The Chemistry and Biochemistry of Glutathione and its Role in Mechanisms of Pulmonary Toxicity

John Timothy Skamarauskas

Submitted to the University of London
for examination for the degree of
Master of Philosophy
1991

Toxicology Unit,
Department of Pharmacology,
School of Pharmacy,
29-39 Brunswick Square,
London. UK

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Abstract

Glutathione, the major cellular nonprotein thiol plays a critical role in cellular defences against oxidative stress and in the detoxification of many electrophiles. A major aim of the studies in this thesis was to assess the relative importance of the protective mechanisms of glutathione in pulmonary toxicity.

The roles of glutathione conjugation and redox cycling were assessed in different cell types in the lung. The toxicity of diquat, 2,3-dimethoxy-1,4-naphthoquinone (2,3-diMeO-NQ), paraquat (redox cycling compounds) and 1,4-benzoquinone (an arylating compound) was investigated using an *in vitro* lung slice model with inhibited glutathione reductase activity. Toxicity was assessed using biochemical and functional markers. Studies using inhibitors of glutathione peroxidase and catalase suggest possible differences in protection against oxidative stress between cell types. These studies suggest the possibility that the role of glutathione conjugation and redox cycling may possibly differ between cell types in the lung.

Adenosine accumulation by rat lung slices was investigated as a potential marker for pulmonary endothelial cell function. Adenosine accumulation was shown to be saturable but does not appear to follow simple Michaelis Menten kinetics. After uptake, adenosine is predominantly phosphorylated to form ATP, but a minor fraction is deaminated to form inosine. The use of metabolic inhibitors suggested that the majority of the metabolism might occur in the cytosol. Microautoradiography showed some localisation of the accumulated adenosine in the region of the pulmonary endothelium. The accumulation of adenosine by rat lung slices may prove to be a useful marker of endothelial cell function *in vitro*.

The influence of raised pulmonary glutathione levels on the *in vivo* pulmonary toxicity of α-naphthylthiourea (ANTU) was investigated. It was shown that ANTU is

toxic to both the pulmonary endothelium and epithelium. Raised glutathione levels appear to protect against the endothelial, but not the epithelial, toxicity of ANTU.

Contents

Figures

Tables

Acknowledgements

I would like to thank Dr Gerry Cohen whose wisdom, enthusiasm and support as a supervisor has been a constant source of encouragement during the past two years. Gerry's patient understanding, together with his scientific expertise, has inspired a deeper interest and furthered my curiosity in the field of toxicology beyond which I could have found elsewhere. I would also like to acknowledge the support I have received from Dr David Upshall whose advice in preparing this thesis has been especially valuable.

Special thanks go to Mary Fagg who helped me out of so many desperate situations with the typewriter and the photocopier.

I would like to express my gratitude to Adrian Rodgers whose technical expertise has guided me steadily through my time as a research student, and whose wisdom and support as a fellow christian has helped my own faith to grow.

I have many happy memories of my fellow colleagues in the laboratory and I am grateful for their support. Firstly to Simon Hardwick for our many stimulating conversations, both scientific and non scientific, and whose friendship has helped me on the road to maturity. To Andrew Jenner, whose sense of humour and amusing anecdotes of midnight cycle tours and exploding ferrets have kept my spirits up in times of need. To Adrian Keene for his infectious liveliness and friendly advice, and Michael Butterworth for his occasional but much needed words of wisdom. To Cathy Waterfield, whose sympathetic support has always made me feel better when things have gone wrong, and Mark Raffray, for his wisdom in the ways of this world, and never being without a funny story.

I am also grateful to John Mooney, Winston Morgan, Simin Ghatineh and Brenda M^{c}Cormack for their friendly support and for putting up with my occasional bouts of drunkenness.

There are many people in other departments at the School of Pharmacy who have provided help, expert advice and an occasional friendly ear, for which I am extremely grateful. To Steve, Donna and Mark in the animal house, whose unfailing cheerfulness, efficiency and advice have helped me a great deal. To Dr Peacock, Curt Homeyer and Graham Florence in the computer unit, who introduced me to the world of computing and patiently guided me through the early frustrations of getting to grips with the various programs. To the librarians Linda Lisgarten, Karen, Elaine and Michelle who patiently endured my frequent visits to the library.

Abbreviations

AA	amino acid
ACE	angiotensin converting enzyme
Ada	Adenosine Deaminase
ADA	5'-amino-5'-deoxyadenosine
Adk	Adenylate Kinase
Adn	adenine
Ado	adenosine
ADP	Adenosine 5'-diphosphate
8AG	8-aminoguanosine
AK	Adenosine Kinase
Am A	Antimycin A
AMP	Adenosine 5'-monophosphate
ANTU	α-naphthyl thiourea
APUD	amine precursor uptake and decarboxylation
AS	ATP synthase
AT	3-amino-1,2,4-triazole
ATA	aurintricarboxylic acid
ATG	aurothioglucose
ATP	Adenosine 5'-triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BHT	butylated hydroxytoluene
BSO	buthionine sulphoxime
cat	carboxyatractyloside

CDNB	1-chloro-2,4-dinitrobenzene
Cys	Cysteine (reduced)
DAPP	P1,P5-di(adenosine) pentaphosphate
DEM	diethyl maleate
2,3-diMeO-NQ	2,3-dimethoxy-1,4-naphthoquinone
DMSO	dimethyl sulphoxide
Dpm	dipyridamole
Glu	Glutamine
Gly	Glycine
H_2O_2	hydrogen peroxide
Hpx	hypoxanthine
5-HT	5-hydroxytryptamine (serotonin)
i.p.	intra peritoneal
IMP	Inosine 5'-monophosphate
Ino	inosine
KCN	potassium cyanide
KRP	Krebs ringer solution, phosphate buffered with glucose
MEM	minimal essential medium
MS	mercaptosuccinic acid
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NPSH	nonprotein sulphydryl
ogm	oligomycin
OTC	L-2-oxothiazolidine-4-carboxylate

PCA	perchloric acid
PNP	Purine Nucleoside Phosphorylase
rot	rotenone
SOG	S-octyl glutathione
t-Bu-OOH	*t*-butyl hydroperoxide
TCA	trichloracetic acid
TLC	thin layer chromatography
UV	ultraviolet radiation

Chapter 1

1.1. Toxicology and the Target Organ

1.1.1. Toxicology and the Lung

Toxicology is traditionally defined as the science of poisons [1]. This definition needs to be further qualified, however, in light of the observation that "All things are poisons.... only the dose differentiates between a poison and a remedy" [2]. A more useful definition has been suggested as "The study of the adverse effects of chemical agents on biological systems" [3]. The growth and diversification of toxicology as a science is a reflection of the increasing range and use of man made chemicals for consumption and appearing in the environment. Examples of this are seen in the range of pharmaceutical products for the treatment of disease, and pesticides to increase crop yields by removing parasitic pests [4]. Some individuals, however, choose a more liberal definition of the term pest, thus practising a more traditional form of toxicology - criminal poisoning [5].

Within a particular organism, all tissues are susceptible to the toxic effects of chemicals [6]. Many chemicals exhibit a marked propensity to selectively damage particular organs. These are referred to as target organs of toxicity [6]. As a target organ, the lung has received considerable attention in recent years. This results from the current rapid industrial and technological developments involving the production and use of new products and processes. Increasing prominence has been given to the significance of airborne pollutants such as SO_2, NO_2, cigarette smoke, and occupationally inhaled chemicals and dusts such as solvents, asbestos, cements, glass fibre and silica. The increasingly widespread use of household products in aerosol form such as hairsprays, furniture polishes and insecticides offers a significant

potential for pulmonary damage by inhalation [7].

In addition to the pulmonary changes induced by inhalation, a large number of drugs and chemicals have been found to produce lung damage following systemic administration [8,9,10]. Lung damage may occur specifically or in addition to damage to other tissues. The complexity of the lung in terms of structure, physiology and biochemistry renders it vulnerable to toxicity via the systemic route [12]. Pulmonary toxicity is an increasing problem due to the increasing use of drugs and chemicals which cause selective damage through some specific feature of the lung. Examples of compounds which damage the lung following systemic administration include the herbicide paraquat, which is actively accumulated by the lung where it exerts its effects [12]. The cytotoxic drugs bleomycin, busulfan and cyclophosphamide also cause lung damage by various mechanisms [8,9], and it is often the case that such toxicity is a limiting factor in their therapeutic usefulness [10].

1.1.2 The Lung as a Target Organ of Toxicity

The lung is primarily an organ of gaseous exchange, although attention has been given to its role as a hæmodynamic, immunological and metabolic organ [13-15]. It is uniquely susceptible as a target organ in terms of its anatomical location and physiological functions [12]. The architectural structure of the lung is designed to provide and protect a vast surface area within the chest cavity which allows the effective exchange of respired gases within the bloodstream. This maintains adequate oxygenation of the blood. Consequently, the lung is exposed to noxious gases, vapours and particles (if small enough) present in the inspired air. Even toxins present at very low concentrations in the atmosphere may present a risk to the lung, especially when one considers that the adult lung respires approximately three tons of air per year [11]. In addition, the lung is at a primary site of exposure to toxic xenobiotic

compounds and their metabolites present in the blood, since the total cardiac output passes through the lung [11].

1.2 The Lung: Structure and Function

1.2.1 Anatomy and Cellular Organisation

In gross morphological terms, the lungs in rats are divided left and right. The left lung forms a complete lobe, whilst the right lung is further subdivided into the superior lobe, the middle lobe, the post caval lobe and the inferior lobe (figure 1.1) [17]. The fine structure of the lung, as revealed by light and electron microscopy, shows much complexity in terms of architecture and cellular heterogeneity [18]. This is illustrated in the numerous different cell types in the lung with specific functions. Over forty individual cell types have been identified [19]. This reflects the multifunctional nature of the organ as well as cell-cell interactions which serve to complicate patterns of injury in terms of biochemistry and pathology which may be associated with toxic insult.

The tracheobronchial lining is a tall columnar, pseudostratified epithelium in which at least thirteen different cell types have been identified by electron microscopy [18]. These cell types include eleven epithelial and two mesenchymal cell types. Most of the epithelium consists of ciliated and mucus secreting cells with intra-epithelial corpuscles referred to as neuroepithelial bodies [18]. Ciliated cells are roughly columnar and possess an electronlucent cytoplasm containing ribosomes, rough endoplasmic reticulum and atypical mitochondria [18]. There are about 250 cilia on the luminal surface of each cell. The cilia beat towards the pharynx thus propelling the tracheobronchiolar secretions in this direction [18]. Goblet cells are abundant in the trachea and bronchi [18].

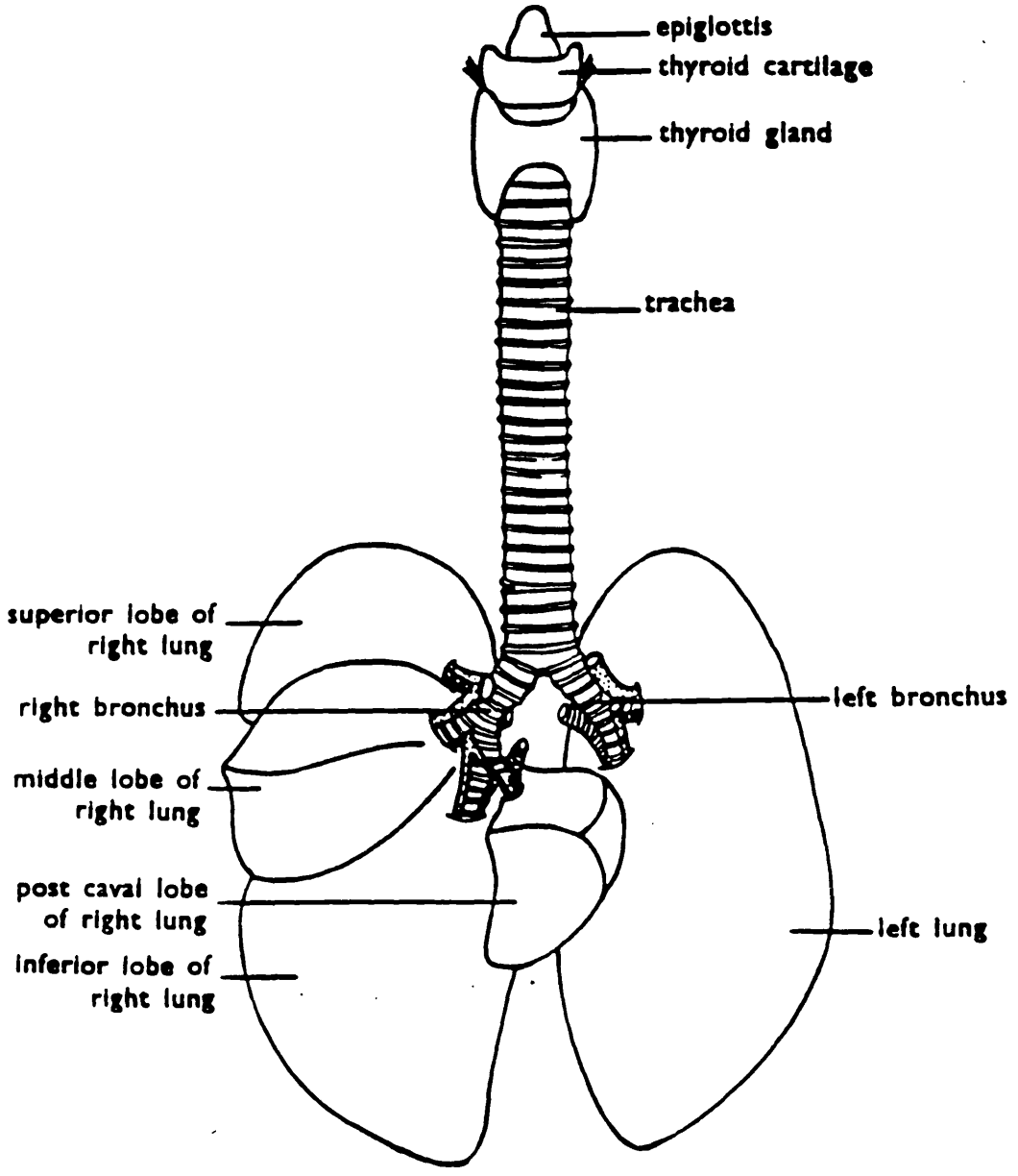


figure 1.1. Diagram showing lobes of rat lung.

The cell is goblet-shaped due to its content of mucous granules. Other cells of the lower respiratory tract include the epithelial serous cell, the 'burst' cell, the basal cell and the K-cell (Kultschitzky cell). The last named cell is believed to function as an APUD (amine precursor uptake and decarboxylation) cell [18]. Although not fully characterised, the cells of the lower respiratory tract have postulated functions incorporating stretch receptors and CO_2 chemoreceptors involved in the regulation of pulmonary circulation under hypoxic conditions [18].

Four important cell types have been investigated with regard to pulmonary toxicology. These are: the non ciliated bronchiolar epithelial (Clara) cell, the type I and the type II pneumocytes and the pulmonary endothelial cell. The epithelium of the bronchioles is composed of low ciliated and taller non ciliated cells, the non ciliated cells being the Clara cells [18]. Light microscopy of the Clara cells show a dome shaped atypical cytoplasm [18]. The cytoplasm contains abundant smooth endoplasmic reticulum and electron dense inclusion granules, although in many species, rough endoplasmic reticulum predominates over smooth endoplasmic reticulum. It is thought that the cytoplasmic granules reflect a secretory function for the Clara cells contributing to a surface active lining layer in the bronchioles (although alveolar surfactant is thought to be produced by type II pneumocytes [20]). The smooth endoplasmic reticulum of Clara cells is believed to be associated with the P-450 dependent mixed function oxidase activity of these cells. When the P-450 isoenzymes are stained for using immunoperoxidase and immunofluorescent methods, it can be shown that the spectrum of P-450 isoenzymes is different to that in the liver [21], thus providing a rationale for selective damage by metabolic activation.

Type I and type II pneumocytes are a set of epithelial cells with defined functions in the lung [13-15, 18, 19, 21]. Type I pneumocytes are highly specialised squamous epithelial cells. They cover most of the alveolar surface with a thin

cytoplasmic layer 0.2-0.5 μM thick [7, 18], thus facilitating gaseous exchange, their primary function. Few organelles can be seen except in the perinuclear cytoplasm [18]. Type I pneumocytes cover more than 90 % of the alveolar surface, although they comprise 8 % of the parenchymal cell population. Type II cells comprise of 16 % of the total lung cell population. Type II pneumocytes function as precursors to type I cells, but they have specialised functions in their own right [16, 18]. An important function of type II cells is the synthesis of surfactant, 2/3 of which is phosphatydylcholine based lipid [16]. It can be shown, using [^{3}H]-palmitate, that fatty acids are taken up and incorporated into phosphatydylcholine in the lamellar bodies of the type II cells. Surfactant is necessary for lung function in order to decrease the surface tension at the air-liquid interface [16, 20]. It is also thought to play a role in the prevention of infection by providing an environment which favours bacterial killing by alveolar macrophages. Type II cells also have a capacity for xenobiotic metabolism by P-450 associated enzymes, although the spectrum of activity found in these cells is more restricted than that found in the liver [21]. One important P-450 enzyme activity that is found in type II cells is ethoxyresorufin-O-deethylase. This activity increases when the cells are pretreated with an inducer such as β-naphthoflavone. A further function of type II cells is transepithelial water movement [16]. This function is necessary to prevent the accumulation of fluid in the alveoli, which would decrease gaseous exchange. This has been investigated in isolated type II cells [16]. Transepithelial water movement in type II cells can be decreased by cooling to 2° C which leads to cellular swelling and the accumulation of intracellular sodium. When the cells are rewarmed to 37° C, the intracellular sodium rapidly decreases with cell volume. It has been postulated that a Na^+/H^+ exchange pathway transports sodium passively into cells while a Na^+/K^+ translocase activity actively extrudes sodium to the basal lateral side [16].

Another cell type of toxicological interest in the lung is the pulmonary endothelial cell [22]. This cell type is typical of capillary endothelial cells in maintaining an active role in vascular homeostasis. The endothelium plays a significant role in arachidonic acid metabolism by the production of prostacyclin, a powerful anti-platelet-aggregating metabolite [23]. The action of endothelial derived prostacyclin is opposed by platelet derived thromboxane [23]. Together, prostacyclin and thromboxane form one of the controlling mechanisms of platelet aggregation [23]. Consequently, a loss of endothelial cell function may be reflected in platelet aggregation and deposition [23]. In the lung, endothelial cells are further specialised in that they are responsible for transport of oxygen to the erythrocytes after diffusion through type I epithelial cells [25]. The location of pulmonary endothelial cells within the pulmonary architecture, together with their specific biochemical and physiological roles, renders them vulnerable to the effects of systemically administered chemicals as well as irritants and high concentrations of oxygen [8, 9, 22, 25, 26, 27]. The inflammatory mediator role of endothelial cells within the lung is thus in a position to be disrupted by the action of irritants and to a number of compounds which appear to selectively compromise these cells.

1.2.2 Biochemistry of the lung

As a complete organ, the lung is metabolically active [28, 29], deriving a significant proportion of its energy from the catabolism of glucose [29]. Lung tissue is also capable of oxidising fatty acids, amino acids, lactate and glycerol [30, 31, 32]. Metabolic energy in the lung is used for producing reducing equivalents (NADH and NADPH), secretion, clearance, biosynthesis, maintenance and repair [29]. Glucose is transported into lung tissue by both active transport and facilitated diffusion [33, 34]. The presence of more than one uptake mechanism for glucose may reflect the

contribution of more than one cell type [29]. Involvement of individual cell types in glucose uptake has not been fully elucidated, although it has been suggested that the epithelium is a likely site for active transport [29].

Glucose is catabolised primarily via the glycolytic and the pentose phosphate pathways [29]. These generate metabolic intermediates for biosynthesis, metabolic energy in the form of ATP, and reducing equivalents, primarily in the form of NADPH. A major fraction of glucose (40 - 50 %) is metabolised to lactate and pyruvate [35]. The distribution between lactate and pyruvate depends upon the redox state of the cell, but typical control lactate : pyruvate ratios are about 10 : 1 in a control situation. A second major fate of glucose is oxidation to CO_2 (22 %) [35]. Of the total CO_2 production, about 1/4 is generated by the pentose phosphate pathway, 1/4 by the action of pyruvate dehydrogenase and 1/2 by the tricarboxylic acid cycle [36]. Other fates of glucose include incorporation of intermediates into protein, nucleic acids, polysaccharides, fatty acids and other lipids. It has been suggested that gluconeogenesis does not occur in lung, hence relying on circulating glucose as a major source of intracellular carbohydrate [29].

The control of glycolysis in the lung is exerted by a range of factors acting in both short and long term [29]. The short term regulation of glycolysis is via the allosteric control of enzyme activities [29]. Two important sources of regulation in this respect are the ATP / ADP+AMP ratio (the cellular energy charge) [29], and the NAD^+ /NADH ratio (the cellular redox state) [29]. The rate of glycolysis increases in response to ATP depletion but ATP is necessary for the initial glucose phosphorylation [29].

1.2.3. Pulmonary metabolism and cytochrome P-450

In addition to intermediary metabolism, the lung also contains enzyme systems that are commonly associated with metabolism of xenobiotics [37]. The most extensively investigated of these systems is the cytochrome P-450 mixed function oxidase system [14, 37, 38]. Pulmonary cytochrome P-450 catalyses the oxidative metabolism of a wide variety of xenobiotics [15]. Studies of this enzyme system have shown a heterogenous distribution of activities between different cell types [21]. The multiplicity of P-450 isoenzymes with overlapping substrate specificities, and their differential induction by different compounds serve to complicate such studies. The sites showing greatest cytochrome P-450 activity in the lung are the Clara cell and the alveolar type II cell [14, 21, 39]. Mixed function oxidase activities have been demonstrated in lung using a number of substrates, including: carbon tetrachloride [40], 1,1-dichloroethylene [41], naphthalene [42], benzphetamine [43], benzo[a]pyrene [44] and 4-ipomeanol [45]. In addition to their properties as substrates demonstrating classical P-450 isoenzyme differences, these compounds are also of toxicological relevance to the lung [37]. Metabolism studies have also shown a differential distribution of isoenzymes within the lung. It has been shown that *in vivo* carbon tetrachloride treatment decreases pulmonary bromobenzene metabolism, whereas *in vivo* naphthalene treatment has no effect [46]. The inference is that naphthalene is preferentially metabolised by Clara cells and bromobenzene is preferentially metabolised by the alveolar epithelium. The differential distribution of cytochrome P450 isoenzymes has also been shown by immunocytochemical studies [21]. Antisera prepared against rat liver cytochrome P-450 isoenzymes are also directed against isozymes found in rat lung. Isoenzymes which have been demonstrated using such antisera include those inducible by β-naphthoflavone, 3-methylcholanthrene and phenobarbital [21].

1.3 PULMONARY TOXICITY

1.3.1. Factors influencing Pulmonary Toxicity

The selective toxicity of chemicals to the lung is influenced by a range of physiological parameters. These include the route of administration of the toxicant, its pharmacokinetic and pharmacodynamic properties and the specific biochemical and physiological characteristics of individual cell types in the lung [11]. The specific susceptibility of the lung may be attributed to the lung's particular profile of isozymes of cytochrome P-450-dependent monooxygenases. This susceptibility may be enhanced by the heterogenous cellular distribution of these enzymes whereby very high levels of activation may take place in certain lung cells [11]. These mechanisms have been well documented in the case of 4-ipomeanol [45], which selectively injures lung cells that are rich in the appropriate cytochrome P-450, namely Clara cells. The factors influencing the selective vulnerability of the lung are summarised below:

1) The route of exposure (i.e. inhalation or via the bloodstream).
2) The mean aerodynamic diameter (particulates).
3) Solubility of inhaled gases (e.g. sulphur dioxide compared with ozone).
4) Selective uptake mechanism (paraquat).
5) Selective metabolic activation (e.g. 4-ipomeanol).
6) Susceptibility of individual cell types.
7) Species susceptibility (e.g. BHT, trialkylphosphorothioates, ANTU, 4-ipomeanol, paraquat).

1.3.2. Chemically Induced Pulmonary Pathology

The lung manifests one of the most diverse ranges of chemically induced pathological lesions of all organ systems [7]. Acute lung damage is typically characterised by the presence of a multiplicity of lesions which serve to complicate the pattern of damage [7]. This complication arises from the differential effects of toxicants on the different cell types, cell-cell interactions as a result of toxic injury, and repair mechanisms. The diversity of chemically induced lesions that can be produced in the lung is shown in table 1.1. Some chemically induced lesions seen in the lung are common to a wide range of compounds whilst some lesions are typically produced by particular compounds [7].

Pulmonary œdema is a common pathological lesion in chemically induced lung damage. The development of pulmonary œdema is a consequence of capillary damage [47]. The endothelial cells represent the first target for toxic agents in the blood and their close proximity to the alveolar air spaces also makes them vulnerable from this route [22]. It is often associated with severe degenerative changes and may occur secondary to such changes. Less commonly, it may present itself as the major finding, or in isolation, as a toxic response. A number of compounds produce pulmonary œdema without degenerative changes, for example, α-naphthylthiourea (ANTU), monocrotaline, adrenaline and ethylchlorvynol. Pulmonary œdema is characterised by the accumulation of fluid within the thoracic cavity. This fluid originates from the pulmonary circulation where the lymphatic drainage of the lung becomes insufficient to drain away fluid leaking from the pulmonary vasculature. Leakage usually occurs as a result of the formation of gaps between the endothelial cells in the pulmonary vasculature. Œdema fluid may be described as serous ('watery'), fibrinous (proteinaceous, with a tendency to clot), or hæmorrhagic depending on the severity of damage [48].

Table 1.1: Chemically induced lesions of the lung

pulmonary œdema
pulmonary congestion
alveolar hæmorrhage
diffuse alveolar damage
interstitial fibrosis
intra-alveolar fibrosis
alveolar epithelialisation
alveolar adenomatous hyperplasia
lipoproteinosis
phospholipidosis
emphysema
embolism
interstitial histiocytosis
aspiration pneumonia
pneumonitis

Diffuse alveolar damage is a term used to describe severe degenerative lesions, usually associated with alveolar epithelial cell damage [49]. Such lesions involve necrosis or injury to alveolar epithelial cells with subsequent further degenerative change, repair or inflammatory response. Pulmonary congestion is often a secondary response to diffuse alveolar damage, commonly associated with injury to the type I pneumocyte, and is accompanied by endothelial changes owing to their close proximity. A wide range of compounds produce this type of lung damage, including hyperbaric oxygen [26], NO_2, ozone [12], nickel carbonyl [50], paraquat [51], phosgene [52], bleomycin [53], BHT [54], O,O,S-trimethyl-phosphorodithioate [55], and cyclophosphamide [56]. If the basement membrane is disrupted, exudation from the capillaries which follows produces an inflammatory reaction. When exudation becomes more severe, fibrin deposition may occur in the alveolar walls. Subsequently, infiltration of the alveoli with inflammatory cells, cell debris, macrophages and fibrin may follow [48].

Epithelialisation of the alveolar walls is characteristic of a repair mechanism involving proliferation of type II cells [57]. This is a response to alveolar wall damage where the basement membrane remains intact following the initial damage. A period of epithelialisation then occurs in which the alveolar walls become lined by a continuous sheet of cuboidal type II pneumocytes [57]. These type II cells may subsequently differentiate into type I cells. If the epithelialisation occurs in excess of that required to maintain repair mechanisms, then this is referred to as hyperplasia [48]. Type II cell hyperplasia is seen in the mouse in response to BHT [54].

Pulmonary fibrosis is the abnormal deposition of collagen in the lungs [58]. It usually occurs subsequent to chronic inflammatory and degenerative changes, and involves the secretion of an extracellular collagenous matrix by fibroblasts [58]. It may be speculated that abnormal basement membrane accumulation plays a role in

the development of pulmonary interstitial fibrosis [7].

1.4 Mechanisms of Pulmonary Toxicity

1.4.1 Metabolic Activation

The interaction of xenobiotics in a particular organism often results in some chemical change. For a reactive compound, chemical changes may occur spontaneously or, more commonly, may be enzyme-catalysed [59]. In such cases, the chemical change is referred to as metabolic activation [14]. This is an important concept in understanding why compounds may selectively damage the lung. There are several consequences of metabolic activation.

1) The rate of elimination of the compound is changed, usually increased.
2) Levels of endogenous compounds *e.g.* coenzymes, may be depleted or otherwise altered.
3) Harmful, endogenously derived by-products may be formed *e.g.* oxygen radicals.
4) Formation of a stable metabolite with the appearance of or change in some pharmacological or toxicological activity.
5) Formation of a chemically reactive species *e.g.* a free radical, which can covalently bind to proteins and other essential constituents.

It is not always clear, however, as to whether metabolic activation results in a chemically reactive compound, or a chemically stable but biologically active compound.

The relationship between metabolic activation and cell damage is not fully

understood, and it appears that there is no single common mechanism [37]. Two general pathways of activation have been proposed. Firstly, an inert substance may be converted to a reactive electrophilic intermediate, capable of interacting with nucleophilic centres within the cell. Secondly, the reduction of an inert chemical can produce an electron rich nucleophile which may reduce critical cellular components [37].

The most widely investigated enzyme system involved in metabolic activation is the cytochrome P450 dependent mono-oxygenases [38]. These have been shown to metabolise a wide variety of compounds including furans [60], bromobenzene and polycyclic hydrocarbons [61] to highly reactive intermediates. Other activating systems have been implicated in pulmonary toxicity. Prostaglandin synthetase has been shown to activate benzopyrene via prostaglandin hydroperoxy endoperoxide G_2 which could co-oxidise benzopyrene and its 7,8-diol to reactive epoxides [62]. A flavin-containing mono-oxygenase, in addition to P450, has been shown to be involved in the oxidative metabolism of amines and organic sulphur compounds [63]. NADPH-cytochrome P450 reductase has been implicated in the toxicity of a number of compounds including paraquat [62], diquat [63] and a number of quinones [64]. This enzyme has been shown to transfer single electrons to certain substrates resulting in the formation of free radicals which reduce molecular oxygen, forming toxic oxygen species [64].

Metabolic activation has been proposed to damage the lung by three principle mechanisms [13]. These are summarised in figure 1.2. Firstly, the parent compound remains inert until it reaches the lung where activation takes place *in situ* leading to lung damage.

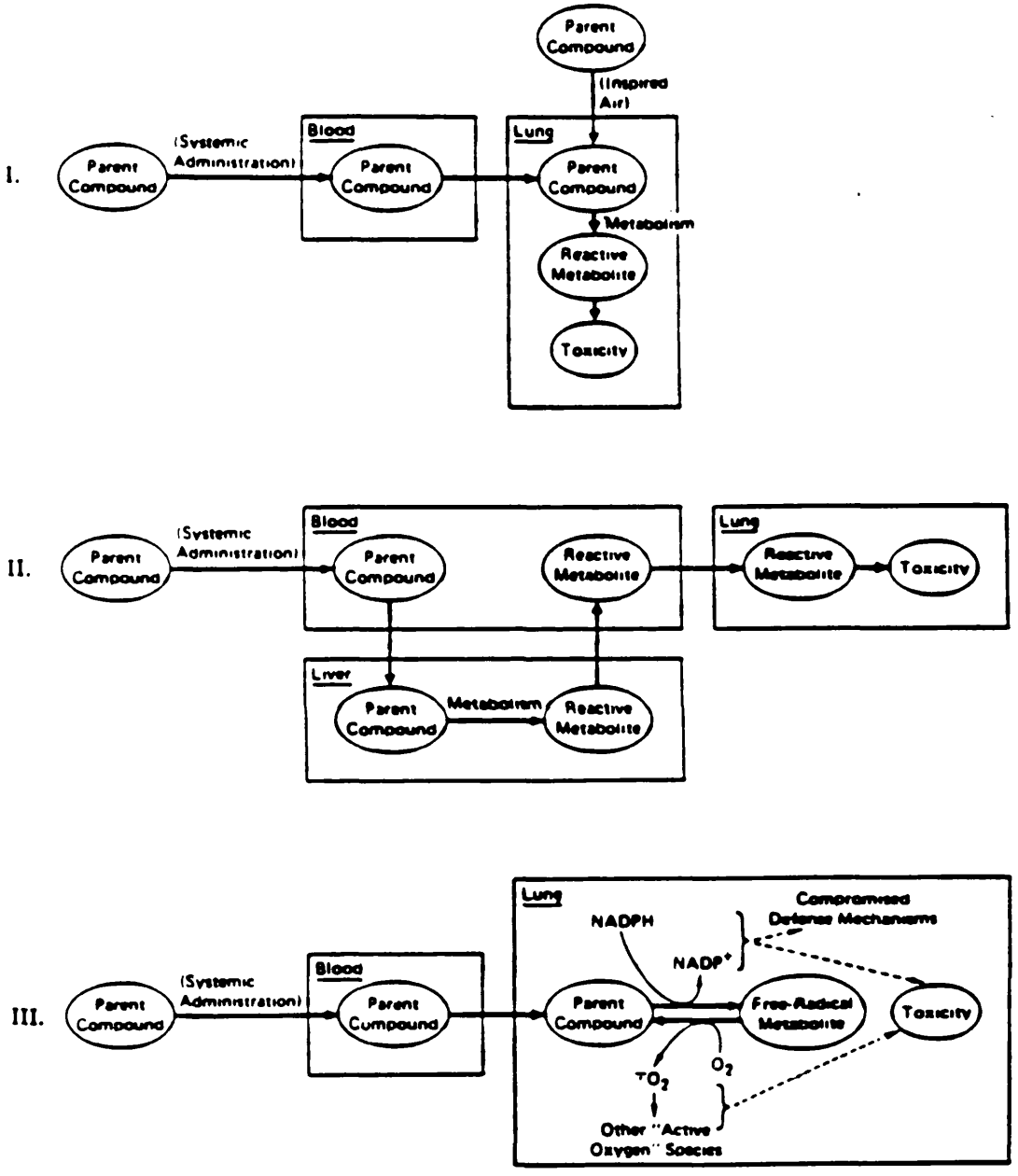


figure 1.2. Diagram showing three proposed mechanisms of pulmonary toxicity

Secondly, activation to an active metabolite takes place in an organ other than the lung (*e.g.* the liver) such metabolites then reach the lung where damage occurs due to direct interaction or further metabolism may take place leading to damage. Thirdly, the compound reaches the lung and cycles between the parent compound and a free radical metabolite at the expense of NADPH, resulting also in the production of toxic oxygen species. The third mechanism is discussed in greater detail in the section on redox cycling. Examples of compounds that damage the lung via these mechanisms are given in table 1.2.

1.4.2 Free Radicals

Pulmonary toxicity can be mediated via free radicals [13]. Free radicals are species capable of independent existence that contains one or more unpaired electrons [69, 70]. The overall charge on the molecule is independent of electron pairing, hence radicals can exist as ions or in the electrically neutral state [69, 70]. Free radicals can result from the homolytic fission of a chemical bond due to thermal homolysis or high energy reduction or photolysis, or by a one electron reduction reaction [71]. Such reactions are commonly encountered in chemistry, for example, The chlorine molecule (Cl-Cl) can be homolytically cleaved with ultraviolet radiation or by high temperatures to form short-lived chlorine free radicals (Cl·) [72].

The most significant free radicals, relevant to biological systems, are those derived from molecular oxygen [69, 70, 73]. In the ground state, oxygen contains two unpaired electrons of parallel spin, each occupying one orbital [70]. The addition of spin-paired electrons from other compounds, in accordance with the Pauli Exclusion Principle, requires the spin inversion of one electron to avoid parallel spin electron occupancy of one orbital [70, 72].

Table 1.2: Targets and Mechanisms of Pulmonary Toxicity

Compound	Primary Target	Species	Probable Mechanism	Ref
BHT	Type I cells	Mouse	I or II	[65]
Bleomycin	Type I and II cells	Mouse	-	[53]
Bromobenzene	Clara cells	Mouse, Rat	I or II	[61]
Carbon Tetrachloride	Clara cells	Rat, Guinea Pig	I	[40]
1,1-Dichloroethylene	Clara cells	Mouse	I or II	[41]
4-Ipomeanol	Clara cells	Rat, Mouse, Guinea Pig, Hamster, Rabbit	I	[13]
3-Methylfuran	Clara cells	Rat, Hamster	I	[13]
Nitrofurantoin	Type I and II cells	Rat	III	[13]
Naphthalene	Clara cells	Mouse	I or II	[61]
Paraquat	Type I and II cells	Mouse, Rat	III	[66]
Pyrrolizidine	Endothelium	Rat	II	[13]
BCNU	Endothelium	Mouse	---	[67]
Oxygen	Endothelium	Rat, Mouse, Human	---	[26, 27]
N-Nitrosobis-(2-hydroxy-propyl)amine	Neuroepithelial cells	Syrian Golden Hamster	---	[68]

This inversion, however, is energetically unfavourable and hence is a limiting factor in the reactivity of the oxygen molecule. A consequence of this is that univalent reduction of oxygen is favoured over divalent reduction hence the formation of the superoxide ion radical [75]. It has been suggested that the superoxide ion radical is not the major oxygen radical damaging species [76]. It is not indiscriminately reactive, although some reactions may occur with reference to cell injury [76]. To illustrate this point further, $O_2^{\cdot-}$ does not react appreciably with free NADH in solution, but when NADH is bound to lactic dehydrogenase, it reacts to form the NAD· radical. Thus a catalytic oxidation of NADH could occur [77].

One reaction pathway of the superoxide ion radical, which has biological significance, is the reduction of the Fe^{3+} ion, followed by the Fenton reaction, to form the hydroxyl radical OH·. These reactions are summarised in terms of the iron catalysed Haber-Weiss reaction:

$$\mathbf{O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot}$$

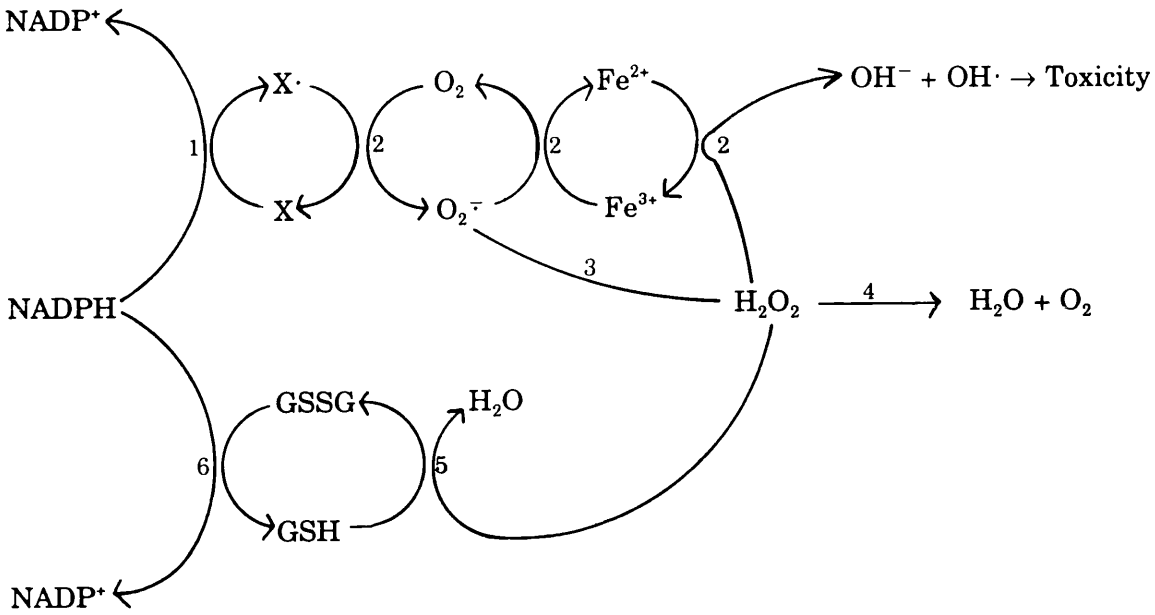
The hydroxyl radical is an extremely reactive species in comparison to the superoxide ion radical, reacting with most organic species at very high rate constants (typically 10^8 - 10^{11} M^{-1} s^{-1} for a range of compounds) [70]. Three types of reaction have been described with relevance to biological systems: hydrogen abstraction, addition and electron transfer [70]. Hydrogen abstraction is the removal of hydrogen from a donor molecule to form water and an organic free radical. Rearrangement of the radical to a lower energy form may then take place. Organic free radicals may themselves participate in hydrogen abstraction from adjacent molecules, giving rise to chain reactions [69-71]. Reactions of lipid radicals, formed in this way, with molecular oxygen can lead to lipid peroxidation [70, 78, 79]. Radical reactions may be

terminated by collision with another radical in by addition onto the molecule [70, 78, 79].

1.4.3 Redox Cycling

Redox cycling is the term used to describe the non stoichiometric oxidation of NADPH, and concomitant oxygen consumption, in relation to the compound present which elicits the process [80]. This results from an initial one electron reduction of the compound, followed by a subsequent oxidation, via molecular oxygen, to form the parent compound and the superoxide ion free radical [81]. The sequential reduction and oxidation of the compound occurs repetitively in a cyclical manner, hence the description redox cycling. A simplified schematic equation for this is shown in figure 1.3. Electrons for the one electron reduction are derived from several sources. Studies with redox active quinones have shown that one electron transfer to the compound may be catalysed by the enzymes NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase, NADH-ubiquinone oxidoreductase and xanthine oxidase [64, 82-84]. The enzymes require the reduced pyridine nucleotides, NADH or NADPH, as a source of electrons depending upon which enzyme is catalysing the transfer. It has been suggested that xanthine oxidase activity does not occur *in vivo* but is an *in vitro* artifact of the preparation of xanthine dehydrogenase [70].

The transfer of one electron to a redox cycling compound results in the formation of a species, usually a relatively stable free radical, which can be reoxidised to the parent compound by molecular oxygen. The molecular oxygen is reduced in this reaction to the superoxide ion free radical ($O_2^{\cdot-}$). This reaction is not enzyme mediated and occurs spontaneously.



Enzymes:

(1) - NADPH-cytochrome P450 reductase
(2) - spontaneous chemical reaction
(3) - superoxide dismutase
(4) - catalase
(5) - glutathione peroxidase
(6) - glutathione reductase

figure 1.3. Schematic pathway of redox cycling

The redox cycling compound can thus be thought of as catalysing the formation of superoxide ion radical from NAD(P)H and molecular oxygen according to the equation:

$$\mathbf{NAD(P)H + O_2 \rightarrow NAD(P)^+ + O_2^{\cdot -}}$$

Whilst the initial studies on redox cycling concentrated on quinones, studies have shown this mechanism to mediate the toxicity of other compounds such as paraquat [85] and diquat [63]. The redox cycling reaction thus depends upon the presence of oxygen and reduced pyridine nucleotide. The redox cycling reaction eventually leads to loss of cell function. This is thought to be due to the formation of oxygen radicals [76] although it has been suggested that toxicity could, in part, be due to depletion of reduced pyridine nucleotides [86].

1.4.4. Mechanisms of Pulmonary Endothelial Toxicity

The pulmonary endothelial cell has a range of physiological and biochemical functions [25]. These include:

1 Maintaining vascular integrity.

2 Participating in gaseous exchange.

3 Uptake, clearance and metabolism of endogenous compounds.

4 Maintaining biochemical and hormonal homeostasis.

5 Participating in the inflammatory response.

6 Maintaining vascular hæmostasis.

Consequently, the pulmonary endothelium is particularly vulnerable to selective damage as can be reflected in the adverse modulation of any of the above

functions [21].

The pulmonary endothelium is remarkably susceptible to oxidative damage [21]. It appears to be the primary target in the toxicity of hyperbaric oxygen and ozone [26]. Some compounds, which exert their toxicity through redox cycling, may preferentially damage other cell types. In some instances, this is due to selective disposition *e.g.* paraquat [12]. It has been also suggested that the generation of oxygen radicals is greater in cell types other that the pulmonary endothelial cell [21].

The mechanism by which oxidative damage occurs to the endothelium is not fully understood. There are numerous effects of such damage. These include: lipid peroxidation, depression of intracellular ATP levels, increase in oxidised sulphydryl formation, increase in cytosolic calcium, and perturbation of the cytoskeleton [21]. Some of the deleterious changes seen in endothelial cell toxicity may reflect a loss of function, thus reactive oxygen species have been shown to affect endothelial cell arachidonic acid metabolism in culture [87]. Prostacyclin production by the endothelial cell is partly responsible for its hæmostatic role [23]. The *in vivo* significance of this is that platelet deposition is seen in hyperbaric oxygen toxicity.

As a reaction to chemically induced tissue injury, the vascular integrity afforded by the pulmonary endothelium may be compromised [48]. This is a physiological response, mediated via the cytoskeleton [48], allowing access of cells involved with inflammation and repair to the site of injury [7]. Such responses also entail a loss of organ function as leakage of vascular fluid and accumulation of inflammatory cells may occur [7, 47], leading to pulmonary hypertension, œdema and inflammatory responses [7, 48]. Although this is essentially a repair mechanism, the loss of lung function may be viewed as a toxicological response [7, 48].

1.5 GLUTATHIONE

1.5.1 Occurrence and Biological Importance

The chemistry and biochemistry of the thiol group is exploited by many different cell types in both endogenous and exogenous metabolism [88-93]. Its importance in these respects can be inferred by its diversity and the concentration of thiol compounds relative to other essential cellular compounds [89]. Examples of the diversity of thiol containing compounds include the active site of enzymes such as glyceraldehyde-3-phosphate dehydrogenase, amino acids methionine, cysteine and the derivative N-acetyl cysteine and some coenzymes such as coenzyme-A [89, 94]. Glutathione is probably the most important thiol compound in respect of cellular function [88], and has been extensively investigated [88, 91, 92]. Glutathione is the tripeptide γ-glutamyl cysteinyl glycine (figure 1.4) and is distinctive because it contains a γ-glutamyl linkage, which is resistant to the action of peptidases, and a reactive thiol moiety forming part of the cysteinyl residue [90]. It appears to occur almost universally in nature and at high intracellular concentrations. Reported values indicate a typical intracellular concentration of 5 mM [89, 92] with some examples, rabbit lens and rat hepatocytes, reaching concentrations of 10 mM [88]. The intracellular concentration of glutathione is higher than the reported intracellular concentrations of other biologically significant molecules such as glucose (5 mM), ATP (3 mM), glutamate (2 mM), NAD (1 mM) and other thiols such as coenzyme-A [95].

Glutathione has many important roles in physiological and toxicological processes.

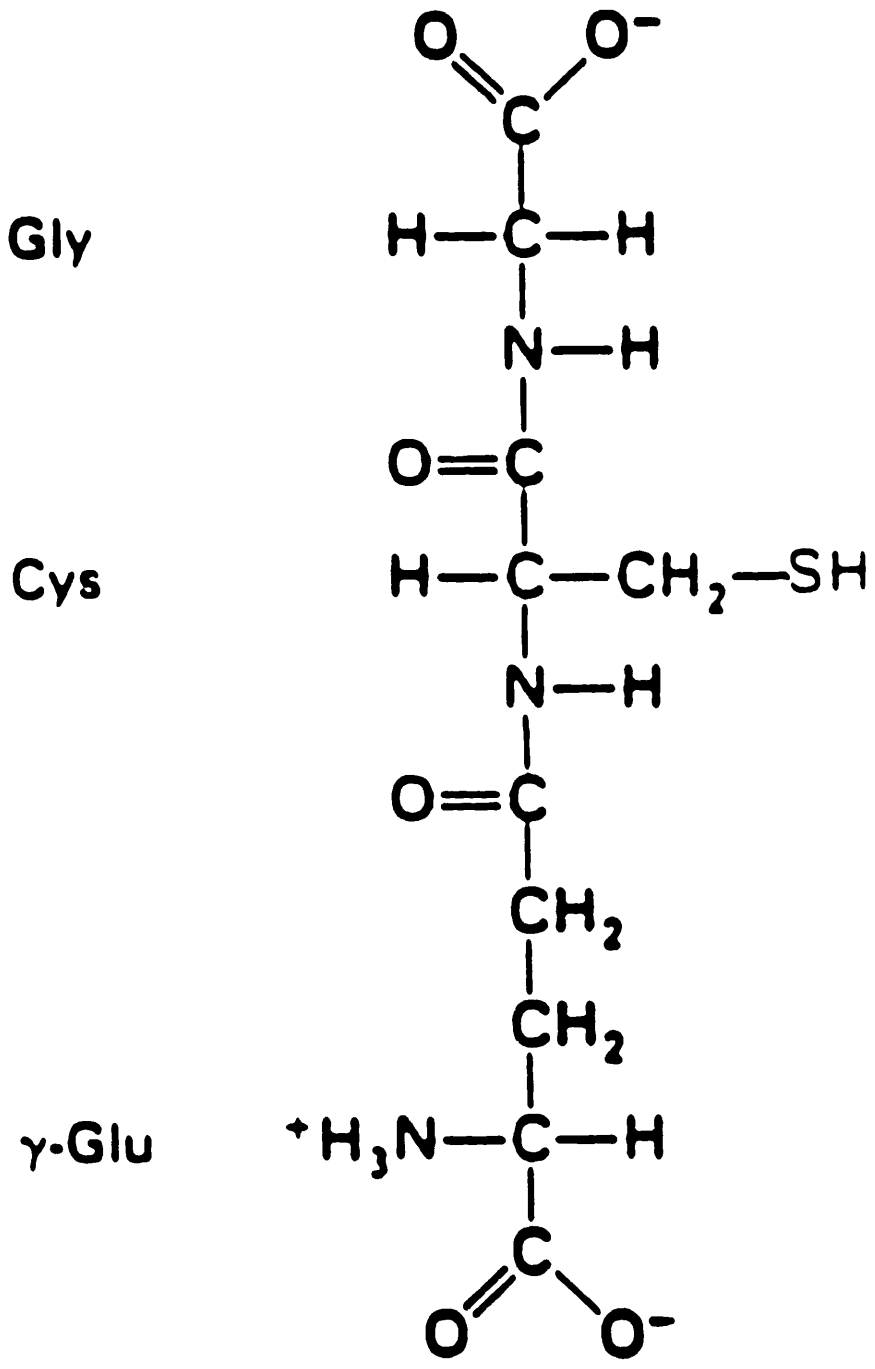
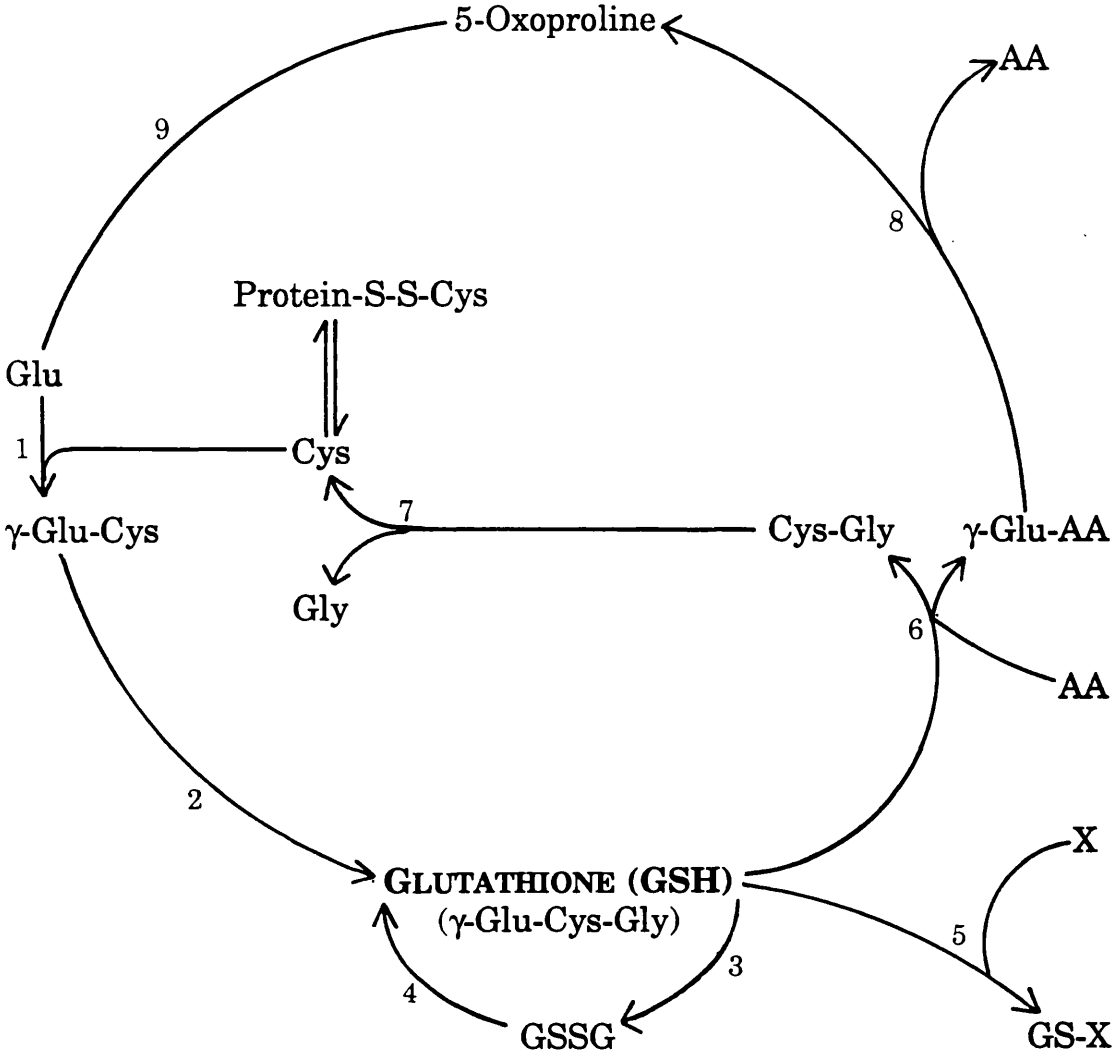


figure 1.4. Glutathione molecule

To illustrate its diversity, it has been shown to take part in biosynthesis [91, 92, 96], endogenous and xenobiotic metabolism [13, 92], Phase II conjugation reactions (detoxification of P-450 activated compounds) [38], free radical and oxygen toxicity [70, 97].

1.5.2 Biosynthesis and Turnover of Glutathione

Studies with selective inhibitors of glutathione associated enzymes have given some insight into its biosynthesis and metabolism with regard to its roles within the cell [91, 98]. The reactions concerning the synthesis and turnover of glutathione, referred to as the γ-glutamyl cycle, are summarised in figure 1.5. Glutathione is synthesised intracellularly from its constituent amino acids by the consecutive actions of the enzymes γ-glutamylcysteine synthetase and glutathione synthetase [92]. The activity of the first enzyme is feedback inhibited by glutathione, hence regulating its concentration [91, 92]. Glutathione, glutathione disulphide and S-substituted glutathione are catabolised by γ-glutamyl transpeptidase which transfer the γ-glutamyl moiety to acceptor amino acids [92]. Whilst GSH is predominantly intracellular, γ-glutamyl transpeptidase activity appears to be located on the external surface of the membrane, with a significant degree of this activity associated with the renal brush border [92]. Intracellular γ-glutamyl amino acids are converted to the free amino acids and 5-oxoproline by the action of γ-glutamyl cyclotransferase, the 5-oxoproline is then converted to glutamate by the action of 5-oxoprolinase which is ATP-dependent. Cysteinylglycine, formed in the transpeptidase reaction, is split by the action of dipeptidase [92].



Abbreviations:

- AA - amino acid
- Glu - Glutamine
- Cys - Cysteine (reduced)
- Gly - Glycine
- X - any conjugating species

Enzymes:

- (1) - γ-glutamyl-cysteine synthetase
- (2) - glutathione synthetase
- (3) - glutathione peroxidase
- (4) - glutathione reductase
- (5) - glutathione-S-transferase
- (6) - γ-glutamyl transpeptidase
- (7) - dipeptidase (non specific)
- (8) - γ-glutamyl cyclotransferase
- (9) - Oxoprolinase

figure 1.5. Pathways of glutathione turnover

1.5.3 Biological roles of Glutathione

The diverse biological roles of glutathione are illustrated by its biochemistry associated with its roles in coenzyme function and conjugation reactions, and in biosynthetic pathways [91]. A frequent reaction of glutathione is conjugation with electrophilic species [89]. This may occur spontaneously with a 'soft' electrophile or may be catalysed by the glutathione-S-transferases [89, 90, 93]. In the case of xenobiotics, the glutathione conjugates are often further metabolised by γ-glutamyl transpeptidase to form the cysteinylglycine residue, dipeptidase to form the cysteine conjugate, and N-acetylase to form the N-acetyl cysteine conjugate [91, 92], for which a specific renal excretion mechanism exists [99]. However, this pathway is also of toxicological relevance in the mechanism of toxicity of hexachlorobutadiene which forms reactive species in the kidney after the formation of the N-acetyl conjugate in the liver [90, 99].

1.5.4 Chemical properties of Glutathione

A physiologically important property of glutathione is its oxidation to form glutathione disulphide (GSSG) at the expense of potentially toxic oxidising species or free radicals [70]. This can occur chemically via the formation of the thiyl radical (GS·) [100]. This can be achieved in a number of ways. Firstly, hydrogen abstraction by an alkyl free radical ($R_3C\cdot + GSH \rightarrow R_3CH + GS\cdot$). This occurs because the C-H bond strength is higher than the S-H bond [100]. Secondly, via a one electron donation of the reaction type:

$$X_n + GS^- \rightarrow X^- + GS\cdot$$

This is important with regard to the superoxide ion radical, the hydroxyl radical and

radicals derived from halogens, azide, sulphate and nucleic acids [100]. Other modes of thiyl radical formation include photochemical production and spontaneous decomposition of conjugates (*e.g.* thionitrites) [100]. These are probably less important in biological systems.

1.5.6 Biochemistry of Glutathione - The Glutathione Redox Cycle

The purely chemical reactions of glutathione, in biological systems, have a limited application because of a lack of specificity of the reactive thiol and the inefficiency of the uncatalysed reactions [89]. A more biologically significant role, in terms of antioxidant defence, is the enzyme catalysed oxidation of GSH to GSSG, whilst reducing hydrogen peroxide to water. The enzyme which catalyses this reaction is glutathione peroxidase. The stoichiometry of the reaction is:

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

Glutathione peroxidase consists of a number of components which may be classified in terms of selenium dependent [101], and non-selenium dependent activities [102]. The selenium dependent glutathione peroxidase occurs freely in the cytosol and the mitochondria and it is thought that the enzyme in both cases is identical [103]. They are both tetrameric proteins [103]. There is evidence that a different form of the selenium dependent enzyme exists in the lipid phase of the cell membrane, which is monomeric [103]. The non selenium dependent component of glutathione peroxidase is associated with isozymes of the glutathione-S-transferases [104]. These isoenzymes are thought to be involved in the reduction of lipid hydroperoxides and hydrogen peroxide as it has been observed that the selenium dependent enzyme activity has a pronounced (although not absolute) substrate specificity for hydrogen peroxide [103].

Glutathione peroxidase activity has also been observed with hæmoproteins [105] although the relevance of this *in vivo* is uncertain. GSSG is reduced intracellularly by glutathione reductase, a flavoprotein containing a tightly bound FAD molecule, whose action is dependent on NADPH, the major source in lung being the hexose monophosphate shunt [29]. The oxidation and subsequent reduction reactions of glutathione in this way are collectively referred to as the glutathione redox cycle.

The role of the glutathione redox cycle is the reduction of hydrogen peroxide, formed intracellularly, by acting as a co-enzyme for glutathione peroxidase [106]. Hydrogen peroxide is formed from toxic oxygen species (the superoxide ion free radical) by the action of the enzyme superoxide dismutase [76]. GSSG is reduced at the expense of NADPH formed as a result of the hexose monophosphate shunt (glucose-6-phosphate dehydrogenase) [106]. This can have implications for compounds that produce toxic oxygen species in quantities that saturate the ability of glutathione peroxidase to remove such species, in that other metabolic pathways may be involved in the sequence of toxic effects observed.

1.5.7 Chemical and Biochemical aspects of Glutathione Conjugation

Conjugation with glutathione represents an important cellular defence mechanism against the toxic action of a wide variety of reactive electrophiles [107]. Glutathione is a nucleophile that can react chemically with electrophiles [89]. At physiological pH, reduced glutathione occurs predominantly as the thiolate ion (GS^-) and it is this which is the active nucleophile [108]. Reactions of glutathione show some selectivity between electrophiles of similar reactivity [93]. The basis for this is described in terms of chemical "hardness" and "softness" of electrophiles and nucleophiles [109]. These are functions of the ability of a polarised charge centre of a molecule or species to undergo further polarisation and can be considered, partly,

in terms of the polarised charge density [93, 109]. The facility of reaction between electrophiles and nucleophiles is restricted to those of similar hardness [109]. This is thought to be due to the energy barrier of the transition state of reaction being greater with nucleophiles and electrophiles of dissimilar hardness [93, 109]. This concept is independent of the strength of an electrophile or nucleophile. Hard electrophiles are highly polarised, have a high positive charge density at the electrophilic centre and thus further polarisation of the molecule is difficult. Soft electrophiles have a low positive charge density that is readily polarisable. The same is true for nucleophiles and negative charge density [109]. Examples of hard and soft electrophiles and nucleophiles are shown in table 1.3. With reference to glutathione, the large atomic volume of sulphur, and thus diffuse electron density, render the thiol group a particularly soft nucleophile [109]. Conjugation of a xenobiotic compound with glutathione can proceed non-enzymatically or, more commonly, via the action of the glutathione-S-transferases [89]. These are a group of isoenzymes, ten of which have been characterised in rat liver [110]. These transferases have an overlapping ability to bind a wide variety of substrates independent of their ability to conjugate them with glutathione [90]. This function led to a previous terminology of 'ligandins' [111]. Glutathione-S-transferases can detoxify very reactive xenobiotic intermediates in their own right by covalent protein modification [90]. The transferases have a dimeric structure in which the monomers fall into three categories as defined by their apparent molecular weights; Y_a (M.Wt. 22,000), Y_b (M.Wt. 23,000) and Y_c (M.Wt. 25000) [90]. Glutathione-S-transferases A, C, D and E are homodimers of Y_b but vary in isoelectric points and substrate specificity which probably indicates that Y_b is a family of polypeptide monomers of similar molecular weight. Transferase AA is a Y_c homodimer while Transferase B is separable into two forms; a Y_a homodimer, and a Y_aY_c heterodimer [90].

Table 1.3: Examples of Hard and Soft Nucleophiles and Electrophiles

Type	Electrophiles	Nucleophiles
Hard	Charged species e.g. Alkyl carbocations Electrophiles containing an asymmetric electronegative group	Oxygen atoms of phosphate groups of nucleic acids.
Soft	asymmetrically polarised C=C double bonds e.g. cyanoethylene carbonyl groups of aldehydes and ketones	Species containing a lone pair of electrons and/or a large atomic volume e.g. amine ($-NH_3$) and thiol (-SH) groups.

Glutathione-S-transferase AA and both forms of transferase B possess glutathione peroxidase activity when measured *in vitro* but the *in vivo* role for these has not been clearly defined [90]. It is likely that the peroxidase activity of these enzymes has a role in the reduction of organic hydroperoxides [103]. Alternative nomenclatures for glutathione-S-transferases have been proposed based on the chronological order in which the subunits have been characterised [110]. The subunits are denoted by a number (1 to 10), and the enzymes are referred to in terms of these subunits *e.g.* 1-1, 2-2 (homodimers), 1-2 and 3-6 (heterodimers) [110]. The relationship between the nomenclatures are shown in table 1.4.

Endogenous roles of glutathione conjugation reactions include the formation of leukotrienes from arachidonic acid metabolites [96], which take part in chemical signalling systems in the immune system. Leukotriene A, for example, forms a glutathione conjugate to give leukotriene C. Removal of the γ-glutamyl moiety to give the cysteinylglycine conjugate results in the formation of leukotriene D [96]. Further metabolism by dipeptidase to form the cysteine-S-conjugate gives leukotriene E. Leukotrienes C, D and E have been identified as Slow Reacting Substance of Anaphylaxis (SRS-A) [112]. The disturbance of such metabolism may have important toxicological implications [96], especially in the lung which is a major site of leukotriene metabolism [26, 96, 112].

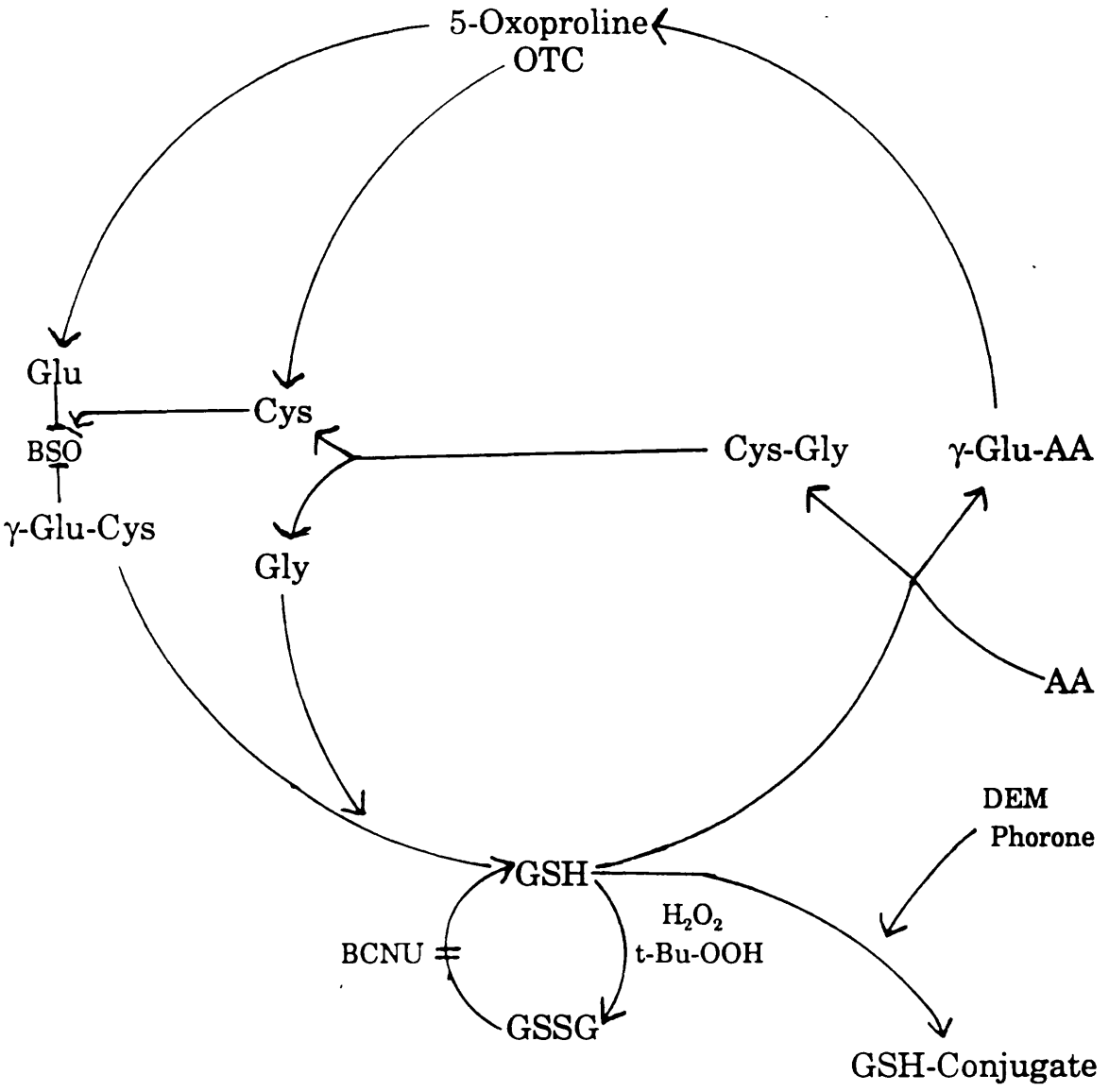
1.5.8 Experimental Modulation of Glutathione Biochemistry

Modulation of thiol status, that is, the functional state of thiol groups, can reveal much information about its role in toxicology and cell injury [107]. Cells within an organism may be exposed to a wide variety of compounds of exogenous origin [107]. Glutathione has been shown to be involved in limiting cell injury due to the deleterious effects of xenobiotic metabolic reactions [107].

Table 1.4: Glutathione-S-Transferase Subunits in the Rat

Chronological Nomenclature	Mobility on SDS-PAGE	Molecular Weight
1	Ya	25,000
2	Yc	28,000
3	Yb^1	26,500
4	Yb^2	26,500
5	***	26,500
6	Yn	26,000
7	Yf or Yp	24,000
8	Yk	24,500
9	Yn	26,000
10	***	25,500

The γ-glutamyl cycle and the GSH-GSSG redox cycle can be modified to alter the absolute levels of GSH and the relative levels of GSH, GSSG and GSH conjugates [98]. γ-Glutamylcysteine synthetase can be inhibited by buthionine sulphoximine which prevents further synthesis of glutathione [113]. Compounds that combine with glutathione, will lower the levels of GSH by depleting the intracellular glutathione pool. Diethyl maleate lowers GSH by this mechanism via the activity of glutathione-S-transferase. Intracellular GSH can also be lowered by oxidation to the disulphide (GSSG), followed by subsequent transport out of the cell. Oxidation of GSH is reversible due to the activity of glutathione reductase [106]. Glutathione levels can also be modulated by the use of the cysteine delivery system, L-2-oxothiazolidine-4-carboxylate (a 5-oxoproline analogue) [98, 115]. This is illustrated in figure 1.6. In the glutathione redox cycle, glutathione peroxidase has proved difficult to selectively inhibit due to the nature of the enzyme although several compounds have been found to interfere with its action. Selenium deficient diets have been used to reduce the activity of the enzyme in model systems but this is difficult to achieve in practice as selenium is difficult to exclude from the diet, and has a low turnover [116]. This means that the time needed to produce a reduction in peroxidase activity in some organs may well be eclipsed by the deleterious effect of such a diet on the whole organism [116]. Chemical inhibitors include cisplatin [117], aurothioglucose [118] and mercaptosuccinate [119]. Aurothioglucose has the greatest potential for selective inhibition, as the other compounds are limited by extreme toxicity or by the interference of non specific effects. Disadvantages of the chemical inhibition of glutathione peroxidase include the unknown distribution of the inhibitor, which may limit its experimental value.



Abbreviations:

AA - amino acid
Glu - Glutamine
Cys - Cysteine (reduced)
Gly - Glycine

Compounds:

BCNU - 1,3-bis(2-chloroethyl)-1-nitrosourea
t-Bu-OOH - *t*-butyl hydroperoxide
H_2O_2 - hydrogen peroxide
DEM - diethyl maleate
BSO - buthionine sulphoxime
OTC - L-2-oxothiazolidine-4-carboxylate

figure 1.6. inhibitors of the γ-glutamyl cycle.

A further difficulty is that the non-selenium dependent activity, associated with the glutathione-S-transferase activities, may assume the role of the inhibited selenium dependent enzyme as the transferase enzymes are inducible [103].

Glutathione reductase can be selectively inhibited by the antitumour agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [120]. This compound produces irreversible inhibition of the reductase enzyme via carbamoylation [121]. Selective modulation of the glutathione redox cycle is an important tool in investigating the role of glutathione in oxidative mechanisms of toxicity. Potentiation of toxicity by inhibiting glutathione reductase activity is a possible indicator of oxidative stress and demonstrates the need for reduced glutathione in maintaining cell integrity.

1.5.9 The Role of Glutathione in Toxicology

Glutathione plays an important part in the cellular defence against toxic chemicals [107]. The role of glutathione and protein thiol depletion has been investigated using model compounds, for example, menadione (2-methyl-1,4-naphthoquinone) [107, 122]. For many toxic chemicals, a reactive electrophile may be formed as a result of the cytochrome P-450 associated mixed function oxidase system [37]. Such electrophiles may react chemically or enzymically with glutathione which results in an effective depletion of functional GSH levels [89]. Some compounds produce toxic oxygen species by a variety of mechanisms involving free radical formation, which usually results in the formation of hydrogen peroxide by the conversion of superoxide by superoxide dismutase [70]. Hydrogen peroxide is removed by the action of glutathione peroxidase and GSH, resulting in the formation of GSSG which is normally reduced back to GSH by glutathione reductase at the expense of NADPH [106]. This system can become saturated when the rate of reactive oxygen species produced increases as a result of toxic stress, especially when NADPH becomes

limiting [106]. In these situations, GSSG accumulates as hydrogen peroxide can chemically as well as enzymically oxidise thiol groups [88, 92]. GSSG and GSH-substituted compounds are actively transported out of the cell by an ATP-dependent mechanism probably involving a translocase protein [123]. In conditions of oxidative stress, mixed disulphide formation and thiol oxidation in proteins may play a significant role in the loss of protein function [107].

It has been observed that with some compounds that produce oxidative stress, cell death is preceded by GSH depletion and oxidation of protein thiol groups [124]. Studies with menadione have shown that the oxidation of GSH to GSSG is the major pathway of GSH depletion. It has also been shown that formation of conjugates of menadione with glutathione, and of mixed disulphides between glutathione and cellular proteins, account for minor fractions of GSH consumption [125]. When this phenomenon is investigated more closely, it can be seen that GSH depletion is an early event whereas the loss of protein thiol groups is a much later event in the development of toxicity [107]. The implication of this is that protein thiol groups are critical for the normal functioning of the protein and their integrity is maintained, to some degree, by GSH [107]. It has been argued that protein thiol group oxidation does not necessarily lead to the expression of cell toxicity, as some protein thiol groups are more important in maintaining protein function than others and represent a 'critical thiol fraction' of total protein thiol groups [107]. It remains uncertain as to how small this proportion of thiol groups is.

During many examples of oxidative stress produced by redox cycling compounds or by diamide, a GSH oxidising compound, numerous blebs appear on the surface of the cell. There is evidence to suggest that this blebbing is due to a redistribution of intracellular calcium [126]. It is of interest to note that in menadione exposure, in hepatocytes, GSH loss precedes calcium loss [107]. In the normal situation, a low free

cytosolic calcium concentration is maintained by an active compartmentation process and by tightly binding to proteins such as calmodulin [126]. Calcium is taken up into mitochondria by an energy dependent mechanism and released by a Ca^{2+}/H^{+} antiport process which is probably mediated by the redox state of intramitochondrial pyridine nucleotides [127]. It has been suggested on the basis of recent evidence that thiols may be important in modulating mitochondrial calcium transport [126]. The transport of calcium across the endoplasmic reticulum and the plasma membrane appears to be mediated by calcium stimulated, magnesium dependent ATPases which rely on the maintenance of free thiol groups to preserve its activity [128]. Menadione impairs the ability of mitochondria to take up and retain calcium by interfering with the protein thiols affecting the uptake mechanism, and by altering the redox state of intramitochondrial pyridine nucleotides [124]. Reticular calcium homeostasis is impaired by the oxidation and the arylation of protein thiol groups, and by the formation of protein mixed disulphides [126].

1.6 General Aims

The primary aim of the work presented in this thesis is to elucidate the involvement of glutathione in mechanisms of pulmonary toxicity. Also, to establish relative differences, between pulmonary cell types, of toxicity, defence enzyme activities and glutathione, and their relative importance in terms of pulmonary toxicity. It is hoped to achieve this in several ways:

1) The evaluation of the use of adenosine accumulation as an indicator of cell specific toxicity.

2) The use of specific enzyme inhibitors in a lung slice system to modulate the toxicity of a number of compounds.

3) The modulation of glutathione levels *in vivo* to assess its role in the endothelial toxicant α-naphthylthiourea (ANTU).

Chapter 2

Materials and Methods

MATERIALS

Animals

Male Wistar rats (180-220g body weight) were obtained from OLAC, Southampton, UK and were used in experiments unless otherwise stated. Animals were maintained in a temperature controlled environment on a 12 hour light/12 hour dark cycle and allowed access to standard laboratory diet and water *ad libitum*. Rats were acclimatised to these conditions for a period of not less than two weeks.

Chemicals

The following were obtained from The Sigma Chemical Company, Poole, Dorset, UK:

Chemicals: adenosine, inosine, hypoxanthine, adenine, adenosine 5'-triphosphate (disodium salt, prepared by phosphorylation of adenosine), adenosine 5'-diphosphate (sodium salt, from equine muscle), adenosine 5'-monophosphate (sodium salt, from equine muscle), inosine 5'-monophosphate, carboxyatractyloside (dipotassium salt, from Actractylis gummifera), ethylene diamine tetra acetic acid dihydrate (disodium salt), aurintricarboxylic acid, 8-aminoguanosine, P1,P5-di(adenosine-5') pentaphosphate (sodium salt), oligomycin, 5'-amino-5'-deoxyadenosine, (p-toluene sulphonate salt), dipyridamole, sodium potassium tartrate tetrahydrate (Rochelle salt), 5,5-dithiobis-(2-nitrobenzoic acid), mercaptosuccinic acid, 3-amino-1,2,4-triazole, o-phthalaldialdehyde, bovine serum albumin.

Enzymes: glutathione-S-transferase

The following were obtained from Aldrich Chemical Company, Gillingham, Dorset, UK:

Chemicals: 1,4-benzoquinone.

The following were obtained from BDH Limited, Poole, Dorset, UK:

Chemicals: sodium chloride, potassium chloride, calcium chloride dihydrate, magnesium sulphate heptahydrate, potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate dihydrate, sodium arsenate heptahydrate sodium carbonate, copper sulphate pentahydrate, sodium hydroxide, Folin and Ciocalteu's phenol reagent, sucrose, hydrogen peroxide.

Solvents: 1-butanol, ethyl acetate, 2-methylpropan-1-ol, pentan-1-ol, 2-ethoxyethanol, ammonia (35% NH_3 w/w).

The following were obtained from Rathburn Chemicals Limited, Peebleshire, Scotland, UK:

Solvents: Methanol.

The following were obtained from Boerhinger-Mannheim, W. Germany:

Chemicals: reduced glutathione, oxidised glutathione.

Enzymes: glutathione reductase.

The following were obtained from Amersham International plc, Buckinghamshire, UK:

Radiolabelled Chemicals: [U-^{14}C]-spermidine (117mCi/mmol), [U-^{14}C]-putrescine (118mCi/mmol), [2-^{3}H]-adenosine (23Ci/mmol).

The following were generous gifts of Mr I Wyatt, ICI plc Macclesfield, Cheshire, UK:

Chemicals: paraquat dichloride, diquat dibromide monohydrate.

The following was a generous gift of Dr Y S Bahkle, The Royal College of Surgeons, Lincolns Inn, London, UK:

Chemicals: alpha-naphthylthiourea.

I am grateful to Mr S Forrow for the preparation of the following:

Chemicals: 2,3-dimethoxy-1,4-naphthoquinone (prepared according to the method of Gant et al [122]).

WORKING SOLUTIONS

Krebs Ringer solution, phosphate buffered with glucose (KRP) [129]

Compound	Concentration	
	mM	mg/L
sodium chloride	130	7597.2
potassium chloride	5.2	387.7
calcium chloride	1.9	279.3
magnesium sulphate	1.29	317.9
sodium phosphate (buffered)	10	1560.1
glucose	11	1981.8

pH adjusted to 7.4 with 1.0 M sodium hydroxide solution

Krebs Ringer solution, phosphate buffered with glucose, supplemented with amino acids (KRP-AA)

Compound	Concentration	
	mM	mg/L
sodium chloride	130	7597.2
potassium chloride	5.2	387.7
calcium chloride	1.9	279.3
magnesium sulphate	1.29	317.9
sodium phosphate (buffered)	10	1560.1
glucose	11	1981.8
L-serine	0.2	21.02
L-methionine	0.2	29.84
L-cysteine	0.2	35.12 (hydrochloride)
L-glutamine	0.2	29.42
glycine	0.67	50.30

Gibco Minimum Essential
Medium amino acids (50x)
without L-glutamine 2% v/v

pH adjusted to 7.4 with 1.0 M sodium hydroxide solution

(Composition of Gibco minimum essential medium amino acids (in mg/L):-
arginine hydrochloride (6320), cysteine (1200), histidine hydrochloride monohydrate (2100), isoleucine (2625), leucine (2620), lysine hydrochloride (3625), methionine (755), phenylalanine (1650), threonine (2380), tryptophan (510), tyrosine (1800), valine (2340).)

TLC Solvent I

1-butanol	42 ml
ethyl acetate	24 ml
methanol	18 ml
ammonia	24 ml

TLC Solvent II

2-methyl-1-propanol	18 ml
1-pentanol	12 ml
ethoxyethanol	36 ml
ammonia	18 ml
water	24 ml

METHODS

Administration of compounds *in vivo*

Phorone was administered as a homogeneous solution in corn oil via the intraperitoneal route such that 300 µl per 200 g body weight yielded the desired dose in mg/kg. Phorone was administered 48 hours prior to further experimentation.

ANTU was administered as a suspension in corn oil via the intraperitoneal route such that 300 µl per 200 g body weight yielded the desired dose. ANTU was administered prior to subsequent assessment until death or recovery for the *in vivo* studies and 4 hours before sacrifice for the *in vitro* studies. Homogeneity of the suspension was ensured by sifting the solid ANTU, suspending in corn oil within one minute of administration and by continuous mixing.

Preparation of Rat Lung Slices

(i) Preparation of control slices

The preparation of rat lung slices for *in vitro* studies is based on the method of Rose *et al.* [129]. There are several advantages in the use of tissue slices over other systems: oxygen and xenobiotics can quickly and freely diffuse through the slice, some of the architecture of the lung is maintained within the slice, The system also allows assessment of different biochemical parameters under a number of different conditions on tissue from the same lung. This system has been characterised previously [129].

Rats were anaesthetised by injection of Nembutal (sodium pentobarbital, 60 mg/ml) 60 mg/kg *i.p.* until the withdrawal reflex was lost. The lungs were perfused *in situ* with KRP aerated with Carbogen using a single pass procedure employing a Watson-Marlow 502-S peristaltic pump. Tissue slices of 0.5 mm thickness were then prepared using a McIlwain tissue chopper (Mickle Ltd, Godalming, Surrey, UK). Slices with two cut surfaces and taken from different lobes were used for each single determination.

(ii) Preparation of slices with inhibited glutathione reductase activity

This procedure, taken from Hardwick *et al.* [130], results in viable lung slices with irreversibly inhibited glutathione reductase activity.

Lung slices were prepared as described above. Slices were preincubated in 5 ml KRP to which 100 µl of BCNU in DMSO had been added to give a final concentration of 100 µM. The buffer was also supplemented with L-cysteine (0.2 mM), glycine (0.67 mM), L-methionine (0.2 mM) and MEM amino acid solution without L-glutamine (Gibco 50X strength, 1 ml per 50 ml of buffer). The slices were incubated for 45 minutes at 37° C in conical flasks shaken at 60 cycles/minute. Control slices were similarly treated except that BCNU and the amino acids were omitted. All the lung slices were washed in amino acid-free KRP and reincubated in fresh KRP for the duration of the experiment.

Incubation of lung slices with compounds

Lung slices were prepared as described and allowed to incubate in 4.9 ml of KRP for 30 minutes. Compounds were dissolved in KRP or DMSO such that 100 µl added to 4.9 ml of KRP yielded the desired final concentration. Exposure to compounds was terminated by removing the lung slice from the medium and washing in fresh KRP before any further treatment. Solvent controls were run for each experiment but the results were shown for these only where they differed from no-solvent controls.

Measurement of Reduced Glutathione (GSH)

(i) Method of Hissin and Hilf [131].

The principle of this method is the chemical formation of a fluorescent adduct (figure 2.1 [132]). The advantage of this method is its sensitivity in that nanomole quantities of glutathione can be detected. A disadvantage of this method is that the assay is not absolutely specific for GSH in that a number of other acid soluble NPSH's are detected by this method [133]. It has previously been shown that the majority of acid soluble thiols in lung tissue (>90%) comprise of GSH [134].

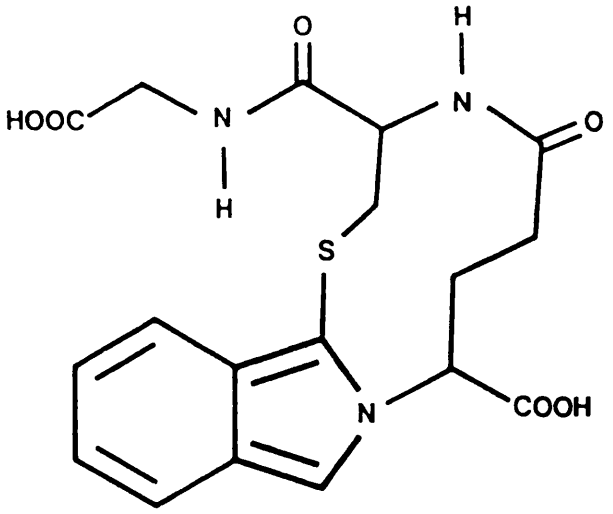


figure 2.1. Structure of adduct formed between glutathione and *o*-phthalaldialdehyde.

Lung slices were homogenised in 0.1 M phosphate buffer/5 mM EDTA, pH 7.6 using a Polytron (Kinematica, Hull, UK.) for 15 seconds in iced water and immediately, an equal volume of ice-cooled 13% TCA was added. The acidified homogenate was then mixed and allowed to stand for 1 hour at 4° C, to ensure complete precipitation of protein. The precipitated protein was removed by centrifugation in a refrigerated centrifuge (Denley BR 401) at 3500 rpm for 15 minutes. GSH standards were prepared in a mixture of equal volumes of 13% TCA and the buffer used for homogenisation. Supernatant or standard (100 µl) was added to 3.0 ml of 0.1 M phosphate buffer/5 mM EDTA, pH 8.0 and was followed by the addition of 100 µl of o-phthalaldehyde (2 mg/ml in methanol, freshly prepared). This was then mixed and allowed to stand at room temperature, in the dark, for 30 minutes. Fluorescence was then measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm using a fluorescence spectrophotometer (Perkin-Elmer LS3). GSH concentration in samples was related to that fluorescence produced by the known GSH concentration in the prepared GSH standards.

(ii) Method of Brigelius *et al.* [135].

This assay relies upon the enzyme catalysed formation of a colorimetric product using glutathione-S-transferase and CDNB to form the glutathione adduct. A much enhanced specificity for GSH is shown by this method in relation to the fluorometric method described above. This assay did not detect GSH related thiols: N-acetylcysteine, cysteine or glutamyl cysteine. Disadvantages of this assay are that the enzyme may be subject to inhibition or other modulation by components in the sample such that an end point is not reached, the assay is not sufficiently sensitive enough to detect GSH in lung slices but is more applicable to whole lung

Lung tissue was clamped in stainless steel tongs cooled to the temperature of

liquid nitrogen, and was then pulverised in a stainless steel percussion mortar, similarly cooled. The resulting powder was transferred to 1.0 M PCA. The suspension formed was allowed to stand for 1 hour at 4° C to ensure complete precipitation of protein. The precipitated protein was removed by centrifugation in a refrigerated centrifuge (Denley BR 401) at 3500 rpm for 15 minutes. GSH standards were prepared in 1.0 M PCA. 1.0 ml of ice-cooled sample or standard was neutralised with ice-cold 1.0 M potassium hydroxide, and the precipitate was removed by centrifugation, keeping the solution between 0 and 4° C. A quartz cuvette was prepared containing 500 µl 0.1 M potassium phosphate buffer, pH 7.0 and 10 µl 10 mM CDNB in ethanol, prewarmed to 37° C. 100 µl of neutralised sample or standard was added to the cuvette and the reaction was initiated by the addition of 10 µl of GSH-S-transferase (35 units/ml) and the absorbance was followed for five minutes at the wavelength pair 340-400 nm using a Sigma ZWS II dual wavelength spectrophotometer.

Measurement of adenosine triphosphate (ATP) [136]

The determination of ATP in lung slices requires a method capable of greater sensitivity than that produced by spectrophotometric methods. The method of choice for these experiments is a bioluminescence assay based on the ATP driven production of photons from luciferin catalysed by luciferase. The luciferin/luciferase is provided by a crude extract from firefly lanterns.

Lung slices were homogenised, acid-precipitated and centrifuged in the same manner as described in the determination of GSH by the method of Hissin and Hilf. An assay buffer was freshly prepared consisting of a mixture of equal volumes of solutions of magnesium sulphate (80 mM), potassium dihydrogen phosphate (10 mM)

and sodium arsenate (100 mM). ATP standards (2.5-40 µM) were prepared in a mixture of equal volumes of 13% TCA and the buffer used for homogenisation. 10 µl of sample or standard was added to 2 ml of the assay buffer in disposable plastic test tubes (75 x 12 mm). The firefly lantern extract was resuspended in distilled water (5 ml/vial) and insoluble material was removed by centrifugation in a refrigerated centrifuge (Denley BR 401). The supernatant formed was maintained at a temperature between 0-4° C to retard decomposition. The bioluminescence reaction was initiated by adding 100 µl of resuspended firefly lantern extract supernatant to the sample or standard in assay buffer. Photons, measured as counts per second corrected for background interference, were counted for 6 seconds after a lag time of 15 seconds after the addition of the lantern extract. ATP concentration was related to those photon counts per second produced by ATP standards of known concentration.

Accumulation of radiolabel

The accumulation of radioactivity by lung slices incubated in the presence of [^{14}C]-spermidine and [^{14}C]-putrescine has been correlated with pulmonary epithelial cell function in previous studies [13]. The accumulation of radioactivity in the presence of [2-^{3}H]-adenosine was used to assess the function of cell types in the lung other than the epithelium, primarily the pulmonary endothelium. The use of [2-^{3}H]-adenosine was based on initial studies by Hardwick [137] and is more fully investigated in chapter 3. The methodology for the accumulation studies was adapted from Gordonsmith [138].

(i) Spermidine

Lung slices were prepared as described and treated according to the procedure used. The slices were washed by brief immersion in fresh KRP and placed into conical flasks containing 4.9 ml KRP. 100 µl of [^{14}C]-spermidine, adjusted with unlabelled spermidine, was added to the flask such that the resultant concentration was 10 µM with a specific activity of 0.1 µCi per 5 ml (flask volume). The lung slices were allowed to incubate for 30 minutes at 37° C in a shaking water bath. The slices were then removed and washed by brief immersion in fresh KRP and blotted to remove excess fluid. The slices were then dissolved by the addition of 0.4 ml 1.0 M sodium hydroxide solution and maintained at 95-100° C for 30 minutes. If dissolution is not complete after gentle agitation, the slices were then maintained for a further 15 minutes at 95-100° C until dissolution was complete. The dissolved lung slices were then allowed to cool and were then neutralised by the addition of 0.4 ml 1.0 M hydrochloric acid. The neutralised solution was then added to 4.0 ml Aquasol in Beta vials and assessed for radioactivity by scintillation counting in a Rackbeta LKB 1216 liquid scintillation counter.

(ii) Adenosine

The procedure for spermidine accumulation was followed but modified in the following ways: [2-^{3}H]-adenosine was used in place of spermidine such that the resultant concentration in which the lung slices were incubating was 10 µM with a specific activity of 1.0 µCi per 5 ml (flask volume). The slices were allowed to incubate for 15 minutes in a shaking water bath at 37° C.

Analysis of nucleotides and nucleosides by TLC

The procedure described here followed that of Shimizu *et al.* [139] for nucleoside separation, and on that of Norman *et al.* [140] for nucleotide separation. These separation procedures were used by Bakhle and Chelliah [141] for the *in vitro* analyses of adenosine metabolites in the isolated perfused rat lung. In this study, the separation of nucleotides and nucleosides was achieved using homogenates from lung slices incubated with [2-^{3}H]-adenosine. Two different solvent systems were employed in order to resolve nucleosides [139] and nucleotides [140].

Lung slices were incubated in a range of concentrations of [2-^{3}H]-adenosine (5.0 µCi/5 ml) for varying time periods. The slices were then homogenised, acid deproteinised and centrifuged as described previously. Nucleotide and nucleoside standards (unlabelled) were prepared using ATP, ADP, AMP, IMP, inosine, adenosine, hypoxanthine and adenine in separate solutions and as a mixture of each of the above. In addition, a radiolabelled mixture of the above was prepared containing [2-^{3}H]-adenosine (1.0 µCi/ml). Each standard was adjusted to a final concentration of 1 mM. Standards were prepared in a mixture of equal volumes of 13% TCA and the buffer used for homogenisation. Supernatant or standard (10 µl) was spotted onto a 20 x 20 cm fluorescent-coated, aluminium backed TLC plate. Spots were placed 2.5 cm from one edge and spaced 4.0 cm apart on duplicate plates to run in two TLC solvent systems (TLC solvent I and TLC solvent II as described in working solutions). The plates were run in TLC solvent tanks until the solvent height reached between 2 and 4 cm from the upper edge. The plates were then removed from the solvents, the solvent height marked and allowed to dry for at least eight hours. The plates on which the unlabelled standards were run were viewed under a uv lamp and each standard, noted as a fluorescent-quenched area, was marked. The plates on which the

labelled standard and samples were run were marked into areas which extended 1 cm either side of the baseline spot perpendicular to the direction of solvent flow, and 0.5 cm either side of the baseline spot parallel to the direction of solvent flow. Successive 2 x 1 cm areas were marked from the baseline spot to the solvent height. Each area was then separated, placed into Beta vials and 4.0 ml of Aquasol liquid scintillant was added. These were then assessed for radioactivity. The resultant radioactivity was plotted against Rf values obtained from the baseline to the central point of each area. The results were compared to those obtained with unlabelled standards.

Statistical Analysis of Data

Quantified data without error bars represent results from one experiment typical of several. Unless otherwise stated, error bars represent the standard error of the mean (sem, from 3 experiments) around which they are centred. Where data differs from its relevant control, or between treatments (as stated in the legend), probability of significance is accepted when greater than 95% (*i e* $P > 0.95$ or, for the null hypothesis that there is no significant difference, $P < 0.05$). Statistical significance was tested according to Student's t-test and is indicated via the null hypothesis in the text ("$p < 0.05$") or, on diagrams, with an asterisk (*). Where comparisons have been made between pretreatments, asterisk superscript 1 ($*^1$) refers to significant difference from controls within a pretreatment group, whilst asterisk superscript 2 ($*^2$) refers to significant difference between two pretreatments.

Chapter 3

The accumulation of adenosine by rat lung slices - its potential as an indicator of cell specific toxicity.

INTRODUCTION

Pulmonary toxicity can manifest itself in terms of histological changes or alterations in some aspect of cellular function [142]. This may be expressed physiologically as a loss of lung function. A limitation in the assessment of toxicity, produced *in vivo* or *in vitro*, is the correlation of functional changes with degrees of injury to particular cell types within such a heterogeneous organ [142]. This problem has been approached *in vitro* by the exploitation of the specificity of cell types within the lung to accumulate certain compounds. The accumulation of oligoamines, for example, has been used to reflect alveolar epithelial function and has been characterised previously [13, 142-144]. The pulmonary endothelium also represents a target for cell-specific toxicity [22]. The accumulation of 5-hydroxytryptamine (5-HT) into rat lung [144], and angiotensin converting enzyme (ACE) activity [145] have been used in studies relating to endothelial toxicity in the appropriate models.

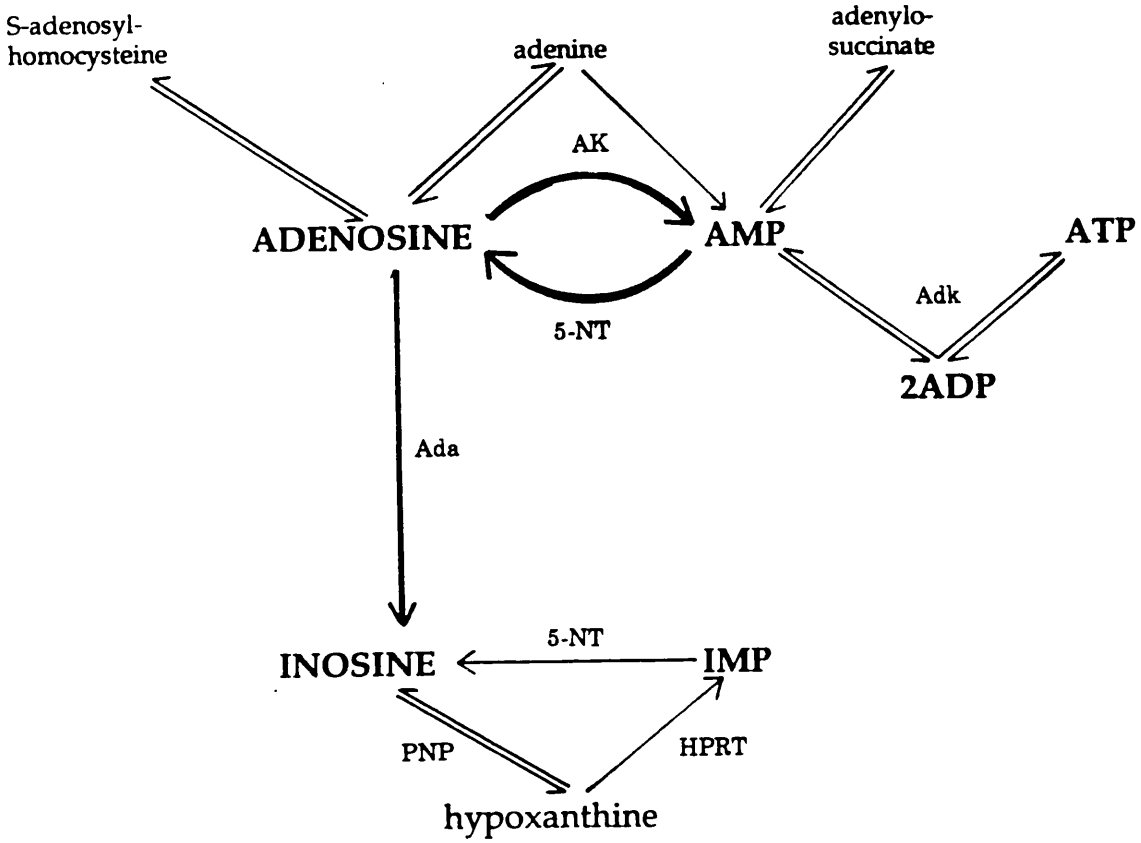
A number of studies have implicated the involvement of adenosine in the control of vascular tone. Early studies on adenosine centred on its role as a vasodilator [146, 147]. Inactivation was thought to occur via deamination [148]. Kolassa *et al* suggested that uptake of adenosine into endothelium could be responsible for its removal from the circulation [149]. Further investigation showed the uptake of adenosine by endothelial cells to be very rapid. In addition, at least two distinct uptake systems have been described, one of high affinity (apparent Km = 3 μM) and a system of lower affinity (apparent Km = 250 μM) [150].

A further suggestion was that transport of adenosine into endothelial cells

represented not just uptake, but also the involvement of a rate limiting metabolic step [150]. Various studies on the metabolism of adenosine by endothelial cells have revealed that several pathways exist [151]. A summary of the known major pathways of adenosine metabolism is shown in figure 3.1. The deamination of adenosine, via adenosine deaminase, has been shown to occur, with localisation of the enzyme on the external surface of the cell [152]. Other workers have shown an adenosine mediated, rapid induction of nucleoside and nucleotide metabolism, with an increased intracellular level of ATP [153]. In heterogeneous systems, it has been shown that most of the adenosine, at low micromolar concentrations of incubation, is sequestered in the endothelium [154].

The accumulation of radioactivity following incubation of rat lung slices with [2-^{3}H]-adenosine was investigated as a possible marker of endothelial cell function in some initial studies by Hardwick [137]. Previous studies relating to endothelial metabolism of adenosine, have been investigated in isolated perfused rat lung [141]. In this model, described by Bakhle and Chelliah, the endothelial cell involvement of adenosine uptake and metabolism was shown by its removal from the perfused vascular space. Subsequent work on this model investigated the effects of α-naphthylthiourea (ANTU) on the pharmacokinetics of adenosine [155], and the metabolism of adenine nucleotides [156].

In the present study, the accumulation of radioactivity by rat lung slices, following incubation with radiolabelled adenosine, was further investigated. To assess its potential as an indicator of endothelial cell function, several aspects of adenosine uptake were examined. These included kinetic studies, studies on the metabolism of adenosine by rat lung slices, the influence of metabolic and specific enzyme inhibition, and localisation by autoradiography. The inhibitors of adenosine metabolism are summarised in figure 3.1.



	Enzyme	Inhibitor	Ref.
AK	Adenosine Kinase	5'-amino-5'-deoxyadenosine	[153]
		5-iodotubercidin	[157]
5-NT	5'-nucleotidase		[151]
Ada	Adenosine Deaminase	deoxycoformycin	[158]
		coformycin	[153]
Adk	Adenylate Kinase	P1,P5-di(adenosine-5'-) pentaphosphate	[159]
PNP	Purine Nucleoside Phosphorylase	8-aminoguanosine	[160]
HPRT	hypoxanthine phosphoribosyl-transferase		[151]
	ATP Synthase	oligomycin	[159]
	Mitochondrial ATP/ADP translocase	carboxyatractyloside	[159]

figure 3.1. Pathways of adenosine metabolism

RESULTS

Time Course of Adenosine Accumulation

The quantity of adenosine accumulated, over 120 minutes, showed a concentration and time dependent increase (figure 3.2). The accumulation of adenosine was approximately linear with respect to incubation time, at an incubation concentration of 10 μM (figure 3.2). Incubation with 100 μM adenosine showed an apparent decrease in adenosine accumulation with respect to incubation time after a period of 15 minutes, compared to that seen between 5 and 15 minutes and became approximately linear between 15 and 120 minutes, the amount of adenosine accumulated remaining elevated over that shown by slices incubated with 10 μM adenosine ($p < 0.05$). Incubation with 500 μM adenosine resulted in a greater initial adenosine accumulation with respect to incubation time ($p < 0.05$) which progressively decreased consistently over the time intervals observed (figure 3.2). This was indicative of saturation of adenosine transport.

Kinetic Parameters of Adenosine Accumulation

The derived apparent K_m of adenosine accumulation varied between 87.6 and 449.3 μM. The derived apparent V_{max} of adenosine accumulation varied between 106.5 and 255.0 μmol/g wet wt./hour. The results from these are shown in table 3.1.

TLC of Rat Lung Slice Homogenates and Incubation Media

Thin layer chromatograms of lung slices incubated with 10 μM [2-^{3}H]-adenosine for 15 minutes were run (figure 3.3 and table 3.2). Analysis of chromatograms from lung slices incubated with [2-^{3}H]-adenosine showed that the majority of the radiolabel appeared as nucleotides (88%, figure 3.4a). Using a solvent system to resolve nucleotides, it was found that 61% of the radiolabel appeared as ATP, 15% as ADP and 6% each appearing as AMP and IMP (figure 3.4b).

Analysis of the chromatograms from the medium in which the lung slices were incubated showed that the majority of the radiolabel remained as adenosine (79%), the remainder of the radiolabel appearing as other nucleosides (figure 3.5).

Time Course of Adenosine Metabolism Followed by TLC

The metabolism of adenosine to nucleotides (figure 3.6a) and ATP (figure 3.6b) was found to be very rapid, reaching a maximum between 5 and 15 minutes then remaining constant. The disappearance of adenosine from the incubation medium showed that the fractional concentration decreased, in an approximately linear fashion, with incubation concentrations of 1 μM and 10 μM adenosine (figure 3.7a). The rate of disappearance was inversely related to the initial adenosine concentration. Metabolism to inosine, the major adenosine metabolite found in the medium, appeared to proceed at a constant rate over a 60 minute time period figure 3.7b). The rate was also inversely related to the initial incubation concentration of adenosine.

Time Course of Adenosine Induced Elevation of ATP Levels in Rat Lung Slices

Total ATP levels in rat lung slices, measured by bioluminescence, showed a concentration dependent increase when incubated with adenosine (figure 3.8). Control lung slices were incubated in KRP in the absence of adenosine. Incubation of lung slices with 20 μM adenosine resulted in a small but not significant increase in ATP levels over those seen in controls (figure 3.8). Incubation of lung slices with 100 μM and 500 μM adenosine resulted in an increase in ATP levels over those seen in controls which was significant by 15 minutes ($p < 0.05$, figure 3.8). A time dependent increase was seen between 0 and 30 minutes by which time a maximum value was achieved.

Effects of Incubating Rat Lung Slices with Nucleosides on ATP Levels

Incubation of lung slices with adenosine, inosine, hypoxanthine and adenine all resulted in elevation of ATP levels figure 3.9). The most marked elevations were seen in those incubated with adenosine and adenine ($p < 0.05$). Adenosine and adenine exhibited a 40% increase in ATP levels over controls ($p < 0.05$) whilst inosine and hypoxanthine resulted in a 20% elevation over controls ($p < 0.05$).

Effect of Metabolic inhibition on Adenosine Accumulation by Rat Lung Slices

Mitochondrial inhibition with cyanide, rotenone or antimycin A reduced the adenosine uptake to about 60% of the control value ($p < 0.05$, figure 3.10). Incubation

of slices at 4° C reduced the uptake of adenosine to 15% of the control value ($p < 0.05$, figure 3.10).

Effect of Metabolic inhibition on the Adenosine Induced ATP Elevation in Rat Lung Slices

Mitochondrial inhibition with cyanide, rotenone or antimycin A reduced the ATP levels in rat lung slices to 67%, 50% and 38% of control values respectively ($p < 0.05$, figure 3.11). The ratio of ATP levels in control incubated lung slices to those incubated with adenosine for control, cyanide, rotenone or antimycin A incubated slices were: 0.67, 0.69, 0.71 and 0.75 respectively. Incubating the slices at 4° C had little effect on ATP levels in control incubated slices, but no increase in ATP was seen on incubation with adenosine at this temperature (figure 3.11).

Effects of Incubating Rat Lung Slices with Proposed Inhibitors of Nucleoside Metabolism Inhibitors on the Adenosine Induced Elevation of ATP Levels in Rat Lung Slices

Inhibition of adenylate kinase, adenosine kinase, purine nucleoside phosphorylase or adenosine transport had little apparent effect on control or adenosine induced elevated ATP levels in rat lung slices (figure 3.12). Inhibition of ATP synthetase markedly reduced the level of ATP in lung slices incubated in the absence of adenosine ($p < 0.05$, figure 3.12). The ratio of ATP in adenosine control slices to adenosine incubated slices remained similar to that seen in controls (figure 3.12). A similar observation was made with inhibition of mitochondrial ATP translocase although the reduction in control ATP levels was much less marked (figure 3.12).

Influence of Glycolysis on the Adenosine Induced Elevation of ATP by Rat Lung Slices

A concentration dependent increase in ATP elevation was seen in lung slices incubated with adenosine (figure 3.13). When glucose was omitted from the incubation medium, this increase in the adenosine induced elevation of ATP in lung slices was abolished ($p < 0.05$, 100 μM). When glucose was replaced with the same concentration of sucrose, the adenosine induced ATP elevation in the slices was similar to that seen in control slices (incubated with glucose supplemented medium, figure 3.13). Incubation of lung slices with aurintricarboxylic acid (ATA), to inhibit phosphofructokinase, resulted in an apparent reduction of adenosine induced elevation of ATP in lung slices (figure 3.13).

Localisation of Accumulated Radiolabelled Adenosine

Lung slices were incubated in the absence of radiolabelled adenosine (figure 3.14a) to show background staining. Lung slices incubated with [2-^{3}H]-adenosine (figure 3.14b) resulted in evidence of labelling in the region of the adventitia of a small arteriole. The autoradiograph also showed the radiolabel to be localised, as very little labelling could be seen in the surrounding connective tissue. Labelling also appeared to be localised within the wall of a venule (figure 3.14c) and much less evident within the epithelium. Localisation of radiolabelled adenosine within the endothelium of a small blood vessel (figure 3.14d) was also evident.

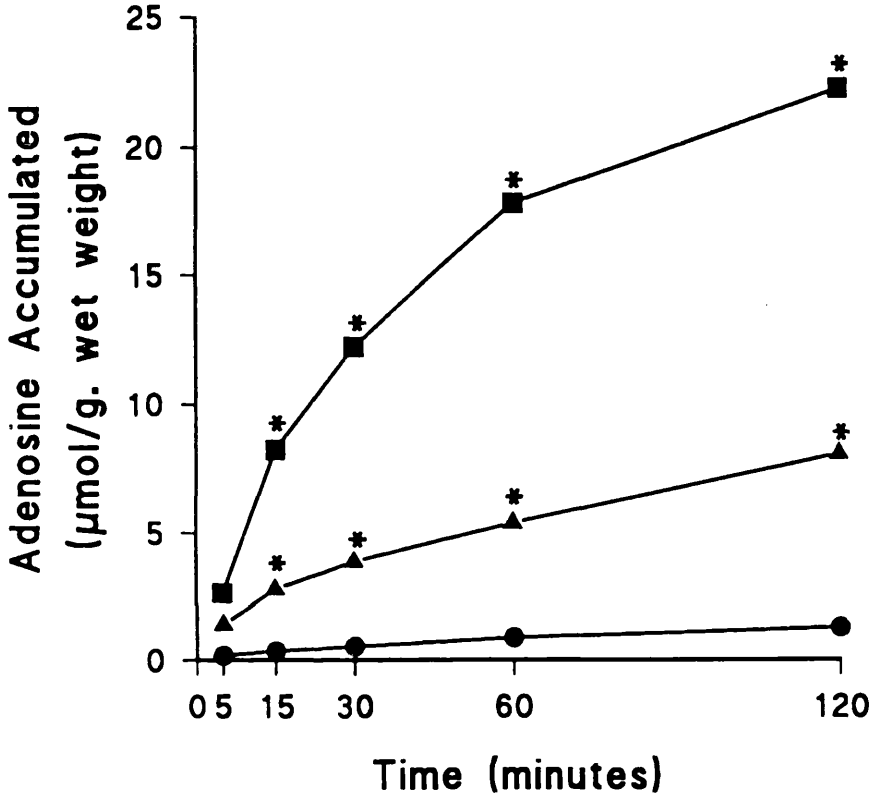


figure 3.2. Time course of Adenosine Accumulation by Rat Lung Slices

Rat lung slices were incubated with [2-^{3}H]-adenosine at a concentration of 10 µM (•), 100 µM (▲) or 500 µM (■). For each experiment, the specific activity was adjusted to 1.0 µCi/5 ml (incubation volume). Adenosine accumulation was assessed by measuring the radioactivity retained by the lung slice. Results represent the mean of 3 experiments (sem < 1% of maximum value). * Denotes significant difference compared with control slices incubated with 10 µM adenosine.

Plot	K_m	V_{max}
Direct Linear	228.0	255.0
Lineweaver-Burk	87.6	106.5
Hanes-Woolf	449.3	327.3
Eadie-Hofstee	137.3	202.0

table 3.1. Kinetic parameters of Adenosine accumulation by Rat Lung Slices

Further kinetic studies utilised a computer programme (ENZPAK) to calculate the apparent K_m and V_{max} for adenosine uptake. The data was derived from measurements taken from rat lung slices incubated with [2-^{3}H]-adenosine at concentrations of 1, 10, 100, 500 and 1000 µM for periods of 5, 15 and 30 minutes. The kinetic parameters were derived from direct linear (curve fitting) plots, and the transformations of Lineweaver-Burk, Hanes-Woolf and Eadie-Hofstee.

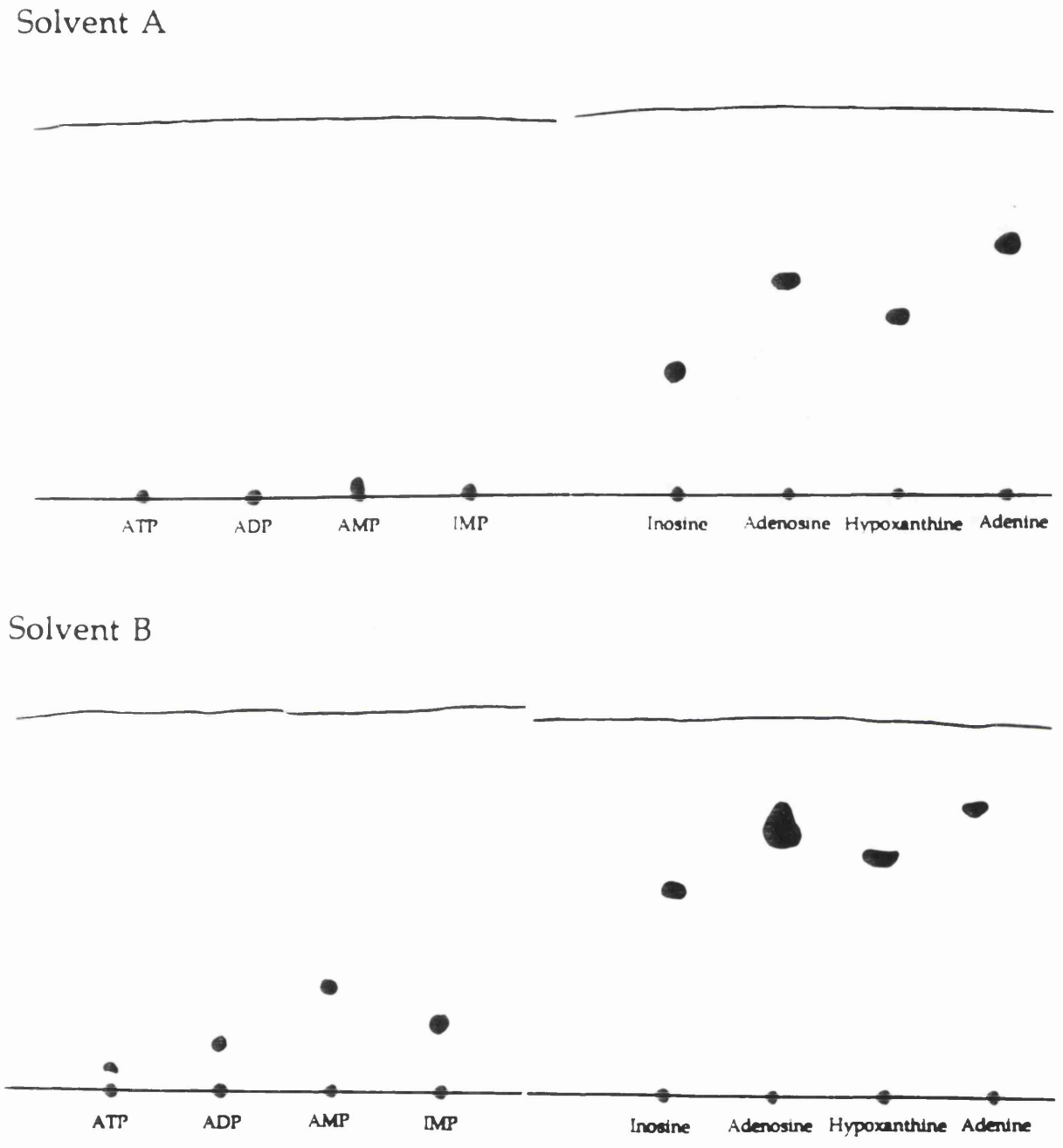


figure 3.3. TLC of nucleotide and nucleoside standards.

1.0 mM solutions of nucleotides and nucleosides were prepared in a mixture of equal volumes of phosphate buffer/EDTA homogenisation buffer and 13% TCA. 10 µl of each standard was spotted onto an aluminium-backed, fluorescent-coated TLC plate and run with TLC solvent A or TLC solvent B. The solvent fronts were marked and the plates allowed to dry. The resulting spots were visualised as quenched areas under UV radiation and marked out.

Standard	Solvent A	Solvent B
Adenosine 5'-triphosphate	--	0.06
Adenosine 5'-diphosphate	--	0.12
Adenosine 5'-monophosphate	--	0.28
Inosine 5'-monophosphate	--	0.18
Inosine	0.32	0.54
Adenosine	0.55	0.72
Hypoxanthine	0.46	0.63
adenine	0.67	0.78

table 3.2. Rf values of nucleotides and nucleosides

Rf values were calculated from the ratio of the distance travelled by the compound to that of the solvent front in the thin layer chromatograms described previously (page 65).

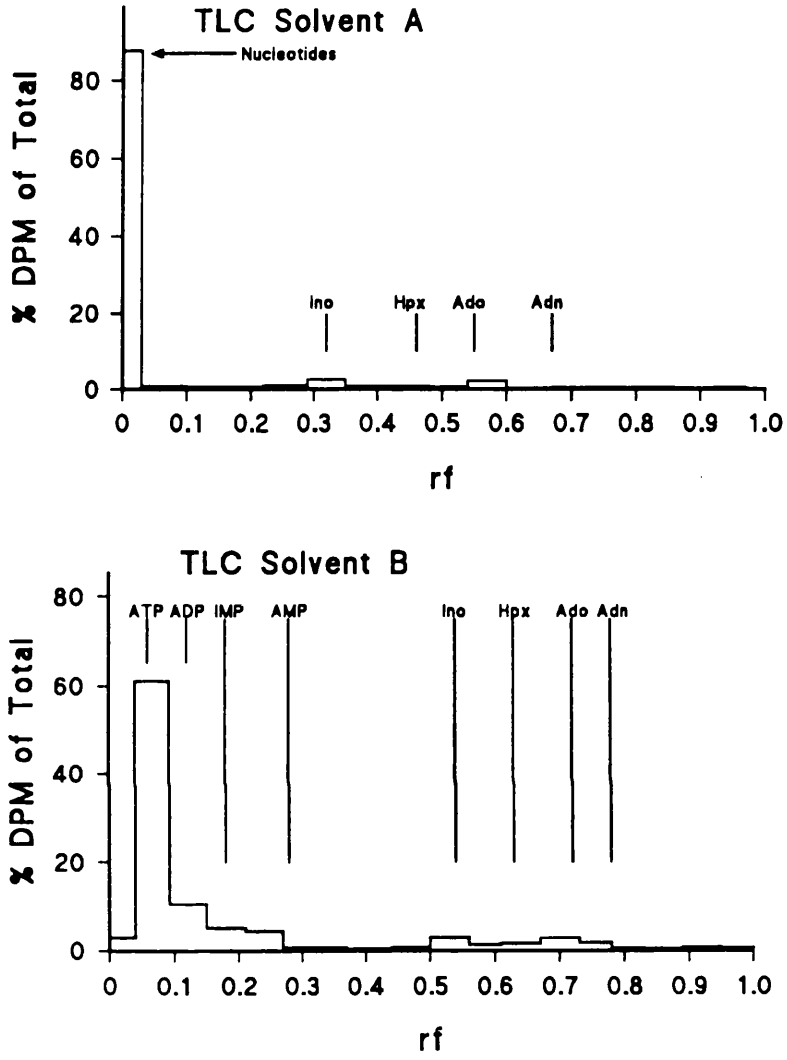


figure 3.4. TLC profile of Rat Lung Slice Homogenates incubated with adenosine.

Rat lung slices were incubated with [2-^{3}H]-adenosine (specific activity adjusted to 10 µCi/5 ml, incubation volume) at a concentration of 10 µM for 15 minutes at 37° C. Slices were then removed, washed and homogenised. The homogenate was deproteinised by the addition of an equal volume of 13% TCA, followed by centrifugation at 3000 rpm for 15 minutes. 10 µl of deproteinised lung slice homogenate was spotted onto an aluminium-backed TLC plate and run in TLC solvent A, to resolve nucleosides (figure 3.4a) or TLC solvent B, to resolve nucleotides (figure 3.4b). The plates were dried and sections 2 cm (width) x 1 cm (height) were taken and measured for radioactivity. Results were expressed as a percentage of total radioactivity found between the original spot and the solvent front.

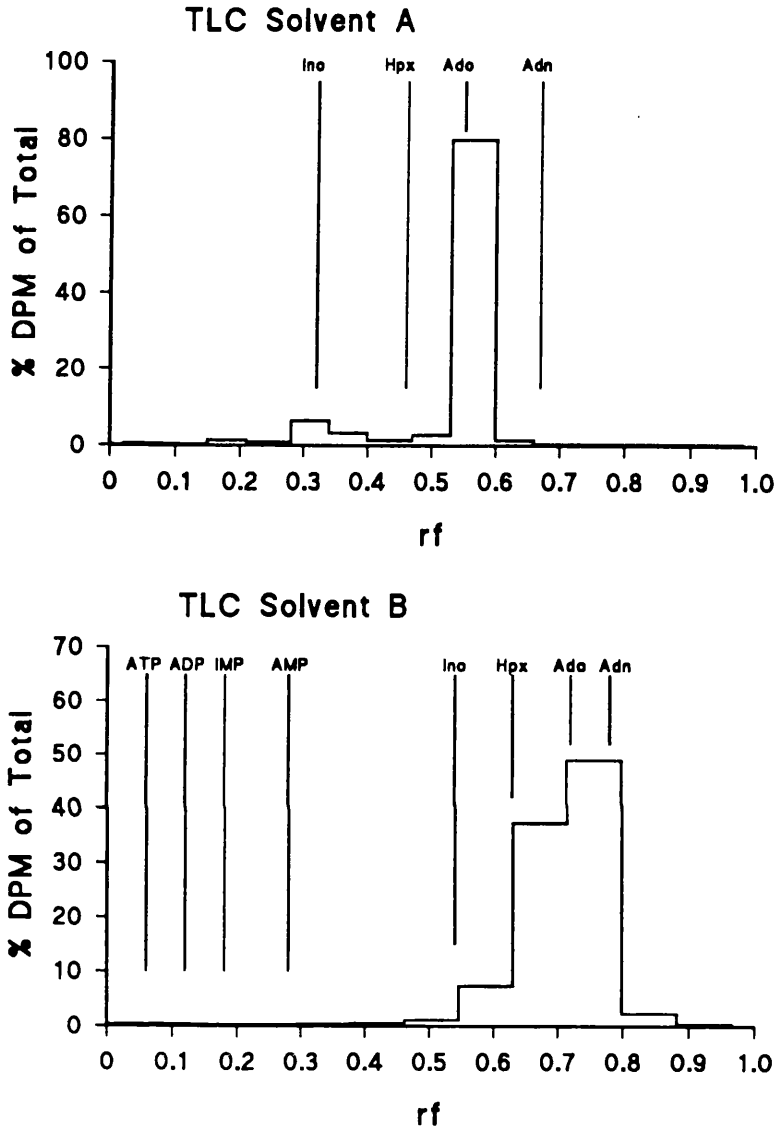


figure 3.5. TLC profile of incubation medium after incubation of rat lung slices with adenosine.

A 1.0 ml aliquot of the incubation medium, from the previous experiment, was taken immediately after removal of the slices. This was then acidified by the addition of an equal volume of 13% TCA. Debris was removed by centrifugation at 3000 rpm for 15 minutes. 10 µl of treated incubation medium was spotted onto an aluminium-backed TLC plate and run in TLC solvent A, to resolve nucleosides (figure 3.5a) or TLC solvent B, to resolve nucleotides (figure 3.5b). The plates were dried and sections 2 cm (width) x 1 cm (height) were taken and measured for radioactivity. Results were expressed as a percentage of total radioactivity found between the original spot and the solvent front.

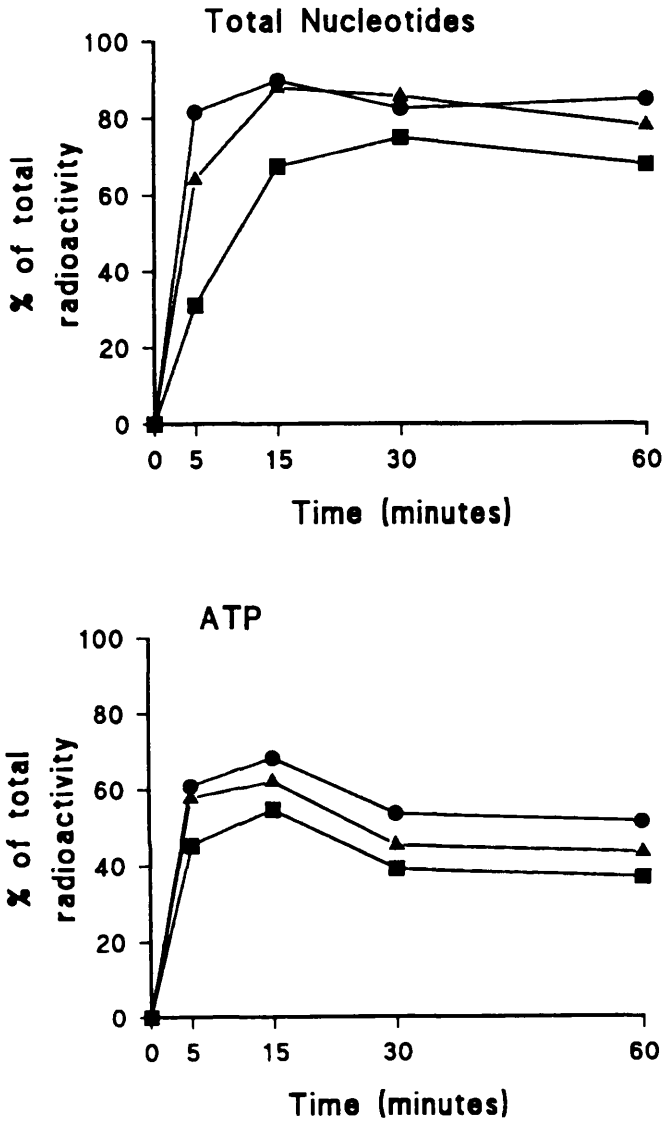


figure 3.6. Time course of adenosine incorporation into nucleotides and ATP.

Rat lung slices were incubated with [2-^{3}H]-adenosine at a concentration of 10 µM (•), 100 µM (▲) or 500 µM (■). For each experiment, the specific activity was adjusted to 1.0 µCi/5 ml (incubation volume) for time periods up to 60 minutes at 37° C. Slices were then removed, washed and homogenised. The homogenate was deproteinised by the addition of an equal volume of 13% TCA, followed by centrifugation at 3000 rpm for 15 minutes. 10 µl of deproteinised lung slice homogenate was spotted onto an aluminium-backed TLC plate and run in TLC solvent A (to show total nucleosides) or TLC solvent B (to resolve nucleotides). The plates were dried and sections 2 cm (width) x 1 cm (height) were taken and measured for radioactivity. Results were expressed as a percentage of total radioactivity found between the original spot and the solvent front appearing as total nucleotides (figure 3.6a) or ATP (figure 3.6b), determined from the standards.

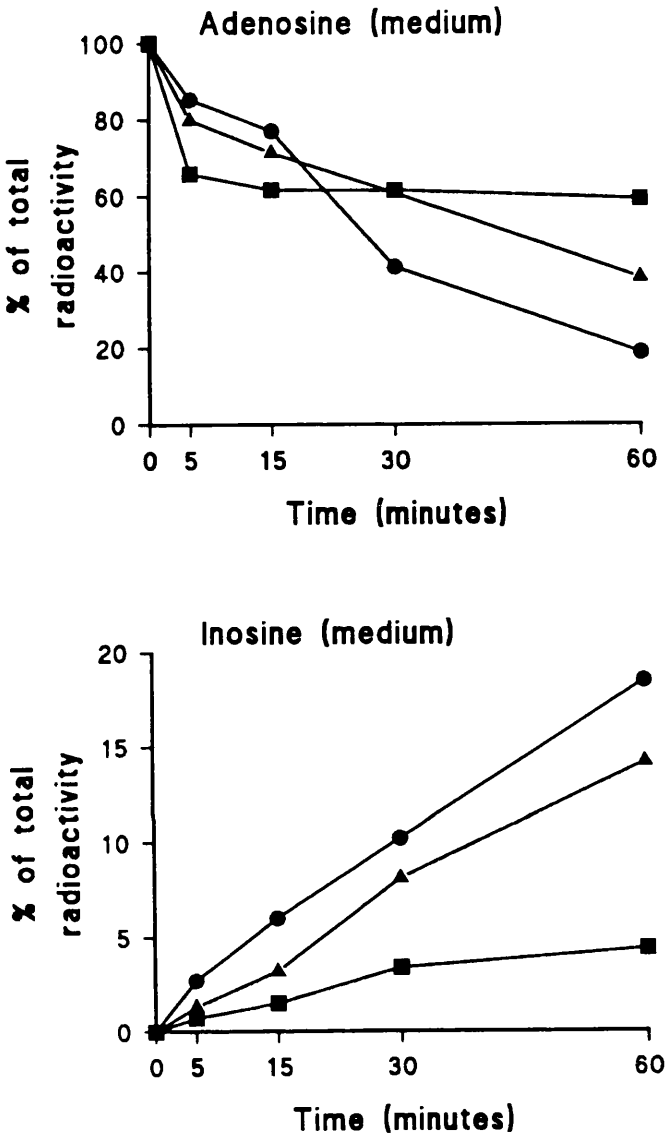


figure 3.7. Time course of disappearance of [2-^{3}H]-adenosine from, and appearance of inosine in medium incubating with rat lung slices.

A 1.0 ml aliquot of the incubation medium, from flasks containing lung slices incubated with [2-^{3}H]-adenosine at a concentration of 10 µM (•), 100 µM (▲) or 500 µM (■), as described in figure 3.6, was taken immediately after removal of the slices. This was then run in TLC solvent A (to resolve nucleosides). The plates were dried and sections 2 cm (width) x 1 cm (height) were taken and measured for radioactivity. Results were expressed as a percentage of total radioactivity found between the original spot and the solvent front appearing as adenosine (fig. 3.7a) or inosine (fig. 3.7b), as determined from the standards.

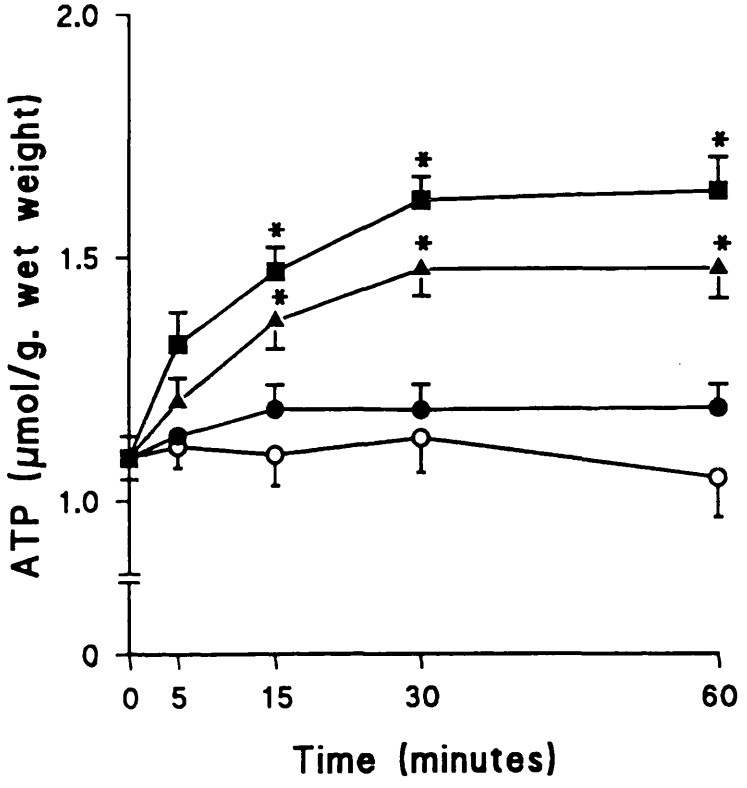


figure 3.8. Time course of adenosine induced elevation of ATP levels in rat lung slices.

Rat lung slices were incubated inthe absence of adenosine (o) or with [2-^{3}H]-adenosine (specific activity adjusted to 1.0 µCi/5 ml, incubation volume) at a concentration of 10 µM (•), 100 µM (▲) or 500 µM (■). After 15 minutes, ATP levels were measured and compared with the corresponding controls (slices incubated in the absence of adenosine. Results represent the mean of 3 experiments ± sem. * Denotes significant difference from controls ($p < 0.05$).

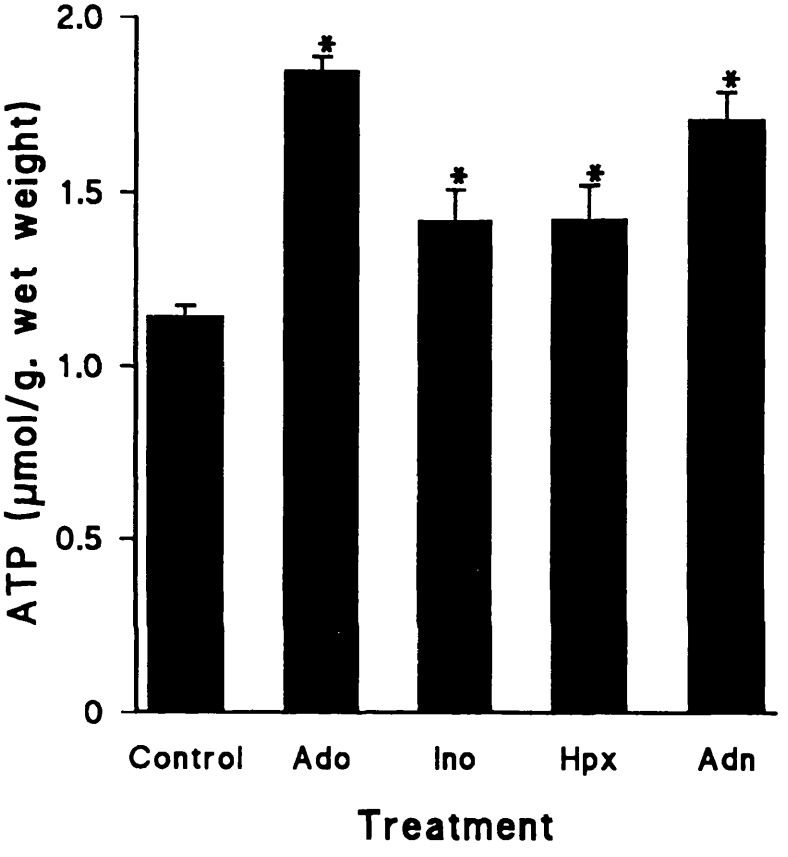


figure 3.9. Effects of nucleosides on ATP levels in rat lung slices.

Rat lung slices were incubated with KRP (con), 100 µM adenosine (Ado), 100 µM inosine (Ino), 100 µM hypoxanthine (Hpx), or 100 µM adenine (Adn) for 30 minutes. ATP levels were measured in the slices. Results represent the mean of 3 experiments ± sem. * Denotes significant difference from controls ($p < 0.05$).

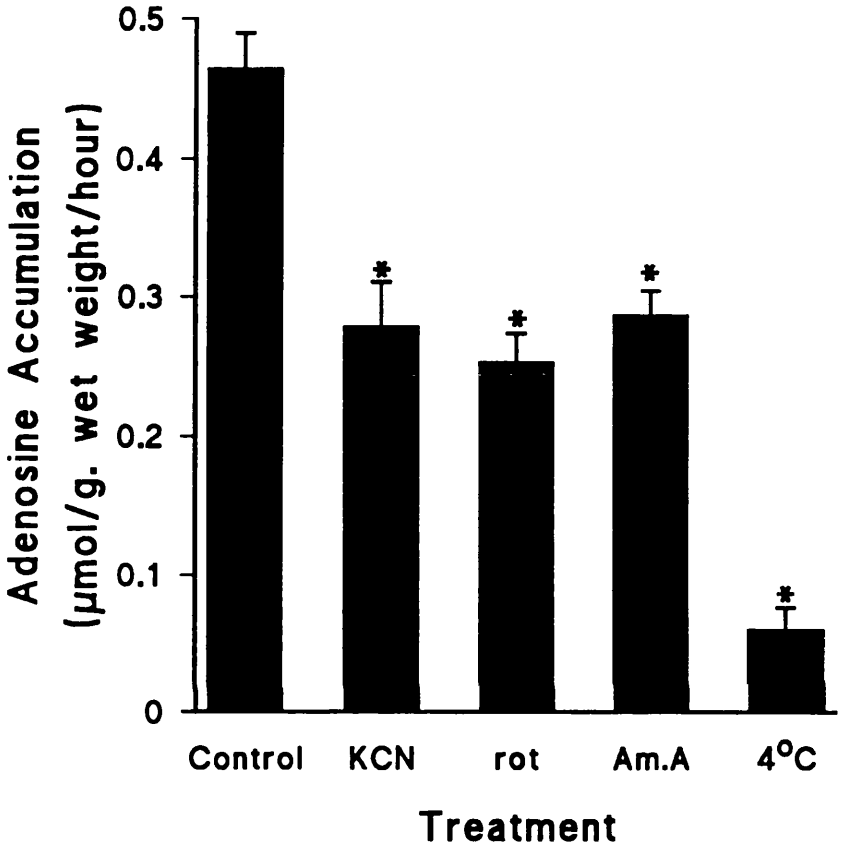


figure 3.10. Effect of metabolic inhibition on adenosine accumulation by rat lung slices.

Rat lung slices were incubated with metabolic inhibitor for 15 minutes, after which, [2-^{3}H]-adenosine was added to give a specific activity of 10 µCi/5 ml, incubation volume at a concentration of 10 µM at 37° C. After 30 minutes, slices were assessed for uptake of radiolabelled adenosine as described. Metabolic inhibition was achieved by incubation with potassium cyanide (KCN, 1 mM), rotenone (rot, 100 µM), antimycin A (Am A, 0.1 µM) and incubating the slices at 4° C throughout. Results represent the mean of 3 experiments ± sem. * denotes significant difference from controls from slices incubated in the absence of inhibitor ($p < 0.05$).

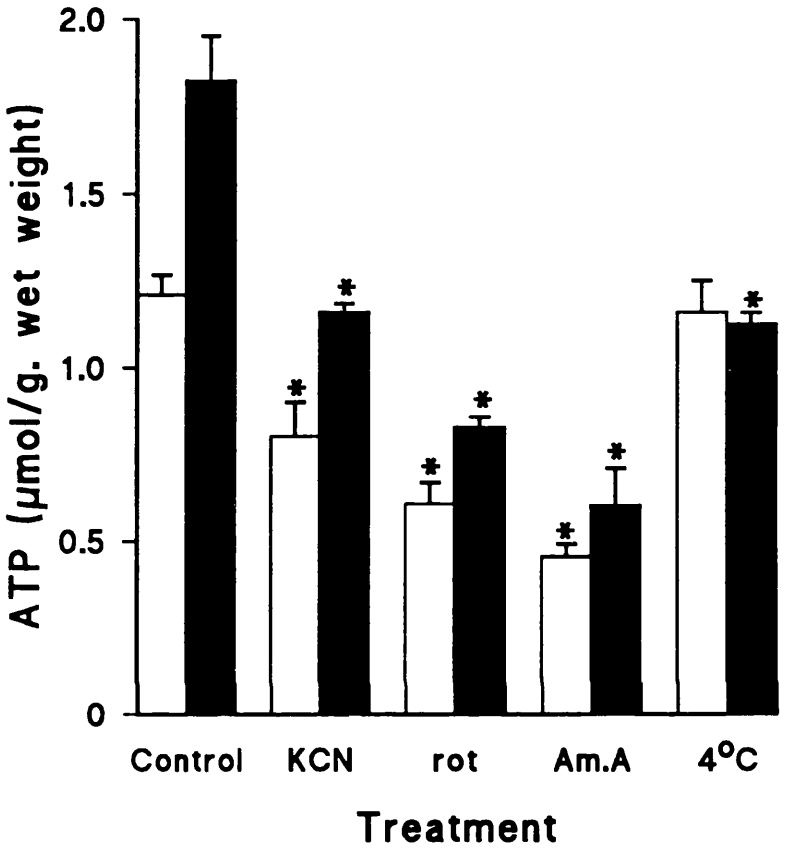


figure 3.11. Effect of metabolic inhibition on adenosine induced elevation of ATP in rat lung slices.

Rat lung slices were incubated with inhibitor at 37° C, for 15 minutes, after which, the slices were incubated in the absence (□) or presence of adenosine (100 µM, ■). After 30 minutes, ATP levels from slices incubated in the presence and in the absence of adenosine were measured in the slices. These were compared with their corresponding controls incubated in the absence of inhibitor. Metabolic inhibition was achieved by incubation with potassium cyanide (KCN, 1 mM), rotenone (rot, 100 µM), Antimycin A (Am A, 0.1 µM) and by incubating the slices at 4° C throughout. Results represent the mean of 3 experiments ± sem. * Denotes significant difference from corresponding controls from slices incubated in the absence of inhibitor ($p < 0.05$).

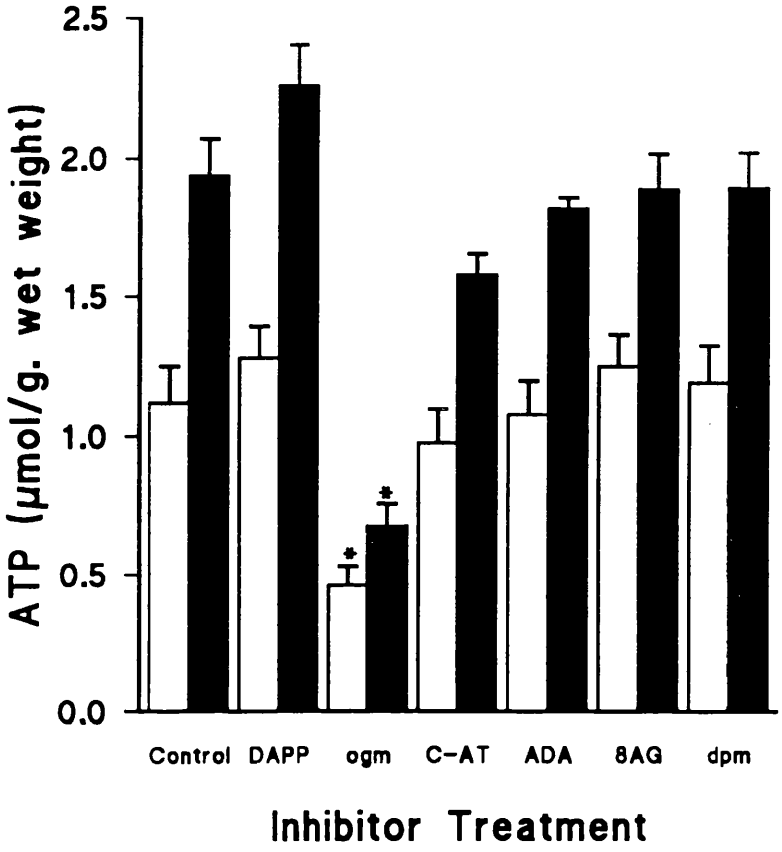


figure 3.12. Effect of inhibition of nucleoside metabolism on adenosine induced elevation of ATP levels in rat lung slices.

Rat lung slices were incubated with inhibitor at 37° C, for 15 minutes, after which, adenosine was added to give a concentration of 100 µM. After 30 minutes, ATP levels were measured in the slices (■), and compared with controls incubated in the absence of adenosine (□). Selective inhibition of enzymes associated with nucleoside transport and metabolism was achieved by incubation of lung slices with P1,P5-di(adenosine) pentaphosphate (DAPP, 0.25 mM, an inhibitor of adenylate kinase [159]), oligomycin (ogm, 5 µM, an inhibitor of ATP synthetase [159]), carboxyatractyloside (cat, 5 µM, an inhibitor of ATP mitochondrial transport [159]), 5'-amino-5'-deoxyadenosine (ADA, 20 µM, an inhibitor of adenosine kinase [153]), 8-aminoguanosine (8AG, 100 µM, an inhibitor of purine nucleoside phosphorylase [160]) and dipyridamole (Dpm, 100 µM, an inhibitor of adenosine transport [161]). Results represent the mean of 3 experiments ± sem. * Denotes significant difference from corresponding controls from slices incubated in the absence of inhibitor ($p < 0.05$).

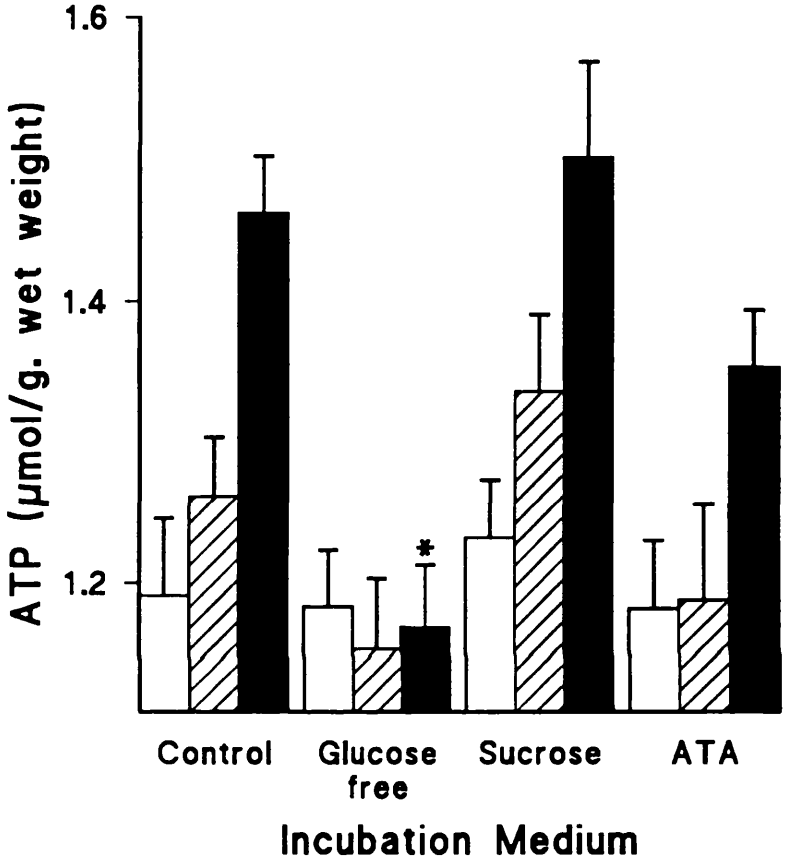


figure 3.13. Influence of glycolysis on the adenosine induced elevation of ATP levels in rat lung slices.

Rat lung slices were incubated for 1 hour in normal KRP (control), glucose-free KRP (glucose free), sucrose KRP (sucrose, 11 mM sucrose replacing glucose) and 0.25 mM aurintricarboxylic acid (ATA, an inhibitor of glycolysis), after which, adenosine was added to give a concentration of 0 µM (□), 20 µM (▨) or 100 µM (■). After 30 minutes, ATP levels were measured in the slices and compared with correspnding controls incubated with KRP with glucose, and in the absence of inhibitor. Results represent the mean of 3 experiments ± sem. * Denotes significant difference from corresponding controls from slices incubated with KRP with glucose, and in the absence of inhibitor ($p < 0.05$).

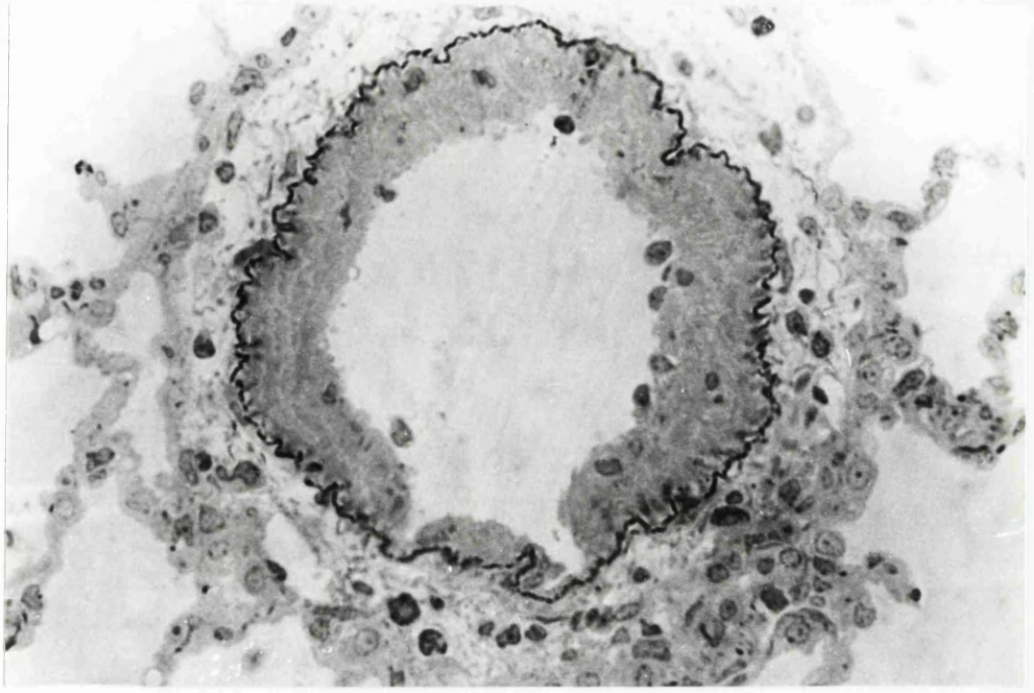
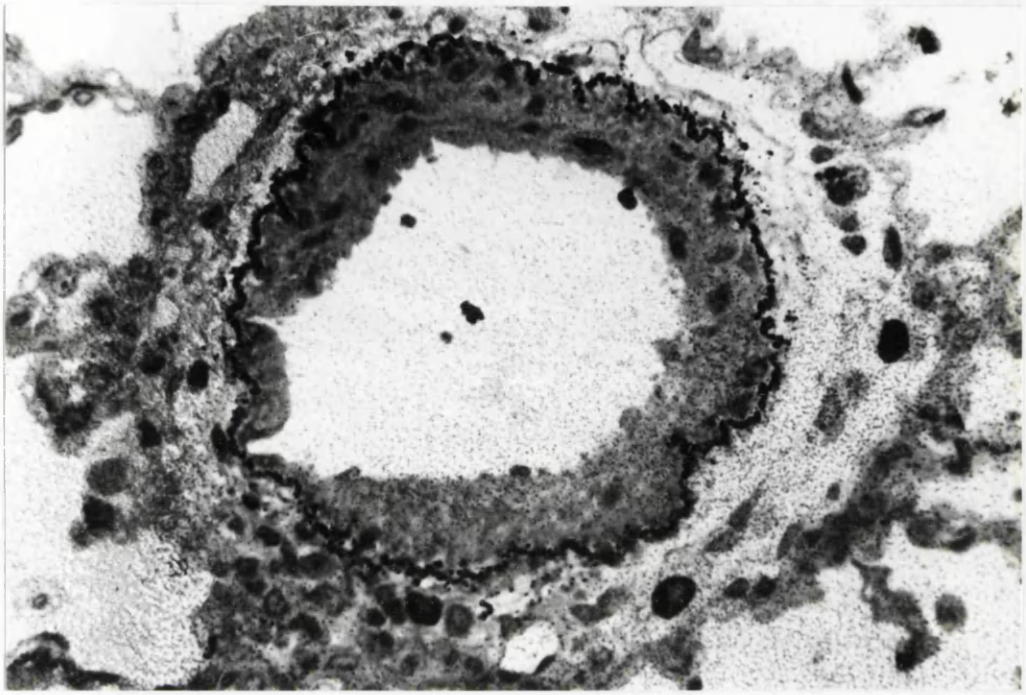
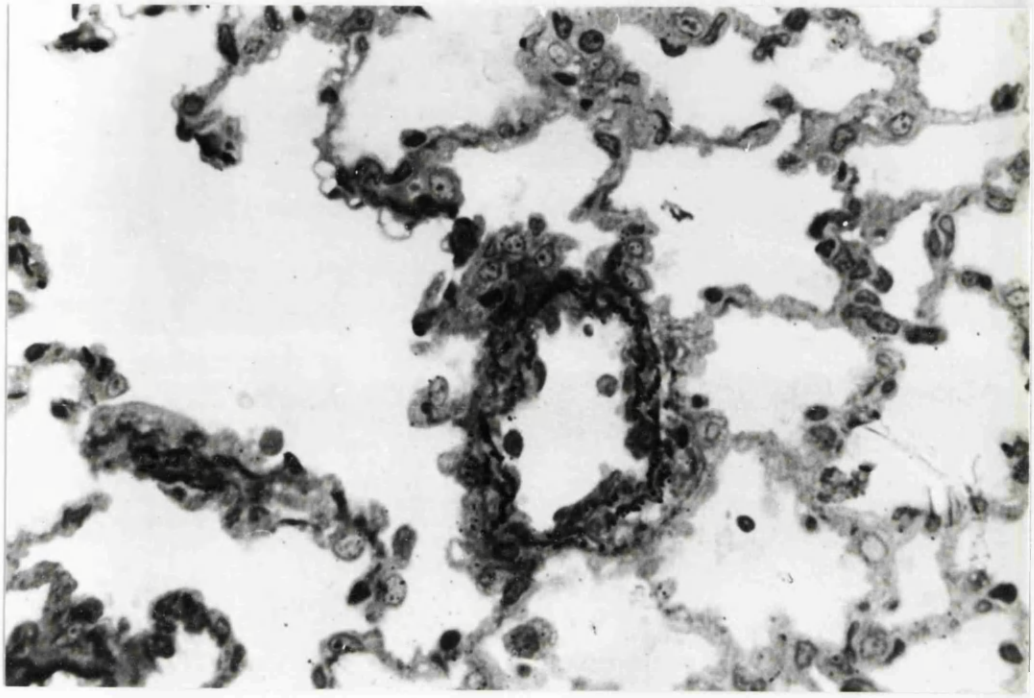


figure 3.14. Autoradiographic localisation of accumulated radiolabel

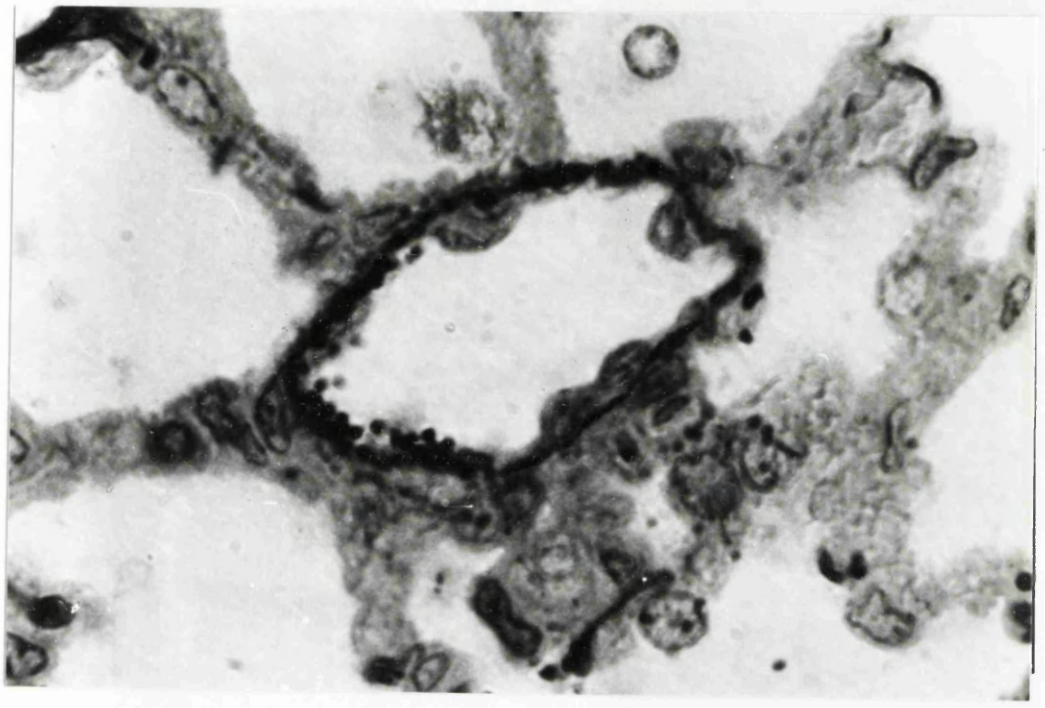
Lung slices were incubated with 10 μM [2-^{3}H]-adenosine, 10 μCi/3 ml, or KRP control, for a period of 15 minutes, and exposed autoradiographically for 4 weeks. After developing, using an appropriate fixative, the sections were then washed, dried and stained with 1% toluidine blue. The KRP control (figure 3.14a) shows a small artery. Toluidine blue staining is evident in the region of the adventitia. Magnification X 640.



The autoradiograph (figure 3.14b) shows a small artery. Labelling, in the region of the adventitia, is shown as dark spots. This occurs to a much lesser extent in the surrounding connective tissue. Magnification X 640.



The autoradiograph (figure 3.14c) shows a venule. Labelling appears to be localised within the wall of the venule. Much less label is evident within the epithelium. Magnification X 640.



The autoradiograph (figure 3.14d) shows the endothelium of a small blood vessel. Labelling appears to be localised within the endothelium. Labelling is not evident within the epithelium. Magnification X 1600.

DISCUSSION

The experiments in this chapter show that rat lung slices are capable of taking up and actively metabolising adenosine. Firstly, it was shown that radioactivity associated with [2-^{3}H]-adenosine is accumulated and retained by rat lung slices, and the accumulation is saturable (figure 3.2). This data supports a similar study by Hardwick [137] which shows that incubation of lung slices with low concentrations of adenosine (10 μM) leads to a linear accumulation of radioactivity with time, over 120 minutes, whereas at higher concentrations, the accumulation steadily diminishes showing saturation. A more detailed approach attempted to derive an apparent K_m and V_{max} for the uptake system (table 3.1). Previous work described adenosine accumulation in terms of Michaelis-Menten kinetics [150]. K_m values for adenosine accumulation in different systems show some degree of consistency, typical values reported being 215 μM for isolated rat lung [141], 250 μM and 0.3-1.1 mM [150] for cultured pig aortic endothelial cells. These values compare well with those values derived from the direct linear curve fitting and the Hanes-Woolf plots (table 3.1). Discrepancies between methods of deriving kinetic data may be due to spacing of derived data [162]. "Clustering" of data, such as that commonly seen with Lineweaver-Burk plots may limit the accuracy of the resulting kinetic data [162].

There are several reasons as to why a Michaelis-Menten approach is too simplistic to be appropriate. It has been shown previously that uptake occurs via more than one system [150]. In addition, adenosine is membrane permeable and can thus diffuse into cells [151]. A third complication is that adenosine is metabolised intracellularly, by several pathways [151]. The enzymes involved in adenosine metabolism are regulated by feedback control [151]. The kinetics of adenosine uptake is complicated by these considerations.

Adenosine is produced, released, taken up and metabolised by most animal tissues [151]. Adenosine has a number of known metabolic fates in animal tissues, however, the relative significance of each, in different tissues, is unknown. The two major fates of adenosine inside the cell are metabolism to inosine (figure 3.5) and adenine nucleotides (figure 3.4) by the enzymes adenosine deaminase and adenosine kinase respectively. When adenosine is taken up into the cell, metabolism occurs with some of the adenosine being incorporated into nucleotides, predominantly ATP [141, 150, 151, 153]. The retention of radioactivity by the cell is likely, in part, to reflect this pathway of metabolism. The main reason for this is that ATP and other nucleotides are membrane impermeable whilst adenosine, with its nucleoside metabolites inosine and hypoxanthine, is membrane permeable [151], and thus would be less likely to be retained intracellularly. This is consistent with the model proposed by Bakhle and Chelliah [141] where the majority of retained radiolabel was found as ATP. This is also confirmed here by the finding that the majority of the radioactivity retained by the slice was determined as nucleotides, predominantly ATP. This also suggests the possibility that this pathway is not rate limiting in the uptake/metabolism/retention process, as efficient incorporation into nucleotides occurs intracellularly from early time points. However, it has been suggested that adenosine kinase is the main controlling factor in adenosine entry into cells, as opposed to some external membrane receptor [151]. This is based on *in vivo* studies where labelled adenosine, administered to animals, was found to be distributed between organs roughly in proportion to the activity of adenosine kinase.

In contrast to the rapid metabolism of adenosine to adenine nucleotides (figure 3.6), the metabolism to inosine, via adenosine deaminase, occurs more slowly and more steadily over 60 minutes (figure 3.7). This can, in part, be rationalised in terms of the K_m values for the primary enzymes of adenosine metabolism. Adenosine kinase (K_m

= 0.5-1.5 μM [151]) phosphorylates adenosine to form AMP which ,it is proposed, is the pathway that results in the increase in ATP. Adenosine deaminase (K_m = 6-60 μM [151]) removes an NH_2 group to form inosine (figure 3.1). Although the involvement of adenosine deaminase in the metabolism of adenosine to inosine has never been questioned, the involvement of adenosine kinase in the incorporation into adenine nucleotides is less conclusive. It has been suggested that other pathways involve an initial de-ribosylation to form adenosine via the enzyme purine nucleoside phosphorylase followed by reaction with 5'-phosphoribosyl-1-pyrophosphate to form AMP, catalysed by adenine phosphoribosyl transferase [151]. Alternatively, adenosine is converted, via inosine, to hypoxanthine, catalysed by hypoxanthine phosphoribosyl transferase [151]. It appears, however, that these pathways are quantitatively unimportant as the activity of purine nucleoside phosphorylase in tissues is relatively low [151]. [^{14}C]-Labelled adenosine has been found to be incorporated into nucleotides without conversion to adenine. There are several lines of evidence for the importance of adenosine kinase in the incorporation of adenosine into nucleotides. These have been reviewed by Arch and Newsholme [151].

Other factors which need to be considered are those suggested by Klenow and Østergaard, that adenosine induces the catabolism of its nucleotides [153]. This would result in the adenosine induced elevation of ATP reaching a maximum level as its catabolism becomes important. Also, the production of inosine could occur via ATP catabolism, with ADP, AMP and IMP as intermediates [153]. It is not clear to what extent this pathway occurs in relation to the production of inosine by adenosine deamination. A further complication is that adenosine deaminase has been found to occur both intracellularly [163] and extracellularly [152], so that inosine found in the medium could be the result of adenosine metabolism to ATP with subsequent catabolism to inosine, intracellular or extracellular deamination of adenosine. The

multiplicity of pathways of adenosine metabolism in lung is supported by the finding that incubation with not only adenosine, but the nucleosides inosine, hypoxanthine and adenine also elevate ATP levels ($p < 0.05$, figure 3.9).

The inhibition of mitochondrial function in rat lung slices produced only a moderate reduction in adenosine accumulation ($p < 0.05$), in comparison to low temperature incubation, which reduced the adenosine accumulation more substantially ($p < 0.05$, figure 3.10). This suggests the possibility that mitochondrial function is of minor importance in the uptake and subsequent processing of adenosine. The adenosine induced ATP elevation in relation to inhibition of mitochondrial function (figure 3.11) is complicated. Whilst mitochondrial inhibitors appear to reduce ATP levels in lung slices *per se*, incubation of those slices with adenosine apparently raises ATP levels in an approximately constant ratio to those incubated without adenosine. These ratios are consistent with the fact that the phosphorylation of adenosine by adenosine kinase is ATP dependent, hence the extent to which adenosine is retained may be partly dependent on the intracellular ATP status.

To further investigate the metabolism of ATP, attempts were made to selectively inhibit the activity of enzymes associated with adenosine metabolism (figure 3.12). In general, little effect could be obtained using specific inhibitors. The interpretation of this is ambiguous. It is uncertain as to whether inhibition occurred but without affecting the ATP elevation, or physiological barriers (for example, impermeable membranes) limited access of the inhibitor to the enzyme, or whether competitive enzyme inhibition occurred but was overcome through an excess of substrate. Further work remains to elucidate these problems. In spite of these difficulties the use of oligomycin, to inhibit ATP synthetase, reduced the ATP levels in control and adenosine incubated lung slices ($p < 0.05$, figure 3.12) but the ratio of ATP elevation was unchanged from the appropriate control thus supporting the earlier

findings.

The possibility of extramitochondrial metabolism having greater significance, at least initially, in the incorporation of adenosine into nucleotides was investigated (figure 3.13). A major source of extramitochondrial ATP production is derived from the glycolytic pathways, in particular, from phosphoglycerate kinase and pyruvate kinase. The finding that the absence of glucose prevented the adenosine induced ATP elevation inferred the possibility of involvement of glycolysis, but this is complicated by the fact that the ATP elevation is restored when glucose is replaced by sucrose. This leads to several possibilities, the influence of osmosis (glucose and sucrose present at 11 mM), the metabolism of sucrose by lung tissue with respect to glycolysis, or the presence of an independent mechanism of ATP elevation. The use of aurintricarboxylic acid to inhibit phosphofructokinase had only a moderate apparent influence on ATP elevation. This, again, needs to be interpreted with caution in that inhibition may not have occurred, been overcome or that inhibition may have occurred but with little consequence. Further work remains to elucidate the influence of glycolysis on the adenosine induced elevation of ATP levels in rat lung slices.

The use of autoradiography supports the hypothesis that adenosine accumulation is localised within lung tissue (figure 3.14). It has been suggested that localisation is primarily endothelial as a result of a number of studies. Adenosine receptors, however are known to be distributed heterogeneously, with several classes of receptor described in pharmacological terms, thus reflecting a diversity of effects in different tissues. However, only a few cell types have been shown to incorporate adenosine into ATP. The apparent localisation of radiolabelling around blood vessels in lung tissue needs to be viewed cautiously as *in vitro* autoradiography is subject to artefacts due to the nature of the fixing process, hence the possibility of selective trapping of radiolabel cannot be excluded. The fact that localisation of adenosine

accumulation occurs in lung tissue is further supported by Hardwick who showed that paraquat (known to be selective for the epithelium) did not significantly affect adenosine accumulation, whereas *in vivo* treatment with α-naphthylthiourea (which causes endothelial damage), markedly reduced adenosine accumulation.

Preliminary work has thus indicated that adenosine accumulation may indicate selective toxicity, possibly to the endothelium, but correlation with endothelial cell function in the lung is difficult to interpret. The suggestion has been made earlier that retention of radiolabelled adenosine could, to a degree reflect its incorporation into nucleotides. If the initial metabolic step is the phosphorylation of adenosine by adenosine kinase, which is dependent on the level of ATP initially present, then a relationship between adenosine accumulation and ATP status could possibly be inferred. The fact that adenosine accumulation and incorporation into ATP has been shown to be energy dependent suggests that the viability of cell types to which this is localised may be inferred. That a relationship to initial cellular ATP status may be a possibility would lead to a greater degree of confidence to which cellular function could be quantified. The scenario is obviously more complex, as adenosine metabolism is regulated by its own metabolites as well as those from other pathways. In spite of the complexities, adenosine uptake appears consistent in lung slices prepared from healthy, untreated rats. The loss of adenosine accumulation in lung slices prepared from rats treated with pulmonary toxicants *in vivo* [137] shows a correlation with damage to cell types in the lung identified histologically [27]. Pathological lesions to the pulmonary endothelium have been shown in the absence of striking changes in assessments of endothelial cell function [164]. The discrepancy between pathology and cell-specific functional markers is further complicated by the extent to which a particular effect represents a physiological or toxicological response, or even to the extent to which these can be delineated.

Further studies on the accumulation of adenosine by rat lung slices would include investigations into cellular localisation, using histology, toxicants selective for particular cell types and selective inhibitors of nucleoside uptake and metabolism. Some useful studies could possibly employ isolated lung cells to assess the participation of individual cell types in this process. A better understanding of the relationship between loss of adenosine accumulation and loss of other cellular functions, associated with toxic injury, is required. This requires a more detailed understanding of the biochemistry and physiology of the cell types associated with adenosine accumulation than is presently known. Also, the relationship between cellular biochemistry, cellular function and cell injury needs to be more fully explored to assess the value of such markers of cell function.

Chapter 4

The biochemistry of glutathione in mechanisms of pulmonary toxicity - the relative roles of conjugation and oxidative stress.

INTRODUCTION

Glutathione is proposed to limit the degree of toxic insult to the lung by two main mechanisms. The first of these is by the detoxication of potentially toxic oxygen species, via enzymic reduction. A number of compounds are substrates in the cell for reduced pyridine nucleotide dependent, one electron reductases. These enzymes reduce such compounds to their respective free radical species, which are unstable and spontaneously return to the parent compound, in the presence of molecular oxygen to form the superoxide ion radical ($O_2^{\cdot-}$). The reduction and oxidation reactions occur in a cyclic manner and is thus referred to as redox cycling (see Introduction). The superoxide anion radical formed is converted to hydrogen peroxide by superoxide dismutase, but this too must be removed if deleterious effects are to be avoided.

There are two enzyme activities present in the cell which are capable of catalysing the breakdown of hydrogen peroxide. Catalase is responsible for the metabolism of hydrogen peroxide produced by the action of peroxisomal enzymes. Glutathione peroxidase, a selenium-containing enzyme is mainly concerned with metabolism of hydrogen peroxide occurring in the cytosol and mitochondria [165]. Some metabolism of organic hydroperoxides also occurs via this enzyme. In addition to the selenium containing enzyme, one of the glutathione-S-transferase isozymes has been shown to possess glutathione peroxidase activity [166].

The glutathione-S-transferase glutathione peroxidase activity is mainly concerned with metabolism of organic hydroperoxides, although some hydrogen peroxide metabolising activity has been shown. Glutathione peroxidase catalyses the following reactions:

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

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

The resulting oxidised glutathione (GSSG) has two possible fates. It may be actively extruded from the cell [167, 168], or it can be reduced back to GSH by GSSG reductase at the expense of NADPH. The ability of the reductase to reduce increased amounts of GSSG is dependent on the supply of NADPH [169] and thus the removal of large quantities of peroxide can lead to elevated levels of GSSG, if the NADPH is limiting, and hence a depletion of intracellular glutathione through the loss of GSSG from the cell. Because of the important role played by glutathione in the protection of cellular thiols its loss has been suggested to be the crucial event in the toxicity of compounds which induce oxidative stress. The level of GSSG reductase activity may therefore be of great significance in determining the susceptibility of a cell to the toxicity of compounds which exert their toxicity by a mechanism of redox cycling.

The second main mechanism of protection from toxicity involves conjugation of glutathione with a compound or its metabolite. Compounds or metabolites can react directly with glutathione. This usually results in a less reactive compound, although in some cases, increased toxicity may occur [170]. Reaction with glutathione can occur spontaneously, or may be catalysed by the glutathione-S-transferases [89]. Conjugation with glutathione represents an important cellular defence mechanism

against the toxic action of a wide variety of reactive electrophiles [90]. Reactions of glutathione show some selectivity between electrophiles of similar reactivity [89]. The basis for this is described in terms of chemical "hardness" and "softness" of electrophiles and nucleophiles [109]. This is more fully discussed in chapter 1.

To assess the role of pulmonary glutathione, and its associated enzymes, in redox cycling, a lung slice model had been employed which has a marked and irreversibly inhibited glutathione reductase activity [130]. This was achieved by pre-incubating the slices with the antitumour agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Nitrosoureas have been used as active-site directed inhibitors of a number of enzymes [121] and BCNU has been found to be a relatively specific inhibitor of GSSG reductase [120]. The specificity of GSSG reductase inactivation was confirmed in erythrocytes in which it was found that nineteen associated enzyme activities were either unaffected or only minimally affected [120]. However, some studies have suggested effects on enzymes such as lactate and malate dehydrogenases, creatine kinase and hepatic esterases [173]. It has been suggested that such effects may possibly result from its carbamoylating activity [174].

It is not the parent compound that is responsible for the inactivation of the enzyme, but the isocyanate product of nitrosourea breakdown (in the case of BCNU, 2-chloroethylisocyanate). The inactivation requires the initial reduction of the enzyme by NADPH and appears to occur through carbamoylation of a distal thiol group at the active site [121].

Both the cytosolic and mitochondrial reductases are inhibited by BCNU [172], and >90% inactivation can be achieved in freshly isolated hepatocytes within 60 minutes incubation with a concentration of 75 μM [174]. It was also found that about 80 % inhibition can be achieved in rat lung slices after 45 minutes incubation with a concentration of 100 μM [130]. The remaining activity however, appears to be fairly

resistant and requires considerable increases in the concentration of inhibitor used and of the length of exposure.

A difficulty in the use of BCNU is the accompanying depletion of glutathione. This may in part be a result of the inactivation of the reductase such that the removal of endogenous hydrogen peroxide leads to a build up, and loss from the cell, of GSSG. The metabolism of BCNU in the cell also leads to the formation of S-(2-chloroethyl) glutathione [174] and hence some glutathione will be lost through conjugation reactions. The effects of a lowered glutathione levels in the cell may influence the toxicity of the compounds. For this reason, it is necessary to make up this loss and maintain normal levels. This is achieved by supplementing the incubation medium with an appropriate amino acid mixture to stimulate glutathione synthesis [169].

The aims of this study were to investigate the influence of inhibition of glutathione reductase in rat lung slices on the toxicity of a number of compounds. Some preliminary studies involving the use of proposed inhibitors of the antioxidant defence enzymes catalase and glutathione peroxidase are presented. The toxicants investigated were diquat and 1,4-benzoquinone. Diquat is presumed to exert toxicity via redox cycling [63], whilst 1,4-benzoquinone has been shown to act via arylation of glutathione and other nucleophiles [175]. The effects of these compounds on glutathione levels were assessed. Toxicity was assessed by measuring ATP levels in lung slices, as ATP reflects the energy status of the cell. To assess the differential toxicity between the epithelial and endothelial cells in the lung, the accumulation of [^{14}C]-spermidine [138] and [2-^{3}H]-adenosine ([137] and chapter 3) were employed respectively.

RESULTS

Time Course of GSH and ATP Depletion in Rat Lung Slices Incubated with Diquat

Incubation of rat lung slices with diquat produced no discernible loss of GSH (figure 4.1a). Similarly, ATP levels were maintained at control values (figure 4.2a) suggesting a lack of toxicity of diquat to rat lung *in vitro*. Incubation of lung slices with 2,3-dimethoxy-1,4-naphthoquinone (a redox cycler) produced a time dependent loss of GSH at the concentration used (50 μM, $p < 0.05$, figure 4.1a). This was most rapid in the first two hours of incubation. An appreciable loss of ATP was also observed but this occurred between 2 and 3 hours ($p < 0.05$, figure 4.2a). Inhibition of glutathione reductase, by pretreatment of the slices with BCNU, resulted in a time dependent and concentration dependent loss of GSH ($p < 0.05$, figure 4.1b) and ATP levels ($p < 0.05$, figure 4.2b) in lung slices incubated with diquat. BCNU pretreatment potentiated a loss of GSH in lung slices incubated with 100 μM diquat ($p < 0.05$), the potentiation of loss of ATP was small but not significant. The loss of GSH and ATP seen in lung slices incubated with 2,3-dimethoxy-1,4-naphthoquinone was markedly potentiated by BCNU pretreatment ($p < 0.05$), especially between 1 and 3 hours.

Time Course of Loss of Accumulation of Spermidine and Adenosine by Rat Lung Slices Incubated with Diquat

Incubation of rat lung slices with diquat produced no discernible loss of spermidine accumulation (figure 4.3a), suggesting a lack of toxicity to the

pulmonary epithelium. Similarly, with diquat, no discernible loss of adenosine accumulation was observed (figure 4.4a). This suggests a lack of toxicity of diquat to the pulmonary endothelium. Incubation of rat lung slices with paraquat (a redox cycler, accumulated by the pulmonary epithelium) produced a small loss of spermidine accumulation at the concentration used ($p < 0.05$, 100 μM, figure 4.3a). This was especially notable in the first two hours of incubation. Incubation with paraquat produced no discernible loss of adenosine accumulation (figure 4.4a). Inhibition of glutathione reductase, by pretreatment of the slices with BCNU, resulted in a time dependent and concentration dependent loss of spermidine accumulation in lung slices incubated with diquat ($p < 0.05$, figure 4.3b). A loss of adenosine accumulation was also observed but only at the higher concentration of diquat (1000 μM, $p < 0.05$, figure 4.4b). The loss of spermidine accumulation seen in lung slices incubated with 100 μM paraquat (figure 4.4) was markedly potentiated by BCNU pretreatment ($p < 0.05$), however, no potentiation of loss of adenosine accumulation was observed.

Time Course of GSH and ATP Depletion in Rat Lung Slices Incubated with 1,4-Benzoquinone

Incubation of lung slices with 1,4-benzoquinone produced a rapid and concentration dependent loss of GSH ($p < 0.05$, figure 4.5a). Similarly, a rapid and concentration dependent loss of ATP was observed ($p < 0.05$, figure 4.6a). The loss of GSH due to incubation with 1,4-benzoquinone was essentially complete within 10 minutes (figure 4.7a). The loss of ATP showed a time dependence up to 30 minutes (figure 4.7b). Inhibition of glutathione reductase, by pretreatment of the slices with BCNU, did not result in a modulation of loss of either GSH (figure 4.5b)

or ATP (figure 4.6b).

Time Course of Loss of Accumulation of Spermidine and Adenosine by Rat Lung Slices Incubated with 1,4-Benzoquinone

Incubation of rat lung slices with 1,4-benzoquinone produced a rapid and concentration dependent loss of spermidine accumulation ($p < 0.05$, figure 4.8a). Similarly, a rapid and concentration dependent loss of adenosine accumulation was observed ($p < 0.05$, figure 4.9a). The loss of both spermidine accumulation and adenosine accumulation, due to incubation with 1,4-benzoquinone, was essentially complete between 20 and 60 minutes (figure 4.10). Incubation with 100 μM 1,4-benzoquinone for 5 minutes resulted in a marked loss of adenosine accumulation ($p < 0.05$, figure 4.10b) but spermidine accumulation did not differ from those seen in controls (figure 4.10a).

Effect of Glutathione Peroxidase Inhibitors on the Depletion of GSH and ATP in Rat Lung Slices Incubated with Compounds that Produce Oxidative Stress

Incubation of lung slices with paraquat produced no appreciable difference in GSH (figure 4.11a) or ATP (figure 4.11b) to controls. Incubation with 2,3-dimethoxy-1,4-naphthoquinone or hydrogen peroxide produced an apparent reduction in both GSH and ATP levels compared with control values. Preliminary studies involved pretreatment plus co-incubation of lung slices with inhibitors of selenium dependent glutathione peroxidase (aurothioglucose [118], mercaptosuccinic acid [119]) or an inhibitor of selenium independent glutathione

peroxidase/glutathione-S-transferase (S-octyl glutathione [176]). All of the inhibitor treatments resulted in no appreciable alteration in the reduction of GSH or ATP levels produced by PQ, DMN or H_2O_2.

Effect of Glutathione Peroxidase Inhibitors on the Loss of Accumulation of Spermidine and Adenosine by Rat Lung Slices Incubated with Compounds that Produce Oxidative Stress

Incubation of rat lung slices with paraquat or 2,3-dimethoxy-1,4-naphthoquinone produced a small but not statistically significant depletion in spermidine accumulation (figure 4.12a). Adenosine accumulation (figure 4.12b) was not appreciably depleted compared to controls. Incubation with hydrogen peroxide produced a more noticeable reduction in both spermidine accumulation and adenosine accumulation compared with control values. Preliminary studies involved pretreatment plus coincubation of lung slices with inhibitors of selenium dependent or selenium independent glutathione peroxidase in the same manner as slices taken for GSH and ATP measurements. Aurothioglucose and mercaptosuccinic acid appeared to potentiat loss of spermidine accumulation, but no modulation of adenosine accumulation was observed. S-Octyl glutathione produced no detectable modulation of spermidine or adenosine accumulation.

Effect of Catalase Inhibitors on the Depletion of GSH and ATP in Rat Lung Slices Incubated with Compounds that Produce Oxidative Stress

Incubation of lung slices with paraquat produced no appreciable difference

in GSH (figure 4.13a) or ATP (figure 4.13b) to controls. Incubation with 2,3-dimethoxy-1,4-naphthoquinone or hydrogen peroxide produced an apparent reduction in both GSH and ATP levels compared with control values. Preliminary studies involved pretreatment plus coincubation of lung slices with inhibitors of catalase (sodium azide [177], 3-aminotriazole [178], cyanamide [179]). Treatment with 3-aminotriazole resulted in a small loss of GSH in control slices (figure 4.13a). Cyanamide produced a small loss of GSH in unstressed control and stressed lung slices. The loss of GSH in control slices means that the increased loss of GSH in cyanamide treated stressed slices cannot necessarily be viewed as potentiation. Sodium azide produced a large depletion of ATP in unstressed control lung slices (figure 4.13a) and thus modulation of loss of ATP in stressed slices was obscured. 3-Aminotriazole produced little or no modulation of ATP levels in stressed and unstressed lung slices. Cyanamide potentiated the ATP loss produced by 2,3-dimethoxy-1,4-naphthoquinone and hydrogen peroxide but not by paraquat.

Effect of Catalase Inhibitors on the Loss of Accumulation of Spermidine and Adenosine by Rat Lung Slices Incubated with Compounds that Produce Oxidative Stress

Incubation of lung slices with paraquat produced no appreciable difference in spermidine accumulation (figure 4.14a) or adenosine accumulation (figure 4.14b) to controls. Incubation with 2,3-dimethoxy-1,4-naphthoquinone or hydrogen peroxide produced a marked reduction in both spermidine accumulation and adenosine accumulation levels compared with control values. Preliminary studies involved pretreatment plus coincubation of lung slices with inhibitors of catalase in the same manner as slices taken for GSH and ATP measurements. Treatment

with sodium azide resulted in a potentiation of loss of spermidine accumulation in all of the stressed slices. Control values for spermidine accumulation were maintained. Treatment with 3-aminotriazole resulted in little or no potentiation of loss of spermidine accumulation in stressed lung slices. Cyanamide potentiated the ATP loss produced by 2,3-dimethoxy-1,4-naphthoquinone and hydrogen peroxide but not by paraquat. Sodium azide and cyanamide potentiated the loss of adenosine accumulation produced by 2,3-dimethoxy-1,4-naphthoquinone and hydrogen peroxide (figure 4.14b) otherwise little effect was seen by the inhibitors.

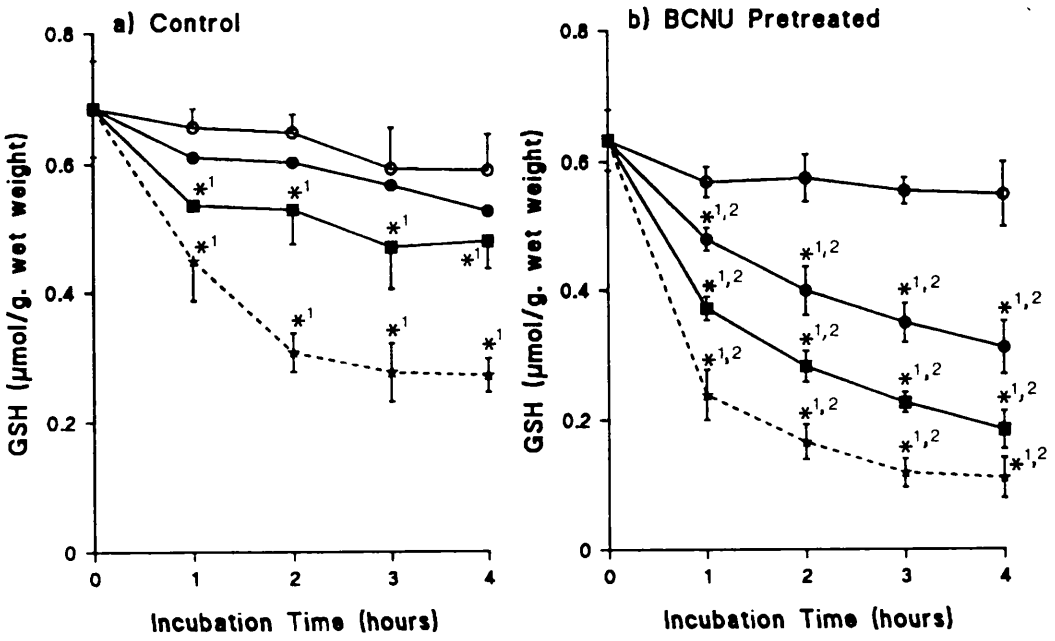


Figure 4.1. Effect of diquat on levels of GSH in a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP only (a), or BCNU (b) for 45 minutes. They were then removed, rinsed in fresh KRP and incubated in the absence of diquat (o——o), or with diquat at concentrations of 200 µM (•——•) or 1000 µM (■——■) or with 2,3-dimethoxy-1,4-naphthoquinone at a concentration of 50 µM (★— —★) for up to 4 hours. GSH was determined (as NPSH) in supernatants from lung slice homogenates by the fluorometric method of Hissin and Hilf [131]. Results represent the mean of 3 experiments ± sem.

$*^1$ Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of diquat for a particular pretreatment.

$*^2$ Denotes significant difference ($p<0.05$) between results obtained from control pretreated and the corresponding BCNU pretreated lung slices.

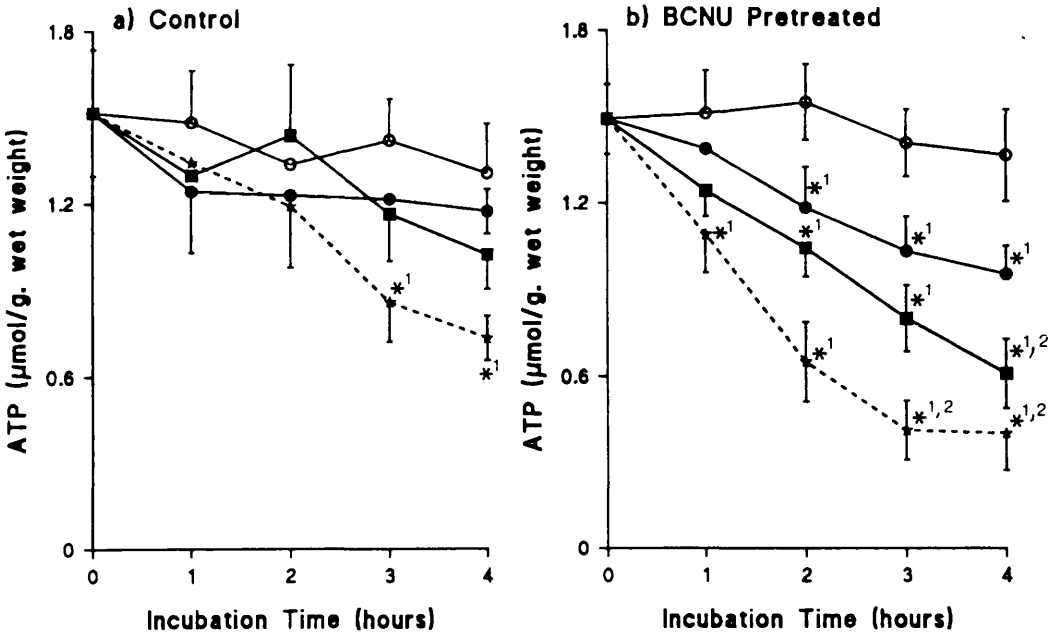


Figure 4.2. Effect of diquat on levels of ATP in a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP only (a), or BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of diquat (o——o), or with diquat at concentrations of 200 µM (•——•) or 1000 µM (■——■) or with 2,3-dimethoxy-1,4-naphthoquinone at a concentration of 50 µM (★——★) for up to 4 hours. ATP was determined in supernatants from lung slice homogenates by the bioluminescence method of LeMasters and Hackenbrock [136]. Results represent the mean of 3 experiments ± sem.
$*^1$ Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of diquat for a particular pretreatment.
$*^2$ Denotes significant difference ($p<0.05$) between results obtained from control pretreated and the corresponding BCNU pretreated lung slices.

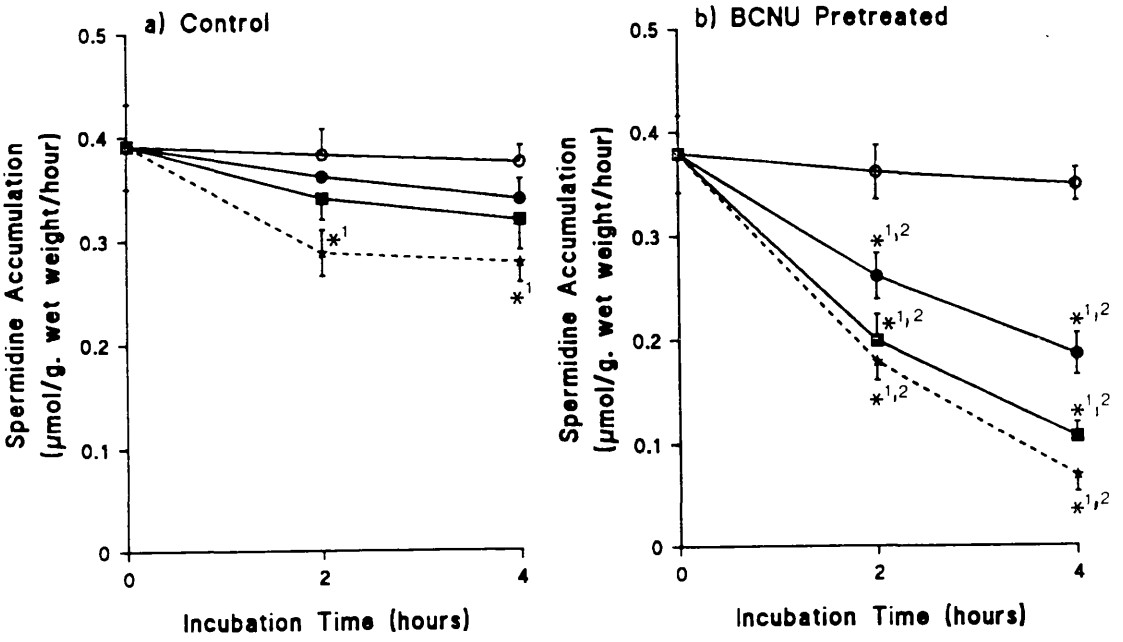


Figure 4.3. Effect of diquat on the accumulation of spermidine by a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP alone (a), or with BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of diquat (o——o), or with diquat at concentrations of 200 µM (•——•), 1000 µM (■——■) or with paraquat at a concentration of 100 µM (★– –★) for up to 4 hours. The lung slices were then removed, briefly immersed in fresh KRP and accumulation of [^{14}C]-spermidine was assessed. Results represent the mean of 3 experiments ± sem.

*[1] Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of diquat for a particular pretreatment.

*[2] Denotes significant difference ($p<0.05$) between results obtained from control pretreated and the corresponding BCNU pretreated lung slices.

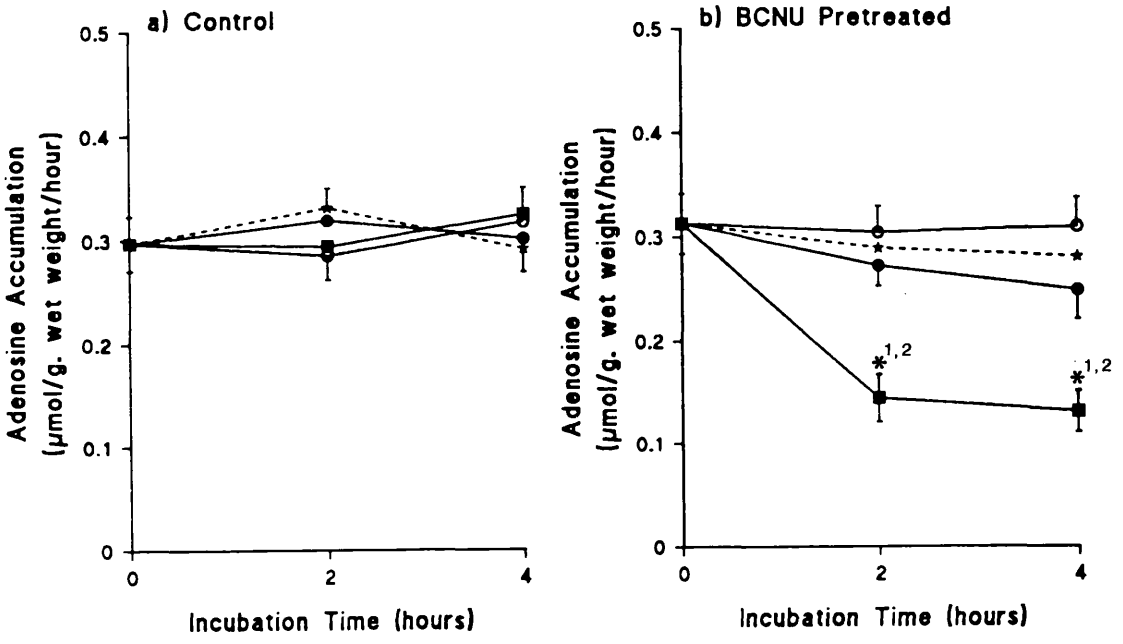


Figure 4.4. Effect of diquat on the accumulation of adenosine by a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP alone (a), or with BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of diquat (o——o), or with diquat at concentrations of 200 µM (•——•), 1000 µM (■——■) or with paraquat at a concentration of 100 µM (★– –★) for up to 4 hours. The lung slices were then removed, briefly immersed in fresh KRP and accumulation of [^{3}H]-adenosine was assessed. Results represent the mean of 3 experiments ± sem.

*[1] Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of diquat for a particular pretreatment.

*[2] Denotes significant difference ($p<0.05$) between results obtained from control pretreated and the corresponding BCNU pretreated lung slices.

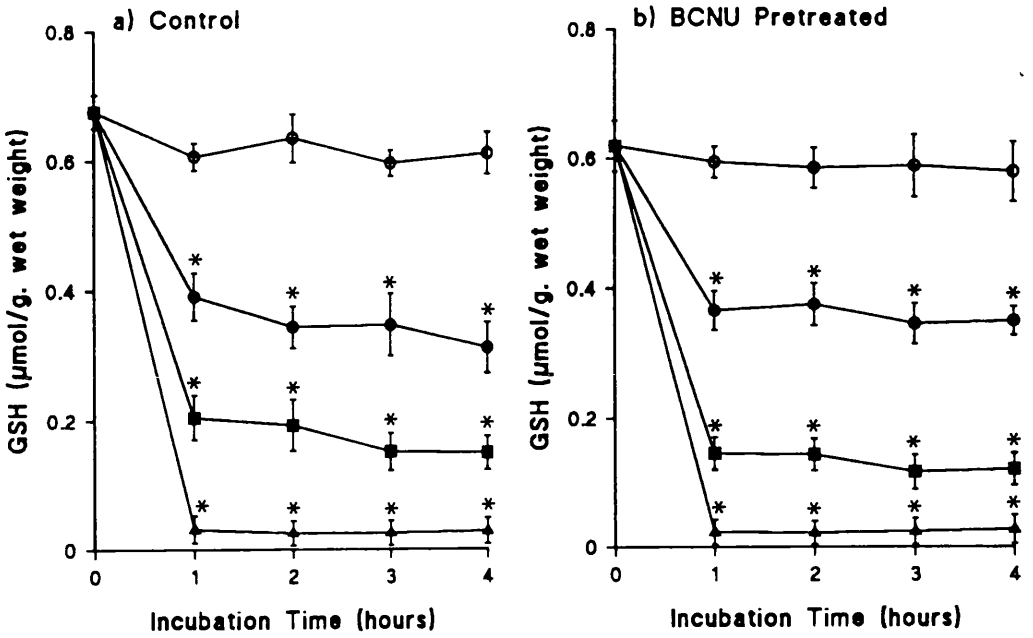


Figure 4.5. Effect of 1,4-benzoquinone on levels of GSH in a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP alone (a), or with BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone at concentrations of 20 µM (•——•), 100 µM (■——■) or 500 µM (▲——▲) for up to 4 hours. GSH was determined (as NPSH) in supernatants from lung slice homogenates by the fluorometric method of Hissin and Hilf [131]. Results represent the mean of 3 experiments ± sem.

* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone for a particular pretreatment.

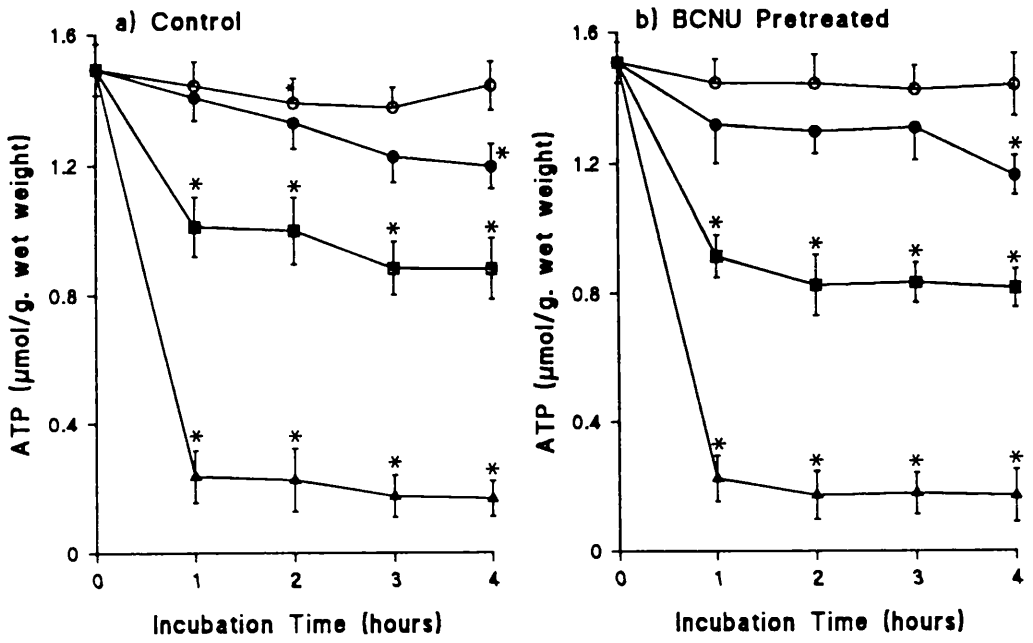


Figure 4.6. Effect of 1,4-benzoquinone on levels of ATP in a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP only (a), or BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone at concentrations of 20 µM (•——•), 100 µM (■——■) or 500 µM (▲——▲) for up to 4 hours. ATP was determined in supernatants from lung slice homogenates by the bioluminescence method of LeMasters and Hackenbrock [136]. Results represent the mean of 3 experiments ± sem.

* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone for a particular pretreatment.

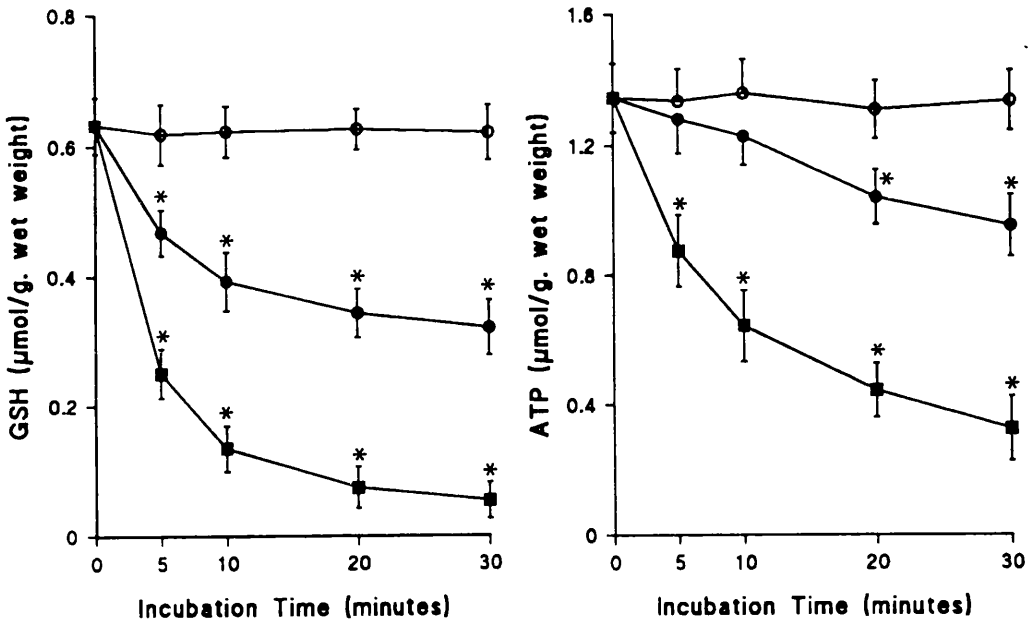


Figure 4.7. Effect of short incubations of 1,4-benzoquinone on levels of a) GSH and b) ATP in rat lung slices.

Lung slices were incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone 100 µM (•——•) or 500 µM (■——■) for 30 minutes. GSH was determined (as NPSH) in supernatants from lung slice homogenates by the fluorometric method of Hissin and Hilf [131]. ATP was determined in supernatants from lung slice homogenates by the bioluminescence method of LeMasters and Hackenbrock [136]. Results represent the mean of 3 experiments ± sem.

* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone.

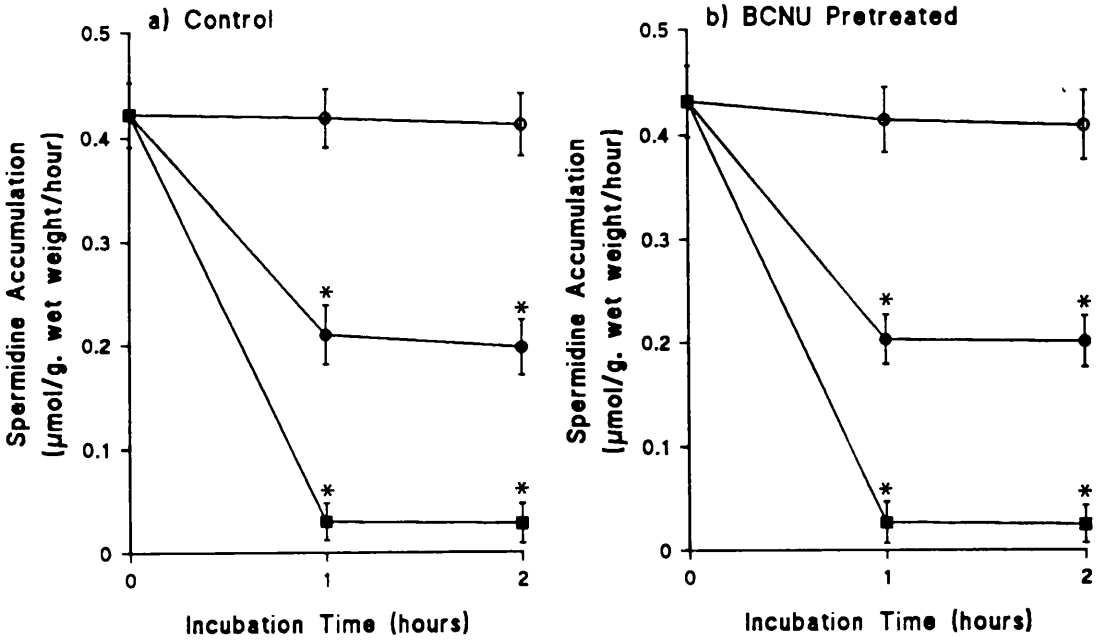


Figure 4.8. Effect of 1,4-benzoquinone on the accumulation of spermidine by a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP alone (a), or with BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone at concentrations of 100 µM (•——•), or 500 µM (■——■) for up to 4 hours. The lung slices were then removed, briefly immersed in fresh KRP and accumulation of [^{14}C]-spermidine was assessed. Results represent the mean of 3 experiments ± sem.
* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone for a particular pretreatment.

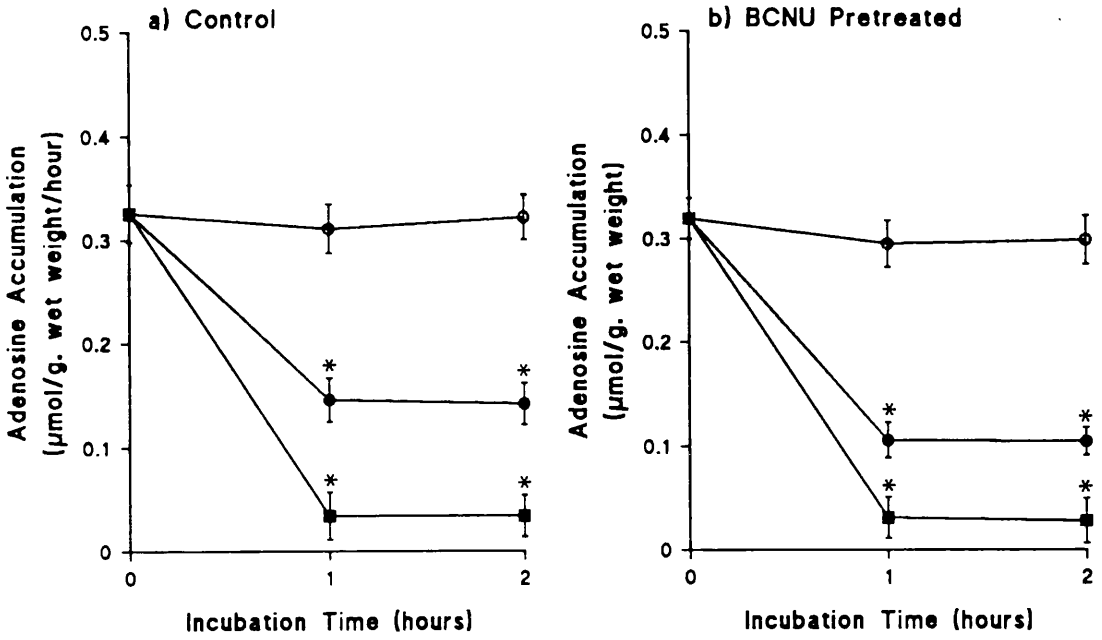


Figure 4.9. Effect of 1,4-benzoquinone on the accumulation of adenosine by a) Control and b) BCNU pre-incubated rat lung slices.

Lung slices were incubated in KRP alone (a), or with BCNU (b) for 45 minutes. They were then removed, briefly immersed in fresh KRP and incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone at concentrations of 100 µM (•——•), or 500 µM (■——■) for up to 4 hours. The lung slices were then removed, briefly immersed in fresh KRP and accumulation of [^{3}H]-adenosine was assessed. Results represent the mean of 3 experiments ± sem.
* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone for a particular pretreatment.

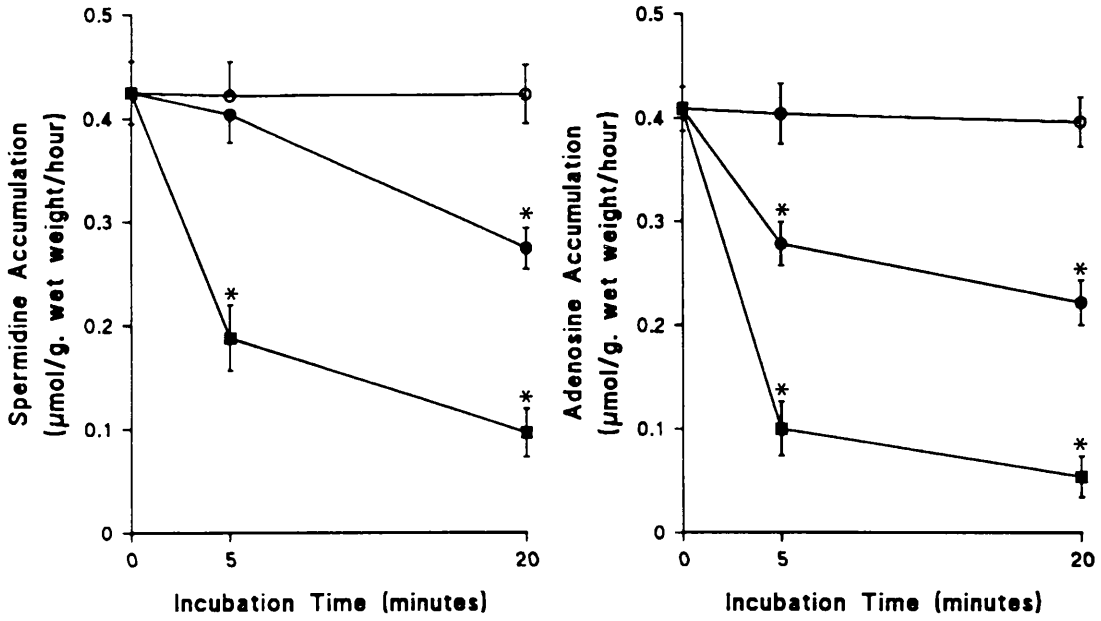


Figure 4.10. Effect of short incubations of 1,4-benzoquinone on the accumulation of a) spermidine and b) adenosine in rat lung slices.

Lung slices were incubated in the absence of 1,4-benzoquinone (o——o), or with 1,4-benzoquinone at concentrations of 100 µM (•——•) or 500 µM (■——■) for 30 minutes. They were then removed, briefly immersed in fresh KRP, and accumulation of [^{14}C]-spermidine or [^{3}H]-adenosine were assessed. Results represent the mean of 3 experiments ± sem.
* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the presence and absence of 1,4-benzoquinone.

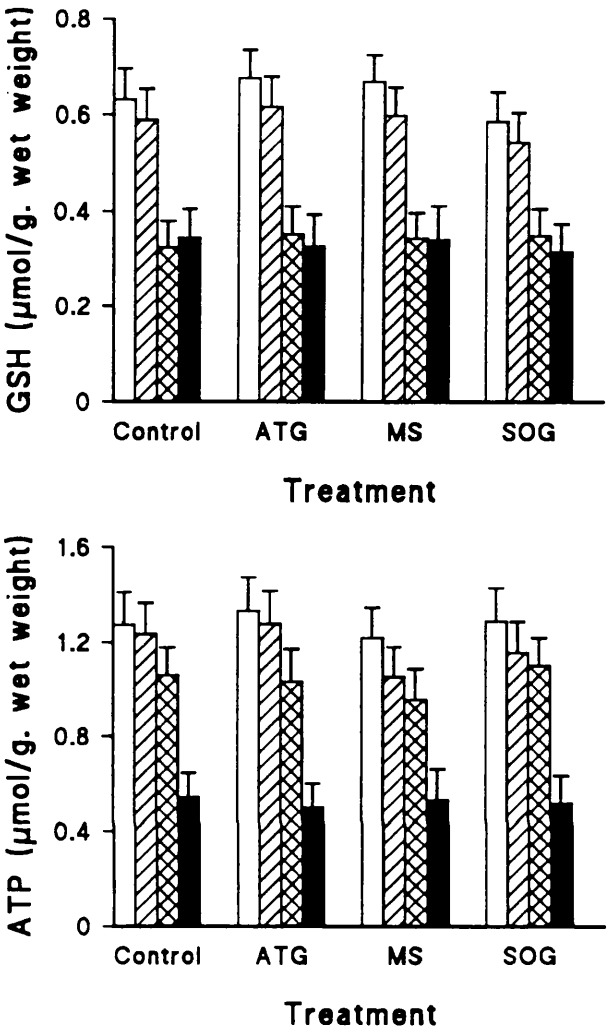


Figure 4.11. Effect of glutathione peroxidase inhibitors on the levels of a) GSH and b) ATP in rat lung slices.

Lung slices were incubated with KRP, aurothioglucose, 100 µM (ATG, an inhibitor of glutathione peroxidase [118]), mercaptosuccinic acid, 200 µM (MS, an inhibitor of glutathione peroxidase [119]), or S-octyl glutathione, 200 µM (SOG, an inhibitor of glutathione-S-transferase [176]), for 30 minutes. For each of these inhibitor pretreatments, the lung slices were incubated with either KRP (□), paraquat, 150 µM (▨), 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (⊠) or hydrogen peroxide, 5 mM (■) for a further 2 hours, co-incubating with the inhibitor. GSH (NPSH) and ATP was determined in supernatants from lung slice homogenates. Results represent the mean of 3 experiments ± sem.

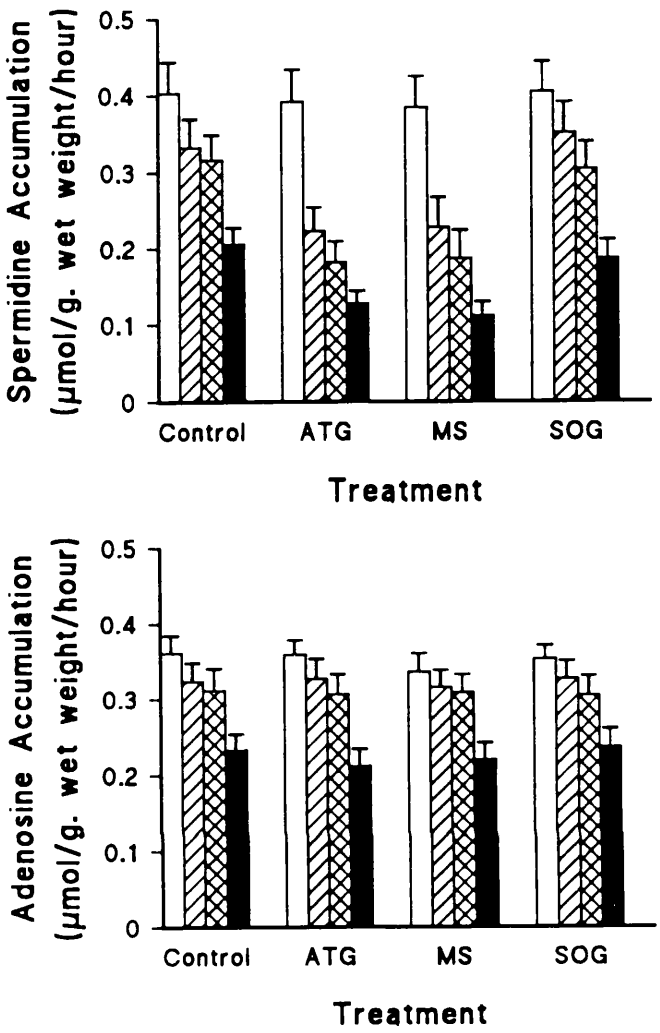


Figure 4.12. Effect of glutathione peroxidase inhibitors on the accumulation of a) spermidine and b) adenosine by rat lung slices.

Lung slices were incubated with KRP, aurothioglucose, 100 µM (ATG, an inhibitor of glutathione peroxidase [118]), mercaptosuccinic acid, 200 µM (MS, an inhibitor of glutathione peroxidase [119]), or S-octyl glutathione, 200 µM (SOG, an inhibitor of glutathione-S-transferase [176]), for 30 minutes. For each of these inhibitor pretreatments, the lung slices were incubated with either KRP alone (□), or with paraquat, 150 µM (▨), 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (▩) or hydrogen peroxide, 5 mM (■) for a further 2 hours, co-incubating with the inhibitor. The lung slices were then removed, briefly immersed in fresh KRP, and accumulation of [^{14}C]-spermidine or [2-^{3}H]-adenosine was assessed. Results represent the mean of 3 experiments ± sem.

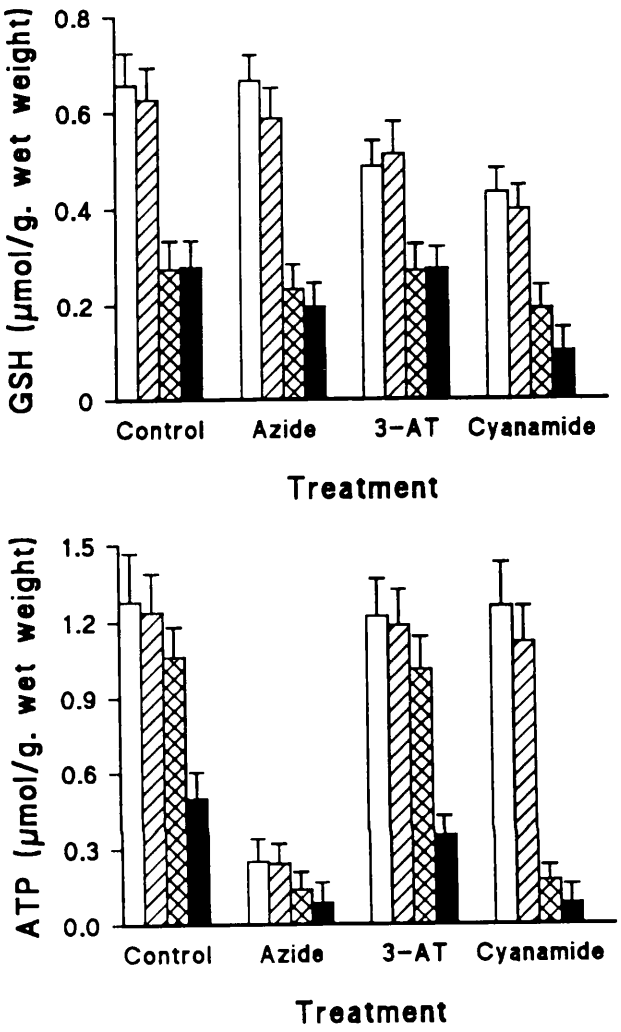


Figure 4.13. Effect of catalase inhibitors on the levels of a) GSH and b) ATP in rat lung slices.

Lung slices were incubated with KRP, sodium azide, 5 mM (Azide, an inhibitor of catalase [177]), 3-amino-1,2,4-triazole, 25 mM (3-AT, an inhibitor of catalase [178]), or cyanamide, 1 mM (Cyanamide, an inhibitor of catalase [179]), for 30 minutes. For each of these inhibitor pretreatments, the lung slices were incubated with either KRP alone (□), or with paraquat, 150 µM (▨), 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (⊠) or hydrogen peroxide, 5 mM (■) for a further 2 hours, co-incubating with the inhibitor. GSH (NPSH) and ATP was determined in supernatants from lung slice homogenates. Results represent the mean of 3 experiments ± sem.

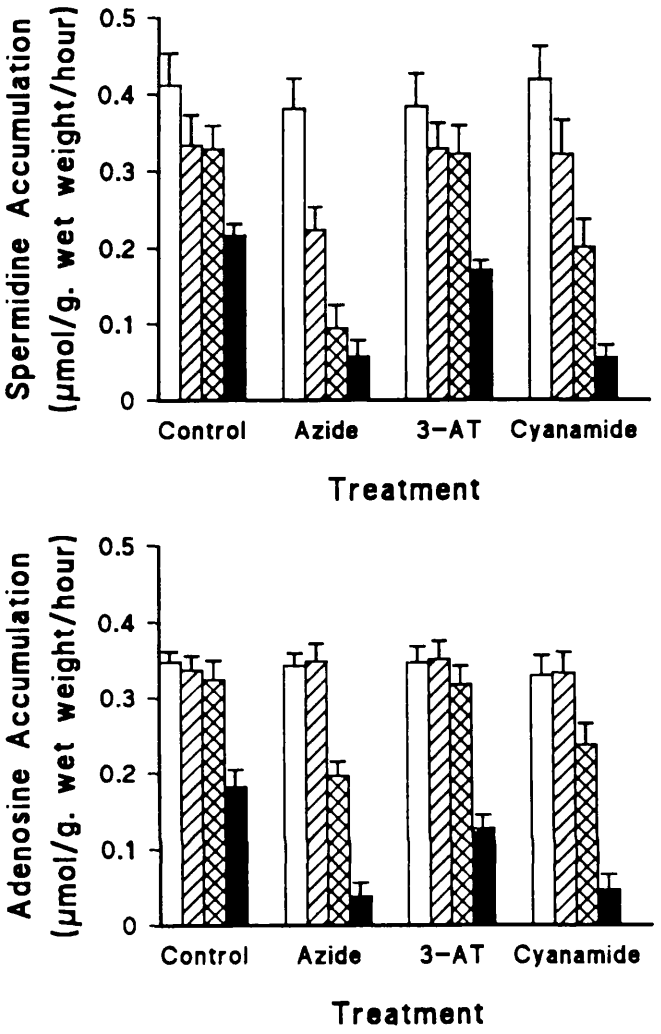


Figure 4.14. Effect of catalase inhibitors on the accumulation of a) spermidine and b) adenosine by rat lung slices.

Lung slices were incubated with KRP, sodium azide, 5 mM (Azide, an inhibitor of catalase [177]), 3-amino-1,2,4-triazole, 25 mM (3-AT, an inhibitor of catalase [178]), or cyanamide, 1 mM (Cyanamide, an inhibitor of catalase [179]), for 30 minutes. For each of these inhibitor pretreatments, the lung slices were incubated with either KRP alone (□), or with paraquat, 150 µM (▨), 2,3-dimethoxy-1,4-naphthoquinone, 50 µM (⊠) or hydrogen peroxide, 5 mM (■) for a further 2 hours, co-incubating with the inhibitor. The lung slices were then removed, briefly immersed in fresh KRP, and accumulation of [^{14}C]-spermidine or [2-^{3}H]-adenosine was assessed. Results represent the mean of 3 experiments ± sem.

DISCUSSION

These studies have attempted to distinguish the relative importance of redox cycling versus glutathione conjugation in the protective role of glutathione in an *in vitro* model of pulmonary toxicity. The compounds studied were diquat (a redox cycling compound [63]) and 1,4-benzoquinone (a nucleophile arylating compound [175]). In order to elucidate the mechanisms of toxicity of these compounds in lung tissue, a rat lung slice model was employed with markedly inhibited glutathione reductase activity using the antitumour agent BCNU [130]. The effects of incubating lung slices with diquat (a redox cycling compound) and 1,4-benzoquinone (an arylating compound) were assessed using biochemical (figures 4.1, 4.2, 4.5-4.7) and functional (figures 4.3, 4.4, 4.8-4.10) indices of toxicity, in terms of cellular function. The influence of inhibiting glutathione reductase on the effects of these compounds was also studied.

BCNU pretreatment of lung slices potentiated the loss of GSH ($p < 0.05$, figure 4.1) and appeared to potentiate the loss of ATP (figure 4.2) in rat lung slices incubated with diquat. The loss of spermidine accumulation ($p < 0.05$, figure 4.3) and adenosine accumulation ($p < 0.05$ for 1 mM, figure 4.4) was also potentiated by BCNU pretreatment. This indicates that the main toxic effects of diquat on the lung slice results from oxidative stress, mediated via redox cycling. The potentiation of loss of both spermidine and adenosine accumulation suggests that there is a lack of cellular specificity of diquat for a particular cell type in the lung. This is in contrast to paraquat, a chemically related compound, which showed a preferential potentiation of loss of spermidine accumulation following BCNU pretreatment ($p < 0.05$, figure 4.3).

The fact that GSH (figure 4.1), ATP (figure 4.2) and both functional markers

(figures 4.3, 4.4) were maintained in the control lung slices suggests that the cellular defences against the proposed oxidative stress produced by diquat [63] is sufficient to maintain GSH in a reduced state. The effect of paraquat, when compared to diquat, on spermidine and adenosine accumulation is consistent with the proposed specificity of paraquat accumulation, considering that paraquat has been shown to redox cycle more slowly than diquat [180].

Diquat is not known to arylate glutathione or other nucleophiles, hence it is thought to be a 'pure' redox cycler. The redox cycling of diquat is thought to occur predominantly in the endoplasmic reticulum, as it has been found not to penetrate the mitochondria [63]. In lung slices, incubation with diquat showed minimal effects on GSH despite its proposed mechanism. This is in contrast to the effects produced by 2,3-dimethoxy-1,4-naphthoquinone (also a 'pure' redox cycler [122]) which shows significant GSH depletion at 50 μM incubation concentration. ($p < 0.05$, figure 4.1). Firstly, the diquat ion, being a charged species, would be less lipophilic than the quinone, and thus would traverse cell membranes less readily. Consequently, for any given incubation concentration, 2,3-diMeO-NQ would achieve a higher intracellular concentration compared with diquat. Secondly, for a given intracellular concentration, the comparative redox cycling efficiency between 2,3-diMeO-NQ and diquat has not been reported. It could be postulated that the site of enzyme-catalysed reduction in the redox cycle may be a factor in redox cycling efficiency. The basis for this is the marked differences in the ability of redox cycling compounds to penetrate the mitochondrial membrane [63].

In contrast to diquat, 1,4-benzoquinone produced a rapid, concentration dependent decrease in both GSH ($p < 0.05$, figure 4.5) and ATP ($p < 0.05$, figure 4.6) that was not potentiated by BCNU inactivation of glutathione reductase. A lack of cellular specificity of the quinone, for a particular cell type in the lung, was

suggested by the observation that loss of both spermidine accumulation ($p < 0.05$, figure 4.8) and adenosine accumulation ($p < 0.05$, figure 4.9) occurred.

1,4-Benzoquinone has also been found to redox cycle very poorly with either purified NADPH cytochrome P-450 reductase, cytochrome b_5 reductase [64], or in isolated hepatocytes [83]. The poor redox cycling of 1,4-benzoquinone is due to its high positive one-electron redox potential, +99 mV [64]. The chemical reaction of 1,4-benzoquinone with glutathione results in the formation of adducts that exibit increasing degrees of glutathione substitution [181]. The reaction is a typical Michael addition across a C=C bond. The primary substitution product is 2-(glutathion-S-yl)hydroquinone. Autoxidation of the substituted hydroquinone usually precedes further substitution. The autoxidation of the glutathione substituted 1,4-benzoquinone proceeds more rapidly than that of the parent quinone [181]. In a reaction mechanism suggested by Brunmark and Cadenas [182], the substituted quinone reacts with molecular oxygen to form the substituted semiquinone and the superoxide ion radical. Further oxidation arises from the reaction of the substituted semiquinone with another molecule of oxygen to form the substituted quinone and a second molecule of the superoxide ion radical. The activity of superoxide dismutase would then result in the formation of two molecules of hydrogen peroxide for each oxidation associated with a glutathione substitution. The reaction of one molecule of 1,4-benzoquinone with glutathione results in the depletion of up to four molecules of glutathione and the formation of up to six molecules of hydrogen peroxide (The oxidation of the tetraglutathionyl hydroquinone is unfavourable [181]). This suggests that the possibility of an oxidative stress mechanism in the toxicity of 1,4-benzoquinone cannot be excluded. A limitation to the interpretation of the investigation of the role of oxidative stress in the toxicity of this quinone, using the reductase inhibited lung slice system, is

the possibility of quinone mediated glutathione reductase inhibition. This remains to be investigated. The GSH depletion observed with 1,4-benzoquinone (figure 4.5) occurs much more rapidly than that seen with redox cycling compounds. In addition, the poor redox cycling of 1,4-benzoquinone, mentioned above, and the proposed non-catalytic formation of hydrogen peroxide, as opposed to the catalytic and continuous formation by redox cycling, suggests that the primary mechanism of benzoquinone toxicity in the lung would be via arylation of critical nucleophiles, notably glutathione.

The preliminary experiments involving the use of compounds acting via oxidative stress with the reported inhibitors of glutathione peroxidase and catalase are difficult to interpret. The possible reasons for this are, firstly, whilst enzyme inhibition has been reported in cell free systems [178, 183], inhibition of glutathione peroxidase or catalase has not yet been fully elucidated in lung slices. The criteria for enzyme inhibition in the lung (*e.g.* incubation concentration and preincubation periods) needs to be more fully characterised. Secondly, some of the inhibitors are known to lack specificity. Azide, for example, inhibits the electron transport chain [177] in addition to its inhibition of catalase. Thirdly, a homogeneous distribution of inhibitor, or homogeneous degree of inhibition cannot necessarily be assumed. The possibility of selective uptake of an inhibitor, by specific cell types within the lung, cannot be excluded. Other interpretational problems centre around whether, for some inhibitors, potentiation of biochemical or functional changes can be viewed as the interaction of related systems (oxidative stress and inhibition of antioxidant defence enzymes) or the combination of independent adverse effects.

The preliminary experiments involving the use of inhibitors of antioxidant defence enzymes (catalase and glutathione peroxidase) attempted to elucidate

possible differences in the activity of these enzymes between different cell types within the lung. The apparent lack of effect of glutathione peroxidase inhibitors on glutathione levels (figure 4.11a) could be consistent with inhibition of glutathione peroxidase if more than one pathway for glutathione depletion via oxidative stress exists. This has been suggested for glutathione peroxidase activity [102, 103] and uncatalysed glutathione depletion via oxidative stress [100]. However, a lack of effect could also imply a lack of inhibition. This ambiguity means that no definite conclusions can be drawn from the effect of the glutathione peroxidase inhibitors on oxidatively mediated glutathione depletion. This argument also applies to effects on ATP levels (figure 4.11b). Apparent differences between the effects of the proposed glutathione peroxidase inhibitors on oxidatively mediated loss of spermidine (figure 4.12a) or adenosine (figure 4.12b) accumulation could suggest possible differences in selenium dependent glutathione peroxidase activity between different cell types. Other possibilities cannot be excluded. A heterogeneous distribution of inhibitor could arise from possible selective uptake mechanisms, or possible differences in cellular permeability between different cell types.

Results obtained from experiments involving the use of proposed inhibitors of catalase are complicated by the reported lack of specificity of the inhibitors for catalase [177, 179] and their complex mechanism of action [178]. Some of the inhibitors appeared to effect small changes in GSH levels themselves (figure 4.13a) which would serve to complicate the pattern of data such that potentiation of GSH loss by oxidative stress cannot necessarily be implied nor ruled out. The complex action of the proposed catalase inhibitors is further shown in modulation of ATP levels (figure 4.13b). The depletion of ATP associated with incubating the slices with azide obscures any modulation of ATP changes associated with oxidative stress. Cyanamide appears to increase the loss of ATP caused by 2,3-dimethoxy-

1,4-naphthoquinone and hydrogen peroxide (figure 4.13b). This would imply that catalase activity might contribute significantly to the antioxidant defence system under conditions of oxidative stress induced by redox cycling compounds. The lack of potentiation of loss of ATP is consistent with the suggestion that the majority of ATP is located in cell types other than those which accumulate paraquat, *i.e.* the pulmonary epithelium [184]. The lack of potentiation seen with 3-aminotriazole is consistent with the proposed complex mechanism of inhibition which also requires hydrogen peroxide [178]. The loss of ability to accumulate spermidine (figure 4.14a) and adenosine (figure 4.14b) due to oxidative stress is modulated by treatment with the proposed catalase inhibitors. It cannot be assumed that the observed effects are due, solely or in part, to inhibition of catalase.

Further work remains to elucidate the influence of antioxidant defence enzyme inhibition on the modulation of the effects of oxidative stress. The characteristics of enzyme inhibition need to be evaluated in terms of specificity and conditions of incubation. Additionally, the non-specific effects of the inhibitors need to be distinguished from the effects of enzyme inhibition. This could be achieved, in part, by the use of an appropriate control compound. In the case of catalase inhibition, the effects of mitochondrial respiration need to be distinguished from catalase activity as the proposed inhibitors are also reported to lack specificity.

Chapter 5

An investigation into the relationship between pulmonary glutathione levels and the toxicity of α-naphthyl thiourea (ANTU)

INTRODUCTION

Glutathione (GSH) is known to attenuate the toxicities of various agents which damage the lung, including 3-methylindole [185] naphthalene [186] and 4-ipomeanol [187]. The relationship between pulmonary glutathione levels and the toxicity of α-naphthylthiourea (ANTU) has been explored previously, where studies have revealed that ANTU-induced lethality, hydrothorax and pulmonary covalent binding were potentiated by pretreatment with diethyl maleate (DEM; a potent GSH depleting agent [188]).

ANTU is a rodenticide which produces a relatively selective pulmonary toxicity. This is expressed pathologically as a fibrin-rich non hæmorrhagic œdema with extensive pleural effusions both in the rat and dog [189]. Meyrick *et al.* [27] showed a blebbing and scalloping of endothelial cells following ANTU administration. Histological investigations of ANTU-induced injury have suggested that œdema results from the reversible formation of gaps in the endothelium [190]. These findings suggest that the pulmonary endothelium is the major target of ANTU toxicity. Chemically-induced damage to the pulmonary endothelium typically leads to an increased pulmonary microvascular permeability to fluids and proteins, which results in marked abnormalities in gas exchange and mechanical properties of the lungs. The consequence of this sequence of events is pulmonary œdema. Damage to the endothelium was also indicated by a reduction in 5-hydroxytryptamine uptake (a marker of endothelial cell function) observed in isolated perfused lungs prepared from rats dosed with ANTU [164]. Epithelial damage has also been observed, though this

has been reported to occur subsequent to endothelial injury [27].

The precise mechanism by which ANTU produces lung damage is uncertain [15]. Following administration of radiolabelled ANTU *in vivo*, covalent binding, to macromolecules in lung and liver, was observed [188]. There is evidence that metabolic activation, involving desulphuration, occurs to form a reactive metabolite [188]. Metabolism, at least in part, by cytochrome-P450 monooxygenase in both lung and liver microsomes increases covalent binding [191]. Pretreatment *in vivo* with piperonyl butoxide (an inhibitor of cytochrome-P450 monooxygenase activity) confers some protection against ANTU toxicity at low doses, supporting the involvement of pathways of metabolic activation [192]. It has also been suggested that flavin-containing monooxygenase may be involved in the metabolic activation of ANTU [193]. Pulmonary endothelial cells are also the primary site of exposure to compounds which access the lung via the systemic route.

In the present study, the influence of elevated pulmonary GSH levels on the toxicity of ANTU *in vivo* in the rat was assessed. A variety of parameters was used to evaluate toxicity. Pulmonary glutathione levels were elevated by prior administration of phorone. Phorone (di-isopropylidene acetone) is an α,β-unsaturated carbonyl compound which conjugates with GSH [194]. This compound has been used in a variety of studies pertaining to the effects of GSH depletion on lipid peroxidation [195]. It has been shown that after phorone administration, the initial GSH depletion is followed by an elevation over control levels. It is presumed that the elevation of GSH levels results from rebound synthesis as GSH levels are self regulated by feedback inhibition of one of the enzymes responsible for its own synthesis - γ-glutamylcysteine synthetase.

In addition, the selectivity of ANTU toxicity *in vitro* was assessed in rat lung slices.

RESULTS

Effect of phorone pretreatment on ANTU-induced lethality

In control animals, ANTU (10 mg/kg) was fatal to 4 out of 8 animals (figure 5.1). This is consistent with previous investigations which have assigned an LD_{50} for ANTU at 7.5-10 mg/kg *i.p.* [188, 191]. The highest dose of ANTU (20 mg/kg was fatal to all 8 animals in the dose group. Both groups showed signs of toxicity including hunching and piloerection. The lowest dose of ANTU (5 mg/kg) was not fatal to any animal in the dose group but toxic signs were still observed though their severity was reduced compared with animals given the higher doses.

In animals given phorone 48 hours prior to ANTU, the ANTU-induced lethality was completely abolished (figure 5.1). This would suggest that elevation of pulmonary GSH levels obtained with phorone pretreatment protects against ANTU-induced lethality.

Effect of phorone pretreatment on ANTU-induced hydrothorax

In control animals, ANTU produced hydrothorax, the volume of which was dose-dependent. Administration of phorone alone (figure 5.2) did not result in any detectable hydrothorax after a 48 hour period. In rats pretreated with phorone (figure 5.2), ANTU administration did not result in detectable hydrothorax. The volumes of fluid were in good accord with those previously reported by Boyd and Neal [188].

By 4 hours, ANTU (10 mg/kg) induced a small increase in the wet/dry weight ratio of whole lung. This increase was offset by prior treatment with phorone (figure 5.3).

Effects of ANTU on pulmonary GSH

In control animals, ANTU produced a little or no change in pulmonary GSH levels after 4 hours (figure 5.4). In rats pretreated with phorone (200 mg/kg, *i.p.*) 48 hours previously, the pulmonary glutathione levels were elevated to 1.7 ±0·1 (n=3) times those seen in control pretreated rats. This finding is in agreement with previous observations by Hardwick [137]. When phorone pretreated rats were given ANTU, the pulmonary GSH levels were depleted to the level of those seen in control pretreated rats (figure 5.4).

Effects of ANTU on adenosine accumulation

Rat lung slices accumulated radioactivity associated with adenosine in a time and concentration dependent manner (chapter 3). Phorone pretreatment produced no significant modulation of the uptake of adenosine (10 μM) into lung tissue compared with lung slices prepared from control animals. Administration of ANTU *in vivo* resulted in the dose-dependent loss of ability to accumulate radiolabel. Pretreatment with phorone completely protected against the loss of adenosine uptake at the lower dose of ANTU (5 mg/kg) and almost completely protected against the loss of adenosine uptake at the higher dose of ANTU (10 mg/kg, figure 5.5a)

Effects of ANTU on spermidine accumulation

Spermidine was accumulated into lung slices from control animals in a manner consistent with that seen in previous studies [138]. Prior treatment with phorone did not affect the uptake of the oligoamine. In the absence of phorone pretreatment, ANTU (5 and 10 mg/kg) resulted in a significant dose dependent reduction in spermidine uptake. Pretreatment with phorone protected partially but not completely against the loss of spermidine uptake caused by ANTU (figure 5.5b).

Effects of ANTU *in vitro*

Spermidine was accumulated into control incubated lung slices in a manner consistent with that seen in previous studies [138]. Incubation of rat lung slices with ANTU *in vitro* for 4 hours, over a concentration range of 1-1000 μM, resulted in no significant modulation in the ability of the slices to accumulate spermidine.

Control incubated rat lung slices accumulated radioactivity associated with adenosine in a time and concentration dependent manner (chapter 3). Incubation of rat lung slices with ANTU *in vitro* for 4 hours, over a concentration range of 1-1000 μM, resulted in a non-linear, concentration dependent loss of ability of the lung slices, to accumulate radiolabel associated with adenosine (10 μM), compared with control incubated lung slices. These findings are in contrast to the toxicity of ANTU seen *in vivo*.

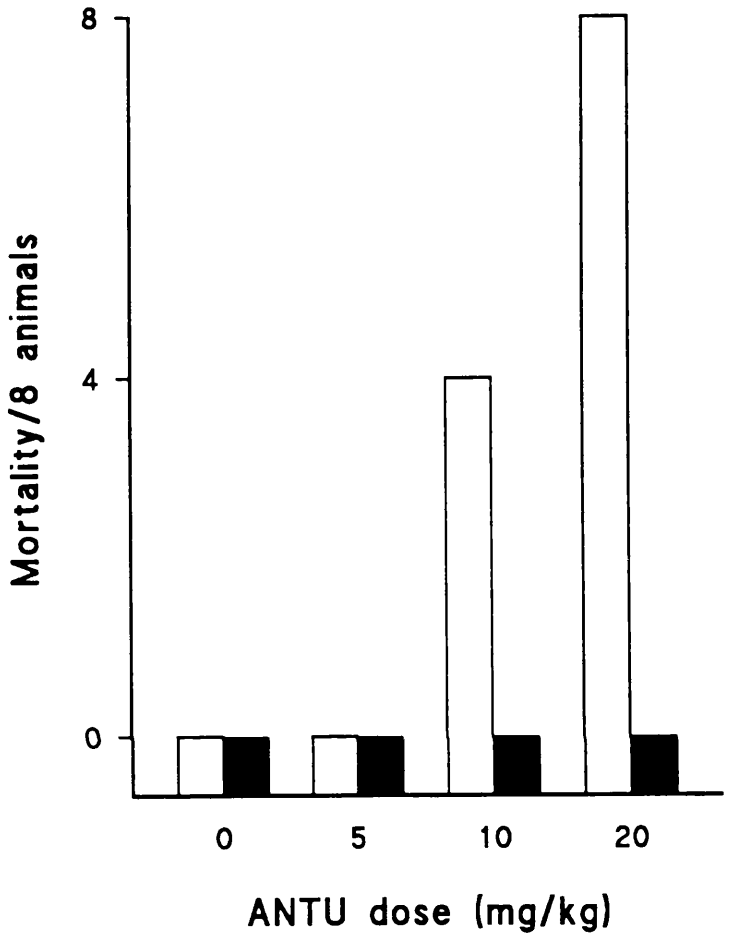


figure 5.1. Effect of phorone pretreatment on ANTU induced lethality.

Animals were injected *i.p.* with corn oil alone (□) or phorone (200 mg/kg, ■) 48 hours prior to administration of ANTU (0, 5, 10 or 20mg/kg). The animals were observed at intervals of 3-6 hours for 48 hours for clinical signs of toxicity and lethality. Subsequently the animals were monitored daily for any further lethality for a period of three weeks following dosing with ANTU.

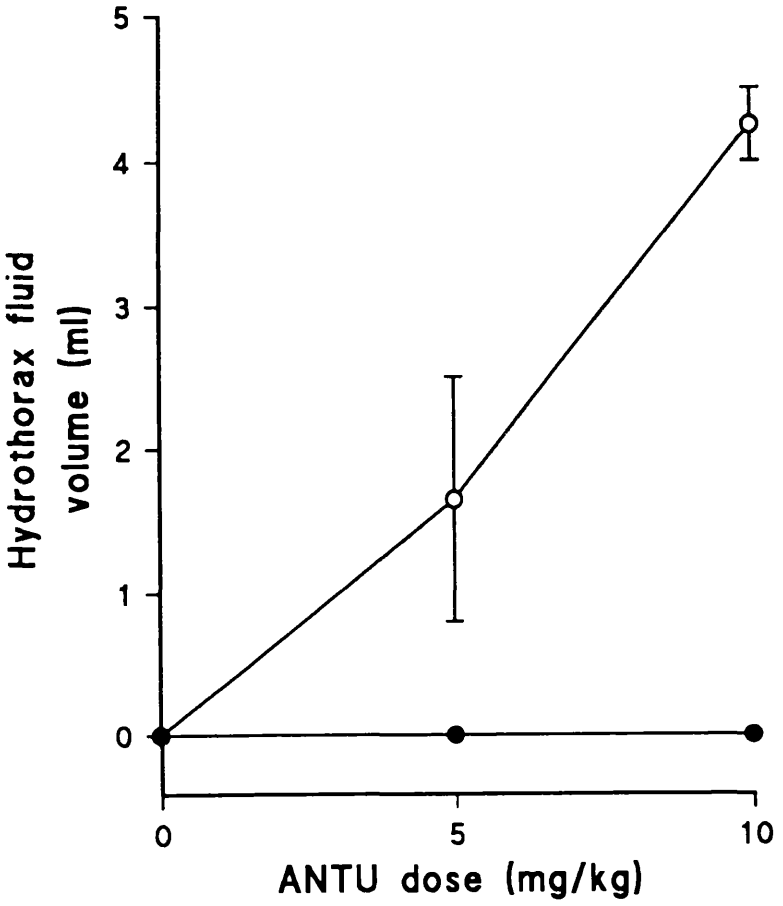


figure 5.2. Effect of phorone pretreatment on hydrothorax associated with ANTU.

Animals were injected *i.p.* with corn oil alone (o) or phorone (200 mg/kg, •) 48 hours prior to administration of ANTU (0, 5 or 10 mg/kg). 4 hours after dosing with ANTU, the animals were sacrificed and the thoracic cavity opened. Hydrothorax fluid volume was reported as the volume of fluid which could be aspirated by pipette as described by Boyd and Neal. Results represent the mean ± maximum and minimum range.

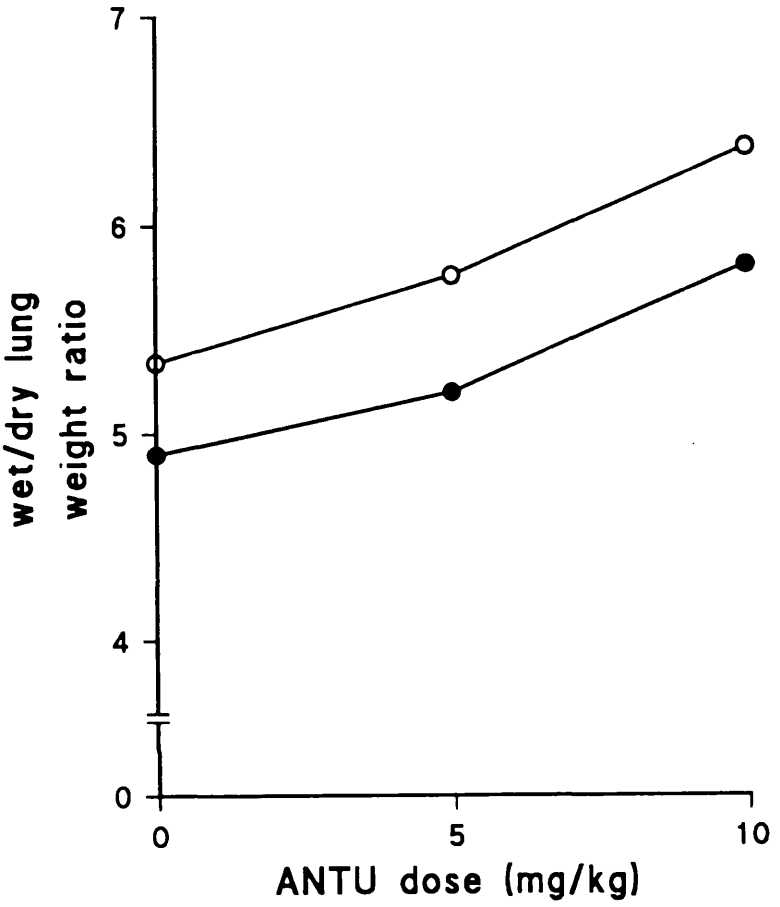


figure 5.3. Effect of phorone pretreatment on wet/dry weight ratio changes associated with ANTU.

Animals were injected *i.p.* with corn oil alone (o) or phorone (200 mg/kg, •) 48 hours prior to administration of ANTU (0, 5 or 10 mg/kg). 4 hours after dosing with ANTU, the animals were sacrificed, the lungs removed and blotted to remove excess external fluid and weighed. The lungs were then heated to 110° C for 48 hours and reweighed. The lungs were then heated to 110° C for a further 24 hours and this was repeated until a constant weight was achieved. The results were expressed as the fraction wet weight/dry weight and represent one experiment typical of several.

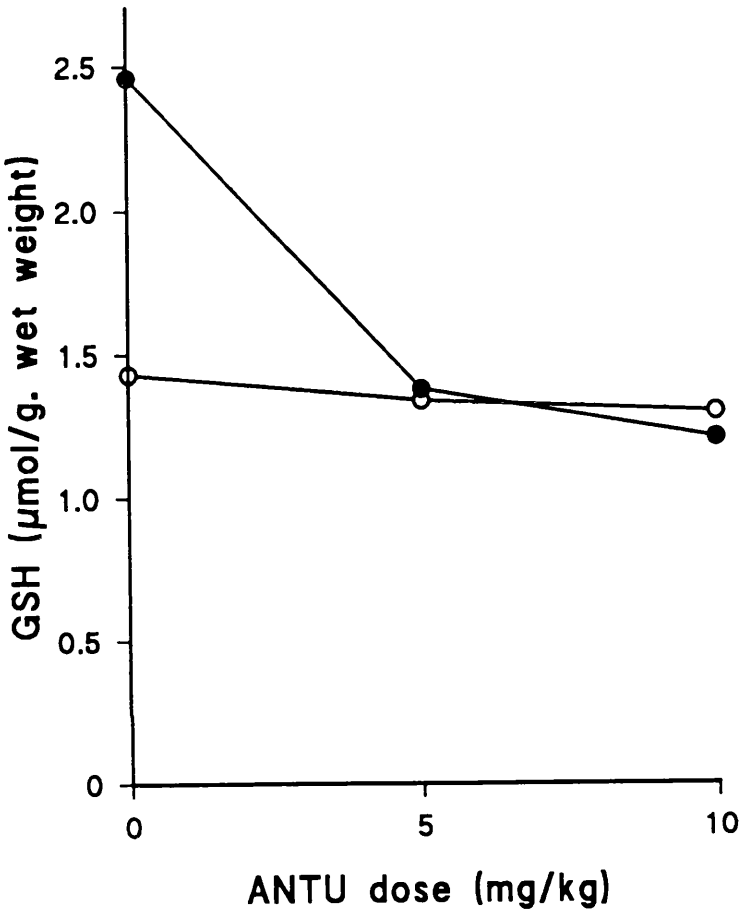


figure 5.4. Effect of phorone pretreatment on GSH changes associated with ANTU.

Animals were injected *i.p.* with corn oil alone (o) or phorone (200 mg/kg, •) 48 hours prior to administration of ANTU (0, 5 or 10 mg/kg). 4 hours after dosing with ANTU, the animals were sacrificed, the lungs removed and GSH levels were determined according to the method of Brigelius *et al.* Results represent the values obtained from one experiment typical of several.

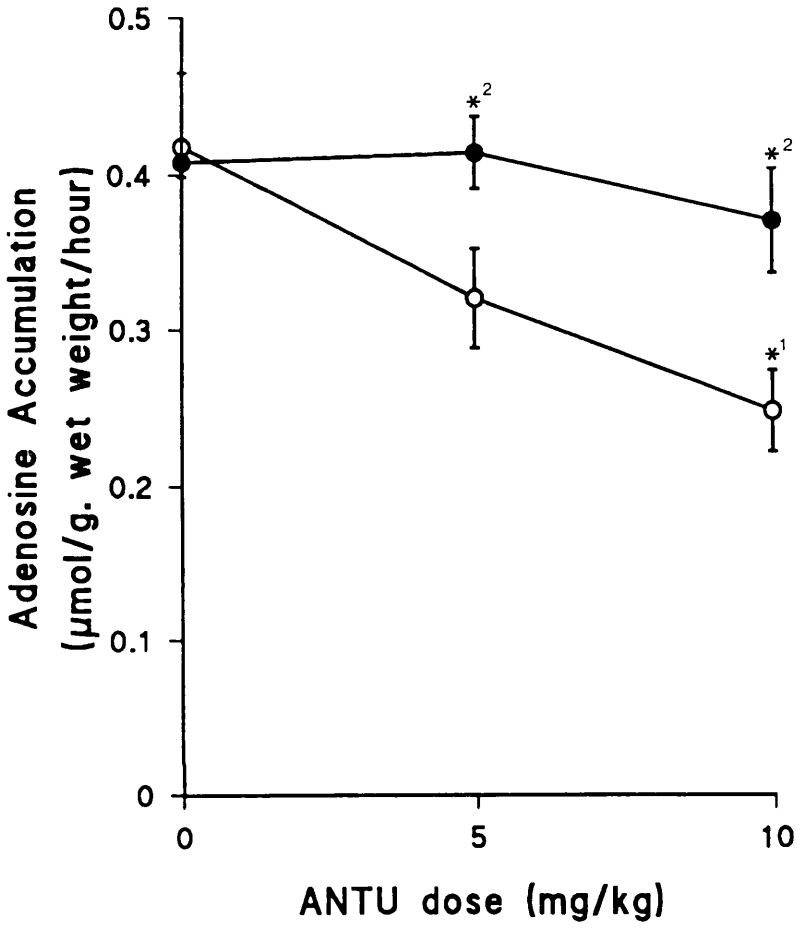


figure 5.5. Effect of phorone pretreatment on changes in adenosine accumulation associated with ANTU.

Animals were injected *i.p.* with corn oil alone (o) or phorone (200 mg/kg, •) 48 hours prior to administration of ANTU (0, 5 or 10 mg/kg). 4 hours after dosing with ANTU, the animals were sacrificed and lung slices were prepared. The accumulation of adenosine was carried out as described in chapter 2. Results represent the mean ± sem of 3 experiments.

$*^1$ Denotes significant difference ($p<0.05$) between results obtained from rats dosed with vehicle control (ANTU controls) for each pretreatment.

$*^2$ Denotes significant difference ($p<0.05$) between results obtained from control pretreated rats (phorone controls) for a particular dose of ANTU.

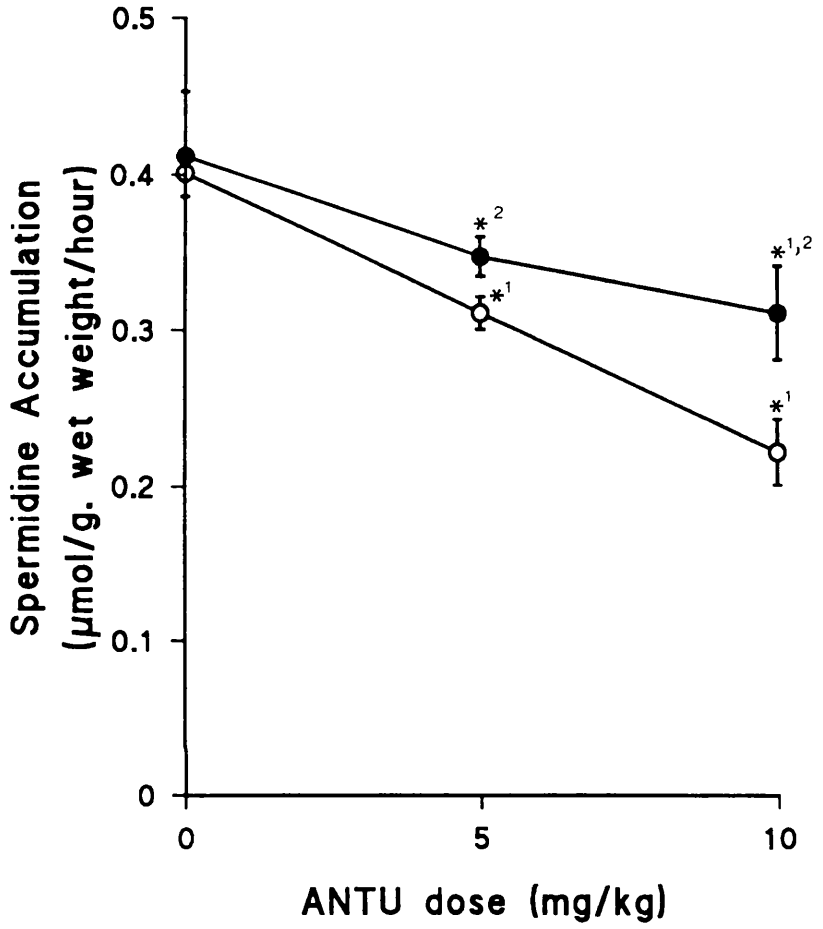


figure 5.6. Effect of phorone pretreatment on changes in spermidine accumulation associated with ANTU.

Animals were injected *i.p.* with corn oil alone (o) or phorone (200 mg/kg, •) 48 hours prior to administration of ANTU (0, 5 or 10 mg/kg). 4 hours after dosing with ANTU, the animals were sacrificed and lung slices were prepared. The accumulation of spermidine was carried out as described in chapter 2. Results represent the mean ± sem of 3 experiments.

$*^1$ Denotes significant difference ($p<0.05$) between results obtained from rats dosed with vehicle control (ANTU controls) for each pretreatment.

$*^2$ Denotes significant difference ($p<0.05$) between results obtained from control pretreated rats (phorone controls) for a particular dose of ANTU.

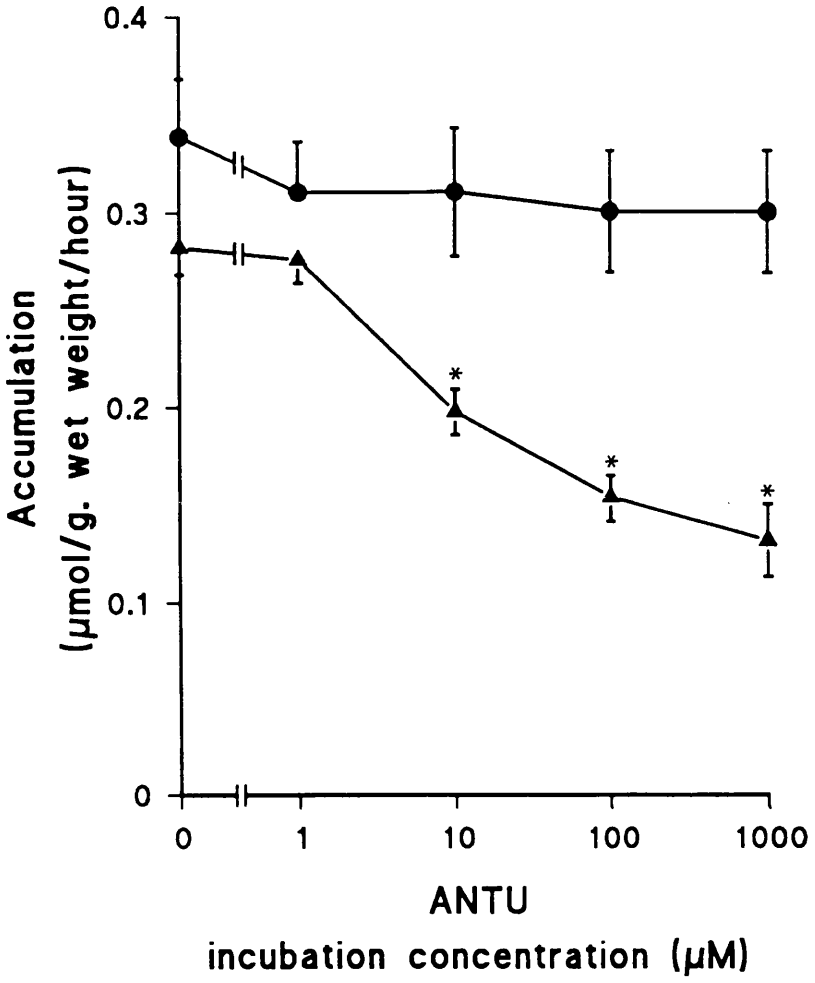


figure 5.7. Effect of ANTU *in vitro* on adenosine and spermidine accumulation

Lung slices were incubated with ANTU at concentrations of 0, 1, 10, 100 and 1000 µM for a period of 4 hours at 37° C. ANTU was dissolved in DMSO such that 100 µl addition to 4.9 ml KRP incubation medium achieved the desired concentration. Spermidine (▲) or adenosine (●) accumulation was then carried out as described in chapter 2. Results represent the mean ± sem of 3 experiments. * Denotes significant difference of uptake from slices incubated in the absence of ANTU.

* Denotes significant difference ($p<0.05$) between results obtained from lung slices incubated in the absence of ANTU

DISCUSSION

The most important finding in the study was that prior treatment with phorone was associated with a marked reduction in the toxicity of ANTU. The indicators used to assess ANTU toxicity were: lethality, hydrothorax, pulmonary GSH levels and changes in adenosine and spermidine uptake.

Whilst the association between phorone pretreatment and observed reduction in indicators of ANTU toxicity was clear, the mechanism for this protection is less so. Lee *et al.* proposed that ANTU is metabolically activated to atomic sulphur, a portion of which, becomes covalently bound to the cysteine side chains of proteins forming the hydrodisulphide [191]. Therefore, GSH may protect by offering an alternative site for covalent binding of the reactive metabolite(s) of ANTU. In this study, pretreatment with phorone was used to increase pulmonary GSH in order to determine if this could protect against the animals against ANTU-induced toxicity. This was based on the observed time course of GSH levels in both rat lung and liver following a single administration of phorone [196] (data not shown). Phorone rather than diethyl maleate was chosen because it has been reported to exert fewer effects on cytochrome-P450 monooxygenase activity [197]. The cellular pulmonary distribution of the elevated GSH following phorone pretreatment is unknown. It has been suggested that much of the GSH is in cell types other than the epithelium, possibly the endothelium [184]. Furthermore, in isolated cell preparations from rabbit lung the rate of *de novo* GSH synthesis is lower in type II cells than Clara cells or alveolar macrophages [198]. Phorone presumably elevates GSH by rebound synthesis which may be low or absent in type II cells. Thus the elevated GSH following phorone pretreatment may have been in part in endothelial cells thus resulting in the observed protection against ANTU. This finding is also supported by the observation that

phorone pretreatment did not protect against paraquat toxicity *in vivo*, which has been shown to damage primarily the alveolar epithelial type I and type II cells [196].

The effect of ANTU on pulmonary GSH levels, observed at 4 hours (figure 5.4) is complicated. The preliminary experiment suggested that no modulation of GSH was seen in ANTU treated rats, but with phorone pretreatment, the elevated GSH levels appeared to be depleted to levels seen in control pretreated rats. Observations with phenylthiourea, a chemically and toxicologically related compound to ANTU, have shown an apparent increase in lung glutathione levels after 2 hours [199]. This could suggest that a complex, perhaps time dependent, relationship between pulmonary GSH levels and ANTU toxicity exists. A time course of pulmonary and hepatic GSH, with and without phorone pretreatment, following ANTU administration may give more detailed information as to the precise relationship between GSH levels and ANTU toxicity.

A further complication in delineating the mechanism of the phorone protection against ANTU-induced toxicity was the striking elevation in hepatic NPSH levels (data not shown) [196]. These changes could alter the pharmacokinetics of ANTU resulting in a decreased availability to the lung and therefore a decreased toxicity. Phorone has been shown to induce hæm oxygenase activity in rat liver [197]. This suggests that phorone may alter the metabolic profile of the liver by selective induction (or inhibition). It is therefore conceivable that hepatic metabolism of ANTU may be modulated, so altering its bioavailability to the lung. In addition, both lung and liver microsomes may activate ANTU [191] so a possible hepatic contribution to the observed protection cannot be excluded.

ANTU (5 and 10 mg/kg) also caused a dose dependent inhibition of spermidine accumulation, in agreement with Nemery *et al.* [144]. As spermidine accumulation occurs in alveolar epithelial as opposed to endothelial cells, these results suggest that

ANTU may also damage epithelial cells, in agreement with the results of Meyrick *et al.* although these histological effects were observed at a dose of 50 mg/kg [27]. In the present study, ANTU did not show any particular selectivity for the endothelium, compared with the epithelium, based on functional markers. This could be the result of assessing these parameters at 4 hours after ANTU administration (when œdema has been shown to be maximal [190]). Altenatively, the relationship between a given change in a functional marker and the degree of toxicity which this represents may vary such that direct comparisons between functional markers could be misleading. Investigations at earlier times might have shown preferential damage to the different cell types.

The effects of ANTU observed *in vitro* are in contrast to those seen *in vivo* (figure 5.6). The effects were seen in the pulmonary epithelium and not in the endothelium. Incubation of lung slices with 10 μM ANTU for 4 hours resulted in a reduction in spermidine uptake ($p < 0.05$). Adenosine uptake remained apparently unaffected by incubation with ANTU for 4 hours, at concentrations up to 1 mM. This could suggest differences in the mechanism of toxicity of ANTU, administered *in vivo* or incubated *in vitro*, to the lung. The differences could arise from a number of factors such as possible hepatic involvement in terms of metabolic activation, changes in pulmonary intercellular relationships between the *in vivo* or the *in vitro* conditions. Other considerations include physiological responses of endothelial cells to mechanical damage, and white cell recruitment. Leukocyte and platelet infiltration of the lung has been shown in oxygen mediated pulmonary endothelial toxicity [26]. Further, changes in solubility of ANTU after metabolism, leading to precipitation, has not been reported. It is possible that such changes could lead to mechanical vascular damage seen *in vivo* which is absent in the *in vitro* situation.

Despite the problems in understanding the precise mechanism of the apparent

protection afforded by phorone pretreatment against ANTU induced toxicity, similar pretreatment may also protect against other types of endothelial cell damage such as that induced by hyperoxia.

Chapter 6

Final Discussion

6.1 Introduction

The aim of these studies was to investigate the biochemistry of glutathione in the mechanisms of pulmonary toxicity. It is well established that glutathione is involved in the detoxification of potentially harmful compounds. The protection afforded by glutathione occurs via two main mechanisms. Firstly, by conjugation with the toxicant *per se* or following 'phase I' (mixed function oxidase type) metabolism. The second main mechanism of protection afforded by glutathione is that of detoxifying reactive oxygen species. Glutathione acts in conjunction with specialised enzyme systems in both mechanisms of protection. A number of questions needed to be addressed to clarify the relevance of glutathione biochemistry in the lung. The lung is a heterogeneous organ in that more than 40 different cell types have been identified. The protection from toxicity to a particular cell type, afforded by glutathione, would be dependent on the intracellular level of glutathione and the activity of the appropriate enzyme which catalyses the detoxifying reactions. Possible differences in either the level of glutathione or its associated enzyme activities, between cell types in the lung could, in part, explain selectivity of toxic effects for some compounds. This hypothesis is further complicated in terms of the differential physiology of cell types within the lung. This is exemplified by the demonstration of an oligoamine uptake system in the lung by the pulmonary epithelium, and the hæmostatic role of the pulmonary endothelium via the production of prostacyclin. Some insight into differences of toxicity between cell types was attempted by the application of selective indicators of pulmonary cell function.

6.2 Adenosine as a marker of pulmonary endothelial cell function.

The use of adenosine as a selective indicator of pulmonary cell function followed some preliminary investigations by Hardwick [137]. The characteristics of adenosine accumulation was investigated in terms of kinetics and metabolism. Autoradiographic studies to show localisation of adenosine accumulation was also attempted (figure 3.18). The accumulation of radioactivity associated with adenosine was, at some concentrations, approximately linear with time (figure 3.2). Saturation of adenosine uptake was also shown. Attempts to express adenosine accumulation in terms of Michaelis-Menten kinetics suggested that this approach was too simplistic, and that other factors, in addition to simple uptake, might be involved (table 3.1). Further characterisation of the accumulation of adenosine revealed that it was rapidly metabolised to ATP. The involvement of adenosine kinase suggested that an initial phosphorylation of adenosine might be dependent on the ATP content of the cell, hence the rate of adenosine uptake and conversion to ATP could be a reflection of the initial ATP levels within the cell. The localisation to cell types other than the pulmonary epithelium, possibly the endothelium, has been suggested on the basis of studies by Hardwick [137], with cell selective toxicants. This has been supported in the preliminary autoradiographic studies (figure 3.18). However, other possibilities, such as initial metabolism at a distant site followed by subsequent transport, cannot be excluded. Whilst preliminary data presented here, and that shown by Hardwick [137], suggests that accumulation of radiolabel by rat lung slices might indicate pulmonary endothelial cell function, further work remains to elucidate the characteristics of adenosine transport more fully in terms of biochemistry and localisation.

6.3. The relative importance of conjugation and antioxidant defences in the lung

The studies from chapter 4 attempted to distinguish the relative importance of redox cycling versus glutathione conjugation in the protective role of glutathione in pulmonary toxicity. In order to elucidate the mechanisms of toxicity of these compounds in lung tissue, a rat lung slice model was employed with markedly inhibited glutathione reductase activity using the antitumour agent BCNU [130]. The toxicity of a number of compounds whose mechanisms of toxicity are known, were investigated. The compounds investigated were assumed to be distributed evenly across the different cell types in terms of concentration apart from paraquat, for which a selective accumulation for the pulmonary epithelium has been demonstrated [13]. Toxicity was indicated by cellular ATP and GSH levels and selective toxicity was inferred by the use of functional markers of spermidine and adenosine accumulation.

BCNU pretreatment of lung slices potentiated the loss of GSH (figure 4.1) and ATP (figure 4.2) in rat lung slices incubated with diquat. The loss of spermidine accumulation (figure 4.3) and adenosine accumulation (figure 4.4) was also potentiated by BCNU pretreatment. This suggests that oxidative stress mediated via redox cycling is involved in the mechanism of toxicity of diquat. The potentiation of loss of both spermidine and adenosine accumulation suggests that there is a lack of cellular specificity of diquat for a particular cell type in the lung. This is in contrast to paraquat, a chemically related compound, which showed a preferential potentiation of loss of spermidine accumulation following BCNU pretreatment (figure 4.3).

In contrast to diquat, 1,4-benzoquinone produced a rapid, concentration dependent decrease in both GSH (figure 4.5) and ATP (figure 4.6) that was not potentiated by BCNU inactivation of glutathione reductase. A lack of cellular specificity of the quinone, for a particular cell type in the lung, was suggested by the

observation that loss of both spermidine accumulation (figure 4.8) and adenosine accumulation (figure 4.9) occurred. The preliminary experiments, involving the use of compounds acting via oxidative stress with the reported inhibitors of glutathione peroxidase and catalase, attempted to show possible differences in the activities of antioxidant defence enzymes between different cell types in the lung. The preliminary nature of these studies poses limitations on the interpretation of these studies. This means that a lack of effect could either be due to ineffective enzyme inhibition, or lack of effect of enzyme inhibition. However, the use of reported inhibitors of antioxidant defence enzymes have suggested that differences in enzyme activities between cell types in the lung could be a possibility. However, from the results of these studies, no definite conclusions can be drawn.

6.4. The relationship between levels of glutathione and ATP, activities of functional markers, and cellular toxicity

The concept of toxicity, as applied to biochemical and functional markers, is not absolutely precise because the relationship between cellular levels of GSH and ATP, and cellular function (as assessed by the activity of functional markers) have not been fully elucidated. Additionally, the point at which a particular parameter may be viewed as a physiological, pharmacological or toxicological response is, to some extent, arbitrary.

Studies have shown that lowering the intracellular GSH levels with BSO, *in vivo* or diethyl maleate, *in vitro* does not necessarily lead to a loss of cell function [113, 114, 126], however, the observation of a chemically induced lowering of GSH may indicate a potentially toxic reaction. Many compounds, including 1,4-benzoquinone, react non specifically with nucleophiles, thus the large excess of GSH within the cell can protect against damage to more critical thiol groups. It has been suggested that the

maintenance of activity of proteins critical to cellular function, depends upon the presence of reduced sulphydryls on the protein [107, 126].

The relationship of ATP levels to loss of GSH has also been investigated. Oxidative stress is proposed to increase levels of oxidised glutathione (GSSG) within the cell prior to an ATP-dependent excretion. A large increase of GSSG formed within the cell could therefore explain the observed ATP depletion. In the case of 1,4-benzoquinone, the mechanism of ATP-depletion is less clear. The non-selectivity of reaction may suggest the possibility of compromising ATP generating systems, or that the quinone may react directly with ATP. The possibility that the interaction of the quinone with cellular components may induce other ATP utilisation mechanisms cannot be excluded.

The evaluation of cellular toxicity by the use of functional markers is a useful means of determining toxicity to particular cell types. The energy dependence of functional markers means that their activity is dependent on the cellular level of ATP. This reflects the energy generation versus energy utilisation of the particular cell type with which they are associated. The precise relationship between different functional markers, the activity of critical cellular components and the level of ATP will depend on the energy dependence of each function relative to each other which, in the majority of situations is far from clear. It has recently been proposed that no simple correlation exists between intracellular ATP levels and cell damage [200].

Functional markers may reflect the adverse effect of direct interaction of the compound with cellular macromolecules. This can lead to a loss of clarity in interpreting cellular toxicity. A compound that damages proteins non-selectively will compromise the parameter being assessed, together with components which serve the more immediate cellular requirements, and is thus a reflection of cellular function.

For selective chemical damage to proteins, functional markers may not be so precisely related to loss of critical cell function.

6.5. The role of glutathione in the toxicity of ANTU

The relationship between pulmonary glutathione levels and the toxicity of ANTU was investigated. It was found that prior treatment with phorone was associated with a marked reduction in the toxicity of ANTU. The indicators used to assess ANTU toxicity were: lethality, hydrothorax, pulmonary GSH levels and changes in adenosine and spermidine uptake.

The effect of ANTU on pulmonary GSH levels, observed at 4 hours (figure 5.4) is complicated. The preliminary experiment suggested that no modulation of GSH was seen in ANTU treated rats, but with phorone pretreatment, the elevated GSH levels appeared to be depleted to levels seen in control pretreated rats. This could suggest that a complex, perhaps time dependent, relationship between pulmonary GSH levels and ANTU toxicity exists. A time course of pulmonary and hepatic GSH, with and without phorone pretreatment, following ANTU administration may give more detailed information as to the precise relationship between GSH levels and ANTU toxicity.

A further complication in delineating the mechanism of the phorone protection against ANTU-induced toxicity was the striking elevation in hepatic NPSH levels in the rat (data not shown) [196]. These changes could alter the pharmacokinetics of ANTU resulting in a decreased availability to the lung and therefore a decreased toxicity. It has also been shown that phorone may change hæm oxygenase activity in rat liver [197] thus possibly altering hepatic metabolism of ANTU so altering its bioavailability to the lung. In addition, both lung and liver microsomes may activate ANTU [191] so a possible hepatic contribution to the observed protection cannot be excluded.

ANTU (5 and 10 mg/kg) also caused a dose dependent inhibition of spermidine accumulation, in agreement with Nemery *et al.* [144]. These results suggest that ANTU may also damage epithelial cells, in agreement with the results of Meyrick *et al.* although these histological effects were observed at doses of 50 mg/kg [27]. In the present study, ANTU did not show any particular selectivity for the endothelium, compared with the epithelium, based on functional markers. This could be the result of assessing these parameters at 4 hours after ANTU administration (when œdema has been shown to be maximal [190]). Investigations at earlier times might have shown preferential damage to the different cell types

The effects of ANTU observed *in vitro* are in contrast to those seen *in vivo* (figure 5.7). Firstly, higher levels of ANTU were required to produce changes in functional markers *in vitro* (up to 1 mM as opposed to an *in vivo* dose of 10 mg/kg). Secondly, the effects were seen in the pulmonary epithelium and not in the endothelium. This could suggest differences in the mechanism of toxicity of ANTU, administered *in vivo* or incubated *in vitro*, to the lung. The differences could arise from a number of factors such as possible hepatic involvement in terms of metabolic activation and changes in pulmonary intercellular relationships. Other considerations include physiological responses of endothelial cells to mechanical damage, and white cell recruitment. Leukocyte and platelet infiltration of the lung has been shown in oxygen mediated pulmonary endothelial toxicity [26]. Further, changes in solubility of ANTU after metabolism, leading to precipitation, has not been reported. It is possible that such changes could lead to mechanical vascular damage seen *in vivo* which is absent in the *in vitro* situation.

6.6. Summary

These studies have shown that glutathione, in conjunction with its associated enzymes, forms an important part of the pulmonary defence mechanisms against toxic insult. The studies using the known redox cycling compounds paraquat, diquat and 2,3-dimethoxy-1,4-naphthoquinone and the arylating compound 1,4-benzoquinone suggest possible differences in toxicity between cell types in the lung. It has been suggested that much of the glutathione in the lung is located in cell types other than the epithelium, possibly the endothelium [184]. If pulmonary glutathione levels formed an important part of pulmonary defence mechanisms *per se*, then this would be inconsistent with the finding that elevated oxygen levels affect primarily the endothelium [26]. Initial studies using proposed inhibitors of enzymes associated with the defence against oxidative stress (glutathione peroxidase and catalase) have suggested that there may be possible differences in enzyme activity between different cell types. This would suggest that glutathione manifests different biochemical roles in different cell types in the lung.

The role of glutathione in the toxicity of ANTU is unclear. It was shown that raised glutathione levels conferred protection against some of the toxic effects of ANTU. The mechanism for this protection, however, remains obscure.

The response of the lung to toxic insult is obscured by a multiplicity of processes involving interrelated and overlapping changes at biochemical, physiological and pathological levels. Pulmonary damage may result from direct effects, interference with biochemical processes or eliciting inappropriate or exaggerated repair responses. Studies of pulmonary toxicity require further understanding into the contribution of biochemical and physiological processes to pulmonary toxicity.

REFERENCES

1 **Sykes, J.B. (ed.).** The concise oxford dictionary of current english. (7th edition).
O.U.P. (1985)

2 **Albert, A.** Xenobiosis.
Chapman and Hall, London. (1986)

3 **Doull, J. and Bruce, M.C.** Origin and scope of toxicology. (from: **Klaasen, C.D., Amdur, M.O. and Doull, J. (eds.).** Casarett and Doull's Toxicology. The basic Science of Poisons. (3rd ed.)).
Macmillan, NY. (1986)

4 **Timbrell, J.A.** Introduction to toxicology.
Taylor and Francis. (1989)

5 **Gee, D.J.** Lecture notes in forensic medicine. (4th ed.)
Blackwell, Oxford. (1984)

6 **Cohen, G.M.** Basic principles of target organ toxicity. (from: **Cohen, G.M.** Target organ toxicity. Volume I.)
CRC Press, Florida. (1986)

7 **Gopinath, C., Prentice, D.E. and Lewis, D.J.** Atlas of experimental toxicological pathology.
MTP Press Ltd., Lancaster, UK. (1987)

8 **Kehrer, J.P. and Kacew, S.** Systemically applied chemicals that damage lung tissue.
Toxicology, **35**: 251-93. (1985)

9 **Yost, G.S., Buckpitt, A.R., Roth, R.A. and McLemore, T.L.** Mechanisms of lung injury by systemically administered chemicals.
Tox. Appl. Pharmacol., **101**: 179-95. (1989)

10 **Cooper, J.A.D. and Matthay, R.A.** Drug-induced pulmonary disease.
Dis. Mon., **33(2)**: 61-120. (1987)

11 **Smith, L.L. and Nemery, B.** Cellular specific toxicity in the lung (from: **De Matteis, F. and Lock, E.A.** Selectivity and molecular mechanisms of toxicity.)
Macmillan Press, UK. (1987)

12 **Mustafa, M.G. and Tierney, D.F.** Biochemical and metabolic changes in the lung with oxygen, ozone and nitrogen dioxide toxicity.
Am. Rev Respir. Dis., **118**: 1061-88. (1978)

13 **Smith, L.L.** The identification of an accumulation system for diamines and polyamines into the lung and its relevance to paraquat toxicity.
Arch. Toxicol., **Suppl. 5**: 1-14. (1982)

14 **Boyd, M.R.** Biochemical Mechanisms in Chemical-Induced Lung Injury: Roles of Metabolic Activation.
C.R.C. Crit. Rev. Tox., **7**: 103-76. (1980)

15 **Boyd., M.R.** Metabolic activation and Lung Toxicity: A basis for Cell-Selective Pulmonary Damage by Foreign Chemicals.
Environ. H. Persp., **55**: 47-51. (1984)

16 **Castranova, V., Rabovsky, J,. Tucker, J.H. and Miles, P.R.** The Alveolar type II epithelial cell: a multifunctional pneumocyte.
Tox. Appl. Pharmacol., **93**: 472-83. (1988)

17 **Rowett, H.G.Q.** Guide to dissection.
John Murray (Publishers) Ltd, London. (1970)

18 **Breeze, R. and Turk, M.** Cellular Structure, function and organisation in the lower respiratory tract.
Environ. H. Persp., **55**: 3-24. (1984)

19 **Sorokin, S.P.** The cells of the lung. (From: **Nettesheim, P. (ed.).** Morphology of experimental respiratory carcinogenesis).
Atomic Energy Commission Symposium Series, **21**: 3-44. (1970)

20 **Rooney, S.A.** The surfactant system and lung phospholipid biochemistry.
Am. Rev. Respir. Dis., **131**: 439-60. (1985)

21 **Baron, J., Burke, J.P., Guengerich, F.P., Jakoby, W.B. and Voigt, J.M.** Sites for Xenobiotic Activation and Detoxication within the respiratory tract: Implications for Chemically Induced Toxicity.
Tox. Appl. Pharmacol., **93**: 493-505. (1988)

22 **Moldeus, P. and Cotgreave, I.** Mechanisms of endothelial lung damage. (from: **Volans, G.N., Sims, J., Sullivan, F.M. and Turner, P. (eds.)** Basic Science in Toxicology. Proceedings of the Vth international congress of toxicology, Brighton, England. 16-21 July 1989.)
Taylor and Francis, London. (1990)

23 **Pittilo, R.M.** Endothelium and the vessel wall. (from: **Pittilo, R.M. and Machin, S.J.** Platelet-vessel wall interactions.)
Springer-Verlag, Berlin. (1988)

24 **Kuhn III, C.** The cells of the lung and their organelles. (from: **Crystal, R.G. (ed.)** The biochemical basis of pulmonary function. (Lung biology in health and disease, Vol. 2.)).
Marcel Dekker inc. NY. (1976)

25 **Ryan, J.W. and Ryan, U.S.** Pulmonary endothelial cells.
Fed. Proc., **36(13)**: 2683-91. (1977)

26 **Crapo, J.D.** Morphologic changes in pulmonary oxygen toxicity.
Ann. Rev. Physiol., **48**: 721-3. (1986)

27 **Mèyrick, B., Miller, J. and Reid, L.** Pulmonary œdema induced by ANTU or by high or low oxygen concentrations in rat - an electron microscopic study. Br. J. Exp. Pathol., **53**: 347-58. (1972)

28 **Tierney, D.F.** Lung metabolism and biochemistry. Ann. Rev. Physiol., **36**: 209-32. (1974)

29 **Fisher, A.B.** Intermediary metabolism of the lung. Environ. H. Perspect., **55**: 149-58. (1984)

30 **Fisher, A.B., Steinberg, H. and Bassett, D.J.P.** Energy utilisation by the lung. Am. Rev. Respir. Dis., **57**: 437-46. (1974)

31 **Rhoades, R.A.** Net uptake of glucose, glycerol and fatty acids by the isolated perfused rat lung. Am. J. Physiol., **226**: 144-9. (1979)

32 **Wolf, R.R., Hochachka, P.W., Trelstad, R.L. and Burke, J.F.** Lactate metabolism in perfused rat lung. Am. J. Physiol., **236**: E276-82. (1979)

33 **Fricke, R.F. and Longmore, W.J.** Effects of insulin and diabetes on 2-deoxy-D-glucose uptake by the isolated perfused rat lung. J. Biol. Chem., **254**: 5092-8. (1979)

34 **Kerr, J.S., Fisher, A.B. and Kleinzeller, A.** Transport of glucose analogs in rat lung. Am. J. Physiol., **241**: E191-5. (1981)

35 **Bassett, D.J.P. and Fisher, A.B.** Metabolic response to carbon monoxide by isolated rat lungs. Am. J. Physiol., **230**: 658-63. (1976)

36 **Bassett, D.J.P. and Fisher, A.B.** Pentose cycle of the isolated perfused rat lung. Am. J. Physiol., **231**: 1527-32. (1976)

37 **Minchin, R.F. and Boyd, M.R.** Localisation of metabolic activation and deactivation systems in the lung: Significance to the pulmonary toxicity of xenobiotics. Ann. Rev. Pharmacol. Toxicol., **23**: 217-38. (1983)

38 **Gibson, G.G. and Skett, P.** Introduction to drug metabolism. Chapman and Hall, London. (1986)

39 **Boyd, M.R.** Evidence for the Clara cell as a site of cytochrome P450-dependent mixed-function oxidase activity in the lung. Nature, **269**: 713-15. (1977)

40 **Boyd, M.R., Statham, C.N. and Longo, N.S.** The pulmonary Clara cell as a target for toxic chemicals requiring metabolic activation.
J. Pharmac. Exp. Ther., **212**: 109-14. (1980)

41 **Krigsheld, K.R., Lowe, M.C., Mimnaugh, E.G., Trush, M.A, Ginsburg, E. and Gram, T.E.** Selective damage to nonciliated bronchiolar epithelial cells in relation to impairment of pulmonary monooxygenase activities by 1,1-dichloroethylene in mice.
Tox. Appl. Pharmacol., **74**: 201-13. (1984)

42 **Warren, D.L., Brown, D.L. and Buckpitt, A.R.** Evidence for cytochrome P-450 mediated metabolism in the bronchiolar damage by naphthalene.
Chem.-Biol. Interact., **40**: 287-303. (1982)

43 **Serabjit-Singh, C.J., Wolf, C.R. and Philpot, R.M.** The rabbit pulmonary monooxygenase system: immunochemical and biochemical characterization of enzyme components.
J. Biol. Chem., **254**: 9901-7. (1979)

44 **Grover, P.L., Hewer, A. and Sims, P.** Metabolism of polycyclic hydrocarbons by rat lung preparations.
Biochem. Pharmacol., **23**: 323-32. (1974)

45 **Boyd, M.R.** Role of metabolic activation in the pathogenesis of chemically induced pulmonary disease: mechanism of action of the lung-toxic furan 4-ipomeanol.
Environ. H. Perspect., **16**: 127-38. (1976)

46 **Fakjian, N. and Buckpitt, A.R.** Metabolism of bromobenzene to glutathione adducts in lung slices from mice treated with pneumotoxicants.
Biochem. Pharmacol., **33(9)**: 1479-86. (1984)

47 **Staub, N.C.** Pathogenesis of pulmonary œdema.
Am. Rev. Respir Dis., **109**: 358-72. (1974)

48 **Glaister, J.** Principles of toxicological pathology.
Taylor and Francis, UK. (1986)

49 **Whimster, W.F. and De Poitiers, W.** The Lung. (from: **Riddell, R.H. (ed.)** Pathology of drug-induced and toxic diseases.)
Churchill-Livingstone, NY. (1982)

50 **Hackett, R.L. and Sunderman Jr, F.W.** Pulmonary alveolar reaction to nickel carbonyl: ultrastructural and histochemical studies.
Arch. Environ. Health., **16**: 349-62 (1968)

51 **Pasi, A.** The toxicology of paraquat, diquat and morfamquat.
Hans Huber, Bern. (1978)

52 **Diller, W.F., Bruch, J. and Dehnen, W.** Pulmonary changes in the rat following low phosgene exposure.
Arch. Toxicol., **57**: 184-90. (1985)

53 **Adamson, I.Y.R.** Pulmonary toxicity of bleomycin.
Environ. H. Perspect., **16**: 119 (1976)

54 **Marino, A.A. and Mitchell,J.T.** Lung damage in mice following intraperitoneal injection of butylated hydroxytoluene.
Proc. Soc. Exptl. Biol. Med., **140**: 122-5. (1972)

55 **Kehrer, J.P., Klein-Szanto, A.J.P., Thurston, D.E., Lindenschmidt, R.C. and Witschi, H.** O,S,S-Trimethyl phosphorothioate-induced lung damage in rats and mice.
Tox. Appl. Pharmacol., **84**: 480-92. (1986)

56 **Patel, J.M., Block, E.R. and Hood, C.I.** Biochemical indices of cyclophosphamide-induced lung toxicity.
Tox. Appl. Pharmacol., **76**: 128-38. (1984)

57 **Witschi, H.** Proliferation of Type II alveolar cells; a review of common responses in toxic lung injury.
Toxicology, **5**: 267-77. (1976)

58 **Reiser, K.M. and Last, J.A.** Early cellular events in pulmonary fibrosis.
Exp. Lung Res., **10**: 331-5. (1986)

59 **Caldwell, J., Sangster, S.A. and Sutton, J.D.** The role of metabolic activation in target organ toxicity. (from: **Cohen, G.M.** Target organ toxicity. Volume I.)
CRC Press, Florida. (1986)

60 **Boyd, M.R.** Biochemical mechanisms in pulmonary toxicity of furan derivatives. (from: **Hodgson, E., Bend, J.R. and Philpot, R.M. (eds.)** Reviews in Biochemical toxicology. Vol. 2.
Elsevier, NY. (1980)

61 **Reid, W.D., Ilett, K.F., Glick, J.M. and Krishna, G.** Metabolism and binding of aromatic hydrocarbons in the lung.
Am. Rev. Respir. Dis., **107**: 539-51 (1973)

62 **Ilett, K.F., Stripp, B., Menard, R.H., Reid, W.D. and Gillette, J.R.** Studies on the mechanism of the lung toxicity of paraquat: comparison of tissue distribution and some biochemical parameters in rats and rabbits.
Tox. Appl. Pharmacol., **28**: 216-26. (1974)

63 **Eklow-Lastbom, L., Rossi, L., Thor, H. and Orrenius, S.** Effects of oxidative stress caused by hyperoxia and diquat. A study in isolated hepatocytes.
Free Rad. Res. Comm., **2(2)**: 57-68. (1986)

64 **Powis, G. and Appel, P.L.** Relationship of the single electron reduction potential of quinones to their reduction by flavoproteins.
Biochem. Pharmacol., **29**: 2567-72. (1980)

65 **Bibich, H.** Butylated hydroxytoluene (BHT): a review.
Environ. Res., **29**: 1-29. (1982)

66 **Smith, P. and Heath, D.** Paraquat.
C.R.C. Crit. Rev. Tox., **4**: 411-45. (1974)

67 **Reznik-Schuller, H.M., Smith, A.C., Therot, J.P. and Boyd, M.R.** Pulmonary toxicity of the anticancer drug bis-chloroethylnitrosourea (BCNU) in rats.
Toxicologist, **4**: 29 (1984)

68 **Tateishi, P. and Ishikawa, O.** The effect of N-nitrosobis (2-hydroxypropyl)amine on pulmonary neuroepithelial cells in syrian golden hamsters.
Am. J. Pathol., **119**: 326-35. (1985)

69 **Pryor, W.A.** The role of free radical reactions in biological systems. (from: **Pryor, W.A. (ed.)** Free radicals in biology. Vol. 1.)
Academic Press, UK. (1976)

70 **Halliwell, B. and Gutteridge, J.M.C.** Free radicals in biology and medicine.
O.U.P. (1985)

71 **Pryor, W.A.** Free radical reactions and their importance in biological systems.
Fed. Proc., **32**: 1862-9. (1973)

72 **Sykes,** Guidebook to mechanism in organic chemistry (6th edition).
O.U.P. (1986)

73 **Sies, H.** Oxidative stress: introductory remarks. (from: **Sies, H. (ed.)** Oxidative stress.
Academic Press, UK. (1985)

74 **Brown, G.I.** A new guide to modern valency theory (4th etition).
Longman, UK. (1980)

75 **Taube, H.** Mechanisms of oxidation with oxygen.
J. Gen. Physiol., **49**: 29-50. (1965)

76 **Fridovich, I.** Superoxide radical: an endogenous toxicant.
Ann. Rev. Pharmacol. Toxicol., **23**: 239-57. (1983)

77 **Bielski, B.H.J. and Chan, P.C.** Kinetic study by pulse radiolysis of the lactate dehydrogenase-catalysed chain oxidation of nicotinamide adenine dinucleotide by $HO_2\cdot$ and O_2^{-} radicals.
J. Biol. Chem., **250**: 318-21. (1975)

78 **Kappus, H.** Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. (from: **Sies, H. (ed.)** Oxidative stress. Academic Press, UK. (1985)

79 **Horton, A.A. and Fairhurst, S.** Lipid peroxidation and mechanisms of toxicity. C.R.C. Crit. Rev. Tox., **18(1)**: 27-79. (1987)

80 **Lind, C., Hochstein, P. and Ernster, L.** DT-Diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. Arch. Biochem. Biophys., **216**: 178-85. (1982)

81 **Kappus, H. and Sies, H.** Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. Experientia, **37**: 1233-41. (1981)

82 **Iyagani, T. and Yamazaki, I.** One and two electron reduction of quinones by microsomal flavins. Biochim. Biophys. Acta, **172**: 370-81. (1969)

83 **Powis, G. Svingen, B.A. and Appel, P.L.** Quinone stimulated superoxide formation by subcellular fractions, isolated hepatocytes and other cells. Mol. Pharmacol., **20**: 387-94. (1981)

84 **Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S.** Metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. J. Biol. Chem., **257(20)**: 12419-25. (1982)

85 **Smith, L.L., Rose, M.S. and Wyatt, I.** The pathology and biochemistry of paraquat. (from: Oxygen free radicals and tissue damage.) Ciba Foundation., **65**: 321-41. (1979)

86 **Brigelius, R., Lenzen, R. and Sies, H.** Increase in hepatic mixed disulphide and glutathione disulphide levels elicited by paraquat. Biochem. Pharmacol., **31**: 1637-41. (1982)

87 **Jackson, R.M., Chandler, D.B. and Fulmer, J.D.** Production of arachidonic acid metabolites by endothelial cells in hyperoxia. J. Appl. Physiol., **61**: 584-91. (1986)

88 **Kosower, N.S., and Kosower, E.M.** The glutathione status of cells. Int. Rev. Cytol., **54**: 109-60. (1977)

89 **Ketterer, B.** The role of nonenzymatic reactions of glutathione in xenobiotic metabolism. Drug Met. Rev., **13(1)**: 161-87. (1982)

90 **Ketterer, B., Coles, B. and Meyer, D.J.** The role of glutathione in detoxication.
Environ. H. Persp., **49**: 59-69. (1983)

91 **Meister, A.** Metabolism and transport of glutathione and other γ-glutamyl compounds. (from: **Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B.** Functions of glutathione: Biochemical, pysiological, toxicological and clinical aspects.)
Raven, NY. (1983)

92 **Meister, A. and Anderson, M.E.** Glutathione.
Ann. Rev. Biochem., **52**: 711-60. (1983)

93 **Coles, B.** Effects of modifying structure on electrophilic reactions with biological nucleophiles.
Drug. Met. Rev., **15(7)**, 1307-34. (1984-85)

94 **Stryer, L.** Biochemistry (2nd edition)
Freeman, NY. (1981)

95 **Gumaa, K.A., Sochor, M., McLean, P. and Greenbaum, A.L.** Effect of quinolinic acid on the metabolic profile and regulation of carbohydrate and lipid metabolisms in the liver of diabetic rats.
Arch. Biochem. Biophys., **206**: 1-7. (1981)

96 **Hammarstrom, S.** Leukotrienes: biologially active molecules formed from polyunsaturated fatty acids and glutathione. (from: **Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B.** Functions of glutathione: Biochemical, pysiological, toxicological and clinical aspects.)
Raven, NY. (1983)

97 **Moldeus, P. and Jernstrom, B.** Interaction of glutathione with reactive intermediates. (from: **Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B.** Functions of glutathione: Biochemical, pysiological, toxicological and clinical aspects.)
Raven, NY. (1983)

98 **Meister, A.** Glutathione and its selective modification.
J. Biol. Chem., **263(33)**: 17205-8. (1988)

99 **Lock, E.A. and Ishmael, J.** Hepatic and renal nonprotein sulphydryl concentration following toxic doses of hexachloro-1,3-butadiene in the rat: the effects of Arochlor 1254 or SKF 525A treatment.
Tox. Appl. Pharmacol., **57**: 79-87. (1981)

100 **Wardman, P.** Conjugation and oxidation of glutathione via thiyl free radicals. (from: **Sies, H. and Ketterer, B. (eds.)** Glutathione conjugation: mechanisms and biological significance.)
Academic Press, London. (1988)

101 **Rotruck, J.T., Pope, A.L., Ganther, H.G., Swanson, A.B., Hafeman, D.G. and Hoekstra, W.G.** Selenium: biochemical role as a component of glutathione peroxidase.
Science, **179**: 588-90. (1973)

102 **Lawrence, R.A. and Burk, R.F.** Species, tissue and subcellular distribution of non Se-dependent glutathione peroxidase activity.
J. Nutr. **108**: 211-5. (1978)

103 **Ursini, F. and Bindoli, A.** The role of selenium peroxidases in the protection against oxidative damage of membranes.
Chem. Phys. Lipids, **44**: 255-76. (1987)

104 **Burk, R.F., Trumble, M.J. and Lawrence, R.A.** Rat hepatic cytosolic glutathione-dependent enzyme protection against lipid peroxidation in the NADPH-microsomal lipid peroxidation system.
Biochim. Biophys. Acta, **618**: 35-41. (1980)

105 **Simonarson, B.** Glutathione peroxidase, selenium and vitamin E in defense against reative oxygen intermediates. (from: **Quintanilha, A. (ed)** Reactive oxygen species in chemistry, biology and medicine.)
Plenum Publishing Corporation. (1988)

106 **Reed, D.J.** Regulation of reductive processes by glutathione.
Biochem. Pharmacol., **35(1)**: 7-13. (1986)

107 **Nicotera, P. and Orrenius, S.** Role of thiols in protection against biological reactive intermediates. (from: **Kosis, J.J., Jollow, D.J., Witmer, C.M., Nelson, J.O. and Snyder, R. (eds.)** Biological reactive intermediates. Vol. 3.)
Plenum Publishing Corporation. (1986)

108 **Douglas, K.** Reactivity of glutathione in model systems for glutathione-S-transferases and related systems. (from: **Sies, H. and Ketterer, B. (eds.)** Glutathione conjugation: mechanisms and biological significance.)
Academic Press, London. (1988)

109 **Pearson, R.G. and Songstad, J.** Application of the principle of hard and soft acids and bases to organic chemistry.
J. Am. Chem. Soc., **89**: 1827-36. (1967)

110 **Ketterer, B., Meyer, D.J. and Clark, A.G.** Soluble glutathione transferase isozymes. (from: **Sies, H. and Ketterer, B. (eds.)** Glutathione conjugation: mechanisms and biological significance.)
Academic Press, London. (1988)

111 **Maruyama, H., Inoue, M., Arias, I.M. and Listowsky, I.** Ligandins or glutathione-S-transferases: a family of multifunctional proteins in the rat. (from: **Larsson, A., Orrenius, S., Holmgren, A. and Mannervik, B. (eds.)** Functions of glutathione: Biochemical, pysiological, toxicological and clinical aspects.)
Raven, NY. (1983)

112 **Taùssig, M.** Processes in pathology and microbiology.
Blackwell, UK. (1984)

113 **Griffith, O.W. and Meister, A.** Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-butyl homocysteine sulfoximine).
J. Biol. Chem., **254**: 7558-60. (1979)

114 **Plummer, J.L., Smith, B.R., Sies, H. and Bend, J.R.** Chemical depletion of glutathione *in vivo*.
Meth. Enzymol. **77**: 51-9. (1983)

115 **Williamson, J.M. and Meister, A.** Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate.
Proc. Natl. Acad. Sci., **78**: 936-9. (1981)

116 **Burk, R.F.** Production of selenium defiiency in the rat.
Meth. Enzymol., **143**: 307-13. (1987)

117 **Milano, G., Caldani, C., Khater, R., Launay, J-M., Soummer, A-M., Namer, M. and Schneider, M.** Time and dose dependent inhibition of erythroyte glutathione peroxidase by cisplatin.
Biochem. Pharmacol., **37(5)**: 981-2. (1987)

118 **Chaudiere, J. and Tappel, A.L.** Interactions of gold(I) with the active site of selenium glutathione peroxidase.
J. Inorg. Biochem., **20**: 313-25. (1984)

119 **Chaudiere, J., Wilhelmsen, E.C. and Tappel, A.L.** Mechanism of Selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acids and other mercaptans.
J. Biol. Chem., **259(2)**: 1043-50. (1984)

120 **Frischer, H. and Ahmad, T.** Severe generalised glutathione reductase deficiency after antitumour chemotherapy with BCNU (1,3-bis(chloroethyl)-1-nitrosourea).
J. Lab. Clin. Med., **89**: 1080-91 (1977)

121 **Babson and Reed, D.J.** Inactivation of glutathione reductase by 2-chloroethyl-derived isocyanates.
Biochem. Biophys. Res. Comm., **83**: 754-62. (1978)

122 **Gant, T.W., Rao, D.N.R., Mason, R.P. and Cohen, G.M.** Redox cycling and sulphydryl arylation; their relative importance in the mechanisms of quinone cytotoxicity.
Chem.-Biol. Interact., **65**: 157-73. (1988)

123 **Srivastava, S.K. and Beutler, E.** The transport of oxidised glutathione from human erythrocytes.
J. Biol. Chem., **244**: 9-16. (1969)

124 **DiMonte, D., Bellomo, G., Thor, H., Nicotera, P. and Orrenius, S.** Menadione-induced cytotoxicity is associated with protein thiol oxidation and alteration in intraellular Ca^{2+} homeostasis.
Arch. Biochem. Biophys., **235(2)**: 343-50. (1984)

125 **DiMonte, D., Ross, D., Bellomo, G., Eklow, L. and Orrenius, S.** Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes.
Arch. Biochem. Biophys., **235(2)**: 334-42. (1984)

126 **Nicotera, P. and Orrenius, S.** Perturbation of thiol and calcium homeostasis in cell injury. (from: **Fowler, B.A. (ed.)** Mechanisms of cell injury: implications for human health
John Wiley & Sons Ltd. (1987)

127 **Lehninger, A.C., Vercesi, A. and Bababunmi, E.P.** Regulation of Ca^{2+} release from mitochondria by the oxidation-reduction state of pyridine nucleotides.
Proc. Natl. Acad. Sci., **75**: 1690-4. (1978)

128 **Moore, L., Chen, T., Knapp, H.R. and Landon, E.J.** Energy dependent calcium sequestration activity in rat liver microsomes.
J. Biol. Chem., **250**: 4562-8. (1975)

129 **Rose, M.S., Lock, E.A., Smith, L.L. and Wyatt, I.** Paraquat accumulation: species and tissue specificity.
Biochem. Pharmacol., **25**: 419-23. (1976)

130 **Hardwick, S.J., Adam, A., Smith, L.L. and Cohen, G.M.** A novel lung slice system with compromised antioxidant defences.
Environ. H. Perspect., **85**: 129-133. (1990)

131 **Hissin, P.J. and Hilf, R.** A fluorometric method for determination of oxidised and reduced glutathione in tissues.
Anal. Biochem., **74**: 214-26. (1976)

133 **Nueschwander-Tetri, B.A. and Roll, F.J.** Glutathione measurement by high-performance liquid chromatography separation and fluorometric detection of the glutathione-orthophthalaldehyde adduct.
Anal. Biochem., **179**: 236-41. (1989)

133 **Jocelyn, P.C. and Kamminga, A.** Development of fluorescence between *o*-phthaldialdehyde and thiols.
Anal. Biochem., **37**: 417-21. (1970)

134 **Moron, M.S., DePierre, J.W. and Mannervick, B.** Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver.
Biochim. Biophys. Acta, **582(1)**: 67-78. (1979)

135 **Brigelius, R., Muckel, C., Akerboom, T.P.M. and Sies, H.** Identification and quantitation of glutathione in hepatic protein mixed disulphides and its relationship to glutathione disulphide.
Biochem. Pharmacol., **32(17)**: 2529-34. (1983)

136 **Lemasters, J.L. and Hackenbrock, C.R.** Firefly luciferase assay for ATP production by mitochondria.
Meth. Enzymol., **57**: 36-50. (1978)

137 **Hardwick, S.J.** The use of adenosine as a marker of the pulmonary capillary endothelium. (from: **Ph. D. Thesis**: The role of glutathione in the biochemical mechanism of toxicity of paraquat and of other pulmonary toxicants.)
University of London. (1990)

138 **Gordonsmith, R.H.** Characterisation of oligoamine uptake into rat lung slices. (from: **Ph. D. Thesis**: Characterisation of the pulmonary uptake of the oligoamines and structurally related comp[ounds.)
University of London. (1988)

139 **Shimizu, H., Creveling, C.R. and Daly, J.** Stimulated formation of adenosine 3' 5' cyclic phosphate in cerebral cortex: synergism between electrical activity and biogenic amines.
Proc. Natl. Acad. Sci., **65**: 1035-40. (1970)

140 **Norman, G.A., Follet, M.J. and Hector, D.A.** Quantitative thin layer chromatography of ATP and its degradation products in meat tissue.
J. Chromatog., **90**: 105-11. (1974)

141 **Bakhle, Y.S. and Chelliah, R.** Metabolism and uptake of adenosine in rat isolated lung and its inhibition.
Br. J. Pharmacol., **79**: 509-15. (1983)

142 **Smith, L.L., Cohen, G.M. and Aldridge, W.N.** Morphological and biochemical correlates of chemical induced lung injury.
Arch. Toxicol., **58**: 214-8. (1986)

143 **Smith, L.L. and Wyatt, I.** The accumulation of putrescine into slices of rat lung and brain and its relationship to the accumulation of paraquat.
Biochem. Pharmacol., **30(10)**: 1053-8. (1981)

144 **Nemery, B., Smith, L.L. and Aldridge, W.N.** Putrescine and 5-hydroxytryptamine accumulation in rat lung slices: cellular localisation and responses to cell-specific lung injury.
Tox. Appl. Pharmacol., **91**: 107-20. (1987)

145 **Lazlo, J.S., Catravas, J.D. and Gillis, C.N.** Reduction in rabbit serum and pulmonary angiotensin converting enzyme activity after subacute bleomycin treatment.
Biochem. Pharmacol., **30**: 2577-84. (1981)

146 **Drury, A.N. and Szent-Gyorgi, A.** The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. J. Physiol., **68**: 213-7. (1929)

147 **Drury, A.N.** The physiological activity of nucleic acid and its derivatives. Physiol. Rev., **16**: 292-325. (1936)

148 **Clarke, D.A., Davoli, J., Philips, F.S. and Brown, G.B.** . J. Pharmacol. Exp. Ther., **106**: 291-302. (1952)

149 **Kolassa, N., Pfleger, K. and Tram, M.** Species differences in action and elimination of adenosine after dipyridamole and hexobendine. Eur. J. Pharmacol., **13**: 320-5. (1971)

150 **Pearson, J.D., Carleton, J.S.,Hutchings, A. and Gordon, J.** Uptake and metabolism of adenosine by pig aortic endothelial and smooth-muscle cells in culture. Biochem. J., **170**: 265-71. (1978)

151 **Arch, J.R.S. and Newsholme, E.A.** The control of the metabolism and the hormonal role of adenosine. (from: **Campbell, P.N. and Aldridge, W.N. (eds.)** Essays in biochemistry. Vol. 14.) The Biochemical Society/Academic Press. (1978)

152 **Meghji, P., Middleton, K., Hassall, C.J.S., Phillips, M.I. and Newby, A.C.** Evidence for the extracellular deamination of adenosine in the rat heart. Int. J. Biochem., **20(12)**: 1335-41. (1988)

153 **Klenow, H. and Ostergaard, E.** Adenosine induction of rapid catabolism of adenine ribonucleotides and independent elevation of the ATP content in quiescent mouse fibroblasts. J. Cell. Physiol., **137**: 565-70. (1988)

154 **Kroll, K., Kelm, M.K.M., Burrig, K-F. and Schrader, J.** Transendothelial transport and metabolism of adenosine and inosine in the intact aorta. Circ. Res., **649(6)**: 1147-57. (1989)

155 **Bakhle, Y.S. and Grantham, C.J.** Effects of pulmonary œdema on pharmacokinetics of adenosine in rat isolated lungs. Br. J. Pharmacol., **91**: 849-56. (1987)

156 **Grantham, C.J. and Bakhle, Y.S.** Effect of acute lung injury on metabolism of adenine nucleotides in rat perfused lung. Br. J. Pharmacol., **94**: 1029-36. (1988)

157 **Henderson, J.F., Paterson, A.R.P., Caldwell, I.C., Paul, B., Chan, M.C. and Lau, K.F.** inhibitors of nucleoside and nucleotide metabolism. Cancer Chemother. Rep. pt 2, **3**: 71-85. (1972)

158 **Agarwal, R.P., Spector, T. and Parks Jr, R.E.** Tight binding inhibitors-IV. Inhibition of adenosine deaminases by various inhibitors.
Biochem. Pharmacol., **26**: 359-67. (1977)

159 **Gauthier, T., Denis-Pouxviel, C., Paris, H. and Murat, J-C.** Study on ATP-generating system and related hexokinase activity in mitochondria isolated from undifferentiated or differentiated HT29 adenocarcinoma cells.
Biochim. Biophys. Acta, **975**: 231-8. (1989)

160 **Scharenberg, J.G.M., Rijkers, G.T., Spaapen, L.J.M., Toebes, E.A.H., Rijksen, G., Duran, M., Staal, G.E.J. and Zegers, B.J.M.** Different pathways for deoxyguanosine toxicity in T-lymphocytes of various developmental stages.
Int. J. Immunopharmacol., **10(6)**: 675-86. (1988)

161 **Dieterle, Y., Ody, C., Ehrensberger,A., Stalder, H. and Junod, A.F.** Metabolism and uptake of Adenosine triphosphate and adenosine by porcine aortic and pulmonary endothelial cells and fibroblasts in culture.
Circ. Res., **42**: 869-76. (1978)

162 **Price, N.C. and Dwek, R.A.** Principles and problems in physical chemistry for biochemists. (2nd edition)
O.U.P. (1982)

163 **Rubio, R., Wiedmeier, V.T. and Berne, R.M.** Nucleoside phosphorylase: localization and role in myocardial distribution of purine.
Am. J. Physiol., **222**: 550-5. (1972)

164 **Block, E.R. and Schoen, F.J.** Effect of alpha naphthylthiourea on uptake of 5-hydroxytryptamine from the pulmonary circulation.
Am. Rev. Respir. Dis., **123**: 69-73. (1981)

165 **Chance, B., Sies, H. and Boveris, A.** Hydroperoxide metabolism in mammalian organs.
Physiol. Rev., **59**: 527-605. (1979)

166 **Sies, H. and Cadenas, E.** Biological basis of detoxification of oxygen free radicals. (from: **Caldwell, J. and Jakoby, W.B. (eds.)** Biological basis of detoxification.)
Academic Press, NY. (1983)

167 **Kondo, T., Dale, G.L. and Buetler, E.** Glutathione transport by inside-out vesicles from human erythrocytes.
Proc. Natl. Acad. Sci., **77**: 6359-62. (1980)

168 **Akerboom, T.P.M., Bilzer, M. and Sies, H.** The relationship of biliary glutathione disulphide efflux and intracellular glutathione disulphide content in perfused rat liver.
J. Biol. Chem., **257**: 4248-52. (1982)

169 **Eklòw, L., Moldeus, P. and Orrenius, S.** Oxidation of glutathione during hydroperoxide metabolism. A study using isolated hepatocytes and the glutathione reductase inhibitor 1,3-bis(2-chloroethyl)-1-nitrosourea.
Eur. J. Biochem., **138**: 459-63. (1984)

170 **Lash, M.W.A.L., Dekant, W., Elfarra, A.A. and Dohn, D.R.** Biosynthesis and biotransformation of glutathione-S-conjugates to toxic metabolites.
C.R.C. Crit. Rev. Tox., **18(4)**: 311-41. (1988)

171 **Dive, C.,Workman, P and Watson, J.V.** Inhibition of intracellular esterases by antitumour chloroethylnitrosoureas. Measurement by flow cytometry and correlation with molecular carbamoylating activity.
Biochem Pharmacol., **37**: 3987-93. (1988)

172 **Meredith, M.J. and Reed, D.J.** Depletion of *in vitro* of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).
Biochem. Pharmacol., **32**: 1383-8. (1983)

173 **Dive, C.,Workman, P and Watson, J.V.** Novel dynamic flow cytoenzymologigal determination of intracellular esterase inhibition by BCNU and related isocyanates.
Biochem. Pharmacol., **36**: 3731-8. (1987)

174 **Babson, J.R., Abell, N.S. and Reed, D.J.** Protective role of the glutathione redox cycle against adriamycin-mediated toxicity in isolated hepatocytes.
Biochem. Pharmacol., **30**: 2299-304. (1981)

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

176 **Tirmenstein, M.A. and Reed, D.J.** Role of a partially purified glutathione S-transferase from rat liver nuclei in the inhibition of nuclear lipid peroxidation.
Biochim. Biophys. Acta, **995**: 174-80. (1989)

177 **Dixon, M. and Webb, E.C.** Enzymes (2nd edition)
Academic Press, NY. (1964)

178 **Chang, J.Y. and Schroeder, W.A.** Reaction of 3-amino-1,2,4-triazole with bovine liver catalase and human erythrocyte catalase.
Arch. Biochem. Biophys., **148**: 505-8. (1972)

179 **Demaster, E.G., Redfern, B., Shirota, F.N. and Nagasawa, H.T.** Differential inhibition of rat tissue catalase by cyanamide.
Biochem. Pharmacol., **35**: 2081-5. (1986)

180 **Adam, A.** An evaluation of the redox cycling potencies of paraquat and nitrofurantoin in microsomal and lung slice systems. (from: **Ph. D. Thesis**: Effects of redox cycling compounds on lungs with normal and compromised oxidative defences.)
University of London. (1989)

181 **Lau, S.S., Hill, B.A., Highet, R.J. and Monks, T.J.** Sequential oxidation and glutathione addition to 1,4-benzoquinone: correlation of toxicity with increased glutathione substitution.
Mol. Pharmacol., **34**: 829-36. (1988)

182 **Brunmark, A. and Cadenas, E.** Reductive addition of glutathione to *p*-benzoquinone, 2-Hydroxy-*p*-benzoquinone, and *p*-benzoquinone epoxides. Effect of the Hydroxy- and glutathionyl substituents on *p*-benzohydroquinone autoxidation.
Mol. Pharmacol., **34**: 829-36. (1988)

183 **Beckman, J.K. and Greene, H.L.** Effects of aurothioglucose on iron-induced rat liver microsomal lipid peroxidation.
Lipids, **23(9)**: 899-903. (1988)

184 **Hardwick, S.J.** Potentiation of the cell specific toxicity of paraquat by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Implications for the heterogenous distribution of glutathione in rat lung.
Biochem. Pharmacol., **39(3)**: 581-9. (1990)

185 **Hanafy, M.S.M. and Bogan, J.A.** Pharmacological modulation of the - pneumotoxicity of 3-methylindole.
Biochem. Pharmacol., **31**: 1765-71. (1982)

186 **O'Brien, K.A.F., Smith, L.L. and Cohen, G.M.** Differences in naphthalene-induced toxicity in the mouse and rat.
Chem.-Biol. Interact., **55**: 109-22. (1985)

187 **Statham, C.N. and Boyd, M.R.** Distribution and metabolism of the pulmonary alkylating agent and cytotoxin, 4-ipomeanol, in control and diethyl maleate-treated rats.
Biochem. Pharmacol., **31**: 1585-9. (1982)

188 **Boyd, M.R. and Neal, R.A.** Studies on the mechanism of toxicity and of the development of tolerance to the pulmonary toxin α-naphthylthiourea (ANTU).
Drug Metab. Dispos., **4**: 314-22. (1976)

189 **Richter, C.P.** The development and use of alpha-naphthylthiourea (ANTU) as a rat poison.
J.A.M.A., **129**: 937-41. (1945)

190 **Cunningham, A.L. and Hurley, J.V.** Alpha-naphthylthiourea-induced pulmonary œdema in the rat: a topographical and electron-microscope study.
J. Pathol., **106**: 25-35. (1972)

191 **Leê, L.L., Arnaud, T. and Neal, R.A.** Metabolism of α-naphthylthiourea by rat liver and rat lung microsomes.
Tox. Appl. Pharmacol., **53**: 164-73. (1980)

192 **van den Brenk, H.A.S., Kelly, H. and Stone, M.G.** Innate and drug-induced resistance to acute lung damage in rats by α-naphthyl thiourea (ANTU) and related compounds.
Br. J. Exp. Path., **57**: 621-36. (1976)

193 **Poulsen, L.L., Hyslop, R.M. and Ziegler, D.M.** S-Oxidation of thioureylenes catalysed by a microsomal flavoprotein mixed-function oxidase.
Biochem. Pharmacol., **23**: 3431-40. (1974)

194 **Van Doorn, R., Leijdekkers, C.M. and Henderson, P.T.** Synergistic effects of phorone on the hepatotoxicity of bromobenzene and paracetamol in mice.
Toxicology, **11**: 225-33. (1978)

195 **Reiter, R. and Wendel, A.** Chemically-induced glutathione depletion and lipid peroxidation.
Chem.-Biol. Interact., **40**: 365-74. (1982)

196 **Hardwick, S.J., Skamarauskas, J.T., Smith, L.L., Upshall, D.G. and Cohen, G.M.** Protection of rats against the effects of alpha-naphthylthiourea (ANTU) by elevation of non-protein sulphydryl levels.
Biochem. Pharmacol. (in press)

197 **Younes, M., Sharma, S.C. and Siegers, C.P.** Glutathione depletion by phorone. Organ specificity and effect on hepatic microsomal mixed-function oxidase system.
Drug Chem. Toxicol., **9**: 67-73. (1986)

198 **Horton, J.K., Meredith, M.J. and Bend, J.R.** Glutathione biosynthesis from sulfur-containing amino acids in enriched populations of Clara and type II cells and macrophages freshly isolated from rabbit lung.
J. Pharmacol. Exp. Ther., **240(2)**: 376-80 (1982)

199 **Scott, A.M., Powell. G.M., Upshall, D.G. and Curtis, C.G.** Pulmonary toxicity of thioureas.
Environ. H. Perspect., **85**: 43-50. (1990)

200 **Kristensen, S.R.** A critical appraisal of the association between energy charge and cell death.
Biochim. Biophys. Acta, **1012**: 272-8. (1989)