles used to administer compounds to rats and mice in the studies presented in this thesis. The dose volumes were 2.5ml/kg and 5ml/kg for rats and mice respectively. CIPE, *N*-acetylcysteine and cysteine were dissolved in saline immediately before use. The pH of the solutions was monitored with a probe pH meter and modified with a small volume of NaOH (5 M) to within the range pH 6.0-7.5. Dissolution of compounds in corn or sesame oil, or propylene glycol was achieved by sonication or gentle heating.

COMPOUND	SPECIES	ROUTE	DOSE RANGE	VEHICLE
paraquat	rat	ip	20-80mg/kg	saline
phorone	rat	ip	200mg/kg	corn oil
bromobenzene	rat	ip	0.5-15mmol/kg	sesame oil
phenobarbitone	rat/mouse	ip	80mg/kg	saline
benzo(a)pyrene	mouse	ip	20mg/kg	sesame oil
paracetamol	mouse	ip	150-750mg/kg	propylene glycol
naphthalene	mouse	ip	200mg/kg	sesame oil
cysteine iso-propyl ester	rat/mouse	ip	1-5-6.0mmol/kg	saline
N-acetylcysteine	mouse	ip	1.5-6.0mmol/kg	saline
cysteine	mouse	ip	1.5-3.0mmol/kg	saline

2.4 Preparation of rat lung slices

Male Wistar rats (180-250 g) were anaesthetized in a bell jar with a rich Halothane atmosphere (3%). When respiratory movement had ceased, the thoracic cavity was opened with a midline incision, exposing the heart and lungs. A small incision was made into the right ventricle of the heart and a cannula inserted, causing the left auricle to expand with oxygenated perfusate. The wall of the left auricle was cut and the lung perfused via the pulmonary artery. The lungs were perfused in this manner using a peristaltic pump until all the the lobes of the lung were cleared of blood. Each lobe was carefully removed at the hilus and any extraneous connective tissue trimmed. Lung slices (0.5 mm thickness) were prepared using a McIlwain tissue chopper (Mickle Ltd, Godalming, Surrey). Lung slices (30-40 mg) were quickly weighed and placed into 25 ml Erlmeyer flasks containing 3 ml of incubation medium. Modified Krebs Ringer phosphate buffer (KRP), pH 7.4 supplemented with glucose was used to perfuse the lungs and as the incubation medium. KRP consisted of NaCl (130mM), CaCl (1.9mM), $MgSO_4.7H_2O$ (1.29mM), KCl (5.2mM), Na_2HPO_4 (10mM) and glucose (11mM).

A Inhibition of lung slice glutathione reductase activity

The activity of glutathione reductase was inhibited using the method of Hardwick *et al* [24]. Lung slices were incubated with BCNU (100μM) for 45 min. The incubation medium was Minimal Essential Medium amino acids diluted with KRP with additional L-cysteine, L-methionine, L-glycine, L-serine and L-glutamine (final concentration of the additional amino acid was 0.2mM). This protocol reduces the activity of the enzyme by ~75%.

B Depletion of lung slices GSH

Lung slices were incubated with diethyl maleate (500μM) (in 0.3% DMSO) for 30 min, which reduced lung slices GSH by ~80%. Increasing the concentration of diethyl maleate (1 and 2mM) did not appreciably enhance the depletion.

C Inhibition of esterase activity

The esterase activity of lung slices and isolated hepatocytes was inhibited by incubating with paraoxon (10μM) or bis(4-nitrophenyl) phosphate (200μM). Both inhibitor concentrations produced maximal inhibition of the hydrolysis of CIPE by rat lung slices after 30 min. Every endeavour was made to perfuse lungs and liver free of blood, as plasma paraoxonase activity reduces the effectiveness of paraoxon as an inhibitor of carboxylesterase activity.

2.5 Measuring the accumulation of radiolabelled putrescine and paraquat by lung slices

The ability of lung slices to accumulate [1,4-^{14}C]-putrescine after damage to type I and II alveolar cells by paraquat, can be used as a marker of their functional integrity [175]. The greater the damage to type I and II cells , the lower the accumulation of radiolabelled putrescine.

After incubation of lung slices with paraquat and potential antidotes, slices were washed, and placed in fresh KRP containing 0.1μCi [1,4-^{14}C]-putrescine and cold putrescine (10μM). After a further 30 min incubation at 37°C, slices were washed, blotted dry and dissolved by boiling in 1.0M NaOH (400μl) for 20-30 min. Samples were neutralised with 1.0M HCl (400μl) and were transferred to ß-vials containing Aquasol scintillant (4ml). Liquid scintillation counting was performed using a Rackbeta 1216 Scintillation Counter (LKB Instruments, Surrey, UK).

Additional experiments were also carried out investigating the effect of several compounds on the uptake of radiolabelled putrescine and paraquat by lung slices. Slices were incubated for 30 minutes with 0.1μCi radiolabel and a range of cold putrescine or paraquat concentrations concomitantly with the potential inhibitors of polyamine uptake. Slices were then treated as outlined above.

2.6 Preparation of isolated rat hepatocytes

Rat hepatocytes were prepared by Dr R Davies by the method

of Seglen [198], which is outlined briefly below.

Male Fischer (F344) rats (200-250g bogy weight) were anaesthetized with Sagital (600μl, i.p.). The portal vein was cannulated and the descending portal vein cut. The liver was initially perfused with Ca^{2+}- and Mg^{2+}-free HBSS with EGTA (0.5mM) for 5 min (pH7.4, 37°). The liver was excised and further perfused for 30 min with collagenase (.008%, w/v) in HBSS containing $CaCl_2$ and $NaHCO_3$. After perfusion, hepatocytes were released by gentle agitation and filtered to remove undigested material. The cells were gently spun down and the cell pellet suspended in KRP. This procedure was repeated twice before the viability was assessed by trypan blue exclusion.

2.7 Analysis of tissue non-protein sulphydryls by the Ellman method

Ellman method is based upon the reaction between 5,5'dithiobis(2-nitrobenzoic acid) and any non-protein sulphydryl (-SH) group, forming a yellow compound which absorbs at 412 nm [199]. The reaction is non-specific and will detect but not distinguish between glutathione, cysteine, CIPE and other cysteine precursors with a free -SH group, but the assay is quick and simple.

Tissues were homogenised in 4% sulphosalicylic acid using a Polytron (Kinematica, Northern media Supply Co, N. Humberside, UK) and spun at 3500 rpm (Denley BR 401 refrigerated centrifuge or Beckman GPR refrigerated centrifuge) for 20 minutes. A 0.5 ml aliquot was added to 4.5 ml KH_2PO_4 (pH 8.0) and mixed with 5,5'dithio-bis(2-nitrobenzoic acid) (50μl,10mM). After allowing the samples to stand for 15 min in the dark, the absorbance was measured at 412nm. An estimate of the free -SH available was obtained by reading against a GSH standard curve, constructed over the range 0-500μM.

2.8 Determination of serum glutamate-oxaloacetate transaminase (SGOT) and glutamate-pyruvate transaminase (SGPT)

Serum GOT and GPT levels were colourimetrically

determined, by making use of the transamination reaction catalysed by each of the enzymes (equations 2.1 and 2.2) [200]. GPT forms pyruvate from L-alanine and α-ketoglutarate, while GOT generates oxaloacetate from L-aspartate and α-ketoglutarate. Both pyruvate and oxaloacetate react with 2,4-dinitrophenylhydrazine under alkaline conditions to produce phenylhydrozones which can be measured at 546 nm.

GPT Equation 2.1.

alanine + α-ketoglutarate -----> pyruvate + glutamate

GOT Equation 2.2.

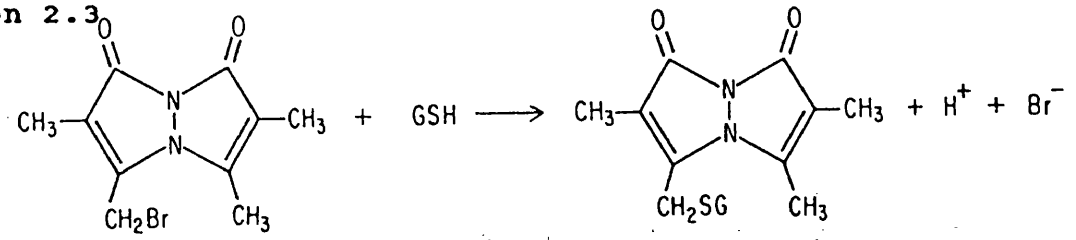
aspartate + α-ketoglutarate -----> oxaloacetate + glutamate

Blood samples taken from rats or mice by cardiac puncture were spun down and 20μl aliquots of plasma were either mixed with GOT substrate (100μl) (100mM aspartic acid and 2mM α-ketoglutaric acid) and incubated for 1 hour at 37°C or with GPT substrate (100μl) (200mM alanine and 2mM α-ketoglutarate) and incubated for 30 minutes at 37°C. The enzyme reactions were stopped by adding the colour reagent 2,4-dinitrophenyl hydrazine (dissolved in 1 M HCl). After mixing and standing for 20 minutes at room temperature, the samples were mixed with 0.4 mM NaOH (1.5ml) and left to stand for a further 5 minutes before reading the optical density at 546 nm. The methods were standardised using standards containing different amounts of pyruvate incubated with relevant transaminase substrate. The standard curves were non-linear.

2.9 Measurement of tissue sulphydryl levels by high performance liquid chromatography

Reduced sulphydryl levels (R-SH) were monitored by HPLC using a modified method of Kower *et al* [201] and Fahey *et al* [202]. Central to the method is the reaction of monobromobimane with sulphydryl groups forming fluorescent adducts (equation 2.3). Monobromobimane is lipid soluble and can cross plasm membranes to label SH groups *in situ*.

Equation 2.3



The use of fluorescence detection sinnificantlyincreases the sensitivity for thiol detection over other HPLC methods dependent on spectrophotometric detection [203]. Monobromobimane will react with GSH, as well as other reduced low molecular weight thiols, such as cysteine, *N*-acetylcysteine [204] and esters of glutathione [202]. As this method depends on thiol labelling, it will not identify sulphydryl oxidation products such as cystine, glutathione-S-conjugates, GSSG or mixed disulphides of GSH with other low molecular weight thiols or proteins. In such cases the thiol component have to be measured following cleavage to the free thiol but in doing so the identity of the original compound is lost [202].

A Sample derivatisation

Tissues from *in vivo* experiments were weighed and immediately homogenised in 2-3 ml of N-ethylmorpholine (pH 8.0). A 450µl aliquot of homogenate was then vigorously mixed with 450µl of monobromobimane (0.5 mg/ml; dissolved in acetonitrile (0.1%) and N-ethylmorpholine pH 8.0 (99.9%)). **After 15 min derivatisation** , preferably in the dark, the reaction was stopped by lowering the pH to 1 by the addition of 10% (v/v) methane sulphonic acid (100µl).

Rat lung slices after various treatments *in vitro*, were washed in fresh KRP and placed directly into monobromobimane (900µl;0.5mg/ml), which being lipid soluble conjugates thiols within cells. Slices were homogenised after the addition of methanesulphonic acid.

Isolated hepatocytes were gently spun down and washed. Intracellular sulphydryls were labelled by vigorously mixing with monobromobimane (900µl;0.5 mg/ml).

An aliquot of extracellular medium taken from lung slices or hepatocytes was derivatised with monobromobimane to estimate

the sulphydryl content.

Much of the unconjugated monbromobimane was extracted from the homogenates and cell suspensions by mixing with dichloromethane (500μl) for 10 seconds. After centrifugation, the aqueous layer was removed for HPLC analysis.

B Analysis of cysteine, GSH, CIPE and CCHE by HPLC

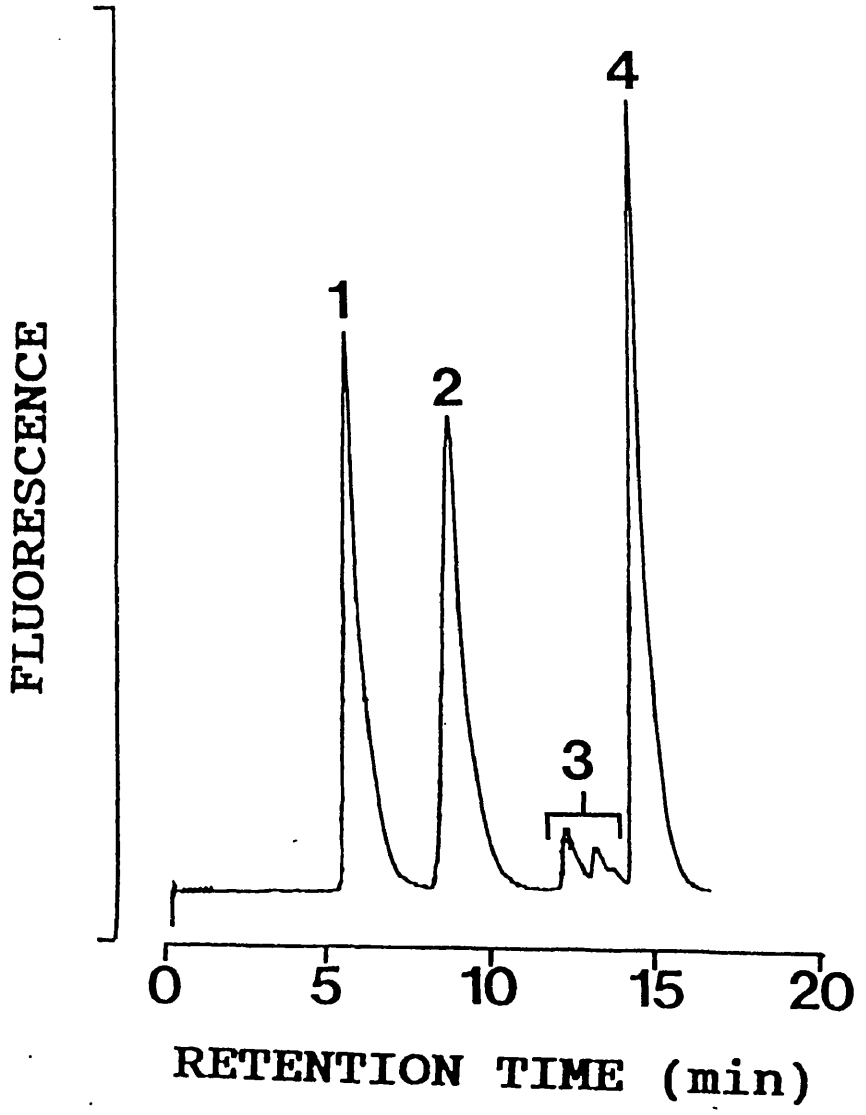
Samples were injected onto a Hypersil ODS 3 micron column (15cm.4.6mm) (HPLC Technology, Macclesfield, UK) fitted with a C-18 guard column (μ-Bondapack C-18, Waters). The column was connected to a Waters 600E HPLC pump. Fluorescence detection was achieved with a Waters 470 scanning fluorescence detector (excitation 390nm, emission 477nm). Derivatised samples were eluted at a flow rate of 1.3 ml/min with a discontinuous gradient, consisting typically of an initial step (12 min) with 8% acetonitrile, .25% acetic acid pH 3.7, then followed by step 2 (7 min) when the acetonitrile concentration was increased to 40% and finally returning to initial conditions for 6 min to equilibrate the column. Figure 2.1 shows a typical chromatogram of cysteine, GSH and CIPE. The analysis of tissue extracts treated with cysteine cyclohexyl ester was achieved using a gradient step up to 70% acetonitrile from the initial solvent. This reduced the retention time of cysteine cyclohexyl ester on the column.

When isolated rat hepatocytes samples were spiked with CIPE (250μM) and derivatised with monobromobimane for 15 min, 99.6% of the CIPE was recovered.

2.10 Statistical Analysis

Statistical significance was tested according to Student's t-test and the probability of a significant difference accepted when greater than 95% ($p<0.05$).

Figure 2.1 - Chromatography of cysteine, GSH and CIPE



A typical thiol standard (**Key to peaks:** cysteine = 1, GSH = 2, monobromobimane = 3 and CIPE = 4) (50μM). Gradient elution was as described in the methods.

Chapter 3 - Screening of potential antidotes to the toxicity of paraquat using a rat lung slice model

3.1 Introduction

Paraquat has been used as a broad spectrum, non-selective contact herbicide for about 25 years [205]. Human fatalities often occur after accidental or suicidal ingestion of paraquat. At low doses, deaths are due to damage to the lungs. Paraquat is selectively accumulated into the pulmonary type I and II alveolar epithelial cells via the polyamine uptake system [206]. Within the type I and II cells paraquat undergoes redox cycling generating reactive toxic oxygen species such as hydroxyl radicals [174], causing lipid peroxidation and depletion of NADPH, leading to cell death.

Studies presented in this thesis are concerned with the prevention of paraquat-induced toxicity following accumulation of paraquat in into type I and II alveolar cells. A number of iron chelators, as well as modulation of lung slice thiol status have been used to attempt to ameliorate the toxicity of paraquat.

A IRON CHELATORS

It is hypothesised that chelation of ferric (Fe^{3+}) ions may prevent the Fenton driven generation of toxic $OH^{\cdot}$ from H_2O_2 following paraquat accumulation into type I and II alveolar cells, by the following reaction:-

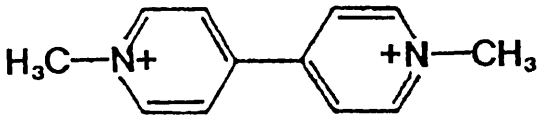
$$Fe^{3+} + e^{-} \text{ -----> } Fe^{2+}$$

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

The studies presented have used several chelators of Fe^{3+} from diverse chemical groups (fig 3.1); desferrioxamine, the hydroxypyridinone CP51, the acyl hydrazone pyridoxal isonicotinyl hydrazone, phytic acid and 2,3-dihydroxybenzoic acid.

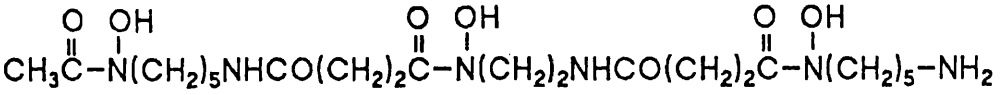
A suitable Fe^{3+} chelator should be specific for and have a high binding constant for iron. Hexadentate ligands often form the more stable complexes with Fe^{3+} compared to bi- or tridentate ligand where there is a possibility of partial

Figure 3.1 Structures of paraquat, putrescine and the iron chelators desferrioxamine, CP51 and 2,3-dihydroxybenzoic acid

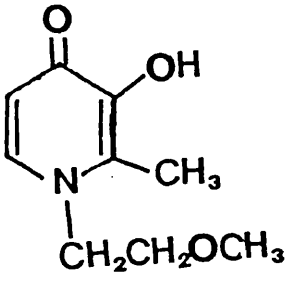


paraquat

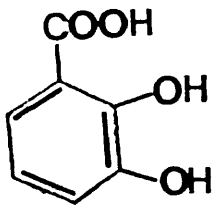
putrescine



desferrioxamine



CP51



2,3-dihydroxybenzoic acid

dissociated complexes at low chelator concentrations generating reactive oxygen species [207].

Desferrioxamine is a hydroxamic acid derivative and has a high affinity for Fe^{3+}, forming complexes with low lipophilicity [208]. It is used clinically in the treatment of iron overload anaemia and has been proposed for use in several disease states including arthritis [209]. To its disadvantage, desferrioxamine is susceptible to gastrointestinal breakdown and must be given by iv and sc infusions.

CP51 [1-(2-methoxyethyl)-2-methyl-3-hydroxypyridin-4-one] is an Fe^{3+} selective ligand [210]. CP51 carries no charge either as the free ligand or when bound to Fe^{3+}, allowing ready cellular uptake of CP51 and mobilization of the CP51-Fe^{3+} complex out of cells. CP51 is relatively free from the toxic side effects, such as hypersalivation or interacting with barbiturates causing retinal damage and death [207], experienced with several other hydroxypyridinones.

2,3-Dihydroxybenzoic acid is a bidentate catecholate ligand [208] and is often found as the iron binding moiety in naturally occurring iron chelators such as enterobactin and synthetic chelators. Pyridoxal isonicotinyl hydrazone (PIH) is a bidentate aryl hydrazone, capable of mobilizing iron from ^{59}Fe-labelled macrophages [211] and reticulocytes [211,212]. In reticulocytes PIH exceeded the ability of desferrioxamine to mobilize ^{59}Fe. The aryl hydrazones are capable of binding both Fe^{3+} and Fe^{2+}, consequently complexes have the potential to redox cycle and generate free radicals.

B THIOL MODULATION

GSH is the major cellular non-protein thiol in the lung and other organs. GSH has several roles in cellular defence including acting as an antioxidant and a redox buffer [1]. Following paraquat intoxication the pulmonary burden of GSH oxidation products (GSSG and GSH mixed disulphides) increases [213,214].

Modulation of pulmonary GSH can influence the result of

oxidant exposure. Depletion of GSH by diethyl maleate or starvation enhances the toxicity of several oxidants [215]. Administration of sulphydryl reagents such as *N*-acetylcysteine and cysteamine protect can protect against oxidants [216].

Three attempts have been made to increase cellular levels of GSH and prevent the toxicity of paraquat to rat lung slices. Exogenous GSH, the rebound synthesis of GSH 48 hr after phorone administration and the GSH precursors cysteine, cysteine isopropyl ester and methionine were used to elevate GSH and thereby ameliorate paraquat toxicity.

To assess the efficacy of the compounds to act as antidotes, the accumulation of ^{14}C-putrescine was used as a functional marker of type I and II cell viability after incubating lung slices with paraquat.

3.2 RESULTS

1. Effect of paraquat on putrescine accumulation by lung slices after control and BCNU-pretreatment

Paraquat produced a time and concentration dependent fall in the ability of rat lung slices to accumulate ^{14}C-putrescine (fig.3.2). Preincubating lung slices with BCNU, to inhibit GSH reductase activity, potentiated the toxicity of paraquat. For example, control slices incubated with paraquat (100μM) experienced a 56% reduction in their ability to sequester putrescine after 4 hr, while GSH reductase-compromised slices experienced a >80% decline in putrescine accumulation (fig.3.2). BCNU pretreatment alone had no effect on the accumulation of putrescine by slices.

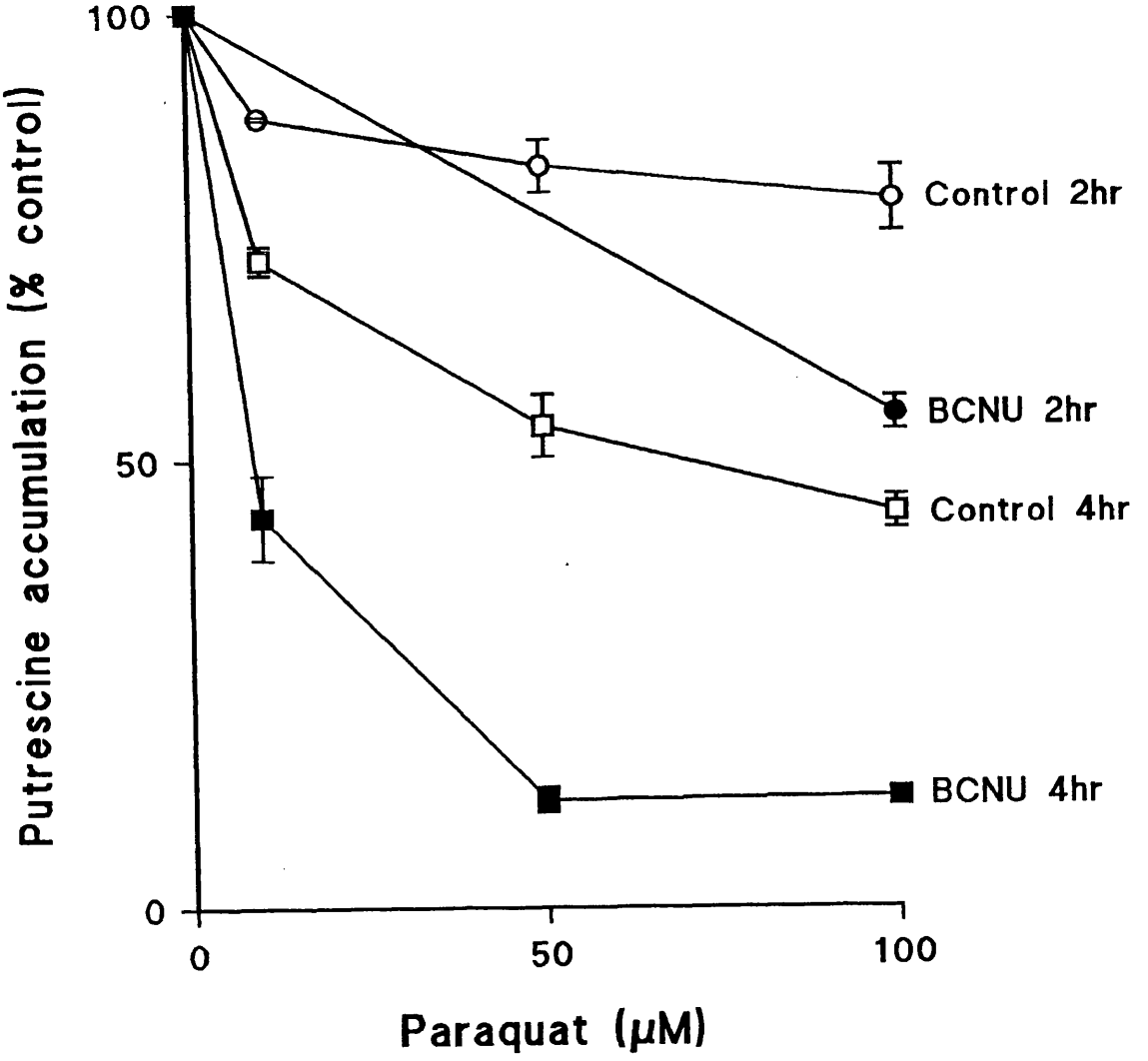
IRON CHELATORS

2. Accumulation of ^{14}C-putrescine by slices incubated with paraquat and desferrioxamine

Paraquat (100μM) significantly reduced the ability of lung slices to accumulate putrescine (fig.3.3) and compromising GSH reductase activity potentiated this decline. However desferrioxamine (500μM) ameliorated the decline in putrescine uptake in both control and BCNU-pretreated slices (fig 3.3). Paraquat (10,50 and 100μM) produced a concentration dependent fall in putrescine uptake, a trend partially reversed by desferrioxamine (500μM) (fig 3.4). Interestingly desferrioxamine (500μM) gave an apparently complete protection against paraquat-induced (10μM) fall in diamine accumulation by slices with full GSH reductase activity. Lung slices incubated with desferrioxamine alone showed no change in their ability to accumulate putrescine.

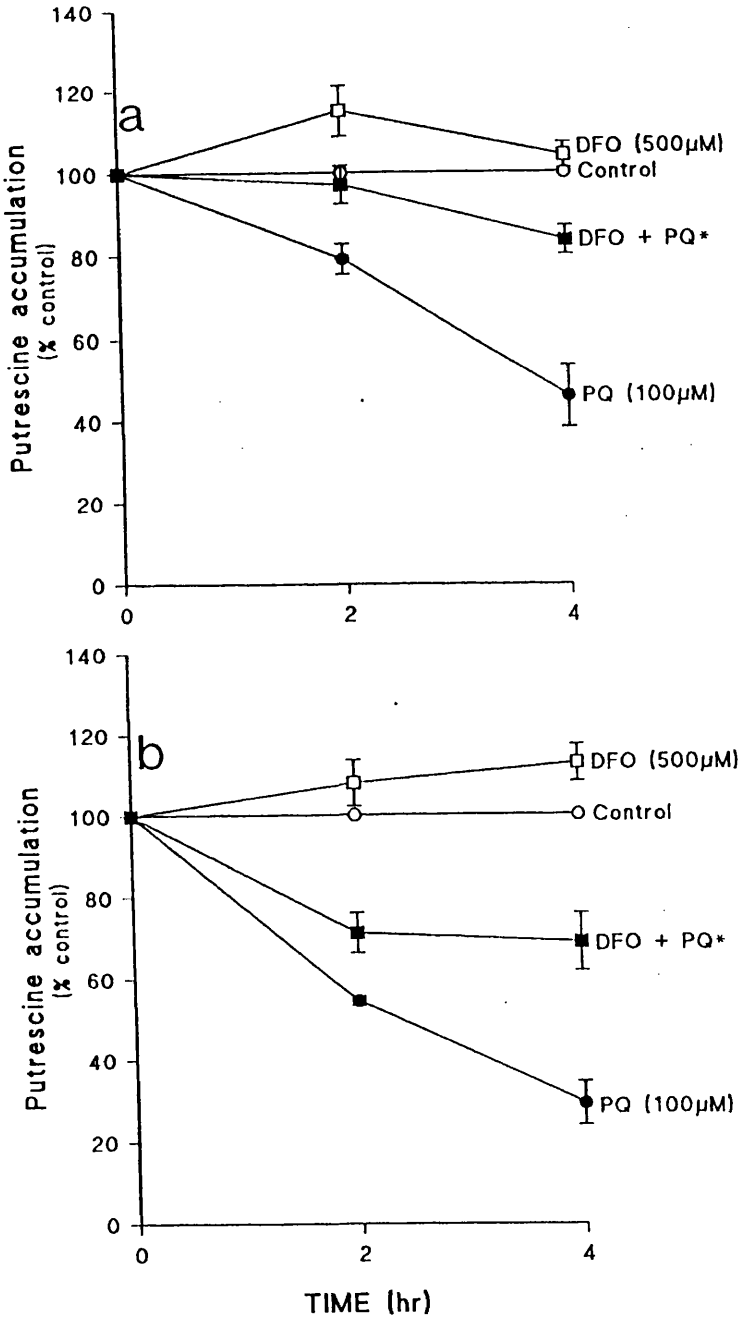
After 4 hr, several concentrations of desferrioxamine ameliorated the toxicity of paraquat (10μM) to slices (fig. 3.5). Concentrations of desferrioxamine in excess of 100μM (100,200 and 500μM) appeared to be equally effective in preventing the paraquat-mediated reduction in putrescine accumulation (fig 3.5).

Figure 3.2 Reduction in the accumulation of ^{14}C-putrescine by control and glutathione reductase-compromised rat lung slices.



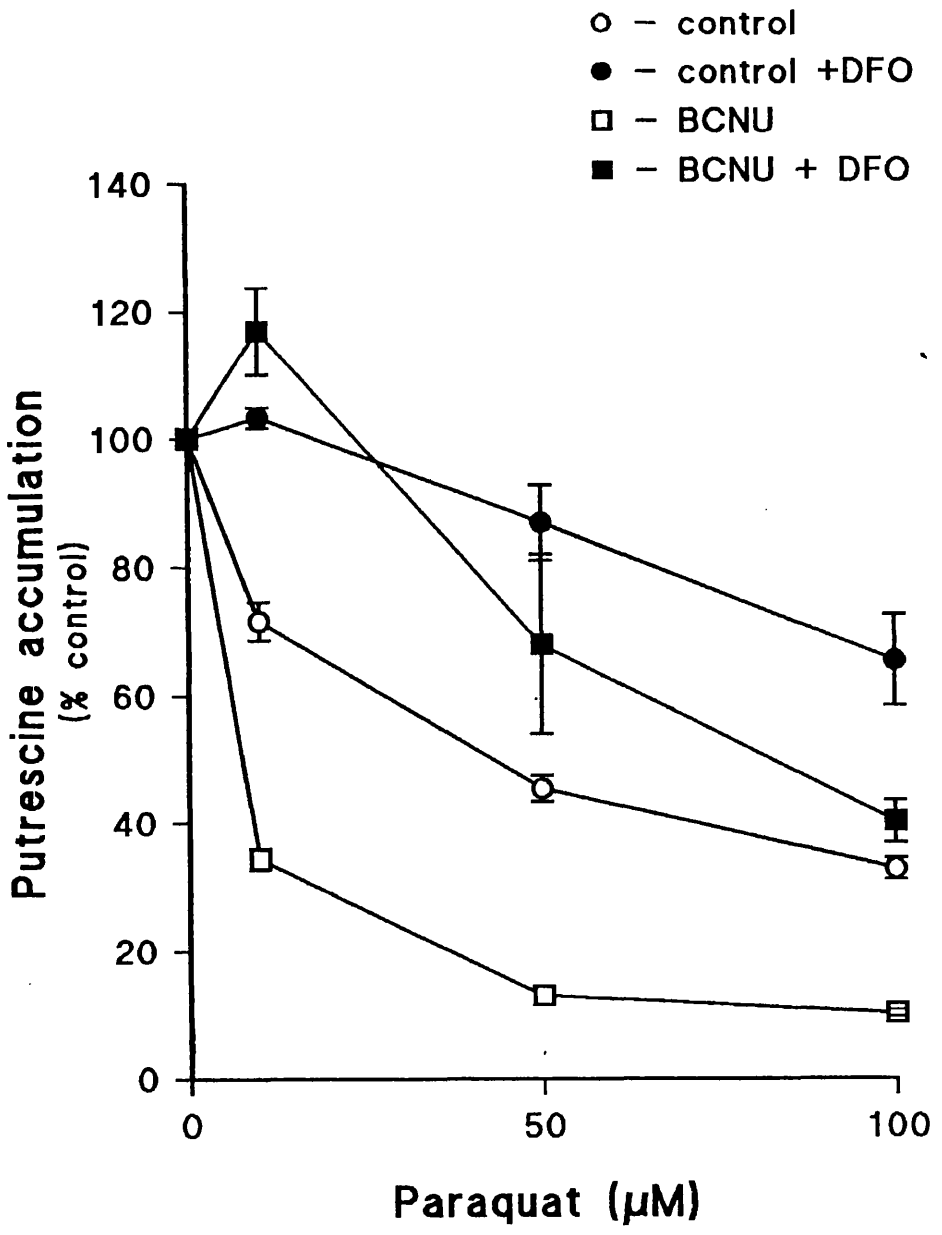
To inhibit GSH reductase activity, slices were preincubated with BCNU (100μM) for 45 min (solid symbols), control slices (open symbols) received no BCNU before being exposed to paraquat for 2 hr (○ and ●) or 4 hr (□ and ■). Data is presented as a percentage of the control at each time point [Control putrescine accumulation (mean±SEM); 2 hr control 195±44.9, BCNU-pretreated 201±42.7; 4hr control 177±34, BCNU 208±21 nmol/g wet lung weight]. Each point represents the mean ±SEM for at least 3 separate experiments.

Figure 3.3 Desferrioxamine reduced the paraquat-mediated decline in putrescine accumulation by control- (a) and BCNU-pretreated (b) lung slices.



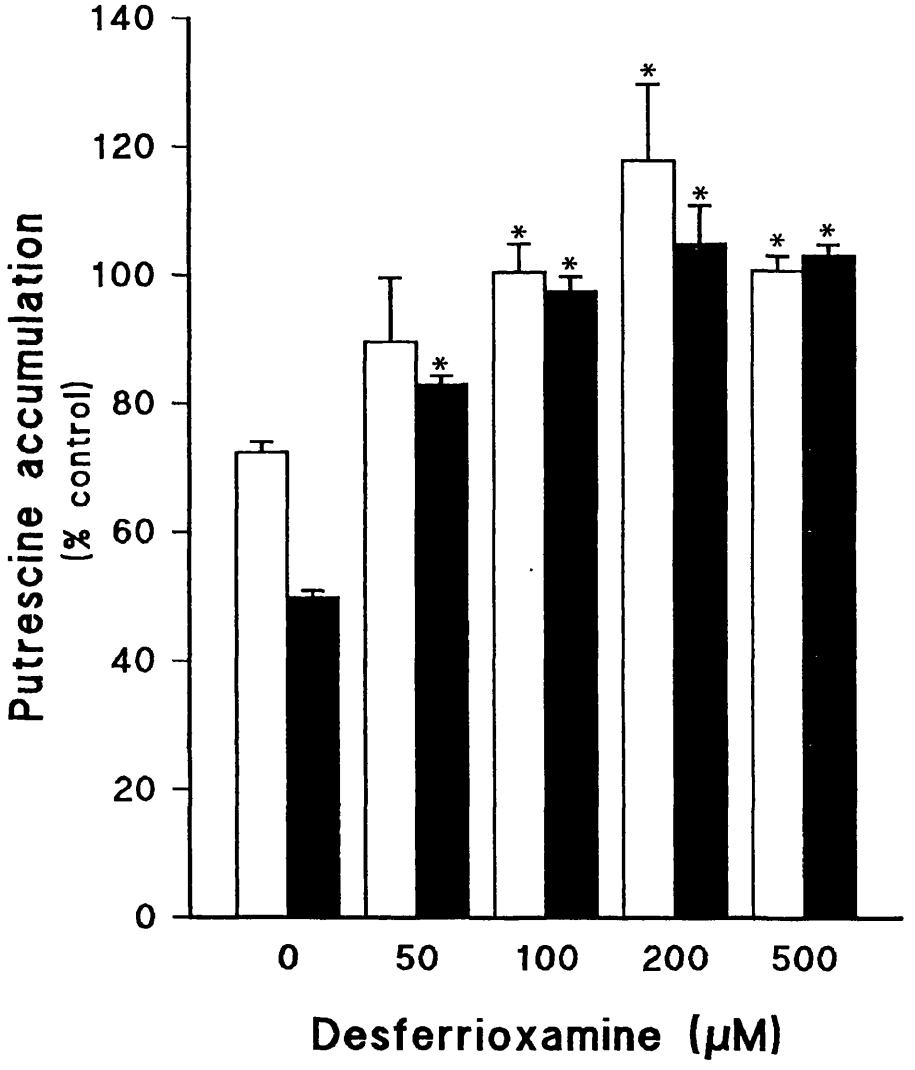
Slices were incubated in the presence or absence of paraquat (100μM), with or without desferrioxamine (500μM). Results are expressed as a percentage of the control values at each time point. Each point represents the mean of at least 3 experiments (±SEM). Initial putrescine uptake - 258.1±9.7 (nmol/g wet wt/30 min). After 4 hr the uptake of putrescine by slices incubated with paraquat and desferrioxamine are significantly greater than slices incubated with paraquat alone, *p<.01.

FIGURE 3.4 Desferrioxamine-mediated protection against several concentrations of paraquat



Control- (○ and ●) and BCNU-pretreated lung slices (□ and ■) were incubated in the presence or absence of paraquat (10, 50 or 100μM) for 4 hr with or without desferrioxamine (500μM). The results are expressed as a percentage of paraquat-free controls. Each point is the mean of at least 3 separate experiments (±SEM). The area under the curve for slices treated with desferrioxamine and paraquat is significantly greater than the area for slices treated with paraquat alone, $p<.01$

FIGURE 3.5 A concentration dependent protection against paraquat toxicity by desferrioxamine



Control (□) and GSH reductase compromised lung slices (■) were exposed to paraquat (10μM) with or without desferrioxamine (50,100,200 and 500μM) for 4 hr. Each bar represents the mean of 3 experiments (±SEM). *p<.05 denotes uptake of ^{14}C-putrescine by slices which is significantly greater than uptake by slices incubated solely with paraquat.

3. Accumulation of putrescine by lung slices incubated with paraquat and CP51

Paraquat (100μM) produced a 58% fall in the ability of slices to accumulate ^{14}C-putrescine (fig. 3.6) after 4 hr. The decline was potentiated by inhibiting GSH reductase activity. Coincident incubation of control slices with CP51 (500μM) and paraquat did not prevent the fall in putrescine accumulation. However in BCNU-pretreated slices, CP51 ameliorated the loss of diamine uptake, significantly, after 4 hr.

Lowering extracellular paraquat (75μM), surprisingly produced a 72% loss in putrescine accumulation after 4 hr (fig 3.7). A modest prevention in the decline was noted at several CP51 concentrations when slices were preincubated for 30 min with CP51. When slices were incubated solely with CP51, no decline in putrescine uptake was observed until a concentration of 3.2 mM (fig. 3.7).

4. Influence 2,3-dihydroxybenzoic acid, pyridoxal isonicotinyl hydrazone and phytic acid on the toxicity of paraquat to lung slices

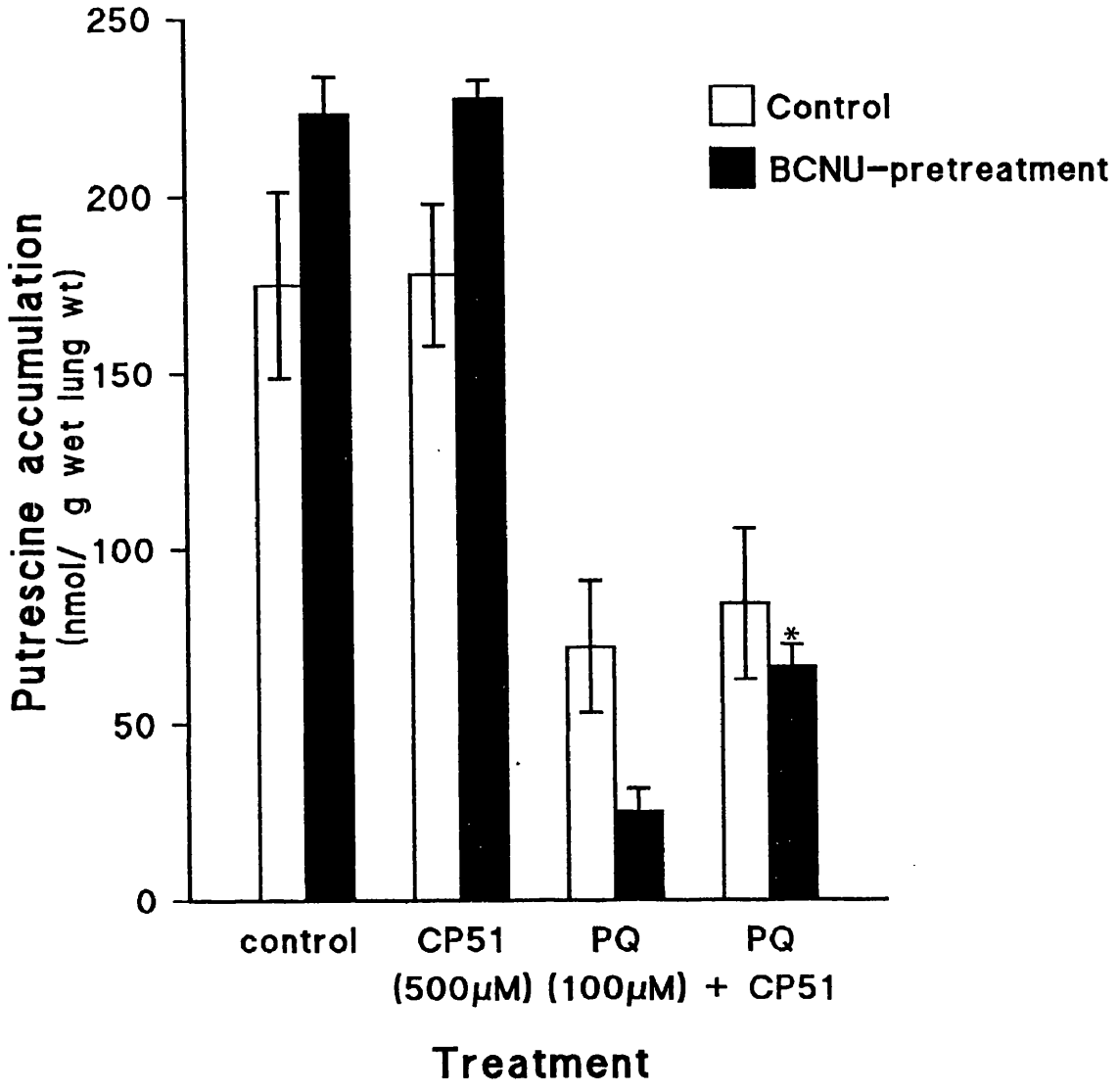
Preliminary experiments with a wide range of PIH and phytic acid (0-2 mM) had no effect on the decline in putrescine accumulation by slices incubated with paraquat (100μM) for 4 hr (fig 3.8). The chelators produced no toxicity when incubated alone with lung slices.

2,3-Dihydroxybenzoic acid (2mM) was able to partially ameliorate the 59% loss in the ability of lung slices to accumulate putrescine after exposure to paraquat (100μM) for 4 hr (fig 3.9). A lower concentration of this chelator (0.5 mM) was ineffective (fig 3.9).

5. The effect of iron chelators on the accumulation of putrescine and paraquat by lung slices

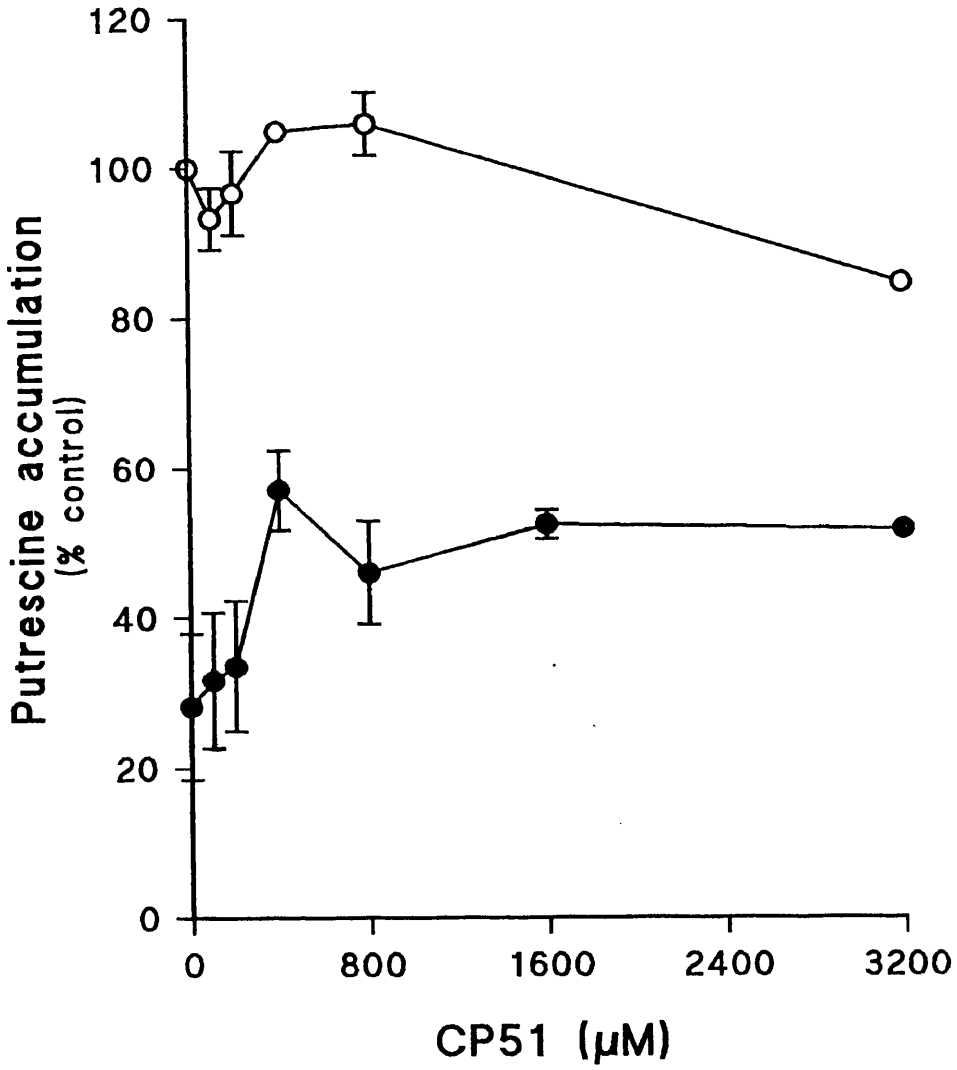
Paraquat and putrescine are proposed to be accumulated bytype I and II alveolar epithelial cells and Clara cells[175] via a polyamine uptake system. Desferrioxamine was found to prevent the accumulation of both radiolabelled putrescine and

FIGURE 3.6 Effect of CP51 on the toxicity of paraquat



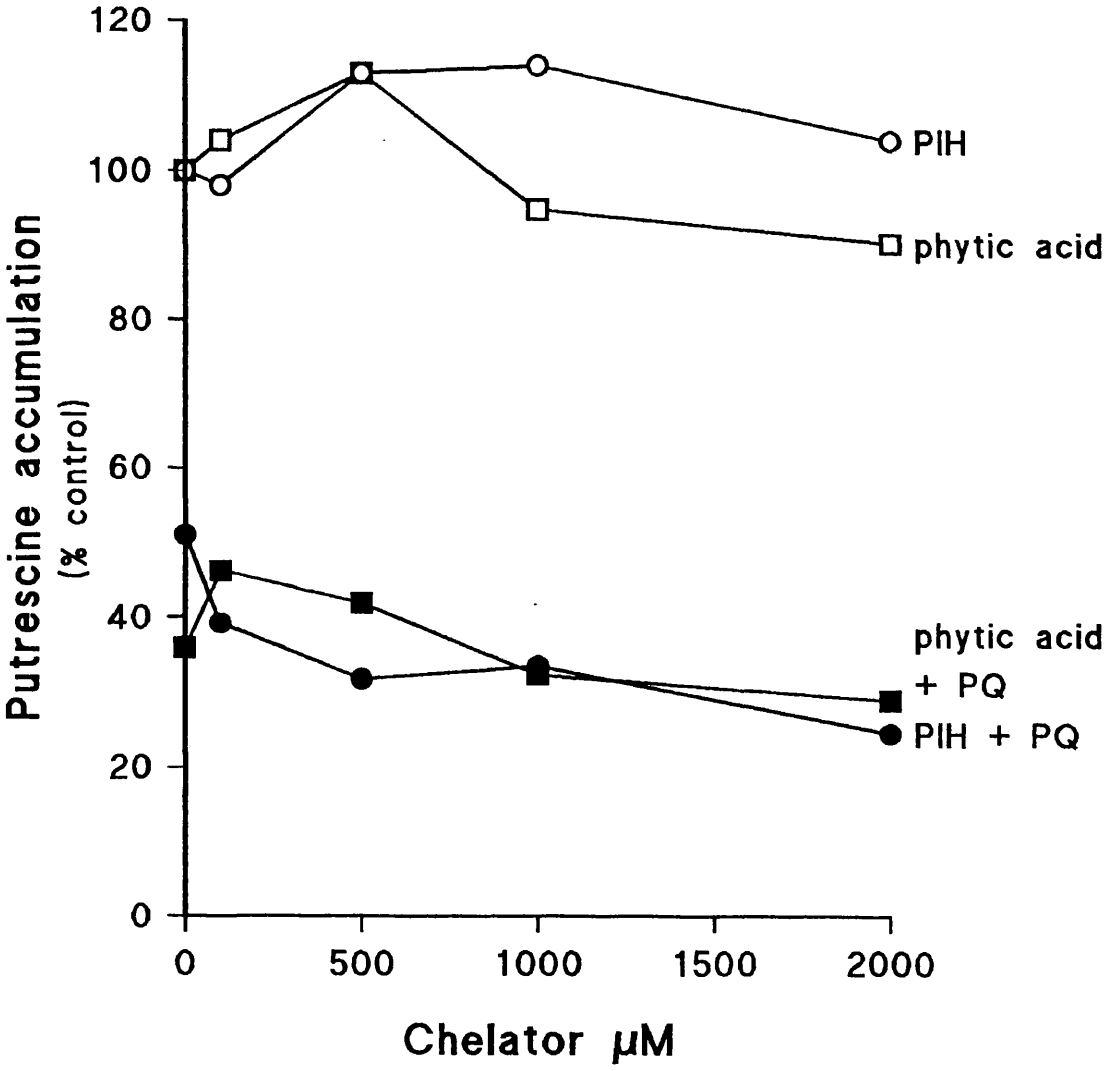
Control (□) and GSH reductase compromised slices (■) were incubated in the presence or absence of paraquat with or without CP51 for 4 hr. Each bar represents the mean of 3 separate experiments. BCNU-pretreated slices incubated with paraquat and CP51 accumulated significantly more putrescine than slices incubated with paraquat alone, $p<.05$.

FIGURE 3.7 Effect of several concentrations of CP51 on the decline in putrescine uptake after incubation with paraquat.



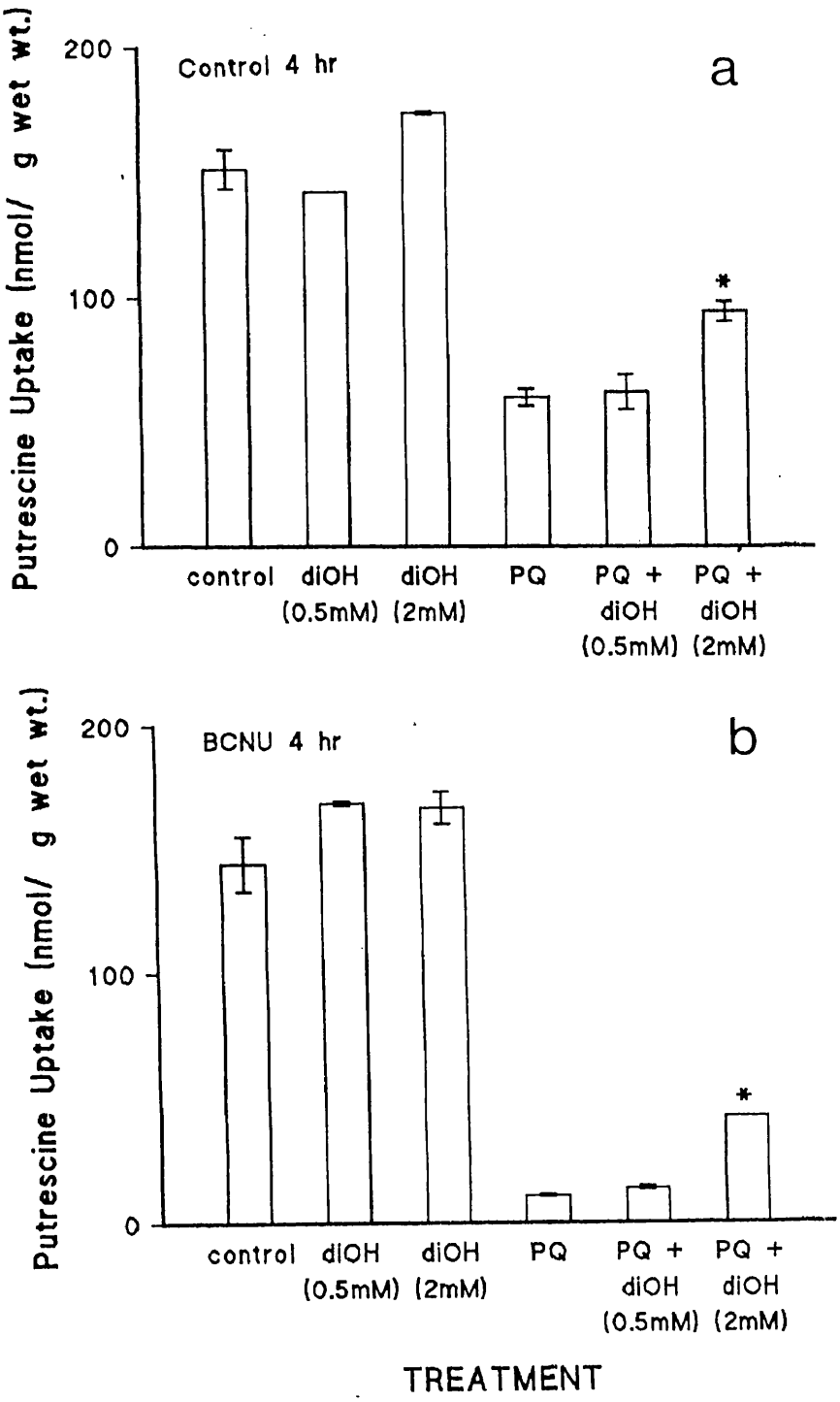
Lung slices were preincubated with CP51 (0.1,0.2,0.4,0.8 1.6, 3.2 mM) for 30 min. After a 4hr incubation, in the presence (●) or absence (○) of paraquat (75μM) with or without CP51, the ability of slices to accumulate putrescine was assessed. Each point represents the mean of 3 experiments (±SEM). Data are expressed as a percentage of control (initial uptake 170.1±7.7 nmol/g wet wt/30 min).

FIGURE 3.8 Phytic acid and pyridoxal isonicotinoyl hydrazone did not prevent the fall in putrescine accumulation by rat lung slices



Preliminary results showing putrescine accumulation by slices which were incubated with in the absence (○ and □) or presence (● and ■) of paraquat with or without phytic acid (squares) or PIH (circles) for 4 hr.

FIGURE 3.9 Effect of 2,3 dihydroxybenzoic acid on the paraquat-mediated decrease in putrescine accumulation by lung slices



Control- (a) and BCNU-pretreated slices (b) were incubated with or without paraquat (100μM) in the absence or presence of 2,3-dihydroxybenzoic acid (0.5 and 2.0 mM) (diOH). Each bar represents the mean of at least 3 experiments (±SEM). Uptake is expressed as nmol of putrescine/g wet lung weight within 30 min.

paraquat by lung slices (fig 3.10). Neither CP51 (fig 3.10) nor preliminary data with 2,3-dihydroxybenzoic acid (not shown), inhibited the polyamine uptake system.

MODULATION OF -SH STATUS

6. Effect of exogenous GSH on the toxicity of paraquat

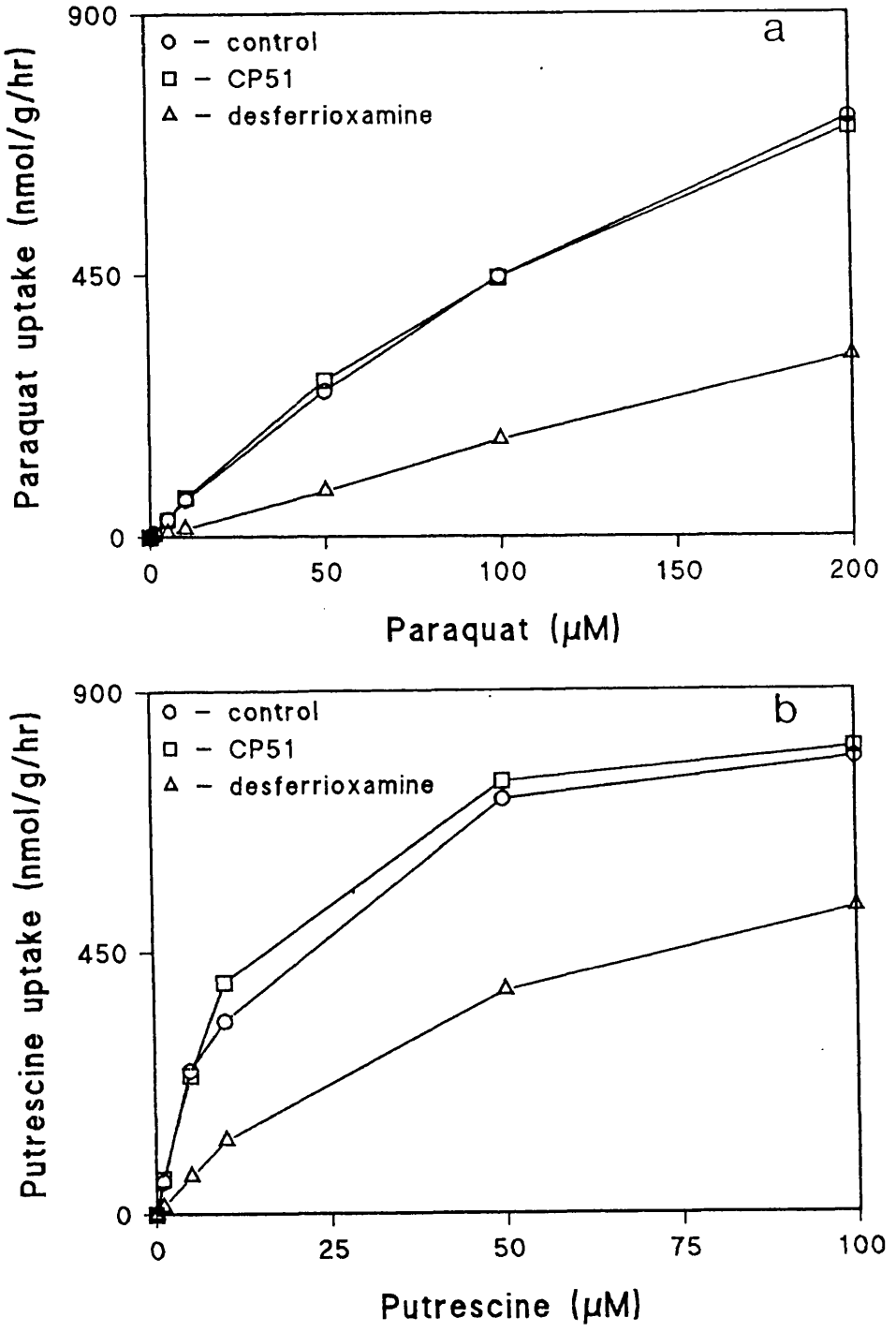
Paraquat (50μM) reduced putrescine uptake by 35 % after 4 hr (fig. 3.11). Coincubating with a range of GSH concentrations (1-5mM) produced small but significant recoveries in putrescine accumulation. These concentrations of GSH did not have a deleterious effect on lung viability as monitored by diamine uptake.

The concentration of GSH in control lung slices declined over 4hr when monitored by HPLC (fig 3.12a), which paraquat (100μM) did not potentiate (fig.3.12b). Incubating slices with GSH (2mM) prevented the decline of intracellular GSH in both control and paraquat-treated lung slices. Surprisingly the levels of cellular GSH after 4 hr were greater than initial levels (fig.3.12b) Slices exposed to GSH also had significantly elevated levels of cysteine after 4 hr (fig.3.12c). Experiments carried out in parallel using putrescine uptake as a functional marker of type I and II cell viability showed paraquat (100μM) caused a 50% fall which was partially ameliorated by GSH (2mM) (fig 3.12.d).

7. Toxicity of paraquat to lung slices from rats pretreated with phorone

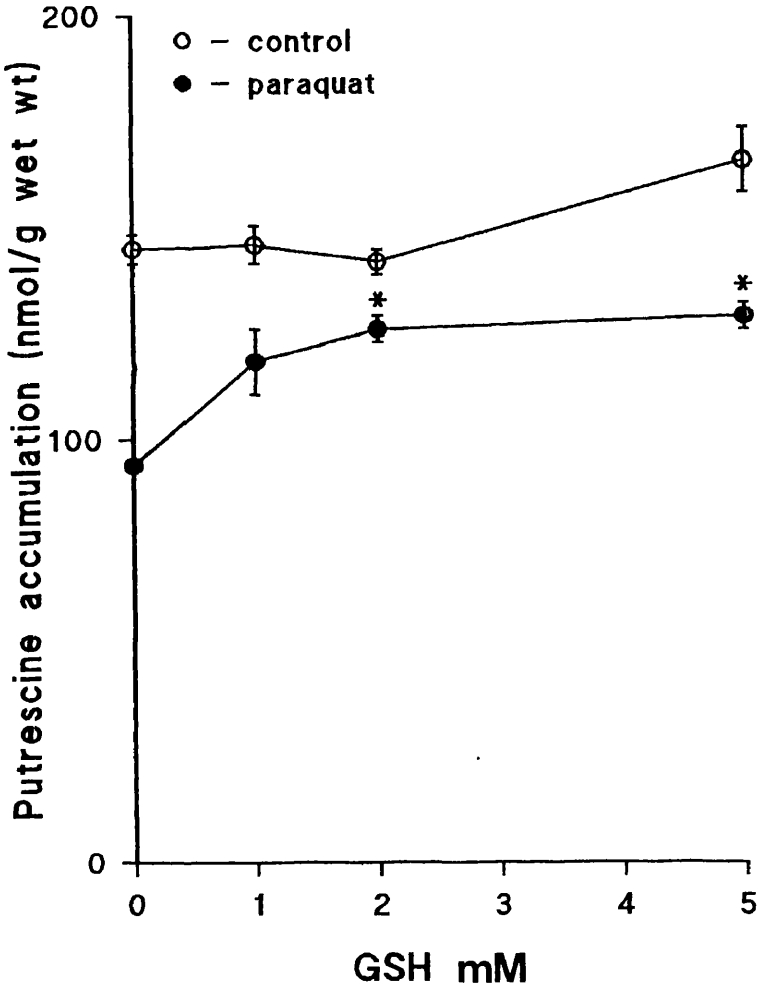
Phorone administration produced a 1.3 to 1.4 fold increase in pulmonary GSH after 48 hours, falling short of the reported 2.5 fold increase [228]. This rise in intracellular GSH did not ameliorate the toxicity of paraquat (10 and 100μM) to slices prepared from the lungs of phorone treated animals (fig 3.13).

FIGURE 3.10 Inhibition of the polyamine uptake system by desferrioxamine but not CP51



Slices were incubated for 30 min with paraquat (1-200μM) or putrescine (1-100μM) with 0.1μCi of paraquat or putrescine. Desferrioxamine (500μM) or CP51 (500μM) were coincubated with slices and the uptake of paraquat or putrescine by the slices determined. The results are the mean of 2-3 experiments and uptake expressed as nmol/g wet wt/hr.

FIGURE 3.11 Effect of GSH on paraquat-induced decline in pulmonary putrescine accumulation



Control- (o) and paraquat-treated slices (●) were incubated with or without GSH (1, 2, 5mM) for 4 hr. Each point is the mean of at least 3 experiments (±SEM). The data is expressed as nmol putrescine accumulated/g wet lung weight within 30 min, *p<.05.

FIGURE 3.12 Relationship between intracellular pulmonary GSH and cysteine with paraquat-mediated toxicity

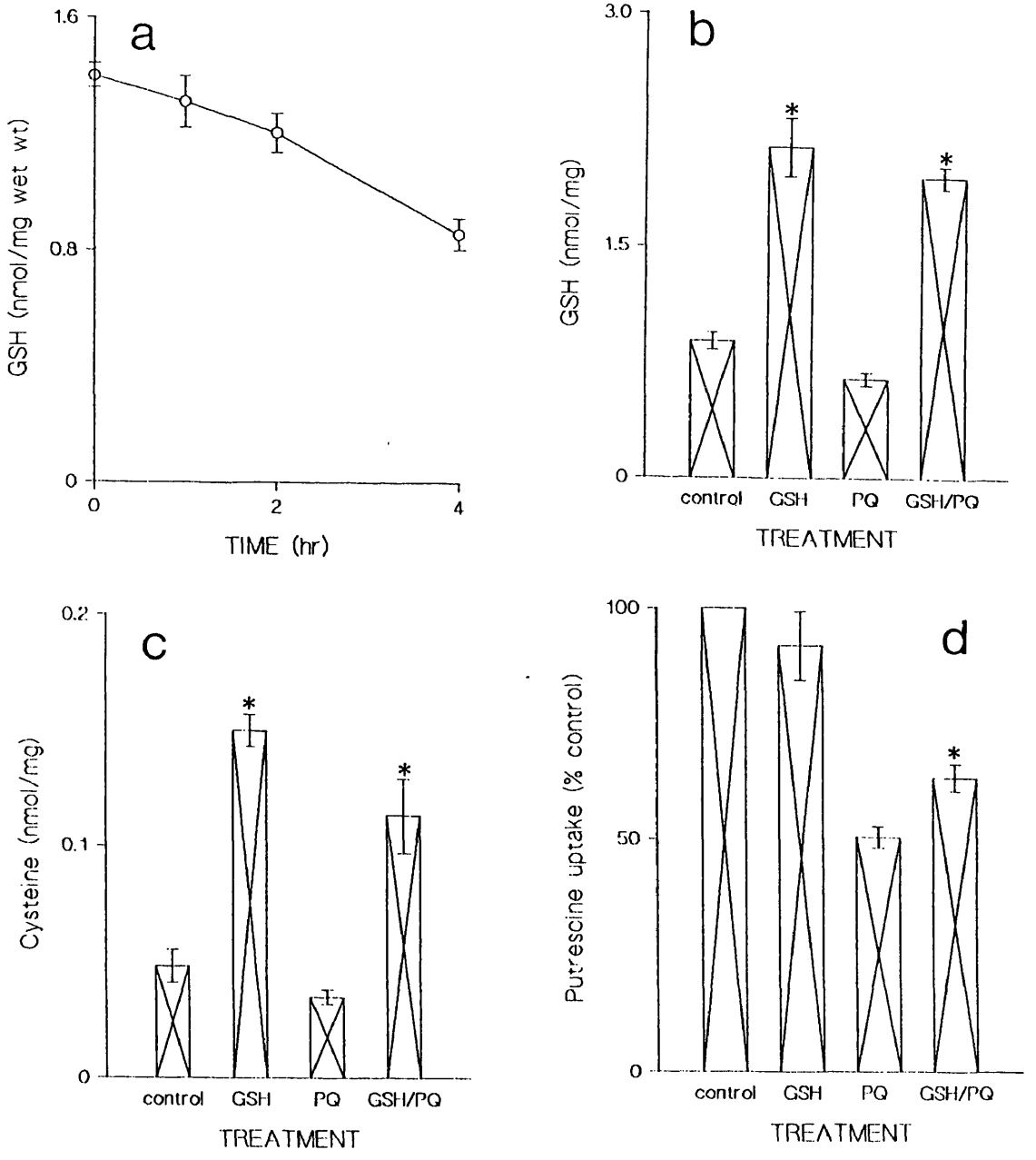


Fig 3.12a - The decline in cellular GSH over a 4 hr incubation.

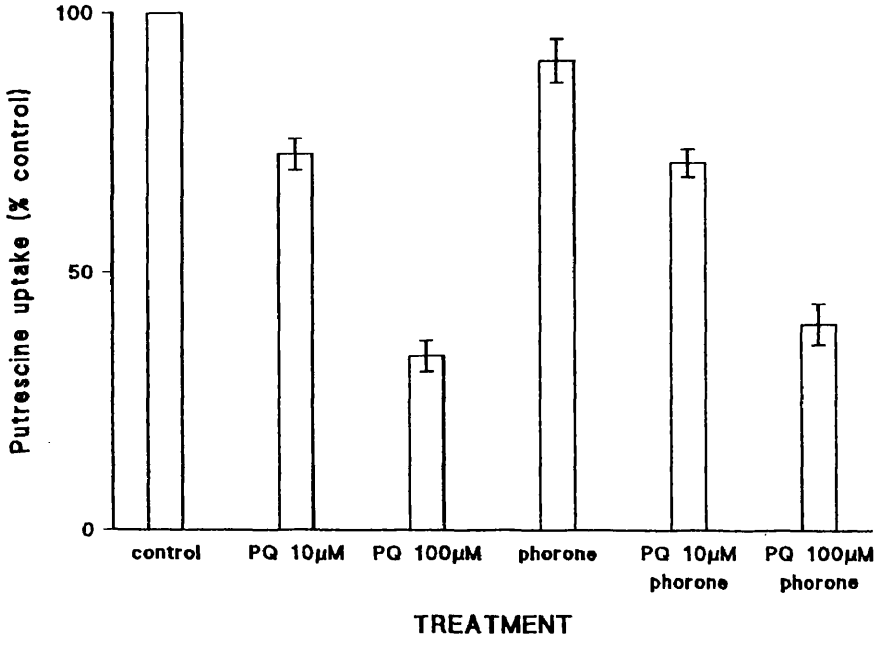
Fig 3.12b - The effect of GSH (2mM) and paraquat (100μM) on the level of GSH in lung slices after 4hr.

Fig 3.12c - The effect of GSH (2mM) and paraquat (100μM) on the levels of cysteine in lung slices.

Fig 3.12d - The effect of GSH (2mM) on the decline of putrescine uptake when exposed to paraquat (100μM).

The levels of GSH and cysteine were assessed by HPLC analysis. Each point represents the mean of 3 experiments (±SEM). *p<.05a denotes significant difference from respective control.

FIGURE 3.13 Effect of phorone pretreatment on paraquat-induced toxicity



Rats were exposed to phorone (200mg/kg. i.p.) for 48 hr. Subsequently control and phorone-pretreated lung slices were prepared and incubated with or without paraquat (10 and 100μM) for 4 hr before estimating the ability of slices to accumulate putrescine. Each bar represents the mean of 3 experiment (±SEM).

8. The effect of cysteine, cysteine isopropyl ester and methionine on paraquat mediated toxicity

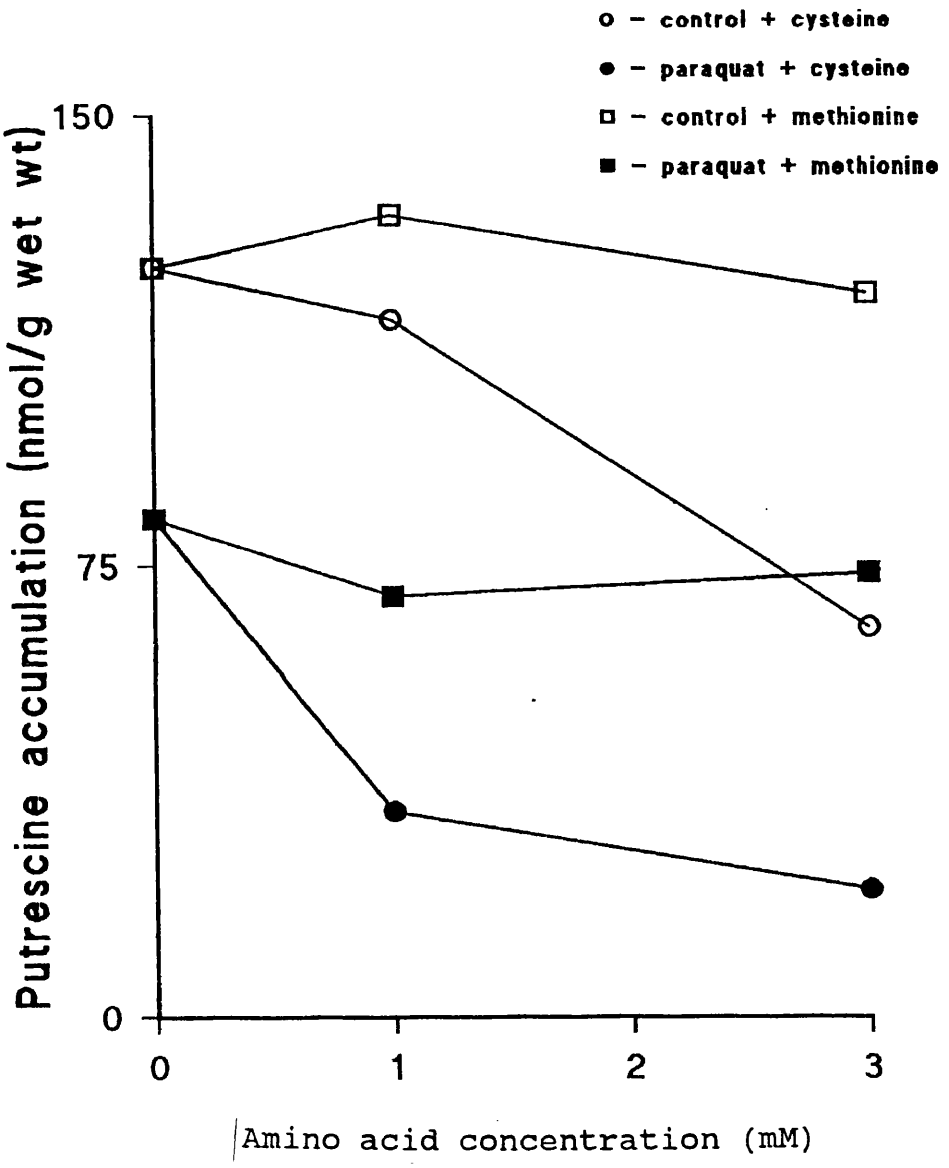
In a preliminary experiment, cysteine (1 and 3mM) causeda decline in putrescine accumulation and potentiated the toxicity of paraquat (100μM) to lung slices (fig 3.14). In contrast, methionine (1 and 3mM) did not induce toxicity, nor potentiate the toxicity of paraquat to slices (fig 3.14). A lower concentration of cysteine (250μM), did not produce overt toxicity to lung slices when monitored by putrescine uptake but still enhanced paraquat-mediated toxicity (result not shown).

CIPE (250μM) had no effect on putrescine accumulation by slices but dramatically potentiated the toxicity of paraquat (10μM) (fig 3.15), from a <20% fall with paraquat alone to a >70% drop. *N*-Acetylcysteine (250μM) neither protected against nor enhanced the toxicity of paraquat to lung slices.

9. Influence of taurine, cystamine and hypotaurine on paraquat induced toxicity to lung slices

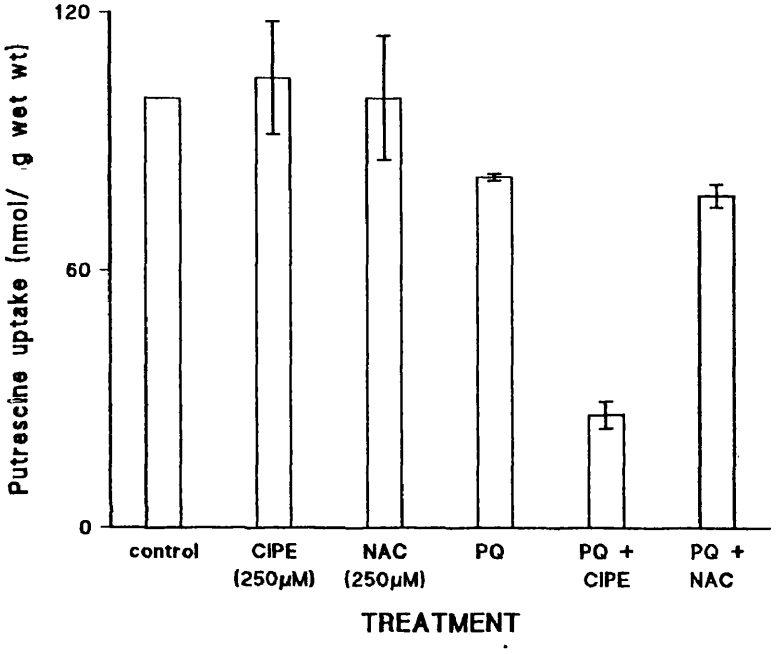
In a preliminary study, paraquat (100μM) reduced putrescine accumulation by 58%. Slices preincubated with cystamine (5 and 100μM) for 30 min and then incubated for a further 4 hr in a cystamine-free medium did not alter this decline (fig 3.16). However including cystamine in the incubation medium for 4 hr, reduced the paraquat-mediated fall in diamine uptake (fig 3.16). Taurine and hypotaurine, possible metabolites of cystamine, did not alleviate the paraquat-induced decline in putrescine accumulation.

FIGURE 3.14 Cysteine potentiates the toxicity of paraquat



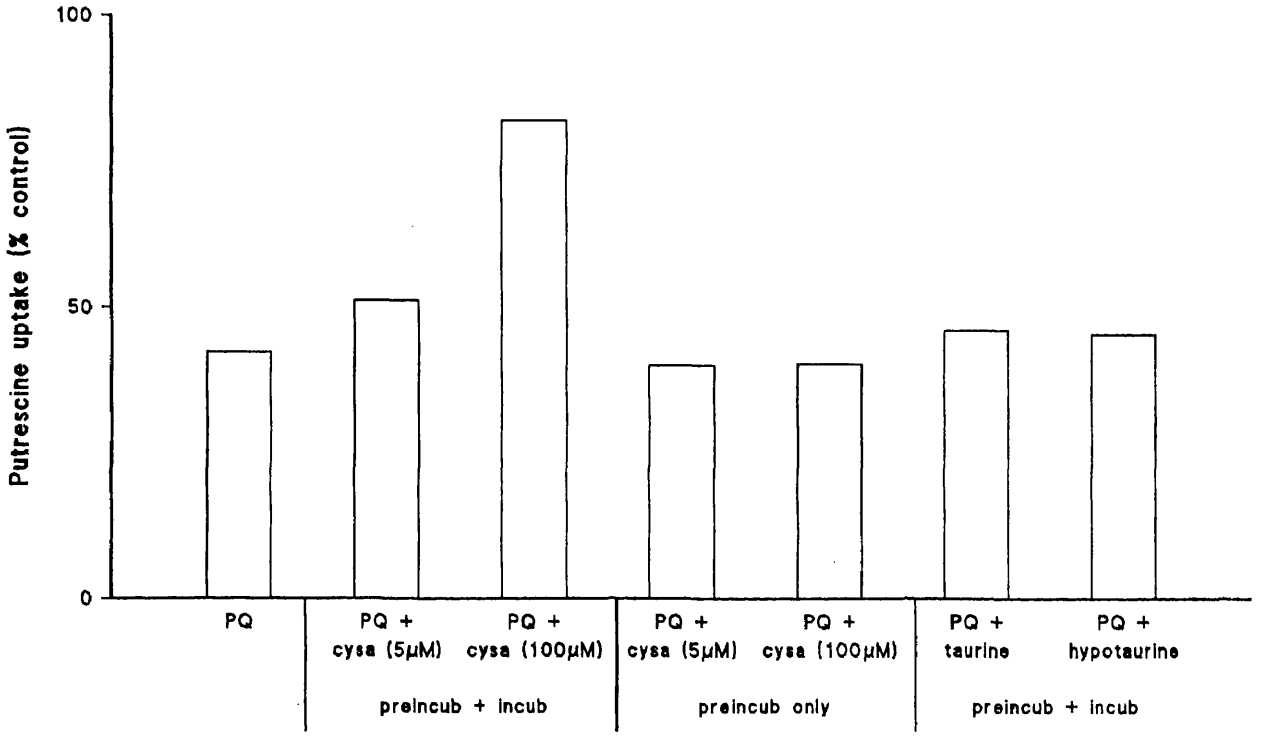
Slices were incubated with or without paraquat (100μM) in the absence or presence of cysteine (o or ●) or methionine (□ or ■). After 4 hr the ability of slices to accumulate putrescine was estimated. The results are from one experiment.

FIGURE 3.15 The effect of cysteine isopropylester and *N*-acetylcysteine on the fall of putrescine accumulation by lung slices incubated with paraquat



Slices were incubated in the absence or presence of paraquat (100μM) with or without cysteine isopropylester (250μM) or *N*-acetylcysteine (250μM). After 4 hr the putrescine accumulation by lung slices was estimated. The results are the mean of 3 experiments (±SEM).

FIGURE 3.16 Cystamine ameliorated the paraquat-mediated decline in putrescine accumulation by rat lung slices



Slices were preincubated with cystamine (5 and 100μM), taurine (100μM) and hypotaurine (100μM) for 30 min. All slices were then incubated with paraquat (100μM) in the presence or absence of cystamine (5 and 100μM) or presence of taurine (100μM) and hypotaurine (100μM). The results are from one experiment only.

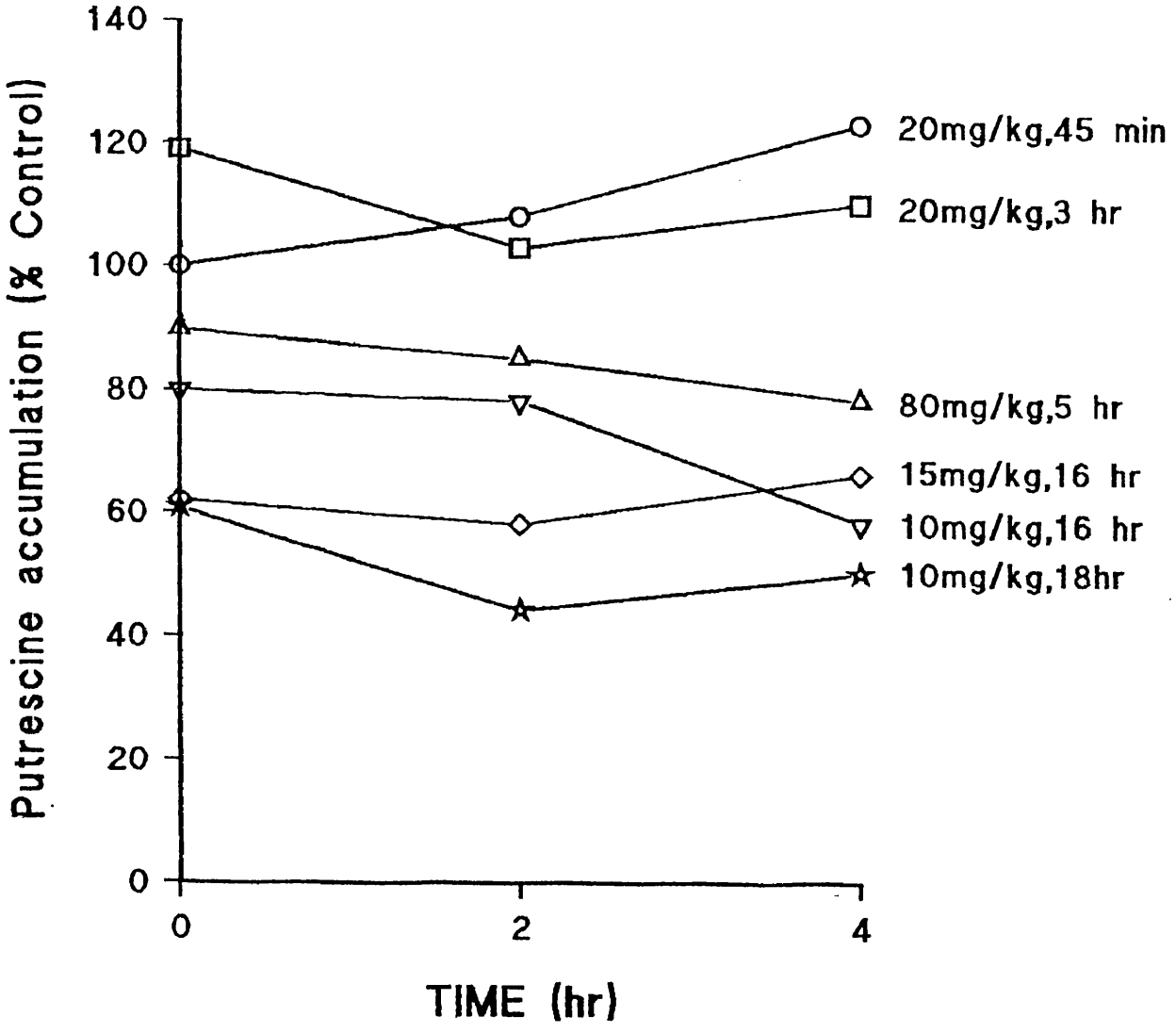
IN VIVO/IN VITRO LUNG SLICE MODEL

10. Development of an alternative lung slice model for paraquat toxicity

Lung slices prepared from animals previously exposed to paraquat *in vivo*, will have tissue concentrations of paraquat similar to levels achieved following intoxication and will a be suitable model in which to screen potential antidotes to paraquat toxicity. Putrescine accumulation was monitored *in vitro* for up to 4 hr in slices prepared from animals exposed to various doses of paraquat for up to 18 hr (fig 3.17). In summary, either exposing rats to large doses of paraquat for 5-6 hr or medium doses for longer periods of time (16-18 hr) reduced putrescine accumulation. No further decline was observed when slices were incubated for 4 hr *in vitro*. Desferrioxamine or CP51 did not ameliorate the drop in putrescine accumulation, nor did GSH reductase inhibition potentiate the decline (data not shown).

FIGURE 3.17 *In vivo* administration of paraquat reduced putrescine accumulation by subsequently prepared lung slices

Paraquat was administered to rats (i.p., see figure for doses and exposure time) and lung slices subsequently prepared. The ability of slices to accumulate putrescine was assessed after 0, 2 and 4 hr incubation in KRP.



3.3 DISCUSSION

Some of the chelators of iron, desferrioxamine, CP51 and 2,3-dihydroxybenzoic acid, were able to alleviate paraquat toxicity to varying extents in either control and/or lung slices with GSH reductase compromised activity (figs 3.3-3.7 and 3.9). Desferrioxamine was the most effective iron chelator.

DFO >> 2,3-diOH benzoic acid = CP51 > PIH = phytic acid

However desferrioxamine also substantially reduced the ability of slices to accumulate paraquat, unlike CP51 (fig 3.10), consequently its ability to chelate iron cannot be the sole explanation for its mechanism of action. Desferrioxamine has a complex polyamine-like structure (fig 3.1), allowing it to interfere with the uptake of paraquat by the polyamine uptake system of type I and II cells. However a role for iron chelation in the protection observed cannot be ruled out. Van der Wal *et al* [220] suggest desferrioxamine protects isolated type II cells by 2 mechanisms; iron chelation and inhibition of paraquat uptake. As CP51 and 2,3-dihydroxybenzoic acid did not inhibit paraquat accumulation, it could be assumed that any diminution of paraquat toxicity is due to chelation of iron.

Desferrioxamine has been reported to prevent the toxicity of paraquat in several animal and microbial systems. It increased the survival time of mice exposed to paraquat, while iron supplements enhanced toxicity [218] and reduced the mortality caused by paraquat when continuously infused to vitamin E deficient rats [219]. In animals with normal levels of vitamin E, desferrioxamine was an ineffective treatment but CP51, continuously infused, reduced paraquat-mediated mortality [220]. In contrast, CP51 was less effective than desferrioxamine in the lung slice model (figs 3.3 and 3.6).

Desferrioxamine also has several antioxidant properties *in vitro* [209], being a radical scavenger which may also play a part in the protection observed.

Not all studies using desferrioxamine have successfully prevented paraquat toxicity [221]. It increased the mortality of paraquat to rats [221]. This is proposed to occur as a result of the paraquat radical cation reducing the Fe^{3+}-desferrioxamine complex to an Fe^{2+} complex. Fe^{2+} can dissociate from the complex and result in the generation of reactive oxygen species, so potentiating the toxicity of paraquat. This has been demonstrated to occur *in vitro* [222], but not *in vivo* [209].

The studies presented did not estimate the cytosolic concentration of chelators, so the relative ineffectiveness of several of them may be due to poor membrane permeability. CP51 carries no charge at pH 7.4 and should pass through cell membranes [211]. Both 2,3-dihydroxybenzoic acid and PIH have been reported to mobilise iron from intracellular depots. However desferrioxamine has a slow rate of penetration into cells [209] which may not be by passive diffusion but by pinocytosis [223]. The subsequent subcellular localisation of desferrioxamine is restricted to lysosomes [223], questioning the role of chelation in desferrioxamine-mediated amelioration of paraquat toxicity.

GSH has several important cellular functions. Several studies have correlated intracellular levels of GSH and sensitivity to oxidative injury. GSH is a cofactor for GSH peroxidase, allowing for H_2O_2 and lipid hydroperoxide reduction. Exogenous GSH produced a small but significant restoration of putrescine accumulation following incubation of lung slices with paraquat (fig 3.11). Extracellular GSH also maintained the pulmonary concentration of GSH and cysteine at or above initial control values after 4hrs incubation (fig 3.12). This suggests that the modest protection observed was due to the maintenance of intracellular levels of GSH. Exogenous GSH protects alveolar type II cells isolated from adult rats against the deleterious effects of paraquat [38] and type II cells isolated from foetal and neonatal rabbits against oxidative injury [224]. Similarly cultured mouse Clara cells were exquisitely sensitive to paraquat-induced damage (6μM)

[225] but high concentrations of GSH reduce the amount of injury.

An extracelluar pool of GSH may support cellular replenishment of GSH in 2 ways; certain epithelial cells including type II cells are able to accumulate GSH intact, alternatively the lung has γ-glutamyl transpeptidase activity [226], capable of initiating the cleavage of GSH into its constitutive amino acids. Following transport across the plasma membrane, GSH may be synthesised from the amino acids. In support of this latter hypothesis, an increase in intracellular cysteine was observed following incubation of lung slices with GSH (fig 3.12). However it has been suggested that much of the pulmonary GSH is distributed in cells other than type I and II cells [25], and type II cells are the least able to generate GSH from cysteine when compared to pulmonary macrophages and Clara cells isolated from rabbit lung [227].

Elevation of pulmonary GSH following phorone administration, while sufficient to protect against the toxicity of the rodenticide α-naphthylthiourea [228], did not reduce the damage produced by paraquat (10 or 100μM) to slices. This may be due to the relatively small increase in GSH, 1.3-1.4 fold, compared to the published 2.5 fold increase in GSH after 48 hours (It is interesting to note the 1.9-2.5 fold increase in GSH when slices were incubated with exogenous GSH for 4 hr(fig 3.12)). Alternatively the rise in GSH after phorone treatment may be in pulmonary cell types not targeted by paraquat .

Methionine, the substrate for the cystathionine pathway, did not prevent paraquat-mediated damage, confirming the absence of this pathway in the lung [227]. Cysteine and cysteine isopropylester both potentiated the toxicity of paraquat to the alveolar epithelial cells. Esters of cysteine are hydrolysed to cysteine [94]. Cysteine can be oxidised to cystine in a metal catalysed reaction, generating superoxide, hydrogen peroxide and hydroxyl radicals [116]. If this occurred in the extracellular medium, it could enhance the toxicity of

paraquat. The addition of cysteine to culture media was highly toxic to cultured cells [111]. However cysteine did increase the survival rate of mice given Gramoxon, a commercial paraquat formulation [229].

Cystamine is a disulphide, accumulated by the polyamine uptake system into lung slices and also by a low affinity uptake system [230]. Cystamine is metabolised via hypotaurine to taurine [230]. Both cystamine and taurine have postulated roles as antioxidants. In the preliminary data presented, only cystamine (fig 3.16) protected against paraquat damage when coincubated with paraquat. Taurine did not alleviate toxicity. Cystamine probably protected by competing with paraquat for the polyamine uptake system, reducing the cellular concentration of paraquat.

Lung slices prepared from animals exposed to paraquat *in vivo* will have paraquat levels which are toxicologically relevant. However once damage had been produced *in vivo*, accumulation of putrescine by subsequently prepared slices was not restored by iron chelators such as desferrioxamine and CP51. Compromising GSH reductase with BCNU activity did not potentiate the fall in diamine accumulation. This suggests that putrescine accumulation, as a functional marker, may not be the best marker for the type of studies carried out. A decline in putrescine uptake is indicative of a loss of cell viability, rather than a means of identifying cells which are toxicologically stressed. The possible use of alternative biochemical markers of alveolar type II injury, such as nucleotide levels and surfactant production were being investigated before the direction of research changed to investigate the esters of cysteine as potential chemoprotectants.

Chapter 4 - Prevention of paracetamol and bromobenzene hepatotoxicity by cysteine isopropylester.

4.1 INTRODUCTION

Glutathione (GSH) has a wide biological distribution and is important in many physiological and toxicological processes. It has a particular role in cellular defense mechanisms. Modulation of tissue GSH levels greatly influences the susceptibility of various organs to different chemicals [12]. Several agents, such as buthionine sulphoximine or diethyl maleate, or a protein deficient diet are capable of depleting tissue GSH and enhancing the toxicity of electrophilic xenobiotics. Exogenous GSH is relatively ineffective as a protectant, being unable to cross cell membranes as the intact tripeptide. A variety of different methods have been utilised to elevate intracellular GSH [29,46-54,72,73].

A key factor in the maintenance of intracellular GSH is the supply of L-cysteine [7]. Unlike GSH, cysteine is transported into several cell types using neutral amino acid transport systems [62]. In this thesis, cysteine esters are considered as novel cysteine delivery systems. Esters of cysteine due to their greater lipophilicity relative to cysteine should enter cells with ease and increase intracellular cysteine after enzymic cleavage by esterases. Administration of cysteine esters may alter the overall thiol defenses of an organ in two ways. Firstly the provision of excess cysteine, a substrate for γ-glutamylcysteine synthetase, will promote synthesis of intracellular GSH. Secondly cysteine may itself be protective as it is known to react directly with reactive electrophiles more rapidly than glutathione [231].

A number of cysteine esters have been synthesized and several including CIPE have been shown to protect against the acute pulmonary oedema induced by perfluoroisobutene, a pyrolysis product of polytetrafluoroethylene (Teflon) [94]. In this chapter the potential of CIPE to protect against the hepatotoxicity of paracetamol and bromobenzene and the pulmonary toxicity of naphthalene are investigated.

4.2 RESULTS

A PHARMACOKINETICS

1. Effect of CIPE on NPSH levels in mice

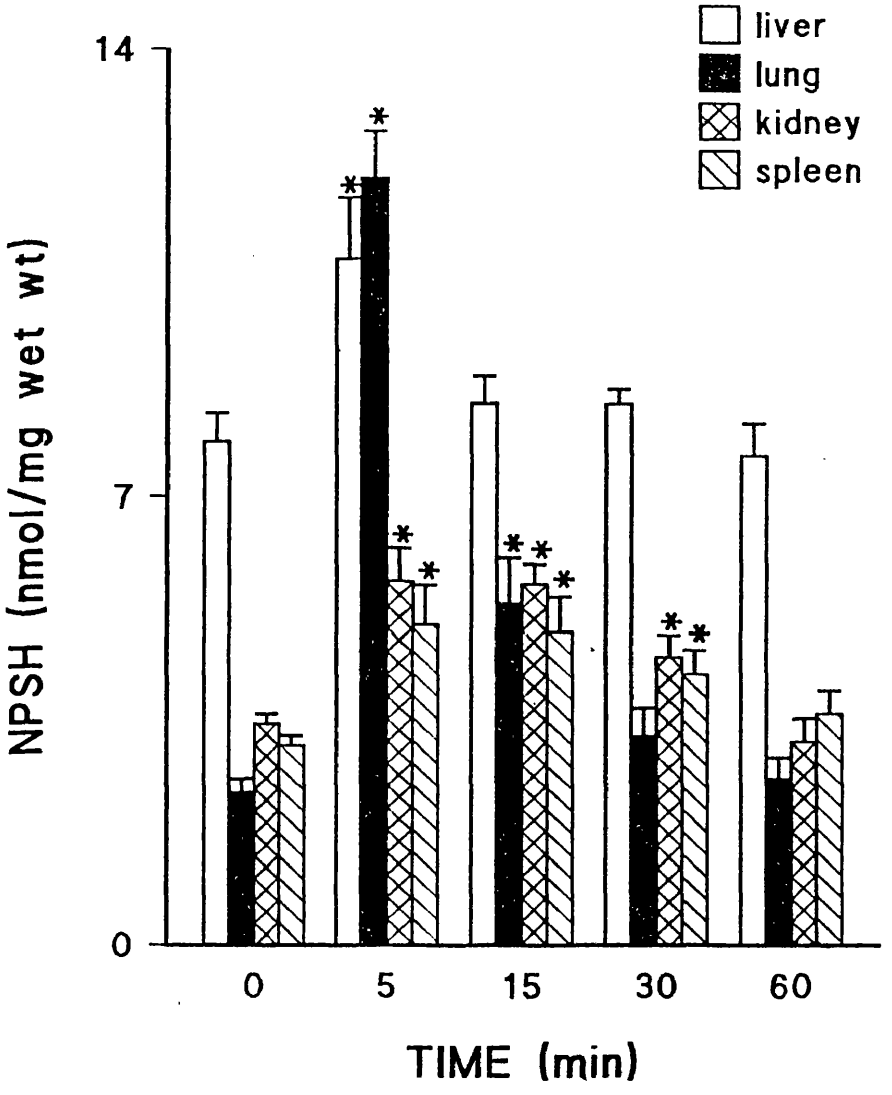
CIPE produced a transient elevation of NPSH levels in liver, lung, kidney and spleen (fig 4.1). Slight increases were also observed in the heart and testis. Maximum increases were observed 5 minutes after administration when the increase in the lung (521% of control) was particularly striking (fig 4.1). These increases were very transient, pulmonary and hepatic NPSH values fell rapidly and approached control values after 30 minutes. The elevated NPSH levels noted in the spleen and kidney, while not as pronounced as rises in pulmonary NPSH, followed a similar pattern, reaching control levels between 30 and 60 minutes.

In the rat, the earliest time point taken to measure NPSH was 30 min after administration of CIPE (3.0mmol/kg) (fig 4.2). There were slight but not significant elevations in hepatic, pulmonary and renal NPSH values. However the elevation of pulmonary and hepatic NPSH and cysteine levels at earlier times following administration of CIPE to rats are similar to those seen in mice [94].

2. Comparison of CIPE, cysteine and N-acetylcysteine in their ability to elevate NPSH levels in mice

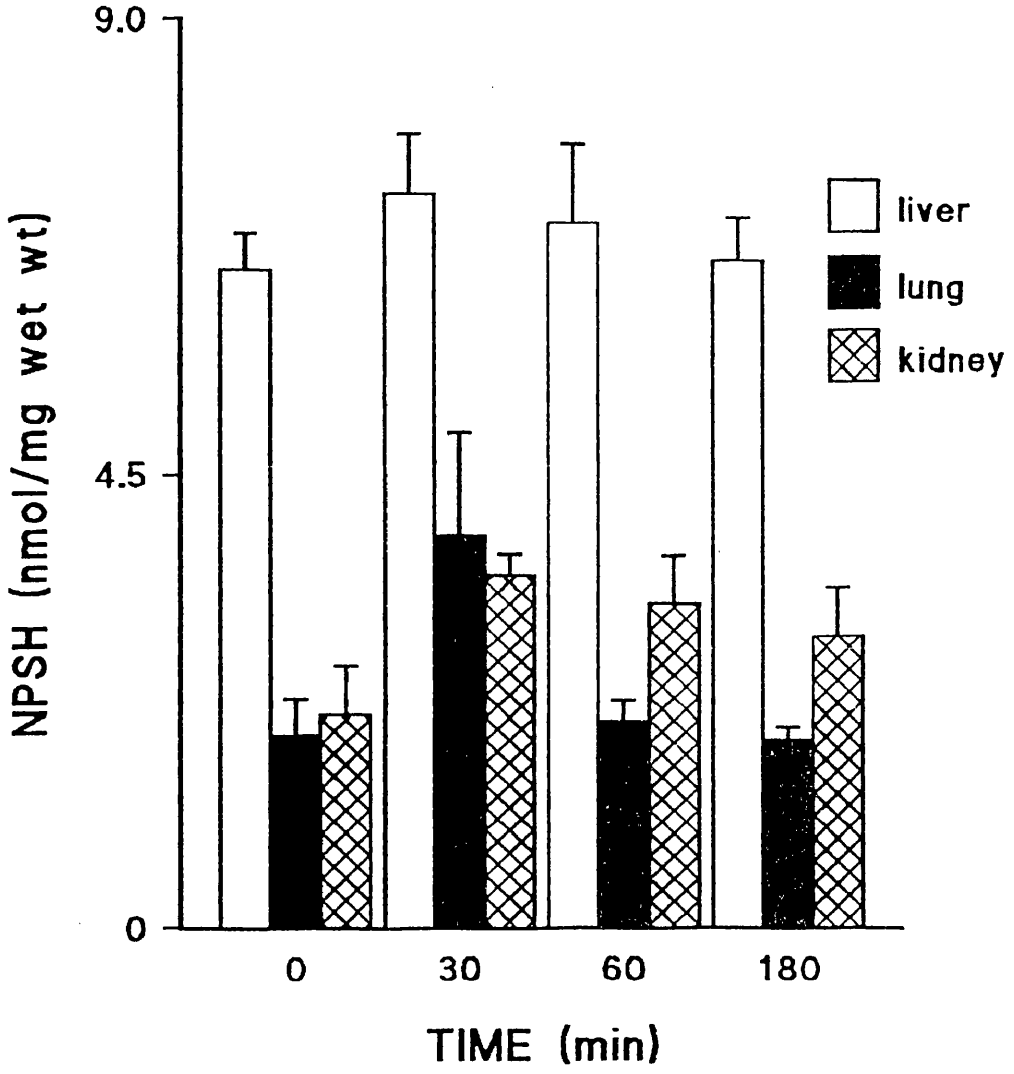
CIPE, cysteine and N-acetylcysteine promoted the NPSH levels of liver, lung, kidney and spleen to varying degrees (fig 4.2 a-d). After 5 min exposure, CIPE produced a pronounced elevation of pulmonary NPSH. N-Acetylcysteine produced substantial rise in renal NPSH. Preliminary data using HPLC analysis of the individual thiols, demonstrated that the bulk of the rise in NPSH by CIPE and N-acetylcysteine was accounted for as cysteine (fig 4.4a). No CIPE was detected in any tissue, and only relatively low amounts of N-acetylcysteine. CIPE rapidly disappeared from the plasma (fig 4.4b), giving rise to a pool of extracellular cysteine. Large amounts of unmetabolised N-acetylcysteine were present at these early time points (fig 4.4b).

Figure 4.1. Elevation of non-protein sulphydryls in several mouse organs following a single administration of CIPE (3.0mmol/kg *i.p.*)



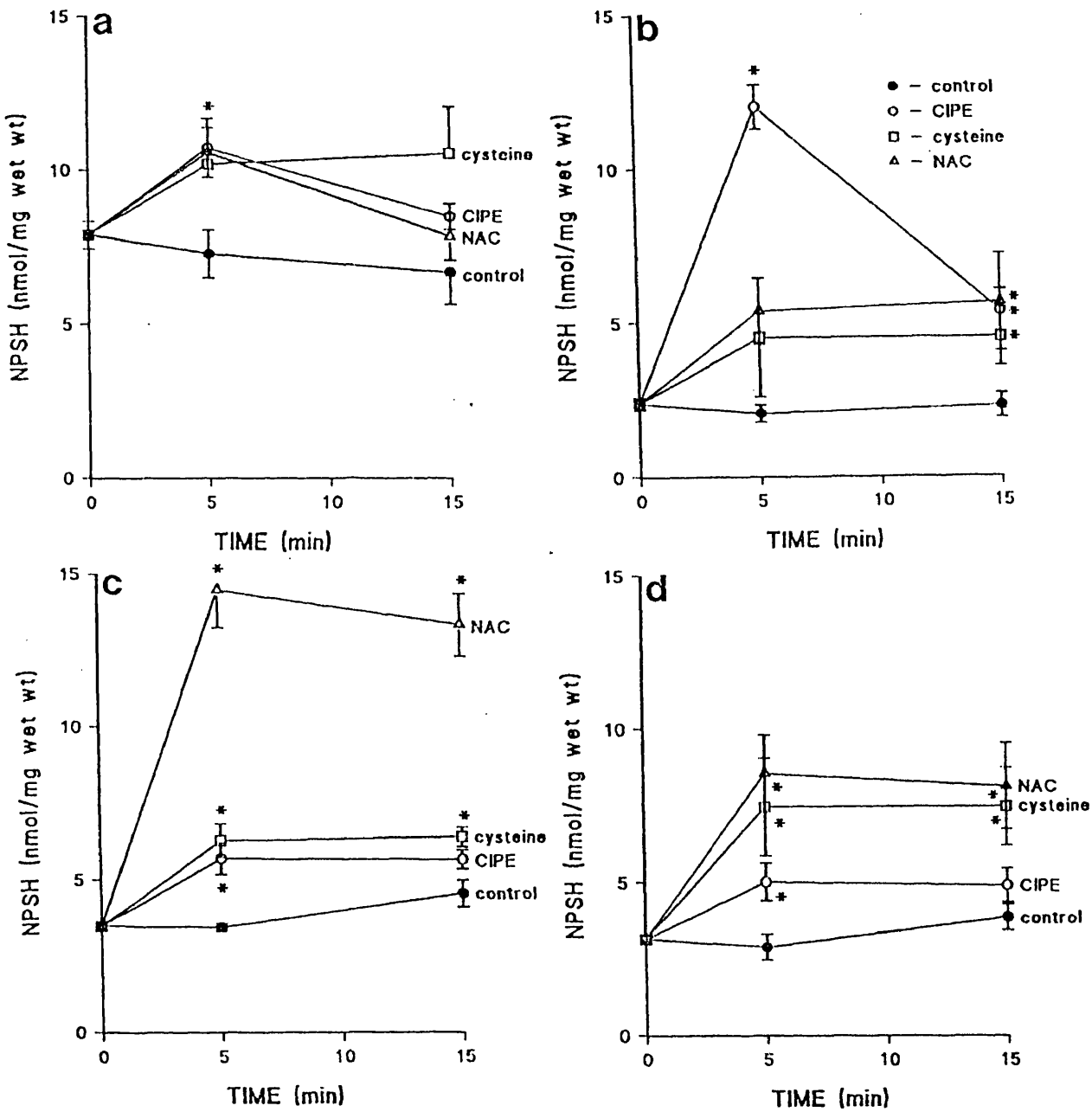
Animals were treated with CIPE and sacrificed at the times indicated. Organ homogenates, liver (□), lung (■), kidney (▨) and spleen (▧), were prepared and NPSH values assessed by the method of Ellman. Each bar represents the mean of 6-11 animals, assessed on separate occasions (±SEM). Each NPSH value from CIPE treated mice was compared with control (0 time), *p<.05.

Figure 4.2. Changes in rat organ NPSH after administration of CIPE (3.0mmol/kg)



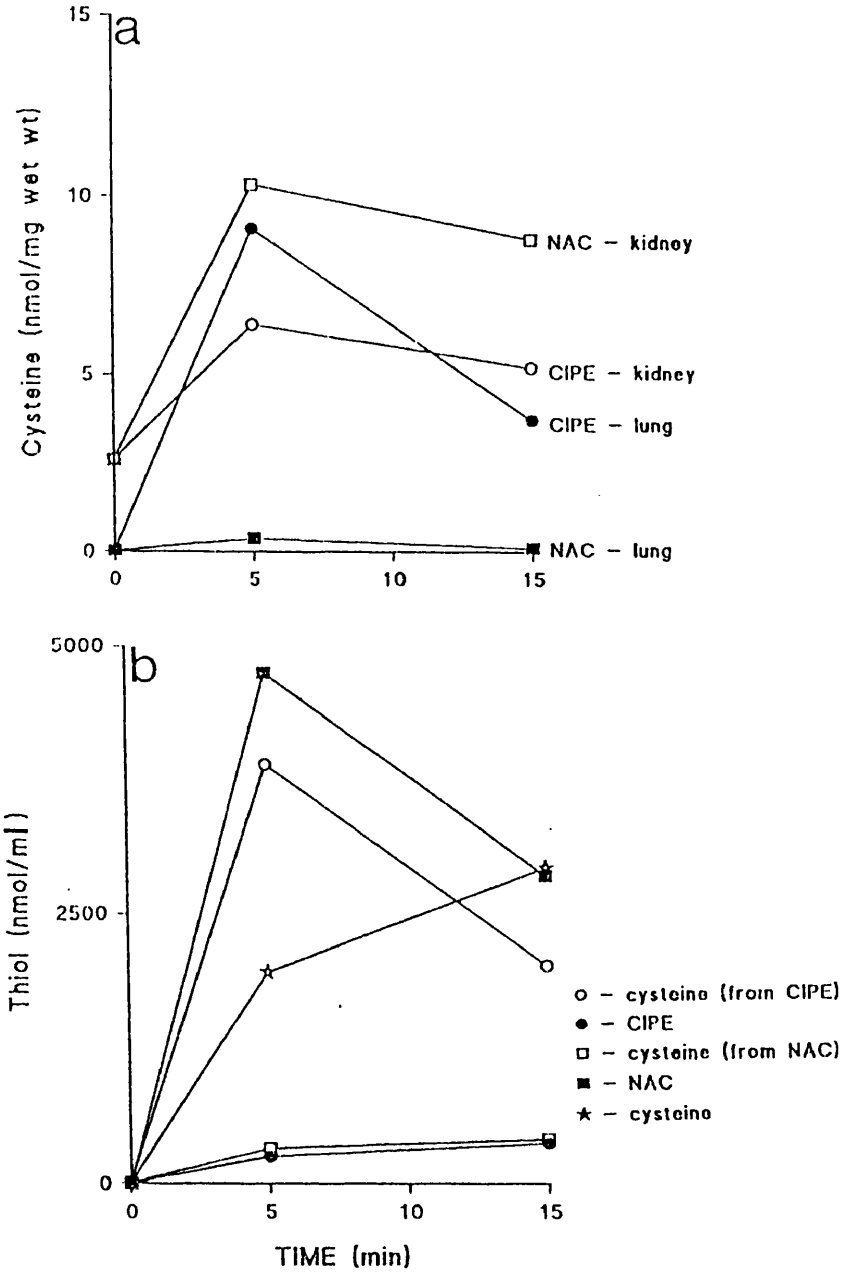
Animals were killed at the times indicated and liver (□), lung (■) and kidney (▨) were homogenized before an assessment of NPSH values by the Ellman method made. Each bar represents the mean of at least 4 animals (±SEM).

Figure 4.3. A comparison of the relative abilities of CIPE, *N*-acetylcysteine and cysteine to promote the NPSH values of liver (a), lung (b), kidney (c) and spleen (d)



CIPE (○), cysteine (□) and *N*-acetylcysteine (△) were given as a single dose (3.0mmol/kg i.p.) and animals killed after 5 and 15 minutes. Each point represents the mean of 3 separate experiments (±SEM). Each time point from thiol-treated animals was compared to its control, * $p<0.05$. At 5 minutes all the treated animals have significantly elevated levels of hepatic NPSH.

Figure 4.4 HPLC analysis of thiol levels in mouse tissues and plasma after exposure to CIPE



Following administration of CIPE, *N*-acetylcysteine and cysteine (3.0mmol/kg i.p.), HPLC analysis showed the elevation of tissue NPSH values were largely comprised of cysteine (a). The plasma levels of CIPE (b) rapidly fell, to be replaced by cysteine. While *N*-acetylcysteine was more persistent and gave rise to a smaller pool of plasma cysteine (fig 4.4b). Animals were sacrificed after 5 and 15 min. Plasma samples, obtained by cardiac puncture and tissue homogenates were derivatised with monobromobimane for HPLC analysis. Each point represents the mean of 2 separate experiments.

The tissue levels of GSH remained unaltered by the thiols (data not shown). However it was perplexing to note the low amounts of GSH and high amounts of cysteine detected in the renal fraction. Confirming the difficulty of analysing intact renal GSH due to the activity of γ-glutamyltranspeptidase [232].

3. Elevation of NPSH in rat and mouse organs after repeated exposure to CIPE

The half life of CIPE after a single exposure was short. It was initially envisaged that to obtain a sustained elevation of tissue thiols, repeated or continuous administration of CIPE would be required. Preliminary experiments investigating the feasibility of repeated dosing regimens were carried out.

The NPSH values of several rat and mouse organs were estimated 30 min after 4 consecutive intraperitoneal administrations of CIPE (3.0mmol/kg), each injection being 30 min apart. Substantial rises in NPSH levels were observed in several organs (fig 4.5), again the elevation of pulmonary NPSH was especially striking. The elevation of NPSH in rat plasma was 3490% greater than control levels.

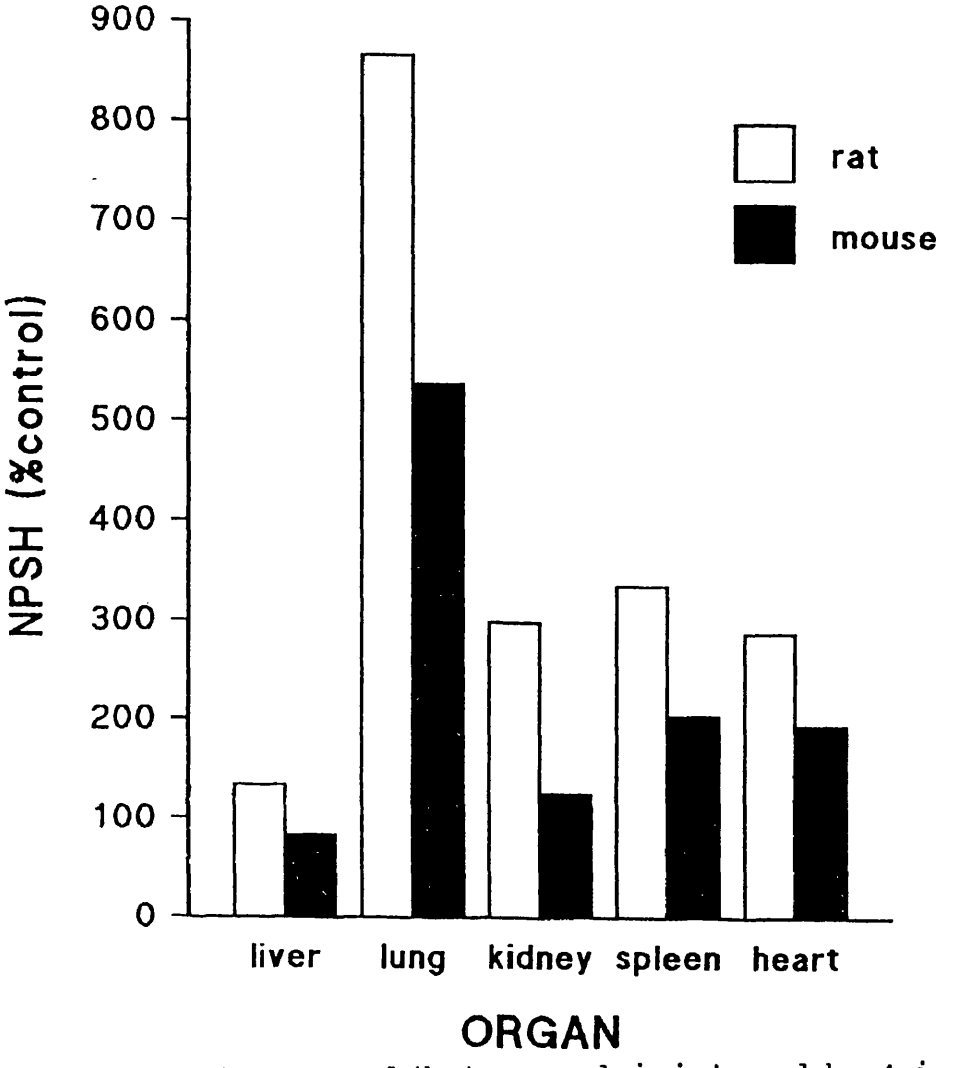
However repeated exposure of mice to CIPE resulted in fatalities within 24 hr, as did repeated exposure to L,2-oxothiazolidine-4-carboxylic acid (OTZ) (data not shown). In rats, both CIPE and OTZ given repeatedly, while not fatal, did potentiate the toxicity of bromobenzene (5.0mmol/kg), causing fatalities (data not shown).

B CHEMOPROTECTION

1. Development of animal models for paracetamol and bromobenzene hepatotoxicity

The hepatotoxicities of paracetamol and bromobenzene are characterised by a decline in GSH [13,158]. When depletion of GSH reaches about 80% of initial values, covalent binding of electrophilic metabolites to alternative nucleophiles can occur [127]. As the elevation in NPSH following administration of CIPE was only transient, it can be rationalised that any

Figure 4.5. Repeated exposure to CIPE produced substantial elevations in several mouse and rat organs.



CIPE (12.0 mmol/kg) was administered by 4 i.p. injections, with a 30 min pause between each injection. Rats (□) and mice (■) were killed by an overdose of halothane anaesthesia 30 min after the last injection, tissues homogenized and an assessment of NPSH values made by the Ellman method. The data presented is the result of 1 experiment.

protection afforded directly by cysteine *per se* may only be of a short duration. I therefore required models of paracetamol and bromobenzene toxicity, which caused a rapid generation of electrophiles NABQI and bromobenzene-3,4-oxide, which would result in a rapid decline of hepatic GSH to coincide with the extra thiol (-SH) groups available either due to CIPE or its hydrolysed product, cysteine.

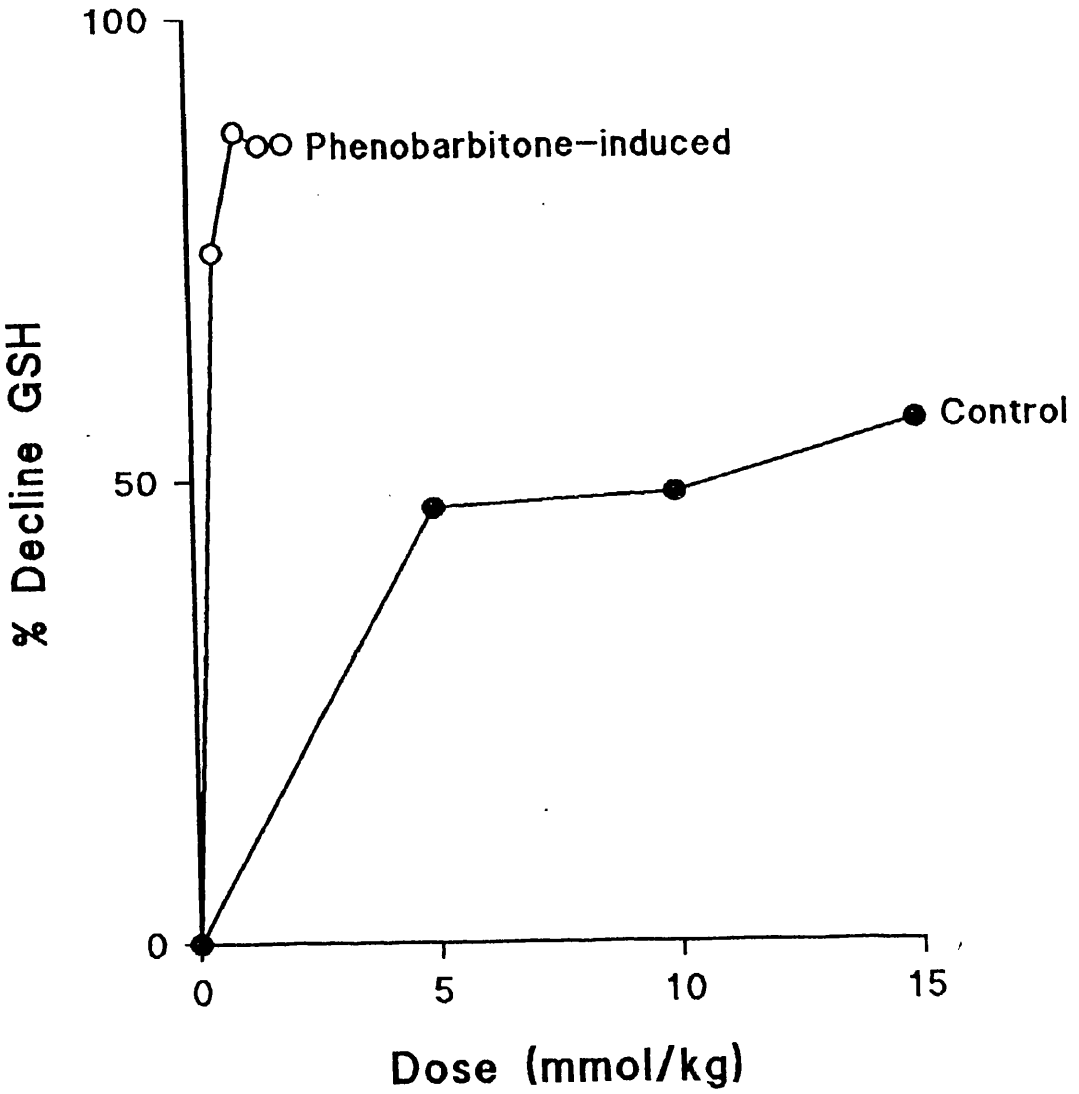
Administration of bromobenzene to rats (5,10 and 15mmol/kg) only produced a 50% depletion of hepatic GSH after 3 hr (fig 4.6), which CIPE did not reverse (data not shown). These doses were also acutely toxic, animals rapidly became moribund and convulsive. When cytochrome P450 activity was induced with phenobarbitone, lower doses of bromobenzene produced a >80% depletion of hepatic NPSH (fig 4.6) without the side effects encountered with higher doses of bromobenzene.

A similar picture emerged when paracetamol was used as a tool to deplete hepatic NPSH. Administration of paracetamol to mice produced a dose dependent decline in hepatic NPSH (fig 4.7), however the nadir was not reached until 3 hours post-dose and levels of NPSH returned to control values after 6 hr at all doses except 750 mg/kg. To deplete hepatic NPSH values rapidly, mice were pretreated with benzo(a)pyrene to induce cytochrome P450, which following paracetamol administration (400mg/kg) produced a rapid and sustained fall in NPSH (>80%) within 30 min (fig 4.7). In contrast when mice were induced with phenobarbitone, paracetamol failed to produce such a rapid and sustained fall in hepatic NPSH (data not shown).

2. Changes in hepatic NPSH, serum enzymes transaminases and mortality after administration of bromobenzene and CIPE

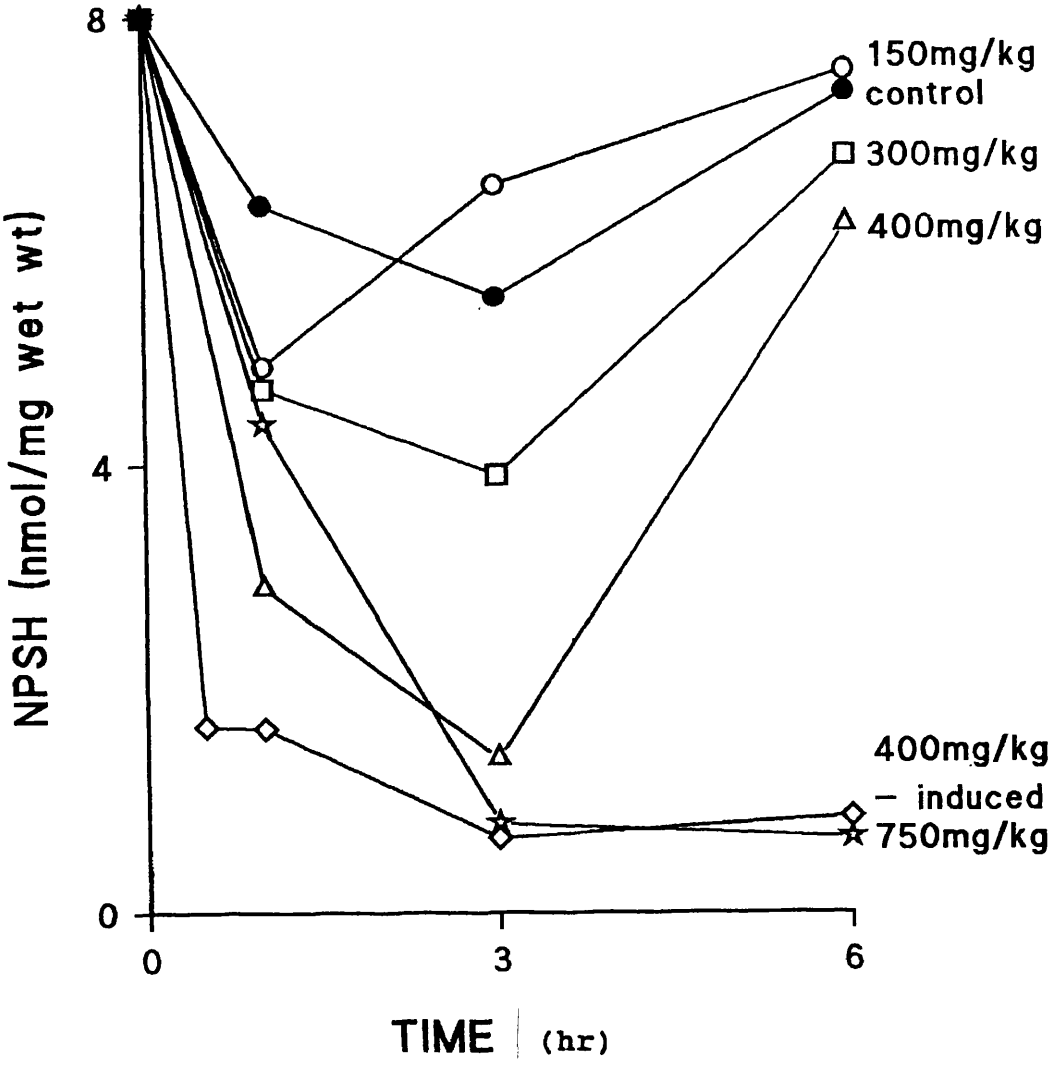
When CIPE was administered coincidently with bromobenzene to rats, the decline in hepatic NPSH was slowed. Levels of hepatic NPSH recovered (fig 4.8), exceeding those observed in control animals after 24 hr. Rats given bromobenzene alone experienced a recovery in hepatic GSH after 6 hr, however the majority of the animals died within 24 hr (14% survival, n=7). No fatalities occurred in animals receiving both bromobenzene

Figure 4.6. Depletion of hepatic GSH by bromobenzene in rats.



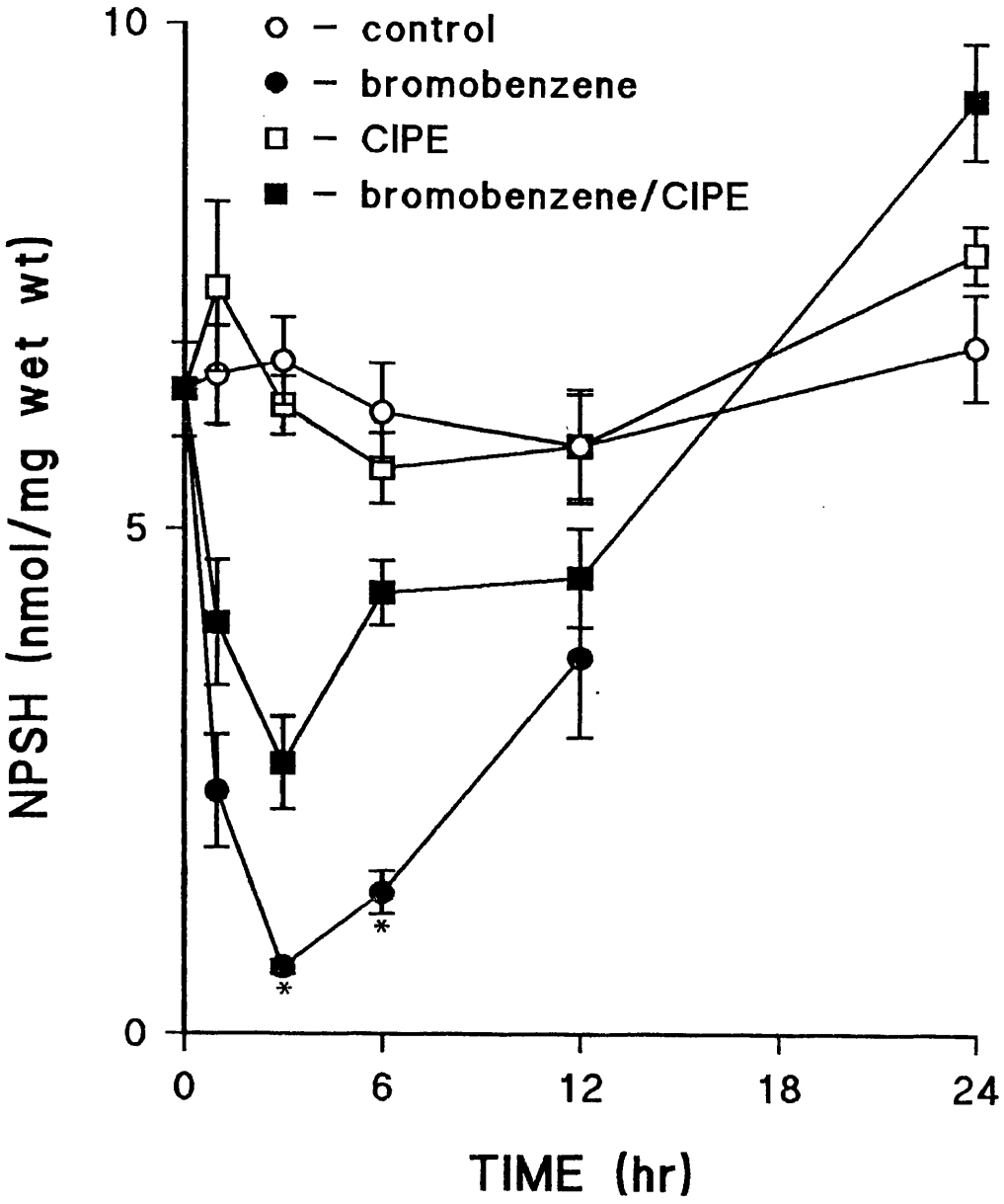
Animals were given phenobarbitone (80mg/kg, i.p.) (O) or saline (i.p.) (●) for 3 consecutive days. On the fourth day animals received bromobenzene; induced animals (0.5,1.0, 1.5 and 2.0 mmol/kg, i.p.) and control animals (5.0, 10.0 and 15.0 mmol/kg, i.p.). After 3 hr animals were sacrificed and the depletion of hepatic NPSH assessed by the method of Ellman. The data represents 1 experiment only.

Figure 4.7. The effect of paracetamol on mouse hepatic NPSH.



Paracetamol (150, 300, 400, 750 mg/kg, i.p.) was given as a single dose to uninduced mice or paracetamol (400mg/kg, i.p.) was administered to mice pretreated with benzo(a)pyrene (20mg/kg, i.p. daily for 3 consecutive days). Animals were killed at the times indicated and subsequent liver homogenates measured for their NPSH levels. The data represents the results from 1 experiment only.

Figure 4.8. Effect of CIPE (3.0mmol/kg, *i.p.*) on hepatic NPSH values in rats exposed to bromobenzene (2.0mmol/kg, *i.p.*)



Bromobenzene and CIPE were co-administered and rats were sacrificed at the times indicated. All rats were induced with phenobarbitone (80mg/kg, *i.p.* for 3 consecutive days). Rats treated with bromobenzene alone died within the first 12 hours of exposure (14% survival). Each point represents the mean of 4 or 5 animals, assessed on separated occasions (±SEM). There is a significant difference in the levels of hepatic NPSH between rats exposed to both CIPE and bromobenzene and those treated with bromobenzene alone, $p<.05$.

and CIPE. An apparent slight diurnal fluctuation in control hepatic GSH occurred (fig 4.8). CIPE, after an initial elevation of NPSH had no significant effect on the amount of GSH in the liver (fig 4.8).

Bromobenzene also reduced the levels of NPSH in the lung and slightly in the kidney (fig 4.9 a and b), the nadir occurring after 3 hr. CIPE delayed and reduced the magnitude of these depletions.

Bromobenzene (2.0mmol/kg) produced a pronounced increase in SGOT and SGPT activities, which was especially striking after 12 hr (fig 4.10). While CIPE (3.0mmol/kg) produced no elevation in either SGOT or SGPT, it substantially ameliorated the rise in transaminase caused by bromobenzene (fig 4.10).

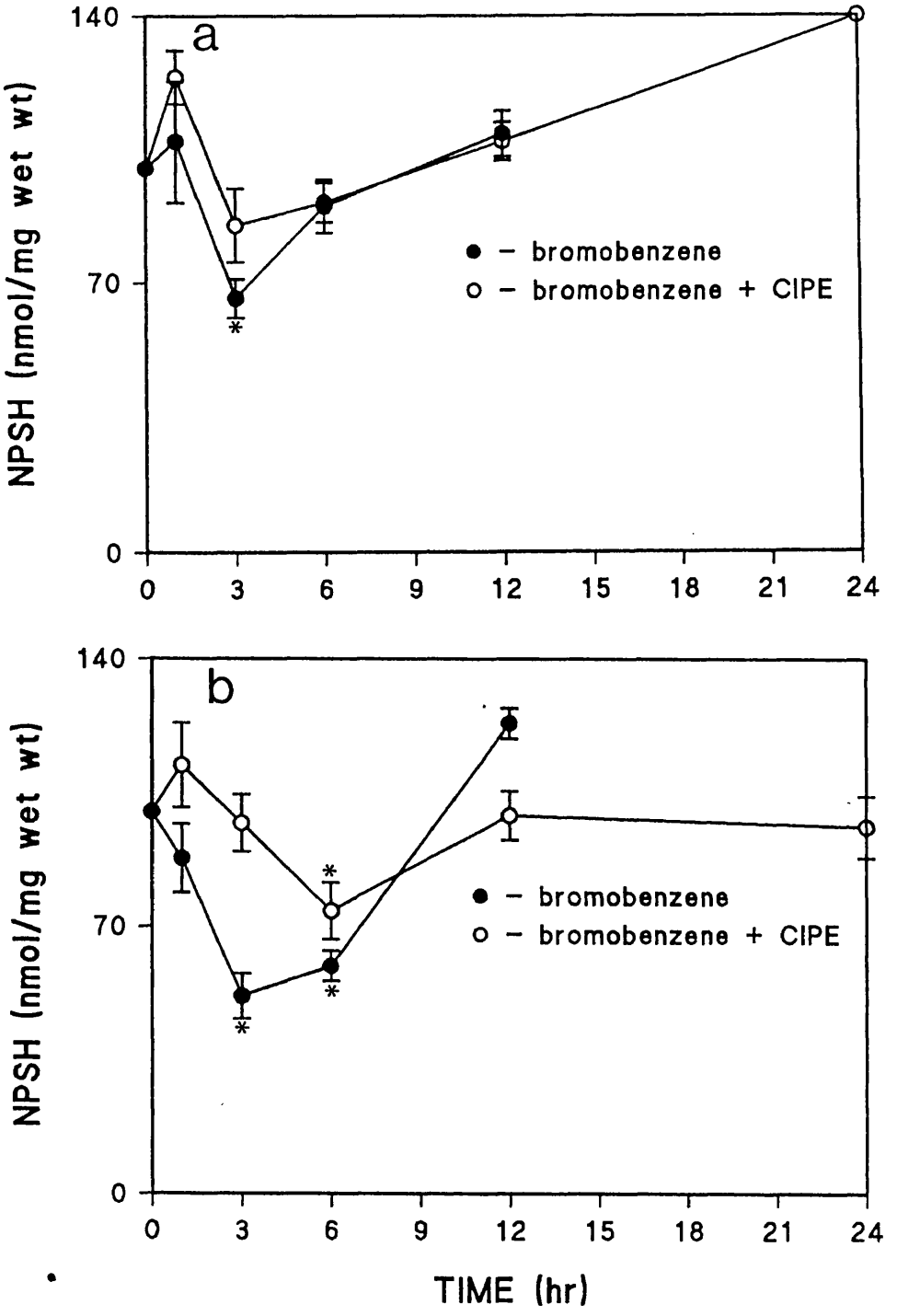
3. Effect of CIPE on bromobenzene induced changes in hepatic and renal histopathology

Bromobenzene (2.0mmol/kg) produced visible hepatic necrosis after 12 hr, coincident with the elevation in serum GOT and GPT., which the administration of CIPE (3.0mmol/kg) prevented. After exposure to bromobenzene for 24 hr, the livers of surviving animals had distinct areas of centrilobular (zone 3) necrosis (fig 4.11), which CIPE prevented (fig 4.11). Some hepatic necrosis was noted in 2 mice treated with both bromobenzene and CIPE after 24 hr. Neither bromobenzene alone nor in combination with CIPE damaged the kidney at 12 or 24 hr (not shown).

4. Effect of CIPE on paracetamol induced changes in hepatic NPSH and serum enzymes

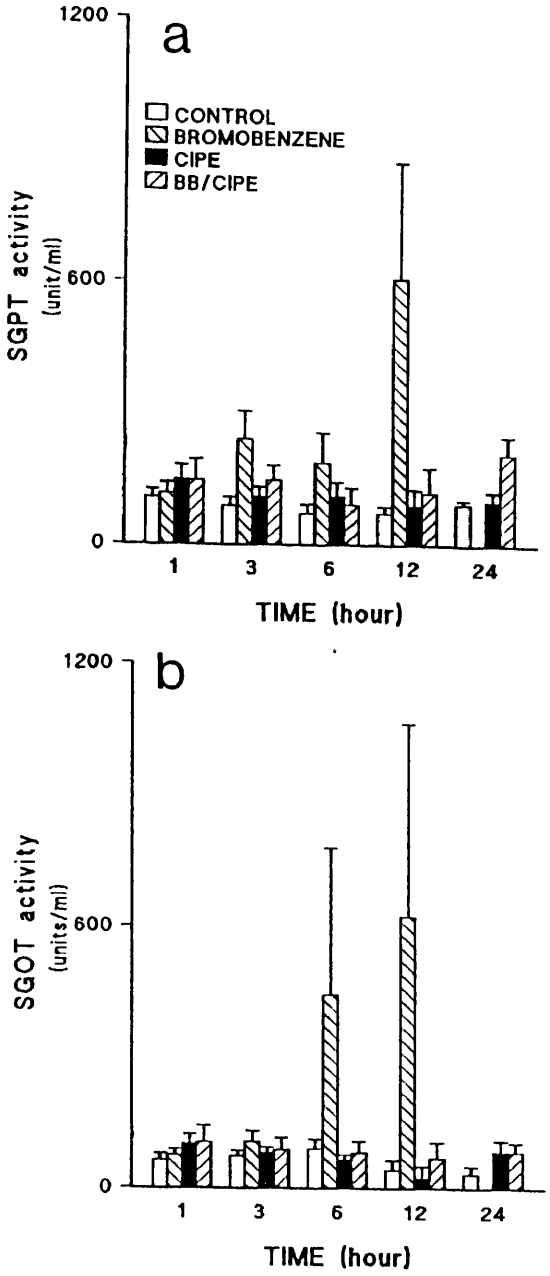
Co-administration of CIPE (3.0mmol/kg) together with paracetamol (400mg/kg) partially prevented the extensive decrease in hepatic NPSH observed with paracetamol alone (fig 4.12). After 1 hour, a 65% decrease in NPSH was observed compared to an 82% fall in mice treated solely with paracetamol and after 6 hours a restoration to 75% of the control value was noted. A diurnal variation in hepatic GSH was observed in control mice (fig 4.12) in agreement with previous findings

Figure 4.9. Bromobenzene-mediated decline in renal and pulmonary NPSH values



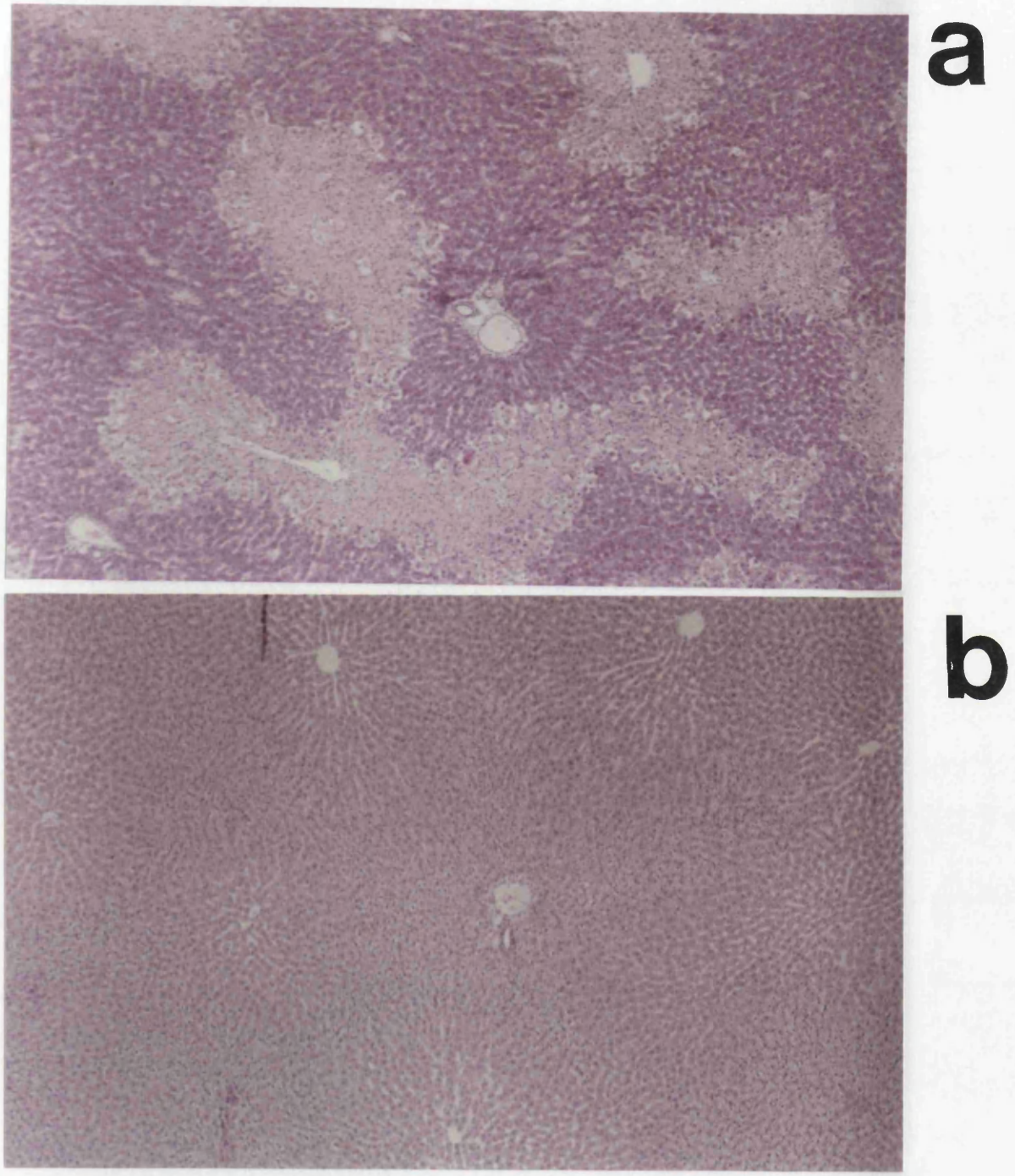
Bromobenzene (2.0mmol/kg, i.p.) and CIPE (3.0mmol/kg, i.p.) were administered at the same time to rats. Renal (a) and pulmonary (b) levels of NPSH were measured. Results are expressed as a percentage of the initial levels of NPSH in the kidney (2.76±.54 nmol/mg) and lung (2.12±.17 nmol/mg). Each point represents the mean (±SEM), n≥4. After exposure to bromobenzene NPSH levels were significantly reduced at 3 hr in the kidney and 3 and 6 hr in the lung, *p<.05.

Figure 4.10. Serum GPT(a) and GOT(b) levels after a single dose of 2.0mmol/kg bromobenzene with and without co-administration of CIPE (3.0mmol/kg)



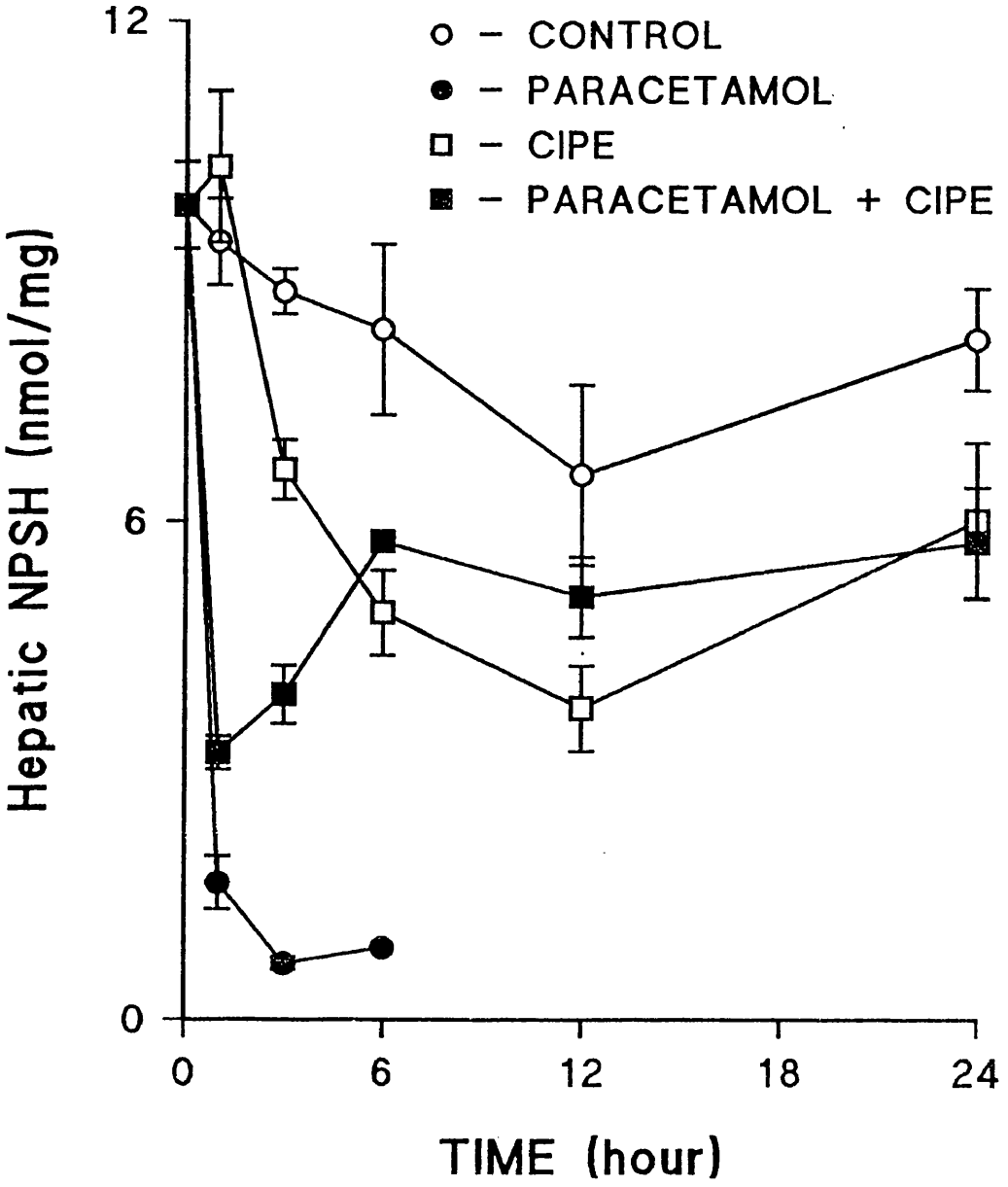
Blood samples were drawn by cardiac puncture at the time indicated and assessed for serum transaminase levels. Bromobenzene alone produced an 85% mortality rate within the first 24 hours of exposure. Each point represents the mean (±SEM), n≥4.

Figure 4.11 Histopathological changes in rat liver following the administration of bromobenzene (a) and protection by CIPE (b)



Rats were pretreated with phenobarbitone (80mg/kg, i.p.) daily for 3 days. Animals were given bromobenzene alone (2mmol/kg, i.p.) (a) or in combination with CIPE (3mmol/kg) (b) and killed after 24hr. The liver was excised and stored in neutral buffered formalin. Sections were cut and stained with haematoxylin and eosin.

Figure 4.12. Effect of CIPE (3.0mmol/kg, *i.p.*) on hepatic NPSH values in mice exposed to paracetamol (400mg/kg, *i.p.*)



Paracetamol and CIPE were co-administered and mice were sacrificed at the times indicated. All mice were induced with benzo(a)pyrene (20mg/kg, *i.p.* for 3 consecutive days). Mice treated with paracetamol alone died within the first 12 hours of exposure. Each point represents the mean of 3 animals, assessed on separated occasions (±SEM). NPSH values were compared to controls at each time point; *<.05. There is a significant difference between mice exposed to both CIPE and paracetamol and those treated with paracetamol alone.

[233]. CIPE (3.0mmol/kg) caused an initial but short lived elevation in hepatic NPSH followed by a prolonged decrease. A full restoration of hepatic levels of NPSH to control values was not observed even at 24 hr (fig 4.12).

Paracetamol (400mg/kg) produced a very marked elevation in both SGOT and SGPT which was maximal 6 hours after treatment (Figs 4.13). CIPE (3.0 or 1.5 mmol/kg) or *N*-acetylcysteine alone had no effect on either of the transaminases but it inhibited the elevation in SGOT and SGPT caused by paracetamol (400mg/kg) after 24 hr (Figs 4.13 and 4.14).

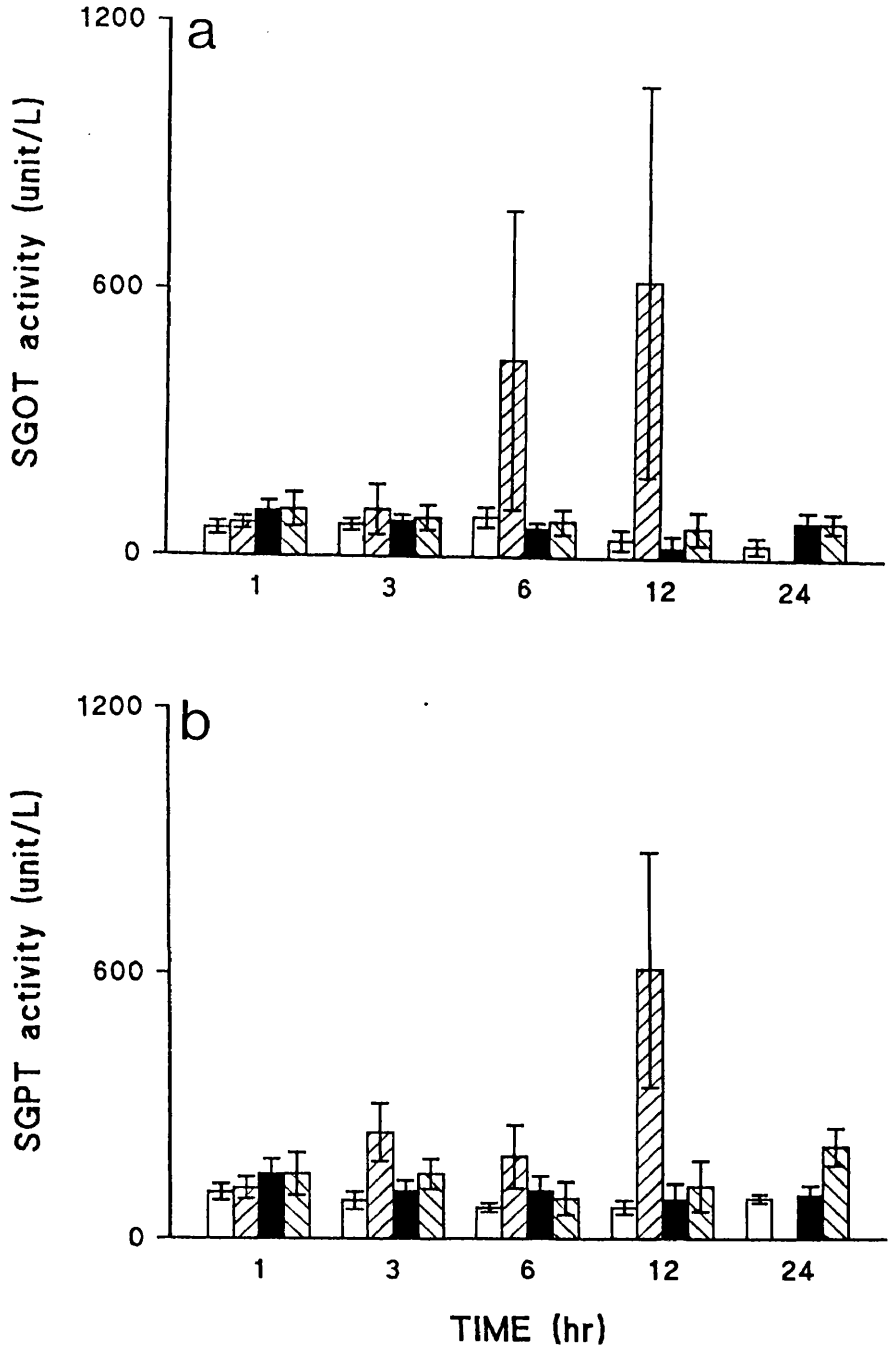
Lower doses of CIPE (0.75 and 0.375 mmol/kg) were also effective in inhibiting the elevation of transaminases by paracetamol (350mg/kg) (Table 4.I). CIPE was as efficient as N-acetylcysteine in preventing the paracetamol-mediated rise in serum enzymes. The amount of CIPE or *N*-acetylcysteine given could be lowered to 0.375mmol/kg before any rise in serum transaminase activity was observed (Table 4.I).

The protection afforded by CIPE against paracetamol-induced loss of NPSH and increases in SGOT and SGPT suggested that it may also protect against paracetamol-induced lethality.

5. Effect of CIPE on paracetamol-induced mortality

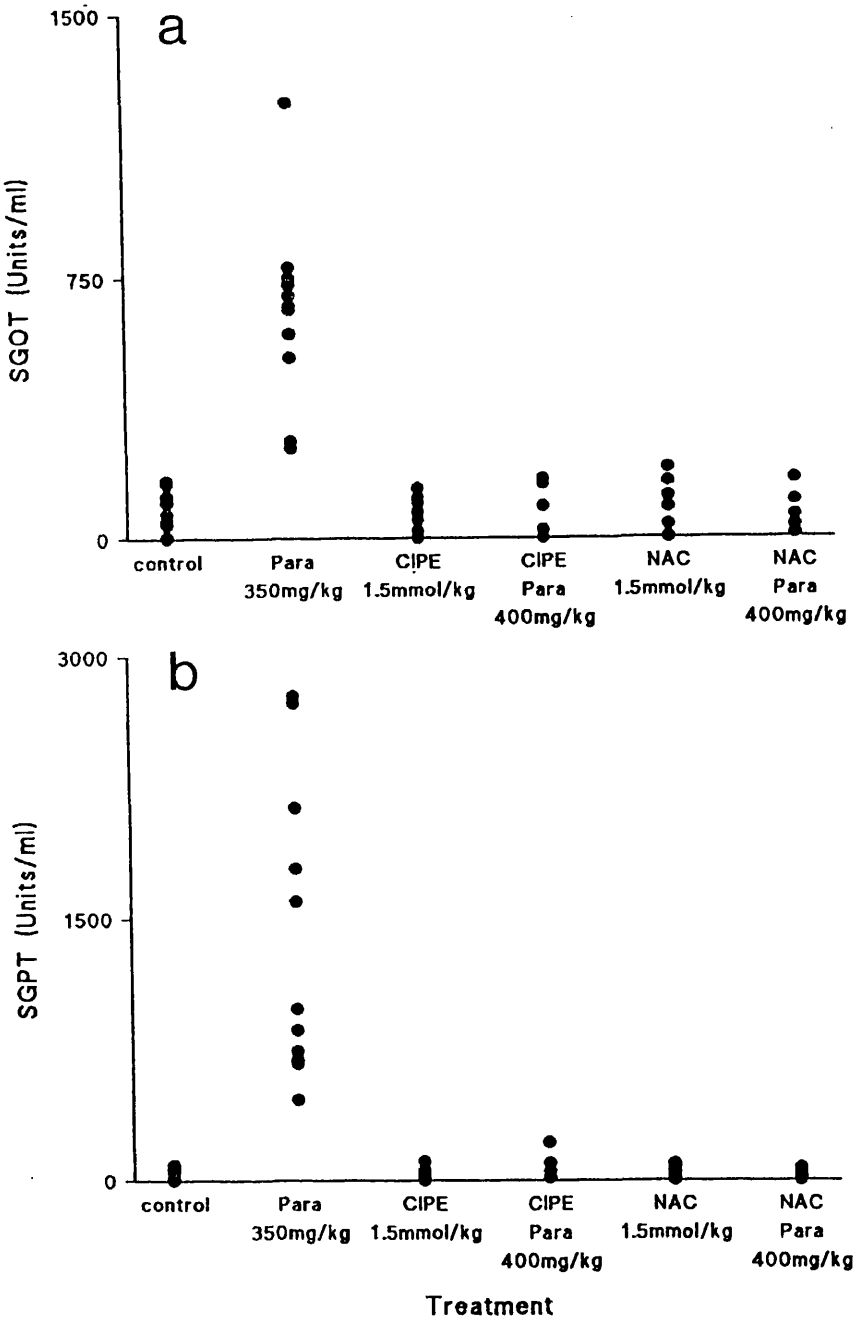
In mice pretreated with benzo(a)pyrene, paracetamol (400mg/kg) caused a 62% mortality within 24 hours. Up to 24 hours, CIPE (3.0mmol/kg) completely prevented the paracetamol-induced lethality (Table 4.II). At these early times no lethality or signs of overt toxicity were observed in animals treated with ester alone. However protection was not evident at later times (Table 4.II), CIPE only delayed mortality due to paracetamol (Table 4.II). Subsequent experiments, lowering the dose of CIPE to 1.5mmol/kg, protected against mortality associated with paracetamol intoxication in mice induced with benzo(a)pyrene as effectively as N-acetylcysteine (1.5mmol/kg) (Table 4.II). After 48 hours, fatalities were noted amongst mice exposed to the higher dose of CIPE and the survivors exhibited a slowed rate of body weight gain. No mortalities were observed in mice treated with CIPE (1.5mmol/kg) alone.

Figures 4.13 Serum GOT (a) and GPT (b) levels after a single dose of 400mg/kg paracetamol with and without co-administration of CIPE (3.0mmol/kg)



Mice received no treatment (□), paracetamol (▨), CIPE (■), or combined exposure to CIPE and paracetamol (▧). Blood samples were drawn by cardiac puncture at the time indicated and assessed for serum transaminase levels. All mice treated with paracetamol alone died within the first 12 hours of exposure. Each point represents the mean of 3 animals, assessed on separate occasions (±SEM).

Figure 4.14 A comparison of CIPE (1.5mmol/kg, i.p.) with N-acetylcysteine (1.5mmo/kg, i.p.) in their abilities to prevent the elevation of serum GOT (a) and GPT (b) 24 hr after a single dose of paracetamol (400mg/kg, i.p.)



Mice exposed to paracetamol (400mg/kg) died within the first 12 hr of the experiment. Transaminase levels from mice treated with 350mg/kg paracetamol (25% mortality) are shown for comparison. Each group contained at least 5 animals.

Table 4.I CIPE and *N*-acetylcysteine (doses .375, .75 and 1.5 mmol/kg, i.p.) prevented the elevation of serum GOT and GPT after exposure to paracetamol (350mg/kg, i.p.) for 24 hr

unit/ml

TREATMENT	+/-	n	SGOT	SGPT
control	-	13	83.3±16.9	55.3±7.2
paracetamol	+	12	622.5±79.5 [a]	1344.8±242.0 [a]
CIPE (1.5mmol/kg)	-	10	79.2±14.0	43.9±8.7
CIPE (.75mmol/kg)	-	4	119.3±62	64.5±9.5
CIPE (.375mmol/kg)	-	4	50±43	55.5±9.0
NAC (1.5mmol/kg)	-	9	71.4±23.0	47.2±10.5
NAC (.75mmol/kg)	-	4	68.0±9.5	82.8±26.1
NAC (.375mmol/kg)	-	4	98.5±55	62.0±10.3
CIPE (1.5mmol/kg)	+	6	11.2±36.0	65.8±19.7
CIPE (.75mmol/kg)	+	6	74.2±15.6	46.0±6.5
CIPE (.375mmol/kg)	+	6	91.0±14.0	478±200.4
NAC (1.5mmol/kg)	+	6	105.5±20.5	86.5±13.5
NAC (.75mmol/kg)	+	6	160.4±25.0	70.7±10.1
NAC (.375mmol/kg)	+	6	208.7±54	94.8±31.1

[a]The elevation of transaminases by paracetamol was significantly higher than controls. Co-administration of both paracetamol and any thiol (all doses) gave significantly lower levels of transaminases than animals given paracetamol alone, $p<.05$. (+/- denotes with or without paracetamol) (mean±SEM)

Table 4.II Effect of cysteine *iso*propylester (CIPE) and N-acetylcysteine on paracetamol-induced mortality

Treatment	% Mice Surviving							
	Days after exposure to paracetamol and/or thiol							
	0	1	2	3	4	5	6	7
Control	100	100	100	100	100	100	100	100
Paracetamol (400 mg/kg)	100	38	24	24	19	19	19	19
CIPE (3.0 mmol/kg)	100	100	100	78	67	67	56	56
Paracetamol + CIPE (3.0 mmol/kg)	100	83	50	33	33	33	33	33
CIPE (1.5 mmol/kg)	100	100	100	100	100	100	100	100
Paracetamol + CIPE (1.5 mmol/kg)	100	92	92	92	92	92	92	92
N-Acetylcysteine (1.5 mmol/kg)	100	100	100	100	100	100	100	100
Paracetamol + N-acetylcysteine	100	100	100	100	100	92	92	92

Mice were dosed with benzo(a)pyrene (20 mg/kg, *i.p.*) for 3 consecutive days. Paracetamol (400 mg/kg, *i.p.*) was administered at the same time as CIPE (1.5 or 3.0 mmol/kg) or N-acetylcysteine (1.5 mmol/kg). Each treatment group consisted of at least 6 animals. Mortalities and body weight changes were monitored over 7 days as indicators of toxicity. The data represents the percentage animals surviving on each day.

The toxicity of CIPE (3.0mmol/kg) was surprising as in initial studies with uninduced mice it was not toxic at this dose level. The effect of enzyme induction on the toxicity of CIPE to mice was therefore investigated.

6. Toxicity of CIPE after induction of cytochrome P450 activity

Pretreatment of mice with benzo(a)pyrene markedly increased the toxicity of CIPE. A dose dependent mortality was observed, 1.5 mmol/kg caused no fatalities, while 3.0 and 6.0 mmol/kg caused 44 and 88% mortality respectively at the end of a 7 day observation period (Table 4.III) . There was also a dose related loss and slowing in the body weight gain over 7 days compared to control animals (fig 4.15c).

In marked contrast, neither control mice nor mice induced with phenobarbitone suffered any mortalities after exposure to CIPE (1.5-6.0 mmoles/kg) (Table 4.III). However some effects were observed on body weight. At the highest dose (6 mmol/kg) of CIPE in control mice, a marked decrease in body weight was observed for several days (4.15a). A similar but not as marked loss was noted in mice pretreated with phenobarbitone (fig 4.15b).

Following administration of CIPE (3.0mmol/kg) to mice induced with benzo(a)pyrene, the hepatic GSH decreased (fig 4.12), which could account for the ester-mediated mortality. However a similar phenomenon occurred when CIPE was administered to uninduced mice but the decline in hepatic NPSH was not as prolonged (fig 4.16). When several other mouse organs were examined, CIPE did not deplete NPSH (data not shown).

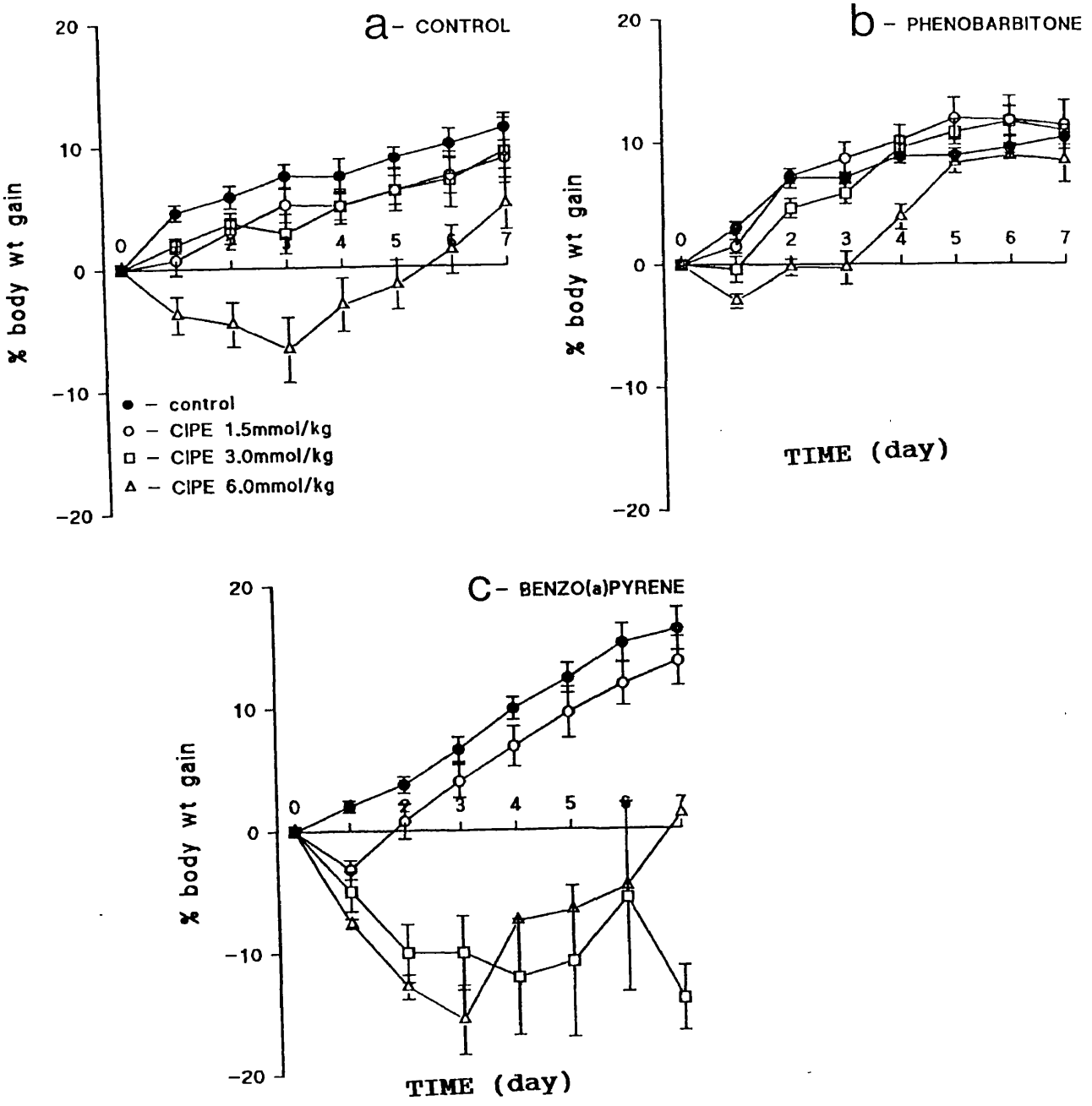
Cysteine (3.0mmol/kg) was extremely toxic, independent of the pretreatment when given to mice (fig 4.17, table 4.IV). No such toxicity was observed when N-acetylcysteine was administered (fig 4.17, table 4.IV).

Table 4.III Effect of hepatic cytochrome P450 inducers on mortality caused by cysteine *iso*propylester in mice

% Survival									
	Control			Phenobarbitone			Benzo(a)pyrene		
dose / Day	1.5	3.0	6.0	1.5	3.0	6.0	1.5	3.0	6.0
1	100	100	100	100	100	100	100	100	89
2	↓	↓	↓	↓	↓	↓	↓	100	89
3	↓	↓	↓	↓	↓	↓	↓	78	44
4	↓	↓	↓	↓	↓	↓	↓	67	11
5	↓	↓	↓	↓	↓	↓	↓	67	11
6	↓	↓	↓	↓	↓	↓	↓	55	11

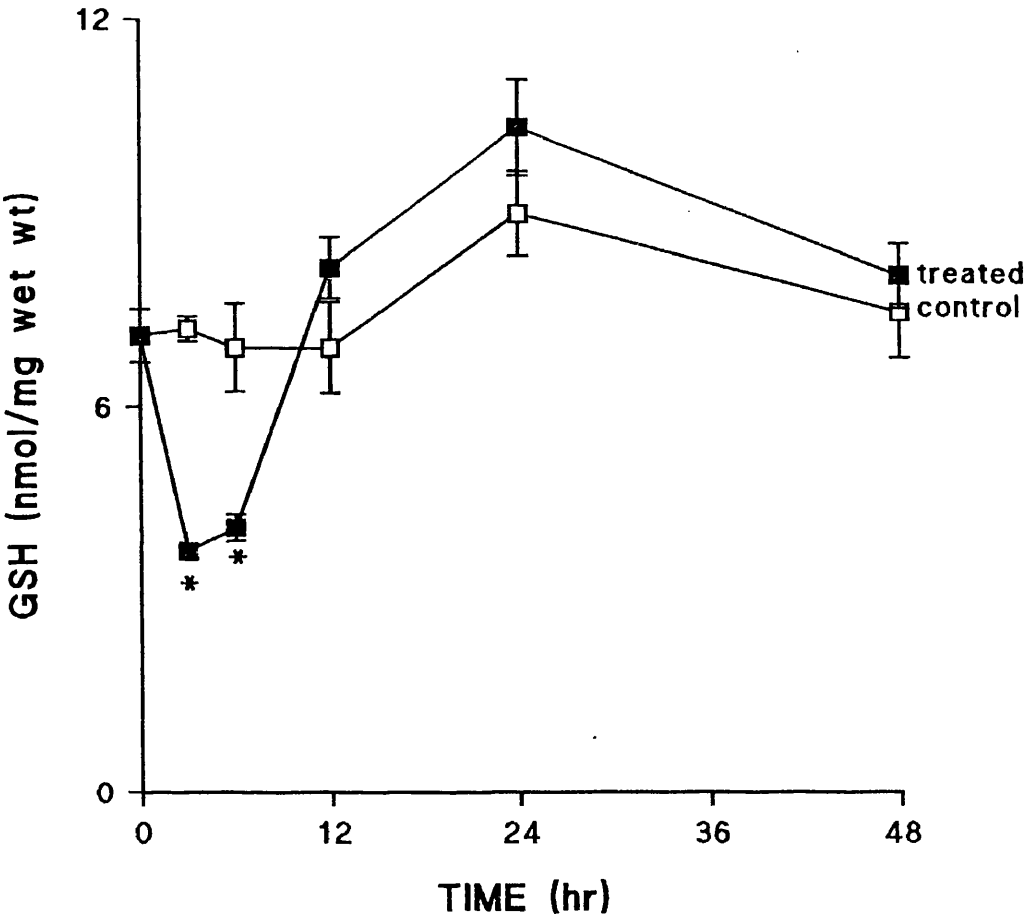
Mice received benzo(a)pyrene (20mg/kg, i.p.), phenobarbitone (80mg/kg, i.p.) or saline daily for 3 consecutive days. On the fourth day animals were dosed with CIPE (1.5, 3.0 or 6.0mmol/kg, i.p.) and the mortalities and body weight changes monitored over 6 days as indicators of toxicity. The data represents the percentage animals surviving on each day. Each treatment group consists of at least 6 animals.

Figure 4.15 Body weight gains of mice exposed to CIPE after induction of hepatic cytochrome P450



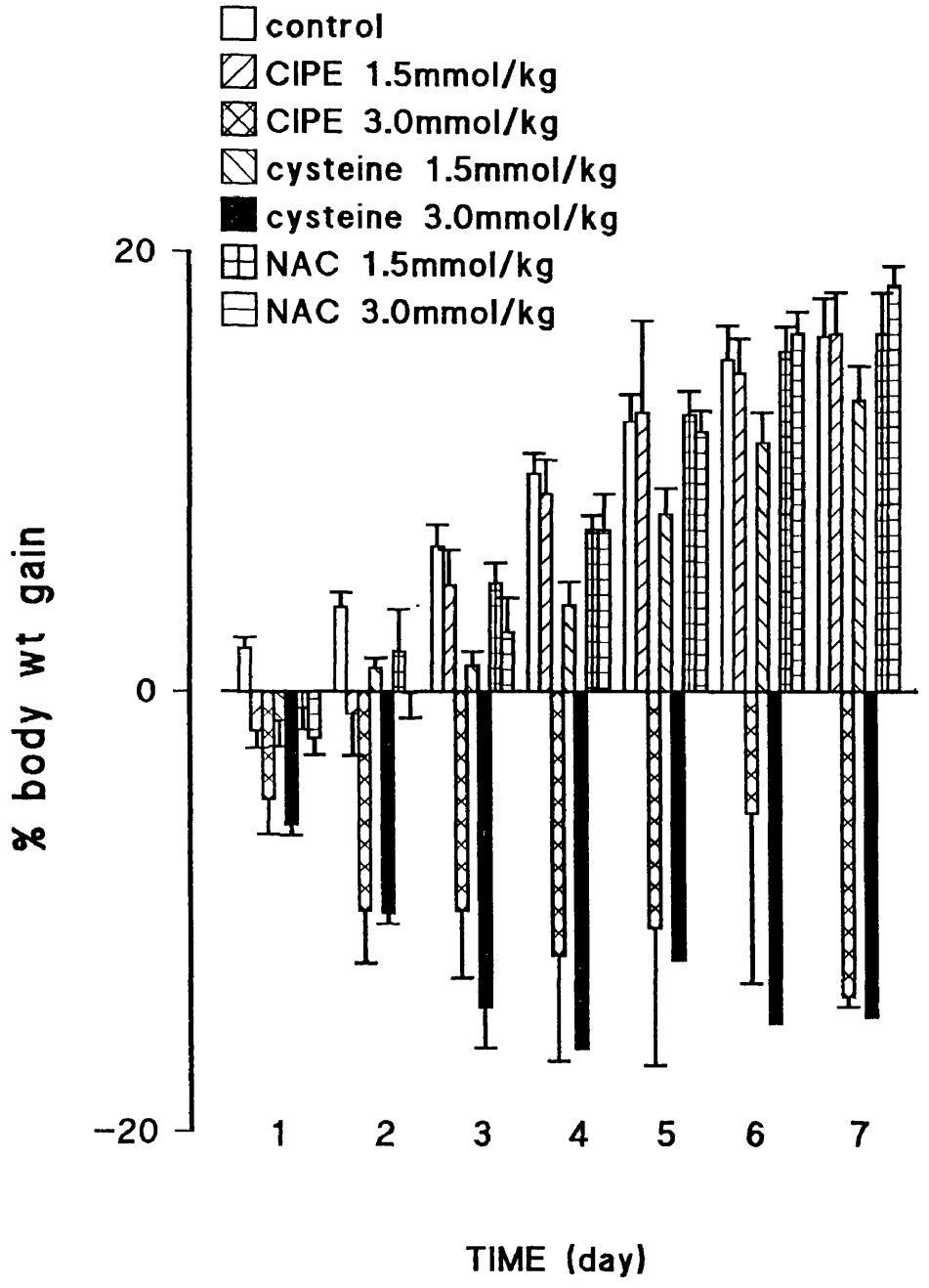
Mice were treated daily with saline (a), benzo(a)pyrene (20mg/kg, i.p.) (b) or phenobarbitone (80mg/kg, i.p.) (c) for 3 consecutive days. On the fourth day animals were given saline (●) or a single dose of CIPE (1.5 (○), 3.0 (□) or 6.0 (△) mmol/kg, i.p.) and body weight gains were monitored over 7 days as indicators of toxicity. The data represents the body weight changes on each day with respect to the initial weight. Each point represents the mean of at least 6 animals (±SEM)

Figure 4.16 Effect of a single CIPE (3.0mmol/kg, i.p.) dose on hepatic GSH levels in uninduced mice



Animals were killed by cervical dislocation at the times indicated and the levels of hepatic GSH assessed by HPLC. Each point represents the mean of 3 animals (±SEM). A significant fall in hepatic GSH was noted at 3 and 6 hr post-dose, *$p<.05$.

Figure 4.17 Effect of CIPE, *N*-acetylcysteine and cysteine on the body weight gains of mice



Animals were given benzo(a)pyrene (20mg/kg, i.p.) for 3 consecutive days. On the fourth day CIPE, *N*-acetylcysteine or cysteine (1.5 or 3.0 mmol/kg, i.p.) were administered as single doses and mortality and body weights monitored over the subsequent 7 days. Each bar represents the mean of at least 5 animals (±SEM).

Table 4.IV The change in survival rates of $BALB_c$ mice exposed to cysteine, *N*-acetylcysteine and CIPE

			% Survival (days)							
THIOL	PRETREATMENT	n	0	1	2	3	4	5	6	7
none	none	6	100	100	100	100	100	100	100	100
cysteine 3.0mmol/kg	none	3	100	100	68	33	0	0	0	0
none	BP	12	100	100	100	100	100	100	100	100
NAC 1.5mmol/kg	BP	5	100	100	100	100	100	100	100	100
NAC 3.0mmol/kg	BP	6	100	100	100	100	100	100	100	100
cysteine 1.5mmol/kg	BP	6	100	100	100	100	100	100	100	100
cysteine 3.0mmol/kg	BP	6	100	100	83	50	33	17	17	17
CIPE 1.5mmol/kg	BP	7	100	100	100	100	100	100	100	100
CIPE 3.0mmol/kg	BP	9	100	100	100	78	78	68	56	56

Mice received saline (none) or benzo(a)pyrene (BP, 20mg/kg, i.p.) pretreatment daily for 3 days. On the fourth day animals received a single intraperitoneal injection of cysteine, CIPE or *N*-acetylcysteine (dissolved in saline and modified to ~pH 7) and mortality monitored over the following 7 days.

7. Effect of CIPE on the morphological changes in mouse liver induced by paracetamol

Paracetamol (400mg/kg) was severely toxic to mice induced with benzo(a)pyrene. Several mice were killed *in extremis* after 6 hr or died. Animals surviving for 24 hr had massive necrosis of the liver, with necrotic areas stretching from the portal tracts (zone 1) to the central veins (zone 3) (fig 4.18). Also some inflammatory cell response was observed. An increase in the number of apoptotic lymphocytes were noted in the white pulp areas of the spleen at 6 and 24 hr. Similar changes were noted when the doses of paracetamol was lowered to 350mg/kg.

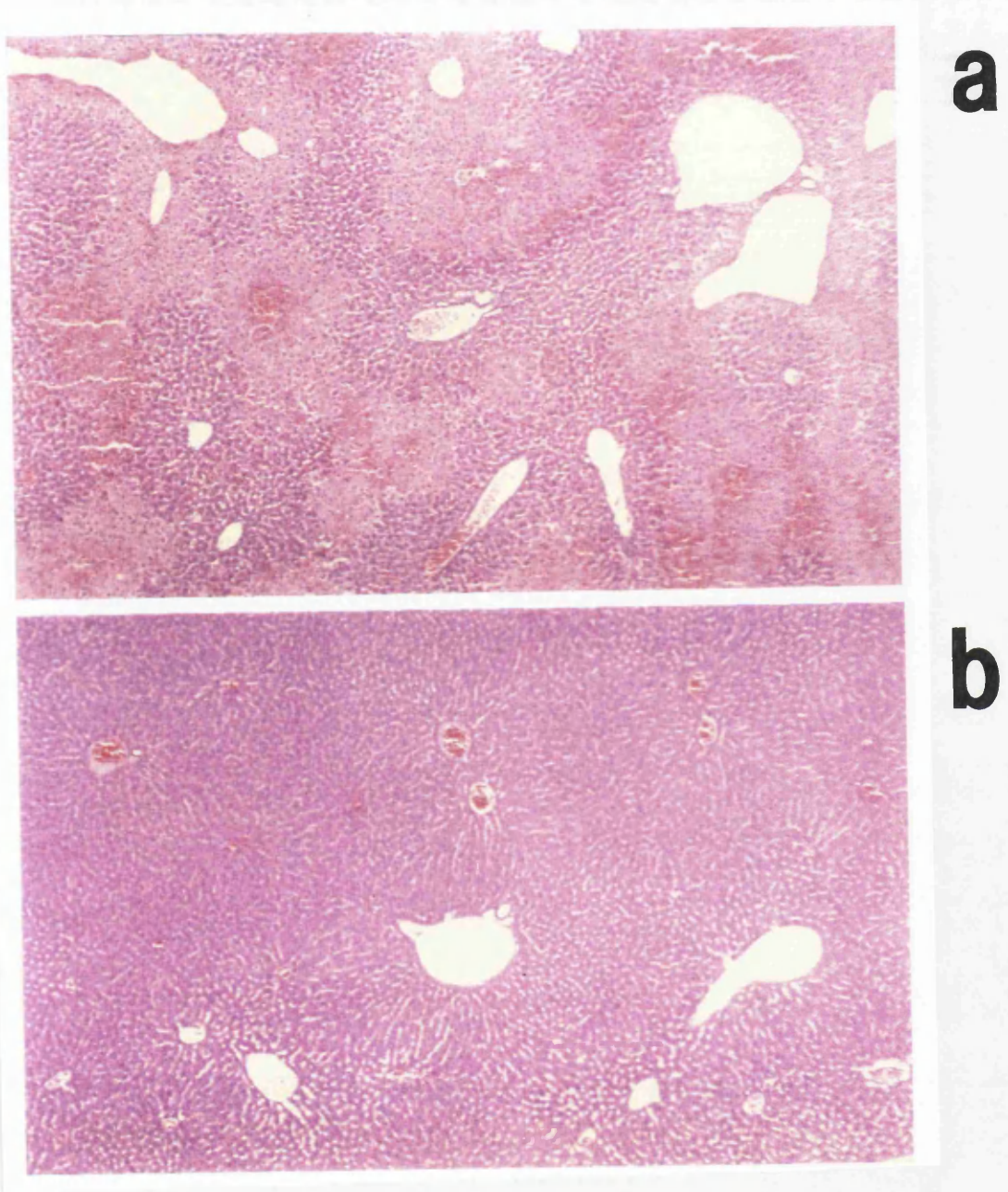
Administering CIPE (1.5 or 3.0 mmol/kg) or *N*-acetylcysteine (1.5mmol/kg), virtually abolished the paracetamol-induced changes to the liver and spleen. Paracetamol alone or in combination with CIPE or *N*-acetylcysteine did not alter the morphology of the kidney (not shown).

8. Assessment of *N*-acetylcysteine and CIPE as precursors of GSH following diethyl maleate administration

Diethyl maleate (600μL/kg) produced an 89% depletion in hepatic GSH (and NPSH values) (fig 4.19) within 30 minutes. In the lung, diethyl maleate depleted GSH (and NPSH) concentrations by 73% (fig 4.20) and a 42% decline in renal NPSH levels (fig 4.19). Administering CIPE (3.0mmol/kg) 30 min after diethyl maleate treatment, significantly elevated the NPSH levels in the liver, lung and kidney (fig 4.19-21) HPLC showed elevated tissue concentrations of cysteine were primarily responsible for the rise in NPSH in the liver and lung within the first hour (fig 4.22). HPLC analysis was not carried out on the kidney. Hepatic and pulmonary levels of GSH were slightly elevated following administration of CIPE, but not significantly even by 1 hr (fig 4.19-20).

N-acetylcysteine increased tissue levels of GSH following diethyl maleate treatment. When monitored over an

Figure 4.18 Protection against the paracetamol induced changes in hepatic morphology by CIPE



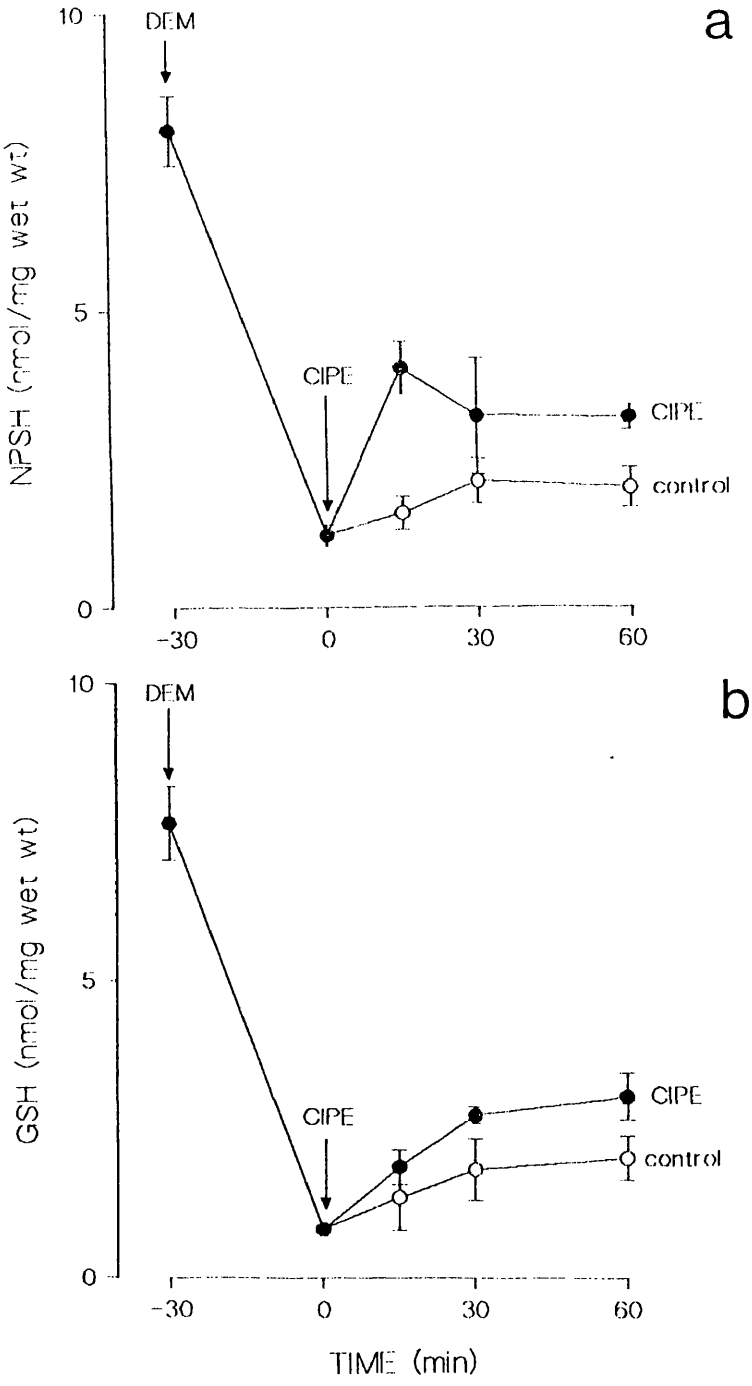
Mice were pretreated with benzo(a)pyrene (20mg/kg, i.p.) daily for 3 consecutive days. Animals received paracetamol (350mg/kg) (a) or received a combination of paracetamol (400mg/kg) with CIPE (1.5mmol/kg) (b). After 24 hr the animals were killed, the liver excised and stored in neutral buffered formalin. Slices were stained with haematoxylin and eosin.

8 hr period, the area under curve for hepatic GSH from mice exposed to *N*-acetylcysteine, was significantly greater than that obtained from animals treated with CIPE (fig 4.23a). The levels of hepatic GSH from mice treated with CIPE followed control levels very closely but after 8 hr were significantly lower (fig 4.23a). Both CIPE and *N*-acetylcysteine were able to replenish pulmonary GSH (fig 4.23b). After 8 hr exposure to diethyl maleate, pulmonary and hepatic levels of GSH were comparable to initial GSH levels (fig 4.23b). Renal levels of NPSH increased 2-3 fold subsequent to diethyl maleate treatment (8 hr) (fig 4.21), which exposure to CIPE or *N*-acetylcysteine did not effect (fig 4.21)

9. Effect of CIPE on the toxicity of naphthalene to Clara cells

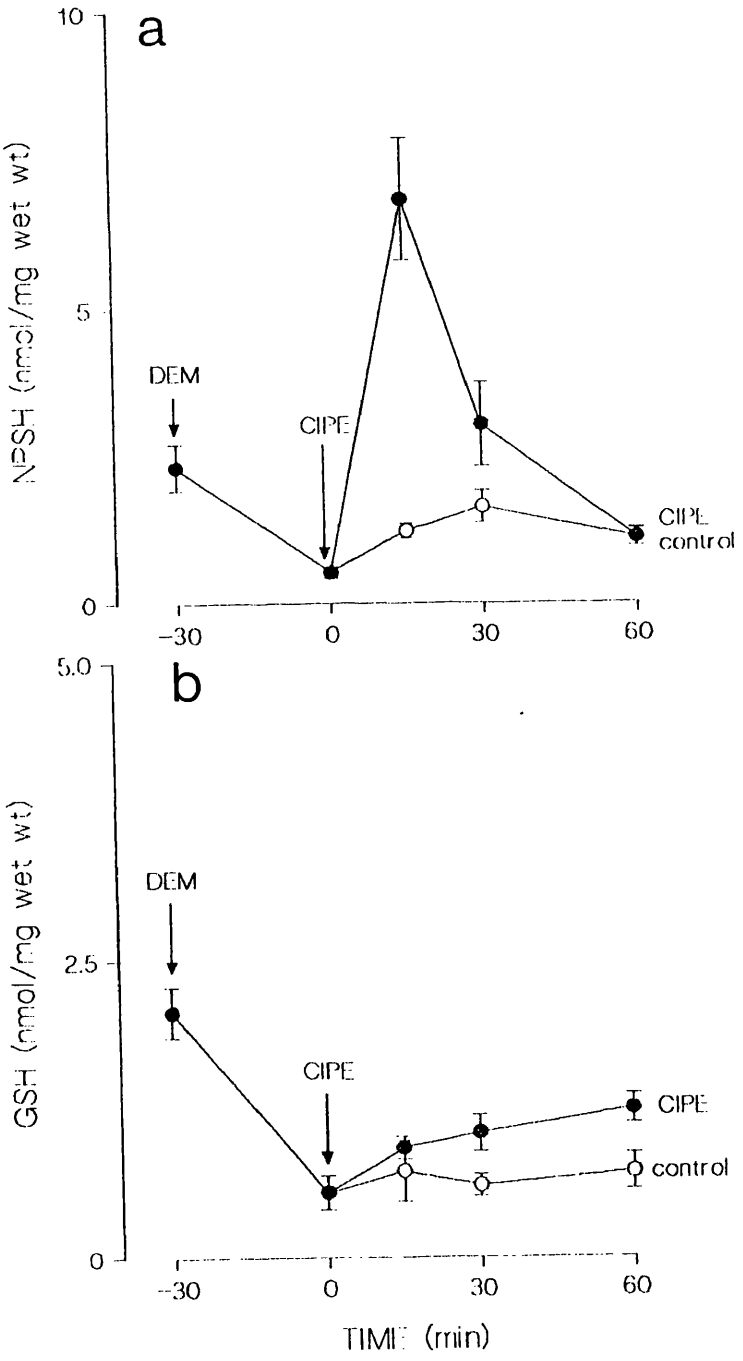
CIPE (3.0mmol/kg) was administered to mice either together with naphthalene (200mg/kg) or 2 hr after exposure to naphthalene and the pulmonary histopathology examined after 24 hr to assess damage to Clara cells. Intraperitoneal administration of a single dose of naphthalene (200 mg/kg) produced exfoliation of Clara cells, the apical projections observed in control sections were absent (fig 4.24) and large numbers of cells were to be found sloughed into the airway lumen. Co-incident or delayed exposure to CIPE (3.0mmol/kg) had very little effect on the pattern of pulmonary necrosis induced by naphthalene (fig.4.24). CIPE alone did not apparently alter the architecture of the lung (data not shown). Naphthalene also reduced the level of hepatic NPSH after 24 hr. CIPE while having no effect itself on hepatic NPSH at 24 hr, potentiated the fall produced by naphthalene (table 4.V).

Figure 4.19 The short term effect of CIPE on hepatic NPSH and GSH following administration of diethyl maleate



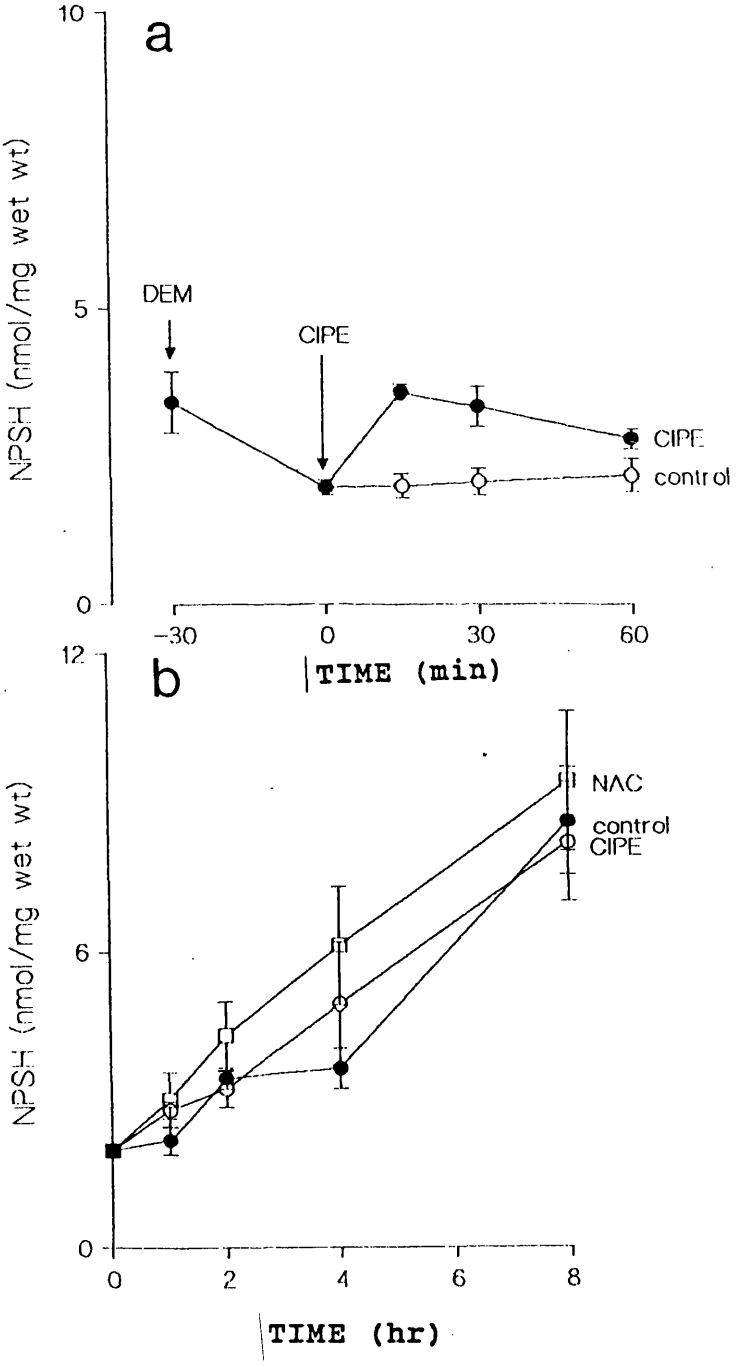
CIPE (3.0mmol/kg,i.p.) was given to mice 30 min after diethyl maleate (600μL/kg,i.p.). Control (O) and treated (●) animals were killed at the time indicated and liver homogenates were assessed for NPSH (a) by the Ellman method and GSH (b) by HPLC. Each point is the mean (±SEM) (n=3 or 4). The rise in NPSH (area under the curve) but not GSH, is significant, $p<.05$.

Figure 4.20 The short term effect of CIPE on pulmonary NPSH and GSH following administration of diethyl maleate



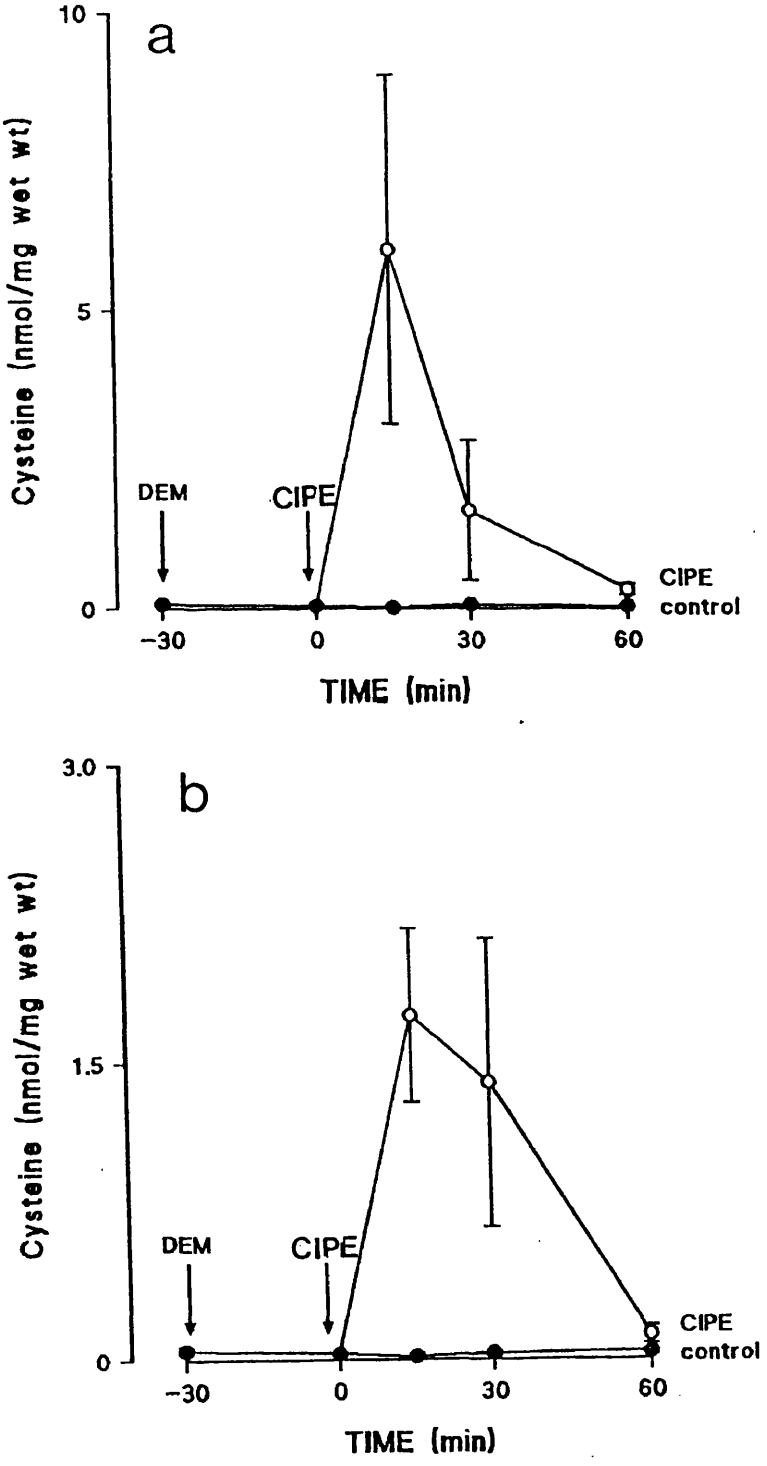
After diethyl maleate (600µl/kg,i.p.) pretreatment, CIPE (3.0mmol/kg,i.p.) was administered. Control (O) and treated (●) animals were killed at the times indicated and liver homogenates were assessed for NPSH (a) by the Ellman method and GSH (b) by HPLC. Each point is the mean (±SEM) (n=3 or 4).

Figure 4.21 Effect of CIPE and *N*-acetylcysteine on renal levels of NPSH following administration of diethyl maleate



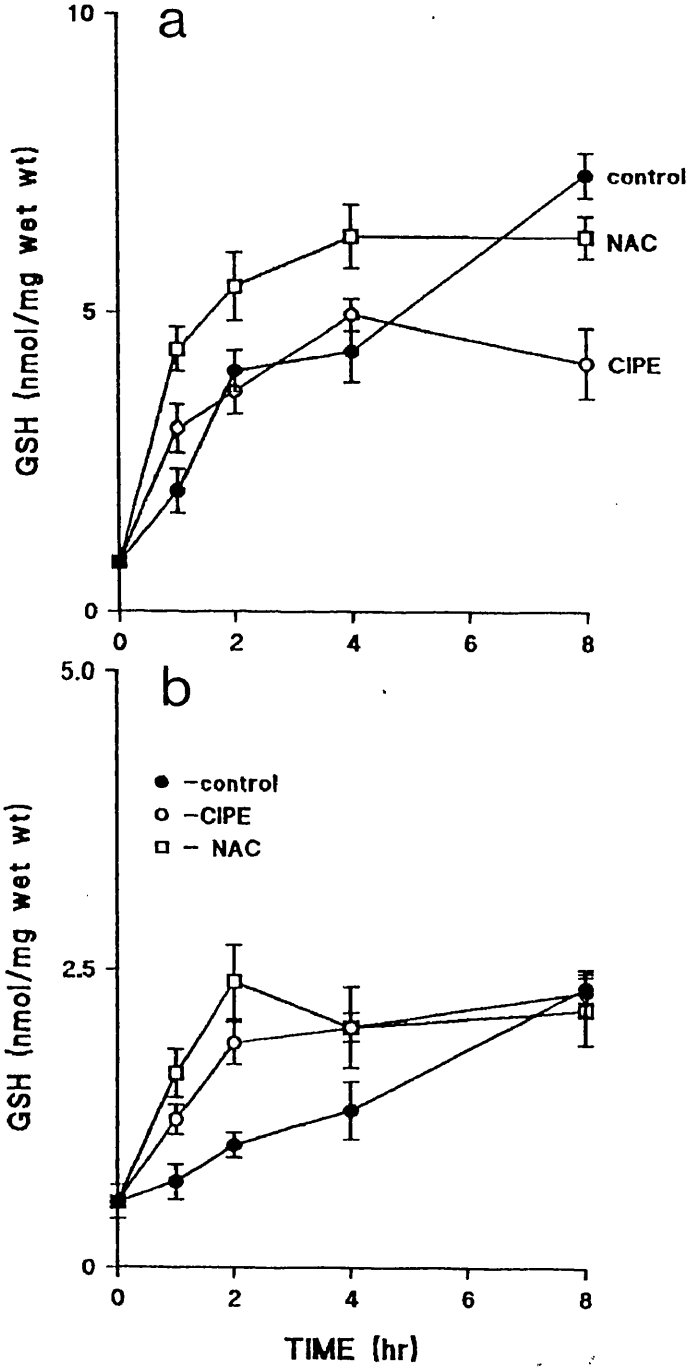
Mice were pretreated with diethyl maleate for 30 min before the administration of CIPE or *N*-acetylcysteine (3.0mmol/kg, i.p.). The early effects of CIPE (a) and the effect of CIPE and *N*-acetylcysteine over 8 hr (b) on NPSH levels were assessed by the Ellman method. CIPE significantly elevated levels of NPSH at early time points (a). At later times (b), neither CIPE nor *N*-acetylcysteine increased renal NPSH values above control. However diethyl maleate pretreatment significantly raised NPSH levels by 8 hr, $p<.05$. Each point is the mean (±SEM) (n=3 or 4).

Figure 4.22 The influence of CIPE on hepatic and pulmonary cysteine: early time points



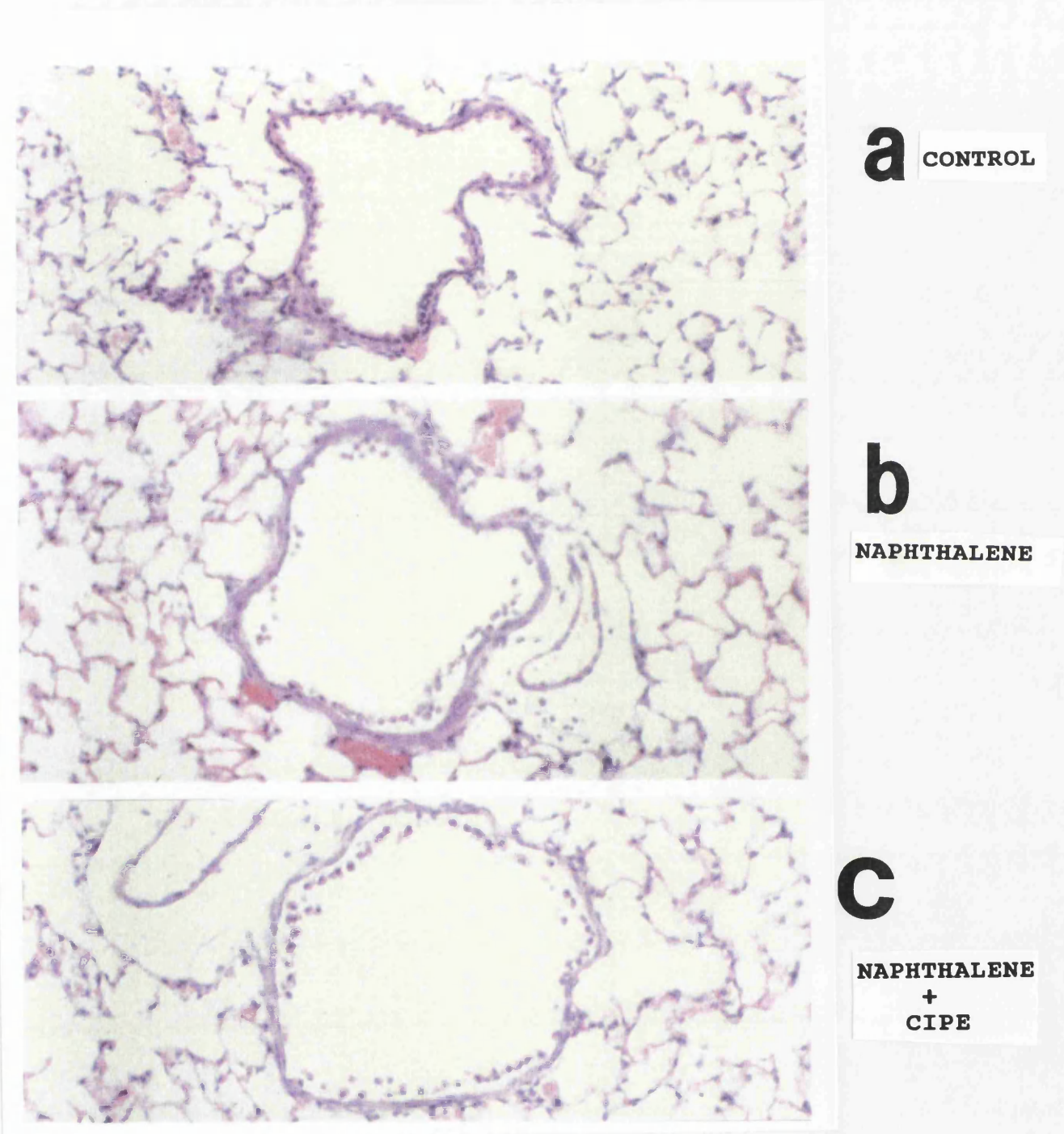
Mice were pretreated with diethyl maleate (600µl/kg,i.p.) for 30 min, when CIPE (3.0mmol/kg, i.p.) (O) was administered. Animals were killed at the times indicated and liver (a) and pulmonary (b) cysteine measured by HPLC. Each point is the mean of 3 or 4 experiments (±SEM).

Figure 4.23 The effect of CIPE and *N*-acetylcysteine on pulmonary (b) and hepatic (a) GSH following exposure of mice to diethyl maleate



30 min after administration of diethyl maleate, CIPE (O) and *N*-acetylcysteine (□) (3.0mmol/kg,i.p.) were given. Animals were sacrificed at the times indicated and hepatic (a) and pulmonary (b) levels of GSH were measured by HPLC. Each point is the mean (±SEM) (n= 3 or 4). The area under curve for hepatic GSH is significantly greater for animals treated with *N*-acetylcysteine than for control or mice treated with CIPE, $p<.05$.

Figure 4.24 Effect of CIPE on the toxicity of naphthalene to Clara cells



Naphthalene (200mg/kg) was given alone to mice (b) or in combination with CIPE (3mmol/kg) (c). After 24 hr, animals were killed and their lung inflated and fixed with formalin. Slices were stained with haematoxylin and eosin. When the administration was delayed until 2 hr after naphthalene was given similar changes in pulmonary morphology were observed.

Table 4.V The effect of naphthalene and CIPE on the hepatic levels of NPSH

TREATMENT	n	hepatic NPSH (% control)
control	5	100
naphthalene (200mg/kg)	6	74 ± 5.4
CIPE (3.0mmol/kg)	5	104 ± 6.8
naphthalene + CIPE (Co-administration)	3	43 ± 1.0
naphthalene + CIPE (2 hr delay)	3	64 ± 1.0

A single injection of CIPE (3.0mmol/kg, i.p.) was given coincidently or 2 hrs after naphthalene (200mg/kg, i.p.). After 24 hr the animals were killed and the hepatic NPSH values measured by the Ellman method. Each point represents the mean ± SEM. The control hepatic NPSH value was 5.77 ± .28 nmol/mg wet liver weight.

4.3 DISCUSSION

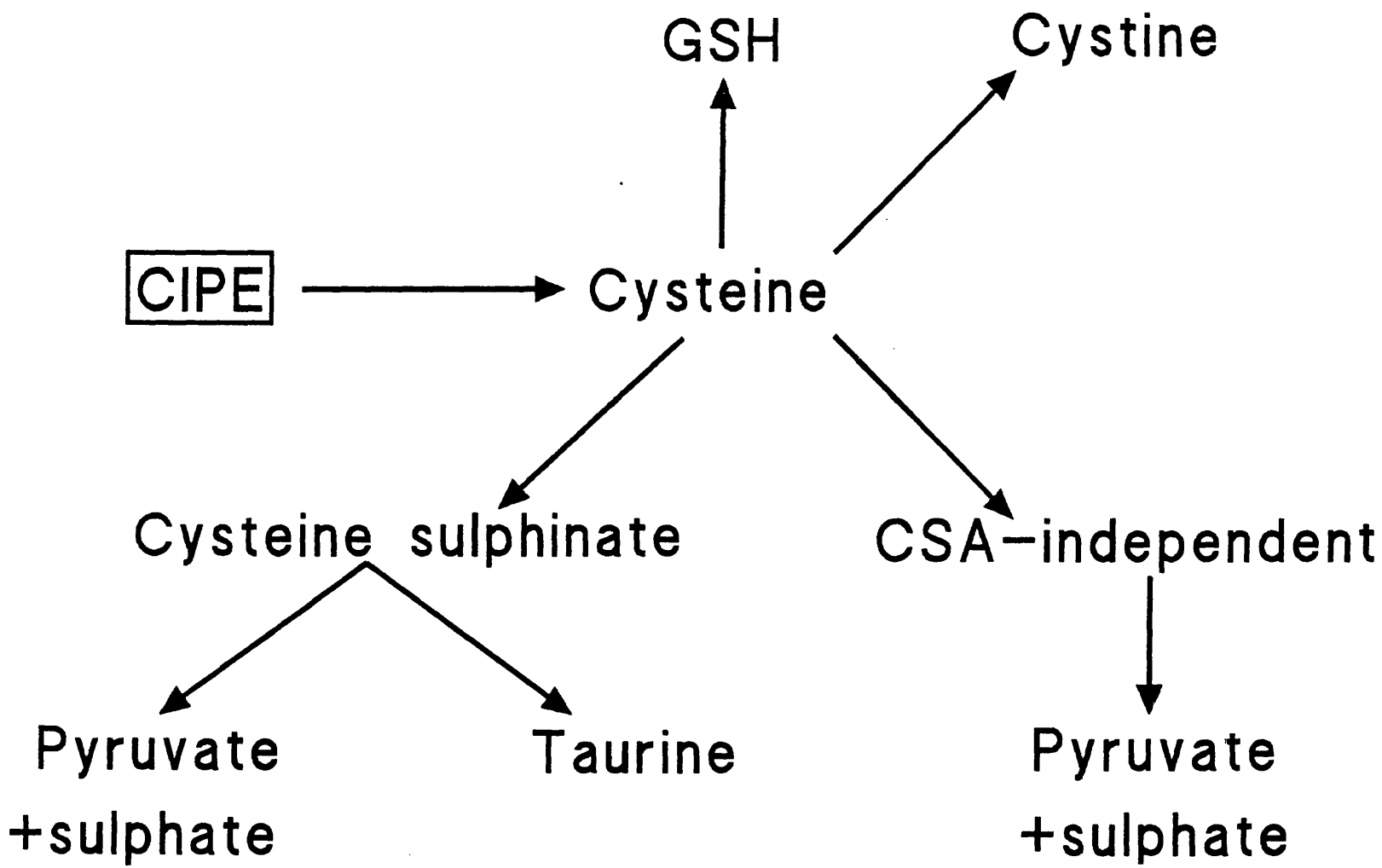
These studies demonstrated that CIPE, caused a transient elevation in NPSH levels in the liver, lung, kidney and spleen (Fig 4.1). The increase in hepatic NPSH, while short lived, reduced the toxicity of paracetamol and bromobenzene to mice and rats induced with either benzo(a)pyrene or phenobarbitone as measured by changes in NPSH, SGOT, SGPT and mortality (figs 4.8-4.14 and Table 4.I and II). Preliminary data using high performance liquid chromatography suggested the elevation in mouse organ NPSH was due to an increase in intracellular cysteine, after hydrolysis of CIPE.

The very transient elevation in NPSH suggested that cysteine was rapidly metabolised (summarised in fig 4.25). Cysteine may be utilised for GSH synthesis, oxidized to cystine or be metabolized by cysteine dioxygenase to cysteine sulphinate and further to taurine or pyruvate and sulphite [33]. Cysteine may also be metabolized by pathways independent of cysteine sulphinate including formation of 3-mercaptopyruvate with subsequent desulphuration or cleaved by cystathionase [33,101]. Desulphuration pathways are proposed to favour cysteine catabolism when cysteine concentrations are high [33].

The studies in this chapter showed no evidence for an increase in GSH up to 24hr following CIPE administration (fig 4.8 and 4.12). The results suggest that cysteine was rapidly metabolised, possibly by one of the above pathways but under the conditions used in the studies was not utilized to any significant extent for GSH enhancement. GSH levels were probably sufficiently high to act as a feedback inhibitor of γ-glutamylcysteine synthetase, the rate limiting step in the biosynthesis of GSH.

CIPE behaved similarly to other esters of cysteine [94,95] and produced an exaggerated elevation in pulmonary NPSH levels. Cysteine ethyl ester has a initially high affinity for the lung, and also the intestinal and gastric

Figure 4.25 - Summary of CIPE and cysteine metabolism



mucosa [95]. Servin *et al* [95] suggest these sites are targeted because of their high mucus content. Mucus is rich in disulphide bridges which are targets for the muceolytic activity of the ethyl ester of cysteine and may explain the selective distribution of cysteine to the lung.

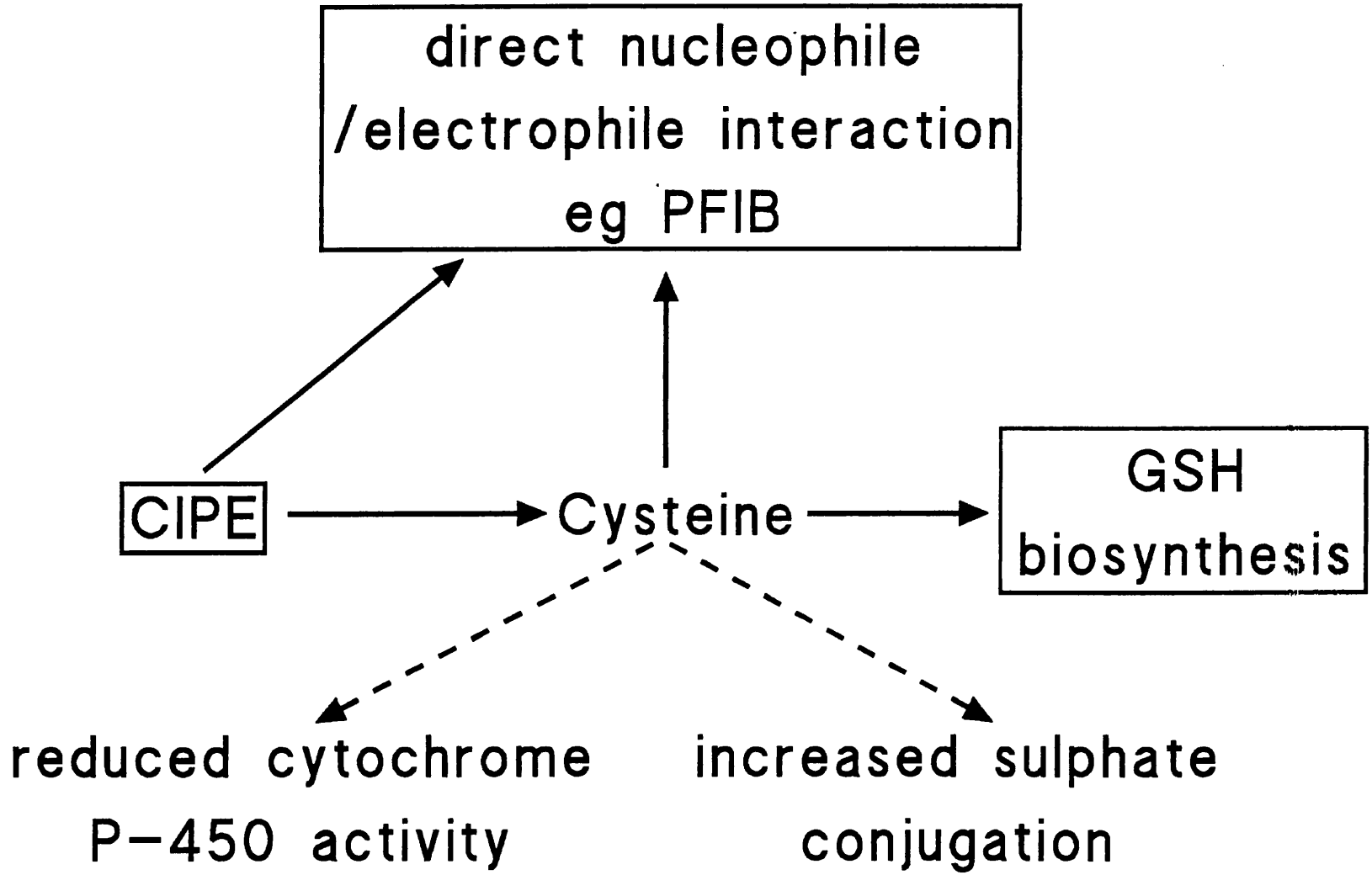
As CIPE was rapidly metabolised by mice and rats, benzo(a)pyrene or phenobarbitone induction of hepatic cytochrome P450 activity was used to ensure the paracetamol- and bromobenzene-induced fall in hepatic GSH was coincident with the life span of CIPE. CIPE (3.0mmol/kg) protected against the hepatotoxicity of paracetamol and bromobenzene at early times (fig 4.8-13 and Table 4.I).

The mechanism of this protection is unclear and may be due to one or more possibilities (summarised in fig 4.26). The most likely was that CIPE or cysteine, derived from hydrolysis, provide alternative nucleophilic centres to interact with reactive electrophiles so preventing the depletion of hepatic GSH below the critical threshold required for the induction of hepatotoxicity (fig 4.8 and 4.12). Data from a variety of studies suggest that depletion of hepatic GSH below approximately 80% of control values is critical for the induction of the hepatotoxicity of a number of compounds including paracetamol [13] and bromobenzene [159]. When GSH is depleted below this critical level, the reactive electrophiles may then bind covalently to critical cellular macromolecules possibly leading to toxicity [12]. Alternatively CIPE by increasing intracellular cysteine may alter paracetamol toxicity by increasing sulphate conjugation either by maintaining the supply of both the cofactor adenosine 3'-phosphate 5'-sulphatophosphate (PAPS) [33,98] and also inorganic sulphate [234]. Both cysteine and *N*-acetylcysteine are precursors of sulphate [234,235]. Administration of sodium sulphate while increasing hepatic PAPS did not protect against the hepatotoxicity of paracetamol to mice [236] or isolated hepatocytes [237].

Thiol compounds have been shown to interact directly

FIGURE 4.26

CYTOPROTECTION BY CIPE



with cytochrome P450, preventing the metabolic conversion of paracetamol to NABQI and inhibiting its covalent binding to hepatic mouse microsomes [238]. Similarly the ester or cysteine derived from ester could have the opportunity of interacting with cytochrome P450, inhibiting the formation of hepatotoxic bromobenzene-3,4-oxide or NABQI.

CIPE appeared to be as effective as *N*-acetylcysteine in preventing paracetamol-induced toxicity (figs 4.14 and table 4.I and 4.II). *N*-Acetylcysteine prevents paracetamol toxicity by increasing the synthesis of GSH, rather than interacting directly with NABQI [28,78,79,80].

An interesting complication of the studies was the increased toxicity of CIPE in mice pretreated with benzo(a)pyrene compared either to controls or animals pretreated with phenobarbitone. The mechanism of this enhanced toxicity is unclear. CIPE alone, following an initial elevation caused a prolonged decrease in hepatic NPSH (fig 4.12), such a decline was not observed in rats. Part of this decrease may be due to the formation of mixed disulphides possibly caused by pretreatment with benzo(a)pyrene, although a similar effect was observed in uninduced mice (fig 4.16). Whilst the enhanced toxicity following induction with benzo(a)pyrene is suggestive of an involvement of cytochrome P450 in the catabolism of cysteine. This is somewhat surprising as cytochrome P450 is not generally considered to play a role in the metabolism of cysteine [33,97,98]. The possibility cannot be excluded that other enzymic activities such as esterase have been modified by the pretreatment with benzo(a)pyrene.

Cysteine is known to be toxic but its mechanism of toxicity has not been satisfactorily elucidated, although it is reported to damage the nervous system of new born rats [112]. In our studies, cysteine (3.0mmol/kg) was equally toxic in both control and benzo(a)pyrene induced mice (table 4.IV), whereas at an equivalent dose of CIPE produced 50% mortality in benzo(a)pyrene induced mice only.

Diethyl maleate depletes GSH by conjugation in a GSH-*S*-

transferase-mediated reaction [239] and the decline in the tissue levels of GSH is in agreement the reported effects of diethyl maleate [240]. The initial substantial increase of NPSH levels in liver and lung following exposure to CIPE (fig 4.19 and 4.20) was due to a transient enhancement of cysteine levels (fig 4.22) and not GSH (4.19 and 4.20). *N*-Acetylcysteine was capable of supporting significant hepatic GSH replenishment, unlike CIPE (fig 4.23). CIPE had a very short half life, within 5 min the majority of circulating ester has been hydrolysed, in contrast to *N*-acetylcysteine (fig 4.4). The inability of CIPE to elevate hepatic GSH beyond control values, following depletion by diethyl maleate, supports the hypothesis that its main mechanism of hepatoprotection was by elevating intracellular cysteine, to act as an alternative short lived nucleophile. While the ability of *N*-acetylcysteine to foster hepatic GSH biosynthesis after diethyl maleate pretreatment, supports its protective role as a precursor of GSH.

Naphthalene produced marked histopathological changes in the lung, against which the administration of CIPE produced no discernable protection. Naphthalene is postulated to be metabolised by pulmonary cytochrome P450 to the electrophilic naphthalene oxides [171], which deplete the levels of GSH in isolated perfused lungs [170]. CIPE replenished pulmonary GSH after exposure to diethyl maleate (fig 4.23), consequently the potential for pulmonary GSH biosynthesis by CIPE after naphthalene administration exists. However the toxicity of naphthalene to the lung was not diminished by CIPE (fig 4.24) Additionally CIPE potentiated the depletion of hepatic levels of NPSH by naphthalene (table 4.V). The failure of CIPE to protect against naphthalene pulmonary toxicity could be explained by the kinetics of CIPE and naphthalene; the elevation of pulmonary NPSH values by CIPE was short lived, while the depletion of pulmonary GSH by naphthalene is prolonged, reaching its nadir between 2 and 4 hr after administration [166], implying the release of naphthalene oxides long after

the disappearance of CIPE and/or cysteine. Esters of cysteine may prevent naphthalene toxicity in animal models capable of rapidly generating naphthalene oxides, perhaps after induction of pulmonary cytochrome P450. CIPE and other esters of cysteine do protect against short lived electrophiles, such as perfluoroisobutene, preventing pulmonary oedema [94]. However in an animal model with induced pulmonary cytochrome P450 activity, other factors, such as coincidentinduction of hepatic cytochrome P450 activity, may influence the pulmonary toxicity mediated by naphthalene.

CIPE had a short life span *in vivo*, as do other esters of cysteine [94], and consequently will be unsuitable as a means to provide extended thiol elevation, unless the pharmacokinetics of the compounds are greatly modified. This may be achieved by repeated exposure or continuous release of esters, either by i.v. infusion or miniosmotic pumps. Repeated intraperitoneal administration of CIPE (3.0mmol/kg) proved to be deleterious to both rats and mice. However as a single dose of CIPE (0.375mmol/kg) was beneficial against the toxicity of paracetamol to mice induced with benzo(a)pyrene, repeated or continuous exposure to low amounts of the esters of cysteine is feasible and may overcome some of the pharmacokinetic problems.

Alternatively, modifying the structure of the esters may reduce their susceptibility to hydrolysis and increase their half life *in vivo*. An ester of *N*-acetylcysteine could be a useful tool to modify cellular thiols, especially GSH. Treatment of cultured human melanoma cells with N,S-diacetyl-L-cysteine methyl ester produced a marked elevation of intracellular levels of GSH [96]. An ester of methionine may provide a chemoprotectant specifically targeted to the liver, as the only organ with substantial cystathionine pathway activity.

In summary, CIPE provides a short lived period of thiol coverage which was sufficient to protect against paracetamol and bromobenzene toxicity but not naphthalene.

The prevention of hepatotoxicity is probably associated with the ability of CIPE or cysteine to react directly with electrophiles or replenishment of GSH. Benzo(a)pyrene pretreatment induced CIPE toxicity not seen in phenobarbitone pretreated or control mice. The mechanism of toxicity still remains to be ascertained but may indicate a role for cytochrome P450 in cysteine metabolism.

Chapter 5 - Elevation of cysteine and replenishment of GSH in rat lung slices by cysteine isopropylester and other cysteine precursors

5.1 INTRODUCTION

The turnover of GSH in any organ can be influenced by several factors; activity of synthetic enzymes, feedback inhibition of γ-glutamylcysteine synthetase by GSH, the rate of GSH degradation and the supply of cysteine. Intracellular cysteine levels can be influenced by uptake of γ-glutamylcysteine [35], or alternatively several cell types accumulate preformed cysteine and cystine using amino acid transport systems [62]. Cells are usually well supplied with glycine and glutamate, the other component amino acids of GSH, although glutamine supplements, deaminated to glutamate in the mitochondria, can preserve hepatic GSH and prevent paracetamol-mediated toxicity [241]. Several cysteine pro-drugs have been used to support the biosynthesis of GSH [29,73].

In order to overcome the problem of a limited supply of cysteine, a series of cysteine esters with increasing lipophilicity have been synthesized [94], including cysteine isopropylester (CIPE) and cysteine cyclohexyl ester (CCHE). These esters should lead to a rapid increase in intracellular cysteine following enzymic hydrolysis by esterases. This may be important in extrahepatic organs such as the lung which, unlike the liver [32], have little or no cystathionine pathway activity, so preventing their use of methionine as a cysteine precursor and making them largely dependent on preformed L-cysteine.

CIPE and related esters have been shown to protect rats against the pulmonary oedema induced by perfluoroisobutene, a pyrolysis product of Teflon [94]. Following *in vivo* administration, CIPE produced transient increases in pulmonary and hepatic non-protein sulphydryl (NPSH) which was primarily due to increases in cysteine (fig 4.1) [94].

Depletion of pulmonary GSH by protein deficient diet or

diethyl maleate, accentuates oxidant-mediated injury to the lungs produced [215], which sulphydryl reagents such as *N*-acetylcysteine or cysteamine will alleviate [216]. Pulmonary GSH also plays a role in determining the outcome of intoxication with α-naphthylthiourea [228], naphthalene [171] and 4-ipomeanol [20]. It could be rationalized that CIPE, or following hydrolysis cysteine, in addition to direct reaction with electrophilic molecules, may also provide an opportunity for pulmonary GSH replenishment and maintenance during a toxic insult to the lung. In order to test this hypothesis, I used a rat lung slice model to compare the abilities of CIPE and other cysteine delivery systems to elevate intracellular cysteine and GSH in both control and GSH-depleted lung slices. Experiments are also presented showing the metabolism of CIPE by isolated hepatocytes and attempting to use CIPE as a chemoprotectant against the toxicity of naphthalene and paraquat in rat lung slices and 2,3-dimethoxy-1,4-naphthoquinone in isolated hepatocytes.

5.2 RESULTS

1. Elevation of intracellular cysteine by cysteine prodrugs

The basal level of cysteine in the control lung slices was 0.059±0.003 nmol/mg wet wt (59μM), which was maintained during a 60 min incubation, in agreement with reported values for tissue concentration of cysteine (10-100μM) [29]. CIPE or CCHE (500μM) produced pronounced elevations of intracellular cysteine, in excess of the rise in pulmonary cysteine concentrations achieved with cysteine, N-acetylcysteine or OTZ (fig 5.1 and 5.3). Elevation of cysteine by CIPE (500μM) was rapid, reaching a maximum within 5 min. However CCHE produced a maximal rise in pulmonary cysteine concentration after 15 min. The relative abilities of the different cysteine delivery systems to elevate intracellular cysteine were:-

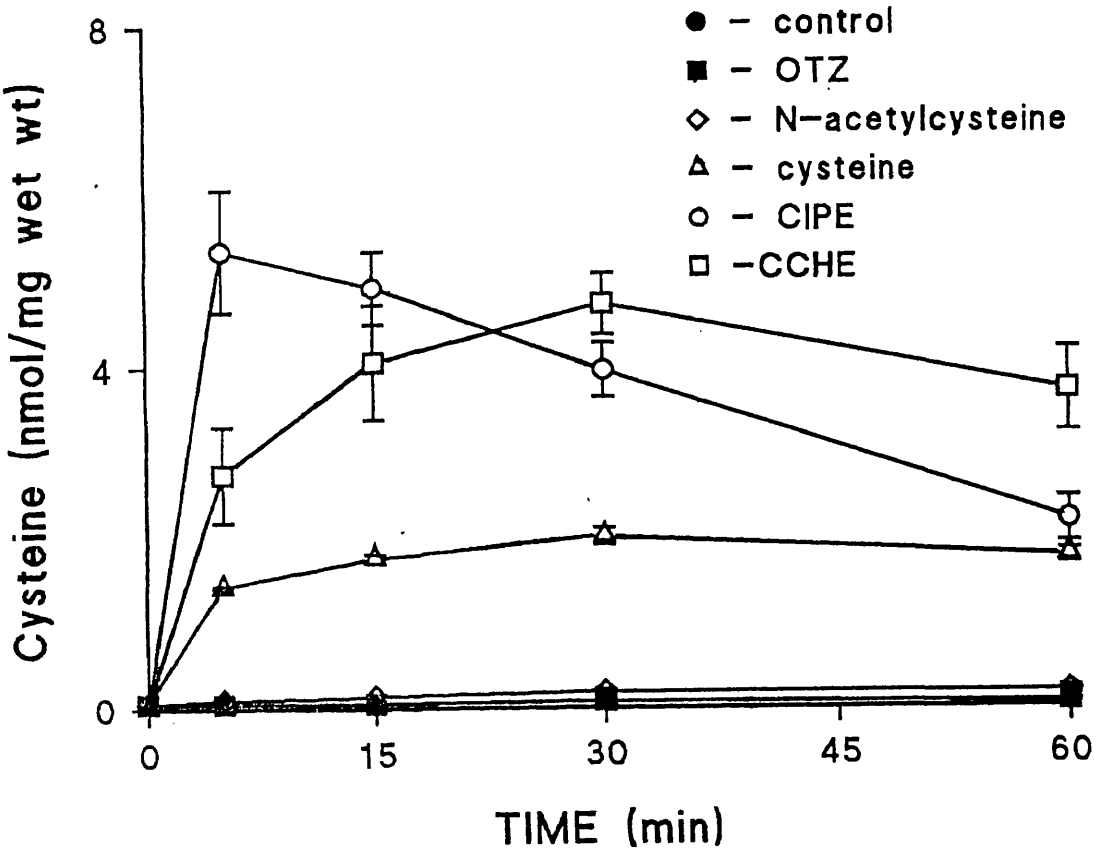
CIPE = CCHE > cysteine > N-acetylcysteine > OTZ

When incubated at 50μM, cysteine, CIPE and CCHE caused a similar elevations of intracellular cysteine (fig 5.2). At 60 min, the cysteine concentration of slices incubated with cysteine (50μM) alone was greater than the levels which resulted from incubation with CIPE or CCHE (fig 5.2).

When lung slices were incubated with CIPE or CCHE, little or no unmetabolised intracellular ester was detected. The intracellular ester levels achieved never exceeded 1% of amount of cysteine ester added (data not shown). This phenomena is investigated further in chapter 6. The t-butylester of cysteine failed to increase intracellularcysteine beyond control values (fig 5.4). In addition the ester could not be detected using HPLC or by the Ellman method. However mass spectral analysis of the ester gave a pattern of positive ions characteristic for cysteine t-butylester (fig 5.5).

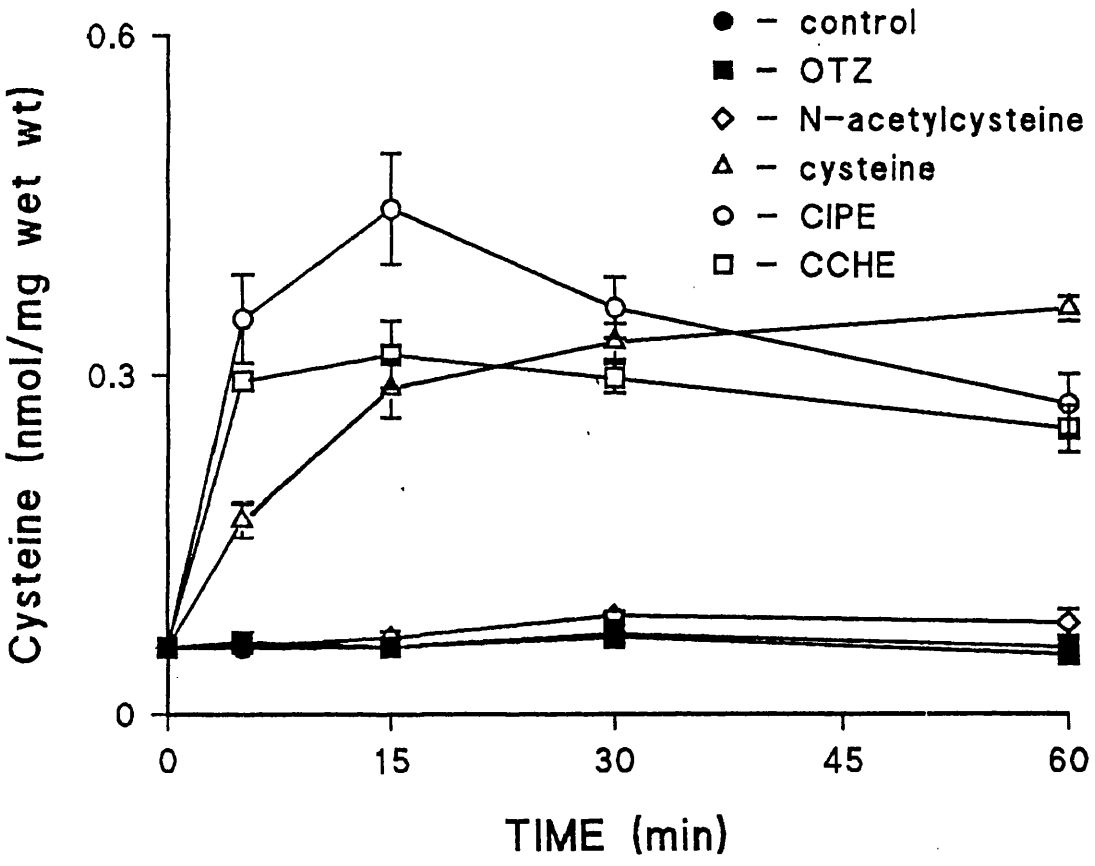
In contrast to the esters of cysteine, a pool of intracellular N-acetylcysteine was detected (0.034±0.013 and

Figure 5.1 Effect of several cysteine delivery systems on the intracellular cysteine levels of rat lung slices



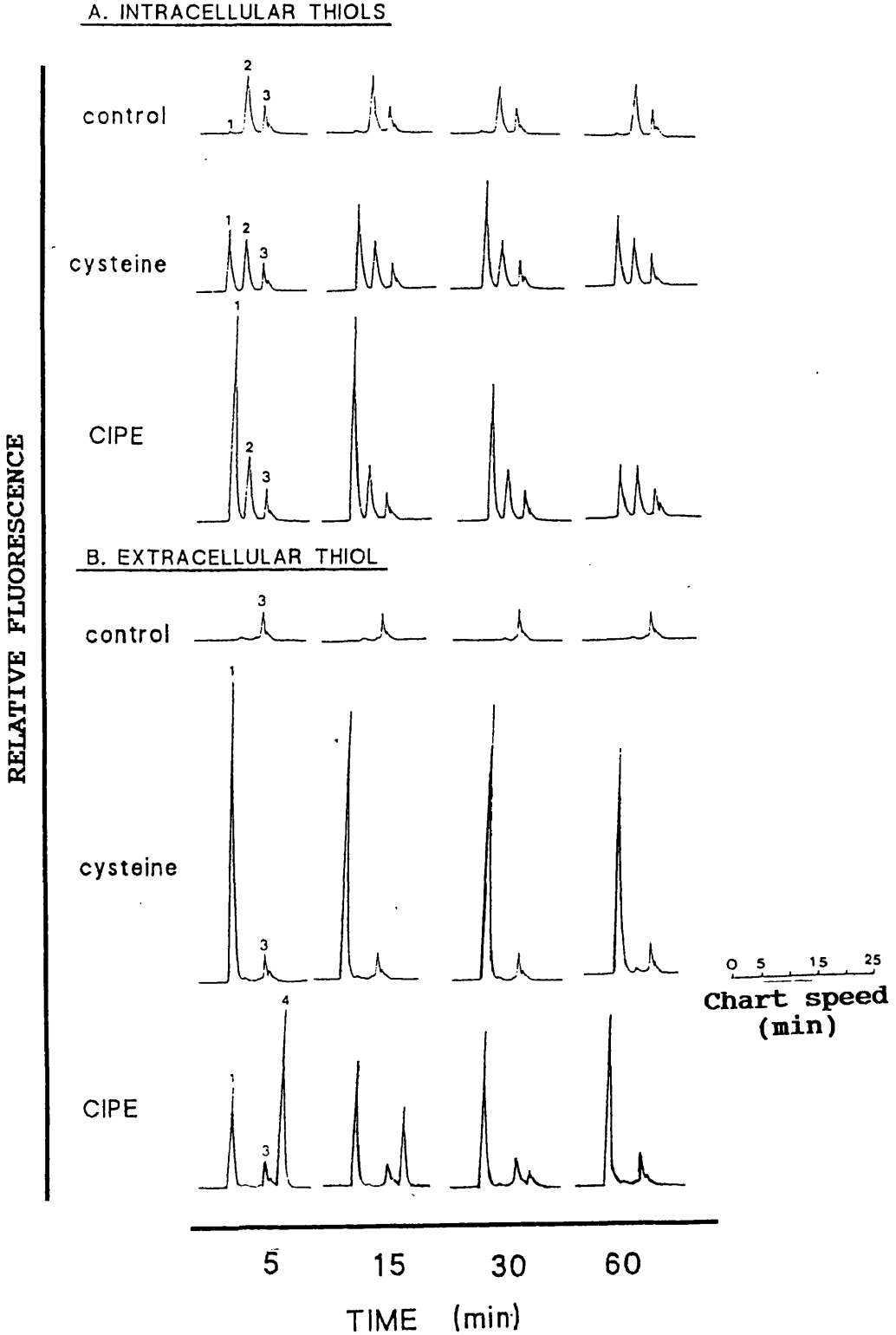
Lung slices were incubated at 500μM with the following compounds; control, OTZ, *N*-acetylcysteine, cysteine, CIPE and CCHE. Intracellular cysteine was then measured by HPLC. Each point represents the mean ± SEM (n>3).

Figure 5.2 Intracellular cysteine levels of lung slices when incubated at low concentrations of cysteine delivery systems



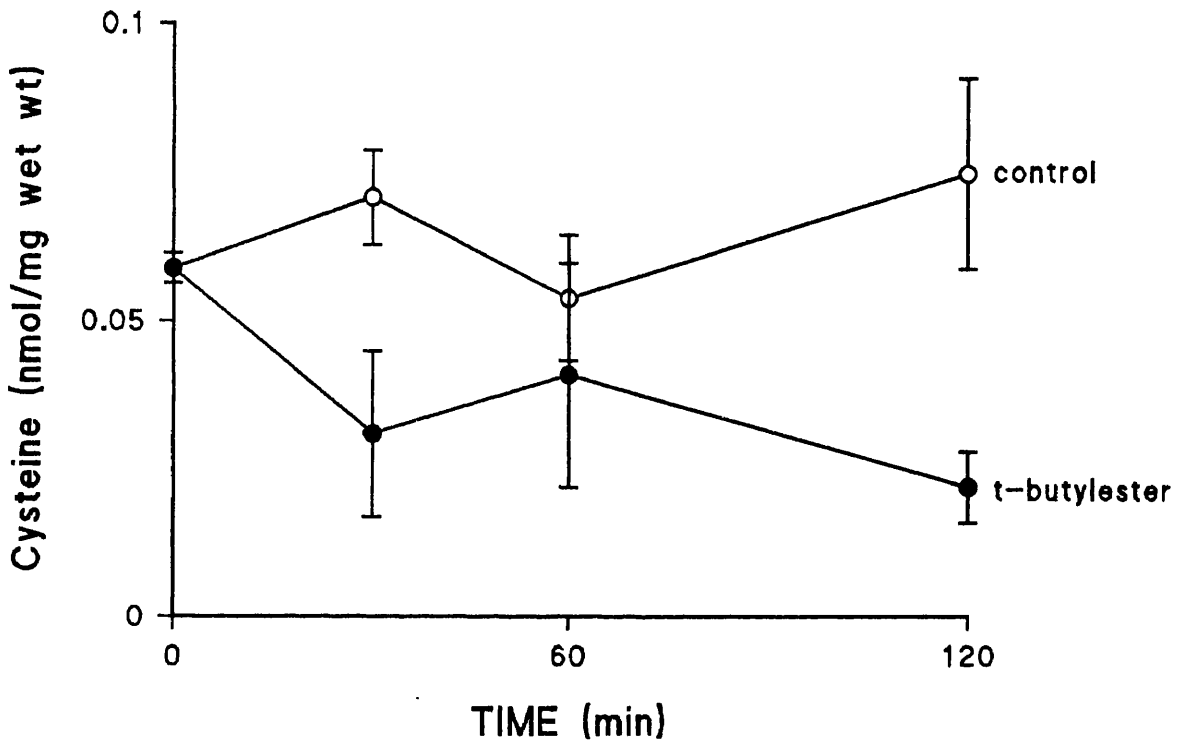
Lung slices were incubated at (50μM) with the following compounds; control, OTZ, *N*-acetylcysteine, cysteine, CIPE and CCHE. Intracellular cysteine was quantified by HPLC. Eachpointrepresents the mean ± SEM (n>3).

extracellular thiols following incubation of rat lung slices with CIPE and cysteine



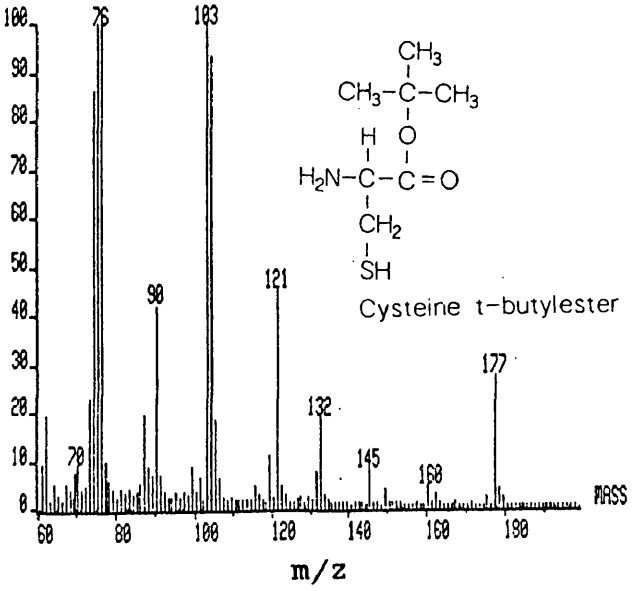
After 5, 15, 30 and 60 min incubation of lung slices with CIPE or cysteine (500μM), levels of intra- (a) and extracellular (b) thiols were measured by HPLC analysis . The data illustrates the HPLC traces obtained (**Key to peaks:** 1 = cysteine, 2 = GSH, 3 = monobromobimane and 4 = CIPE). Note the absence any detectable intracellular CIPE. Chromatographic conditions are described in material and methods.

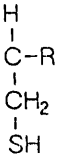
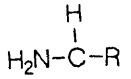
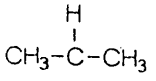
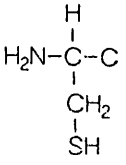
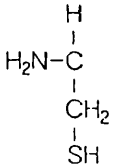
FIGURE 5.4 The t-butylester of cysteine did not elevate intracellular levels of cysteine



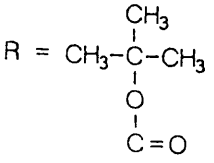
Lung slices were incubated with cysteine t-butylester (500μM) (●), or no cysteine prodrug (O). An estimate of intracellular cysteine was made by HPLC. Each point represents the mean of 3 experiments (±SEM)

FIGURE 5.5 The mass spectrum of t-butylester of cysteine



m/z ratio	possible positive ions
177	molecular ion
160	
145	
132	
121	
103	
90	
76	

KEY



0.43±0.09 nmol/mg wet weight for 50 and 500μM respectively after 60 min incubation). *N*-Acetylcysteine produced only a small rise in intracellular cysteine compared to CIPE, CCHE or cysteine (fig 5.1 and 5.2). OTZ has no free -SH groups and is unable to form a fluorescent conjugate with monobromobimane. Nor did OTZ produce a detectable rise in intracellular cysteine in lung slices (fig 5.1 and 5.2). Exogenous cysteine (500μM) maximally elevated intracellular cysteine within 30 minutes.

There was no significant difference in the profiles of intracellular cysteine between control lung slice and lung slices preincubated with diethyl maleate (data not shown).

2. Changes in extracellular CIPE, CCHE, cysteine and *N*-acetylcysteine when incubated with rat lung slices.

A time dependent decrease in extracellular CIPE and CCHE (500μM) (fig 5.6) was accompanied by a rise in extracellular cysteine (fig 5.7). Less than 10% of the unmetabolized esters remained after 60 minutes. The decrease of extracellular CCHE was significantly slower than that of CIPE.

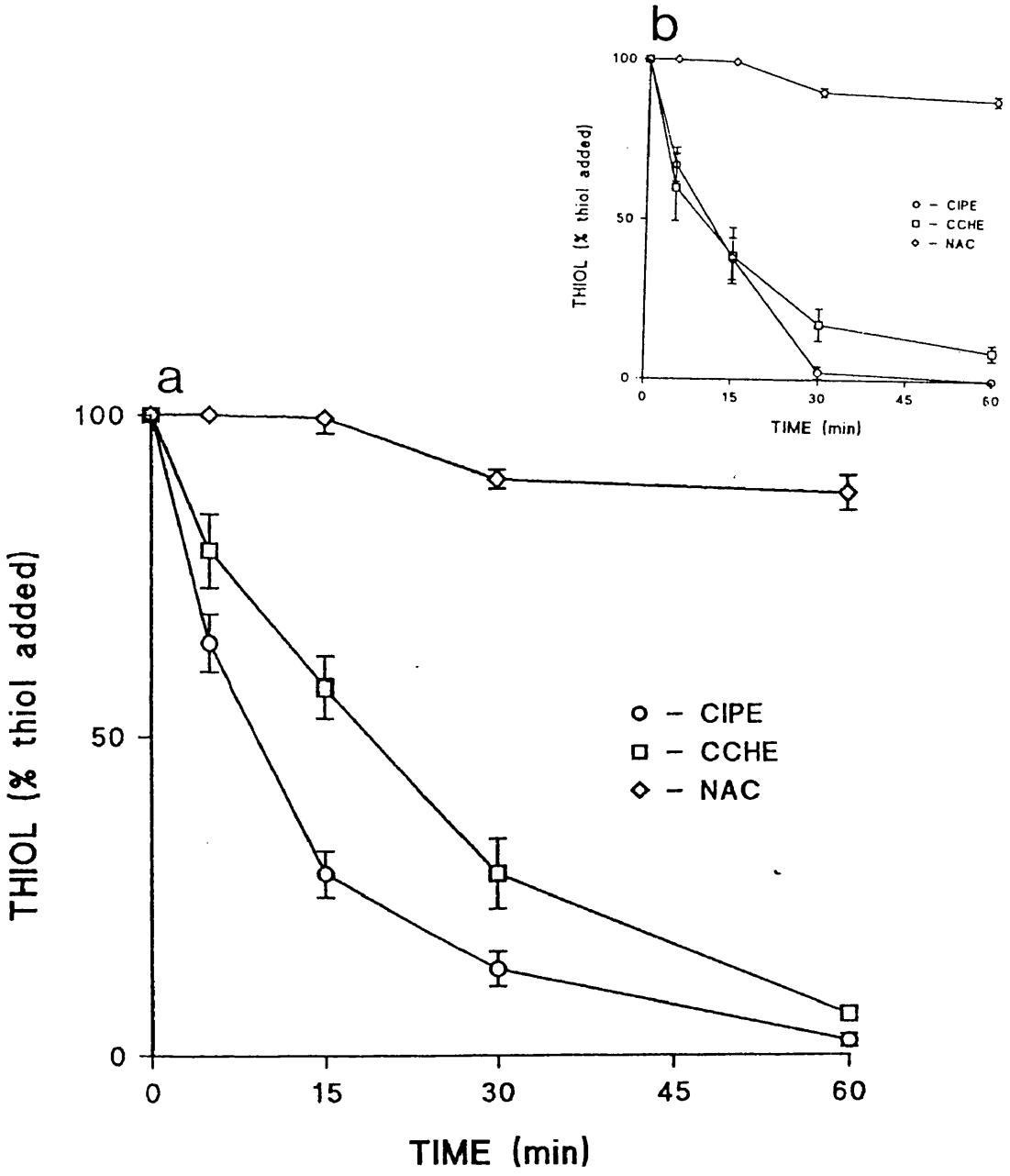
The extracellular pool of *N*-acetylcysteine (500μM) remained relatively constant over 60 min (fig 5.6). A limited rise in extracellular cysteine was noted (fig 5.7).

After 60 min incubation of rat lung slices with cysteine (500μM), extracellular cysteine declined by 21% (fig 5.7). When cysteine was incubated in the absence of lung slices there was no appreciable decline in cysteine over 60 minutes (fig 5.8). OTZ had no effect on extracellular thiol levels.

A similar pattern of extracellular thiols was observed with lower concentrations of the cysteine pro-drugs (50μM) (fig 5.6b and 5.7b). A comparison of extracellular thiolsfrom slices preincubated with diethyl maleate showed a similar trend to control medium.

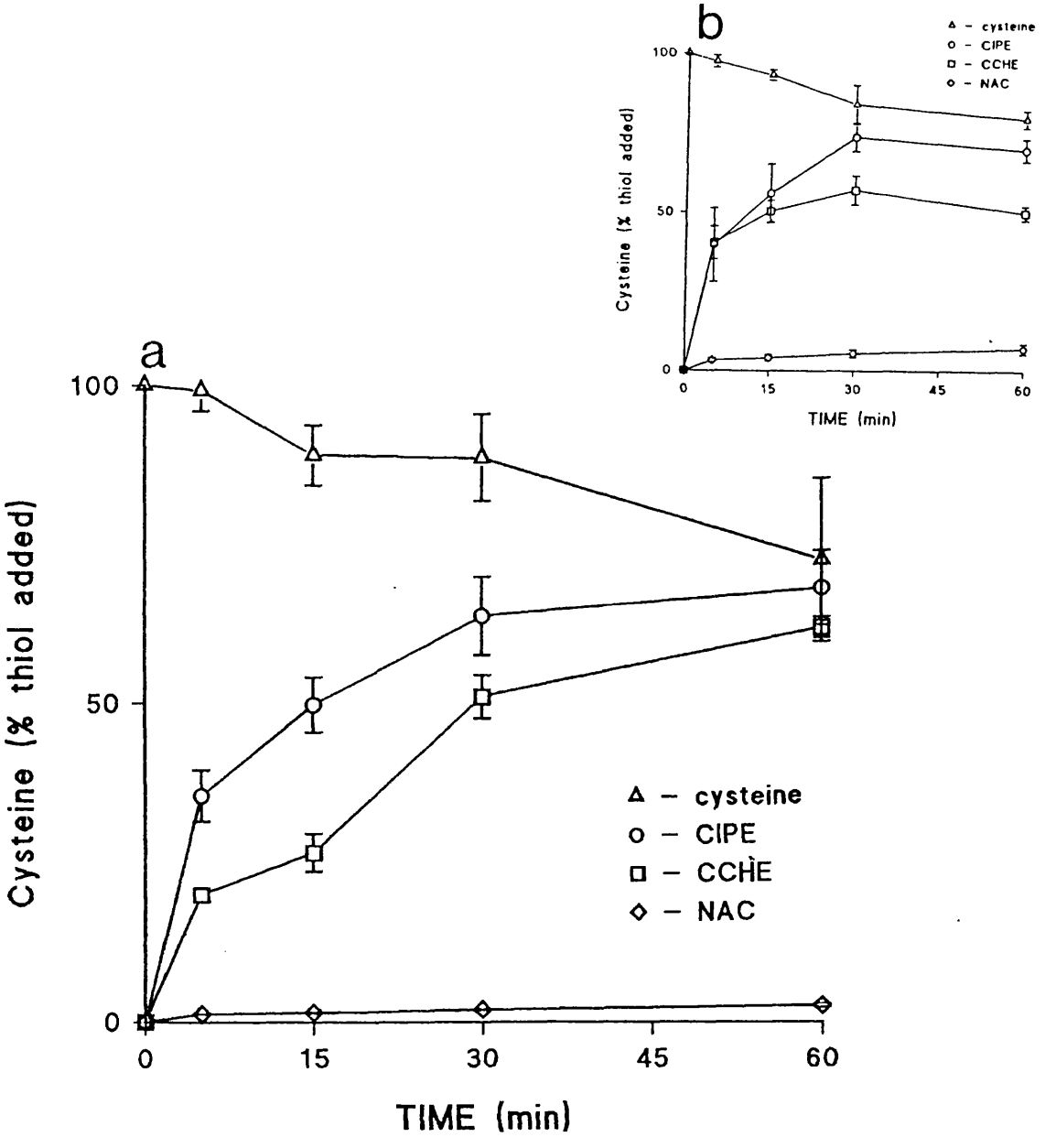
The total amount -SH present after 60 min was used as a marker of metabolism of cysteine pro-drugs by lung slices

FIGURE 5.6 Changes the in extracellular pool of CIPE, CCHE and *N*-acetylcysteine



Disappearance of CIPE (○), CCHE (□) and *N*-acetylcysteine (◇) from the extracellular medium was assessed by HPLC. The data is expressed as a percentage of the amount of thiol added to the medium, either (a) 500μM or (b) 50μM. Each point represents the mean ± SEM (n>3). [initial sulphydryl levels (nmol/ml); CIPE (500μM) = 521±23.5, CIPE (50μM) = 53.2±5.2, CCHE (500μM) = 504±35.4, CCHE (50μM) = 57.8±7.3, cysteine (500μM) = 582±43.3, cysteine (50μM) = 57.8 ±4.7, N-acetylcysteine (500μM) = 525±29.3, N-acetylcysteine (50μM) = 53.7 ± 3.5].

FIGURE 5.7 The effect of lung slices on extracellular cysteine



The concentration of cysteine was measured in an aliquot of extracellular medium from slices incubated with cysteine (Δ), CIPE (○), CCHE (□) or *N*-acetylcysteine (◇) at either 500μM (a) or 50μM (b). Results are expressed as a percentage of the amount of SH initially added to the medium. Initial SH values are the same as for fig 5.5. Each point is the mean ± SEM of at least 3 separate determinations.

(fig 5.8). When *N*-acetylcysteine, cysteine or the esters of cysteine were incubated at 37° in the absence of slices, 5-10% of the -SH initially available had disappeared after 60 min (fig 5.8). In general, the loss of -SH was greater in the presence of lung slices, especially for the esters of cysteine (fig 5.8).

3 Effect of CIPE and other cysteine precursors on the GSH levels of rat lung slices

Control lung slices had initial GSH levels of 1.23±0.12 nmol/mg wet weight which were maintained over a 3 hourincubation period (fig 5.9) These values are in good agreement with published data [29,242]. No elevation of pulmonary GSH was observed in control lung slices incubated with CIPE, CCHE, N-acetylcysteine, OTZ or cysteine (all 500μM) (Table 5.I).

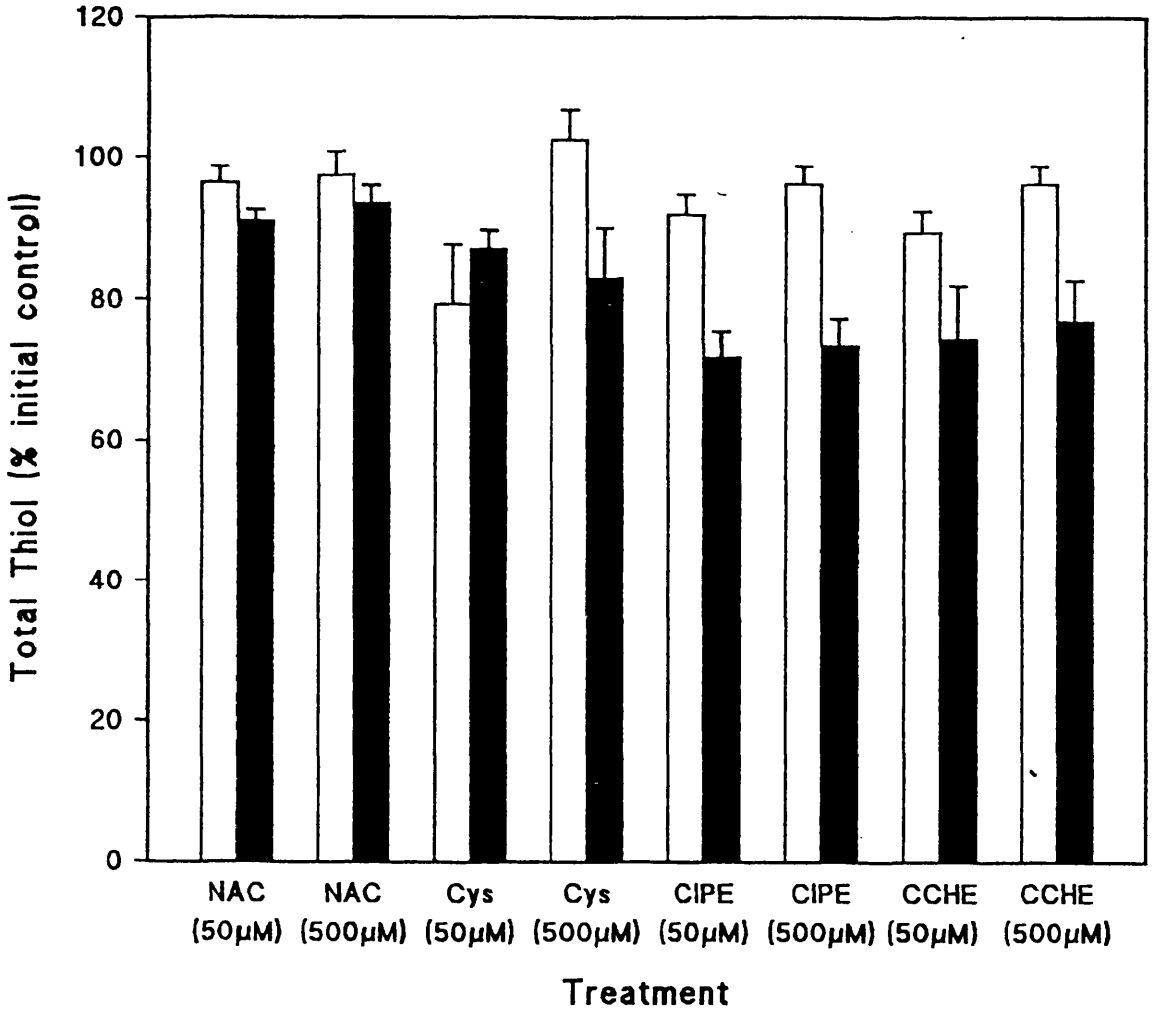
Incubation of rat lung slices with diethyl maleate (500μM) for 30 minutes caused a 75% fall in pulmonary GSH (fig 5.9) GSH levels did not recover when slices were incubated in fresh KRP for a further 3 hours (fig 5.9).

Interestingly, in GSH depleted lung slices, all five cysteine delivery systems supported resynthesis of GSH to varying extents (fig 5.10). The greatest increases were observed with CIPE, CCHE, N-acetylcysteine and cysteine, whilst OTZ was apparently less effective (fig 5.10).

4. Metabolism of CIPE by isolated rat hepatocytes

CIPE (500μM) rapidly disappeared from the extracellular medium of isolated rat hepatocytes (fig 5.11b). Within 2 min, the majority of the ester had vanished. The disappearance was coincident with a considerable elevation of intracellular cysteine and the appearance of a large extracellular pool of cysteine (fig 5.11). No ester was detected intracellularly. There was no loss of viability, as assessed by trypan blue exclusion, by exposing to CIPE (500μM) [initial viability - 79.7±1.9%, after 60 min - 76.5±1.7%]. CIPE (10mM) resulted in a 56% loss of viability

FIGURE 5.8 The total amount of SH remaining 60 min after incubation of cysteine delivery systems with or without lung slices



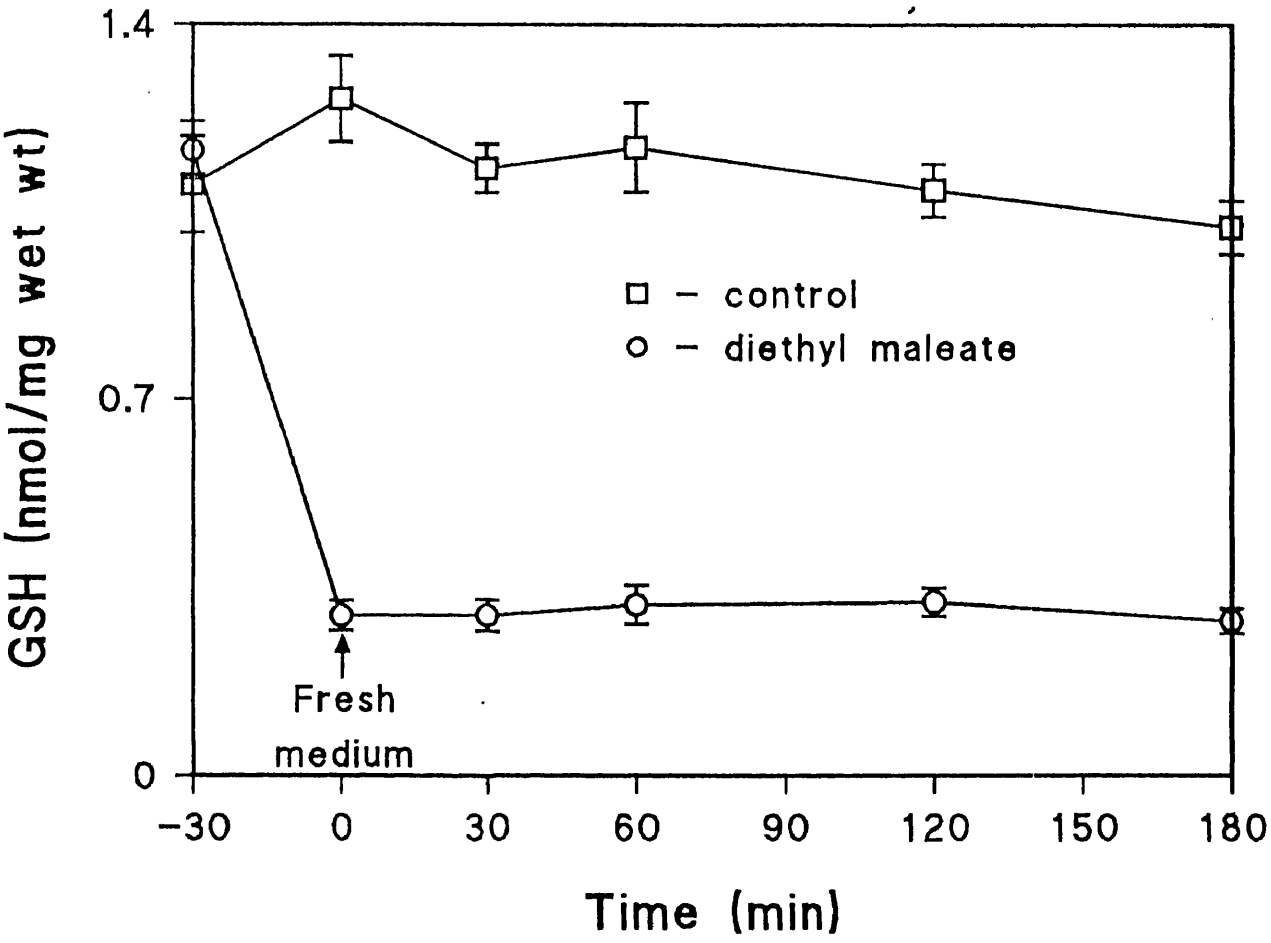
Lung slices were incubated with *N*-acetylcysteine, cysteine, CIPE or CCHE either at 500μM of 50μM (■), or these compounds were incubated in the absence of slices (□). The total free SH available to react with monobromobimane was the sum of the levels of intra- and extracellular cysteine or cysteine delivery system. The results are expressed as a percentage of the initial amount of sulphydryl added (nmol). Bars represent the mean ± SEM (n>3).

TABLE 5.I Effect of CIPE, CCHE, *N*-acetylcysteine, cysteine and OTZ (500μM) on the levels of GSH in rat lung slices

	GSH (nmol/mg wet wt)		
Treatment	Incubation time (min)		
	15	30	60
control	1.39±.07	1.26±.08	1.35±.05
CIPE	1.30±.09	1.29±.08	1.27±.08
CCHE	1.19±.10	1.15±.04	1.26±.09
N-acetylcysteine	1.20±.13	1.12±.13	1.05±.20
cysteine	1.41±.14	1.25±.14	1.19±.07
OTZ	1.17±.19	1.15±.17	1.05±.12

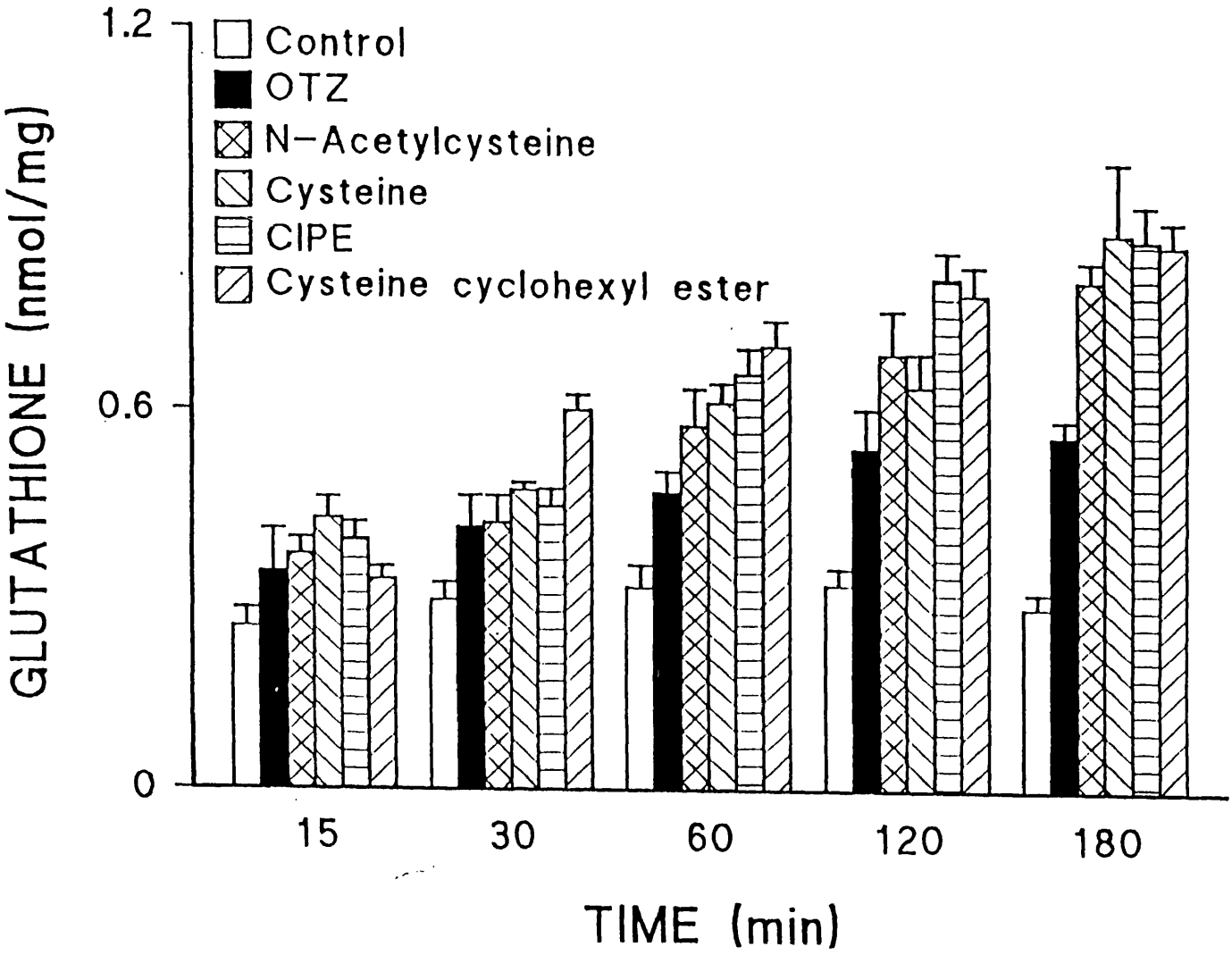
Initial pulmonary concentration of GSH = 1.29 ± .03 nmol/mg wet wt. Each point is the mean ± SEM, n=3.

FIGURE 5.9 Depletion of pulmonary GSH by diethyl maleate



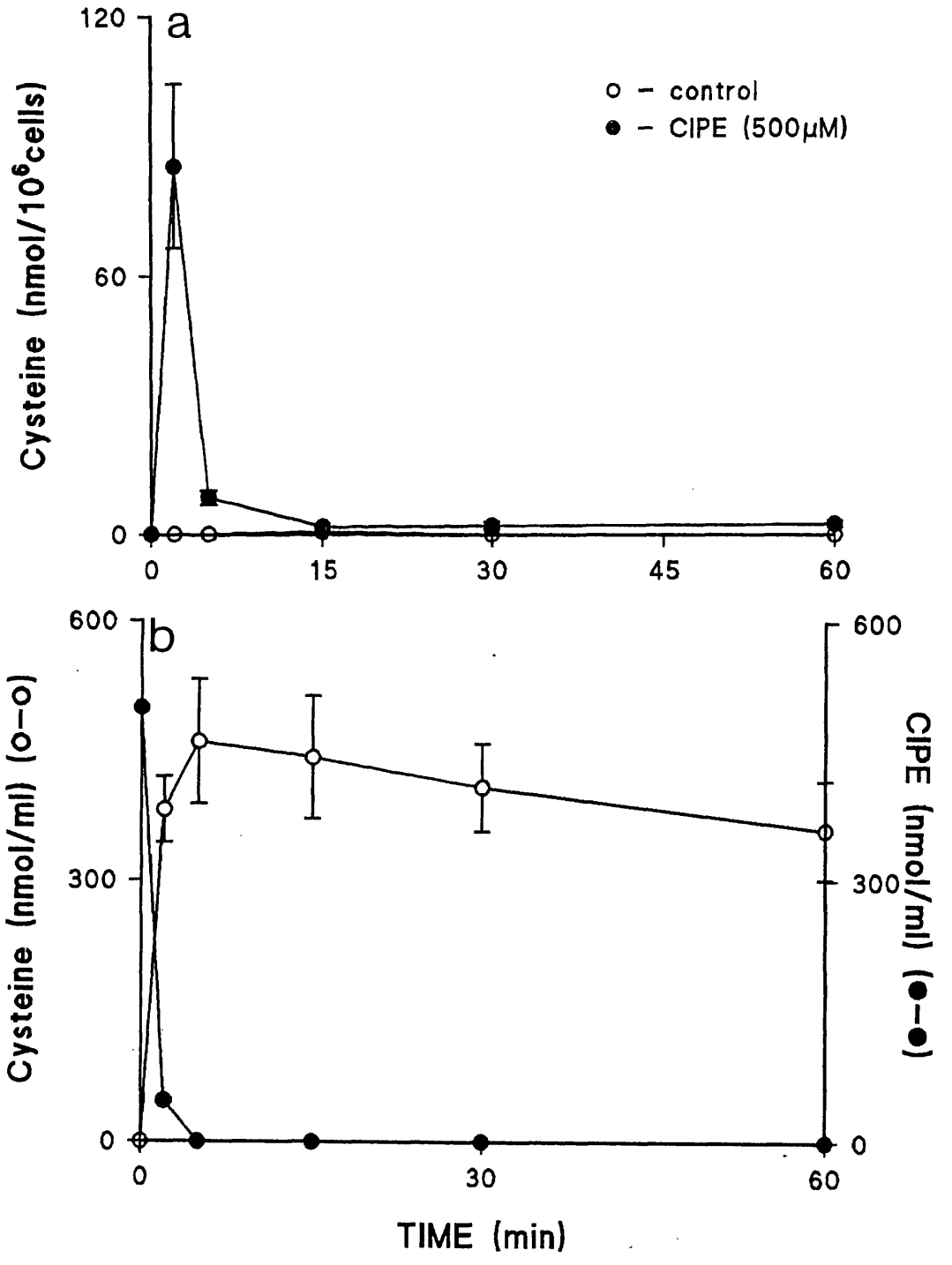
Lung slices were incubated with diethyl maleate (500μM) for 30 min and then incubated for a further 3 hr in fresh KRP. Control slices were incubated in diethyl maleate-free medium. Each point is the mean ± SEM (n>4)

FIGURE 5.10 Replenishment of pulmonary GSH by cysteine delivery systems



Levels of GSH were depleted by diethyl maleate and lung slices transferred to fresh KRP containing OTZ, *N*-acetylcysteine, cysteine, CIPE, CCHE or no cysteine delivery system for a further 3 hr. Bars represent the mean ± SEM (n = 4 or 5).

FIGURE 5.11 The metabolism of CIPE by isolated rat hepatocytes



Isolated hepatocytes (1×10^6 cells/ml) were incubated with CIPE (500μM) for 60 min and the intracellular cysteine levels (a) and extracellular cysteine and CIPE concentrations (b) were measured by HPLC. Each point represents the mean ± SEM (n=4)

after 60 min (not shown). CIPE (500μM) had no effect on the levels of intracellular GSH levels of hepatocytes.

2,3-Dimethoxy-1,4-naphthoquinone (500μM), a pure redox cycling agent [243], decreased hepatocyte viability over 4 hr (fig 5.12). While CIPE (500μM) had no effect on hepatocyte viability, co-administration with 2,3-dimethoxy-1,4-naphthoquinone potentiated the decline in viability mediated by the quinone. A second addition of CIPE (500μM) after 60 min reduced hepatocyte viability further (fig 5.12).

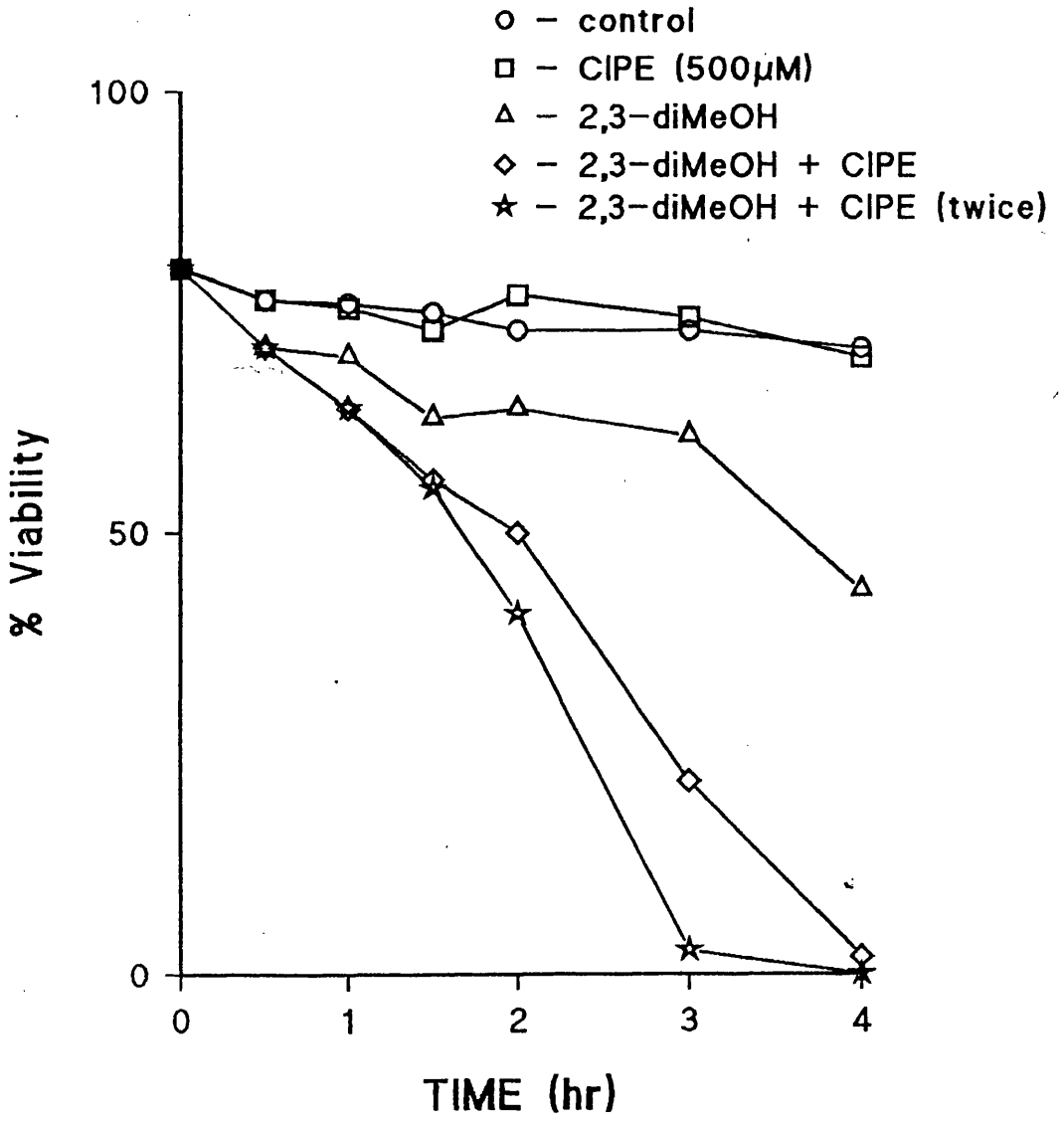
5. Metabolism of CIPE by rat plasma

In a preliminary study blood samples were drawn from rats by cardiac puncture and spun down. The plasma was diluted (1:1) with Krebs-Ringer phosphate buffer and incubated at 37° with CIPE (500μM). CIPE rapidly disappeared, within 15 min <1% remained (fig 5.13). As CIPE declined so cysteine appeared in the solution (fig 5.13). The amount of cysteine present began to decline after 5 min. GSH levels also fell from 30.0 nmol/ml to 12.7 nmol/ml after 45 min.

6. Effect of CIPE and *N*-acetylcysteine on the toxicity of naphthalene to rat lung slices

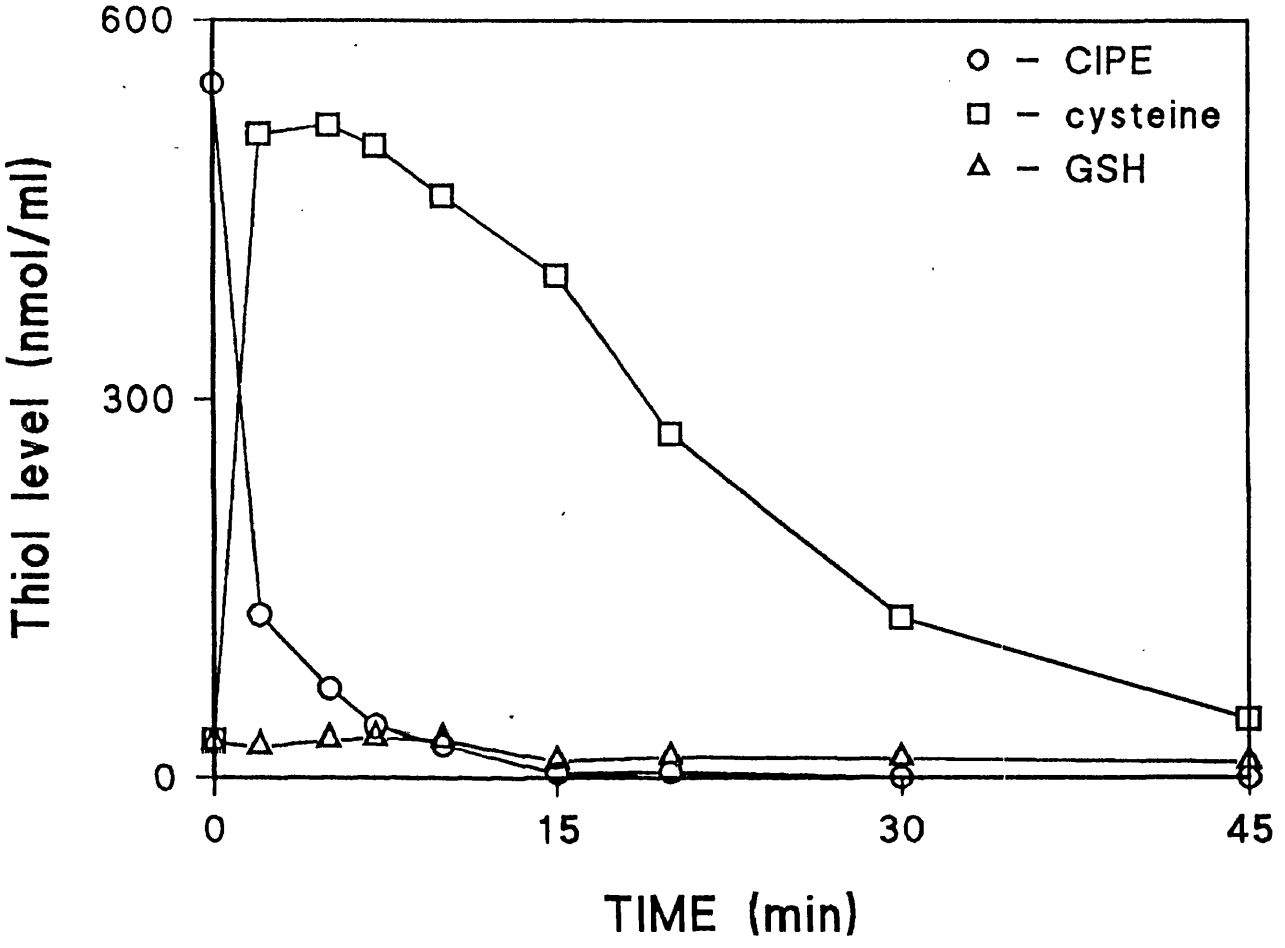
Naphthalene (1mM) produced a significant decline in pulmonary GSH after 1 hr (fig 5.14). There was no recovery or enhancement of this loss when slices were incubated for a further 3 hr in presence of naphthalene (Fig 5.14). Interestingly, the level of GSH in control lung slices also declined over 4 hr (fig 5.14). CIPE (250μM) was able to prevent the loss of GSH in both control and slices incubated with naphthalene (fig 5.14). Slices incubated with both naphthalene and CIPE for 1, 2 and 4 hr had levels of GSH significantly greater than those incubated with naphthalene alone. *N*-Acetylcysteine was less effective at replenishing levels of GSH. The area under the curves for slices incubated with *N*-acetylcysteine were significantly lower

FIGURE 5.12 Effect of CIPE and 2,3-dimethoxy-1,4-naphthoquinone on the viability of isolated rat hepatocytes



The viability of isolated rat hepatocytes incubated in the presence or absence of 2,3-dimethoxy-1,4-naphthoquinone (500μM) (2,3-diMeOH) with or without CIPE (500μM) was estimated using their ability to exclude Trypan blue (.4%). The data presented is the mean of 2 experiments.

Figure 5.13 The metabolism of CIPE by rat plasma



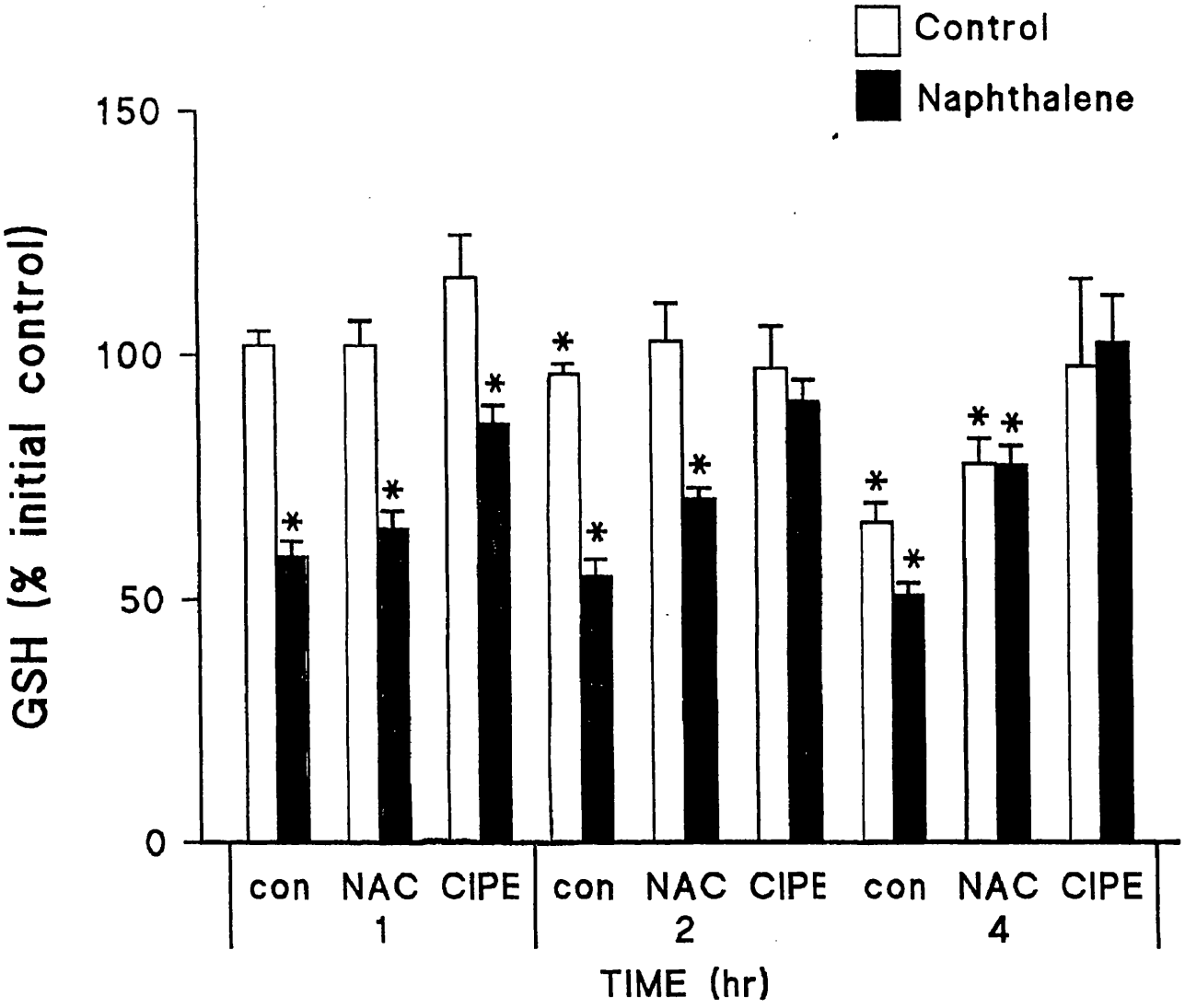
Rat plasma was diluted with KRP and incubated with CIPE (500μM). HPLC was used to monitor changes in the levels of CIPE (o), cysteine (□) and GSH (Δ). The data is preliminary.

than the areas for slices exposed to CIPE, for both control and slices subjected to naphthalene.

CIPE produced a considerable elevation of cellular cysteine (fig 5.15) which was unaffected by naphthalene. However naphthalene reduced the levels of intracellular cysteine in both control and slices incubated *N*-acetylcysteine (fig 5.15). Naphthalene did not alter the size of the extracellular pool of cysteine from slices incubated with CIPE (fig 5.16) but significantly reduced the extracellular concentration of cysteine derived from *N*-acetylcysteine (fig 5.16). Naphthalene had no significant effect on the levels of intracellular *N*-acetylcysteine (data not shown)

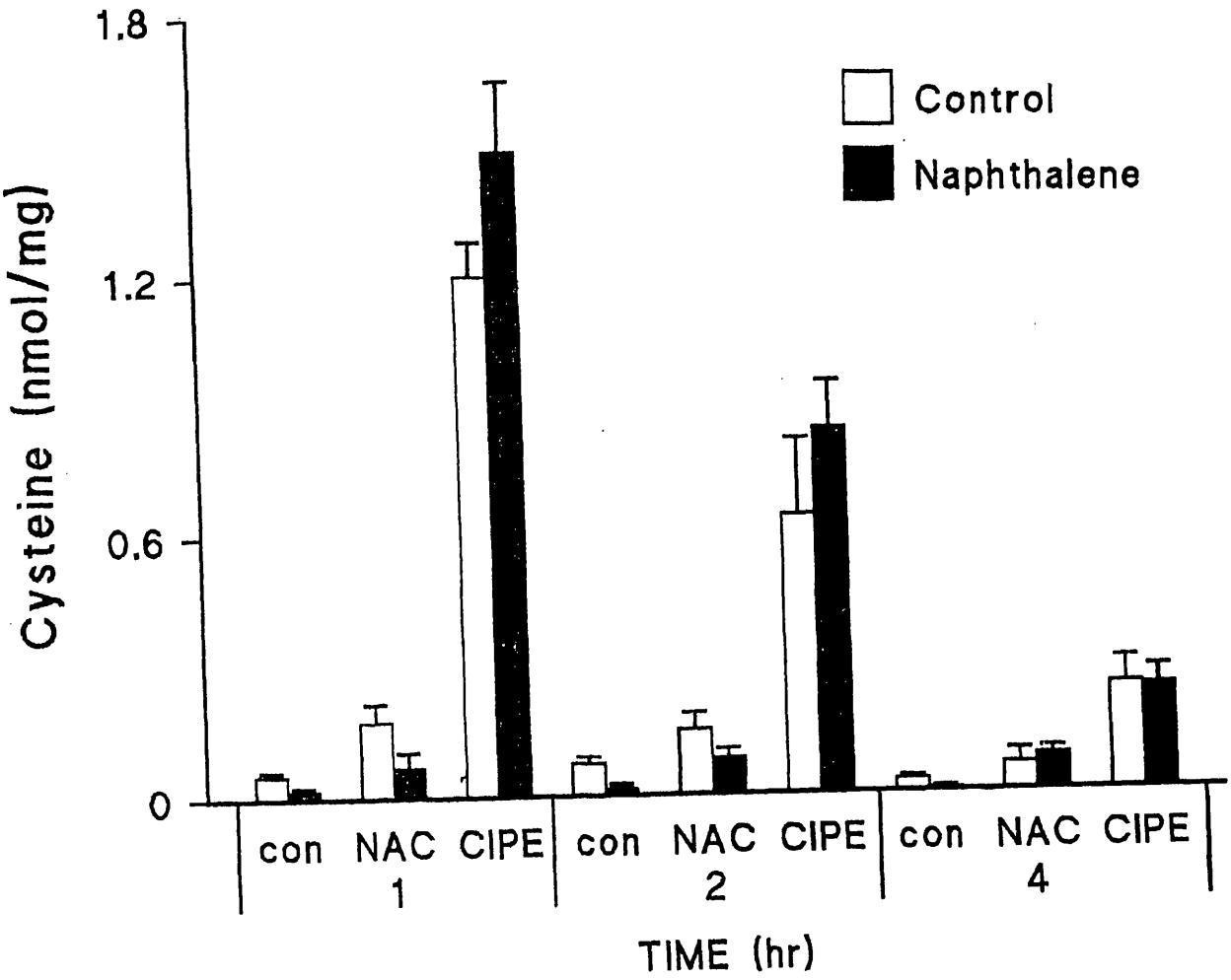
Naphthalene (1mM) had no detrimental effect on the ability of lung slices to accumulate putrescine (figure 5.17), unlike paraquat (10μM). While CIPE further reduced the ability of lung slices to accumulate putrescine when exposed to paraquat, it did not effect putrescine uptake when incubated with naphthalene (fig 5.17).

FIGURE 5.14 Depletion of pulmonary GSH by naphthalene



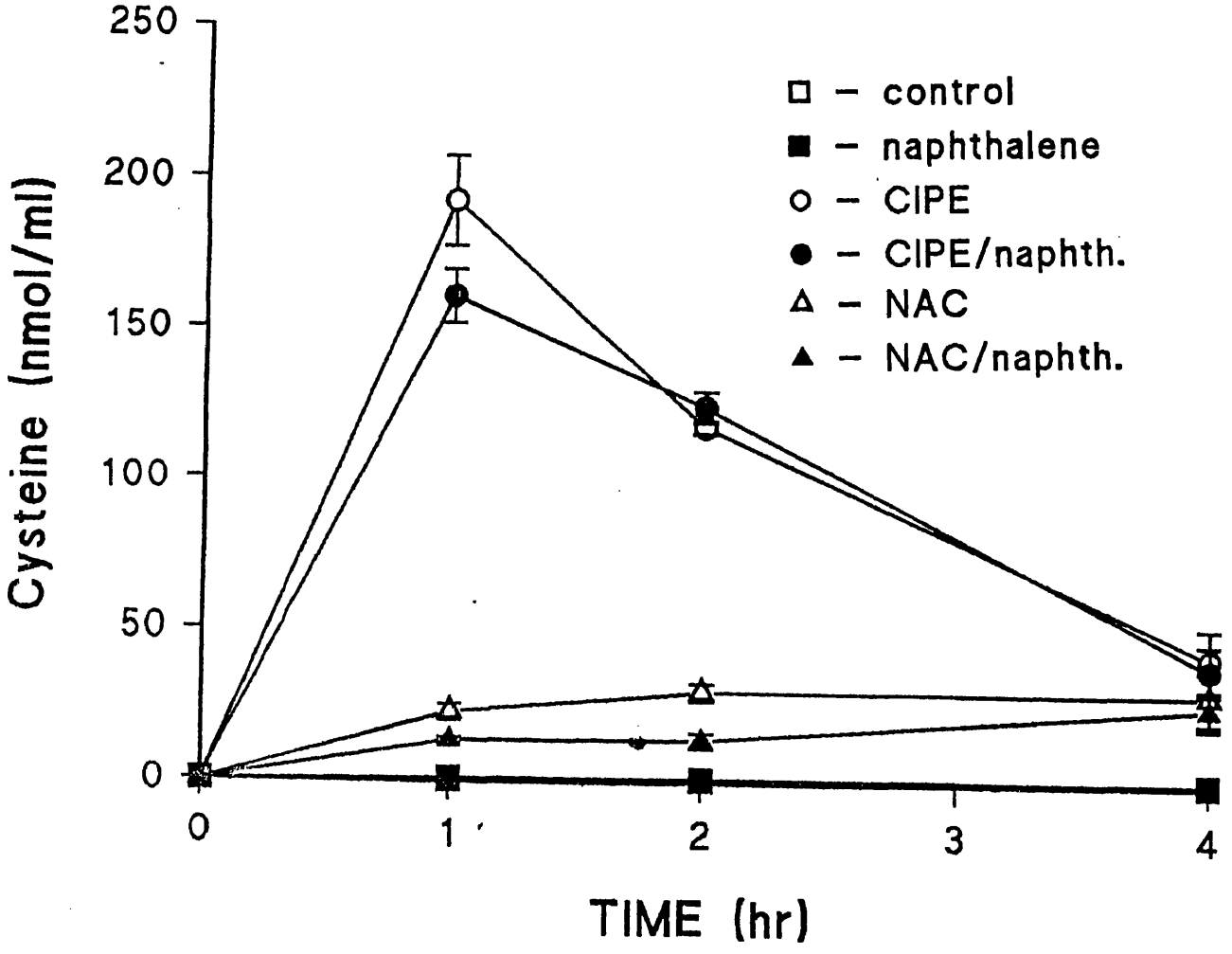
Lung slices were incubated with or without naphthalene (1 mM) in the presence or absence of CIPE or *N*-acetylcysteine (250μM) and the levels of pulmonary GSH monitored by HPLC. Naphthalene was dissolved in KRP by prolonged, gentle mixing. The data is expressed as a percentage of the initial GSH values, 1.40 ± .04 nmol/mg wet wt. Bars represent the mean ± SEM ($n \geq 4$). *denotes significant difference from the initial GSH value, $p \leq .05$.

FIGURE 5.15 Effect of CIPE and *N*-acetylcysteine on the naphthalene-induced decline in the cysteine concentration of rat lung slices



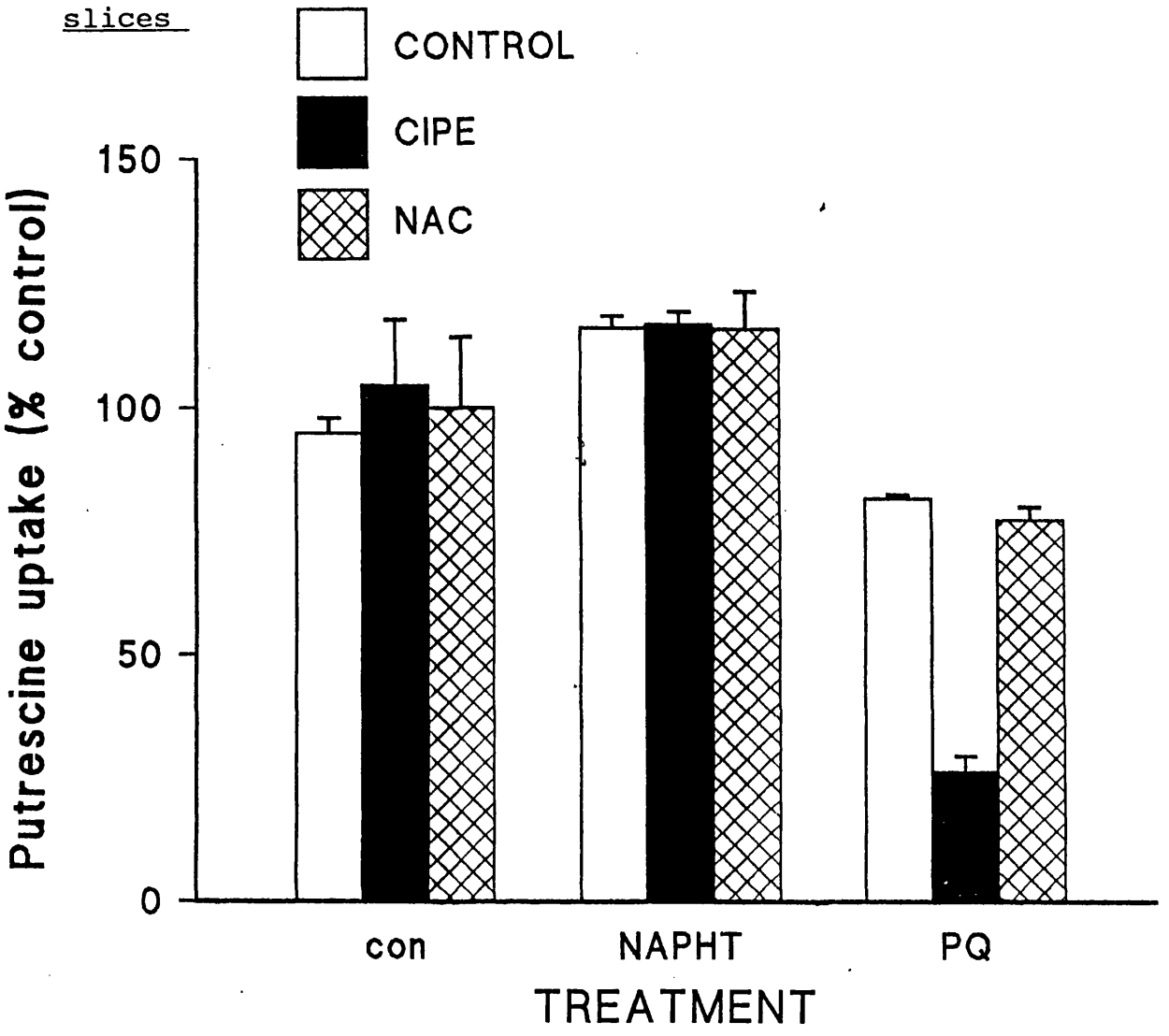
Control- and naphthalene-treated lung slices were incubated with or without CIPE (250μM) or *N*-acetylcysteine (250μM) and intracellular levels of cysteine measured by HPLC. Each bar is the mean ± SEM (n≥4). The levels of intracellular cysteine from slices incubated solely with naphthalene (1mM) were significantly lower than control slices, $p<.05$.

FIGURE 5.16 Effect of naphthalene on the CIPE or *N*-acetylcysteine-mediated increase in extracellular cysteine



The extracellular cysteine from lung slices incubated with or without CIPE or *N*-acetylcysteine in the presence or absence of naphthalene was measured by HPLC. Each point is the mean ±SEM, n≥4. The area under the curve for slices treated with naphthalene and *N*-acetylcysteine were significantly lower than that for slices incubated with *N*-acetylcysteine alone, $p<.05$.

FIGURE 5.17 A comparison of the abilities of naphthalene and paraquat to inhibit putrescine accumulation by lung slices



Lung slices were incubated with naphthalene (1mM) (Napht) and paraquat (10μM) (PQ), with or without CIPE or *N*-acetylcysteine for 4 hr and the ability of slices to accumulate radiolabelled putrescine was assessed, as a marker of type I and II alveolar epithelial cell viability. Bars represent the mean ± SEM (n=3). Control values for putrescine accumulation were 95±5.2 nmol/g wet lung wt.

5.3 DISCUSSION

These studies demonstrated that the esters of cysteine elevated pulmonary cysteine levels to a greater extent than other cysteine delivery systems (fig 5.1 and 5.3). The relative efficacies of these systems being:-

CIPE = CCHE >cysteine >N-acetylcysteine >OTZ = t-butylester

CIPE and CCHE were particularly efficient cysteine delivery systems, producing a rapid burst in intracellular cysteine. The elevated levels of cysteine were fairly short lived (fig 5.1) and declined as the esters were metabolised and removed from the extracellular pool. Cysteine added either exogenously or derived from hydrolysis of the esters was probably accumulated into lung cells by neutral amino acid uptake systems [56,62].

The esters of cysteine acted independently of the neutral amino acid uptake system, as the initial increases in intracellular cysteine were much greater than those observed when slices were incubated solely with cysteine (fig 5.1 and 5.5). The loss of intracellular cysteine following its generation from the cysteine delivery systems was probably due to one or more possibilities. The data presented suggested that much of the cysteine was exported from lung slices and especially rapidly from hepatocytes. Once free from the reducing environment of the cell, oxidation of cysteine to cystine would account for the gradual disappearance of cysteine from the extracellular pool. Alternatively , the intracellular cysteine may contribute to the turnover of pulmonary GSH [227], or may possibly undergo catabolic metabolism, ultimately generating taurine or pyruvate and sulphite [33]. However, the majority of studies on the metabolic fate of cysteine have been carried out with mammalian liver and may not be applicable to its fate in the lung. Additionally, SO_3^{2-} or SSO_3^{2-} are labelled by monobromobimane [203], but were not

detected by HPLC in samples from slices or hepatocytes treated with CIPE, suggesting catabolism does not play an important role in the disappearance of cysteine in these *in vitro* models.

Significant differences were observed in intracellular cysteine profiles following exposure of lung slices to the esters of cysteine. CIPE produced a rapid increase in cellular cysteine, with maximum levels being observed within 5 min, whilst CCHE produced a slower but more sustained elevation in intracellular cysteine (fig 5.1). This corresponded to the longer half life of CCHE in the extracellular medium (fig 5.6) and may have been due to a slower hydrolysis by pulmonary esterases caused by the bulkier cyclohexyl group.

The t-butylester of cysteine failed to elevate intracellular cysteine (fig 5.4) and could not be detected by HPLC but when analyzed by mass spectroscopy the t-butylester appears to be present intact (fig 5.5). Several esters of cysteine protected against lethal doses of inhaled perfluoroisobutene and elevated plasma levels of NPSH in rats except cysteine t-butylester [94]. Possibly the t-butyl moiety stabilises the structure of the molecule or in some way prevents the expression of the reactive thiolate anion ($R\text{-}S^-$), accounting for its inability to react with perfluoroisobutene or monobromobimane.

In control lung slices, none of the cysteine precursors produced an increase in GSH, most probably because of a negative feed back on γ-glutamylcysteine synthetase by normal levels of GSH [27]. All the cysteine delivery systems were capable of replenishing lung slice GSH after exposure to diethyl maleate (fig 5.10). The largest increases in GSH occurred after 1 hour incubation, when the majority of the esters had already disappeared (fig 5.6) and intracellular cysteine levels for slices incubated with the esters, approached levels experienced by slices incubated with cysteine alone. This may account for the failure of the cysteine esters to offer any advantage over cysteine in

the replenishment of GSH.

N-Acetylcysteine and OTZ were relatively ineffective in elevating intracellular cysteine in control lung slices (fig 5.1 and 5.2), but both promoted GSH generation in diethyl maleate treated slices (fig 5.10). In slices incubated with *N*-acetylcysteine, cysteine levels were consistently below 0.3 mM, the reported K_m value of renal and erythrocyte γ-glutamylcysteine synthetase for cysteine [30]. In isolated hepatocytes low cellular concentrations of cysteine, such as those produced by OTZ or methionine, tend to be differentially partitioned to the biosynthesis of GSH rather than alternative routes of cysteine metabolism [108]. As the lung is an heterogeneous organ, the location of pulmonary GSH synthesis supported by N-acetylcysteine or OTZ may be limited to cells possessing deacetylase or prolinase activity. Whilst the apparent intracellular cysteine concentration for the whole lung slices is below the K_m value for γ-glutamylcysteine synthetase, selectively distributed enzyme activity could generate cysteine in cells best able to generate GSH. Lung homogenates deacetylate N-acetylcysteine [74] but the ability of the lung to use OTZ for the generation of cysteine is variable [29].

Several of the cysteine precursors studied have previously been shown to slow the depletion of GSH after a toxic insult and support GSH resynthesis. The L-isomers of CIPE, N-acetylcysteine and OTZ protect against the hepatotoxicity of paracetamol [chapter 4,72,87]. *N*-Acetylcysteine increases the biosynthesis of GSH in isolated perfused lungs [226] and when administered in the drinking water increases pulmonary GSH 2 fold [244]. *N*-Acetylcysteine has also been proposed to have a role in treating human pulmonary disorders, being a mucolytic agent [74].

The rate of hydrolysis of CIPE by hepatocytes, relative to lung slices, was very much greater (fig 5.11), reflecting the substantial reserve of hepatic esterase activity. Additionally preliminary studies showed plasma

to have abundant esterase activity (fig 5.13), a finding confirmed by colleagues at the Chemical and Biological Defence Establishment. These 2 facts show the transient elevation of pulmonary NPSH by CIPE *in vivo* (fig 4.1) was probably due to factors distant from the lung, possibly hepatic and plasma metabolism, rather than pulmonary.

Lung slices incubated with naphthalene produced a 50 % decline in intracellular GSH within 1 hr (fig 5.14), a trend which CIPE but not *N*-acetylcysteine quickly reversed. Naphthalene depleted pulmonary cysteine in control slices (fig 5.15), demonstrating a possible direct interaction between cysteine and naphthalene oxide. CIPE greatly elevated the concentration of intracellular cysteine, allowing cellular GSH to be preserved by 2 mechanisms (summarised in fig 5.18); either the excess cysteine could act as a suitable alternative nucleophile for naphthalene oxide to react with, so sparing GSH or cysteine could be a substrate for γ-glutamylcysteine synthetase, replenishing GSH levels.

CIPE potentiated the toxicity of both paraquat and 2,3-dimethoxy-1,4-naphthoquinone (fig 5.17 and 5.12). The rationale behind the use of CIPE was its potential to supplement intracellular GSH and consequently combat the oxidative stress imposed by paraquat and 2,3-dimethoxy-1,4-naphthoquinone by providing a substrate for GSH peroxidase to remove H_2O_2 or lipid hydroperoxides. However CIPE was rapidly hydrolysed to cysteine. Cysteine (1mM) is toxic to hepatocytes [245]. Cysteine may become oxidised to its disulphide cystine via its thiyl radical and disulphide radical anion, with concomitant formation of superoxide radical, hydrogen peroxide and hydroxyl radical [116]. The extra redox stress imposed by CIPE through cysteine may account for the enhanced toxicity of paraquat and 2,3-dimethoxy-1,4-naphthoquinone in the presence of CIPE (summarised in fig 5.19)

In summary, the esters of cysteine, CIPE and CCHE, produced a marked elevation of cysteine in rat lung slices

Figure 5.18 Possible routes by which CIPE and *N*-acetylcysteine may protected against naphthalene toxicity

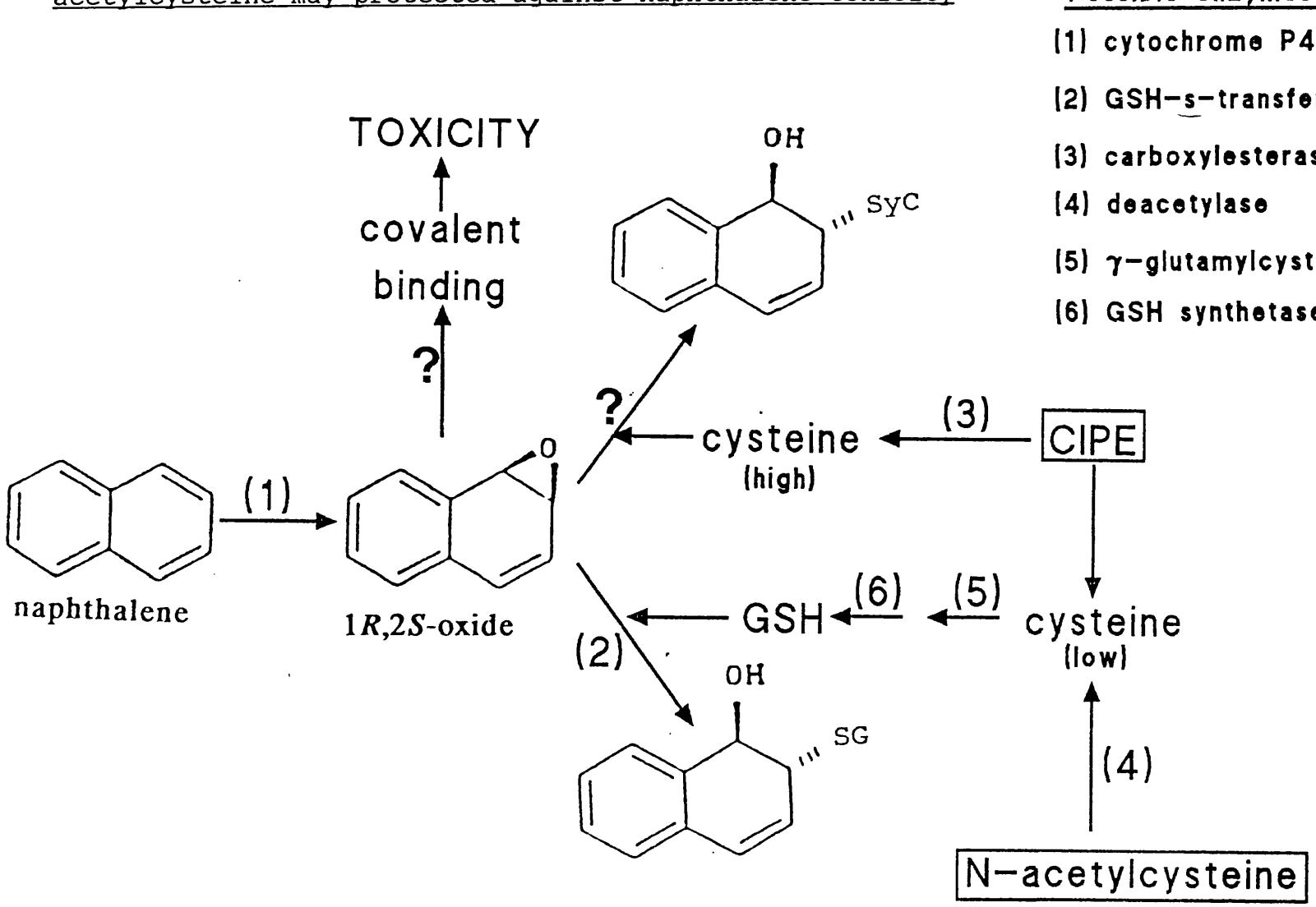
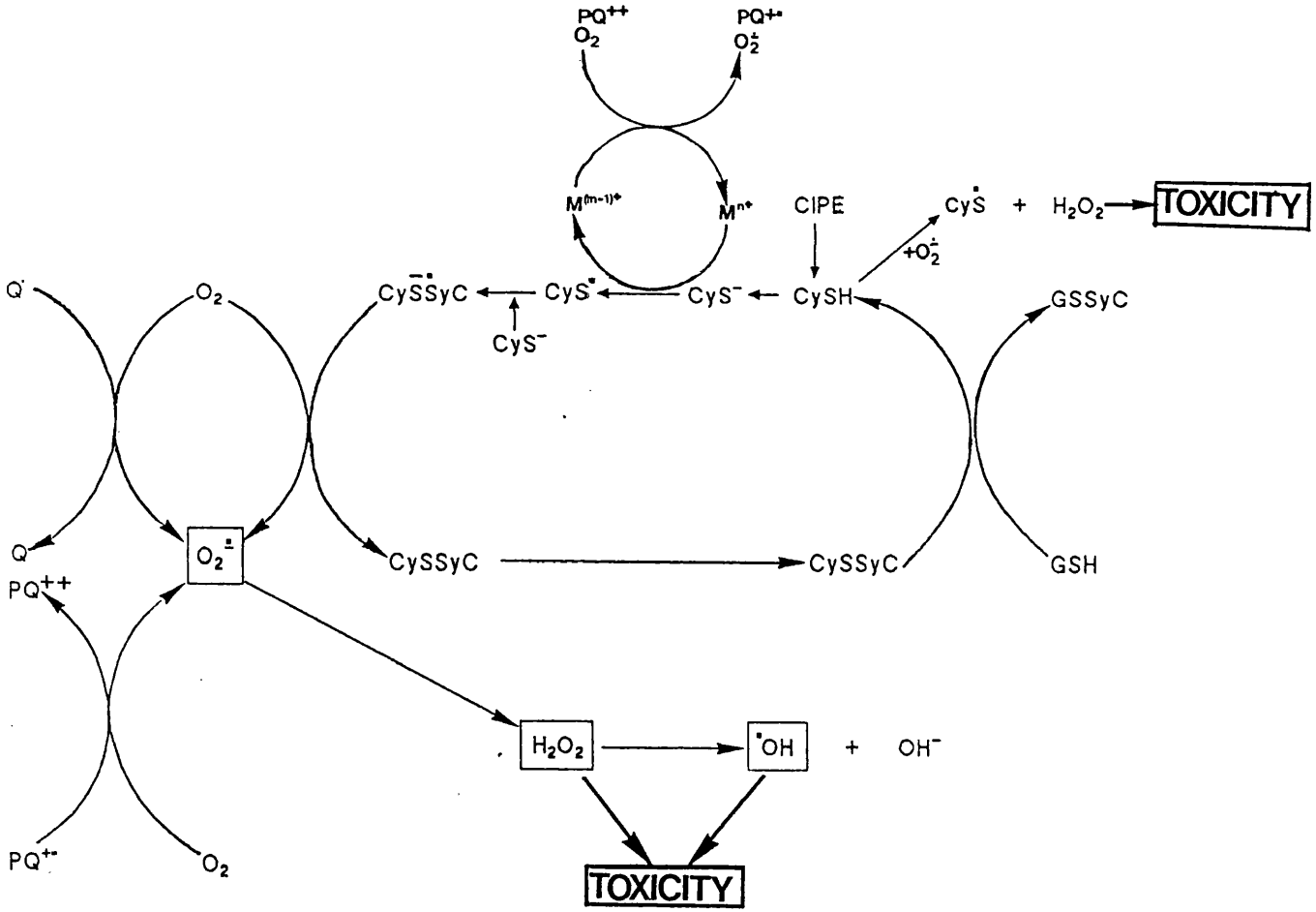


Figure 5.19 Potentiation of paraquat (PQ) and 2,3-dimethoxy-1,4-naphthoquinone (Q) toxicity by CIPE and cysteine



compared to cysteine or *N*-acetylcysteine, but without offering any advantage in their ability to replenish pulmonary GSH. The esters of cysteine have been observed to protect against rapidly generated electrophiles, such as inhaled perfluoroisobutene in rats pretreated with L-buthionine sulphoximine [94] and paracetamol in mice with induced cytochrome P450 activity (table 4.II). The protection observed, is probably associated with the rapid burst in intracellular cysteine. The cysteine acting as an alternative focus for electrophilic attack, sparing GSH and preventing electrophile interaction with other critical nucleophilic sites.

Chapter 6 - Esterase-mediated accumulation of cysteine esters by rat lung slices and isolated hepatocytes

6.1 INTRODUCTION

Previous experiments presented in this thesis demonstrated esters of cysteine, such as cysteine isopropyl or cyclohexyl ester (CIPE and CCHE), to be efficient cysteine delivery systems. After enzymic cleavage, the esters of cysteine should provide a source of cellular cysteine which may act either as a direct chemoprotectant and/or support GSH biosynthesis. However little or none of the ester was detected within the intracellular compartment *in vivo* and *in vitro*. In this chapter the effects of inhibitors of carboxylesterase activity on the metabolism of CIPE and CCHE by rat lung slices and isolated hepatocytes are investigated.

Cysteine is an amino acid necessary for the synthesis of proteins and the tripeptide glutathione (GSH, γ-glutamyl-cysteinylglycine) [1]. GSH has several metabolic and protective functions within cells [1]. The supply of cysteine is often rate limiting in GSH biosynthesis [7]. Consequently several cysteine prodrugs; methionine, OTZ and *N*-acetylcysteine, have been utilized to elevate cellular cysteine and so protect against the toxicity of various chemicals by supporting GSH replenishment. Cysteine can enter many cells via the neutral amino acid transport systems, designated systems A, ASC and L [56,62]. The Na^+-dependent system ASC is the predominant system in mammalian cells [56]. Extracellular levels of cystine far exceed those of cysteine, and may be transported into several cell types [62] via a specific Na^+-independent system, X^-_c [66]. Under normal conditions, the reducing environment of the cell generates cysteine from cystine.

Part of the rationale for the synthesis of cysteine esters was that their increased lipophilicity relative to cysteine should facilitate their entry into cells, increasing the bioavailability of cysteine. Several esters

of cysteine were effective in protecting against the pulmonary toxicity of inhaled perfluoroisobutene [94] and cysteine isopropylester protected against paracetamol and bromobenzene-induced hepatotoxicity (figs 4.8-13). The proposed mechanism of protection was a direct interaction between reactive electrophiles and the esters and/or cysteine cleaved from esters. However little or no intracellular ester was detected either *in vivo* or *in vitro* experiments, suggesting rapid hydrolysis by esterases. A rat lung slice model and isolated hepatocytes have been used to investigate the role of esterases in the metabolism of CIPE and CCHE, using the organophosphate inhibitors of carboxylesterase activity, paraoxon and bis(4-nitrophenyl) phosphate.

6.2 RESULTS

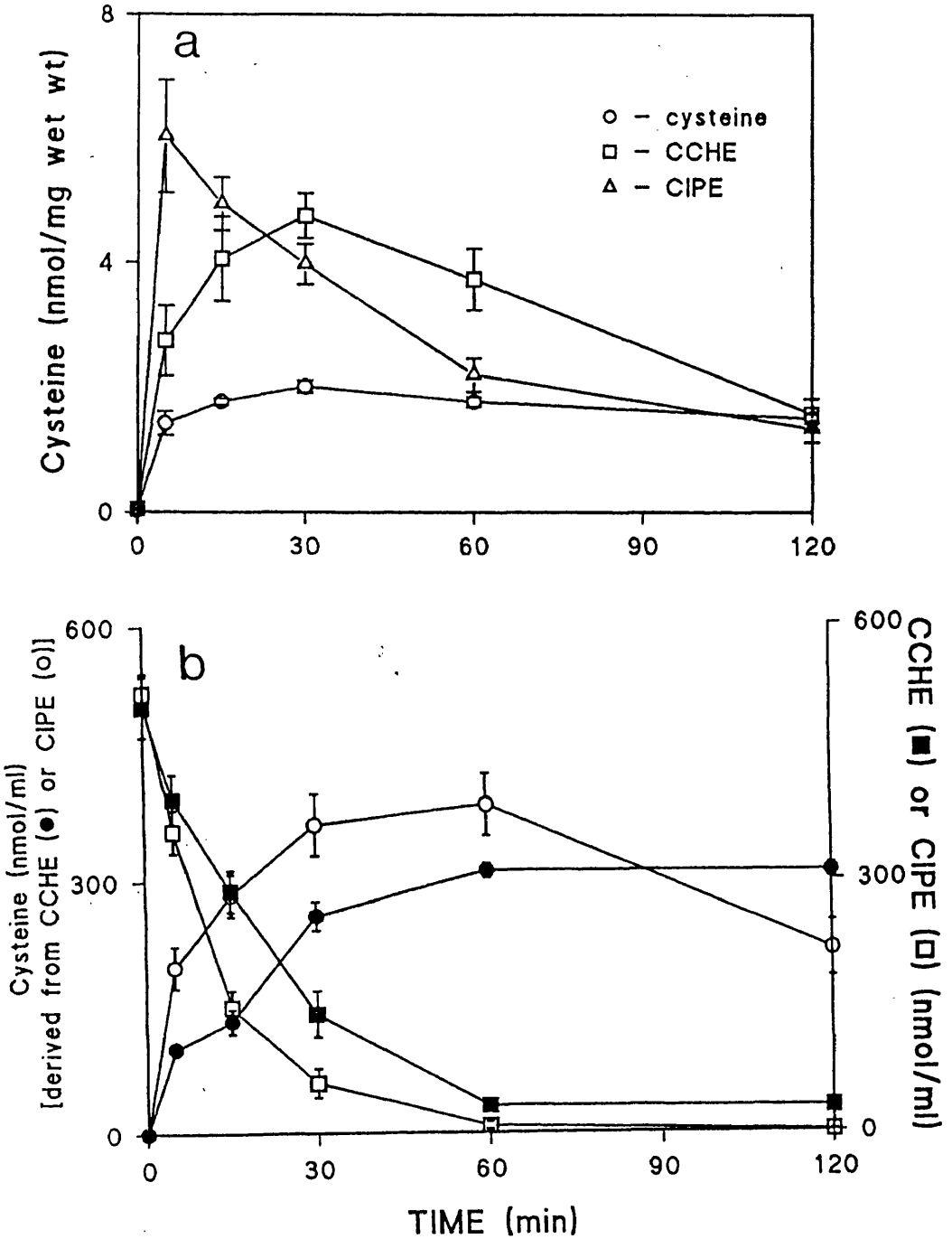
1. Metabolism of CIPE and CCHE by rat lung slices after esterase inhibition

Addition of exogenous cysteine, CIPE or CCHE (500μM) to rat lung slices caused a marked elevation in intracellular cysteine (fig 6.1). The elevation produced by both esters of cysteine were significantly greater than cysteine. The elevation in intracellular cysteine following incubation with both CIPE and CCHE was accompanied by a rapid disappearance of extracellular esters (<10% remained after 60 min) and the concomitant appearance of extracellular cysteine (fig 6.1). Little (≤1% of the CCHE added) or no unmetabolised CIPE was detected intracellularly at any time. It was clear from these results that esterase activity was a major factor effecting the disposition of the esters. In order to investigate this, I used 2 inhibitors of esterase activity bis(4-nitrophenyl) phosphate and paraoxon.

Lung slices were preincubated with both inhibitors for 30 min to inhibit esterase activity. This resulted in a marked alteration in the disposition of the esters (fig 6.2-6.5). The rate of hydrolysis of CIPE to cysteine was reduced, the half life of extracellular CIPE prolonged (fig 6.4) and the rise in extracellular cysteine slowed (fig 6.5). After 60 min, 195±15 nmol/ml CIPE (500μM) remains compared to only 29±5.5 nmol/ml when bis(4-nitrophenyl) phosphate was omitted. Inhibition of the esterase activity slowed the rate of intracellular cysteine accumulation but cellular cysteine levels continued to rise for a longer period of time compared to control lung slices (fig 6.2). Inhibition of esterase activity did not have such a profound effect on the metabolism of CCHE as CIPE, but a similar pattern was observed (fig 6.3-6.5).

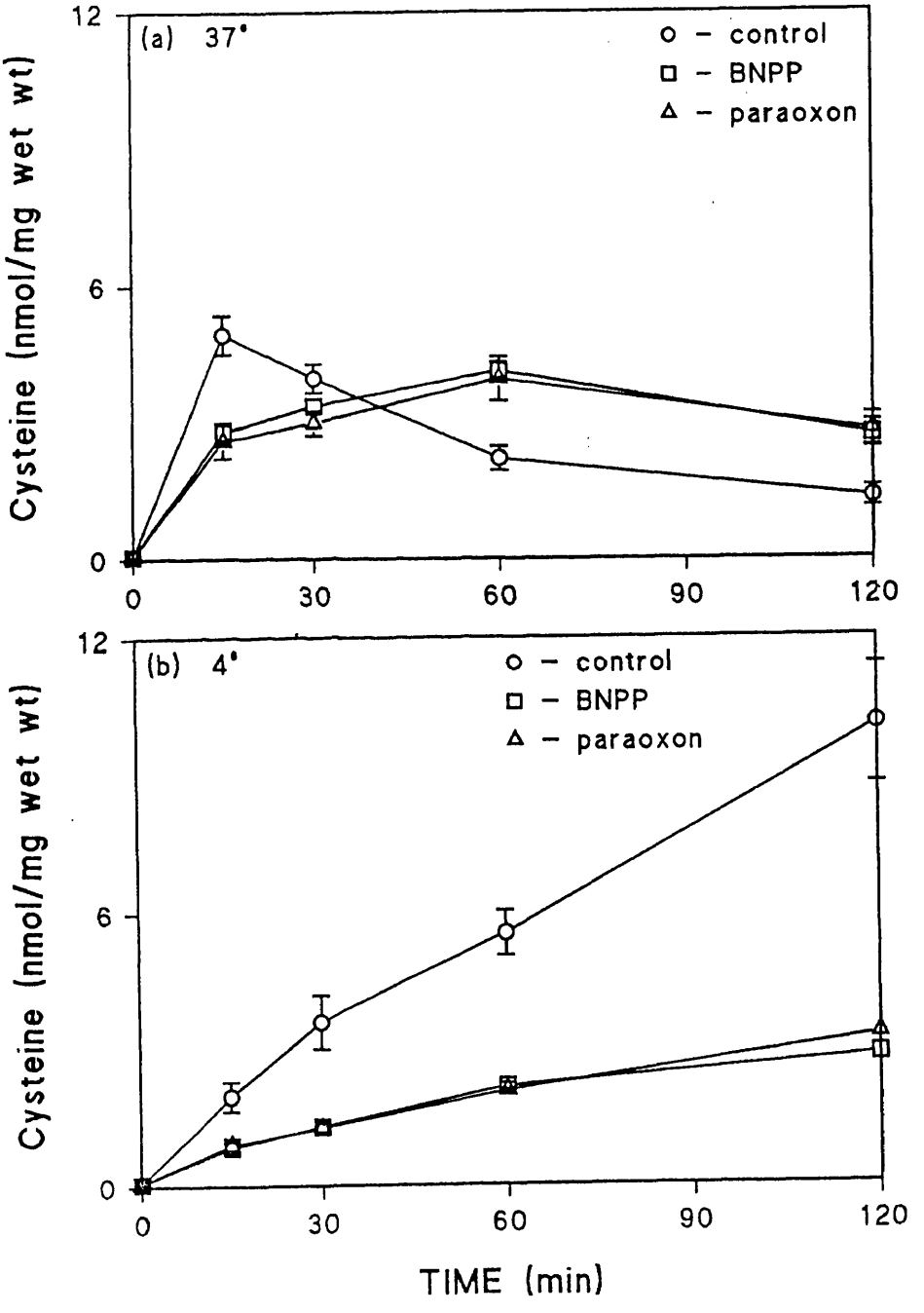
Despite the large pool of extracellular CIPE or CCHE due to esterase inhibition, only a slight elevation in the level of intracellular ester were detected (≤1% of the thiol

FIGURE 6.1 The effect of CIPE, CCHE and cysteine on intracellular levels of cysteine in lung slices



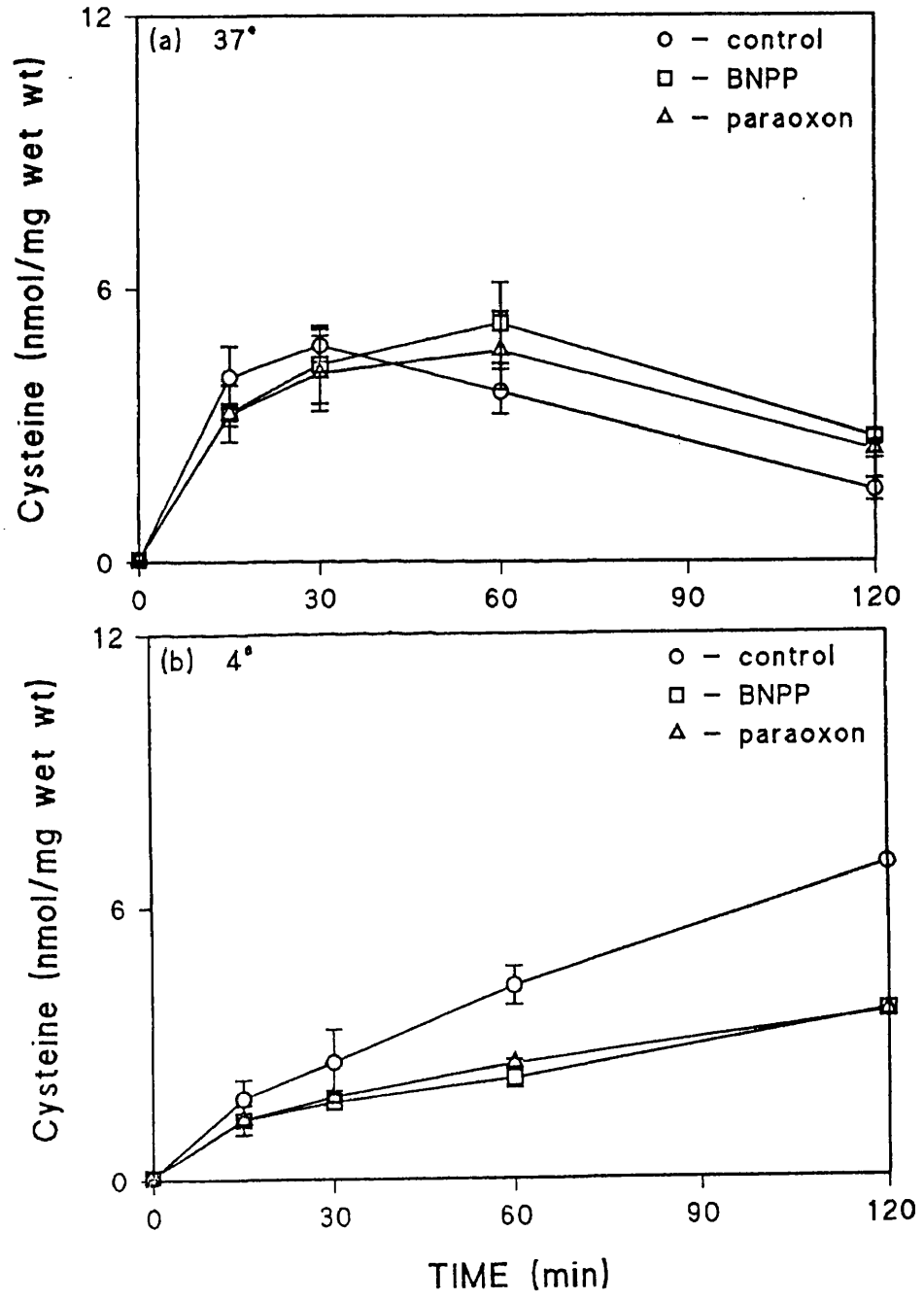
Lung slices were incubated with CIPE, CCHE and cysteine (500μM) for up to 120 min. The intracellular concentration of cysteine (a) and the external levels of cysteine and ester (b) were assessed by HPLC. Each point is the mean (±SEM) (n≥3). All points are significantly greater than control slice levels of cysteine _____, $p<.05$. Intracellular levels of cysteine for CIPE incubated slices are significantly different from those for CCHE at 5 and 60 min, $p<.05$.

Figure 6.2 The effect of paraoxon and bis(4-nitrophenyl) phosphate on the elevation of intracellular cysteine by CIPE at 4° and 37°



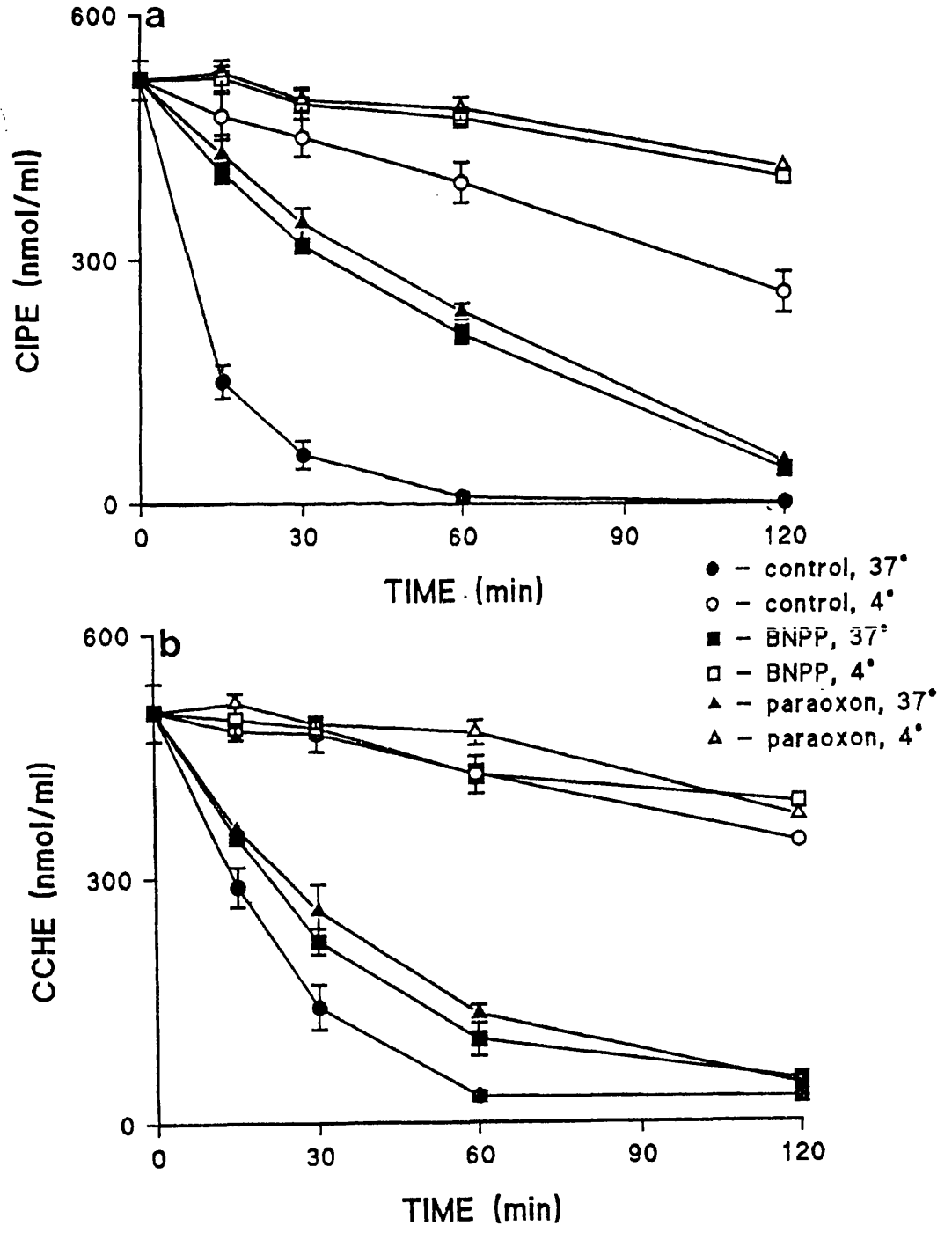
Lung slices received no pretreatment (o) or were preincubated with paraoxon (10μM) (Δ) or bis(4-nitrophenyl) phosphate (200μM) (□) at 37° for 30 min. Slices were transferred to fresh medium chilled to 4° (b) or at 37° (a) with CIPE (500μM) in the presence or absence of esterase inhibitors. Each point is the mean of 4-6 experiments (±SEM) .

Figure 6.3 The effect of paraoxon and bis(4-nitrophenyl) phosphate on the elevation of intracellular cysteine by CCHE at 4° and 37°



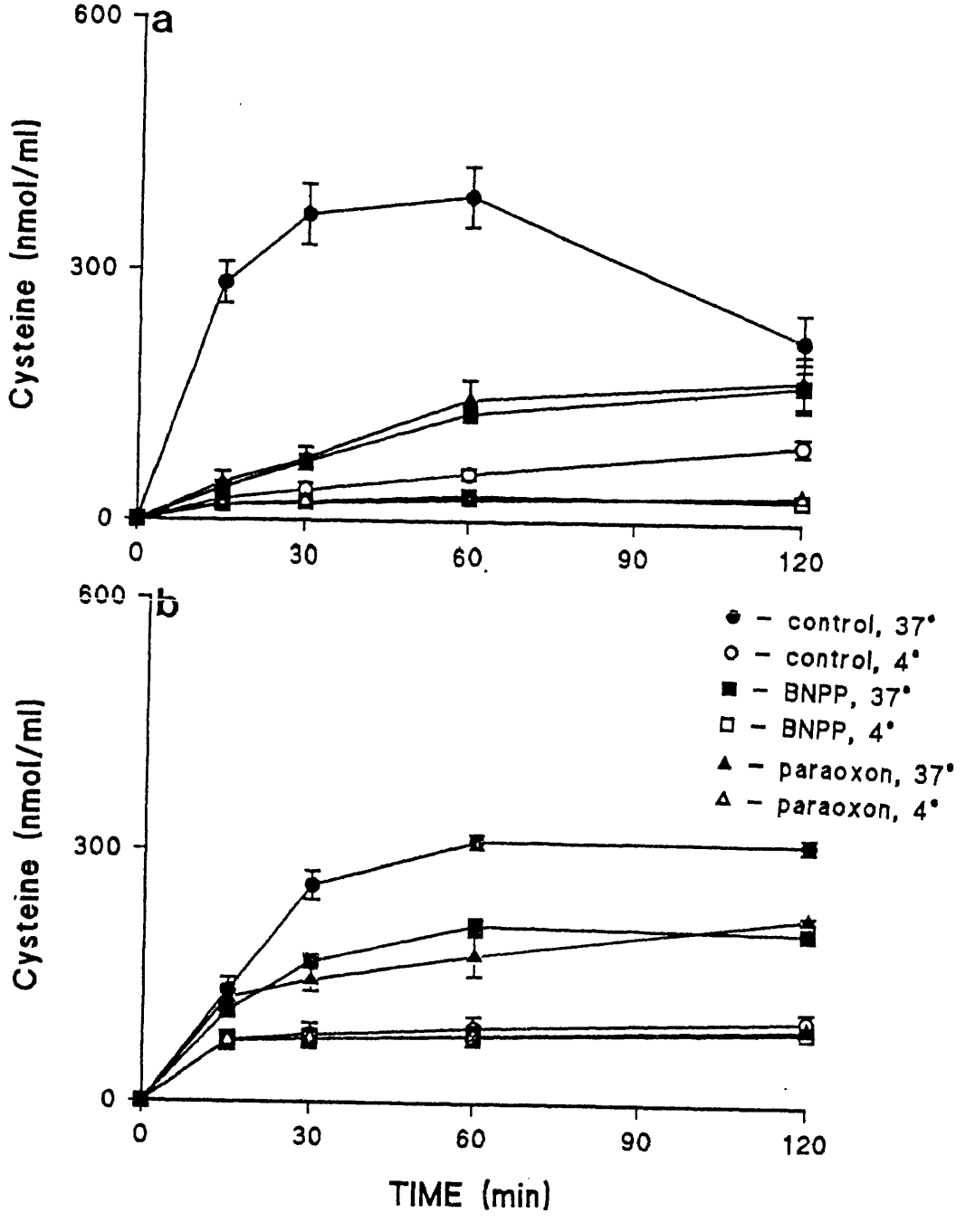
Lung slices received no pretreatment (O) or were preincubated with paraoxon (10μM)(Δ) or bis(4-nitrophenyl) phosphate (200μM) (□) at 37° for 30 min. Slices were transferred to fresh medium chilled to 4° (b) or at 37° (a)with or without CCHE (500μM) in the presence or absence of esterase inhibitors. Each point is the mean of 4-6 experiments (±SEM) .

Figure 6.4 Effect of paraoxon and bis(4-nitrophenyl) phosphate on the disappearance of CIPE and CCHE from extracellular medium of lung slices incubated at 4° and 37°



Extracellular CIPE (a) and CCHE (b) were monitored by HPLC analysis after lung slices were preincubated with paraoxon (10μM) (▲ or △) or bis(4-nitrophenyl) phosphate (200μM) (■ or □) and then incubated with esters of cysteine at 4° (open symbols) or 37° (closed symbols). Each point is the mean of 4-6 experiments (±SEM).

Figure 6.5 Influence of esterase inhibition on the appearance of cysteine in the extracellular medium of lung slices incubated with CIPE or CCHE at 4° or 37°



Lung slices were preincubated with paraoxon (10μM) (▲ or △) or bis(4-nitrophenyl)phosphate (200μM) (■ or □) before being exposed to CIPE (a) or CCHE (b) at 4° (open symbols) or 37° (closed symbols) and extracellular cysteine estimated using HPLC. Each point represents the mean of 4-6 experiments (±SEM). elevation in the pulmonary concentration

elevation
added) (fig 6.6). The rises were considerably less than the^ of cysteine observed when slices with intact esterase activity were incubated with CIPE or CCHE.

2. Elevation of intracellular cysteine by CIPE and CCHE at 4° after esterase inhibition

The very rapid initial rate of cysteine enhancement by CIPE in control lung slices at 37° (fig 6.2) was dramatically slowed at 4°, but nevertheless cysteine was steadily accumulated over a 2 hr incubation period (fig 6.2). The continued rise in intracellular levels of cysteine is most probably due to the continued presence of ester in the medium (fig 6.4). CIPE produced a greater elevation in intracellular cysteine than CCHE at 4° (fig 6.2 and 6.3). At 4° slices with compromised esterase activity, despite high levels of extracellular CIPE or CCHE (fig 6.4), showed significantly lower levels of intracellular cysteine compared to control slices at 4° (fig 6.4) and esterase compromised slices at 37° (fig 6.2). Again no dramatic rises in intracellular CIPE or CCHE were detected at 4° (fig 6.6) levels were ≤1% of the sulphydryl added.

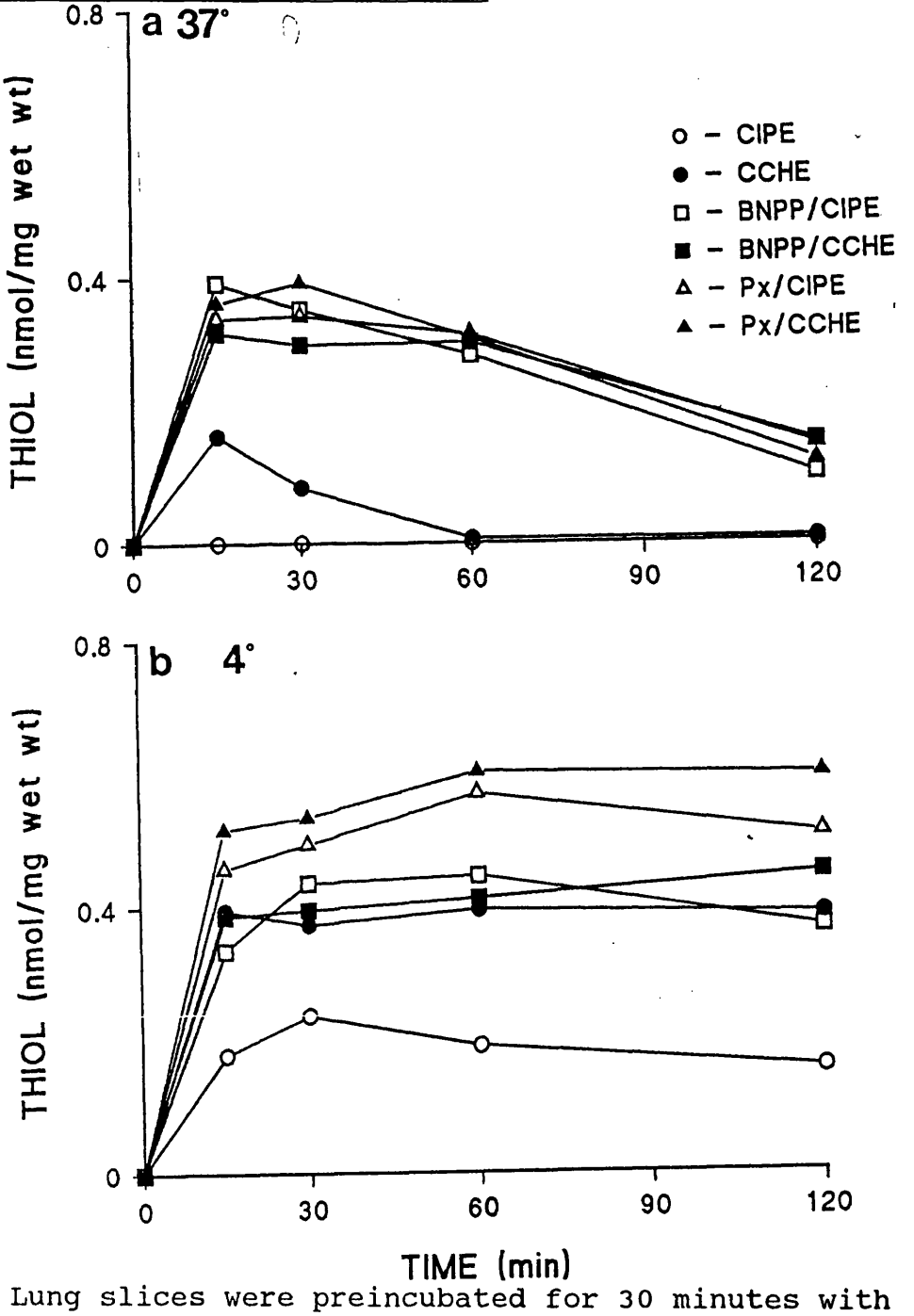
The hydrolysis of CIPE and CCHE (500μM) to cysteine in both control and esterase-compromised lung slices was markedly inhibited when incubated at 4° (fig 6.4). At 4°, extracellular cysteine levels increased slowly (fig 6.5), mirroring the small drop in the level of extracellular ester (fig 6.4). The hydrolysis of CIPE at 4° could be slowed even further by inhibiting esterase activity (fig 6.4).

Slices incubated with cysteine (500μM) at 4° had a reduced capacity to accumulate cysteine from the external medium (fig 6.7) (~50% reduction).

3. Metabolism of CIPE by isolated hepatocytes after esterase inhibition

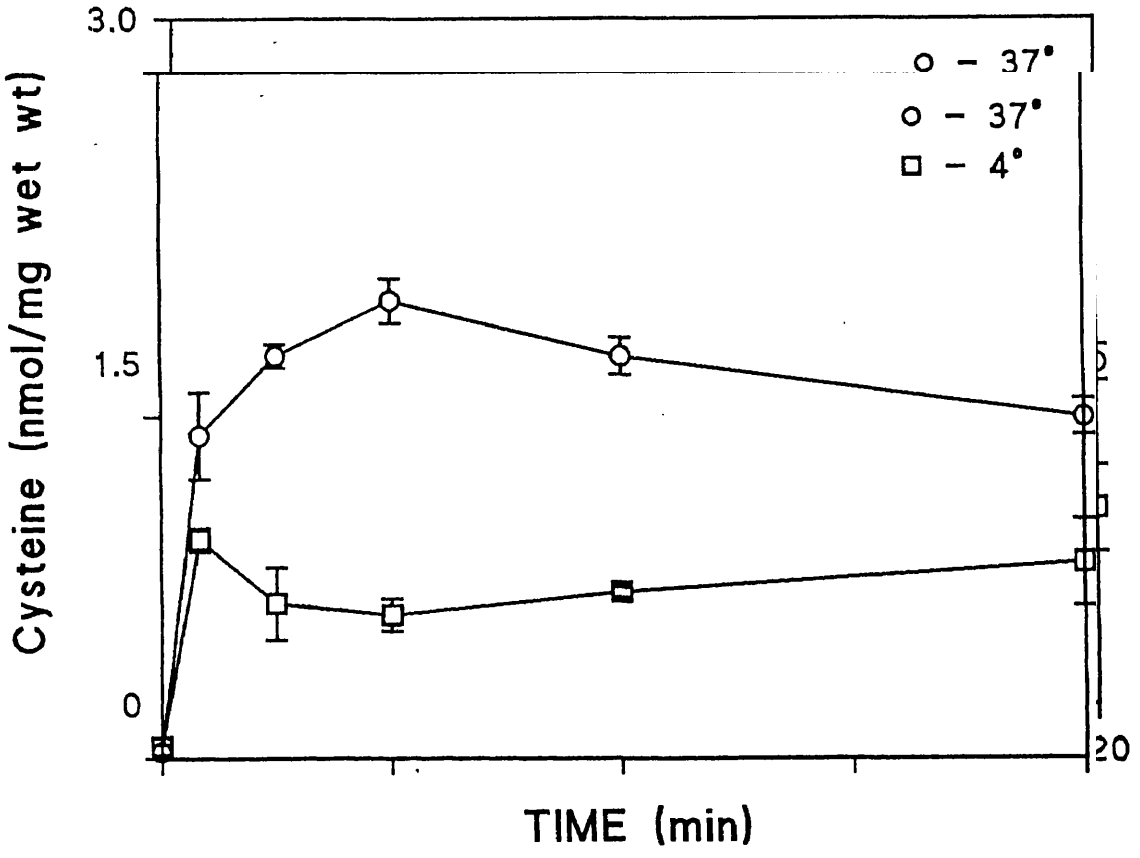
The liver is probably the main site of metabolism *in vivo* [1]. Consequently isolated hepatocytes should be a more relevant model to study the role of esterases in the

Figure 6.6 Elevation of intracellular cysteine esters following incubation of esterase-compromised lung slices with CIPE and CCHE at 4° and 37°



Lung slices were preincubated for 30 minutes with paraoxon (Px) (10μM) (▲ or △), bis(4-nitrophenyl) phosphate (BNPP) (200μM) (□ or ■), or received no pretreatment (o or ●), before being exposed to CIPE (open symbols) or CCHE (closed symbols) (500μM) at 4° (b) or 37° (a). Each point represents the mean of 4-6 experiments. Some error bars are >25% of the mean and for clarity bars have been omitted. (0.4nmol CIPE or CCHE/mg wet lung weight ≈ 0.8% of the initial SH added).

Figure 6.7 The effect of temperature on the uptake of cysteine by rat lung slices



Rat lung slices were incubated with cysteine (500μM) at 37° (O) or 4° (□) for up to 2 hrs. The intracellular cysteine was measured by HPLC analysis. Each point represents the mean ± SEM for 3 experiments. When comparing areas under the curve, slices incubated at 4° accumulated significantly less cysteine than those incubated at 37°, $p<.05$.

disposition of cysteine esters.

The metabolism of CIPE by control rat hepatocytes was extremely rapid. Within 2 min all the ester had been metabolized (fig 6.8c) and appeared primarily as extracellular cysteine (fig 6.8b). The metabolism of CIPE was coincident with a sudden, transient elevation in intracellular cysteine (fig 6.8a), far in excess of the levels achieved when incubated with cysteine alone. Inhibition of esterase activity with bis(4-nitrophenyl)phosphate or paraoxon prolonged the half life of CIPE in the extracellular medium (fig 6.8c) which was accompanied by a slow rise in extracellular cysteine (fig 6.8b) and a prolonged elevation in intracellular cysteine (fig 6.8a). No CIPE was detected intracellularly in control or esterase-compromised hepatocytes.

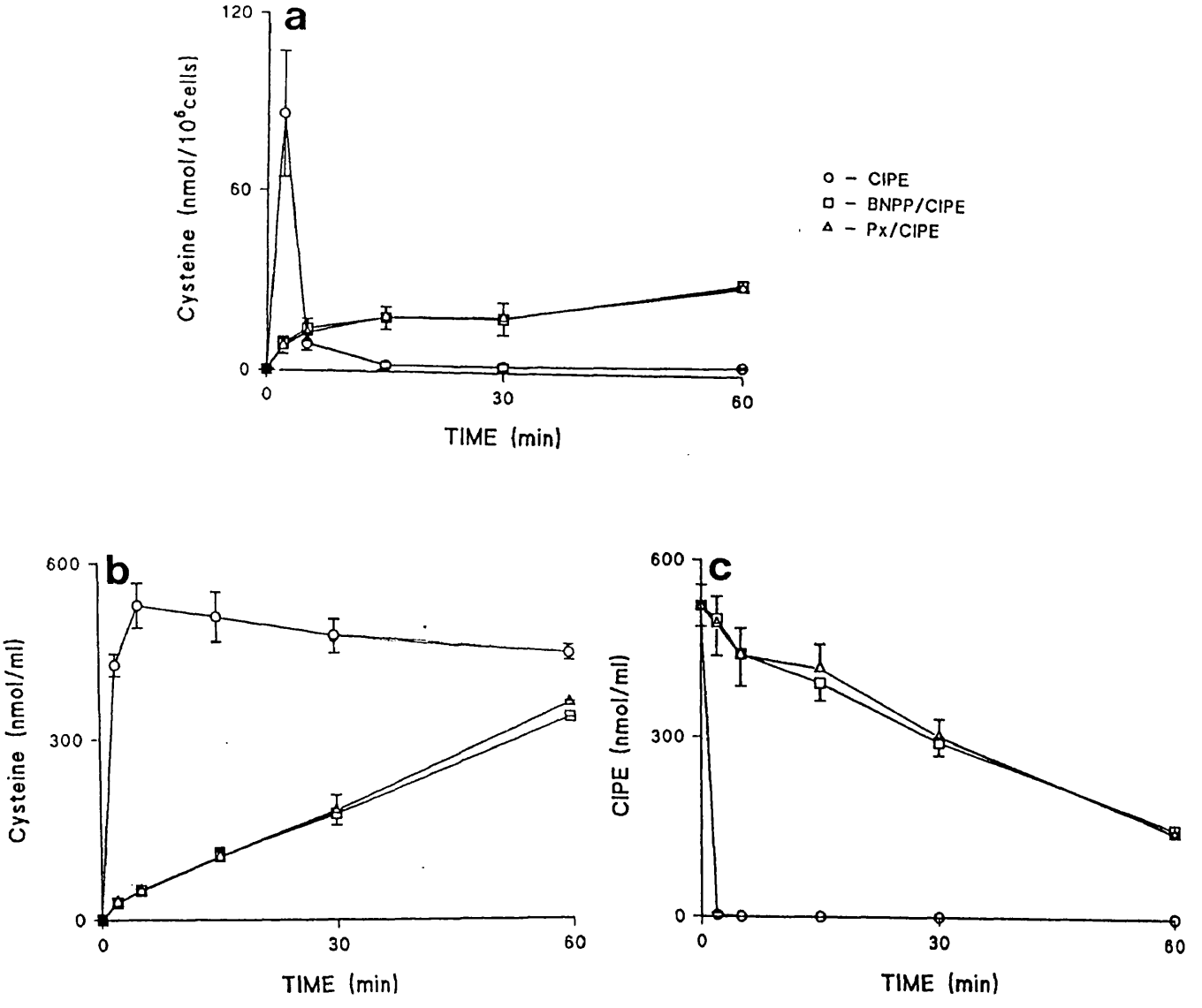
After 2 minutes, control hepatocytes incubated with CIPE were bathed solely in cysteine, which produced a slight elevation of intracellular levels of the amino acid (fig 6.8a). Cellular levels of GSH remained relatively constant over the 60 minute incubation.

All hepatocytes had similar viabilities as assessed by trypan blue exclusion after incubations of 1 hour.

Incubating hepatocytes at 4° had a profound effect on the metabolism of CIPE and especially its ability to increase intracellular cysteine (fig 6.9). Preliminary experiments showed CIPE increased intracellular cysteine to a massive 190nmol/10^6 cells after 15 min. The disappearance of CIPE from the extracellular medium (fig 6.10a and b) was slowed, without an increase in cellular levels of the ester. However after 60 min the majority of the extracellular CIPE had disappeared and the levels of intracellular cysteine declined (fig 6.9).

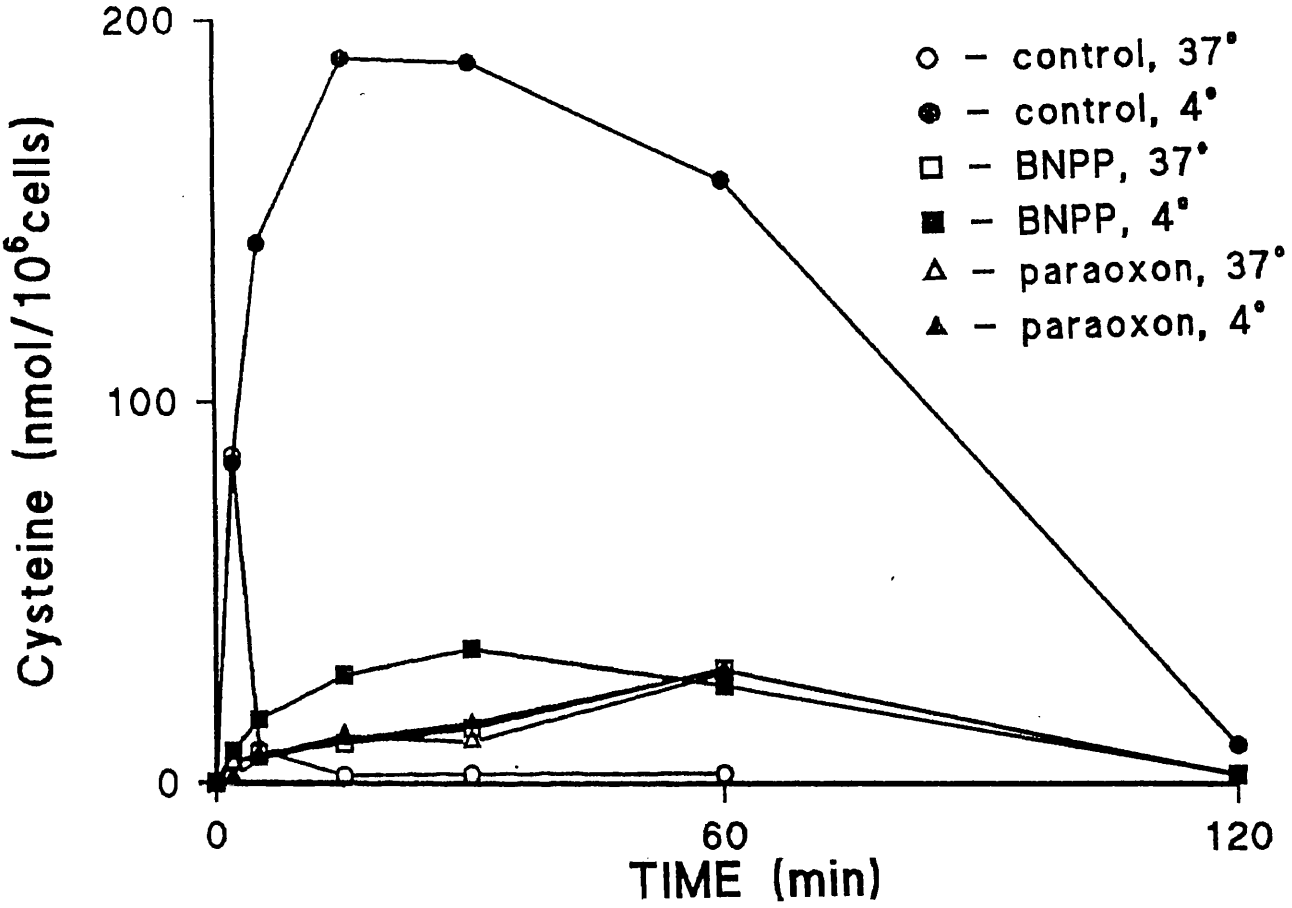
When hepatocytes with compromised esterase activity were incubated at 4° with CIPE, the rise in intracellular cysteine was considerably reduced (fig 6.9). Also the pool of extracellular CIPE persisted for longer and the rate of

Figure 6.8 Metabolism of CIPE by isolated rat hepatocytes with reduced esterase activity



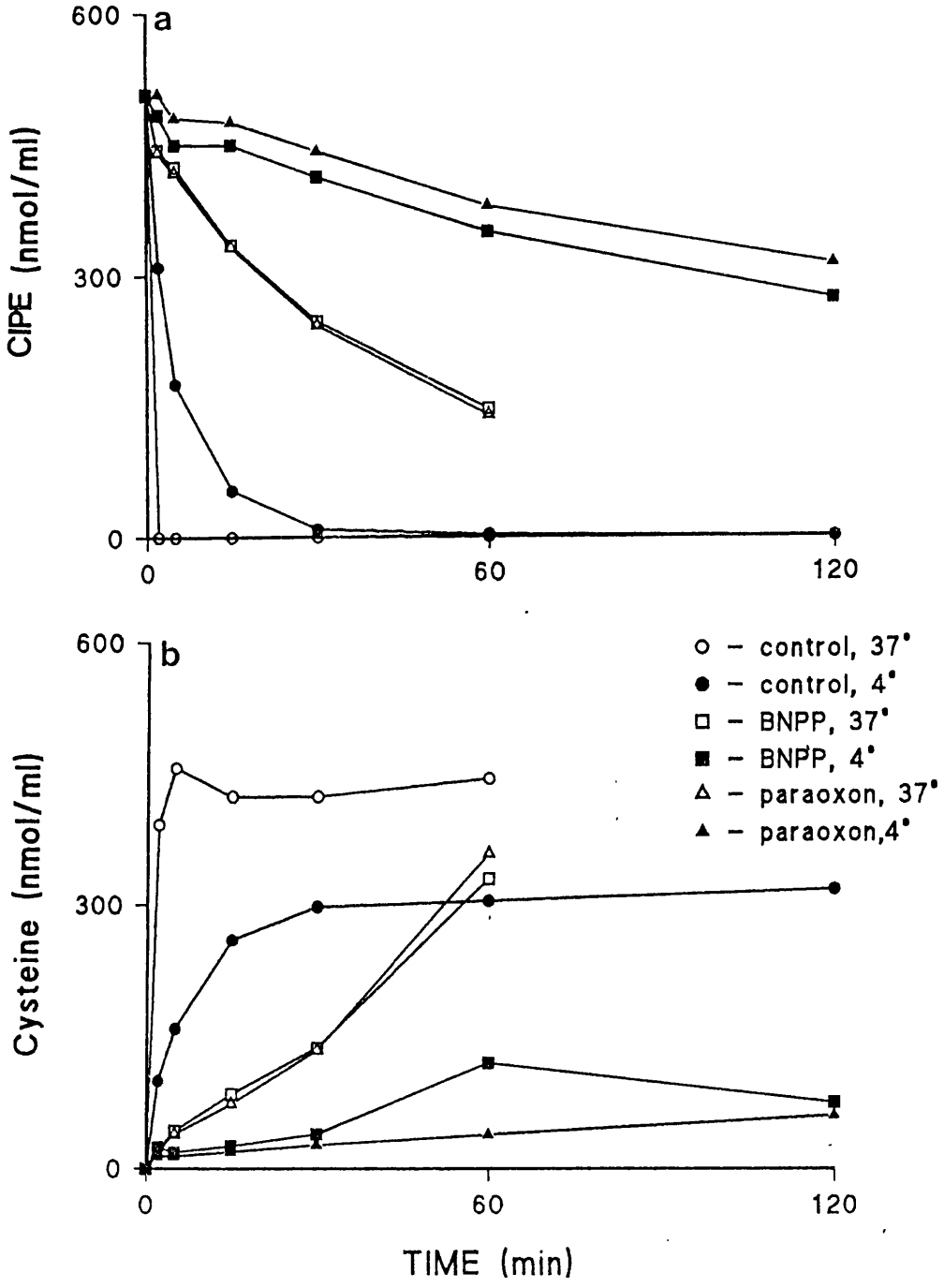
Isolated hepatocytes (1×10^6 cell/ml) were preincubated for 30 min with paraoxon (10μM) (△), bis(4-nitrophenyl) phosphate (200μM) (□) or received no esterase inhibitor (o), before being treated with CIPE (500μM). Hepatocyte levels of cysteine (a) and external levels of cysteine (b) and CIPE (c) were assessed by HPLC analysis. Each point is the mean (±SEM) of three separate experiments. The areas under the curves for external cysteine and CIPE were significantly lower and greater, respectively for esterase-compromised cells when compared to controls, $p<.05$.

Figure 6.9 The influence of esterase inhibitors on the metabolism of CIPE by isolated rat hepatocytes at 4°



Isolated hepatocytes (1×10^6 cells/ml) were preincubated with paraoxon (10μM), bis(4-nitrophenyl) phosphate (200μM) at 37° for 30 min before being exposed to CIPE (500μM) at either 4° or 37°. Cellular levels of cysteine were measured by HPLC. The results are from 1 experiment only.

Figure 6.10 The effect of esterase inhibitors on the levels of extracellular cysteine and CIPE when hepatocytes are incubated with CIPE at 4°



Isolated rat hepatocytes were preincubated with paraoxon (10μM) or bis(4-nitrophenyl) phosphate (200μM) at 37° for 30 min, before being exposed to CIPE (500μM) at either 4° or 37°. Extracellular levels of CIPE (a) and cysteine (b) were measured by HPLC. The results are from 1 experiment only.

hydrolysis to cysteine was further reduced (fig 6.10a and b).

4. Promotion of cellular cysteine by exogenous cysteine and CIPE in the presence of inhibitors of the neutral amino acid uptake

Several tools were used to block the neutral amino acid uptake systems, to investigate the possible role of the uptake systems in the elevation of intracellular cysteine by esters of cysteine and to determine which of the systems, A, ASC or L, contributes to the accumulation of exogenous cysteine by lung slices.

Slices incubated with a range of cysteine concentrations (up to 500μM) produced a linear increase in intracellular cysteine after 15 minutes (fig 6.11). The accumulation of cysteine appeared not to be saturated at 500μM. 2-(Methylamino) isobutyric acid (25mM), a specific inhibitor of the type A neutral amino acid uptake system, had no significant effect on the intracellular concentrations of cysteine (fig 6.11). Lower concentrations of 2-(methylamino)isobutyric acid also had no effect on cysteine accumulation by lung slices (data not shown).

Removing the Na^+ from the extracellular medium, to characterize the ASC uptake system for cysteine, substantially reduced the ability of lung slices to sequester cysteine from the extracellular medium (fig 6.11).

2-Aminobicyclo(2,2,1)heptane-2-carboxylic acid in a Na^+-free environment, an inhibitor of the type L neutral amino acid uptake system, further reduced the ability of cysteine to elevate cellular levels of the amino acid but only to a small extent (fig 6.12).

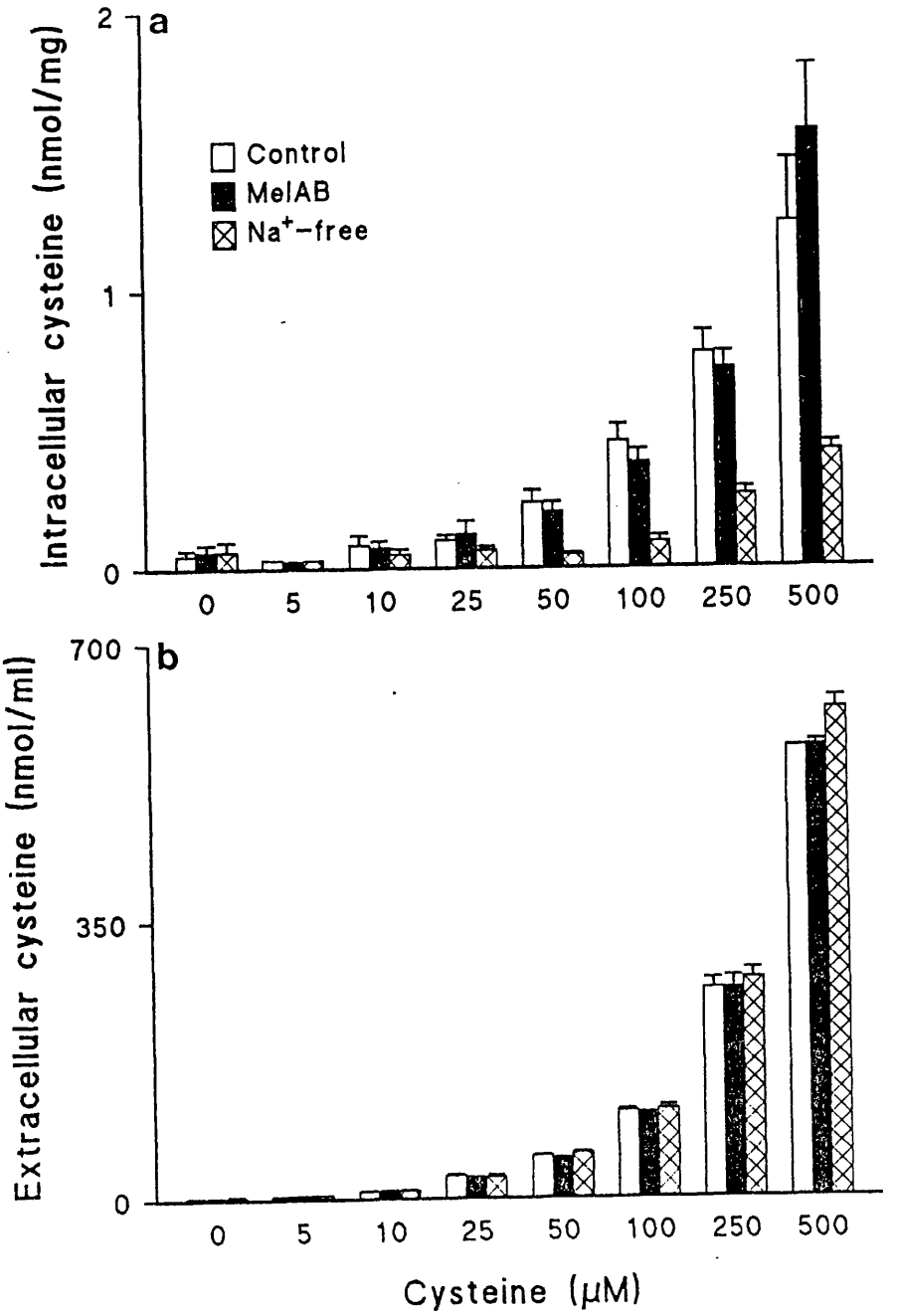
CIPE (up to 250μM) elevated intracellular cysteine levels of slices in a concentration dependent manner and to a greater extent than achieved with cysteine alone (fig 6.13). Coincident exposure of slices to 2-(methylamino)isobutyric acid did not affect levels of intracellular cysteine (fig 6.11). The levels of

intracellular cysteine in slices incubated in Na^+-free medium were not significantly different from the controls (fig 6.13).

5. The effect of paraoxon, Na^+-free medium and 4° on the metabolism of CIPE by lung and liver homogenates

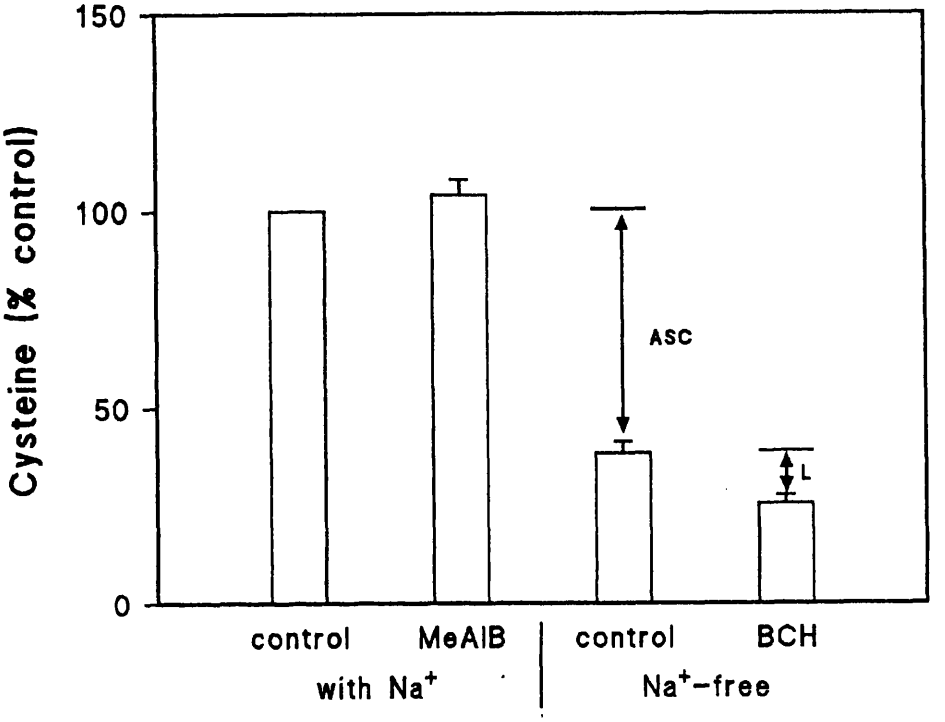
To investigate the possibility that Na^+-free medium may have an inhibitory effect on esterase activity and alter the metabolism of CIPE, the hydrolysis of CIPE by lung and liver homogenates incubated in Na^+-free medium was studied. Paraoxon and incubations at 4° are used as positive controls. At 37°, CIPE was rapidly hydrolysed by both lung and liver homogenates to cysteine (fig 6.14). A similar rate and extent of hydrolysis was observed when homogenates suspended in Na^+-free medium were incubated with CIPE (fig 6.14). Incubating lung and liver homogenates at 4° or with paraoxon slowed the generation of cysteine from CIPE (fig 6.14).

Figure 6.11 The effect of 2-(methylamino)isobutyric acid and Na^+-free medium on the uptake of cysteine by rat lung slices



Slices were incubated with cysteine (up to 500μM) (□) alone or together with either 2-(methylamino)isobutyric acid (25mM) (MeIAB) (■) or in Na^+-free medium (⊠). NaCl was replaced by cholineCl and Na^+phosphate buffer by a K^+phosphate buffer in the medium. Intracellular (a) and extracellular (b) cysteine were measured by HPLC. Bars represent the mean ± SEM ($n \geq 3$).

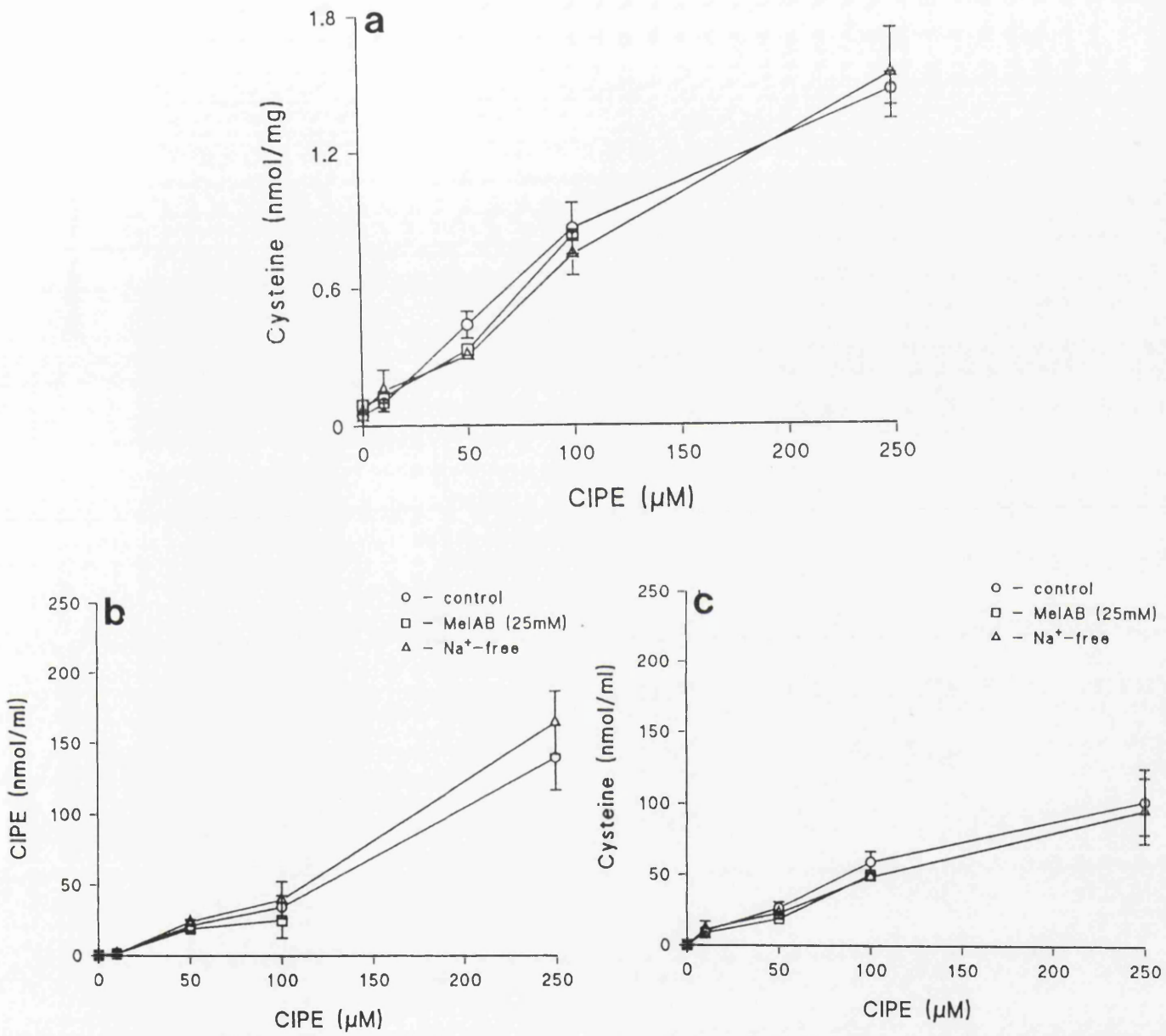
Figure 6.12 Contribution of the A, ASC and L neutral amino acid systems to the elevation of intracellular cysteine by exogenous cysteine



Uptake system	% contribution to cysteine uptake
A	0
ASC	62
L	12
Not known	21

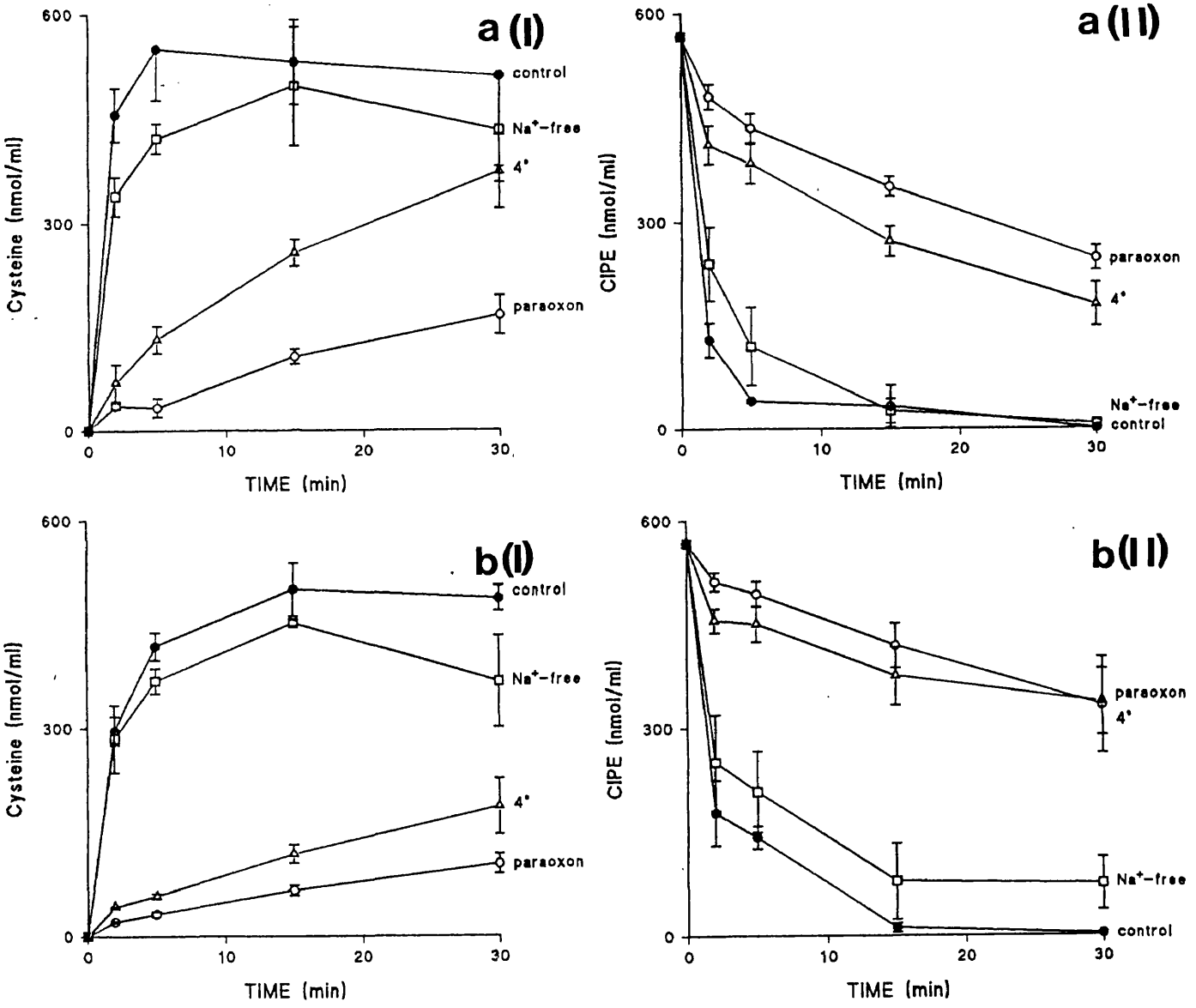
Lung slices were incubated with cysteine (250μM) together with or without 2-(methylamino)isobutyric acid (25mM) (MeIAB), without any effect on cysteine accumulation. Slices were also incubated in Na^+-free medium. The resultant decline in cysteine accumulation was attributed to inhibition of the ASC system. 2-Aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (10mM) (BCH) further suppressed the uptake of cysteine by lung slices incubated in Na^+-free medium. This effect was characteristic of inhibition of the L system for cysteine uptake.

Figure 6.13 Enhancement of intracellular cysteine by CIPE when the A and ASC uptake systems are inhibited



Lung slices were incubated with several concentrations of CIPE (up to 250μM) in the presence or absence of 2-(methylamino)isobutyric acid (25mM) or in Na^+-free medium. After 15 min the intracellular cysteine (a) and external levels of CIPE (b) and cysteine (c) were assessed by HPLC. Each point is the mean of 3 experiments (±SEM).

Figure 6.14 The metabolism of CIPE by liver and lung homogenates



Rat liver (a) and lung (b) were perfused with ice cold KRP and homogenised. Lung (20mg tissue/ml) and liver (10mg/ml) homogenates incubated with CIPE (500μM) after preincubation with paraoxon (10μM) for 30 min or no pretreatment, or in Na^+-free medium or at 4°. Levels of cysteine (I) and CIPE (II) were measured by HPLC analysis. Each point represents the mean of 3 experiments (±SEM).

6.3 DISCUSSION

Inhibition of esterase activity profoundly affected the metabolism of the cysteine esters by both rat lung slices and isolated hepatocytes and demonstrated a critical role for esterase in the disposition of the cysteine esters. Esters had longer half lives in the extracellular medium (fig 6.4 and 6.8) and the speed at which they elevated the levels of intracellular cysteine was reduced (fig 6.2 and 6.8). Esterase inhibition did not greatly increase intracellular levels of the unmetabolised ester.

The esters of cysteine are aliphatic esters and are probably substrates for the carboxylesterases (Type B-esterase) [246]. The liver has the highest concentration of esterase activity towards simple aliphatic compounds, but also the lung has significant amounts of B-type carboxylesterase activity [247]. A number of carboxylesterases have been identified in mammalian liver, within the cytosol and the endoplasmic reticulum, with a wide spectrum of specificities for substrates and inhibitors [246].

Paraoxon and bis(4-nitrophenyl) phosphate act by irreversible phosphorylation of the active site of the enzyme and have an inhibitory effect on a variety of carboxylesterases [248]. Although paraoxon has been reported to be a stronger inhibitor of carboxylesterase activity than bis(4-nitrophenyl) phosphate in isolated enzyme fractions and hepatocytes [248,249,250], no differences were observed between these inhibitors in slowing the rate of hydrolysis of CIPE and CCHE by hepatic and pulmonary esterases (fig 6.4, 6.5, 6.8 and 6.9).

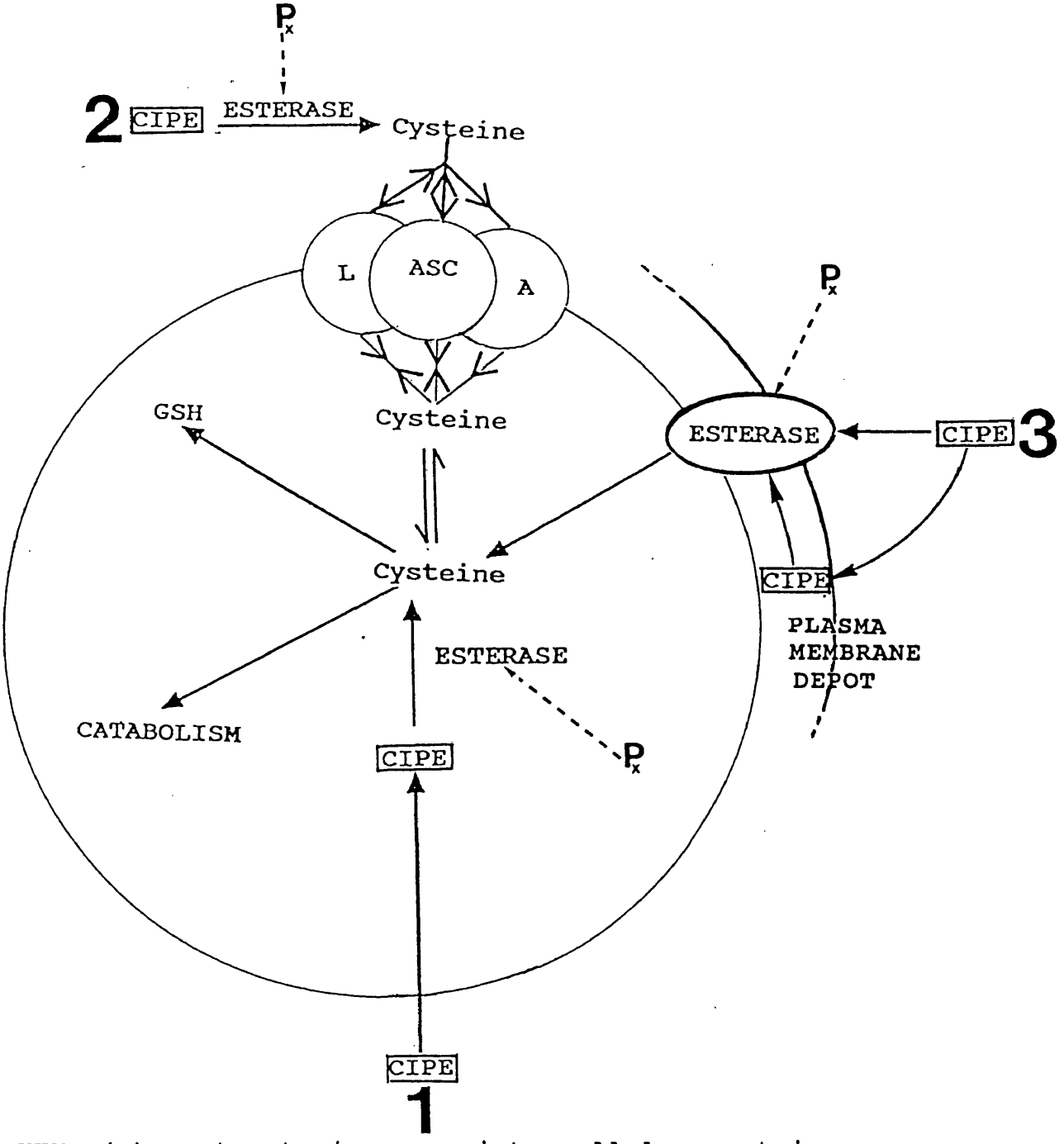
In none of the studies were significant amounts of either ester detected intracellularly. The initial hypothesis was that esterification of cysteine should increase its lipophilicity, so facilitating its cellular accumulation, by obviating the need for cysteine to be accumulated by the neutral amino acid uptake system.

Following uptake, it was rationalised that the esters should be hydrolysed by intracellular esterases, enhancing cellular cysteine levels, in a manner analogous to the elevation of cellular GSH by esters of GSH. The esters of cysteine elevated the levels of cysteine in lung slices to a greater degree than cysteine alone (fig 6.1) and *in vivo* transiently promoted levels of pulmonary cysteine (fig 4.1-2), but without increasing intracellular ester levels. In order to help understand why unmetabolised esters of cysteine are not detected intracellularly, two inhibitors of esterase activity bis(4-nitrophenyl) phosphate and paraoxon were used. These clearly prevented the hydrolysis of CIPE and CCHE (fig 6.4 and 6.8) but they also reduced the efficiency of CIPE to rapidly elevate intracellular cysteine (fig 6.2, 6.3 and 6.8).

There are three possible mechanisms by which esters of cysteine may promote cellular cysteine levels:- (i) their inherent lipophilicity, (ii) cysteine generated from the esters extracelluarly being accumulated by the neutral amino acid uptake system or (iii) possibly by a plasma membrane associated esterase.

Firstly, our initial hypothesis (scheme 1, fig 6.15) that the increased lipophilicity of the esters relative to cysteine, should allow the esters to cross the plasma membrane by diffusion, which following esterase-mediated hydrolysis would release intracellular cysteine. If cellular carboxylesterase activity is inhibited, the levels of intracellular cysteine ester should increase with little or no elevation in the levels of cellular cysteine, dependent on the degree of esterase inhibition. The situation should be similar to that observed with paraoxon-pretreated hepatocytes incubated with acetyl salicylate, when intracellular levels of the ester and salicylate are increased and decreased respectively [250]. However in the present studies, despite high levels of extracellular ester (fig 6.4), no large elevation in intracellular levels of cysteine ester (fig 6.6), to match the ester-mediated rise

Figure 6.15 - Possible routes by which CIPE may enter cells



KEY (a) routes to increase intracellular cysteine:

1 - lipophilicity

2 - extracellular hydrolysis and accumulation via neutral amino acid uptake

3 - esterase-mediated uptake
either - directly
or - via a plasma membrane depot

(b) inhibitor

Px - esterases susceptible to inhibition by paraoxon or bis(4-nitrophenyl) phosphate

in cysteine was observed.

A second possibility (scheme 2, fig 6.15) suggests that the esters are cleaved in the extracellular medium, releasing cysteine, which is then taken up by the neutral amino acid uptake systems (A, ASC or L) [56,62]. In this model, the esters of cysteine should only be as effective as cysteine in elevating intracellular cysteine. This suggestion is not supported by the data in fig 6.1 . In addition, lung slices incubated in Na^+-free medium were less able to accumulate cysteine (fig 6.11) whereas the elevation of intracellular cysteine by CIPE was unaffected by Na^+-free medium (fig 6.13).

A third possibility (scheme 3, fig 6.15) proposes that the esters of cysteine would be cleaved at the plasma membrane, liberating cysteine to the intracellular compartment. Inhibiting esterase activity should both prolong the life span of the ester in the medium and also slow the elevation of intracellular cysteine. The data obtained with both CIPE and CCHE in lung slices and isolated hepatocytes exposed to bis(4-nitrophenyl) phosphate or paraoxon are compatible with this hypothesis (fig 6.2-6.5, 6.8 and 6.9). Inhibition of esterase activity reduced the ability of the esters of cysteine to enhance cellular levels of cysteine, especially at 4° (fig 6.2, 6.3 and 6.9), without a compensatory increase in intracellular CIPE and greatly increased the half life of extracellular esters. I therefore propose that a plasma membrane associated esterase is involved in the elevation of tissue concentrations of cysteine by esters of cysteine. This is a novel physiological role for esterases in the cell, as the majority of carboxylesterase activity is located in the microsomal fraction [247,248].

Linked to the latter hypothesis (scheme 3, fig 6.15), is the possibility of a plasma membrane depot into which the esters could accumulate due to their lipophilic nature, prior to hydrolysis by plasma membrane-associated esterases. This hypothesis would explain the small pool of ester

detected when lung slice esterase was inhibited. If such a plasma membrane depot has a limited capacity, it should be readily saturated. At 4°, the extracellular pool of ester persisted over 2 hr and the apparent intracellular levels of ester quickly reached a plateau (fig 6.6b) (≤1% of the CIPE or CCHE added) which remained static over 2hr. This demonstrates the possible existence of a small saturable pool of ester associated with the lung slices. As the supply of ester wanes, as at 37°, even in the presence of paraoxon or bis(4-nitrophenyl) phosphate, the apparent intracellular levels of ester fell (fig 6.6a).

The vastly reduced levels of intracellular cysteine when lung slices were incubated with cysteine in Na^+-free KRP (fig 6.11 and 6.12), suggested the ASC system for amino acid uptake was largely responsible for the accumulation of cysteine by rat lung. The ineffectiveness of 2-(methylamino)isobutyric acid, a specific inhibitor of the A system [56], was indicative of low or no expression of this system in the lung. This activity increases in response to amino acid starvation [59]. However preliminary experiments with lung slices prepared from rats fasted for 24 hr showed no enhanced accumulation of cysteine or susceptibility to 2-(methylamino)isobutyric acid (data not shown).

2-Aminobicyclo (2,2,1)heptane-2-carboxylic acid further reduced the levels of cysteine accumulated by lung slices incubated in Na^+-free medium (fig 6.12), which suggested the L system may also contribute to cysteine uptake.

The elevation of cellular cysteine by CIPE was independent of the neutral amino acid uptake systems, as 2-(methylamino)isobutyric acid, a Na^+-free environment (fig 6.13) or 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (not shown) had no significant impact on the promotion of cellular cysteine by CIPE after 15 min. Nor did Na^+-free medium slow the hydrolysis of CIPE by liver or lung homogenates (fig 6.14) However, preliminary experiments showed as CIPE was hydrolysed to cysteine, its ability to promote cellular cysteine was diminished by Na^+-free medium

(data not shown).

Lowering the incubation temperature appeared to be almost as effective as inhibiting esterase activity with paraoxon or bis(4-nitrophenyl)phosphate (fig 6.2-6.5, 6.9) in slowing the hydrolysis of CIPE. Incubating hepatocytes and slices at 4° with CIPE or CCHE, forced a massive burden of cysteine on to cells, possibly by modifying the export of cysteine from the intracellular compartment. Lowering the temperature to 20° also retards the efflux of cysteine from kidney cortex slices [251]. Lower temperatures also reduced the accumulation of cysteine by lung slices (fig 6.7), suggesting the activity of the ASC or L uptake systems are sensitive to temperature.

Both ASC and L neutral amino acid systems are capable of being *trans*stimulated, that is the external presence of cysteine will accelerate the exodus of cysteine previously accumulated by cellular systems [56,62]. For example, aminoisobutyric acid, a substrate for the ASC system, *trans*stimulates the export of both aminoisobutyric acid and 2-(methylamino)isobutyric acid from Erhlich ascites tumour cells when external concentrations of aminoisobutyric acid are high [56]. By lowering the temperature to 4°, inhibiting the ASC system, the export of cysteine via *trans*stimulation would diminish and force extraordinary levels of intracellular cysteine to build up.

In summary an important role for esterase activity in the disposition of CIPE and CCHE was observed. Reducing esterase activity prolonged the half life of the cysteine esters, reduced their ability to increase intracellular cysteine but without an elevation of cellular ester levels. I propose a novel role for esterases as plasma membrane bound enzymes which mediate the elevation of intracellular cysteine by the esters of cysteine.

Chapter 7 - Discussion

7.1 Introduction

The aims of this thesis were:

(1) To assess cysteine isopropylester (CIPE) as a chemoprotectant.

(2) To compare CIPE with other cysteine delivery systems in their abilities to increase intracellular cysteine and replenish GSH.

(3) To investigate the role of esterases in the elevation of intracellular cysteine by the esters of cysteine.

(4) To use an *in vitro* lung slice model of paraquat toxicity to assess iron chelators and other compounds as potential antidotes to paraquat. The outcome of these studies are fully discussed in chapter 3.

7.2 Pharmacokinetics of CIPE

The half life of CIPE *in vivo* was very short. CIPE produced a transient enhancement in the level of NPSH in several mouse organs by briefly increasing tissue levels of cysteine. The rise in pulmonary NPSH was particularly outstanding. Administration of cysteine did not elevate pulmonary levels of cysteine to the same extent, so the rise in pulmonary cysteine mediated by CIPE was a characteristic of the ester and not cysteine hydrolysed from the ester (fig 4.2).

7.3 CIPE as a chemoprotectant

Despite the rapid metabolism of CIPE, the elevation of intracellular cysteine prevented the hepatotoxicity of paracetamol and bromobenzene in animals with induced cytochrome P450 activity (fig 4.8-13). Pretreatment of mice and rats with benzo(a)pyrene or phenobarbitone ensured a sudden and rapid burst of electrophile from paracetamol or bromobenzene, which would be coincident with the elevation of cysteine. The cysteine may react directly with the toxic electrophiles or alternatively may sustain the biosynthesis of GSH. However CIPE did not support the replenishment of

hepatic GSH following its depletion by diethyl maleate. *N*-Acetylcysteine did sustain the replenishment of GSH. This would suggest that the main mechanism by which CIPE deflects paracetamol and bromobenzene toxicity is by offering a transient, alternative nucleophilic centre to interact with electrophiles.

In general CIPE appears to offer best protection against electrophilic attacks of short duration, coinciding with the elevated levels of tissue cysteine. It was less effective against compounds capable of redox cycling, potentiating the toxicity of paraquat and 2,3-dimethoxy-1,4-naphthoquinone *in vitro*.

When administered to mice, CIPE did not prevent the morphological changes induced by naphthalene to Clara cells (fig 4.24) but prevented the decline and restored levels of GSH in rat lung slices incubated with naphthalene (fig 5.14). The failure of CIPE to protect *in vivo* is probably due to its short half life relative to that of naphthalene and the transient increase in pulmonary cysteine compared to the more persistent elevation of naphthalene oxide. While in the *in vitro* lung slice model, CIPE had a longer half life and the increase in intracellular cysteine was maintained over 4 hrs, which offered a steady pool of nucleophile to react with naphthalene oxide or support the biosynthesis of GSH.

The esters of GSH have also been used to modulate tissue levels of GSH, to protect against tissue damage mediated by reactive electrophiles. The models used are often weighted to favour the protectant. For example, tissue levels of GSH are usually depleted by pretreatment with buthionine sulphoximine or fasting, in order to show the ability of the esters of GSH to increase intracellular levels of GSH [42-44, 47-50]. Also the esters of GSH are only effective as chemoprotectants when administered prospectively. In contrast, OTZ may be given concomitant with paracetamol or administration delayed, to afford protection [58]. In common with OTZ, the esters of cysteine

were given concomitantly with paracetamol (or bromobenzene) to prevent hepatotoxicity, but unlike the thiazolidine, CIPE greatly elevated intracellular levels of cysteine, again suggesting a possible protective role of the esters by acting as direct nucleophiles. OTZ does not substantially promote cellular cysteine and protects by stimulating the biosynthesis of GSH [29,87].

7.4 Further studies to elucidate the mechanism of protection by esters of cysteine

Several studies could be carried out to enhance the understanding the mechanism by which the esters and cysteine protect against electrophile mediated toxicity. The esters of cysteine could be prevented from contributing to the turnover of GSH, either by inhibiting γ-glutamylcysteine synthetase with buthionine sulphoximine or using the unnatural D-isomer of the esters. If protection against electrophilic attack was still observed, it would point to the esters/cysteine acting as direct chemoprotective agents. Secondly, HPLC analysis of urinary paracetamol conjugates should show an increase in cysteine-paracetamol conjugates, if cysteine directly conjugates NABQI.

7.5 Toxicity of cysteine and CIPE

It was interesting to note the doses of CIPE and *N*-acetylcysteine as low as .75mmol/kg prevented the rise in serum levels of GOT and GPT by paracetamol. Bearing in mind the rapid metabolism of CIPE to cysteine, these doses are several times lower than those used to study the toxicity of cysteine. The doses of cysteine used (10-12mmol/kg, [112, 113]) are not only physiologically excessive but are also probably in excess of the levels required to act as chemoprotectants. In dietary studies, comparing the effects of several amino acids, cysteine produced fatalities [100]. However, the cysteine was given as a drinking solution, as the ethyl ester and solutions only changed every third day [252]. This would allow for oxidation of cysteine to

cystine, giving rise to reactive oxygen species such as H_2O_2 which may have accounted for the toxicity observed [100]. The neurotoxicity of cysteine has been reported in neonatal rats [112] when the blood brain barrier is incomplete. However the way in which cysteine is toxic still remains to be clarified. Induction of cytochrome P450 activity with benzo(a)pyrene enhanced the toxicity of CIPE to mice (table 4.II). This was an odd event as there is no evidence in the literature to support the involvement of cytochrome P450 activity in the metabolism of cysteine. Possibly benzo(a)pyrene pretreatment alters esterase activity, so modifying the pharmacokinetics of CIPE and releasing cysteine at sites especially sensitive to its toxicity or devoid of enzymes for its metabolism.

7.6 Potential therapeutic uses of cysteine esters

Further studies are warranted on the chemoprotective potential of CIPE and other esters of cysteine which are not as prone to hydrolysis by esterases. The esters of cysteine have other potential therapeutic uses. As radioprotectants, administration of ester prior to radiotherapy would transiently increase the sulphydryl content of several tissues, protecting healthy cells from radiation damage. The responsiveness of the immune system in individuals with human immunodeficiency virus (HIV) has been linked to depleted levels of GSH in lymphocytes and the supply of cysteine [253]. Esters of cysteine, by increasing the availability of cysteine, have the potential to replenish GSH, inhibiting HIV transcription and slowing the progression of the disease. However, as esters also have some pro-oxidant activity, their use as therapeutic agents may be limited against inflammatory or degenerative diseases and tumour development which involve the generation of reactive oxygen species.

7.7 Comparison of several cysteine prodrugs as cysteine delivery systems

When compared to several cysteine delivery systems, the esters of cysteine, CIPE and CCHE, produced the greatest elevation of cellular cysteine in rat lung slices (fig 5.1,5.2 and 6.1. After 5 min incubation, CIPE produced over a 100 fold increase in intracellular cysteine, while cysteine produced a 26 and *N*-acetylcysteine a 1.9 fold increase. Yet each of these thiols replenished GSH levels in diethyl maleate pretreated lung slices to a similar extent, despite the large differences in their relative abilities to promote intracellular cysteine (fig 5.10). Even OTZ, which produced no discernable rise in intracellular cysteine, sustained some GSH biosynthesis (fig 5.10). The heterogeneity of cell types in lung slices makes an assessment of the sites at which the esters, *N*-acetylcysteine and cysteine supported GSH biosynthesis difficult. Type II, Clara cells and macrophages isolated from rabbit lung were able to use cysteine as a precursor for GSH, macrophages have the fastest rate of cysteine utilisation, type II cells the slowest [227].

7.8 Novel role of esterases in the enhancement of intracellular cysteine by esters of cysteine

The very low level of intracellular ester even in the presence of inhibitors of esterase activity is puzzling (fig 6.6). Reducing esterase activity by lowering the temperature, or using inhibitors such as paraoxon or bis(4-nitrophenyl)phosphate greatly extended the life span of the esters of cysteine in the extracellular medium of lung slices and isolated hepatocytes but slowed the increases in intracellular cysteine, without a notable increase in intracellular levels of the ester. In chapter 6, the possible novel role of esterases as plasma membrane bound mediators of the increase in cysteine by the esters was proposed and the evidence strongly supports this role. However a problem with such a model appears when considering

the behaviour of the esters *in vivo*. CIPE was administered as an intraperitoneal injection, so in order to reach the circulation, the ester has several membrane barriers to cross. If this process were solely mediated by an esterase then only cysteine would reach the circulation. However, CIPE was detected in the plasma (fig 4.4a) and the kinetics of CIPE were very much different from cysteine administered to mice (fig 4.3). This would suggest that the lipophilicity of the ester relative to cysteine must still play a part in the uptake of cysteine esters and not rely solely on membrane bound esterase activity.

Several esters of GSH are effective cellular delivery systems for GSH. GSH monethyl ester promotes GSH levels after intracellular hydrolysis *in vivo* and in several isolated cell systems [47-54]. The effect of esterase inhibition by paraoxon or bis(4-nitrophenyl)phosphate on the supplementation of cellular levels of GSH bears investigation.

7.9 Summary

1. CIPE has a very short half life *in vivo* and transiently elevates the level of cysteine in several mouse organs.
2. CIPE protects against rapidly generated electrophiles. It prevented the hepatotoxicity of paracetamol and bromobenzene but not the pulmonary toxicity of naphthalene.
3. When compared to several other cysteine prodrugs, the esters of cysteine were best able to promote intracellular levels of cysteine in a rat lung slice model.
4. The activity of esterases plays an important role in the elevation of intracellular levels of cysteine by the esters in *in vitro* models.

Conclusion

Esters of cysteine protect against the toxicity of short lived electrophiles. The protection is probably mediated by the increase in intracellular levels of cysteine, which acts as a direct nucleophilic protectant. However, their application as therapeutic agents will be limited by their kinetics.

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