THE ROLE OF GLUTATHIONE IN THE BIOCHEMICAL MECHANISM OF PARAQUAT TOXICITY ~~OF PARAQUAT~~ AND OF OTHER ~~PULMONARY~~ TOXICANTS. IN THE LUNG

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ABSTRACT

Paraquat and nitrofurantoin are proposed to exert their effects by redox cycling and oxidative stress. The aim of the studies presented in this thesis was to clarify the role of glutathione status in the toxicity of these agents.

Mice and rats were treated with the glutathione reductase inhibitor 1,3 -bis (2-chloroethyl)-1-nitrosourea (BCNU). The lethality of paraquat was potentiated in both experimental species. BCNU was then employed in studies to develop a novel rat lung slice model with a compromised glutathione reductase activity but normal levels of ATP, NADP(H) and glucose oxidation along with a modest reduction in thiol levels in BCNU pretreated slices.

The effects of paraquat, nitrofurantoin and 2, 3-dimethoxy-1, 4-naphthoquinone (a control compound, a 'pure' redox cycler) were assessed in the BCNU compromised model. From these studies it was concluded that, on the basis of biochemical (GSH and ATP levels) and functional assessments (oligoamine and 5-hydroxytryptamine uptake) of toxicity, the effects of paraquat, nitrofurantoin and the quinone were potentiated as a result of inhibition of glutathione reductase, thereby implicating GSH in the toxicity. In addition, it was concluded that paraquat selectively affected the alveolar type I and II cells, whereas nitrofurantoin and the quinone exerted effects throughout the lung slice. Furthermore, it was considered on the basis of these studies that much of the pulmonary GSH is in cell types distinct from the alveolar epithelium. The studies highlighted the insights which can be gained from use of the

appropriate functional marker and underline the problems faced when investigating biochemical mechanisms of toxicity in a heterogeneous organ such as the lung.

Adenosine uptake into rat lung slices was studied in an attempt to develop a method for assessing endothelial function, as an alternative to 5-hydroxytryptamine accumulation. Studies revealed that α-naphthylthiourea (ANTU), an endothelial damaging agent, reduced adenosine uptake, whereas paraquat failed to produce a loss of adenosine uptake, suggesting that adenosine uptake was specific for the endothelium. Studies of adenosine uptake into BCNU compromised lung slices confirmed previous studies with regard to the selectivity of paraquat and the quinone. Furthermore, incubation of lung slices with adenosine resulted in an elevation of pulmonary ATP levels, which may provide another exciting tool for toxicological studies, in vitro.

In an attempt to raise pulmonary thiols, animals were treated with phorone which resulted in a significant elevation of GSH in lung and liver of rat and mouse after 48 h. Phorone pretreatment did not confer protection against paraquat-induced lethality in mouse in vivo or in rat in vivo or in vitro. Elevation of thiols did, however, confer protection against the endothelial damaging agent ANTU as evidenced by the abolition of mortality, hydrothorax and loss of functional markers. It was concluded that the lack of protection against paraquat may arise from a failure to elevate thiols in the cell types which constitute a target for paraquat.

These studies implicate GSH in the toxicity of these agents which

exert their effects by oxidative stress and suggest that type I and II cells contain relatively small amounts of GSH. Furthermore, the work has established novel methods for investigating mechanisms of pulmonary toxicity.

"All our ancient history is no more than accepted fiction"

-Voltaire.

PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS

AA / aa : amino acids
ACE : angiotensin converting enzyme
ado : adenosine
ADP : adenosine 5'-diphosphate
AMP : adenosine 5'-monophosphate
ANTU : α-naphthylthiourea
ATP : adenosine 5'-triphosphate

BCNU : 1, 3- bis (2-chloroethyl)-1- nitrosourea
BSO : buthionine sulphoximine

CHO cells : Chinese hamster ovary cells

DEM : diethyl maleate
DFO : desferrioxamine
2, 3-diOMe : 2, 3 -dimethoxy-1, 4-naphthoquinone.
DMSO : dimethyl sulphoxide
DQ : diquat
DSPC : disaturated phosphatidylcholine
DTNB : 5, 5'- dithiobis (2-nitrobenzoic acid)

EAT cells : Ehrlich Ascites Tumour cells
EDTA : ethylenediaminetetraacetic acid

GSH : reduced glutathione, or γ-glutamylcysteinylglycine
GSSG : oxidized glutathione, or glutathione disulphide

HEPES : N- [2- hydroxyethyl] piperazine N'- [2- ethane sulphonic acid]
HMPS : hexose monophosphate shunt pathway
5-HT : 5-hydroxytryptamine
HX : hyperoxia

IDP : inosine 5'-diphosphate
IMP : inosine 5'-monophosphate
i. p. : intra-peritoneal
4-ipo : 4-ipomeanol
i. v. : intra-venous
ITP : inosine 5'-triphosphate

KCN : potassium cyanide
KRB : Krebs Ringer Phosphate medium

LPO : lipid peroxidation

MEM : Minimal Essential Medium
3-MI : 3-methyl indole

N-acetyl-cys-S-R : mercapturic acid derivative
NAD : nicotinamide adenine dinucleotide, oxidised form
NADH : nicotinamide adenine dinucleotide, reduced form
NADP : nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH : nicotinamide adenine dinucleotide phosphate, reduced form
NF : nitrofurantoin
NPSH : non-protein sulphydryls
NX : normoxia

PCA : perchloric acid
PQ : paraquat

s. c. : sub-cutaneous
SOD : superoxide dismutase

tBHP : tert- butyl hydroperoxide
TCA : trichloroacetic acid
TCA cycle : tricarboxylic acid cycle

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CHAPTER 1 - GENERAL INTRODUCTION

1. 1. OVERVIEW

The primary function of the lung is gaseous exchange for which it is ideally suited, possessing a large surface area (70 m^2) [1], and the entire cardiac output passes through the lung. In contrast to the erstwhile held view of the lung as a 'bellows', the lung is now known to be involved in a variety of processes and is metabolically active. It is ideally situated to remove unwanted substances from the circulation and also for synthesis and release. The pulmonary endothelium modifies several vasoactive amines either through modification by enzymes on the luminal surface or active accumulation and metabolism [2].

The lung is a prominent target organ for chemical-induced damage. A toxic insult may reach the lung either from the atmosphere via the inspired air, or from the systemic circulation [3]. The lung damage resulting from toxicity may lead to loss of gaseous exchange due to oedema or from fibrosis [4].

The lung possesses an array of enzymes capable of activating a number of inert chemicals to toxic species [5]. In addition, the lung is a heterogeneous organ and several toxicants exhibit a high degree of selectivity for particular cell types. Lung tissue accumulates a variety of compounds, both endogenous substances and xenobiotics [6] and may greatly increase the bioavailability of a toxicant. Cell injury may occur via a variety of biochemical mechanisms such as covalent binding to macromolecules and oxidant stress and is dependent on the balance of activating and deactivating pathways and the availability of substrates and cofactors and the nature of the metabolites formed [7]. The lung possesses a variety of defences such as vitamin E, glutathione and the

enzymes of the antioxidant defence system.

A wide variety of compounds are capable of inducing lung damage including herbicides, antibiotics, anti-tumour agents, cigarette smoke, anti-hypertensives, irritant gases and mineral dusts.

1. 2 CELLULAR ORGANISATION OF THE LUNG

The lung is a heterogeneous organ, comprising over 40 different cell types [8]. Of these cell types, four are unique to the lung : Type I and II cells of the alveolar epithelium, the nonciliated bronchiolar epithelial (Clara) cell and the alveolar macrophage. Fig. 1/ 1 shows a stylised representation of the mammalian alveolus.

Morphologically, lung cells can be divided into two distinct groups :

a) Parenchyma : 86 % of total lung cells. This group includes the alveolar, duct and capillary cells.

b) Non-parenchyma : cells of the conducting airways, blood vessels and connective tissue [8]. In this group are the Clara cells which have been intensively studied [9, 10].

Type I pneumocytes cover approximately 95 % of the alveolar surface of the respiratory bronchiole and account for 9 % of total parenchymal cells in the lung [11]. Type I cells are characterised due to their extended cytoplasm and the paucity of organelles. This attenuated cytoplasm is ideal for performing the major function of this cell type i. e. gaseous exchange. These highly specialised squamous cells form a very thin cytoplasmic layer (0. 2-0. 5 µm). Many pinocytotic vesicles are present. Type I cells are very sensitive to injury by inhaled and blood-borne agents.

Type II cells comprise 16 % of the total lung parenchyma, though covering a mere 5 % of the alveolar surface. In common with the type I

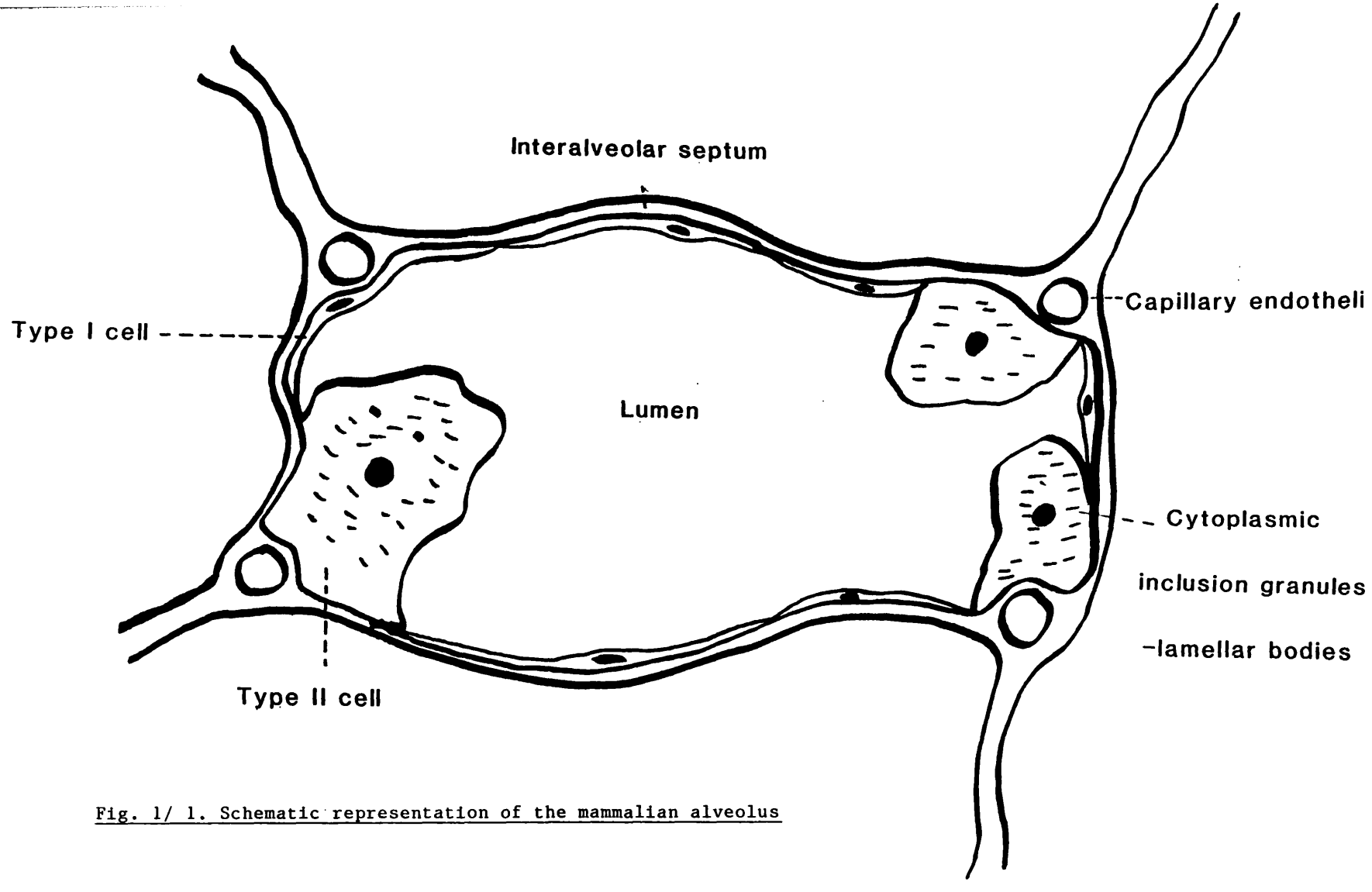


Fig. 1/ 1. Schematic representation of the mammalian alveolus

cells, type II pneumocytes remain on the epithelial side of the basal lamina. Type II cells are cuboidal and project into the lumen of the alveolus often from niches in the alveolar septum [12]. Their lower lateral borders are often covered by cytoplasmic extensions of type I cells to which they are joined by tight cell junctions [11]. Characteristically, lamellar inclusions are present in the apical part of the cell, from where they are extruded into the lumen. These inclusions constitute pulmonary surfactant and contain phospholipids such as phosphatidyl choline and phosphatidyl glycerol [12]. Surfactant permits gaseous exchange and prevents collapse of the air sacs [13]. Furthermore, the type II cell acts as a progenitor of the type I cell and proliferates in response to extensive damage to type I cells [14].

The Clara cells are situated in the bronchiolar epithelium. They possess abundant smooth endoplasmic reticulum and cytoplasmic inclusion granules. The cytoplasmic granules reflect a secretory function for Clara cells and contain a protein or lipoprotein which may contribute to a surface active lining layer in the bronchioles [11]. The abundant smooth endoplasmic reticulum is believed to be the site of NADPH-cytochrome P-450 dependent mixed function oxidase metabolism of xenobiotics reaching the lung via the air or in the blood [15]. Clara cells are, therfore, a target for pneumotoxic, mutagenic or carcinogenic chemicals requiring metabolic activation [16].

Immunohistochemical studies with antibodies for cytochrome P-450 and NADPH-cytochrome P-450 reductase [17, 18, 19] revealed that both type II and Clara cells possess both activities with the highest activity in the Clara cell.

The pulmonary capillary endothelium is complete, nonfenestrated and metabolically active. These cells contain many surface caveolae and

pinocytotic vesicles and it appears that their surfaces are the site of metabolism of adenine nucleotides, vasoactive amines, peptides and prostaglandins, including 5-hydroxytryptamine, bradykinin and angiotensin I [20].

1. 3 PULMONARY INTERMEDIARY METABOLISM AND SURFACTANT SYNTHESIS

The lung is metabolically active [13, 21]. The lung is capable of oxidising glucose, fatty acids, amino acids, lactate and glycerol [22, 23, 24], the rate of glucose oxidation being greatest.

There is more than one transport system for hexoses in the lung and both facilitated diffusion and active transport may occur [25, 26]. It has been suggested that the presence of these multiple uptake systems may reflect contributions from more than one cell type [21].

The major catabolic pathways include glycolysis and the hexose monophosphate shunt (HMPS) producing glycerol 3-phosphate, lactate, carbon dioxide, pyruvate and ribose which can be utilised as precursors of amino acids, nucleic acids and lipids and oxidation via the TCA cycle. Glucose breakdown results in the generation of NADPH (essential for reductive biosynthesis) and NADH which is utilised by the electron transport chain. Approximately 50 % of glucose carbons are metabolised to lactate and pyruvate [27]. Under control conditions, lactate production is ten-fold greater [27], but the lactate : pyruvate ratio depends on the redox state of the cell.

Total oxidation to carbon dioxide represents the second major fate of glucose carbons in the lung and occurs via the HMPS and the mitochondrial TCA cycle. Of total carbon dioxide production, 1/ 4 results from the HMPS, 1/ 4 from the pyruvate dehydrogenase reaction and 1/ 2 from TCA cycle [28].

Glucose carbons are readily incorporated into proteins and

nucleoprotein [29]. Complex lipids are also synthesised from glucose carbons which appear in the fatty acyl moiety (from dihydroxy acetone phosphate/ glycerol 3-phosphate). Small quantities of glucose carbons are also incorporated into the side chains of phospholipids. The rate of gluconeogenesis in lung tissue is presumed to be very low since the activity of phosphoenolpyruvate carboxykinase, a rate limiting enzyme, is very low [30].

Flux through the glycolytic pathway is controlled by short-term regulators (in addition to the long-term hormonal influences). In other tissues, glycolytic flux increases in response to ATP depletion and decreases as NAD^+ is depleted. In lung ventilated with carbon monoxide,ATP was significantly decreased. Glycolysis was stimulated. Carbon monoxide stimulated glycolysis even with an elevated NADH/ NAD^+ ratio. The effect of redox status was further investigated using phenazine methosulphate to oxidise reduced nucleotides, whilst the energy status remained unchanged, glycolysis was stimulated. These findings suggest that pulmonary glycolytic activity can be affected independently by redox state and ATP levels [31, 32].

The capacity of the lung to esterify free fatty acids and to incorporate acetate into lipids is relatively great [13]. Few cell types in the lung show clear morphological evidence of extensive fatty acid metabolism. The alveolar type II cells are the exception and secretion of pulmonary surfactant has been attributed to this cell type [33]. It has been shown by autoradiography that the type II cells are the only cell type to be highly labelled following the injection of tritiated palmitate into mice [34]. Macklin [35] proposed that the alveolar epithelium was covered by a mucoid film which is vital to normal lung function. The major function is to decrease the surface tension at the

air-liquid interface of the lung. This results in decreased work required to inflate the lung, increased alveolar stability, and the prevention of oedema [13]. Surfactant also has bactericidal properties. This lining material was found to be a lipoprotein complex [36] which contains nonserum proteins, phospholipids and neutral lipids. Approximately 2/ 3 of the lipid in surfactant is phosphatidyl choline and 2/ 3 of this being saturated i. e. disaturated phosphatidyl choline (DSPC) [36].

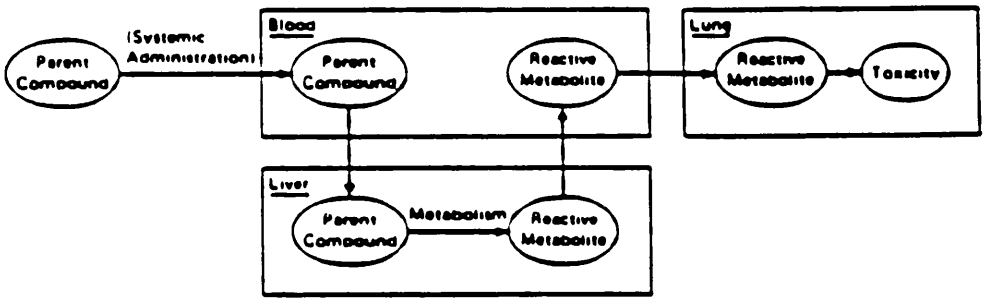
Askin and Kuhn [37] injected tritiated palmitate into rats. Lung autoradiography indicated fatty acid incorporation into DSPC in the lamellar bodies of type II cells. Long chain fatty acids can be obtained from uptake of circulating fatty acids, synthesis within the lung from precorsors and also the recycling of exogenous fatty acids. The type II cell is capable of a high de novo synthetic rate and lactate is the preferred substrate [38].

1. 4 THE LUNG AS A TARGET ORGAN

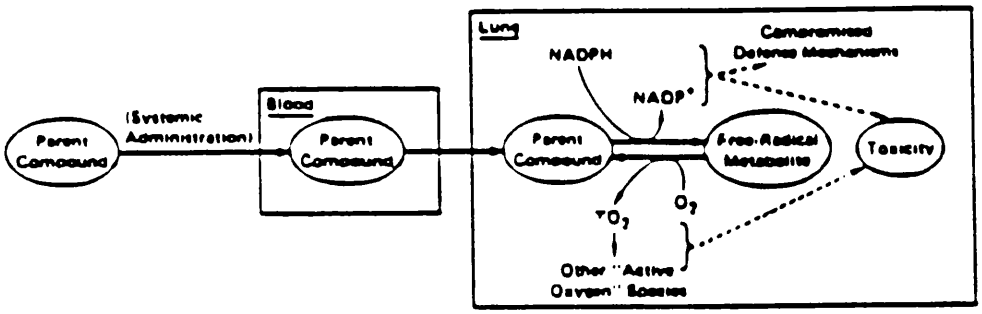
The lung is a target for a large number of toxicants [39, 40]. Several distinct types of lung injury can be seen and Boyd has elegantly reviewed mechanisms of chemical-induced pulmonary toxicity [41] (fig. 1/ 2).

In the first type, the mechanism of toxicity is proposed to involve transport of the parent compound to the lung in the circulation. Upon arrival in the lungs, the parent molecule undergoes in situ metabolic activation via cytochrome P-450 dependent mixed-function oxidase reactions to yield a highly reactive species such as an epoxide which is capable of covalent binding to tissue macromolecules. Such covalent binding leads to cell death and tissue injury. Examples of compounds which exert their effects by such a mechanism include 4-ipomeanol [42] and 3-methylindole [43]. Since the Clara cells contain a high titre of cytochrome P-450 mixed

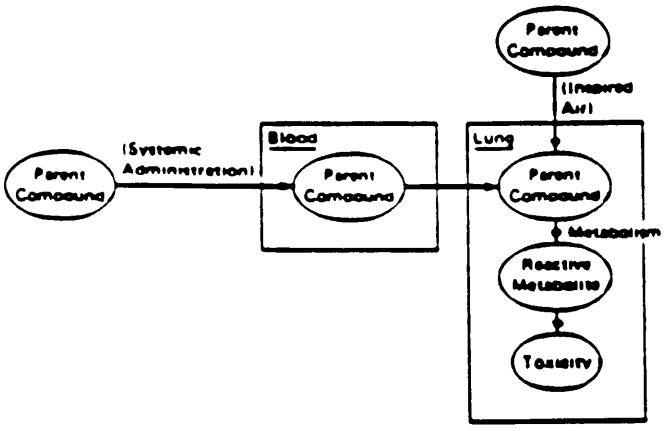
Fig. 1/ 2. Biochemical mechanisms of pulmonary toxicity involving metabolic activation of chemicals
From Boyd [41].



Pulmonary toxicity model involving reactive metabolites formed primarily in liver.



Pulmonary toxicity model involving "oxygen activation."



Pulmonary toxicity model involving *in situ* metabolism to reactive metabolite.

function oxidase activity [15], these compounds are relatively selective for this cell type,especially at low doses [16].

The second type of lung injury results from oxidative stress. Upon accumulation of agents into a susceptible cell type in the lung, these compounds undergo a one-electron reduction to yield radical species. This reduction is catalysed by NADPH-cytochrome P-450 reductase. The radical species formed is unstable and is reoxidised in the presence of molecular oxygen reforming the parent species with the concomitant production of activated oxygen species leading to cell death via a variety of routes such as lipid peroxidation and/ or depletion of vital cellular cofactors [44, 45]. Paraquat and nitrofurantoin are examples of two such compounds.

The third mechanism of metabolic activation resulting in pulmonary toxicity is exemplified by the pyrollizidine alkaloid, monocrotaline. This compound primarily damages the endothelium. By virtue of its location at a site of maximum exposure to reactive metabolites reaching the lung via the circulation the pulmonary endothelium would be expected to be a primary site for toxic lung damage from preformed reactive intermediates reaching the lungs. The production of endothelial toxicity by monocrotaline is consistent with this view since Mattocks [46] has shown that metabolism occurs via the hepatic cytochrome P-450 mixed function oxidase system.

1. 5 ANTIOXIDANT DEFENCES IN THE LUNG

Aerobic life presents problems since oxygen can be activated to reactive species including free radicals which can damage biomolecules, and can result in a number of physiological conditions including aging, carcinogenesis, inflammation, immune disorders and drug-induced toxicity [47]. By definition a free radical possesses an odd number of electrons and can be positive, negative or neutral [47]. The electronic structure

of molecular oxygen predisposes it to a univalent pathway of reduction. Molecular oxygen possesses two unpaired electrons with parallel spins. A spin reversal of one of these two electrons is required before a two electron reduction can occur, which is not favoured kinetically. One electron reductions are not restricted [48].

The intermediates encountered during successive univalent reductions of molecular oxygen are the superoxide anion ($O_2^{\cdot -}$),hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$).

$$O_2 \longrightarrow O_2^{\cdot -} \longrightarrow H_2O_2 \longrightarrow HO^{\cdot} \qquad [49]$$

There are several sources of superoxide anion [50]. Firstly, auto-oxidation reactions involving flavins, quinones, paraquat, aromatic nitro compounds, thiols, tetrahydropteridines and iron chelates. Secondly, enzymatic reactions such as those catalysed by cytochrome P-450, indoleamine and tryptophan dioxygenases, NADH-b_5 reductase, cytochrome P-450 reductase and xanthine oxidase. Thirdly, cellular sources including the electron transport chain, mitochondria, microsomes, bacteria, leucocytes and macrophages. Finally, environmental factors such as U. V. and γ-radiation.

Superoxide anion can dismutate spontaneously

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

Hydrogen peroxide can also be formed through non-radical two electron reduction of molecular oxygen via oxidases e. g. monoamine oxidases [50].

Superoxide anion can be detected using radical scavengers e. g. cytochrome c [51] or by spin traps (detection via electron spin resonance

spectroscopy) [52]. Though superoxide anion can react with biomolecules [53] it possesses only mild oxidative abilities as does hydrogen peroxide and the toxicity of the two species is believed to result from formation of hydroxyl radicals [51]. The hydroxyl radical species is a strong oxidant and is extremely reactive and is capable of interactions with lipids, polysaccharides, proteins and nucleic acids.

Haber and Weiss [54] proposed that $OH^{\cdot}$ is formed by reaction of superoxide anion with hydrogen peroxide. The reaction is slow [55] and is thought to be catalysed by iron salts and complexes.

$$O_2^{\cdot -} + H_2O_2 \longrightarrow HO^{\cdot} + O_2 + OH^- \quad [53]$$

$$O_2^{\cdot -} + Fe^{+++} \longrightarrow O_2 + Fe^{++} \quad [56]$$

$$Fe^{++} + H_2O_2 + H^+ \longrightarrow Fe^{+++} + H_2O + HO^{\cdot}$$

(Fenton reaction) [57].

Pulmonary defences are multifaceted [58]. The first defence is avoidance of the univalent pathway of reduction effected by enzymes capable of the tetravalent reduction of molecular oxygen to water without release of active intermediates e. g. cytochrome oxidase [57]. The second layer of defence is provided by metalloenzymes called superoxide dismutases (SOD), which catalyse the reaction :

$$2O_2^{\cdot -} \longrightarrow 2H_2O + O_2$$

[58, 59].

The third layer of defences involves the enzyme catalase which dismutates hydrogen peroxide into water and oxygen by catalysing the reaction :

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

Hydrogen peroxide can also be eliminated by peroxidases that catalyse the reduction of hydrogen peroxide from several electron donors. The most significant of these is the seleno-enzyme glutathione peroxidase [60, 61], which catalyses the reaction :

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

Reduction of hydrogen peroxide occurs at the expense of reduced glutathione (GSH) and results in the formation of oxidised glutathione (GSSG). Glutathione reductase maintains thiol status by reducing GSSG, using NADPH as a cofactor [62]. Glutathione peroxidase is specific with regard to GSH as a cofactor. The degree of hydroperoxide specificity is far less stringent. The enzyme can catalyse the reduction of fatty acid hydroperoxides. Catalase is located in peroxisomes whilst glutathione peroxidase is situated throughout the cytosol. In addition, the Km of glutathione peroxidase for hydrogen peroxide is lower than that exhibited by catalase [60]. This may suggest that catalase is less important in the removal of hydrogen peroxide. If the rate of intracellular hydrogen peroxide generation is increased, however, then catalase may become more important.

A number of GSH-S-transferase isozymes show a peroxidase activity which is selenium-independent and their substrate specificity is restricted to organic hydroperoxides [61].

A further component of the antioxidant defence system of mammalian cells is vitamin E, which can act to break the chain reactions of lipid peroxidation which result in membrane damage [63].

Exposure to the hyperoxic conditions used to treat respiratory insufficiency also increase the rate of formation of superoxide anion and hydroxyl radical in the lung [64, 65]. In addition, infiltration of inflammatory cells may increase the rate of pulmonary oxygen radical formation.

1. 6 LIPID PEROXIDATION

Lipid Peroxidation (LPO) has been observed as a consequence of administration of a variety of toxic agents in vitro and in vivo [66].

The initiation step in LPO [67, 68] involves the abstraction of a hydrogen atom from a polyunsaturated fatty acid via the action of hydroxyl radical

$$LH + HO^{\bullet} \longrightarrow L^{\bullet} + H_2O$$

Molecular oxygen reacts with the lipid radical forming a lipid peroxy radical

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$$

Following initiation, these lipid peroxy radicals result in a chain reaction in which there is interaction between the peroxy radical and another molecule of unsaturated lipid along with a lipid monohydroperoxide

$$LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH$$

The hydroperoxide undergoes breakdown in the presence of an iron

catalyst :

$$LOOH + Fe^{++} \longrightarrow LO^{\cdot} + Fe^{+++} + OH^{-}$$

(alkoxy radical)

$$LOOH + Fe^{+++} \longrightarrow LOO^{\cdot} + Fe^{++} + H^{+}$$

[68, 69].

Termination of the chain reactions occur with interaction between $L^{\cdot}$ and $LOO^{\cdot}$ and a second radical [51].

$$LOO^{\cdot} + LOO^{\cdot} + H^{+} \longrightarrow LOH + {}^{1}O_{2} + LO^{\cdot}$$

In the absence of termination, membrane disintegration occurs [47].

1. 7 GLUTATHIONE

Glutathione is a ubiquitous tripeptide (γ-Glu-CysH-Gly) widely distributed in animal tissues, plants and microorganisms.

Glutathione exists in two forms. The predominant form is reduced glutathione, or GSH and is present in mammalian cells at concentrations ranging from 0. 5 - 10 mM. GSH is the most prevalent cellular thiol and low molecular weight peptide. The two characteristic structural features are the γ-glutamyl linkage and the sulphydryl group which promote intracellular stability and are intimately associated with its functions. The structure of GSH is shown in fig. 1/ 3.

The oxidised form, GSSG (glutathione disulphide), is present in micromolar concentrations. In rabbit lung the GSH/ GSSG ratio is in the region of 240/1[70]. This value is similar to that in liver [71].

Protein glutathione disulphides (prot-SSG) are also present in micromolar concentrations and can be formed as follows :

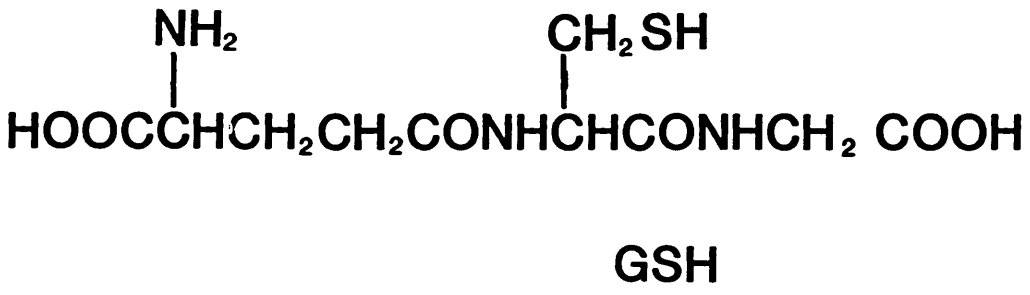


Fig. 1/ 3. Structure of Glutathione (GSH).

prot-SH + GSSG $\rightleftharpoons$ prot-SSG + GSH.

The amounts of mixed disulphides present depend on age, tissue, nutritional state and diurnal variations [72, 73].

Actual values of these components, as reported in the literature, vary since they depend on the methodology employed in their analysis. In rat lung, for example, values of GSH / GSSG as low as 6:1 have been reported [74].

1. 8 THE γ-GLUTAMYL CYCLE

GSH is synthesised by the sequential actions of γ-glutamylcysteine synthetase and GSH synthetase (fig. 1/ 4). γ-Glutamylcysteine synthetase is feedback inhibited by GSH [75]. GSH is degraded by γ-glutamyl transpeptidase, which effects the transfer of the γ-glutamyl group of GSH to acceptors (amino acids, dipeptides, GSH, H_2O). Cysteinylglycine formed via transpeptidation, is split by peptidases forming glycine and cysteine. The γ-glutamyl amino acids formed in transpeptidation are substrates for γ-glutamyl cyclotransferase which converts them to 5-oxoproline and the corresponding amino acids. 5-Oxoprolinase catalyses the conversion of 5-oxoproline to glutamate [76].

1. 9 METHODS FOR MODULATING THIOL STATUS

Since GSH has been implicated in cellular protective functions against a variety of toxic agents, the modulation of intracellular glutathione status is an area of great interest. Chemically induced depletion of GSH can be accomplished using several strategies, reviewed by Plummer et al. [77]. Fig. 1/ 5 illustrates the factors affecting cellular or tissue GSH levels.

GSH levels can be lowered by conjugate formation (mediated by GSH-

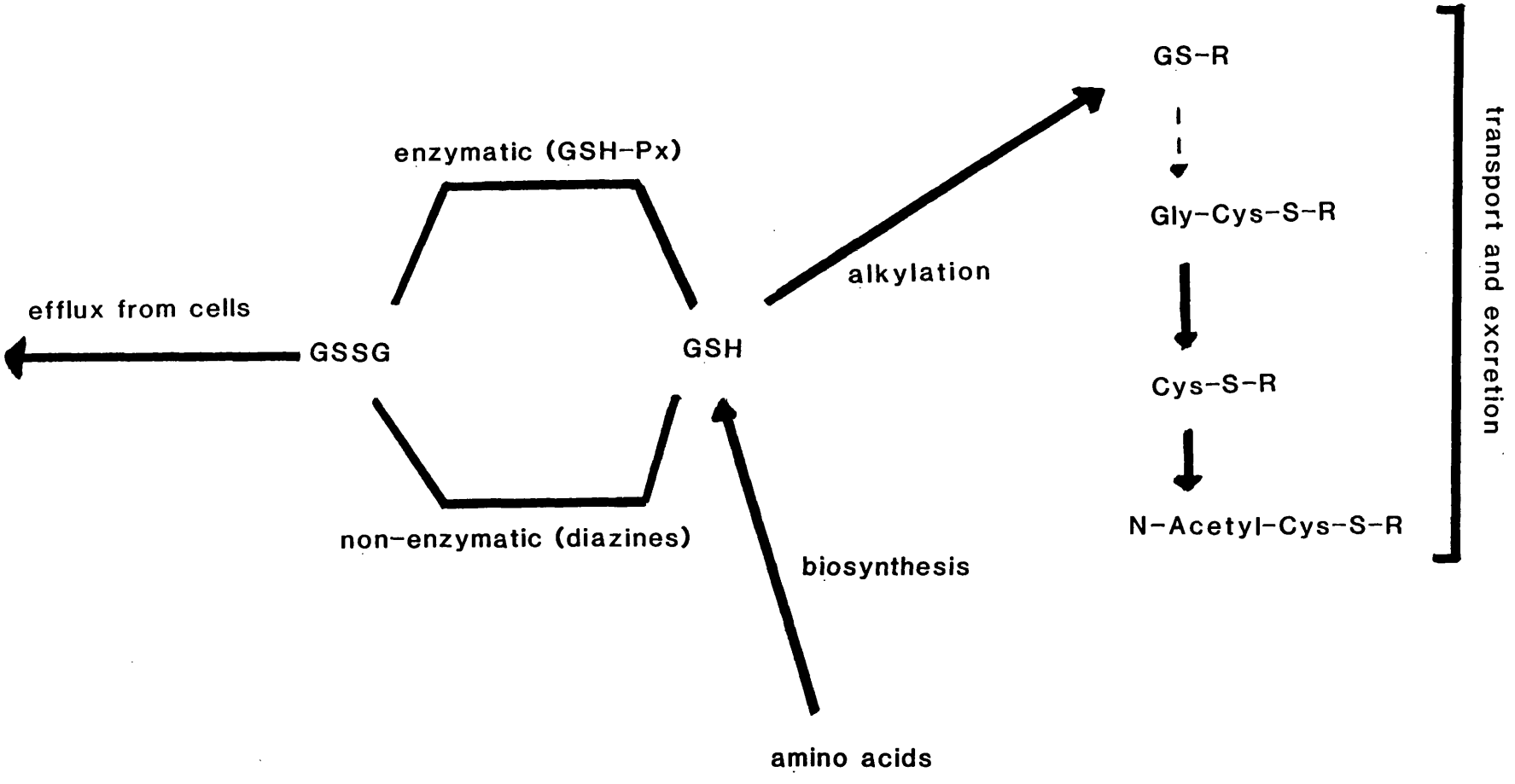


Fig. 1/ 5. Factors affecting cellular GSH levels
(Adapted from Plummer et al. [77].

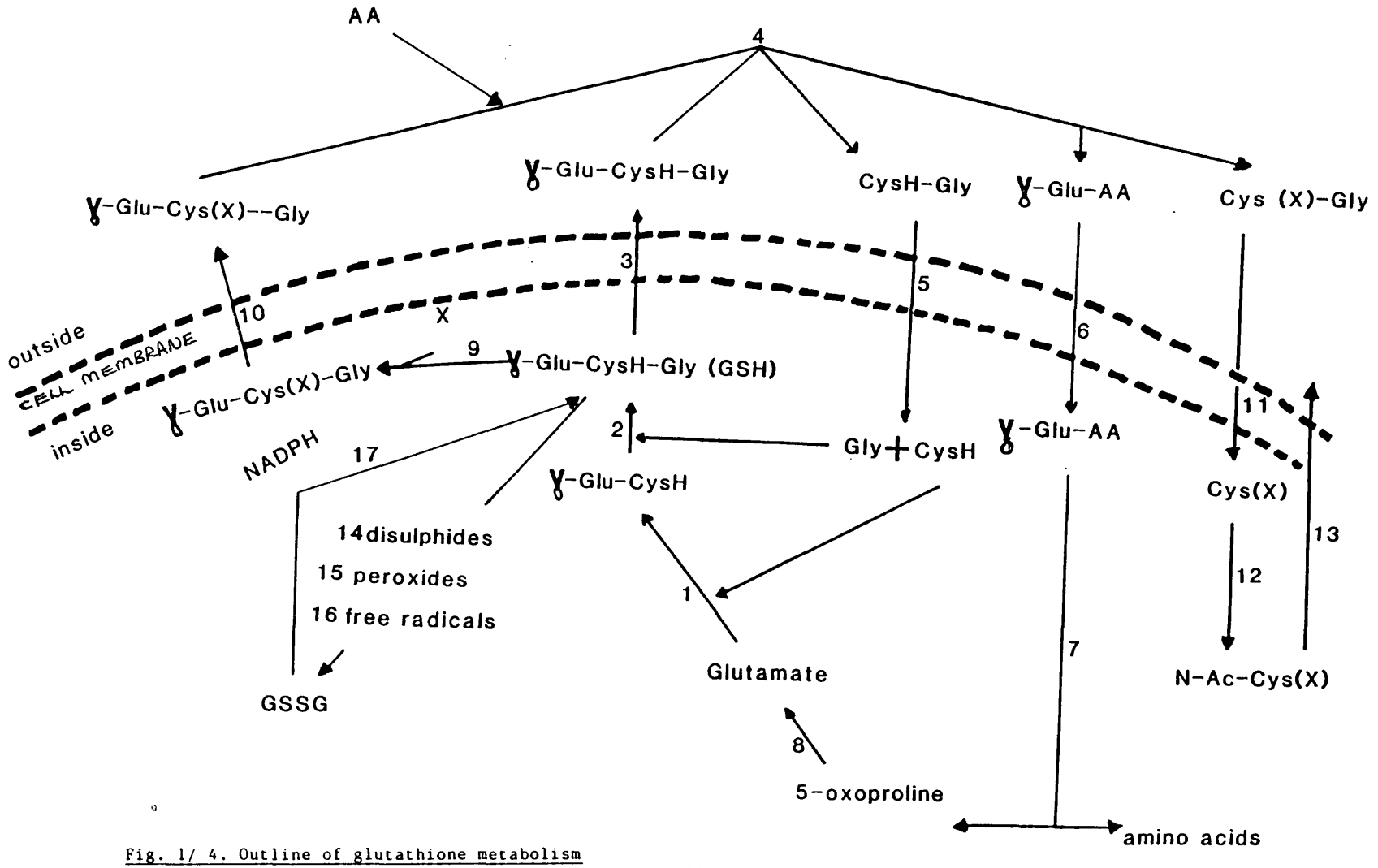


Fig. 1/ 4. Outline of glutathione metabolism

(1) γ-Glutamyl cysteine synthetase (2) GSH Synthetase (3) Conversion by membrane bound γ-glutamyl transferase (4) Cysteinylglycine and γ-glutamyl amino acids (5) Membrane bound dipeptidase (6) γ-Glutamylcyclo-transferase (7) Amino acids and 5-oxoproline (8) 5-Oxoprolinase (9) GSH-S-transferase (10) Transport (11) Transport (12) Acetylase (forms mercapturic acids) (13) Traansport (14) Transhydrogenase (15) GSH-peroxidase (16) Free radicals (17) Glutathione reductase. X : electrophiles , AA : amino acids

S-transferase activity) or by inhibition of biosynthesis. Agents which oxidise GSH to GSSG have also been employed.

a. Substrates for GSH-S-Transferase. Diethyl maleate (DEM) and phorone are the two best characterised of this group of agents. They are α,β-unsaturated carbonyl compounds, weak electrophiles that react with GSH in the presence of transferase enzyme [78].

DEM is the most commonly used agent in this class of compound and depletes GSH in rat liver and in other organs such as the lung and heart [79]. In addition to its use in vivo, DEM has been used as a glutathione depletor in microsomal preparations and also in isolated hepatocytes. Phorone decreases hepatic GSH in rat [80] and in mouse [81].

The use of DEM and phorone is, however, associated with unwanted side effects unrelated to their GSH-depleting activities. With DEM these effects include inhibition of aryl hydrocarbon hydroxylase activity [82] and decreased p-nitroanisole demethylase activity along with an increase in NADPH-cytochrome c reductase and cytochrome P-450 content in rat liver microsomes [83]. Phorone and DEM have been reported to induce ornithine decarboxylase activity in rat liver [84, 85]. Phorone has been reported to induce heme oxygenase activity in rat liver [86].

b. Thiol Oxidants. Oxidants which convert GSH to GSSG have been used mainly in isolated cell preparations. Srivastava et al. [87] studied glutathione metabolism using the diazenecarboxylic acid derivative, diamide, an agent developed by the Kosowers [88]. This compound is potent and GSH is greatly reduced in a variety of systems such as adipocytes, EAT cells, CHO cells and leukaemic cell lines. Diamide has also been used to diminish GSH levels in rat lung slices [89].

The use of diamide has been associated with several undesirable

effects including membrane damage [90], inhibited protein kinase, sodium-potassium ATPase and glucose 6-phosphatase, along with inhibition of protein synthesis.

Enzymatic oxidation of GSH is effected by treatment with organic hydroperoxides such as tert-butyl hydroperoxide (TBHP) which has been used in a variety of studies to modulate GSH levels in to facilitate studies of intermediary metabolism and effects on pyridine nucleotides [91] in perfused liver. TBHP has also been employed to produce thiol oxidation and oxidant stress in hepatocytes [92, 60].

Another approach for the modulation of glutathione levels involves the use of specific inhibitors of enzymes involved in glutathione biosynthesis. Buthionine sulphoximine (BSO) irreversibly inhibits γ-glutamylcysteine synthetase [93] and has been used widely. The administration of BSO to animals results in a rapid decrease in the GSH levels in the kidney, plasma and liver. In order to decrease pulmonary GSH with BSO, sustained administration is required (15 days in drinking water for mice) [94]. BSO pretreatment has increased the susceptibility to toxic agents in a variety of systems. Cell lines show increased sensitivity to radiation [95], chemotherapeutic agents [96], and oxygen radical mediated cytolysis [97].

BSO is most effective in an organ which possesses a rapid turnover rate of glutathione such as the kidney or liver. Since depletion results from inhibition of de novo GSH synthesis, organs such as the brain and the lung are affected less markedly by BSO [94].

The upper level of GSH is regulated by feedback inhibition, but cellular levels also depend upon precursor availability, especially of cysteine [98]. L-cysteine, even at moderate doses, is toxic. Compounds have been employed which act as intracellular cysteine delivery systems

and which, after being readily transported, are effectively converted to cysteine. One of these compounds is a substrate for 5-oxoprolinase, L-2-oxothiazolidine-4-carboxylate [99]. This compound increases the rate of resynthesis of GSH in mouse liver following starvation [99] and protects against paracetamol [100]. However, GSH levels in other organs such as the brain are only slightly increased.

These systems are limited by the feedback inhibition of γ-glutamyl-cysteine synthetase, though this can be overcome by the provision of substrates for the GSH synthetase. Kidney which has the greatest glutathione turnover rate and possesses a transport system for γ-glutamyl amino acids, transports γ-Glu-CysH and γ-Glu-$(Cys)_2$ when they are given intact and are used by GSH synthetase to increase GSH levels.

Monoesters of GSH in which the glycine carboxyl group is esterified are effectively transported and are hydrolysed [101] are useful for GSH delivery.

GSH exists in both mitochondrial and cytosolic pools. The mitochondrial pool in liver does not arise from intramitochondrial synthesis, but rather from the cytoplasm, by a process characterised by a slow net transport, and a more rapid exchange transport [102].

Glutathione turnover was studied in the perfused rabbit lung along with the effect of exogenous glutathione. The kidney is the primary organ which utilises circulating plasma GSH by virtue of a high γ-glutamyl transpeptidase activity. Dawson et al. found that isolated perfused rat lung utilised external GSH for conjugation [103]. In rabbit lung, however, GSH was not effectively utilised or metabolised [104].

1. 10 THE GLUTATHIONE REDOX CYCLE : INTERACTION WITH THE HEXOSE MONOPHOSPHATE SHUNT AND MAINTENANCE OF NADPH LEVELS

The detoxification of lipid hydroperoxides and hydrogen peroxide via glutathione peroxidase activity results in the formation of GSSG which is either extruded from the cell or is reduced by glutathione reductase activity to maintain GSH levels. Clearly, the presence of hydroperoxides in a cell would lead to rapid and extensive GSH depletion in the absence of the reductase enzyme. Glutathione peroxidase and reductase are located in both the cytosol and the mitochondrial matrix [60].

Glutathione reductase activity results in the consumption of NADPH. Studies of NADPH utilisation by a variety of cellular systems suggested that the glutathione reductase enzyme is potentially the largest single site of NADPH oxidation. It has been suggested [105] that under conditions of oxidative stress, the ability to maintain thiol status is dependent on the level of NADPH.

NADPH is derived from several sources (table 1. I). Studies of drug oxidation in liver revealed an increase in 2-oxoglutarate along with decreases in citrate and isocitrate [106]. In liver, requirements for NADPH are, in part, met from extramitochondrial isocitrate dehydrogenase. Inhibition of HMPS activity with 6-aminonicotinamide (an inhibitor of 6-phosphogluconate dehydrogenase) did not alter hepatic mixed function oxidase activity [107].

Table 1. I. Rates of generation and utilisation of NADPH in rat liver (from Reed [105]).

GENERATION	RATE (μmol/ min/ g)	UTILISATION	RATE (μmol/ min/ g)
Isocitrate DH	22. 4	Fatty acid synthesis	1-2
G-6-P DH	1. 42	Mixed-function oxidation	1-2
6-PG DH	2. 84	Glutathione reductase	8-10
Malic enzyme	1.27		

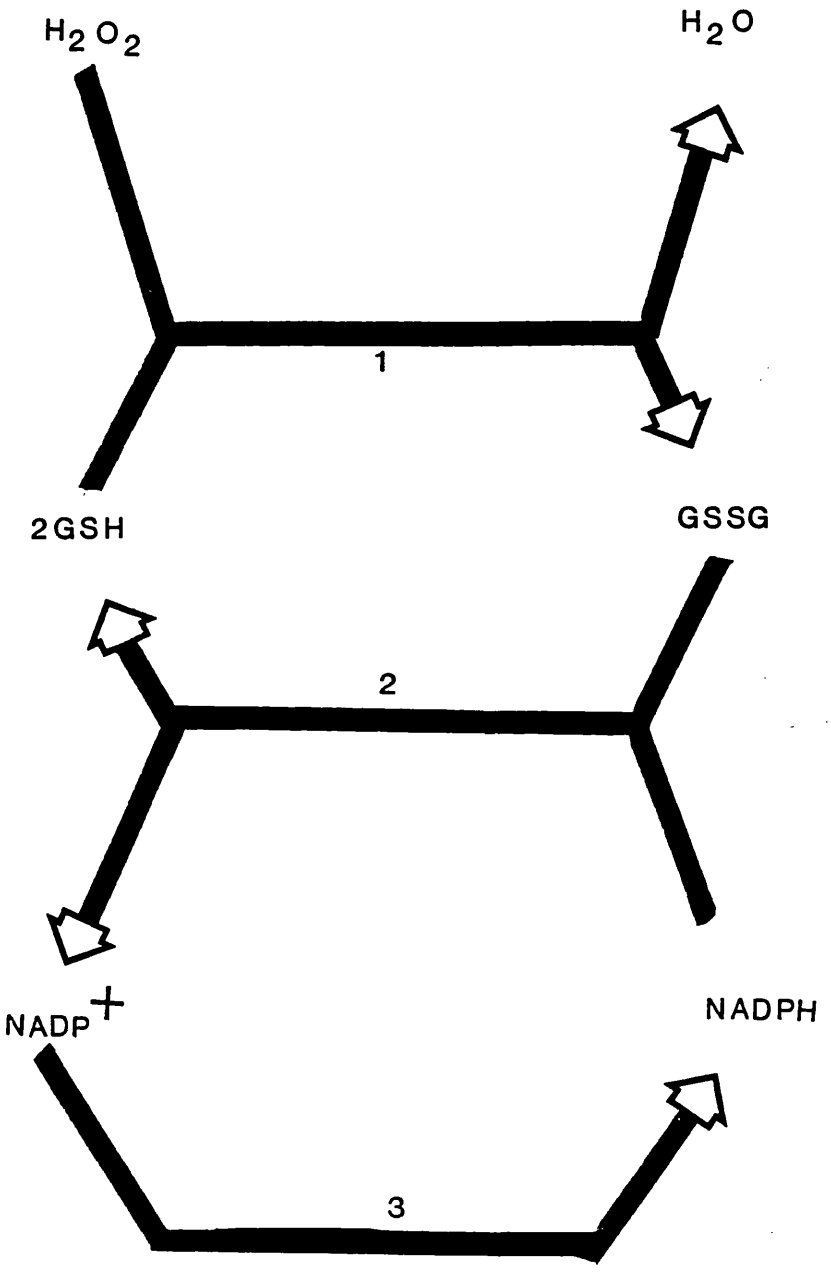


Fig. 1/ 6. Relationship between the glutathione redox cycle and the hexose monophosphate shunt

(1) Glutathione peroxidase (2) Glutathione reductase (3) Glucose-6-phosphate dehydrogenase.

(G-6-P DH: Glucose-6-phosphate dehydrogenase; 6-PG DH: 6-phosphogluconate dehydrogenase). All incubations were at 25°.

In the lung, the activity of 'malic' enzyme is very low [108] and hence, maintenance of NADPH levels is more reliant on the activity of the HMPS.

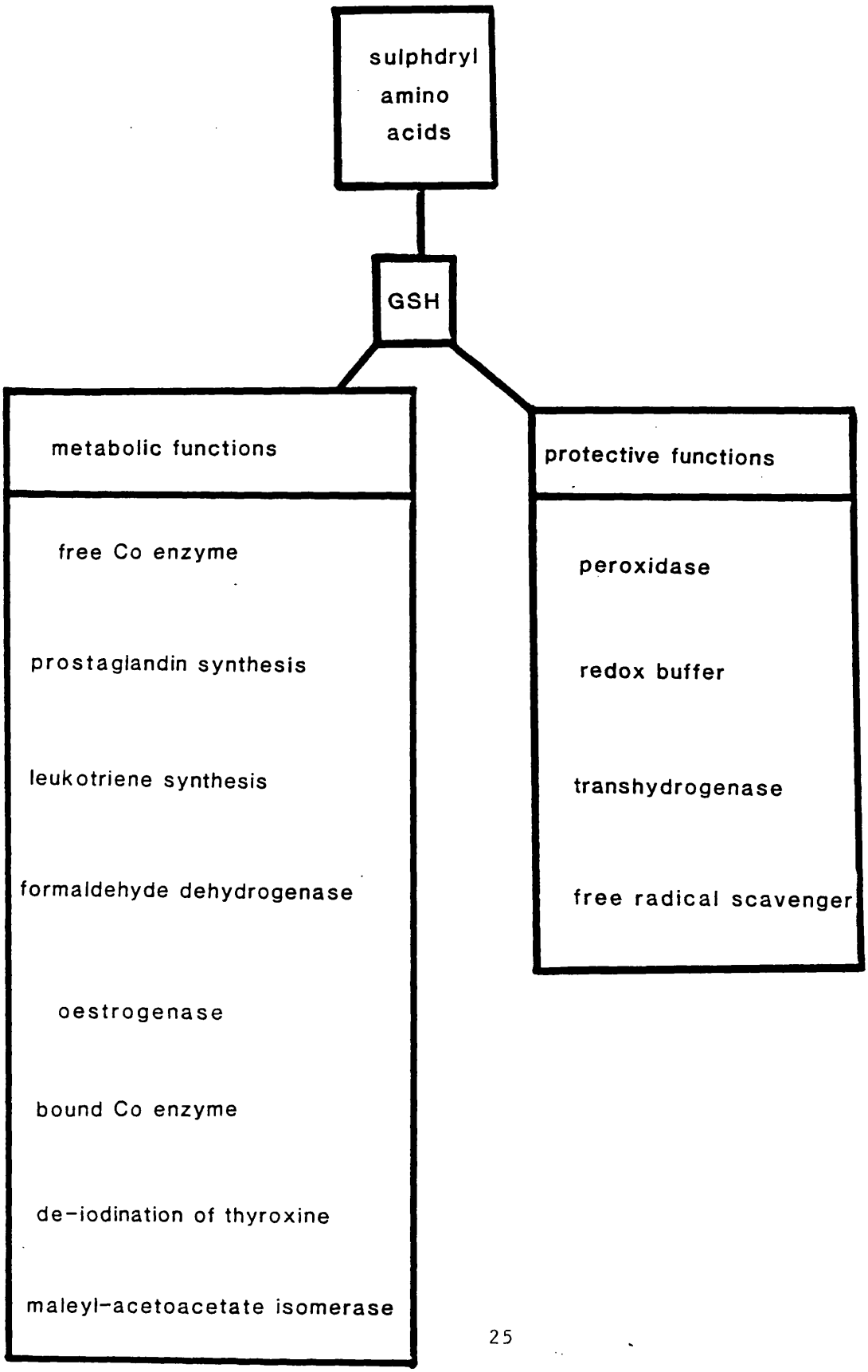
Glucose 6-phosphate dehydrogenase catalyses the first step in the pathway (fig. 1/ 6). This step is the major site of control. The enzyme is inhibited by NADPH and inhibition in vitro is virtually complete at NADPH / $NADP^+$ ratios greater than 9 [109]. As this ratio is considered to be around 100 in tissues under control conditions [110], other factors have been implicated in the regulation. Eggleston and Krebs [109] proposed that GSSG could reverse the inhibition by physiological NADPH/ $NADP^+$ ratios. It has been proposed that this effect is mediated by a thiol transferase activity [71]. However, the area is one of controversy [111] since GSSG is reduced by glutathione reductase activity, which intrinsically lowers the NADPH/ $NADP^+$ ratio.

The link between glutathione oxidation and activity of the HMPS was investigated by Brigelius [112]. Perfusion of livers with t-butyl hydroperoxide decreased GSH levels and increased GSSG with an increase in glutathione mixed disulphides, in both fed and fasted rats. In fasted rats HMPS activation occurred upon t-BHP infusion. Flux through the pathway was unchanged in fed animals [112]. In rat leukocytes hydrogen peroxide metabolism directly stimulated glucose oxidation via the HMPS [113].

1. 11 ROLE OF GLUTATHIONE IN PULMONARY METABOLISM AND TOXICOLOGY

GSH serves as a cofactor in a variety of metabolic reactions [114]. In the lung, its role as a cofactor and substrate in the metabolism of leukotrienes and prostaglandins is of particular importance since these mediators are linked to pulmonary hypertension and oedema. Fig. 1/ 7 shows the involvement of GSH as a cofactor and its protective functions.

Fig. 1/ 7. Protective and metabolic functions of glutathione
Taken from Patterson and Rhoades [114].



Normal alveolar epithelial lining fluid was found to have a high level of GSH (0.43 mM which is approximately 70 times higher than the GSH concentration in plasma) [115] and it has been proposed that the cells of the alveolar epithelium secrete GSH into this lining fluid.

GSH responds to toxic changes induced by oxygen therapy, ozone, nitrogen dioxide or paraquat in two ways. Firstly, it returns cellular proteins proteins to their normal redox state (maintaining biological activity) and secondly, detoxifies lipid hydroperoxides.

Hyperoxia results in changes in GSH status, and glutathione related enzymes. Kimball et al. [116] showed that exposure of rats to 90 % oxygen resulted in increased activities of superoxide dismutase, glucose-6-phosphate dehydrogenase, glutathione reductase and glutathione peroxidase. The levels of GSH and nonprotein sulphydryl compounds were also elevated. Pre-exposure to 90 % oxygen also protected significantly when these animals were then exposed to 100 % oxygen.

The role of GSH in the pulmonary toxicity of oxygen was investigated by Kehrer and Paradathaithu [117]. Pretreatment of rats with the glutathione reductase inhibitor, BCNU enhanced hyperoxia induced pulmonary injury in mice.

The therapeutic effects of N-acetyl cysteine and cysteamine were investigated with respect to oxygen induced mortality [118]. Cysteamine and N-acetyl cysteine significantly reduced mortality by 30 % and 65 % respectively. Oxygen, per se, caused a 50 % decrease in nonprotein sulphydryl levels and loss of thiols was ameliorated by continuous infusion of cysteamine. In contrast, N-acetyl cysteine administration actually potentiated paraquat induced mortality [118], possibly due to N-acetyl cysteine being capable of donating electrons directly to paraquat. Rats treated with DEM showed accelerated mortality when exposed to 100 %

oxygen [114].

GSH was shown to protect isolated rat lung cells from the toxic effects of 1-chloro-2, 4-dinitrobenzene by enhancing intracellular GSH related detoxification [103].

Butylated hydroxytoluene (BHT), a dietary antioxidant, selectively damages the type I cells of mouse lung [119]. Dietary cysteine (given 3 days prior to BHT) conferred a small but significant protection against pulmonary enlargement and reduced covalent binding of [^{14}C]-BHT in lung and liver [120].

3-Methylindole (3MI) is present in cigarette smoke and is produced in the rumen of cattle. 3-MI produces Acute Bovine Pulmonary Emphysema ('fog fever') in ruminants such as cows, sheep and goats. The reactive metabolite of 3MI was investigated using microsomal preparations prepared from the lungs of cattle. Nucleophilic thiol agents, GSH, L-cysteine and N-acetyl cysteine, protected microsomal protein against alkylation. The cytosolic fraction from cattle lung increased this protection (suggesting mediation via the GSH-S-transferase). Pretreatment of sheep with DEM increased the severity of 3MI induced lung injury, whereas pretreatment with L-cysteine reduced the severity of the lesion [121].

4-Ipomeanol (present in mouldy sweet potatoes) produces marked pulmonary injury in experimental species. The primary cell type affected (as with 3MI) is the Clara cell of the bronchiolar epithelium. Toxicity is associated with covalent binding to tissue macromolecules following *in situ* metabolic activation. Pretreatment of rats with DEM increased the toxicity of 4-ipomeanol and the extent of covalent binding [42].

Previous work assessed GSH and GSSG levels in the plasma of rats given diquat, nitrofurantoin and adriamycin. None of these agents significantly influenced plasma concentrations of GSH. Diquat and

nitrofurantoin increased plasma GSSG by 4 to 13-fold [122].

Various antitumour agents are toxic to the lung. 1, 3-Bis (2-shloroethyl)-1-nitrosourea (BCNU) is a good example and is discussed more fully in Chapter 3. Another such compound is the soluble glycopeptide complex, bleomycin [123]. Pulmonary fibrosis develops in DBA strain mice within several days following a single intratracheal instillation of bleomycin [123]. Following chronic administration of the compound, pulmonary protective enzymes are altered in mice [124]. Increases were observed in catalase, glutathione reductase and glutathione peroxidase. Total lung glutathione was unchanged [124]. Bleomycin interacts with oxygen and iron (II) to form an 'activated' ternary complex, which is thought to produce both tumour destruction and fibrosis. Oxygen species are generated which result in lipid peroxidation and DNA damage [125].

1. 12. a PARAQUAT

Paraquat (1, 1'-dimethyl-4, 4'-bipyridylium) is an effective broad spectrum herbicide. The compound is associated with numerous human fatalities when ingested accidentally or with suicidal intent. The organs damaged are primarily the lung and kidney and lung damage has been observed in a variety of experimental species e. g. rat, mouse and monkey [126].

1. 12. b HERBICIDAL PROPERTIES

Following spraying onto plants, paraquat is readily taken into leaf cells and competes for electrons with photosystem I to be reduced to the free radical. In the presence of molecular oxygen this results in the production of superoxide anion and hydrogen peroxide which leads to cell

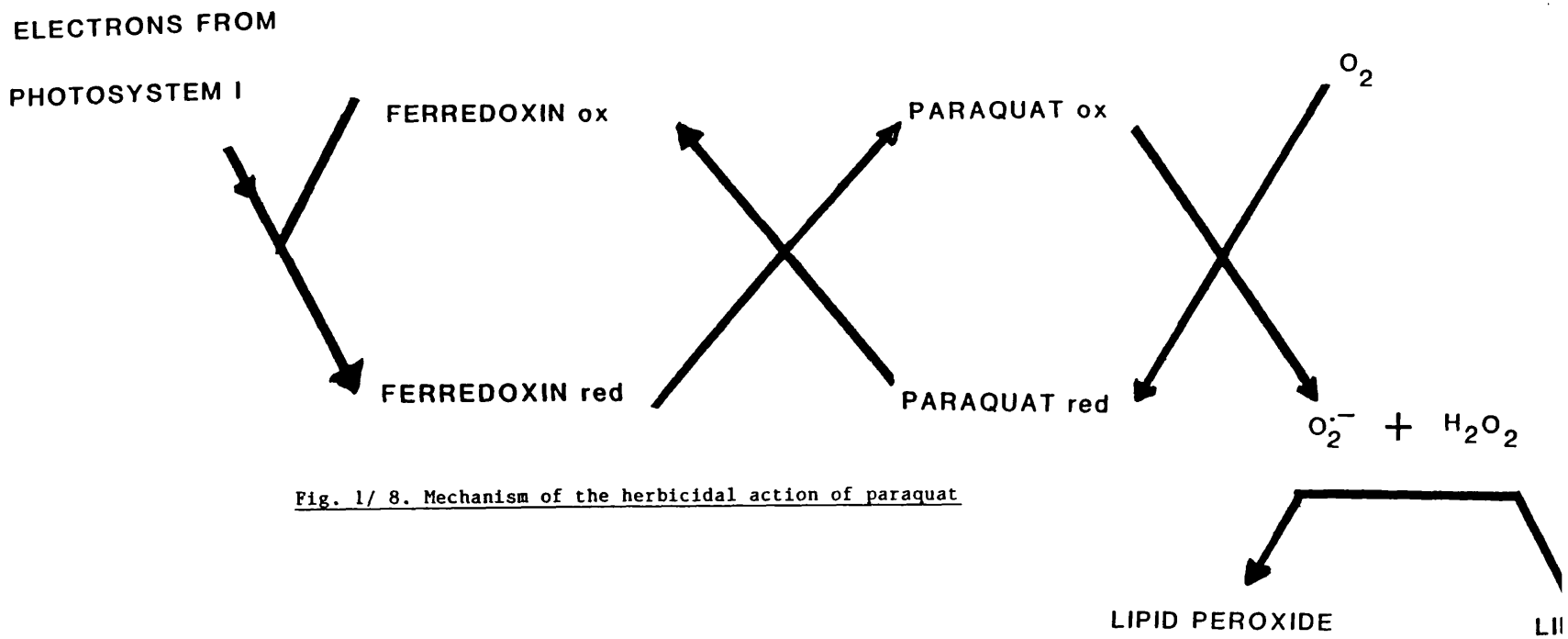


Fig. 1/ 8. Mechanism of the herbicidal action of paraquat

membrane damage and tonoplast disruption leading to cell death (fig. 1/ 8) [127]. Paraquat has the advantage of leaving no residue and is strongly adsorbed onto clay particles [127] enabling sowing soon after use.

1. 12. c PATHOLOGY OF PARAQUAT IN THE LUNG

Lung damage has been most extensively studied in the rat. After an LD50 dose, the type I and type II alveolar epithelial cells are damaged initially, along with the development of alveolar oedema and haemorrhage into the air spaces. Inflammatory cell infiltration occurs , without apparent damage to the endothelium. This is the initial destructive phase and is an alveolitis. Within 2-3 days areas of the alveolus appear denuded of epithelium. Many of the rats which die do so during this initial phase. Animals surviving this initial phase, proceed to the proliferative phase, characterised by fibroblast proliferation in the interstitium and intra-alveolar spaces. Extensive collagen deposition can lead to loss of alveolar architecture and disruption of gaseous exchange. Fibrosis can result in death from anoxia [128] and may occur after several weeks.

1. 12. c BIOCHEMICAL MECHANISM OF TOXICITY

Paraquat is proposed to be toxic via a mechanism involving redox and oxidative stress. Redox cycling is presumed to occur following the pulmonary accumulation of paraquat and has been shown to result in cofactor depletion and/ or lipid peroxidation. (Fig. 1/ 9).

a) Accumulation of Paraquat into Lung Tissue

Paraquat is accumulated into rat lung by an energy-dependent process which can be inhibited by KCN, rotenone and iodoacetate [129] and obeys saturation kinetics (Km = 70 µM; Vmax= 300 nmol/ g lung / h).This uptake process is specific to the lung [130] and also occurs

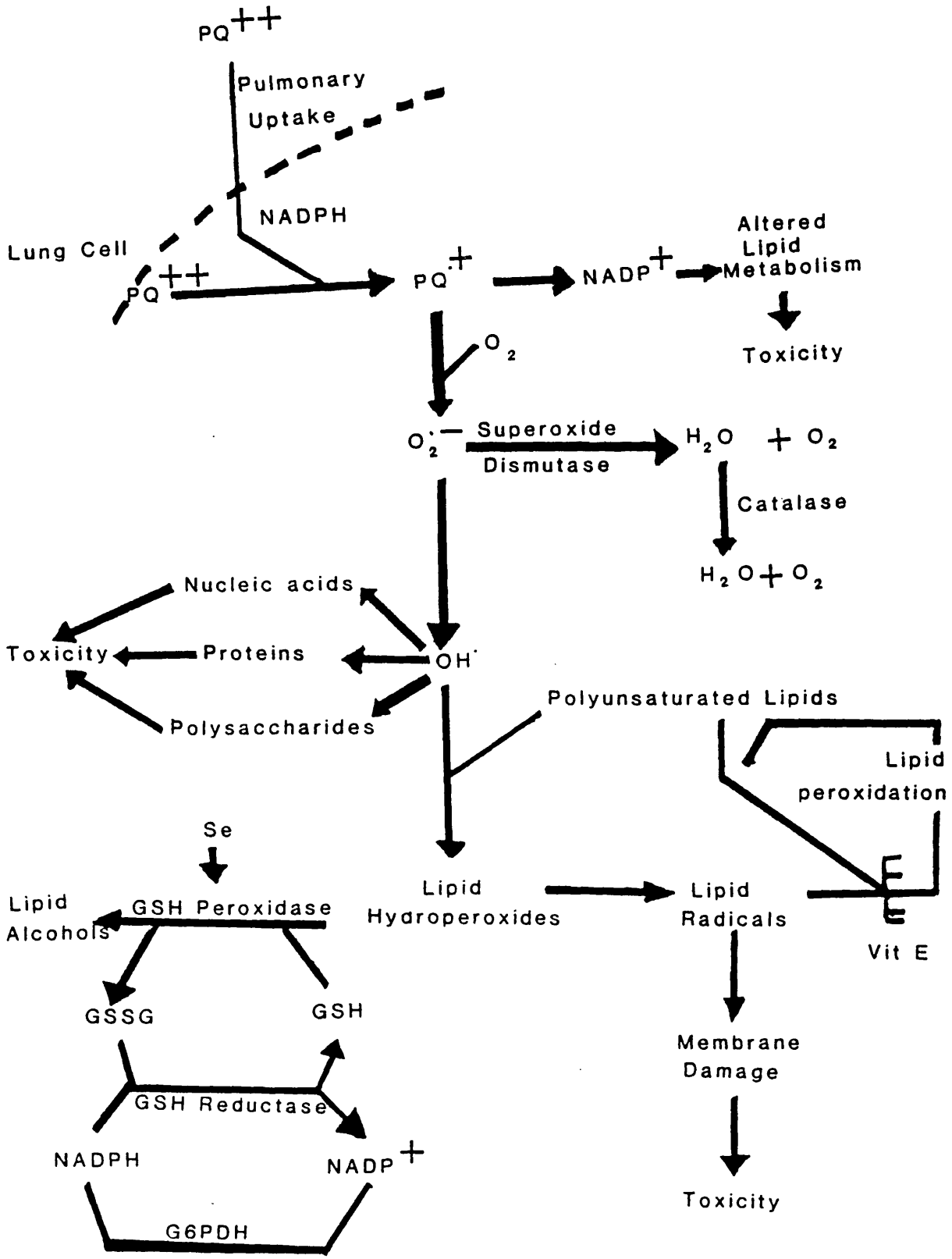


Fig. 1/ 9. Proposed biochemical mechanism of paraquat toxicity

in vivo. After oral administration of paraquat in the rat, the concentration in the plasma remains relatively constant between 2 and 30 h. Over this same time period the paraquat concentration in the lung increases progressively until, at 30 h, it can be 6-fold higher than that of the plasma.

Initial studies [131] suggested that paraquat and the monoamine 5-hydroxytryptamine (5-HT; which is accumulated into the alveolar endothelium were taken up into distinct cell types. Since the Type I and II cells of the alveolar epithelium are the initial sites of paraquat-induced lung damage [128] it was likely that uptake occurred into these cell types. This was confirmed by the work of Sykes et al. [132]. Paraquat was used to selectively damage the epithelium in vivo. As time after dosing increased (reflecting increasing cell damage) so the uptake of paraquat into lung slices prepared from animals dosed with paraquat, decreased. Uptake of 5-HT into paraquat dosed rats was not affected [132].

Attempts were made to find compounds which would effectively reduce paraquat uptake. Ross and Krieger [133] concluded that α,β-diamino-alkanes were the most potent inhibitors having two quaternary nitrogen atoms at pH 7. 4. Gordonsmith et al. [134] showed that the receptor for the transport process recognises quaternary nitrogen atoms with an optimal spacing of around 7 Å. The oligoamines were the most effective inhibitors. The diamine putrescine is accumulated with a Km of 11 μM and a Vmax of 720 nmol/ g lung/ h and is a very potent inhibitor of paraquat uptake. The polyamines spermine and spermidine exhibit similar kinetic parameters and inhibitory potencies.

Recent autoradiographic studies [135] showed that putrescine uptake occurs into type I and II cells and also into the Clara cells in vivo in rat. The uptake of paraquat was assigned to type II cells. Waddell and

Marlowe [136] studied the distribution of [^{14}C]-methyl paraquat in mice following an i. p. dose. The lung showed a preferential accumulation; tentatively assigned as an uptake into Type II cells.

1. 12. d REDOX CYCLING AND ACTIVE OXYGEN SPECIES GENERATION

Under anaerobic conditions, paraquat can undergo a one-electron reduction from the cation to form a stable free radical. In the presence of oxygen, this radical is reoxidised to reform the parent species with the concomitant generation of superoxide anion (fig. 1/10). Providing there is a source of electrons the paraquat will cycle with the generation of superoxide anion.

Gage [137] demonstrated that the microsomal fraction of rat liver supported reduction of paraquat to its radical. The one electron reduction is dependent upon NADPH and enzymic catalysis. Homogenates from tissues other than the lung are also capable of supporting the reduction [138]. Production of superoxide anion leads to production of hydrogen peroxide, either as a result of superoxide dismutase activity, or by a spontaneous dismutation reaction. If hydrogen peroxide comes into contact with a transition metal (in particular iron) then the highly reactive hydroxyl radical will be formed via an iron-catalysed Haber-Weiss reaction [139].

It has been proposed that the pulmonary toxicity of paraquat is related to the ability of the compound to form the free radical which can then interact with molecular oxygen in the manner described above, which may lead to cell damage via several mechanisms including lipid peroxidation and depletion of reduced cofactors. It is, however, the redox cycling which is accepted to be the critical event in paraquat-induced cytotoxicity.

Two major hypotheses have been proposed to account for the primary

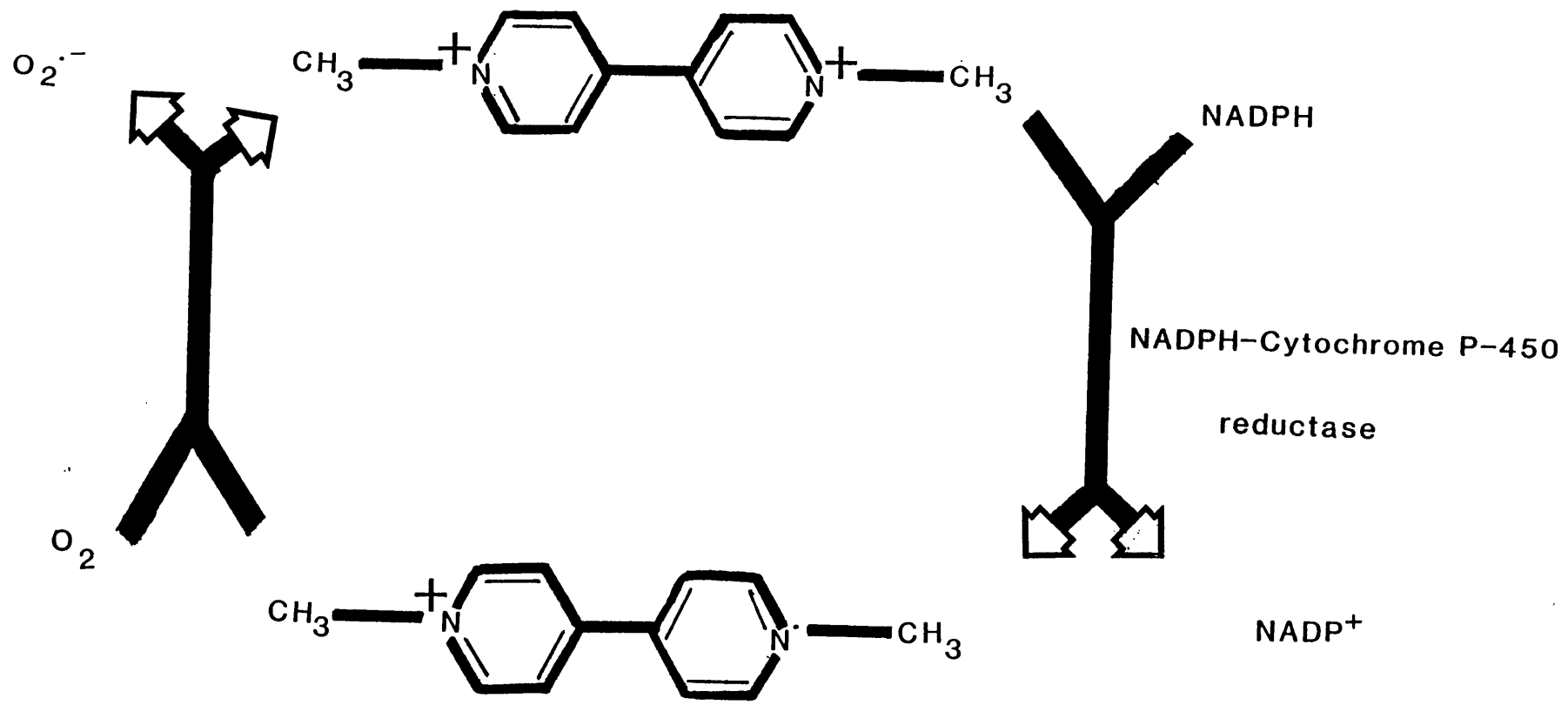


Fig. 1/ 10. The redox cycling of paraquat

biochemical mechanism of paraquat toxicity. These are lipid peroxidation and/ or NADPH depletion. Since lipid peroxidation would be enhanced under conditions of oxidant stress, these two hypotheses need not be mutually exclusive [7].

1. 12. e LIPID PEROXIDATION, NADPH DEPLETION AND THE INVOLVEMENT OF GLUTATHIONE IN PARAQUAT TOXICITY

Bus et al. [140] demonstrated that paraquat could stimulate lipid peroxidation in a reconstituted microsomal system. Subsequent studies have have produced conflicting results. Trush et al. observed that paraquat stimulated lipid peroxidation in rat lung microsomes without the addition of exogenous iron, though this was dependent upon adequate NADPH, aerobic conditions and duration of incubation [141]. Kornbrust and Mavis [142] investigated microsomal lipid peroxidation in vivo and in vitro. Their results indicated that paraquat does not cause toxicity by initiating lipid peroxidation.

In vivo the data for paraquat-induced lipid peroxidation is tenuous. Shu et al. [143] concluded that peroxidation of polyunsaturated fatty acids may not be responsible for the in vivo toxicity of paraquat since pretreatment of mice with N, N'-diphenyl-p-phenylene diamine reduced peroxidation but did not ameliorate toxicity.

In those cells in which paraquat is accumulated, the concentration may be very high and result in fast rates of NADPH depletion. If this rate exceeds the rate of regeneration by the HMPS pathway, depletion of NADPH will occur. Early studies revealed oxidation of NADPH in vivo resulting from paraquat administration [45]. Further investigations confirmed this finding and also revealed a linear relationship between stimulation of the HMPS and the reduction in fatty acid synthesis (both being indicative of

an extensive NADPH depletion) [74].

The role of glutathione in the pulmonary toxicity of paraquat is unclear. Bus et al. [144] observed no depletion of GSH in mouse lung following paraquat administration. Similarly, Keeling et al. [74] observed no GSH depletion in rat lung,though dosing with paraquat did result in an increase in GSSG and protein mixed disulphides. Brigelius et al. [70] have recently reported a decrease in the GSH/ GSSG ratio in isolated perfused rabbit lung (reflecting increased levels of GSSG) perfused with paraquat.

The effects of modulating glutathione peroxidase (selenium deficient diet) activity on paraquat toxicity has been investigated in several studies. Omaye et al. [145] showed enhanced toxicity and Glass et al. observed that selenium deficiency potentiates paraquat induced lipid peroxidation in isolated perfused rat lung and concluded that an inverse relationship existed between GSSG efflux and MDA accumulation from paraquat perfused lungs [146].

1. 13 ALPHANAPHTHYLTHIOUREA (ANTU)

Alphanaphthylthiourea (ANTU) is a rodenticide [147] which at low doses, produces a relatively selective pulmonary toxicity. This toxicity is manifested by fibrin-rich pulmonary oedema and extensive pleural effusions in rats [148] and dogs [149]. Pathological investigations of ANTU-induced pulmonary injury in rats suggests that the oedema results from the formation of reversible gaps in the endothelium [150]. Meyrick et al. [151] showed a blebbing and scalloping of endothelial cells following administration of ANTU. Endothelial effects were also indicated by a reduction in the uptake of 5-hydroxytryptamine (5-HT; a marker of endothelial function) observed in isolated perfused lung prepared from rats dosed with ANTU [152]. Epithelial damage can also be observed though

this occurs subsequent to the endothelial damage [151].

The precise mechanism by which ANTU produces lung damage is unknown There is evidence that metabolic activation involving desulphuration occurs to form the reactive metabolite. Dosing of rats with radiolabelled ANTU leads to covalent binding of radioactivity to the macromolecules of the lung and liver. In vitro, ANTU is metabolised by lung and liver microsomes to an intermediate which covalently binds to macromolecules [153]. This is mediated by cytochrome P-450 mixed-function oxidase activity and in vivo pretreatment with piperonyl butoxide (an inhibitor of cytochrome P-450) confers some protection against ANTU toxicity in rats [154] supporting the involvement of metabolic activation [16].

The role of glutathione in the toxicity of ANTU has been studied [155]. Lethality, hydrothorax, and pulmonary covalent binding activities of ANTU were potentiated by diethylmaleate, a potent GSH depleting agent.

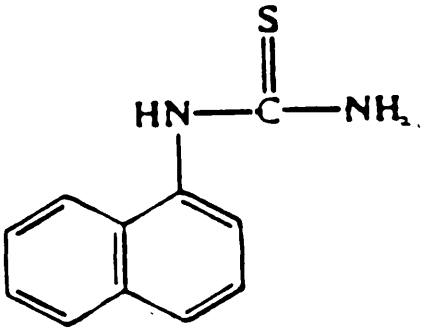
1. 14 NITROFURANTOIN

The 5-nitrofuran derivative, nitrofurantoin (N-[5-nitro-2-furfuryl-idene]-1-aminohydantoin) is a widely used antibiotic employed in the treatment of urinary tract infections [156]. Its use is frequently associated with serious pulmonary complications, including fibrosis [157].

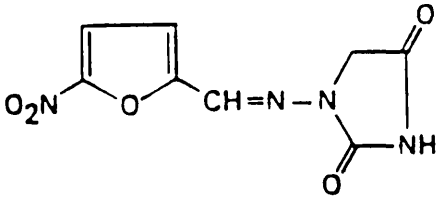
Nitrofurantoin has been shown to undergo metabolic activation. Boyd et al. [158] observed that microsomal and soluble fractions of rat lung and liver mediated the covalent binding of [^{14}C]-nitrofurantoin to tissue macromolecules in vitro. Oxygen strongly inhibited binding in both microsomal and soluble fractions. Carbon monoxide failed to inhibit binding indicating that activation resulted from nitroreduction and not from oxidation of the furan ring. An antibody against NADPH-cytochrome c reductase inhibited the microsomal nitroreduction and covalent binding of

Fig. 1/ 11. Structures of ANTU (A), nitrofurantoin (B) and diquat (C)

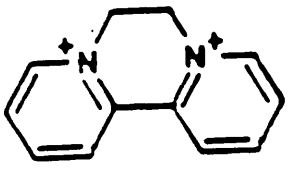
A)



B)



C)



nitrofurantoin.

Mason and Holtzmann [159] suggested similarities between nitrofurantoin and paraquat in terms of their in vitro microsomal metabolism. These studies predicted that superoxide anion would be produced from the cyclic reduction/ oxidation of nitrofurantoin in vitro under aerobic conditions. Sasame and Boyd [160] investigated superoxide anion and hydrogen peroxide production along with NADPH production in microsomes incubated with nitrofurantoin. Nitrofurantoin enhanced the oxidation of NADPH in lung microsomal suspensions along with production of superoxide anion and formation of hydrogen peroxide.

Peterson et al. [161] investigated the metabolic activation of oxygen by nitrofurantoin in chicks. Lipid peroxidation is also observed in lung microsomes [162]. Nitrofurantoin caused an elevation in GSSG in the isolated perfused rabbit lung [163, 164].

Nitrofurantoin can also inhibit mitochondrial respiration [165] an effect which may contribute to its toxicity.

1. 15 DIQUAT

Diquat (1, 1'-ethylene-2, 2'-dipyridilium dichloride) is, like paraquat, a member of the bipyridylium herbicides. Diquat with a redox potential of -348 mV, is more easily reduced then paraquat (with a redox potential of -446 mV) via a one electron reduction.

Diquat, in common with paraquat, stimulates oxygen consumption in rat liver cell fractions [137]. The LD50 for diquat in rats is similar to that for paraquat. However, these herbicides produce strikingly different pathological changes in mammals. Diquat produces no striking toxicity to the lung. The lack of pulmonary toxicity is considered to result from the absence of pulmonary accumulation of the compound [166], since striking

pulmonary injury is observed when diquat is administered via an intra-tracheal route [167]. It has been proposed that diquat can enter cells via passive diffusion. In studies with lung slices [168] both diquat and paraquat elicit stimulation of the HMPS at similar concentrations.

Diquat induces oxidative stress in a variety of systems. In addition to lung slices its effects have been investigated in isolated hepatocytes. The compound is generally considered to be toxic via redox cycling, depletion of reduced pyridine nucleotides [169] and lipid peroxidation.

1. 16 GENERAL AIMS

The aims of the present work were as follows:

i) To investigate the role of glutathione reductase in the toxicity of paraquat *in vivo* and *in vitro*.

ii) To investigate the cellular selectivity of toxic agents in lung tissue using a combination of biochemical and functional markers of cellular injury.

iii) To investigate the influence of elevated pulmonary thiols on the toxicity of paraquat and ANTU.

iv) To attempt to use adenosine accumulation into lung tissue as a marker of pulmonary endothelial damage.

v) To investigate the influence of a variety of conditions such as hyperoxia and desferrioxamine on the toxicity of paraquat *in vitro*.

CHAPTER 2 - MATERIALS AND METHODS

2.1. MATERIALS

2.1.a CHEMICALS.

All chemicals were of the highest grade of purity available and were purchased from the Sigma Chemical Co.Ltd (Poole, Dorset) unless otherwise stated.

Halothane was purchased from May and Baker Ltd. (Dagenham).

Dimethyl sulfoxide (DMSO) was obtained from BDH Ltd. (Poole, Dorset).

Petroleum ether, methanol and ethanol were acquired from Rathburn Chemical Company (Peebleshire, Scotland).

Aquasol for liquid scintillation counting was obtained from NEN Research Products (Dupont; Boston, MA).

The following radiochemicals were purchased either from NEN or from Amersham International plc (Amersham,Bucks) :-

D-[^{14}C(U)]-glucose (304. 7 mCi/ mmol, 99 % pure).

D-[1-^{14}C]-glucose (54. 6 mCi/ mmol, $>$ 99 % pure).

D-[6-^{14}C-glucose (55. 0 mCi/ mmol, 99 % pure).

[2-^{3}H]-adenosine (20 Ci/ mmol, 98. 5 % pure).

5-Hydroxy [side-chain-2-^{14}C]-tryptamine creatinine sulphate (54 mCi/ mmol, 99 % pure).

[1, 4-^{14}C]-putrescine dihydrochloride (111 mCi/mmol, 97% pure).

[^{3}-H]-putrescine dihydrochloride (120 mCi/ mol, 98 % pure).

[^{14}C]-spermidine trihydrochloride (117 mCi/ mmol, 98 % pure).

[Methyl-^{14}C]-paraquat dichloride (111 mCi/ mmol, 98 % pure).

[U^{14}C]-acetic acid (50-60 mCi/ mmol).

1, 3-Bis (2-chloroethyl)-1-nitrosourea (BCNU/BiCNU/Carmustine) was obtained from Bristol-Myers Oncology (Uxbridge).

Paraquat dichloride and diquat dibromide were a generous gift of Dr Lewis Smith (Imperial Chemical Industries plc, Central Toxicology Laboratories, Alderley Park, Cheshire) and were > 98 % pure.

2, 3-Dimethoxy-1, 4-naphthoquinone was prepared in the laboratory by Mr Stephen Forrow, using an established technique [170]. The compound was purified by HPLC (99. 99 % pure) and the identity was ascertained using Mass Spectroscopy.

Diethyl maleate was obtained from BDH Ltd. as a 99 % solution.

Phorone was acquired from Aldrich Chemical Co. (Gillingham, Dorset) as a 95 % solution.

Nitrofurantoin was obtained from Sigma and α-naphthylthiourea was a generous gift of Dr Y. S . Bakhle (Royal College of Surgeons, Lincolns' Inn Fields).

Corn oil was obtained from Safeways Foodstores (marketed as pure corn oil).

2.1.b ANIMALS

Male Wistar-derived rats (180-250 g) were employed in these studies having been obtained either from Harlan-Olac (Bicester) or from ICI (the Alderley Park strain Wistar rat).

Male mice (25-30 g; strains TO and BALB/ c) were obtained from Bantin and Kingman (Hull). Male Alderley Park strain mice of a similar weight range were obtained from ICI.

2.1.c AMINO ACID SUPPLEMENTS AND BIOCHEMICALS

Minimal Essential Medium (MEM) amino acids were purchased from

Gibco Ltd. (Paisley, Scotland as a (50-fold solution). The composition of the medium is as follows :-

AMINO ACID	FINAL CONCENTRATION (mM)
L-arginine HCl	0. 36
L-aspartate	0. 45
L-glutamate	0. 91
L-histidine	0. 83
L-isoleucine	0. 17
L-leucine	0. 33
L-lysine	1. 15
L-phenylalanine	0. 26
L-proline	0. 38
L-threonine	0. 55
L-tryptophan	0. 17
L-tyrosine	0. 19
L-valine	0. 48

In the medium employed in the studies described with BCNU pre-treated rat lung slices (Chapter 3), additional supplements were made :-

L-cysteine, L-methionine, L-glycine, L-serine and L-glutamine all at a final concentration of 0. 2 mM.

Glutathione, in both its reduced and oxidised forms and glutathione reductase (E. C. 1. 6. 4. 2.) were obtained from Boehringer-Mannheim (Lewes, East Sussex).

2.2. METHODS

2.2.a PREPARATION OF RAT LUNG SLICES

Lung slices were prepared from male rats (180-250 g). Animals were placed in a jar containing halothane. Upon cessation of respiratory movements, the thoracic cavity was opened and the lungs exposed. The lungs were then perfused free of blood, in situ. This technique is best performed whilst the heart is still beating. Following removal of the lungs, perfusion was continued until the lungs were white. Perfusion was carried our using a Luer cannula inserted into the right ventricle, the perfusate leaving via the left atrium (which was cut). This method employed a re-circulating system, maintained by a Watson-Marlow 502-S peristaltic pump, along with a bubble trap, allowing buffer aeration.

The perfusate was Krebs Ringer Phosphate buffer, pH 7. 4 with glucose (KRB) at 37^{o}. KRB contained :-

NaCl (130 mM),

$CaCl_2$(1. 9 mM),

$MgSO_4*7H_2O$ (1. 29 mM)

KCl (5. 2 mM)

Na_2HPO_4 (10 mM),

glucose (11 mM).

The above chemicals were all obtained from BDH Ltd.

Following perfusion, lung lobes were separated and connective tissue removed using scissors. Slices (0. 5 mm thick) were prepared using a Mc Illwain tissue chopper (Mickle Ltd. Godalming, Surrey). The basic method for preparation of lung slices followed that of Rose et al. [171].

The preparation of BCNU pretreated rat lung slices is described fully in Chapter 3.

2.2.b RADIOCHEMICAL STUDIES WITH LUNG SLICES

Lung slices were incubated with the appropriate radiolabel and

concentration of 'cold' compound. Following incubation, slices were washed briefly, carefully blotted dry and then dissolved in 0. 4ml 1M NaOH (30 min incubation in a boiling water bath). Samples were then neutralised with 0. 4ml 1M HCl. Aquasol scintillant (4 ml) was then added. Liquid scintillation counting was performed on an LKB Rackbeta-1216 counter. All results are expressed as dpm/ mg or nmol/ g, calculated from quench curves obtained from the internal standards programmed into the counting procedure. This general method was employed in uptake studies.

Incubations were performed in 25 ml conical flasks and 20-40 mg lung slices were required. The polyamine uptake studies are similar to the methods of Gordonsmith [172].

The rate of glucose oxidation was determined under a variety of conditions. Three substrates were employed : D-[1-^{14}C]-glucose, D-[6-^{14}C]-glucose and D-[^{14}C(U)]-glucose. In each case the procedure was the same. Lung slices were incubated for the appropriate time in a sealed centre well flask (containing 10 % KOH to absorb the carbon dioxide produced). Slices were killed by injecting 20 % TCA through the suba-seal. Following incubation for a further 30 min to ensure maximal carbon dioxide absorption, the seals were removed, the filter paper (Whatman No. 541) wicks ($2cm^2$) were removed and placed in a Betavial. Then the centre well was washed twice with distilled water using a pasteur pipette, and the water added to the Betavial. $^{14}CO_2$ was determined following addition of Aquasol. 1 µCi of 'hot' substrate was employed per flask.This method follows closely that of Katz and Wood [173]. Incubation was carried out at 37^{o} in a shaking water bath.

2.2.c ACETATE INCORPORATION INTO FATTY ACIDS IN LUNG SLICES

Approximately 300 mg of lung slices were incubated in 7 ml KRB under

air in 50ml conical flasks in a shaking water bath at 37^{0} . After a 20min preincubation period, 4 µCi [^{14}C]-acetic acid and 40 µmoles 'carrier' sodium acetate were added to the medium. After a further 90 min incubation period, 1 ml 15M NaOH was added to the incubation mixture containing lung slices and the tissue was digested in a boiling water bath for 2 h.

The alkaline digest was extracted three times with 20 ml petroleum ether ($40-60^{0}$) and the aqueous phase retained. This was then acidified to pH 1 with 12M sulphuric acid and extracted three times with 20 ml petroleum ether. The aqueous layers were discarded and the bulked petroleum ether fraction washed twice with 10 ml 2 % acetic acid and then twice with 10 ml ethanol (50 %). The petroleum ether layer was evaporated to dryness by blowing air into a weighed glass scintillation vial. The vial was reweighed and the mass of fatty acids calculated. Aquasol (10 ml) was added to the vial before radioactivity measurement. This is basically the method of Gould [174].

2.2.d GLUTATHIONE REDUCTASE ASSAYS

Glutathione reductase activity was assessed in tissue homogenates by the method of Carlberg and Mannervik [175]. In this coupled assay, the rate of oxidation of NADPH was monitored at 340 nm on a Shimadzu MPS-2000 spectrophotometer (with a Shimadzu PR3 graphic printer ; from V. A. Howe and Co. Ltd. London) and a heated cell block to maintain a constant temperature of 37^{0} during the analysis. Final concentrations in the cuvette were :-

1 mM oxidised glutathione

0. 1 mM NADPH (tetrasodium salt).

0. 1M KH_2PO_4/ 5 mM EDTA buffer, pH 7. 6

Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma.

A sufficient volume of tissue extract was added in order to produce a steady, linear oxidation of the pyridine nucleotide. The reaction was initiated by the addition of 100 µl of NADPH solution. The activity was assessed over a 5 min incubation period. Over the same period, a cuvette was monitored which contained GSSG, NADPH but no tissue extract. This is to correct for any oxidation occurring other than that resulting from the enzymic activity i. e. the 'blank'.

2.2.e NONPROTEIN SULPHYDRYL / GSH ASSAYS

Thiols were assessed by three different methods :-

1) The fluorometric procedure of Hissin and Hilf [176].

Tissue extract (75 µl) was added to 2745 µl KH_2PO_4/5mM EDTA, pH 8.0 buffer. Sodium hydroxide (30 µl) was added in order to maintain the final reaction mixture at a pH greater than 8. 0. Then 150 µl o-phthaldialdehyde was added. After 30 min incubation at room temperature the fluorescence was measured using a Perkin-Elmer Fluorescence Spectrophotometer. The wavelength of excitation was 350 nm, that of emission 420 nm. The o-phthaldialdehyde was made up in methanol (1 mg/ ml).Reduced glutathione standards were made up in 6. 5 % TCA.

2) The spectrophotometric method of Ellman [177].

This method has the advantages of speed and simplicity but is not as specific or sensitive as the fluorometric or enzymic methods. The method is primarily designed for use with tissue homogenates. Following the appropriate homogenisation, addition of sulphosalicylic acid (4 %) and centrifugation, 0. 5 ml of supernatant was added to 4. 5ml phosphate buffer, pH 8. 0. Then 50 ul 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was added. The samples were vortexed and allowed to stand for 15 min at room temperature in the dark. The absorbance was then determined using a Pye

Unicam SP6-550 UV/ vis spectrophotometer (Pye Unicam Ltd. Cambridge).

3) The glutathione-S-transferase method of Brigelius et al. [178].

Briefly, the largest lung lobe (c. a. 300 mg) was weighed and freeze clamped in liquid nitrogen and then powdered in a percussion mortar. PCA (1. 0 M) was added to the powder. After vortexing, the suspension was spun at 800 g for 5 min at 4^{o}. Supernatants were then kept on ice. Following addition of 1. 0 M KOH (to neutralise) and centrifugation, 100 µl of supernatant was added to a cuvette along with 0. 1 M potassium phosphate buffer, pH 7. 0; 0. 5 ml) and 1-chloro-2, 4-dinitrobenzene (10 mM; 10 µl). GSH-S-transferase (equine; 35 U/ml; 20 µl) was added. The cuvette was incubated at 37^{o}. Conjugate formation was monitored at the wavelength-pair 340-400 nm for 5 min using a Sigma ZWS II dual wavelength spectrophotometer.

Important points with regard to the methodology for measuring pulmonary sulphydryls were outlined by Moron et al. [179]. Firstly, perfusion is essential since otherwise a 50 % error can be incurred due to contaminating erythrocytes. In the perfusion system outlined in their work, the error was assessed as approximately 10 %. In the studies presented here, the error would presumably be still low, since lungs were perfused until completely white.

2.2.f ASSAY OF ADENOSINE 5'-TRIPHOSPHATE IN LUNG SLICES

ATP was assayed using the bioluminescence technique of Stanley and Lemasters [180]. This method employs firefly luciferase. Bioluminescence was detected using a Thorn-EMI Photon Detection System coupled to Amstrad PC 1512 software. Luciferase extract and ATP (disodium salt, from equine heart) were obtained from Sigma Ltd. Following reconstitution with 5 ml distilled water, the luciferase extract was spun (5 min at 1000 rpm

and 4^{o}) in order to remove nonsoluble debris. The tissue extracts and suitably diluted standards were added to 12x75 mm plastic tubes (10 µl).To this was added 2 ml of the assay buffer :-

Magnesium sulphate heptahydrate, 80 mM/ potassium dihydrogen orthophosphate, 10 mM/ disodium hydrogen arsenate heptahydrate, 100 mM. The final pH was 7. 4

The reaction was started by the addition of luciferase (100µl). The tubes were then vortexed briefly and the bioluminescence determined. The principle of the assay :-

Luciferase (E) + Luciferin (LH_2) + ATP ---> E. LH_2AMP + PPi

E. LH_2AMP + O_2 ---> E + CO_2 + AMP + oxyluciferin + light.

2. 2. g TISSUE HOMOGENISATION

Samples for enzyme assays were homogenised in an appropriate volume of one of the following buffers :

Tris-HCl, 5 mM / NaCl, 0. 15 M / EDTA, 5 mM, pH 7. 4

KH_2PO_4, 0. 1 M / EDTA, 5 mM, pH 7. 6.

Homogenates were then spun at 3500 rpm in a Denley BR 401 refrigerated centrifuge at 4^{o} for 15 min. The supernatants were then filtered through nylon mesh to remove any suspended debris.

Samples for assay of ATP, and GSH (via the fluorometric method) were homogenised in 0. 1M KH_2PO_4/ 5 mM EDTA, pH 7.6, to which was added an equal volume of 13 % TCA. Samples were spun at 2500 rpm at 4^{o} for 15 min and supernatants filtered and stored for assay.

When GSH was assayed by the enzymic method of Brigelius, PCA (0. 5M)[178] was the protein precipitant of choice. In the method of Ellman,[177], sulphosalycylic acid (4 %) was used.

Homogenisation of tissue samples was carried out using a Polytron

(Kinematica, Northern Media Supply Co. , North Humberside) set at high speed for 20 sec. Homogenisation was performed in plastic tubes, in iced water.

2. 2. h STORAGE OF SAMPLES

In most cases GSH and ATP assays were performed on the same day as sample preparation. When storage was unavoidable, no degradation was observed at -20^{o} when left overnight.

Glutathione reductase was stable at -20^{o} for at least a week.

2. 2. i PROTEIN ASSAYS

The protein assay of choice was the method of Lowry et al, [181]. Folin-Coicalteau reagent was obtained from BDH Ltd. The two reagents required for the assay were prepared directly before use. Copper sulphate (1 %) and potassium/ sodium tartrate (2 %) were prepared in advance (stored at 4^{o}). They were diluted in sodium carbonate (2 %) in a final ratio of 1 : 1 : 100 for use. The Folin-Coicalteau reagent was diluted 1 : 2 with distilled water. Duplicate 0. 4 ml aliquots of samples/ standards (Bovine Serum Albumin was obtained from Sigma) were placed into 75x12mm glass tubes, 2 ml of copper sulphate/ tartrate solution was added and the tubes were mixed. After 10 min at room temp. 0. 2 ml of Folin reagent was added with immediate vortexing. After 30 min standing, the absorbance at 720 nm was determined using a Pye Unicam SP6-550 spectrophotometer.

CHAPTER 3- THE USE OF 1,3-BIS (2-CHLOROETHYL)-1-NITROSOUREA (BCNU) IN VIVO AND IN THE DEVELOPMENT OF A NOVEL LUNG SLICE SYSTEM WITH COMPROMISED ANTIOXIDANT DEFENCES

3. 1. AIMS

The aims of the work presented in this chapter were two-fold. Firstly, to assess the consequences of glutathione reductase inhibition on the toxicity of paraquat to rats and mice in vivo and, secondly, to develop an rat lung slice model with marked inactivation of glutathione reductase activity and accompanied by only minimal modulations of other pulmonary biochemical functions. The studies employed the anti-tumour agent 1, 3-bis (2-chloroethyl)-1-nitrosourea (BCNU), a relatively specific inhibitor of glutathione reductase activity.

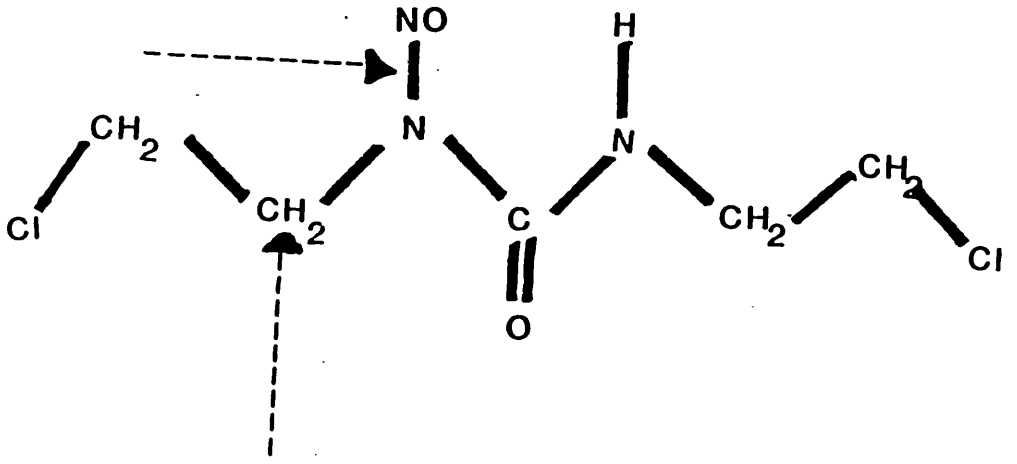
3. 2. INTRODUCTION

3. 2. a 1, 3-BIS (2-CHLOROETHYL)-1-NITROSOUREA (BCNU)

BCNU is a member of the chloroethylnitrosourea family of compounds and is used clinically as an antitumour agent. BCNU has demonstrated activity against lymphomas and other major uses include therapy against gliomas, small-cell carcinoma of the lung, and melanoma [181]. Studies in man, mice and monkeys revealed rapid degradation following administration and BCNU is stable under acidic conditions, well absorbed orally and its high lipid solubility allows it to cross the blood-brain barrier.

3. 2. b DECOMPOSITION AND METABOLISM OF BCNU

BCNU (fig. 3/ 1) is a bifunctional molecule possessing carbamoylating



1,3-BIS(2-CHLOROETHYL)-1-NITROSOUREA (BCNU)

Fig. 3/ 1. Structure and sites of metabolism of BCNU
Arrows indicate sites of NADPH-mixed function oxidase activity.

properties in addition to its alkylating activity. Chloroethylnitrosoureas decompose spontaneously under physiological conditions. Colvin et al. investigated the decomposition of BCNU in aqueous solution [182]. BCNU labelled with ^{14}C in the chloroethyl groups produced vinyl chloride, acetaldehyde, dichloroethane and chloroethanol consistent with the existence of chloroethylcarbonium ions. The formation of isocyanates during the degradation of BCNU was established in early studies [183]. Biologically, the compound undergoes denitrosation which is catalysed by liver microsomes in the presence of NADPH [184].

3. 2. c MECHANISM OF ACTION

It is generally accepted that the antitumour activity of BCNU results from its alkylating activity. The compound has been shown to interact with DNA resulting in extensive formation of alkali-labile sites (sites of single strand scission) [185]. Alkylation, in addition to possible, mispairing or miscoding effects, has been observed to lead to the production of interstrand cross links [186].

The carbomylating activity resulting from the formation of 2-chloroethyl isocyanate has been proposed to be responsible for the unwanted toxicities associated with the compound. Carbamoylation has been observed with proteins but not with nucleic acids. Kann et al. [187] reported a marked inhibition of repair of single strand breaks,produced by 2-chloroethyl isocyanate. BCNU and its isocyanate have also been shown to inhibit DNA polymerase II from rat liver [188].

3. 2. d INHIBITION OF GLUTATHIONE REDUCTASE

In clinical trials, patients receiving BCNU acquired a profound deficiency of erythrocytic glutathione reductase activity. In contrast,

the activities of nineteen additional enzymes were unaffected including glucose-6-phosphate dehydrogenase, glutathione peroxidase, phosphofructokinase, ATPase and glyceraldehyde-3-phosphate dehydrogenase [189]. Babson and Reed [190] showed that inactivation of glutathione reductase only occurs when the enzyme is in the reduced state (i. e. NADPH reduction) and two thiol groups are present at the active site. The inactivation is proposed to occur via an active site directed carbamoylation reaction. The loss of catalytic activity occurs as a thiocarbamate adduct forms between the isocyanate and the distal thiol group at the active site.

Replenishment of activity is thought to result from de novo protein synthesis, since the inactivation process is irreversible. Recovery of activity in L1210 cells has been shown to be dependent upon protein synthesis and to be independent of DNA synthesis [191].

Since BCNU is an effective and relatively specific inhibitor of glutathione reductase, the compound has been employed in a variety of toxicological investigations, both in vivo and in vitro.

3. 2. e BCNU IN THE LUNG IN VIVO

BCNU has been shown to cause a profound loss of pulmonary glutathione reductase activity when administered to rats and mice [192, 193]. Smith and Boyd [192] showed a preferential effect of BCNU on pulmonary glutathione reductase in rat. Single doses inhibited in a dose- and time-dependent manner. Loss of activity persisted in the lung for up to 8 days following 20 mg BCNU/ kg. This was in marked contrast to the liver where activity returns to control levels after approximately 48 h. Kehrer [193] showed similar effects in mice. A single dose of 50 mg BCNU/ kg significantly inhibited glutathione reductase activity in liver within

10 min and in lung and heart within 30 min. Inhibition was maximal at 4 h in all tissues with activity remaining depressed in lung and heart for 96 h, whilst liver activity was normal at 48 h. In these studies no depletion of pulmonary GSH was observed, though a depletion of hepatic GSH in mouse has been reported as a result of BCNU administration [194].

Kehrer and Paradathaithu [117] administered a maximal non-lethal dose of BCNU to mice, which resulted in enhanced susceptibility to the effects of hyperoxia.

Following administration of butylated hydroxytoluene to mice, lung collagen synthesis was enhanced by a single dose of BCNU, suggesting the exacerbation of pre-existing lung damage [195].

BCNU is, itself, a lung toxicant. Pulmonary fibrosis is observed in a small but significant number of patients receiving chemotherapeutic doses [196, 197]. No damage is seen in rodent lung following a single dose of the drug, but when rats are maintained on a multiple dose regimen, striking pulmonary damage is produced. Smith and Boyd reported a decrease in pulmonary angiotensin converting enzyme (ACE) activity (a marker of endothelial integrity) in rats given small multiple doses [198]. Pulmonary fibrosis was was observed with the first morphologically detectable changes occurring in the alveolar type II cells with subsequent endothelial damage, accompanied by plasma cell infiltration [199].

Other toxicities observed clinically and in experimental animals include gastro-intestinal effects, bone marrow depression [200] and some hepatotoxicity [201].

3. 2. f USE OF BCNU IN VITRO

BCNU has been employed *in vitro* in studies with isolated hepatocytes. Babson et al. incubated cells with BCNU (75 μM) resulting in

90 % inactivation of glutathione reductase and a 60 % depletion of intracellular GSH levels [202].

Later studies [203] employed an initial incubation with BCNU (50 μM) for 30 min in a modified Krebs-Henseleit buffer, pH 7. 4 supplemented with HEPES and an amino acid mixture containing serine, glutamine, cysteine and methionine (all at 0. 2 mM) and other amino acids at the concentrations specified by Waymouth and Jackson [204]. This treatment resulted in a 90 % inhibition of glutathione reductase activity (though it was never complete). Following this initial treatment the hepatocytes were incubated in fresh amino acid-supplemented medium for a further 2 h, in the absence of BCNU, to allow recovery of GSH levels. It was concluded that the effects of BCNU were relatively selective and specific [203].

The cytotoxicities and glutathione depleting activities of a variety of compounds which are proposed to be toxic via a mechanism involving oxidative stress, have been assessed in BCNU compromised hepatocytes. These include adriamycin [205], diquat [206], paraquat [206], menadione [207], nitrofurantoin [208] and hydroperoxides [203].

RESULTS INHIBITION OF GLUTATHIONE REDUCTASE *IN VIVO* IN RAT AND MOUSE : BIOCHEMICAL INVESTIGATIONS AND LETHALITY STUDIES WITH BCNU AND PARAQUAT

The work presented in this section relates to attempts to inhibit pulmonary glutathione reductase activities *in vivo* in mouse and rat. The goal of these studies was to define the maximal non-lethal dose of BCNU in both experimental species along with the extent and duration of the inhibitory effects. This information would then be utilised to assess the consequences of inhibiting glutathione reductase on the toxicity

of paraquat.

3. 3. a INHIBITION OF PULMONARY GLUTATHIONE REDUCTASE ACTIVITY IN MOUSE : DOSE RESPONSE WITH BCNU

Inactivation of lung glutathione reductase activity in mouse was dose-dependent with regard to BCNU (fig. 3/ 2). The administration of 15 mg BCNU/ kg in a single dose (i. p.) was sufficient to result in a 40 % inhibition of activity. BCNU, at a dose of 60 mg/ kg produced inhibition of 66 %. These results were obtained from animals sacrificed at 2 h following drug administration. (Previous workers [193] have shown that the nadir of activity occurs 2 h subsequent to a single dose in mouse).

3. 3. b BCNU INDUCED LETHALITY IN MOUSE

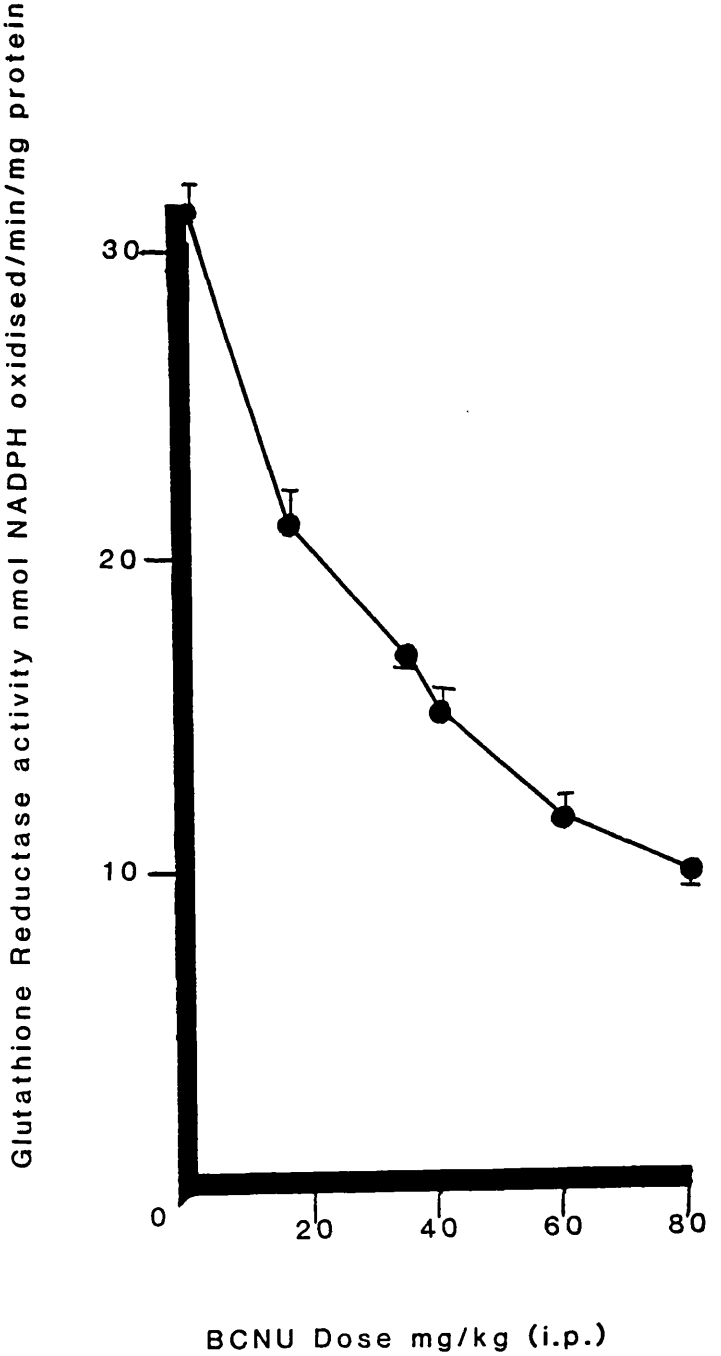
BCNU produced severe toxicity in mice at doses in excess of 40 mg/ kg. Animals dosed with 60 and 80 mg BCNU/ kg were all dead within seven days (8/ 8 in each group) table 3. I). Severe toxic signs were exhibited. These included :- Inertia, ocular problems and diarrhoea which was a common feature of all animals dosed with BCNU and which was also associated with a marked, dose-dependent weight loss (not shown). This suggested a gastro-intestinal toxicity. A copious hypotonic urine was produced along with a reduction in grooming behaviour. No lethality (0/ 8) was observed over the 14 days of the study at 20 or 40 mg BCNU/ kg.

From these results a maximal non-lethal dose was tentatively assigned at 35 mg/ kg for use in subsequent studies.

3. 3. c THE EFFECT OF A SINGLE MAXIMAL NONLETHAL DOSE OF BCNU ON THE ACTIVITIES OF HEPATIC AND PULMONARY GLUTATHIONE REDUCTASE IN MOUSE

Fig. 3/ 2. Dose-response: Inhibition of glutathione reductase activity in mouse with BCNU

Mice were dosed with BCNU in a corn oil vehicle in a single i. p.(100µl) injection. Animals were sacrificed after 2 h. Following perfusion lungs were excised and homogenised and glutathione reductase activity assessed. Results represent the mean activities of three animals per data point ± S. E.



The effects of the maximal non-lethal dose of BCNU (35 mg/ kg, as assessed from the data in 3. .. b) were investigated over 96 h following administration of the compound. Glutathione reductase activity was assayed in both lung and liver and interesting patterns of inhibition were revealed (fig. 3/ 3). The enzyme was very rapidly inhibited in both organs with approximately 40 % inhibition in lung and around 50 % in liver at 2 h compared to control animals given the corn-oil vehicle only. Glutathione reductase activity was at its lowest at 2 h in good agreement with previous workers [193]. After 24 h, however, hepatic activities in BCNU treated animals were similar to those of control animals. In contrast, pulmonary activities recovered only slowly and showed a significant (20 %) inhibition at 96 h following administration of BCNU. Therefore, there appeared to be a preferential effect of BCNU on pulmonary glutathione reductase.

3. 3. d PARAQUAT INDUCED LETHALITY IN MOUSE

Paraquat at 37. 5 and 50 mg paraquat ion/ kg (s. c.) was fatal to all mice (8/ 8 at each dose. Mortality was associated with an early weight loss. No loss of weight, or mortality was observed in control animals given 0. 9 % saline only. No deaths (0/ 8) were recorded at 12. 5 mg paraquat ion/ kg whereas 25 mg/ kg was fatal to 3/ 8 mice.

The weight losses referred to above were dose-dependent as were times of lethality. Toxic signs included copious urine production and in animals fatally intoxicated, respiratory movements were extremely laboured In addition, production of faeces was reduced. Pathologically, the lungs of paraquat treated animals appeared plum-coloured and haemorrhagic.

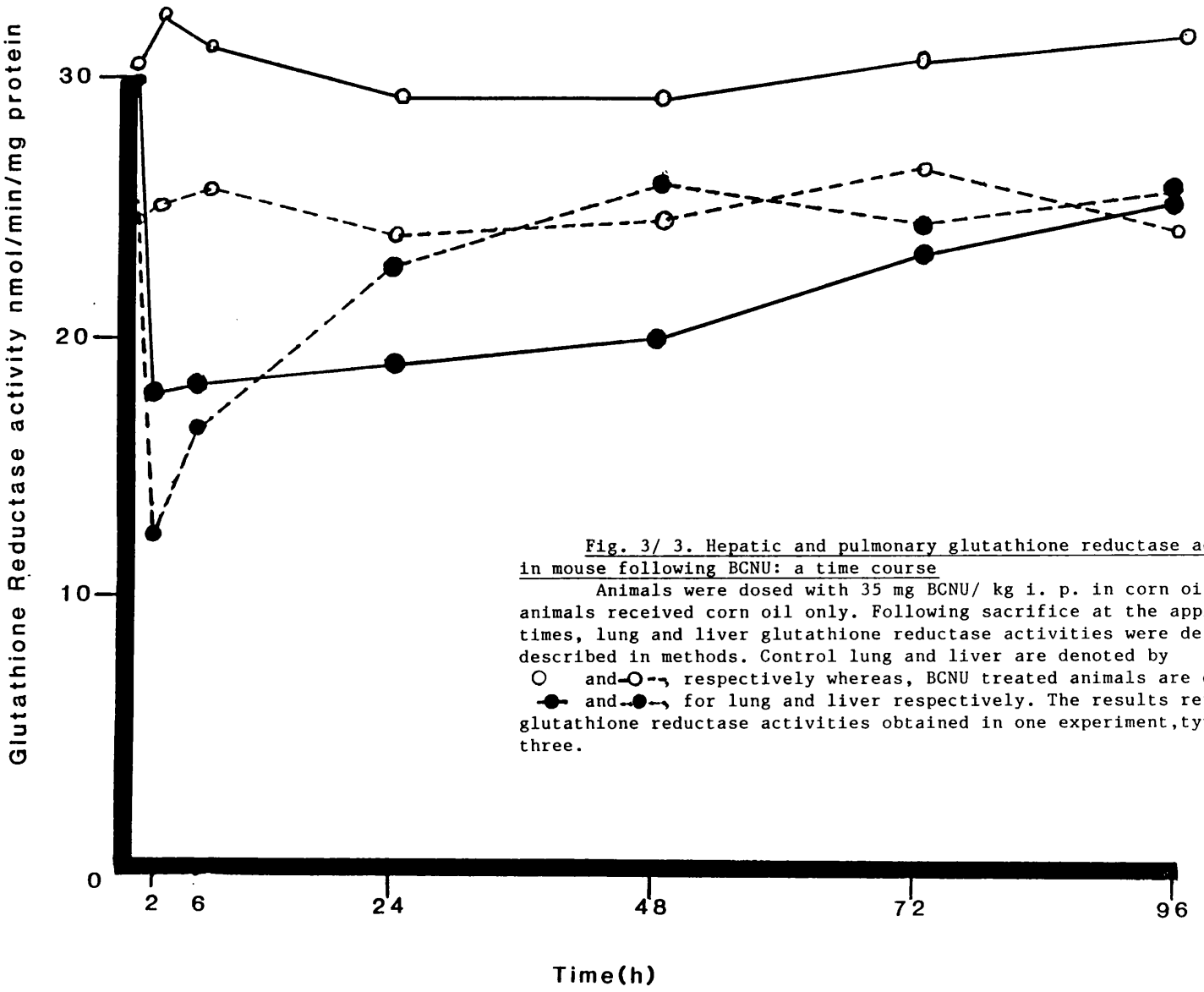


Fig. 3/ 3. Hepatic and pulmonary glutathione reductase activities in mouse following BCNU: a time course

Animals were dosed with 35 mg BCNU/ kg i. p. in corn oil. Control animals received corn oil only. Following sacrifice at the appropriate times, lung and liver glutathione reductase activities were determined as described in methods. Control lung and liver are denoted by ○ and -○-, respectively whereas, BCNU treated animals are denoted by ●- and -●-, for lung and liver respectively. The results represent the glutathione reductase activities obtained in one experiment, typical of three.

3. 3. e LETHALITY STUDY IN MOUSE : CO-TREATMENT STUDIES WITH BCNU AND PARAQUAT

BCNU enhanced the toxicity of paraquat as assessed by lethality and weight changes. Clearly (fig. 3/ 4), 12. 5 mg paraquat ion/ kg was not lethal to mice either in the presence, or absence of a BCNU pretreatment. Lethality was increased at the higher dose of paraquat (25 mg/ kg) in the BCNU treated mice i. e. 8/ 14 died compared to control lethality, 3/ 14.

In addition, the times to onset of mortality were decreased as a result of BCNU pretreatment. Control animals given the higher dose of paraquat which died did so on the third day after dosing, whereas the BCNU treated animals which succumbed, did so on the second day after dosing.

The weight changes are interesting (fig. 3/ 4). The results represent the changes occurring between dosing and the second day of the of the study, a period when all the animals were alive. Control animals given corn oil and saline vehicles gained weight as did control animals given the lower dose of paraquat. Administration of the higher dose of paraquat caused weight loss in mice as did dosing with BCNU per se.

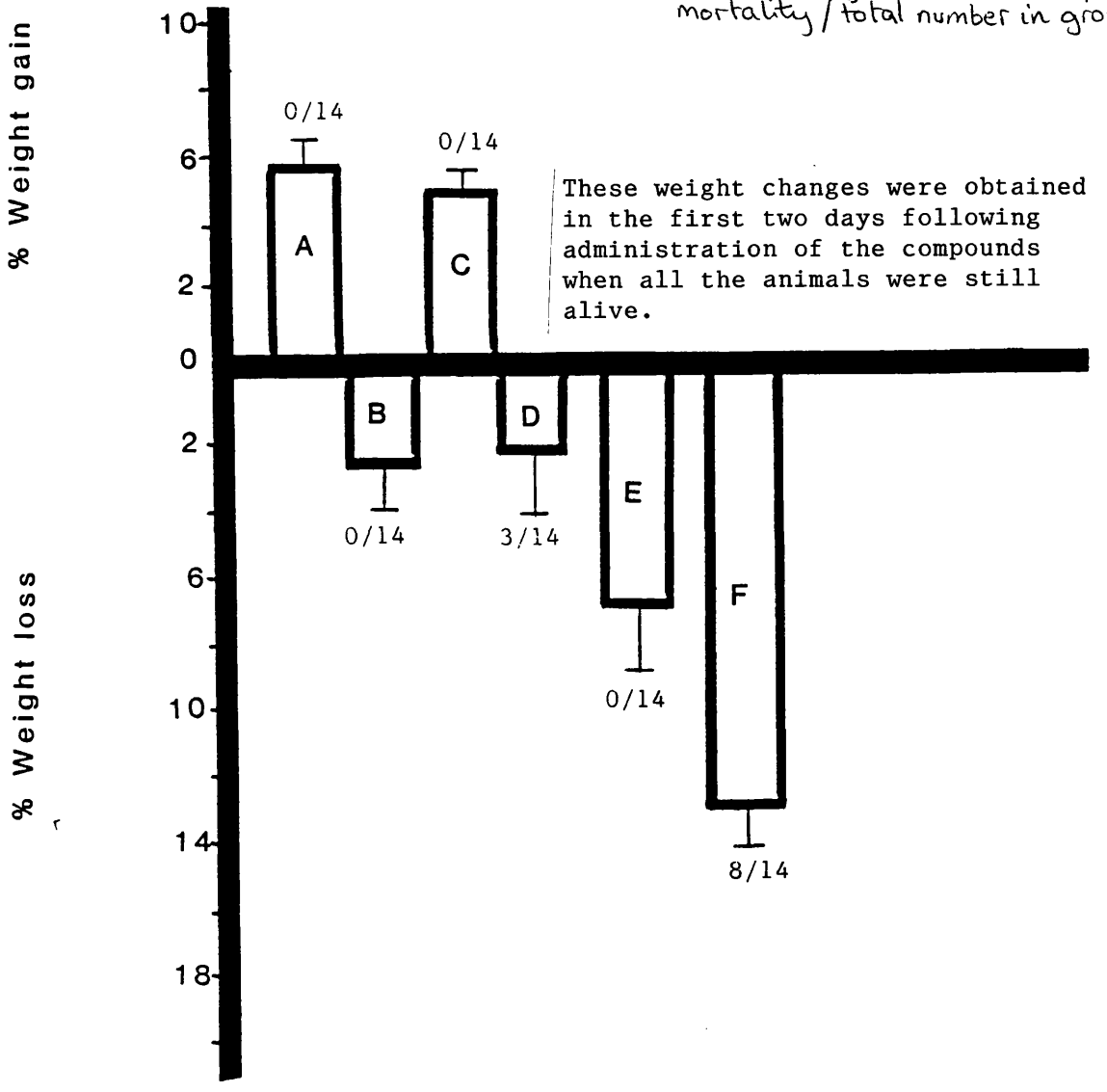
BCNU pretreatment potentiated weight loss at the higher dose of paraquat and produced a significant weight loss at the lower dose.

Therefore, from these results, BCNU appeared to potentiate the toxicity of paraquat in vivo in mouse. Pathological inspection of fatally intoxicated animals revealed plum-coloured oedematous lungs with haemorrhagic patches. It was not possible to state with any great objectivity whether these consequences of paraquat treatment were enhanced in animals which had been given BCNU prior to the paraquat. Such pathological signs were not observed in BCNU pretreated animals in the absence of paraquat.

A : Control animals (vehicles only).
B : BCNU (35 mg / kg).
C : 12. 5 mg paraquat ion / kg.
D : 25. 0 mg paraquat ion / kg.
E : BCNU + 12. 5 mg paraquat ion / kg.
F : BCNU + 25. 0 mg paraquat ion / kg.

Fig. 3/ 4. BCNU and paraquat co-treatment study in mice: mortality and weight changes

BCNU (35 mg/ kg i. p.) was given in corn oil 2 h prior to paraquat (12. 5 and 25 mg paraquat ion/ kg s. c.). Animals were weighed twice daily and assessed for toxic signs three times daily. Figures above SE represent mortality / total number in group.



3. 3. f PARAQUAT TOXICITY IN THE RAT : INFLUENCE OF BCNU

Using a parallel procedure to that employed in the studies with mice a maximal non-lethal dose of BCNU was assigned as 30 mg/ kg i. p. in corn oil in rat. Doses above 40 mg BCNU/ kg proved resulted in fatality to all rats (6/ 6 in each group at 60, 80 and 100 mg/ kg). The dose-dependency of BCNU-induced glutathione reductase inhibition in rat lung is shown in fig. 3/ 5.

Paraquat (25 mg paraquat ion/ kg s. c.) resulted in death in 4/ 8 rats whereas 12. 5 mg ion/ kg did not prove fatal to any rats. Toxicity was associated with a dose-dependent weight loss (data not shown).

BCNU administration 2 h prior to paraquat resulted in an apparent enhancement of the effects of paraquat. In BCNU-pretreated animals 12. 5 mg/kg paraquat ion proved fatal to 3/ 8 rats whereas 6/ 8 rats succumbed at the higher dose of paraquat (25 mg/ kg). Paraquat, in control rats, resulted in mortality and weight loss consistent with the previous study.

3.4 THE USE OF BCNU IN THE DEVELOPMENT OF A LUNG SLICE SYSTEM WITH COMPROMISED ANTIOXIDANT DEFENCES

The use of BCNU with lung tissue in the manner described here is novel. In vivo studies are complicated by the dose-limiting toxicity of the compound. A maximal non-lethal dose of the drug results in an inhibition of pulmonary glutathione reductase activity of around 60 % in mice and rats. This is markedly lower than the degree of inhibition obtained in hepatocyte systems ($>$ 90 %).

Several investigations have suggested that a glutathione reductase activity lower than normal is sufficient to maintain glutathione status in the lung and in other organs [117, 192], in the absence of oxidant stress.

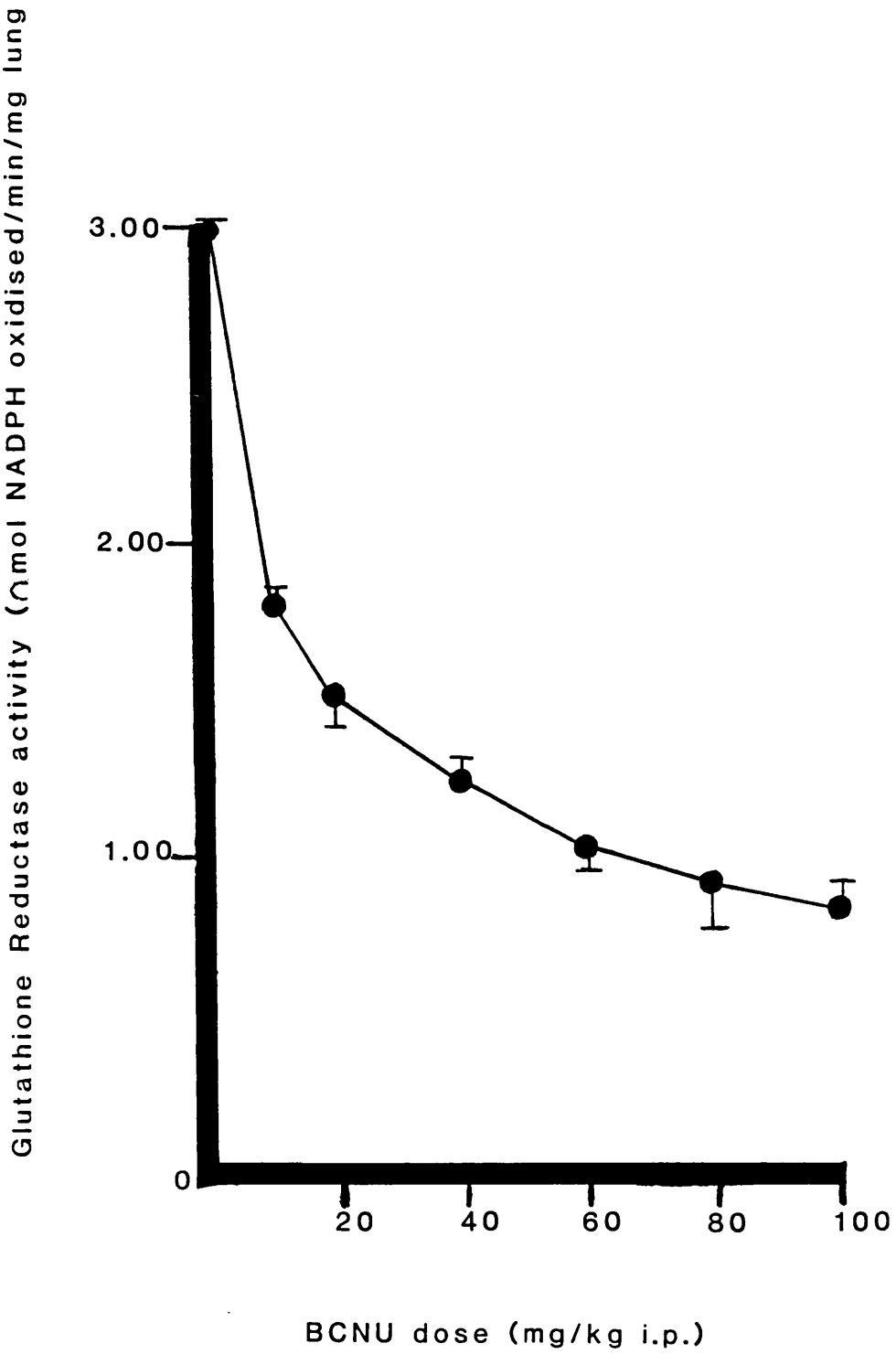


Fig. 3/ 5. Inactivation of glutathione reductase in rat lung with BCNU: dose-dependency

Animals were given BCNU in corn oil via a single i. p. injection. Following sacrifice (2 h), lungs were homogenised as described in Methods and glutathione reductase activities assessed. The values presented are the mean of four animals per data point.

The primary aim of the work presented in this section was to prepare rat lung slices which possessed markedly inactivated glutathione reductase activity with a degree of inhibition comparable to that seen in isolated hepatocytes.

3.4. a PULMONARY GLUTATHIONE REDUCTASE ACTIVITY : CONCENTRATION AND TIME DEPENDENT RESPONSE OF INHIBITION WITH BCNU

Rat lung slices were prepared as described (Methods) and treated with BCNU. Following 60 min incubation slices were removed from BCNU and glutathione reductase activities assessed. Concentrations of BCNU ranged from 40-1500 μM. However, inactivation was near-maximal at 100 μM BCNU. (Fig. 3/ 6). BCNU at 1. 0, 1. 3 and 1. 5 mM failed to increase the extent of inhibition, which was in the region of 75-80 %. Potent inactivation was obtained at 40 μM BCNU. This early work suggested that a concentration of 100 μM was the most potentially useful.

Lung slices were incubated for up to 60 min with 100 μM BCNU. The inactivation was near-maximal at 45 min (fig. 3/ 7). Obviously, it was intended that the incubation time should be minimised to reduce the possibility of unwanted side-effects of the drug.

3.4. b THE EFFECTS OF BCNU ON GSH LEVELS

BCNU (100 μM) caused a depletion of GSH in lung slices over a 5 h period of incubation. This loss of GSH is marked and occurs at early time points (up to 1 h). GSH levels in control slices, in contrast, remain stable over the 5 h (fig. 3/ 8). In order to attempt to prevent this loss of thiols a strategy similar that used in studies with isolated hepatocytes was

Fig. 3/ 6 Glutathione reductase inhibition in rat lung slices: a concentration-response with BCNU

Following 1 h of incubation at 37°, slices were homogenised and glutathione reductase activity assessed. Results are the mean of three experiments. Control activities (lung slices incubated in the absence of BCNU) were 2. 84 nmol NADPH oxidised/ min/ mg lung ± 0. 11 (3).

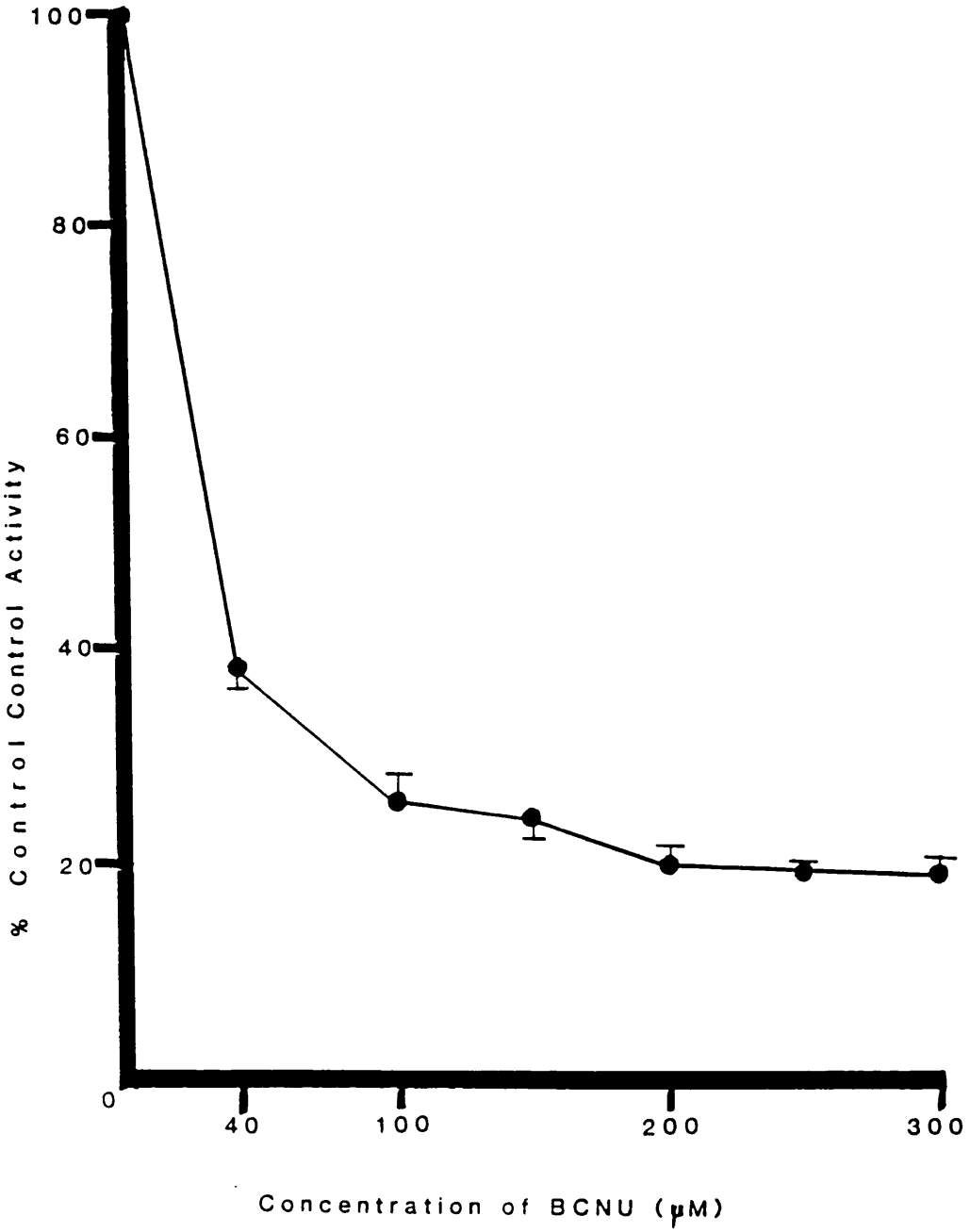


Fig. 3/7.Glutathione reductase activities resulting from treatment with BCNU (100 µM): a time course

Lung slices were incubated in KRB with BCNU (100 µM) for times up to 1 h. Results are the mean glutathione reductase activities in nmol NADPH oxidised/ min/ mg lung ± S. E. (n= 4-6 animals per data point). Control activities were 2. 92 ± 0. 025 (5).

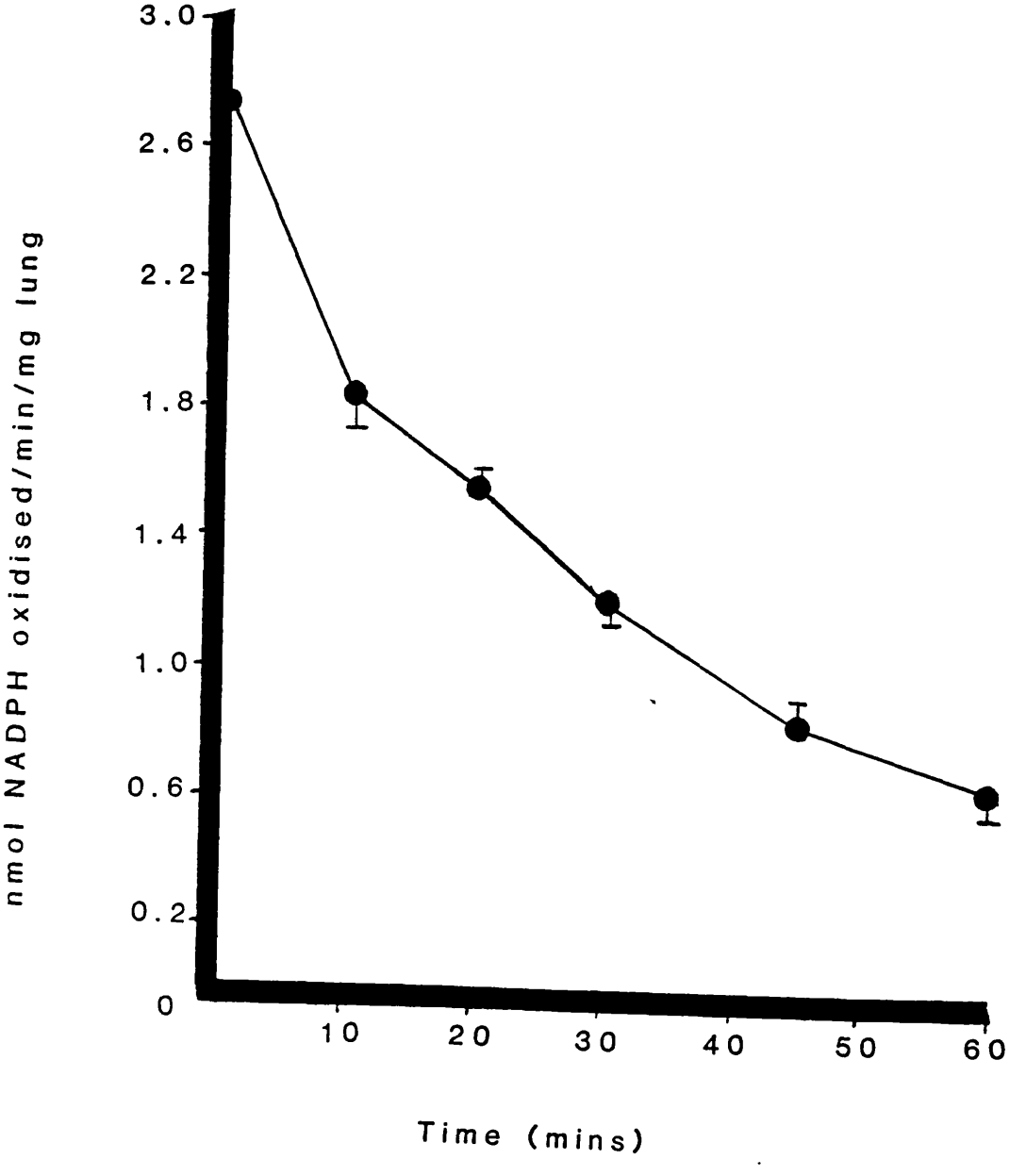
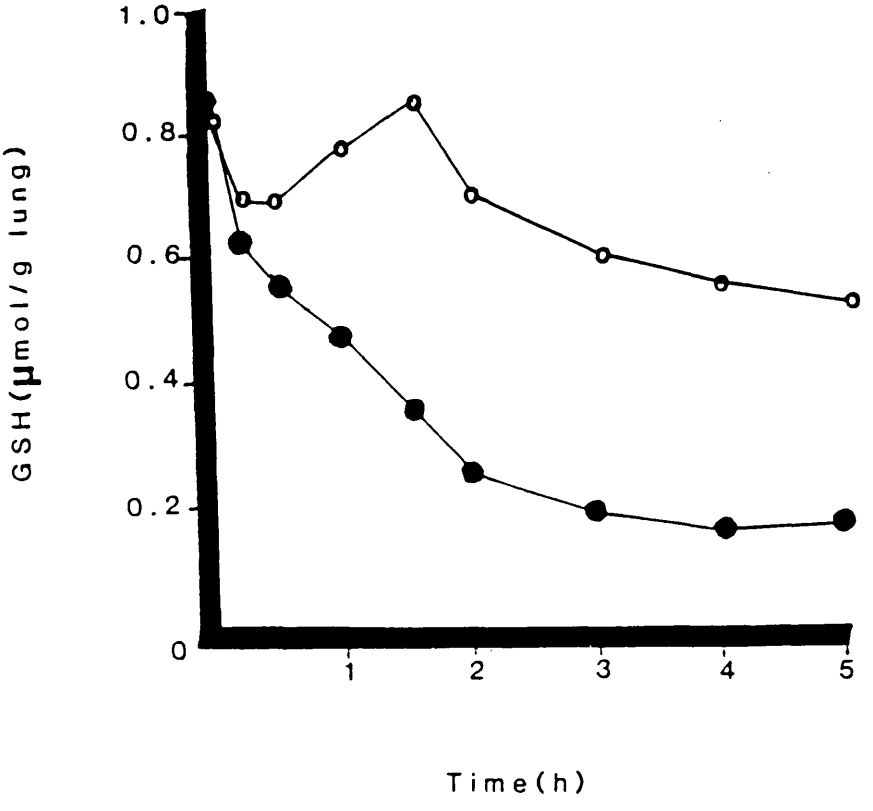


Fig. 3/ 8 GSH levels in lung slices treated with BCNU

The results shown are from one experiment typical of three. Lung slices were incubated in KRB following incubation for 60 min either under control conditions (KRB, 0.3 % DMSO, ○) or with BCNU (100 μM, ●)for up to 5 h.



employed. Studies employed BCNU (100 μM) in the presence of Krebs-Ringer phosphate buffer (KRB) supplemented with Minimal Essential Medium (MEM) amino acids and additionally supplemented with L-cysteine, L-methionine, L-serine, L-glycine and L-glutamine (all at 0. 2 mM). Initial studies with amino acid supplemented KRB indicated protection of GSH levels following incubation with BCNU under a variety of conditions. In one study slices were incubated for 30 min and 90 min with BCNU (100 μM) in the presence and absence of amino acid supplements and sulphydryl levels were in the series : Control (0. 3% DMSO) > BCNU / amino acids / 30min > BCNU / 30 min > BCNU / amino acids / 90 min > BCNU / 90 min. (Data not shown).

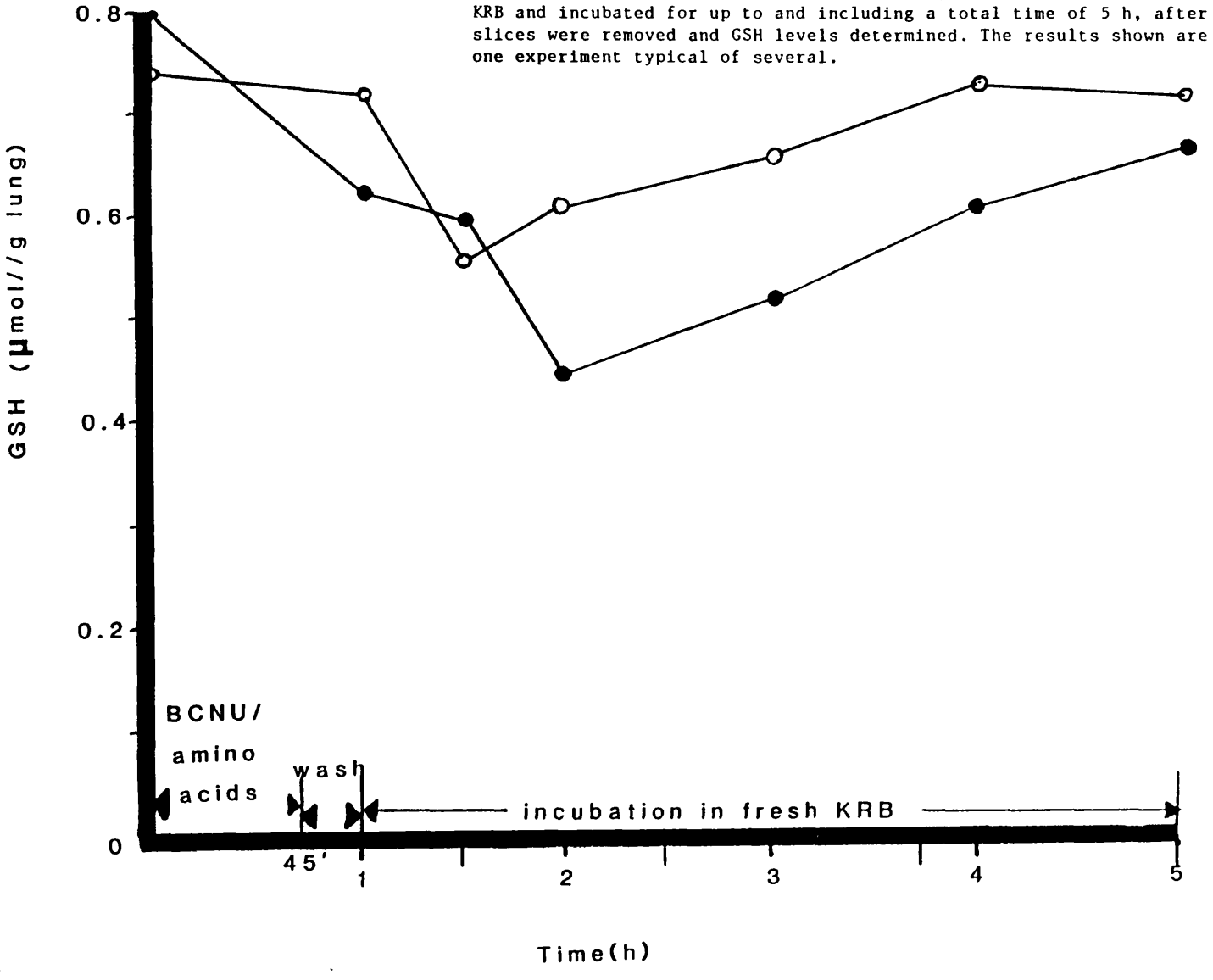
Supplementing KRB with amino acids in the manner described above also protected against extensive loss of thiols in lung slices incubated with BCNU for periods up to 3 h. (Data not shown).

Since the studies of glutathione reductase inactivation described above had suggested optimal incubation conditions of 100 μM BCNU and a time of 45 min, the effects of the amino acid supplements were assessed under these conditions. A small reduction in GSH levels was observed (approximately 15 %) though the GSH remained stable at this slightly lower level up to, and including, 4 h (fig. 3/ 9). A washing step was performed following incubation with BCNU to avoid quenching of the fluorometric assay (for thiol determination) by the amino acids e. g. L-cysteine. This entailed a gentle shaking in KRB (without amino acids). Slices were placed in fresh KRB (in the absence of amino acids and BCNU) and reincubated at 37^{o}.

One study employed incubation either with MEM supplemented KRB or with buffer supplemented solely with N-acetylcysteine (1 mM). Assessment of GSH levels indicated that N-acetylcysteine was not effective in

Fig. 3/ 9 . GSH levels in rat lung slices incubated with BCNU: effect of amino acids

Rat lung slices were incubated either as controls (0. 3 % DMSO; ○) or with BCNU (100 μM in the presence of amino acid supplemented KRB; ●). Following the appropriate pretreatment, lung slices were replaced in fresh KRB and incubated for up to and including a total time of 5 h, after which slices were removed and GSH levels determined. The results shown are from one experiment typical of several.



maintaining thiols, (data not shown) in contrast to the MEM amino acid supplements.

All lungs were perfused in situ with KRB, pH 7. 4, using a re-circulating buffer system as described in Methods in order to minimise erythrocyte contamination.

The following results represent a more detailed characterisation of the compromised lung slice model.

3. 4. c COMPROMISED SLICE GLUTATHIONE REDUCTASE ACTIVITY

The incubation of perfused rat lung slices with BCNU (100 μM) in the presence of amino acid-supplemented KRB for 45 min in a shaking water bath resulted in marked inactivation of glutathione reductase activity. Following washing and placing in fresh KRB, the activity of the enzyme remained profoundly inhibited ($>$ 75 %) over 4 h of incubation (fig. 3/ 10).

There was no loss of enzyme activity in control slices incubated in the absence of BCNU over this time period.

3. 4. d THIOL LEVELS IN COMPROMISED LUNG SLICES

Compromised slices showed a small diminution of thiols (c. a. 15%), though levels remained stable over the 4 h incubation period investigated (fig. 3/ 11). Despite use of an amino acid supplemented incubation medium this small loss of thiols could not be prevented,though the significance of this modest depletion is not clear.

3. 4. e ATP LEVELS IN BCNU COMPROMISED SLICES

Compromised slices exhibited only small perturbations of ATP

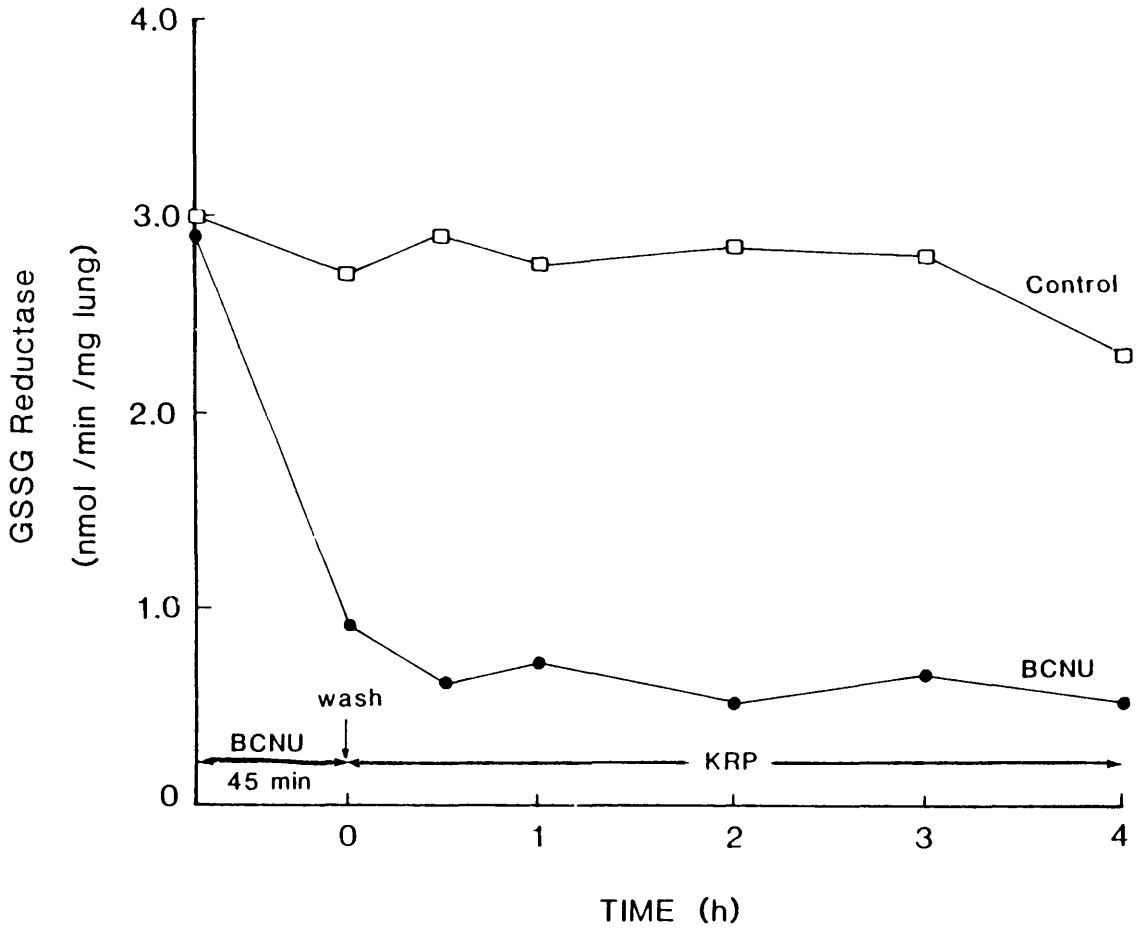


Fig. 3/ 10. Effects of BCNU pretreatment on glutathione reductase activity

Lung slices were treated with DMSO (□) or with BCNU (0. 1 mM in amino acid-supplemented KRB, ●) for 45 min, then washed in KRB and replaced in fresh medium and incubated for up to 4 h. Results shown are from one experiment, typical of three.

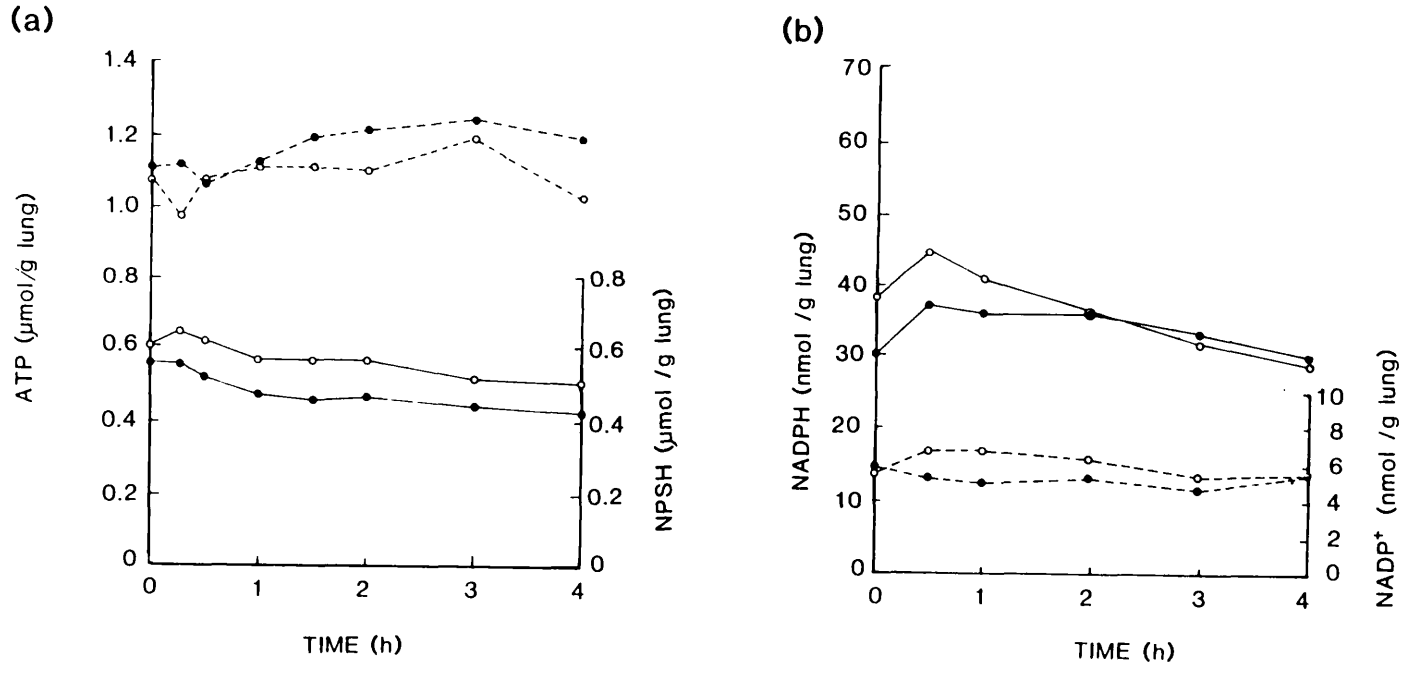


Fig. 3/ 11. Levels of NPSH, ATP and NADP(H) in BCNU compromised slices

Lung slices were incubated either as controls (O) or with BCNU (●). After 45 min incubation and washing with fresh KRB, the slices were incubated in KRB for periods up to 4 h and NPSH (-), ATP (---) (a), NADPH (-) and NADP (---) (b) were measured. The results represent the means of at least seven determinations per data point. Control slices were incubated with 0·3% DMSO (w/v).

The NADP(H) data was obtained by Aishah Adam [231].

levels. No significant difference existed between control and compromised slices at any time up to and including, 4 h of incubation following BCNU pretreatment (fig. 3/ 11).

Measurements of ATP levels have been used in previous biochemical investigations of toxicity as an index of cell viability [209]. The absence of significant modulation suggests that BCNU pretreated lung slices maintain functional integrity and this is in accord with the absence of significant effects on total glucose oxidation (3. 4. f).

3. 4. f D-[^{14}C(U)]-GLUCOSE OXIDATION

The ability of the BCNU compromised lung slice to oxidise glucose was employed as a biochemical assessment of viability. Following pre-treatment of lung slices with BCNU, slices were incubated in fresh KRB for up to 4 h in the presence of 1 μCi D-[^{14}C(U)]-glucose.

As a control i. e. an index of toxicity for comparative purposes, control lung slices were incubated with rotenone (100 μM), an inhibitor of the mitochondrial electron transport chain.

Clearly, glucose oxidation was linear with time in control and in BCNU compromised lung slices. In addition, the rates of oxidation were virtually identical. Glucose oxidation was virtually abolished in slices incubated with rotenone (fig. 3/ 12).

Oxidation of D-[^{14}C(U)]-glucose gives an assessment of total glucose oxidation. Though its use affords only a broad indication of viability, the results presented suggested that BCNU pretreatment does not impair the ability of the lung slices to utilise glucose.

Using this method, preliminary work had shown that BCNU (75 and 100 μM) had little effect on glucose oxidation. BCNU (1 mM) also failed to markedly reduce $^{14}CO_2$ production (fig. 3/ 13). The lower concentrations

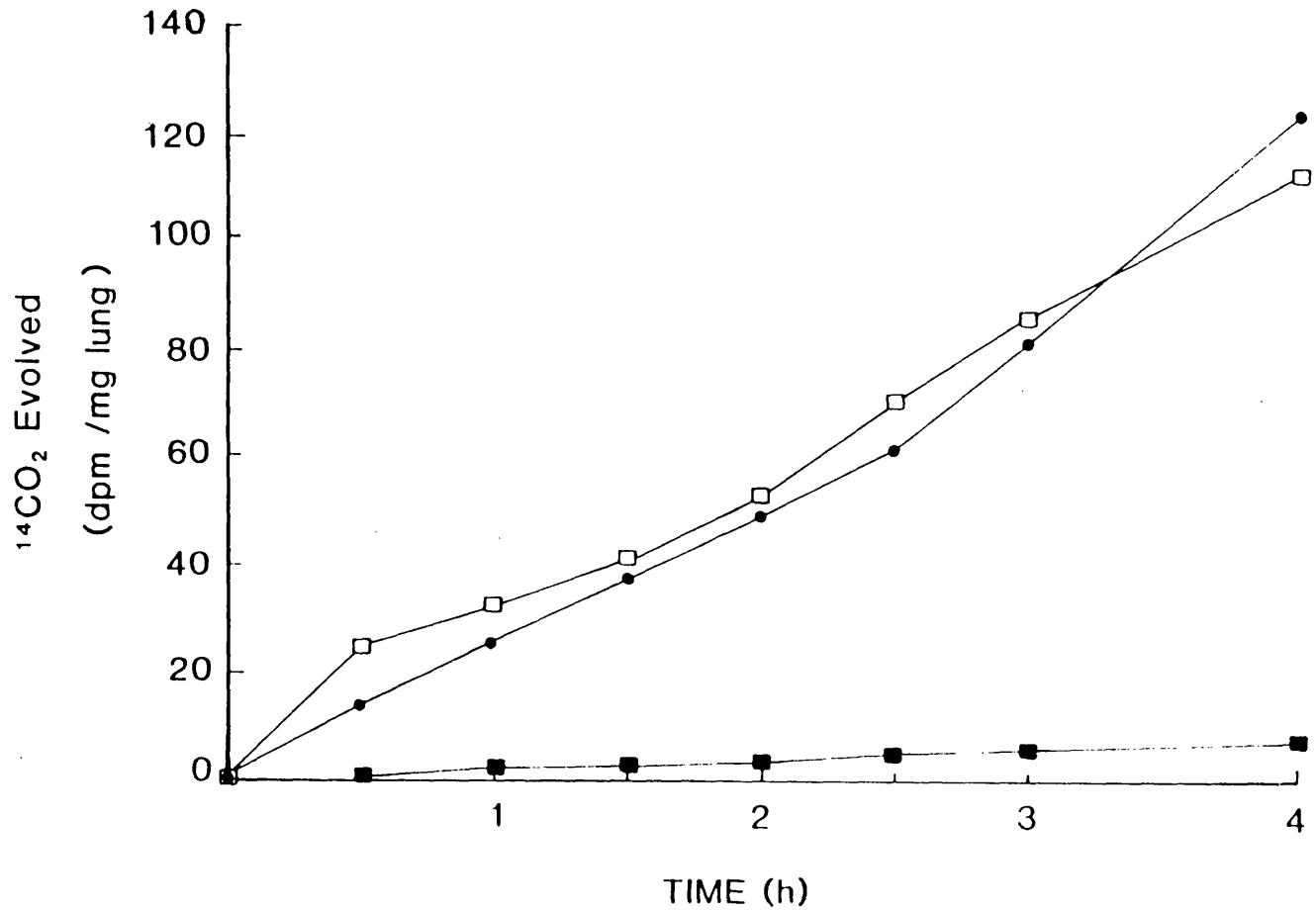
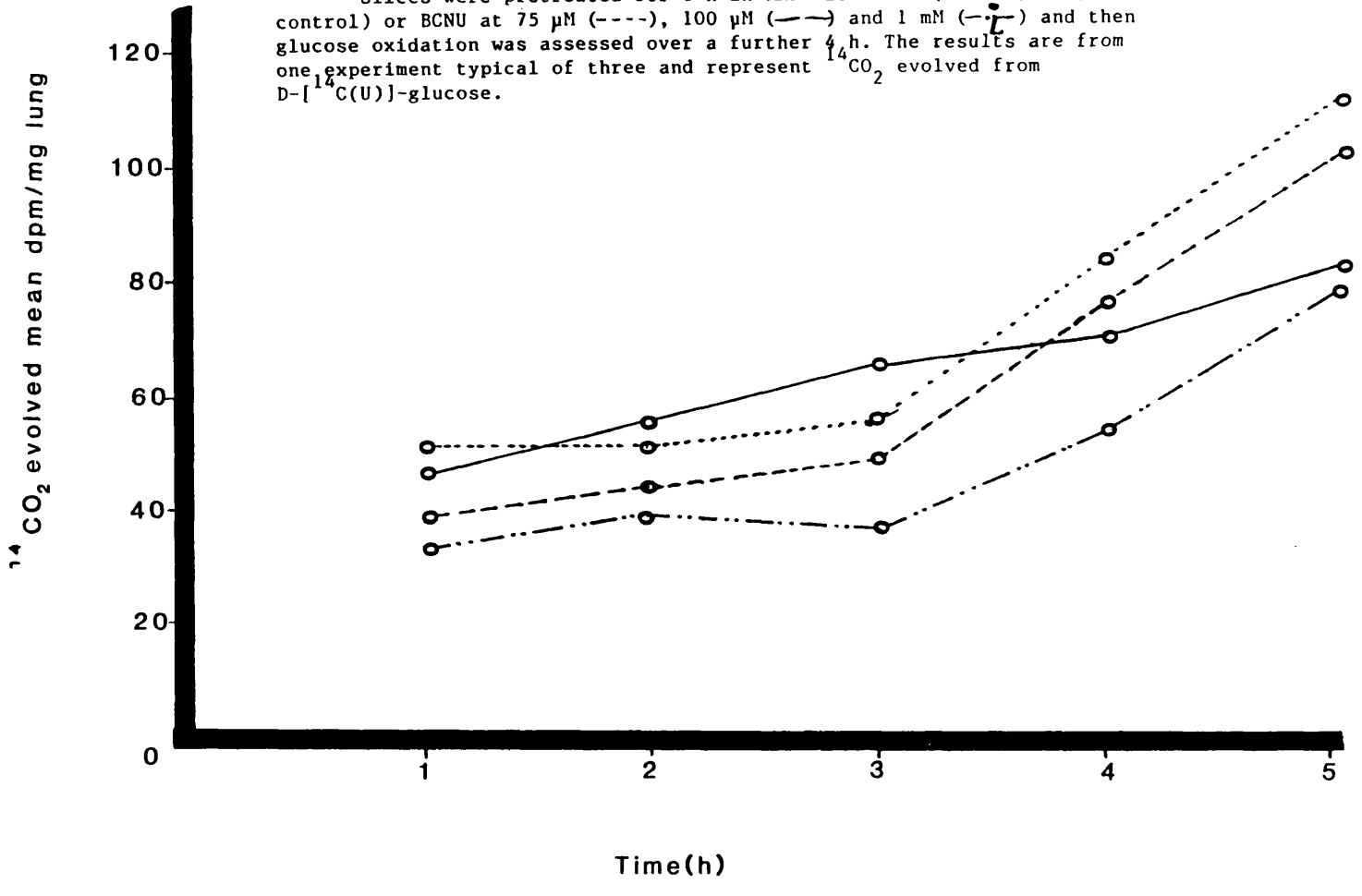


Fig. 3/ 12. D-[^{14}C(U)]-glucose oxidation in compromised slices

Control (□) and compromised slices (●) were prepared as described previously. . In addition, a set of control slices was treated with rotenone (0. 1 mM; ■ $^{14}CO_2$). Results are the means of three experiments. The basal amounts of $^{14}CO_2$ collected at 0 h have been subtracted from the subsequent means. Standard errors have been omitted since they were small i. e. < 10 %. (n= 4). Rotenone was added in DMSO (0·3% final concentration of DMSO).

Fig. 3/14. Glucose oxidation in rat lung slices following incubation with BCNU

Slices were pretreated for 1 h in KRB with DMSO (0.3 %; — ; control) or BCNU at 75 µM (----), 100 µM (— —) and 1 mM (— · —) and then glucose oxidation was assessed over a further 4 h. The results are from one experiment typical of three and represent $^{14}CO_2$ evolved from D-[^{14}C(U)]-glucose.



of BCNU appeared to slightly enhance glucose oxidation at the later time points. The biological relevance and origin of this effect is unknown.

DISCUSSION

BCNU was an effective inhibitor of glutathione reductase *in vivo* and *in vitro* in these studies. BCNU is generally accepted to be a relatively specific inhibitor of the flavoenzyme. However, recent *in vivo* studies suggest effects on other enzymes [210, 211] including lactate and malate dehydrogenases, creatine kinase and hepatic esterases. Such effects would presumably result from the carbamoylating activity. Considerations of the influence of BCNU should not, therefore, exclude the possibilty of actions other than that of inhibition of glutathione reductase.

The magnitude and time course of reductase inactivation were in good general agreement with literature results in rat [192] and mouse [193]. Following administration of the maximal nonlethal dose of the compound, a preferential inhibition of pulmonary glutathione reductase activity was observed after 24 h compared to hepatic activities in both species. In these studies the nitrosourea was administered 2 h prior to dosing with paraquat. Since paraquat is known to be also toxic to the liver and kidney [126], the possibility of having potentiated the effects in an extrapulmonary site cannot be excluded. Other studies (data not shown) involved pretreating mice with BCNU 24 h prior to paraquat administration. At the time of dosing with paraquat pulmonary glutathione reductase is inhibited to a greater extent than in the liver (fig. 3/ 3). However, paraquat mortality was not modulated compared to BCNU treatment 2 h prior to paraquat administration. This tends to support the conjecture that BCNU is potentiating the effects of paraquat in the lung.

In vivo lethality studies revealed a similar maximal nonlethal

dose of BCNU (35 mg BCNU/ kg) to that previously reported in mice [117]. The Wistar rat appeared to be less susceptible to the toxic effects of BCNU compared to data obtained by other workers in the F344 rat [192]. In the paraquat and BCNU cotreatment studies in rat a maximal nonlethal dose of 30 mg/ kg nitrosourea was employed (compared to an LD50 of 17 mg/ kg in F344 rat [192]). At higher doses of the compound, similar toxic signs were observed to those reported previously in rat, mouse, and other experimental species.

BCNU appeared to enhance paraquat toxicity to mouse and rat, based on the results of initial weight loss and also lethality, at two doses of paraquat. It could be suggested that these results simply reflected the effects of two extremely toxic agents acting independently.

It is tentatively suggested that the toxicity of paraquat is being enhanced by the dosing of animals 2 h previously with the nitrosourea since the potentiation of weight loss was dose-dependent with respect to paraquat.

The use of BCNU with rat lung slices in the manner described is novel. The lung slice represents a convenient system for study,since preparation is simple and rapid. Lung slices have been successfully employed in a variety of biochemical investigations of pulmonary toxicology. Their use presents some drawbacks: Normal blood-air relationships are lost and portions may become anoxic. However, they have an advantage over isolated cell preparations in that cell-cell contacts are maintained. Much information with regard to the biochemical effects of of paraquat has been obtained in lung slices particularly pertaining to uptake [129, 134] and effects on intermediary metabolism [212].

The primary aim of the <u>in vitro</u> work was satisfied i. e. the

preparation of rat lung slices with a compromised glutathione reductase activity. The development of the lung slice model has been described. Following perfusion of lungs, incubation of lung slices with BCNU (100 μM) in the presence of amino acid supplemented KRB for 45 min at 37°C and then washing in fresh KRB (without amino acids and BCNU) resulted in approximately 80 % inhibition of glutathione reductase activity. This loss of activity was accompanied by only modest decreases in GSH, normal levels of ATP and pyridine nucleotides and unchanged rates of glucose oxidation, when compared with control slices. The use of amino acid supplements is similar to the compromised hepatocyte model [206, 203] and ameliorates grievous loss of thiols. BCNU has been shown to deplete cytosolic and and mitochondrial pools of GSH in isolated rat hepatocytes [205] and this limitation was overcome by allowing resynthesis by incubation with amino acid rich Krebs-Heinseleit medium according to the specifications of Seglen [213]. In the studies with lung slices it was not possible to completely recover the GSH: Incubation with amino acids reduced the depletion. This finding possibly reflects a lower rate of de novo GSH biosynthesis in lung compared with the liver. It is believed that the minimal reductions of GSH incurred, are of only limited toxicological significance since several organs e. g. liver can withstand very severe GSH depletions and continue to function normally and without discernible evidence of toxic insult [214].

The BCNU compromised lung slice model represents a useful model for the study of oxidative stress. Since the lung slice is heterogeneous in terms of cell types present, this model offers potential for the study of the cellular selectivity of toxic agents.

CHAPTER 4 - BIOCHEMICAL AND FUNCTIONAL MARKERS OF TOXICITY : THE EFFECTS OF TOXICANTS IN BCNU COMPROMISED RAT LUNG SLICES

4. 1 AIMS

The aims of the work presented in this chapter were as follows :

a) to assess whether glutathione reductase inactivation potentiates the toxicity of paraquat and a number of other toxicants.

b) to compare biochemical and functional markers of toxicity in the compromised lung slices in an attempt to distinguish the specificity of particular toxicants for certain cell types and c) to gain greater insight into biochemical mechanisms of toxicity in lung tissue and the distribution of GSH in different cell types

4. 2. INTRODUCTION

Paraquat is proposed to be toxic to mammalian lung via a mechanism involving redox cycling and oxidative stress [45]. Paraquat is not metabolised, other than its ability to undergo one electron reduction catalysed by NADPH-cytochrome P-450 reductase. Oxidative stress is presumed to be responsible for the cytotoxicity of diquat and certain members of the quinone family of compounds [215]. Quinones which have been characterised in toxocological studies include the antitumour agents mitomycin C and adriamycin [216, 205], and menadione [207, 217].

Another member of the quinone family is of particular interest in the work presented in this chapter : 2, 3-dimethoxy-1, 4-naphthoquinone. This compound is a 'pure' redox cycler, undergoing the one electron reduction. The agent does not react directly with GSH in contrast to menadione which arylates, in addition to possessing the ability to redox

cycle [218]. In studies in isolated hepatocytes, BCNU pretreatment of cells potentiated loss of GSH, ATP and loss of viability resulting from incubation with 2, 3-dimethoxy-1, 4-naphthoquinone [219].

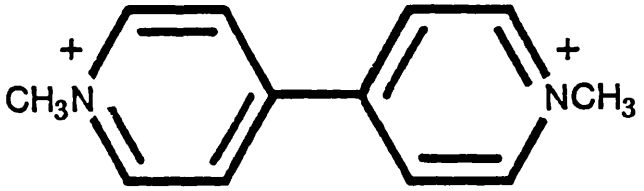
The GSH depletion associated with diquat is markedly potentiated in BCNU compromised hepatocytes along with loss of cellular viability [206].

Histologically, paraquat in vivo appears to damage, initially the type I and II cells of the alveolar epithelium [128]. Cellular selectivity of toxic agents presents some difficulties in biochemical investigation of mechanisms of toxicity in a heterogeneous organ such as the lung. It has been suggested [220] that biochemical changes, though critical in a target cell population, could remain undetected as a result of contributions from cell types other than those affected by the toxicant. Functional assessments offer a strategy whereby such difficulties may be circumvented and additional insights gained [221].

A functional marker of the integrity of the alveolar epithelium involves the ability to accumulate oligoamines such as putrescine, spermine and spermidine (fig. 4/ 1). These endogenous amines and paraquat are accumulated into lung tissue via an energy dependent process [129]. Recent work has shown that putrescine uptake is localised in the type I and II cells of the alveolar epithelium and the Clara cells [135]. Early studies revealed a loss of ability to accumulate spermidine into lung slices prepared from rats given paraquat [132]. More recently, polyamine uptake has been used to investigate cellular selectivity of a variety of toxic agents [221].

The uptake of 5-hydroxytryptamine (5-HT) has similarly been used to assess endothelial function [221]. Decreased 5-HT uptake has been observed following exposure to hyperoxia [222] and α-naphthylthiourea [152]. Uptake and processing have been localised in the endothelium [223, 224].

Fig. 4/1 Structures of Paraquat and some endogenous oligoamines.



paraquat

$NH_2(CH_2)_4NH_2$ putrescine

$NH_2(CH_2)_4NH(CH_2)_3NH_2$ spermidine

$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ spermine

The metabolism has been studied mainly in isolated perfused lung from various species. Alabaster and Bakhle [225] reported that 92 % of 5-HT perfused through the rat lung in vitro was removed and metabolised to 5-hydroxy-indolyl-acetic acid. Junod [223] revealed that 5-HT was accumulated and metabolism was intracellular. Uptake of 5-HT into lung slices has been investigated by Lock et al. [226] and Wikberg and Hede [227].

The work presented in this chapter includes measurements of GSH and ATP levels, rates of glucose oxidation, incorporation of acetate into fatty acids along with uptake of spermidine, putrescine and 5-HT. These biochemical and functional markers were assessed in an attempt to investigate the role of glutathione reductase in the effects of the toxicants paraquat, nitrofurantoin and 2, 3-dimethoxy-1, 4-naphthoquinone.

4. 3 RESULTS

4. 3. a PRELIMINARY WORK IN CONTROL LUNG SLICES : EFFECTS ON GSH

Paraquat (100 µM) and diquat (100 µM) produced no depletion of pulmonary GSH, over 4 h of incubation. Similarly, lung slices incubated in the absence of compound, showed stable GSH levels over the 4 h incubation period investigated. In contrast, nitrofurantoin (100 µM) produced a significant loss of GSH over 4 h. Menadione (100 µM) exerted a more extensive depletion than nitrofurantoin. (Fig. 4/ 2).

4. 3. b GSH LEVELS IN CONTROL AND BCNU COMPROMISED LUNG SLICES : EFFECTS OF PARAQUAT

Fig. 4/ 3 shows the effects of incubation of lung slices with

Fig. 4/ 2. Preliminary studies of modulation of sulphydryls in rat lung slices

Lung slices were incubated as controls (0. 3 % DMSO, O-O) or with paraquat (100 µM, ▲), diquat (100 µM, ●), menadione (100 µM, ✱) or nitrofurantoin (100 µM, ■) for up to 4 h. After incubation the slices were removed and GSH levels assessed. The results represent GSH levels from one experiment of two.

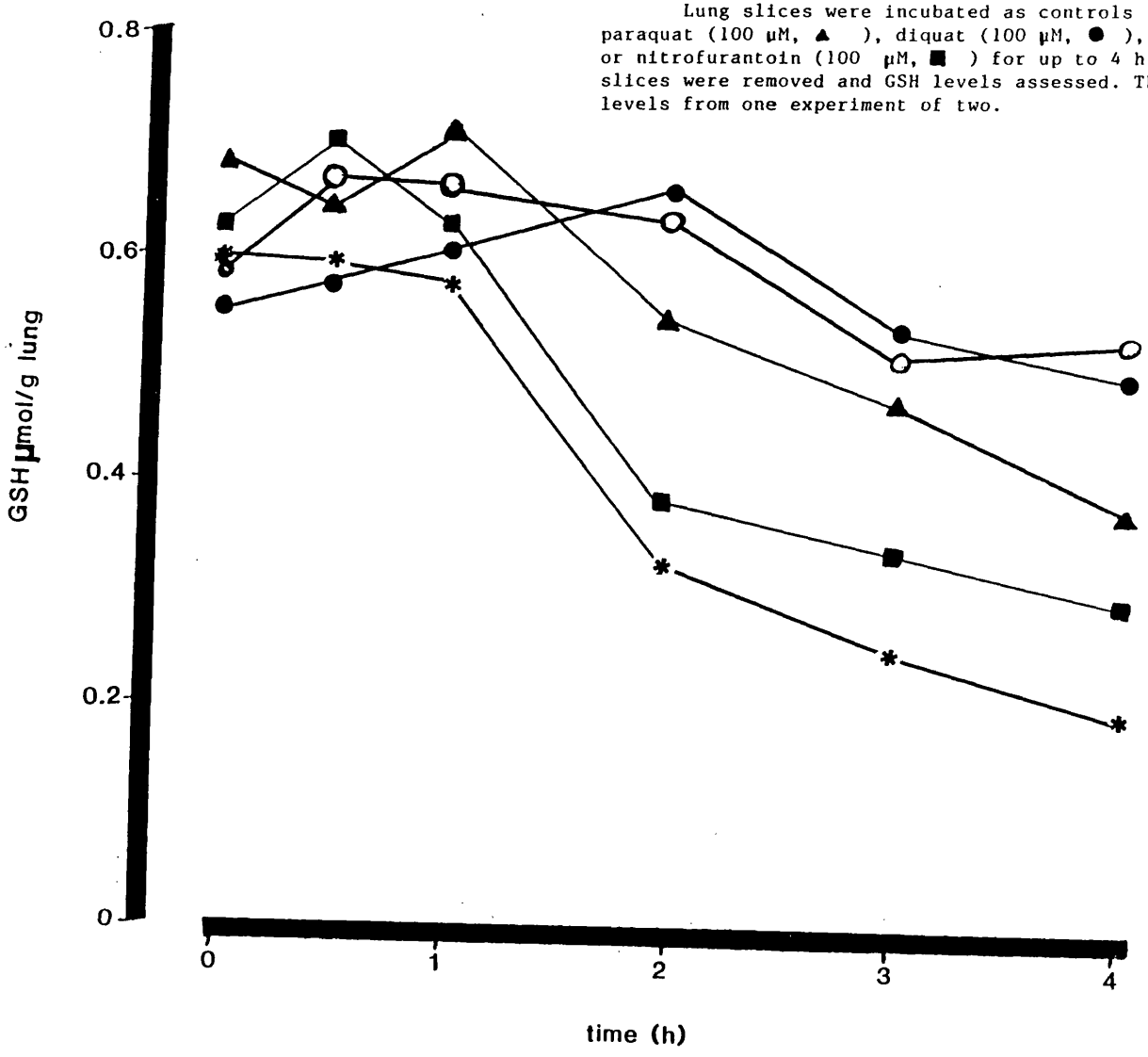
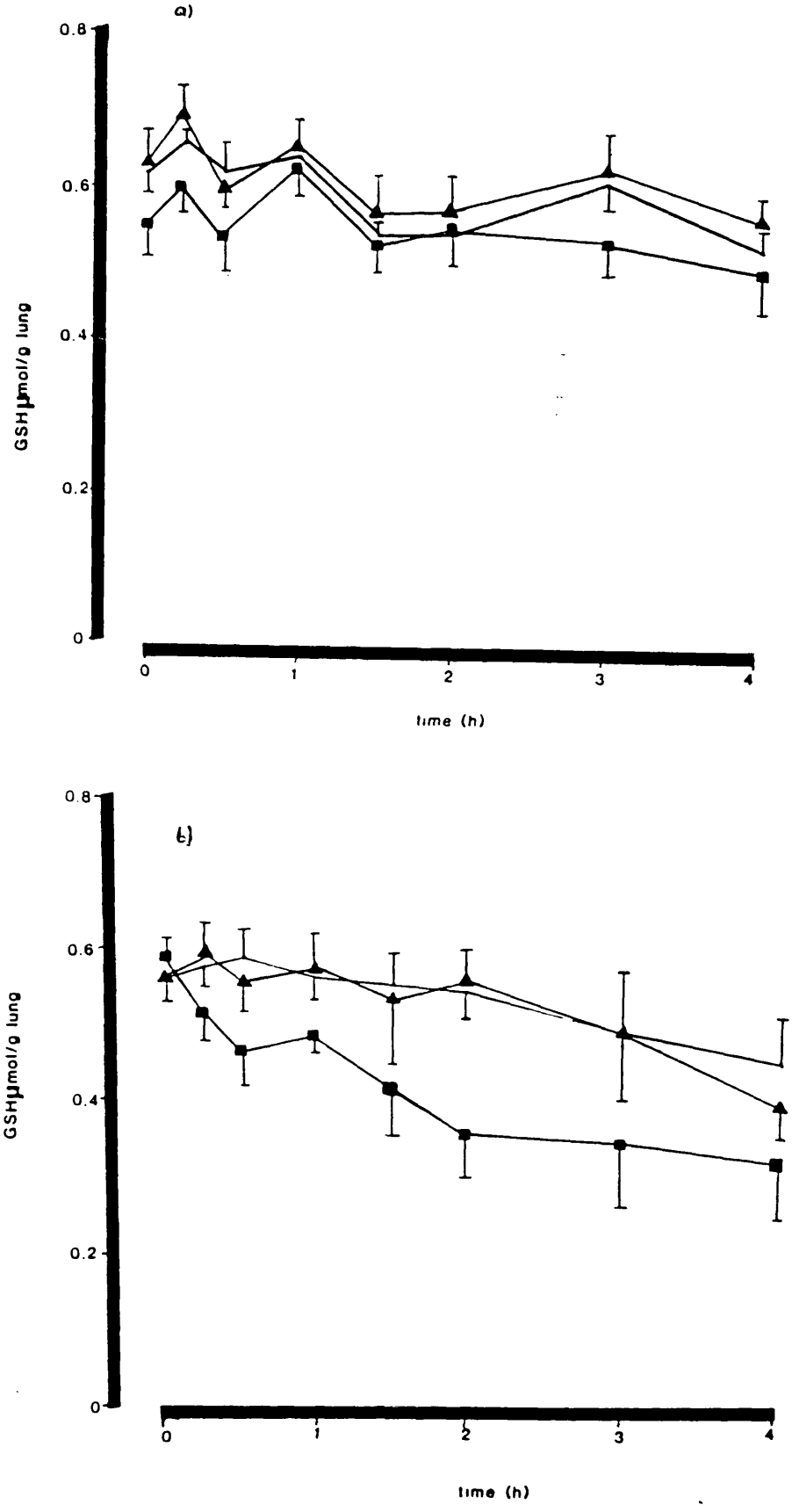


Fig. 4/ 3. Effects of paraquat on GSH levels in control and BCNU compromised lung slices

Control lung slices (a) or BCNU pretreated slices (b) were incubated in KRB either alone (-) or with paraquat (10μand 0. 1 mM; ▲ and ■ , respectively) for up to 4 h. The results shown represent the means of three experiments ± S. E.



paraquat for up to 4h on thiol levels. BCNU compromised and control slices exhibited stable thiol levels in the absence of paraquat.

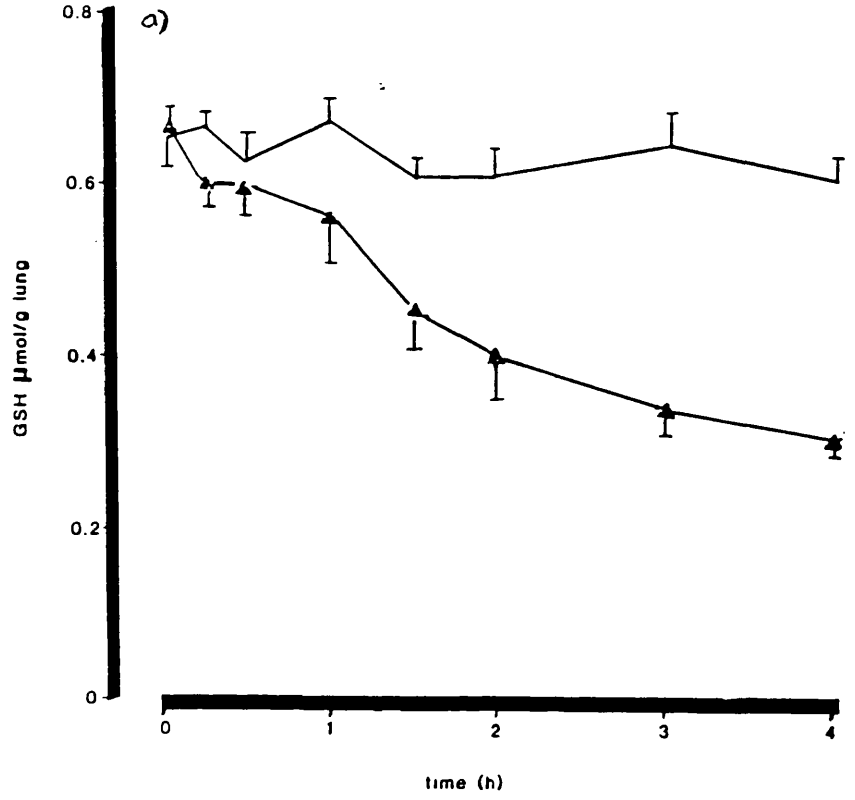
In control slices paraquat (10 and 100 μM) had only minimal effects. In slices possessing an inhibited glutathione reductase activity, these minimal effects were only slightly enhanced. The absence of a marked BCNU-potentiable GSH depletion was also noted at 1, 50 and 500 μM paraquat (data not shown).

4. 3. c 2, 3-DIMETHOXY-1, 4-NAPHTHOQUINONE (2, 3-diOMe) IN CONTROL AND COMPROMISED LUNG SLICES : GSH LEVELS

2, 3-DiOMe produced a time and concentration-dependent GSH depletion (figs. 4/ 4 and 4/ 5) in control and compromised lung slices. The quinone (50 μM) produced a steady loss of GSH which was strikingly potentiated in slices possessing inactivated glutathione reductase activity. This potentiation was particularly evident at early time points (up to 1 h). The extent of the potentiation at 75 μM was not as great as at 50 μM since the effect in control slices was more extensive.

4. 3. d NITROFURANTOIN IN CONTROL AND BCNU COMPROMISED LUNG SLICES : EFFECTS ON GSH

Initial studies revealed that nitrofurantoin (50-500 μM) depletes GSH in a time- and concentration- dependent manner (fig. 4/ 6). Low concentrations (50 μM) had no effect on GSH levels in control slices when compared with control slices incubated in the absence of nitrofurantoin. Following maintenance of stable GSH levels for 1 h, a steady depletion was observed with 100 μM nitrofurantoin. Higher concentrations (200 and 500 μM) depleted thiols to a greater extent. On the basis of these initial studies, 200 and 500 μM nitrofurantoin were selected for investigation in



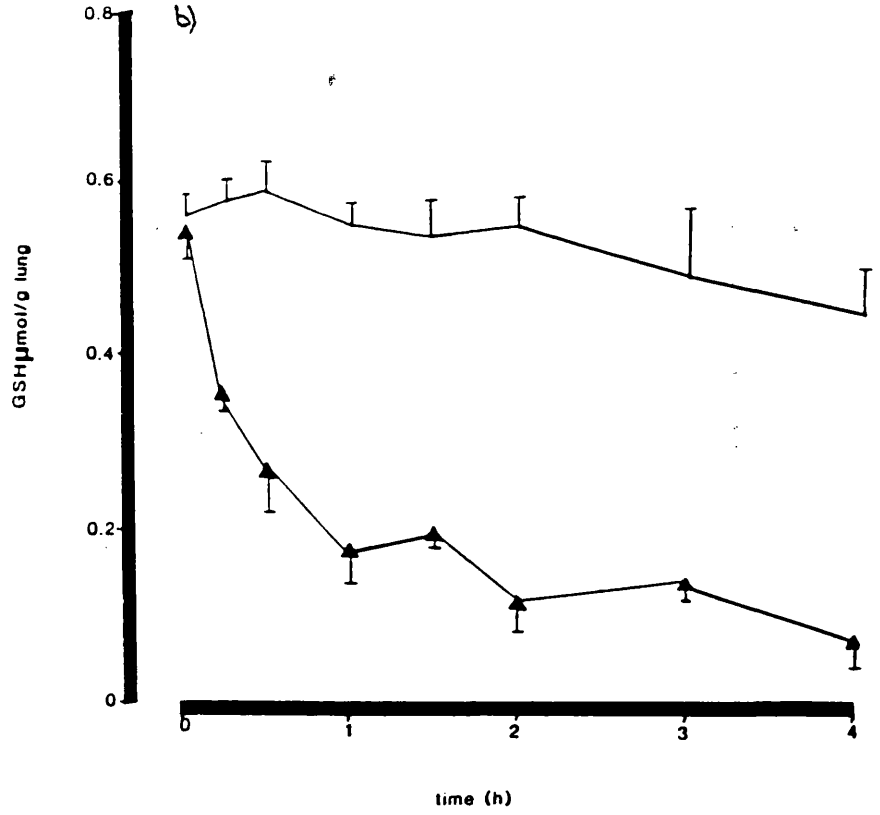
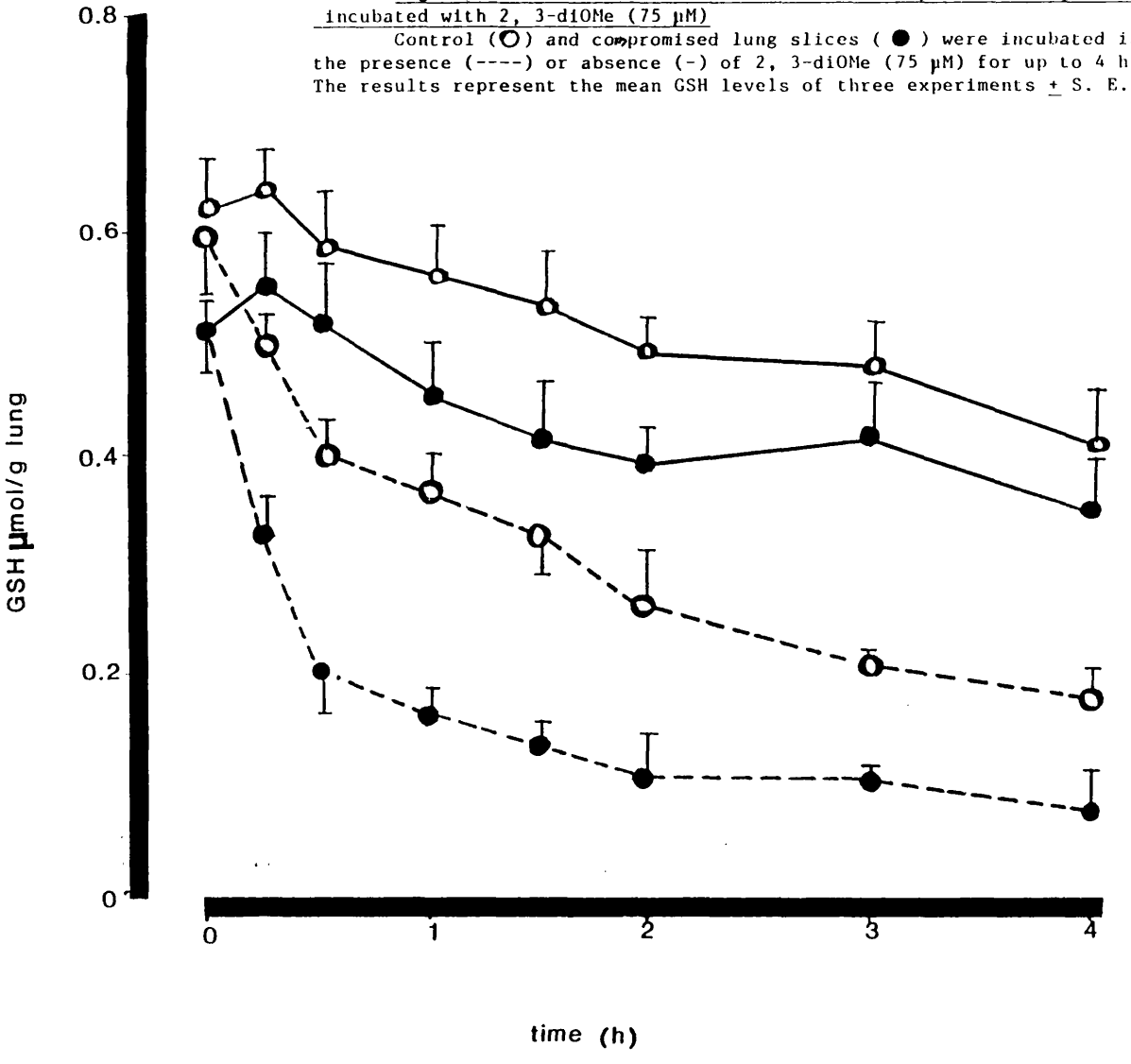


Fig. 4/ 4. GSH levels in control and compromised slices incubated with 2, 3-dimethoxy-1, 4-naphthoquinone

Control (a) or BCNU pretreated lung slices (b) were incubated in the absence (-) or presence (▲) of 2, 3-diOMe (50 µM) for up to 4 h. The results represent mean GSH levels in umol/ g lung ± S. E. of three experiments).

Fig. 4/ 5. GSH levels in control and BCNU compromised lung slices incubated with 2, 3-diOMe (75 µM)

Control (○) and compromised lung slices (●) were incubated in the presence (----) or absence (-) of 2, 3-diOMe (75 µM) for up to 4 h. The results represent the mean GSH levels of three experiments ± S. E.



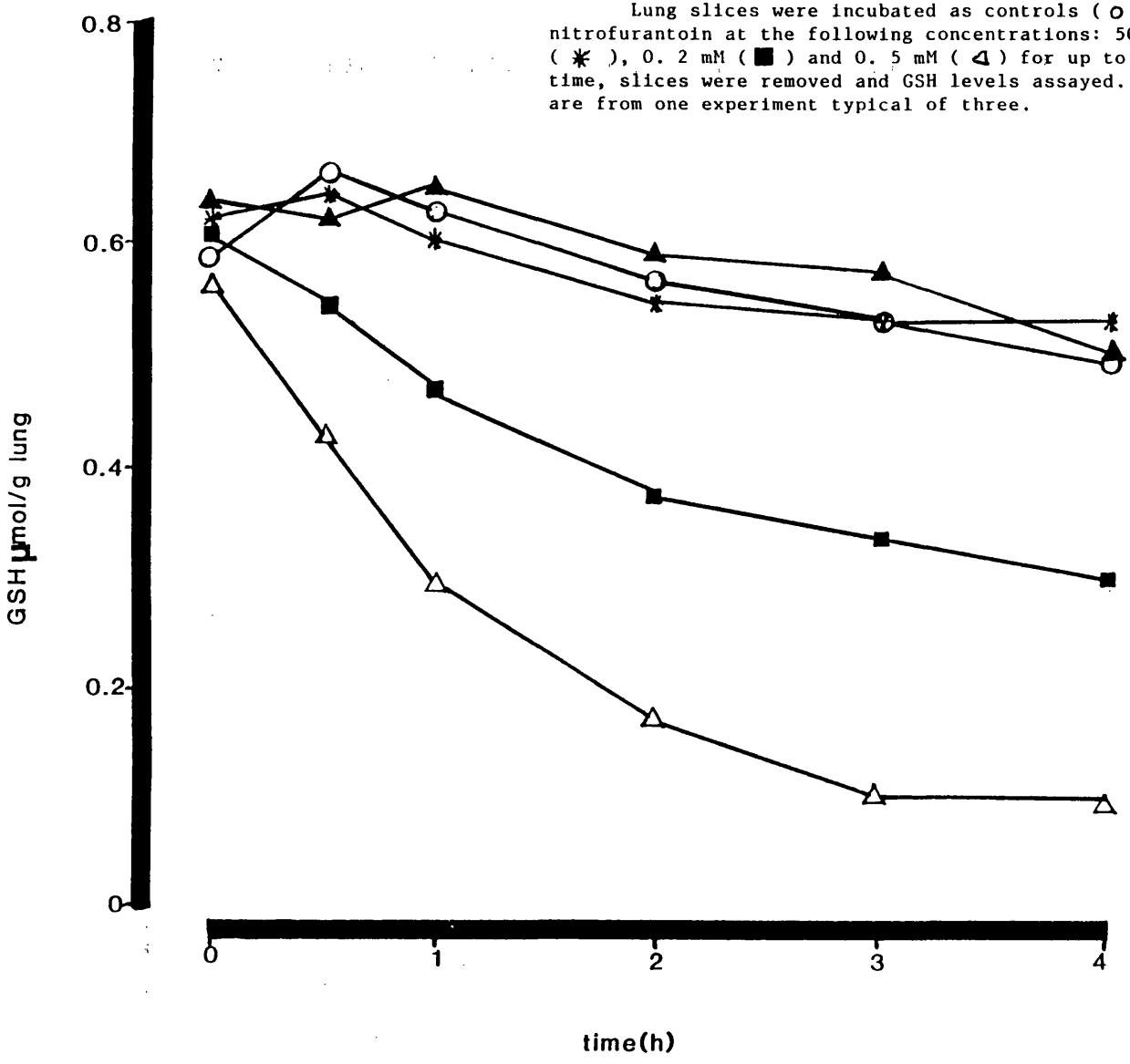


Fig. 4/ 6. Nitrofurantoin in control lung slices: GSH levels

Lung slices were incubated as controls (o , 0. 3 % DMSO) or with nitrofurantoin at the following concentrations: 50 µM (▲), 0. 1 mM (✱), 0. 2 mM (■) and 0. 5 mM (◁) for up to 4 h. At the appropriate time, slices were removed and GSH levels assayed. The results presented are from one experiment typical of three.

BCNU compromised slices.

The GSH depletion induced by 200 and 500 µM nitrofurantoin was potentiated in lung slices possessing an inhibited glutathione reductase activity (fig. 4/ 7). The thiol depletions exerted in control slices in these studies were comparable to those in the initial studies (fig. 4/ 6).

4. 3. e PARAQUAT IN CONTROL AND BCNU PRETREATED SLICES : EFFECTS ON ATP

ATP levels were stable over the 4 h incubation period assessed in control or BCNU pretreated slices incubated in the absence of paraquat. In control slices, paraquat (10 µM) produced minimal effects on ATP levels. At the higher concentrations (100 µM), a small reduction in ATP was observed after 3-4 h of incubation (fig. 4/ 8). The minimal effects of paraquat were not potentiated in lung slices possessing inhibited glutathione reductase activity and the small reduction in ATP observed with 100 µM paraquat was not enhanced (fig. 4/ 8).

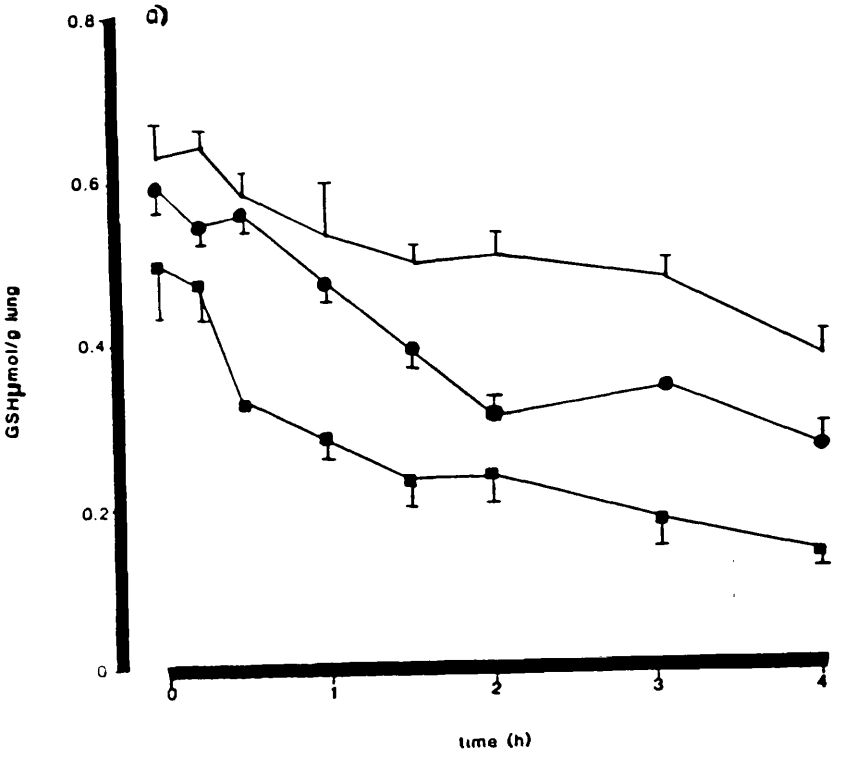
In addition, paraquat at 1, 50 and 500 µM also failed to produce marked effects on ATP levels in either control or compromised lung slices (data not shown).

4. 3. f NITROFURANTOIN IN CONTROL AND COMPROMISED SLICES : ATP LEVELS

Nitrofurantoin (200 and 500 µM) depleted ATP in control slices in a time and concentration dependent manner. In slices with inhibited glutathione reductase, these ATP depletions were potentiated, the effect being most striking at 500 µM at early time points. (Fig. 4/ 9).

Fig. 4/ 7. Nitrofurantoin in control and compromised lung slices -effects on GSH levels

Control (a) or BCNU compromised (b) lung slices were incubated in the absence (-) or presence of 0. 2 and 0. 5 mM nitrofurantoin (● and ■ ,respectively). All incubations were carried out in KRB up to and including 4 h. Following homogenisation, GSH levels were determined. The results represent mean GSH levels in µmol/ g lung ± S. E. (n= 3/ 4).



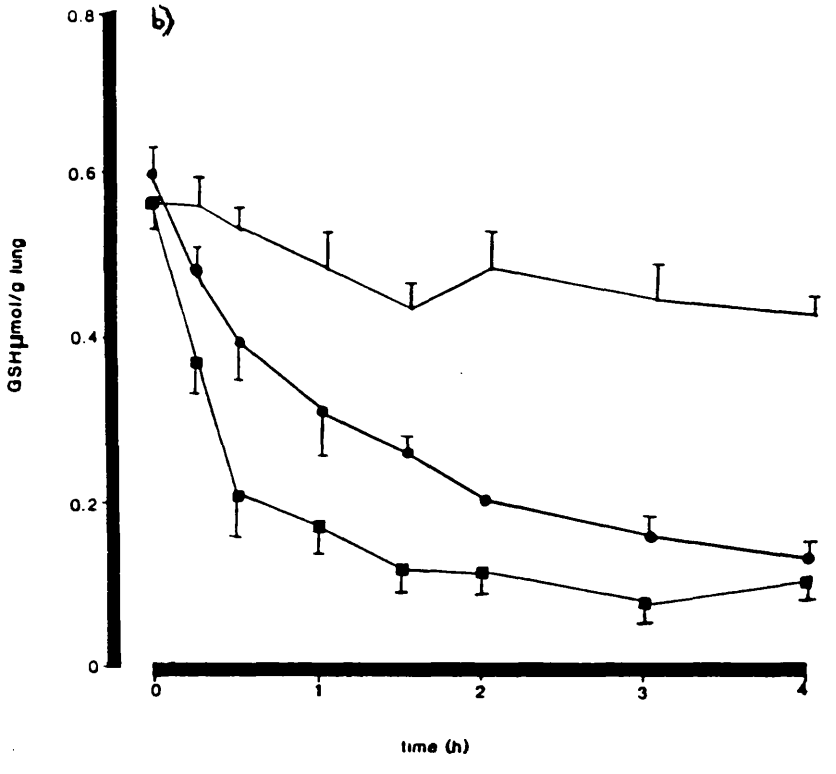
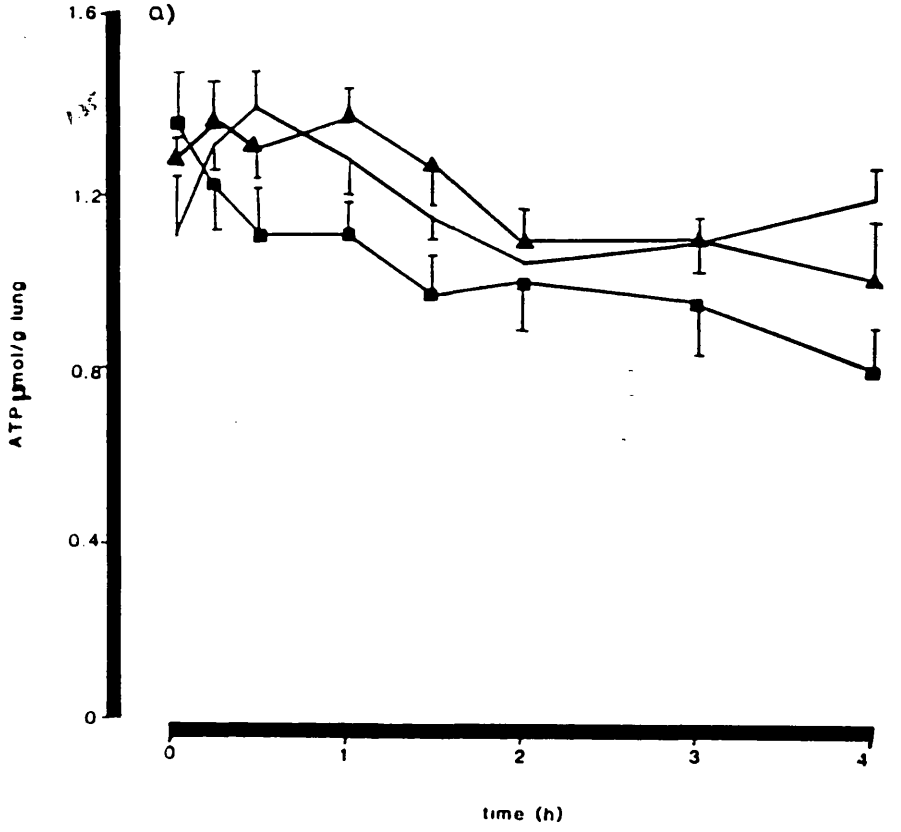


Fig. 4/ 8. ATP levels in control and compromised lung slices: effects of paraquat

Control (a) and compromised lung slices (b) were incubated in the absence (-) or presence of paraquat (10 µM, ▲ and 100 µM, ■) for up to 4 h. ATP levels were determined as described in methods. Results represent mean ATP levels expressed in µmol/ g lung ± S. E. (n = 3).



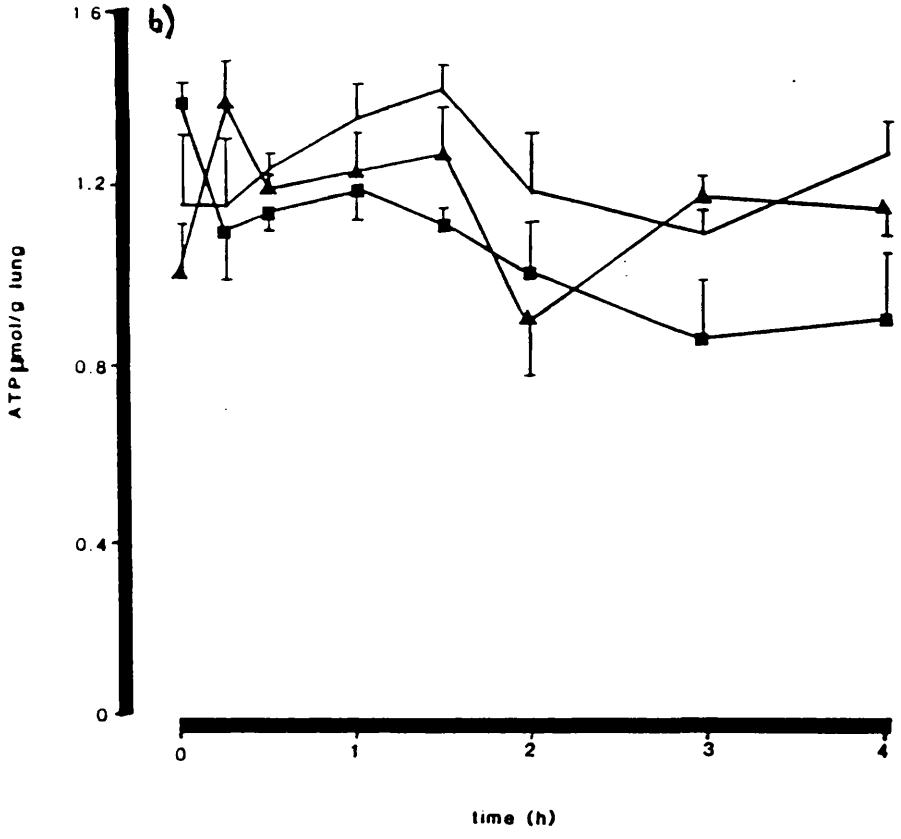
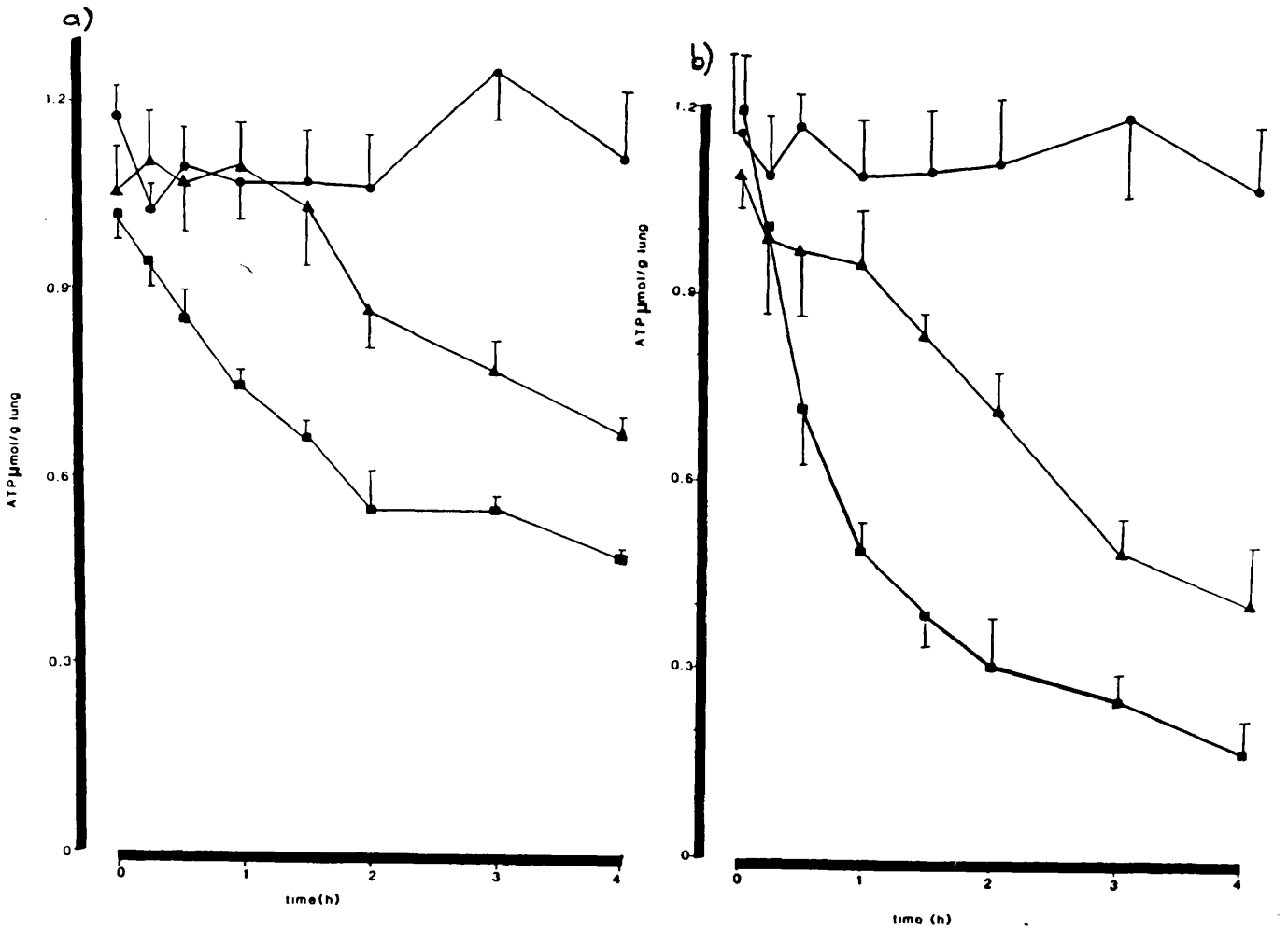


Fig. 4/ 9. ATP levels in control and compromised lung slices incubated with nitrofurantoin

Control (a) and BCNU pretreated slices (b) were incubated with KRB in the absence (●) or presence of nitrofurantoin (200 µM, ▲ and 500 µM, ■). Following the appropriate period of incubation, slices were removed and ATP levels determined. The results represent the mean ATP levels in µmol/ g lung of three experiments ± S. E.



4. 3. g 2, 3-DIMETHOXY-1, 4-NAPHTHOQUINONE AND ATP LEVELS IN CONTROL AND BCNU PRETREATED SLICES

In control slices 2, 3-diOMe (50 μM) produced only slight effects on ATP over 4 h of incubation. In BCNU pretreated lung slices, however, there was a clear, striking potentiation of loss of ATP which was near-maximal at around 2-3 h (fig. 4/ 10). ATP levels were maintained over the 4 h in control and compromised slices, in the absence of the quinone, and no significant difference was detected between the two sets of slices (fig. 4/ 10).

4. 3. h PARAQUAT IN CONTROL AND COMPROMISED LUNG SLICES : EFFECTS ON D-[1-^{14}C]-GLUCOSE OXIDATION

Control and compromised slices, incubated in the absence of paraquat, exhibited little difference in their ability to oxidise glucose via the hexose monophosphate shunt (HMPS). This similarity was consistent with the virtually identical rates of D-[^{14}C(U)]-glucose oxidation described in Chapter 3, suggesting that BCNU treatment, alone, did not appear to severely inhibit the enzymes of the HMPS such as glucose-6-phosphate dehydrogenase.

In control slices, paraquat produced a concentration-dependent stimulation of D-[1-^{14}C]-glucose oxidation, near-maximal at a paraquat concentration of 10 μM (fig. 4/ 11). With the three concentrations of paraquat, oxidation i. e. $^{14}CO_2$ evolution was approximately linear for the 2 h period investigated.

In compromised lung slices, the HMPS was maximally stimulated at a much lower concentration of paraquat, and the extent of activity resulting from incubation with paraquat was lower than that observed in control slices similarly incubated. Once again, $^{14}CO_2$ evolution was broadly

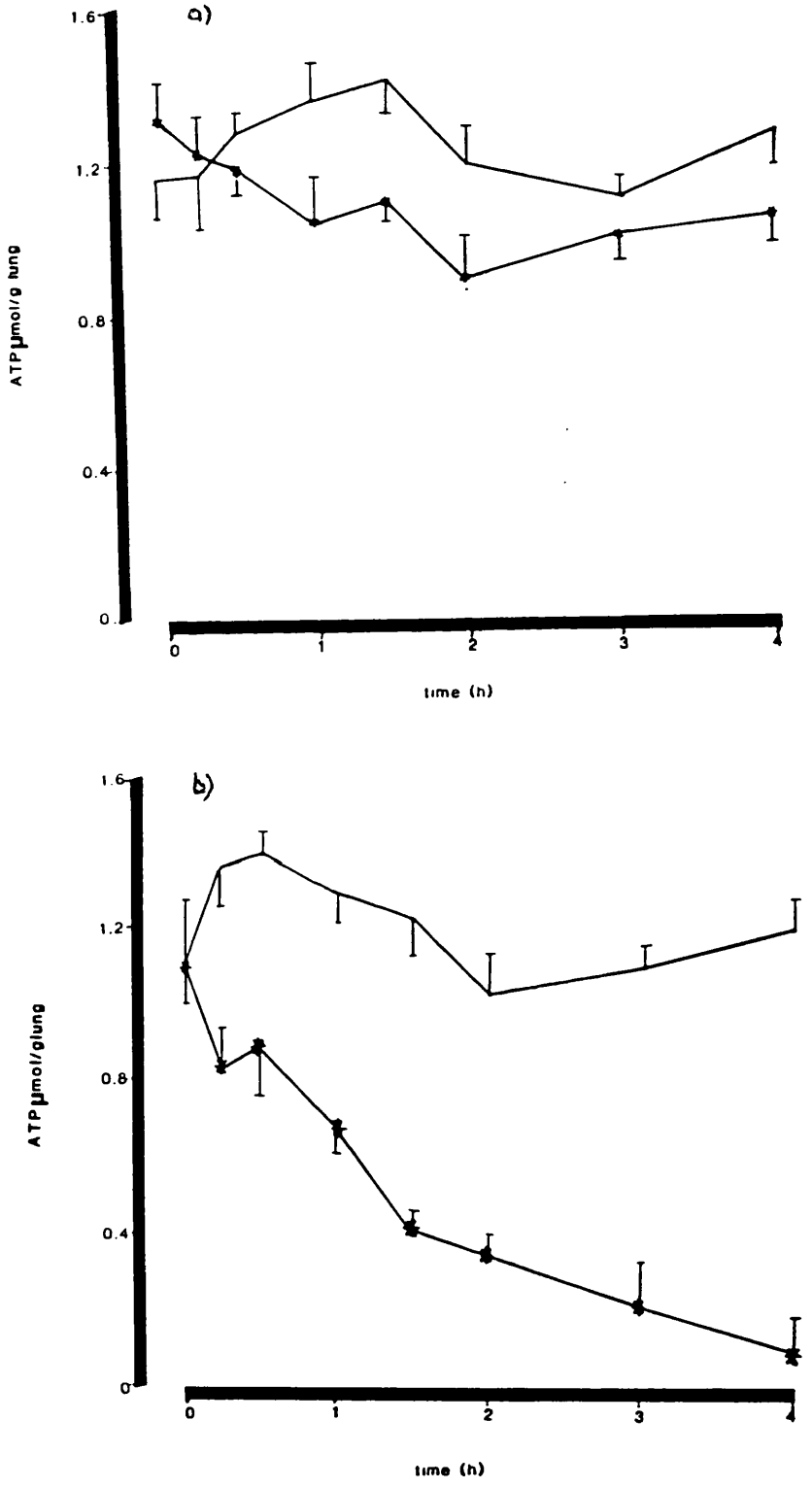
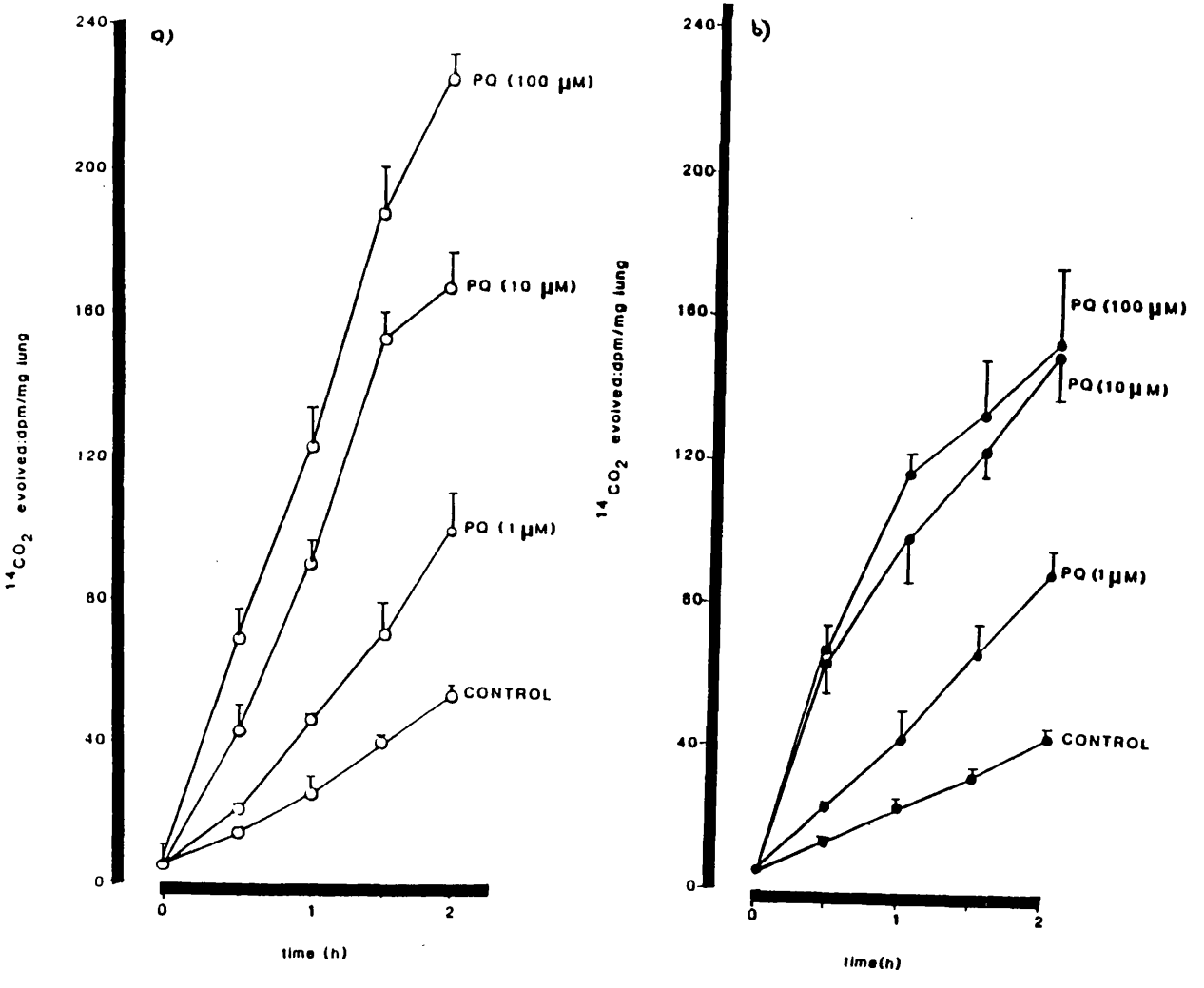


Fig. 4/ 10. 2, 3-Dimethoxy-1, 4-naphthoquinone : effects on ATP levels in control and compromised lung slices

Control (a) or BCNU pretreated slices (b) were incubated in the absence (-) or presence of 2, 3-diMeO (50 µM, *-*) for up to 4 h after which, slices were removed, homogenised and assay for ATP using the bioluminescence method (described in Methods). Results are expressed as mean µmol ATP/ g lung ± S. E. (n= 3).

Fig. 4/ 11. D-[1-^{14}C] glucose oxidation in control and BCNU compromised lung slices : effects of paraquat

Control (a) or BCNU compromised lung slices (b) were incubated in KRB as controls or with paraquat (1, 10 and 100 µM) for up to 2 h with 1 uCi radiolabelled glucose in a total glucose concentration of 11 mM. Results represent $^{14}CO_2$ evolved expressed as dpm/ mg lung ± S. E. (n=3).



linear at each concentration of paraquat. Paraquat at 1 μM stimulated HMPS activity similarly in both control and compromised lung slices (fig. 4/ 11). In control slices, 10 μM paraquat resulted in 4-5 fold activation of the shunt after 1 h.

4. 3. i D-[6-^{14}C]-GLUCOSE OXIDATION : EFFECTS OF PARAQUAT IN CONTROL AND COMPROMISED LUNG SLICES

Glycolytic activity was assessed using $^{14}CO_2$ evolution from D-[6-^{14}C]-glucose. Glycolytic activities in compromised and control slices were similar, without paraquat (fig. 4/ 12), suggesting a lack of effect of BCNU on the enzymes of the glycolytic pathway.

In control slices, paraquat did not produce any significant effect on glycolysis. Similarly, $^{14}CO_2$ evolution in BCNU treated slices was unaffected by paraquat.

4. 3. j D-[1-^{14}C]-GLUCOSE OXIDATION IN CONTROL AND COMPROMISED SLICES INCUBATED WITH NITROFURANTOIN

In preliminary experiments, low concentrations of nitrofurantoin, (1 and 10 μM), did not enhance HMPS activity. Nitrofurantoin (100 μM) elicited a 4-fold elevation of the shunt in control and BCNU treated slices after 1 h. Due to the preliminary nature of these experiments, it was not possible to state whether the increase in nitrofurantoin-induced activity at 90 min was a true observation (fig. 4/ 13). It is, however, tentatively suggested that nitrofurantoin is capable, like paraquat, of stimulating the shunt, the near-maximal stimulation occurring at a higher concentration of nitrofurantoin, than paraquat.

4. 3. k D-[6-^{14}C]-GLUCOSE OXIDATION IN CONTROL AND BCNU PRETREATED

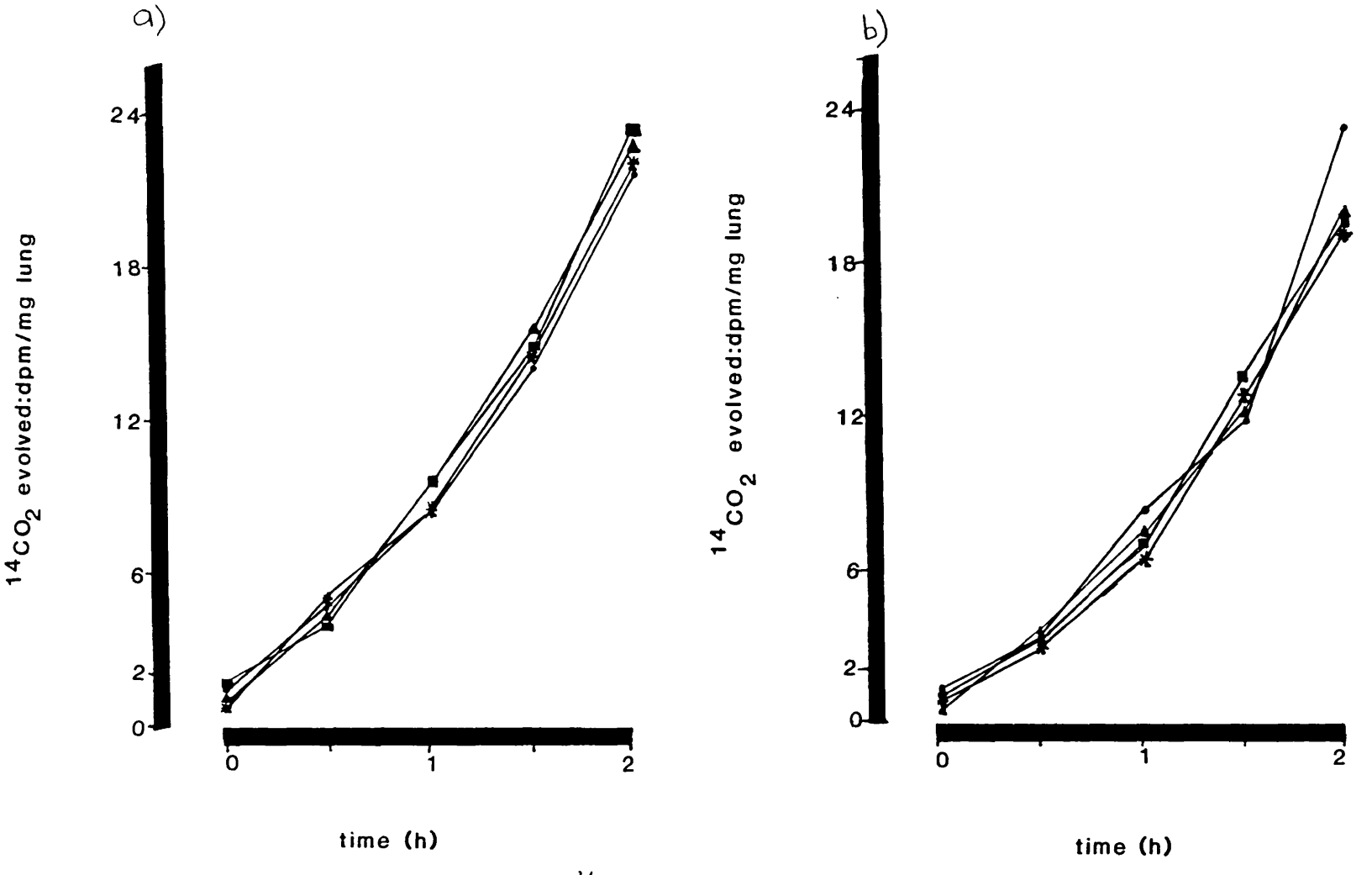


Fig. 4/ 12. D-[6-^{14}C]-glucose oxidation in compromised lung slices incubated with paraquat

Control (a) or BCNU pretreated lung slices (b) were incubated in KRB in the absence or presence of paraquat (1 µM, ▲ ; 10 µM, ■ and 100 µM, ✳) for up to 2 h with 1 µCi D-[6-^{14}C]-glucose (11 mM). Results are expressed as mean dpm/ mg $^{14}CO_2$ evolved ± S. E. M. (n= 3).

Slices incubated in the absence of paraquat are denoted by ●.

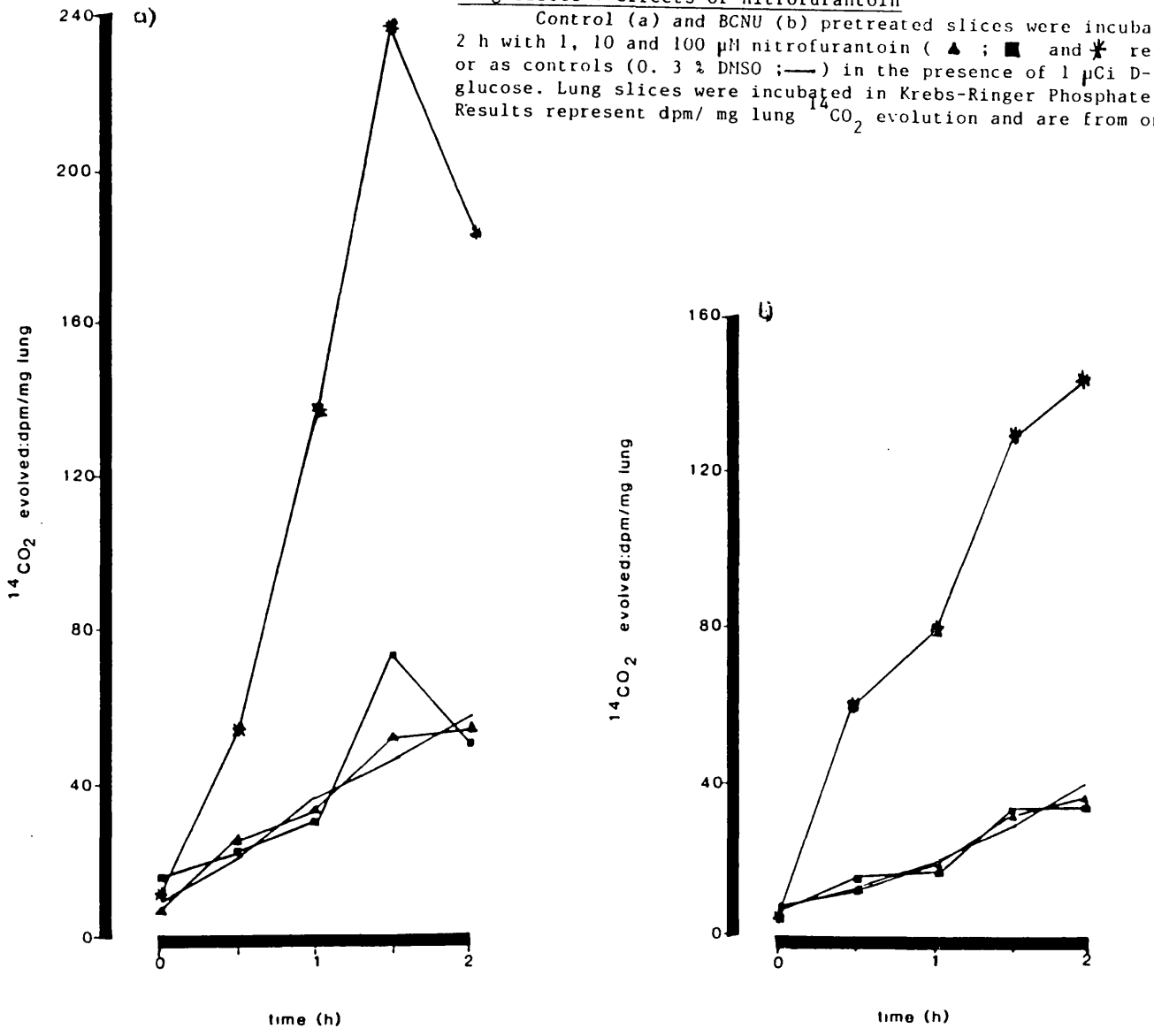


Fig. 4/ 13. D- [1-^{14}C]-glucose oxidation in control and compromised lung slices : effects of nitrofurantoin

Control (a) and BCNU (b) pretreated slices were incubated for up to 2 h with 1, 10 and 100 µM nitrofurantoin (▲ ; ■ and ✱ respectively) or as controls (0. 3 % DMSO ;——) in the presence of 1 µCi D-[1-^{14}C]-glucose. Lung slices were incubated in Krebs-Ringer Phosphate medium. Results represent dpm/ mg lung $^{14}CO_2$ evolution and are from one experiment.

SLICES INCUBATED WITH NITROFURANTOIN

In contrast to previous work [228] no inhibition of glycolysis was observed at 1, 10 or 100 μM nitrofurantoin in either control or BCNU compromised slices.

4. 3. 1 [^{14}C]-ACETATE INCORPORATION INTO COMPROMISED LUNG SLICES : INFLUENCE OF PARAQUAT

Paraquat reduced the extent of acetate incorporation into fatty acids (fig. 4/ 14) in both control slices and in BCNU pretreated slices. Of interest was the finding that BCNU treatment, in the absence of paraquat, appeared to result in a greater incorporation of acetate,compared to control slices incubated similarly. Paraquat (10 μM) significantly reduced acetate incorporation in control slices, but this diminution was potentiated in lung slices possessing an inhibited glutathione reductase activity.

A similar trend was observed with 1 μM paraquat (data not shown).

4. 3. m UPTAKE OF PUTRESCINE INTO RAT LUNG SLICES

Preliminary studies using putrescine accumulation were in accord with previous results. In control slices (incubated with KRB, without prior BCNU treatment), uptake of putrescine was linear with time (0-60 min) at a range of putrescine concentrations (1-100 μM). These characteristics of putrescine uptake were similarly exhibited by lung slices following BCNU pretreatment (table 4. I).

4. 3. n PUTRESCINE ACCUMULATION IN CONTROL AND BCNU COMPROMISED LUNG SLICES : KINETIC PARAMETERS

The oligoamine transport system which is present in lung tissue and is responsible for the accumulation of putrescine is known to obey

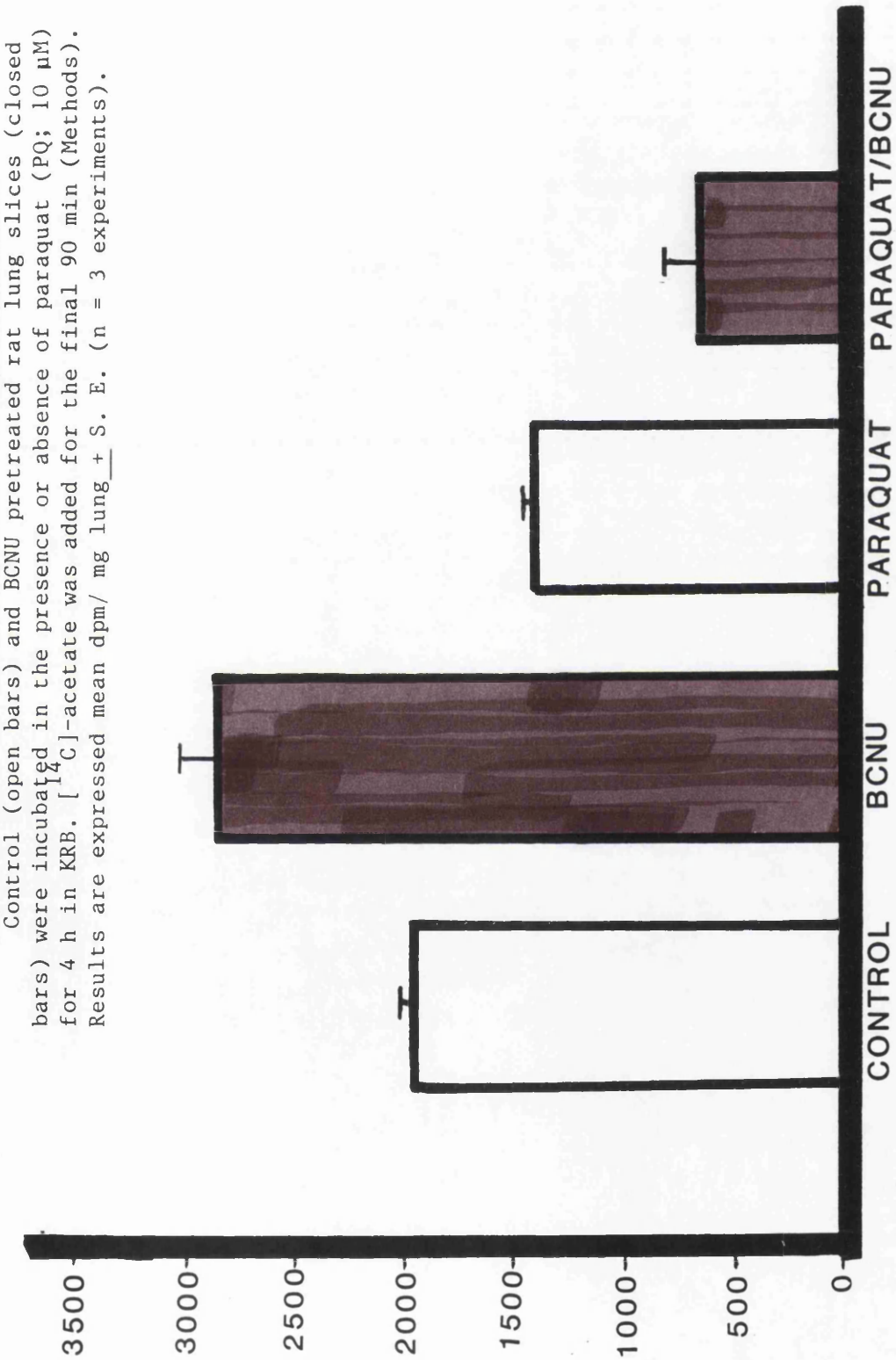


Fig. 4/ 14. Effect of paraquat on fatty acid synthesis

Control (open bars) and BCNU pretreated rat lung slices (closed bars) were incubated in the presence or absence of paraquat (PQ; 10 μM) for 4 h in KRB. [^{14}C]-acetate was added for the final 90 min (Methods). Results are expressed mean dpm/ mg lung ± S. E. (n = 3 experiments).

Table 4. I . Putrescine uptake in control and compromised lung slices : linearity with concentration and time.

Putrescine accumulation nmol/ g lung

[Putrescine]	Control			Compromised		
(uM)	15 min	30 min	60 min	15 min	30 min	60 min
1	13. 1	26. 2	38. 5	10. 6	24. 4	40. 4
5	38. 2	81. 9	167. 6	47. 5	78. 8	132. 3
10	65. 1	136. 3	290. 3	59. 8	125. 9	257. 8
50	123. 6	215. 7	513. 8	140. 9	188. 3	397. 1
100	145. 3	235. 3	472. 9	175. 2	261. 2	406. 7

Control and BCNU pretreated lung slices were incubated with putrescine (1, 5, 10, 50 and 100 μM) for 15, 30 and 60 min with 0. 1 μCi radiolabelled putrescine. The results are from one experiment typical of several.

Table 4. II. Kinetic parameters of putrescine accumulation in control and BCNU pretreated lung slices

Treatment	Apparent Km (μM)	Vmax (nmol/ lung/h)
Control	13.5 + 0.4	718 ± 30.2
Compromised	15.6 + 0.1	720 ± 23.7

Control or compromised lung slices were incubated with putrescine (1, 5, 10, 50, and 100 μM) for up to 60 min and uptake determined as described in materials and methods. Results are expressed as mean ± S.E. (n=3). Km and Vmax values were derived from Hanes-Woolf plots.

saturation kinetics. Control and BCNU pretreated slices exhibited virtually identical kinetic parameters of Km and Vmax, suggesting that BCNU treatment does not, per se, affect putrescine uptake. The values presented in table 4. II are in good agreement with values reported elsewhere [134].

4. 3. o PUTRESCINE ACCUMULATION FOLLOWING INCUBATION WITH PARAQUAT AND 2, 3-DIMETHOXY-1, 4-NAPHTHOQUINONE : EFFECTS IN CONTROL AND COMPROMISED SLICES

Fig. 4/ 15 shows the effects of incubation of control and compromised lung slices under various conditions, on putrescine uptake. Incubation of lung slices in control medium for 4 h, did not impair their ability to accumulate putrescine (10 µM).

Control slices incubated with paraquat (10 and 100 µM) for 4h showed a concentration dependent loss of ability to accumulate putrescine. In slices with an inhibited glutathione reductase activity, this paraquat-induced loss of putrescine uptake was significantly potentiated. Significant potentiation was also observed at paraquat concentrations of 5 and 50 µM and a slight effect was noted at 1 uM (data not shown).

Similarly, the redox cycling, lipophilic quinone 2, 3-diOMe (50 µM) reduced putrescine accumulation following incubation for 4 h in control slices. This effect was strongly potentiated by BCNU pretreatment of lung slices.

4. 3. p PUTRESCINE ACCUMULATION INTO CONTROL AND COMPROMISED SLICES : INFLUENCE OF NITROFURANTOIN

Putrescine uptake was assessed over a 30 min incubation period, after 2 h incubation in the presence of 200 µM nitrofurantoin -fig. 5/ 16. Putrescine accumulation in the absence of nitrofurantoin was identical in

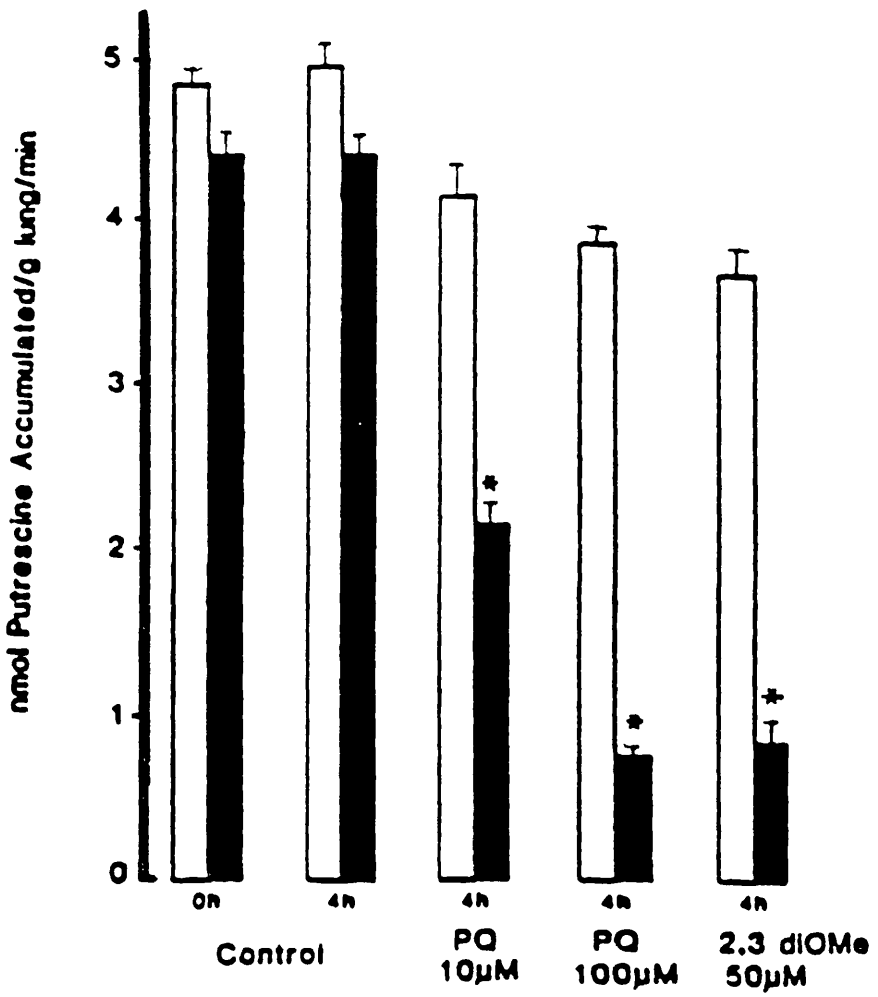


Fig. 4/ 15. Putrescine uptake in control and BCNU treated slices following incubation with paraquat

Putrescine accumulation was assessed following incubation of slices with paraquat (PQ) and 2, 3- dimethoxy- 1, 4- naphthoquinone (2, 3- diOMe). Open and closed bars denote control and BCNU pretreated slices respectively. Slices were incubated with 0. 1 μCi [^{14}C] putrescine (10 μM) for 30 min at 37^{o}. Results shown represent mean ± S. E. (n=4). (*) Significant at $P < 0.05$ using a paired t- test comparing BCNU pretreated slices to their corresponding controls.

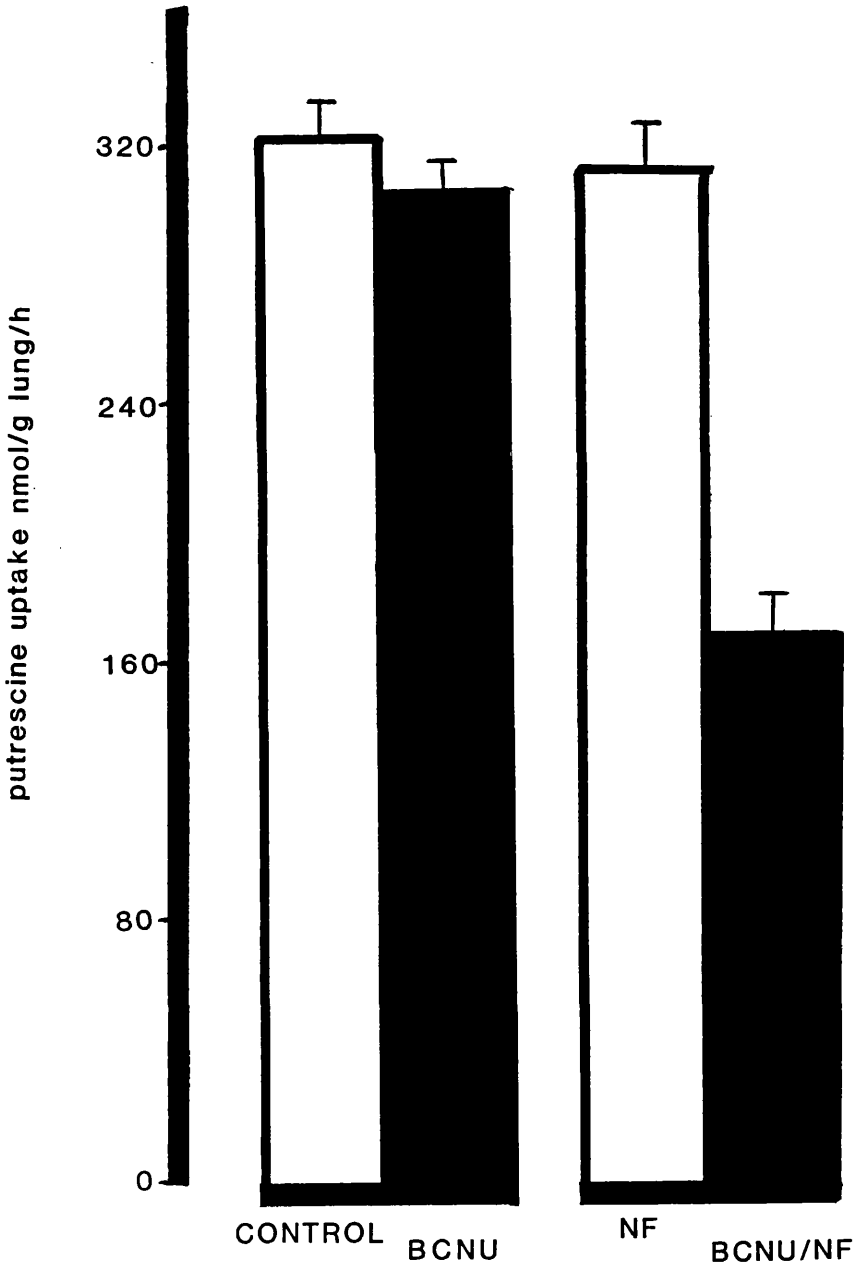


Fig. 4/ 16. Putrescine uptake in control and compromised slices: effect of nitrofurantoin

Control (open bars) and BCNU pretreated (closed bars) were incubated in the presence or absence of nitrofurantoin (200 μM) for 2 h. Putrescine uptake was then assessed over a 30 min incubation period using 0. 1 μCi in a total concentration of 10 μM putrescine.

control and compromised slices. BCNU pretreated slices, when incubated with nitrofurantoin, exhibited a marked loss of ability to accumulate putrescine. Control slices, similarly incubated with nitrofurantoin (200 μM) exhibited no such loss of diamine uptake. (Fig. 5/ 16).

4. 3. q LOSS OF PUTRESCINE ACCUMULATION IN BCNU COMPROMISED LUNG SLICES INCUBATED WITH PARAQUAT : TIME COURSE

In the absence of paraquat, BCNU pretreated and control slices exhibited virtually identical putrescine uptake, over a 4 h incubation period (fig. 4/ 17). In control slices, paraquat (100 μM) produced a gradual but significant decrease in ability to accumulate the diamine whereas in compromised slices a rapid and much more extensive loss of putrescine uptake was observed. In these experiments, a somewhat greater inhibition of putrescine uptake, presumably resulting from prior incubation with paraquat, was observed compared to fig. 4/ 15. However, in all experiments, paraquat-induced loss of uptake was much greater in lung slices possessing a marked inactivation of glutathione reductase activity.

4. 3. r UPTAKE OF SPERMIDINE IN CONTROL AND COMPROMISED LUNG SLICES

The uptake of spermidine was assessed in BCNU compromised slices and compared to control slices. Paraquat (10 and 100 μM) caused a reduction in spermidine accumulation which was potentiated in BCNU pretreated lung slices, similarly incubated. In the absence of paraquat, uptake into control and compromised slices was similar. Thus, the effects with spermidine (table 4. III) reflected those obtained with putrescine. In addition,loss of spermine uptake was observed following incubation with paraquat and 2, 3-diOMe (data not shown-Walther,Hardwick and Cohen-unpublished results) and this loss of uptake was potentiated in

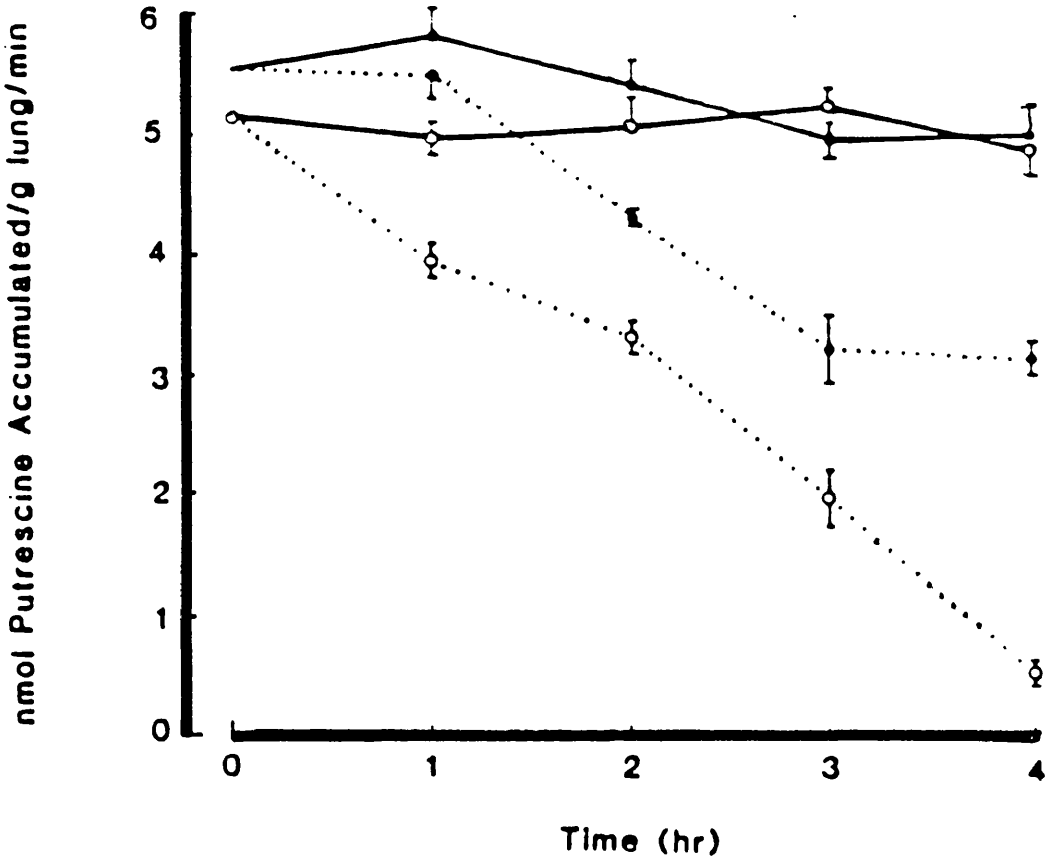


Fig. 4/ 17. Time course of loss of putrescine accumulation in BCNU compromised lung slices incubated with paraquat

Control lung slices (•) or BCNU pretreated slices (o) were incubated either in the absence (-) or the presence (····) of paraquat (100 μM). At the indicated times, slices were removed and putrescine accumulation determined over 30 min of incubation with 0. 1 μCi [^{14}C] putrescine (10 μM). Results are the mean of four experiments ± S. E.

Table 4. III. Uptake of spermidine into control and compromised lung slices following incubation with paraquat.

[PQ] (μM)	Time (h)	Spermidine Uptake nmol/ g lung/ min Control	Compromised
-	0	4. 95 ± 0. 29	4. 91 ± 0. 07
-	4	4. 70 ± 0. 23	4. 48 ± 0. 27
10	4	4. 09 ± 0. 09	2. 95 ± 0. 08*
100	4	2. 73 ± 0. 14	1. 00 ± 0. 11*

*$\leq$0. 05 using a paired 't- test'.

Control and BCNU pretreated lung slices were incubated in the in the presence or absence of paraquat (100 μM). Following the appropriate incubation, slices were removed and incubated for a further 30 min with 0. 1 μCi labelled spermidine in a total spermidine concentration of 10 μM. The results represent the mean ± S. E. of three experiments.

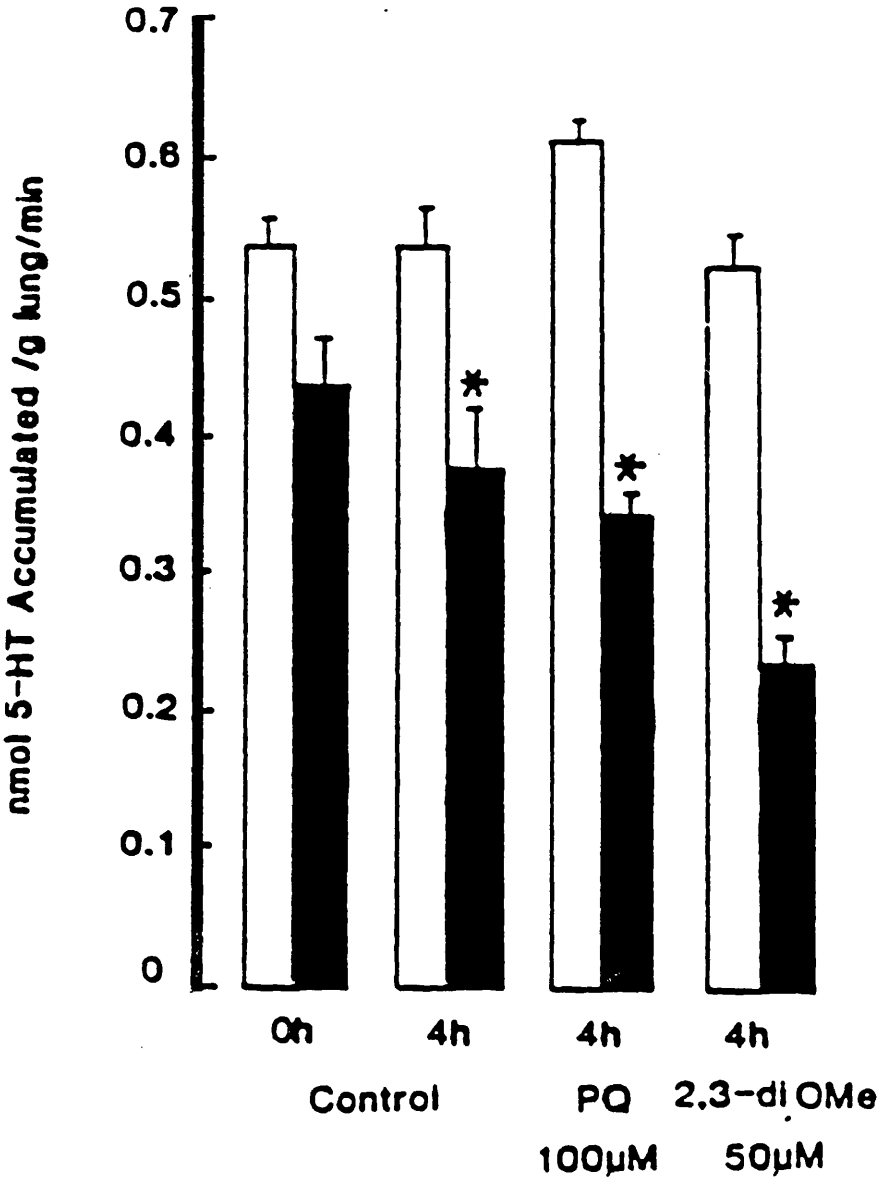


Fig. 4/ 18. Uptake of 5- HT in control and compromised slices

Lung slices were incubated with 0. 1 μCi [^{14}C] 5- HT (1 μM) for 20 min, in the presence of iproniazid (0. 5 mM), following 4 h incubation with either paraquat (PQ, 100 μM) or 2, 3- dimethoxy- 1, 4- naphthoquinone (2, 3-diOMe, 50 μM). Open and closed bars represent control and BCNU pretreated slices respectively. Results are the means ± S. E (n=3). (*) Significant at $P < 0.05$ using a paired t- test comparing BCNU pretreated slices to their corresponding controls.

lung slices possessing an inhibited glutathione reductase activity.

4. 3. s EFFECTS OF PARAQUAT AND 2, 3-DIMETHOXY-1, 4-NAPHTHOQUINONE ON UPTAKE OF 5-HYDROXYTRYPTAMINE (5-HT)

Incubation of control slices for 4 h did not affect the uptake of 5-HT (fig. 4/ 18). Lung slices pretreated with BCNU exhibited a small but significant reduction both initially and after 4 h, which could have been due to a toxic effect of BCNU on the endothelium. Paraquat slightly increased 5-HT uptake into control slices. In compromised slices incubated with paraquat, uptake was reduced, but was not significantly lower than the lung slices exposed to BCNU alone.

In control slices, 2, 3-diOMe (50 μM) did not affect the pulmonary accumulation of 5-HT. However, in BCNU pretreated slices a significant inhibition of uptake was observed (fig. 4/ 18).

4. 3. t NITROFURANTOIN AND 5-HT UPTAKE

Assessment of endothelial uptake using 5-HT accumulation following incubation with nitrofurantoin, yielded results which were unclear. Nitrofurantoin (200 μM) produced no significant effects on 5-HT uptake in control slices, but an elevated accumulation was observed in BCNU treated lung slices incubated with nitrofurantoin (200 μM)(table 4. IV).

4. 4. DISCUSSION

The generally accepted mechanism of the pulmonary toxicity of paraquat involves initial uptake into the epithelium, where it then exerts its toxicity by redox cycling. However, if redox cycling is intimately associated with toxicity it is difficult to reconcile the absence of pulmonary thiol depletion observed in mice [144] and rats [74]. In the

Table 4. IV. 5-Hydroxytryptamine accumulation into BCNU compromised lung slices.

TREATMENT	5-HT UPTAKE (nmol/ g/ h)
Control	34. 4 ± 1. 0
BCNU	28. 3 ± 3. 9
Nitrofurantoin	33. 6 ± 5. 8
BCNU/ nitrofurantoin	40. 7 ± 2. 9

Control and BCNU compromised lung slices were incubated for 2 h with or without nitrofurantoin (200 μM). After this initial treatment, the slices were removed and 5-hydroxytryptamine uptake assessed (0. 1 μCi 5-HT in a total of 1 μM) in the presence of iproniazid (500 μM) for a further 20 min. Results represent mean uptakes of three experiments and are expressed in nmol/ g lung/ h ± S. E.

latter study, no increase in GSSG was observed, though an increase in pulmonary mixed protein disulphides was noted.

BCNU pretreatment of lung slices did not markedly potentiate the effects of paraquat on pulmonary GSH (fig. 4/ 3) or ATP (fig. 4/ 8) but did potentiate the inhibitory effects on the pulmonary uptake of the oligoamines putrescine, spermidine and spermine. The absence of a marked potentiation with regard to GSH and ATP with paraquat, was in contrast to the striking potentiation of 2, 3-diOMe induced loss of both ATP and GSH in BCNU pretreated lung slices (figs 4/ 4 and 4/ 10).

In hepatocytes and rat liver microsomes, it has been shown that 2, 3-diOMe is a potent redox cycling compound, causing a concentration dependent depletion of GSH and increase in GSSG, which precedes cytotoxicity in hepatocytes [219].

The most likely explanations for the differential effects of paraquat and 2, 3-diOMe on GSH levels in BCNU pretreated slices are :- (i) paraquat redox cycles much more slowly than the quinone and therefore the small amount of residual glutathione reductase activity may be capable of reducing any GSSG formed in the presence of paraquat and (ii) paraquat is accumulated by alveolar type I and II cells and Clara cells whereas 2, 3-diOMe, because of its lipid solubility would be expected to diffuse into all cell types in the lung slice. Thus, the much more extensive GSH depletion obtained with the quinone is compatible with the hypothesis that only a small fraction of pulmonary GSH is present in alveolar type I and II cells. This hypothesis is further substantiated by the observations that BCNU pretreatment of rat lung slices potentiates the toxicity of paraquat,based upon the use of a functional assessment of viability of the alveolar epithelium i. e. putrescine and spermidine uptake in contrast to the lack of effects on GSH and ATP. Thus, consideration of the GSH

levels in compromised slices in the presence of paraquat argues against the potentiation of an oxidative stress-induced toxicity, whereas use of a functional assessment of alveolar epithelial damage indicates that paraquat is toxic by redox cycling.

These findings highlight the problems encountered when investigating biochemical mechanisms of toxicity in such a heterogeneous organ.

The conclusion that much of the pulmonary GSH is in cell types other than the alveolar epithelium is further substantiated by studies with isolated rabbit lung cells which showed a wide variation in GSH levels ,and rates of de novo synthesis ,

macrophages > Clara cells > type II cells [229]. In this study some of the observed differences may have been caused by losses during isolation procedures.

In the lung slices used in these studies a significant proportion of the cells are endothelial cells which, it is hypothesised contain a significant proportion of the pulmonary GSH. The function of this glutathione is unknown but may be related to leukotriene biosynthesis [230] If pulmonary GSH has an uneven distribution, as suggested by these studies then this may have important implications in the susceptibility of individual cell types to toxic injury.

A similar consideration of the data with paraquat and 2,3-diOMe on ATP in control and BCNU pretreated slices suggests that much of the pulmonary ATP is also present in the endothelium.

The data with 5-HT suggests that paraquat does not have a major effect on the endothelium. Paraquat caused a small increase in uptake in lung slices similar to that reported by others following dosing with paraquat in vivo, though the origin of this effect is unknown [221]. In

contrast, the quinone did not alter amine uptake in control lung slices but in compromised slices caused a significant reduction in accumulation (fig. 4/ 18) suggestive of toxic interactions within the endothelium. Interestingly, the BCNU control groups show reduced accumulation at 0 h and 4 h compared to control slices, suggesting a toxic effect of BCNU, per se. Endothelial damage in lung tissue following BCNU administration in vivo has been reported [198].

Other studies [231] have investigated the effects of paraquat, nitrofurantoin and 2, 3-diOMe on the levels of pyridine nucleotides in BCNU pretreated slices. Using similar considerations, it was concluded that much of the pulmonary NADPH is in the alveolar type II cell [231]. Inhibition of glutathione reductase activity failed to result in a conservation of NADPH levels in slices incubated with paraquat.

The following hypothesis offers a possible explanation of the results : Paraquat is actively accumulated into the type I and II cells. It is a redox cycler of low potency [232] but it reaches very high intracellular concentrations. Presumably, there is significant conversion of GSH present to GSSG which, in control slices is reduced to GSH, at the expense of NADPH. Thus NADPH is utilised at two levels : by NADPH-cytochrome P-450 reductase and via glutathione reductase. It can be postulated that if glutathione reductase was inhibited, the NADPH conserved could be utilised by NADPH-cytochrome P-450 reductase to support more extensive redox cycling of paraquat (assuming the NADPH concentration is rate-limiting).

The other consequence in compromised slices could be a failure to maintain GSH levels with a consequent loss of ability to detoxify lipid hydroperoxides and hydrogen peroxide. Thus, BCNU pretreatment may increase the rate of generation of active oxygen species and also reduce the

capacity of the target cell to deal with these species.

The results of the glucose oxidation suggest that HMPS activity is not stimulated to as great an extent in compromised slices,compared to control slices. There are two alternative explanations for this finding. Firstly, that there is conservation of NADPH, or secondly, that reduction is due to potentiation of the effects of paraquat. From the work of Adam [231] the first explanation appears unlikely.

The stimulation of the pentose phosphate pathway with paraquat, in the absence of BCNU pretreatment was in agreement with previous results in lung slices [233, 74], in perfused lung [234] and in isolated lung cells [235]. Paraquat-induced stimulation of the hexose monophosphate shunt was near-maximal at a lower concentration of paraquat in compromised slices compared to control slices. This effect could arise from two possible mechanisms. Firstly, conservation of NADPH due to minimal glutathione reductase activity. Since the $NADP^+$/ NADPH ratio is considered to be the major control of the shunt [236], then this might be plausible. However, compromised slices incubated with paraquat exhibit extensive oxidation of pyridine nucleotides. The second potential mechanism is that the results reflect toxicity in the critical 'target' cells. Since 58 % of lung glucose-6-phosphate dehydrogenase is in the type II cell in rat [237], then it is reasonable to propose that loss of shunt activity reflects toxicity to this cell type.

The extensive loss of NADPH in BCNU compromised lung slices incubated with paraquat observed by Adam [231] is consistent with the results obtained with [^{14}C]-acetate incorporation (fig. 4/ 14). Paraquat (10 μM) results in lower fatty acid synthesis in BCNU compromised slices compared with control slices and is supportive of a potentiation of an oxidative stress induced mechanism of toxicity. The method used in

these studies is not specific for surfactant (and, hence, type II cells) unlike some other methods currently used [238] and future studies could pursue this area.

It was concluded from these studies with paraquat that in BCNU compromised slices, GSH depletion did occur in the target cell types, but that it was not possible to measure these modulations. One area of further investigation would involve measurement of GSSG levels, since loss of GSH would, presumably, be reflected by increased GSSG, which would be detectable since the concentration would be low in cell types unaffected by the toxicant. A decrease in the GSH/ GSSG ratio, reflected by increases GSSG has been observed in isolated rabbit lung perfused with paraquat [70].

The effects of 2, 3-diOMe in compromised lung slices have other facets. Clearly, from figs 4/ 4, 4/ 10 and 4/ 15, inactivation of glutathione reductase potentiates the toxicity of the quinone as assessed by loss of GSH,ATP and uptake of putrescine and 5-HT. However, in control slices the compound (50 μM) produces a steady depletion of GSH over 4 h of incubation. Adam [231] observed that NADPH depletion is maximal at 10 uM, at around 1 h. This could suggest that loss of NADPH precedes a thiol depletion and is the critical biochemical event in the cytotoxicity As with paraquat, the quinone results in extensive NADPH depletion in BCNU compromised lung slices, once again suggesting the possibility of more extensive redox cycling. The quinone was not selective for a particular cell type, evidenced by a diminution of uptake of 5-HT and putrescine.

The epithelial effects of nitrofurantoin are potentiated as a result of BCNU pretreatment (fig. 4/ 16), whilst endothelial effects are less clear (table 4. IV). This enigma may reflect limitations in the use of 5-HT as a marker.

The actual potentiation of loss of thiols and ATP is small, whilst potentiation of loss of putrescine is clear, could suggest that the measurable GSH changes occurring represent an event independent of the loss of epithelial integrity. This would be supportive of the hypothesis that much of the pulmonary GSH in cell types distinct from the alveolar epithelial type I and type II cells. Since nitrofurantoin is lipid soluble, ,non-selectivity would be anticipated.

Two other factors complicate studies with nitrofurantoin. Firstly, the agent possesses an arylating activity and may diminish GSH via two distinct mechanisms : redox cycling and direct conjugation. Secondly, nitrofurantoin has been reported to inhibit glutathione reductase activity [239]. Both factors complicate assessment of nitrofurantoin-induced effects in lung slices. In previous studies of nitrofurantoin-induced oxidative stress in isolated rat hepatocytes [208] the occurrence of high GSSG levels and GSH depletion was attributed to the reversible inhibition of glutathione reductase and this factor must be taken into account.

The potentiations of toxicity are consistent with previous observations in the literature with systems employing BCNU. For example, the interaction of stimulated polymorphonuclear leukocytes (PMN) with lung cells is considered to play a role in the pathogenesis of lung disease. PMN-mediated cell damage has been attributed to reactive oxygen metabolites as the major cytotoxic species. The importance of the glutathione redox cycle for the resistance of lung epithelial cells against a PMN-mediated attack was investigated by Suttorp and Simon [240].

Lung epithelial cells were incubated with BCNU or aminotriazole, or were isolated from selenium-deficient rats. Lung cells deficient in glutathione reductase displayed a dramatically enhanced suseptibility to PMN-mediated attack, whereas catalase inhibition (with aminotriazole) resulted in only minor increases in susceptibility. However, inhibition of glutathione peroxidase substantially increased the vulnerability of lung cells against an oxidant attack from extracellular hydrogen peroxide [241]. Selenium deficiency and BCNU treatment enhanced the toxicity of hydrogen peroxide to isolated hepatocytes [241]. Since paraquat and other compounds which induce conditions of oxidative stress are proposed to generate hydrogen peroxide,the potentiation of their toxicities with BCNU is in accord with these results.

CHAPTER 5 - THE USE OF ADENOSINE AS A FUNCTIONAL MARKER OF THE PULMONARY CAPILLARY ENDOTHELIUM

5. 1 AIM

The primary aim of the work presented in this chapter involved an attempt to employ adenosine accumulation as an endothelial marker in lung slices.

5. 2 INTRODUCTION

Pulmonary endothelial cells are involved in a number of metabolic reactions which are independent of gaseous exchange (table 5. I). The uptake of several compounds have been employed as functional markers of the endothelium. The best characterised involves assessment of 5-HT uptake into lung tissue. A variety of markers of endothelial integrity have been employed including prostacyclin formation [242],angiotensin converting enzyme activity [243],and metabolism of adenine nucleotides [244, 245].

Several studies have employed 5-HT uptake as a marker [221, 152] but there are conflicting reports in the literature, particularly with regard to data obtained in isolated perfused lung compared with lung slices [221]. Block and Schoen [152] observed a reduction in 5-HT uptake following the administration of ANTU (which damages the pulmonary endothelium). Nemery et al. [221] observed an increased uptake of 5-HT following paraquat and O,S,S-Me (a trialkyl phosphorodithioate compound), which was hard to reconcile when the cellular targets were considered.

Nemery et al. suggested several alternative hypotheses to account for these findings. The simplest was that epithelial damage results in the breakdown of cellular barriers exposing additional sites for 5-HT uptake.

Table 5. I. Non- respiratory functions of the pulmonary endothelium.

Drug accumulation	e. g. Quinine, chlorpromazine.
Blood coagulation	i. e. Modulates activity of Factor VIII and is a source of heparin. Also activates plasminogen.
Handling of bioactive amines	Inactivation of 5- hydroxytryptamine, tryptamine, noradrenaline, acetylcholine, bradykinin and reno active peptide. Activation of angiotensin I and reno active peptide.
Prostaglandin metabolism	Arachidonic acid activation. Inactivation of prostaglandins of the E and F type.
Nucleotides	Inactivation of AMP, ATP and circulating adenosine.

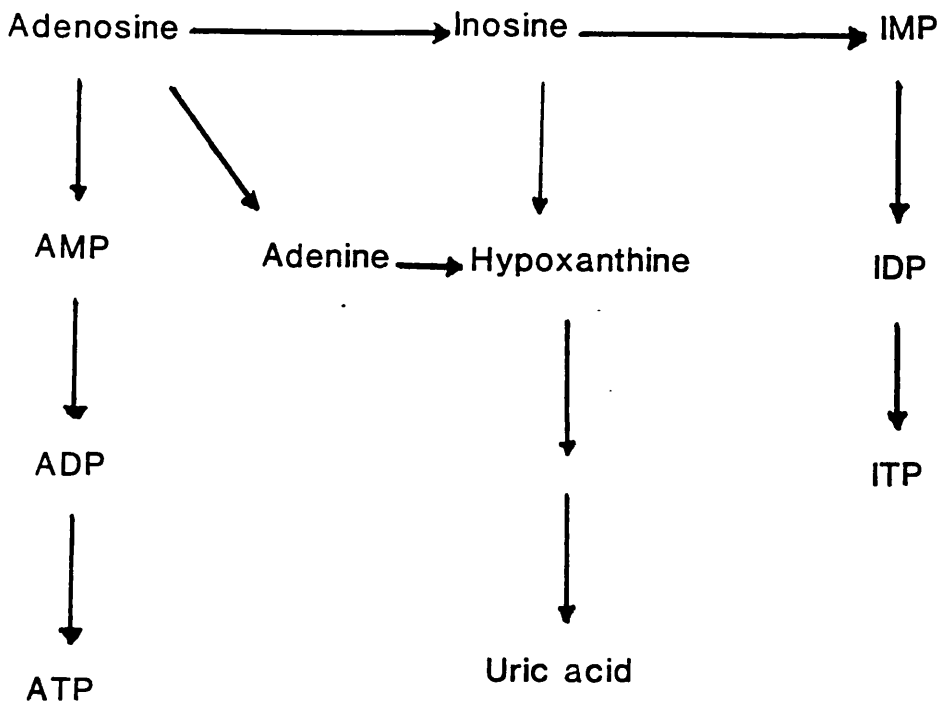
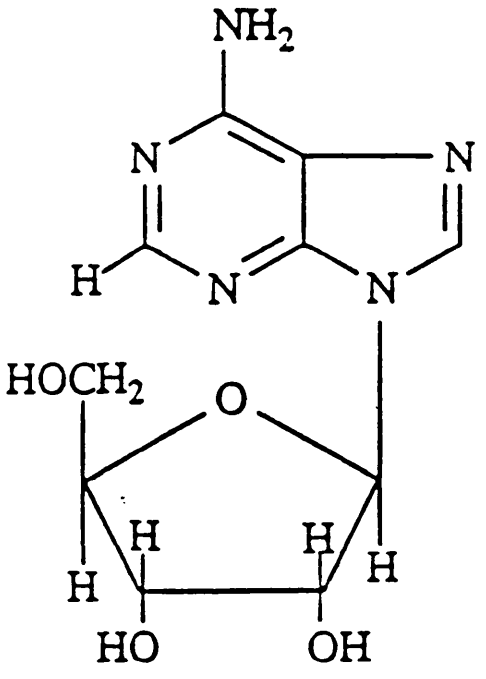


Fig. 5/ 1. Structure and metabolic fate of adenosine

ADENOSINE



Nemery et al. further speculated that the isolated perfused lung does not necessarily give correct results [221], since in studies with paraquat and hyperoxia the data is not consistent. Furthermore, some forms of definite lung injury are not accompanied by a decrease in 5-HT uptake [246],whereas 5-HT uptake may be decreased without evidence of endothelial injury,with halothane and trichloroethane [247].

Previous investigations [245] revealed alterations in the pharmacokinetics of adenosine in the isolated perfused rat lung following ANTU administration. Adenosine is anti-aggregatory and its removal from the vascular space into cells is important in the maintenance of vascular haemostasis. Adenosine is metabolically inactivated (to hypoxanthine and inosine) or is retained in the lung as ATP. Adenosine is taken up and attacked by adenosine deaminase to yield inosine and nucleoside phosphorylase to form hypoxanthine or by nucleotide kinases to form adenine nucleotides [245] (fig. 5/ 1).

ATP is eliminated during passage through the lungs. ATP and 5'-AMP are metabolised during passage through the lungs. Nucleotides are metabolised by hydrolysis of the phosphate ester and by deamination. Cellular uptake is not required and the enzymes needed are not secreted. These processes are known to occur in caveolae open to the vascular lumen. Previous studies [245] investigated metabolism of adenine nucleotides in rat perfused lung, and the effect of ANTU. Treatment with ANTU did not change the proportion of [^{3}H]-ADP surviving a single passage through the pulmonary circulation and they concluded that, in spite of considerable endothelial cell damage, the ectoenzymes catalysing ADP and AMP hydrolysis were relatively little affected by ANTU.

Previous studies have assigned adenosine metabolism as a function of the pulmonary endothelium [245]. The studies in this chapter describe

attempts to characterise and employ adenosine uptake into lung slices as a functional marker of the endothelium, as an alternative to uptake of 5-HT.

5. 3 RESULTS

5. 3. a PRELIMINARY STUDIES WITH ADENOSINE ACCUMULATION INTO RAT LUNG SLICES

Initial studies revealed that the uptake of adenosine into lung slices was approximately linear with time for up to, and including, 60 min at a range of adenosine concentrations 0. 1-10 μM (fig. 5/ 2). From this initial study the concentration of 10 μM adenosine was selected for use in further studies. From other preliminary work (data not shown), the amount of radiolabelled adenosine neccessary to yield reproducible radioactivity in lung slices incubated with 10 μM 'cold'adenosine was 1 μCi [2-^{3}H]-ado.

5. 3. b THE EFFECT OF α-NAPHTHYLTHIOUREA (ANTU) ON ADENOSINE ACCUMULATION

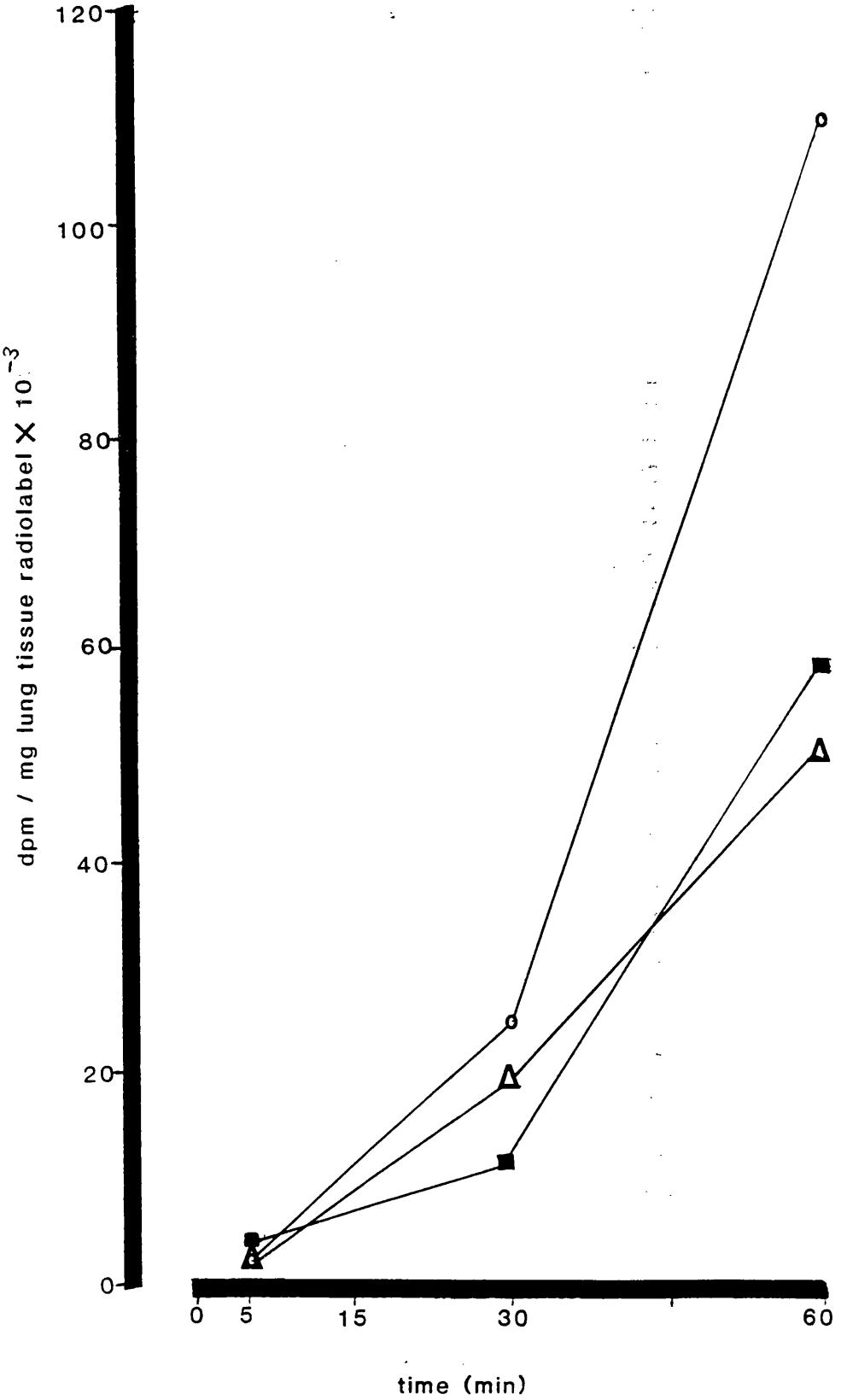
The pulmonary endothelial-damaging agent ANTU had striking effects when administered at 15 mg/ kg i. p. in corn oil. Animals were sacrificed at 4 h following insult (when oedema is near-maximal). Lungs from ANTU-treated rats exhibited approximately 60 % reduction in radioactivity accumulated in slices incubated with adenosine for 5 and 15 min, compared to lung slices prepared from control rats and incubated similarly (fig. 5/ 3).

5. 3. c ASSESSMENT OF THE CELLULAR SPECIFICITY OF ANTU TOXICITY IN RAT LUNG

In addition to effects on adenosine accumulation (a presumed

Fig. 5/ 2 Uptake of adenosine into lung slices

Lung slices were incubated with adenosine at 0. 1 μM (▲); 1 μM (■) and 10 μM (O) in KRB for up to 60 min with 0. 1 μCi [2-^{3}H]-adenosine. Approximately 20-40 mg of tissue per flask was employed in these studies. The results are expressed as dpm/ mg lung and are from one experiment.



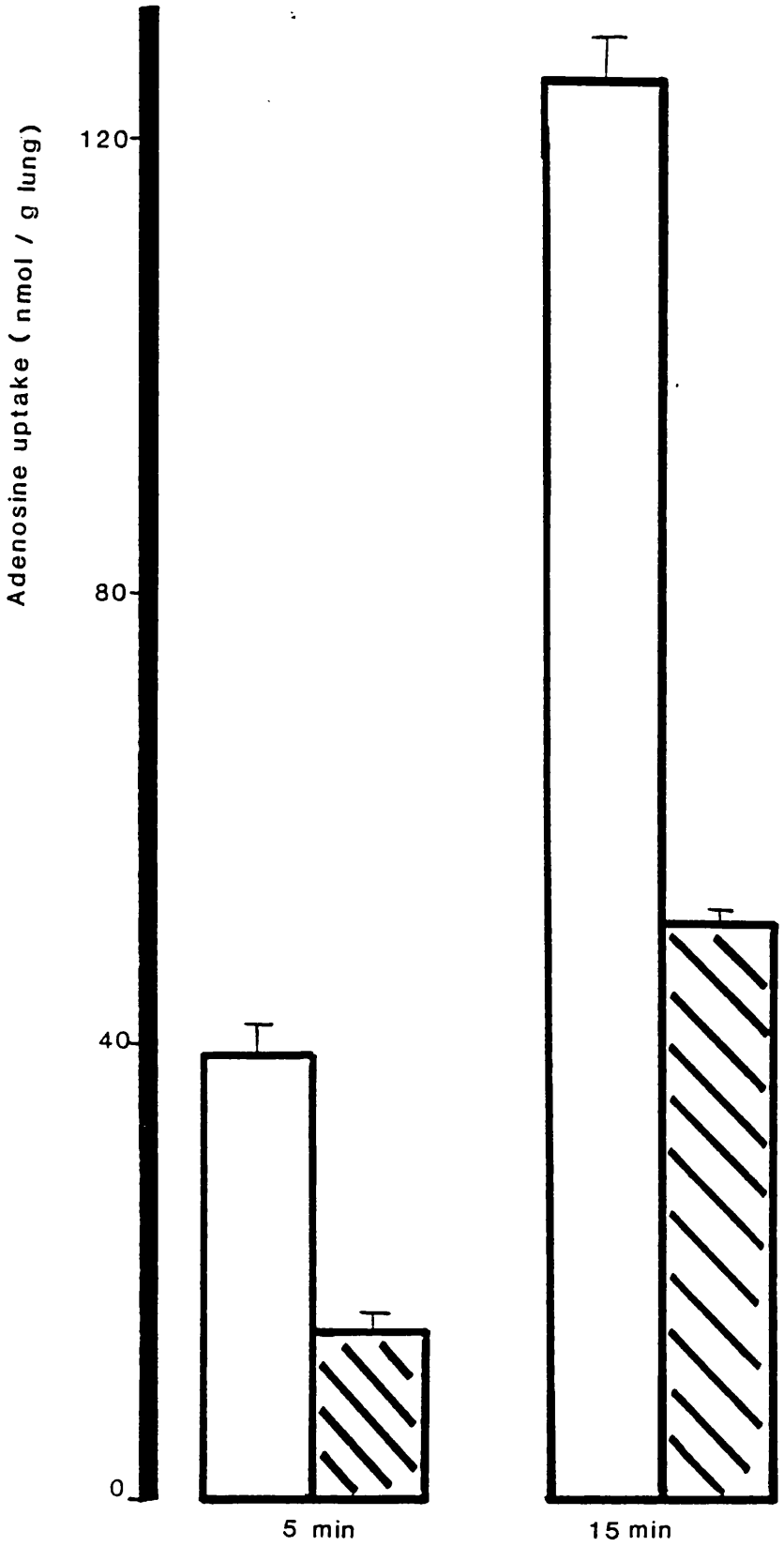


Fig. 5/ 3 Effect of 15 mg ANTU/ kg on adenosine uptake

Lung slices were prepared from control animals (open bars, corn oil vehicle only) and also from animals to which 15 mg ANTU/ kg had been administered 4 h previously (closed bars). In each case, slices were incubated with tritiated adenosine (1 µCi) in KRB (total adenosine concentration of 10 µM) for 5 and 15 min. The results represent the mean uptake in nmol/ g lung + S. E. (n = 3).

function of the pulmonary endothelium), the modulation of epithelial function following dosing with ANTU was assessed using uptake of the oligoamine spermidine.

ANTU (15 mg/kg i. p.) diminished adenosine uptake at both 5 and 15 min in accord with the results presented in fig. 5/ 3. However, this loss of adenosine accumulation was accompanied by a loss of spermidine uptake which was quantitatively similar. (Fig. 5/ 4).

5. 3. d ASSESSMENT OF THE CELLULAR SPECIFICITY OF ADENOSINE UPTAKE IN RAT LUNG

Administration of 10 mg ANTU/ kg i. p. resulted in loss of adenosine accumulation and also in loss of spermidine uptake when slices were prepared at 4 h following ANTU (fig. 5/ 5).

In contrast, administration of paraquat to rats (30 mg paraquat ion/ kg i. p.) resulted in significant loss of spermidine accumulation 24 h following dosing (approximately 40 %) compared to lungs prepared from control animals (fig. 5/ 5). Paraquat administration did not,however, result in accompanying loss of adenosine uptake (fig. 5/ 5).

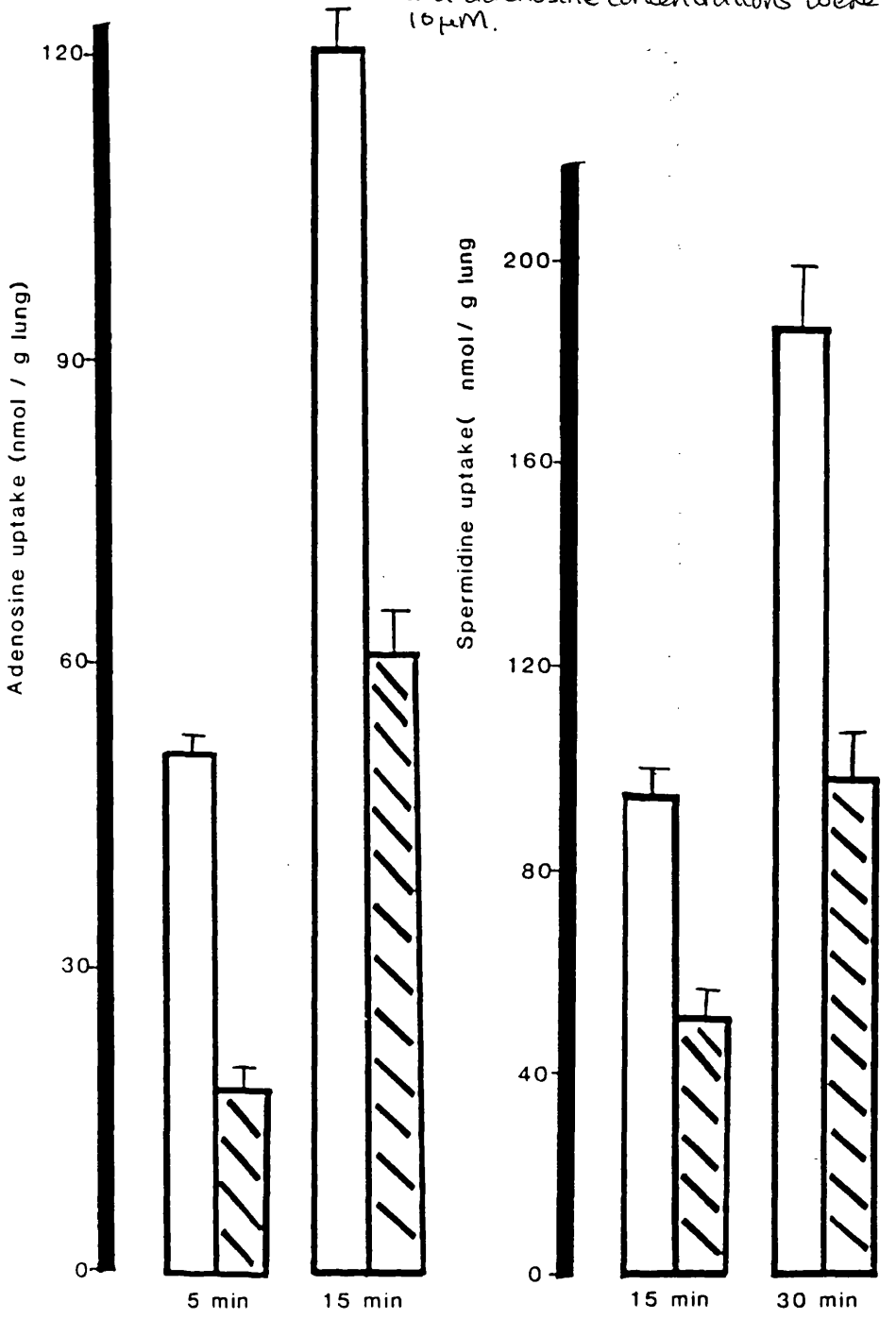
5. 3. e THE INFLUENCE OF METABOLIC INHIBITORS ON ADENOSINE ACCUMULATION INTO LUNG TISSUE

Adenosine uptake into control slices very closely resembled the data presented above for 5 and 15 min of incubation with adenosine. Preincubation for 30 min at 4^{o} and subsequent incubation (also at 4^{o}) with adenosine resulted in greatly reduced accumulated radioactivity (to around 10 % of control values-fig. 5/ 6).

Pretreatment of lung slices (30 min) and subsequent incubation with rotenone (100 µM) and KCN (1 mM) resulted in significantly inhibited uptake

Fig. 5/ 4 Uptake of spermidine and adenosine into lung slices: effect of 15 mg ANTU/ kg

Following dosing with ANTU (15 mg/ kg i. p. in corn oil, hatched bars) or corn oil vehicle (open bars) rats were sacrificed after 4 h and lung slices prepared and incubated with tritiated adenosine and [^{14}C]-spermidine for 5 and 15 min and 15 and 30 min respectively. Results are the mean uptakes in nmol/ g lung ± S. E. (n= 4 experiments). Total spermidine and adenosine concentrations were 10 μM.



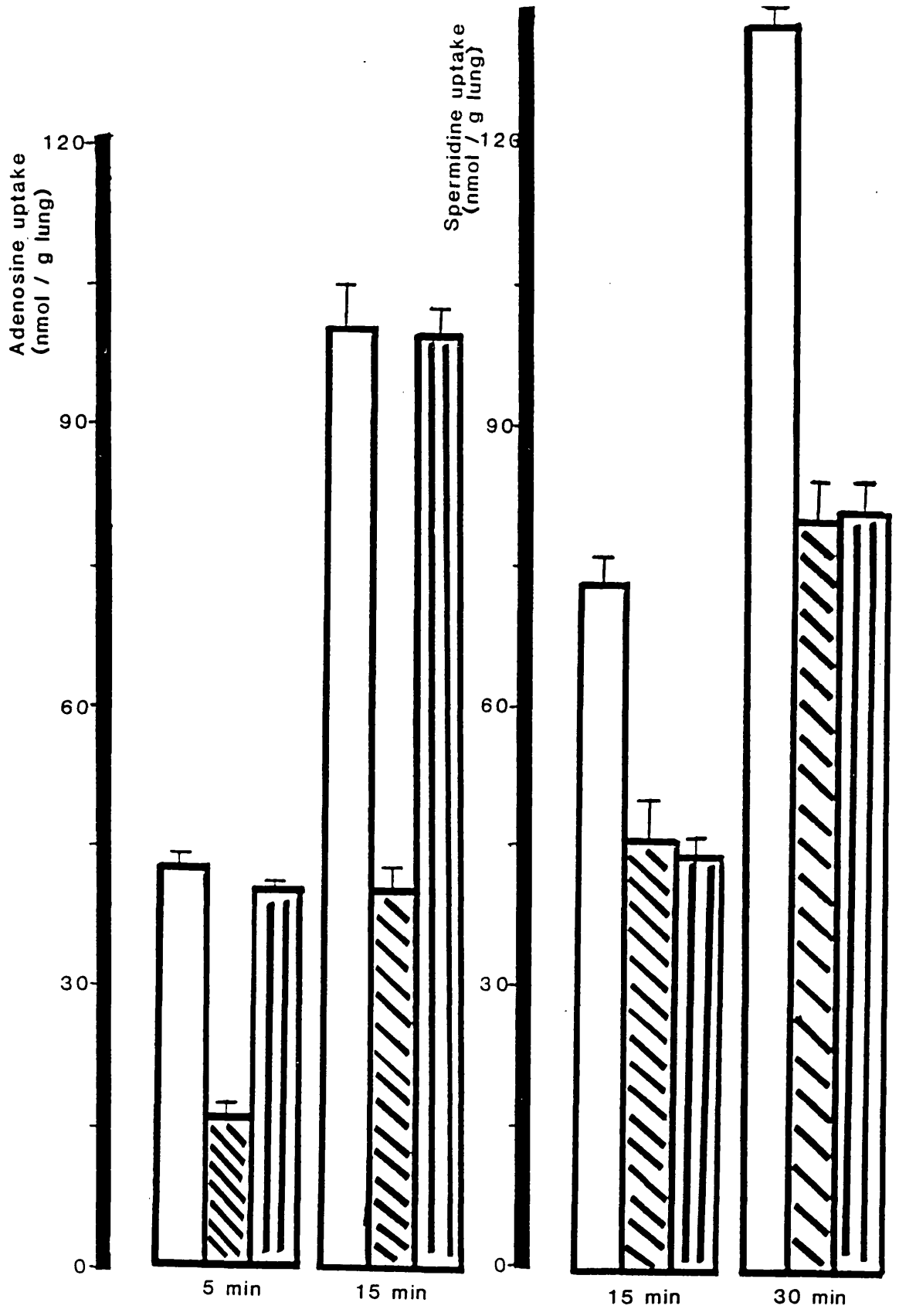
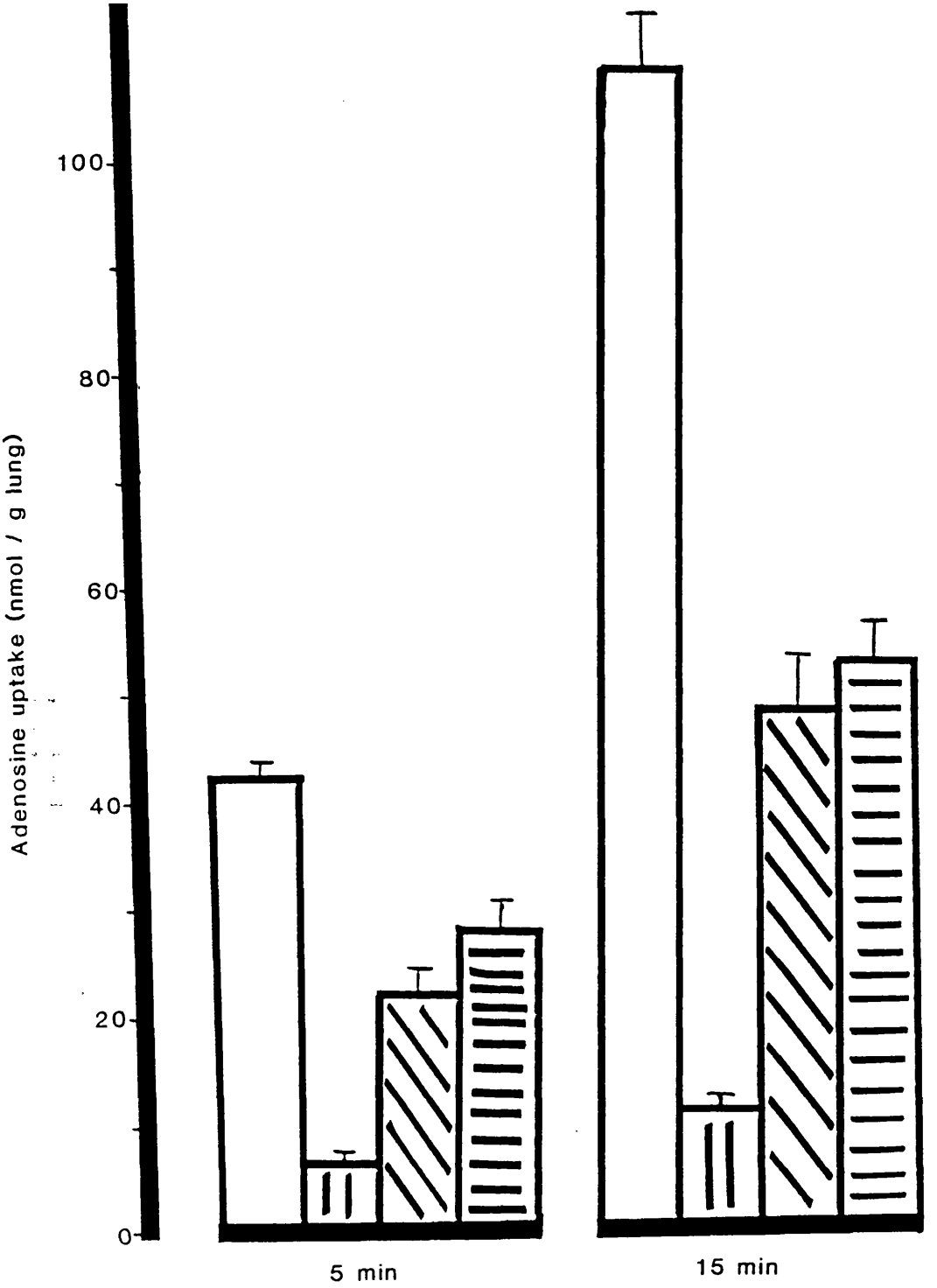


Fig. 5/ 5. The effects of ANTU and paraquat administration on adenosine and spermidine accumulation into lung slices

Animals were treated as controls (0. 9 % saline/ corn oil, open bars) or given paraquat (30 mg paraquat ion/ kg i. p. , |||) 24 h prior to sacrifice. The third group was given 10 mg ANTU/ kg 4 h prior to sacrifice (\\\ ). Lung slices were prepared and adenosine and spermidine uptake determined over 5 and 15 min for adenosine and 15 and 30 min for spermidine accumulation. The results are expressed in nmol accumulated/ g lung ± S. E. (n= 4).

Fig. 5/ 6. Effect of metabolic inhibitors on adenosine uptake

Lung slices were incubated as controls (□), or pretreated for 30 min with rotenone (0. 1 mM, ▨), potassium cyanide (1 mM, ▤), or preincubated for 30 min at 4° (▥). The slices were then transferred to flasks containing the appropriate treatment for a further 15 min in the presence of tritiated adenosine (total concentration of adenosine was 10 μM). All incubations were carried out in KRB. Results are the mean nmol uptake/ g lung + S. E. (n= 3).



(fig. 5/ 6). The effect was slightly greater at 15 min than at 5 min. However, inhibition is not nearly so great as that observed with 4^{o}. In addition, rotenone and KCN much more greatly affected spermidine uptake than adenosine accumulation (data not shown).

5. 3. f SPERMIDINE AND 5-HYDROXYTRYPTAMINE : EFFECTS ON THE UPTAKE OF ADENOSINE IN RAT LUNG

Spermidine (1. 0-1000 μM) failed to significantly inhibit the accumulation of adenosine into rat lung slices (table 5. II), suggesting it was not accumulated by the oligoamine uptake system present in the alveolar epithelium. In addition, 5-hydroxytryptamine (0. 1-100 μM) failed to reduce radioactivity in slices following incubation with adenosine, suggesting that 5-hydroxytryptamine and adenosine do not share the monoamine oxidase transport system. Adenosine accumulation was in good agreement with that previously measured in control slices (incubated in the absence of spermidine of 5-HT). (Table 5. II).

5. 3. g ADENOSINE ACCUMULATION INTO CONTROL AND BCNU COMPROMISED LUNG SLICES

Control slices exhibited linear accumulation of adenosine over the 30 min incubation period assessed. Adenosine accumulation into BCNU pretreated lung slices was not significantly different from control uptake at 5, 15 or 30 min (fig. 5/ 7).

5. 3. h THE USE OF ADENOSINE AS A FUNCTIONAL MARKER IN CONTROL AND BCNU COMPROMISED LUNG SLICES

BCNU pretreatment of lung slices did not impair their ability to accumulate radioactivity when compared to control slices. Incubation for

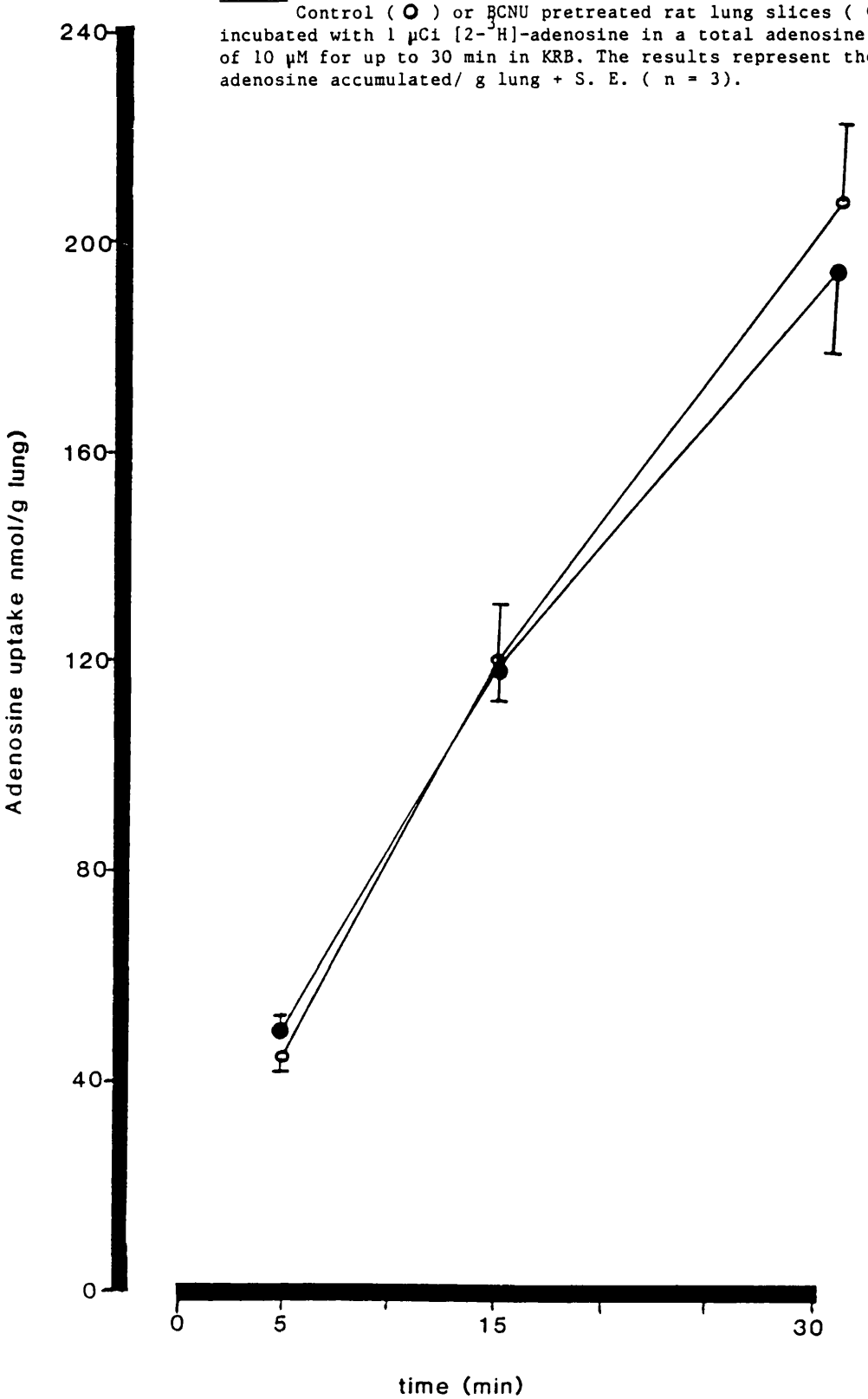
Table 5. II. The effects of spermidine and 5-hydroxytryptamine on adenosine incubation into rat lung slices.

[Spermidine] (μM)	Adenosine uptake (nmol/ g lung)	[5-HT] (μM)	Adenosine uptake (nmol/ g lung)
0	106. 1 ± 5. 4	0	115. 4 ± 16. 2
1	109. 8 ± 4. 6	0. 1	123. 7 ± 13. 2
10	111. 5 ± 11. 5	1. 0	118. 7 ± 15. 2
100	107. 4 ± 18. 6	10	103. 6 ± 11. 0
1000	131. 1 ± 9. 6	100	105. 6 ± 3. 2

Rat lung slices were incubated with 0. 1 μCi tritiated adenosine (total adenosine concentration 10 μM) along with 0- 1 mM spermidine, or 0- 0. 1 mM 5-hydroxytryptamine. Uptake was determined after 15 min of incubation. Results represent the mean of three experiments.

Fig. 5/ 7. Uptake of adenosine into control and compromised lung slices

Control (O) or BCNU pretreated rat lung slices (●) were incubated with 1 µCi [2-^{3}H]-adenosine in a total adenosine concentration of 10 µM for up to 30 min in KRB. The results represent the mean nmol of adenosine accumulated/ g lung + S. E. (n = 3).



4 h failed to inhibit the process since values obtained at this time were in very good agreement with values presented earlier (fig. 5/ 8).

Paraquat (100 μM) did not produce significant effects on adenosine uptake in either control or compromised slices, after 4 h of incubation. Incubation with nitrofurantoin (200 μM) resulted in a significant slight enhancement in control slices, but this was not observed in slices with inactivated glutathione reductase activity. Incubation of slices with the redox cycling quinone 2, 3-dimethoxy-1, 4-naphthoquinone (50 μM) for 4 h resulted in a significant reduction in radioactivity acccumulated in control slices which was markedly potentiated in BCNU compromised slices. Similarly, diquat (1 mM) resulted in significant loss of adenosine uptake in control slices which was also potentiated significantly in BCNU pre-treated slices (fig. 5/ 8). The findings with respect to diquat and paraquat were confirmed by Skamarauskas (unpublished results).

5. 3. i THE INFLUENCE OF EXOGENOUS ADENOSINE ON PULMONARY ATP LEVELS

Initial studies revealed that incubation of lung slices with 1-50 μM adenosine for 15-60 min resulted in an increase in ATP levels in lung tissue (fig. 5/ 9). 1 uM has little significant effect on ATP levels over 60 min of incubation. Adenosine at 50 μM results in increases in ATP of approximately 60 % at 2 h (fig. 5/ 9). These initial studies were expanded. Higher concentrations of adenosine (200 and 500 μM)were employed (fig. 5/ 10). Both concentrations of adenosine elevated ATP significantly. The elevation resulting from incubation with 500 μM adenosine was approximately 60 % at 15 min and was near-maximal at this time. Control ATP levels showed some scatter, but were in accord with previous results (fig. 5/ 9).

Fig. 5/ 8. Uptake of adenosine into control and compromised lung slices: effects of various agents

Control (open bars) and BCNU compromised lung slices (■) were incubated as controls or with paraquat (PQ), nitrofurantoin (NF), 2, 3-dimethoxy-1, 4-naphthoquinone (2, 3-diOMe) or diquat (DQ) for 4 h. Following the appropriate incubation, slices wre incubated for a further 15 min with 1 µCi tritiated adenosine in a total concentration of 10 µM. The results are expressed as the mean nmol accumulated/ g lung ± S. D. of three experiments.

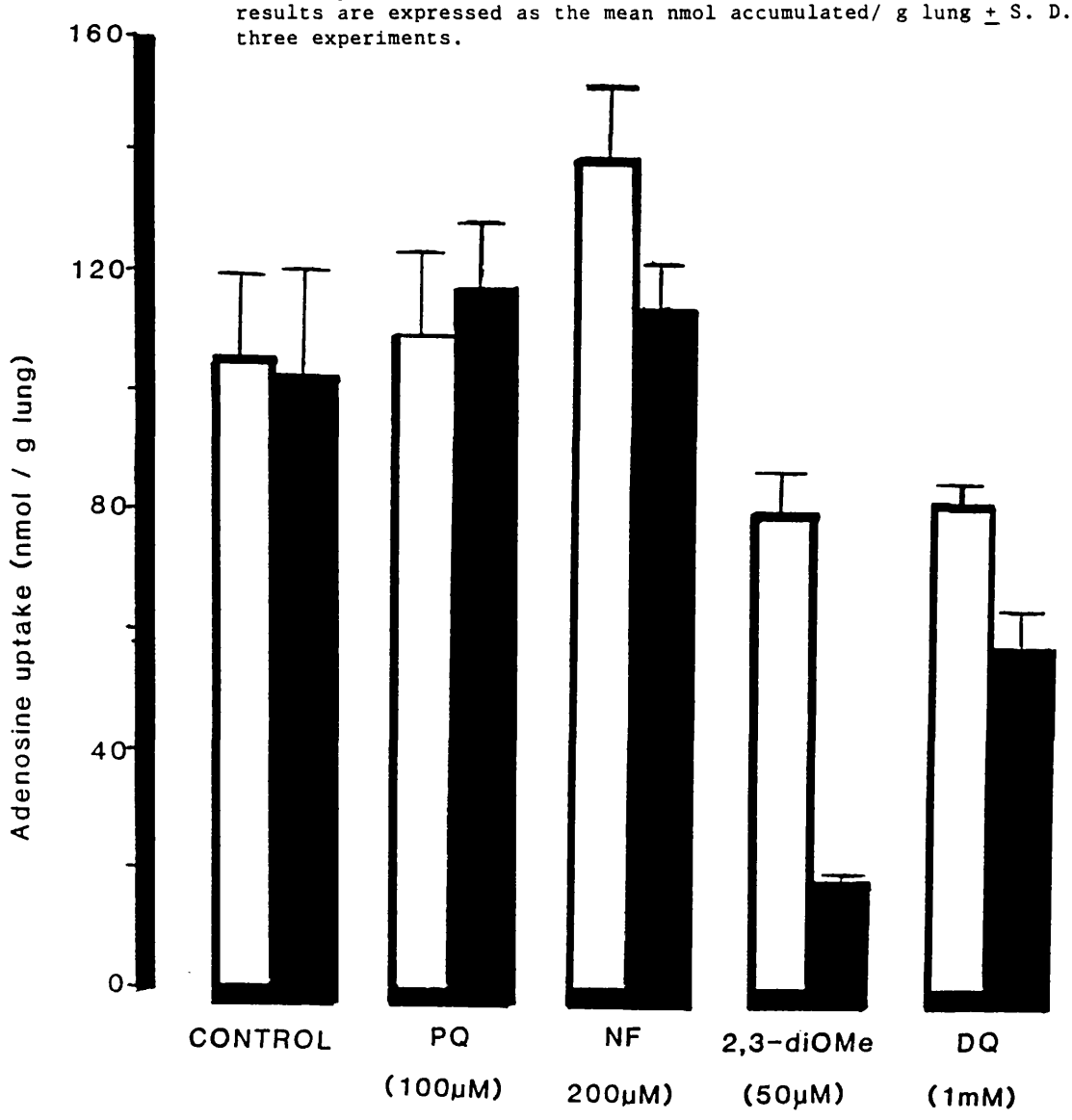


Fig. 5/ 9. ATP levels in lung slices following incubation with adenosine

Lung slices were incubated with adenosine for up to 60 min with 1 μM (▲), 10 μM (■) and 50 μM (✱–✱) adenosine, or in the absence of adenosine (●). ATP was determined using the bioluminescence assay described in methods.

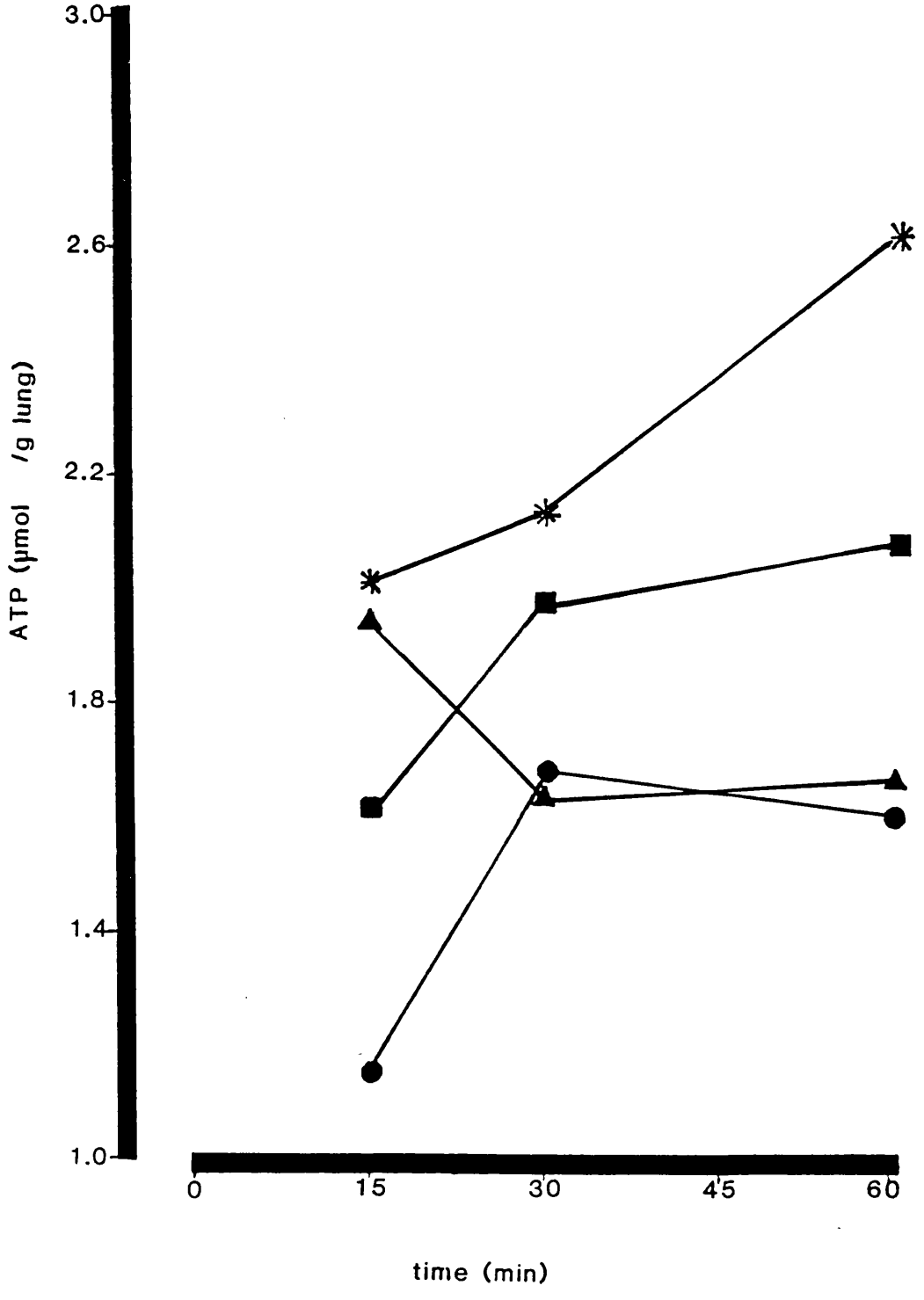
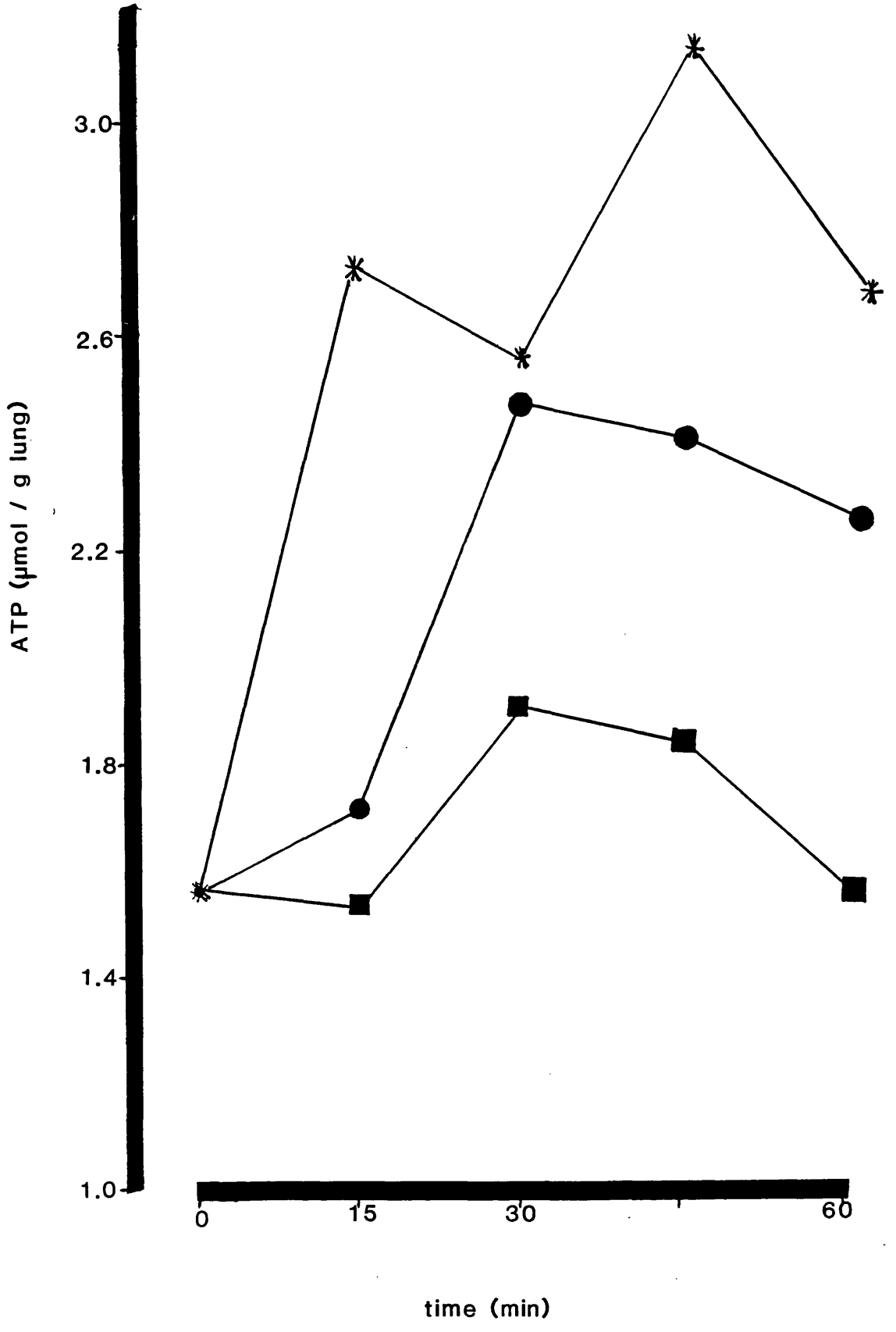


Fig. 37 Exogenous adenosine and ATP levels in lung slices

Lung slices were incubated in the KRB in the presence of adenosine (0. 2 and 0. 5 mM; ● and ✳, respectively) or in the absence of adenosine (■) for up to 60 min. After the appropriate incubation, ATP levels were determined. The results presented are from one experiment typical of several.



5. 4 DISCUSSION

The work presented in this chapter represents an attempt to employ adenosine uptake in rat lung slices as a marker of endothelial function. In these studies, adenosine uptake was calculated from the tissue radioactivity following incubation with [2-^{3}H]-adenosine. Further studies would involve a study of the identity of the compound(s) labelled.

Initial studies (fig. 5/ 2) revealed an approximately linear aaccumulation at adenosine concentrations, 0. 1-10 μM. Previous studies [245] derived an apparent Km for adenosine accumulation into isolated perfused rat lung of 215 μM. Hence, in the lung slices system employed in these studies, adenosine at 10 μM was well below the Km and the uptake process was not, therefore, saturated.

α-Naphthylthiourea (ANTU) was employed in an attempt to identify the pulmonary endothelium as the site of adenosine uptake. Since ANTU is generally considered to be primarily an endothelial toxicant, modulation of adenosine uptake as a result of ANTU administration would be suggestive of endothelial dysfunction. Lung slices prepared from rats dosed with 15 mg ANTU/kg i. p. exhibited striking loss of adenosine accumulation compared to slices prepared from control rats. These results suggest that adenosine uptake is decreased by an endothelial damaging agent (fig. 5/ 3).

Measurement of spermidine accumulation, however, revealed a loss of epithelial function following ANTU administration (fig. 5/ 4) which posed the problem of specificity. The first possibility is that adenosine accumulation is not specific for the pulmonary endothelium or, secondly, that the ANTU-induced lung damage is not confined to the endothelium. Previous work [151] revealed effects on the epithelium following ANTU administration. This is not too surprising since type I and the capillary

endothelial cells are adjacent and ANTU-induced injury to the endothelium might be expected to result in 'knock-on' effects on the fragile type I pneumocyte. In these studies the loss of spermidine uptake paralleled the modulations in adenosine accumulation (fig. 5/ 4). Paraquat ,in vivo, resulted in a loss of spermidine accumulation but did not affect adenosine uptake (fig. 5/ 5), whereas 10 mg ANTU/ kg i. p. reduced the uptake of both compounds (fig. 5/ 5). Since paraquat is presumed to primarily affect the type I and II cells, these results tend to support the conjecture that ANTU affects both the epithelium and the endothelium and that losses of adenosine accumulation reflect metabolic dysfunction in the endothelial cell.

Metabolic inhibitors (fig. 5/ 6) were employed to further characterise the uptake process. Clearly, incubation of slices at 4^{o} resulted in greatly reduced uptake compared with control slices. However, rotenone and KCN less effective in their ability to inhibit adenosine uptake (fig. 5/ 6). This is similar to the situation with 5-HT [221],where rotenone and KCN at similar concentrations inhibited 5-HT uptake to a lesser extent then putrescine accumulation.

Spermidine failed to inhibit adenosine accumulation as did 5-HT (table 5. II) suggesting that adenosine uptake did not occur via the oligoamine uptake system or via the monoamine transport process.

The accumulation of adenosine into control and BCNU compromised rat lung slices was virtually identical, both initially (fig. 5/ 7) and after 4 h (fig. 5/ 8), suggesting a lack of effect of BCNU pretreatment on the endothelium to accumulate adenosine. This data is in conflict with the 5-HT data which revealed a reduction of monoamine uptake into the BCNU compromised slice (Chapter 4).

Paraquat (100 μM) did not produce significant effects on adenosine

uptake in either control or compromised slices which is consistent with the cellular specificity previously described (Chapter 4 and fig. 5/ 8). In contrast to paraquat and nitrofurantoin which did not induce markedly potentiated loss of adenosine uptake, incubation with the quinone 2, 3-dimethoxy-1, 4-naphthoquinone (which resulted in a BCNU potentiable loss of 5-HT uptake in Chapter 4) induced a significant reduction in adenosine uptake in control slices which was markedly potentiated in BCNU compromised slices. The effects of diquat were potentiated in BCNU treated slices (fig. 5/ 8). The results confirm the cellular selectivity of paraquat-induced toxicity, the ubiquitous effects of the quinone, and the non-selective effects of diquat.

Previous studies have shown that adenosine can be metabolised to ATP in lung tissue. The increases in ATP levels observed following incubation of lung slices with adenosine (fig. 5/ 9 and fig. 5/ 10) are in accord with these previous findings and present the intriguing possibility of elevating pulmonary ATP, in vitro and assessing the influence of raised ATP levels in the toxicity of a variety of agents. Such experiments would form the basis of future investigations. The elevation of ATP levels in lung slices by incubation with adenosine is consistent with recent observations in mouse liver in which intraperitoneal administration of adenosine resulted in total liver ATP levels [248].

The reductions of adenosine accumulation following ANTU are marked. The question of whether this occurs as a result of damage to the wall of the capillary endothelium, or a disruption of metabolism remains unclear. Further studies are essential to determine the various fates of the adenosine, since throughout these studies a reduction in accumulated label has been accepted to be indicative of toxicity. This may be too simplistic. Investigations of differential distribution of radiolabel between the

various metabolites could also reveal endothelial cell dysfunction.

CHAPTER 6-PROTECTORS AND POTENTIATORS OF PULMONARY TOXICITY

6. 1 AIMS

Firstly, to attempt to elevate pulmonary thiols by prior treatment of rats and mice with phorone. Secondly, to assess the influence of elevated lung sulphydryls on paraquat toxicity in vitro and in vivo and on the effects of the oedematogenic compound ANTU. Thirdly, to investigate the influence of exogenous GSH, DEM, hyperoxia and desferrioxamine on the effects of paraquat in rat lung slices.

6. 2 INTRODUCTION

The effect of sulphydryl pretreatments on paraquat toxicity is enigmatic. Exogenous GSH protects isolated type II cells against paraquat induced loss of viability [249]. In contrast, i. v. infusion of cysteamine and N-acetyl cysteine failed to reduce paraquat -induced mortality in rats, whereas oxygen-induced mortality was ameliorated. In addition, exogenous GSH has been shown to protect isolated small intestinal cells from oxidant injury induced by t-butyl hydroperoxide and menadione [250].

The elevation of lung sulphydryls with butylated hydroxyanisole treatment (BHA) has been shown, in our laboratory, to confer some protection against paraquat [228] though the mechanism of protection was uncertain. A problem of sulphydryl pretreatments may lie in an inability to take up and utilise exogenous GSH by cell types susceptible to paraquat. An alternative approach is to utilise the rebound synthesis phase of treatment with an α, β-unsaturated carbonyl compound such as phorone to elevate sulphydryls since the literature with regard to the ability of lung to take up and utilise glutathione is unclear [103, 104].

In these studies, phorone was employed for in vivo treatments, since it has been reported to exert fewer unwanted effects than diethylmaleate (DEM).

DEM enhanced the effects of paraquat on rat liver [251] and has also been shown to enhance the pulmonary toxicity of the oedematogenic compound ANTU [155]. Protection against ANTU-induced injury has been obtained with various pretreatments (e. g. prior exposure to small doses of ANTU).

Hydrogen peroxide produced by the redox cycling of paraquat may then be reduced to hydroxyl radical in an iron-catalysed Fenton reaction, which is extremely reactive and results in a variety of toxic events.

It has recently been shown that desferrioxamine, a chelator that blocks the catalytic activity of iron in oxygen radical generation, reduced paraquat mortality in laboratory mice [252]. In addition, several other studies have described the protective effects of DFO in oxygen radical mediated injury [253]. DFO has been shown to significantly reduce the cytotoxicity of diquat in isolated hepatocytes [254].

6. 3 RESULTS

6. 3. a PHORONE AND THIOL LEVELS IN THE RAT

Administration of phorone (200 mg/ kg i. p.) to Wistar rats (approximately 200 g) resulted in striking depletion in pulmonary and hepatic GSH levels (fig. 6/ 1 and 6/ 2). A similar pattern was observed in both organs with significant depletion detected at 2 h, and maximal at 4 h, followed by significant recovery at 6 h. Thiol levels were significantly elevated over control at 24 h, but the greatest elevation was obtained at 48 h. At 48 h levels of GSH were approximately 2. 5-fold higher than in control lungs,compared with a 4-fold increase in livers of phorone treated

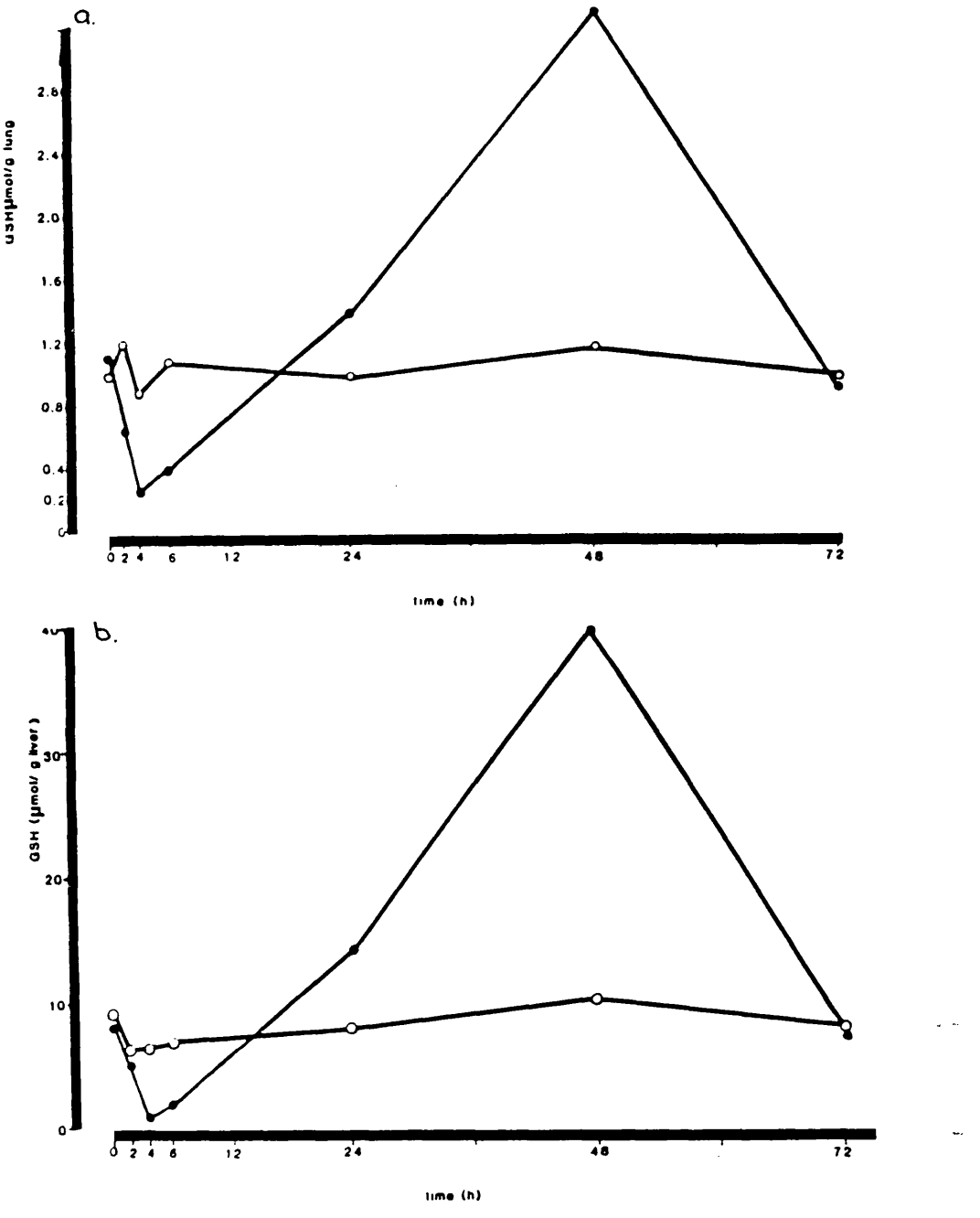


Fig. 6 / 1 and 6/ 2. Effect of phorone treatment on pulmonary and hepatic non-protein sulphydryls in the rat

Animals (c. a. 200 g in weight) were given 200 mg phorone / kg in corn oil via an i. p. route and sacrificed at the appropriate times up to and including 72 h (●). Control animals were given corn oil vehicle only (○).

Lungs and livers were perfused in situ with 0. 9 % sodium chloride (w/ v) and then homogenised (as described in methods). Thiol levels were assessed using the fluorometric assay. Results are expressed in in umol/ g lung and values represent the means of three experiments. Standard errors were small i. e. < 10 % and have, therefore, been omitted to facilitate presentation. Lung results are shown in fig. 6/ 1 (a) and liver results are presented in fig. 6/ 2 (b).

animals (compared to control livers) (figs. 6/ 1 and 6/ 2). The magnitude of the initial depletion and subsequent rebound synthesis was dose-dependent with respect to phorone. Phorone at 125 mg/ kg produced a more modest initial depletion, with only minimal rebound, whilst 250 mg/ kg resulted in an even greater GSH depletion at 2 h (data not shown). However, toxic signs and mortality were noted in some animals at the higher dose (250 mg/kg i. p.)and hence, for future studies, phorone at a dose of 200 mg/ kg was selected in order to raise pulmonary thiols.

6. 3. b PULMONARY THIOLS IN MICE FOLLOWING PHORONE ADMINISTRATION

Aministration of 400 mg phorone/ kg i. p.) increased pulmonary thiols by approximately 4. 5-fold, 24 h after dosing. A depletion of GSH was observed in the lung at 2 h,but no other early time points were investigated (fig. 6/ 3). This elevation is greater than that observed with rat lung and occurs earlier. At 650 mg/ kg phorone produced lethality and toxic signs (data not shown).

6. 3. c PARAQUAT TOXICITY IN MICE : INFLUENCE OF PHORONE

Animals were given phorone (400 mg/ kg i. p.) 24 h prior to the administration of paraquat. Elevation of pulmonary thiols with phorone failed to reduce paraquat induced mortality in mice (table 6. I), or weight loss (data not shown). In addition, the survival time was not prolonged (table 6. I). Mortality, survival time and weight loss were dose-dependent with respect to paraquat. Whilst it was not possible to exactly identify pulmonary injury as the cause of death, all fatally intoxicated animals exhibited extremely laboured breathing and plum-coloured lungs.

Fig. 6/3. Phorone in mice-pulmonary non-protein sulphydryl levels

Male mice (BKTO strain; 25-30 g) were dosed with 400 mg phorone / kg i. p. in corn oil. The results are expressed as percentage of controls calculated from the mean of values obtained with three animals per time point. GSH levels were determined at the appropriate time periods. Following sacifice, lungs were perfused with isotonic saline in situ, then removed and homogenised. GSH levels were assayed via the fluorometric method described in (Methods). Control pulmonary NPSH was 1. 85 ± 0. 1 μmol/ g lung (n = 3 experiments).

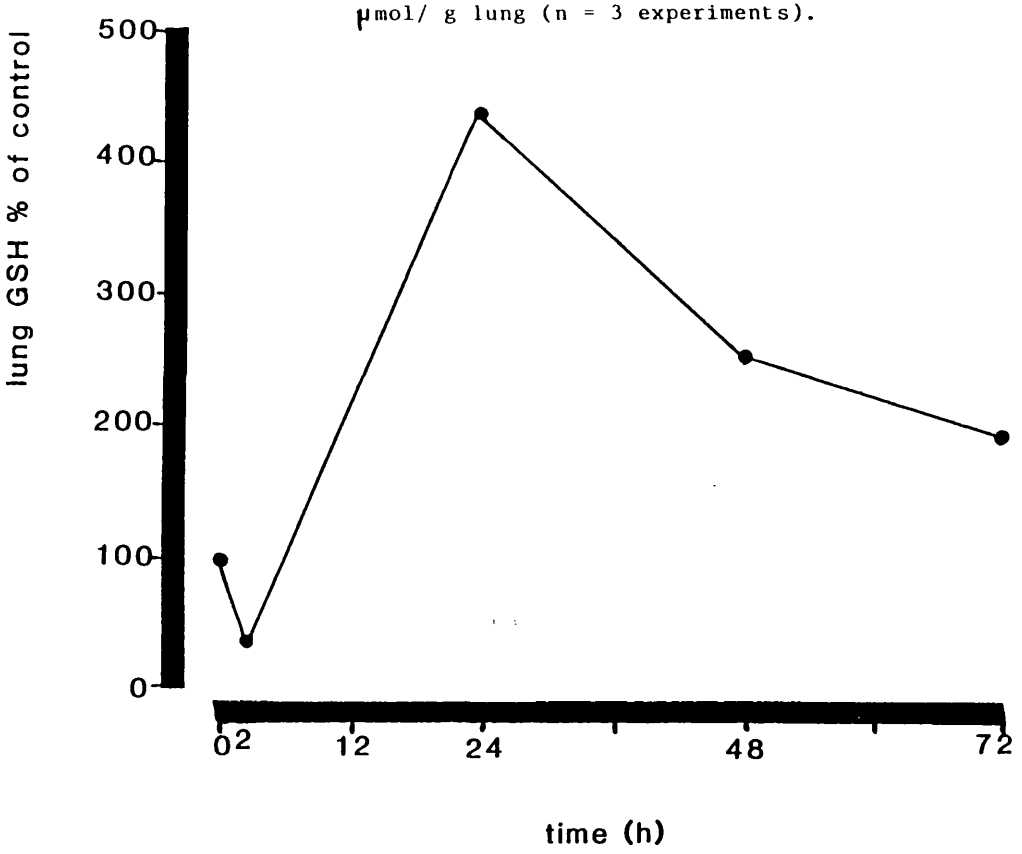


Table 6. I. Influence of phorone on paraquat-induced mortality in mouse and rat.

MOUSE			RAT		
Treatment	Mortality	Times of death	Treatment	Mortality	Times of death
Control	0/ 8	-	Control	0/ 6	-
Phorone (400 mg/ kg)	0/ 8	-	Phorone (200 mg/kg)	0/ 6	-
20 mg PQ ion/kg	0/ 8	-	10 mg PQ ion/ kg	0/ 6	-
30 mg PQ ion/kg	4/ 8	2 : T+4 2 : T+5	20 mg PQ ion/ kg	2/ 6	2 : T+3
35 mg PQ ion/kg	8/ 8	6 : T+4 2 : T+5	Phorone + 10 mg/ kg	0/ 6	-
Phorone + 20 mg PQ ion/kg	0/ 8	-	Phorone + 20 mg/ kg	3/ 6	2 : T+3 1 : T+4
Phorone + 30 mg PQ ion/kg	4/ 8	2 : T+4 2 : T+5			
Phorone + 35 mg PQ ion/kg	8/ 8	4 : T+4 4 : T+5			

Phorone was administered to male BKTO strain mice (25- 30 g) in corn-oil (400 mg/kg i. p.). After 24 h, the appropriate paraquat dose was administered s. c. in saline. Control animals received both vehicles only. Male Alderley Park strain Wistar rats (180 g) were dosed with 200 mg phorone/ kg and paraquat was given after 48 h.

6. 3. d PARAQUAT TOXICITY IN RAT : INFLUENCE OF PHORONE

Pretreatment of animals with phorone did not result in lethality. Prior treatment with phorone failed to ameliorate the effects of paraquat, consistent with the results of the study in mice. Times to the onset of mortality were not increased, mortality was not reduced and the paraquat-induced weight losses were not modulated.

6. 3. e THE INFLUENCE OF PHORONE PRETREATMENT ON α-NAPHTHYLTHIOUREA (ANTU) TOXICITY IN THE RAT

Rats (approximately 200 g) were dosed appropriately as shown in table 6. II. ANTU resulted in a dose dependent hydrothorax after 4 h which was not present in phorone pretreated animals dosed similarly with ANTU (table 6. II). The hydrothorax fluid was non-haemorrhagic. ANTU at a dose of 10 mg/ kg i. p. was fatal to 4/ 8 animals. At this dose of ANTU, phorone pretreatment protected completely against lethality. ANTU at 5 mg/ kg was not fatal to any animal, though the toxic signs exhibited in phorone pretreated animals were ameliorated. Phorone treatment did not per se, result in measurable hydrothorax, toxic signs or lethality.

In whole lung there was an increase in rat lung wet/ dry weight ratios as a result of ANTU at 4 h following administration. This effect of ANTU was ameliorated by phorone pretreatment. In sliced lung, there was little difference in wet/ dry weight ratios exhibited by the different treatment groups (table 6. II).

There was no significant difference in adenosine uptake into lung slices prepared from phorone pretreated rats, compared to uptake into lung tissue from control animals. ANTU produced a dose dependent loss of adenosine uptake (fig. 6/ 4). ANTU (5 mg/ kg) reduced adenosine uptake by

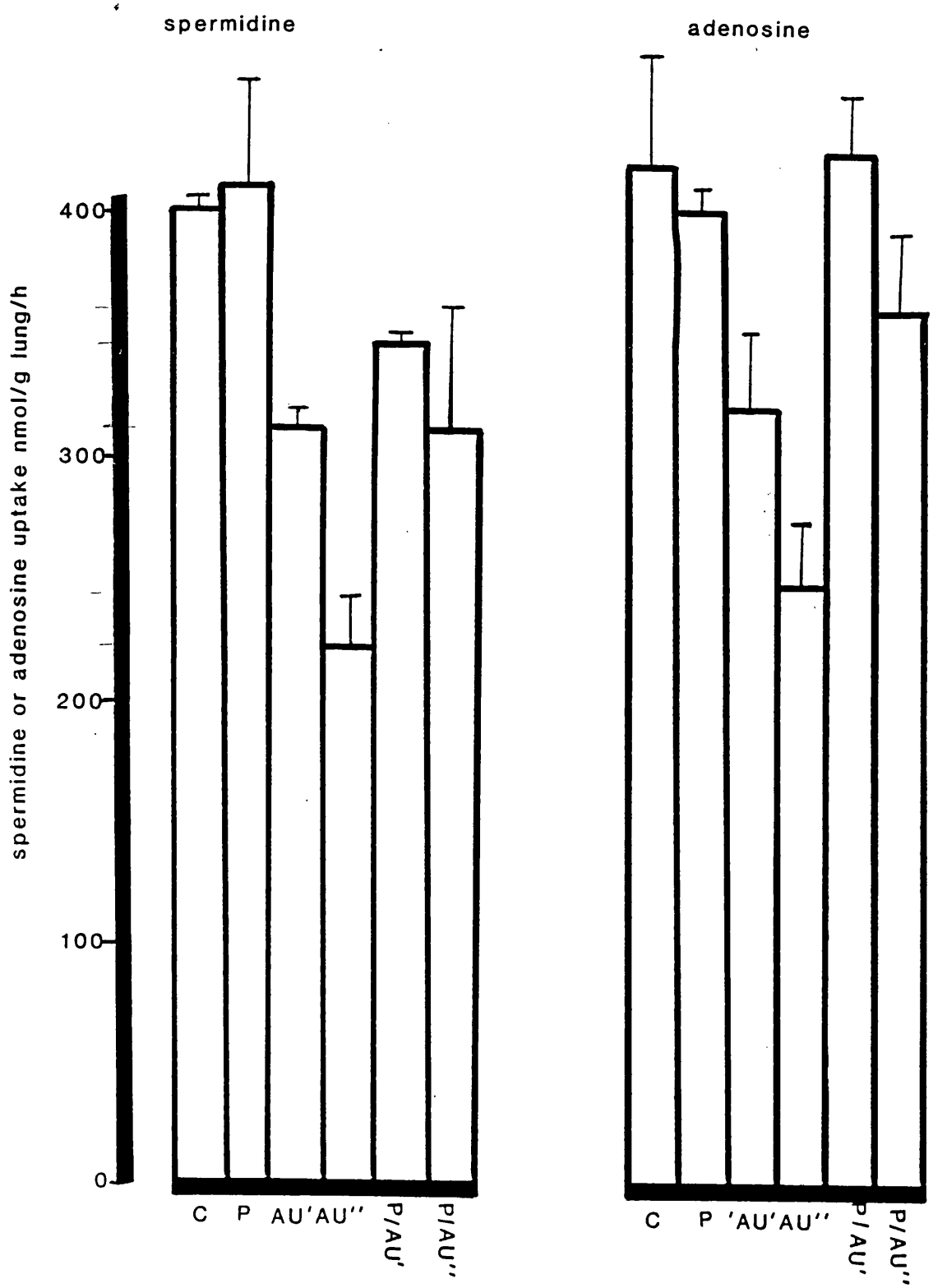


Fig. 6/ 4. Effect of ANTU on adenosine and spermidine accumulation into lung slices prepared from control and phorone pretreated rats

Control animals were given corn oil vehicle only, whereas phorone pretreated rats received 200 mg phorone/ kg i. p. After 48 h, both groups received either ANTU (5 or 10 mg/ kg i. p.) and corn oil vehicle. At 4 h after ANTU, animals were sacrificed and the uptake of spermidine and adenosine was determined over 30 and 15 min, respectively. The results are expressed in nmol accumulated/ g lung/ h ± S. E. (n = 3). Abbreviations: C, control; P, phorone pretreated control; A', 5 mg/ kg ANTU; A'', 10 mg/kg ANTU.

P/ AU' : Phorone + 5 mg ANTU / kg. P/ AU" : Phorone + 10 mg ANTU / kg.

Table 6. II. The Influence of Phorone Pretreatment on ANTU Toxicity in Rat Lung.

Animals were given 200 mg/kg phorone ip or treated as controls. After 48 h, ANTU was administered.

Treatment	Mortality	Hydrothorax (ml)	Lung wet/dry weight ratios Whole lung	Sliced lung	[GSH] µmol/g lung	Spermidine Accumulation nmol/g/h	Adenosine Accumulation nmol/g/h
Control	0/8	ND	5.44	4.14	1.43	401±3.5(3)	418±47(3)
Phorone	0/8	ND	4.62	5.17	2.46	412±41(3)	400±9.2(3)
5mg/kg ANTU	0/8	0.8-2.5	5.76	5.19	-	311±8.4(3)	320±32(3)
10mg/kg ANTU	4/8	4.0-4.5	6.38	5.49	-	222±21(3)	248±26(3)
20mg/kg ANTU	8/8	-	-	-	-	-	-
Phorone + 5mg/kg ANTU	0/8	ND	5.20	5.10	-	347±2.6(3)*	424±23(3)*
Phorone + 10mg/kg ANTU	0/8	ND	5.81	5.16	-	311±50(3)*	358±34(3)*
Phorone + 20mg/kg ANTU	0/8	-	-	-	-	-	-

ND = not detected
* = statistically different from control pretreated ANTU dose ($p = < 0.5$ % using an unpaired t-test).

approximately 1/ 4, whilst 10 mg ANTU/ kg reduced uptake by nearly 1/ 2 after 4 h. Phorone pretreatment reduced, significantly, the loss of adenosine accumulation produced by ANTU.

In animals dosed with ANTU, a marked loss of spermidine accumulation was observed which paralleled the losses of adenosine accumulation observed following administration of ANTU. In phorone pretreated rats this loss of spermidine uptake was ameliorated (fig. 6/ 4). Protection was not complete, but was substantial.

Thiol levels were elevated in phorone pretreated rat lung compared with the lungs of control animals, in the absence of ANTU (table 6. II). GSH was measured by two methods,though both revealed a 2-fold increase which was a little lower than the results presented in fig. 6/ 1. The specific enzymatic method of Brigelius gave higher values than the less specific method of Ellman which uses dithionitrobenzoic acid.

6. 3. f GSH LEVELS IN LUNG SLICES INCUBATED WITH DIETHYL MALEATE OR PHORONE

GSH levels remained stable in control slices over the 60 min of incubation assessed. Phorone (1 mM) produced a steady GSH depletion which was approximately linear. At 60 min, depletion of approximately 2/ 3 was observed. However, a near maximal depletion of GSH was observed at 15 min with 1 mM DEM. There was no significant difference at 15, 30 and 60 min between 0.5 mM and 1 mM DEM, which resulted in a GSH depletion of approximately 85 % at 15 min (fig. 6/ 5).

6. 3. g GSH LEVELS FOLLOWING INCUBATION WITH DEM

Incubation with 0. 5 mM DEM for 15 min resulted in a thiol depletion which was consistent with that observed in the studies in fig.

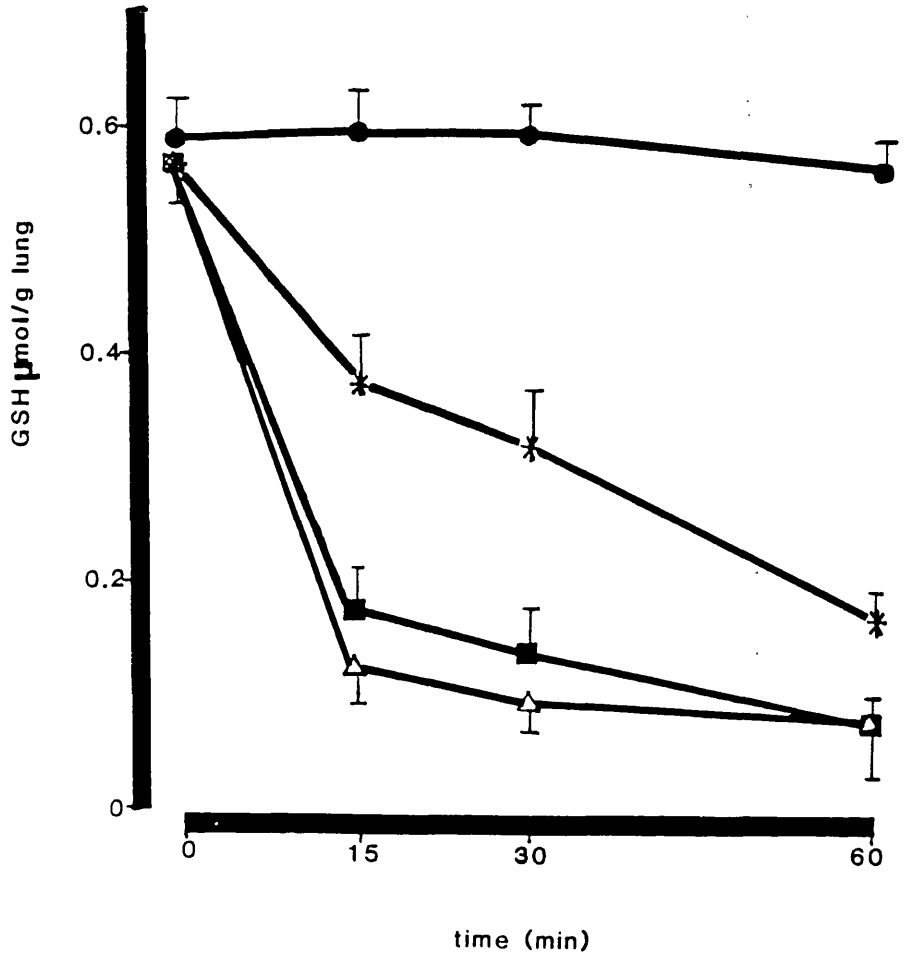
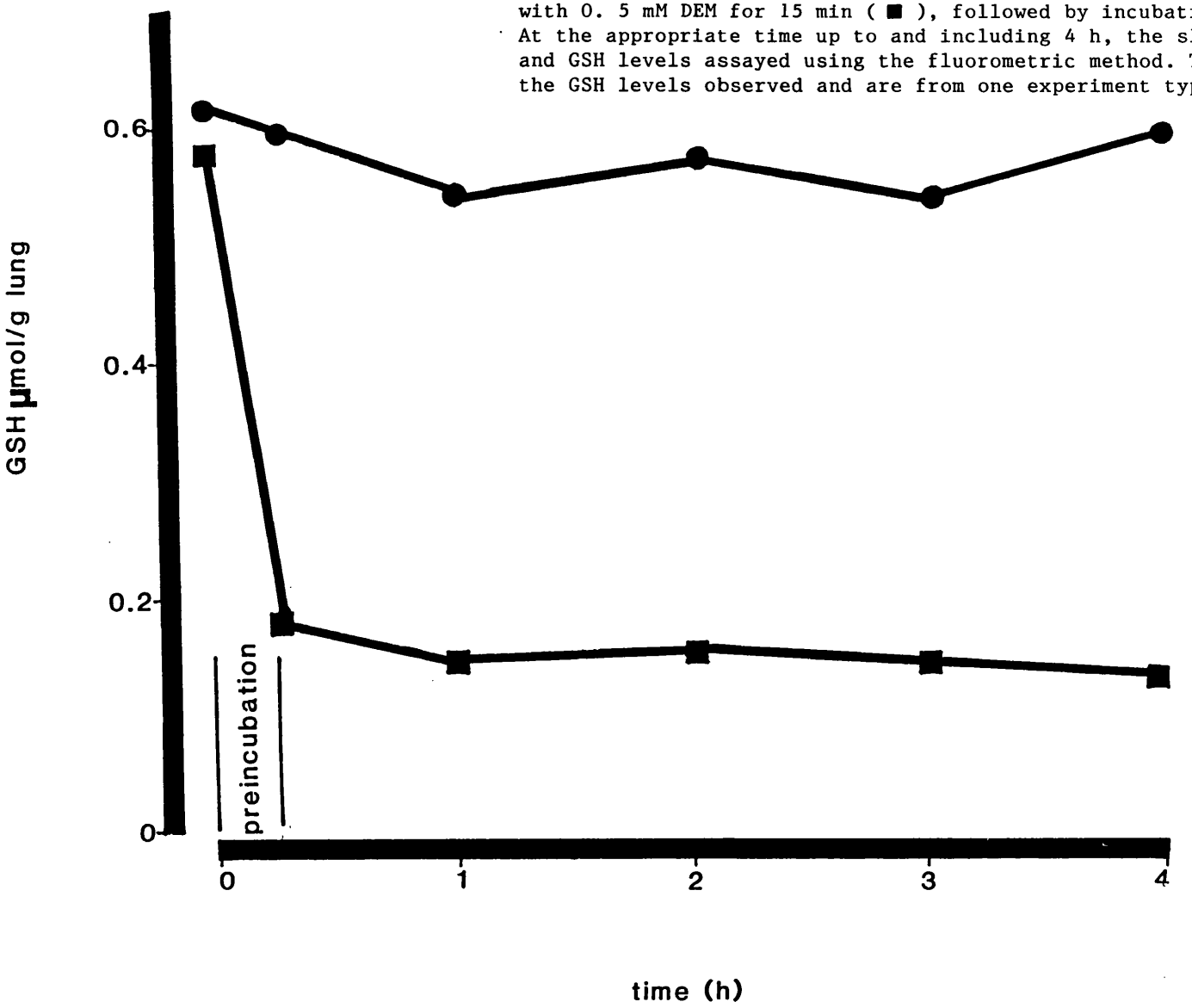


Fig. 67 5. DEM and phorone in lung slices : effects on GSH levels
Rat lung slices were incubated in KRB as controls (0. 3 % DMSO, ●), with 1 mM phorone (✱), 0. 5 mM DEM (■) or 1 mM DEM (Δ) for up to 60 min. Following inubation slices were removed, homogenised and GSH levels determined via the fluorometric method. Results are expressed in mean µmol GSH/ g lung ± S. E. (n= 3).

Fig. 6/6. GSH levels in lung slices following incubation with DEM

Perfused rat lung slices were incubated as controls (0. 3 % DMSO; ●) for 15 min and then replaced in fresh KRB and incubated for 4 h, or with 0. 5 mM DEM for 15 min (■), followed by incubation in fresh KRB. At the appropriate time up to and including 4 h, the slices were removed and GSH levels assayed using the fluorometric method. The results represent the GSH levels observed and are from one experiment typical of two.



6/ 5). The depletion was maintained over 4 h of incubation (fig. 6/ 6) and there was no rebound of sulphydryl levels.

6. 3. h INFLUENCE OF DIETHYLMALEATE PRETREATMENT ON PARAQUAT TOXICITY IN CONTROL AND COMPROMISED LUNG SLICES AS ASSESSED BY PUTRESCINE UPTAKE

The pretreatment of lung slices with diethyl maleate did not result in a loss of putrescine uptake in either control tissue or in BCNU compromised slices (fig. 6/ 7). Paraquat (100 μM) resulted, in control slices, in a loss of putrescine accumulation similar to that seen previously. This loss of putrescine uptake was potentiated in control slices pretreated with diethyl maleate and similarly incubated with paraquat. In BCNU compromised slices, paraquat resulted in a marked loss of diamine accumulation, in accord with previous studies (Chapter 4), that constituted a significant potentiation of toxicity as a result of the inactivation of glutathione reductase (fig. 6/ 7). At 4 h, this loss of putrescine uptake was too extensive for a marked potentiation to be observed with diethyl maleate. A small potentiation was observed which, though statistically significant, was of dubious biological relevance (fig. 6/ 7).Previous studies revealed a significant enhancement of paraquat induced loss of putrescine uptake in BCNU compromised slices at 2 h as a result of preincubation with diethyl maleate (data not shown).

6. 3. i THE EFFECT OF PHORONE PRETREATMENT ON PARAQUAT TOXICITY IN RAT LUNG SLICES : EFFECTS ON PUTRESCINE UPTAKE

Slices prepared from the lungs of control animals exhibited a concentration-dependent (with respect to paraquat) and time dependent loss of diamine uptake following incubation with paraquat (10 and 100 μM)

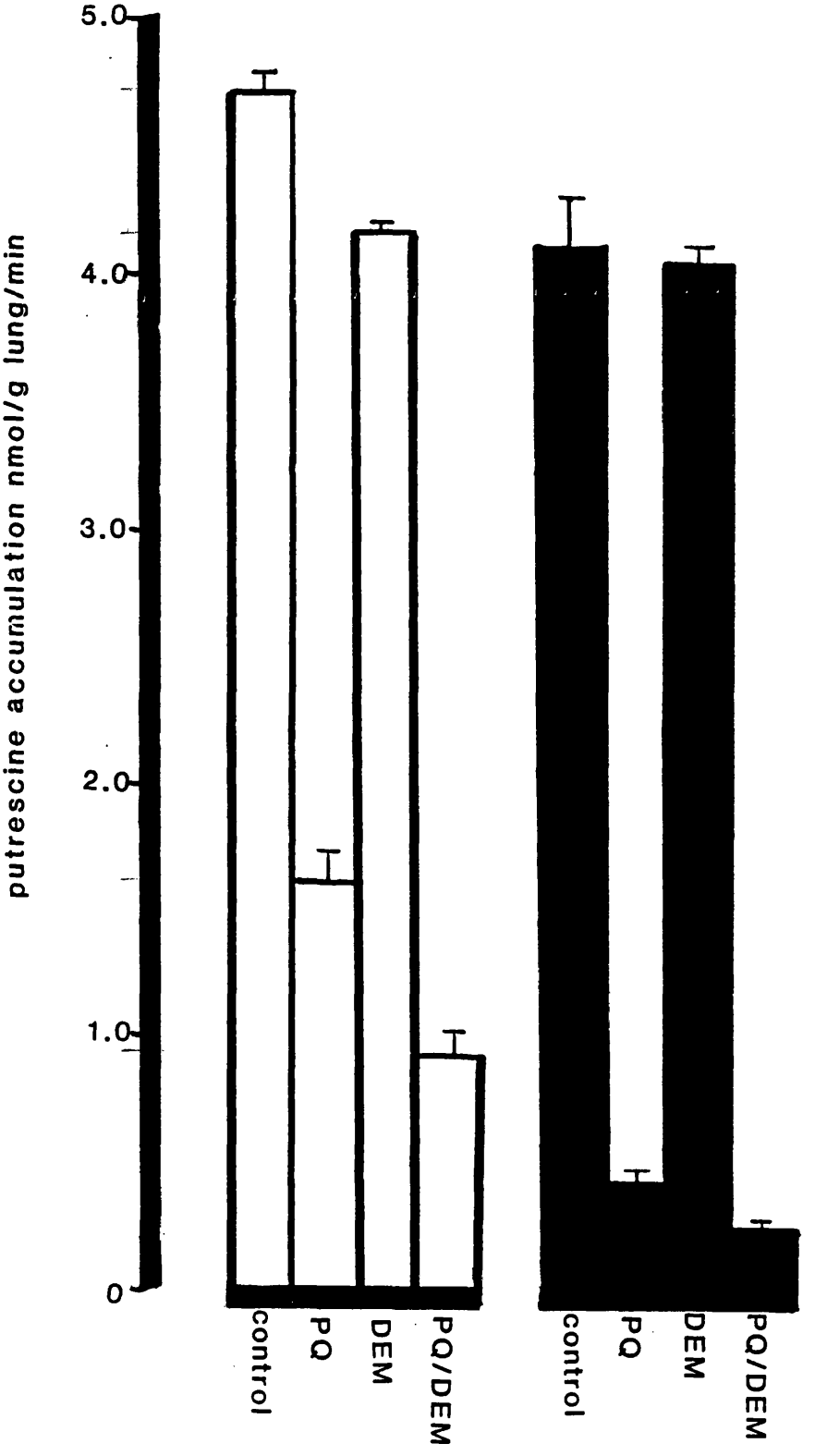


Fig. 6/ 7. Effect of DEM pretreatment of lung slices on paraquat toxicity as assessed by putrescine uptake

Control (open bars) and BCNU compromised slices (hatched bars) were incubated in the presence or absence of diethylmaleate (0. 5 mM) for 15 min and then incubated for a further 4 h in the presence or absence of paraquat (100 μM). Putrescine uptake was then determined as described in methods. Results are expressed as mean nmol putrescine accumulated/ g lung/ min ± S. E. (n = 3 experiments).

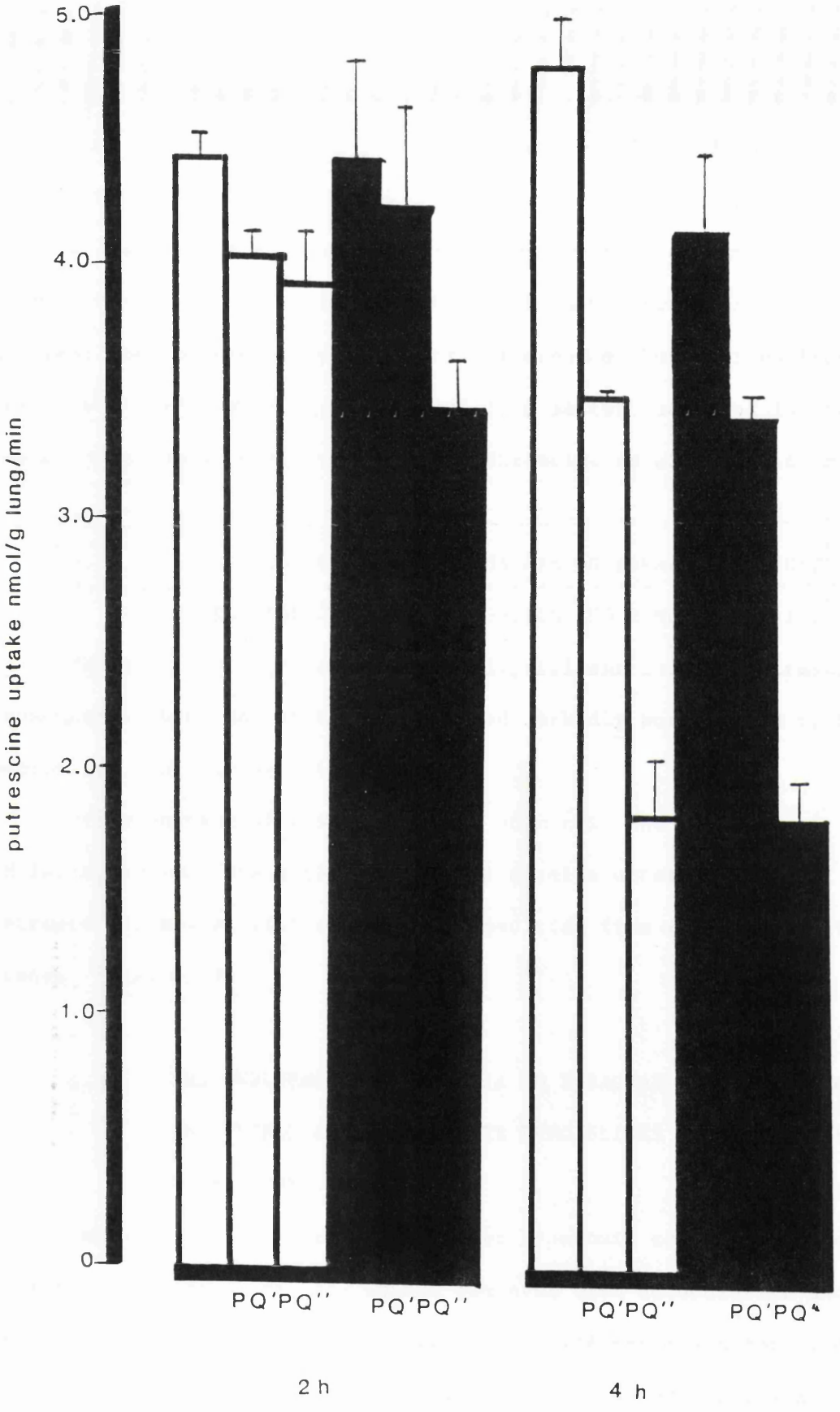


Fig. 6/ 8. The effects of paraquat in lung slices prepared from phorone pretreated rats.

Control lung slices (open bars: prepared from rats given corn oil vehicle 48 h previously) and slices prepared from phorone pretreated rats (closed bars; 200 mg phorone / kg i. p. 48 h previously) were incubated in the absence or presence of paraquat (PQ, 10 and 100 μM) for 2 and 4 h in KRB. Slices were then removed and putrescine uptake was assessed. The results are expressed as mean nmole putrescine accumulated/ g lung/ min ± S. E. (n = 3).

PQ' : 10 μM PQ" : 100 μM.

(fig. 6/ 8).

Phorone treatment of rats (200 mg/kg i. p. 48 h prior to sacrifice, preparation of slices and subsequent incubation) elevated pulmonary thiols approximately 2-fold (data not shown). However, slices prepared from the lungs of phorone treated animals exhibited diamine accumulation following incubation with paraquat (10 and 100 μM) which was not statistically different from control slices similarly incubated. Lung slices from phorone pretreated animals,and control lung slices incubated in the absence of paraquat exhibited similar putrescine uptake (fig. 6/ 8).

6. 3. j THE INFLUENCE OF EXOGENOUS GSH ON PARAQUAT TOXICITY : PUTRESCINE UPTAKE INTO CONTROL AND BCNU PRETREATED SLICES

Paraquat (100 μM) resulted in a significant loss of putrescine accumulation which was time dependent and markedly potentiated in BCNU compromised lung slices (fig. 6/9).

Preincubation of lung slices for 30 min in the presence of 1 mM GSH failed to ameliorate these losses of diamine uptake in control or BCNU pretreated slices at either 2 or 4 h, resulting from incubation with paraquat (fig. 6/ 9).

6. 3. k THE INFLUENCE OF HYPEROXIA ON PARAQUAT AND DIQUAT TOXICITY IN CONTROL AND COMPROMISED LUNG SLICES AS ASSESSED BY PUTRESCINE UPTAKE

Incubation of control slices under hyperoxic conditions resulted in a significant loss of diamine uptake compared with accumulation under normoxic conditions, whereas no significant difference was observed in BCNU pretreated slices incubated normoxically or under hyperoxia.

Paraquat-induced loss of putrescine uptake was potentiated by BCNU

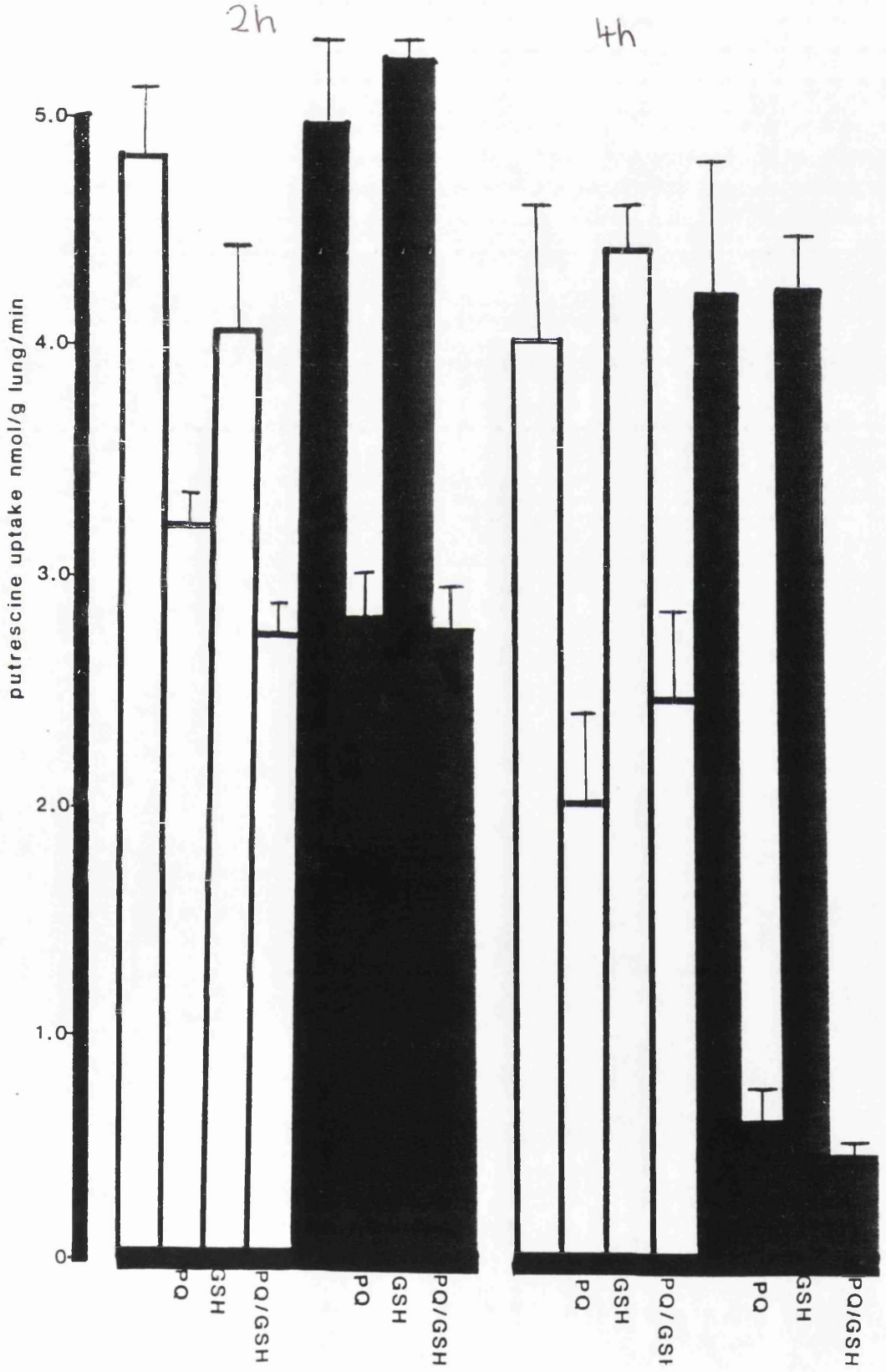


Fig. 6/ 9. Influence of exogenous GSH on the effects of paraquat in control and BCNU compromised lung slices

Control (open bars) and BCNU pretreated slices (b) were incubated for 30 min in KRB in the presence or absence of 1 mM GSH. Slices were then replaced in fresh KRB and incubated for 2 and 4 h with paraquat (PQ, 100 μM). Following the appropriate incubation the ability to accumulate putrescine was assessed over 30 min. (n = 3)

pretreatment, though hyperoxia significantly ameliorated this loss of uptake in both control and compromised lung slices (fig. 6/ 10).

In contrast, diquat -induced loss of putrescine accumulation was potentiated by inhibition of glutathione reductase activity, but hyperoxia did not modulate the response to diquat in control or compromised lung slices (fig. 6/ 10).

Previous work revealed that incubation of control and compromised slices under hyperoxic conditions did not affect paraquat uptake compared to slices incubated in room-air (data not shown).

6. 3. 1 EFFECT OF DESFERRIOXAMINE (DFO) ON PARAQUAT TOXICITY IN CONTROL AND COMPROMISED LUNG SLICES

Paraquat (100 μM) significantly reduced putrescine accumulation into control slices after 4 h of incubation, and this loss of diamine uptake was markedly potentiated in BCNU compromised slices. DFO (500 μM) ameliorated loss of diamine uptake, significantly, in both control and BCNU compromised slices after 4 h of incubation. A similar trend was observed at 2 h (data not shown). Incubation of lung slices in the absence of paraquat with 0. 5 mM DFO did not affect subsequent putrescine accumulation in either control or BCNU compromised slices (fig. 6/ 11).

6. 3. m DFO AND PARAQUAT TOXICITY : THE EFFECTS OF A RANGE OF PARAQUAT CONCENTRATIONS IN CONTROL AND COMPROMISED LUNG SLICES

In control slices paraquat (10, 50 and 100 μM) resulted in a concentration-dependent loss of putrescine uptake following 4 h of incubation. A similar trend was observed in compromised slices,though the effects of 50 and 100 μM paraquat were not different statistically.

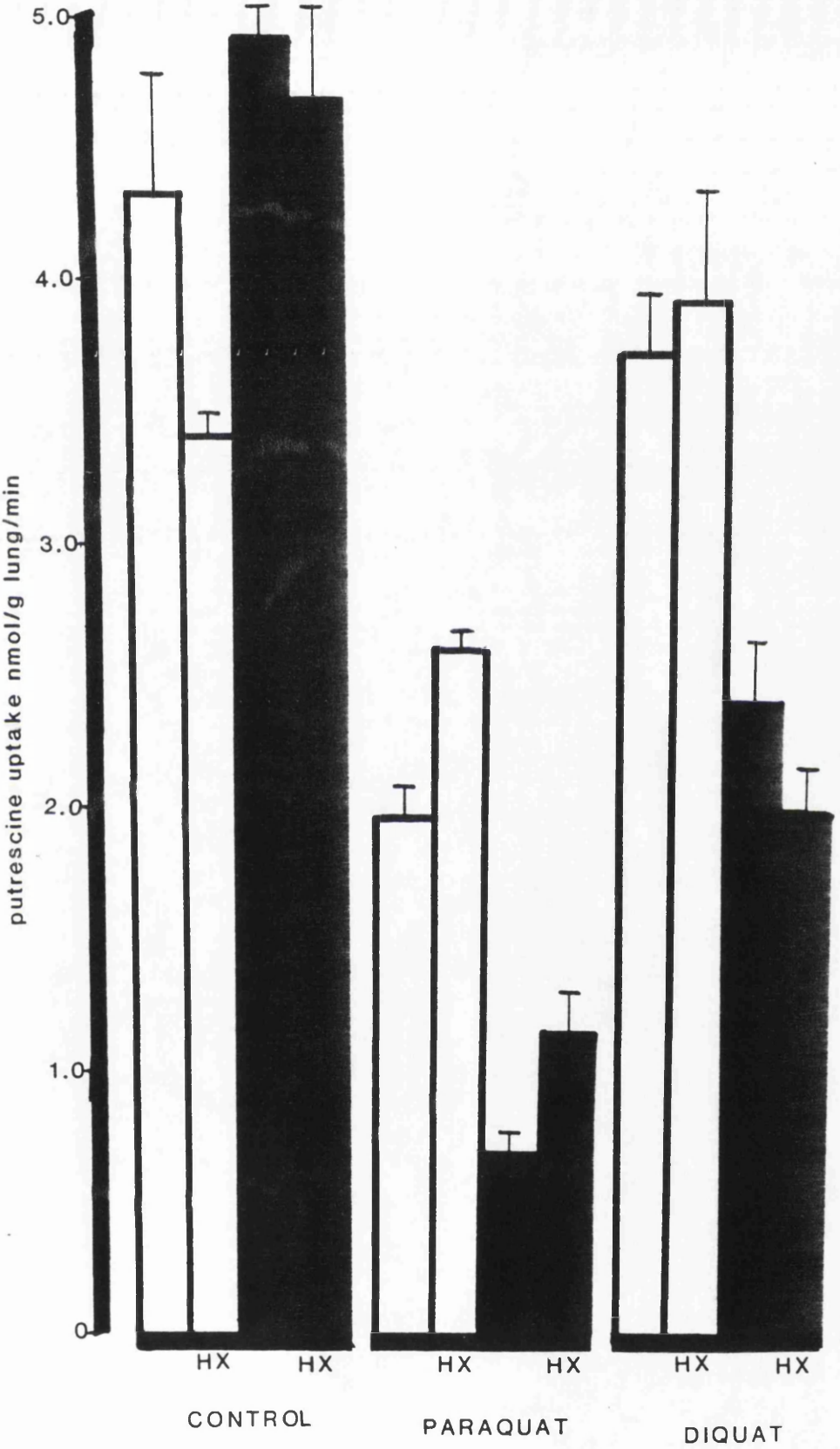


Fig. 6/ 10. Influence of hyperoxia in control and compromised lung slices : studies with paraquat and diquat.

Control (open bars) and compromised slices (closed bars) were incubated either as controls or with paraquat (100 µM) or diquat (200 µM) for 4 h either under room air or under hyperoxic (HX) conditions. After the appropriate incubation period, putrescine uptake was assessed over 30 min (as described in methods). Results represent mean nmole putrescine accumulated/ g lung/ min ± S. E. (n = 3). Hyperoxia was produced by constant gassing with 95% O_2/5% CO_2.

1 : No paraquat and no DFO.
2 : 10 μM PQ, no DFO.
3 : 100 μM DFO alone.
3 : 500 μM DFO alone.
5 : 10 μM PQ and 50 μM DFO.
6 : 10 μM PQ and 100 μM DFO.
7 : 10 μM PQ and 200 μM DFO.
8 : 10 μM PQ and 500 μM DFO.

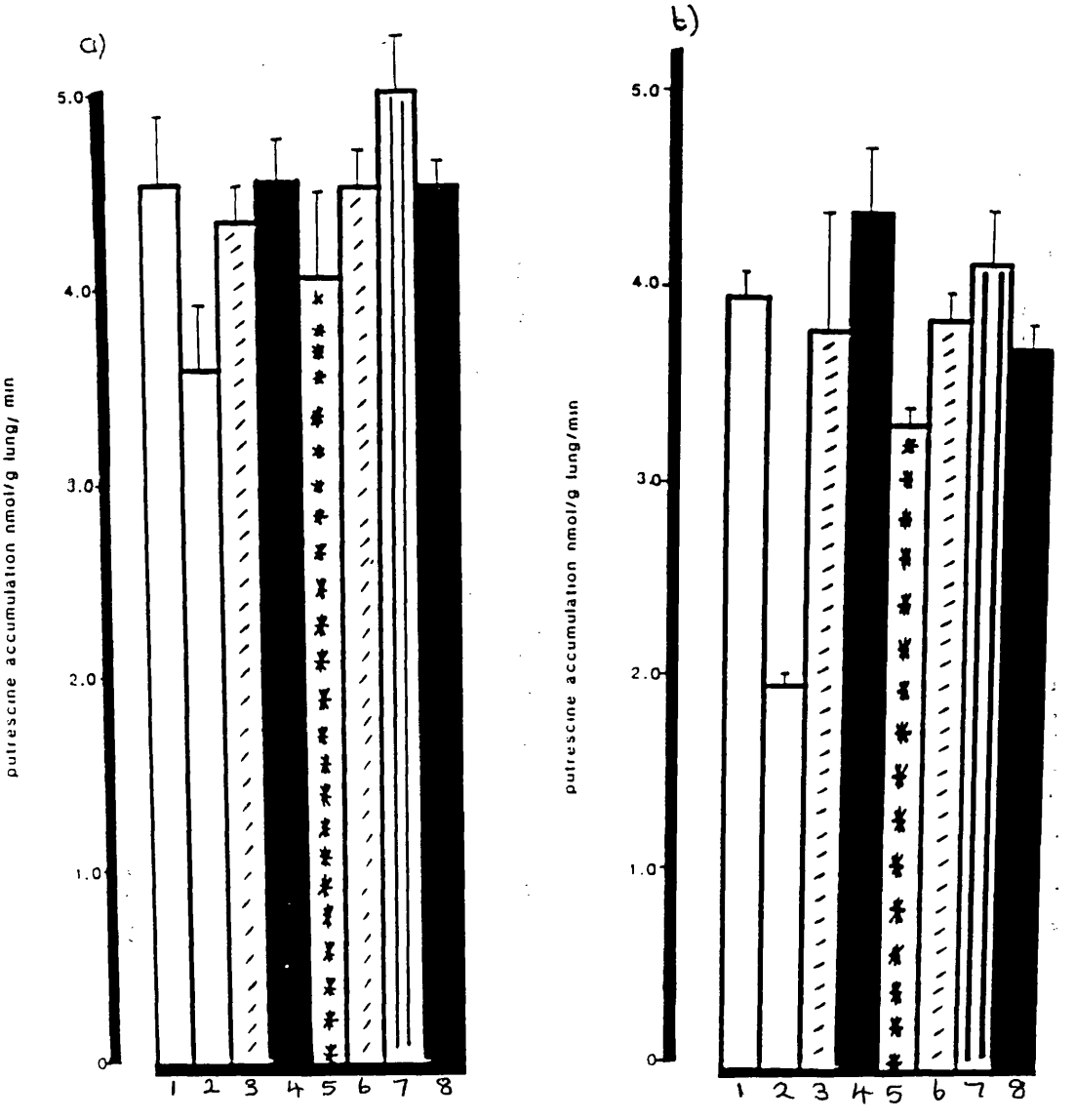


Fig. 6/ 11. Paraquat in control and compromised lung slices : the influence of desferrioxamine on putrescine accumulation

Control (a) and BCNU compromised lung slices (b) were incubated in KRB for 4 h in the presence or absence of paraquat (10 μM). Slices were incubated in the presence or absence of desferrioxamine (**, 50 μM; //, 100 μM; || , 200 μM and ■ , 500 μM). Following the appropriate incubation putrescine accumulation was assessed (as described in methods). Results are expressed as mean nmol putrescine accumulated/ g lung/ min ± S. E. (n = 3 experiments).

DFO (0. 5 mM) resulted in significant protection of putrescine uptake at each concentration of paraquat in both control and BCNU pre-treated lung slices (fig. 6/ 12).

6. 3. n DFO AND PARAQUAT (10 μM) : EFFECTS OF A RANGE OF DFO CONCENTRATIONS ON PUTRESCINE ACCUMULATION INTO CONTROL AND BCNU COMPROMISED LUNG SLICES

Paraquat (10 μM) reslted in a significant loss of diamine uptake in control slices after 4 h of incubation, in accord with previous results Incubation of control slices with DFO at 100 and 500 μM, in the absence of paraquat, did not affect putrescine uptake after 4 h of incubation. In the presence of paraquat, DFO ameliorated, significantly, loss of putrescine accumulation at DFO concentrations (100 -500 μM), with marginal protection at 50 uM. (Fig. 6/ 13).

In compromised slices paraquat (10 μM) resulted in a much more marked loss of diamine uptake than that observed in control slices, representing a similar potentiation to that observed above. Once again,incubation of slices with DFO (100 and 500 μM) in the absence of paraquat for 4 h failed to modulate subsequent putrescine accumulation. In the presence of paraquat (10 μM), DFO ameliorated loss of uptake at all concentrations with complete protection obtained at DFO concentrations 100-500 μM. (Fig. 6/ 13). The optimal protection was obtained at 200 μM DFO.

6. 3. o INFLUENCE OF DESFERRIOXAMINE ON THE UPTAKE OF PARAQUAT AND PUTRESCINE INTO LUNG SLICES.

Incubation of rat lung with DFO (0. 5 mM) resulted in significant inhibition of putrescine and paraquat uptake into lung slices (table 6. III).

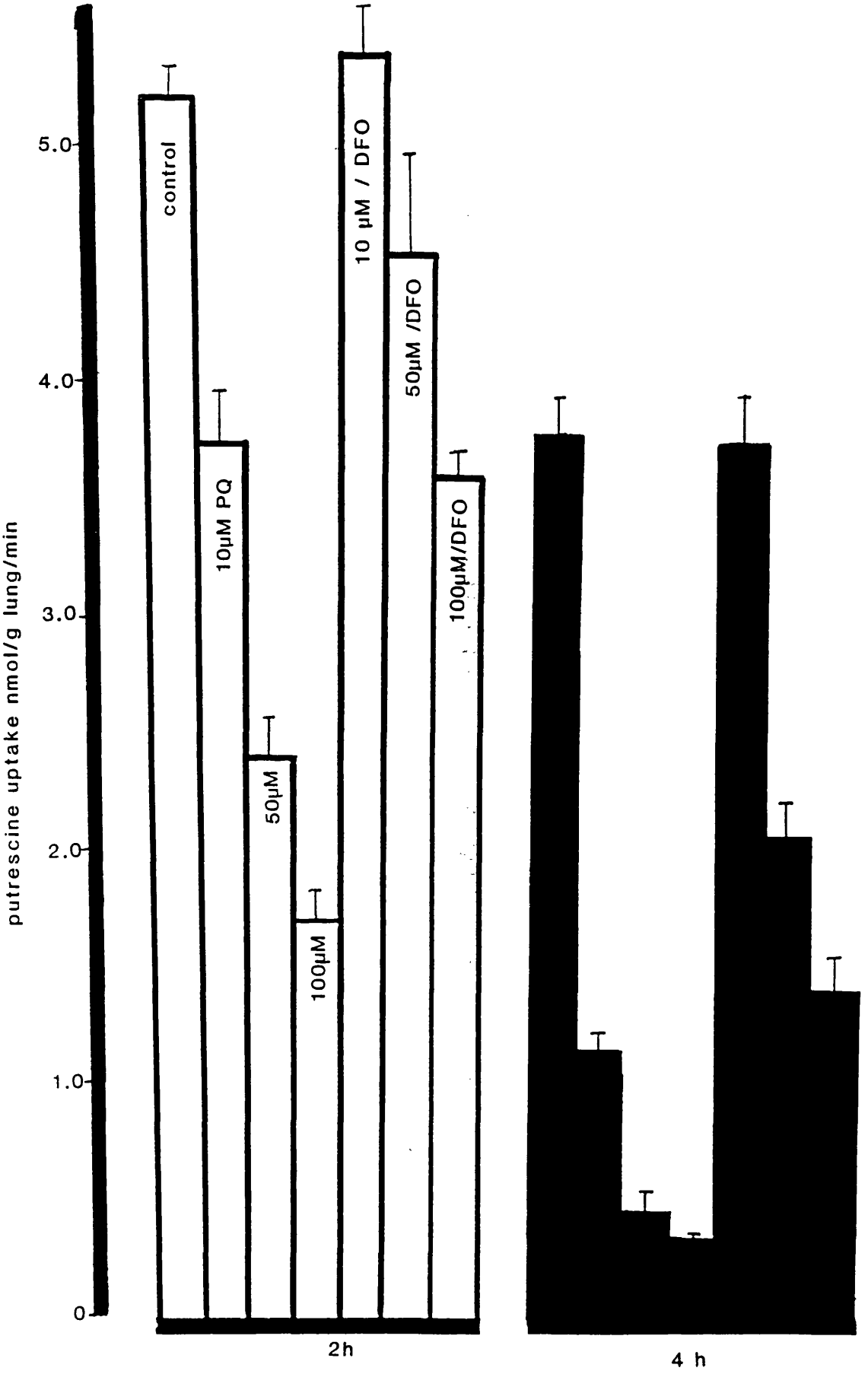


Fig. 6/ 12. Desferrioxamine-mediated protection against paraquat : a dose response with paraquat in control and compromised lung slices

Control (open bars) and BCNU pretreated lung slices (closed bars) were incubated in the presence or absence of paraquat (10, 50 and 100 μM), with or without desferrioxamine (0. 5 mM). Following 4 h of appropriate incubation, lung slices were removed and putrescine uptake assessed over a 30 min period (as described in methods). The results are expressed as mean nmol putrescine accumulated/ g lung/ min ± S. E. (n = 3 experiments).

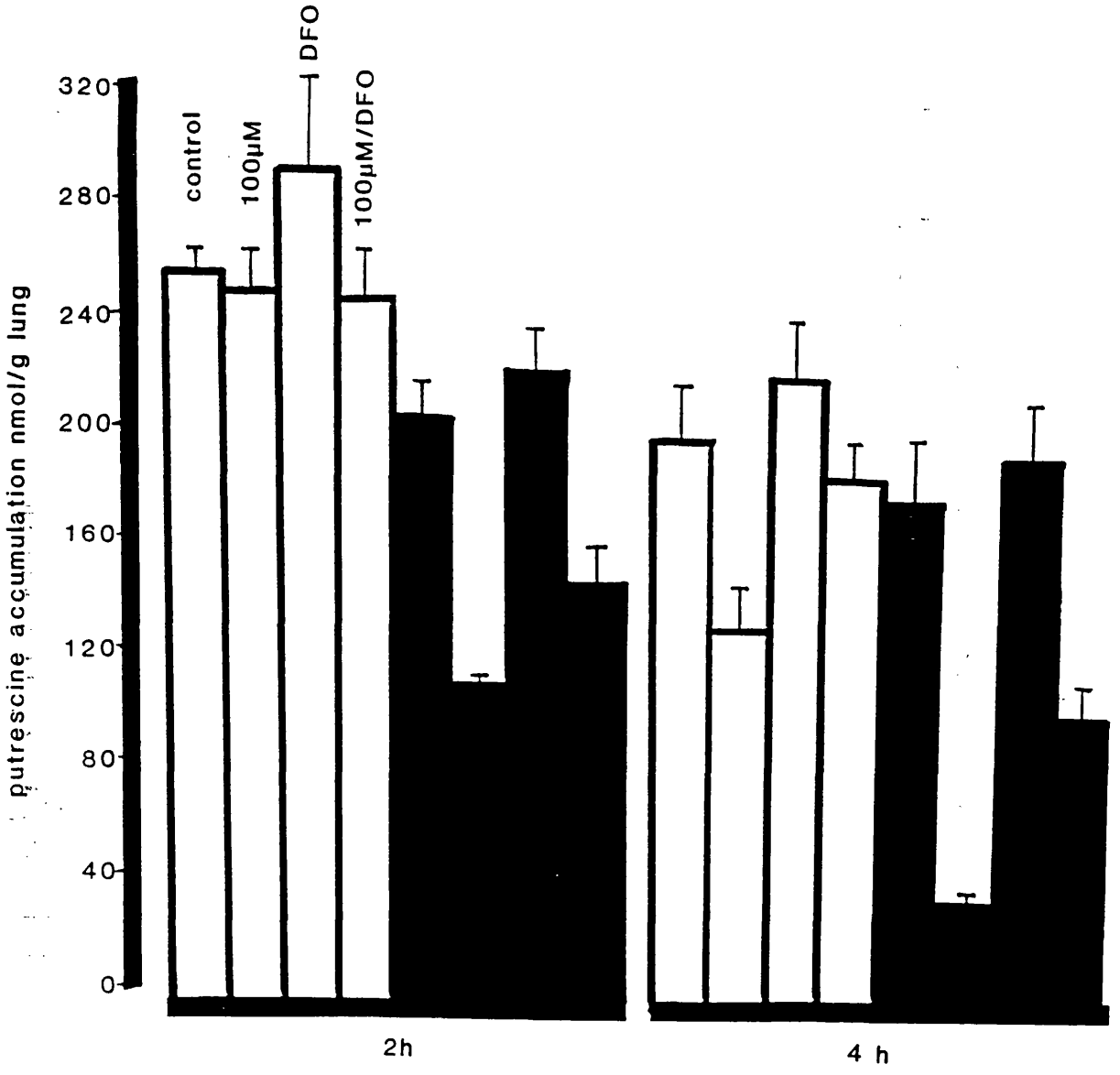


Fig. 6/ 13. Influence of desferrioxamine (0. 5 mM) on the effects of paraquat in control and compromised lung slices

Control (open bars) and BCNU pretreated lung slices (closed bars) were incubated in KRB in the presence or absence of paraquat (100 µM) with or without 0. 5 mM desferrioxamine. Incubations were carried out for 2 h and 4 h and then putrescine accumulation was assessed over 30 min. The results are expressed as nmol putrescine accumulated/ g lung ± S. E. (n = 3 experiments).

Table 6. III. The effect of desferrioxamine on the uptake of putrescine and paraquat into rat lung slices.

[Paraquat]	% difference		[Putrescine]	% difference	
(μM)	15 min	30 min	(μM)	15 min	30 min
1	66. 1	93. 0	1	81. 8	75. 3
50	63. 0	51. 6	5	68. 4	75. 6
100	60. 8	81. 0	10	53. 4	65. 7
500	31. 2	12. 3	50	60. 3	38. 3
			100	9. 5	46. 9

Rat lung slices were prepared as described in methods. They were then incubated for up to 30 min with either paraquat (1- 500 μM) or putrescine (1- 100 μM) in the presence of 0. 1 μCi of the appropriate radiolabel. Slices were incubated with or without desferrioxamine (DFO) and the uptake determined. The results are from one experiment and represent the % difference of uptake into lung slices incubated with DFO compared to accumulation into control slices similarly incubated.

This preliminary data revealed that the extent of inhibition decreased with increasing paraquat and putrescine concentration.

DISCUSSION

Clearly, phorone in mice (fig. 6/ 3) and rats (figs. 6/ 1 and 6/ 2) resulted in a significant elevation of pulmonary and hepatic thiols after 48 h. This elevation was preceded by a significant and marked loss of sulphydryls in both organs presumably leading to the increase in thiols observed as a result of rebound synthesis. The magnitude of the elevation of pulmonary thiols was slightly higher in mouse (4-fold) compared with rat (3-fold) (figs. 6/ 3 and 6/ 1). The initial depletion of glutathione in liver was consistent with Younes et al. [80], however, these workers did not observe a depletion of pulmonary thiols with phorone, in contrast to data presented here.

Phorone pretreatment failed to ameliorate the effects of paraquat in vivo in mouse and rat. Similarly, lung slices from phorone pretreated rats exhibited a loss of putrescine uptake identical to that seen in control lung slices when incubated with paraquat in vitro.

Prior treatment with phorone did, however, significantly protect rats against the effects of ANTU. Whilst ANTU affected both adenosine and spermidine uptake suggesting epithelial effects in addition to the endothelial damage, consistent with previous investigations [151] which noted such effects. It is reasonable to suggest that as type I cells and capillary endothelium are adjacent, then loss of endothelial function is likely to result in direct/ indirect effects on the type I cells. Phorone treatment ameliorated loss of spermidine and adenosine uptake. In addition, ANTU-induced mortality was abolished by prior treatment of rats with

phorone (table 6. II). Furthermore, protection was conferred against increases in wet/ dry lung weight ratios (table 6. II).

In control animals dosed with ANTU there was marked oedema at 4 h (near-maximal in previous studies [150]). However, the physical action of tissue slicing released much of the fluid in oedematous lungs (table 6. II). This was an important finding since otherwise the effects on uptake of adenosine and spermidine (as expressed as nmol / g lung wet weight) could simply be attributed to increased wet lung weight rather than a loss of function of particular cell types.

Phorone pretreatment resulted in total absence of the hydrothorax produced by ANTU in control animals (table 6. II), once again suggestive of a protection as a result of elevated pulmonary thiols. Such a protection would be consistent with previous work [155] when prior depletion of pulmonary GSH with diethyl maleate resulted in enhanced ANTU-induced mortality.

One cautionary note must be sounded, however. Whilst the simplest explanation is that increased pulmonary thiols are responsible for the protection against ANTU, it may not be correct. Increased hepatic thiols were observed as a result of phorone pretreatment. Previous work has revealed metabolism of ANTU by rat hepatic and lung microsomes [153] and hence, protection may result from an interaction in the liver,which reduces the bioavailability of ANTU in the lung. The finding that DEM enhanced toxicity is compatible with this explanation since the thiol depleting activity would not be specific for the lung and this must be considered carefully. These two possible mechanisms need not be mutually exclusive. Boyd and Neal [155] concluded that covalent binding following microsomal incubation with ANTU occurred via two distinct mechanisms : an NADPH-dependent, enzymatic process and an NADPH-

independent non-enzymatic process. Boyd and Neal suggested that the NADPH-binding might reflect non-enzymatic mixed disulphide formation between ANTU and tissue protein sulphydryl groups. It would, therefore, be reasonable to propose that a possible mechanism for phorone-mediated protection against the effects of ANTU involves the provision of alternative sites for covalent binding (by elevation of lung sulphydryls), for disulphide formation via the NADPH-independent mechanism for covalent binding. This might also account for the observation that the protection was not complete, since the NADPH-dependent pathway would not be influenced. Younes et al. [80] reported the absence of effects of phorone on the hepatic microsomal mixed-function oxidase system. Of particular interest in these studies is the possibility of effects on the pulmonary mixed function oxidase system. Induction, for instance, could lead to the enhancement of the NADPH-dependent pathway of covalent binding following ANTU administration and hence, the results obtained may represent the interplay of several discrete mechanisms .

The results are interesting since they tend to argue against an involvement of thiols in paraquat toxicity, in contrast with the work presented in Chapter 4. It is suggested that the lack of protection from phorone pretreatment could result from a failure to elevate thiol in the alveolar epithelium as a result of rebound synthesis. This is consistent with previous work [229] which revealed that type II cells possess a very limited ability to utilise precursors for glutathione biosynthesis compared to Clara cells or macrophages and a slow replenishment of GSH levels following diethyl maleate treatment was also observed [229].

In these studies GSH was depleted very effectively in rat lung slices (fig. 6/ 5) in accord with previous studies using DEM and on an equimolar basis was more potent than phorone in vitro. Thiol

levels remained low over 4 h of incubation following a 15 min pre-incubation (fig. 6/ 6).

Preincubation of lung slices (15 min) with DEM resulted in a small enhancement of paraquat toxicity in vitro as assessed by putrescine uptake in both control and compromised lung slices (fig. 6/ 7) which implicates, once again, GSH in the toxicity of paraquat. DEM is lipophilic and would deplete thiols throughout a lung slice.

Previous results have shown that the in vivo toxicity of paraquat is enhanced by elevated concentrations of inspired oxygen [255]. Paraquat uptake into lung slices incubated under conditions of hyperoxia was not modulated [256]. In the work presented here, hyperoxia failed to potentiate the toxicity of paraquat as assessed with putrescine uptake in control or BCNU compromised lung slices (fig. 6/ 10) . Indeed, hyperoxia slightly enhanced uptake into lung slices incubated with paraquat, possibly reflecting the enhancement of viability of portions of the slice which would otherwise become hypoxic. The effects of diquat were not modulated in contrast to previous results with hyperoxia and diquat toxicity in isolated hepatocytes [254].

In these studies preincubation of lung slices with exogenous GSH failed to protect against the effects of paraquat (fig. 6/ 9). Presumably, this reflects a lack of entry of GSH into the target cell types and is in conflict with results obtained in isolated type II cells [249], though the preincubation was performed in a similar manner.

The chelating agent desferrioxamine (DFO) protected against paraquat in both control and compromised lung slices. This protection was concentration dependent with respect to paraquat, significant protection being observed at 10, 50 and 100 uM paraquat, in both control and compromised lung slices, after 4 h of incubation with 0. 5 mM DFO. Lower

concentrations of DFO also conferred protection. DFO (0. 2 mM) protected completely with 10 μM paraquat. DFO, as anticipated from its structure inhibited paraquat uptake into lung slices (table 6. III), which could in part account for the protection observed. The protection is in accord with previous studies which revealed protection in bacterial systems and in mice.

CHAPTER 7 - FINAL DISCUSSION

The results of the studies presented in this thesis reflect an attempt to clarify the role of glutathione in the mechanism of toxicity of paraquat and of other agents in lung tissue.

7. 1 THE USE OF BCNU *IN VIVO* AND *IN VITRO*

In vivo studies with BCNU revealed an inhibition of glutathione reductase activity of around 60 % following a maximal non-lethal dose in rat and mouse lung (figs. 3/3 and 3/5). Animals treated with BCNU showed potentiated paraquat-induced mortality and weight loss (fig. 3/ 4). However, the site of this toxic interaction could not be exclusively assigned to the lung.

In order to overcome the potential limitation of organ-specificity and to obtain other advantages offered by an *in vitro* system, the BCNU compromised rat lung slice model was developed.

BCNU pretreated rat lung slices possessed a markedly inhibited glutathione reductase activity (c. a. 80 %; fig. 3/ 10) normal levels of ATP (fig. 3/ 11) and NADP(H). Glucose oxidation as assessed by $^{14}CO_2$ evolution from D-[^{14}C(U)]-, D-[1-^{14}C]- and D-[6-^{14}C]-glucose (fig.3/12, 4/11 and 4/12 respectively) yielded virtually identical results to those obtained in control slices. A modest depletion of GSH was noted (fig. 3/ 11) along with an elevated fatty acid incorporation (fig. 4/ 14). Oligoamine uptake was indistinguishable from that exhibited by control slices (tables 4. II and 4. III). 5-Hydroxytryptamine accumulation (fig. 4/ 18) was reduced but adenosine uptake was unchanged (fig. 5/ 9).

In addition, the activities of catalase and cytochrome c reductase were indistinguishable from those exhibited by control slices (data not shown). This apparent specificity of BCNU in the compromised lung slice

model is a very important facet, but cannot be taken as absolute. BCNU breaks down under physiological conditions to yield the highly reactive 2-chloroethyl isocyanate [190]. Nitrosourea-derived isocyanates are associated with a variety of effects on cellular proteins [187, 188, 209, 210].

The compromised lung slice model has several major assets, apart from the profound inactivation of glutathione reductase obtained. Firstly, speed and simplicity of preparation and secondly, it facilitates the study of the selectivity of toxicants in a heterogeneous cell population. Also, cell-cell interactions are maintained (a distinct advantage over isolated cell preparations).

Furthermore, the BCNU pretreated rat lung slice model could represent a useful starting point for study of antioxidant defences in oxidative stress. Further modifications could include the use of, for example, 3-aminotriazole (to inhibit catalase), or 6-aminonicotinamide (to inhibit the hexose monophosphate shunt), in order to assess the relative importance of each component. Initial, preliminary work of this nature has been carried out using BCNU pretreatment and the glutathione depletor DEM (fig. 6/ 6).

7. 2. THE EFFECTS OF TOXICANTS IN THE COMPROMISED SLICE

Investigations of the effects of paraquat, 2, 3-dimethoxy-1, 4-naphthoquinone and nitrofurantoin in BCNU pretreated slices yielded interesting results.

Biochemical measurements i. e. GSH and ATP levels failed to suggest the potentiation of an oxidative stress-related toxicity as a result of BCNU pretreatment. In contrast, the quinone exhibited loss of GSH and ATP as a result of glutathione reductase inactivation.

The use of oligoamine uptake (a marker of epithelial integrity) revealed that the effects of paraquat were indeed potentiated by BCNU pre-treatment . This finding would have been overlooked if biochemical measurements alone had been made and illustrates the additional insight which can be obtained by the use of the appropriate functional marker. (Fig. 4/ 15 and table 4. III). The loss of putrescine uptake following incubation of slices with the quinone was potentiated similarly.
The GSH and ATP depleting activities of nitrofurantoin were potentiated in BCNU pretreated slices as was loss of putrescine and 5-HT accumulation. The measurable GSH/ ATP depletions were considered to occur as a process distinct from the loss of oligoamine uptake. (Figs. 4/7, 4/9 and 4/16).

These agents differed in their effects on the functional marker of the endothelium, 5-hydroxytryptamine uptake. Paraquat did not modulate accumulation into compromised slices and produced a slight enhancement of uptake in control slices (in accord with previous work [221]). (Fig. 4/18). The quinone produced a loss of uptake which was enhanced by BCNU treatment (fig. 4/ 18).

a) IMPLICATIONS FOR THE CELLULAR SELECTIVITY OF TOXIC AGENTS

These results imply that paraquat exerts its effects on the cells of the alveolar epithelium whereas the quinone and nitrofurantoin exert effects throughout the lung slice. Paraquat is known to be taken up into lung tissue by an active process [129] by type I and II cells of the alveolar epithelium and also the Clara cells of the bronchiolar epithelium [135]. The quinone and nitrofurantoin are lipophilic and can permeate throughout the slice.

The potentiation of the endothelial effects of the quinone by

BCNU pretreatment further implies that there are glutathione reductase and NADPH-cytochrome P-450 reductase activities associated with the endothelium.

The cellular selectivity of paraquat in the lung slice model is consistent with previous work [131, 221]. Nemery et al. [221] also reported a selective loss of putrescine accumulation into lung slices following the administration of paraquat to rats. Nemery et al. also suggested that under the conditions of incubation of lung slices cellular barriers may become permeable. The results of the present study tend to disagree with this argument.

b) IMPLICATIONS FOR THE DISTRIBUTION OF PULMONARY GSH

From the results obtained in compromised slices it would seem reasonable to conclude that much of the pulmonary GSH is in cell types other than the alveolar epithelium. Since paraquat toxicity was potentiated as a consequence of glutathione reductase inactivation, it was inferred that a GSH depletion did occur in the cell types affected by paraquat, but that this depletion was not detectable because of large 'background' contents in cell types other than those affected by the toxicant.

A low GSH content in the alveolar epithelium could have important ramifications under conditions of oxidative stress. Clearly, if the cellular GSH content were low, then these cell types would be dependent upon glutathione reductase to maintain thiol status. This would be consistent with previous work which showed that inhibition of glutathione reductase or glutathione peroxidase greatly enhanced susceptibility of isolated lung epithelial cells to hydrogen peroxide and polymorphonuclear leucocyte-mediated attack [240].

One question is whether the low GSH content arises from a low concentration on a per cell basis or by virtue of the type I and II cells constituting, together, about 1/ 4 of lung parenchyma. The alveolar capillary endothelium constitutes 40 % of total lung parenchyma [8]. Therefore, there is a distinct possibility that much of GSH is in the endothelium simply because this cell type predominates. A combination of the two hypotheses cannot be excluded. Work in isolated rabbit lung cells [229] revealed a low GSH content in type II compared with Clara cells or macrophages (expressed on a per cell basis), and also exhibited a very slow rate of de novo GSH synthesis. In a lung slice preparation Clara cells will be present and cannot be ignored when considering the site of pulmonary GSH.

A limitation of these studies is that no measurements of oxidised glutathione (GSSG) have been made. Previous studies in isolated perfused rabbit lung revealed a paraquat-induced increase in GSSG [70].

In these studies it was assumed that glutathione depletions reflected oxidation to GSSG and increases would, presumably, have been detectable since the 'background' level of GSSG is so low.

7. 3. ADENOSINE ACCUMULATION IN VITRO

These results were of preliminary investigations of the use of adenosine uptake into rat lung slices as a functional marker of endothelial integrity.

ANTU, an agent which causes oedema and endothelial damage resulted in a loss of adenosine accumulation (fig. 5/ 3). However, loss of spermidine uptake was also seen (fig. 5/ 4). The question arises as to whether the uptake of adenosine is restricted to the endothelium, or ANTU induced damage is specific for the endothelium. Prior administration of paraquat failed to diminish adenosine accumulation, but did reduce

spermidine uptake (fig. 5/5), suggesting that ANTU was not acting solely on the endothelium. This is consistent with previous investigations which revealed epithelial effects [151]. Future investigations might involve the use of monocrotaline (selective for the pulmonary endothelium) to verify the specificity of adenosine uptake.

A limitation of the present study was that animals were sacrificed 4 h after ANTU. Further work would investigate effects at early time points e. g. 1 h , since it may be possible to differentiate endothelial and epithelial effects.

Another limitation is the absence of characterisation of the fate of radiolabelled adenosine in the lung slice following a variety of treatment conditions. Additional investigations would pursue this area.

An interesting finding was that incubation of lung slices with adenosine elevated ATP levels (figs. 5/ 9 and 5/ 10). This poses some intriguing questions. If this represents an endothelial effect, then could this imply that much of the pulmonary ATP is endothelial ? Is there transport of ATP between different cell types ? When ATP levels are elevated, what happens to the levels of AMP and ADP ?

Enhancing levels of ATP could provide an exciting opportunity to assess the influence on the effects of a variety of toxicants.

7. 4. PROTECTORS AND POTENTIATORS

Phorone was used successfully to elevate pulmonary and hepatic thiols in rat and mouse. It is assumed that this increase in thiols resulted from rebound synthesis, since γ-glutamyl cysteine synthetase is regulated by feedback inhibition from GSH [75]. Phorone pretreatment failed to protect mice, in vivo, or rats against paraquat in vivo or in vitro. In contrast, phorone pretreatment did protect rats against the effects of

ANTU as reflected by the amelioration of loss of adenosine and spermidine uptake (fig. 6/ 4) and table 4.II), mortality and hydrothorax (table 4. II).

The failure to protect rat lung slices from phorone pretreated animals against the effects of paraquat appears to argue against the involvement of GSH in paraquat toxicity . However, type II have a low rate of de novo synthesis [229] and hence the thiol rebound could represent an effect in cell type(s) distinct from the alveolar epithelium. This could be consistent with the data in table 4. II in which ANTU-induced loss of adenosine accumulation was protected to a slightly greater extent by phorone pretreatment than was spermidine uptake which could be indicative of differential thiol rebound in the endothelium compared with the epithelium.

The elevation of hepatic sulphydryls was striking and presents a further opportunity for toxicological studies. Future work, in vitro, could involve preparation of isolated hepatocytes from phorone pretreated rats and the toxicity of compounds such as quinones assessed in cells with elevated thiols.

One area of investigation in which phorone pretreatment of rats might reveal useful insights could be in the study of oxygen toxicity. Such a study was beyond the scope of the present investigation. Since hyperoxia-induced pulmonary damage is thought to occur initially in the endothelium with subsequent effects on the epithelium, then elevation of pulmonary sulphydryls should ameliorate this toxicity, particularly if the phorone pretreatment is effecting elevation of non-protein sulphydryls predominantly in the endothelium.

A protection against paraquat was observed in vitro with the iron chelator desferrioxamine. Significant protection was observed in both control and compromised lung slices (figs. 6/ 11, 12 and 13). DFO protected

optimally at 0. 2 mM. The chelator inhibited uptake of paraquat into lung slices though this was not considered to account entirely for the protection obtained.

SUMMARY

These studies clearly implicate oxidative stress in the mechanism of paraquat toxicity in lung tissue. The work has also further confirmed the accepted biochemical mechanism of toxicity shown in fig. 1/ 9. These studies implicate glutathione and glutathione reductase in paraquat toxicity and adds a further facet to account for the susceptibility of the alveolar epithelium to the herbicide : In addition to possessing an uptake system which can accumulate the compound, a high titre of NADPH-cytochrome P-450 reductase and much of the pulmonary NADPH [231] (which supports the redox cycling, it is also proposed that the epithelial cell types possess low glutathione contents.

The elevation of pulmonary glutathione levels clearly conferred protection against the oedematogenic agent, ANTU. Though the mechanism of this protection is uncertain, there is a clear role for GSH in the toxicity, consistent with previous observations.

The effects of nitrofurantoin and diquat were enhanced by glutathione reductase inactivation, suggesting potentiation of oxidative stress related mechanisms of toxicity.

The effects of paraquat were restricted to the epithelium based on the use of functional markers, whereas ANTU resulted in epithelial and endothelial effects. Nitrofurantoin, diquat and the redox cycling quinone 2, 3-dimethoxy-1, 4-naphthoquinone were not selective for a particular cell type.

APPENDIX

PREPARATION OF BCNU COMPROMISED RAT LUNG SLICES

Rat lungs were perfused *in situ* and slices prepared as described in methods. Lung slices were incubated with BCNU (100 uM) in KRB supplemented with amino acids for 45 min at 37^{o} in a shaking water bath. Then the slices were washed by brief immersion in fresh KRB (without amino acids), and then reincubated with fresh KRB medium (again, without amino acids) at 37^{o} in a shaking water bath for up to, and including, 4h.

The BCNU was solubilised with DMSO and was added in a total volume of 0. 1 ml to a 25 cm^{3} flask containing 2. 9 ml KRB (with amino acids) and lung tissue. The final concentration of DMSO was 0. 3 % (w/ v).

ADDITION OF COMPOUNDS TO INCUBATIONS WITH LUNG SLICES

Paraquat, diquat, 5-hydroxytryptamine, 5-iproniazid, reduced glutathione, potassium cyanide and the oligoamines were all added to the appropriate incubation in 0. 1 ml KRB to 2. 9 ml KRB (both without amino acids).

Desferrioxamine and adenosine were similarly added in KRB (without amino acids).

Rotenone, 2, 3- diOMe, menadione, nitrofurantoin, diethyl maleate, and phorone were added to the appropriate flask in 3 % DMSO (w/ v), such that the final DMSO concentration was 0. 3 % (w/ v).

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