STUDIES ON THE METABOLISM AND TOXICITY OF HYDRAZINE ~~IN THE RAT~~

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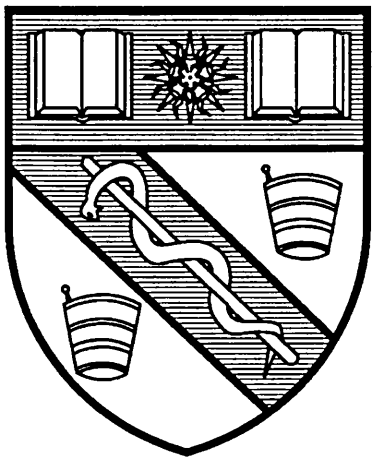

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" Life is a bitch and then you die "

Anon.

Hydrazine is extensively utilised in industry and is a minor metabolite of the clinically used drugs isoniazid and hydralazine. It is toxic, carcinogenic and mutagenic, but the metabolism and biochemical mechanisms of toxicity are not yet fully understood.

Isolated rat liver microsomes incubated with both 2.0 mM and 0.2 mM hydrazine in the presence of NADPH and oxygen at 37°C resulted in the disappearance of hydrazine, which was demonstrated to be due to both enzymatic and chemical oxidation. Boiled microsomes increased the proportion of chemical disappearance whereas incubating the microsomes on ice effectively eliminated it. Further *in vitro* microsomal studies therefore incorporated samples incubated on ice as controls, allowing the microsomal enzymatic metabolism of hydrazine to be calculated.
Absence of NADPH and oxygen markedly reduced microsomal hydrazine metabolism, as did the presence of each of the cytochrome P450 inhibitors carbon monoxide, piperonyl butoxide and metyrapone, thus indicating that microsomal hydrazine metabolism is catalysed by cytochrome P450. Methimazole, an inhibitor of flavin monooxygenase, also diminished hydrazine metabolism, whereas NADH in the presence of NADPH, but not alone, increased metabolism.
Microsomes prepared from either β-naphthoflavone, acetone or isoniazid pretreated rats did not show significantly increased hydrazine metabolism compared to control microsomes per g protein. However phenobarbitone pretreatment did increase metabolism.
Hydrazine metabolism was 20-70% lower in human microsomes prepared from 3 individuals compared to control rats.

The dose response for hydrazine hepatotoxicity *in vivo*, as manifested by triglyceride increase and depletion of ATP and glutathione (GSH), was measured in control rats 6 hr after an i.p. dose. This was then compared to animals which had been pretreated with various inhibitors and inducers of cytochrome P450. Pretreatment with the inhibitor piperonyl butoxide resulted in an increase in hepatotoxicity, while induction by phenobarbitone (inducer of P450IIB) or β-naphthoflavone (inducer of P450IA) decreased hepatotoxicity. In contrast, acetone or isoniazid (inducers of

P450IIE) induction caused an increase in hepatotoxicity. The modulation of hydrazine hepatotoxicity by such pretreatments indicates that different isozymes of cytochrome P450 catalyse the metabolic transformation of hydrazine toxicity by various mechanisms.

6 hr after an acute i.p. hydrazine dose, certain dose related alterations in hepatic microsomal enzyme activity were measured, including a depletion in ethoxyresorufin *O*-deethylase and *p*-nitrophenol hydroxylase activity.

Repeated administration of 0.78 mM hydrazine in drinking water (2.5 $mg.kg^{-1}.day^{-1}$) had a significant effect on several hepatic biochemical parameters and microsomal enzyme activities after 1,5 and 10 days. This indicated hydrazine to be a probable inducer of cytochrome P450IIE1.

Hepatic biochemical parameters and activities of microsomal enzymes were virtually unchanged after repeated administration of 65 µM hydrazine in drinking water (0.25 $mg.kg^{-1}.day^{-1}$) for 5 or 10 days.

In the presence of over 5 µM hydrazine, ATP synthesis in isolated mitochondria was inhibited. Inhibition up to 100 µM hydrazine was found to be dose related and reached a maximum 20-30% inhibition of control. However, above this concentration further inhibition did not occur.

Hydrazine was also found to be metabolised by isolated mitochondria, which was not significantly decreased in the presence of either the monoamine oxidase A inhibitor, clorgyline or B inhibitor, pargyline.

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ABBREVIATIONS

ADP - Adenosine diphosphate
ANOVA - Analysis of variance
ATP - Adenosine triphosphate
AUC - Area under curve
BNF - β-naphthoflavone
BSA - Bovine serum albumin
CCCP - *m*-chloro-phenyl-hydrazone
CCl_4 - Carbon tetrachloride
CDP - Cytidine diphosphate
CO - Carbon monoxide
CoA - Coenzyme A
DMBA - *p*-Dimethylaminobenzaldehyde
DMSO - Dimethylsulphoxide
DNA - Deoxyribonucleic acid
DPPD - Diphenyl-*p*-phenylenediamine
DTNB - 5,5-Dithiobis-(2-nitrobenzoic acid)
EROD - Ethoxyresorufin *O*-deethylase
FMO - Flavin-containing monooxygenase
GABA - γ-aminobutyric acid
GAD - Glutamate decarboxylase
GC - Gas Chromatography
GSH - Glutathione (reduced)
MAO - Monoamine oxidase
MDA - Malonaldehyde
MFO - Mixed function oxidase
mRNA - Messenger RNA
MS - Mass spectrometry
NADH - Nicotine adenine dinucleotide (reduced form)
NADPH - Nicotine adenine dinucleotide phosphate (reduced form)
NMR - Nuclear magnetic resonance
NPD - Nitrogen phosphorous Detector
NPH - *p*-Nitrophenol hydroxylase
P450 - Cytochrome P450

PAP - Phosphotidate phosphohydrolase

PCA - Perchloric acid

PEPCK - phosphoenolpyruvate carboxykinase

PFB - Pentafluorobenzaldehyde

PFPH - Pentafluorophenylhydrazine

PLP - Pyridoxal-5'-phosphate

PROD - Pentoxyresorufin *O*-deethylase

RCR - Respiratory control ratio

RNA - Ribonucleic acid

TBA - Thiobarbituric acid

TCA - Trichloroacetic acid

TFT - Tetraformyltrisazine

THOPC - 1,4,5,6 Tetrahydro-6-oxo-3-pyridazine carboxylic acid

UHQ - Ultra high quality

VLDL - Very-low-density-lipoprotein

1.1 PHYSICAL PROPERTIES, HISTORY AND APPLICATIONS OF HYDRAZINE

Hydrazine NH_2NH_2 is a colourless, fuming hygroscopic liquid (m.p. 2.0°C, b.p. 113.5°C). It dissolves in polar solvents such as water, alcohols, ammonia and amines and is combustible, burning with a blue flame. Hydrazine is not known to occur naturally except perhaps in the tobacco plant.

Hydrazine, in the form of its derivatives, the hydrazo-compounds, has been known since 1863, but it was not until 1887 that the German, T Curtius, succeeded in isolating hydrazine itself by the action of alkali on diazoacetic ester. However there was little interest in hydrazine until the discovery that hydrazine hydrate could be employed as a rocket fuel when mixed with oxidising agents such as nitric acid, hydrogen peroxide or oxygen. It was this discovery that stimulated the expansion of hydrazine production in Germany during the Second World War in order to power its V-2 rockets and ME-163 fighter planes. Today hydrazine production remains substantial (in excess of 35K tonnes in 1981), fuelled by its extensive use in the chemical manufacturing industry, producing hydrazine derivatives that are utilised as:

1) Blowing Agents -

These are used in the manufacture of expanded plastics and rubber and account for a large share of hydrazine consumption, for example azodicarbonamide (Fig 1.1).

2) Herbicides -

maleic hydrazide (Fig. 1.1) is a widely used plant growth suppressant and is particularly used in tobacco plantations.

3) Clinical Drugs -

a) Isoniazid (isonicotinic acid hydrazide Fig 1.1) is a very effective antitubercular agent and is commonly used in combination with rifampicin.

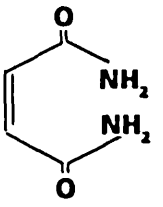
b) Hydralazine is an antihypertensive agent that acts by lowering diastolic blood pressure while increasing renal blood flow.

c) Phenelzine (β-phenylethylhydrazine) and iproniazid are both antidepressive agents due to their monoamine oxidase (MAO) inhibitor action.

Figure 1.1 Chemical structure of some routinely used hydrazine derivatives

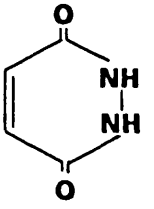
Azodicarbonamide

Blowing agent



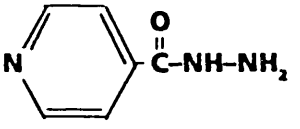
Maleic Hydrazide

Plant growth regulator



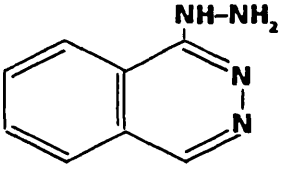
Isoniazid

Antitubercular agent



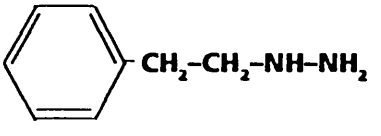
Hydralazine

Antihypertensive agent



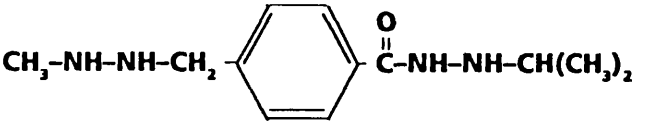
Phenelzine

Antidepressive agent (MAO inhibitor)



Procarbazine

Antineoplastic agent



treatment of Hodgkins disease in combination therapy.

4) Rocket Propellants -

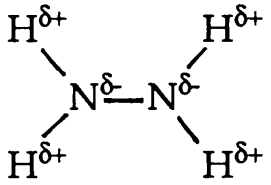
Methylhydrazine, unsymmetrical dimethyl hydrazine and also hydrazine are used, often in combination, to power spacecraft and military aircraft.

The main use of hydrazine at present is as a high pressure water system additive in order to prevent oxidative corrosion of reinforced steel used in boiler linings. Recently hydrazine sulphate has been successfully incorporated into cancer therapy to counteract the cachexia often seen in cancer patients (Chlebowski *et al* 1984 and 1987, Gold 1987).

1.2 HYDRAZINE CHEMISTRY

1.2.1 Electronic and chemical structure

Hydrazine is the simplest possible diamine and possesses four covalent N-H bonds that are polar and one non-polar covalent N-N bond. A dipole moment is generated with the negative charge centre between the two adjacent nitrogen atoms, due to each having a lone pair of free, unshared electrons. These nitrogen lone pair electrons make hydrazine a strong nucleophile and consequently a very reactive and powerful reducing agent.



1.2.2 Oxidation and reduction reactions

In spite of hydrazine's strong reducing properties, in the presence of a strong reducing agent such as Raney nickel or Zinc and HCl, it can be reduced to ammonia.

$$NH_2\text{-}NH_2 \xrightarrow{2[H]} 2NH_3$$

Oxidising agents react with hydrazine to give nitrogen gas and water. This rapid

reaction appears to proceed via diimide (NH=NH) and is the basis of the use of aqueous hydrazine solution for the deoxygenation of boiler feed water.

$$NH_2\text{-}NH_2 \xrightarrow{[O]} NH{=}NH + H_2O \xrightarrow{[O]} N_2 + H_2O$$

Many metals catalyse the oxidation of hydrazine by air to a considerable extent including copper and other metals that possess more than one valency, and their salts. This catalysis is effective only in alkaline solution and can be suppressed by calcium oxide, magnesium oxide, zinc oxide, cadmium oxide, aluminium oxide and most effective of all sulphur in minute doses.

1.2.3 Hydrazide formation

Hydrazine can react with acylating agents such as acid halides, acid anhydrides, esters or amides to form hydrazides, either mono or disubstituted.

$$NH_2\text{-}NH_2 + R\text{-}CO\text{-}X \longrightarrow R\text{-}CO\text{-}NH\text{-}NH_2 + HX$$

$$R^1\text{-}CO\text{-}NH\text{-}NH_2 + R^2\text{-}CO\text{-}X \longrightarrow R^1\text{-}CO\text{-}NH\text{-}NH\text{-}CO\text{-}R^2 + HX$$

Esters and Amides are generally less reactive, but in all cases the highly polar hydrazine precursor is converted into less polar solid N-acyl derivatives. Lower alkyl hydrazides have low melting points and are soluble in water, while the aryl and higher alkyl hydrazides are insoluble in water. The hydrazide (-CO-NH-NH) moiety of aryl derivatives is acidic, as it is also in the diacyl-hydrazines, and confers solubility in NaOH. Primary hydrazides can react with carbonyl groups to produce acyl hydrazones.

1.2.4 Hydrazone and azine formation

Carbonyl compounds may react with one or both amino groups of hydrazine to rapidly form hydrazones.

$$NH_2\text{-}NH_2 + R^1\text{-}CO\text{-}R^2 \longrightarrow R^1,R^2C{=}N\text{-}NH_2 + H_2O$$

$$R^1,R^2C{=}N\text{-}NH_2 + R^3\text{-}CO\text{-}R^4 \longrightarrow R^1,R^2C{=}N\text{-}NH{=}CR^3,R^4 + H_2O$$

aldehyde hydrazones react with a second molecule of aldehyde more rapidly than hydrazine itself. Ketazines, on the other hand, require the presence of an excess of ketone together with acetic or formic acid as catalyst. Aliphatic hydrazones are strongly basic, and the aromatic weakly basic; both can condense with a new carbonyl compound to form mixed azines.

With an acyl compound hydrazones produce acyl hydrazones which can be extracted from aqueous solutions by nonpolar organic solvents such as ethyl acetate.

1.3 BIOCHEMICAL REACTIVITY AND METABOLISM OF HYDRAZINE

Owing to it's high chemical reactivity, hydrazine interacts with various cellular components and endogenous compounds, which accounts for much of its metabolism and many of its toxic properties.

1.3.1 Pyridoxal phosphate interaction

Hydrazine and it's derivatives that have a free amino group react with the aldehyde group of pyridoxal-5'-phosphate (PLP) (Castagne *et al* 1987) to form the corresponding hydrazone via initial formation of a Schiff-base. PLP is an important coenzyme involved in several major metabolic pathways including amino acid decarboxylation and transamination. Formation of the thermodynamically stable hydrazone is considered to be the reason for the inhibition of PLP dependent enzymes by hydrazine (Medina 1963), although it has also been observed that pyridoxal hydrazones are potent inhibitors of pyridoxal phosphokinase (McCormick and Snell 1961).

1.3.2 Flavoprotein interaction

The use of phenelzine and iproniazid as antidepressant drugs followed the observations that these compounds, together with other hydrazines, bind to mammalian monoamine oxidase (MAO) of the outer mitochondrial membrane and interact with the flavin prosthetic group. The enzymatic mechanism of substrate oxidation involves flavin reduction with formation of a Schiff-base and the resultant reduced flavoprotein requires molecular oxygen to revert to it's original oxidised state. Studies on purified MAO by Patek and Hellerman (1974) demonstrated that the irreversible inhibition and depletion of flavin absorbance (bleaching) by

irreversible inhibition and bleaching also occurred in the presence of phenyldiazene, formed by chemical means. Nagy *et al* (1979) used isolated bacterial trimethylamine dehydrogenase, which has an identical cysteinyl flavin prosthetic group to MAO and is similarly irreversibly inhibited by several hydrazines with flavin bleaching. They isolated the cysteinyl flavin moiety after phenylhydrazine treatment by protease digestion and demonstrated that the flavin group was arylated at the C4a position with a phenyl group. These results indicate that flavoproteins can oxidise hydrazines to form diazenes, and if sufficient aryl- or alkyl- diazine exist, they may lead to addition of the corresponding aryl- or alkyl- radical to the C4a position of the flavin. Recent information is available on the interaction of hydrazine with the flavin prosthetic group indicating that hydrazine can indeed bind and react with flavoproteins. Noda *et al* (1988) reported that hydrazine oxidation by microsomes isolated from phenobarbitone induced rats was remarkably accelerated by addition of flavin adenine dinucleotide. In addition they demonstrated that purified cytochrome P450 reductase (fp_2), a flavoprotein, catalysed hydrazine oxidation and that this could be inhibited by addition of antibodies raised against fp_2.

1.3.3 Hemoprotein interaction

In 1890 Heinz bodies were first described after treatment of erythrocytes with phenylhydrazine and this alteration of red cell function explained the decrease in tissue oxygenation *in vivo*. Phenylhydrazine has since been demonstrated to react with oxyhemoglobin (Fe^{2+}) to form hemoglobin with arylated heme moieties as well as hemoglobin with arylated apoprotein (Moloney and Prough 1983). Hemoglobin interacts with various other hydrazines and similarly hydrazine derivatives have been reported to alkylate other heme proteins including horseradish peroxidase, catalase and various cytochromes. Hydrazines such as phenylhydrazine, iproniazid and phenelzine can lead to heme destruction by forming an irreversible heme complex.

In 1982 Nelson and Gordon demonstrated that hydrazine incubated with blood in the presence of air is oxidised rapidly to N_2 gas and they indirectly ascertained diimide (HN=NH) as a probable intermediate. These findings were consistent with the observations of Buckmaster (1913) and Springer *et al* (1981) that hemoglobin converted hydrazine to N_2. Hydrazine is a much less potent inducer of red blood cell

is largely responsible for the hemolytic reaction. Thornalley (1984) trapped hydroxyl radicals ($OH^{\bullet}$) during the reaction of hydrazine with hemoglobin and this was relatively independent of superoxide, hydrogen peroxide and radicals that had also been trapped, indicating that the autooxidation of hydrazine is initiated by heme-bound intermediates but thereafter is autocatalytic.

Biological oxidation of hydrazine derivatives by cytochrome P450 has been reported to lead to formation of diazine intermediates and there is crystallographic evidence (Mansuy *et al* 1982) supporting the contention that a diazine can bind to the 6-coordination position of the cytochrome P450 heme moiety (Hines and Prough 1980, Jonen *et al* 1982).

1.4 HYDRAZINE DISPOSITION, METABOLISM AND EXCRETION

Compared to compounds containing carbon, elucidating the metabolic fate of hydrazine is extremely difficult due to the absence of an appropiately radiolabelled analogue for detection purposes. The nitrogen radioisotope [^{13}N] has a very short half life ($t_{½}$ = 9.96 min) rendering itself redundant and the use of tritium is futile as the hydrogen atoms of hydrazine are readily exchangeable. The only alternative in the study of hydrazine is the use of ^{15}N labelled material which can be monitored by Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR).

1.4.1 Hydrazine disposition

Hydrazine is rapidly absorbed via all routes of exposure including the skin. Smith and Clark (1972) demonstrated that maximum serum concentrations were reached 1-3 hr after application to uncovered canine skin. After an i.p. or subcutaneous dose in rats and mice peak serum concentrations were measured almost immediately and hydrazine was then rapidly distributed in most tissues (Dambruskas and Cornish 1964, Nelson and Gordon 1982) as would be expected for such a small, water soluble molecule. Highest levels of hydrazine after an i.p. dose were measured in the kidneys and other tissues had much lower levels.

Recent studies using GC.MS have demonstrated that the increase in the peak plasma level and in the area under the plasma concentration time curve after administration of ^{15}N-hydrazine to rats is not directly proportional to the dose (Preece *et al* 1992). Also the ratio of plasma to liver hydrazine varied with dose suggesting that there

may be an active uptake of hydrazine from the plasma into the liver which is saturable.

1.4.2 Hydrazine metabolism and excretion

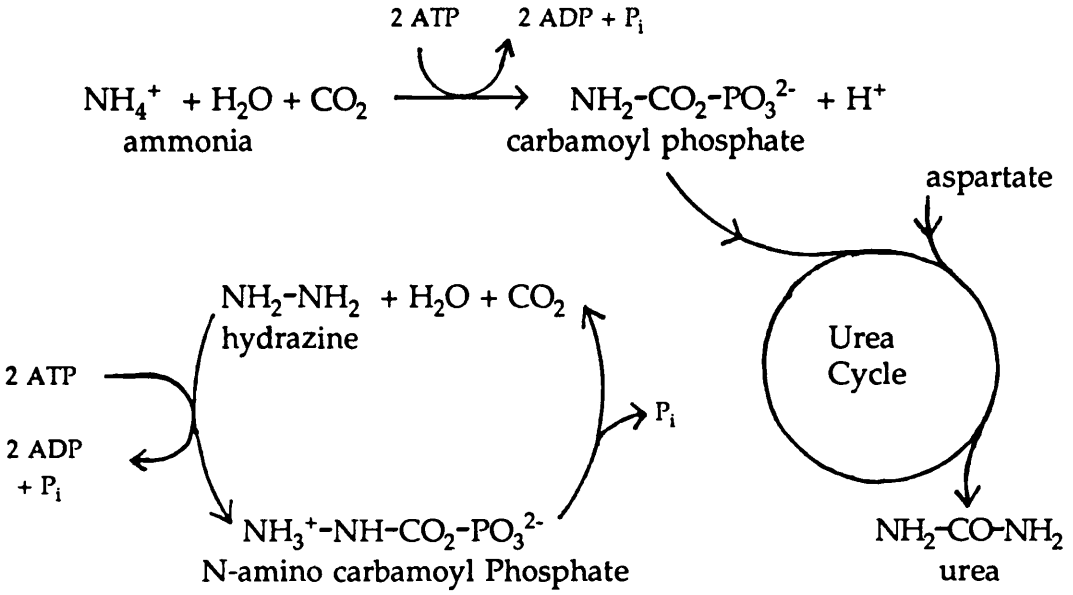
Excretion of hydrazine is also rapid. Springer *et al* (1981) found that 15% of a 32 $mg.kg^{-1}$ hydrazine dose in rats was expired as N_2 gas in the first 30 min, compared to 25% after 24 hr. Similarly Nelson and Gordon (1982) after administration of 32 $mg.kg^{-1}$ [^{15}N] hydrazine to mice showed that 21% was converted to N_2 gas which after 48 hr rose to 35%. The initial rapid phase of N_2 expiration followed by a much slower rate correlates with the observed elimination of hydrazine from plasma. N_2 gas is the major metabolite of hydrazine and its formation is considered to be predominantly catalysed by oxyhemoglobin (Springer *et al* 1981, Nelson and Gordon 1982) and also liver microsomal monooxygenases (Nelson and Gordon 1982, Noda *et al* 1985).

A significant proportion of administered hydrazine has been demonstrated to be excreted unchanged in the urine of dogs and rabbits (McKennis *et al* 1955, 1959 and 1961), rats (Springer *et al* 1981) and mice (Nelson and Gordon 1982). Total hydrazine excreted unchanged in the urine varies between studies depending on the dose, route of administration, species and also the method used for analysis, but is between 10 to 30% (Dambruskas and Cornish 1964, Wright and Timbrell 1978, Springer *et al* 1981, Nelson and Gordon 1982). Urine of hydrazine treated animals also contains acid-hydrolysable metabolites and only within the past 10 years have some of these compounds been identified. McKennis *et al* (1959) initially found evidence for the presence of diacetylhydrazine in hydrazine treated rabbits, but not dogs and in later studies (Wright and Timbrell 1978, Nelson and Gordon 1982) very small amounts (1-2%) of acetyl- and diacetyl- hydrazine were detected after derivatisation. The proportion of hydrazine and acetylhydrazine excreted in the urine has recently been shown to decline with dose (Preece *et al* 1992).

Nelson and Gordon (1982) also reported having detected in mice urine the presence of the pyruvate and α-ketoglutarate hydrazones and 1,4,5,6 terahydro-6-oxo-3-pyridazine carboxylic acid (THOPC), which is probably formed by dehydrative cyclisation of the α-ketoglutarate hydrazone. Very recently nuclear magnetic resonance (NMR) analysis of the ^{1}H and ^{15}N nuclei have identified urinary

hydrazine (Fig. 1.2). Preece *et al* (1991) identified all the urinary metabolites detected in past studies plus the presence of ^{15}N labelled ammonia and urea, indicating that the N-N bond is cleaved *in vivo*. The labelled urea is presumably synthesised from labelled ammonia entering the urea cycle. Although hydrazine can act as an alternative substrate to ammonia in the first step of urea synthesis, catalysed by carbamoyl-P synthetase (McKinley *et al* 1967), an unstable N-amino carbamoyl phosphate analogue is formed (Fig. 1.2) which is unable to enter the urea cycle.

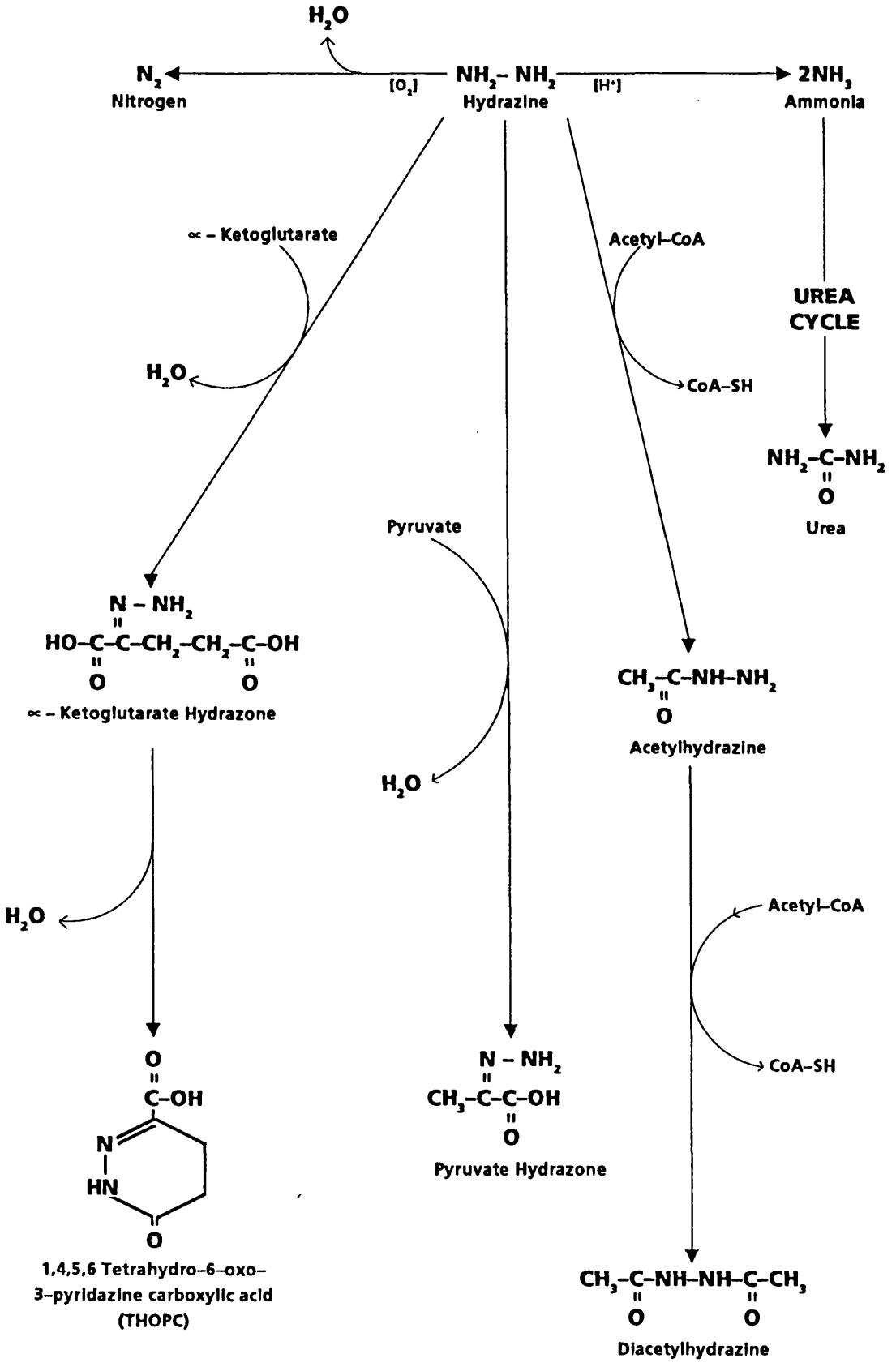
Figure 1.2 Catalytic conversion of ammonia and hydrazine by carbamoyl phosphate synthetase



Springer *et al* (1981) however did not detect any labelled ammonia in the urine of rats administered [^{15}N] hydrazine and agreed with Floyd (1980) that the hyperammonia generated in hydrazine treated dogs was caused by hydrazine's effect on endogenous metabolic pathways. In isolated rat hepatocytes incubated with [^{15}N] hydrazine, Preece *et al* (1990b) observed the metabolites; acetylhydrazine, THOPC and very small amounts of diacetylhydrazine.

Because of the problems outlined in the study of hydrazine metabolism, about 25% of administered hydrazine still remains unaccounted for. Hydrazine is virtually absent from the animal carcass after 24 hr and no evidence exists that derivatives of hydrazine bind to tissue components to the extent of 25%.

Figure 1.3 Metabolic pathways for hydrazine (Preece *et al* 1991)



1.5.1 Human exposure to hydrazine

Hydrazine can be judged as posing little hazard for the general population at normal ambient levels. However in the work-place, such as facilities producing hydrazine and its derivatives, rocket and aircraft refuelling sites, plants using high-pressure boilers, and under conditions of accidental exposure, for example from bulk storage, handling, transport and improper waste disposal, hydrazine can be released into the atmosphere presenting a significant health hazard. Workers normally exposed to hydrazine are provided with respiratory and skin protection.

Hydrazine, in addition, has been detected in the plasma of human patients during the metabolism of the two drugs isoniazid and hydralazine (Blair *et al* 1985). Several studies have also detected hydrazine in the urine of human patients after administration of isoniazid (Timbrell *et al* 1977b, Noda *et al* 1978) and hydralazine (Timbrell and Harland 1979) while hydrazine has been detected in urine after iproniazid administration, but only in rabbits and rats (Hsu *et al* 1980). The toxicological significance of hydrazine exposure during ingestion of hydrazine-based drugs has yet to be fully assessed.

1.5.2 Human and experimental animal toxicity

Human exposure to hydrazine vapour has been reported to result in nausea, vomiting, local eye and exposed skin irritation, plus irritation of mucous membranes of the upper respiratory tract with respiratory distress. Skin lesions caused by direct contact with hydrazine have often been described, as well as the frequent reports of inflammatory skin conditions in workers exposed to hydrazine, for example contact dermatitis (Hovding, 1967) and allergic eczema. The low odour threshold of hydrazine (3-4 ppm) and distinct ammoniacal smell of the vapour means the risk of inadvertent acute hydrazine intoxication is small, but chronic intoxication can readily ensue and may ultimately lead to death (Sotaniemi *et al* 1971) if exposure is repeated continuously.

Human ingestion of liquid hydrazine, reported in accident case studies, leads to local irritation and vomiting followed by disturbances of central nervous origin. Such disturbances include somnolence, ataxia, restlessness, incoordination and paraesthesia which may be accompanied by transient respiratory and cardiac rhythm disorders, that are probably also of nervous system origin (Reid 1965).

experimental animals allowing the mechanism of hydrazine toxicity to be studied. Early animal studies have shown that hydrazine is acutely toxic, the LD_{50} being approximately 60 $mg.kg^{-1}$ in rats and mice (Witkin 1956). The main symptoms of acute hydrazine toxicity in animal studies are hypopnoea followed by increased excitability and tonoclonic convulsions, decreased blood pressure and nerve conduction disturbances. Hydrazine administration has also been shown to cause nephrotoxicity, resulting in a quick reduction of creatinine clearance and rate of glucose absorption (Wong 1966). Histopathological investigations have revealed fatty metamorphosis of primarily the liver and kidneys (Scales and Timbrell 1982, Patrick and Back 1965, Yard and McKennis 1955, Clark *et al* 1970) but also the myocardium (Amenta and Johnston 1962) and skeletal muscle (Patrick and Back 1965).

1.5.3 Induction of convulsions

It has been proposed that the inhibition of the PLP dependent enzyme glutamate decarboxylase (GAD), which is responsible for synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), is much greater than the inhibition of the PLP dependant enzyme GABA-transaminase, which is responsible for GABA metabolism. Hence the resulting decrease in GABA is responsible for the convulsive effect of hydrazine. Studies by Medina (1963) illustrated that brain GAD activity is indeed severely inhibited *in vivo* by hydrazine as well as several other hydrazine derivatives (Killam and Bain 1957), but Medina (1963) also found that GABA transaminase activity is severely inhibited *in vivo* and unlike several hydrazine derivatives which reduced total brain levels of GABA, hydrazine actually increased them. Matsuyama *et al* (1983) also found increased levels of GABA in the brain after hydrazine administration.

Further investigations of individual regions of the brain, which vary in GABA concentration, have been performed by Horton *et al* (1979) but no direct relationship could be demonstrated between seizures and GABA concentration or metabolism after administration of various hydrazides. Wood *et al* (1980) isolated synaptosomes (nerve endings) from rats exposed to various convulsant and anticonvulsant agents and demonstrated an excellent linear relationship between synaptosomal GABA content and the convulsant potential of the compound. This suggests that the GABA concentration at a particular intracellular site of nerve cells may play an important

role in modulation of brain excitability. Unfortunately in the same study a convulsant dose of hydrazine did not cause any significant reduction in synaptosomal GABA content and was the only exception to the linear relationship. Other possible reasons for hydrazine induced seizures include:

1) Hyperammonia - Elevated levels of ammonia are known to cause convulsions and are generated rapidly after hydrazine administration.

2) Severe hypoglycaemia - Depletion of blood glucose is known to promote convulsions via brain nutrient starvation and occurs quickly following hydrazine administration. Lewis and Izume (1926) have demonstrated that infusion of glucose to hydrazine treated animals can prevent convulsions.

1.6 DISTURBANCE OF INTERMEDIARY METABOLISM BY HYDRAZINE

1.6.1 Hypoglycaemia and glycogen depletion

Initial experiments by Underhill (1911) demonstrated, in hydrazine treated dogs, an elevation of blood glucose in fed animals, and only a marked hypoglycaemia in starved animals. Later work by Lewis and Izumi (1926) and Fortney (1966) showed that in fed, hydrazine treated rabbits and dogs respectively the hyperglycaemia was a transient phenomenon, followed by a pronounced depression in blood glucose and eventually convulsions which could be prevented by infusion of glucose. Dost (1975) and Fortney (1966) showed that parenteral glucose could not be converted into liver glycogen. It is interesting that if the animal survives after convulsions then the hypoglycaemia can reverse in 2 to 3 days and the liver glycogen levels return to normal.

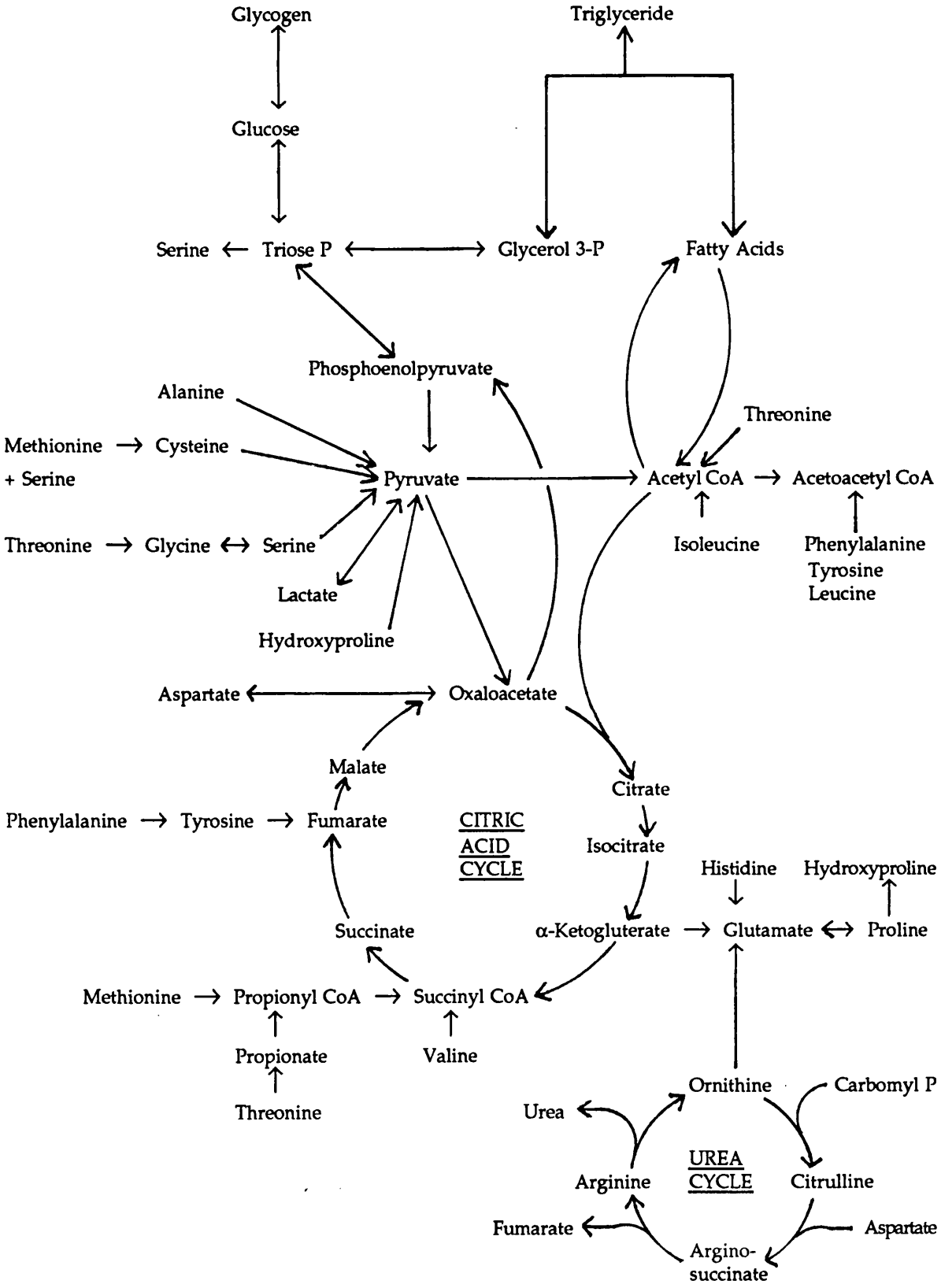
During the period of hypoglycaemia Taylor (1966) observed that liver and muscle glycogen levels were rapidly depleted until exhausted. Blood glucose declined followed by convulsions. Serum lactate and pyruvate levels were highly elevated at the onset of hypoglycaemia and glycogen depletion. Fortney (1966) and Fortney *et al* (1967) found acidosis developed after several hours in dogs and rats respectively and that the rate of lactate and pyruvate accumulation after hydrazine administration in dogs was dose related up to 50 $mg.kg^{-1}$. Together these results clearly indicate that hydrazine causes a massive utilisation of glucose in most tissues at the expense of liver and muscle glycogen and that the maintenance of blood glucose by gluconeogenesis in liver may be impaired. Two mechanisms have been established for the suppression of gluconeogenesis by hydrazine:

1) Inhibition of gluconeogenic conversion of citric acid cycle intermediates - As a result of increased free fatty acid presence, acetyl CoA should not be limiting in the liver during hydrazine treatment (Fig. 1.4). *In vitro* CO_2 production, via the citric acid cycle, from succinate in the presence of hydrazine was not altered (Fortney *et al* 1967), indicating that sufficient acetyl-CoA was indeed present. Smith (1965) demonstrated a reduction of *in vivo* and *in vitro* CO_2 in the presence of high concentrations of pyruvate and the amino acids glycine, alanine and aspartate revealing an inability of these substrates to be converted into citric acid cycle intermediates. The prevention of pyruvate entering the citric acid cycle is assumed to be due to an inhibition of the pyruvate dehydrogenase complex (Fortney *et al* 1967). Although there is no direct evidence to support this hypothesis, the enzyme complex does contain flavin as a prosthetic group, with which hydrazine is known to react.

Inhibition of the pyridoxal phosphate dependent transaminases prevents the formation of α-keto acids which can subsequently enter the citric acid cycle and act as gluconeogenic substrates (Fig. 1.4). Indeed it has been demonstrated that incorporation of [^{14}C] into glucose from radiolabelled alanine and aspartate, as well as pyruvate, is markedly inhibited by hydrazine administration (Fortney *et al* 1967). It is highly critical that citric acid cycle intermediates are replenished if any are to be drawn off for biosyntheses. Since oxaloacetate is considered to be a limiting factor in citric acid cycle function and there is a lack of anaplerotic (replenishment) mechanisms to raise mitochondrial levels, the cycle could be slowed by a reduction in oxaloacetate concentration (Fig. 1.4). This may be caused during hydrazine exposure by rapid formation of hydrazones with α-keto acids and inhibition of transamination. Roberts *et al* (1965) significantly protected against hydrazine lethality in mice by pretreatment with oxaloacetate and α-ketogluterate.

2) Inhibition of Gluconeogenesis - Decreased levels of oxaloacetate would mean that its participation in the citric acid cycle would take preference over its gluconeogenic conversion to phosphoenolpyruvate. In addition, the enzyme catalysing this gluconeogenic reaction, phosphoenolpyruvate carboxykinase (PEPCK), has been shown by Ray *et al* (1970) to be directly inhibited by hydrazine.

precursors


Glycogen
Triglyceride
Glucose
Serine
Triose P
Glycerol 3-P
Fatty Acids
Phosphoenolpyruvate
Alanine
Threonine
Methionine → Cysteine
+ Serine
Pyruvate
Acetyl CoA → Acetoacetyl CoA
Threonine → Glycine ↔ Serine
Isoleucine
Phenylalanine
Tyrosine
Leucine
Lactate
Hydroxyproline
Aspartate
Oxaloacetate
Malate
Citrate
Phenylalanine → Tyrosine → Fumarate
CITRIC ACID CYCLE
Isocitrate
Histidine
Hydroxyproline
Succinate
α-Ketogluterate → Glutamate ↔ Proline
Methionine → Propionyl CoA → Succinyl CoA
Propionate
Valine
Threonine
Ornithine
Carbomyl P
Urea
UREA CYCLE
Arginine
Citrulline
Fumarate
Aspartate
Argino-succinate

Wells demonstrated as early as 1908 that hydrazine administered to dogs, cats and guinea pigs resulted in fatty livers and this has been reported in all subsequent studies of hydrazine hepatotoxicity. Lipid levels were shown to be elevated in rats 4 hr after hydrazine administration, using light microscopy and biochemical analysis (Amenta and Johnston 1962). The hepatic lipid accumulation appeared to be midzonal and periportal and was reversible. Scales and Timbrell (1982) observed midzonal and periportal accumulation of lipids in rat liver as early as 30 min after hydrazine administration. Fatty infiltration of kidney and heart has also been reported by Amenta and Johnston (1962), Dominguez *et al* (1962) and Patrick and Black (1965). Amenta and Johnston (1962) showed that the hydrazine induced higher lipid levels in the liver were directly proportional to the hypoglycaemic effect and that the time course of liver lipid accumulation was inversely proportional to that of glycogen depletion.

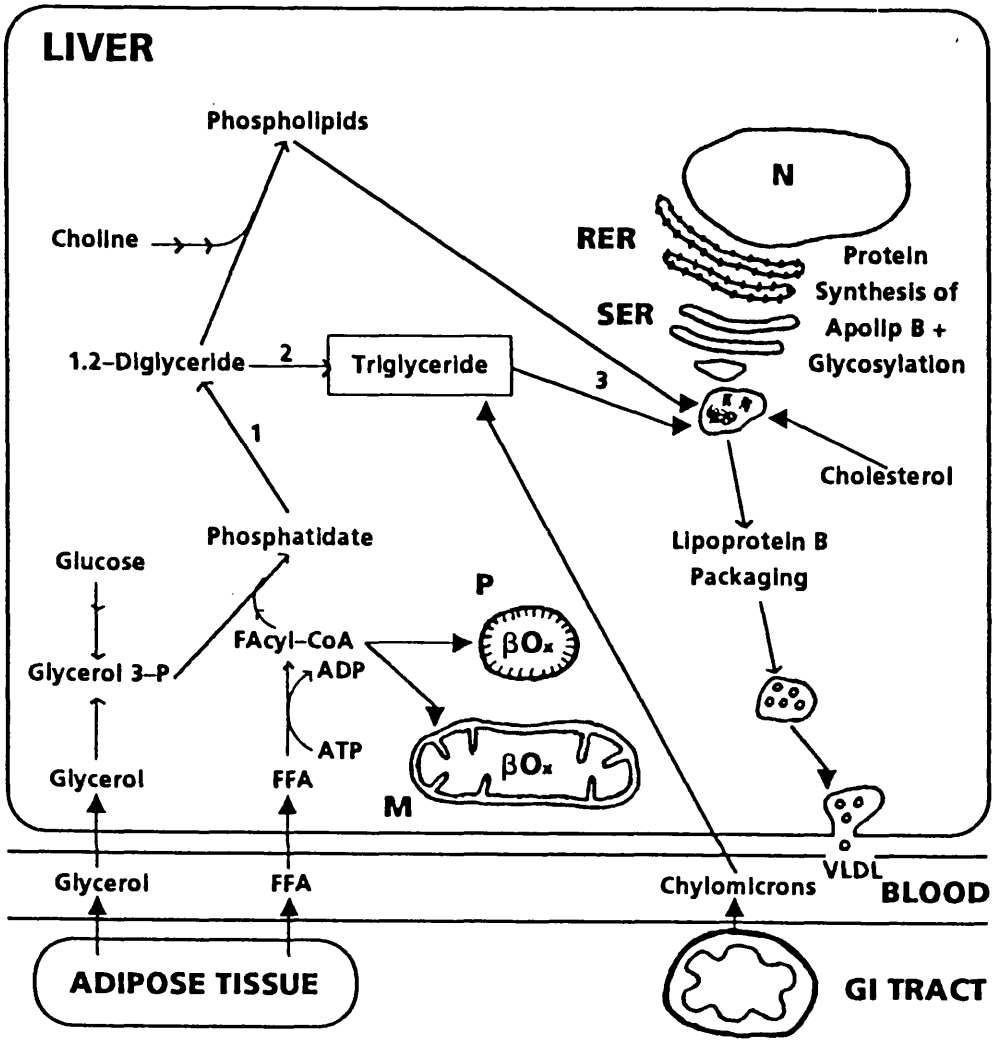
Trout (1964) showed that hydrazine induced lipid accumulation is primarily triglyceride and is accompanied by a rise in blood free fatty acids. Trout (1966) also demonstrated that the free fatty acid elevation in rats administered hydrazine was greatly reduced by feeding glucose. Clark *et al* in 1970 showed the increase in serum free fatty acids was only transient but hepatic triglyceride levels were increased approximately 10 fold and were accompanied by a 30-40% increase in liver weight. In the same study, liver and serum levels of cholesterol ester or phospholipid remained relatively constant, indicating little accumulation of cholesterol esters and phospholipids occurs relative to triglyceride synthesis. The maximal increase in liver triglycerides occurs 24 hr after a single dose of hydrazine (Amenta and Johnston 1962, Clark *et al* 1970, Haghighi and Honarjou 1987) and these levels return to normal between 48 and 72 hr (Amenta and Johnston 1962, Haghighi and Honarjou 1987).

Over the past 30 years, work has been focused on the synthesis of liver triglycerides and phospholipids and their packaging into lipoproteins, the major vehicle for lipid disposition. This research has allowed further studies to be carried out on the mechanism of hydrazine induced liver triglyceride accumulation.

Triglyceride synthesis (Fig. 1.5) is regulated by the key enzyme, phosphatidate phosphohydrolase (PAP) which cleaves phosphatidic acid to form the 1,2-diglyceride. This 1,2-diglyceride is then either esterified at the 3-position to form triglyceride or reacts with CDP-choline to form phosphatidylcholine or lecithin. At low diglyceride concentrations the latter reaction catalysed by phosphorylcholine-glyceride transferase proceeds normally allowing subsequent formation of phosphatidylserine or phosphatidylethanolamine, while the synthesis of triglyceride is slow.

Liver microsomes prepared from hydrazine treated rats were shown by Lamb and Banks (1979) to have much higher activities of PAP and *sn*-glycerol 3-phosphate acyltransferase and the time course of enzyme activation (maximal for both at 16 hr) was demonstrated to precede the *in vivo* triglyceride accumulation. *In vitro* exposure of control rat liver microsomes to hydrazine did not increase triglyceride synthesis or PAP activity (Lamb and Banks 1979) but hepatocyte monolayers exposed to 0.1 mM hydrazine for 24 hr exhibited an increased synthesis of triglyceride from diglyceride and a rise in neutral lipid/phospholipid ratio. Therefore it seems likely that hydrazine or a metabolite increases the enzymatic rate of triglyceride synthesis directly.

Unpublished preliminary observations of Lamb (cited in Lamb and Banks 1979) of a 50% reduction in the hydrazine induced accumulation of rat liver triglyceride due to cycloheximide (2 $mg.kg^{-1}$) administration, indicate that protein synthesis may be involved in the response of PAP to hydrazine. Similar conclusions have been reached previously concerning a rise in this enzyme's activity following partial hepatectomy (Vaurecka *et al* 1969) and ethanol exposure (Fallon *et al* 1979).



Abbreviations: **M** - mitochondrion, **P** - peroxisome, **βOx** - β oxidation, **N** - nucleus, **RER** - rough endoplasmic reticulum, **SER** - smooth endoplasmic reticulum, **G** - golgi body, **GI TRACT** - gastro intestinal tract.

FFA - free fatty acid, FAcyl-CoA - fatty acyl-CoA, Apolip B - apolipoprotein B VLDL - very-low-density-lipoprotein.

1 - phosphotidate phosphohydrolase, 2 - *sn*-glycerol 3-phosphate acyltransferase 3 - phosphorylcholine-glyceride transferase.

Amenta and Dominguez 1965) and their increased uptake by the liver (Clark *et al* 1970, Trout 1965) may induce PAP activity. Haghighi and Honarjou (1987) have shown that the hydrazine induced increase in PAP activity of the soluble liver fraction, and the hepatic triglyceride accumulation, was paralleled with a rise in adrenal catecholamine concentration. They also showed that hydrazine induced liver triglyceride accumulation could be completely abolished by adrenalectomy, and in further unpublished experiments demonstrated that in adrenalectomised rats, hepatic responses to hydrazine can be restored by administration of corticosteroids.
Preatment with the α-adrenergic blocking agent, phenoxybenzamine, inhibited the hydrazine induced lipid accumulation at 4 hr but not at 24 hr (Amenta and Dominguez 1965). Cooling *et al* (1979) have also demonstrated that circulating concentrations of corticosterone and insulin are increased and decreased respectively in the serum of rats exposed to hydrazine. They proposed that the increased mobilisation of free fatty acids from adipose tissue might be caused by the effects of hydrazine on the sympathetic nervous system and on levels of adrenal hormone, possibly in response to hydrazine induced hypoglycaemia. Cooling *et al* (1979) also proposed that increased activity of PAP may be a direct result of increased corticosterone levels. This data suggests that adrenal hormones may have a role in the mechanism of hydrazine induced fatty liver.

In addition to triglyceride synthesis, the formation and secretion of lipoprotein, the packaged form by which the cell exports triglyceride has been investigated. It has been suggested that the liver lipid accumulation induced by another hepatotoxin CCl_4 may be due to decreased lipoprotein formation, as well as increased triglyceride synthesis (Recknagel and Glende 1973). Amenta and Dominguez (1965) using triton WR-1339 *in vivo* as a tool to prevent uptake of triglyceride into the liver from plasma, demonstrated a slight decrease in triglyceride and phospholipid secretion into serum at 4 hr and no difference at 24 hr after hydrazine administration, while Trout (1965) detected a slight increase in secretion for fasted animals only, between 1½ and 3 hr. These data illustrate that hydrazine induced changes in the rate of lipid secretion are very minor hence the assumption that very-low-density lipoprotein

triglyceride synthesis. It is possible that VLDL is formed at a maximal rate and is rate limiting or alternatively that the activation of lipoprotein synthesis due to the stimulus of triglyceride accumulation may be prevented by hydrazine. This could be caused by inhibition of the synthesis of selected phospholipids or apolipoprotein B, both of which are essential components of VLDL (Fig. 1.5).

Depletion of liver ATP and GSH after hydrazine exposure, tog% '^T20%(th the alterations in intermediary metabolism may also have a role in triglyceride accumulation due to a limitation in lipoprotein synthesis and/or VLDL packaging. Triglyceride accumulation after hydrazine administration is much more rapid than after administration of other lipidaemic agents. During the first 4-6 hr, hepatic triglyceride levels are 70% of the peak value that occurs 24 hr after hydrazine administration, but following this period relatively small triglyceride increases occur for hydrazine compared to the progressive accumulation during the first 24 hr of other hepatotoxic agents, such as carbon tetrachloride (Recknagel and Glende 1973).

1.6.3 ATP depletion

ATP was depleted in isolated rat hepatocytes, both in suspension (Preece *et al* 1990a) and primary culture (Ghatineh and Timbrell 1990), as well as *in vivo* (Preece *et al* 1990a) after hydrazine administration. Thus confirming a previous study of several toxic compounds that were found to deplete ATP (Story *et al* 1983). Preece *et al* (1990a) showed the ATP depletion to be dose dependent both *in vitro* and *in vivo*. In addition, depletion in isolated hepatocytes occurred at a non-cytotoxic concentration and preceded toxicity at higher concentrations indicating that ATP depletion may be a cause rather than a consequence of hydrazine toxicity. The non lethality after 3 hr of ATP depleting doses *in vivo* (Preece 1990a) is consistent with this hypothesis, although some ATP may be utilised as a result of metabolic insult or possibly oxidative stress after a prolonged period of hydrazine intoxication. Evidence suggests that ATP depletion by hydrazine may involve several different mechanisms, the relative importance of which are as yet unknown.

by hydrazine in various metabolic pathways (as discussed in section 1.6) will lead to depletion of substrates available for mitochondrial oxidative phosphorylation.

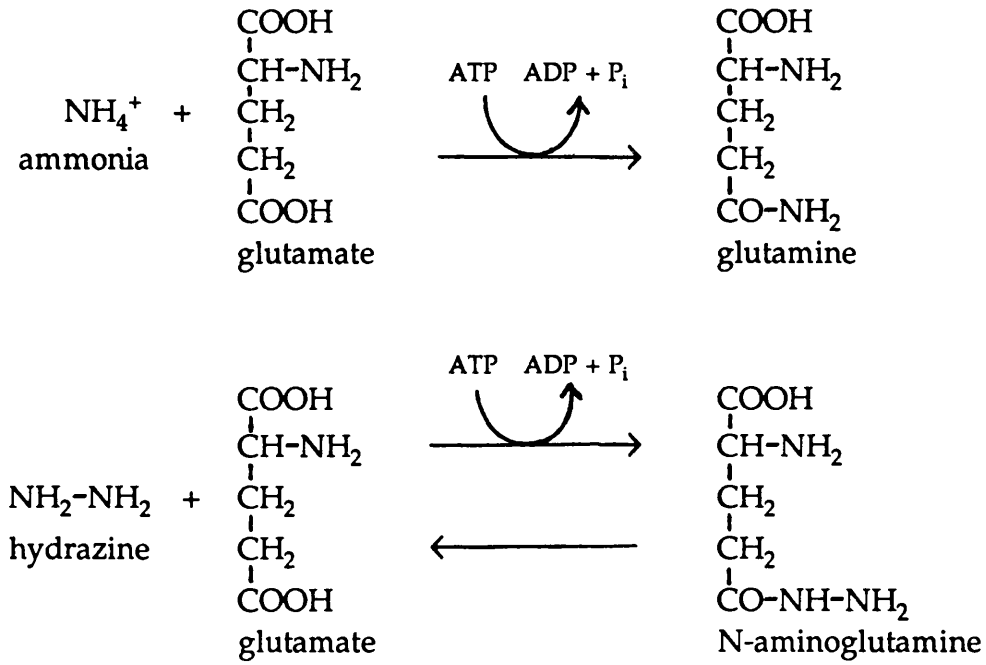
Hydrazine has been established to physically interact with the electron transport chain and disturb respiratory control. Isolated rat liver mitochondria were demonstrated to be uncoupled by hydrazine in a phosphate dependent manner (Hadler and Cook 1978) and hydrazine was shown to bind to cytochrome oxidase *in vitro*, resulting in the inhibition of cytochrome c oxidation (Markosyan *et al* 1988). However, it has not been definitively proven that hydrazine exerts the same effects *in vivo*, yet it is likely that hydrazine may interact with the flavin, haem or copper prosthetic groups common to the redox enzymes of the electron transport chain.

Hydrazine administered to rats resulted in mitochondrial swelling as soon as 1 hr after a 60 $mg.kg^{-1}$ i.p. dose (Scales and Timbrell 1982). Rats placed on a 1% hydrazine diet for 3 days had slightly enlarged mitochondria and after 7 days mega-mitochondria were observed (Wakabayashi *et al* 1987). After 3 days mitochondrial increases in total phospholipid, relative acid phospholipids, Ca^{2+} content, water content and thermotropic lipid phase transition temperatures were measured. After 7 days these parameters were further increased, suggesting that mega-mitochondrial formation may be due to fusion of adjacent swollen mitochondria by Ca^{2+}-acidic phospholipases in order to stabilise mitochondrial membranes. In another study Wakabayashi *et al* (1983) prepared mega-mitochondria from mice receiving 10% hydrazine in the diet for 3 days and rats receiving 20% hydrazine in their diet for 8 days. They showed that succinate and glutamate oxidation coupling efficiency, P/O ratio, ATPase activity and cytochrome c oxidase were all slightly decreased, suggesting that respiration and oxidative phosphorylation in mega-mitochondria is inhibited to an extent.

Another mechanism by which hydrazine could deplete ATP is by the increased utilisation of ATP. Hydrazine can act as an alternative substrate to ammonia *in vitro* in the reaction catalysed by either glutamine synthetase (Speck 1949) or carbamoyl

hydrazine treatment ATP may be wasted by futile cycling in the formation of labile N-amino analogues of glutamine or carbamoyl phosphate that decompose back to hydrazine (Figs. 1.2 and 1.6)

Figure 1.6 Catalytic conversion of ammonia and hydrazine by glutamine synthetase



1.6.4 Glutathione depletion

The tripeptide glutathione (GSH) is an important low molecular weight thiol that is involved in the Phase II metabolism of many xenobiotics and is protective against toxic reactive electrophiles. Depletion of such a cellular defensive compound would compromise normal cell physiology and lead to toxicity. It is possible that hydrazine may deplete GSH after oxidation by conjugating with it, since certain monosubstituted hydrazines have been discovered to generate glutathione adducts in the presence of NADPH, oxygen and liver microsomes (Nelson *et al* 1976 and 1978, Spearman *et al* 1984). This is believed to result from formation of the diazine which decomposes to the carbon centred free radical. Under the same conditions GSH resulted in stimulation of alkane formation during oxidative metabolism of alkylhydrazines, presumably via abstraction of a hydrogen from the thiol by the

products has been demonstrated by Kosower (1971). Nucleophilic attack of thiols on azo linkages substituted with electron withdrawing groups on one or both nitrogens leads to reduction of the azine and regeneration of the parent hydrazine. For the latter two reactions previously described, glutathione is oxidised to glutathione disulphide (GSSG).

Timbrell *et al* (1982) demonstrated that the dose dependent depletion of GSH measured 24 hr after hydrazine administration was not due to GSH oxidation. The ratio of oxidised (GSSG) : reduced (GSH) glutathione was not altered by hydrazine administration but absolute levels of both GSH and GSSG were reduced to half of the control levels. These data suggest that the depletion of glutathione may be due to specific inhibition of glutathione synthesis by hydrazine or to the general disruption of intermediary metabolism. Glutathione formation requires 2 molecules of ATP, one of which is necessary in the rate limiting step catalysed by γ-glutamylcysteine synthetase. Consequently synthesis may be influenced by depletion of ATP although this mechanism has yet to be proven. Disturbance of amino acid metabolism during hydrazine exposure may lead to depletion of amino acids required for glutathione synthesis and result in reduced glutathione levels, similar to the situation after fasting.

1.6.5 Alteration of ammonia and urea levels

Synthesis of urea via the urea cycle (Fig. 1.4) was inhibited in rat liver homogenates by hydrazine as was the synthesis of another urea cycle intermediate, citrulline (Roberge *et al* 1971). However, in rats treated with 32 $mg.kg^{-1}$ hydrazine for 4 days they found that the activity of urea cycle enzymes was not affected except for arginosuccinase where an increase was noted. Roberge *et al* (1971) also demonstrated that hydrazine inhibits ornithine-ketoacid transaminase, a pyridoxal-phosphate dependent enzyme. Thus accounting for the accumulation of ornithine in the liver of hydrazine treated rats that had previously been reported by Simonsen and Roberts (1967), Cornish and Wilson (1968) and Banks (1970). Roberge *et al* (1971) proposed that the increase in urea synthesis they found *in vivo* is due to the presence of a high

reported in dogs by McKennis *et al* (1961) and Floyd (1980). The accumulation of citrulline after hydrazine administration in mice (Simonsen and Roberts 1967) is claimed to be a consequence of the rate limiting citrulline-aspartate reaction. In contrast to Roberge *et al* (1971), Lewis and Izume (1926) concluded that urea production was not greatly altered by hydrazine and Simonsen and Roberts (1967) showed a decreased urea production in mice. Floyd (1980) did not see as large a rise in urea formation in hydrazine treated dogs as would be expected from the elevation in ammonia and this may be due to the competitive inhibition of carbamoyl phosphate synthetase by hydrazine (Fig. 1.2). The differences seen in the effect of hydrazine on urea synthesis may be due to different dosing regimes, species and dose levels utilised in experiments.

1.6.6 Alteration of amino acid levels

Several studies have indicated that hydrazine produces an amino acid imbalance in experimental animals. Amenta and Johnston (1963) found that conversion of ^{14}C labelled glycine or alanine to $^{14}CO_2$ was impaired by 60-70% in liver slices from hydrazine treated rats. The conversion of amino acids to keto acids *in vitro* via transamination has also been shown to be inhibited by hydrazine and this inhibition can be reversed by addition of pyridoxal phosphate to the reaction mixture (Killam and Blain 1957, McCormick and Snell 1961). Simonsen and Roberts (1967) have reported increased levels of alanine, glutamate, aspartate, ornithine, glycine and citrulline in livers of hydrazine treated rats and Cornish and Wilson (1968) found increased levels of tyrosine and α-amino acids in brain, plasma and liver of rats due to hydrazine. Korty and Coe (1968) in addition, reported the elevation of free amino acids in plasma and urine of dogs exposed to hydrazine. All such findings demonstrate the known inhibition of amino acid metabolism by hydrazine, due to binding of the coenzyme pyridoxal phosphate which is contained in all transaminases. It has also been reported that hydrazine interferes with the synthesis of pyridoxal phosphate *in vitro* (McCormick and Snell, 1961) and *in vivo* (Chatterjee and Sengupta 1980)

In contrast to the decreased amino acid metabolism, the incorporation of labelled amino acids into cellular protein following hydrazine administration has been found to be increased by many authors, including Banks and Stein (1965) and Banks (1970) *in vivo* and Amenta and Johnston (1963) *in vitro*. The latter suggest that hydrazine blocks amino acid metabolism but not the assimilation of amino acids into protein. Banks and Stein (1965) have also demonstrated that liver RNA and liver protein, when compared to liver DNA levels (Banks 1970), are concomitantly elevated in hydrazine treated rats. Free amino acid levels measured by Banks (1970) indicate that after hydrazine treatment the skeletal muscle could serve as the amino acid supply source for the liver.

In contrast to previous data, Lopez-Mendoza and Villa-Trevino (1971), using radiolabeled leucine and glycine uptake studies, observed an inhibition of *in vivo* rat liver protein synthesis 8½ hr after hydrazine administration, although they too detected increased levels of free amino acids. The discrepancy in amino acid incorporation could not be explained, but they suggested that the reported increase in total liver protein by Banks and Stein (1965) could be due to a decrease in protein breakdown or a defect in protein exportation from the liver.

1.7 HYDRAZINE CARCINOGENICITY

It has long been known that hydrazine is a carcinogen in laboratory animals (Roe *et al*, 1967) but the incidence and origin of tumours depends on the dose and the period and route of exposure. Oral administration of hydrazine at a growth depressing dose induced lung adenocarcinomas and hepatocellular carcinomas in male rats (Severi and Biancifori, 1968) hepatocellular carcinomas in mice, but no lesion in hamsters (Biancifori, 1970). However, Bosan *et al* (1987) did detect hepatocellular carcinomas in hamsters at the highest dose they had administered in the drinking water.

The carcinogenic potential for humans has been studied in a few epidemiological investigations of occupational hydrazine exposures. The observed mortality from all causes or specific causes, for example from lung cancer, suggests no adverse effects of hydrazine exposure (Wald *et al* 1984). However caution must be applied to these

detect more extreme hydrazine induced changes.

The most useful trials for assessing occupational carcinogenicity are long term inhalation studies. Vernot *et al* (1985) in chronic inhalation studies found increased incidences of nasal epithelium tumours in rats exposed to hydrazine. However it is known that the epithelium of the nasal cavity of rodents is highly sensitive to the local effects of carcinogens and therefore it would be difficult to extrapolate these results to the human situation.

At present the carcinogenic effects of hydrazine have been demonstrated with only maximally tolerated, unambiguously toxic doses or locally irritating concentrations (inhalation studies). In some of the few studies carried out according to currently acceptable standards, hydrazine administered to rats (Steinhof and Mohr 1988) and mice (Steinhof *et al* 1990) in drinking water could only demonstrate a weak carcinogenic effect at the highest dose, solely as the very late development of small, mostly benign, hepatocellular tumours. In accordance with this study and all other past studies the MAK (Maximale Arbeitsplatz Konzentration) manifesto states "Hydrazine proved to be weakly carcinogenic in the rat, mouse and hamster only after practically life-long administration of toxic doses".

1.8 HYDRAZINE MUTAGENICITY/GENOTOXICITY

Hydrazine has been shown to induce gene mutations and/or chromosome aberrations in a variety of test systems (Kimball 1977) including plants, bacterial phages, bacteria, fungi, insects and mammalian cells *in vitro*. Most authors report an observed effect both with and without microsomal activation (Noda *et al* 1986) but in a few studies microsomal activation was an absolute requirement (World Health Organisation (WHO) task group 1987). Hydrazine has also been shown to increase the transformation of baby hamster kidney cells and human cells (Purchase *et al* 1978) and in addition DNA single-strand breaks have been detected in the liver and lung cells of hydrazine dosed mice (Parodi *et al* 1981). Although hydrazine is capable of reacting *in vitro* with pyrimidine bases under critical conditions (Kimball 1977, Cashmore and Peterson 1978) no hydrazine-DNA adducts have been reported to

However, administration of single hepatotoxic doses of hydrazine induces the dose dependent formation of 7-methylguanine and O^6-methylguanine in liver DNA of rats, hamsters, mice and guinea pigs (Barrows and Shank 1981, Shank *et al* 1980, Shank 1983, Shank 1984, Barrows *et al* 1983, Bosan and Shank 1983, Shank 1987). Formation of 7-methylguanine but not O^6-methylguanine was detected in the liver DNA of rats repeatedly administered a daily oral dose of 3 $mg.kg^{-1}$ hydrazine for 4 days (Becker *et al* 1981). Other carcinogens such as dimethylnitrosamine, 1,2-dimethylhydrazine and methylnitrosurea as well as the two hepatotoxins carbon tetrachloride and ethanol produce similar patterns of liver DNA guanine methylation and it is proposed that DNA methylation constitutes the initiation process in carcinogenesis (Bosan *et al* 1987). It was initially suggested (Barrows and Shank, 1981) that DNA methylation after hydrazine administration was dependent upon endogenous S-adenosylmethionine, but little or no evidence could be obtained to support this idea or the proposal that hydrazine was methylated within hepatocytes to monomethylhydrazine and then metabolically activated to a methylating agent.

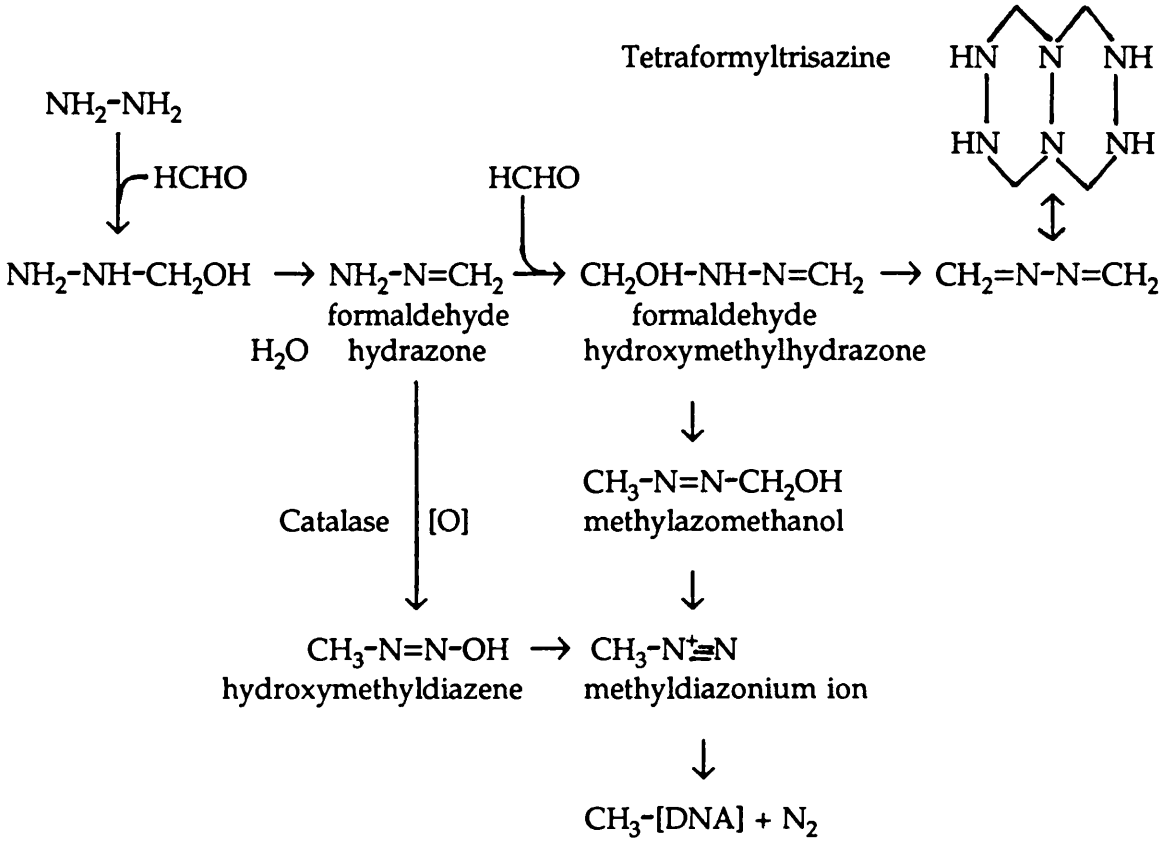
Shank *et al* (1984) using 9,000*g* liver supernatants detected much greater amounts of 7-methylguanine and O^6-methylguanine after hydrazine addition when formaldehyde was also added. This activity in cytosolic and microsomal fractions was demonstrated to be heat labile (Bosan *et al* 1986). This is consistent with *in vivo* data from the same study which indicates a role of alcohol dehydrogenase and/or aldehyde dehydrogenase.

Tetraformyltrisazine (TFT), a major reaction product of hydrazine and formaldehyde, produced greater amounts of DNA guanine methylation than hydrazine both *in vivo* and *in vitro* (Lambert *et al* 1986) and lead to the hypothesis that TFT is an important intermediate in the methylation of DNA guanine following administration of hydrazine to animals.

Hydrazine and formaldehyde, at concentrations that approach those *in vivo* after hydrazine administration, were shown to react *in vitro* to form formaldehyde hydrazone by Lambert and Shank (1988). These coworkers also demonstrated that catalase, but not cytochrome P450 or flavin monooxygenase, is involved in the *in vitro* methylation of DNA by hydrazine. They propose that formaldehyde hydrazone

catalase and/or other hepatic enzymes to a methylating agent responsible for DNA guanine methylation (Fig. 1.7).

Figure 1.7 Proposed metabolic pathways for the genotoxic activation of hydrazine to a DNA methylating reagent



Doses of hydrazine that were toxic for the mother have been reported to produce adverse effects on embryos and fetuses of rats and mice (Keller 1988), such as reduced fetal weight, increased resorptions and perinatal mortality plus increased incidences of litters and fetuses with abnormalities. These abnormalities included mainly supernumary and fused ribs, delayed ossification, modest hydronephrosis and slight brain ventricle dilation which were considered to be minor by the authors. Destruction of male gonadal epithelium has been observed after 6 months of oral hydrazine exposure (WHO task group 1987).

Xenobiotic metabolism has evolved in order to increase polarity of foreign compounds, enabling them to be excreted more quickly and consequently reduce their toxicity. However, xenobiotic metabolites may be formed which are more toxic than the parent compound. Biotransformation of xenobiotics has therefore a major influence on the toxicological activity and it's duration.

The general aim of this study was to investigate the metabolism of hydrazine and determine its significance on the biochemical toxicity of hydrazine. This research will provide further comprehension of the mechanisms of hydrazine toxicity and may lead to the development of a metabolic strategy which prevents or alleviates hydrazine toxicity. Such a system would enable the risks of occupational hydrazine toxicity to be reduced.

2.1 MATERIALS

2.1.1 Chemicals

Hydrazine hydrate, *p*-dimethylaminobenzaldehyde (DMBA) [crystalline], pentafluorophenylhydrazine, pentafluorobenzaldehyde, acetylhydrazine (free base), bovine serum albumin (BSA) [fraction V], ferric cytochrome c [Type III: from horse heart], NADH [dipotassium salt], NADPH [tetrasodium salt, type I], NADP [sodium salt from yeast], glucose-6-phosphate dehydrogenase [type VII from yeast], D glucose-6-phosphate [monosodium salt], metyrapone, methimazole, ADP [potassium salt, grade XIX, equine], ATP [disodium salt equine], pargyline, clorgyline, catalase [crystalline suspension], oligomycin, carbonyl cyanide *m*-chloro-phenyl-hydrazone, β-naphthoflavone, isoniazid, 7-ethoxyresorufin, 7-pentoxyresorufin, resorufin [sodium salt], *p*-nitrophenol [crystalline spectrophotometric grade], *p*-nitrocatechol [crystalline spectrophotometric grade], GSH [Crystalline free acid], 5,5-dithiobis-[2-nitrobenzoic acid] (DTNB), luciferase [firefly lantern extract], 4,5-dihydroxynaphthalene-2,7-disulphonic acid [disodium salt], zeolite, sodium metaperiodate, sodium arsenite were all obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

Piperonyl butoxide was obtained from Koch-Light (Colnbrook, UK) and all solvents were supplied by Rathburn Chemicals Ltd. (Walkerburn, Scotland).
All other reagents were obtained from BDH Ltd. (Poole, Dorset, UK).

2.1.2 Animals

Male Sprague-Dawley rats (240 - 300 g) were purchased from Charles River (UK) and allowed food and water *ad libitum* prior to each study.

2.2.1 Colorimetric determination of hydrazine

Reagents :-

i) 6% (w/v) *p*-Dimethylaminobenzaldehyde (DMBA) in methanol.

This assay utilises the condensation reaction of DMBA with hydrazine at pH 1 to produce a vivid yellow azine (Fig. 2.1) and is adapted from a method devised by Reynolds and Thomas (1965) for measuring hydrazine in blood.

Urine Assay

Samples of urine from animals administered hydrazine, together with calibration standards (0-10 mM), were diluted 1:150 with 10% (w/v) trichloroacetic acid (TCA). The TCA mixtures were centrifuged at 1,500*g* (3,000 rpm) and 1.25 ml supernatant added to 2.0 ml of 6% (w/v) DMBA. The samples were immediately vortexed and after 10 min the absorbance was measured at 470 nm. Calibration curve linearality was good (r= 0.99). Repeated assays on spiked urine samples indicated reproducibility to be ± 4.8% in the 1 - 10 mM urine concentration range (n=60).

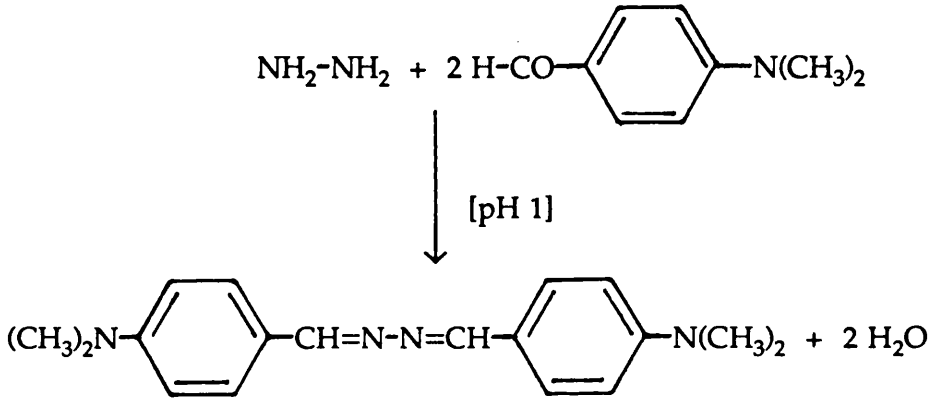
Liver Microsomal/Mitochondrial Assay

Samples of microsomal/mitochondrial suspension incubated with hydrazine together with calibration samples (0-0.2 mM or 0-2.0 mM) were diluted 1:4 or 1:40 with 10% (w/v) TCA. The TCA mixtures were centrifuged at 1,500*g* (3,000 rpm) and 0.8 ml supernatant was further diluted by addition of 1.8 ml 40% (w/v) TCA. 1.25 ml of 6% (w/v) DMBA in methanol was added, followed by immediate vortexing and after 10 min the absorbance was measured at 470 nm. Calibration curve linearality was good (r= 0.99). Repeated assays on spiked microsomal/TCA samples indicated reproducibility to be ± 3.9% in the 25 - 200 µM microsomal concentration range (n=80) and ± 4.8% in the 0.25 - 2 mM microsomal concentration range (n=60).

Absorbance was found to be linear over a final assay concentration range of 0-70 µM for both urine and microsomal suspensions and the detection limit was $\leq$ 5µM. Acetylhydrazine was found to cause no interference in this assay and sample/TCA mixtures were found to be stable at -20°C for up to 48 hr.

microsomal/mitochondrial samples was found to be necessary in order to prevent subsequent clouding upon the addition of DMBA in methanol. This interference in absorbance was probably due to precipitation of previously acid solubilised proteins, either by the presence of methanol or by the reduction in acid concentration. The latter assumption was based on the fact that microsomal samples spiked with 2.0 mM hydrazine required more initial dilution with 10% (w/v) TCA than 0.2 mM hydrazine spiked samples and did not become cloudy in the presence of DMBA in methanol.

Figure 2.1 Colorimetric reaction of hydrazine and *p*-dimethylaminobenzaldehyde



2.2.2 Determination of hydrazine and acetylhydrazine by gas chromatography

Reagents :-

i) 20 mM Pentafluorophenylhydrazine (PFPH internal standard) in nitrogen degassed 50% (v/v) Ultra High Quality (UHQ) water/methanol [HPLC grade].

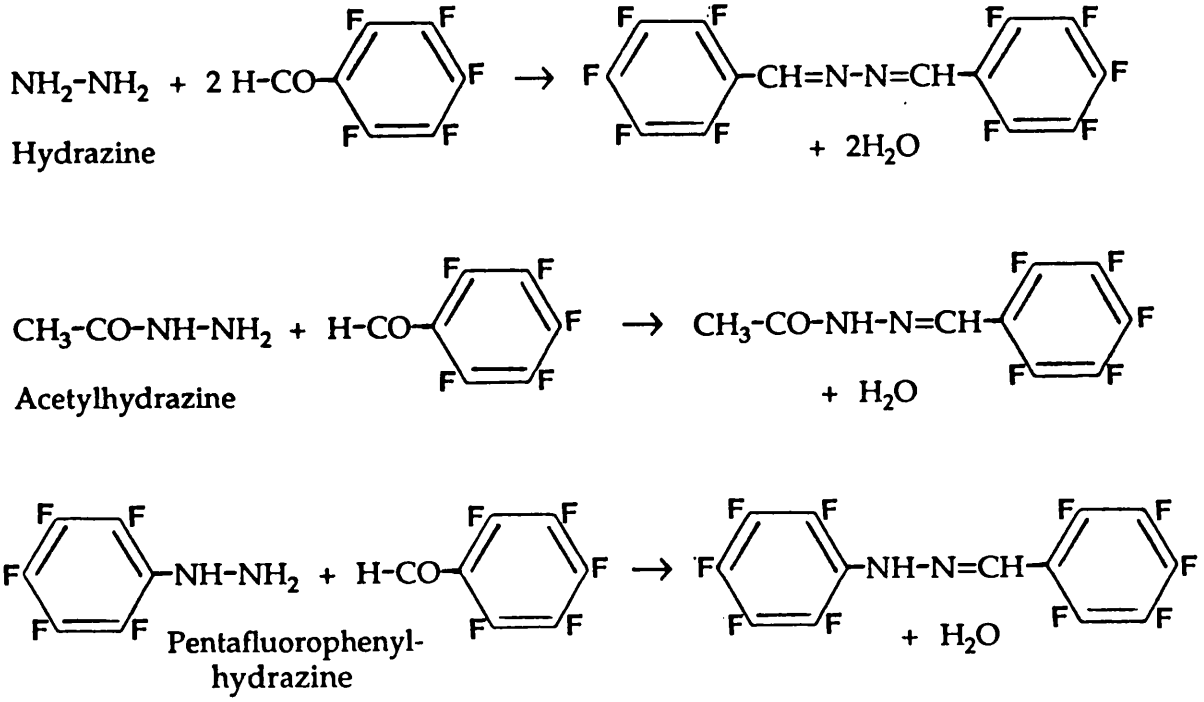
ii) 1 M Pentafluorobenzaldehyde (PFB derivatising solution) in nitrogen degassed methanol [HPLC grade].

Aliquots of acidified sample or standard were mixed thoroughly with 150 µl of 20 mM PFPH and after 10 min 100 µl of 1 M PFB was added and immediately vortexed. After 30 min 2.5 ml of ethyl acetate was added and the samples were shaken intermittently for a further 30 min. The solvent layer was extracted and stored at 4°C.

The PFB derivatives were measured using a Nitrogen Phosphorous Detector (NPD) after 0.5 µl had been injected into a gas chromatograph. Details of GC analysis

Peak areas were measured by an integrator. Hydrazine and acetylhydrazine were quantified against standards by comparison with the internal standard. Calibration curves showed good linearality. Reproducibility of repeated assays on spiked samples was calculated to be ± 3.1% for urine samples in the range 1 - 10 mM (n=72) and ± 4.5% for microsomal samples in the range 0.25 - 2.0 mM (n=60).

Figure 2.2 Derivatisation reactions of hydrazine, acetylhydrazine and pentafluorophenylhydrazine with pentafluorobenzaldehyde



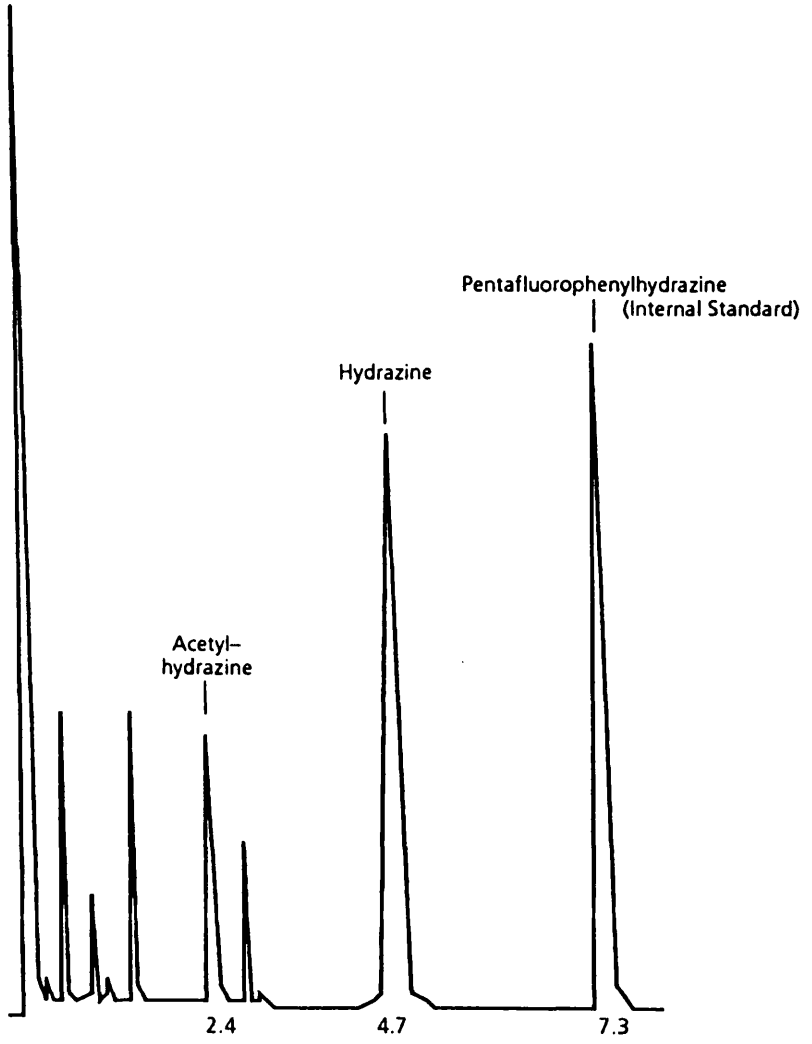
Stock PFB adducts were synthesised by mixing each hydrazine or hydrazide with PFB derivatising solution (Fig. 2.1) in 50% (v/v) UHQ water/methanol [HPLC grade] and then dissolved by warming. The adducts were isolated after crystallisation at 4°C (ice) by vacuum flask filtration. Finally the adducts were purified by dissolving them in methanol, allowing them to recrystallise and then filtering them off and drying them overnight in a vacuum desiccator. The PFB adducts, dissolved in ethyl acetate, were used to identify and check GC retention times.

pentafluorophenylhydrazine after derivatisation with pentafluorobenzaldehyde

Column Type	Wall Coated, Open Tubular, Capillary
Packing	Bonded Phase 1 on OV 1 Fused Silica
Column Length	12m
Internal Diameter	0.22mm
Injection Temperature	300°C
Oven Temperature	140°C
Detector Temperature	350°C

NPD DECTECTOR

Carrier Gas	Helium
Flow Rate	1.0ml/min



Retention Time (minutes)

5 g of acetylhydrazine free base was dissolved in 10 ml of chloroform and warmed to 60°C. Anhydrous sodium sulphate (20 g) was then added to remove any water present and after 5 min the warm chloroform was filtered. Anhydrous diethyl ether (20 ml) was added and the mixture cooled in ice. Fine needles of acetylhydrazine were filtered off and dried in a vacuum desiccator overnight.

2.2.4 Isolation of rat liver microsomes

Reagents :-

i) Homogenisation buffer: 0.25M sucrose, 10 mM tris, 1mM EDTA (pH 7.4).

ii) 0.1 M Phosphate buffer: 0.1 M K_2HPO_4 + 0.1 M KH_2PO_4 (pH 7.4).

The following procedures were all carried out at 4°C.

Male Sprague Dawley rats were killed by cervical dislocation. The liver was perfused via the hepatic portal vein with 20 ml homogenisation buffer (pH 7.4) and then rapidly removed, weighed and washed in 4°C homogenisation buffer. It was then added to 4 volumes of homogenisation buffer, scissor chopped into small pieces and subsequently homogenised on ice in a Polytron blender.

The homogenate was then centrifuged at 10,000*g* (11,000 rpm) for 20 min at 4°C. The resultant supernatant was carefully decanted and centrifuged at 100,000*g* (33,000 rpm) for 70 min. The microsomal pellet was resuspended in 0.1 M phosphate buffer (pH 7.4), using a few gentle hand passes of the Potter-Elvehjem homogeniser, and centrifuged again at 100,000*g* for 70 min. Finally the microsomal pellet was resuspended in 0.1 M phosphate buffer containing 20% (v/v) glycerol (pH 7.4) (approximately 4 volumes) and stored at -80°C until required. Microsomal protein content was assessed by the method of Lowry *et al* (1951).

2.2.5 Determination of liver homogenate and microsomal protein content

Reagents :-

i) Solution A: 2% (w/v) Na_2CO_3 in 0.1M NaOH.

ii) Solution B_1: 2% (w/v) sodium potassium tartrate.

iii) Solution B_2: 1% (w/v) $CuSO_4.5H_2O$.

iv) BSA stock solution: 200 $\mu g.ml^{-1}$ bovine serum albumin [fraction V].

Protein content of samples was determined by the method of Lowry *et al* (1951) Microsomes were diluted 1:250 in 0.1 M phosphate buffer, while 10% (w/v) liver

with 1 M HCl and then diluted 1:100 with 0.1 M phosphate buffer. A range of BSA standards were constructed at the same time in the range 0-200 $\mu g.ml^{-1}$ from the BSA stock solution. 5.0 ml of fresh assay reagent (solution B_1, solution B_2, solution A, 1:1:98) was added to 0.5 ml aliquots of samples and standards. After 10 min 0.5 ml of 50% (v/v) Folin's phenol reagent was added followed by immediate vortexing. After 30 min the absorbance of was measured at 750 nm.

2.2.6 Spectral determination of microsomal cytochrome P450

The cytochrome P450 content of microsomes was assessed by the method of Omura and Sato (1964).

Stock microsomal samples were diluted 1:5 (final concentration 3 mg $protein.ml^{-1}$) in 0.1 M phosphate buffer, containing 20% (v/v) glycerol (pH 7.4). 2.0 ml was then added to both matched sample and reference cuvette, which were placed in the forward position of a split-beam spectrophotometer. A baseline spectrum was recorded between 400 and 500 nm. A few grains of sodium dithionite were then added to both cuvettes and gently mixed with a cuvette stirrer. The sample cuvette was gently gassed (approximately 1 $bubble.sec^{-1}$) with carbon monoxide for 60 sec and the cuvettes were rescanned to give a reduced difference spectrum. The cytochrome P450 content was calculated according to the following equation, assuming an extinction coefficient of 91 $mM^{-1}.cm^{-1}$:

$$\text{nmoles cytochrome P450.sample}^{-1} = \frac{\Delta E \text{ (450 nm - 490 nm)}}{91} \times 10^3$$

2.2.7 Spectral determination of microsomal cytochrome b_5

The cytochrome b_5 content of microsomes was assessed by the method of Omura and Sato (1964).

Stock microsomal samples were diluted 1:5 (final concentration 3 mg $protein.ml^{-1}$) in 0.1 M phosphate buffer, containing 20% (v/v) glycerol (pH 7.4). 2.0 ml was then added to both matched sample and reference cuvette, which were placed in the forward position of a split-beam spectrophotometer. A baseline spectrum was recorded between 400 and 500 nm. 25 µl of 2% (w/v) NADH was added to the test cuvette only and the spectrum re-recorded.

The cytochrome b_5 content was calculated according to the following equation,

$$\text{nmoles cytochrome } b_5.\text{sample}^{-1} = \frac{\Delta E \text{ (424 nm - 490 nm)}}{112} \times 10^3$$

2.2.8 Determination of microsomal NADPH cytochrome P450 reductase activity

Reagents :-

i) Ferric cytochrome c solution: 5 $mg.ml^{-1}$ oxidised cytochrome c [equine]

The enzyme activity was assessed by measuring the rate of exogenous cytochrome c (oxidised, ferric form) reduction and is based on the method of Yasukochi and Masters *et al* (1976).

Reactions were carried out in the forward position of a split-beam spectrophotometer at 37°C. Microsomes were diluted in 0.1 M phosphate buffer (pH 7.4) to give a concentration of 0.1 mg $protein.ml^{-1}$ and a 3.0 ml aliquot was placed in a sample cuvette and a matched reference cuvette. 0.3 ml of ferric cytochrome c solution was added to both cuvettes and the reaction was initiated by addition of 25 µl of 2% (w/v) NADPH to the sample cuvette only. The rate of reduced ferrous cytochrome c produced was monitored at 550 nm and recorded on a chart recorder until it became non-linear.

Specific NADPH cytochrome P450 reductase activity was calculated according to the following equation, using an extinction coefficient of 29.5 $mM^{-1}.cm^{-1}$ (Sigma catalogue) for reduced cytochrome c:

$$\text{Activity (nmol cytochrome c reduced.sample}^{-1}) = \frac{\Delta E \text{ (550 nm).min}^{-1}}{29.5} \times 10^3$$

2.2.9 Determination of microsomal ethoxyresorufin and pentoxyresorufin *O*-deethylase activity (EROD and PROD)

Reagents :-

i) 1 mM 7-Ethoxyresorufin in dimethylsulphoxide (DMSO).

ii) 1 mM 7-Pentoxyresorufin in DMSO.

iii) 10 µM Resorufin [sodium salt] in DMSO.

The rate of *O*-deethylation of the two phenoxazone alkyl ethers was assessed using a modified method of Burke *et al* (1985) by measuring the increase in fluorescence due to the formation of the common product resorufin. EROD activity is specific for

for cytochrome P450IIB1 (induced by phenobarbitone).

Reactions were carried out in quartz cuvettes at 37°C using a fluorescence spectrometer set at 585 nm (emission) and 530 nm (excitation). Microsomes were diluted in 0.1 M phosphate buffer (pH 7.5) to give a concentration of either 1 mg protein.ml^{-1} (control microsomes) or 0.1 mg protein.ml^{-1} (induced microsomes) in a total volume of 2.0 ml. 10 µl of either 1 mM ethoxyresorufin in DMSO or 1 mM pentoxyresorufin in DMSO was added and the reaction initiated by addition of 25 µl of 40 mM NADPH. The rate of increase in fluorescence was recorded on a chart recorder until it became non-linear.

A series of calibration measurements were carried out on 5 µl aliquots of resorufin in DMSO (1 or 10 µM depending on the fluorometer sensitivity setting) added sequentially to a cuvette containing 2.0 ml of buffer, microsomes and substrate. The deethylase activity was expressed as resorufin synthesised.mg protein^{-1}.min^{-1}.

2.2.10 Determination of microsomal *p*-nitrophenol hydroxylation (NPH) activity

Reagents :-

i) 5 mM *p*-Nitrophenol [crystalline spectrophotometric grade]

ii) 1 mM *p*-Nitrocatechol [crystalline spectrophotometric grade]

A modified method of Koop (1986) and (Reinke and Moyer 1985) was used in order to measure the rate of *p*-nitrophenol hydroxylation by spectrophotometrically assessing the formation of the product *p*-nitrocatechol, which was found to have an E_{max} of 510 nm. NPH activity is specific for cytochrome P450IIE1 (induced by acetone or isoniazid).

Microsomes (1 mg protein.ml^{-1}) were incubated in 0.1 M phosphate buffer (pH 7.4) at 37°C in a shaking water bath with 60 µl of 5 mM *p*-nitrophenol (initial concentration 125 µM) to give a total reaction volume of 2.0 ml. The reaction was initiated by addition of 40 µl of 50 mM NADPH and after 10 min the reaction was terminated by addition of 0.5 ml of 0.5 M perchloric acid (PCA). *p*-Nitrocatechol standards were constructed (range 0-60 µM) from samples containing microsomes, PCA and substrate but not NADPH. The PCA mixtures were centrifuged at 1,500g (3,000 rpm) in order to remove precipitated proteins. 1.0 ml of the resultant

nitrocatechol was immediately measured at 510 nm. NPH activity was expressed as *p*-nitrocatechol synthesised.mg^{-1} protein.min^{-1}.

2.2.11 Liver freeze-clamping

The clamping apparatus was pre-cooled in liquid nitrogen. Animals were sacrificed by cervical dislocation and the liver quickly removed. Liver (0.4 - 0.6 g) was cut from the edge of the central lobe and immediately placed on one of the aluminium blocks, whereupon the large tongs were quickly snapped shut. The frozen clamped liver was carefully scraped into the mortar and the pestle was hammered down on top. The liver was scraped into a powder and emptied into a preweighed pot containing 12.0 ml of 0.5 M PCA. The pot was finally stored at -80°C for up to 24 hr. The sample was thawed, centrifuged at 1,500*g* (3,000 rpm) for 10 min and the supernatant analysed for ATP (1 in 3 dilution) and reduced glutathione (no dilution).

2.2.12 Determination of reduced glutathione (GSH)

Reagents :-

i) GSH stock solution: 2 mM GSH [Crystalline free acid] in 0.5 M PCA.

ii) DTNB solution: 10 mM 5,5-Dithiobis-(2-nitrobenzoic acid) in phosphate buffer (pH 7.4).

GSH was determined by a modification of the spectrophotometric method of Ellman et al (1959). The assay is based on the reduction of DTNB by the thiol (-SH) group of GSH and other non protein sulphydryl groups to yield TNB [5-Thio,2-nitrobenzoic acid] which absorbs light at 412 nm. Removal of protein after acid precipitation prevents the reaction of DTNB with protein thiol groups. Hydrazine was not found to interfere with this assay.

To each 0.5 ml sample in 0.5 M PCA or GSH standard (range 0-0.5 mM) in 0.5 M PCA, 4.5 ml of 0.1 M phosphate buffer (pH 9.0) was added and then 50 µl of DTNB solution. The samples were immediately vortexed and allowed to stand at room temperature in the dark for 15 min, after which the sample absorbance was measured at 412 nm.

Reagents :-

i) Assay buffer: 80 mM $MgSO_4$, 10 mM KH_2PO_4, 100 mM Na_2AsO_4; 1:1:1 (pH 7.4).

ii) Luciferase: 5 ml of water at 4°C was added to firefly lantern extract and the supernatant collected after centrifugation at 500*g* (1,000 rpm) and 4°C for 5 min.

The assay, using a modified method of Lemasters and Hackenbrock (1978), relies on the reaction of ATP with the luciferase extract of firefly tails to form adenyl-luciferin, which is oxidised by air with light emission. The rate of light emitted is proportional to the amount of ATP present for the assay conditions used (one molecule of ATP consumed produces one photon), allowing calibration of a photon detection system for the measurement of ATP in cells and tissues. Because the rate of light emission is not constant and depends on time after luciferase addition, it is very important to measure the rate at a constant time interval after enzyme addition. An optimal time was found to be 15 sec. The reaction is carried out in standardised plastic tubes in order to maintain the area of liquid for counting constant.

Samples and ATP standards were diluted in 0.5 M PCA (range 0-40 µM) and 10 µl was added to 2.0 ml of fresh assay buffer (pH 7.4) in standardised 12 x 75 mm plastic tubes. The bioluminescence reaction was initiated by addition of 100 µl luciferase with immediate vortexing. The tube was placed inside the light tight sample compartment and 15 sec after the addition of luciferase the luminescence was measured for 6 sec by a photon detection system (power supply/control unit, photomultiplier tube housed at -25°C, forced air controller). Data was collected by a microcomputer which controlled the system.

2.2.14 Determination of liver triglycerides

Reagents :-

i) 0.2% (w/v) Chromotropic acid reagent: 0.229% (w/v) 4,5-dihydroxynaphthalene-2,7-disulphonic acid [disodium salt] in concentrated sulphuric acid/water (3:2).

ii) Zeolite: activated by heating at 110°C for 18 hr.

This assay measures glycerol spectrophotometrically after the saponification of triglycerides and is based on the method of Butler *et al* (1960).

zeolite moistened with 8.0 ml of chloroform. A further 12.0 ml of chloroform was added and after 10 min of intermittent shaking the mixture was filtered through coarse, fat-free, filter paper. An aliquot of the chloroform filtrate (0.2-1.0 ml depending on estimated triglyceride content) was pipetted into three glass test tubes in order to give approximately 0.05 mg of triglycerides. Triglyceride standards were constructed in triplicate (range 0 - 0.09 $mg.ml^{-1}$) from a stock 0.10 $mg.ml^{-1}$ corn oil chloroform solution.

Chloroform was evaporated from all samples and standards by maintaining them at 80°C in a water bath. Two of the replicates were saponified by addition of 0.5 ml of 0.4% (w/v) KOH in 95% ethanol and 0.5 ml of 95% ethanol was added to the third unsaponified replicate. All tubes were maintained at 60 - 70°C for 20 min and then 0.5 ml of 0.2 N sulphuric acid was added to every tube. Ethanol was removed by placing the tubes in a gently boiling water bath for 20 min after which they were cooled to room temperature. To each tube 0.1 ml of fresh 0.05 M sodium metaperiodate was added and immediately vortexed. After 10 min 0.2 ml of 1 M sodium arsenite was added and left for a further 10 min. Finally 5.0 ml of 0.2% (w/v) chromotropic acid reagent was added, immediately vortexed and heated for 30 min in a boiling water bath in the absence of excessive light. After cooling to room temperature the absorbance was measured at 570 nm. Sample triglyceride content was calculated according to the following equation:

$$\text{Triglyceride content (mg)} = \frac{20 \times 10}{\text{Vol.of chloroform extract (ml)}} \times R \times 0.05 \text{ mg}$$

$$\text{Where:} \quad R = \frac{\Delta E570 \text{ nm (sapon. sample - unsapon. sample)}}{\Delta E570 \text{ nm (sapon. 0.05 mg std - unsapon. 0.05 mg std)}}$$

2.2.15 Statistical analysis of results

Comparison of a single treatment with a control was calculated using the unpaired Student's t-Test as described by Gad and Weil (1989).

Significance of treatment was obtained from Student's t tables.

single control group was assessed initially using one way ANOVA and then Dunnet's t-Test.

Significant difference among the groups was initially established using one way ANOVA (Gad and Weil 1989), where the F ratio value obtained was compared with a table of F values. Subsequently the significance of each treatment with the control was calculated using Dunnett's t-Test (Dunnett 1964, Gad and Weil 1989) which incorporates the mean square within groups calculated during ANOVA. Probability values for the t statistic were obtained from Dunnett's t tables (Dunnett 1964).

Dose response curves in Chapter 4 were compared using 2 way ANOVA (Calcutt and Boddy), which compares the variance attributable to dose or pretreatment with the residual variance, taking into account any interaction. Significance of variation was calculated by comparing the F ratios with a table of F values.

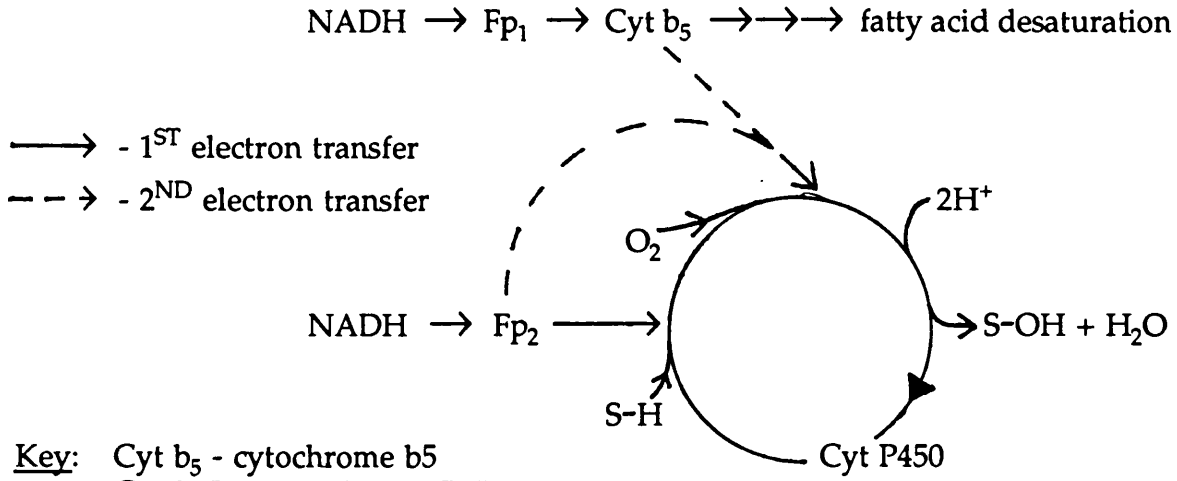
3.1 INTRODUCTION

Xenobiotic metabolism can often be divided into two phases. Phase I biotransformations involving oxidation, reduction or hydrolysis generally result in the formation of metabolites that undergo Phase II biotransformations, involving conjugation. The major enzyme system involved in phase I xenobiotic metabolism is the mixed function oxidase (MFO) system which is responsible for the metabolism of over 250,000 drugs, pesticides, toxins, carcinogens and mutagens in addition to the biotransformation of a number of endogenous substrates including fatty acids, lipid hydroperoxides, cholesterol, bile acids, eicosanoids, retanoids and steroid hormones (Coon *et al* 1992).

The greatest levels of mammalian MFO enzymes occur in the liver and to a lesser extent in the kidney, brains, intestine, blood, skin and nasal epithelium and within the cell, MFO enzymes are located predominantly in the endoplasmic reticulum. The isolation of endoplasmic reticulum in the microsomal fraction of liver homogenates thus provides a convenient source of MFO enzymes for *in vitro* study of xenobiotic metabolism.

The MFO system comprises three major components: Cytochrome P450 (hemoprotein with an iron-porphyrin moiety), NADPH cytochrome P450 reductase (flavoprotein) and a lipid component. This multicomponent electron transport system has an absolute requirement for NADPH and molecular oxygen. Electrons are shuttled from NADPH to cytochrome P450 the terminal oxidase via NADPH cytochrome P450 reductase (Fig. 3.1). Electrons can also be synergistically donated from NADH to the MFO pathway in the presence of NADPH and this involves the essential interaction of cytochrome b_5, another hemoprotein, via the flavoprotein, cytochrome b_5 reductase. The involvement of cytochrome b_5 depends on the MFO substrate.

Microsomes also contain another Phase I monooxygenase flavin-containing monooxygenase (FMO) which is responsible for the specific oxygenation of soft nucleophiles (Ziegler 1991). Commonly, substrates for FMO are also substrates for cytochrome P450.



Key: Cyt b_5 - cytochrome b5
Cyt P450 - cytochrome P450
Fp_1 - cytochrome b_5 reductase
Fp_2 - cytochrome P450 reductase
S-H - cytochrome P450 substrate

Furthermore both FMO and cytochrome P450 require NADPH and oxygen for activity and are localised in the microsomal fraction. Therefore the contribution of FMO requires careful analysis in order to distinguish it from that of P450. In contrast to P450, FMO oxygen activation occurs before binding of the substrate enabling the catalysed oxygenation of structurally dissimilar compounds (Ziegler 1990).

Another important location of Phase I enzymes are mitochondria which contain monoamine oxidases (MAO) that are responsible for the oxidative deamination of endogenous neurotransmitters. These flavoproteins have also been demonstrated to metabolise a variety of xenobiotics including phenelzine (Juchau and Horita 1972) and the 1,2-disubstituted hydrazines, procarbazine and 1,2-dimethylhydrazine (Coomes and Prough 1983) and are inhibited by several hydrazine derivatives including iproniazid (Smith *et al* 1963), isocarboxazid (Juchau and Horita 1972), hydralazine (Lyles *et al* 1983), phenelzine (Andree and Clark 1982), phenylhydrazine (Patek and Hellerman 1974) and safrazine (Eto *et al* 1988).
In addition mitochondrial membranes contain the MFO enzyme system including cytochrome P450 (Shayiq and Avadhani 1989, Ohyama *et al* 1991). Isolation of mitochondria from liver homogenates therefore yields another useful source of Phase

retain their ATP synthetic activity in the presence of suitable substrates and therefore provide a useful tool for further investigating the mechanism of ATP depletion by hydrazine.

3.1.1 Aims

A simple method for the accurate measurement of hydrazine in isolated cell fractions was developed in order that *in vitro* hydrazine metabolism by microsomes and mitochondria could be assessed. The assay was used to characterise the various cofactors and enzyme systems involved in the *in vitro* metabolism of hydrazine. In addition the effect of hydrazine on *in vitro* mitochondrial respiration and ATP synthesis was evaluated.

3.2 METHODS

3.2.1 *In vitro* metabolism of hydrazine by microsomes or mitochondria

Reagents :-

i) Glucose-6-phosphate dehydrogenase [type VII from bakers yeast].

ii) D Glucose-6-phosphate [crystalline monosodium salt].

iii) NADP [sodium salt].

Microsomes (4 mg protein.ml^{-1}) or mitochondria (3 mg protein.ml^{-1}) were incubated in a 25 ml glass conical flask at 37°C, using a shaking water bath. Microsomes were incubated in 0.1 M phosphate buffer (pH 7.4) and NADPH was provided, when necessary, by a regeneration system (final concentration 10 mM $MgCl_2$, 1.5 mM NADP, 20 mM glucose-6-phosphate, 1.5 units.ml^{-1} glucose-6-phosphate dehydrogenase). The following microsomal inhibitors and cofactors were utilised: 1.5 mM NADH, 1 mM metyrapone, 0.5 mM piperonyl butoxide, 1 mM methimazole. Gaseous atmospheres of nitrogen and carbon monoxide/oxygen (8:2) were created by displacement of air with the required gas and were maintained by rubber seals. Mitochondria were incubated in 0.25 M sucrose, 5 mM tris, 20 mM K_2HPO_4, 2 mM $MgCl_2$, 15 mM ADP buffer (pH 7.5) with 20 mM succinate as substrate. The following mitochondrial inhibitors were utilised: 100 µM pargyline, 0.5 µM clorgyline.

Metabolism was initiated by addition of 50 µl hydrazine (20 or 200 mM) to give a final hydrazine concentration of either 2.0 or 0.2 mM in a final volume of 5.0 ml.

to 10% (w/v) TCA in order to stop metabolism and prevent hydrazine breakdown.

3.2.2 Isolation of rat liver mitochondria

Reagents :-

i) Homogenisation buffer: 0.25 M sucrose, 10 mM tris, 1 mM EDTA (pH 7.4).

The following procedures were all carried out at 4°C.

Male Sprague Dawley rats were killed by cervical dislocation. The liver was perfused via the hepatic portal vein with 20 ml homogenisation buffer and then rapidly removed, weighed and washed. It was then added to 4 volumes of homogenisation buffer, scissor chopped into small pieces and subsequently homogenised in an overhead motor-driven Potter-Elvehjem (Teflon pestle) homogeniser (0.35 mm clearance). The homogenate was then centrifuged at 500*g* (1,000 rpm) for 10 min at 4°C. The resultant supernatant was centrifuged for 7 min at 12,500*g* (12,000 rpm) and 4°C. The mitochondrial pellet was resuspended in homogenisation buffer, using a few gentle hand passes of the Potter-Elvehjem homogeniser, and centrifuged again at 12,500*g* for 7 min. The mitochondrial pellet was finally resuspended in homogenisation buffer again (approximately 4 volumes) and stored on ice. Immediately afterwards protein content was determined by the Biuret method and mitochondrial coupling state was assessed using a YSI Oxygen Electrode System.

3.2.3 Determination of mitochondrial protein content

Reagents :-

i) BSA stock solution: 8 $mg.ml^{-1}$ Bovine serum albumin [fraction V].

ii) Biuret reagent A: 4.5% (w/v) sodium potassium tartrate, 0.5% (w/v) KI, 1.5% (w/v) $CuSO_4.5H_2O$, 1.12% (w/v) NaOH.

iii) Biuret reagent B: 8% (w/v) NaOH, 5% (w/v) KI.

Mitochondrial protein content was assessed using the Biuret method .

Mitochondria were diluted 1:4 in homogenisation buffer and a range of BSA standards were constructed in the range 0-8 $mg.ml^{-1}$ from the BSA stock solution. 2.5 ml of Biuret working solution (reagent A, reagent B, water, 5:2:18) was then added to 0.5 ml aliquots of samples and standards and mixed. After 30 min the absorbance at 545 nm was measured.

Reagents :-

i) Respiration buffer: 0.25 M sucrose, 5 mM KH_2PO_4, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl

ii) Catalase suspension [crystalline suspension: diluted to 230 units.ml^{-1}]

iii) H_2O_2 solution: 30% (w/v) H_2O_2, diluted 1 in 250.

The functional integrity of isolated mitochondria was assessed by how tightly respiration was coupled to ATP synthesis. Oxygen uptake in the presence of the substrate succinate (20mM) was measured before and after addition of ADP and the respiratory control ratio (RCR) calculated.

The following procedure was used to calibrate the oxygen electrode system. 2.0 ml of nitrogen saturated respiration buffer was added to the chamber, maintained at 30°C, and the oxygen electrode probe immediately replaced on top removing any air bubbles. 10 µl of catalase suspension was injected into the cell and then 5 µl of H_2O_2 solution was repeatedly added as often as the chart recorder pen deflection would allow. Each 5 µl aliquot of H_2O_2 produces 88.2 nmol of O_2.

Fresh isolated mitochondria were diluted in the electrode chamber, maintained at 30°C, with fully aerated respiration buffer to give 2 mg.ml^{-1} protein in a total of 2.0 ml. The oxygen electrode was placed over the suspension removing any air bubbles and the magnetic stirrer started. Endogenous respiration (state 1) was recorded for 2 min and then unlimited respiration substrate (20 µl) was added which stimulates the respiration to state 4. The RCR was calculated as the ratio of oxygen consumption in the presence (state 3) and absence (state 4) of 20 mM ADP (20 µl). The effect of hydrazine on mitochondrial respiration was compared to known inhibitors of mitochondrial function.

3.2.5 Determination of *in vitro* mitochondrial ATP synthesis

Mitochondria (3 mg protein.ml^{-1}) were incubated in 0.25 M sucrose buffer containing 5 mM tris, 20 mM K_2HPO_4, 2 mM $MgCl_2$, 15 mM ADP (pH 7.5). The incubations were carried out in 25 ml glass conical flasks at 30°C, using a shaking water bath. Mitochondrial oxidative phosphorylation was initiated by addition of 50 µl succinate (1 M) to give a final concentration of 20 mM in a final flask volume of 2.5 ml.

Aliquots of incubation sample were removed after various time intervals and added to 0.5 M perchloric acid (PCA) in order to stop respiration and prevent ATP

and the supernatant was analysed for ATP content (section 2.2.13).

3.3 RESULTS

3.3.1 Effects of microsomes, NADPH and oxygen on *in vitro* hydrazine disappearance

Hydrazine disappearance in samples incubated with 2.0 mM hydrazine was initially analysed by gas chromatography (GC) (Table 3.1). Substantial degradation of hydrazine occurred when incubated only in the presence of phosphate buffer (made from distilled water) and this was significantly increased by the addition of NADPH ($p<0.01$). Microsomes caused much more hydrazine to disappear than in buffer alone ($p<0.01$) and also when NADPH was present, compared to phosphate buffer and NADPH ($p<0.05$). Using microsomes which had previously been boiled, the disappearance of hydrazine was not as great as with normal microsomes and this was also the case when NADPH was present. However boiled microsomes caused significantly more hydrazine disappearance compared to buffer alone ($p<0.05$) and this was also true when each sample type had NADPH present ($p<0.05$). GC analysis of microsomal samples demonstrated that the metabolite acetylhydrazine was not generated by this particular cell fraction.

Microsomal samples incubated with hydrazine and analysed by GC were compared with studies in which samples were analysed by colorimetry for hydrazine determination (Table 3.1). In the latter studies Ultra High Quality (UHQ) water was used to make all buffers and microsomes were given an extra washing in buffer during their preparation. This resulted in reduced hydrazine disappearance for samples of microsomes and samples of buffer (in the presence or absence of NADPH) that were utilised in colorimetric studies. Hydrazine disappearance measured by colorimetry was diminished in all types of sample when incubated in a nitrogen environment, but the reduction was not as substantial in samples of boiled microsomes (with or without NADPH).

3.3.2 Effect of NADPH and temperature on chemical degradation of hydrazine

Chemical degradation of hydrazine in buffer after 60 min (Table 3.2) was found to be negligibly increased (<1% initial hydrazine concentration) at 37°C compared to 4°C (ice) for concentrations of hydrazine $\geq$200 µM. However at hydrazine

represented a much larger fraction of the initial hydrazine content in the buffer.

There was no chemical degradation of hydrazine (initial concentration = 200 μM) in phosphate buffer at either 37°C or 4°C (ice) in the absence of NADPH (Fig. 3.2). However when NADPH was present there was a significant decrease (14-18%) in hydrazine content at 4°C and 37°C in the first 10 min ($p<0.01$), but this hydrazine degradation did not continue. Over the 60 min incubation period the difference in chemical degradation in the presence of NADPH due to temperature was not found to be statistically significant.

In order to account for, and subtract, the effect of chemical degradation from *in vitro* hydrazine metabolism experiments, duplicate samples were subsequently incubated at 4°C (ice) as chemical degradation controls.

3.3.3 Effect of various cofactors, inhibitors and GSH on *in vitro* microsomal hydrazine metabolism

Absence of NADPH or oxygen (nitrogen atmosphere) and presence of the cytochrome P450 inhibitor, carbon monoxide, considerably reduced hydrazine metabolism at concentrations of 2.0 mM hydrazine at all time points (Fig. 3.3) and 0.2 mM at 20 - 60 min (Fig. 3.7). The specific cytochrome P450 inhibitors, metyrapone and piperonyl butoxide, decreased hydrazine metabolism significantly at 0.2 mM hydrazine at 20 - 60 min (Fig. 3.6) but at 2.0 mM (Fig. 3.5), metyrapone did not significantly inhibit hydrazine metabolism while piperonyl butoxide did at all time points. Presence of the microsomal flavin-containing monooxygenase inhibitor, methimazole, reduced hydrazine metabolism at 2.0 mM at 10, 15, 30 and 60 min (Fig. 3.5). When methimazole was added with metyrapone (Fig. 3.6) there was a further significant reduction (Student's t-Test) in metabolism of 0.2 mM hydrazine at 5 and 60 min, compared to microsomes incubated with metyrapone alone. For 2.0 mM hydrazine, microsomal metabolism in the presence of NADH alone was not significantly increased above that of incubations with no nucleotides (Fig. 3.4). However when both NADH and NADPH were present hydrazine metabolism was significantly increased compared to microsomes incubated with NADPH alone. The actual increase was much greater than would be expected from the sum of the metabolism data of the individual nucleotides. At 0.2 mM hydrazine, metabolism with NADPH was increased with the additional presence of NADH (Fig. 3.7),

Microsomal hydrazine metabolism was unaffected by the presence of either 1 or 2 mM GSH (Fig. 3.8) and during the 60 min incubation period, GSH at both concentrations was not significantly reduced (Fig. 3.9).

3.3.4 *In vitro* metabolism of hydrazine by hepatic microsomes prepared from rat and human

Rat liver microsomes were isolated from two groups of male Sprague-Dawley strain, which had similar body weights of between 250 and 275 g. Both groups were identical in their housing and treatment conditions. Human liver microsomes were prepared from frozen samples, obtained from renal transplant donors (Table 3.3). All tissue samples were shown to have normal histology and their use in microsomal studies had local Research Ethics Committee permission and coroner's approval.
Group A rat liver microsomes metabolised hydrazine at a higher rate than rat group B (Fig. 3.10) but this only became significant at 40 min and after ($p<0.05$). Microsomes isolated from human liver sample 8 had the highest rate of human metabolism. Human liver sample 6 had the lowest rate of microsomal metabolism (Fig. 3.10) which was statistically significant after 20 min compared to the 2 rat samples and the 2 other human samples ($p<0.01$ and <0.05 respectively). Microsomes from human liver samples 8 and 9 had significantly lower rates of metabolism compared to rat group A after 40 and 10 min respectively, but neither were significantly different to rat group B. Compared to the mean rate of rat microsomal metabolism the human rate was 30 - 80% less, depending on the individual human sample.

3.3.5 Effect of monoamine oxidase (MAO) inhibitors on *in vitro* mitochondrial hydrazine metabolism

Isolated rat liver mitochondria were found to metabolise hydrazine (Fig. 3.11) and this was not significantly affected by the presence of either of the monoamine oxidase (MAO) inhibitors clorgyline or pargyline.

3.3.6 Effect of hydrazine on *in vitro* mitochondrial respiration and ATP synthesis

ATP synthesis after 5, 10 and 20 min was unaffected up to a hydrazine concentration of 5 μM, but at concentrations of hydrazine $\geq$100 μM was significantly reduced

μM hydrazine, ATP synthesis was inhibited in a concentration dependent manner up to the maximum achieved at ≥100 μM.

The normal respiratory control of freshly isolated mitochondria utilising succinate as substrate was altered by addition of uncoupler or ATP synthetase inhibitors. These characteristic respiratory responses were used to monitor respiratory control during oxygen electrode experiments on isolated mitochondria and enabled the use of only coupled isolated mitochondria (Fig. 3.13 Trace A). Respiration is stimulated by ADP in coupled mitochondria but is inhibited in coupled mitochondria by addition of the ATPase/synthase inhibitor oligomycin. Addition of an uncoupler like carbonyl cyanide *m*-chloro-phenyl-hydrazone (CCCP) to coupled mitochondria produces a dramatic increase in respiration.

The normal RCR (Respiratory Control Ratio) was slightly increased in the presence of hydrazine at concentrations of 100 μM and above, but this effect was eliminated by prior addition of ADP (Fig. 3.13 Traces B,C and D).

buffer under various conditions

Sample Type	GC (Air)	Colorimetry (Air)	Colorimetry (N_2)
Microsomes + NADPH	2.13 ± 0.13	1.89 ± 0.02	0.42 ± 0.03
Microsomes	1.56 ± 0.08	0.48 ± 0.06	0.46 ± 0.08
Boiled microsomes + NADPH	1.89 ± 0.04	2.11 ± 0.19	1.79 ± 0.25
Boiled microsomes	1.46 ± 0.10	1.80 ± 0.21	1.73 ± 0.43
Phosphate buffer + NADPH	1.56 ± 0.09	1.40 ± 0.03	0.22 ± 0.02
Phosphate buffer	1.11 ± 0.07	0.77 ± 0.03	0.16 ± 0.02

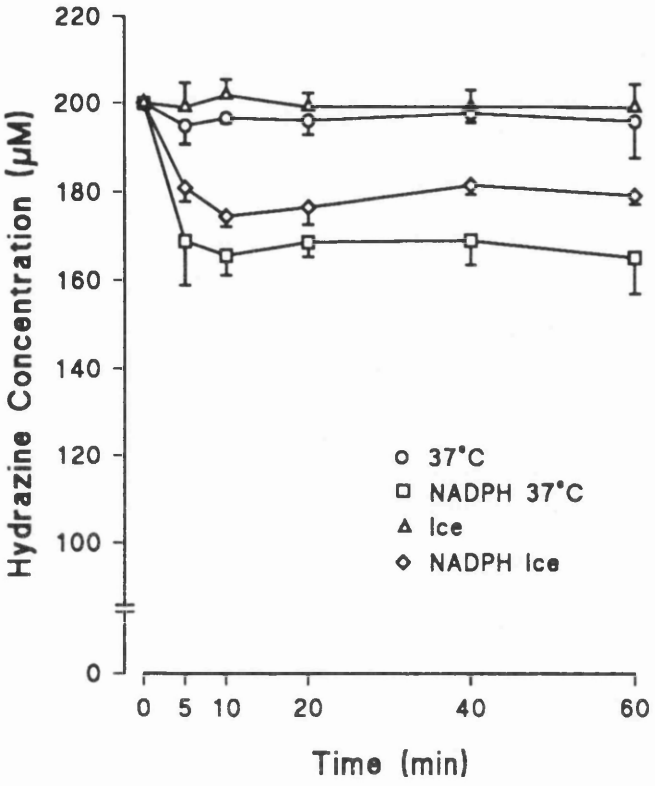
2.0 mM hydrazine was incubated for 45 min under various conditions. Data points represent the mean ± SEM (n=4). Units = µmol. hydrazine disappeared/2.5 ml . Statistical comparisons between samples were assessed using the unpaired Student's t-Test.

Table 3.2 The effect of temperature and hydrazine concentration on the chemical degradation of hydrazine in 0.25 M sucrose buffer (pH 7.5) containing NADPH

Hydrazine Concentration (µM)	37°C Hydrazine Degradation		4°C (Ice) Hydrazine Degradation		Difference in Hydrazine Degradation due to Temperature
	$nmol.ml^{-1}$	%	$nmol.ml^{-1}$	%	%
10	8.04 ± .49	80.4	6.08 ± .45	60.8	32.3
25	19.20 ± 1.24	76.8	17.28 ± .97	69.1	11.2
50	11.28 ± .73	22.5	9.64 ± .61	19.2	17.1
100	28.56 ± 1.51	28.6	27.24 ± 1.42	27.2	4.9
250	36.00 ± 1.70	14.5	35.72 ± 1.86	14.3	0.8

Various concentrations of hydrazine were incubated with 0.25 M sucrose buffer for 60 min at two different temperatures in the presence of NADPH. The data points represent the mean ± SEM (n=4). Percentage values were calculated relative to the initial hydrazine concentration.

the chemical degradation of 0.2 mM hydrazine in 0.1 M phosphate buffer

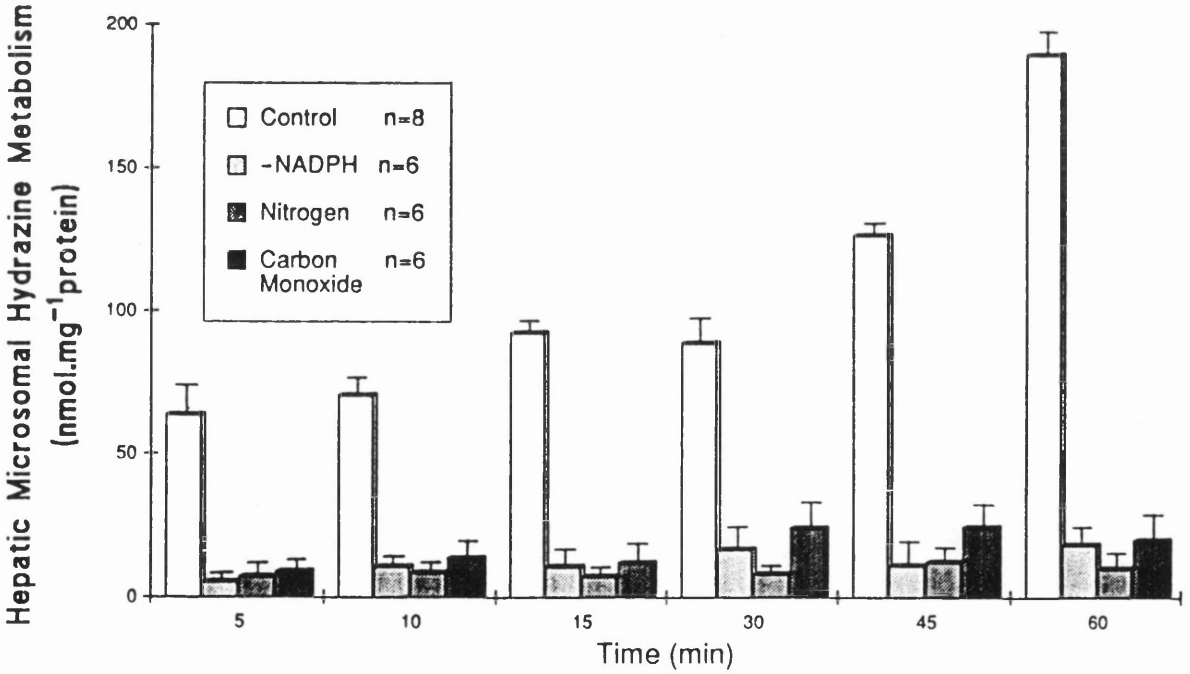


0.2mM hydrazine was incubated with 0.1 M phosphate buffer (pH 7.4) at two different temperatures in the presence and absence of NADPH.

Data points represent the mean ± SEM from four incubations.

Statistical significance of temperature and NADPH on the chemical degradation of hydrazine was assessed using the unpaired Student's t-Test.

Fig. 3.3 The effect of cofactors and gaseous atmosphere on the *in vitro* metabolism of 2.0 mM hydrazine by rat liver microsomes



Hepatic microsomes from male Sprague-Dawley rats were incubated with 2.0 mM hydrazine in the presence of cofactors and different gaseous environments. The data represents the mean ± SEM from n incubations. The statistical significance of the different conditions on microsomal hydrazine metabolism was assessed using one way ANOVA and then Dunnett's t-Test. Control = complete test incubate with NADPH.

by rat liver microsomes

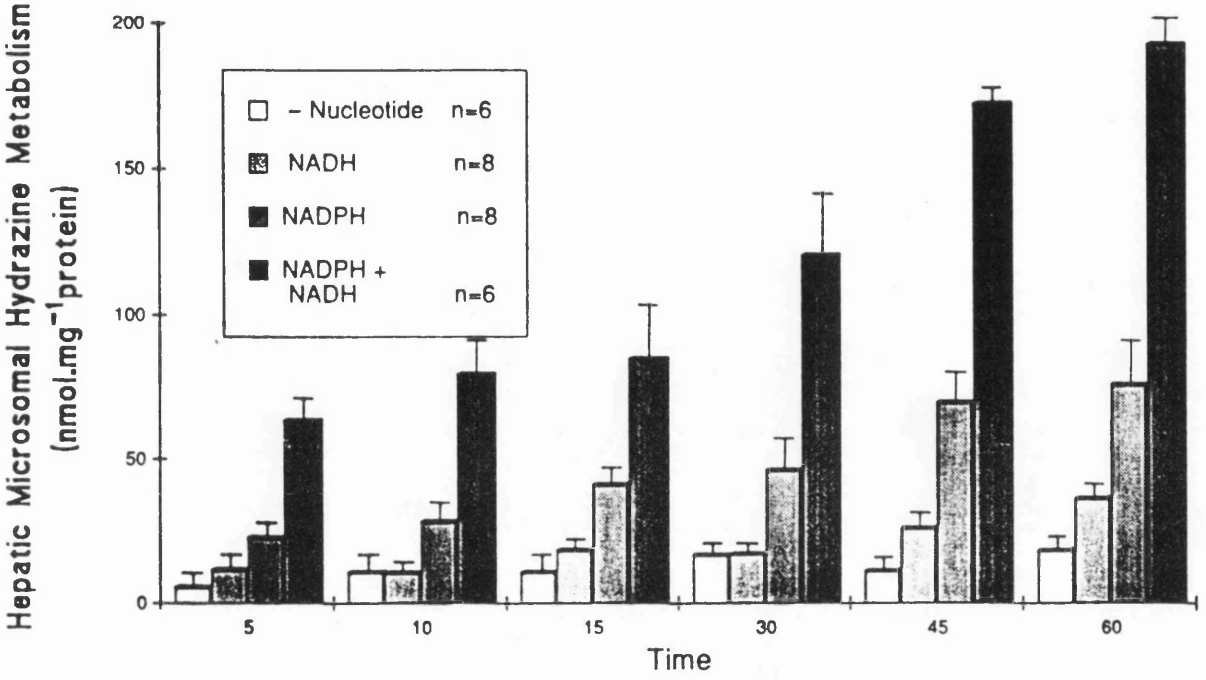
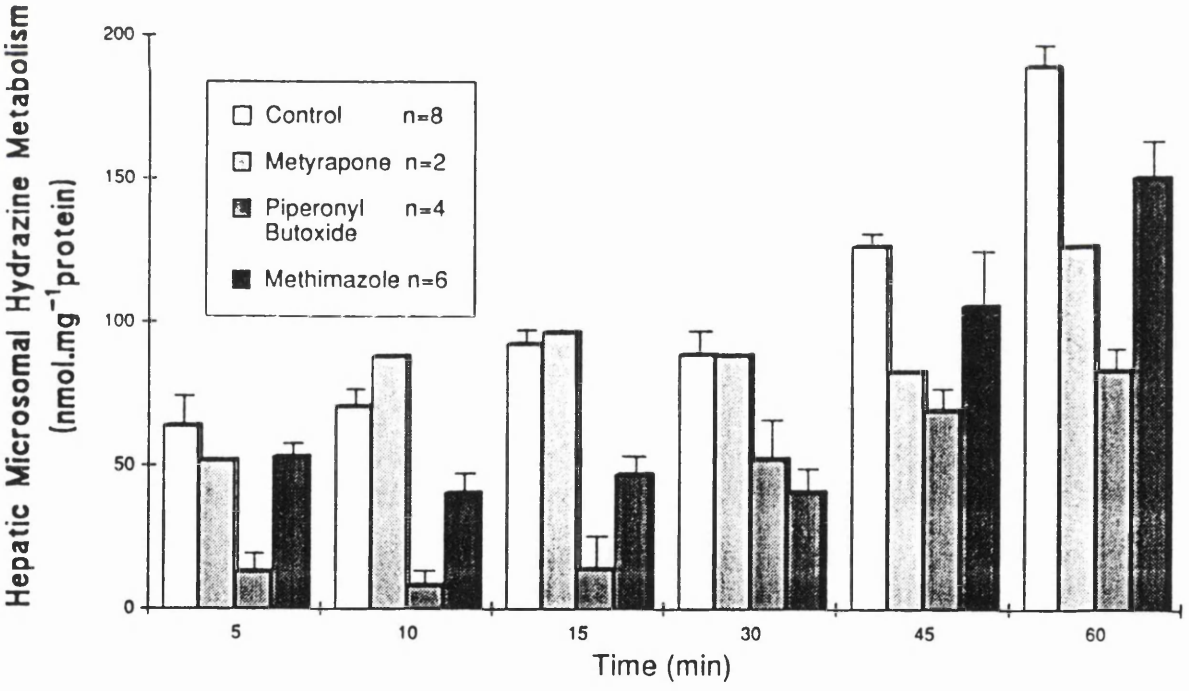
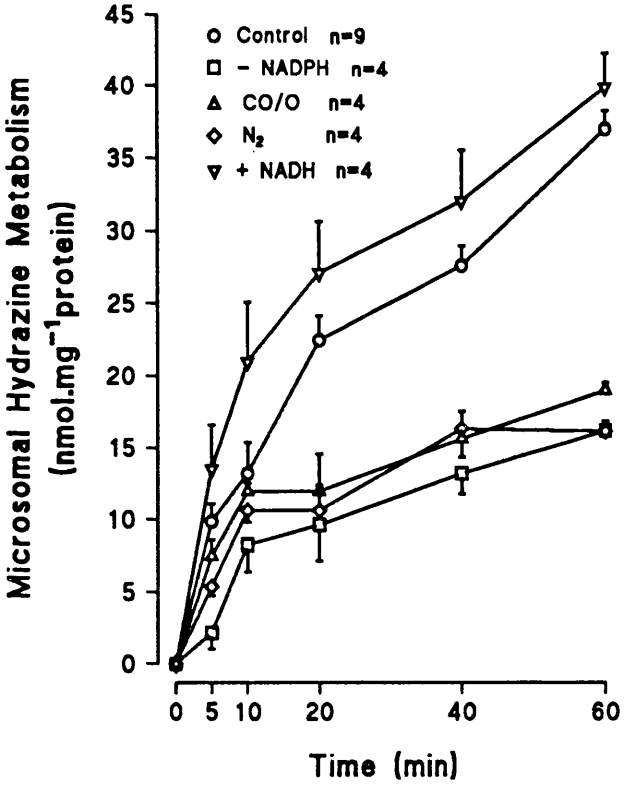


Fig. 3.5 The effect of various inhibitors on the *in vitro* metabolism of 2.0 mM hydrazine by rat liver microsomes



Hepatic microsomes from male Sprague-Dawley rats were incubated with 2.0 mM hydrazine in the presence of cofactors and different gaseous environments. The data represents the mean ± SEM from n incubations. The statistical significance of the different conditions on microsomal hydrazine metabolism was assessed using one way ANOVA and then Dunnett's t-Test. Control = complete test incubate with NADPH.

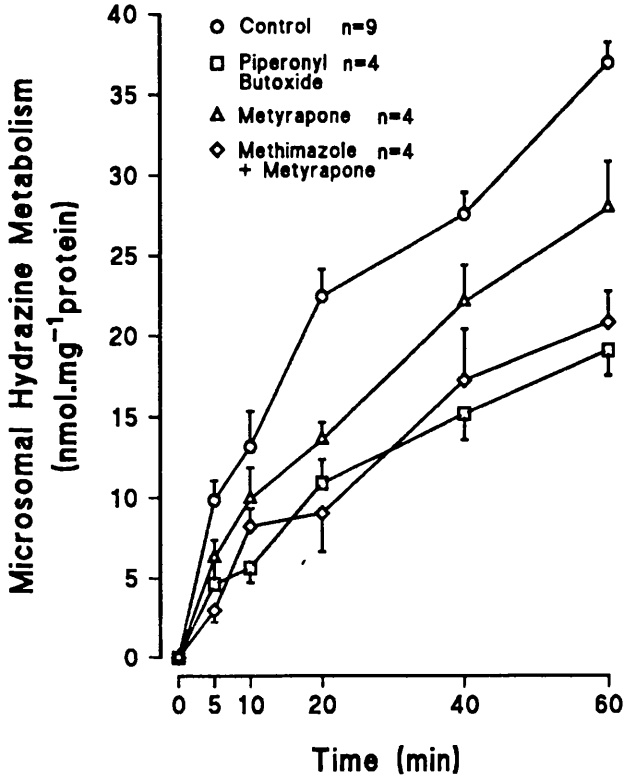
Fig. 3.6 The effect cofactors and gaseous atmosphere on the *in vitro* metabolism of 0.2 mM hydrazine by rat liver microsomes



Hepatic microsomes prepared from male Sprague-Dawley rats were incubated with 0.2 mM hydrazine in the presence of different cofactors and inhibitors.
Control = complete test incubation with NADPH.

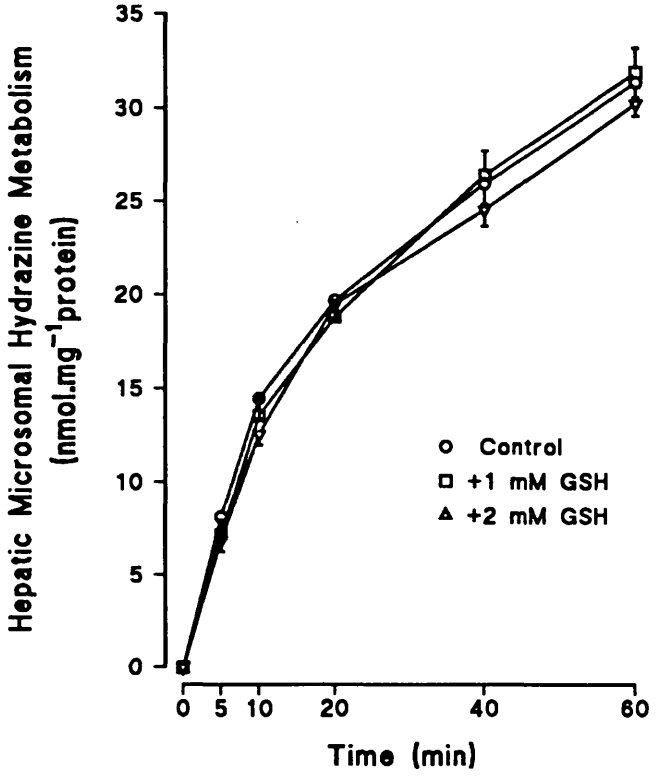
Data points represent the mean ± SEM from n incubations.

Fig. 3.7 The effect of various inhibitors on the *in vitro* metabolism of 0.2 mM hydrazine by rat liver microsomes



Statistical significance of the different conditions on microsomal hydrazine metabolism was assessed using one way ANOVA and then Dunnett's t-Test.

of 0.2mM hydrazine by rat liver microsomes



Hepatic microsomes prepared from male Sprague-Dawley rats were incubated with 0.2 mM hydrazine in the presence of NADPH and various concentrations of GSH. Control = complete test incubate without GSH.

Data points represent the mean ± SEM from n incubations.

Statistical significance of GSH on microsomal hydrazine metabolism. was assessed using one way ANOVA

Fig. 3.9 The *in vitro* depletion of GSH from by rat liver microsomes incubated with 0.2 mM hydrazine

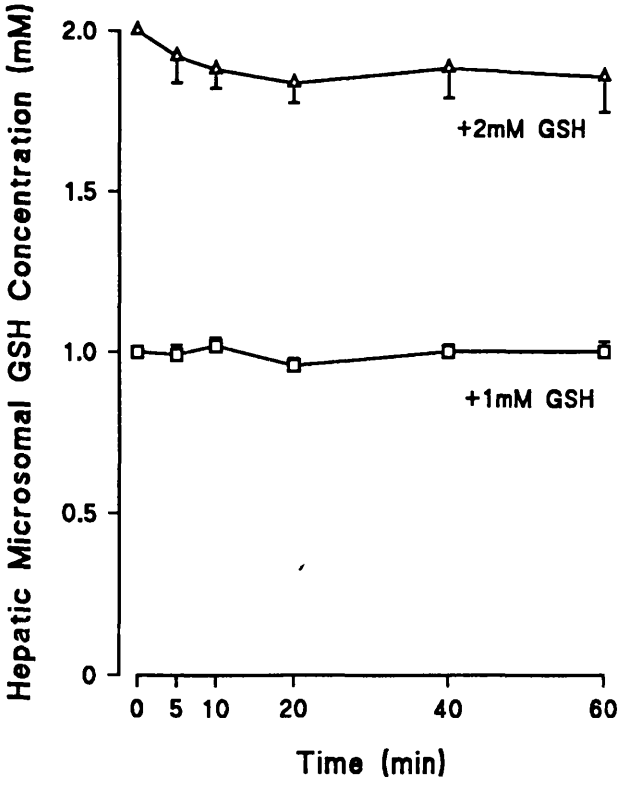
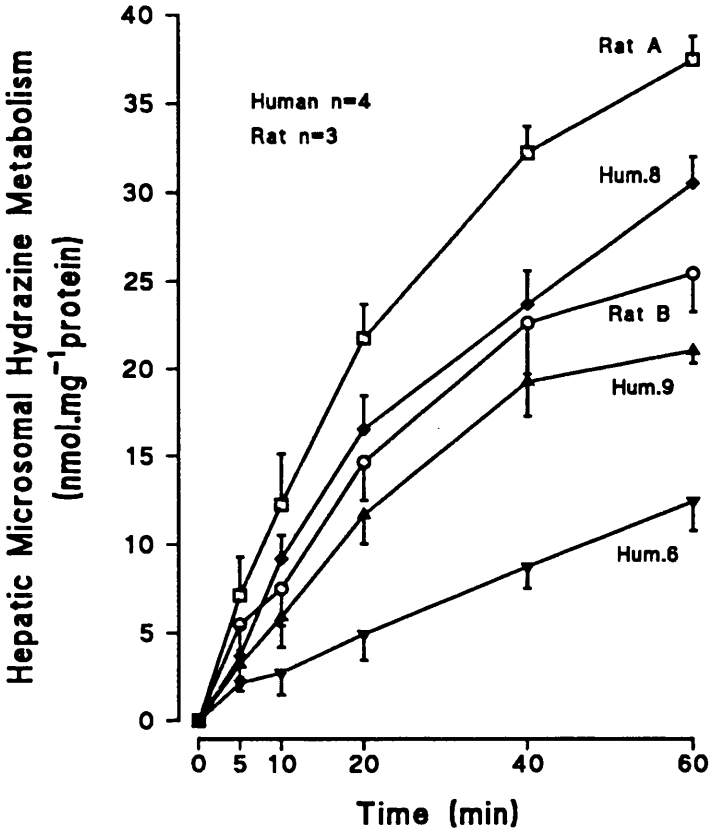


Fig. 3.10 The *in vitro* metabolism of 0.2 mM hydrazine by microsomes prepared from rat liver and from 3 individual human liver samples



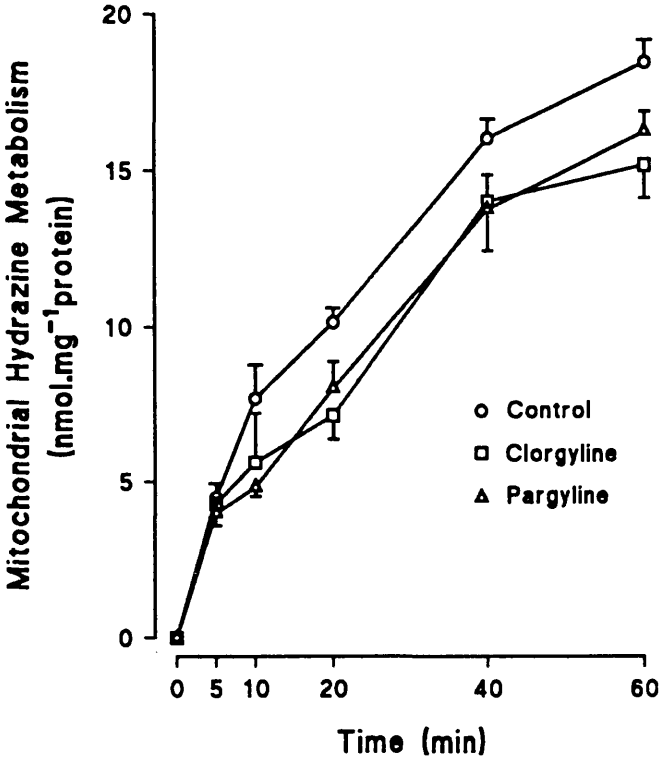
Hepatic microsomes prepared from male Sprague-Dawley rat or from individual human samples were incubated with 0.2 mM hydrazine in the presence of NADPH.

Data points represent the mean ± SEM from n incubations.

Table 3.3 Details of human liver samples

Sample Code	Sex	Age (years)	Cause of death	Drugs administered
Hum.6 (03006)	♂	64	Road Traffic Accident	Dexamethasone
Hum.8 (03008)	♀	38	Sub-arachnoid haemorrhage	None
Hum.9 (03009)	♀	16	Cardiac arrest, coma	Dexamethasone

the *in vitro* metabolism of 0.2 mM hydrazine by rat liver mitochondria

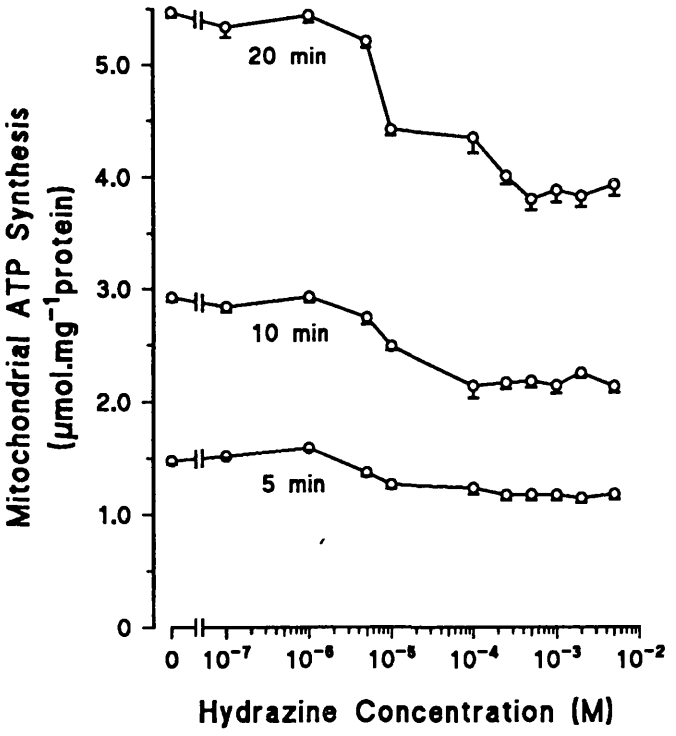


Hepatic mitochondria prepared from male Sprague-Dawley rats were incubated with 0.2 mM hydrazine in the presence of NADPH and two different MAO inhibitors.
Control = complete test incubate.

Data points represent the mean ± SEM from six incubations.

Statistical significance of inhibition on mitochondrial hydrazine metabolism was assessed using one way ANOVA.

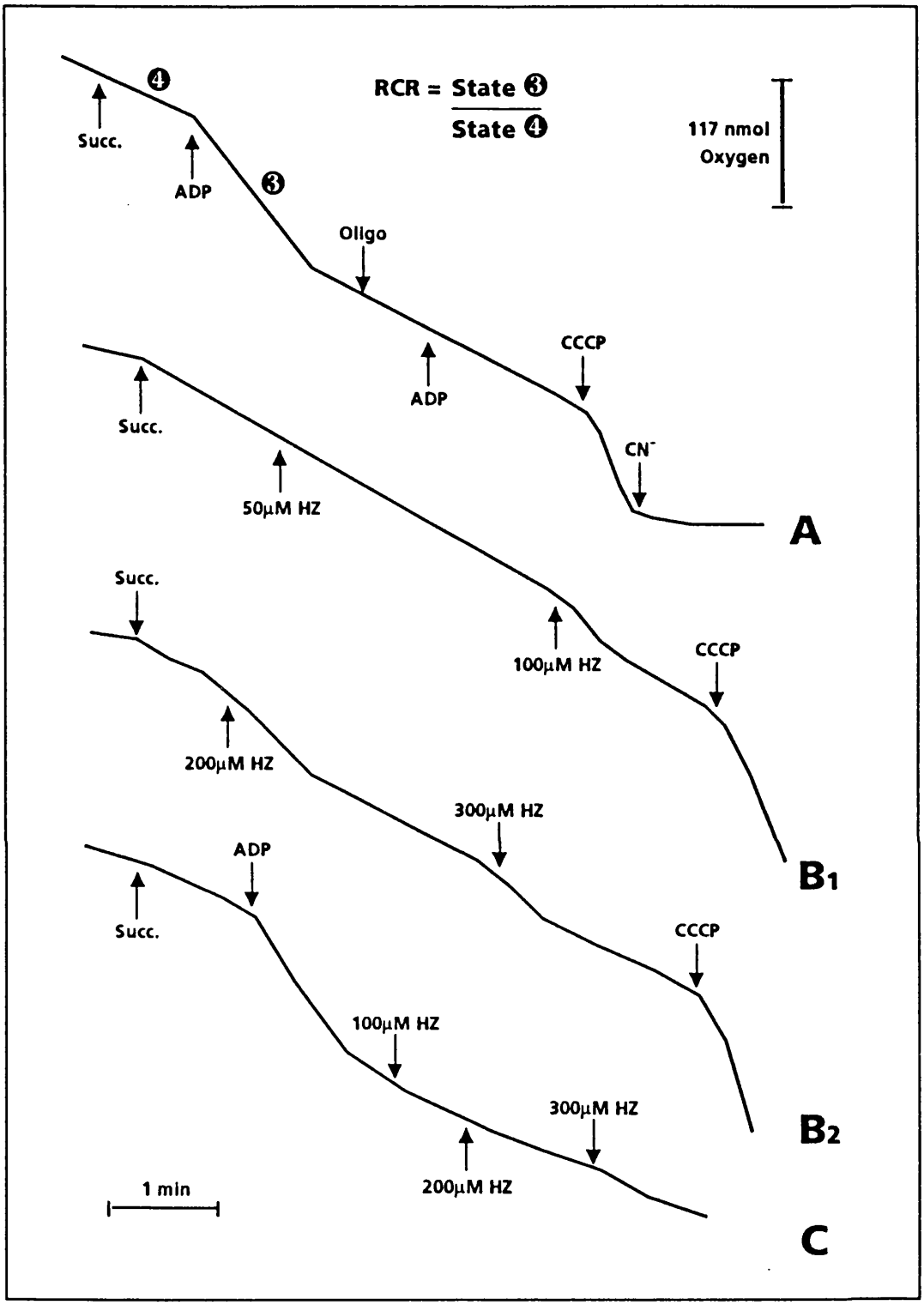
Fig. 3.12 The effect of hydrazine on the *in vitro* synthesis of ATP by rat liver mitochondria



Hepatic mitochondria prepared from male Sprague-Dawley rats were incubated with various concentrations of hydrazine in the presence of succinate, ADP and P_i.
Data points represent the mean ± SEM from n incubations.
n=18 for control.
n=6 for 0.1-10 µM.
n=4 for 100 µM.
n=8 for 0.25-5.0 mM.

Statistical significance of hydrazine on mitochondrial ATP synthesis was assessed after one way ANOVA using Dunnett's t-Test.

Fig. 3.13 The measurement of mitochondrial respiratory control ratio (RCR) and the effect of hydrazine on mitochondrial respiration



Mitochondrial respiration was monitored over time, while various substates, inhibitors and uncouplers were added.
Trace A demonstrates the calculation of RCR.
Trace B_1 and B_2 demonstrate the uncoupling effects of hydrazine.
Trace C demonstrates that the uncoupling effects of hydrazine are abolished by ADP.

Abbreviations: Succ - 10 mM succinate, Oligo - 2 μM oligomycin,
CCCP - 0.5 μM carbonyl cyanide *m*-chloro-phenyl-hydrazone,
CN^- - 250 μM cyanide.

Previous microsomal hydrazine metabolism studies (Nelson and Gordon 1982, Timbrell *et al* 1982) used boiled microsomes as a control without any apparent loss of hydrazine. However, the chemical degradation at hydrazine concentrations of 2 mM was too significant to ignore and the use of boiled microsomes as a control was unsuitable because of increased chemical degradation, due probably to the release of free metal ions from the prosthetic groups of metalloproteins which catalysed hydrazine oxidation.

The use of 1) UHQ (Ultra High Quality) water to make all buffers, 2) Washed microsomes, 3) Larger incubation volumes and continuous sampling, all helped reduce the chemical degradation. At hydrazine concentrations of 0.2 mM and above chemical degradation in buffer with NADPH was unaffected by reducing the temperature from 37°C to 4°C (ice) thus enabling the utilisation of microsomal samples incubated on ice as suitable controls. The temperature dependent increase in the fraction of initial hydrazine that is lost at concentrations below 0.2 mM is believed to be due to the presence of a buffer component, which catalyses hydrazine degradation independently of hydrazine concentration.

Early microsomal experiments used an initial hydrazine concentration of 2.0 mM. However, a concentration of 0.2 mM was utilised subsequently, once it had been discovered that this was the maximal liver concentration achieved after hydrazine administration to rats. (Preece *et al* 1992). Analysis of microsomal hydrazine metabolism by gas chromatography revealed that no acetylated metabolites were formed, which was expected because *N*-acetyltransferases are located in the cytoplasm and not the microsomal fraction.

The dependence of microsomal hydrazine metabolism on NADPH and oxygen indicated in this study is in agreement with the findings of other workers (Nelson and Gordon 1982, Timbrell *et al* 1982, Noda *et al* 1985). However in the absence of either of these two cofactors some metabolism of hydrazine remains. This was also demonstrated in studies by Nelson and Gordon (1982) and Timbrell *et al* (1982), indicating that a minor proportion of hydrazine metabolism is independent of oxygen or NADPH. The synergistic increase in metabolism caused by the additional

donate electrons, via cytochrome b_5, to the MFO pathway and cytochrome P450 in addition to the absolute requirement of electrons from NADPH via cytochrome P450 reductase. The fact that NADH, in the absence of NADPH, did not significantly increase metabolism of 2 mM hydrazine supports this contention. Diminished hydrazine metabolism in the presence of oxygen, NADPH and either carbon monoxide or one of the specific cytochrome P450 inhibitors, piperonyl butoxide or metyrapone, confirms the findings of Noda *et al* (1985) that the NADPH dependent oxidation of hydrazine involves the hemoprotein cytochrome P450.

Carbon monoxide is perhaps the most widely used inhibitor due to its ability to coordinate with the reduced cytochrome P450 iron, while metyrapone inhibits P450 by binding reversibly to the substrate binding site of P450 and inhibits the metabolism of many compounds including aminopyrene, hexobarbital and morphine. The competitive action of this inhibitor may explain why metyrapone inhibition of hydrazine metabolism was decreased at the higher substrate concentration of hydrazine. *In vitro* studies suggest that the inhibition of metabolism of numerous compounds by piperonyl butoxide (Anders 1968) is due to the oxidative formation of a stable metabolite-cytochrome P450 complex (Franklin 1972). Prior studies by Noda *et al* (1987b) demonstrated that hydrazine metabolism in hepatocytes isolated from normal rats was reduced in the presence of piperonyl butoxide or metyrapone. Inhibition of hydrazine metabolism by metyrapone was not found to be significantin hepatocytes isolated from normal rats, but was significant in hepatocytes isolated from phenobarbitone pretreated rats. In addition Noda *et al* (1985) has revealed that the absorption peak at 448 nm generated by the difference spectrum of hydrazine with cytochrome P450 in microsomes or hepatocytes isolated from phenobarbital pretreated rats is significantly inhibited by metyrapone.

Many derivatives of hydrazine have been reported to spectrally interact with cytochrome P450 which may be brought about by the compound's action as a competing ligand (substrate) or the formation of an abortive metabolite complex. Studies by Moloney *et al* (1984) and Hines and Prough (1980) indicate that in the presence of NADPH and oxygen hydrazine derivatives may be metabolised to an

Jonen *et al* (1982), based on the chemistry of hydrazine oxidation (Mansuy *et al* 1982), postulate that oxidative metabolism of most hydrazines proceeds via nucleophilic azo or diazene intermediates which form stable iron porphyrin-diazenyl complexes at the 6-co-ordination position of the hemoprotein (Prough and Moloney 1985). The resulting complexes would rearrange and/or decompose depending on their chemistry (Moloney *et al* 1985) which may result in the generation of radicals (Kalyanaraman and Sinha 1985).

Noda *et al* (1985 and 1987b) has deduced that the characteristic NADPH dependent hydrazine cytochrome P450 difference spectrum in hepatic microsomes and isolated hepatocytes from phenobarbitone pretreated rats is due to formation of the intermediate complex diimide (NH=NH).

During oxidative metabolism of hydrazine in rat liver microsomes and in the presence of NADPH, Noda *et al* (1985) spin trapped the hydrazyl radical ($^{\bullet}NH\text{-}NH_2$) and proposed this to be a precursor of diimide. Generation of the cytochrome P450 diimide complex in isolated hepatocytes and microsomes incubated with hydrazine diminished over time after initially reaching a maximum. The longevity of this complex would delay binding of another hydrazine molecule to cytochrome P450, inhibiting metabolism, which would explain the decreasing rate of microsomal hydrazine metabolism over time that is present in these studies.

Hydrazine has been demonstrated to reduce phenytoin metabolism and cytochrome P450 content (determined by CO-difference spectrum) in rat hepatocytes by 16 and 14% respectively (Noda *et al* 1987a). Noda *et al* (1988) have also reported that the flavoprotein cytochrome P450 reductase (Fp_2) metabolises hydrazine in isolated rat liver microsomes and when purified in the presence of oxygen and NADPH. Hydrazine was oxidised by purified Fp_2 to the hydrazyl radical by elimination of one electron. Thus it is possible that more than one enzyme of the MFO system may be required for the formation of intermediates during hydrazine oxidation that lead to the ultimate metabolite, nitrogen gas (Nelson and Gordon 1982).

Other hemoproteins may also be involved in oxidative metabolism of hydrazine,

The contribution to microsomal hydrazine metabolism of another monooxygenase, flavin containing monooxygenase (FMO) requires careful analysis of metabolism using selective inhibitors and the consideration that the liver enzyme is inactivated above 30°C in the absence of NADPH (Ziegler 1988). This unusual thermal lability could not explain why the rate of microsomal hydrazine metabolism at 37°C decreases over time, since NADPH would not be depleted. Addition of methimazole, a specific FMO inhibitor at the low concentration used, to microsomes decreased hydrazine metabolism and illustrated that FMO may also be involved in microsomal hydrazine metabolism.

In order to compare 0.2 mM hydrazine metabolism by methimazole treated microsomes, the metabolic contribution of cytochrome P450 was removed by addition of metyrapone as a precaution because methimazole and some of its metabolites at higher concentrations are known to interfere with cytochrome P450. Earlier studies (Prough 1973, Prough *et al* 1981) have shown that FMO can catalyse the N-oxidation of several substituted hydrazines, but not hydrazones or hydrazides. Evidence supports the existence of a diazene intermediate that may result from the dehydration of an N-hydroxy metabolite (Prough *et al* 1981).

The lack of any effect of reduced glutathione (GSH) on microsomal hydrazine metabolism and the fact that this tripeptide is not depleted, indicates that GSH does not participate in hydrazine metabolism. Adducts of glutathione have been detected after incubation of isopropylhydrazine or acetylhydrazine with microsomes and NADPH (Nelson *et al* 1976 and 1978), while under the same conditions GSH promoted the production of propane from iproniazid (Spearman *et al* 1984). For both GSH conjugation and GSH hydrogen abstraction reactions, GSH is depleted. Spearman *et al* (1984) after adding cytosolic protein to the microsomal incubation detected a detected a decrease in alkane production and promotion of glutathione adduct generation due to the added presence of particular cytosolic glutathione S-transferases. No evidence exists for the metabolism of hydrazine by such cytosolic glutathione S-transferases and studies *in vivo* (Timbrell *et al* 1982) indicate that

synthesis.

The different activities for hydrazine metabolism demonstrated by microsomes isolated from two groups of male rats was not as great as the variation revealed between microsomes prepared from three individual human liver samples. Human liver microsomal activity for hydrazine metabolism was of the same order of magnitude as that of rat liver, but was comprehensively lower due to the interspecies differences in the activity and distribution of orthologous hepatic enzymes. The variation in human activity was not surprising in view of the different age, sex, lifestyle, for example diet, xenobiotic exposure, and genetic origin which have been demonstrated to have profound effects on xenobiotic metabolism (Jordon and Woolf 1987, Yoo *et al* 1990, Kalow 1991, Yang *et al* 1992). This is in sharp contrast to the high stock control of laboratory animals where the diet, housing and genetic stock of the laboratory animals are carefully regulated. Details of age, sex and cause of death of the human individuals were reported by Sesardic *et al* (1990) and are summarised in Table 3.3.

The publication includes a detailed microsomal monooxygenase activity profile for each individual that highlights the inter-individual variation. The rank order of hepatic human microsomal hydrazine metabolism was actually inversely proportional to total liver cytochrome P450 and certain specific monooxygenase activities. However no significance can be attributed to this, based on the results obtained from merely 3 individuals. In addition certain enzyme activities are known to be affected by extended storage of human liver at freezer temperatures (Powis 1989) and this may have to some extent influenced the evaluation of the results. Few studies have investigated species differences of hydrazine metabolism, and it is not possible to know from the results whether the microsomal products are the same for rat and man.

Isolated rat liver mitochondria were found to metabolise hydrazine but at only approximately half the rate of metabolism by rat liver microsomes. The most predominant phase I oxidase located in mitochondria is monoamine oxidase (MAO)

(Patek and Hellerman 1974, Collins and Youdim 1975). It is proposed that the irreversible inhibition of MAO by several substituted hydrazines is due to oxidation of the hydrazine to form an unstable diazene intermediate which covalently modifies the flavin prosthetic group (Patek and Hellerman 1974). However certain substituted hydrazines, including phenelzine, procarbazine, 1,2-dimethylhydrazine and 2-phenylethylhydrazine, are metabolised by MAO without, or only partially, inactivating MAO (Juchau and Horita 1972, Coomes and Prough 1983, Patek and Hellerman 1974). This preservation of activity is believed to be caused by formation of a more stable diazene intermediate which upon decomposition does not alter the flavin moiety.

Both A and B forms of MAO are located in the outer mitochondrial membrane (Russel *et al* 1979) and would thus be expected to be accessible to the hydrazine molecule, but neither the A or B form of MAO was found to be significantly involved in mitochondrial hydrazine metabolism. The minor role of MAO A or B in metabolism of hydrazine by mitochondria therefore indicates that other mitochondrial enzymes are responsible for the majority of metabolism. Such enzyme candidates must include the mitochondrial MFO system (Shayiq and Avadhani 1989, Ohyama *et al* 1991) plus the heme, copper and flavin containing redox enzymes of the electron transport chain. Indeed Markosyan *et al* (1988) have found that cytochrome oxidase possesses hydrazine oxidase activity.

The characteristic respiratory responses of ADP, oligomycin and carbonyl cyanide *m*-chloro-phenyl-hydrazone (CCCP) were maintained during oxygen electrode experiments on isolated mitochondria and verified that appropiate mitochondrial respiration was sustained.

The slight transient increase in RCR (Respiratory Control Ratio) by addition of hydrazine at concentrations of 100 μM and above indicates that hydrazine may be an uncoupler. Normal stimulation of uncoupled respiration by CCCP after addition of hydrazine demonstrated that hydrazine was not acting directly on the electron transport chain. The elimination of the uncoupling effect of hydrazine by prior addition of ADP is consistent with similar studies by Hadler and Cook (1978) who

concluded that the phosphate dependent transitory uncoupling is due to the interaction of hydrazine with an electrophilic site, presumably involving activated phosphate.

The demonstration that hydrazine inhibits the synthesis of ATP by isolated rat liver mitochondria in this study has not been previously reported. The inhibition of mitochondrial ATP synthesis *in vitro* is concentration dependent between 5 and 100 μM hydrazine, reaching a maximum 20-30% inhibition $\geq$100 μM. This decrease in ATP *in vitro* compares with the depletion of ATP by hydrazine exposure in suspended isolated hepatocytes (Preece *et al* 1990a), primary hepatocyte culture (Ghatineh an Timbrell 1990) and in rat liver (Preece *et al* 1990a, Chapter 4) which are also dose dependent. Inhibition of ATP synthesis may be due to the uncoupling of mitochondrial respiration by hydrazine or alternatively by depletion of substrates for oxidative phosphorylation and citric acid cycle intermediates due to hydrazines disturbance of mitochondrial metabolism. In addition to an inhibition of ATP synthesis in isolated mitochondria, hydrazine may cause an increased utilisation of mitochondrial ATP such as that caused by futile cycling of hydrazine by the mitochondrial enzymes, carbamoyl phosphate synthetase (Fig. 1.2) and glutamine synthetase (Fig. 1.6).

CHAPTER 4. INFLUENCE OF INDUCERS AND INHIBITORS OF CYTOCHROME P450 ON *IN VIVO* HYDRAZINE HEPATOTOXICITY AND RENAL EXCRETION

4.1 INTRODUCTION

The significant excretion of unchanged hydrazine suggests that it is not as rapidly metabolised or as biochemically unstable as might have been expected from its chemical nature. Experiments have been carried out to study the role of metabolism in hydrazine toxicity (hydrazine metabolism is outlined in Fig. 1.3). Most work has been performed on the minor urinary hydrazine metabolite acetylhydrazine because it is also a metabolite of isoniazid (Timbrell 1977a & b, Peretti *et al* 1987) and has been demonstrated to be the toxic species responsible for isoniazid hepatotoxicity in experimental animals (Mitchell *et al* 1975, Timbrell 1979a & b, Timbrell *et al* 1980) and humans (Timbrell 1978, Lauterburg *et al* 1985a). Studies *in vitro* (Nelson *et al* 1976, Augusta *et al* 1981) and *in vivo* (Timbrell *et al* 1980) show that the hepatotoxin acetylhydrazine is metabolically activated to a reactive acetylating intermediate by the microsomal MFO that covalently binds to macromolecules of liver and other tissues. Pretreatment of animals with the microsomal MFO inducer phenobarbitone increased the degree of hepatic necrosis exhibited after acetylhydrazine administration (Bahri *et al* 1981) and conversely inhibitors of microsomal enzymes decreased necrosis (Timbrell *et al* 1980, Woodward and Timbrell 1984, Lauterburg 1985b). These changes in extent of hepatic necrosis paralleled the changes in the proportion of hepatic covalent binding *in vivo* (Timbrell *et al* 1980, Woodward and Timbrell 1984) and correlated with covalent binding in isolated microsomes that had been inhibited or previously induced (Nelson *et al* 1976).

Administration of acetylhydrazine produces fatty liver (McKennis *et al* 1959) in addition to other toxic properties similar to those of hydrazine exposure. However the minor amounts of acetylhydrazine generated after hydrazine administration and the lack of any hydrazine generated hepatic necrosis in monkeys (Warren *et al* 1984) and rats (Amenta and Johnston 1962, Reinhardt and Dinman 1965) indicates that acetylation to acetylhydrazine plays a very minor role in hydrazine toxicity.

In hydrazine treated animals diacetylhydrazine, another minor urinary metabolite, is formed by acetylation of acetylhydrazine. This biotransformation is a major metabolic pathway for animals administered acetylhydrazine and is considered to be a detoxification reaction owing to diacetylhydrazine's much diminished toxicity (McKennis *et al* 1959). This latter fact coupled with the low amounts of diacetylhydrazine generated after hydrazine exposure rules out any influence of this metabolite in hydrazine toxicity. Similarly pyruvate hydrazone, detected as a minor metabolite in urine from hydrazine treated animals, is very much less toxic than the parent compound (Timbrell *et al* 1982). No other studies have been carried out for any of the other minor urinary metabolites which at such low levels are believed to contribute little to hydrazine toxicity and it has been postulated that the toxicity of hydrazine may be due to the parent compound (Timbrell *et al* 1982).

Oxidation of different hydrazine derivatives has been demonstrated *in vitro* to involve the generation of carbon centred free radicals, for example methyl- and dimethyl-hydrazine (Albano *et al* 1989), hydrazine, acetylhydrazine and isoniazid (Sinha 1987), iproniazid (Ortiz de Montellano *et al* 1983), phenelzine (Sinha 1983) and phenylhydrazine, iproniazid and isoniazid (Kalyanaraman and Sinha 1985). Oxygen centred free radicals have also been detected, for example iproniazid (Sinha 1983) and procarbazine (Sinha 1984). These radicals are believed to be responsible for several toxic properties possessed by some of these hydrazines. Sinha (1983) has also detected the nitrogen centred hydralazyl radical after addition of hydralazine to either horseradish peroxidase or prostaglandin synthetase.

Oxidation of hydrazine, generating N_2 gas, is the major route of hydrazine metabolism and is thought to proceed via nitrogen centred free radicals which may be responsible for hydrazine's toxicity. The mixed function oxidase system is an important enzyme system in xenobiotic oxidation and has been reported to catalyse the formation of hydrazine radical ($^{\bullet}NH\text{-}NH_2$) from hydrazine incubated *in vitro* with rat hepatic microsomes (Noda *et al* 1985 and 1988).

Cytochrome P450, the terminal oxidase of the mixed function oxidase (MFO) system, has been found to comprise a superfamily of related enzymes in humans and experimental animals. It has been demonstrated that these multiple forms (isozymes)

al 1982) which enables the MFO system to possess such broad substrate specificity and to catalyse a wide variety of oxidative reactions.

The relative proportions of individual isozymes and their specific activities may govern the metabolic fate and hence the toxification of a particular compound (Guengerich 1992a). Stimulation of the synthesis of cytochrome P450 has been demonstrated after administration of a large variety of chemical compounds, each producing a characteristic pattern of individual isozyme activity (Hammond and Fry 1990). At least 38 different cytochrome P450 genes have been characterised in rats (Guengerich 1992a) and for this thesis the standardised nomenclature, published in 1987 by Nebert *et al*, has been used (Table 4.1)

Table 4.1 Xenobiotic induced cytochrome P450 isozyme families

Family	Sub-family	Gene	Inducing Compound
P450I	A	1-2	2,3,7,8-tetrachlorodibenzoparadioxin, benzo(a)pyrene, 3-methylcholanthrene, β-naphthoflavone
P450II	A	1-3	3-methylcholanthrene
	B	1-2	phenobarbitone, dieldrin, DDT
	C	1-10	[mostly constitutive and sex specific] phenobarbitone
	D	1-2	
	E	1-2	ethanol, acetone, isoniazid, pyridine
P450III	A	1-3	steroids, pregnenolone 16α-carbonitrile, rifampicin, dexamethasone, phenytoin
P450IV	A	1	phthalate esters, clofibrate

Definitions:

(1) Any protein in one gene family is ≥74% dissimilar to a protein in any of the other families (diverged 600-900 million years ago).

(2) Within a gene family of a given species, any protein in one subfamily is approximately 45-60% dissimilar to a protein in any of the other subfamilies (diverged within the past 150 million years).
Percent similarity of the alignment of amino acid sequences is calculated using an algorithm.

Noda *et al* (1985) has reported that induction of cytochrome P450 in rabbits by pretreatment with rifampicin resulted in reduced $AUC_{0\text{-}8\ hr}$ plasma levels of hydrazine, following administration of an acute dose of hydrazine i.v..

Using (i) microsomes from animals pretreated with various inducers, (ii) purified isozymes of cytochrome P450 reconstituted with NADPH cytochrome P450 reductase and lipid and (iii) specific P450 isozyme antibodies, Prough *et al* (1985) demonstrated that the rate of formation of 2 different azoxy metabolites, due to procarbazine *N*-oxidation, was dependent on the specific P450 isozyme present.

4.1.1 Aims

The aims of the studies in this chapter were to assess the significance of *in vivo* hydrazine metabolism by the MFO system on hydrazine hepatotoxicity, as indicated by 3 biochemical parameters known to be affected by hydrazine: ATP and GSH depletion, accompanied by triglyceride accumulation. In particular the importance of inducible cytochrome P450 isoenzymes was investigated.

During these studies the opportunity was taken to assess the effect of acute hydrazine doses on hepatic microsomal enzymes and to determine the effect of cytochrome P450 induction on *in vitro* microsomal hydrazine metabolism.

4.2 METHODS

4.2.1 Hydrazine metabolism/toxicity *in vivo*

Male sprague Dawley rats (250 - 300 g) were housed 3 days prior to hydrazine administration in clean plastic metabowls and allowed food and water *ad libitum*. The animals were either pretreated with an inhibitor/inducer or non-pretreated, receiving vehicle only. Pretreated and non-pretreated animals then received 0 - 60 $mg.kg^{-1}$ of hydrazine in saline (i.p.). Due to the large number of animals involved, the treatments were randomized over 3 consecutive time periods.

Following hydrazine dosing, animals were allowed only water and were sacrificed 6 hr later by cervical dislocation. The 6 hr time period was used because depletion of GSH and ATP due to starvation was reduced, compared to longer time periods, whilst the hepatic biochemical changes induced by hydrazine remained significant. Urine was collected into 0.1 ml concentrated HCl, cooled over ice during the 6 hr period and finally stored at -20°C.

4.2.2 Animal pretreatment

a) Piperonyl Butoxide: administered as a single 500 $mg.kg^{-1}$ dose in corn oil (i.p.), 30 min before hydrazine dosing (Timbrell *et al* 1982).

b) Sodium Phenobarbitone: administered as three daily 75 $mg.kg^{-1}$ doses in saline (i.p.), the last dose 24 hr before hydrazine dosing (Timbrell *et al* 1982).

c) β-Naphthoflavone: administered as a single 80 $mg.kg^{-1}$ dose in sesame seed oil (i.p.), 48 hr before hydrazine dosing (Murray 1991 personal communication).

d) Acetone: administered as a 20% (v/v), 5 $ml.kg^{-1}$ dose (p.o), 20 hr before hydrazine dosing (Lorr *et al* 1984).

e) Isoniazid: administered in drinking water as 0.1% (w/v) (7.29 mM), for 10 days preceding hydrazine dosing (Hammond and Fry 1990).

4.2.3 Tissue sample collection and processing

After cervical dislocation and determination of body weight the liver was rapidly removed, blotted and weighed. Adjacent liver sections were removed from the major lobe and processed within 10 min for the following:

(a) ATP and glutathione (GSH) determination: approximately 0.6 g of liver was freeze clamped and stored in 0.5 M perchloric acid (PCA) at -80°C for 12 hr.

(b) Triglyceride Determination: approximately 0.4 g of liver was homogenised for 30 sec. in 3.6 ml 0.1 M phosphate buffer (pH 7.0) using a Polytron blender and stored at -80°C.

The remaining liver was, where appropiate, utilised for the isolation of microsomes.

4.3 RESULTS

4.3.1 Effect of piperonyl butoxide pretreatment

In both piperonyl butoxide pretreated and untreated animals, hepatic ATP and GSH were depleted whilst triglycerides increased in a dose dependent manner with respect to hydrazine. Pretreatment with piperonyl butoxide resulted in a statistically significant increase in liver triglyceride accumulation as measured by the shift in dose response curve (Fig. 4.1 ANOVA $p<0.05$) and also the difference at the 20 and 30 $mg.kg^{-1}$ hydrazine dose levels. However no significant difference was detected in GSH or ATP depletion (Figs. 4.2 and 4.3) after hydrazine dosing due to piperonyl butoxide treatment.

A statistically significant reduction in renal excretion of unchanged hydrazine over

butoxide pretreatment (Fig. 4.4 ANOVA $p<0.05$). Renal excretion in both pretreated and non-pretreated animals exhibited a linear relationship with hydrazine dose ($r = 0.97$ and 0.99 respectively).

4.3.2 Effect of phenobarbitone pretreatment

Phenobarbitone pretreatment caused significant shifts in all hydrazine dose biochemical response curves, including decreased GSH depletion (Fig. 4.6 ANOVA $p<0.05$), decreased ATP depletion (Fig. 4.7 ANOVA $p<0.001$) and decreased triglyceride accumulation (Fig. 4.5 ANOVA $p<0.05$). Only hepatic GSH depletion was not significantly altered at individual hydrazine doses by pretreatment. All three biochemical indices of hydrazine hepatotoxicity increased in a dose dependent manner for both pretreated and non-pretreated animal groups.

Excretion of hydrazine in urine (Fig. 4.8) increased in a linear fashion with increasing hydrazine dose for both phenobarbitone pretreated ($r = 0.98$) and non-pretreated animals ($r = 0.99$). However there was no significant difference in hydrazine excretion caused by pretreatment of animals with phenobarbitone.

4.3.3 Effect of β-naphthoflavone (BNF) pretreatment

For BNF pretreated and untreated animals, hepatic ATP and GSH were depleted and triglycerides increased in a hydrazine dose dependent manner. No difference due to pretreatment was detected in GSH or ATP depletion (Figs. 4.10 and 4.11) after hydrazine dosing, but a statistically significant decrease in liver triglyceride accumulation was measured by the shift in dose response curve (Fig. 4.9 ANOVA $p<0.05$) and also the individual difference at the 45 and 60 $mg.kg^{-1}$ hydrazine dose levels.

Urinary excretion in both BNF pretreated and non-pretreated animals (Fig. 4.12) showed a linear relationship with respect to dose ($r = 0.99$ and 0.99 respectively). There was no significant shift in urinary excretion following hydrazine administration due to pretreatment of animals with BNF, but pretreatment did result in a significant decrease in renal hydrazine excretion ($p<0.01$) at the 45 $mg.kg^{-1}$ hydrazine dose level.

Acetone pretreatment produced a statistically significant increase in liver triglyceride accumulation at the 20, 30, 45 and 60 $mg.kg^{-1}$ hydrazine dose levels and as measured by the shift in dose response curve (Fig. 4.13 ANOVA $p<0.001$). No significant difference due to pretreatment was observed in the GSH or ATP depletion (Figs. 4.14 and 4.15) after hydrazine dosing. For acetone pretreated and untreated animals, hepatic ATP and GSH were depleted whilst triglycerides increased in a dose dependent fashion with respect to hydrazine.

No significant difference in urinary hydrazine excretion occurred following hydrazine dosing due to acetone pretreatment. Renal excretion in both pretreated and non-pretreated animals displayed a linear relationship with respect to dose ($r = 0.99$ and 0.99 respectively).

4.3.5 Effect of isoniazid pretreatment

Hepatic ATP and GSH were depleted and triglycerides increased by hydrazine in a dose dependent manner for isoniazid pretreated and untreated animals. No difference due to pretreatment was demonstrated for the hydrazine induced GSH or ATP depletion (Figs. 4.18 and 4.19), but a statistically significant increase in liver triglyceride accumulation was measured by the shift in dose response curve (Fig. 4.17 ANOVA $p<0.001$). The difference in triglycerides at the 45 and 60 $mg.kg^{-1}$ hydrazine dose levels was also found to be significant.

The significance of isoniazid pretreatment could not be assessed because of assay interference in the analysis of urine from isoniazid pretreated animals.

4.3.6 Effect of animal pretreatment on rat liver microsomal cytochrome P450

Hepatic microsomal cytochrome P450 content as well as PROD, EROD and NPH activity were demonstrated to be statistically affected by animal pretreatment (Table 4.4). Subsequent statistical examination by Dunnett's t-Test revealed that total cytochrome P450 content was increased by phenobarbitone pretreatment (188% $p<0.05$) and β-naphthoflavone pretreatment (173% $p<0.05$).

It was further demonstrated that PROD activity was elevated by phenobarbitone pretreatment (49 fold $p<0.001$), EROD activity was increased by β-naphthoflavone pretreatment (25 fold $p<0.001$) and NPH activity was raised by acetone pretreatment (2.96 fold $p<0.05$) and isoniazid pretreatment (4.88 fold $p<0.01$).

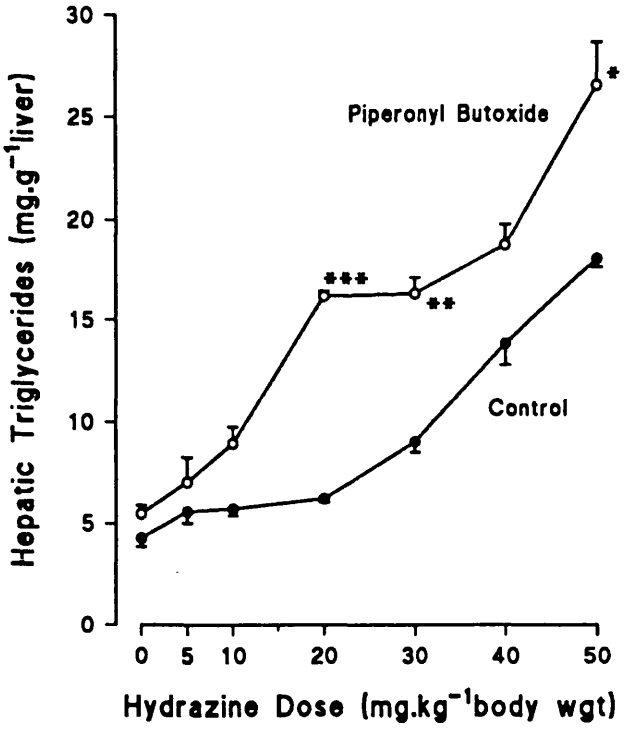
metabolism

Induction of microsomal enzymes by the *in vivo* administration of β-naphthoflavone (BNF), acetone or isoniazid did not affect the rate of *in vitro* microsomal hydrazine metabolism at a hydrazine concentration of 0.2 mM (Fig. 4.20). Phenobarbitone pretreatment did however significantly increase *in vitro* microsomal hydrazine metabolism ($p<0.05$) when incubated with 0.2 mM hydrazine.

4.3.8 Acute dose response of hydrazine *in vivo* on hepatic microsomal enzymes

Statistical examination by one way ANOVA demonstrated that microsomal cytochrome P450 and cytochrome b_5 content plus NADPH cytochrome P450 reductase and PROD activity (Figs. 4.21, 4.22 and 4.24) were all unaffected 6 hr after administration of hydrazine, up to dose levels of 60 $mg.kg^{-1}$. EROD (Fig. 4.24) and NPH (Fig. 4.23) activity were demonstrated by one way ANOVA to be significantly altered 6 hr after hydrazine administration. Further statistical analysis by Dunnett's t-Test revealed that NPH activity was significantly reduced by 40% at the 30, 45 and 60 $mg.kg^{-1}$ hydrazine dose levels ($p<0.05$) while EROD activity was also significantly reduced by 40% at the 20, 45 and 60 $mg.kg^{-1}$ dose levels ($p<0.05$).

Fig. 4.1 The effect of piperonyl butoxide pretreatment on the *in vivo* hydrazine dose dependent accumulation of rat liver triglycerides
(ANOVA $p<0.05$)

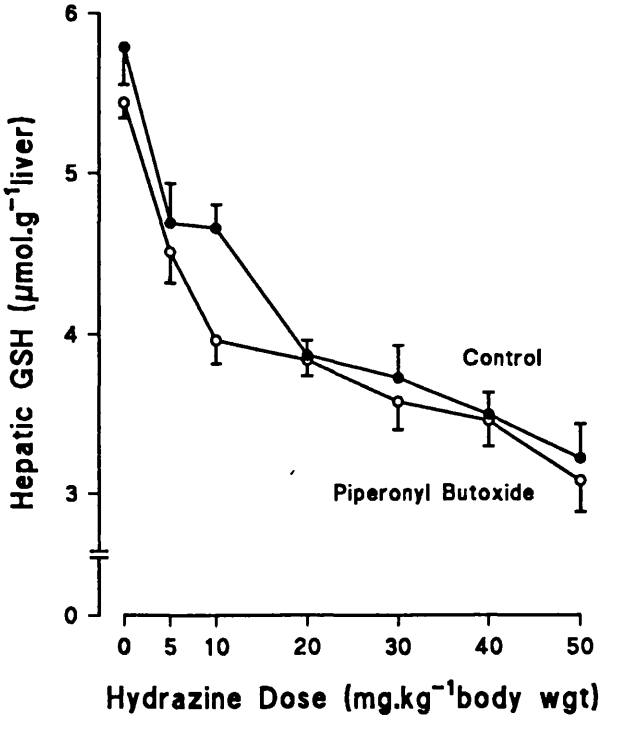


Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either piperonyl butoxide (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of hydrazine was assessed using:

Fig. 4.2 The effect of piperonyl butoxide pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver GSH
(ANOVA p not significant)

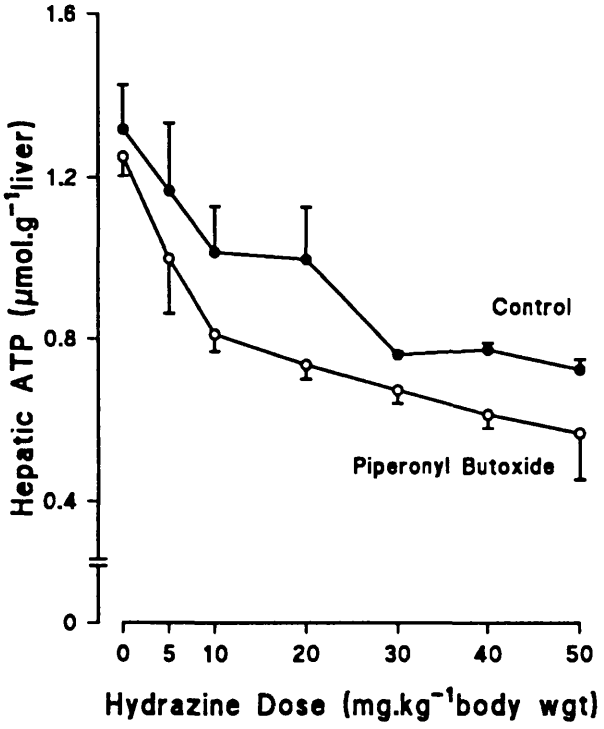


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   * $p<0.05$
   ** $p<0.01$
   *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

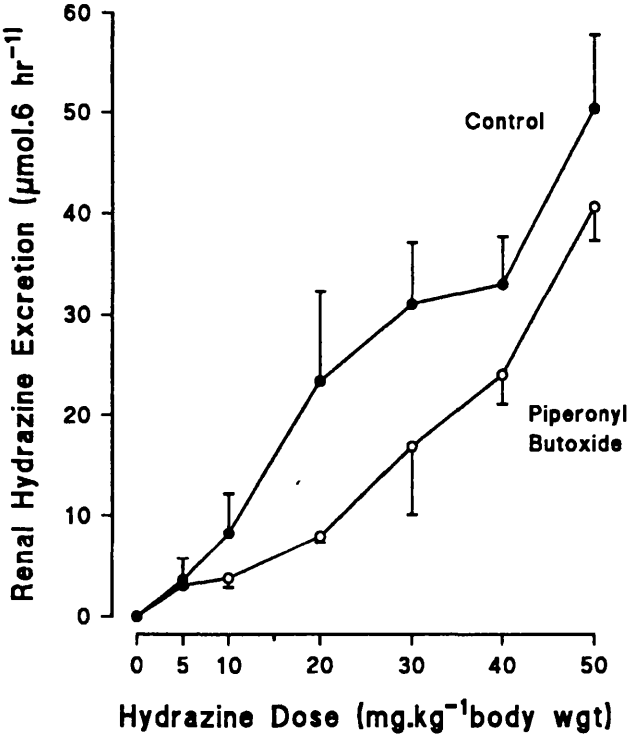
Fig. 4.3 The effect of piperonyl butoxide pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver ATP
(ANOVA p not significant)



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either piperonyl butoxide (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of hydrazine was assessed using:

Fig. 4.4 The effect of piperonyl butoxide pretreatment on the hydrazine dose dependent renal excretion of hydrazine in rat
(ANOVA p<0.05)

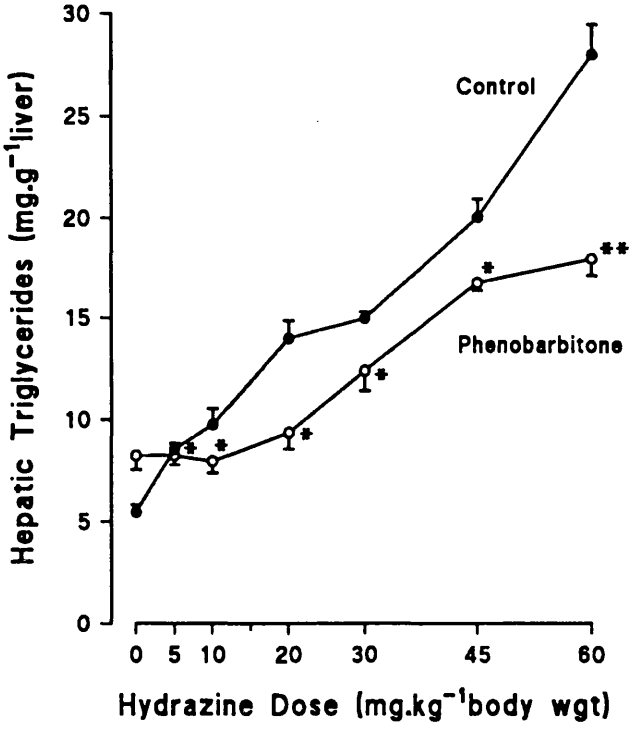


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
* p<0.05
** p<0.01
*** p<0.001

3) Comparison of AUC. (Table 4.2)

Fig. 4.5 The effect of phenobarbitone pretreatment on the *in vivo* hydrazine dose dependent accumulation of rat liver triglycerides (ANOVA $p<0.05$)

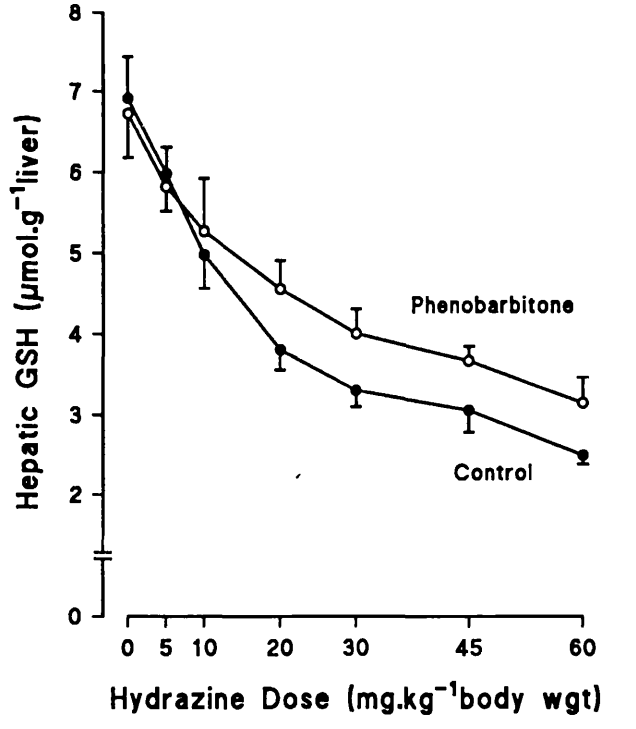


Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either phenobarbitone (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of hydrazine was assessed using:

Fig. 4.6 The effect of phenobarbitone pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver GSH (ANOVA $p<0.05$)

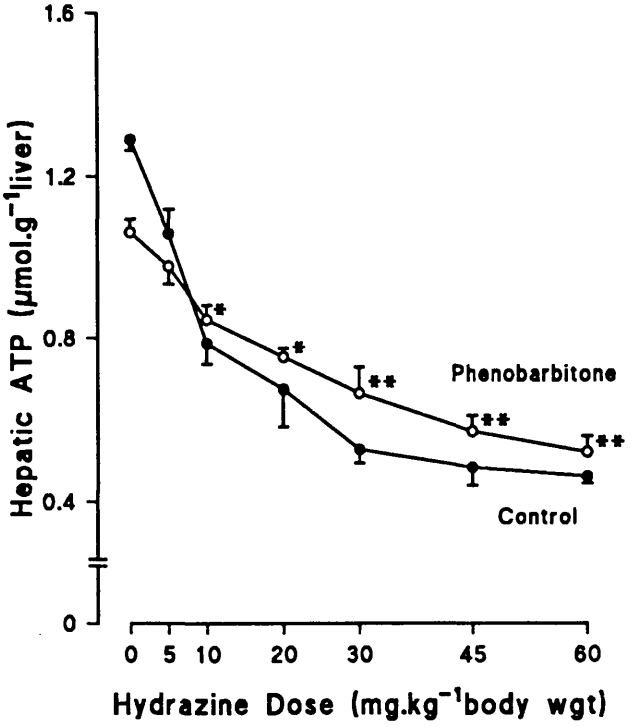


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   - * $p<0.05$
   - ** $p<0.01$
   - *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

Fig. 4.7 The effect of phenobarbitone pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver ATP
(ANOVA $p<0.001$)

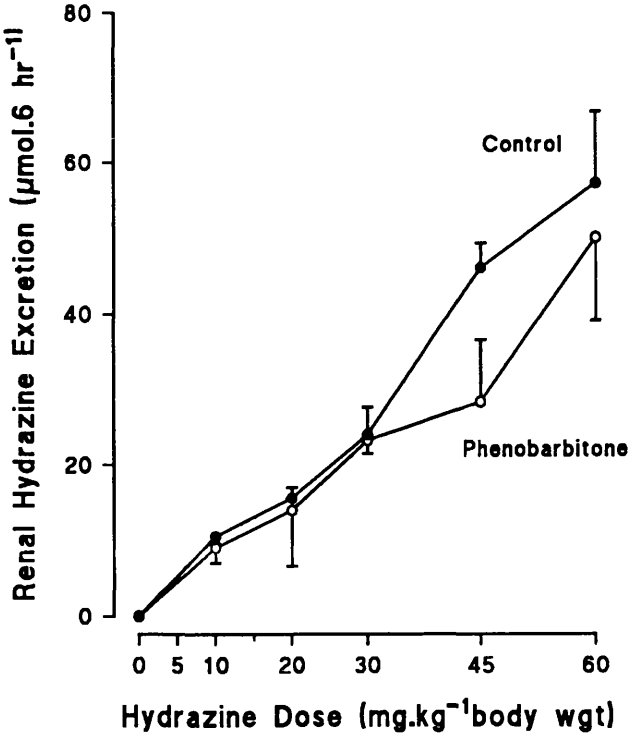


Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either phenobarbitone (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of hydrazine was assessed using:

Fig. 4.8 The effect of phenobarbitone pretreatment on the hydrazine dose dependent renal excretion of hydrazine in rat
(ANOVA p not significant)

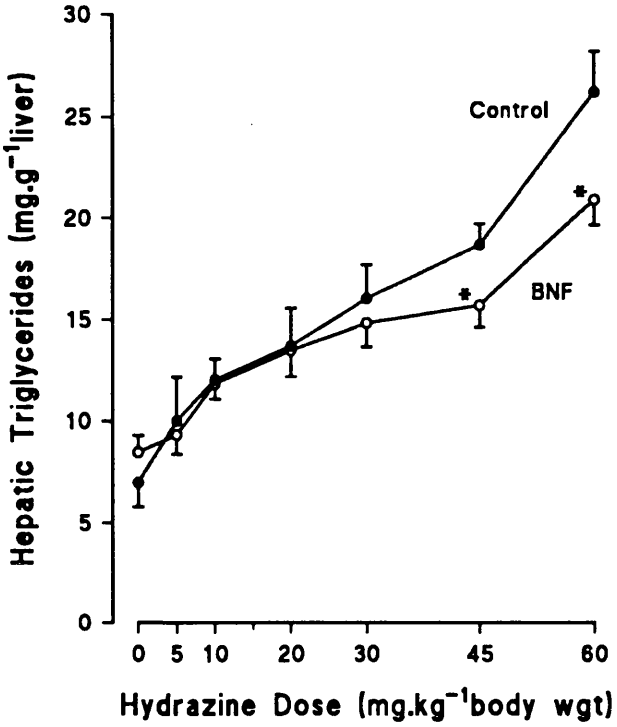


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   * $p<0.05$
   ** $p<0.01$
   *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

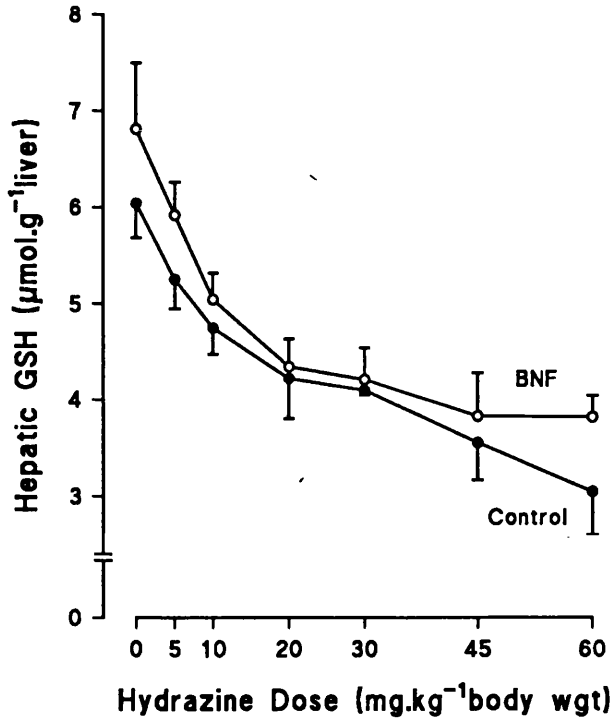
Fig. 4.9 The effect of β-naphthoflavone pretreatment on the *in vivo* hydrazine dose dependent accumulation of rat liver triglycerides
(ANOVA $p<0.01$)



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either β-naphthoflavone (BNF O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of hydrazine was assessed using:

Fig. 4.10 The effect of β-naphthoflavone pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver GSH
(ANOVA p not significant)

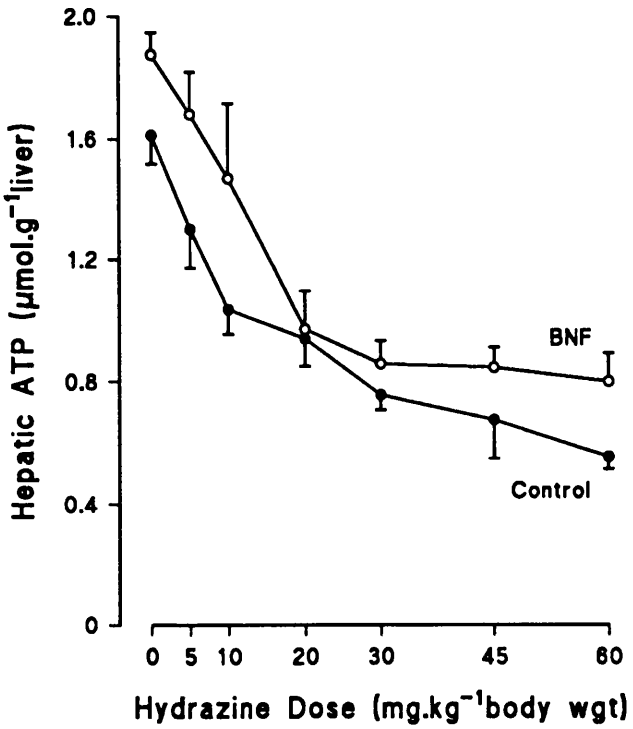


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   * $p<0.05$
   ** $p<0.01$
   *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

Fig. 4.11 The effect of β-naphthoflavone pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver ATP
(ANOVA p not significant)

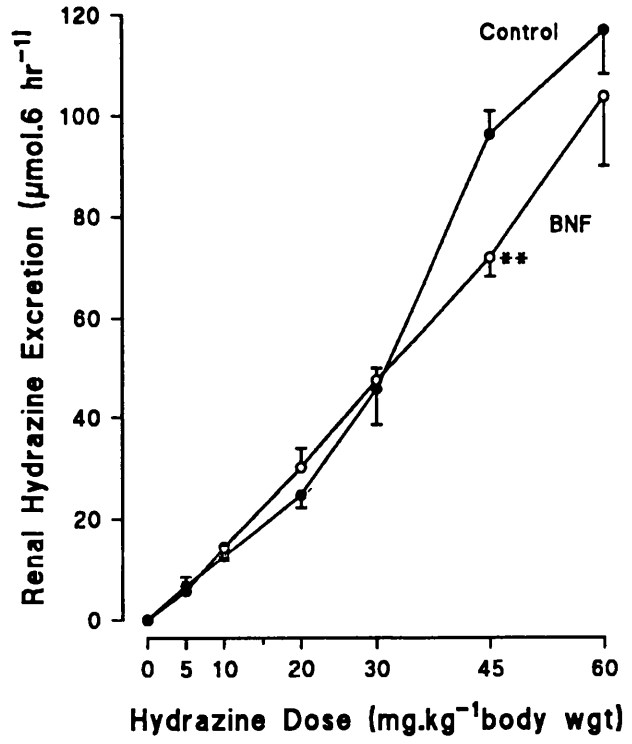


Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either β-naphthoflavone (BNF O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of hydrazine was assessed using:

Fig. 4.12 The effect of β-naphthoflavone pretreatment on the hydrazine dose dependent renal excretion of hydrazine in rat
(ANOVA p not significant)

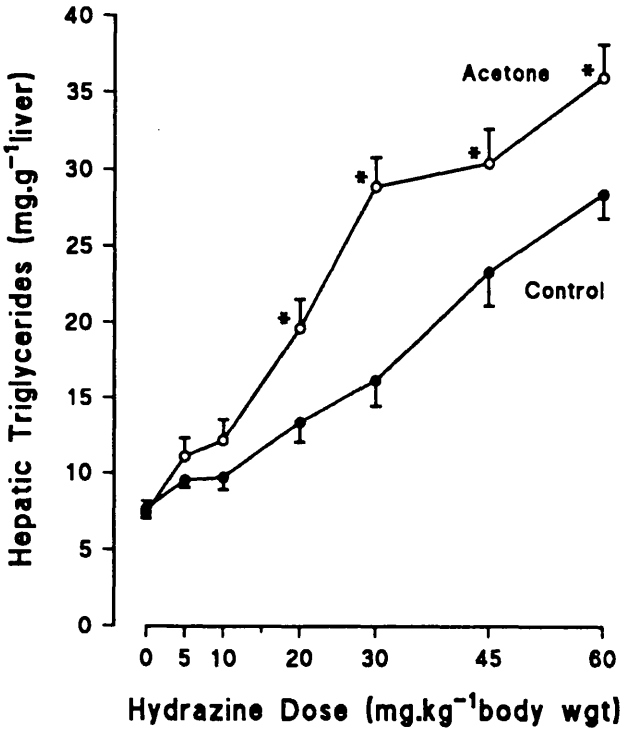


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   * $p<0.05$
   ** $p<0.01$
   *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

Fig. [illegible] the *in vivo* hydrazine dose dependent accumulation of rat liver triglycerides (ANOVA $p<0.001$)

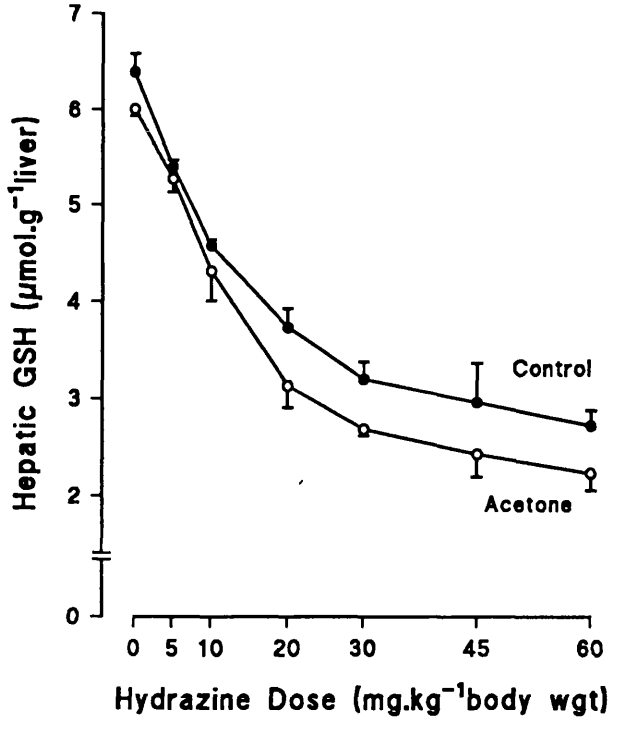


Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either acetone (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of hydrazine was assessed using:

Fig. 4.14 The effect of acetone pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver GSH (ANOVA p not significant)

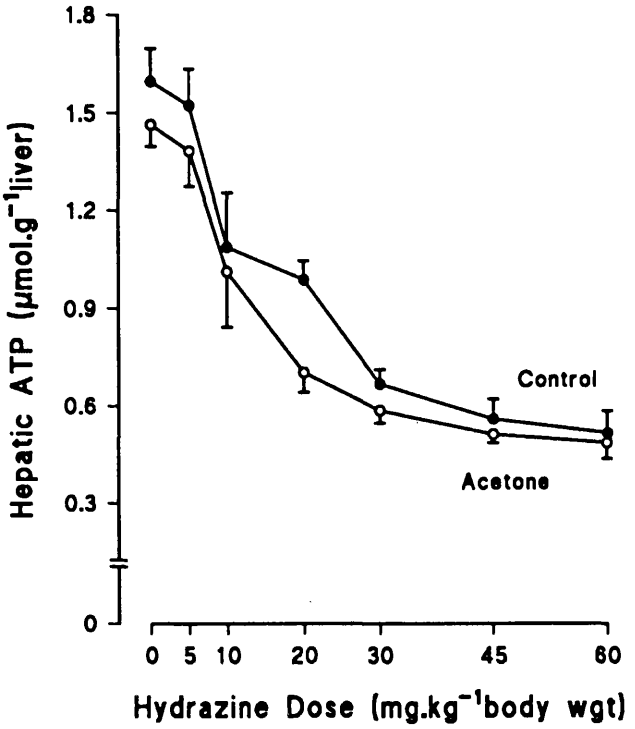


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   - * $p<0.05$
   - ** $p<0.01$
   - *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

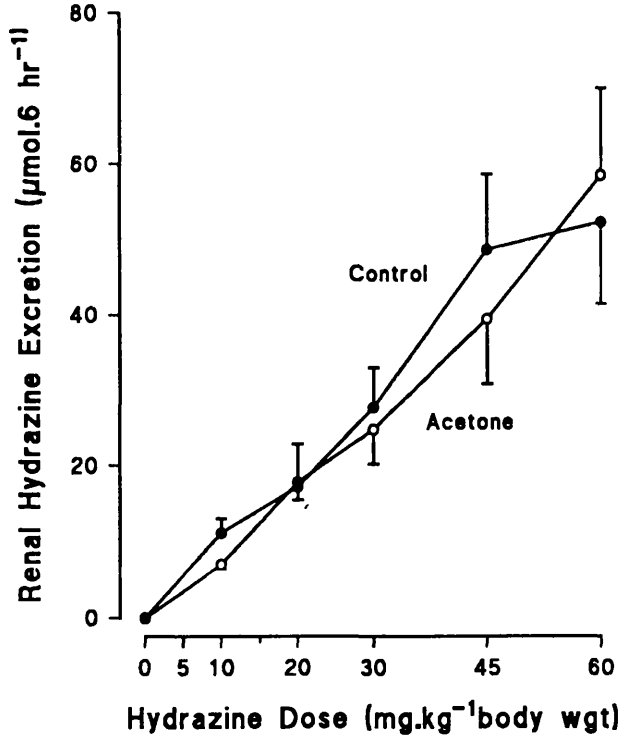
in vivo hydrazine dose dependent depletion of rat liver ATP
(ANOVA p not significant)



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either acetone (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of hydrazine was assessed using:

Fig. 4.16 The effect of acetone pretreatment on the hydrazine dose dependent renal excretion of hydrazine in rat
(ANOVA p not significant)

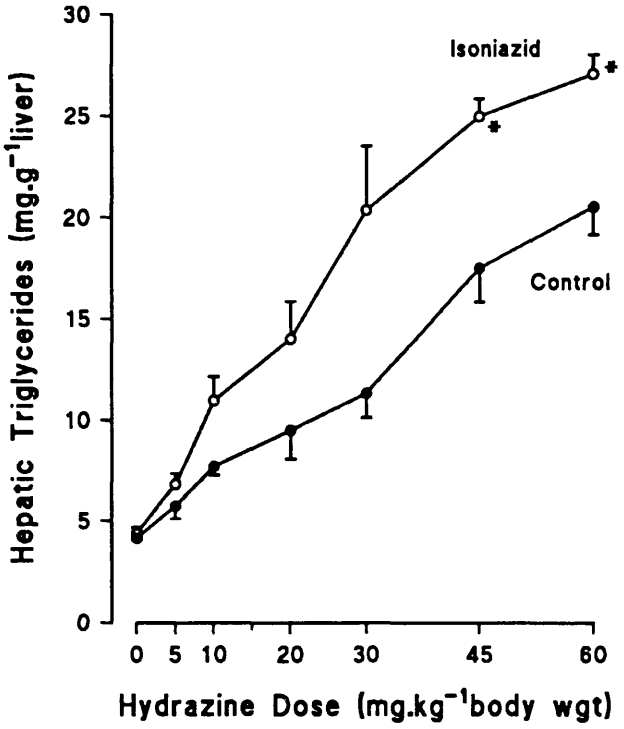


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.

3) Comparison of AUC. (Table 4.2)

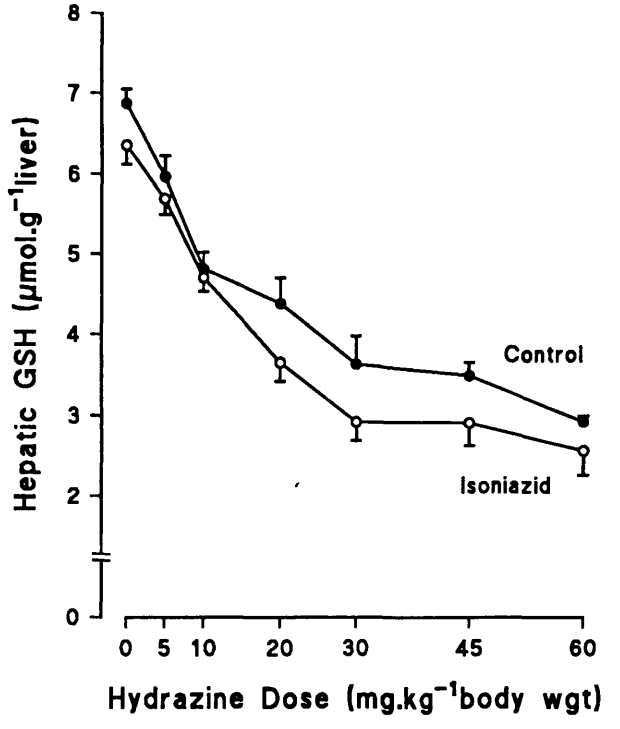
in vivo hydrazine dose dependent accumulation of rat liver triglycerides
(ANOVA $p<0.001$)



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either isoniazid (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity of hydrazine was assessed using:

Fig. 4.18 The effect of isoniazid pretreatment on the *in vivo* hydrazine dose dependent depletion of rat liver GSH
(ANOVA p not significant)

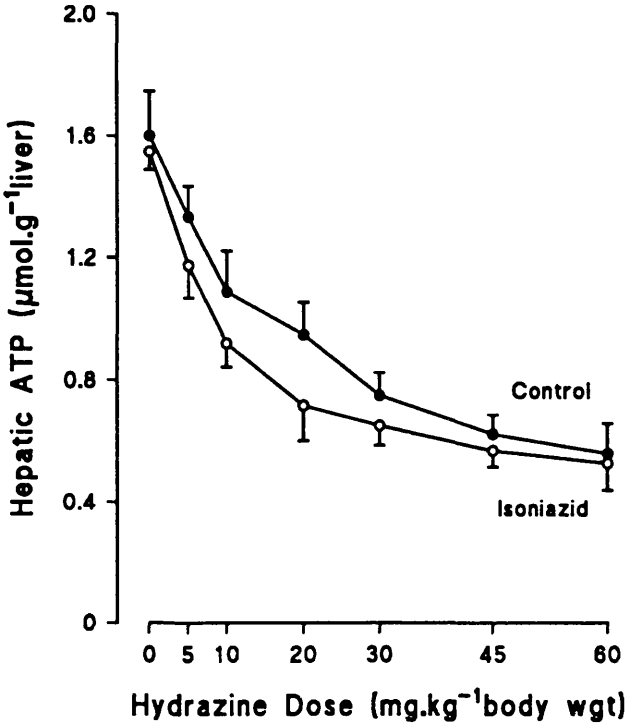


1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.
   * $p<0.05$
   ** $p<0.01$
   *** $p<0.001$

3) Comparison of AUC. (Table 4.2)

in vivo hydrazine dose dependent depletion of rat liver ATP
(ANOVA p not significant)



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. after pretreatment with either isoniazid (O) or vehicle only (control ●). Animals were starved and then sacrificed 6 hr later.

Each data point represents the mean ± SEM from three animals.
After the effect of pretreatment alone had been eliminated from the data, the statistical significance of pretreatment on the hepatotoxicity and excretion of hydrazine was assessed using:

1) 2 way ANOVA for shifts in hydrazine dose response.

2) Unpaired Student's t-Test for individual hydrazine doses.

3) Comparison of AUC. (Table 4.2)

Table 4.2 Comparison of hydrazine dose response Area Under Curve (AUC) between pretreated and non-pretreated animals

Pretreatment	% AUC Difference (compared to non-pretreated)			
	Hepatic ATP	Hepatic Triglycerides	Hepatic GSH	Renal Hydrazine Excretion
Piperonyl Butoxide	-18.4	+39.2	-4.9	-39.0
Phenobarbitone	+9.7	-21.8	+13.7	-21.4
β-Naphthoflavone	+22.9	-10.6	+8.1	-11.6
Acetone	-12.7	+39.5	-12.6	-9.4
Isoniazid	-13.2	+46.5	-12.6	- -

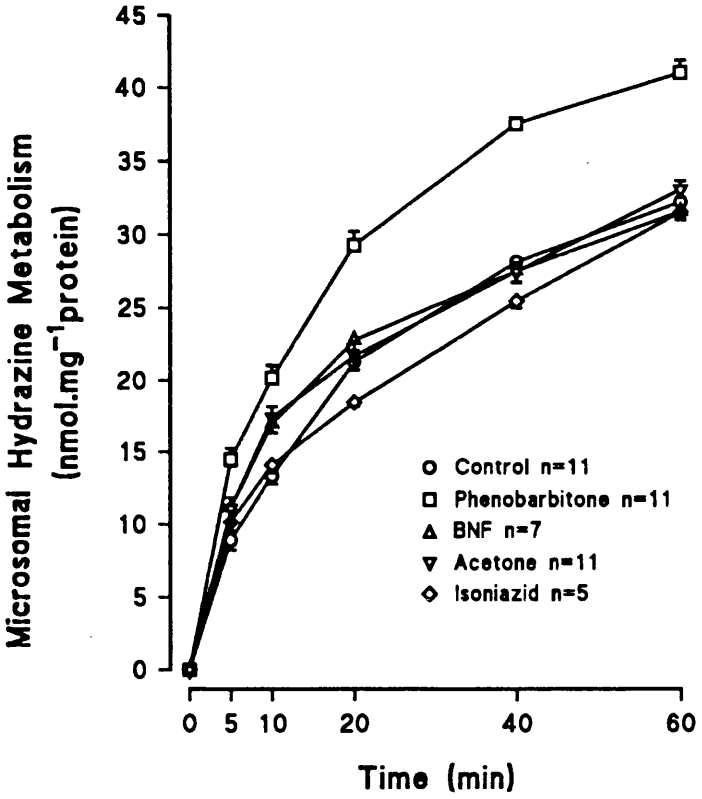
Area Under Curve (AUC) was calculated from Figs. 4.1 - 4.20.
Statistical significance could not be assessed because it was not possible to calculate the variation of AUC.

Table 4.3 A summary for the effect of pretreatment on hydrazine hepatic biochemistry

Pretreatment	Hydrazine Dose ($mg.kg^{-1}$)	Liver Content ($g\ tissue^{-1}$)		
		ATP (μmol)	Triglycerides (mg)	GSH (μmol)
None (Control)	0 ††	1.43 ± .05	5.7 ± 0.5	6.40 ± .17
None	50	0.73 ± .02	18.0 ± 0.4	3.22 ± .22
Piperonyl Butoxide	50	0.57 ± .11	26.7 ± 2.1*	3.08 ± .20
None	60 †	0.50 ± .03	25.7 ± 1.7	2.80 ± .12
Phenobarbitone	60	0.52 ± .04**	17.9 ± 0.9**	3.15 ± .32
β-Naphthoflavone	60	0.80 ± .09	20.9 ± 0.3*	3.82 ± .22
Acetone	60	0.41 ± .04	36.0 ± 2.2*	2.34 ± .17
Isoniazid	60	0.53 ± .09	27.1 ± 1.0*	2.57 ± .30

Data = Mean ±SEM (n=3, ††n=15, †n=12 [from 5†† or 4† individual pretreatment studies]).
Statistical significance of animal pretreatment was assessed using the Student's t-Test on the results of each individual pretreatment study *$p<0.05$, **$p<0.01$

the *in vitro* metabolism of hydrazine by rat liver microsomes



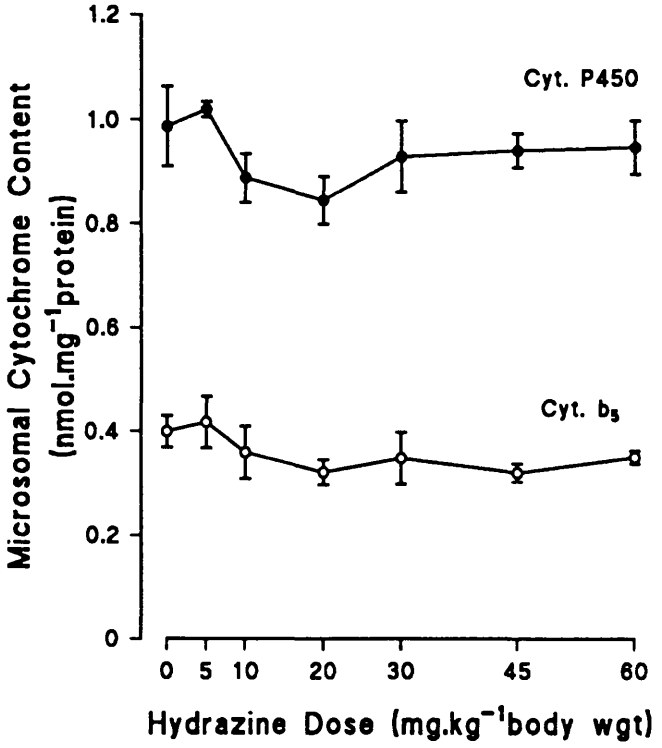
Hepatic microsomes prepared from male Sprague-Dawley rats pretreated with different inducers of cytochrome P450 were incubated with 0.2 mM hydrazine in the presence of NADPH. Data points represent the mean ± SEM from n incubations. Statistical significance of induction on microsomal hydrazine metabolism was assessed after one way ANOVA using Dunnett's t-Test

Table 4.4 The effect of animal pretreatment on rat liver microsomal cytochrome P450

Pretreatment	Total P450 Content (nmol.mg protein^{-1})	Specific P450 activity (pmol.mg protein^{-1}.min^{-1})		
		PROD	EROD	NPH (x 10^3)
Control	0.93 ± 0.06	8 ± 2	35 ± 5	0.68 ± 0.06
Phenobarbitone	1.75 ± 0.13*	392 ± 43***	41 ± 6	0.89 ± 0.07
β-naphthoflavone	1.61 ± 0.11*	11 ± 3	872 ± 61***	0.75 ± 0.06
Acetone	1.09 ± 0.08	31 ± 7	39 ± 4	2.01 ± 0.19*
Isoniazid	1.14 ± 0.09	15 ± 4	79 ± 10	3.32 ± 0.25**

Data = Mean ± SEM (n=3) Statistical significance was assessed using Dunnett's t-Test
*$p<0.05$, **$p<0.01$ ***$p<0.001$

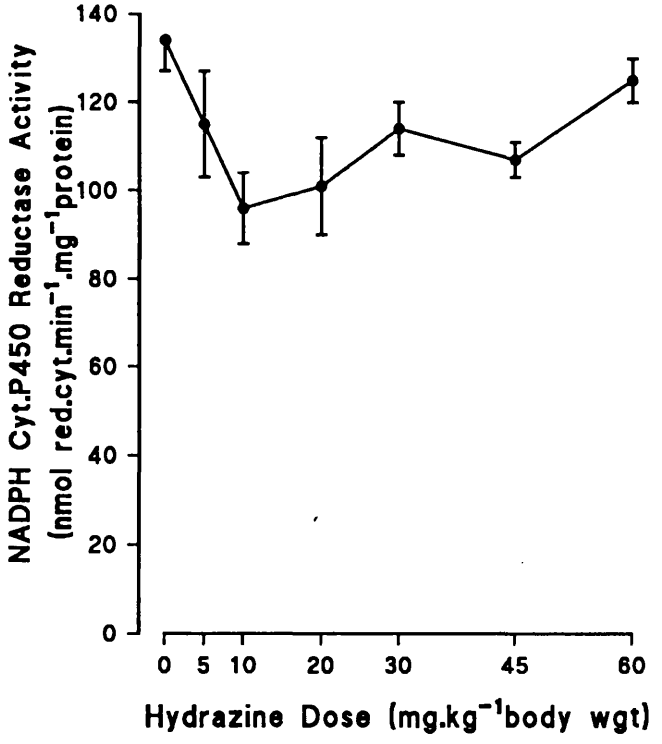
vivo on rat liver microsomal cytochrome P450 and cytochrome b_5



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. and then starved. Animals were sacrificed 6 hr later.

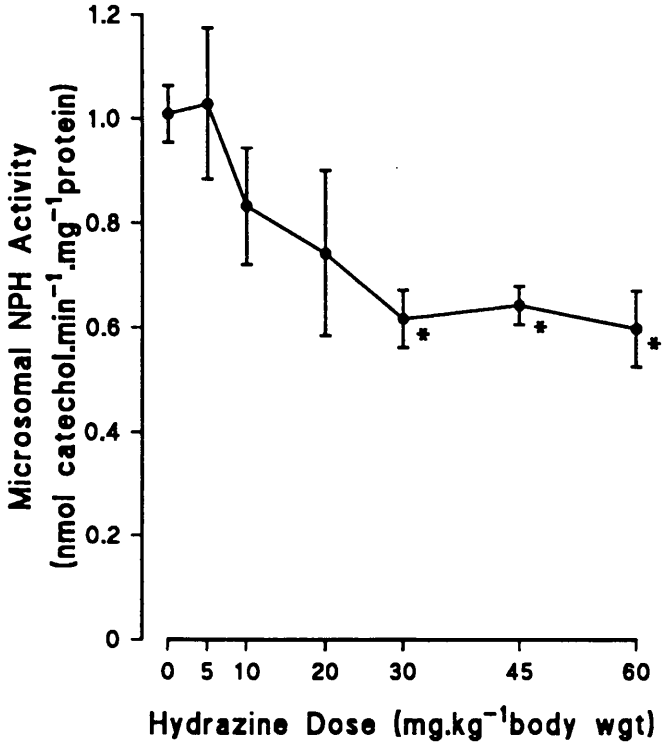
Data points represent the mean ± SEM from three animals.

Fig 4.22 The dose dependent effect of hydrazine *in vivo* on rat liver microsomal NADPH cytochrome P450 reductase activity



The statistical significance of each dose of hydrazine was assessed initially by one way ANOVA.

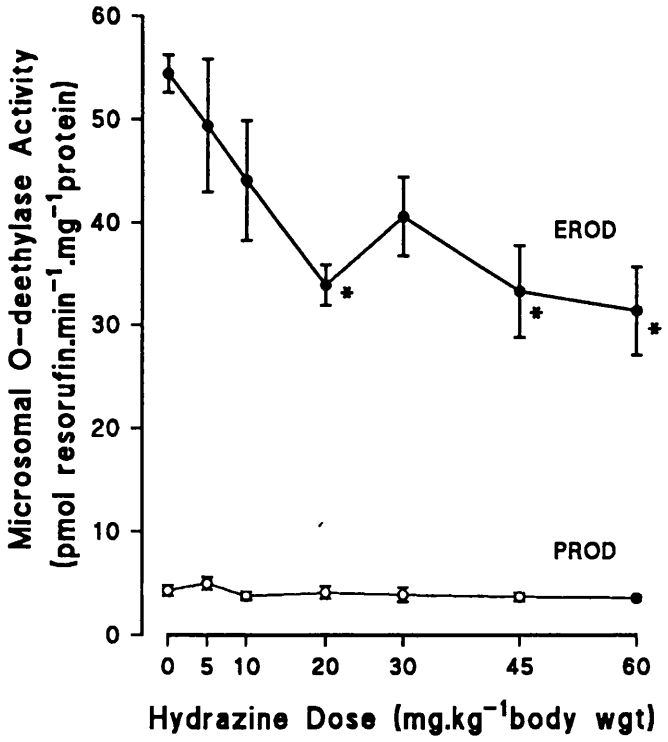
vivo on rat liver microsomal NPH activity



Male Sprague-Dawley rats were administered various doses of hydrazine i.p. and then starved. Animals were sacrificed 6 hr later.

Data points represent the mean ± SEM from three animals.

Fig 4.24 The dose dependent effect of hydrazine *in vivo* on rat liver microsomal EROD and PROD activity



The statistical significance of each dose of hydrazine was assessed initially by one way ANOVA and then Dunnett's t-Test

* $p<0.05$
** $p<0.01$
*** $p<0.001$

The hepatotoxicity of hydrazine in all five individual animal pretreatment studies after 6 hr exposure corresponded to that reported in previous studies, namely triglyceride accumulation (Wells 1908, Amenta and Johnston 1962, Clark *et al* 1969) accompanied by depletion of glutathione (Timbrell *et al* 1982, Alvarez de Laviada *et al* 1987) and ATP (Preece *et al* 1990a). All three indices of hepatotoxicity were dose related after 6 hr and this dose dependency was not eliminated by animal pretreatment. This is comparable with the dose dependent triglyceride accumulation and glutathione depletion detected in rats 24 hr after hydrazine administration (Timbrell *et al* 1982) and the dose dependent depletion of ATP in rats measured 3 hr after dosing (Preece *et al* 1990a).

The hydrazine dose dependent increase in liver : body weight ratio measured after 24 hr (Timbrell *et al* 1982), was absent in these 6 hr studies, due presumably to the time difference. However, analysis 6 hr after hydrazine administration had the advantage over 24 hr that depletion of ATP and GSH due to starvation was reduced (Fig. 5.2 and 5.3), whilst triglyceride accumulation accompanied by ATP and GSH depletion due to hydrazine remained significant. Indeed the apparent dose threshold at 6 hr for hydrazine induced triglyceride accumulation was either the same or slightly lower than that previously observed at 24 hr (10 - 20 $mg.kg^{-1}$, Timbrell *et al* 1982).

The increased accumulation of triglyceride due to hydrazine after pretreatment with the cytochrome P450 inhibitor, piperonyl butoxide, is in full agreement with the results of Timbrell *et al* (1982) who sacrificed rats 24 hr after hydrazine exposure. This data suggests that cytochrome P450 in untreated rats metabolises hydrazine by a detoxification pathway. Studies by Atal *et al* (1985) indicate that the piperine class of chemical compounds have very low toxicity and in this study piperonyl butoxide itself was not demonstrated to be hepatotoxic as measured by ATP and GSH depletion or triglyceride accumulation. Piperonyl butoxide is commonly used in untreated animals to inhibit cytochrome P450 *in vivo* and prolongs hexobarbital sleeping time (Anders 1968). Pretreatment with piperonyl butoxide has also been demonstrated to prevent acetaminophen hepatic necrosis and reduce covalent binding due to the inhibition of cytochrome P450 mediated metabolic activation (Jollow *et al* 1973). Administration of piperonyl butoxide has been demonstrated to

Dalvi 1991) and *in vitro* studies suggest that this is due to the oxidative formation of a stable metabolite-cytochrome P450 complex (Franklin 1972).

Induction of cytochrome P450 by the classic inducer phenobarbitone was demonstrated to reduce hydrazine hepatotoxicity as indicated by the reduction of GSH and ATP depletion as well as the diminished triglyceride accumulation. In previous studies pretreatment of rats with phenobarbitone also resulted in a reduced accumulation of triglyceride 24 hr after hydrazine administration (Timbrell *et al* 1982). Phenobarbitone predominantly induces cytochrome P450IIB1 (PROD activity Table 4.3) and IIB2, but also to a lesser extent isozymes of the cytochrome IIC family. Induction of either one or both of these cytochrome P450 families by phenobarbitone is responsible for catalysing the metabolic detoxification of hydrazine. Although phenobarbitone pretreatment alone resulted in a statistically significant depletion of ATP this was considered to be due to the well documented large increase in protein synthesis and endoplasmic reticulum proliferation (Orrenius *et al* 1965) and not as a result of any hepatotoxicity. GSH and Triglyceride were unchanged by phenobarbitone treatment alone.
Pretreatment with phenobarbitone has also been demonstrated to potentiate iproniazid and isopropylhydrazine necrosis (Nelson *et al* 1978), because of the dramatic increase in cytochrome P450IIB isozymes.

The reduced accumulation of triglyceride in animals pretreated with β-naphthoflavone (BNF) indicates that hydrazine hepatotoxicity is reduced by the induction of the cytochrome P450IA family which catalyses the metabolic detoxification of hydrazine. BNF pretreatment results in the potent induction of the P450IA1 isozyme (EROD activity Table 4.3) and to a lesser extent the P450IA2 isozyme. This is the first report of the effect of cytochrome P450IA induction on hydrazine hepatotoxicity.
Although BNF has been reported to have very low overt toxicity of its own (McKillop and Case 1991), as a consequence of the increase in cytochrome P450IA family pretreatment with this flavonoid metabolically activates several polycyclic aromatic hydrocarbons such as benzo(a)pyrene into ultimate carcinogenic and mutagenic metabolites (Ioannides and Parke 1987, McKillop and Case 1991). In this

biochemical indices.

Acetone and isoniazid are also inducers of cytochrome P450. In contrast to the two isoenzymes discussed earlier, the isozyme induced by both these compounds, P450IIE1 (NPH activity Table 4.3) catalysed the metabolic toxification of hydrazine as indicated by the enhanced accumulation of triglycerides. This is the first report to demonstrate that induction of cytochrome P450IIE1 enhances the hepatotoxicity of hydrazine. Previous studies have demonstrated the potentiation of hepatotoxicity of various other compounds, including *N*-nitrosodimethylamine (Lorr *et al* 1984), carbon tetrachloride (CCl_4) (Hewitt *et al* 1980, Malling *et al* 1975, Lindros *et al* 1991), thiobenzamide (Chieli *et al* 1990) and *N*-methylformamide (Hyland *et al* 1992) by pretreatments that selectively induce P450IIE1.

For both inducers of cytochrome P450IIE1 enhanced depletion of ATP and GSH did not accompany the increased accumulation of triglyceride, a phenomenon similar to that of piperonyl butoxide. It is possible that GSH and ATP depletion are maximally effected by hydrazine, whereas triglyceride accumulation is not. Alternatively triglyceride accumulation may be independent of other hydrazine induced biochemical changes or simply a more sensitive marker of hydrazine hepatotoxicity.

Acetone is commonly used as an inducer of P450IIE1 and this event has been well characterised (Ronis *et al* 1991, Ueng *et al* 1991) but it also induces the P450IIB1 form (Johansson *et al* 1988), although not to the large extent that phenobarbitone pretreatment does. Isoniazid is a more specific and potent inducer of cytochrome P450IIE1.

This type of high spin P450 form can reduce oxygen in the absence of substrate and compared to other forms of cytochrome P450 exhibits higher rates of oxidase activity (Tindberg and Ingelman-Sundberg 1989). This enhanced activity results in the increased production of both superoxide and hydrogen peroxide which generate more reactive hydroxyl radicals propagated by the iron catalysed Fenton reaction (1).

$$^{\bullet}O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

(1) $$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

dimethylsulphoxide is uniquely exhibited by P450IIE1 in reconstituted systems. The increased capacity to generate active oxygen species causes microsomes or liposomes enriched in P450IIE1 to possess enhanced rates of lipid peroxidation and this can be inhibited by addition of antibody to P450IIE1 (Ekstrom and Ingelman-Sundberg 1989). However, the role of the oxidase reaction *in vivo* remains controversial and it has yet to be demonstrated that P450IIE1 is uncoupled *in vivo*.

Di Luzio *et al* in 1973 demonstrated that pretreatment of rats with the lipid soluble antioxidant, diphenyl-*p*-phenylenediamine (DPPD) inhibited hepatic ultrastructural damage and triglyceride accumulation but not hypoglycaemia 6 hr after hydrazine administration. The mechanism by which DPPD reduces hydrazine induced triglyceride accumulation has yet to be established. In the same study increased malonaldehyde (MDA) formation by rat liver homogenates prepared from hydrazine dosed animals after 6 hr indicated enhanced lipid peroxidation, which was reduced in similar animals pretreated with DPPD. Increased hepatic lipid peroxidation was also demonstrated by Di Luzio and Stege (1977) by monitoring chemiluminescence *in vitro* 6 hr after hydrazine administration to rats. Measurement of MDA production in liver homogenates by the previously used thiobarbituric acid (TBA) method in the same study however showed no statistical increase compared to controls. Preece and Timbrell (1989) also could not detect any increase of MDA formation in rat liver 3 hr after a hydrazine dose nor in kidney and spleen. Neither could they detect any increase of *in vivo* ethane and pentane expiration due to lipid peroxidation indicating that hydrazine does not cause lipid peroxidation *in vivo*.

Reduction of renal hydrazine excretion by piperonyl butoxide pretreatment was unexpected since it would be anticipated that the inhibition of metabolism by cytochrome P450 in control animals would increase plasma levels of hydrazine and consequently renal excretion. Pretreatment with piperonyl butoxide may directly affect the the kidneys or possibly increase hydrazine metabolism by an alternative route to that mediated by cytochrome P450.

The lack of any significant effect of all the animal pretreatments that induce cytochrome P450 except for BNF at the 45 $mg.kg^{-1}$ hydrazine dose level, on urinary hydrazine excretion suggests that the rate of renal excretion of unchanged hydrazine

over 6 hr is unaffected by induced cytochrome P450 mediated metabolism. Metabolism of hydrazine by induced isozymes of cytochrome P450 may only generate minor metabolites of hydrazine for urinary excretion, which account for only an insignificant fraction of hydrazine excreted compared to the urinary excretion of unchanged hydrazine. Variation in individual urinary hydrazine excretion and inaccuracies of urine collection however, would be expected to be greater at this relatively early 6 hr time point compared to later collection of urine at 24 hr, especially since hydrazine is initially excreted rapidly. This may have concealed any significance of pretreatment.

Modulation of hydrazine toxicity by induction of different isozymes of cytochrome P450 may be due to either an increase in the rate of catalysis of a particular metabolic pathway and/or the generation of different metabolites which possess various degrees of toxicity.

In spite of the increase in total cytocrome P450 content caused by all 4 microsomal enzyme inducers, only phenobarbitone pretreatment resulted in an increase in hydrazine metabolism activity, thus illustrating that the *in vitro* rate of microsomal hydrazine metabolism depends on the particular isozymes of cytochrome P450 present. The increase in hepatic microsomal hydrazine metabolism *in vitro* due to phenobarbitone pretreatment could increase the formation of a less toxic metabolite, which would explain the reduction of hydrazine hepatotoxicity. The specific induction of P450IIB1 activity (PROD) by phenobarbitone corresponds with the increase in microsomal hydrazine metabolism, but phenobarbitone pretreatment also results in the induction of P450IIB2 and certain isozymes of the P450IIC family which may alternatively or additionally explain the rise in microsomal hydrazine metabolism. The relative increase in microsomal hydrazine metabolism due to phenobarbitone pretreatment above that of control is much lower than the relative increase in total cytochrome P450 content which suggests that microsomal metabolism in control microsomes may be catalysed at different rates by different P450 isozymes and enzymes other than cytochrome P450.

Compared to control microsomes, microsomes prepared from phenobarbitone pretreated animals have also been demonstrated to possess enhanced *N*-methylhydrazine demethylase activity (Witkop *et al* 1969), 1,1-disubstituted

methylhydrazine-P450 complex formation (Hines and Prough 1980) and free radical formation from isoniazid (Tomasi *et al* 1983).

The increase in P450IA1 activity (EROD) by β-naphthoflavone pretreatment or P450IIE1 activity (NPH) by acetone and isoniazid pretreatment does not correspond with an increase in microsomal hydrazine metabolism. However, induction of these P450 isozymes may provide alternative pathways for hydrazine metabolism which change the extent of toxicity.

Many derivatives of hydrazine have been reported to interact with and inhibit cytochrome P450 *in vitro* (Muakkassah and Yang 1981, Jonen *et al* 1982, Moloney *et al* 1984) probably via the generation of an intermediate which forms an abortive metabolite complex (Ortiz de Montellano *et al* 1983, Sinha 1983). Muakkassah *et al* (1981) has demonstrated that the hydrazine moiety of isoniazid is essential for the *in vitro* depletion of microsomal cytochrome P450 content.
However, relatively few studies have analysed the effect of acute doses of hydrazine derivatives to animals on microsomal enzymes and their activities. Koshkaryan *et al* (1988) demonstrated that aniline *p*-hydroxylation and aminopyrene *N*-demethylation activities were reduced 24 hr after a single dose of various hydrazine derivatives.

In this study, cytochrome P450 catalysed EROD and NPH activity were reduced 6 hr after acute doses of 20 - 60 $mg.kg^{-1}$ hydrazine, but this was not accompanied by any other significant alteration of microsomal enzyme content or activity, including PROD activity. This illustrates that hydrazine inhibition is selective for different isozymes of cytochrome P450, namely IA1 and IIE1. Inhibition may be due to the generation of more unstable intermediates of hydrazine (or a metabolite) by these isozymes of cytochrome P450 that form abortive metabolite complexes or cause covalent protein or heme modification.

CHAPTER 5: EFFECT OF ACUTE AND REPEATED EXPOSURE TO LOW DOSES OF HYDRAZINE ON HEPATIC MICROSOMAL ENZYMES AND BIOCHEMICAL PARAMETERS *IN VIVO*

5.1 INTRODUCTION

Past studies of hydrazine have concentrated on either the acute biochemical and toxicological effects of higher single doses or the chronic carcinogenic effect of lower level hydrazine exposure. Very few investigations have been carried out into the effects of low hydrazine doses, which are likely to occur much more frequently during occupational handling. In addition little is known of the development of hydrazine induced alterations beyond 24 hr.

Cytochrome P450 activities have been demonstrated to be induced by a wide variety of chemicals, that are most often also substrates, for example phenobarbitone, β-naphthoflavone, and/or inhibitors, for example piperonyl butoxide (Dalvi and Dalvi 1991), isoniazid (Rice and Talcott 1979), of cytochrome P450. Activities of certain cytochrome P450 isozymes have also been demonstrated to be regulated by diet and hormonal status (Jordan and Woolf 1987). In particular, the isozyme cytochrome P450IIE1 catalyses the metabolism of structurally dissimilar, but collectively small, relatively polar compounds (Guengerich *et al* 1991), and is regulated by the fat-carbohydrate nutritional status of the animal (Yoo *et al* 1991). This isozyme has also been established to be selectively induced by the hydrazide isoniazid (Ryan *et al* 1985).

Consequently, hydrazine is a potential modulator of cytochrome P450 and possibly other microsomal enzymes which may play an important role in the metabolism and toxicity of hydrazine.

5.1.1 Aims

The studies performed set out to determine the effects of an acute low hydrazine dose on hepatic biochemistry and to assess the effect of repeated exposure to low hydrazine doses on the activity of cytochrome P450 isozymes and other microsomal enzymes in addition to measuring liver biochemical changes.

exposure, was carried out in order to distinguish whether any hydrazine induced effects were actually caused indirectly by diet restriction.

5.2 METHODS

5.2.1 Time course of acute hydrazine administration *in vivo*

Male Sprague-Dawley rats (240 - 270 g) were administered 3 $mg.kg^{-1}$ i.p. hydrazine while controls were dosed at the same time with saline. After dosing animals were allowed only water and sacrificed at 1½, 3, 6, 9, 12 and 24 hr time intervals.

5.2.2 Repeated hydrazine exposure *in vivo*

Male Sprague Dawley rats (250 - 300 g) received hydrazine in their drinking water (either 0.78 mM: 2.5 $mg.kg^{-1}.day^{-1}$ or 65 µM: 0.25 $mg.kg^{-1}.day^{-1}$) while control animals were untreated. Rats exposed to 0.78 mM hydrazine and their controls were housed individually, while those exposed to 65 µM hydrazine and their controls were housed in groups of four. Animals were allowed food and water *ad libitum* and were sacrificed at 1 (not 65 µM), 5 and 10 day time intervals. Food (not 65 µM), water and body weight were monitored daily throughout the study.

5.2.3 Animal diet restriction

Male Sprague Dawley rats (250 - 300 g) received a reduced food intake of 20 $g.day^{-1}$ for 5 days in order to mimic the decreased food intake of rats receiving 0.78 mM hydrazine. Control animals were allowed food *ad libitum* and all animals were allowed water *ad libitum*. Food intake and body weight were monitored daily throughout the study.

5.2.4 Tissue sample collection and processing

After cervical dislocation and determination of body weight the liver was rapidly removed, blotted and weighed. Adjacent liver sections were removed from the major lobe and processed within 10 min for the following:

(a) ATP and GSH determination: approximately 0.6 g of liver was freeze clamped immediately after animal sacrifice and stored in 0.5 M perchloric acid (PCA) at -80°C

(b) Triglyceride Determination: approximately 0.4 g of liver was homogenised for 30 sec. in 3.6 ml 0.1 M phosphate buffer (pH 7.0) using a Polytron blender and stored at -80°C.

The remaining liver was utilised for the isolation of microsomes.

5.3 RESULTS

5.3.1 Time dependent effects of a single 3 $mg.kg^{-1}$ hydrazine dose i.p. on hepatic biochemistry *in vivo*

Hepatic triglycerides (Fig. 5.1) were reduced in both control and dosed animals for 6 hr following dosing and withdrawal of food and although the reduction was not as great in dosed animals it was not found to be significant. After 6 hr triglyceride levels in control animals remained relatively constant while levels in hydrazine dosed animals started to rise and were found to be significantly accumulated after 9 (97% $p<0.05$), 12 (95% $p<0.01$) and 24 hr (169% $p<0.001$).

For both control and dosed animals hepatic GSH (Fig. 5.2) was depleted for 12 hr following dosing and withdrawal of food. Depletion over the first 9 hr was greater in hydrazine dosed animals but this was not found to be statistically significant. GSH levels after 12 hr were almost identical for both groups as were the increased levels measured after 24 hr.

An initial increase in hepatic ATP (Fig. 5.3) for control animals, up to 3 hr following dosing and withdrawal of food, was not seen for dosed animals but this was not demonstrated to be statistically significant. However depletion of ATP started to occur after 3 and 6 hr in control and dosed animals respectively and levels of ATP were significantly lower in dosed animals, compared to controls, after 9 (36% $p<0.05$) and 12 hr (34% $p<0.05$). ATP after 24 hr was depleted further but to similar levels in both animal groups.

5.3.2 Hepatic biochemical and microsomal effects of 0.78 mM hydrazine in drinking water

Hydrazine resulted in a decrease in hepatic GSH (Fig. 5.5) after 1 (15% $p<0.01$), 5 (14% $p<0.05$) and 10 (41% $p<0.001$) days exposure, but this was not accompanied by

increased after 1 day hydrazine exposure (33% $p<0.05$), unchanged after 5 days and significantly decreased after 10 days exposure (38% $p<0.05$). Total hepatic protein content was unaffected by hydrazine dosing throughout the whole study (Fig. 5.7). Total microsomal cytochrome P450 (Fig. 5.8) was significantly elevated 1 day after hydrazine exposure (12% $p<0.05$), significantly reduced after 5 days (17% $p<0.05$) and significantly increased after 10 days exposure (30% $p<0.01$), while microsomal cytochrome b_5 content (Fig. 5.9) was unaltered after 1 and 5 days, but significantly increased after 10 days hydrazine exposure (24% $p<0.05$). No difference in the activity of NADPH cytochrome P450 reductase (Fig. 5.10) was detected after 10 days exposure to hydrazine, but was significantly reduced after 1 (15% $p<0.05$) and 5 (26% $p<0.05$) days exposure.

Exposure to hydrazine caused a significant, time dependent, induction of microsomal *p*-nitrophenol hydroxylase activity (Fig. 5.11) that increased from 1.55 fold after 1 day exposure to 1.91 fold after 5 days exposure (both $p<0.05$) and 2.82 fold after 10 days exposure ($p<0.001$). Microsomal ethoxy- and pentoxy- resorufin *O*-deethylase (EROD and PROD) activity (Figs. 5.12 and 5.13) were both significantly lower after 5 days hydrazine exposure (41% $p<0.001$ and 40% $p<0.01$ respectively). This was preceded by a significant reduction (30% $p<0.01$) in PROD activity after exposure for 1 day, while after the same period EROD activity was unchanged. However after 10 days EROD and PROD activity were unchanged by hydrazine exposure.

5.3.3 Effects of 0.78 mM hydrazine in drinking water on daily food and water intake and body weight of rats

Figs. 5.14 and 5.16 illustrate the feeding and drinking habits of the 5 and 10 day study groups of hydrazine exposed and control rats while Fig. 5.15 shows the rate of body weight increase. Compared to controls, food and water intake decreased immediately following exposure to hydrazine and much more dramatically for the 10 day study group. Both food and water intake fell to approximately 65% for the first 3-4 days of hydrazine exposure in the 10 day study group, while intake of food and water by the 5 day study group exposed to hydrazine fell to approximately 75% for the first 3 days.

control levels as did the food intake of the 10 day exposed study group after 4 days. Water intake of the animal group exposed to hydrazine for 10 days increased to approximately 80% of the control level after 3 days and after 8 days was similar to controls.

The normal increase in body weight recorded in all animals before any hydrazine exposure was arrested immediately after exposure to hydrazine. Body weight actually decreased for 1-2 days in hydrazine exposed animals but then returned to a normal linear rate of body weight gain ($r = 0.99$, 7.58 $g.day^{-1}$) as assessed by comparison of growth rates using the Student's t-Test.

The daily hydrazine dose received varied between individual animals and different days due to the variation in animal body weight and water intake. The mean daily dose was calculated to be 2.5 $mg.kg^{-1}$ (minimum = 2.10 $mg.kg^{-1}$, maximum = 2.76 $mg.kg^{-1}$).

5.3.4 Effect of 5 day restricted diet on hepatic biochemistry and animal body weight

Normal increase in body weight was recorded (Fig. 5.18) in all animals when fed *ad libitum* and during this period there was no difference in food intake (Fig. 5.18) between the study groups. Following restriction of daily food intake to 20 g, body weight immediately decreased compared to animals fed *ad libitum* whose mean daily food intake during this study was 31 $g.day^{-1}$. After 3 days the decline ceased and body weight gradually increased, but at a much lower rate than the normal linear ($r = 0.99$) increase in body weight exhibited by the control group throughout the study (5.64 $g.day^{-1}$). Liver triglycerides (Fig 5.17) were not significantly altered after 5 days restricted diet compared to control rats fed *ad libitum*, but microsomal NPH activity (Fig. 5.19) was significantly reduced by 32% ($p<0.01$).

5.3.5 Hepatic biochemical and microsomal effects of 65 µM hydrazine in drinking water

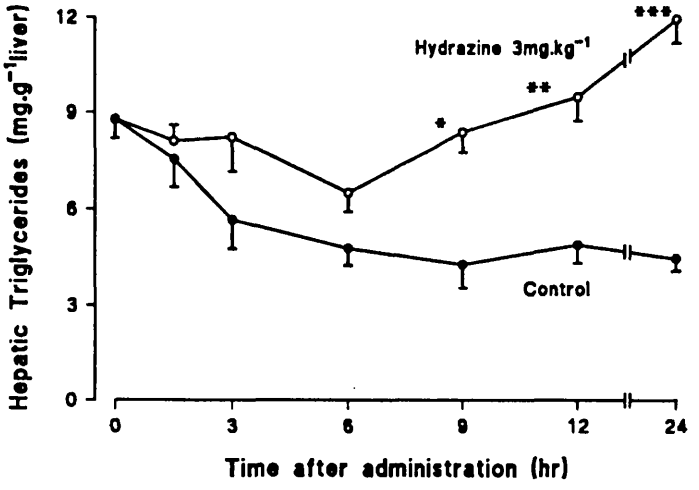
Liver triglycerides (Fig. 5.20), GSH (Fig. 5.22) and protein content (Fig. 29) were all unaltered by hydrazine exposure. Hepatic ATP (Fig. 5.21) was significantly increased after 5 days hydrazine exposure (15% $p<0.01$ respectively), but unchanged after 10

Total microsomal cytochrome P450 (Fig. 5.23) and cytochrome b_5 (Fig. 5.25), as well as all activities of hepatic microsomal enzymes (Figs. 5.26-5.28) were not significantly affected by hydrazine exposure except for a 17% increase in microsomal NPH activity (Fig. 5.28) after 5 days ($p<0.05$).

5.3.6 Effects of 65 µM hydrazine in drinking water on daily water intake and body weight of rats

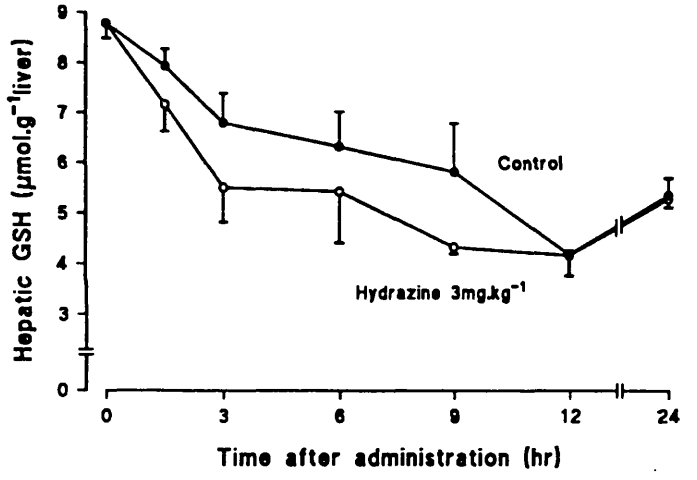
Water intake was not altered by the administration of hydrazine (Fig. 5.31). The linear rate ($r = 0.99$) of body weight increase (Fig. 5.30) in rats exposed to hydrazine (7.82 $g.day^{-1}$) parallelled the linear rate ($r = 0.99$) in control rats (7.53 $g.day^{-1}$). The daily hydrazine dose received was calculated to be 0.25 $mg.kg^{-1}$ (minimum = 0.217 $mg.kg^{-1}$, maximum = 0.286 $mg.kg^{-1}$).

accumulation *in vivo* following administration of 3 mg.kg^{-1} body weight hydrazine



Male Sprague-Dawley rats were administered 3 mg.kg^{-1} hydrazine i.p. (O) while control animals (●) were administered saline only.

Fig. 5.2 The time-course of rat liver GSH depletion *in vivo* following administration of 3 mg.kg^{-1}body weight hydrazine

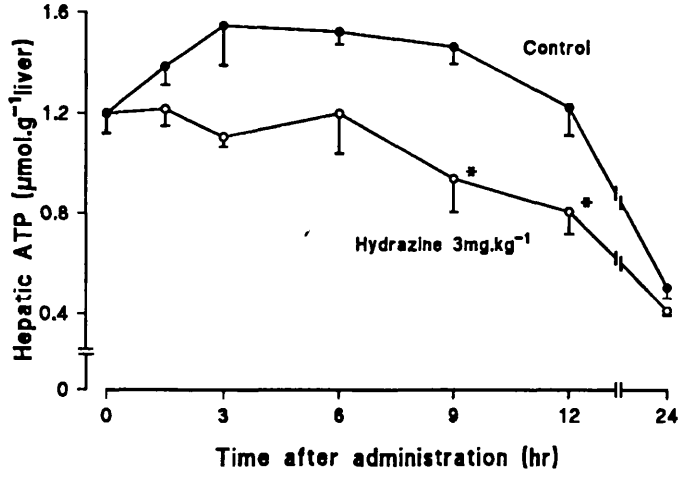


After dosing animals were starved and then sacrificed at different time intervals.

Data points represents the mean ± SEM from three animals.

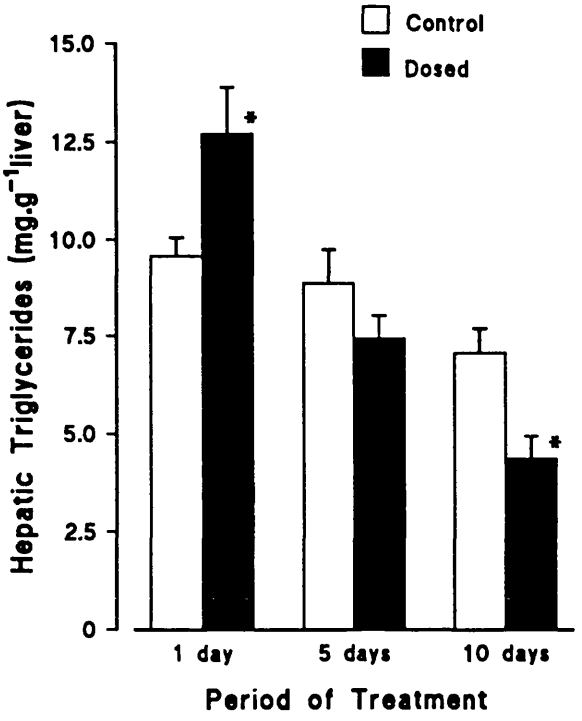
Statistical significance of hydrazine dosing was assessed using the unpaired Student's t-Test.

Fig. 5.3 The time-course of rat liver ATP depletion *in vivo* following administration of 3 mg.kg^{-1}body weight hydrazine



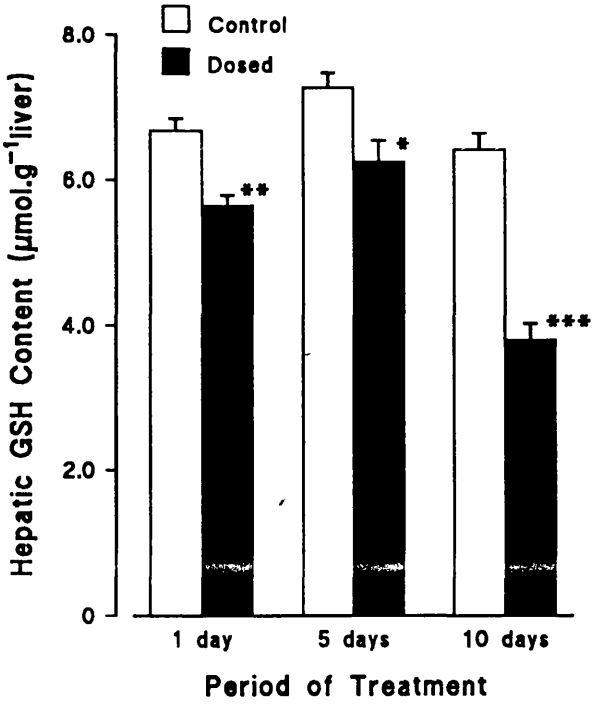
* $p<0.05$
** $p<0.01$
*** $p<0.001$

Fig. 5.4 The effect of 0.78 mM hydrazine in drinking water on rat liver triglycerides



Male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (2.5 mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 1,5 and 10 days. Data represents the mean ± SEM from four animals.

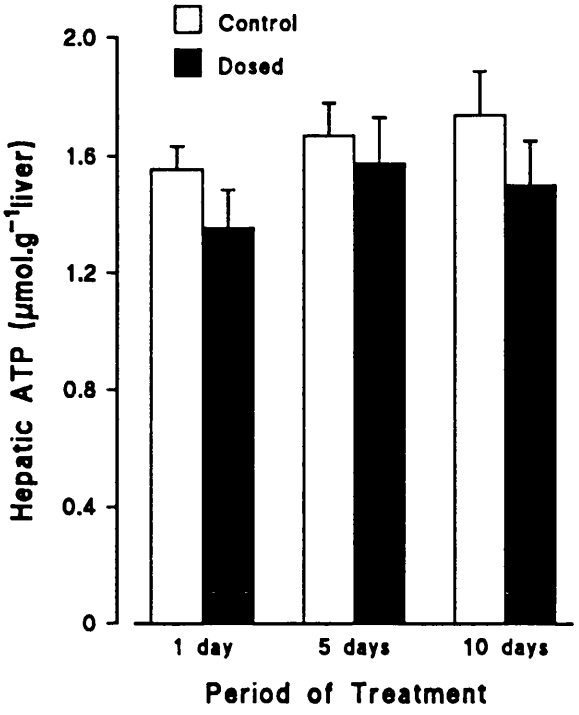
Fig. 5.5 The effect of 0.78 mM hydrazine in drinking water on rat liver GSH



Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

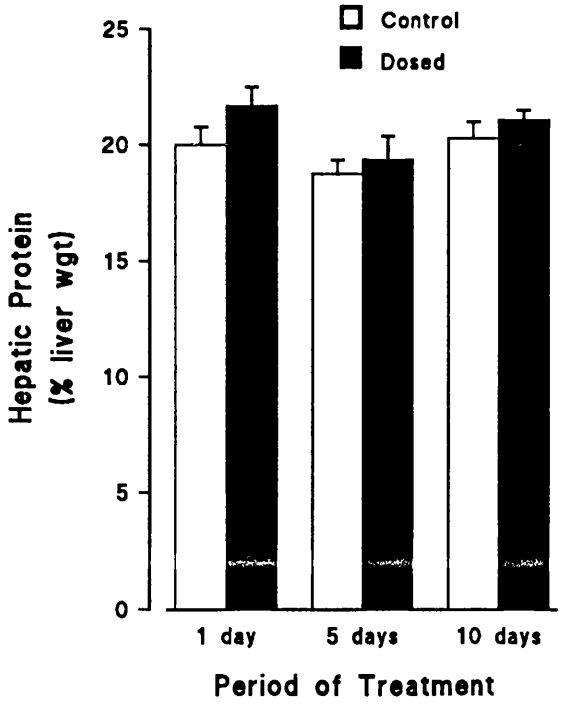
*	p<0.05
**	p<0.01
***	p<0.001

Fig. 5.6 The effect of 0.78 mM hydrazine in drinking water on rat liver ATP



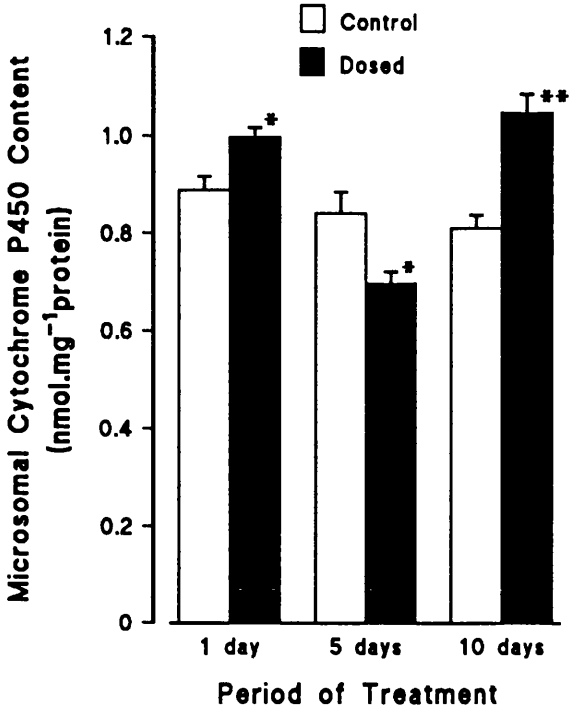
Male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (2.5 $mg.kg^{-1}$ $.day^{-1}$) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 1,5 and 10 days. Data represents the mean ± SEM from four animals.

Fig. 5.7 The effect of 0.78 mM hydrazine in drinking water on rat liver protein



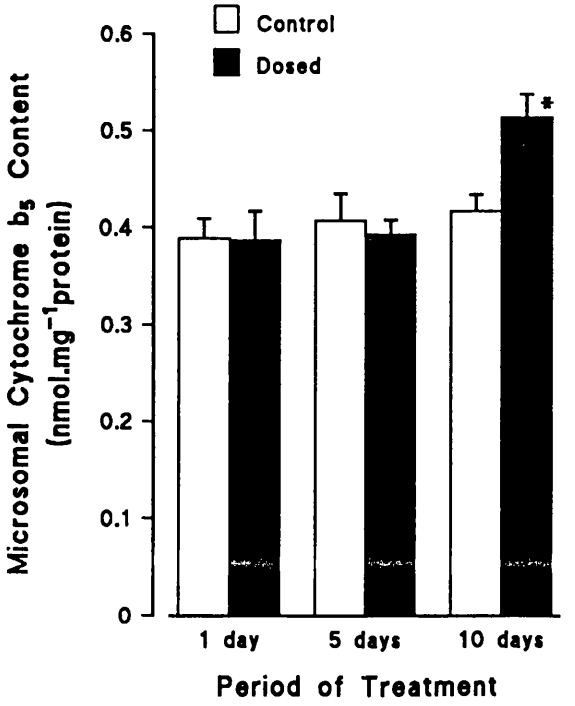
Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

Fig. 5.8 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal cytochrome P450



Male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (2.5 mg.kg^{-1} .day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 1,5 and 10 days. Data represents the mean ± SEM from four animals.

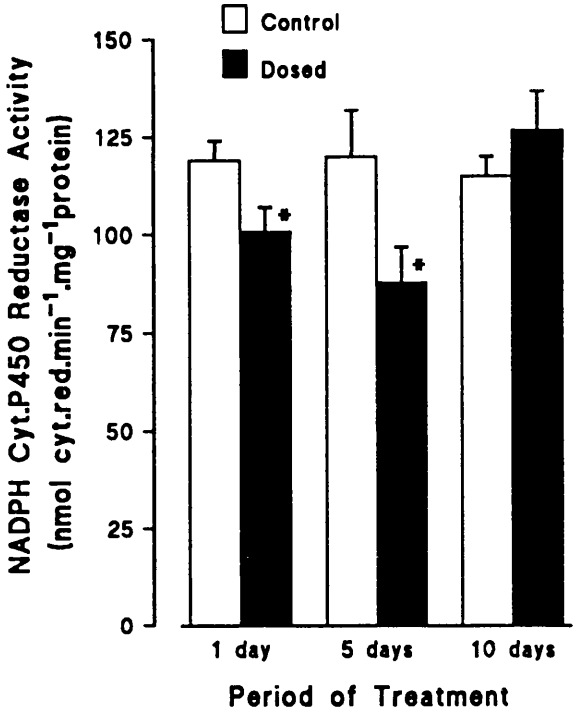
Fig. 5.9 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal cytochrome b_5



Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

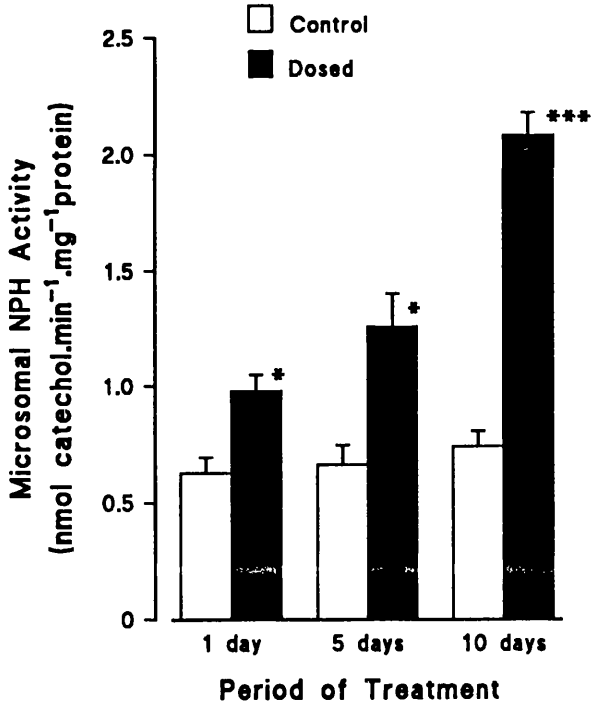
*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.10 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal NADPH cytochrome P450 reductase activity



Male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (2.5 mg.kg^{-1} .day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 1,5 and 10 days. Data represents the mean ± SEM from four animals.

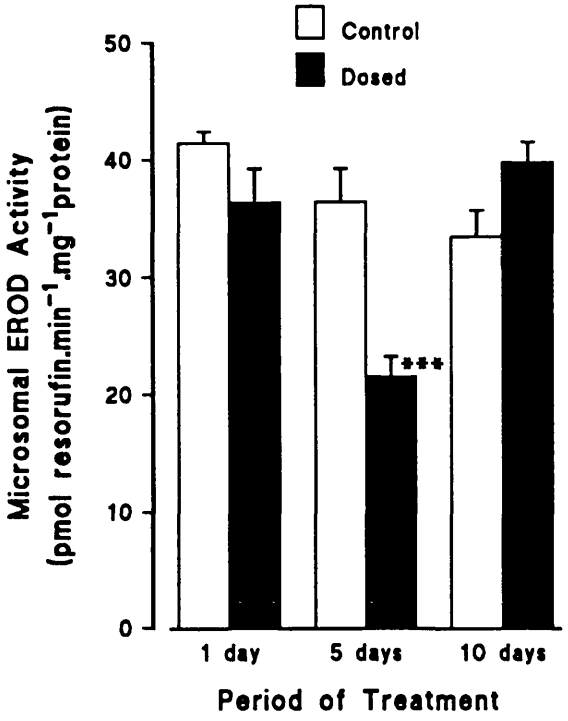
Fig. 5.11 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal NPH activity



Statistical significance of repeated hydrazine dosing was assessed using the unpaired Students t-Test.

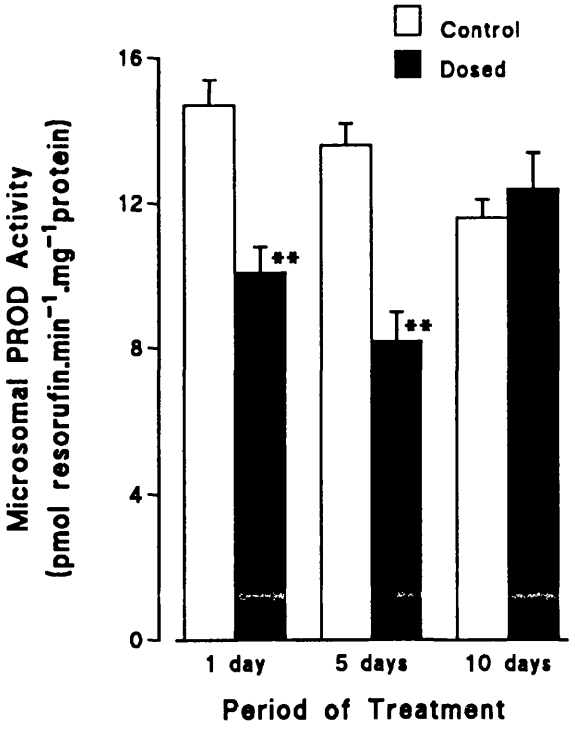
*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.12 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal EROD activity



Male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (2.5 mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 1,5 and 10 days. Data represents the mean ± SEM from four animals.

Fig. 5.13 The effect of 0.78 mM hydrazine in drinking water on rat liver microsomal PROD activity



Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.14 The effect of 0.78 mM hydrazine in drinking water on daily rat food intake

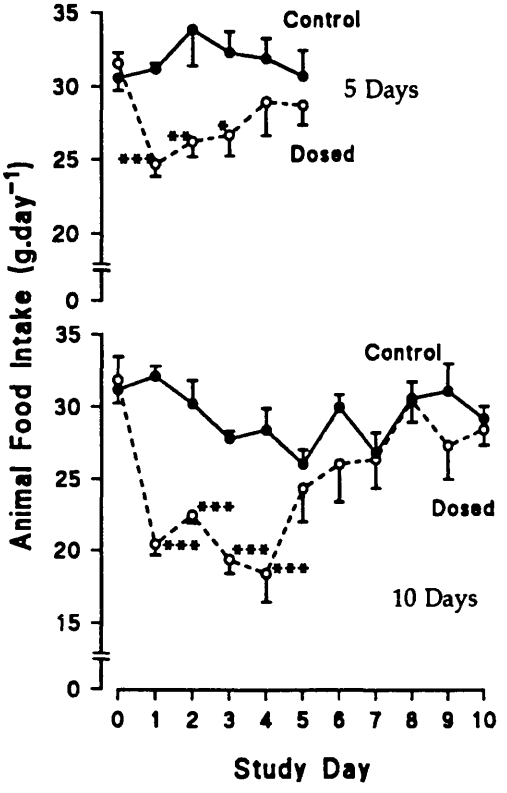


Fig. 5.15 The effect of 0.78 mM hydrazine in drinking water on rat body weight

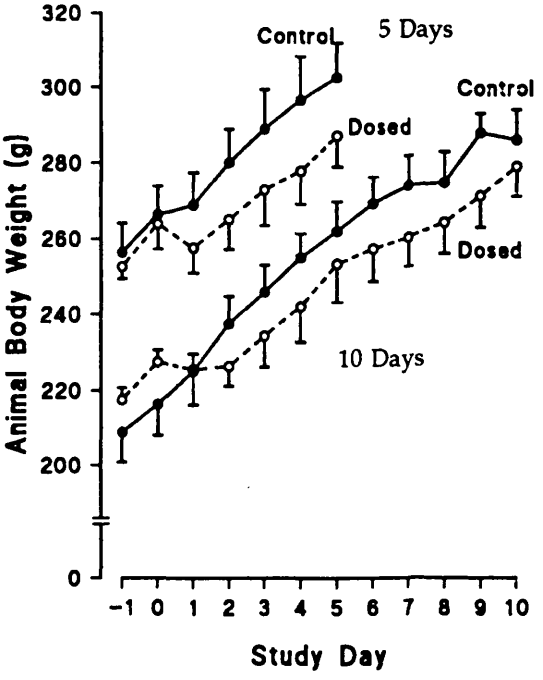
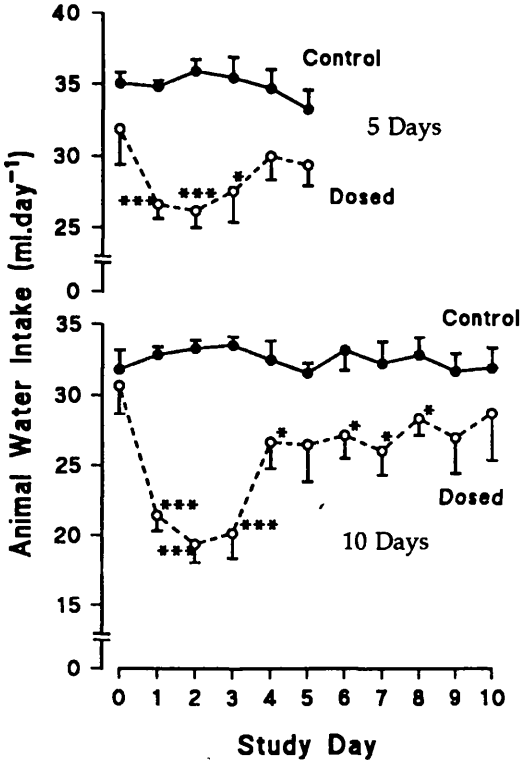


Fig. 5.16 The effect of 0.78 mM hydrazine in drinking water on daily rat drinking water intake



On study day 0 male Sprague-Dawley rats received 0.78 mM hydrazine in their drinking water (O) (2.5 $mg.kg^{-1}.day^{-1}$) while control animals (●) were untreated. Animals were fed *ad libitum* and sacrificed after 5 and 10 days.

Data points represent the mean ± SEM from four animals. Drinking water and food intake was measured each study day for the preceding 24 hr.

Statistical significance of repeated hydrazine dosing was assessed using the unpaired Students t-Test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.17 The effect of 5 days restricted diet (20 g food.day^{-1} on rat liver triglycerides

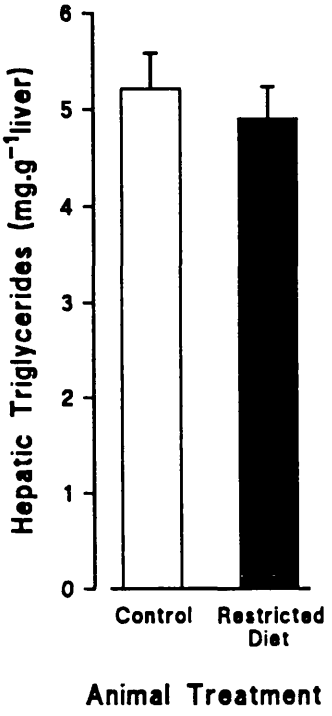


Fig. 5.18 The effect of 5 days restricted diet (20 g food.day^{-1}) on rat body weight

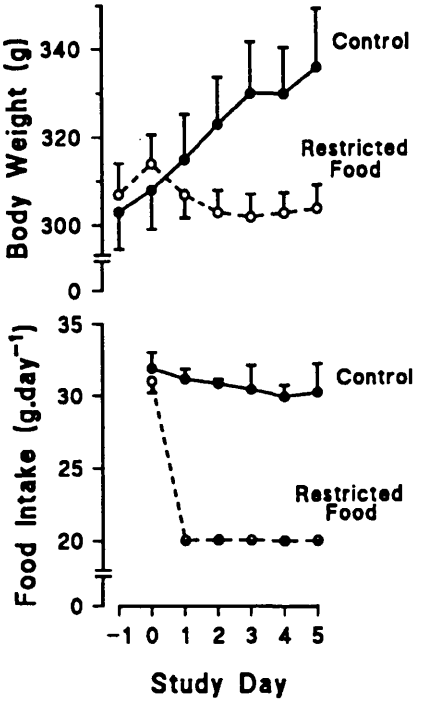
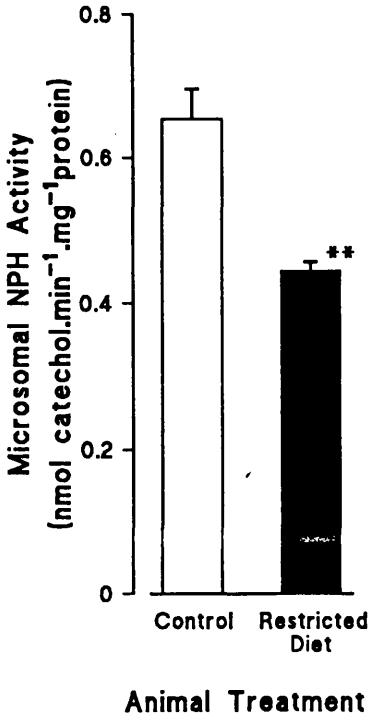


Fig. 5.19 The effect of 5 days restricted diet (20 g food.day^{-1}) on rat liver microsomal NPH activity



On study day 0 male Sprague-Dawley rats received a restricted diet (20 g.day^{-1}) while control animals (●) were fed *ad libitum*. Animals were sacrificed after 5 days.

Data represents the mean ± SEM from four animals. Food intake was measured each study day for the preceding 24 hr.
Statistical significance of diet restriction was assessed using the unpaired Students t-Test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.20 The effect of 65 µM hydrazine in drinking water on rat liver triglycerides

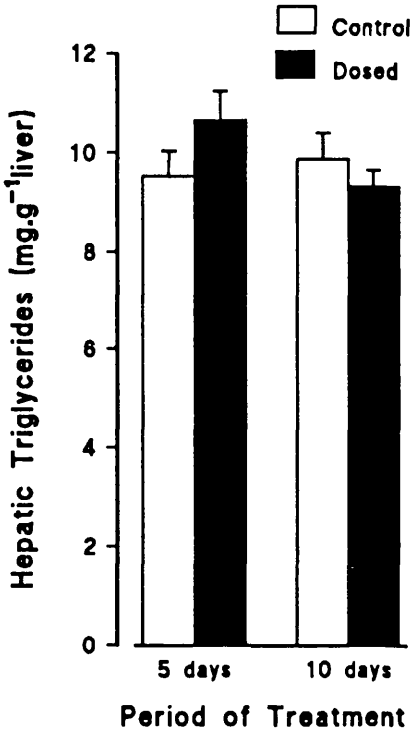


Fig. 5.21 The effect of 65 µM hydrazine in drinking water on rat liver ATP

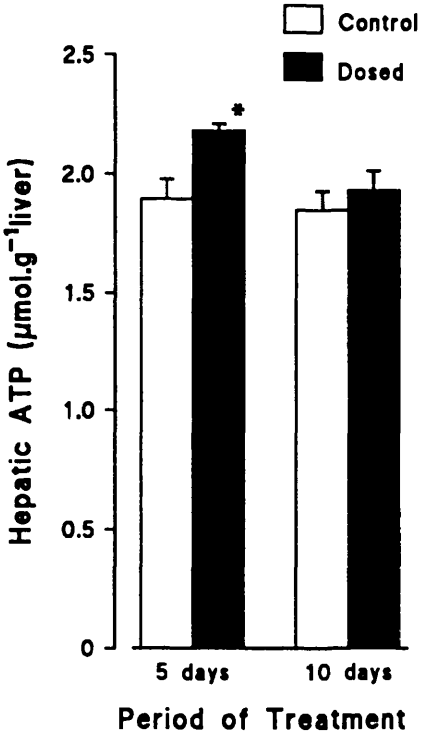
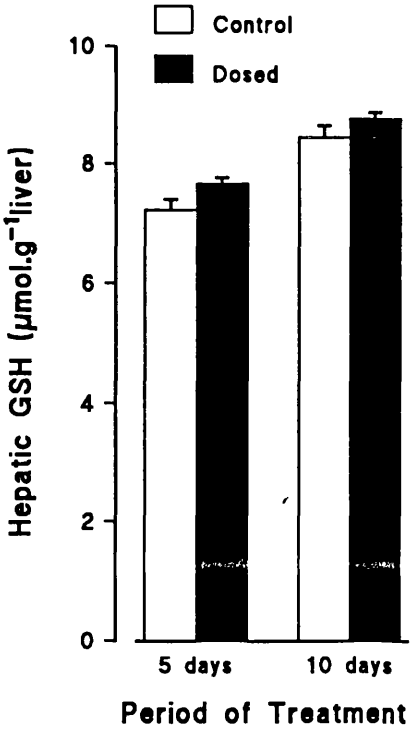


Fig. 5.22 The effect of 65 µM hydrazine in drinking water on rat liver GSH



Male Sprague-Dawley rats received 65 µM hydrazine in their drinking water (0.25 mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 5 and 10 days.

Data represents the mean ± SEM from four animals.

Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.23 The effect of 65 µM hydrazine in drinking water on rat liver microsomal cytochrome P450

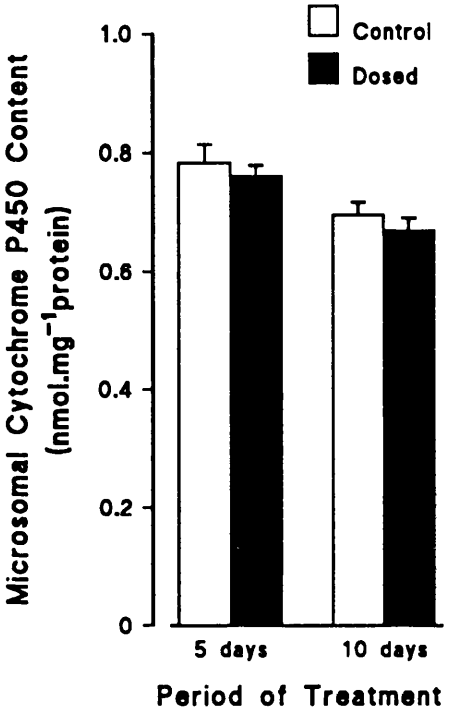


Fig. 5.24 The effect of 65 µM hydrazine in drinking water on rat liver microsomal NADPH cyt. P450 reductase activity

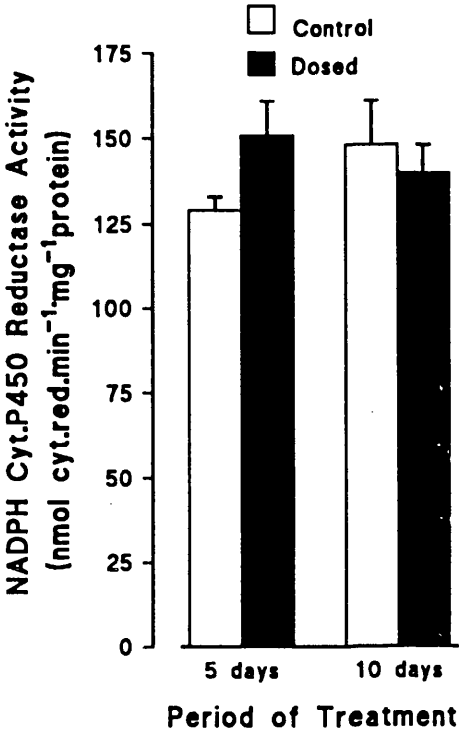
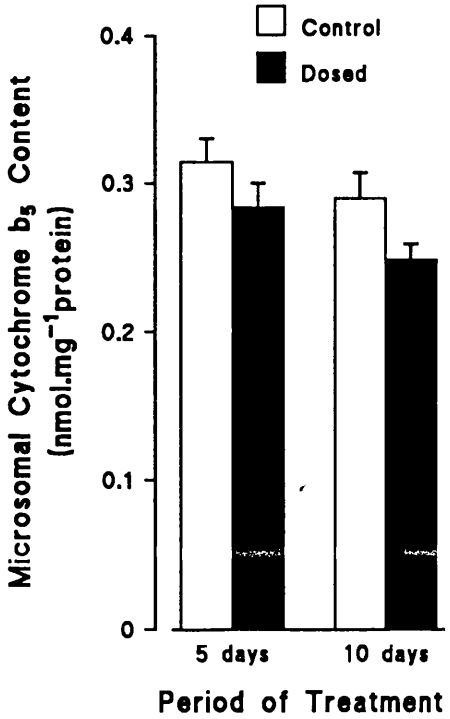


Fig. 5.25 The effect of 65 µM hydrazine in drinking water on rat liver microsomal cytochrome b_5



Male Sprague-Dawley rats received 65 µM hydrazine in their drinking water (0.25 mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 5 and 10 days.

Data represents the mean ± SEM from four animals.

Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

Fig. 5.26 The effect of 65 µM hydrazine in drinking water on rat liver microsomal EROD activity

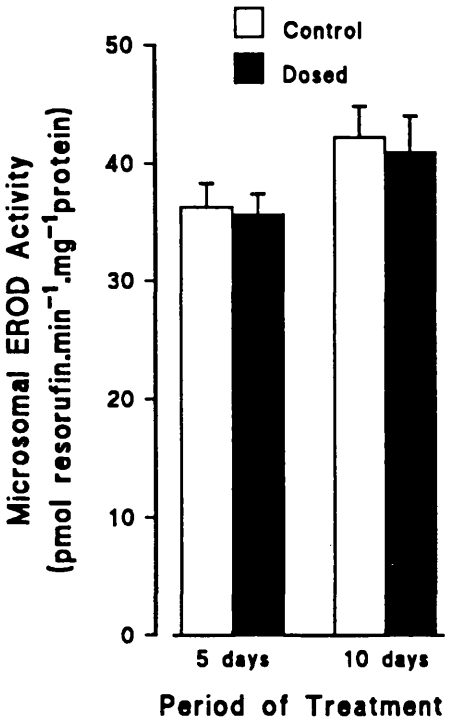


Fig. 5.27 The effect of 65 µM hydrazine in drinking water on rat liver microsomal PROD activity

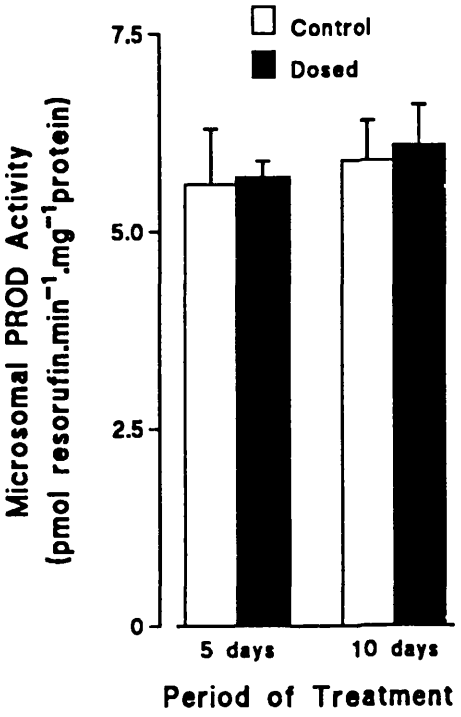
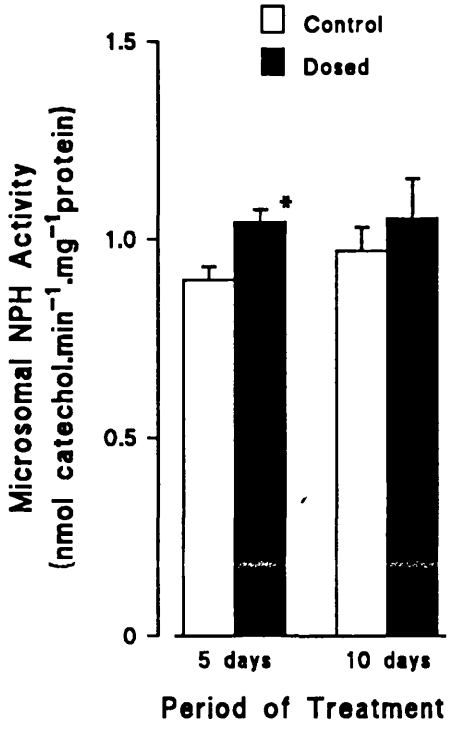


Fig. 5.28 The effect of 65 µM hydrazine in drinking water on rat liver microsomal NPH activity



Male Sprague-Dawley rats received 65 µM hydrazine in their drinking water (0.25 mg.kg^{-1}.day^{-1}) while control animals were untreated. Animals were fed *ad libitum* and sacrificed after 5 and 10 days.

Data represents the mean ± SEM from four animals.

Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

Fig. 5.29 The effect of 65 µM hydrazine in drinking water on rat liver protein

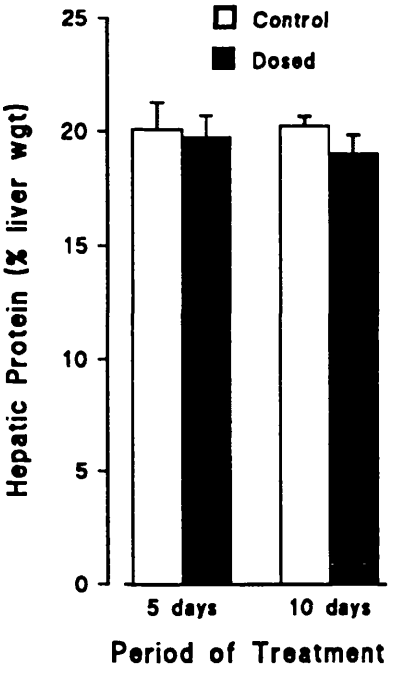


Fig. 5.30 The effect of 65 µM hydrazine in drinking water on rat body weight

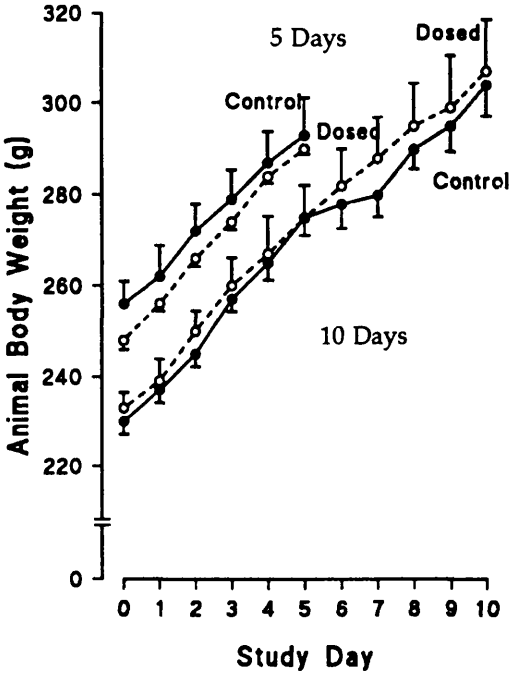
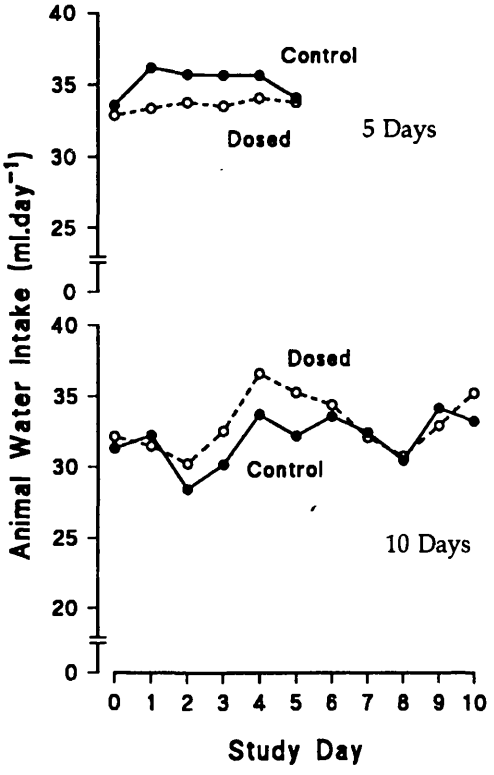


Fig. 5.31 The effect of 65 µM hydrazine in drinking water on rat water intake



On study day 0 male Sprague-Dawley rats received 65 µM hydrazine in their drinking water (O) (0.25 $mg.kg^{-1}.day^{-1}$) while control animals (●) were untreated. Animals were fed *ad libitum* and sacrificed after 5 and 10 days.

Data represents the mean ± SEM from four animals. Drinking water intake was measured each study day for the preceding 24 hr.

Statistical significance of repeated hydrazine dosing was assessed using the unpaired Student's t-Test.

5.4 DISCUSSION

The time dependent steatosis after administration of an acute 3 $mg.kg^{-1}$ hydrazine dose i.p. was accompanied by a significant depletion of ATP. Both events became significant after 9 hr indicating that they are possibly connected and indeed it has been postulated that hydrazine induced fatty liver is the result of an inhibition of lipoprotein synthesis and subsequent secretion due to ATP depletion.

Previous studies by Timbrell *et al* (1982) have reported the dose threshold for hepatic triglycerides accumulation after 24 hr to be 10-20 $mg.kg^{-1}$ and this higher limit may be due to the wider body weight range of smaller rats used (125-240 g) in their study compared to the narrow range of rats used (240-260 g) in this study. The time dependent ATP and GSH depletion caused by starvation may quite possibly have concealed or reduced the significance of hydrazine administration on depletion of ATP and GSH. Only recently has it actually been demonstrated that hydrazine administration results in hepatic ATP depletion (Preece *et al* 1990a) so no studies have been carried out to experimentally investigate the association of this biochemical alteration with others induced by hydrazine, such as triglyceride accumulation, hypoglycaemia and GSH depletion.

Accumulation of hepatic triglycerides after 24 hr in animals fed *ad libitum* and exposed to a daily dose of hydrazine (2.5 $mg.kg^{-1}$) in drinking water was similar to the accumulation recorded in starved rats 24 hr after an acute 3 $mg.kg^{-1}$ dose. In contrast to the animals dosed i.p. there was a significant GSH depletion after 24 hr in rats repeatedly exposed to hydrazine but there was a comparable lack of any ATP depletion after 24 hr hydrazine exposure. Presuming the small difference in hydrazine dose is not responsible, the disparity in results is due either to food restriction or course of hydrazine exposure. Hydrazine is absorbed rapidly via all routes of administration which appear to have little influence on acute hydrazine toxicity (Witkin 1956). However few studies have monitored the pharmacokinetics of low doses. The exact pattern of exposure to hydrazine via drinking water will depend on the animals drinking habits, but over time will consist of lower but repeated doses which will generate a more uniform exposure to hydrazine than

single acute doses.

Continued repeated exposure to 0.78 mM hydrazine (2.5 $mg.kg^{-1}.day.^{-1}$) after 24 hr caused a distinction in liver biochemical changes, compared to acute hydrazine doses. Liver levels of GSH and ATP remained depleted and unchanged respectively after 5 and 10 days exposure, while triglyceride levels reverted to control levels after 5 days and after 10 days were further reduced below control. This steady decline of triglycerides during repeated hydrazine exposure after initial accumulation has not previously been reported. Previous studies have focused on higher acute doses of hydrazine for up to 24 hr after exposure. The possibility that depletion of triglyceride after 24 hr was due to the reduction of food intake in hydrazine exposed animals was ruled out, since animals not exposed to hydrazine but with a similar reduced diet to that of dosed animals had similar levels of hepatic triglyceride to control animals.

The significant depletion of GSH without any concomitant reduction in ATP throughout the 10 days of 0.78 mM hydrazine exposure indicates that hydrazine does not decrease GSH levels by depletion of ATP, which is necessary for the synthesis of GSH and is required in the rate limiting step. In a similar manner the 24 hr triglyceride accumulation was unaccompanied by a depletion of ATP suggesting that hydrazine steatosis may not be due to decreased ATP levels. However it is possible that ATP was depleted before the 24 hr time point which was the case for rats dosed i.p. with 3 $mg.kg^{-1}$ hydrazine and may precede triglyceride accumulation.

The disparate time dependent effects of repeated exposure to 0.78 mM hydrazine on total hepatic microsomal cytochrome P450 are clearly due to the differential impact of hydrazine on individual isoenzymes of cytochrome P450, as indicated by the time dependant alterations in isoenzyme activity. Reduction of specific cytochrome P450IA1 and IIB1 activities (EROD and PROD respectively, Burke *et al* 1985) after 5 days hydrazine exposure may explain the simultaneous decrease in cytochrome P450. Levels of these two cytochrome P450 isozymes are very low in control animals and only become prominent in induced animals. Therefore it is quite probable that

depletion of other constitutive isoenzymes also account for the 5 day depletion of total cytochrome P450. The lack of any alteration in total hepatic protein levels suggests that hydrazine exposure did not result in any general gross changes of protein synthesis and that any induction or depletion of microsomal enzymes were specific.

Akin and Norred (1978) and Ghatineh *et al* (1990a) have both demonstrated the reduction of total cytochrome P450 after 4 consecutive days administration of hydrazine i.p. to male rats at doses of 50 and 17.5 $mg.kg^{-1}.day^{-1}$ respectively. In the same studies Ghatineh *et al* (1990a) too found that EROD activity was reduced, while Akin and Norred detected a depletion of benzo(a)pyrene hydroxylase activity which is catalysed by cytochrome P450 IA1 and also IA2. The latter cytochrome P450 isozyme is expressed at very low levels in non-induced rats too. In contrast to this study Ghatineh *et al* (1990a) did not detect any change in PROD activity.

The consistent and time dependant elevation of NPH activity, with no accompanied induction of PROD activity during 0.78 mM hydrazine exposure, suggests that hydrazine is a specific inducer of cytochrome P450 IIE1, since NPH activity is reported as a good indicator of P450IIE1 (Koop *et al* 1989, Koop 1992). This discovery is affirmed by the previously reported hydrazine induction of aniline *p*-hydroxylation (Akin and Norred 1978, Ghatineh *et al* 1990a), which is non-specifically catalysed by cytochrome P450IIE1 and also to a lesser extent by P450IIB isozymes. However several additional criteria need to be satisfied in order to demonstrate isozyme induction. This requires isolation of the induced isozyme and comparison of characteristics such as catalytic properties, peptide maps, electrophoretic migration, spectral properties and immunological activity.

The intensity of elevation compared to other classical IIE1 inducers, such as acetone and isoniazid (Ryan *et al* 1985) (Table 4.2), also suggests that hydrazine may be a potent inducer. Further induction above that demonstrated in this study may be possible by future investigation into the dose level and time course of hydrazine exposure. Hydrazine a minor metabolite of isoniazid (Timbrell *et al* 1977b, Noda *et al* 1978, Noda *et al* 1982, Blair *et al* 1985, Peretti *et al* 1987) may be partially responsible for the induction of P450IIE1 by isoniazid pretreatment.

Cytochrome P450IIE1 is a constitutive isoenzyme in non-induced rats and the significant induction of P450IIE1 during 0.78 mM hydrazine exposure may account for a proportion of the concurrent increase in total cytochrome P450 after 1 and 10 days. The significant reduction in total cytochrome P450 after 5 days despite the considerable elevation of cytochrome P450IIE1 at that same time confirms that hydrazine must deplete other constitutive forms of cytochrome P450 apart from the very minor forms that were monitored. Depletion of cytochrome P450 by hydrazine must be due to inhibition or destruction.

Cytochrome P450IIE1 is the only member of the P450IIE family in rats mice and humans as determined by Southern blot analysis. Rabbits also possess the second highly related form P450IIE2. P450IIE1 is readily inducible by the administration of a variety of structurally diverse chemicals, including gaseous oxygen (Tindberg and Ingelman-Sundberg 1989), that are generally all small relatively polar compounds (Guengerich *et al* 1991). Induction of this specific class of P450 gene is unique among other P450 gene families that are induced by transcriptional activation of the gene. In contrast, induction of P450IIE1 does not entail transcriptional gene activation except at birth. Within a few hours of birth significant levels of P450IIE1 mRNA were detected and these levels continued to rise reaching a maximum after 6 days (Umeno *et al* 1988). No P450IIE1 was detected in neonatal rat liver. Studies reveal that the enzyme concentration following birth can be regulated by a combination of the following 3 different mechanisms, depending on the inducer or treatment:

1) mRNA stabilisation
2) Increased translation of existent mRNA
3) Inhibition of protein degradation

Xenobiotics, such as ethanol, acetone, imidazole, pyrazole and isoniazid induce P450IIE1 primarily by stabilisation of existing protein (Koop and Tierney 1990). The turnover of P450IIE1 is very rapid in untreated animals and has been reported to be biphasic, with estimated half-lives of about 7 and 37 hr in rat (Song *et al* 1989) which suggests there may be two populations of enzyme. In the same study continuous administration of acetone to rats resulted in only a slow rate of degradation similar

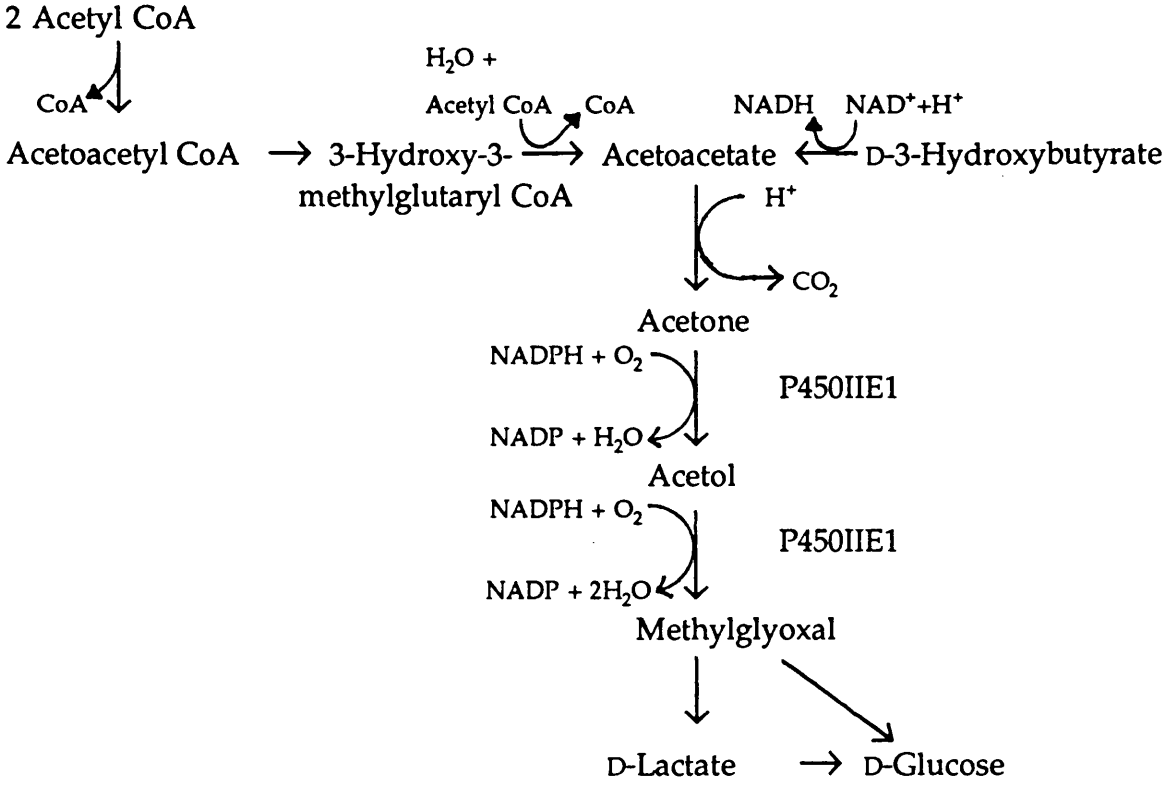
prepared from P450IIE1 induced animals, have demonstrated that when the cells are cultured in the presence of various P450IIE1 ligands, including ethanol, imidazole and dimethylsulphoxide, the subsequent loss of enzyme in culture is reduced (Eliasson *et al* 1988). The protection afforded by each ligand tested was related to the spectral binding constant of the ligand for the purified isozyme indicating that enzyme ligation is important for stabilisation. Inhibition of NPH activity 6 hr after a single large hydrazine dose ($\geq$30 mg.kg^{-1}, Chapter 4) could be due to ligation of P450IIE1 by hydrazine and would explain the induction of the enzyme at lower repeated doses with time. Diminished NPH induction during 65 μM hydrazine exposure may be due to a significant reduction of hydrazine below the concentration required for P450IIE1 stabilisation. However, it is also conceivable that a metabolite of hydrazine is responsible for the inductive effect rather than hydrazine itself.

P450IIE1 is also readily inducible by changes in the hormonal and metabolic status of animals. In almost all cases P450IIE1 mRNA is increased where hormonal changes are involved. Induction of P450IIE1 in either spontaneous or chemically provoked (alloxan or streptozotocin administration) diabetic rats (Ma *et al* 1989) is widely reported to be due to mRNA stabilisation and the rise in enzyme and mRNA levels are both reversed by administration of insulin (Koop and Tierney 1990). Growth hormone, known to regulate the concentration of several rat P450 forms (Gonzalez 1989) is lower in diabetic animals, but results of experiments examining its importance in P450IIE1 regulation are ambiguous (Koop and Tierney 1990). Studies inspecting the role of the male specific hormone, testosterone are also inconclusive. Initial suggestions were made that P450IIE1 induction by the diabetic state was due to increased levels of ketone bodies in the circulation and it is currently believed that there is probably more than one mechanism in P450IIE1 induction by diabetes.

Koop and Casazza (1985) have recognised that P450IIE1 can catalyse the oxidation of acetone to acetol and then to methylglyoxal and demonstrated that this activity is induced by acetone and ethanol administration. This pathway is also induced by starvation (Casazza *et al* 1984) and can ultimately lead to the synthesis of glucose.

It is therefore proposed to be an emergency gluconeogenic pathway for acetone during fasting, providing the only possible route that acetyl CoA can be channelled into glucose formation (Fig. 5.32).

Figure 5.32 Proposed gluconeogenic pathway for acetyl CoA and acetone



Lack of effect of exposure to 65 μM hydrazine on any hepatic microsomal enzyme, except for a slight increase in NPH activity, compared to the changes in NPH and other microsomal enzyme activity after exposure to 0.78 mM hydrazine, indicates that the dose threshold of hydrazine for alterations in hepatic microsomal enzymes is between 65 and 780 μM. The identical threshold for changes in hepatic biochemistry after repeated exposure to hydrazine, suggests that elevation of NPH activity may be due to hydrazine's disturbance of normal liver biochemistry, particularly triglyceride and carbohydrate metabolism.

The decline of hepatic triglycerides 24 hr after 0.78 mM hydrazine exposure may represent triglyceride catabolism, possibly as a compensation mechanism for the inhibition by hydrazine of gluconeogenesis from its normal precursors and the

induced hypoglycaemia.

Lipolysis of triglyceride yields glycerol, which can subsequently enter the conventional gluconeogenic pathway and fatty acids that are sequentially oxidised to form acetyl CoA. Normally when fat and carbohydrate degradation are appropiately balanced acetyl CoA would enter the TCA cycle by reacting with oxaloacetate to form citrate, but under fasting or diabetic conditions oxaloacetate is used to generate glucose. Under these circumstances acetyl CoA is diverted to the formation of acetoacetate, acetone and D-3-hydroxybutyrate (ketone bodies) and with the accompanied induction of P450IIE1 thus provides a means for the formation of glucose from fatty acids. Yoo *et al* (1991) demonstrated that rats fed diets with higher fat : carbohydrate ratios produced raised levels of serum acetone and hepatic microsomal P450IIE1 and Nakajima *et al* (1982) observed that the metabolism and toxicity of carbon tetrachloride, a P450IIE1 substrate, is enhanced by carbohydrate deficient as well as high fat : carbohydrate diets. A high fat diet was also demonstrated to increase rat liver microsomal 1,2-dimethylhydrazine *N*-demethylase activity (Pence *et al* 1991).

Rats made ketotic by administration of medium chain triglycerides exhibited enhanced P450IIE1 activity, but this was accompanied by large increases in PROD and NADPH P450 reductase activity (Yoo *et al* 1990). Exposure of rats to 0.78 mM hydrazine resulted in no such increases in PROD or NADPH P450 reductase activities indicating that ketosis due to triglycerides may not be the major mechanism of P450IIE1 induction by hydrazine. Ketosis during starvation has been shown to be too moderate to account for the entire induction of P450IIE1 during fasting which is accompanied by increased levels of mRNA. It is possible that mechanisms of P450IIE1 induction other than ketosis may play a role during hypoglycaemia.

The prospect that reduction of food intake by animals administered hydrazine was the cause of increased NPH activity, was discounted when animals on a similar reduced diet, to that caused by exposure to 0.78 mM hydrazine, demonstrated an actual decrease in activity compared to rats fed *ad libitum*. The latter finding suggests that rats fed *ad libitum* may be to some degree obese, since it has been demonstrated that hepatic microsomal P450IIE1 protein content and activity is increased in obese overfed rats which have elevated serum ketone levels (Raucy *et al* 1991).

1990). Evidence suggests that ethanol, pyridine and pyrazole may increase mRNA translation but further more rigorous experiments need to be performed in order to confirm these results.

Increased hepatic cytochrome b_5 levels after 10 days repeated exposure to 0.78 mM hydrazine may be associated with induction of P450IIE1 activity since this hemoprotein can significantly alter the activity towards many P450IIE1 substrates by affecting both V_{max} and K_m (Koop 1986) and may be co-induced with P450IIE1.

Microsomal metabolism of hydrazine was demonstrated to be predominantly dependent on NADPH and oxygen and catalysed by cytochrome P450 in common with previous studies (Timbrell *et al* 1982, Noda *et al* 1985). Studies by Noda *et al* (1985) have indicated that a hydrazine radical may be formed, catalysed by the cytochrome P450 system, with an electron donated by cytochrome P450 reductase. They propose that this radical is a precursor of diimide whose presence has also been ascertained during microsomal hydrazine metabolism (Noda *et al* 1985 and 1987b, Nelson and Gordon 1982).

Unfortunately analysis of microsomal hydrazine metabolism in this study was unable to corroborate these findings as such investigation requires the use of spin trapping agents and measurement of electron spin resonance (ESR) by sophisticated and expensive apparatus.

Using ^{15}N labelled hydrazine and mass spectrometry (MS), production of ^{15}N nitrogen was detected *in vitro* (Nelson and Gordon 1982) during microsomal metabolism and *in vivo* studies have shown that the major portion (35%) of a dose of hydrazine is exhaled as nitrogen. N_2 generation could be used as a suitable end point of oxidative metabolism, but the metabolism of hydrazine via oxidative routes remains difficult to measure.

The measurement of hydrazine disappearance during *in vitro* metabolism studies by colorimetry of DMBA (*p*-dimethylaminobenzaldehyde) provided a much quicker and more accessible method for analysing hydrazine metabolism. However, this assay is limited by the fact that only hydrazine disappearance is measured. Further work on isolated microsomes is necessary to quantitatively measure free radicals derived from hydrazine. It is hoped that this coupled with the measurement of nitrogen gas will provide a more detailed assay of microsomal hydrazine metabolism.

In vitro studies have indicated for the first time that in addition to the significant cytochrome P450 mediated metabolism in rat liver microsomes there may be a smaller component of metabolism catalysed by the flavin dependent monooxygenases (FMO). These enzymes are responsible for oxidation of a wide

range of structurally diverse soft nucleophiles (Ziegler 1991) and is the major enzyme catalysing the oxidation of 1,1-disubstituted hydrazines (Prough 1973, Prough *et al* 1981). Investigation of the significance of FMO on hydrazine metabolism and toxicity *in vivo* is unfortunately difficult, since its activity is not induced by xenobiotics and a selective mechanism-based inhibitor is not available. However, FMO antibodies currently being isolated will provide an essential tool for further investigation of *in vitro* hydrazine metabolism by FMO.

Human liver microsomes seem to metabolise hydrazine at a similar but lower rate (20 - 70%) to rat liver microsomes and suggest that the rat may be a suitable model for the study of hydrazine metabolism. However, few studies have investigated species differences of metabolism and the products may not be the same.
Future use of isolated cells especially hepatocytes from human tissues, as well as cell lines transfected with human genes for xenobiotic metabolic enzymes, will provide essential information on human hydrazine metabolism and its significance to hydrazine toxicity.

This is the first study to illustrate that isolated mitochondria also metabolised hydrazine *in vitro*, but to only approximately half the extent that the microsomal fraction does (comparing equal protein). Other hydrazine derivatives, such as phenelzine, procarbazine, 1,2-dimethylhydrazine and 2-phenylethylhydrazine, are metabolised by monoamine oxidase (MAO) (Juchau and Horita 1972, Coomes and Prough 1983, Patek and Hellerman 1974). However, neither MAO form A or B was found to be responsible for any hydrazine metabolism, in spite of this enzyme being the predominant mitochondrial Phase I enzyme. Other mitochondrial enzymes are therefore proposed to metabolise hydrazine, such as the mitochondrial mixed function oxidase system and perhaps redox enzymes of the electron transport system.
Transitory uncoupling of mitochondrial respiration by hydrazine in isolated mitochondria is believed to have contributed to the inhibition of mitochondrial ATP synthesis by hydrazine *in vitro* that was demonstrated for the first time in these studies. This information demonstrates that hydrazine depletes ATP at the

mitochondrial level and confirms that ATP depletion is a cause and not a consequence of hydrazine toxicity. Further studies utilising isolated mitochondria and purified mitochondrial enzymes will need to be carried out in order to define the mechanisms of mitochondrial hydrazine toxicity and metabolism.

The depletion of ATP *in vitro* compares with the hepatic depletion of ATP *in vivo* 6 hr after an acute hydrazine dose. This study has established that ATP depletion *in vivo* after 6 hr is a suitable dose dependent biochemical marker of hydrazine toxicity, together with depletion of GSH and accumulation of triglycerides.

Pretreatment of animals with various inducers and inhibitors of cytochrome P450 resulted in different effects on these 3 biochemical parameters of hydrazine hepatotoxicity. Pretreatment with the cytochrome P450 inhibitor, piperonyl butoxide, resulted in increased triglyceride accumulation, indicating that certain isozymes of P450 in non-induced animals are responsible for metabolism that reduce hydrazine toxicity. Pretreatment with phenobarbitone reduced ATP depletion and triglyceride accumulation caused by hydrazine, while β-naphthoflavone (BNF) pretreatment decreased triglyceride accumulation. This correlated with marked induction of P450IIB1 isozyme and P450IA1 isozyme by phenobarbitone and BNF respectively, indicating that these P450 isozymes may also catalyse metabolism that reduces hydrazine toxicity. P450 isozymes of the IIB and IIC family also induced by phenobarbitone and P450IA2 induced by BNF may catalyse the metabolic detoxification of hydrazine too. P450IIE1 induction by pretreatment with acetone or isoniazid increased the hepatotoxicity of hydrazine as measured by triglyceride accumulation, indicating that this particular isozyme of P450 catalyses the metabolic toxification of hydrazine. These findings confirm the studies of Timbrell *et al* (1982) which found that hydrazine hepatotoxicity was increased by piperonyl butoxide pretreatment and decreased by pretreatment with phenobarbitone. However the results obtained from pretreatment with BNF, acetone and isoniazid in this study indicate the initial hypothesis by Timbrell *et al* 1982 that hydrazine undergoes metabolism by microsomal enzymes which decreases toxicity is not necessarily true. These studies have demonstrated for the first time that hydrazine toxicity is influenced by the particular isozyme of cytochrome P450 induced and in contrast to

all other induced isozymes investigated, only P450IIE1 results in an increase in hydrazine hepatotoxicity.

The modulation of hydrazine toxicity by distinct isozymes of cytochrome P450 may be due to either an increase in the rate of catalysis of a particular metabolic pathway or the generation of different metabolites which possess various degrees of toxicity. Future studies of the influence of cytochrome P450 isozymes on hydrazine toxicity should explore the corresponding changes of hydrazine metabolism.

Only phenobarbitone pretreatment resulted in an increase in hepatic microsomal hydrazine metabolism *in vitro* which based on the *in vivo* studies indicate increased formation of a less toxic metabolite. Lack of an increase in microsomal metabolism by β-naphthoflavone, acetone or isoniazid pretreatment suggests that the cytochrome P450 isozymes induced by these pretreatments provide alternative pathways for hydrazine metabolism which change the extent of toxicity. These alternative metabolic pathways may involve generation of novel metabolites.

The manipulation of isolated hepatocytes from control and pretreated animals will provide a useful model for further study of the influence of cytochrome P450 isozymes on hydrazine hepatotoxicity and metabolism. GC.MS and NMR analysis will be very useful for detecting changes in the metabolic profile.

ESR studies will be able determine if free radical intermediates are formed during hydrazine metabolism by microsomes and hepatocytes and will help illustrate their role in hydrazine toxicity.

Detailed studies investigating hydrazine metabolism by different isozymes of cytochrome P450 may help account for the indeterminate, missing proportion of hydrazine (25%) *in vivo* and better explain the mechanisms underlying the toxic effects of hydrazine.

Different effects on hepatic biochemistry and microsomal enzymes during repeated hydrazine exposure above a particular dose threshold may have a role in the development of chronic hydrazine toxicity. Depletion of GSH was demonstrated continuously over the 10 day hydrazine repeated exposure period (0.78 mM), while ATP levels were unchanged.

Long term GSH depletion may result in the loss of protection normally afforded by GSH against toxicity and especially DNA damage, which may arise by generation of free radicals or other reactive intermediates from hydrazine. GSH depletion may thus play an important role in tumour development during long term hydrazine carcinogenicity studies and should be investigated in further experimental trials.
In vitro microsomal hydrazine metabolism in the presence of GSH indicated that depletion of GSH is not associated with its conjugation or oxidation. Depletion of GSH by hydrazine, which is not as extensive as that seen with some toxic compounds, is more likely due to an inhibition of GSH synthesis and this could be investigated by experiments on hydrazine's modification of different reactions involved in the glutathione synthetic cycle.

Depletion of microsomal EROD and PROD activity by hydrazine must be due to inhibition or reduction of cytochrome P450IA1 and P450IIB1 respectively. Distinct activation of hepatic microsomal NPH activity by repeated 0.78 mM hydrazine exposure indicated the induction of cytochrome P450IIE1, which may be due to hydrazine itself or to hydrazine's effect on fat metabolism. Inhibition of hepatic microsomal NPH activity 6 hr after administration of higher acute doses suggests that hydrazine may form a stable ligand-P450IIE1 complex which inhibits catalytic activity, but protects the enzyme from degradation. This hypothetical scheme has been put forward to explain the lack of a significant increase in hepatic P450IIE1 mRNA for all chemical inducers examined in adult animals (Koop and Tierney 1990). Further studies of hydrazine exposure are capable of characterising the precise changes in individual cytochrome P450 isozymes and their mRNA and would help explain the mechanism of hydrazine induced changes in isozyme activity, particularly P450IIE1. The induction of NPH activity may be optimised in future experiments by altering hydrazine dose levels and exposure period. Comparison of hepatic acetone and hydrazine levels with the levels of cytochrome P450 isozymes during hydrazine exposure may assist identification of the mechanisms that control P450 isozyme activity, particularly P450IIE1.

Cytochrome P450IIE1 is readily inducible in humans (Guengerich *et al* 1991) and its

activity varies between individuals (Sesardic *et al* 1990). It has been demonstrated to metabolise low molecular weight compounds that are also substrates in animal models (Guengerich *et al* 1991).

The discovery that hydrazine is very probably an inducer of P450IIE1 at low doses during repeated exposure and that hydrazine steatosis is potentiated by increased levels of P450IIE1, means that workers regularly exposed to low levels of hydrazine may be at greater risk to toxic effects of hydrazine, especially during accidental high concentration exposure. In addition, workers induced by hydrazine will be potentially more at risk from exposure to common solvents, such as benzene, *N*-methylformamide and carbon tetrachloride, and components in cigarette smoke, such as benzene and alkylnitrosamines, that are also metabolised by P450IIE1 to toxic metabolites. Of particular importance are patients receiving isoniazid therapy whose P450IIE1 levels are known to be elevated (Yang *et al* 1992) and are exposed to low levels of hydrazine as a consequence of isoniazid metabolism (Timbrell *et al* 1977b, Noda *et al* 1978, Blair *et al* 1985).

Human individual lifestyle may also play a major role in the level of P450IIE1 and thus the susceptibility to toxicity by chemicals that are bioactivated by this enzyme. High fat or low carbohydrate diets, starvation and regular alcohol consumption induce P450IIE1 while certain dietary components, including organic sulphur compounds (Brady *et al* 1991a & b), are known to reduce P450IIE1 activity. Recently regular exercise has also been reported to reduce P450IIE1 activity (Day and Weiner 1991). The potential use of chlorzoxazone as a nontoxic, noninvasive probe for estimating P450IIE1 activity in humans, may prove useful in establishing whether hydrazine exposure increases human P450IIE1 activity and for monitoring individuals at higher risk to hydrazine exposure.

In vivo intra peritoneal (i.p.) hydrazine dose preparation

10 ml of N_2 degassed UHQ water was pipetted into glass Mcartney bottles fitted with a rubber seal that had previously been N_2 degassed. Various aliquots of hydrazine hydrate were pipetted into each bottle while under a stream of N_2 gas. Doses were administered within 1 hr of preparation. The different volumes required to produce the range of hydrazine concentrations necessary for correct animal dose levels (2 $ml.kg^{-1}$ body weight) are shown in the following Table.

Animal Hydrazine Dose ($mg.kg^{-1}$body weight)	Hydrazine Conc'n. (mM)	Volume of Hydrazine Hydrate (µl)
5	78.00	38.01
10	156.01	76.32
20	312.01	153.81
30	468.02	232.51
40	624.02	312.43
45	702.03	352.87
50	780.03	393.61
60	936.04	476.08

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The 4TH Stowe School symposium on drug metabolism, Buckinghamshire, UK, April 1990.

The Autumn meeting of the British Toxicology Society (BTS), Oxford, UK, 1990

The Summer meeting of the Drug Metabolism Group (DMG), Nottingham, UK, 1991.

The 3RD International ISSX meeting, Amsterdam, The Netherlands, 1991.

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