THE ENTEROHEPATIC CIRCULATION OF GLUTATHIONE CONJUGATES OF XENOBIOTICS IN THE RAT

BY

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ABSTRACT

The relationships between the excretion of xenobiotics in bile as glutathione S-conjugates and in urine as mercapturic acids have been investigated in the rat using three compounds, naphthalene, 1-chloro-2,4-dinitrobenzene and bromsulphthalein.

These three compounds have been shown to be excreted in the bile of rats as the respective glutathione S-derivatives following intravenous administration of the parent xenobiotic. Following intraduodenal administration of the radiolabelled glutathione S-conjugates of naphthalene and 1-chloro-2,4-dinitrobenzene, radioactive metabolites were absorbed from the gastrointestinal tract and excreted in urine, especially, and in bile, principally as the corresponding mercapturic acids. Absorption and biliary excretion of material derived from the infused glutathione conjugates was observed to be rapid. A very low absorption and excretion of metabolites derived from bromsulphthalein-glutathione was observed i.e. metabolites of bromsulphthalein showed negligible enterohepatic circulation.

The mechanisms involved in the enterohepatic circulation of the glutathione S-conjugates of naphthalene and 1-chloro-2,4-dinitrobenzene were investigated. Intestinal bacteria did not seem to be involved in the absorption and metabolism of these conjugates, since oral antibiotic-pretreatment of rats did not significantly alter the enterohepatic recycling of material derived from the conjugates.

Possible tissue sites for the metabolism of the glutathione conjugates of naphthalene and 1-chloro-2,4-dinitrobenzene were investigated in

incubations of isolated kidney, small intestine and liver cells. Isolated kidney cells extensively hydrolysed both S-(1,2-dihydrohydroxy-naphthyl)glutathione and S-(2,4-dinitrophenyl)glutathione to the respective cysteine derivatives. Kidney cells were also active in the formation of the mercapturic acid derivative of naphthalene. Isolated small intestinal and liver cells metabolised the glutathione S-conjugates to the corresponding cysteine derivatives, although at significantly lower rates than kidney cells. The extent of hydrolysis of the glutathione conjugates by the isolated cell preparations was in agreement with the activity of γ-glutamyltransferase measured for the different cell types.

The metabolic cooperation of the kidney, small intestine and liver in the metabolism of glutathione S-conjugates and excretion as mercapturic acids is discussed in the light of the results presented and data in the literature.

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ABBREVIATIONS

ATP	- adenosine 5-triphosphate
BSP	- bromsulphthalein
BSP-GSH	- S-glutathione conjugate of bromsulphthalein
CYS, Cys	- cysteine
CYSGLY	- cysteinylglycine
DDT	- 2,2-di(p-chlorophenyl)-1,1,1-trichloroethane
DNP	- 2,4-dinitrophenyl
EDTA	- ethylenediaminetetraacetic acid
EGTA	- ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
EI	- electron impact
FAB	- fast atom bombardment
γ-GCNA	- γ-glutamylcarboxynitroanilide
GSH	- reduced glutathione
GSSG	- oxidised glutathione
Hepes	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
h.p.l.c.	- high performance liquid chromatography
i.d.	- intraduodenal
i.p.	- intraperitoneal
i.v.	- intravenous
MA	- mercapturic acid
NADH	- reduced nicotinamide-adenine dinucleotide
NADPH	- reduced nicotinamide-adenine dinucleotide phosphate
NMR	- nuclear magnetic resonance
p.c.	- paper chromatography
S.D.	- standard deviation
t.l.c.	- thin-layer chromatography
Tris	- tris(hydroxymethyl)aminomethane

ENZYME NOMENCLATURE

Enzyme names and classification numbers are used as recommended by the Nomenclature Committee of the IUB on the Nomenclature and Classification of Enzymes (1978), Enzyme Nomenclature, Academic Press, N.Y.

Cystathionase	- EC 4.4.1.1
Cysteine conjugate β-lyase	- EC 4.4.1.13
Leucine aminopeptidase	- EC 3.4.11.1

Glucose-6-phosphate dehydrogenase - EC 1.1.1.49
γ-Glutamyltransferase - EC 2.3.2.2
Glutathione peroxidase - EC 1.11.1.9
Glutathione S-transferase - EC 2.5.1.18

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CHAPTER ONE

INTRODUCTION

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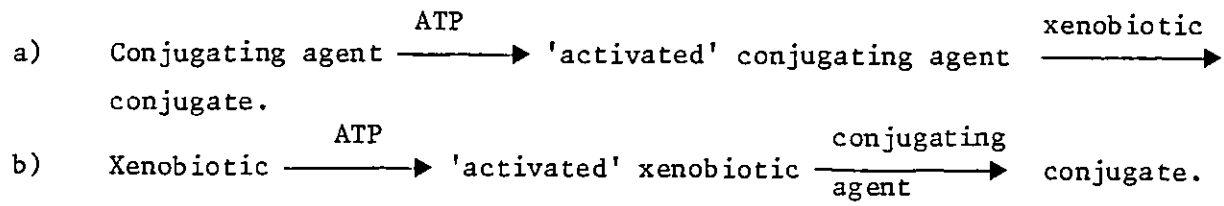
1.1 Introduction - Glutathione

Chemical compounds entering the organism which are unable to be utilised as a food source by that organism are termed foreign compounds or xenobiotics. Such xenobiotics may include drugs, naturally occurring compounds, environmental contaminants or metals. The majority of these compounds are metabolised and transformed into other substances by processes within the organism. The metabolic conversions such xenobiotics undergo are referred to as detoxication mechanisms and are now reasonably well understood (Williams, 1959; Parke, 1968).

The nature of the biotransformation reactions which exist for xenobiotics have been regarded as occurring in two phases (Williams, 1959). In the first phase, the xenobiotic acquires suitable groups such as hydroxyl, carboxyl, sulphydryl or amino by reactions which may be classified as oxidations, reductions and hydrolyses. During the second phase, these groups are the centres for synthetic reactions, usually referred to as conjugations. Each of these phases is designed to increase the polarity of the compound and the products of conjugation are usually fairly strong organic acids, which are readily excreted by the kidney or the hepatobiliary system.

The Phase II, or conjugation, reactions which xenobiotics undergo include condensation with glucuronic acid, sulphuric acid, certain amino acids and glutathione. These latter so-called conjugating agents are synthesised by the organisms. Since conjugations are synthetic reactions, they require a source of energy which is supplied *via* adenosine 5-triphosphate (ATP). These reactions are characterised by the occurrence of an activated nucleotide as an intermediate and a transferring enzyme which catalyses the final step between nucleotide

and the conjugation product. The activated nucleotide can contain in its molecule either the conjugating agent or the foreign compound, so that two kinds of conjugation reactions occur which can be represented as follows:



Glucuronic acid and sulphuric acid conjugations belong to category (a) and conjugation with amino acids to category (b). Glutathione conjugation is more correctly assigned to category (b) than (a), since reaction depends on initial microsomal oxidation of the foreign compound, to a reactive product which may often react with little enzymic assistance with glutathione. However, synthesis of glutathione requires ATP (see Meister, 1975) and often reacts with xenobiotic compounds which possess sufficient inherent reactivity to not necessitate an activation step. This scheme may be thought of as belonging to category (a). The reaction of foreign compounds with glutathione plays a major role in the metabolism of xenobiotics, since the list of compounds with which the thiol reacts includes a number of highly reactive chemical species, including mutagens and carcinogens.

The importance of glutathione <u>in vivo</u> and its role in the detoxification of reactive metabolites of xenobiotics will be illustrated in the following sections. Also, it will become apparent why we require a full understanding of the functions and metabolism of this important endogenous compound in the protection of cellular components against damage by reactive chemical species.

1.2 Historical aspects

In 1888, the French scientist, de Rey-Pailhade isolated a substance from yeast which was involved in the hydrogenation of sulphur (de Rey-Pailhade, 1888a). This substance, which he named philothion (literally "fond of sulphur"), was found in a number of animal tissues, including bovine liver and muscle, brain tissue of sheep, lamb small intestine and fish muscle and in certain plants, although its absence was noted from lamb bile and normal human urine (de Rey-Pailhade, 1888a, b). Over 30 years later Frederick Hopkins in Cambridge isolated an autoxidisable constituent of yeast cells, which had similar properties to that of de Rey-Pailhade's philothion (Hopkins, 1921). This substance was suggested by Hopkins to be a dipeptide of glutamic acid and cysteine and was therefore named glutathione. Glutathione was noted to contain practically all of the non-protein organically bound sulphur in the cell (Hopkins, 1921). Further investigation by Hopkins revealed that glutathione isolated from yeast and red blood corpuscles was not a dipeptide but a tripeptide of glycine, glutamic acid and cysteine (Hopkins, 1929). This structure was confirmed in the same year by other workers, who crystallised the substance and suggested a sequence of glutamyl-glycine-cysteine for the three amino acids (Kendall et al., 1929). The structure of glutathione was finally settled as γ-glutamylcysteinylglycine, a sequence which had been tentatively suggested by Pirie and Pinhey in 1929 working with Hopkins in Cambridge (Pirie and Pinhey, 1929), following synthesis and comparison with the substance isolated from natural sources (Harrington and Mead, 1935).

Since these early discoveries, a great deal of work has been undertaken on glutathione and progress in this area may be assessed by reference

to a number of reviews (Knox, 1960; Meister, 1975, 1981a, b; Meister and Tate, 1976) and conferences (Colwick et al., 1954; Crook, 1959; Flohé et al., 1973; Arias and Jakoby, 1976).

1.2 ii Localisation and Levels

Glutathione is a major constituent of the cell, present in plants, bacteria and animal tissues in concentrations which range from about 0.4 to 12 mM (Meister, 1975). Virtually all of the glutathione found in animals is intracellular and present in the cytosol. Rat liver has high levels of glutathione with respect to extrahepatic tissues (Table 1.1). Thus, levels of glutathione have been reported to be 2-4 fold higher in liver when compared with other tissues of the same species (Davidson and Hird, 1964; Jerina and Bend, 1977). Using a histochemical technique for the localisation of glutathione in tissues, the tripeptide was shown to be uniformly distributed in hepatic lobules, but not in other tissues, in which localisation was apparent in epithelial cells of lungs and vas deferens, proximal convoluted tubular cells of kidney cortex and in the periphery of the lens (Asghar et al., 1975). The relatively high levels of glutathione found in normal human lens tissue have been shown to be age-dependent, decreasing steadily from 3.5 μmole/g of lens at 20 years of age to 1.8 μmole/g of lens at 65 years (Harding, 1970). It is important to realise that values reported for intracellular levels of glutathione are dependent upon the method used (see Meister, 1975) and upon physiological state (Knox, 1960). Thus, levels of glutathione tissues vary as a function of nutritional status (Edwards and Westerfield, 1952; Batalden et al., 1968) and as a consequence of exposure to xenobiotics (see later). Glutathione levels in mouse and rat liver also show a diurnal variation with the highest and lowest values

TABLE 1.1 Levels of reduced glutathione (GSH) in liver and some extrahepatic tissues of rat and rabbit

Tissue	µg GSH/g wet wt	
	Rat	Rabbit
Liver	2884	1879
Brain	584	
Lung	480	843
Small Intestine	620	878
Muscle	338	
Kidney	216	154

From Davidson and Hird (1964) and Jerina and Bend (1977)

reported at 8 a.m. (rat) or 12 noon (mouse) and 8 p.m., respectively (Beck et al., 1958). Levels of glutathione in foetal mouse liver are lower than those found in adult mouse liver and adult levels are attained approximately 10 days after birth (Lambert and Thorgeirsson, 1976).

Glutathione is the most abundant intracellular γ-glutamyl compound (except perhaps glutamine, which also occurs extracellularly) and is usually present in the cell as the reduced thiol (usually represented as GSH). Reduced glutathione can be oxidised to glutathione disulphide (GSSG; equation (1)) either non-enzymatically by disulphide exchange reactions or in reactions involving oxygen, or enzymatically by the action of an organic hydroperoxide and glutathione peroxidase (EC 1.11.1.9) or glutathione oxidase and molecular oxygen (see Meister, 1975).

$$2GSH \rightleftharpoons GSSG \qquad (1)$$

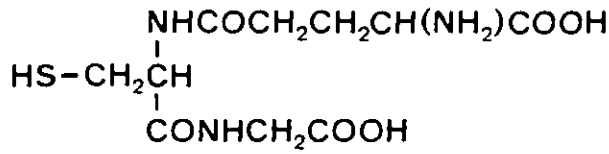
The conversion of GSSG into GSH is catalysed by glutathione reductase, a NADPH-requiring enzyme. The concentration of oxidised glutathione in the cell is usually very low (approximately 0.3% of the levels of reduced glutathione in rat liver, Akerboom et al., 1982a), although levels may increase on exposure to hydrogen peroxide or organic hydroperoxides or during drug oxidations (Sies and Summer, 1975; Sies et al., 1978a).

1.2 iii Structure

Glutathione is a tripeptide of glutamic acid, cysteine and glycine (Figure 1.1). It possesses two types of amide bonds, one between the α-amino group of glycine and the α-carboxyl group of cysteine,

the other between the α-amino group of cysteine and the γ-carboxyl group of glutamic acid.

Figure 1.1 Structure of glutathione



γ-glutamylcysteinylglycine

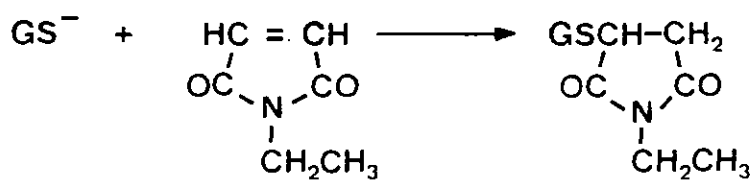
The high number of hydrophilic functional groups combined with a low molecular weight (307.3) leads to a high water solubility for glutathione. The proton dissociation constants (pKa values) for the functional groups in reduced glutathione have been measured by titration and found to be SH, 9.62; NH_3^+, 8.66; COOH, 3.53; COOH, 2.12 (Pirie and Pinhey, 1929). These values have since been confirmed by a method utilising the substantial increase in ultra-violet absorption by the thiolate ion at 225nm (Benesch and Benesch, 1955), although it has also been realised that the NH_3^+ and SH groups affect each other with respect to proton dissociation and a composite pKa value of 9.2 has been measured (Jung et al., 1972). The isoelectric point of glutathione is pH2.8.

The reactions of glutathione are essentially those of a thiol (Wieland, 1954; Kosower, 1976). However, the γ-glutamyl linkage of glutathione provides glutathione with a number of unique properties and is the important group concerned in the participation of glutathione in several biological systems. The γ-glutamyl group may make the molecule

resistant to attack by the usual proteases, which only catalyze the hydrolysis of α-amino bonds. The only known enzyme capable of catalysing the hydrolysis of the γ-glutamyl linkage is γ-glutamyltransferase (EC 2.3.2.2), an enzyme which will be discussed in greater detail later (Section 1.4 ii).

The main driving force behind the reaction of glutathione with compounds is a consequence of the fact that all compounds undergoing conjugation possess, to a greater or lesser extent, an electrophilic centre (Hutson et al., 1976). Kosower (1976) has classified the nucleophilic reactions of glutathione as nucleophilic addition, nucleophilic addition-elimination or nucleophilic displacement. Nucleophilic addition (equation(2)) occurs across carbon-carbon double (or triple) bonds and reaction proceeds at a greater or lesser rate dependent upon activating groups (cyano, carbonyl, ester, etc.) in the molecule. Quinones may be regarded as α,β-unsaturated carbonyl compounds and are particularly reactive towards glutathione (Chasseaud, 1979).

glutathione + N-ethylmaleimide

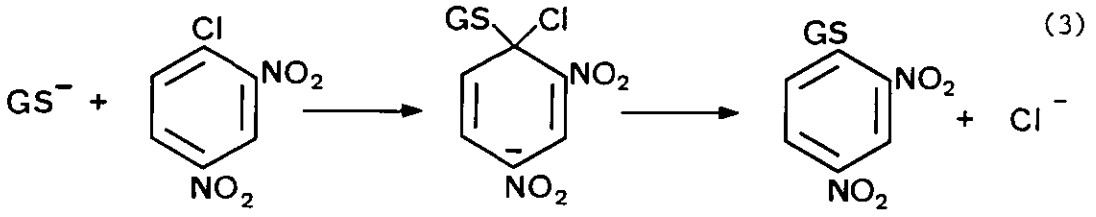


(2)

Nucleophilic addition-elimination (equation (3)) (as distinct from nucleophilic displacement) is the route taken by many if not all aromatic substituents replaced by glutathione. Displacement of halide or nitro groups occurs on aromatic rings provided that the ring system is disposed towards nucleophilic substitution i.e. has an appropriate number of electron-withdrawing substituents. The important difference between nucleophilic addition-elimination and nucleophilic displacement reactions is that the former proceeds via an intermediate which

can often be isolated.

glutathione + 1-chloro-2,4-dinitrobenzene

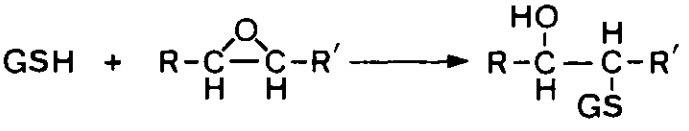


(3)

Nucleophilic displacement reactions (equation (4)) can involve monovalent leaving groups or divalent groups which remain bonded to the molecule attacked by glutathione. The latter includes the example of epoxides (equation (5)).

glutathione + hexyliodide

(4)



(5)

In all the preceding examples it is important to note that nucleophilic attack proceeds most readily with the thiolate anion, the neutral thiol being much less reactive. Since the pKa for the thiol group of glutathione is approximately 9.2 (see above), at physiological pH very little glutathione will exist in an ionised form. Consequently, non-enzymic reactions tend to occur at a very low rate. Reactions between glutathione and electrophilic substrates are, therefore, more generally catalysed by a group of enzymes, the glutathione S-transferases (EC 2.5.1.18). This very important group of enzymes will be discussed later with respect to the role of glutathione in xenobiotic metabolism.

1.2 iv Functions

A large number of important physiological functions have been ascribed to glutathione. These include maintenance of protein sulphydryl groups,

catalysis of disulphide exchange reactions, detoxification functions, and involvement as a coenzyme in a number of enzymic transformations (Table 1.2; Flohé et al., 1973; Arias and Jakoby, 1976). Glutathione may also play a role in the uptake of amino acids by cells (Meister, 1981a). Specific cellular functions may be ascribed to glutathione. Thus, the glutathione of erythocytes appears to function to protect haemoglobin and the cell membrane and abnormalities of the enzymes involved in glutathione metabolism are associated with haemolytic anemia. The glutathione of the lens appears to be essential for the functional integrity of this organ and decreases in the concentration of glutathione in the lens are associated with cataract formation (Meister, 1975).

1.2 v Protective role of glutathione

Over recent years it has become increasingly more apparent that many foreign compounds are metabolized in the body to potent reactive compounds (Gillette et al., 1974; Mitchell and Jollow, 1975). Studies have demonstrated that reactive metabolites of a number of foreign compounds can produce serious tissue lesions including neoplasia, hepatic and renal necrosis and other injuries in man and experimental animals. These studies have indicated a protective role of sulphydryl-containing compounds, particularly glutathione, against toxic reactions.

The analgesic drug paracetamol (acetaminophen) produces hepatic and renal necrosis when given at very high doses to man and experimental animals (Mitchell et al., 1973a). It also produces a dose-dependent depletion of hepatic glutathione in mice and hamsters, since a minor metabolite of the drug combines with glutathione to form a readily excreted mercapturic acid (Mitchell et al., 1973b; Potter et al., 1974).

TABLE 1.2 Functions of glutathione

Physiological functions:-

- Coenzyme for glyoxylase
  - Maleylacetoacetate isomerase
  - Formaldehyde dehydrogenase
  - Indolylpyruvic acid enol-keto tautomerase
  - Maleic acid isomerase
  - Δ^5-3-ketosteroid isomerase
- Maintenance of sulphydryl groups in proteins and other molecules
- Destruction of hydrogen peroxide
- Synthesis of important endogenous agents eg. leukotrienes
- Storage form of cysteine
- Amino acid translocation across cell membranes (?)
- Control in melanin synthesis

Extra-physiological functions:-

- Mercapturic acid synthesis
- Detoxification of reactive intermediates of xenobiotics
- Reduction of organic nitrates and thiocyanates
- Reductive dehalogenation of eg. DDT

Depletion of glutathione levels in mouse liver was not associated with hepatic necrosis until the concentration of glutathione was less than 80% of normal levels. Hepatic necrosis induced by paracetamol is associated with covalent binding of metabolites of the drug and covalent binding was not observed until glutathione levels were almost exhausted (Mitchell et al., 1973b). Increase in hepatic concentrations of glutathione by pretreatment of animals with cysteine, decreased hepatic damage and covalent binding. Pretreatment of animals with diethylmaleate to decrease glutathione levels increased covalent binding of paracetamol metabolites and potentiated hepatic necrosis (Mitchell et al., 1973b; Potter et al., 1974). Similar results have been obtained in studies of the hepatic necrosis seen with phenacetin and renal toxicity seen with thiophene and furan analogues (Mitchell et al., 1976).

Another example of a compound which induces hepatic necrosis is bromobenzene. Depletion of hepatic glutathione by bromobenzene was associated with covalent binding of a metabolite of bromobenzene (believed to be the bromobenzene-3,4-epoxide) to liver macromolecules at low glutathione levels (Jollow et al., 1974). The importance of glutathione in the protection of cellular macromolecules against drug-induced damage is illustrated by the fact that 75% of a non-toxic dose of bromobenzene (0.01 mmole/kg, i.p.) was conjugated with glutathione and excreted in urine as the mercapturic acid, whereas with a toxic dose (10 mmole/kg, i.p.) only 41% of the dose was found as a mercapturic acid in urine (Jollow et al., 1974). The protection against hepatotoxic injury by reactive metabolites of drugs seen following pretreatment with compounds such as cysteine (see above) has been exploited in the use of compounds such as cysteamine (β-mercaptoethylamine) in the successful treatment of severe cases of paracetamol overdosage in humans (Prescott et al., 1974). N-Acetyl cysteine has also been

used in this type of treatment (Golden et al., 1981). Both N-acetyl-cysteine and cysteamine probably act against paracetamol toxicity by acting as intracellular precursors of glutathione.

Glutathione also appears to protect against the hepatotoxic effect of 1,1-dichloroethylene in rats. Interestingly, since glutathione levels in rat liver show a diurnal rhythm, rats were found to be particularly sensitive to the toxic effects of 1,1-dichloroethylene when glutathione concentration was at or near its minimum (between 7 p.m. and 1 a.m.) (Jaeger et al., 1973).

Glutathione may also play a role in the protection of cells against the potentially toxic metabolites of carcinogens such as benzo(a)pyrene and aflatoxin B_1. Mutagenic epoxides of both these compounds react with glutathione to form stable conjugates, which can be excreted in bile (Chasseaud, 1976; Degen and Neumann, 1978). Indeed, the mutagenic activities of benzo(a)pyrene and aflatoxin B_1 towards Salmonella typhimurium are significantly reduced in the presence of glutathione and glutathione S-transferases (Malaveille et al., 1981). Recently, glutathione has been reported to cause regression of aflatoxin B_1-induced liver tumours in female rats when administered 16 months after the carcinogen and has been proposed as a potential antitumour drug in humans (Novi, 1981).

Of interest to the protective role of glutathione in carcinogen metabolism, is the finding that pretreatment of mice with the antioxidants 2(3)tert-butyl-4-hydroxyanisole and 2(3)-tert-butyl-4-hydroxy-toluene, caused inhibition of benzo(a)pyrene-induced neoplasia of the forestomach (Wattenberg, 1978, 1981). This inhibition is believed to be due to induction of glutathione S-transferases brought about by these

antioxidants (Benson et al., 1978, 1979).

Recently many reports on the detection of exposure to electrophilic compounds by measurement of urinary thioether concentrations have appeared (see van Doorn et al., 1981 and references therein). Glutathione conjugation results in the formation of thioethers which are excreted as premercapturic acids and mercapturic acids in urine (see later). However, whether there is any quantitative relationship between the measured thioether values in urine and the degree of exposure to electrophilic compounds is debatable and results obtained by measurement of urinary thioether levels should be assessed in the light of other non-specific exposure assays, such as a test for the presence of mutagens in urine.

1.2 vi Activation by reaction with glutathione

In the majority of reactions in which compounds react with reduced glutathione, the resultant glutathione derivatives are less pharmacologically active and less toxic than their parent compounds or metabolites i.e. glutathione conjugation is a true detoxification mechanism. However, a number of examples exist in which the reaction of glutathione with xenobiotics has been shown to lead to the production of mutagenic and potentially carcinogenic species. Recently, an increase in the pharmacological activities of endogenous fatty acids on reaction with glutathione has also been reported. Both of these types of activation have important consequences in foreign and endogenous compound metabolism.

1.2 vi (a) Xenobiotic activation

1,2-Dichloroethane (DCE) is one of the major components of waste

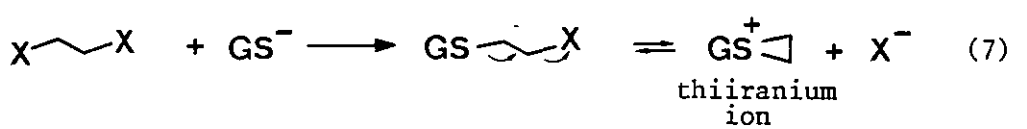
products from vinyl chloride industries. DCE gave a weak direct mutagenic effect when tested for mutagenicity on Salmonella typhimurium strain TA 1535 (Rannug et al., 1978). The mutagenic effect of DCE was enhanced in the presence of either postmitochondrial rat liver S9 supernatant or glutathione and isolated glutathione S-transferases. Furthermore, no enhancement of mutagenic activity was observed when glutathione was replaced by L-cysteine, N-acetyl-L-cysteine or 2-mercaptoethanol (Rannug et al., 1978). When the bile collected from isolated rat liver preparations perfused with DCE or following administration of DCE to mice by i.p. injection, was tested for mutagenicity towards Salmonella typhimurium strains TA 1530 and 1535, bile was found to be highly mutagenic (Rannug and Beije, 1979). In the light of their previous results and the biliary excretion of glutathione conjugates, these workers proposed that a glutathione derivative formed from DCE may be responsible for the mutagenic activity of DCE. Indeed the reaction of glutathione with DCE proceeds via the substitution of a chlorine atom by glutathione to produce a β-halogen thioether (equation (6)). Such compounds are highly reactive and have been recognized for some time for their mutagenic and carcinogenic properties. The chemical warfare agent, mustard gas ($ClCH_2CH_2SCH_2CH_2Cl$) carries the same leaving group as the product of the reaction of glutathione with DCE.

$$\underset{\text{DCE}}{Cl-CH_2-CH_2-Cl} + GSH \longrightarrow \underset{\text{a β-halogen thioether}}{Cl-CH_2-CH_2-SG} + Cl^- \qquad (6)$$

The brominated analogue of DCE, 1,2-dibromoethane (DBE), has been shown to be mutagenic towards Salmonella typhimurium (Rannug et al., 1978) and to induce squamous cell carcinomas of the stomach in rats and mice (Olson et al., 1973). The mutagenic effect of DBE required

a NADP-independent reaction similar to that of DCE (Rannug et al., 1978) and mice administered DBE by i.p. injection secreted bile which was found to be highly mutagenic (Rannug and Beije, 1979). DBE is activated as DCE to a toxic derivative by reaction with glutathione, forming a related β-halogen thioether. Interestingly, the β-halogen-thioether formed from glutathione and DBE has been shown to be detoxified by reaction with another molecule of glutathione (Reichert, 1981), illustrating both activation and detoxification by glutathione with the same substrate.

Compounds, such as DCE and DBE, with good leaving groups situated on vicinal carbon atoms are generally converted to mutagenic species via reaction with glutathione (van Bladeren et al., 1979). The mechanism involved in the formation of a reactive compound from vicinal dihalogen compounds following reaction with glutathione is believed to involve intramolecular rearrangement of the unstable β-halogen thioethers to produce a reactive thiiranium ion (equation (7)). An ethylene S-glutathionylepisulfonium ion is believed to be the reactive intermediate produced during glutathione-dependent biotransformation of 1,2-dihaloethanes to ethylene (Livesey et al., 1982).



(7)

It is the electrophilic thiiranium ion which is believed to be the species responsible for covalent binding and toxic effects of the parent compounds (van Bladeren et al., 1981). Dihalogenated compounds such as cis-1,2-dichlorocyclohexane (van Bladeren et al., 1979) and dibromo- and diiodomethane (van Bladeren et al., 1980) have been shown to form reactive electrophilic compounds on incubation with

glutathione. Glutathione has also been reported to enhance the microsomal-mediated mutagenicity of acrylonitrile towards Salmonella typhimurium strain TA 1530 (Duverger-van Bogaert et al., 1982).

1.2 vi (b) Endogenous compound activation

Only a few examples of endogenous compounds which form glutathione derivatives have been recognized (see Section 1.4 i). However, possibly the most important of the endogenous glutathione derivatives which have been identified to date are the conjugates included in the group of biologically active compounds known as leukotrienes (Samuelsson et al., 1979). This group of compounds, originally known as slow-reacting substance of anaphylaxis, are believed to be involved in allergic and anaphylactic reactions. The glutathione conjugate (leukotriene C) formed by reaction of glutathione with an epoxide metabolite of arachidonic acid (Corey et al., 1980) shows a high pharmacological activity in the guinea pig ileum assay and is under investigation for a possible role in immunological reactions such as occur in asthma and cystic fibrosis.

1.3 i Mercapturic acid formation

Mercapturic acids were first identified as metabolites of xenobiotics as long ago as 1879 when, following adminsitration of bromo- and chlorobenzene to dogs, acid-labile complexes yielding mercapturic acids were isolated from urine (Jaffe, 1879; Baumann and Preusse, 1879). The term "mercapturic acid" was coined by Baumann and Preusse (1879) who noted that the compounds in urine decomposed to thiophenols when treated with strong alkali. Since then mercapturic acids have been identified as urinary metabolites of a very large number of xenobiotics (Boyland

and Chasseaud, 1969).

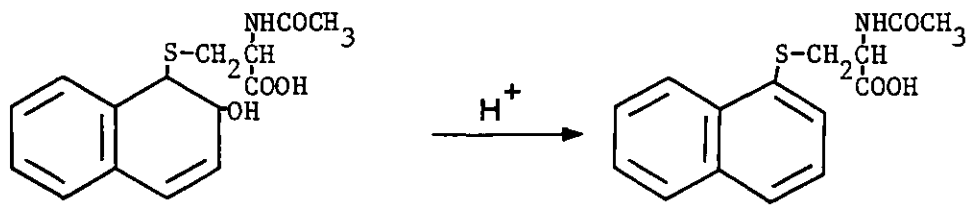
The chemical structure of the cysteine moiety of mercapturic acids was achieved by Friedman as early as 1903 (Wood, 1970) but the origin of the cysteine group remained for a long while unknown. The connection between glutathione conjugation and mercapturic acid synthesis was only made through a number of *in vivo* and *in vitro* studies. A decrease in liver glutathione levels had been observed in rabbits given bromobenzene and this decrease was related to the dose administered (Wood, 1970). Similarly in the rat a number of workers had measured decreases in liver glutathione levels following administration of mercapturic acid precursors (Barnes *et al.*, 1959; Johnson, 1965; Suga *et al.*, 1966). Administration of the glutathione conjugates derived from *p*-bromobenzyl bromide (Stekol, 1941a) and ethyl methane sulfonate (Roberts and Warwick, 1958) led to excretion of the corresponding mercapturic acids in urine. Similarly Foxwell and Young (1964) identified mercapturic acids in urine following subcutaneous administration of *S*-methyl-, *S*-ethyl- and *S*-propylglutathione to rats. More directly, following the administration of the mercapturic acid precursors, *p*-chlorobenzylchloride (Bray *et al.*, 1959a) and methyl iodide (Johnson, 1965) to rats, the corresponding glutathione conjugates could be isolated from the liver of the treated animals. Tissue homogenates of liver and kidneys were found to be effective in catalysing the breakdown of glutathione conjugates (Bray *et al.*, 1959a; Booth *et al.*, 1960), involving a "glutathionase" now known to be γ-glutamyltransferase and peptidases (see later). Further *in vitro* work led to the identification of enzymes which catalysed the conjugation of glutathione with several xenobiotics, some of which are metabolised to mercapturic acids (Booth *et al.*, 1961).

The breakdown of glutathione conjugates to mercapturic acids was considered to proceed in the order glutathione, cysteinylglycine, cysteine and N-acetylcysteine, since all of these conjugates, and not the γ-glutamylcysteine derivative could be found in the bile of rats given large amounts of naphthalene (Boyland et al., 1961), phenanthrene (Boyland and Sims, 1962), pyrene (Boyland and Sims, 1964a) and benzanthracene (Boyland and Sims, 1964b). The last step of mercapturic acid formation i.e. N-acetylation of cysteine to form the N-acetyl cysteine conjugate, was shown to occur in a number of in vitro incubations and in vivo following administration of the cysteine derivative by isolation of the N-acetylcysteine conjugate in urine (Bray et al., 1959b; Booth et al., 1960; Thomson et al., 1963; Barnsley et al., 1966).

1.3 ii Premercapturic Acids

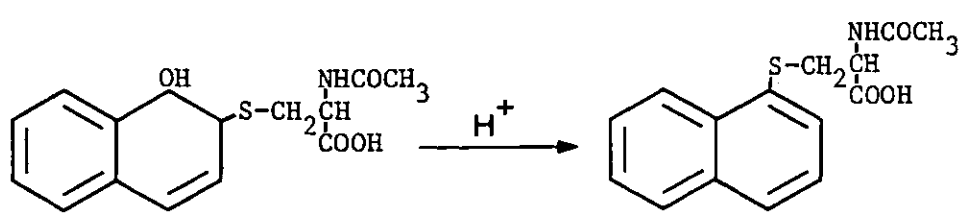
Early work involved with the excretion of mercapturic acids of foreign compounds reported by Baumann and Preusse (1879) and Jaffe (1879), indicated the presence of mercapturic acids of bromo- and chlorobenzene in the urine of dogs as acid-labile precursors. These precursors were not identified for a long time in the urine of animals administered aromatic mercapturic acid precursors because of the practice of isolating mercapturic acids by solvent extraction following acidification of urine (Wood, 1970). The term "premercapturic acid" was first coined by Knight and Young (1957, 1958) for the compounds which produced mercapturic acids on treatment of urine from animals dosed with mercapturic acid precursors with mild acid. N-Acetyl-dihydrohydroxyarylcysteine compounds could be detected following mild acid treatment of urine from animals given naphthalene and a number of substituted and unsubstituted aromatic hydrocarbons (Knight and Young, 1958).

The classic mercapturic acids of aromatic compounds were, therefore, identified as artefacts of isolation procedures. The premercapturic acid derivative of naphthalene was isolated by acidification of urine to pH 4 and absorption onto charcoal (Boyland and Sims, 1958). This compound was identified as 1,2-dihydro-2-hydroxy-1-naphthyl mercapturic acid since it formed 1-naphthyl mercapturic acid on mild acid treatment (equation (8)).



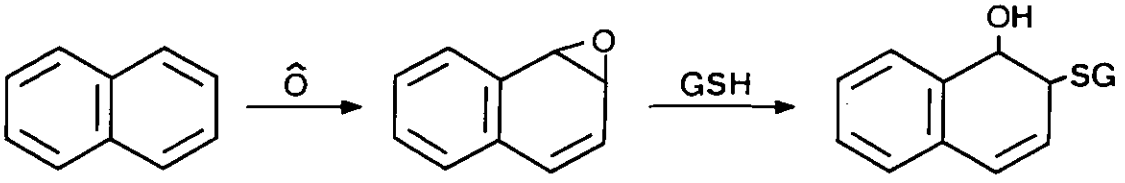
(8)

From this time premercapturic acids found in urine of animals dosed with aromatic compounds were believed to be 1,2-dihydro-2-hydroxy-1-substituted compounds and it was not until later work by Jeffery and Jerina (1975) that it was realised that the position of the hydroxy group and xenobiotic moiety had been wrongly assigned and were in fact reversed i.e. were 1,2-dihydro-1-hydroxy-2-substituted compounds. Migration of the xenobiotic substituent occurred on acid treatment and aromatization resulting in the formation of the corresponding 1-arylmercapturic acid (equation (9)).



(9)

Premercapturic acids are formed from aromatic compounds due to the reaction of glutathione with an epoxide intermediate (equation (10); Jerina et al., 1970).



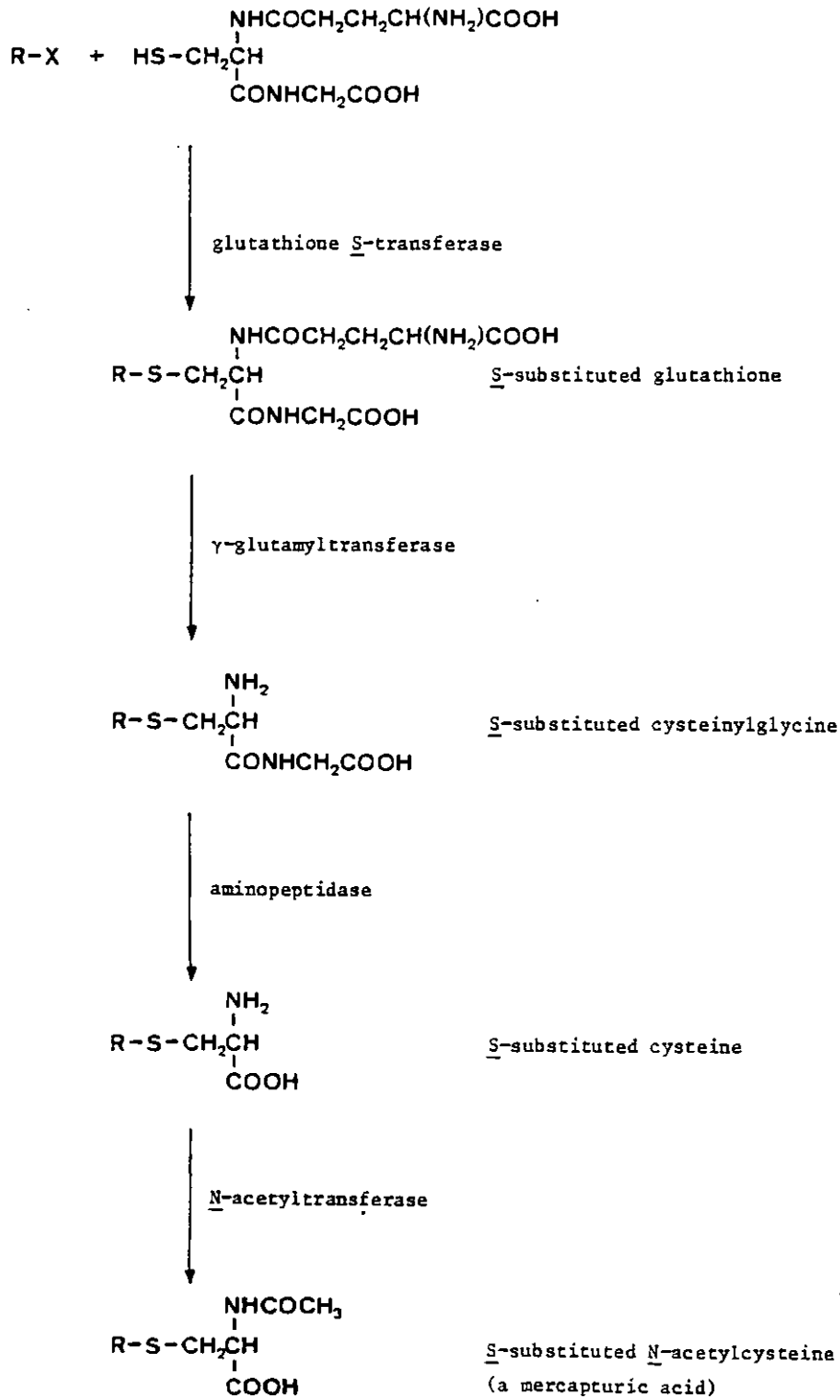
(10)

Studies with the epoxides derived from aromatic compounds such as benzo(a)pyrene (Hernandez et al., 1980), styrene (Pachecka et al., 1979) and bromobenzene (Monks et al., 1982) are consistent with the proposed mechanism of enzyme-catalysed conjugations with glutathione which involves a direct attack of the nucleophilic sulphur atom of glutathione on the electrophilic centre of the epoxide. Usually two positional isomeric conjugates result from nucleophilic attack of glutathione on epoxides and the glutathione is trans to the hydroxyl group. It is now believed that all aromatic compounds which form glutathione conjugates do so via the pathway outlined in equation (10) viz. parent ⟶ epoxide ⟶ dihydrohydroxyglutathione conjugate. Subsequent metabolism of S-dihydrohydroxyaryl glutathione derivatives by the enzymes of mercapturic acid synthesis (see later) produces the corresponding premercapturic acids. Premercapturic acids are the true excretion products in the metabolism of aromatic compounds.

1.4 Enzymology of the mercapturic acid pathway

The evidence acquired by systematic studies into the involvement of glutathione in the formation of mercapturic acids (Wood, 1970) has resulted in the formulation of a scheme as outlined in Figure 1.2. Thus, a xenobiotic possessing a suitably electrophilic centre will react with glutathione, usually in a reaction catalysed by the

FIGURE 1.2 Mercapturic acid pathway



glutathione S-transferases. Subsequent hydrolysis of the glutathione S-conjugate occurs to form the S-cysteinylglycine and S-cysteinyl derivatives. These two reactions are catalysed by the enzymes γ-glutamyltransferase and aminopeptidases, respectively. Finally, N-acetylation of the xenobiotic S-cysteine conjugate results in the formation of a N-acetylcysteine derivative i.e. a mercapturic acid. This final reaction is catalysed by a N-acetyltransferase which utilises acetyl coenzyme A as the acetyl donor. The enzymes which are involved in the synthesis and degradation of glutathione S-conjugates will now be discussed.

1.4 i Glutathione S-transferases

Although there is, with some substrates, considerable non-enzymic reaction between reduced glutathione and electrophilic compounds, the major proportion of glutathione conjugates are formed in reactions catalysed by the glutathione S-transferases, a group of fairly selective multifunctional proteins (Chasseaud, 1979; Jakoby and Habig, 1980). These enzymes are responsible for a relatively large proportion of the proteins in the cell and it has been estimated that they represent approximately 10% and 2% of the soluble fraction of rat and human liver, respectively (Jakoby et al., 1976; Habig et al., 1976a). A large body of work on these enzymes over the last 20 years has provided us with a fairly detailed description of these enzymes with respect to their localisation, physical properties, distribution and physiological functions. A number of reviews on these enzymes have been published (Boyland and Chasseaud, 1969a; Chasseaud, 1974; Jakoby, 1978; Jakoby and Habig, 1980).

1.4 i (a) Nature of glutathione S-transferases

Early work on the nature of the glutathione S-transferases focussed on the types of second substrate involved and these studies indicated a multiplicity of transferases (Chasseaud, 1974). Thus, terms such as glutathione S-aryltransferase (Booth et al., 1961; Grover and Sims, 1964), S-epoxidetransferase (Boyland and Williams, 1965), S-alkyltransferase (Johnson, 1966), S-alkenetransferase (Boyland and Chasseaud, 1969b) were used to signify the enzymes on the basis of the type of second substrate involved. Purification of these enzymes to homogeneity (Fjellstedt et al., 1973; Pabst et al., 1973; Habig et al., 1974a; Askelöf et al., 1975) showed a number of glutathione S-transferases possessing broad and overlapping second substrate specificities (Ketley et al., 1975; Habig et al., 1976a; Jakoby et al., 1976a). The major work on the identification of the transferases has been undertaken using rat liver forms of the enzymes, although information on forms from other tissues and from human studies is now available.

Rat forms

Most of the glutathione S-transferases isolated from rat liver possess an alkaline isoelectric point and have been resolved into six distinct species on carboxymethylcellulose (Habig et al., 1974a). These proteins were named glutathione S-transferase AA, A, B, C, D and E on the bases of the reverse order in which they eluted during this procedure.

Purification of all of the cationic transferases, except D, from rat liver (Fjellstedt et al., 1973; Habig et al., 1974a; Pabst et al., 1974; Jakoby et al., 1976) has led to characterisation of some of

the physical properties of the enzymes. Although the separate transferases were isolated from different liver preparations, all of the transferases have been isolated from a single rat liver (Habig et al., 1976b). The transferases are proteins with a molecular weight of approximately 47000 daltons and are composed of two subunits of approximate equal molecular weight.

In 1974, glutathione S-transferase B was shown to be identical to ligandin (Habig et al., 1974b), the organic anion-binding protein present in the Y fraction of rat liver (Litwack et al., 1971; see later). Ligandin, molecular weight 46000, was believed to exist as a dimer composed of two identical subunits, molecular weight 23000 (see Arias et al., 1976). However in 1976 ligandin was shown to exhibit two non-identical subunit species on discontinuous polyacrylamide gel electrophoresis in sodium dodecylsulphate (Litowsky et al., 1976). These subunits were further investigated and the sulphobromophthalein-binding fraction of rat hepatic cytosol was shown to contain three major subunit bands designated subunits Ya (M.W. 22000), Yb(M.W. 23500) and Yc (M.W. 25000) in ascending order of size (Bass et al., 1977). This led to the proposal that the subunits of ligandin (Ya and Yc) were in fact monomers of two distinct proteins, YaYa and YcYc(Bass et al., 1977). Ligandin was also resolved into two proteins possessing glutathione S-transferase activity.

Carne et al.

(١٩٨٩) showed that ligandin consisted of two proteins of subunit composition YaYa and YaYc in contrast to the supposition of Bass et al. (1977). Despite strong similarities in structure and immunology showing a close relationship between the Ya and Yc subunits (Bhargawa et al., 1980), different, discrete functions could be ascribed to

each subunit. Thus, the high affinity binding of cholic acid (Hayes et al., 1980) was particularly associated with the Ya subunit (Hayes et al., 1981; Bhargawa et al., 1980).

The Ya subunits of YaYa and YaYc glutathione S-transferases are identical when analysed by tryptic digestion mapping, as are the Yc subunits of YcYc and YaYc transferases (Hayes et al., 1981; Beale et al., 1982). Subunit c had been observed to be converted into subunit a by limited proteolysis (Hayes et al., 1979). This latter finding, together with the fact that transferase AA is a homodimer of subunit Yc (Scully and Mantle, 1980), led to the formulation of a proteolytic model for the generation of multiple forms of the transferases, in which a primary gene product (YcYc) was converted into other transferases (e.g. YaYc or YbYb) (Scully and Mantle, 1981). However, no experimental evidence has been presented to support this model and recent immunological and enzymic digestion evidence is inconsistent with this scheme.

The immunological relationships between the subunits of the glutathione S-transferases has been investigated by Kitahara and Sato (1981). Thus, ligandin (YaYa), transferase B (YaYc) and transferase AA (YcYc) all share common antigenic determinants, but transferase A (YbYb) had no immunological relationship with the other three forms (Kitahara and Sato, 1981). Tryptic digestion of Yaand Yc subunits as YaYc, YaYa and YcYc (formed by rehybridization of YaYc following dissociation in 7 M urea, Hayes et al., 1981), indicated that these subunits were the products of at least two different but related genes (Beale et al., 1982).

Most recently six forms of the basic glutathione S-transferases have

been classified into two groups based on their reaction to antibodies to glutathione S-transferase B and to glutathione S-transferase C (Mannervik and Jensson, 1982). The proteins in the first group are composed of the two subunits Ya and Yc in the three possible combinations; the three proteins in the second group consist of subunits Yb and Yb' Thus, glutathione S-transferases A, AA, B and C correspond to the dimers YbYb, YcYc, YaYc and YbYb', respectively. The relative specific activities obtained with different substrates distinguished the various forms from each other. Thus, the Ya subunit has high transferase activity with Δ-androsten-3,17-dione and p-nitrophenylacetate and low activity with ethacrynic acid: subunit Yc has opposite activities; the heterodimer YaYc has activities between those of the dimers YaYa and YcYc (Mannervik and Jensson, 1982). These workers proposed the latest system of nomenclature for the transferases in which the subunits Ya, Yb, Yb' and Yc are designated L, A, C and B and the enzymes denoted as glutathione S-transferases A_2, BL etc. according to subunit composition (Table 1.3).

Human forms

Five cationic transferases similar to those found in rat liver have been identified from human liver and are named α, β, γ, δ, and ε on the basis of their elution from carboxymethylcellulose (increasing isolectric points) (Kamisaka et al., 1975a; Habig et al., 1976a). These enzymes also possess an alkaline isoelectric point and are dimeric proteins of 49000 molecular weight. Acidic transferases have been reported in human erythrocytes (transferase ρ; Marcus et al., 1978) and human placenta (transferase π; Guthenberg et al., 1979). Recent evidence (Guthenberg and Mannervik, 1981) indicates that these two enzymes may be identical. Two other anionic transferases (ω, pI4.6

TABLE 1.3 Classifications used for the nomenclature of the basic glutathione S-transferases of rat liver cytosol

Nomenclature of Boyland and Chasseaud, 1969[1]	Nomenclature of Jakoby and Habig, 1980[2]
aryl	AA
alkyl	A
aralkyl	B, ligandin
alkene	C
epoxide	D
	E

Nomenclature of Bass et al., 1977 and Hayes et al., 1981[3]	Nomenclature of Mannervik and Jensson, 1982[4]
YaYa	L_2
YaYc	BL
YcYc	B_2
YbYb	A_2
Yb'Yb	AC
Yb'Yb'	C_2

(1) Classified on the basis of the carbon skeleton of the electrophilic molecule or specific leaving group involved.

(2) Classified on the basis of reverse order of elution from carboxy-methylcellulose.

(3) Classified on the basis of subunit composition by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

(4) Classified on the basis of subunit composition by affinity chromatography and chromatofocusing.

and ψ, pI 5.4), apparently different from the erythrocyte enzyme, have been isolated from human liver (Awasthi et al., 1980). These are immunologically related to the cationic forms, but differ in that they do not possess glutathione peroxidase activity as seen in the cationic forms (Awasthi et al., 1980).

The human liver transferases are obviously very closely related, since their amino acid compositions are almost identical (Jakoby, 1978). It has been proposed that all the cationic liver transferases are the products of a single gene (Kamisaka et al., 1975a) and the differences between them are a reflection of differential deamidation (Habig et al., 1976b). The interrelationships and functions of the different human glutathione S-transferases are an area of great interest at present.

1.4 i (b) Mode of action

In contrast to other major conjugation processes, such as sulphation or glucuronidation (see Parke, 1968), glutathione conjugation does not require the initial formation of a high energy intermediate involving ATP, although synthesis of glutathione from its component amino acids (Meister, 1975) and N-acetylation of cysteine conjugates (Green and Elce, 1975) do utilize ATP.

As stated earlier (Section 1.2 iii), the reactivity of glutathione is due to the inherent nucleophilicity of the thiolate, GS^-, anion. Thus, the rate of the nonenzymic reaction of glutathione with a second substrate increases in a parallel fashion to the increase in the proportion of glutathione ionised as a function of pH (Chasseaud, 1974). The glutathione S-transferases act by specifically binding

glutathione and lowering the pKa of the thiol group (pKa9.2), making it a stronger nucleophile at physiological pH (Jakoby, 1978). More importantly the transferases bind any of a large number of second substrates at a locus, characterised by hydrophobicity, adjacent to the glutathione binding site (Keen et al., 1976). Compounds possessing sufficiently reactive electrophilic centres and which are capable of binding to the second site are subject to nucleophilic attack by bound glutathione and are released as thioethers or other sulphur adducts. The only apparent specificity encountered is for the nucleophile, glutathione. L-Cysteine, 2-mercaptoethylamine, dithiothreitol and N-acetyl-L-cysteine do not replace glutathione as substrate in any of the reactions tested (Fjellstedt et al., 1973, Keen et al., 1976).

1.4 i (c) Cellular localisation

Early work on the purification of glutathione S-transferases from rat and human liver showed that they were a group of cytosolic enzymes (see Jakoby et al., 1976; Habig et al., 1976a) and little or no activity was noted in other organelles. Booth et al. (1961) had detected low activities of transferases wtih 3,4-dichloronitrobenzene in nuclear, mitochondrial and microsomal fractions of rat liver. Glatt and Oesch (1977) pointed out that the transferases imposed a severe limitation by their localisation in the cytosol in the detoxication of microsome-produced benzo(a)pyrene-4,5-oxide and other reactive metabolites. Subsequent work led to the finding that microsomal glutathione S-transferase activity was present in rat liver and represented approximately 10% of the soluble transferase activity (Morgenstern et al., 1980a). This activity was shown not to be associated with cytosolic contamination nor with ribosomes (Morgenstern et al., 1980b) and the transferases were firmly bound as integral

membrane proteins.

Studies of the microsomal enzymes showed a similar pattern on isoelectric focussing to that found in the cytosol (Friedberg et al., 1979). However, significant differences have been found in the induction patterns of the enzymes from the two sources. Phenobarbital increased cytosolic glutathione S-transferase activity in rat liver (Habig et al., 1974b), but this pretreatment had no effect on the microsomal activity (Friedberg et al., 1979). Conversely, sulphydryl reagents, such as N-ethylmaleimide, specifically increased the activity of the microsomal enzymes to approximately 25-30% of the total transferase activity of rat liver towards 1-chloro-2,4-dinitrobenzene (Morgenstein et al., 1979), a treatment which has no effect on the cytosolic enzymes. These differences in the induction patterns of the enzymes from the two fractions may reflect differences in the presence of different glutathione S-transferases (Mukhtar et al., 1981).

Friedberg et al. (1979) has pointed out that the microsomal activity may be important in the control of reactive metabolites produced in the microsomal membranes by the cytochrome P-450 system. These metabolites are hydrophobic and would preferentially stay dissolved in the endoplasmic reticulum. Glutathione S-transferases located in this organelle are ideally suited to catalyse the conjugation of potentially reactive drug oxidation products.

1.4 i (d) Tissue distribution

As was stated earlier, glutathione is a ubiquitous compound and, not surprisingly, the glutathione S-transferases are found in almost every tissue so far examined (Chasseaud, 1979). Thus, although early

work on the transferases was concerned with the liver, activity has been examined in some detail in almost all mammalian tissues. In general the activity of the transferases has been found to be higher in the liver when compared with other tissues of the same species (Table 1.4). The glutathione S-transferase activity towards 1,2-dichloro-4-nitrobenzene has been reported to be 20- and 35-fold higher in rat liver when compared with kidney and lung, respectively (Litterst et al., 1975).

TABLE 1.4 Glutathione S-transferase activity towards styrene oxide in several tissues of rat and rabbit*

Tissue	Rat	Rabbit
Liver	142	31
Lung	12	6.5
Intestine	13	4.4
Kidney	82	7.9

* Specific activity expressed an nmol conjugate/mg protein/min
From Jerina and Bend (1977)

Tissue distributions of various types of glutathione S-transferases in rat and humans have revealed important differences in isoenzyme type. Different substrate specificities of the transferases in different tissues may have important consequences with regards to toxicity of some compounds in certain cell types.

1.4 i (e) Substrates of glutathione S-transferases

A very wide range of compounds exist which react with glutathione in the absence and/or presence of glutathione S-transferases. As stated previously, the predominant feature of reactive compounds is the possession of a sufficiently electrophilic site which acts as a centre for attack by the nucleophilic glutathione. Thus, glutathione has been demonstrated to react with compounds which include synthetic chemicals, Phase I biotransformation products and naturally occurring and endogenous compounds.

Exogenous compounds

Compounds originating from outside the human body be they synthetic chemicals, environmental pollutants or compounds found naturally in plants, form the largest and best studied group of compounds with respect to glutathione conjugation. A large range of such compounds possessing sufficiently strong electrophilic centres exist (Chasseaud, 1979). Nucleophilic attack by glutathione usually originates at carbon centres but attack at other sites such as nitrogen (e.g. nitrate esters), sulphur (e.g. organic thiocyanates) and oxygen (e.g. peroxides) atoms has been observed (Jakoby and Habig, 1980).

Endogenous compounds

Most substrates which have been identified to react with glutathione are exogenous in origin and until recently, few endogenous compounds were recognized which formed glutathione conjugates. A number of compounds involved in steroid and prostaglandin metabolism have been identified as glutathione conjugates. Incubation of oestradiol-17β

with glutathione in the presence of rat liver supernatant resulted in glutathione S-conjugates which were identified as 1- and 4-glutathione mono and diethers of 2-hydroxyoestradiol-17β and 2-hydroxyoestrone (Kuss, 1969, 1971). Glutathione conjugates of 2-hydroxyoestradiol-17β were excreted in rat bile following intraperitoneal administration of the parent steroid (Elce and Harris, 1971). When one of the glutathione conjugates found in bile, namely, S-(2-hydroxyoestradiol-1-yl) glutathione, was administered intraperitoneally to rats, this conjugate together with the cysteinylglycine, cysteine and N-acetylcysteine derivatives were found in bile, and the N-acetylcysteine derivative was identified in urine (Elce, 1971). An oestrogen glutathione conjugate was reported as a urinary metabolite in rats following large doses of 3-deoxyoestrone (Nambara and Numazawa, 1971).

The cyclopentenone moiety of prostaglandin A_1 has been shown to be subject to attack by reduced glutathione and two glutathione conjugates of prostaglandin A_1 have been identified in human red blood cells (Cagen et al., 1976). These reactions are also catalysed by homogenous hepatic glutathione S-transferases (Cagen et al., 1975). Another compound possessing an α,β -unsaturated ketone group, 15-keto-prostaglandin $F_{2\alpha}$ also formed a glutathione conjugate on incubation with glutathione or with guinea pig liver cytosol (Chaudhari et al., 1978).

One of the most important endogenous glutathione conjugates identified to date has been that reported in the identification of the leukotrienes. These compounds are a recently discovered group of biologically active compounds derived from polyunsaturated fatty acids (Samuelsson et al., 1979). The group comprises "slow reacting substance" of anaphylaxis, a presumed mediator of allergic and anaphylactic reactions (Austen, 1978).

The structure of a slow reacting substance from murine mastocytoma cells, leukotriene C, has been identified as a glutathione conjugate derived from arachidonic acid and has the structure 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid (Hammarström et al., 1979, 1980). Leukotriene C is converted to the cysteinylglycine derivative, leukotriene D, a compound identified as a slow reacting substance from rat basophilic leukemia cells, (Örning et al., 1980). Leukotriene D has a greater biological activity on guinea pig ileum on a molar basis than leukotriene C (Örning et al., 1980).

1.4 i (f) Ligandin

The glutathione S-transferases have been shown to bind a number of lipophilic compounds which are not substrates (Smith and Litwack, 1980). Early work had established the presence of a major protein from rat liver 100000 g supernatant fraction which had independently been named azodye carcinogen-binding protein (Ketterer et al., 1967), corticosteroid binder I (Morey and Litwack, 1969; Singer et al., 1970) and Y protein (Levi et al., 1969). It was subsequently realised that all three groups were dealing with the same protein, which was termed ligandin on the basis of its binding properties (Litwack et al., 1971). Ligandin was shown to possess glutathione S-transferase activity and was believed to be identical to glutathione S-transferase B (Habig et al., 1974b). Separation of ligandin and glutathione S-transferase B has been demonstrated on the basis of lithocholic acid-binding (Hayes et al., 1979). Glutathione S-transferase B exists as a pair of related transferases with subunit composition YaYa and YaYc and these findings have suggested an abandonment of the term ligandin, the transferases being identi-

fied by their subunit composition (Hayes et al., 1980, Section 1.4 i (a)).

The demonstration that glutathione S-transferase B bound a number of non-substrate ligands has been extended to include the other transferases (Ketley et al., 1975). Thus, all of the transferases tested were capable of binding a diverse group of ligands, including bilirubin, indocyanine green, hematin, 3,6-dibromosulphophthalein and cephalothin.

Non-covalent binding

The glutathione S-transferases bind a number of non-substrate ligands often with high affinity (Table 1.5). One of the best studied interactions is the binding between the transferases and bilirubin. Bilirubin has been shown to bind to rat ligandin primarily at a single high affinity binding site with an association constant of 0.7 μM (Kamisaka et al., 1975b). Following the separation of glutathione S-transferase B into YaYa and YaYc proteins, it was apparent that the Ya subunit possessed a high affinity binding site for bilirubin. Hence, the YaYa homodimer bound two molecules of bilirubin/molecule of protein, whereas the herodimer YaYc bound only one molecule bilirubin/molecule of transferase (Bhargawa et al., 1980). Further studies, with purified glutathione S-transferases A and B, indicated the presence of a secondary low-affinity binding site for bilirubin possessing a dissociation constant of 5 and 8 μM for transferases A and B respectively.

The secondary, nonspecific site

TABLE 1.5 Compounds which bind to the glutathione S-transferases (ligandins) as non-substrates

Non-covalently bound:-

azo dye glutathione conjugate
bilirubin
bilirubin glucuronide
bromsulphthalein
bromsulphthalein glutathione conjugate
cephalexin
cephalothin
cholecystographic agents
corticosteroids and metabolites
dibromsulphthalein
ethacrynic acid
glutathione
hematin
indocyanine green
methylcholanthrene
nitrofurantoin
pencillin
phylloerythrin
probenecid
rose bengal
tetracyclines
triiodothyronine and tetraiodothyronine
vasoflavine
various sulphonamides and fatty acids

Covalently bound:-

1-chloro-2,4-dinitrobenzene
ethacrynic acid
methylcholanthrene metabolite
N,N-dimethyl-4-aminoazobenzene

is part of the transferase-catalytic site. No cooperative interactions were apparent between the two sites.

Covalent binding

Ligandin was first isolated from rat liver as a protein which covalently bound a metabolite of the hepatocarcinogenic azo dye 4-dimethyl-aminoazobenzene (Ketterer et al., 1967). Since that time ligandin has been shown to interact covalently with several compounds including 3-methyl-cholanthrene metabolites (Singer and Litwack, 1971) and ethacrynic acid (Yamada and Kaplowitz, 1980).

Some of these compounds are carcinogens and the covalent binding of certain carcinogens to ligandin may play a key role in carcinogen metabolism. N,N-dimethyl-4-aminoazobenzene is believed to bind covalently to the sulphydryl groups of cysteines in the ligandin molecule (Ketterer and Christodoulides, 1969; Ketterer et al., 1976).

1.4 i (g) Importance of ligand binding by the transferases

The glutathione S-transferases possess a dual function with respect to drug metabolism. Firstly, the transferases act in a catalytic manner to catalyse the formation of mercapturic acid precursors, namely, glutathione conjugates. These conjugates usually possess less reactivity towards cellular molecules than their parent compounds and thus are detoxification products. This function has previously been described. Secondly, the transferases bind a broad spectrum of lipophilic non-substrate ligands. By reason of their propensity for binding, the transferases have been proposed

to serve as storage or transport proteins in hepatocytes (Arias et al., 1976; Wolkoff et al., 1979). The need for storage is illustrated by bilirubin, a toxic compound which is poorly soluble in physiological solutions. Bilirubin is transported in plasma as a complex with albumin, but in the hepatocyte, where albumin is not available, bilirubin would sediment if the transferases were not available for binding. This binding allows for quite large intracellular concentrations of bilirubin to exist (Wolkoff et al., 1979). Evidence for a transport "receptor" function for the transferases is more difficult to obtain, but have resulted from observations that renal and hepatic cells selectively transport compounds which bind to the transferases in these cell types (see Kaplowitz, 1980). Low levels of binding and reduced transport are seen in newborn animals (Hales and Neims, 1976), whilst phenobarbital-treated animals have both increased binding and transport (Kaplowitz and Clifton, 1976). However, no direct observations regarding a clear-cut uptake receptor function for the transferases are available and the true role of binding to transferases in uptake and sequential intracellular binding vs. storage remains to be more fully defined.

The significance of the covalent binding of non-substrate ligands by the transferases is unclear. Since the transferases account for 5-10% of the cytosolic protein of the hepatocyte (Habig et al., 1974b), the suggestion has been made that a large reservoir exists in the cell to serve a "scavenger" function by which reactive metabolites may be detoxified by covalent binding to the transferases (Jakoby et al., 1976). Covalent binding of reactive compounds, including carcinogens, by the transferases suggests a pathway for the irreversible detoxification of such compounds, although it is not clear whether covalent binding

occurs prior to or following denaturation of the protein (see Smith and Litwack, 1980). The transferases may also have a further role in the protection against cellular damage by reactive electrophiles, including certain carcinogens, by their propensity to bind a number of steroids which may affect the metabolism of reactive compounds (Smith and Litwack, 1980).

1.4 ii γ-Glutamyltransferase

γ-Glutamyltransferase (γ-glutamyltranspeptidase) (EC 2.3.2.2.) is the only known enzyme capable of catalysing the hydrolysis of the γ-glutamyl linkage of glutathione. The enzyme catalyses three types of reactions, hydrolysis (equation (11)), transpeptidation (equation (12)) and autotranspeptidation (equation (13)).

Hydrolysis

$$\gamma\text{-Glu-X} + H_2O \longrightarrow \text{glutamate} + HX \qquad (11)$$

Transpeptidation

$$\gamma\text{-Glu-X} + \text{acceptor} \rightleftharpoons \gamma\text{-glu-acceptor} + HX \qquad (12)$$

Autotranspeptidation

$$\gamma\text{-Glu-X} + \gamma\text{-glu-X} \rightleftharpoons \gamma\text{-glu-}\gamma\text{-glu-X} + HX \qquad (13)$$

X represents cysteinylglycine in glutathione and S-derivatives of cysteinylglycine in glutathione S-conjugates. A variety of amino acids and dipeptides can serve as acceptors of the γ-glutamyl moiety in transpeptidation (Meister, 1975; Meister and Tate, 1976).

The distribution of γ-glutamyltransferase activity in nature has been studied by a number of investigators. Table 1.6 shows the tissue distribution of γ-glutamyltransferase activity in a number of rat tissues. Kidney has the maximum activity in tissues of rat and other

TABLE 1.6 Distribution of γ-glutamyltransferases in rat tissues

Tissue	Activity[a]	
	units/mg protein	% kidney activity
Kidney		
Cortex	2.68	-
Medulla	2.15	-
Epididymus		
Caput	0.64	26.5
Cauda	0.09	3.7
Seminal veside	0.10	4.1
Jejunal epithelial cells		
Crypt	0.02	0.8
Villus tip	0.08	3.3
Pancreas	0.48	19.9
Liver	0.005	0.2
Spleen cells	0.005	0.2

(a) Activity determined using L-γ-glutamyl-p-nitroanilide and glycylglycine

From Tate (1980)

mammals (Revel and Ball, 1959; Goldbarg et al., 1960). High γ-glutamyltransferase activity is associated with cells with a secretory or absorptive function. Cells such as pancreatic acinar and ductile epithelial cells and epithelial cells of renal proximal tubule, jejunum, bile duct, epididymus and a number of other tissues have been shown to contain high enzyme activities (Rosalki, 1975). Histochemical localisation of γ-glutamyltransferase using N-(γ-L-glutamyl) β-naphthylamide has revealed uneven distribution of activity in cell types of liver and jejunum (Glenner et al., 1962). These histochemical techniques have indicated a cellular localisation of γ-glutamyltransferase in brush border membranes, a site supported by findings in subcellular fractionation of rabbit kidney (George and Kenny, 1973; Booth and Kenny, 1974), rat small intestine (Curthoys and Shapiro, 1975) and rat kidney (Hughey et al., 1978). Other evidence at the electron microscopic level and experiments using enzyme digestion and immunological techniques are in accord with the location of γ-glutamyltransferase on the outer surface of cell membranes (Tate, 1980). Localisation of the enzyme in brush border membranes and its orientation within that membrane is important in the metabolism of glutathione (see below).

Preparations of γ-glutamyltransferase from mammalian tissues show considerable heterogeniety of polyacrylamide gel electrophoresis (Tate and Meister, 1976). 12 distinct species, with isoelectric points in thepH range 5-8 have been identified. The isozymes appear to be dimeric proteins of molecular weight approximately 68000, consisting of two non-identical subunits, molecular weights 46000 and 22000. All of the detergents-solubilised forms of the enzyme appear to contain appreciable amounts of carbohydrate (Tate and Meister, 1976). However, molecular weight and carbohydrate composition

etc. tend to be a function of the source of the enzyme and the isolation technique employed (Tate, 1980).

γ-Glutamyltransferase catalyses two fundamental types of reaction, hydrolysis and transpeptidation. Obviously hydrolysis and transpeptidation depend to an extent on the presence of an acceptor, but also on pH. Different, broad, pH optima exist for the two processes, hydrolysis occurring maximally at pH 6-8, transpeptidation at 8-9 (Tate and Meister, 1974; McIntyre and Curthoys, 1979). It generally appears that hydrolysis and not transpeptidation is the major reaction catalysed by γ-glutamyltransferase *in vivo* (McIntyre and Curthoys, 1979).

The importance of γ-glutamyltransferase in the present discussion is its physiological function with respect to glutathione metabolism and its involvement in the metabolism of glutathione *S*-conjugates. Since γ-glutamyltransferase is the only known enzyme capable of hydrolysing the γ-glutamyl moiety of glutathione, it would perhaps be expected that this enzyme also catalyses the first step in the hydrolysis of glutathione *S*-conjugates to mercapturic acids. Recent studies with isolated cell preparations and perfusion techniques provide evidence for the participation of the transferase in the degradation of glutathione *S*-conjugates en route to mercapturic acid formation. Thus, isolated kidney and small intestinal epithelial cells rapidly hydrolysed a paracetamol glutathione conjugate in a process involving γ-glutamyltransferase (Jones *et al.*, 1979; Grafström *et al.*, 1980). Data from perfused organs and *in vivo* studies have indicated that the γ-glutamyltransferase of kidney is the primary site of glutathione and glutathione *S*-conjugate hydrolysis (Wendel *et al.*, 1978; Hahn *et al.*, 1978; Inoue *et al.*, 1982). Thus, isolated kidney cells possess much higher γ-glutamyltransferase activities than hepatocytes or intestinal cells

(Grafström et al., 1979) and consequently metabolised glutathione and a glutathione S-conjugate at a faster rate than isolated liver or small intestinal cells (Moldéus et al., 1978; Grafström et al., 1980). Also, following administration of radiolabelled glutathione or a glutathione S-conjugate to mice, radioactivity accumulated primarily (within 1 to 2 min) in the kidney as cysteine or as a cysteine S-conjugate (Hahn et al., 1978; Inoue et al., 1982).

1.4 iii Aminopeptidases

It seems beyond doubt that the removal of the γ-glutamyl moiety of glutathione and its derivatives is catalysed in vivo by γ-glutamyl transferase. Less certainty exists with regards to the enzyme or enzymes responsible for the hydrolysis of cysteinylglycine and its S-substituted derivatives. A number of exopeptidases exist which exhibit broad substrate specificities and which possess the potential to cleave cysteinyl peptides. These peptidases, both intracellular and plasma membrane-bound, have only in a very few cases being tested for activity towards S-substituted cysteinylglycines (Tate, 1980).

Aminopeptidases with the capacity of catalysing the hydrolysis of S-cysteinylglycine derivatives in the mercapturic acid synthesis pathway are fairly widespread in mammalian tissues. For example, dipeptidases exist in the cytosolic, lysosomal and microsomal fractions of several tissues (McDonald and Schwabe, 1977). However, the only studies involving the metabolism of mercapturic acid precursors are with the forms of the enzyme from rat kidney. A particulate peptidase from the outer stripe region of rat renal medulla tissue was assayed with S-benzyl cysteine-p-nitroanilide (Hughey et al., 1978). Further fractionation of this enzyme activity revealed its presence, along

with that of γ-glutamyltransferase, in brush border membranes. The purified enzyme showed a high activity towards a range of S-substituted cysteinylglycine substrates. For example, the activity with S-benzylcysteinylglycine was 4x that seen with cysteinylglycine itself (Hughey et al., 1978). No activity was observed with reduced glutathione. The peptidase activity seen with S-benzylcysteine-p-nitroanilide was similar to that observed for aminopeptidase M isolated from rabbit kidney brush border membranes (Green and Kenny, 1973). Recent investigations of the renal catabolism of glutathione has revealed the presence of a second aminopeptidase activity (with cysteinylglycine), separate from aminopeptidase M, in rat proximal tubule brush borders (McIntyre and Curthoys, 1982). Cooperation between the two forms of aminopeptidases resulted in the total hydrolysis of cysteinylglycine.

Interestingly the aminopeptidase in rat renal brush border preparations appears to share a similar topological distribution to that of γ-glutamyltransferase and is believed to act, as the transferase, on external substrates (Okajima et al., 1981). The close proximity of the two enzymes may indicate that they act in sequence whereby the products of the transferase are utilised rapidly by the peptidases. This structural integrity of the two enzymes may be an explanation of the reason why few cysteinylglycine S-conjugates of xenobiotics have been identified as in vivo metabolites relative to cysteine derivatives.

1.4 iv N-Acetyltransferase

N-Acetyltransferase is the least understood enzyme in the mercapturic acid pathway. It catalyses the transfer of an acetyl group from

acetylcoenzyme A to S-substituted cysteines (Equation (14)).

$$\text{cysteine-S-conjugate} + \text{acetylcoenzyme A} \longrightarrow \text{N-acetylcysteine-S-conjugate} + \text{CoA} \qquad (14)$$

Early studies indicated that liver and kidney slices had the capacity to catalyse the N-acetylation of S-substituted cysteines (see Wood, 1970). The enzyme is associated with the microsomal fraction of rat liver and kidney (Green and Elce, 1975), although it differs from γ-glutamyltransferase and aminopeptidases by localisation in the endoplasmic reticulum rather than in brush border membrane (Hughey et al., 1978). The microsomal enzyme differs from the soluble N-acetyltransferases known to exist for glutamate, aspartate and various amines (Green and Elce, 1975) and the only known physiological role which so far can be ascribed to the microsomal enzyme is in mercapturic acid formation. The microsomal enzyme is specific for acetyl coenzyme A as the acetyl group donor, although the acceptor specificity appears to be somewhat broader. The relative activities of the rat liver enzyme towards some S-substituted cysteines were as follows: S-benzyl-L-cysteine 100, S-butyl-L-cysteine 88, S-diphenylmethyl-L-cysteine 76, S-benzyl-D-cysteine 17 (Green and Elce, 1975). Similar relative activities exist in kidney. The specific activity of the enzyme form found in rat kidney was higher than that of liver, although total activity was higher in liver than in kidney (Green and Elce, 1975).

1.5 Metabolic degradation of mercapturic acids

Premercapturic and mercapturic acids have been considered to be end products of metabolism, although evidence exists for their further metabolism. The major metabolic reactions these acids may undergo in vivo include deacetylation, cleavage of the thioether

linkage and sulphur oxidation. The detection of such metabolic processes *in vivo* does not necessarily give any information on their relative importance and in most cases these reactions have been reported as only minor pathways of xenobiotic metabolism.

1.5 i Deacetylation

The final reaction in mercapturic acid synthesis is N-acetylation of S-substituted cysteines catalysed by N-acetyltransferase, a reaction which is irreversible. Evidence that a system may exist *in vivo* for the reverse of this reaction i.e. deacetylation, was found following administration of mercapturic acids to animals. Parke and Williams (1951) reported that rabbits excreted 14% of a dose of phenylmercapturic acid as S-phenylcysteine formed by metabolic deacetylation of the mercapturic acid. Similarly, Marsden and Young (1958) found that only 33% of a dose of S-naphthyl mercapturic acid was excreted unchanged in rat urine and the neutral sulphur fraction was believed to contain the deacetylated derivative. N-Acetyl-containing drugs such as butanilicane (butacetoloid) and phenacetin have been shown to be hydrolysed by esterases present in mammalian tissues (Heymann, 1982).

In direct *in vivo* investigations into the metabolic fate of mercapturic acids, Bray and James (1960) analysed urine following the administration of a number of mercapturic acids to rats, rabbits and guinea pigs. No deacetylated mercapturic acids could be detected in the urine of the three species following the administration of N-acetyl-S-benzyl-cysteine, -(2-chloro-4-nitrophenyl) cysteine and -butylcysteine. However, liver and kidney extracts of all three species deacetylated the mercapturic acids to the corresponding cysteine derivatives. The deacetylase activity of kidney extracts towards the mercapturic

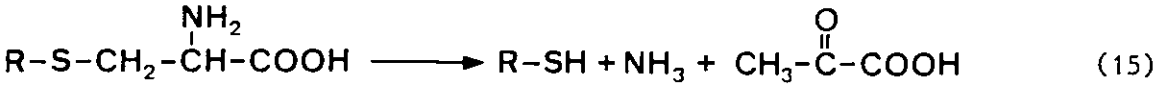
acids was found to be greater than that of liver extracts. The lack of correlation between the *in vitro* and *in vivo* results remained unexplained (Bray and James, 1960). Similar *in vitro* results were obtained using *n*-propyl mercapturic acid, which was deacetylated to *S*-propyl-cysteine on incubation with extracts of rat liver and kidney (Grenby and Young, 1960).

Recently, an enzyme catalysing the *N*-deacetylation of a number of *S*-aryl- and *S*-aralkylmercapturic acids has been purified and characterised from rat liver 105000 g supernatant (Suzuki and Tateishi, 1981). The enzyme was shown to be distinct from four of the five mammalian enzymes known to catalyse the hydrolysis of *N*-acetyl amino acids, but to have an analogous substrate specificity to acylase III purified from rat kidney cytosol. The substrate specificity of the liver deacetylase enzyme is interesting since high activities were observed for aromatic but not for aliphatic mercapturic acids. This is the same substrate specifity observed for liver and kidney cysteine conjugate β-lyase (Tateishi *et al.*, 1978; see Section 1.5 ii) and the results with the liver deacetylase have led to a suggestion that the enzymes may act in succession in the further metabolism of aromatic mercapturic acids conjugates to the corresponding thiol- or methylthio-containing metabolites (Suzuki and Tateishi, 1981).

1.5 ii Cysteine conjugate β-lyase

Until 1978, the only metabolic fate of the cysteine conjugates of various xenobiotics was *via* *N*-acetylation to form the corresponding mercapturic acid. However, an enzymic activity was discovered in rat liver which catalysed the cleavage of the β*C*-*S* linkage in the cysteine moiety of certain cysteine conjugates (Tateishi *et al.*, 1978).

The enzyme responsible for this hydrolysis has been designated cysteine conjugate β-lyase (EC 4.4.1.13) and catalyses the following reaction,



$$R\text{-}S\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{C}}H\text{-}COOH \longrightarrow R\text{-}SH + NH_3 + CH_3\text{-}\overset{\displaystyle O}{\overset{\|}{C}}\text{-}COOH \qquad (15)$$

The enzyme was purified from rat liver cytosol and shown to be distinct from the mammalian cystathionase (EC 4.4.1.1) (Tateishi et al., 1978). Various thioether-containing compounds were tested to define the substrate specificity of the enzyme (Table 1.7). Cysteine conjugates of aromatic compounds were found to be good substrates for the lyase enzyme whereas cysteine derivatives of aliphatic or alicyclic compounds, together with glutathione conjugates and mercapturic acids were inactive. A similar specificity has been observed recently with a purified preparation of β-lyase from rat kidney towards the cysteine and glutathione and N-acetylcysteine derivatives of hexachloro-1,3-butadiene (Green et al., 1983).

Involvement of cysteine conjugate β-lyase in the further metabolism of cysteine conjugates of xenobiotics has been postulated following the identification of a number of methylthio, methylsulphinyl and methylsulphonyl compounds as excretion products of mercapturic acid precursors (see Stillwell, 1981). Thus, compounds such as naphthalene (Stillwell et al., 1978a), bromobenzene (Mizutani et al., 1978) and propachlor (2-chloro-N-isopropylacetanilide; Bakke and Price, 1979) are all excreted as methylthio compounds and as mercapturic acids. In the case of propachlor, intestinal microflora are regarded as the primary site of β-lyase activity, since the production of methylthio-containing derivatives is decreased in germ-free (Bakke et al., 1980) or antibiotic pre-treated animals (Larsen and Bakke, 1981).

TABLE 1.7 Substrate specificity of cysteine conjugate β-lyase from rat liver cytosol

Substrate*	Relative activity (%)
DNP-L-cysteine	100
DNP-D-cysteine	0
DNP-mercapturic acid	0
DNP-glutathione	0
NBZ-L-cysteine	10
BP-L-cysteine	145
BP-mercapturic acid	0
S-Cyclohexyl-L-cysteine	0
S-Ethyl-L-cysteine	0
S-Methyl-L-cysteine	0

* Abbreviations: DNP, 2,4-dinitrophenyl; NBZ, p-nitrobenzyl; BP, p-bromophenyl.
From Tateishi and Shimizu, 1980.

These latter findings are supported by the direct demonstration of a cysteine conjugate β-lyase showing a similar substrate specificity to the hepatic enzyme in the microorganisms populating the large intestine (Suzuki et al., 1982; see Section 1.8 i (b)).

S-Methyltransferases exist in mammalian tissues which catalyse the methylation of thiols (Weisiger and Jakoby, 1980). The resultant methylthio derivatives can be oxidised, in a similar manner to xenobiotic sulphides, to methylsulphinyl-(MeSO) and methylsulphonyl-($MeSO_2$)

containing metabolites (Stillwell, 1981). These latter oxidation products are often found as urinary metabolites of xenobiotics following the action of cysteine conjugate β-lyase.

The production of thiols from thioethers catalysed by cysteine conjugate β-lyase has important considerations with respect to the formation of reactive metabolites. Thiols are highly reactive compounds by virtue of the presence of a powerful nucleophilic sulphur atom (see discussion on glutathione) and S-oxidation can lead to further bioactivation and protein binding (Hutson, 1981). A cysteine conjugate β-lyase present in rat renal brush border membranes appeared to play a significant role in the activation of hexachloro-1,3-butadiene to a reactive thiol (Green et al., 1983). Thus, the mutagenicity of the cysteine derivative of hexachloro-1,3-butadiene was shown to result from the action of a purified β-lyase preparation and the resultant metabolite was believed to be responsible for the depletion of glutathione and marked necrosis produced in rat kidney by hexachloro-1,3-butadiene. Similarly, metabolism of thioether metabolites of propachlor catalysed by a gut microflora β-lyase led to the production of products which could not be extracted from faeces (Bakke et al., 1980), suggesting that the thiol product may be a reactive species. Cysteine conjugate β-lyase may play an important role in the metabolism of certain xenobiotics which form large amounts of a cysteine derivative, under certain conditions (e.g. deacetylation of mercapturic acids or in species such as the guinea pig (Bray et al., 1959b) deficient in N-acetylation) but is unlikely to be involved directly in the metabolism of glutathione conjugates or mercapturic acids.

1.5 iii S-oxidation

A relatively limited number of mercapturic acids containing oxidised sulphur have been isolated as xenobiotic metabolites. Ethyl and n-propyl mercapturic acid sulphoxides were identified in the urine of rats given bromoethane and 1-bromopropane, respectively (Barnsley et al., 1964, 1966). Similarly, the corresponding alkylmercapturic acid sulphoxides were excreted in the urine of rats following subcutaneous administration of S-methyl-L-cysteine and S-ethyl-L-cysteine (Barnsley, 1964; Barnsley et al., 1964). No sulphoxides could be found in the metabolism of higher alkylhalides (James, 1961). The possibility exists that sulphoxides are artefacts arising from chromatography of the corresponding sulphides, although in the study of Barnsley et al. (1964), this factor was investigated by measurement of oxidation of ethylmercapturic acid in urine of different pHs and the sulphoxides can be considered true metabolites. No S-oxidation products of aromatic mercapturic acids have been reported.

It is well established that a large number of sulphur-containing xenobiotics are oxidised in vivo to sulphoxides and sulphones (see Ziegler, 1982). These latter oxidation products are more polar than the parent sulphide and increased polarity permits more rapid translocation and excretion. In general, oxidation of sulphides appears to be a route for detoxication. Whether alkylmercapturic acids undergo S-oxidation in a similar manner to that of thioether-containing xenobiotics is unknown, but is is apparent that S-oxidation of mercapturic acids is probably only a very minor pathway of drug metabolism.

1.6 Excretion of conjugated xenobiotics

The two major routes of excretion for the majority of xenobiotics are urine and faeces, although other channels such as expired air, sweat or milk may exist for some compounds (Levine, 1983). Elimination of xenobiotics in faeces is usually the result of biliary excretion and because of their molecular weights and polar nature, a large number of xenobiotic compounds conjugated with β-glucuronic acid or glutathione are eliminated in bile. This has important consequences since a number of metabolic processes may occur during the passage of biliary-excreted compounds down the intestinal tract.

1.7 Biliary excretion

Bile is a fluid of electrolytes of a composition similar to that found in plasma, although exact composition may vary amongst species and under different physiological conditions within a species. The principal cation in bile is sodium, and bile acids, chloride and bicarbonate contribute to the anion composition. Species differences exist in the principal bile acid found in bile, but can be roughly correlated with diet. Thus, herbivores, with the exception of bovoids, have primarily dihydroxy- or monohydroxy-monoketo bile acids conjugated with glycine, whereas carnivores have trihydroxy bile acids and taurine conjugates (Klaassen et al., 1981).

Although little information is available concerning the mechanisms responsible for the biliary excretion of xenobiotics and their metabolites, systematic studies in various species have indicated some physicochemical factors which influence the elimination in bile of organic anions and cations (Millburn, 1976). Based upon correlations

between molecular weight and the extent of biliary excretion, an approximate threshold molecular weight for compounds has been established below which compounds are only minimally excreted into bile (i.e. less than 10% of the administered dose). For organic anions this threshold varies with species, being 325±50 for the rat (Millburn et al., 1967), 400±50 for guinea pigs and 475±50 for rabbits (Hirom et al., 1972a). The value for man appears to be about 500-600 (Hirom et al., 1972a). Organic cations have threshold molecular weights of approximately 200±50 for monoquaternary ammonium compounds and 500-600 for diquaternary ammonium derivatives and no species differences are observed (Hughes et al., 1973a, b). Bile and urine are complementary pathways for excretion i.e. urinary excretion is greatest for compounds of low molecular weight and tends to decrease with increasing molecular weight: vice versa for biliary excretion (Hirom et al., 1972a). Above the threshold molecular weights there appears to be no direct relationship between molecular weight and extent of excretion in bile (Hirom et al., 1972b).

Although molecular weight considerations are important in determining the extent of biliary excretion of most foreign compounds, the possession of a strongly polar group, either anionic or cationic, in the molecule can combine with molecular weight in determining hepatobiliary disposition (Smith, 1973). The presence of a strongly ionizable group either inherent in the structure of the xenobiotic itself (e.g. bromsulphthalein) or acquired by the xenobiotic by metabolism (e.g. by conjugation with glucuronic acid or glutathione), allows compounds to be readily soluble in body fluids, including bile, at physiological pH.

Studies of compounds of a similar molecular weight and polarity have

indicated that a third structural factor affecting the lipid solubility or shape of the molecule may be important in biliary excretion (Hirom et al., 1972b; Hirom et al., 1974). Slight modifications in the structure of a number of N-acyl sulphonamides markedly altered the affinity of compounds for biliary elimination in the rat (Hirom et al., 1969).

Comparisons of the biliary and urinary excretion in rats of 30 aromatic compounds with molecular weights in the range 100-850 revealed that compounds could be roughly classified into three groups with respect to their pattern of elimination. Thus, group I (molecular weights <300) represent compounds whose major route of elimination was via the urine. Compounds in group 2 (molecular weights 450-850) were excreted predominantly in bile, whereas group 3 compounds (molecular weights 350-450) were extensively eliminated in both bile and urine (Hirom et al., 1976). Obstruction (by ligation of renal pedicles or bile ducts) of the major route of elimination for compounds in groups 1 and 2, did not produce a significant increase in the amount of drug appearing in the minor excretory route for these compounds. However, for compounds in group 3 blockage of one route of elimination was compensated for by an increase in drug excretion by the other route (Hirom et al., 1976). These latter results illustrate the complementary nature of bile and urine as routes of elimination of xenobiotics.

A number of biological factors have been described which influence the biliary excretion of compounds. These include differences in species, sex, age and metabolic transformation and have been described in detail by Smith (1973). Perhaps the most important of these biological factors is metabolic transformation.

Conjugations of phase I metabolism products with endogenous compounds such as glutathione and glucuronic acid result in the formation of highly polar metabolites, which are ionized at physiological pH and soluble in media such as bile and urine. Also conjugation significantly increases the molecular weight of compounds relative to the parent compound or phase I metabolites, since glucuronic acid and glutathione have molecular weights of 177 and 307 respectively. The properties of high polarity and favourable molecular weight, make glutathione and glucuronic acid conjugates ideally suited for excretion in bile and consequently conjugated metabolites of a large number of xenobiotics have been identified in the bile of animal species and man (see Smith, 1973; Klaassen et al., 1981).

1.7 i Biliary excretion of glutathione conjugates

Table 1.8 shows some examples of the diversity of types of drugs and other foreign compounds which have been identified in bile as metabolites conjugated with glutathione. Compounds range from aromatic hydrocarbons, such as naphthalene and pyrene, to carcinogens, such as N- hydroxy-2-acetylaminofluorene and aflatoxin B_1, and from drugs such as the diuretic ethacrynic acid to metals such as mercury, cadmium and zinc. These compounds are excreted in bile when administered to animals by intravenous or subcutaneous injection or can be demonstrated in the bile from isolated liver preparations, perfused with the parent compounds. Most of the compounds in Table 1.8 can be identified in bile as the glutathione S-conjugate, together with the corresponding S-cysteinyl-glycine, S-cysteine and N-acetyl-S-cysteine derivatives.

The mechanisms involved in the secretion of glutathione S-conjugates into bile are unclear, but it is believed they are similar to those

TABLE 1.8 Examples of compounds excreted in bile as glutathione conjugates

Group	Example	Reference
Polycyclic aromatic hydrocarbons	Naphthalene	Boyland et al., 1961
	Phenanthrene	Boyland and Sims, 1962
	Pyrene	Boyland and Sims, 1964a
	Benz(a)anthracene	Boyland and Sims, 1964b
Halogenobenzenes	1-Chloro-2,4-dinitrobenzene*	Wahlländer and Sies, 1979
	Pentachloromethylthiobenzene	Bakke et al., 1981b
Phthaleins	Sulphobromophthalein	Combes, 1957
		Krebs, 1959
Methylmercaptotriazines	Cyanazine	Crayford and Hutson, 1972
	Cyanatryn	Crawford et al., 1980
Alkylhalides	1-Bromobutane	James et al., 1968
	Iodomethane	Johnson, 1966
	1,3-Dibromopropane	James et al., 1981
Arylhalides	Bromobenzene	Sipes et al., 1974
Alkylmethanesulphonates	Methylmethanesulphonate	Pillinger et al., 1965
Epoxides	Styrene oxide*	Ryan and Bend, 1977
	1,2-epoxy-3-(p-nitrophenoxy)propane	James et al., 1978
γ,β-Unsaturated compounds	Acrylamide	Edwards, 1975
	Ethacrynic acid	Klaassen and Fitzgerald, 1974
	Diethylmaleate	Barnhardt and Combes, 1978
Steroids	2-Hydroxyestradiol-17-β	Elce and Harris, 1971
Aralkyl halides	Propachlor	Bakke et al., 1980
		Larsen and Bakke, 1981
Arylamines	Paracetamol	Grafström et al., 1979
	Paracetamol*	Hinson et al., 1982

TABLE 1.8 (Cont'd)

Group	Example	Reference
Carcingens	N-Hydroxy-2-acetylaminofluorene	Meerman et al., 1982
	Aflatoxin B_1	Degen and Neumann, 1978
	Benzo(a)pyrene-4,5-oxide	Plummer et al., 1980
	Benzo(a)pyrene-4,5-oxide*	Smith and Bend, 1979
Carbamates	Urethane	Boyland and Nery, 1965
Metals	Mercury	Refsvik and Norseth, 1975
	Cadmium	Cherian and Vostal, 1977
	Zinc	Alexander et al., 1981
Others	3-Phenyl-5-methyl-1,2,4-oxadiazole	Schickendantz et al., 1976
	Azothioprine*	de Miranda et al., 1973
	4'-(9-Acridinylamino)methanesulfon-meta-anisidide	Shoemaker et al., 1982

Most of the above compounds have been identified as glutathione conjugates in bile from bile duct-cannulated rats, although a few (*) have been demonstrated in the bile of isolated perfused rat liver preparations.

involved in glutathione efflux into bile. The isolated perfused rat liver releases reduced glutathione into both bile and perfusate, whereas glutathione disulphide is excreted into bile alone (Sies and Summer, 1975; Sies et al., 1978a). Thus, rat liver released reduced glutathione into bile and the caval perfusate at rates of 1 and 14 nmol/min/g liver, respectively, but secreted oxidised glutathione into bile at a rate of only 0.4 nmol/min/g liver (Akerboom et al., 1982a). The release of glutathione disulphide into bile is increased on exposure of the liver to perfused organic hydroperoxides and hydrogen peroxide and during oxidation of aminopyrine, ethylmorphine and hexobarbital (Sies and Summer, 1975; Sies et al., 1978b).

Glutathione S-conjugates are released from isolated perfused rat liver preparations into both bile and perfusate. Thus, the glutathione S-derivatives of styrene oxide (Ryan and Bend, 1977), paracetamol (Grafström et al., 1979), 1-chloro-2,4-dinitrobenzene (Wahlländer and Sies, 1979) and benzo(a)pyrene-4,5-oxide (Smith and Bend, 1979) are all released into both bile and the perfusate of isolated liver perfusion systems. Release into the two systems may be a function of the rate of formation of the conjugate, since bile is a preferred route of release at low rates of conjugate formation, although release into the perfusate becomes increasingly more important at higher rates of conjugate formation (Wahlländer and Sies, 1979; Grafström et al., 1979). Glutathione disulphide itself can be regarded as a glutathione S-conjugate of glutathione (Sies et al., 1978a) and mechanisms involved in the release of the disulphide and S-conjugates into bile may be similar. Indeed, a direct competition has recently been demonstrated between the biliary output of glutathione disulphide and S-(2,4-dinitrophenyl) glutathione and these findings were consistent with a shared pathway for the transport of the two types of compounds into bile

(Akerboom et al., 1982b).

The cellular mechanisms of bile formation are still unclear, although the role of bile acid secretion in bile flow is fairly well documented (see Blitzer and Boyer, 1982). In all vertebrate species examined there is a close relationship between canalicular bile formation and bile acid excretion rates. Briefly, bile acids are purported to be transported across the canalicular membrane and concentrated in bile by mechanisms poorly understood. This then creates osmotic and electrical gradients favouring the movement of ionic sodium and obligated water from the intercellular space into the canalicular lumen. This scheme is complicated by the evidence of nonosmotic factors altering the magnitude of the bile acid-dependent bile flow (Blitzer and Boyer, 1982). Studies involving the biliary excretion of certain foreign compounds as glutathione conjugates have helped in the understanding of the mechanisms of bile formation with respect to bile acid secretion. The glutathione S-conjugate of bromsulphthalein, which is excreted extensively in the bile of rats and other animal species administered bromsulphthalein (Krebs, 1959), produced an increase in bile flow related to its biliary excretion (Whelan et al., 1970). Similarly, glutathione S-derivatives of ethacrynic acid (Klaassen and Fitzgerald, 1974), diethylmaleate (Barnhart and Combes, 1978) and 1-chloro-2,4-dinitrobenzene (Wahlländer and Sies, 1979), have all been identified in bile following administration of the parent compound. Biliary excretion of each of these conjugates was associated with stimulation of bile flow (Shaw et al., 1972; Barnhart and Combes, 1978; Wahlländer and Sies, 1979). Chloresis associated with the biliary excretion of glutathione S-conjugates may be of an osmotic nature similar to that observed with bile acids.

1.8 Intestinal metabolism of conjugates of xenobiotics

As has been mentioned, the major metabolites of xenobiotics which are eliminated in bile are β-glucuronic acid and glutathione conjugates. These conjugates are not simply excreted in the faeces following entrance into the gastro-intestinal tract, but may be subject to metabolic reactions which originate at several sites. Metabolism may be catalysed by host enzymes secreted into the gastro-intestinal tract, which normally exist in the hydrolysis of foodstuffs for absorption. However, little evidence is available in the literature to suggest that these host enzymes are of any significant importance in the metabolism of xenobiotics in the gut lumen (Renwick, 1982).

More evidence is available to suggest that metabolism of xenobiotic conjugates is alternatively catalysed by the intestinal microflora which populate the gastrointestinal tract (Brewster, 1981). Similarly enzymes present in the epithelial cells which line the intestinal tract have the capacity to metabolise conjugated xenobiotics. Each of these two important sites will be discussed with respect to xenobiotics conjugated with glutathione or glucuronic acid.

1.8 i Bacterial metabolism

The microorganisms which inhabit the mammalian gastrointestinal tract are capable of a wide range of metabolic transformations of foreign compounds. Some of these reactions are similar to those found in mammalian tissues, whilst others exist which are unique to the gut microflora (Scheline, 1973, 1980; Goldman, 1981). The abundance of examples of metabolism of xenobiotics by intestinal microflora should be viewed with respect to their quantitative importance *in vivo*.

Intestinal microfloral metabolism has considerable significance in the pharmacological and toxicological properties of compounds (see Williams, 1972) as well as their enterohepatic circulation (see later).

1.8 i (a) Hydrolysis of glucuronides by intestinal microflora

A large number of studies have shown that high levels of β-glucuronidase are present in the intestinal contents and faeces of man and experimental animals. The enzymic activity towards the glucuronic acid conjugates of xenobiotics such as diethylstilboestrol and morphine can be considerably diminshed in antibiotic-treated animals (Fischer et al., 1973; Walsh and Levine, 1975). A decrease in the hydrolysis of glucuronides leads to a decrease in the absorption of xenobiotics from the intestinal tract. Glucuronidase activity in the intestinal contents of rat and man are principally bacterial in origin (Kent et al., 1972) and hydrolysis of a number of glucuronides can be demonstrated in incubations of single species and mixed cultures (see Illing, 1981).

The microbial species involved in β-glucuronide hydrolysis are facultative anaerobes and maximal enzyme activity is usually associated with the caecum, which contains large quantities of anaerobic microorganisms in a favourable environment (Williams, 1972). Deconjugation of the glucuronides of potentially reactive xenobiotic metabolites may have important considerations with respect to the aetiology of a number of forms of cancer (Hill, 1980). Indeed it has been demonstrated that during the bacterial hydrolysis of 3-hydroxy-benzo(a)pyrene glucuronide an active intermediate was formed which readily bound to DNA and was thus potentially carcinogenic (Kinoshita and Gelboin, 1978). The hydrolysis of β-glucuronic acid conjugates of xenobiotics by bacteria is important in the persistence and enterohepatic recycling

of compounds (see Section 1.9 i).

1.8 i (b) Metabolism of glutathione conjugates and related compounds by intestinal microflora

Despite the large volume of information known regarding the biliary excretion of glutathione S-conjugates of xenobiotics and their hydrolysis products, very little information is available with regard to the ability of gut microflora to metabolise these compounds.

The first step in the hydrolysis of glutathione S-conjugates, as already mentioned, is the removal of glutamic acid in a reaction catalysed by γ-glutamyl transferase (see Section 1.4 ii). Although this enzyme has been reported to occur in bacteria (Talalay, 1954), it is unknown whether the enzyme is active with glutathione or its S-conjugates. Also, gastrointestinal bacteria possess enzymes capable of hydrolysing α-amide linkages (e.g. in glycine conjugates of bile acids), although again no incubations have been performed with glutathione or its S-substituted derivatives (Scheline, 1973).

As previously described (see Section 1.5 iii), intestinal microorganisms found in the large intestine of rats do possess an active cysteine conjugate β-lyase enzyme (Suzuki et al., 1982). This enzyme possessed high activity towards the cysteine conjugate of bromobenzene to produce the corresponding thiophenol but showed no activity towards the N-acetyl-L-cysteine or glutathione conjugates of bromobenzene.
This bacterial enzyme has been implicated in the formation of methyl sulphur derivatives of a number of xenobiotics. Thus, derivatives of propachlor (2-chloro-N-isopropylacetanilide) in which the chloro group is replaced by a methylsulphonyl moiety have been found as

urinary metabolites in the rat (Bakke and Price, 1979). These metabolites are absent in germ-free (Bakke et al., 1980) and antibiotic-pretreated (Larsen and Bakke, 1981) animals. The methyl sulphonyl derivatives are derived from the cysteine and, possibly, the N-acetylcysteine conjugates of propachlor following oral administration (Bakke et al., 1981a). Intestinal microbial hydrolysis of the cysteine and N-acetylcysteine derivatives of propachlor which are excreted in bile, followed by absorption, S-methylation and oxidation, has been proposed as the major source of methyl sulphonyl-containing compounds excreted in the urine of rats administered propachlor. Involvement of intestinal microfloral cysteine conjugate β-lyase has been implicated in the formation of methylsulphur-containing metabolites of pentachloronitrobenzene (Bakke et al., 1981b), caffeine (Rafter and Nilsson, 1981) and 2-acetamide-4-(chloromethyl)thiazole (Rafter and Bakke, 1982).

Since the substrate specificity of the bacterial cysteine conjugate β-lyase appears to be limited to cysteine S-conjugates and these often represent a low proportion of biliary metabolites of xenobiotics, due consideration should be taken in the quantitative importance of the bacterial reaction in vivo.

1.8 ii Intestinal tissue metabolism

The use of intestinal tissue preparations such as in situ isolated perfused gut loops, everted intestinal sacs, tissue slices, intestinal explants in culture, intestinal mucosal cell suspensions and subcellular fractionation of tissue homogenates have permitted the recognition of a wide variety of drug metabolism reactions in the gut (Caldwell and Marsh, 1982). These include phase I reactions such as carbon oxidation, hydroxylation, dealkylation, nitrogen and sulphur oxidation,

desulphuration, reduction and hydrolysis as well as many typical phase II conjugation reactions. Enzyme activities in the small intestine of rats are usually less than that detected in liver but the ratio of hepatic to small intestinal activity is highly variable. Thus, the ratio of UDP-glucuronyltransferase is close to 1, but that for epoxide hydrase is far greater (Vainio and Hietanan, 1980). Such differences in enzymic activities between liver and small intestine tissues suggests that different patterns of drug metabolism may occur in the two tissues. The bioavailability of xenobiotic metabolites to either of these tissues is also important in determining the contribution of each of the tissues to the overall metabolic disposition of xenobiotics.

1.8 ii (a) Hydrolysis of glucuronides by intestinal tissue

The majority of enzymic reactions occurring in intestinal tissue are synthetic in nature. Of importance to the discussion is the contribution, which intestinal tissue may make to the further metabolism of ß-glucuronides present in the gastrointestinal tract. The presence of ß-glucuronidase activity apparently associated with intestinal mucosa cells has been observed in the caeca of germ-free rats (Eriksson and Gustafsson, 1970; Weisburger et al., 1970). This ß-glucuronidase had a pH optimum similar to that of the mammalian form of the enzyme (pH 5), rather than the bacterial form (pH 7), and thus appeared to be present in the intestinal mucosa tissue (Weisburger et al., 1970). The inability of antibiotics to completely eliminate all ß-glucuronidase activity from the intestinal tract of treated rats (see e.g. Parker et al., 1980) could be explained by the presence of ß-glucuronidase in intestinal mucosa cells.

1.8 ii (b) Metabolism of glutathione conjugates by intestinal tissue

In 1959, Bray and co-workers demonstrated the presence of an enzymic activity in extracts of rat small intestine which catalysed the hydrolysis of S-(p-chlorobenzyl)glutathione (Bray et al., 1959). This enzyme activity, described as a glutathionase, was later identified as γ-glutamyltransferase, the first enzyme in the mercapturic acid synthetic pathway (see earlier).

The activity of γ-glutamyltransferase in intestinal tissue has been found to be much higher than that seen in liver. Thus, levels of γ-glutamyltransferase were 10 fold higher in jejunal epithelial cells when compared with hepatocytes (Tate, 1980). The enzyme showed a gradient profile of activity in small intestinal epithelial cells which was similar to that of alkaline phosphatase, a marker for villus tip cells, in contrast to the profile of galactosyltransferase, localized primarily in the crypt cells of the small intestine. γ-Glutamyltransferase activity in rat small intestine is then localized principally in the villus tip cells, the activity of these cells being 2-3 times higher than that of crypt cells (Curthoys and Shapiro, 1975).

Indications of a role of the γ-glutamyltransferase of intestinal epithelial cells in the in vivo metabolism of S-substituted glutathione conjugates of xenobiotics has come from experiments with isolated small intestinal epithelial cells and in situ closed gut loops. Freshly isolated rat small intestinal cells rapidly hydrolysed paracetamol-S-glutathione to paracetamol-S-cysteine, which was slowly acetylated to the N-acetylcysteine derivative (Grafström et al., 1979). The involvement of γ-glutamyltransferase in this hydrolysis was indicated by stimulation or inhibition of breakdown with methionine or serine-borate, respectively.

Installation of paracetamol-S-glutathione into the lumen of an *in situ* closed intestinal sac preparation, resulted in a hydrolysis similar to that seen with isolated cells and the cysteine derivative was identified in plasma (Grafström *et al.*, 1979). Hydrolysis of leukotriene C_3, an endogenous glutathione S-conjugate (see Section 1.4 i (e)), to the corresponding cysteine derivative (leukotriene D_3) has been observed at low rates in incubations with isolated epithelial cells of intestinal mucosa (Ormstad *et al.*, 1982). Thus, it appears that intestinal tissue has the ability to catalyse the hydrolysis of glutathione S-conjugates and the resultant derivatives may be absorbed from the intestinal tract into plasma.

1.9 i Enterohepatic circulation of β-glucuronic acid conjugates

A compound which undergoes hepatobiliary excretion is either eliminated in faeces or reabsorbed from the intestine. Compounds which are secreted in bile and reabsorbed from the intestine are said to undergo enterohepatic circulation (Plaa, 1975; Smith and Millburn, 1975). The majority of xenobiotics which are secreted in bile are in a water-soluble form. However, lipid-soluble compounds are more readily absorbed from the intestine than more hydrophilic compounds (see Schanker, 1971) and thus undergo a more extensive enterohepatic circulation than water-soluble compounds. Metabolism of hydrophilic derivatives of xenobiotics, such as glutathione and β-glucuronic acid conjugates, to more lipid-soluble forms may be carried out by biotransformation processes by bacteria or intestinal mucosal tissue, as described in the preceding sections. Enterohepatic circulation of metabolites of a number of xenobiotics is known to occur following the excretion in bile as β-glucuronides (Table 1.9). Thus, biliary elimination of radioactivity in rats after i.p. injection of

TABLE 1.9 Examples of compounds which undergo enterohepatic circulation following biliary excretion as β-glucuronides in the rat

Compound	Reference
Stilboestrol	Clarke et al., 1969
Mestranol	Brewster et al., 1977
17β-Oestradiol	Brewster et al., 1977
HP-505 (1,3-dihydro-3-phenyl-spiro[isobenzo-furan-1,4-piperidine])	Johnson and Rising, 1978
Phenolphthalein	
Morphine	Parker et al., 1980
Lysergic acid diethylamide	
Diphenylacetic acid	
Aniline mustard (N,N-di-2-chloroethylamine)	Chipman et al, 1980
4'-Hydroxy-3-phenoxybenzoic acid	Huckle et al., 1981

(^{3}H)-phenolphthalein, (^{3}H)-morphine, (^{14}C)-lysergic acid diethylamide and (^{14}C)-diphenylacetic acid was 90%, 45%, 75% and 57% of the doses respectively, predominantly as glucuronides (Parker et al., 1980). Intraduodenal infusion of the bile from these animals demonstrated enterohepatic circulation, amounting to 85%, 41%, 28% and 66% of the infused doses of the β-glucuronic acid conjugates of phenolphthalein, morphine, lysergic acid diethylamide and diphenylacetic acid, respectively (Parker et al., 1980). Similar data has been obtained following the intraduodenal infusion of the β-glucuronides of a number of other drugs and steroids (see Table 1.9). Interestingly, following intestinal hydrolysis of the β-glucuronide derived from 4-hydroxy-3-phenoxybenzoic acid, the aglycone was absorbed and excreted in urine, principally as the sulphate ester derivative, illustrating an enterohepatorenal disposition for metabolites of 3-phenoxybenzoic acid in the rat (Huckle et al., 1981).

The hydrolysis of β-glucuronic acid conjugates of the compounds listed in Table 1.9 appeared to be principally microfloral in origin, since antiobiotic pretreatment of animals significantly reduced the extent of enterohepatic circulation of these compounds (Illing, 1981). However, antibiotic-pretreatment of rats did not completely eliminate the enterohepatic circulation and this may have indicated the participation of enzymes present in the intestinal mucosal cells (see Section 1.8 ii (a)).

1.9 ii Enterohepatic circulation of glutathione S-conjugates

Although the metabolism of a number of glutathione S-conjugates of xenobiotics has been investigated in vivo, most studies have involved intravenous or subcutaneous administration of the conjugates to

experimental animals (see Wood, 1970). Whilst providing information on the metabolism of glutathione conjugates of xenobiotics to mercapturic acids - in fact this type of study played a large part in the elucidation of glutathione as the origin of cysteine moiety in mercapturic acid synthesis (see Section 1.3 i) - these experiments have provided little information on the fate of glutathione conjugates which enter the gastro-intestinal tract in bile. Information regarding the metabolic fate of glutathione conjugates of xenobiotics following biliary excretion is lacking. Only two studies exist to date in the literature in which S-substituted derivatives of xenobiotics have been administered to animals by an enteric route. These involve the metabolism of the glutathione derivatives of p-bromobenzylchloride and benzylisothiocyanate (Stekol, 1941a, b; Brüsewitz et al., 1977).

Before the significance of glutathione in mercapturic acid formation was fully appreciated, Stekol performed a number of experiments to try to elucidate the mechanisms involved in mercapturic acid formation in vivo (see Stekol, 1941a). Amongst the xenobiotics this investigator used was p-bromobenzylchloride, a compound which was found to be excreted as N-acetyl-S-p-bromobenzylcysteine in the urine of rats (Stekol, 1941b). The mercapturic acid derivative could also be found in urine following the administration of p-bromobenzylcysteine and p-bromobenzylglutathione, in rat food (Stekol, 1941b). Thus, oral administration of the glutathione and cysteine conjugates of p-bromobenzyl chloride to rats was followed by absorption, metabolism and the excretion of the corresponding mercapturic acid derivative in urine.

Studies by Brüsewitz have shown that the metabolic disposition of the naturally occurring benzylisothiocyanate was similar to that of p-bromobenzylchloride. The mercapturic acid derivative of benzylisothio-

cyanate is excreted in rat urine following intravenous or oral administration of the parent compound (Brüsewitz et al., 1977). The mercapturic acid could also be detected in the urine of rats following oral administration of the glutathione conjugate of benzylisothiocyanate.

Both of these studies, involving p-bromobenzylchloride and benzylisothiocyanate, represent examples of compounds which are excreted in urine as N-acetylcysteine derivatives, or mercapturic acids, presumably after reaction with glutathione. Although in neither case has the relevant glutathione derivative been demonstrated to be eliminated in bile (the appropriate studies have not been reported), molecular weight considerations indicate that this is a possibility. The molecular weight of the glutathione conjugates of p-bromobenzylchloride and benzylisothiocyanate are 463 and 456, respectively, in excess of the approximate threshold molecular weight for appreciable biliary elimination in the rat (see Section 1.7), and the conjugates may therefore be expected to be found in rat bile, as is seen for other glutathione conjugates of suitable molecular weight (see Chasseaud, 1979). Apart from these studies no data appears to be available in the literature which give direct in vivo information with respect to the fate of xenobiotic glutathione conjugates which undergo hepatobiliary elimination.

1.10 Purpose of the study

It has been seen, therefore, in the preceding sections, that a large number of xenobiotics exist which are excreted in bile as conjugates of glucuronic acid and glutathione. For glucuronic acid conjugates, it is fairly well understood that metabolism, principally by microflora

which populate the gastrointestinal tract, results in the hydrolysis of the conjugates to metabolites which are absorbed into the portal blood system and may be further metabolised to compounds which are excreted in bile or urine. The situation with respect to xenobiotics which are eliminated in bile as glutathione conjugates and the fate of these metabolites in the intestine is unclear. Circumstantial evidence indicates that metabolites derived from the conjugates may undergo enterohepatic circulation, but few direct results have been obtained in experiments performed in vivo. Additionally, little information is available with respect to the relationships which exist between the excretion of xenobiotics in bile as glutathione conjugates and their excretion in urine as mercapturic acids.

The purpose of the work described in this thesis was to provide further information on the biliary excretion of glutathione conjugates of xenobiotics and their metabolites and to study the metabolism and disposition of these compounds in the gastrointestinal tract. Enterohepatic recycling of metabolites derived from the glutathione conjugates was to be investigated and related to the urinary excretion of mercapturic acids. For these studies, three xenobiotic compounds were investigated in the rat; bromsulphthalein, 1-chloro-2,4-dinitrobenzene and naphthalene. Each of these compounds was known to have a fairly simple metabolic profile, which included glutathione, and was known to be excreted in rat bile.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

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CHAPTER TWO

TABLE TITLE

2.1 Chemicals

Standard laboratory reagents were of AnalaR grade or of the highest purity available. H.p.l.c.-grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland. Water used for h.p.l.c. was AnalaR grade, obtained from BDH Chemicals Ltd., Poole, Dorset. β-Glucuronidase was obtained from either W. R. Warner and Co. Ltd., Eastleigh, Hants. (Ketodase®) or Sigma Chemical Co. Ltd., Poole, Dorset (Glucurase®). γ-Glutamyltransferase (porcine kidney) and reduced glutathione (GSH) were obtained from Sigma.

2.2 Animals and Treatment

Male Wistar rats (200-300 g) (Lions Lab., Ringwood, Hants. or Olac 1976 Ltd., Shaw's Farm, Bicester, Oxon.) were used throughout the studies. Animals were maintained on Labsure Diet 41B-modified (RHM, Poole, Dorset) and water ad libitum.

Bile duct cannulations were performed on rats anaesthetised with sodium pentobarbitone (Sagatal ; May and Baker Ltd., Dagenham, Essex; 60 mg/kg., i.p.). Anaesthesia was maintained by the administration of further pentobarbitone as required. Cannulations were performed using Portex polythene tubing (i.d. 0.4 mm, o.d. 0.8 mm) (A. R. Horwell Ltd., Kilburn High Road, Kilburn, London).

For intravenous (i.v.) administration, compounds, in appropriate vehicles, were administered to anaesthetised animals by injection into the femoral vein.

For enterohepatic recycling experiments, bile samples were collected for 1 h following adminstration of radio-labelled compounds or purified compounds were dissolved in control bile. These bile samples were infused (1 ml over 1 h), using a slow infusion apparatus (Scientific and Research Instruments, Edenbridge, Kent), into the duodena of another group of anaesthetised rats fitted with bile duct cannulae. For this purpose, a second cannula was inserted, at the time of bile fistula surgery, into the duodenum *via* the bile duct.

Bile duct-cannulated rats used in acute experiments (<6 h) were maintained under anaesthesia on a heating table set at 38°C. Urine was taken from the bladder at the end of these experiments. For longer experiments, rats were allowed to recover from anaesthesia and then restrained without food, but with water *ad libitum* for a period of 24 h, with provisions for the separate collection of bile and urine. Bile was collected at hourly intervals on ice up to 6 h, followed by a 6-24 h pooled sample, although occasionally hourly fractions to 24 h were collected using a 2112 Redirac fraction collector (LKB, S. Croydon, Surrey).

Rats were treated with oral antibiotics to suppress gut microflora by administering a suspension of 100 mg neomycin sulphate (Upjohn Co. Ltd., Crawley, Sussex), 50 mg tetracyclin HCl (Cynamid of Great Britain Ltd., Gosport, Hampshire) and 50 mg bacitracin (Sigma) in 0.5 ml 5% (v/v) "Tween" 80 (polyoxyethylene (20) sorbitan monooleate; BDH Chemicals Ltd.) in water daily for three days, the last dose being administered 4 h before bile-duct fistula surgery. Control rats received 5% "Tween" 80 only.

2.3 Analysis of Metabolites

Bile and urine samples were analysed directly without prior clean-up, although when a precipitate was present in some urine samples (especially following freezing and thawing), samples were centrifuged (10 min, 12000 r.p.m.) and the supernatants analysed. Following washing with water, no radioactivity was found to be associated with the pellet in any of the samples analysed. Samples were analysed as soon as possible after collection and stored at -20°C.

2.3 i Measurement of Radioactivity

For measurement of radioactivity, bile and urine samples (0.01-0.02 ml) were added to 4 ml of scintillation fluid. The scintillant used consisted of 2,5-diphenyloxazole (PPO, 0.5% (w/v)) and 1,4-di-2-(5-phenyloxazolyl)benzene (POPOP, 0.03% (w/v)) in toluene (scintillation grade; BDH Chemicals Ltd.) mixed in a ratio of 2:1 (v/v) with Triton X-100. Toluene scintillator (Packard Instruments, Caversham, Berks.), which contained 0.5% (w/v) PPO and 0.01% (w/v) POPOP in toluene, was used alternatively, mixed 2:1 (v/v) with Triton. Parkard Tri-Carb liquid scintillation counter Model Nos. 3255 and 240 CL/D were used and the efficiency of counting assessed by using an external standard and quench-correction curve, constructed using ^{14}C-toluene (Amersham International p.l.c., White Lion Road, Amersham, Bucks.)

Radioactivity in faeces and tissues was measured following homogenisation in 3 vol. (w/v) water using an Ultra-Turrax homogeniser (Janke u. Kunkel, Staufen im Breisgau, West Germany; 3 x 30 sec, half max. speed). Samples (0.2 ml) of homogenates were digested using NCS® (Amersham International; 1 ml) and radioactivity measured by liquid scintillation

counting, following the addition of scintillant (20 ml).

2.3 ii Enzymic hydrolysis

In attempts to determine the proportion of conjugates of glucuronic acid present, β-glucuronidase (Ketodase or Glucurase) was added (2500 Sigma units/ml) directly to bile and urine samples and incubated with shaking at 37°C for 24 h. Control incubations contained 0.1 M sodium acetate buffer (pH 5.0) in place of the β-glucuronidase. γ-Glutamyltransferase incubations (1 unit/ml) were performed in a Krebs-Henseleit buffer, pH 7.4, supplemented with 25 mM Hepes, containing 50 mM glycylglycine as a γ-glutamyl acceptor. Incubations were carried out at 37°C for 30 min. Control incubations contained buffer in place of the enzyme.

2.3 iii Chromatography

To avoid breakdown or oxidation of metabolites, bile and urine samples (0.01-0.02 ml) were routinely dried, following application to chromatograms, using a stream of oxygen-free nitrogen.

Thin-layer chromatography (t.l.c.) was performed using pre-coated silica-gel (0.2 mm) Merck F_{254} plates (type 5214; BDH Chemicals Ltd.) using solvent systems A, B, C and D (Table 2.1). Metabolites and standards were visualised by quenching of background fluorescence of plates under short-wave U.V. light (254 nm; ChromatoVue® cabinet model CC-20G, Ultra-Violet Products Inc., California, U.S.A.)

Paper chromatography (p.c.) was performed using Whatman No. 1 chromatography paper (Whatman Ltd., Springfield Mill, Maidstone, Kent)

TABLE 2.1 Solvents systems used for chromatography

T.l.c. and p.c.:-

A - Butan-1-ol / acetic acid / water, 11/4/5 (by vol.)

B - Butan-1-ol /propan-1-ol / water / aq. ammonia (sp.gr. 0.88), 4/1/1/1 (by vol.)

C - Propan-1-ol / water / acetic acid, 10/5/1 (by vol.)

D - 2-Methyl-propan-2-ol / water, 3/1 (by v/v)

E - Butan-1-ol / acetic acid / water, 2/1/1 (by vol.)

F - Butan-1-ol / propan-1-ol / aq. ammonia (sp.gr. 0.88), 2/1/1 (by vol.)

H.p.l.c.:-

I - Water containing 0.5% (v/v) acetic acid to acetonitrile containing 0.5% (v/v) acetic acid over a linear gradient of 40 min at a flow rate of 1 ml/min.

II - Water to methanol over a linear gradient of 30 min at a flow rate of 1 ml/min.

and developed (16-18 h) by descending chromatography using solvent systems E and F (Table 2.1).

Sulphur-containing metabolites could be detected on chromatograms by spraying with 0.1 M potassium dichromate:acetic acid (1:1, v/v), followed by 0.1 M silver nitrate (Knight and Young, 1958). The presence of divalent sulphur or a sulphoxide moiety produced an orange spot on a red-brown background.

Amino acid conjugates were detected on chromatograms using ninhydrin (0.2% (w/v) in acetone) containing 2% (v/v) pyridine, followed by heating to 110°C (Smith, 1960), a blue-purple colour indicating the presence of a free amino group. Both the dichromate/silver nitrate and ninhydrin/pyridine reagents detected compounds at a limit of approximately 5 µg.

Localisation of radioactive bands on developed chromatograms was achieved by scanning of chromatograms using a Packard Radiochromatogram Scanner, Model No. 7200, with an inert gas consisting of helium (98.7%) and butane (1.3%). Radioactive bands on thin-layer chromatograms were also visualised by autoradiography (1 to 3 weeks' exposure) using Fuji X-ray film, followed by development in Phenisol (Ilford Ltd., Basildon, Essex). Quantitation of radioactivity on thin-layer chromatograms was achieved by scraping 0.25 or 0.5 cm wide bands of silica into methanol (0.5 ml) and the radioactivity measured, following the addition of scintillation fluid (4 ml), by liquid scintillation counting.

Reverse-phase high performance liquid chromatography (h.p.l.c.) analyses were performed using a Waters Associates (Hartford, Cheshire)

h.p.l.c. equipped with Model 6000A solvent delivery systems, Model 660 solvent programmer and Model 440 U.V. absorbance detector (254 nm). Samples were injected (U6K injector; Waters Associates) on to a μBondapak C_{18} column (3.9 mm i.d. x 30 cm) and eluted using either solvent system I or II (Table 2.1). Fractions of the eluate were collected using a 2112 Redirac fraction collector at appropriate time intervals into scintillation vial inserts and radioactivity was measured following the addition of scintillation fluid (4 ml). All samples analysed by h.p.l.c. were filtered through a sample clarification kit (Waters Associates; pore size 0.5 μm) before injection.

2.4 Mass spectrometry

Normally, non-volatile compounds to be analysed by mass spectrometry require derivitisation to increase their volatility. Methylation using diazomethane of the glutathione (especially), cysteine and N-acetyl-cysteine conjugates investigated in these studies produced breakdown of the conjugates. These compounds were therefore, analysed using the relatively new technique of fast atom bombardment (FAB) mass spectrometry (Barber et al., 1981). This technique has been found to be very useful in the analysis of polar compounds, since derivitisation is not required. Molecular weight information is usually obtained from $[M + H]^+$ ions in positive ion spectra and from $[M - H]^-$ ions in negative ion spectra. Little fragmentation is observed, except with high molecular weight molecules.

FAB and electron impact (EI) mass spectrometry were carried out by Dr. F. Cottee. FAB spectra were obtained using a VG 7070E high resolution, double focussing mass spectrometer using Xenon. Samples (approximately 1 μg) were dissolved in glycerol for application to the

sample probe. EI spectra were obtained using a MS50 instrument with an electron beam energy of 70eV. Proton nuclear magnetic resonance (^{1}H-n.m.r.) mass spectrometry was carried out by Dr. B. Regan and spectra were obtained using a Bruker WH-360 n.m.r. spectrometer operating in the F.T. mode.

CHAPTER THREE

GLUTATHIONE CONJUGATION AND ENTEROHEPATIC CIRCULATION IN THE METABOLISM OF NAPHTHALENE

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3.1 MATERIALS AND METHODS

3.1 i Chemicals and Radiochemicals

NADPH (tetrasodium salt), glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and leucine amino-peptidase (EC 3.4.11.1; cytosolic form from porcine kidney) were obtained from Sigma Chemical Co. Ltd.

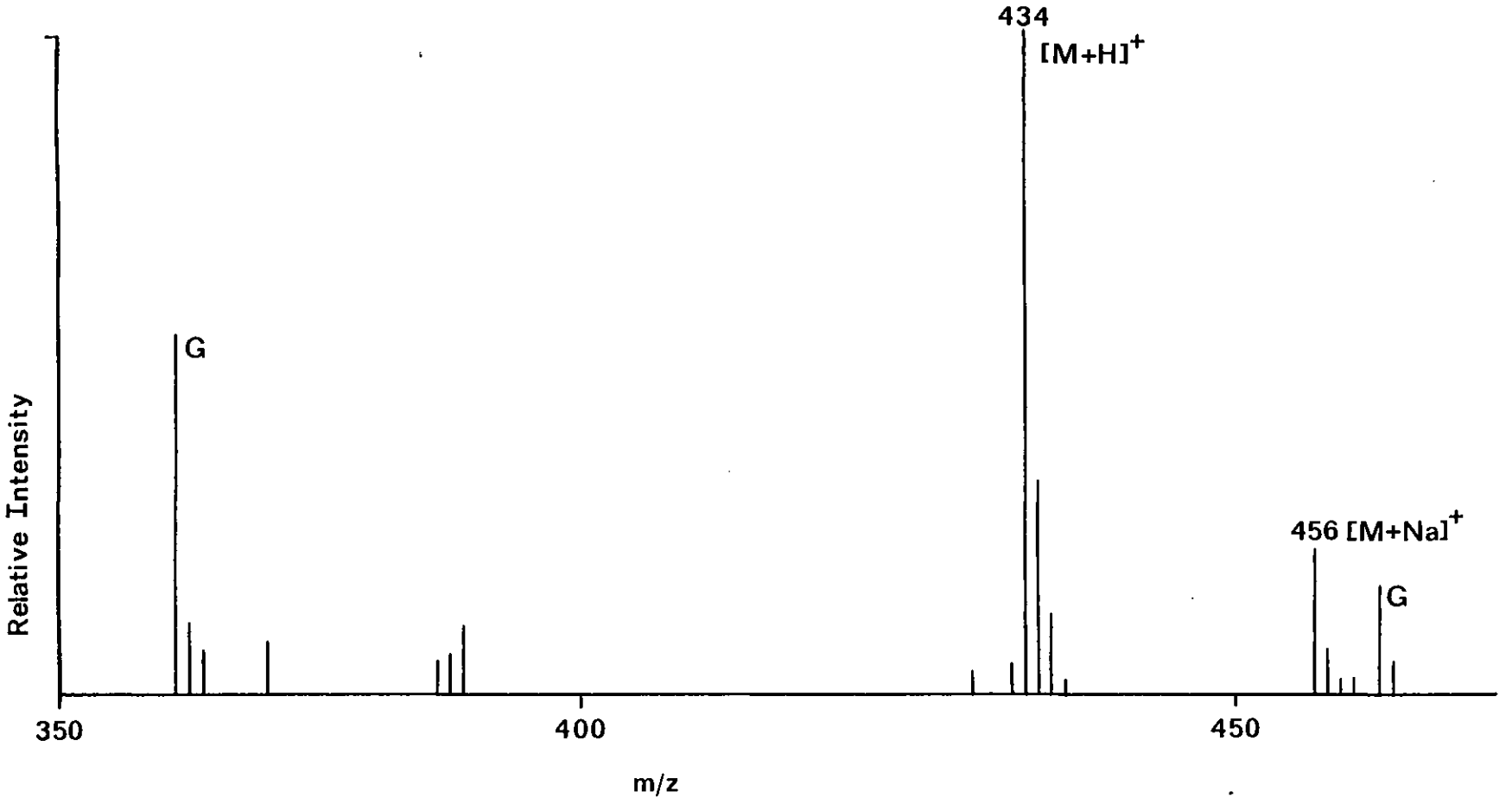
(1-^{14}C)-Naphthalene (specific radioactivity 5 mCi/mmole) was purchased from Amersham Internatonal p.l.c. and, following assessment of purity by t.l.c. in hexane (Rf 0.71), was used as supplied (radiochemical purity 97%).

S-(1-Naphthyl)glutathione was obtained from Dr. P. Sims of The Chester Beatty Research Institute, London, following synthesis by the method of Booth et al. (1960). This compound was analysed by FAB mass spectrometry in the positive ion mode (Figure 3.1a). Molecular ion peaks at m/z 434 ($[M + H]^+$) and 456 ($[M + Na]^+$) were observed in the spectrum. ^{1}H-N.m.r. of the synthesised conjugate gave a spectrum consistent with its structure as S-(naphthyl)glutathione, although it was not possible to confirm the position of attachment of the glutathione moiety to the aromatic ring.

3.1 ii Synthesis of standards

S-(1-Naphthyl)cysteine was synthesised by the method of du Vigneaud et al. (1941), in which diazotised α-naphthylamine was reacted with a cuprous mercaptide derived from cysteine. The product was recrystallised from aqueous ethanol to yield pale brown needle-like crystals

FIGURE 3.1 (a) FAB mass spectrum (+ve ion mode) of S-(naphthyl)glutathione. Peaks labelled G are from the solvent (glycerol) ± Na^+



which had a melting point of 182-184°C (decomp.: 182-183°C, Parke and Williams, 1951). Analysis by FAB mass spectrometry in the positive ion mode gave a spectrum with a molecular ion peak at m/z 248 ($[M + H]^+$) and a fragmentation peak at m/z 159 (thionaphthalene ion) (Figure 3.1b). ^{1}H-N.m.r. of the conjugate revealed the same ring pattern as that seen for the glutathione conjugate (above), showing identical substitution on the naphthyl ring, together with three other protons as expected for the cysteine derivative.

N-Acetyl-S-(1-naphthyl)cysteine was prepared by acetylation of S-(1-naphthyl)cysteine, using acetic acid and acetic anhydride. Thus, 2 mg (8 μmole) S-(1-naphthyl)cysteine was dissolved in 0.5 ml acetic acid in a 10 ml pear-shaped flask fitted with a condenser in the reflux position. Acetic anhydride (0.5 ml, 5 mmole) was added and the mixture heated for 1 h at 50°C. After cooling, the reaction mixture was concentrated under reduced pressure to yield a yellowish oil. This was dissolved in a small amount of methanol and the solution applied to t.l.c. plates. Following development of the plates in solvent system A, the product, Rf 0.70, was visualised under short-wave U.V. light and eluted from the silica into methanol. The product was further purified by reversed-phase h.p.l.c. (solvent system I, Rt 25.7 min) and the appropriate fractions containing the conjugate were pooled and lyophilised. The resultant pale brown solid was subjected to FAB mass spectrometry (positive ion mode) and gave a spectrum with molecular ion peaks at m/z 290 ($[M + H]^+$) and m/z 312 ($[M + Na]^+$) (Figure 3.1c). This spectrum also showed a major fragmentation peak at m/z 159, corresponding to a thionaphthalene ion, which was also observed in the FAB mass spectrum of S-(1-naphthyl) cysteine (see Figure 3.1b).

FIGURE 3.1 (b) FAB mass spectrum (+ve ion mode) of S-(naphthyl)cysteine

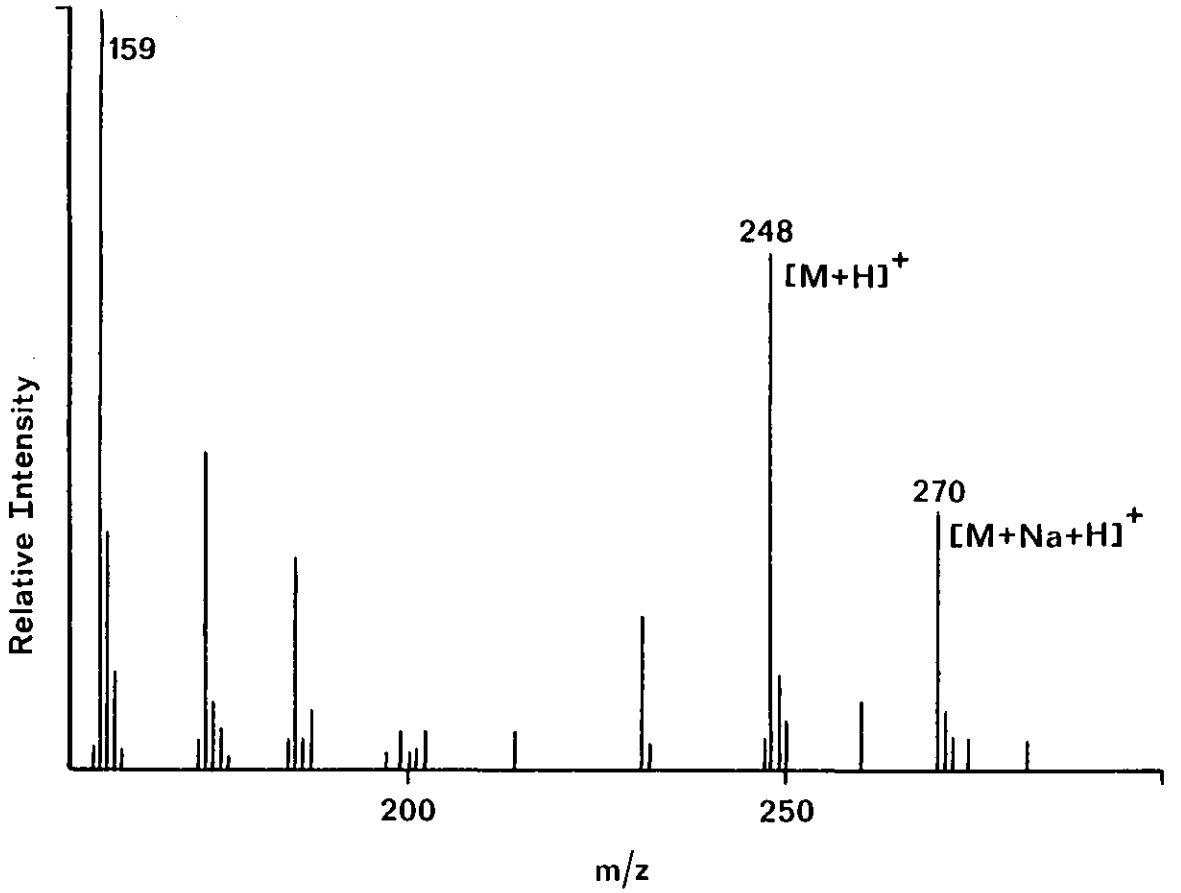
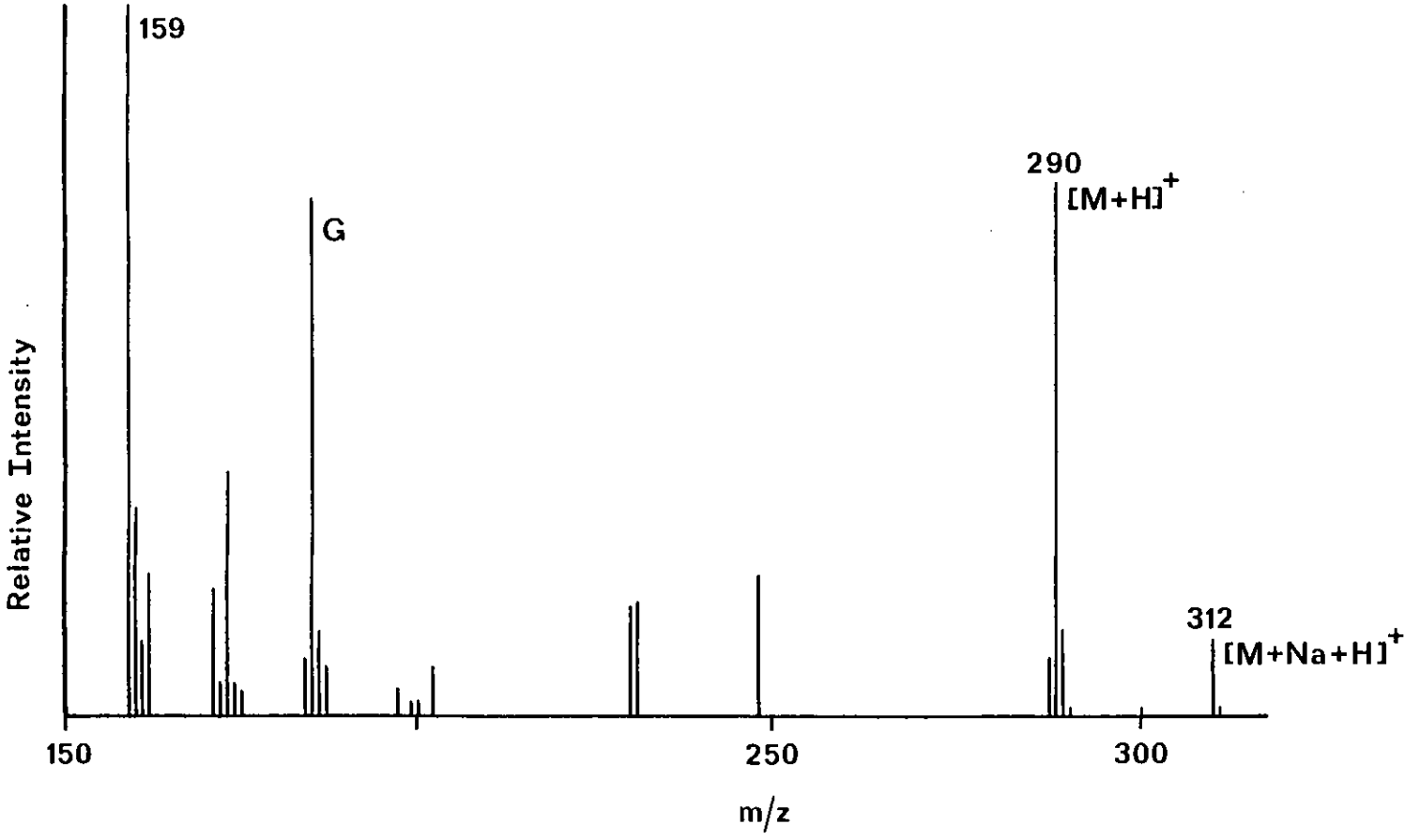


FIGURE 3.1 (c) FAB mass spectrum (+ve ion mode) of N-acetyl-S-(naphthyl)cysteine. Peaks labelled G are from the solvent (glycerol) ± Na^+



The Rf values of the synthesised naphthalene metabolites are shown in Table 3.1.

3.1 iii Metabolism of naphthalene by rat liver supernatant

Synthesis of S-(1,2-dihydrohydroxynaphthyl)glutathione was undertaken using an *in vitro* incubation of naphthalene with 10000 g rat liver supernatant supplemented with GSH.

Preparation of rat liver 10000 g supernatant - Rat liver 10000 g supernatant was prepared from animals which had been treated with phenobarbital (Na^+ salt, 40 mg/kg, i.p.) daily for 4 days, in order to induce high levels of the microsomal monooxygenase system and glutathione S-transferases (Arias *et al.*, 1976; Remmer, 1972). Rats were killed by cervical dislocation and the livers excised. The livers were washed with distilled water, weighed and homogenised in 4 vols. (w/v) ice cold 0.1 M sodium phosphate buffer (pH 7.4), using a Hiedeloph homogeniser (10 strokes, max. speed). The homogenate was then centrifuged at 10000 g for 20 min at 4°C using a Sorvall RC-5 superspeed refrigerated centrifuge (Dupont Instruments). The supernatant was removed and stored on ice until used.

Incubation conditions - The standard naphthalene incubation conditions were similar to those used by Jerina *et al.* (1970) and contained 2 ml pyrophosphate buffer (0.1 M, pH 8.0), 50 µmole nicotinamide, 25 µmole glucose-6-phosphate, 2.5 units glucose-6-phosphate dehydrogenase, 1.2 µmole NADPH, 20 µmole magnesium chloride, 30 µmole GSH, 1.0 ml rat liver 10000 g supernatant and 4 µmole naphthalene (in 0.1 ml 2-ethoxyethanol). Incubations were performed with shaking at 37°C for 30 min, in sealed 15 ml centrifuge tubes. Incubations were

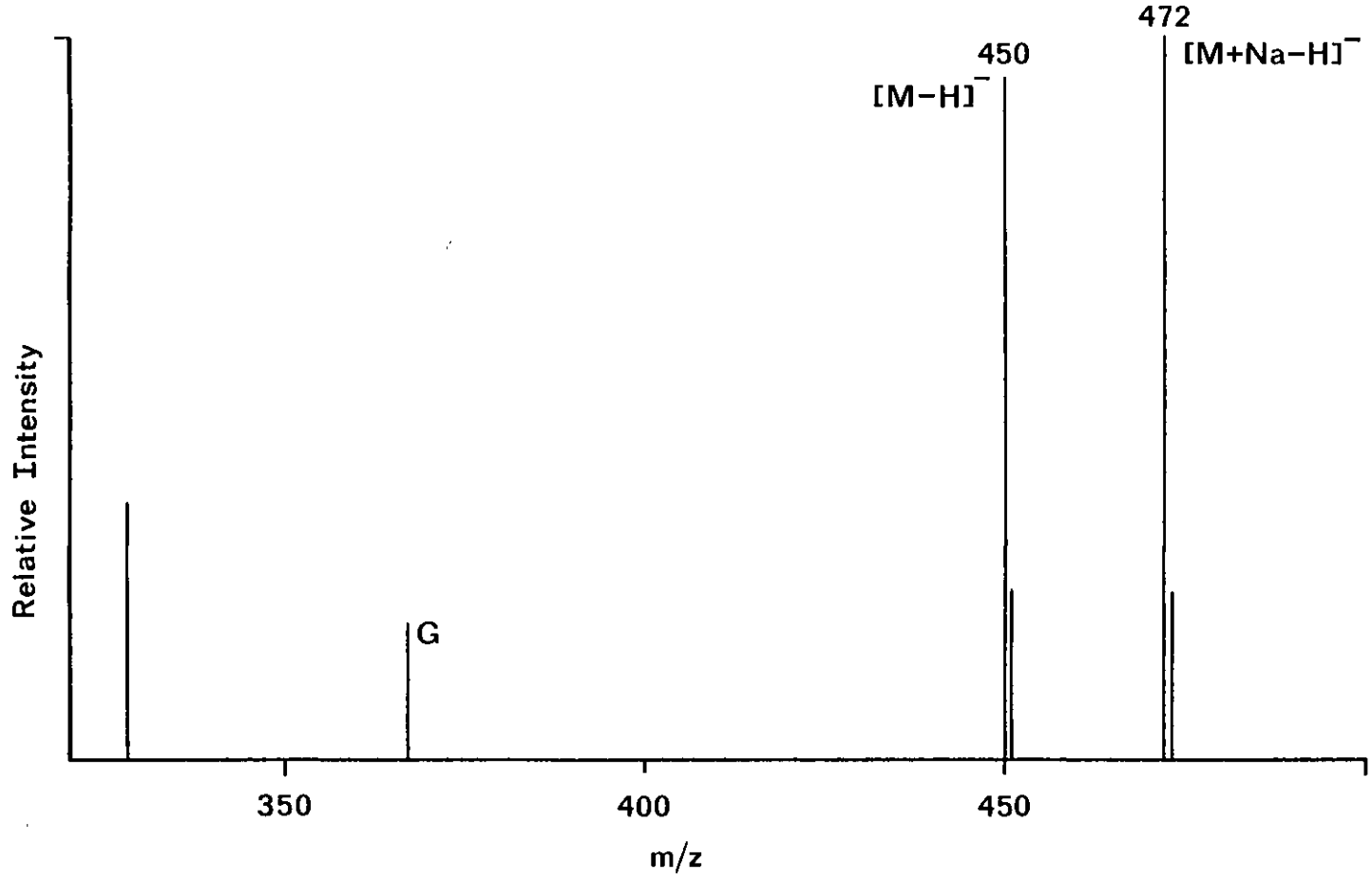
TABLE 3.1 Thin-layer (t.l.c.) and paper chromatography (p.c.) of aromatic amino acid conjugates of naphthalene

	Rf value			
	T.l.c.		p.c.	
Solvent system [a] / Conjugate	A	B	E	F
S-(1-Naphthyl) glutathione	0.46	0.33	0.71	0.12
S-(1-Naphthyl) cysteine	0.64	0.46	0.78	0.43
N-Acetyl-S-(1-naphthyl) cysteine	0.70	0.54	0.90	0.55

(a) See Table 2.1 for composition of chromatography solvent systems.

terminated by placing tubes on ice and by the addition of acetic acid (0.2 ml). The tubes were then centrifuged (MSE Minor 'S': 5 min, 3000 r.p.m.) and the supernatants removed and pooled. Activated charcoal (1 g) was added to the supernatants and shaken well. This mixture was centrifuged (10 min, 3000 r.p.m.) and the supernatant discarded. The charcoal was washed with water (40 ml) and filtered under vacuum. The products from the incubations were then eluted from the charcoal with 100 ml methanol/benzene/aq. ammonia (sp. gr. 0.88), 87/10/3 (by vol). This eluate was concentrated under reduced pressure and analysed by t.l.c. (solvent systems A and B) and h.p.l.c. (solvent system I). The glutathione conjugate had Rf values of 0.42 and 0.21 on t.l.c. in solvent systems A and B, respectively, and was shown to be positive to both ninhydrin and potassium dichromate/silver nitrate reagents (see Section 2.3 iii). On treatment with mild acid and subsequent t.l.c., the mobility of the conjugate increased in both systems (Rf 0.40 to 0.48 in A and Rf 0.21 to 0.30 in B) and co-chromatographed with standard S-(1-naphthyl)glutathione. Similarly, on h.p.l.c. (solvent system I), the conjugate (Rt 15.8 min) increased its retention on acid treatment to a product (Rt 20.2 min), which co-chromatographed with S-(1-naphthyl)glutathione. The synthesised conjugate, following purification by h.p.l.c., was shown to be hydrolysed on incubation with γ-glutamyltransferase, an enzyme specific for the cleavage of the γ-glutamyl linkage of glutathione (see Chapter 1). The conjugate was finally purified by h.p.l.c. in solvent system I, followed by solvent system II, and analysed by negative ion mode FAB mass spectrometry. The spectrum obtained gave molecular ion peaks at m/z 450 ($[M - H]^-$) and m/z 472 ($[M + Na - H]^-$) (Figure 3.2). This mass spectrum was consistent with the structure of the conjugate as S-(dihydrohydroxynaphthyl)glutathione.

FIGURE 3.2 Molecular ion region of FAB mass spectrum of the product of incubation of naphthalene with 10000g rat liver supernatant. Peaks labelled G are from the solvent (glycerol) ± Na^+



Naphthalene has been shown to react with GSH on incubation with rat liver microsomes in the presence of liver 100000g supernatant and GSH to form a glutathione S-conjugate, assigned the structure S-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione (Booth et al., 1967). More recent work (Jeffery and Jerina, 1975) has indicated that this early nomenclature of dihydrohydroxynaphthyl amino acid conjugates was incorrect and that amino acid conjugation occurred via position 2 of the naphthalene ring with the hydroxyl group at position 1 (see Section 1.3 ii). Acid dehydration of these conjugates was accompanied by migration of the amino acid substituent and the formation of S-(1-naphthyl) derivatives (Jeffery and Jerina, 1975). Thus, the structure of the in vitro product of naphthalene formed under the incubation conditions reported here, is likely to be S-(1,2-dihydro-1-hydroxy-2-naphthyl)glutathione.

(^{14}C)-Labelled S-(1,2-dihydro-1-hydroxy-2-naphthyl)glutathione was prepared from an in vitro incubation using (^{14}C)-naphthalene (sp. radioactivity 0.62 μCi/μmole). The yield for two such preparations was found to be low (15-20%), in agreement with Jerina et al. (1970). The radiolabelled product, when compared with the non-labelled conjugate, was shown to have identical properties on t.l.c. (solvent systems A and B) and h.p.l.c. (solvent system I), and to undergo identical hydrolysis on incubation with γ-glutamyltransferase and dehydration on mild acid treatment.

S-(Dihydrohydroxynaphthyl)cysteine and S-(dihydrohydroxynaphthyl) cysteinylglycine conjugates were synthesised from S-(dihydrohydroxy-naphthyl)glutathione by hydrolysis of the glutathione side chain using γ-glutamyltransferase. This enzyme is specific for the cleavage of the γ-glutamyl linkage of glutathione and its action on a glutathione

conjugate yields the corresponding cysteinylglycine adduct (see Section 1.4 ii). When S-(dihydrohydroxynaphthyl)glutathione was incubated with purified γ-glutamyltransferase and the incubation analysed by h.p.l.c. (solvent system I), the glutathione conjugate (Rt 15.8 min) was found to be hydrolysed to one product (Rt 13.6 min) (Figure 3.3a). This compound underwent dehydration upon mild acid treatment, and its retention increased (to Rt 19.8 min) in this chromatography system. This peak co-chromatographed with S-(1-naphthyl)cysteine, suggesting that the product of the action of γ-glutamyltransferase was S-(dihydrohydroxynaphthyl)cysteine. As it was possible that the mild acid conditions used to elicit dehydration could also have cleaved the cysteinylglycine bond, an enzymic hydrolysis of the product of the action of γ-glutamyltransferase was undertaken by incubation of the product with leucine aminopeptidase. This enzyme is one of a number of fairly non-specific enzymes capable of cleavage of the amide bond between cysteine and glycine in S-cysteinylglycine conjugates (see Tate, 1980 and Section 1.4 iii). Incubations were set up using leucine aminopeptidase (3 units) in 0.1 M Tris-HCl buffer (pH 8.0) and carried out at 37°C for 60 min. The incubations were analysed by h.p.l.c. (solvent system I). The product of γ-glutamyltransferase action on S-(dihydrohydroxynaphthyl)glutathione was not metabolised on incubation with leucine aminopeptidase, suggesting that the product of γ-glutamyltransferase action was the corresponding cysteine S-adduct.

The possibility that the hydrolysis of the glutathione S-conjugate to the corresponding cysteine S-conjugate was brought about by the consecutive action of γ-glutamyltransferase and an aminopeptidase present as an impurity in the γ-glutamyltransferase preparation was investigated by inhibition of aminopeptidase activity using

EDTA*. Aminopeptidases have been shown to be divalent metal-requiring enzymes and are inhibited by certain chelating agents, including EDTA (Starnes and Behal, 1974; Tate, 1980), a compound which has not been reported as having any effect on purified γ-glutamyltransferase activity (Tate, 1980). γ-Glutamyltransferase was pre-incubated (5 min, 37°C) in the absence or presence of 10 mM EDTA in 0.1 M phosphate buffer (pH 7.4), before addition to an incubation containing (^{14}C)-labelled S-(dihydrohydroxynaphthyl)glutathione. On analysis by h.p.l.c., a radiolabelled peak (Rt 15.6 min) was observed in the incubation in the presence of EDTA, which was not present on incubation without EDTA (Figure 3.3b). EDTA produced a 96% inhibition of the formation of the cysteine conjugate (Table 3.2).

TABLE 3.2 Effect of EDTA on the formation of S-(1,2-dihydrohydroxynaphthyl)cysteine from S-(1,2-dihydrohydroxynaphthyl)glutathione on incubation with γ-glutamyl transferase

Incubation time (min)	% Incubation metabolites as cysteine derivative	
	-EDTA	+EDTA
0	0.0	0.0
5	87.5	3.3
10	94.4	3.4

* The preparation of γ-glutamyltransferase used for these experiments (obtained from Sigma) was a crude preparation from porcine kidney. On inquiry, it was found that this enzyme was purified as Orlowski and Meister (1965) only as far as step 4, and there was a possibility that an aminopeptidase would not have been removed up to this step in the purification. γ-Glutamyltransferase and aminopeptidase M have been shown to share a similar topological distribution in cell membranes (Okajima et al., 1981). Hence, care should be taken in the assumption that the product of γ-glutamyltransferase incubations of glutathione conjugates is necessarily the corresponding cysteinylglycine conjugate.

FIGURE 3.3 H.p.l.c. profile of incubation of S-(1,2-dihydrohydroxy-naphthyl) glutathione with γ-glutamyltransferase in the absence (a) and presence (b and c) of EDTA

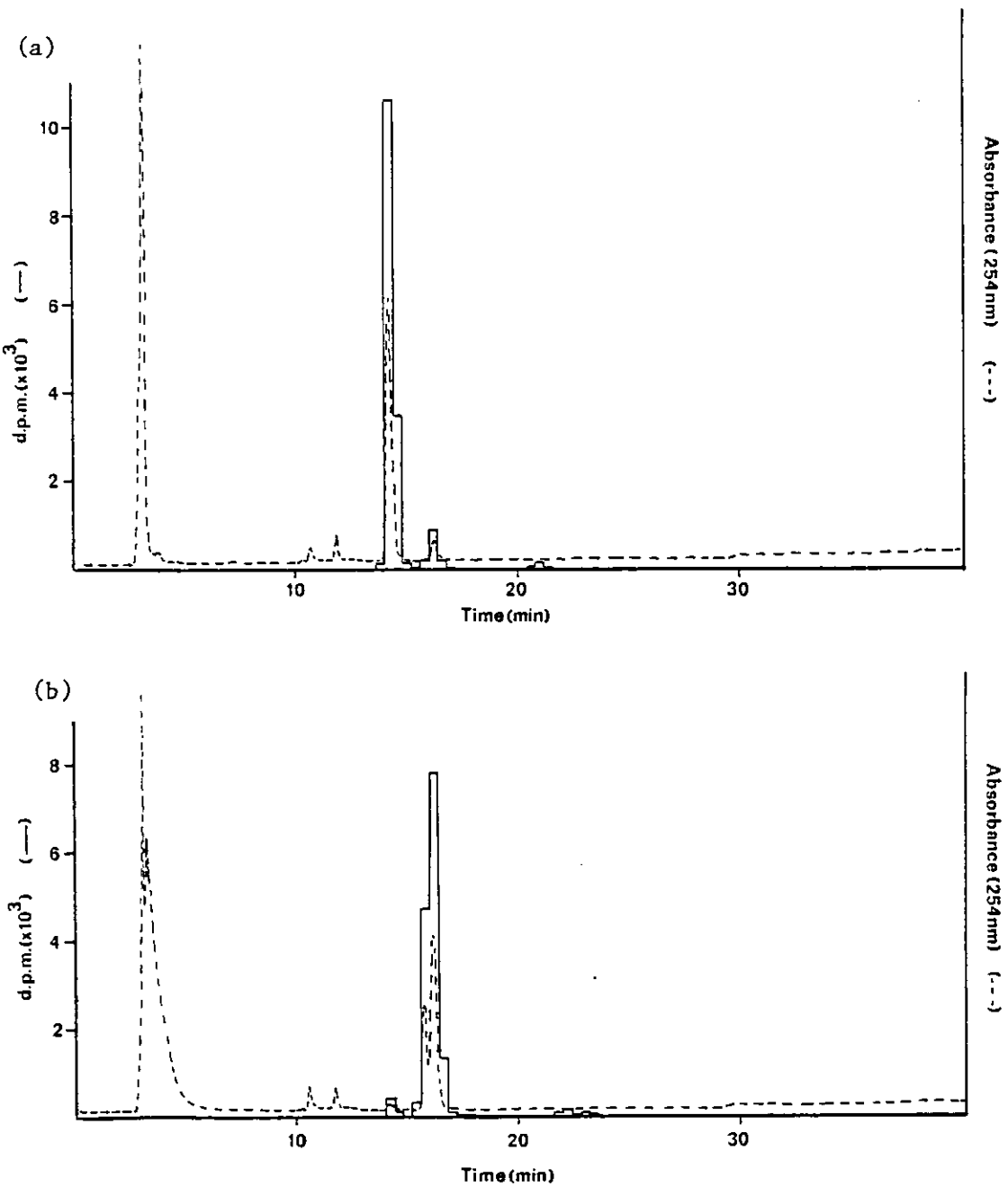
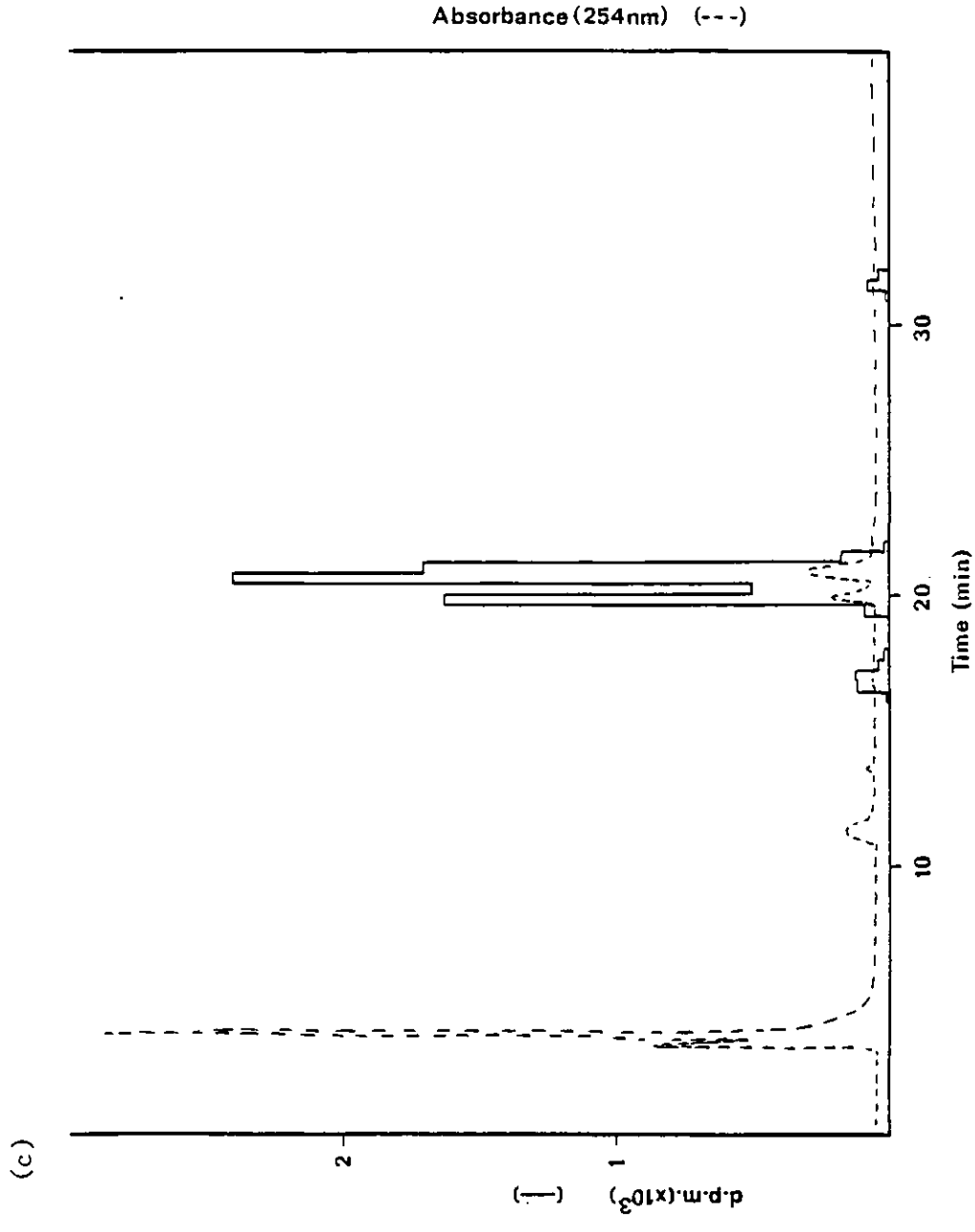


FIGURE 3.3 (Cont'd)



The glutathione, and putative cysteinylglycine conjugates could be resolved to a greater extent by h.p.l.c. using an elution system of a linear gradient of water to 50:50 (v/v) acetonitrile/water, containing 0.5% (v/v) acetic acid (Figure 3.3c). The peaks with retention times of 15.6 min and 13.6 min, on h.p.l.c. in solvent system I, were, thus, tentatively identified as S-(dihydrohydroxynaphthyl) cysteinylglycine and S-(dihydrohydroxynaphthyl)cysteine, respectively.

3.1 iv Animals

For administration to animals, naphthalene (Hopkin and Williams Ltd., Chadwell Heath, Essex) was dissolved in Mulgofen® EL-719 (GAF Ltd., Tilson Road, Wythenshawe, Manchester): ethanol (1:1, v/v) and (^{14}C)-naphthalene in ethanol added to obtain a specific radioactivity of 0.67 µCi/µmole. Distilled water was then added such that the final concentration of Mulgofen was 10%. (^{14}C)-Naphthalene dose solutions were 30 mM. Rats were administered (^{14}C)-naphthalene (30 µmole/kg, 5 µCi) by i.v. injection or, for the purpose of metabolite isolation, by i.p. injection (540 or 600 µmole/kg, 5 µCi). In the latter experiments naphthalene and (^{14}C)-naphthalene were dissolved in "Tween" 80: ethanol, (3:1, v/v). These solutions were at a concentration of 100 mM.

For enterohepatic circulation experiments involving biliary metabolites of naphthalene, bile (1 ml) collected 0-1 h from rats administered (^{14}C)-naphthalene (30 µmole/kg, 5 µCi) was infused intraduodenally over 1 h into another group of bile duct-cannulated animals. These bile samples contained approximately 2.5 µmoles (2 µCi) biliary metabolites of naphthalene.

The major biliary metabolite of naphthalene isolated by t.l.c.

(solvent system B) was dissolved in control bile (1 ml) and infused into bile duct-cannulated rats over 1 h. These bile samples contained 0.5 μmole (0.7 μCi) (^{14}C)-labelled major naphthalene biliary metabolite.

Non-bile duct-cannulated rats used for naphthalene excretion balance studies were kept, after dosing, in individual glass metabolism cages ('Metabowls'; Jencons (Scientific) Ltd., Leighton Buzzard, Beds.), to allow for the separate collection of urine and faeces, for 5 days. These rats were supplied with food and water ad libitum.

3.1 v Analysis of metabolites

Bile, urine and faeces from rats administered (^{14}C)-naphthalene were assayed for radioactivity by liquid scintillation counting as described in Chapter 2. Analyses of metabolites in bile and urine of rats administered (^{14}C)-naphthalene were performed by t.l.c. (solvent systems A and B), p.c. (solvent systems E and F) and h.p.l.c. (solvent system I). It has been established that naphthalene is metabolised to dihydrohydroxynaphthyl derivatives and these derivatives are 1,2-dihydro-1-hydroxy compounds (see Sections 1.3 ii and 3.1 iii). These, so called "pre-conjugates", can be dehydrated to the full aromatic derivatives by mild acid treatment. Since the available standards were the aromatic derivatives, it was necessary to convert metabolites in excreta to aromatic derivatives before investigating co-chromatography. This dehydration was achieved by adjusting bile and urine to pH 0-1 using HCl (2.0 M) and leaving the sample at ambient temperature for approximately two hours. The pH of treated solutions was then adjusted back to 7 (2.0 M NaOH) before chromatographic analyses.

Radioactive metabolites in bile and urine were quantitated from t.l.c.

in solvent system A, as described in Chapter 2. This method reproducibly accounted for >95% of the radioactivity applied to chromatograms.

3.2 RESULTS

3.2 i Excretion of (^{14}C)-naphthalene in intact and bile duct-cannulated rats

Following i.v. administration of (^{14}C)-naphthalene to rats (30 µmole/kg, 5 µCi), radioactivity was quantitatively recovered in urine and faeces over a 5-day period (Table 3.3). The major route of excretion was

TABLE 3.3 Excretion of radioactivity in urine and faeces following i.v. administration of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi)

Time (days)	% Dose excreted* Urine	Faeces
0-1	80.3±8.2	6.5±3.1
1-2	5.7±1.6	1.4±1.0
2-3	2.4±0.6	1.4±0.7
3-4	0.8±0.3	0.5±0.2
4-5	0.5±0.2	0.2±0.1

* Values are mean±S.D. (n=3)

urinary (89.8±5.9% of the dose)[a], with the remainder, 10.0±3.8% of the dose, being recovered in faeces. The majority (87%) of the dose excreted in both urine and faeces was recovered in 0-24 h samples.

[a] All data are expressed as mean ±S.D. (n=3), unless otherwise stated.

When the same dose of (^{14}C)-naphthalene (30 μmole/kg, 5 μCi) was administered intravenously to bile duct-cannulated rats, 63.9±12.9% (n=7) of the dose was recovered in 0-6 h bile (Figure 3.4). Urinary excretion accounted for 13.5±10.5% (n=5) of the dose. Figures for the excretion of radioactivity in 24 h were similar to the figures for 6 h; bile, 68.3±4.5% of the dose and urine, 14.1±6.6% of the dose. Biliary excretion of radioactivity, following i.v. administration of (^{14}C)-naphthalene, was rapid (37.0±11.4% (n=7) of the dose in 0-1 h bile samples), indicating a rapid uptake and clearance of naphthalene by the liver. Comparison of the amount of naphthalene metabolites excreted in bile with the amount recovered in the faeces of non-bile duct-cannulated rats (Table 3.3), suggested that an enterohepatic recycling of naphthalene metabolites occurred in intact animals. This hypothesis was further supported by the low urinary excretion of naphthalene metabolites seen in bile duct-cannulated rats when compared with the urinary excretion of non-bile duct-cannulated rats.

3.2 ii Enterohepatic recycling of naphthalene metabolites

The possibility that naphthalene metabolites may undergo enterohepatic recycling was investigated by intraduodenal (i.d.) infusion of bile, collected for 1 h from rats administered (^{14}C)-naphthalene (30 μmole/kg, 5 μCi i.v.), into another group of bile duct-cannulated rats and bile and urine of the recipient rats analysed for radioactivity.
As is shown in Table 3.4, 85.0±6.4% of the infused dose was absorbed and excreted in bile and urine over a 24 h period. The majority of this excretion occurred in urine (58.4±8.9% of the dose), with approximately half this amount found in bile (26.6±3.5% of the dose). The profile of biliary excretion seen in these animals, suggested a rapid uptake and excretion of radioactivity since highest concentrations were seen in bile collected 1-3 h after infusion (Table 3.4).

FIGURE 3.4 Cumulative biliary excretion of radioactivity following i.v. administration of (^{14}C)-naphthalene (30μmol/kg, 5μCi). Values are mean±S.D.(n=7)

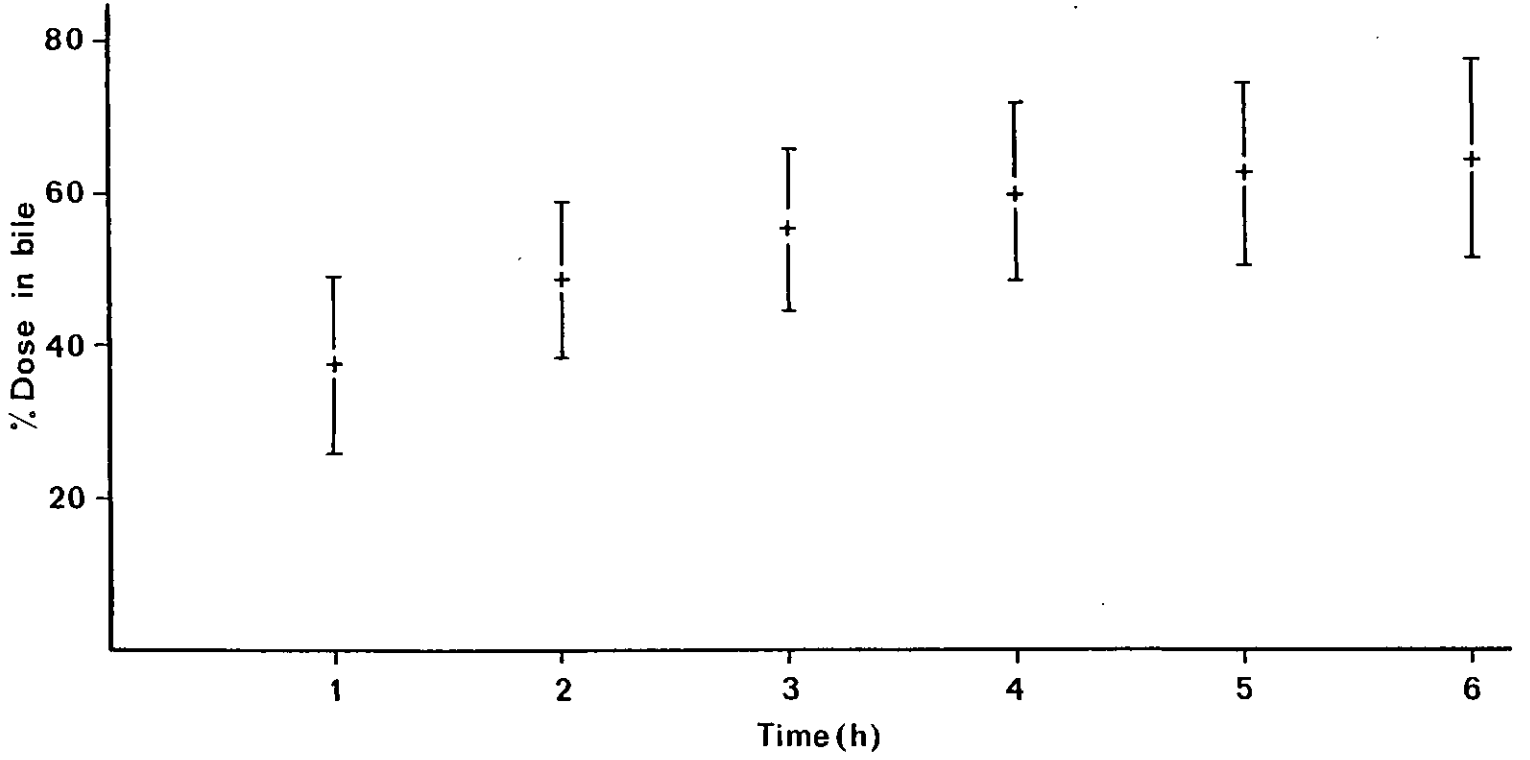


TABLE 3.4 Excretion of radioactivity following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene (11.1 μmole/kg, 2 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	1.9 ± 0.7
	1 - 2	9.0 ± 1.6
	2 - 3	6.3 ± 0.4
	3 - 4	3.6 ± 0.6
	4 - 5	2.0 ± 0.7
	5 - 6	1.1 ± 0.4
	6 - 24	2.7 ± 0.9
Urine	0 - 6	44.6 ± 6.6
	0 - 24	58.4 ± 8.9
Total	0 - 24	85.0 ± 6.4

Values are mean ± S.D. (n = 3)

3.2 iii Analyses of metabolites

Bile and urine from the above experiments were analysed by chromatographic methods as described in 3.1 v.

3.2 iii (a) Intact rats

Analysis of 0-24 h urine from non-bile duct-cannulated rats administered (^{14}C)-naphthalene revealed four radioactive bands on t.l.c. and p.c. (Table 3.5). The major metabolite, Rf 0.63 (t.l.c., solvent system A), which represented approximately 43% of the urinary radioactivity, did not co-chromatograph with any of the available naphthalene standards. This metabolite was found to be ninhydrin negative but positive to the spray reagent for the presence of divalent sulphur groups.

TABLE 3.5 T.l.c. analysis* of urine collected for 24 h following i.v. administration of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi)

Rf	% Total ^{14}C in urine
0.43	9.7±1.2
0.51	23.7±4.8
0.56	15.1±2.3
0.63	43.1±5.8

* Solvent system A was used
Values are mean±S.D. (n=3)

Mild acid treatment of the urine resulted in the [illegible] of this major metabolite, with the resultant appearance of a radioactive com-

pound which co-chromatographed with authentic N-acetyl-S-(naphthyl) cysteine. The metabolite in urine was, thus, tentatively assigned the structure N-acetyl-S-(1,2-dihydrohydroxynaphthyl)cysteine. This compound has been identified from the urine of animals administered naphthalene in a number of studies (Bourne and Young, 1934; Stekol, 1937; Corner and Young, 1954; Boyland and Sims, 1958; Chen and Dorough, 1979), and has previously been termed naphthalene pre-mercapturic acid (Knight and Young, 1958).

β-Glucuronidase incubation of 0-24 h urine from rats administered naphthalene (30 μmole/kg, 5 μCi, i.v.), followed by ethyl acetate extraction of the incubation at pH 7, resulted in 21.7±4.9 (n=4)% extraction of the urinary radioactivity into the organic phase. Analysis of the incubation by t.l.c. (solvent system A), revealed the hydrolysis of the metabolite, Rf 0.51 (Table 3.5). This glucuronic acid conjugate was identified by isolation and characterisation of the aglycone produced from β-glucuronidase treatment of urine. For this purpose, a rat was administered naphthalene (540 μmole/kg, 5 μCi), intraperitoneally, and the 0-24 h urine used for isolation of the aglycone. Urine from this rat gave a qualitatively similar metabolic profile when analysed by t.l.c. as was observed in urine following administration of 30 μmole/kg naphthalene intravenously.

Urine (0-24 h, from a rat administered 540 μmole/kg naphthalene, i.p.) was incubated with β-glucuronidase as described (Section 2.3 ii) and extracted 3 times with an equal volume of diethyl ether, following adjustment of the pH of the incubation to 7.0 with HCl (1 M). The extract was concentrated and analysed by t.l.c. in a solvent system of benzene/ethyl acetate, 85/15 (v/v) (Horning et al., 1980). The aglycone band (major product) was visualised under short-wave U.V.

light and eluted from the chromatogram into methanol. The methanolic extract was concentrated under reduced pressure and analysed by reverse-phase h.p.l.c. using solvent system II. In this system, the aglycone gave a single radioactive peak (Rt 22.6 min) and the fractions containing this peak were pooled from a number of h.p.l.c. applications. The purity of this peak was examined on analysis by h.p.l.c. in the recycle mode using an isocratic solvent system consisting of 40:60 (v/v) methanol:water. In this system the aglycone had a retention time of 7.4 min and could not be resolved into any further peaks after 5 recycles through the column (Figure 3.5). The aglycone collected from h.p.l.c. was subject to EI and ^{1}H-n.m.r. mass spectrometry. The EI mass spectrum of this compound is shown in Figure 3.6a and gave a molecular ion peak at m/z 162, together with fragmentation peaks at m/z 144 ($M-H_2O$), 116 (C_9H_8), 115 ($[C_9H_8]^+$) and 91 ($[C_7H_7]^+$). Fragmentation below 144 was similar to that reported for naphthol (Figure 3.6b).

Thus, the aglycone was assigned the structure 1,2-dihydro-1,2-dihydroxy naphthalene. A mass spectrum of 1,2-dihydro-1,2-dihydroxy-naphthalene similar to the spectrum obtained for the metabolite (Figure 3.6a) has been reported (Chen and Dorough, 1979). ^{1}H-N.m.r. of the aglycone gave a spectrum confirming the structure and H-H coupling of 10Hz for the α-OH protons showed *trans* substitution. The aglycone was therefore identified as *trans*-1,2-dihydro-1,2-dihydroxynaphthalene (naphthalene dihydrodiol). The O-glucuronide present as the metabolite of naphthalene in urine could have glucuronic acid attached *via* either the hydroxy group at position 1 or position 2 of naphthalene dihydrodiol (Bakke *et al.*, 1982).

FIGURE 3.5 H.p.l.c. recycle profile of the aglycone isolated from rat urine following i.p. administration of (^{14}C)-naphthalene (540 μmole/kg, 5 μCi)

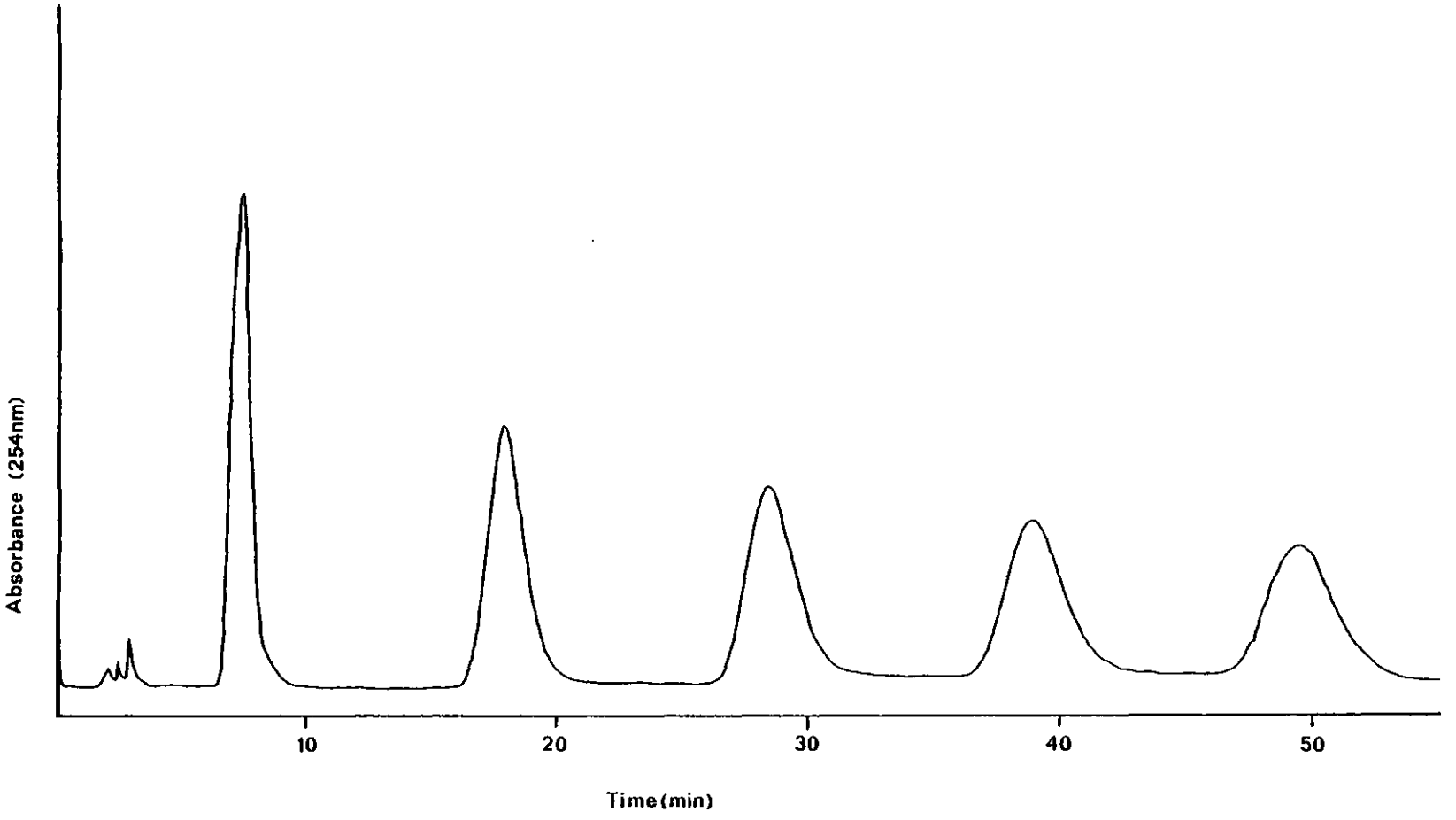
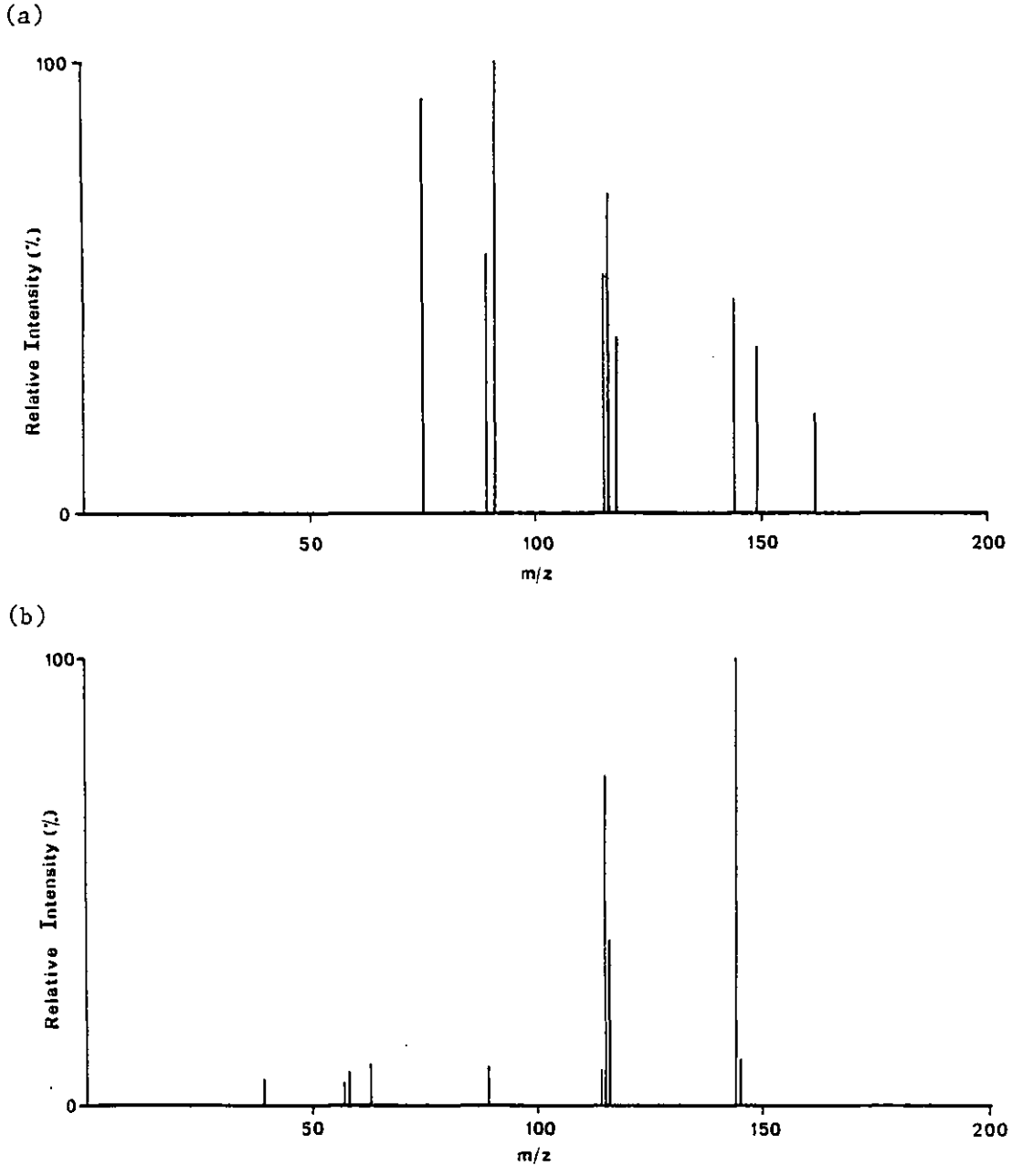


FIGURE 3.6 EI mass spectra of (a) aglycone isolated from rat urine following i.p. administration of (^{14}C)-naphthalene (540 μmole/kg, 5 μCi) and (b) naphth-1-ol



3.2 iii (b) Bile duct-cannulated rats

Bile collected 0-1 h from rats administered (^{14}C)-naphthalene contained one major metabolite when analysed by t.l.c. (solvent systems A and B; Table 3.6), p.c. (solvent systems E and F) and h.p.l.c. (solvent system I; Figure 3.7). This metabolite was found to be positive to ninhydrin and potassium dichromate/silver nitrate reagent sprays (see Chapter 2). The metabolite co-chromatographed on t.l.c. (solvent systems A and B) and h.p.l.c. (solvent system I) with the product of an *in vitro* incubation of naphthalene, identified as *S*-(1,2-dihydro-hydroxynaphthyl)glutathione (see Section 3.1 iii). Following mild

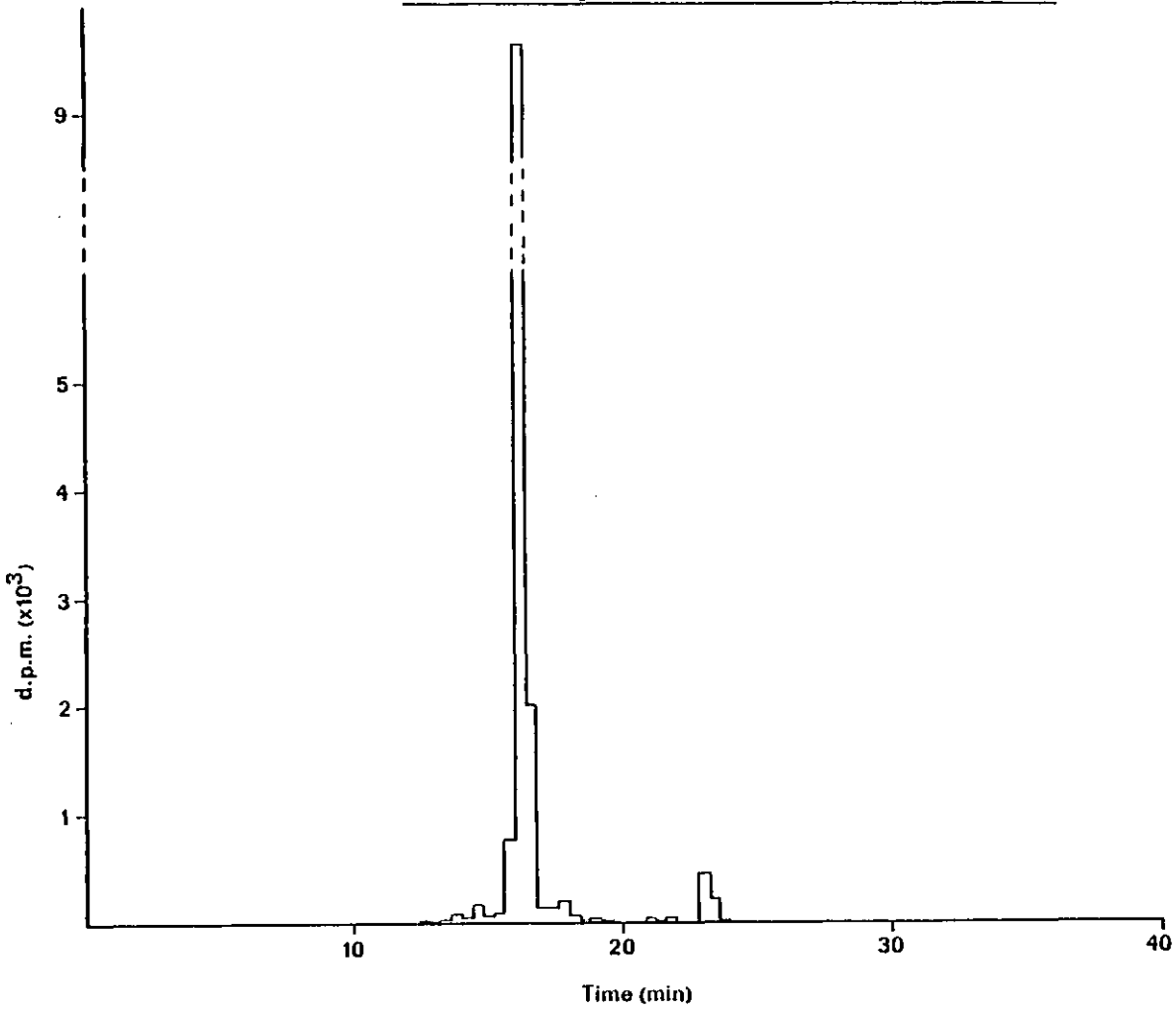
TABLE 3.6 T.l.c. analysis* of bile (0-1 h) following i.v. administration of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi)

Rf	% Total ^{14}C in bile
0.10	2.4 ± 0.2
0.26	5.8 ± 0.3
0.39	76.0 ± 2.6
0.47	13.7 ± 2.5

* Solvent system A was used
Values are mean ± S.D. (n=3)

acid treatment, the metabolite co-chromatographed with *S*-(1-naphthyl) glutathione when analysed by t.l.c. (solvent systems A and B) and p.c. (solvent systems E and F). The metabolite was purified from bile collected for 1 h following i.v. administration of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi) by t.l.c. in solvent system B. Mild acid treatment of this purified metabolite and analysis by t.l.c., confirmed co-chromatography with standard *S*-(1-naphthyl)glutathione.

FIGURE 3.7 H.p.l.c. profile of bile collected for 1 h following i.v. administration of (^{14}C)-naphthalene (30 μmole/kg, 5 μCi)



Stronger acid hydrolysis of the purified metabolite by reflux in 6 N HCl for 3 h resulted in the production of one radioactive band on t.l.c. (solvent system A), which co-chromatographed with S-(1-naphthyl) cysteine, together with ninhydrin-positive compounds which co-chromatographed with standard glutamic acid and glycine.

During further purification by h.p.l.c. (solvent system I) of the metabolite from bile, some breakdown to a less polar compound was observed. This product was further purified by h.p.l.c. using solvent system II and analysed by negative ion FAB mass spectrometry. The spectrum contained molecular ion peaks at m/z 432 and m/z 454, together with a fragmentation peak at m/z 159. This latter peak was probably due to a thionaphthalene ion, seen in FAB mass spectra of other naphthalene adducts (see Section 3.1 iii) and the mass spectrum suggested the structure of the product to be S-(naphthyl)glutathione (cf. positive ion mode spectrum of standard S-(naphthyl) glutathione, Figure 3.1a). All of this evidence indicated that the major metabolite observed in bile of naphthalene-treated rats was S-(1,2-dihydro-1-hydroxy-2-naphthyl)glutathione.

Another naphthalene metabolite present in bile following i.v. administration of (^{14}C)-naphthalene (30 μmole/kg, 5 μCi) (Rf 0.47, Table 3.6), remained unidentified. The Rf values of this metabolite on t.l.c. (solvent systsms A and B) were increased by mild acid treatment suggesting that it was a dihydrohydroxy naphthyl derivative, although it did not co-chromatograph with any available standards. It gave positive reactions to both ninhydrin and potassium dichromate/silver nitrate reagents, indicating that it contained divalent sulphur and a free amino group. It was not hydrolysed to organic solvent-soluble metabolites on incubation with the purified β-glucuronidase enzymes

used. The metabolite may be a cysteinylglycine conjugate of naphthalene, identified by Boyland et al. (1961) in the bile of rats administered naphthalene. However, no peak corresponding to the tentative S-(dihydrohydroxynaphthyl)cysteinylglycine standard was observed when the bile was analysed by h.p.l.c. (solvent system I). No naphthyl pre-mercapturic acid, identified in the urine of bile duct-cannulated (vide infra) and intact animals (Section 3.2 iii (a)), could be detected in bile.

Urine collected for 6 h from bile duct-cannulated rats administered (^{14}C)-naphthalene (30 μmole/kg, 5 μCi), contained three radioactive metabolites on analysis by t.l.c. (solvent systems A and B, Table 3.7) and p.c. (solvent systems E and F). These metabolites had Rf values

TABLE 3.7 T.l.c. analysis* of urine (0-6 h), of bile duct-cannulated rats following i.v. administration of (^{14}C)-naphthalene (30 μmole/kg, 5 μCi)

Rf	% Total ^{14}C in urine
0.43	9.7 ± 3.1
0.50	37.2 ± 3.8
0.63	48.3 ± 1.0

* Solvent system A was used
Values are mean ± S.D. (n=3)

similar to three of the metabolites in urine collected for 24 h from non-bile duct-cannulated rats administered (^{14}C)-naphthalene (Table 3.5). The major metabolite (Rf 0.63) had the same chromatographic properties as the major metabolite found in the 0-24 h urine of non-bile duct-cannulated rats and co-chromatographed with standard

N-acetyl-S-(1-naphthyl)cysteine, following mild acid treatment of urine. It was, thus, believed to be N-acetyl-S-(dihydrohydroxynaphthyl)cysteine.

β-Glucuronidase incubation of urine, collected for 6 h from bile duct-cannulated rats administered naphthalene, followed by ethyl acetate extraction of the incubation at pH 7, resulted in 29.6±6.9% extraction of the urinary radioactivity into the organic phase. The aglycone, resulting from β-glucuronidase incubation, was isolated and characterised from urine collected for 24 h from a rat administered (^{14}C)-naphthalene (600 μmole/kg, i.p.), in the same manner as that used for the aglycone from intact rat urine (Section 3.2 iii (a)). The final product, purified by h.p.l.c. (solvent system II), gave an EI mass spectrum identical to that observed for the aglycone from the urine of non-bile duct-cannulated rats and is shown in Figure 3.8. This aglycone was, therefore, believed to be 1,2-dihydro-1,2-dihydroxynaphthalene and the urinary metabolite, 1,2-dihydro-1,2-dihydroxynaphthalene-O-β-glucuronide.

3.2 iii (c) Enterohepatic circulation experiments

Bile collected 1-2 h following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene (11.1 μmole/kg, 2 μCi) contained the highest concentration of radioactivity and was, therefore, analysed by t.l.c., p.c. and h.p.l.c. Chromatographic analyses showed the presence of 3 radioactive bands (Table 3.8). The metabolite at Rf value 0.61 had chromatographic properties on t.l.c., p.c. and h.p.l.c. similar to those of the major metabolites in the urine of bile duct-cannulated and intact rats given naphthalene (Tables 3.5 and 3.7). This metabolite co-chromatographed with standard N-acetyl-S-(1-naphthyl)cysteine following mild acid treatment and was tentatively identified

FIGURE 3.8 EI mass spectrum of a metabolite of naphthalene isolated following β-glucuronidase treatment of urine from bile duct-cannulated rat administered ^{14}C-naphthalene (600 μmole/kg, 5 μCi)

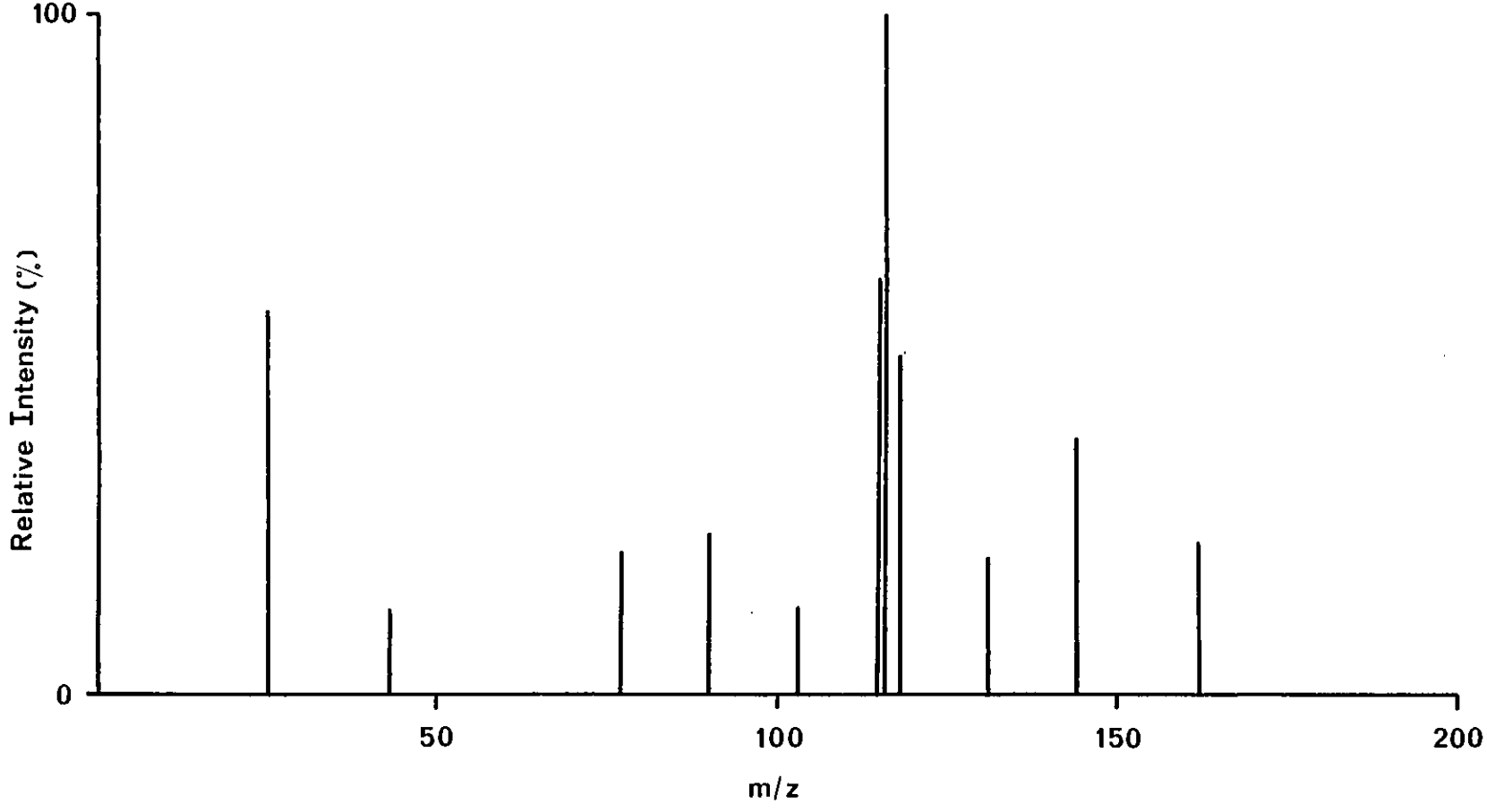


TABLE 3.8 T.l.c. analysis* of bile (1-2 h) following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene (11 µmole/kg, 2 µCi)

Rf	% Total ^{14}C in bile
0.24	49.4 ± 8.0
0.38	13.3 ± 5.7
0.61	20.4 ± 2.4

* Solvent system A was used
Values are mean ± S.D. (n=3)

as N-acetyl-S-(dihydrohydroxynaphthyl)cysteine. The metabolite, Rf 0.38, had properties on t.l.c., p.c. and h.p.l.c. similar to the major biliary metabolite of naphthalene, previously shown (Section 3.2 iii (b)) to be S-(1,2-dihydrohydroxynaphthyl)glutathione. The major radiolabelled metabolite in bile collected 1-2 h following i.d. infusion of (^{14}C)-naphthalene biliary metabolites (11.1 µmole/kg, 2 µCi) was a compound of higher polarity than that seen for S-(1,2-dihydrohydroxynaphthyl)glutathione. The Rf values of this compound were not altered on analysis by t.l.c. or p.c. by mild acid treatment or by β-glucuronidase incubation. Boyland et al. (1961) showed the presence of polar amino acid conjugates in the bile of rats administered high doses of naphthalene (approximately 250 mg/kg., i.p. daily for 5 days). These workers suggested that the polar compounds may be taurine conjugates of S-dihydrohydroxynaphthyl glutathione, since they yielded S-(1-naphthyl)cysteine, glutamic acid, glycine and taurine on acid hydrolysis. These compounds were present in small amounts relative to other conjugates. A major polar metabolite of naphthalene has been tentatively identified in rat bile by Bakke as an O-glucuronide of S-(dihydrohydroxynaphthyl)cysteine (Bakke, 1982). This compound

should be hydrolysed by β-glucuronidase, an enzyme which had no effect on the major biliary metabolite in the studies reported in this thesis. However, the β-glucuronidase from bovine liver used in these experiments may not be active in the hydrolysis of the unusual glucuronide reported by Bakke (1982).

Analysis by t.l.c. and p.c. of urine collected for 24 h following i.d. infusion of the biliary metabolites of (^{14}C)-naphthalene (11.1 μmole/kg, 2 μCi) showed the presence of one major radioactive metabolite, together with two minor metabolites (Table 3.9). The major metabolite (Rf 0.62), which accounted for 81.6±4.0% of the urinary radioactivity, co-chromatographed with standard N-acetyl-S-(1-naphthyl) cysteine, following mild acid treatment. The metabolite in urine was thus, identified as N-acetyl-S-(1,2-dihydrohydroxynaphthyl)cysteine.

TABLE 3.9 T.l.c. analysis* of urine (0-24 h) following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene (11 μmole/kg, 2 μCi)

Rf	% Total ^{14}C in bile
0.39	4.9 ± 0.3
0.55	9.5 ± 2.7
0.62	81.6 ± 4.0

* Solvent system A was used
Values are mean ± S.D. (n=3)

3.2 iv Enterohepatic circulation of (^{14}C)-naphthalene biliary metabolites in antibiotic treated rats

To investigate the influence of intestinal microflora on the enterohepatic circulation of naphthalene metabolites, biliary metabolites of (^{14}C)-naphthalene were infused intraduodenally (0.3 µmole/kg, 0.5 µCi) into a group of bile duct-cannulated rats, which had been previously treated with oral antibiotics to suppress intestinal microflora.

Table 3.10 shows the profile and extent of excretion of radioactivity in the bile and urine of carrier- and antibiotic-treated rats, following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene. No significant differences ($p<0.05$; Analysis of variance test) were found between the excretion in urine and bile nor in total excretion over 24 h when antibiotic-treated rats were compared with carrier-treated animals. Thus, following i.d. infusion of the biliary metabolites of (^{14}C)-naphthalene into antibiotic- and carrier-treated animals, peak levels of radioactivity were found in bile samples collected 1-2 h following infusion. Excretion of radioactivity in bile collected for 24 h from antibiotic-treated animals was 32.3±10.0% of the dose, and in carrier-treated, excretion was 32.4±4.2% of the dose. Similarly, urinary excretions over 24 h in antibiotic- and carrier-treated animals were similar (51.6±8.4% and 27.9±20.3% of the dose, respectively).

On analysis by t.l.c. (solvent system A), the major metabolite in urine collected for 24 h from both antibiotic- and carrier-treated rats following infusion of naphthalene biliary metabolites, was naphthyl pre-mercapturic acid. Similarly, the t.l.c. profiles of radiolabelled naphthalene metabolites in bile collected 1-2 h following i.d. infusion into antibiotic- and carrier- treated rats were qualitatively similar

TABLE 3.10 Excretion of radioactivity following i.d. infusion of biliary metabolites of (^{14}C)-naphthalene (0.3 μmole/kg, 0.5 μCi) in carrier-and antibiotic-treated rats

Route	Time (h)	% Dose excreted in	
		Carrier-treated	Antibiotic-treated
Bile	0 - 1	7.4 ± 1.9	3.5 ± 1.6
	1 - 2	15.0 ± 3.8	15.6 ± 2.1
	2 - 3	4.9 ± 1.5	6.8 ± 1.6
	3 - 4	1.6 ± 0.8	2.7 ± 2.2
	4 - 5	0.9 ± 0.4	1.4 ± 1.0
	5 - 6	0.6 ± 0.3	0.7 ± 0.5
	6 - 24	1.9 ± 1.2	1.5 ± 1.3
Urine	0 - 24	27.9 ± 20.3	51.6 ± 8.4
Total	0 - 24	60.3 ± 20.6	84.0 ± 1.6

Values are mean ± S.D. (n = 3)

to each other.

3.2 v Enterohepatic recycling of the major biliary metabolite of naphthalene

The major biliary metabolite of naphthalene, shown to be S-(1,2-dihydrohydroxynaphthyl)glutathione by methods described earlier in this chapter, was isolated by t.l.c. (solvent system B) from bile, collected for 1 h from rats administered (^{14}C)-naphthalene (30 μmole/kg, 5 μCi). The purified (^{14}C)-labelled conjugate was dissolved in bile (1 ml) and infused intraduodenally (2.1 μmole/kg, 0.7 μCi) over 1 h into a group of bile duct-cannulated rats to investigate uptake and excretion of radioactivity derived from this metabolite separate from other naphthalene biliary metabolites. Table 3.11 shows that excretion following i.d. infusion of the isolated conjugate was similar to that seen following i.d. infusion of a mixture of naphthalene biliary metabolites (cf. Table 3.4). Peaks concentrations of radioactivity were observed in 1-3 h bile and total excretion in bile and urine over 24 h was 82.2±4.8% of the infused dose.

Radiolabelled metabolites in bile and urine of rats infused with the (^{14}C)-labelled purified conjugate were qualitatively similar to those seen in excreta of rats infused with a mixture of biliary metabolites, when analysed by t.l.c. Thus, the major metabolites present in bile and urine were an unknown polar compound and naphthyl pre-mercapturic acid, respectively, which were derived from S-(1,2-dihydrohydroxynaphthyl) glutathione.

3.3 DISCUSSION

The metabolism and excretion of (^{14}C)-naphthalene have been investigated

TABLE 3.11 Excretion of radioactivity in bile duct-cannulated rats following i.d. infusion of (^{14}C)-labelled major biliary metabolite of naphthalene (2.1 μmole/kg, 0.7 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	2.5 ± 2.0
	1 - 2	13.3 ± 4.9
	2 - 3	11.4 ± 2.3
	3 - 4	3.5 ± 1.7
	4 - 5	1.8 ± 0.8
	5 - 6	1.1 ± 0.4
	6 - 24	4.2 ± 1.4
Urine	0 - 24	44.5 ± 2.5
Total	0 - 24	82.2 ± 4.8

Values are mean ± S.D. (n = 3)

in intact rats and rats fitted with bile duct cannulae. Following i.v. administration of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi), radiolabelled metabolites were excreted predominantly in the urine of non-bile duct-cannulated animals and identified as a mercapturic acid and as a glucuronic acid conjugate. Excretion of radioactivity in faeces accounted for only 10% of the administered dose. The mercapturic acid was tentatively identified as N-acetyl-S-(1,2-dihydro-1-hydroxy-2-naphthyl)cysteine. This compound has been termed a pre-mercapturic acid (Knight and Young, 1958), since it can undergo dehydration to a mercapturic acid on treatment with mild acid. The glucuronic acid conjugate found as the other major urinary metabolite of naphthalene was identified as an O-β-glucuronide of trans-1,2-dihydro-1,2-dihydroxy-naphthalene (naphthalene dihydrodiol). These findings confirm original observations made by a number of workers that naphthyl pre-mercapturic acid and naphthalene dihydrodiol glucuronide are major urinary metabolites of naphthalene in the rat (Young 1947; Corner and Young, 1954; Boyland and Solomon, 1955; Boyland and Sims, 1958). Having established that the metabolism of naphthalene in intact animals was in accordance with that previously reported by other workers, and that thioether derivatives were found as naphthalene metabolites, naphthalene was used to study the excretion of thioether-containing compounds in bile and their fate in the intestine.

It was found (Figure 3.3) that approximately 64% of an i.v. dose of (^{14}C)-naphthalene (30 µmole/kg, 5 µCi) was excreted in bile within 6 h of dosing, principally as a glutathione conjugate. The glutathione conjugate was identified as S-(1,2-dihydro-1-hydroxy-2-naphthyl) glutathione (Table 3.6, Figure 3.4). These data are in agreement with the findings of Boyland et al. (1961), who identified a number of amino acid conjugates of naphthalene in the bile of rats given high

doses of naphthalene (approximately 250 mg/kg, i.p. daily for 5 days). These workers observed that the relative amounts of the amino acid conjugates, as judged by the sizes and intensities of their spots on paper chromatograms, were somewhat variable, but usually the glutathione derivative was the predominant metabolite. No data were presented on the extent to which naphthalene metabolites were excreted in bile.

Another amino acid conjugate of naphthalene was identified in the bile of rats administered naphthalene, although no N-acetylcysteine derivative (naphthyl pre-mercapturic acid) was noticed (Table 3.6). The presence of naphthyl pre-mercapturic acid reported by Boyland et al. (1961) in the bile of rats administered naphthalene, may have been a consequence of the relatively high doses used and of the collection times employed. Thus, naphthalene was found to be excreted in rat bile predominantly as a glutathione conjugate. The fate of this conjugate in the intestine was investigated following the infusion of a mixture of biliary metabolites of naphthalene and of the purified conjugate itself.

Following intraduodenal infusion of the biliary metabolites of (^{14}C)-naphthalene (collected for 1 h from rats administered ^{14}C - naphthalene, 30 μmole/kg, i.v. and containing principally the glutathione conjugate; Table 3.6), approximately 85% of the infused radioactivity was absorbed from the intestine and excreted in bile and urine in 24 h following infusion (Table 3.4). The fact that absorption and excretion resulted partly from the glutathione conjugate was illustrated by i.d. infusion of the isolated conjugate. Results obtained with the isolated conjugate were similar to those when a mixture of biliary metabolites were infused (Table 3.11 cf. Table 3.4). Thus, the principal metabolite found following i.d. infusion of either S-(1,2-

dihydrohydroxynaphthyl)glutathione or a mixture of biliary metabolites of naphthalene was the premercapturic acid (N-acetyl-S-(1,2-dihydro-hydroxynaphthyl)cysteine), which was excreted in urine (approximately 50% of the infused dose) and in bile (approximately 5% of the infused dose) (Tables 3.8 and 3.9). The glutathione conjugate was also metabolised (approximately 13% of the infused dose) to a more polar compound (as judged by t.l.c. and p.c.) excreted solely in bile. This compound remained unidentified, but may have resulted from further oxidative metabolism and conjugation with, for example, glucuronic acid as has been reported recently in studies with naphthalene (Bakke, 1982, Bakke et al., 1982). Hence, a major metabolite of naphthalene in the rat is a glutathione conjugate, which is excreted in bile. Metabolism of this conjugate on enterohepatic circulation is the major source of the corresponding pre-mercapturic acid excreted in urine.

The pattern of uptake and excretion in bile of metabolites derived from the glutathione conjugate of naphthalene, when administered by infusion into the duodenum, differed markedly from the enterohepatic recycling of the other major group of conjugates of xenobiotics found in bile, namely, β-glucuronides. Absorption and excretion in bile of radioactivity derived from intraduodenal infusion of the biliary metabolites of (^{14}C)-naphthalene or (^{14}C)-labelled S-(1,2-dihydro-hydroxynaphthyl)glutathione, occurs maximally approximately 1-3 h following infusion (Tables 3.4 and 3.11). However, in the case of intraduodenal infusion of a group of glucuronides, maximal excretion of metabolites was observed in bile collected 5-7 h after intraduodenal infusion (Parker et al., 1980). This latter time period was thought to be that required for the passage of glucuronides down the intestine to the large intestine, where gut bacteria β-glucuronidase activity

was required for hydrolysis of the glucuronides. Uptake of the released aglycone, was followed by glucuronidation in either the liver or intestine and release of the resynthesised glucuronide into bile. Antibiotic pretreatment of animals greatly diminished the enterohepatic circulation of the glucuronides, but not the corresponding aglycones (Parker et al., 1980). Antibiotic treatment of rats did not significantly affect the enterohepatic recycling of the biliary metabolites of naphthalene or alter the metabolic profile of naphthalene metabolites present in the bile and urine of recipient animals (Table 3.10). The antibiotic regime used in these experiments has been shown to suppress the intestinal microflora of rats involved in the enterohepatic recycling of glucuronic acid conjugates of phenolphthalein, morphine and diphenylacetic acid (Parker et al., 1980) and of 3-phenoxybenzoic acid (Huckle et al., 1981). Provided that the antibiotic treatment had the same effects on the gut bacteria in experiments with the biliary metabolites of naphthalene as it did with experiments with glucuronides, these results (Table 3.10) do not indicate a role of gut microflora in the enterohepatic recycling of naphthalene metabolites, and, hence, the enterohepatic recycling of S-(1,2-dihydrohydroxynaphthyl)glutathione.

Gut bacteria are believed to be involved in the further metabolism of thioether-containing metabolites of naphthalene to methylthio compounds (Stillwell et al., 1978b; Section 1.8 i). Pretreatment of rats with neomycin led to a decrease in the formation of a number of methythio derivatives of naphthalene excreted in urine, although these compounds only represented <1% of a dose of 100 mg/kg of naphthalene (Stillwell et al., 1978a) and, therefore, represent a minor pathway of naphthalene metabolism in the rat. In addition, formation of methylthio derivatives and further oxidation products (methylsulphinyl and methylsulphonyl derivatives) of xenobiotics by gut bacteria is believed

to occur *via* the action of gut bacterial C-S lyase (Bakke *et al.*, 1980), an enzyme which has no activity with glutathione conjugates (Suzuki *et al.*, 1982; Table 1.7).

Uptake of glutathione conjugate may proceed by direct uptake without metabolism in the gastro-intestinal tract, since the glutathione conjugate is excreted in bile following i.d. infusion of a mixture of biliary metabolites of naphthalene, containing principally the glutathione conjugate (Table 3.8). The process of direct uptake of the glutathione conjugate of naphthalene from the intestine may occur in parallel with metabolism on uptake (see Chapter 7).

CHAPTER FOUR

GLUTATHIONE CONJUGATION AND ENTEROHEPATIC CIRCULATION IN THE METABOLISM OF 1-CHLORO-2,4-DINITROBENZENE

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4.1 MATERIALS AND METHODS

4.1 i Chemicals and radiochemicals

1-Chloro-2,4-dinitro (U-^{14}C) benzene (sp. act. 5.87 mCi/mmole) and 1-fluoro-2,4-dinitro (U-^{14}C) benzene (sp. act. 21 mCi/mmole) were obtained from Amersham International p.l.c. and used as supplied (radiochemical purities, 98% and 99% respectively). 1-Fluoro-2,4-dinitrobenzene was obtained from Sigma Chemical Co. Ltd. and 1-chloro-2,4-dinitrobenzene from BDH Chemicals Ltd. N-Acetylcysteine was obtained from Sigma Chemical Co. Ltd.

4.1 ii Synthesis of standards

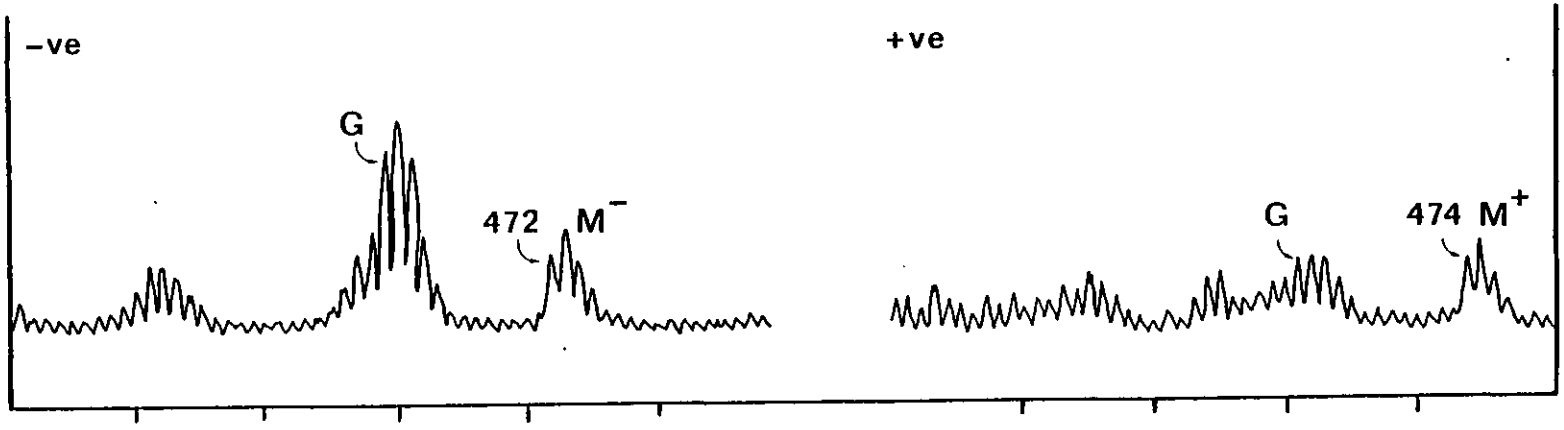
S-(2,4-Dinitrophenyl)glutathione was synthesised as follows. 1-Chloro-2,4-dinitrobenzene (0.2 g, 1 mmole) was added in methanol to 0.92 g (3 mmole) GSH in water (20 ml). The pH of the solution was adjusted to 9 with NaOH (5 M) and the mixture left to stir at ambient temperature overnight. Unreacted 1-chloro-2,4-dinitrobenzene was extracted with chloroform (3 x 10 ml) and the aqueous phase adjusted to pH 4. This solution was left at 4°C and the yellow needle-like crystals which formed were collected by filtration and dried (P_2O_5). The product was recrystallised from water and gave a melting point of 210°C (decomp.: 211°C, Sokolovsky et al., 1964).

(^{14}C)-Labelled S-(2,4-dinitrophenyl)glutathione was synthesised on a micropreparative scale from (^{14}C)-1-fluoro-2,4-dinitrobenzene and GSH as described by Sokolovsky et al. (1964). Thus, (^{14}C)-1-fluoro-2,4-dinitrobenzene (25 µCi), diluted with 1-fluoro-2,4-dinitrobenzene to give a specific radioactivity of 0.37 µCi/µmole, was added to 21 mg

(68 μmole) GSH in 1 N potassium bicarbonate (0.27 ml). This was shaken for 10 min by hand and then acidified using 3 drops of 5 M HCl, mixing thoroughly after each drop. The resultant yellow precipitate was collected by centrifugation and the pellet washed successively with 0.01 M HCl, acetone and diethylether (2 x 0.5 ml each). The pellet was dried under a stream of oxygen-free nitrogen and then *in vacuo*. The product was found to be 98.8% radiochemically pure following t.l.c. analysis in solvent systems A (Rf 0.45) and B (Rf 0.30). FAB mass spectrometry of this compound in positive and negative ion modes gave molecular ion peaks at *m/z* 474 ($[M + H]^+$) and *m/z* 472 ($[M - H]^-$), respectively (Figure 4.1a). The spectrum obtained from ^{1}H-n.m.r. analysis of the product was consistent with its structure as *S*-(2,4-dinitrophenyl)glutathione in the sense that sufficient signals were present for the amino acids and aromatic ring, with shifts expected for 2,4-dinitro substitution.

S-(2,4-Dinitrophenyl)cysteine was synthesised by a modification of the method of Zahn and Traumann (1954). Cysteine HCl (2.6 g, 20 mmole) was dissolved in 10 ml water with stirring under a stream of oxygen-free nitrogen, followed by the addition of 8.2 g sodium acetate in 30 ml water and 40 ml 2 N sodium acetate buffer, pH 5.2. 1-Fluoro-2,4-dinitrobenzene (3.3 g, 20 mmole) in methanol (50 ml) was then added over 2-3 min and, following discontinuation of the nitrogen stream, stirred strongly at ambient temperature. After 2 h, the intense yellow precipitate was filtered and washed consecutively with water (10 ml), acetone (5 ml) and diethylether (10 ml). The yellow product remaining was dried to give a crude yellow-orange solid. The cysteine conjugate was further purified by solubilisation in 1 M HCl by stirring for 1 h at ambient temperature. Undissolved material was removed by centrifugation (3000 r.p.m., 5 min) and the pH of the supernatant

FIGURE 4.1 (a) Molecular ion region of the FAB mass spectra (-ve and +ve ion modes) of S-(2,4-dinitrophenyl)glutathione. Peaks labelled G are from the solvent (glycerol) ± Na^+

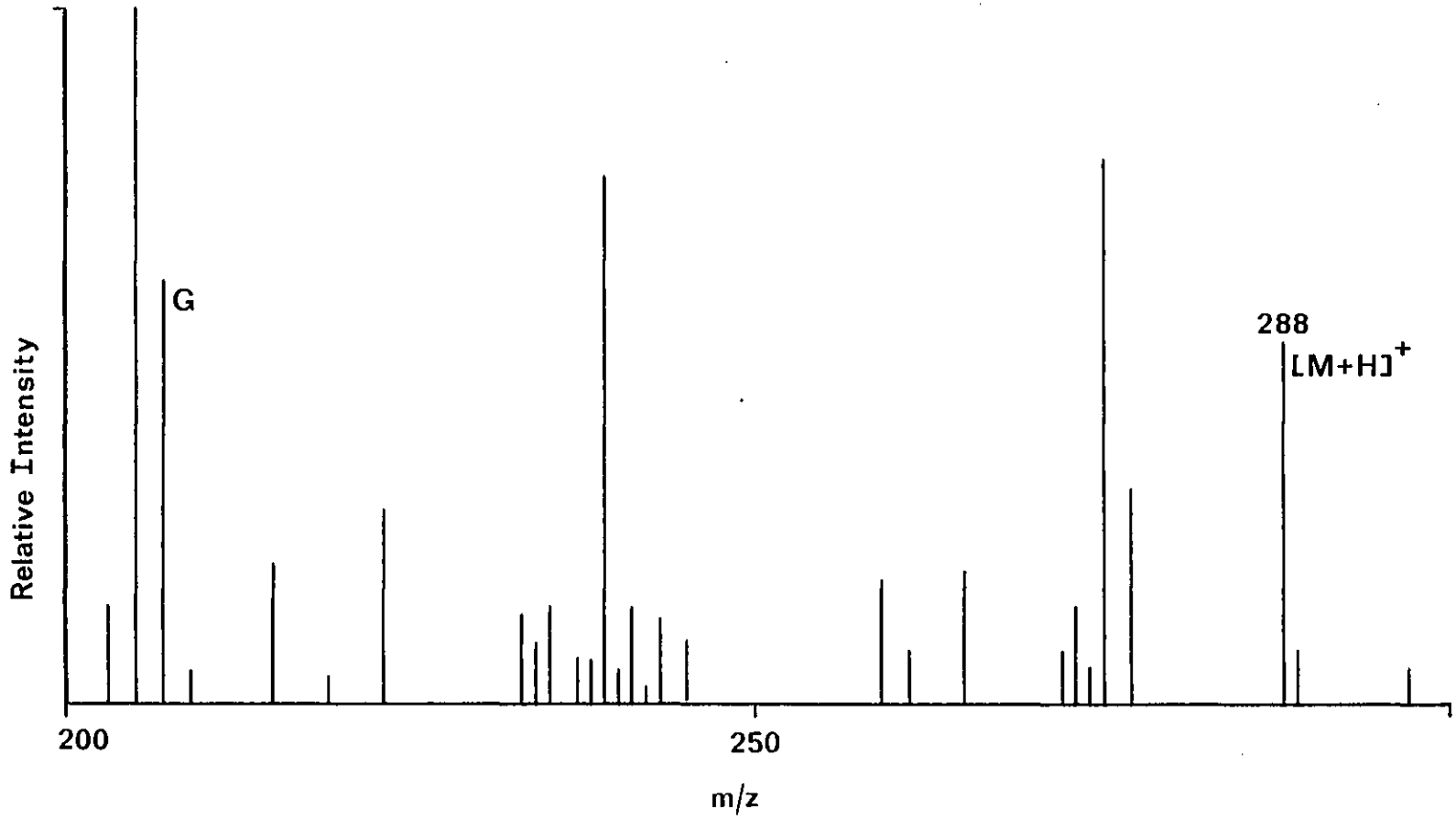


was slowly adjusted to 5-6 by dropwise addition of 1.0 M NaOH, which resulted in the formation of a dense yellow precipitate. The precipitate was collected by centrifugation, washed with water and dried *in vacuo*. The conjugate was dissolved in methanol and gave a single U.V.-quenching spot on t.l.c. in solvent system A (Rf 0.57), which gave a positive reaction with a ninhydrin reagent (see Chapter 2). This compound on reverse-phase h.p.l.c. (solvent system II) gave only one U.V.-absorbing peak (Rt 20.65 min). Following isolation of this peak from a number of h.p.l.c. applications, the relevant fractions from the column were pooled and lyophilised. Positive ion mode FAB mass spectrometry of the purified conjugate gave a molecular ion peak at *m/z* 288 ($[M + H]^+$) (Figure 4.1b), identifying the conjugate as *S*-(2,4-dinitrophenyl) cysteine.

N-Acetyl-*S*-(2,4-dinitrophenyl)cysteine (dinitrophenyl mercapturic acid) was synthesised by the method Lamoureux and Davison (1975) in which 1-chloro-2,4-dinitrobenzene is reacted with *N*-acetylcysteine at pH 9. The final yellow product was dried (P_2O_5) and subject to analysis by EI mass spectrometry. This spectrum showed a parent molecular ion at *m/z* 328 (M-1; 0.2%) and fragmentation mass ions at *m/z* 200 (dinitrophenylthiol ion, 47%), *m/z* 129 ($CH_2 = C(COOH)NHCOCH_3$, 34%) and *m/z* 87 (100%). The EI mass spectrum obtained agreed with the spectrum for *N*-acetyl-*S*-(2,4-dinitrophenyl)cysteine reported by Chen and Dorough (1980).

The cysteinylglycine conjugate of 1-chloro-2,4-dinitrobenzene was synthesised from *S*-(2,4-dinitrophenyl)glutathione by incubation with γ-glutamyltransferase. A problem similar to the case of naphthalene was found on incubation of the glutathione conjugate with γ-glutamyl

FIGURE 4.1 (b) FAB mass spectrum of S-(2,4-dinitrophenyl) cysteine. Peaks labelled G are from the solvent (glycerol) ± Na^+



transferase, since the product of this incubation co-chromatographed on t.l.c. (solvent system A) and h.p.l.c. (solvent system I) with S-(2,4-dinitrophenyl)cysteine. When the incubation was performed using γ-glutamyltransferase preincubated with 10 mM EDTA to inhibit aminopeptidase activity (see Section 3.1 iii), the product of the incubation had a mobility intermediate between that of the glutathione and cysteine conjugates (Figure 4.2 and Table 4.1). This compound was tentatively assigned the structure S-(2,4-dinitrophenyl)cysteinyl glycine.

TABLE 4.1 Thin-layer (t.l.c.) and reversed-phase high performance liquid chromatography (h.p.l.c.) of synthesised conjugates of 1-chloro-2,4-dinitrobenzene

Standard	$Rf_{T.l.c.}$(a)	$Rt(min)_{H.p.l.c.}$(b)
S-(2,4-Dinitrophenyl)glutathione	0.42	19.5
S-(2,4-Dinitrophenyl)cysteinylglycine	0.49	19.0
S-(2,4-Dinitrophenyl)cysteine	0.51	17.8
N-Acetyl-S-(2,4-dinitrophenyl)cysteine	0.60	23.0

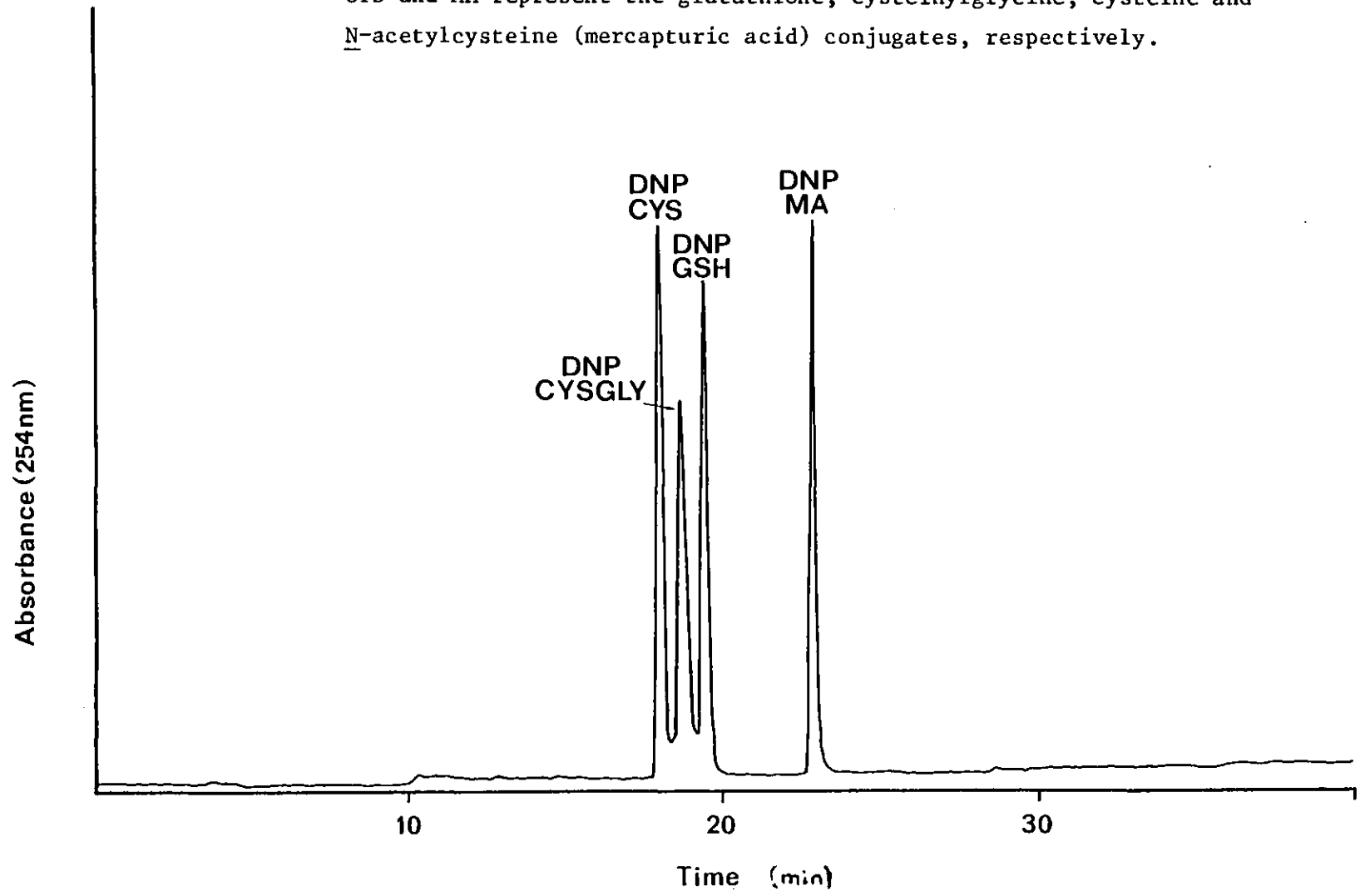
(a) Solvent system A was used

(b) Solvent system I was used

The mobilities of the synthesised dinitrophenyl S-conjugates on t.l.c. and h.p.l.c. are shown in Table 4.1 and Figure 4.2.

(^{14}C)-Labelled N-acetyl-S-(2,4-dinitrophenyl)cysteine (2.47 μCi/μmole)

FIGURE 4.2 H.p.l.c. separation of dinitrophenyl S-conjugates. DNPGSH, CYSGLY, CYS and MA represent the glutathione, cysteinylglycine, cysteine and N-acetylcysteine (mercapturic acid) conjugates, respectively.



was isolated from urine collected for 1 h following i.v. administration of (^{14}C)-1-chloro-2,4-dinitrobenzene to bile duct-cannulated rats (5 μmole/kg, 3 μCi), by extraction into diethylether at pH 1-2. The extract was purified by t.l.c. (solvent system A) and found to be 96.6% radiochemically pure.

4.1 iii Administration of compounds

For administration to rats, (^{14}C)-1-chloro-2,4-dinitrobenzene was solubilised following dilution with unlabelled 1-chloro-2,4-dinitrobenzene in 20% (v/v) Mulgofen:ethanol (1:1 v/v) in water as described for naphthalene (Chapter 3). Dose solutions of (^{14}C)-1-chloro-2,4-dinitrobenzene were at a concentration of 10 μmole/ml (sp. act. 2.47 μCi/μmole) and rats were administered (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi) by i.v. injection.

(^{14}C)-Labelled S-(2,4-dinitrophenyl)glutathione (sp. act. 0.37 μCi/μmole) was dissolved in control bile (1 μmole/ml) for infusion into the duodena of rats (4.3 μmole/kg, 0.33 μCi).

(^{14}C)-Labelled N-acetyl-S-(2,4-dinitrophenyl)cysteine (sp. act. 2.47 μCi/μmole) was dissolved in control bile (0.3 μmole/ml) for i.d. infusion experiments in rats (1.2 μmole/kg, 0.75 μCi).

4.1 iv Analysis of excreta

Bile and urine from rats administered (^{14}C)-1-chloro-2,4-dinitrobenzene or conjugates were analysed by liquid scintillation counting as described in Chapter 2. Analysis of bile and urine for metabolites were performed by t.l.c. (solvent system A) and h.p.l.c. (solvent

system I) and metabolites were identified by co-chromatography with available standards.

Quantitation of radioactive metabolites was performed as described in Section 2.3 iii following t.l.c. of bile and urine in solvent system A. This method reproducibly accounted for >94% radioactivity applied to chromatograms.

4.2 RESULTS

4.2 i Biliary excretion and enterohepatic circulation of (^{14}C)-1-chloro-2,4-dinitrobenzene

Following i.v. administration of (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi) to bile duct-cannulated rats, approximately one-third of the dose (35.5±5.3% of the dose) was excreted in bile collected for 24 h (Table 4.2). Urinary excretion accounted for approximately half of the administered dose (50.6±10.0% of the dose) over the same time period. Excretion in both bile and urine appeared to be rapid following i.v. administration, since an additional experiment with the same dose of (^{14}C)-1-chloro-2,4-dinitrobenzene showed that a large proportion of the radiolabel excreted was recovered in 0-1 h bile (27.1 ±1.8% of the dose) and urine (48.6±5.3% of the dose).

To examine whether metabolites of 1-chloro-2,4-dinitrobenzene, excreted in bile, could be absorbed from the intestinal tract, bile collected for 1 h from rats administered (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi, i.v.) was infused intraduodenally into a second group of bile duct-cannulated rats and bile and urine analysed for radioactivity. As is shown in Table 4.3, 61.6±11.3% of the infused

TABLE 4.2 Excretion of radioactivity following i.v. administration of (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	30.4 ± 5.3
	1 - 2	2.4 ± 0.4
	2 - 3	0.9 ± 0.1
	3 - 4	0.5 ± 0.1
	4 - 5	0.3 ± 0.1
	5 - 6	0.2 ± 0.1
	6 - 24	1.0 ± 0.2
Urine	0 - 24	50.6 ± 10.0
Total	0 - 24	86.1 ± 14.9

Values are mean ± S.D. (n = 3)

TABLE 4.3 Excretion of radioactivity following i.d. infusion of biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene (1.3 μmole/kg, 0.8 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	3.0 ± 2.2
	1 - 2	12.4 ± 3.8
	2 - 3	5.1 ± 2.5
	3 - 4	3.2 ± 1.2
	4 - 5	2.0 ± 0.3
	5 - 6	1.2 ± 0.1
	6 - 24	3.4 ± 0.6
Urine	0 - 24	31.3 ± 3.0
Total	0 - 24	61.6 ± 11.3

Values are mean ± S.D. (n = 3)

dose was absorbed from the intestinal tract and excreted in bile and urine over 24 h. Approximately equal amounts were excreted in bile (30.3±9.5% of the dose) and urine (31.3±3.0% of the dose). Absorption and biliary excretion appeared to be rapid, since peak concentrations of radioactivity were found in bile samples collected 1-2 h following infusion (Table 4.3).

4.2 ii Analysis of metabolites

Table 4.4 shows the metabolic profiles of bile and urine collected for 1 h following i.v. administration of (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi) following analysis by t.l.c. (solvent system A). Two metabolites, accounting for approximately 82% of the biliary radioactivity, were found to be the major metabolites in bile.

TABLE 4.4 T.l.c. analyses of bile and urine (0-1 h) following i.v. administration of (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi)

Rf[a]	% Total ^{14}C in Bile	% Total ^{14}C in Urine
0.36	2.9 ± 1.8	n.d.
0.41	37.9 ± 4.9	n.d.
0.48	5.4 ± 1.2	n.d.
0.54	n.d.[b]	6.3 ± 0.9
0.61	44.4 ± 3.7	85.5 ± 2.0

(a) Solvent system A was used

(b) n.d. = not detected

Values are mean ± S.D. (n=3)

The metabolite, Rf 0.41, was tentatively identified by co-chromatography as S-(2,4-dinitrophenyl)glutathione. Similarly, the metabolite, Rf 0.61, which co-chromatographed with standard dinitrophenyl mercapturic acid, was tentatively identified as N-acetyl-S-(dinitrophenyl) cysteine. Co-chromatography of the major metabolites with standard glutathione and N-acetylcysteine S-conjugates was confirmed by h.p.l.c. (Figure 4.3).

Urine collected for 1 h from bile duct-cannulated rats administered (^{14}C)-1-chloro-2,4-dinitrobenzene, (5 μmole/kg, 3 μCi) showed only two radioactive bands on analysis by t.l.c. (Table 4.4). The major metabolite (Rf 0.61), representing 86% of the urinary radioactivity, co-chromatographed with standard N-acetyl-S-(2,4-dinitrophenyl) cysteine, on analysis by t.l.c. (solvent system A) and h.p.l.c. (solvent system I). The major metabolite was purified from urine by extraction into diethylether, following adjustment of urine to pH 1-2 with 0.1 M HCl. The conjugate was purified from the organic extract by t.l.c. (solvent system A), followed by h.p.l.c. (solvent system I). Fractions containing the conjugate were pooled and lyophilised, and the resultant yellow solid was analysed by positive ion FAB mass spectrometry. A molecular ion peak was observed at m/z 330 ($[M + H]^+$) (Figure 4.4). The major urinary metabolite of 1-chloro-2,4-dinitrobenzene in bile duct-cannulated rats was, therefore, identified as N-acetyl-S-(2,4-dinitrophenyl)cysteine.

A much more complicated metabolite profile was found in bile and urine following i.d. infusion of the biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene (Table 4.5). The major metabolite (Rf 0.61) in bile representing 75% of the biliary radioactivity, was shown by co-chromatography to be N-acetyl-S-(2,4-dinitrophenyl)cysteine.

FIGURE 4.3 H.p.l.c. analysis of 0-1 h bile following i.v. administration of (^{14}C)-1-chloro-2,4-benzene (5 μmole/kg, 3 μCi)

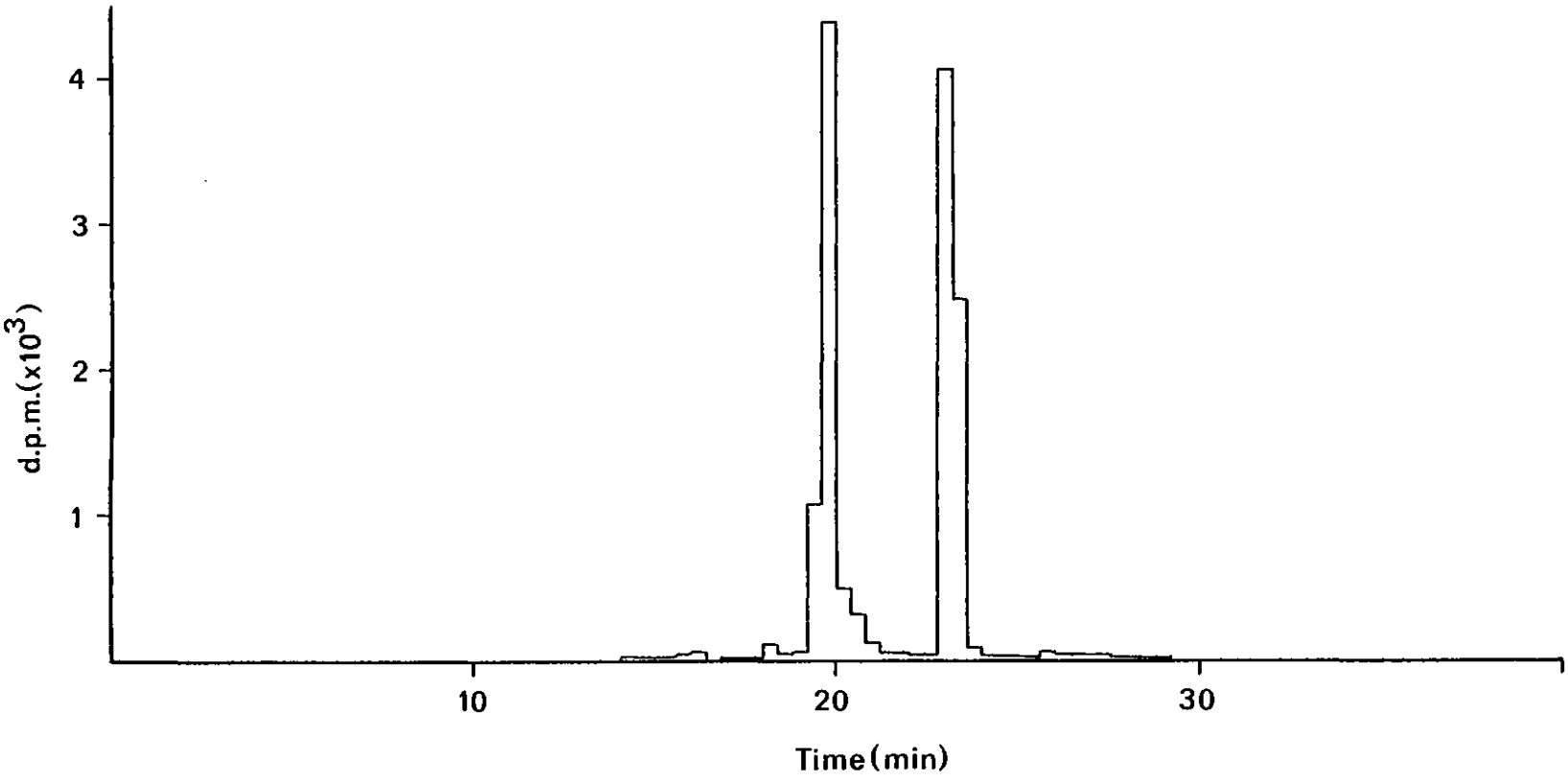


FIGURE 4.4 FAB mass spectrum of the major urinary metabolite of 1-chloro-2,4-dinitrobenzene isolated from bile duct-cannulated rats administered (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi). Peaks labelled G are from the solvent (glycerol) ± Na^+

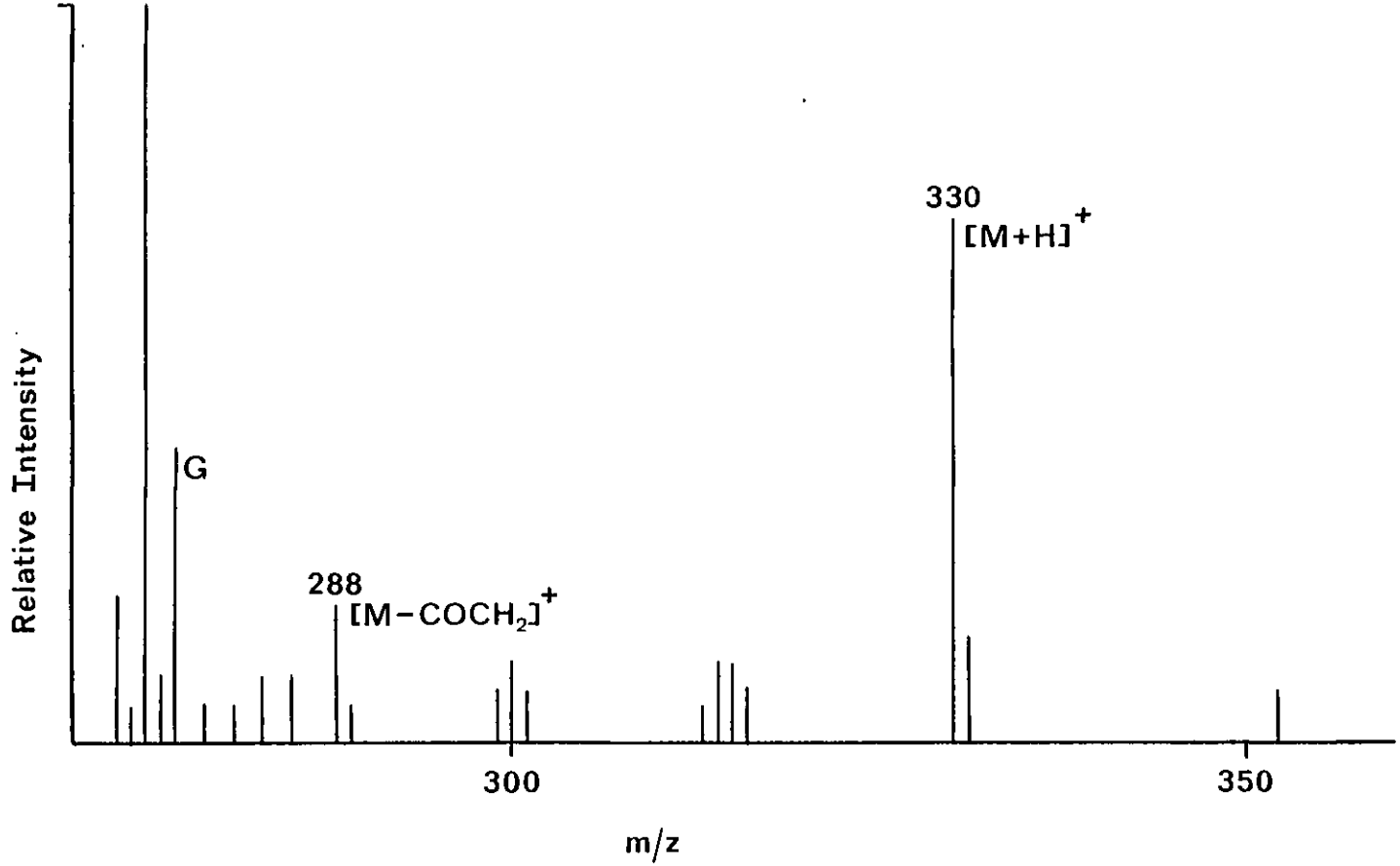


TABLE 4.5 T.l.c. analyses of bile (1-2 h) and urine (0-24 h) following i.d. infusion of biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene (1.3 μmole/kg, 0.8 μCi)

Rf[a]	% Total ^{14}C in Bile	Urine
0.41	n.d.[b]	8.3 ± 0.9
0.44	7.4 ± 0.8	n.d.
0.51	3.2 ± 0.9	7.5 ± 1.0
0.56	3.3 ± 0.8	14.4 ± 2.1
0.61	75.2 ± 4.2	51.7 ± 5.6
0.69	n.d.	7.5 ± 1.0

(a) Solvent systen A was used

(b) n.d. = not detected

Values are mean ± S.D. (n=3)

Small amounts (7.4±0.8% of the biliary radioactivity) of a compound (Rf 0.44) co-chromatographing (t.l.c. and h.p.l.c.) with S-(2,4-dinitrophenyl)glutathione were found in bile following i.d. infusion of the biliary metabolites of 1-chloro-2,4-dinitrobenzene and this may have arisen directly from the glutathione conjugate in the bile infused (see Table 4.4 for composition of infused bile).

The dinitrophenyl mercapturic acid (N-acetyl-S-(2,4-dinitrophenyl) cysteine)was identified by co-chromatography as the major radiolabelled metabolite in urine (51.7±5.6% of the urinary radioactivity) of rats infused intraduodenally with biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene (Table 4.5). The metabolite, Rf value 0.51, found in both bile (3.2±0.9% of the biliary radioactivity) and urine (7.5±1.0% of the urinary radioactivity) was identified by co-chromatography as

S-(2,4-dinitrophenyl)cysteine. A number of other metabolites, notably one with Rf value 0.56, remained unidentified.

To investigate the absorption and excretion of radioactivity derived individually from the dinitrophenyl glutathione and mercapturic acid conjugates, these (^{14}C)-labelled compounds were infused, in separate experiments, intraduodenally into bile duct-cannulated rats and bile and urine analysed for radioactivity.

4.2 iii Enterohepatic recycling of S-(2,4-dinitrophenyl)glutathione

Following i.d. infusion of (^{14}C)-labelled S-(2,4-dinitrophenyl)glutathione (4.3 μmole/kg, 0.3 μCi), 63.8±10.1% of the infused radioactivity was absorbed and excreted in bile and urine (Table 4.6). Approximately equal amounts were excreted in bile (28.5±6.3% of the dose) and urine (35.3±11.1% of the dose). The profile of biliary excretion was similar to that seen following i.d. infusion of the biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene, with highest concentrations of radioactivity appearing in bile collected 1-2 h following infusion.

Bile (1-2 h) and urine (0-24 h) following i.d. infusion of (^{14}C)-S-(2,4-dinitrophenyl)glutathione (4.3 μmole/kg, 0.3 μCi) were analysed by t.l.c. (Table 4.7) and h.p.l.c. The major metabolite in both bile and urine accounting for 83.3±1.5% and 68.0±4.2% of the biliary and urinary radioactivity, respectively, was identified as N-acetyl-S-(2,4-dinitrophenyl)cysteine by co-chromatography. Bile also contained a compound (Rf 0.45; 12.9±2.4% of the biliary radioactivity) which co-chromatographed with standard S-(2,4-dinitrophenyl)glutathione. The metabolite, Rf 0.53, found in both bile and urine co-chromatographed with the standard cysteine conjugate. Thus, i.d. infusion of

TABLE 4.6 Excretion of radioactivity following i.d. infusion of (^{14}C)-labelled S-(2,4-dinitrophenyl)glutathione (4.3 μmole/kg, 0.33 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	4.1 ± 2.6
	1 - 2	14.3 ± 5.4
	2 - 3	5.3 ± 0.8
	3 - 4	1.7 ± 0.3
	4 - 5	0.8 ± 0.2
	5 - 6	0.4 ± 0.1
	6 - 24	1.8 ± 0.2
Urine	0 - 24	35.3 ± 11.1
Total	0 - 24	63.8 ± 10.1

Values are mean ± S.D. (n = 3)

TABLE 4.7 T.l.c. analyses of 1-2 h bile and 0-24 h urine following i.d. infusion of (^{14}C)-1-labelled-S-(2,4-dinitrophenyl)glutathione (4.3 μmole/kg, 0.33 μCi)

Rf[a]	% Total ^{14}C in Urine	% Total ^{14}C in Bile
0.45	n.d.[b]	12.9 ± 2.4
0.53	5.8 ± 2.5	2.1 ± 0.5
0.56	18.4 ± 0.9	n.d.
0.60	68.0 ± 4.2	83.3 ± 1.5

(a) Solvent system A was used

(b) n.d. = not detected

Values are mean ± S.D. (n=3)

S-(2,4-dinitrophenyl)glutathione resulted in metabolism of the glutathione conjugate to the cysteine and N-acetylcysteine derivatives, which were excreted in both bile and in urine. I.d. infusion of the glutathione conjugate also led to the biliary excretion of the glutathione conjugate and this may have arisen by absorption and excretion without metabolism (see Discussion).

4.2 iv Enterohepatic recycling of N-acetyl-S-(2,4-dinitrophenyl)cysteine

Table 4.8 shows the results of an experiment in which purified (^{14}C)-labelled N-acetyl-S-(2,4-dinitrophenyl)cysteine was infused intraduodenally (1.2 μmole/kg, 0.75 μCi) into bile duct-cannulated rats. Approximately 55% of the infused dose was absorbed and excreted in bile and urine over 24 h. Excretion in bile was rapid, with highest concentrations found in bile collected 1-4 h following i.d. infusion (Table 4.8).

TABLE 4.8 Excretion of radioactivity following i.d. infusion of (^{14}C)-labelled N-acetyl-S-(2,4-dinitrophenyl)cysteine (1.2 μmole/kg, 0.75 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	1.6 ± 0.9
	1 - 2	5.9 ± 1.2
	2 - 3	5.4 ± 1.9
	3 - 4	6.3 ± 3.4
	4 - 5	2.6 ± 0.5
	5 - 6	1.8 ± 0.1
	6 - 24	3.7 ± 1.6
Urine	0 - 24	28.1 ± 2.4
Total	0 - 24	55.3 ± 5.3

Values are mean ± S.D. (n = 3)

Bile and urine, collected 1-2 h and 0-24 h respectively, following i.d. infusion of (^{14}C)-N-acetyl-S-(2,4-dinitrophenyl)cysteine were analysed by t.l.c. and h.p.l.c. The major radioactive metabolite in both bile and urine was identified by co-chromatography as N-acetyl-S-(2,4-dinitrophenyl)cysteine (Table 4.9). This compound represented approximately 86% of the biliary radioactivity and approximately 47% of the urinary radioactivity. A metabolite with Rf value 0.50 (Table 4.9)

TABLE 4.9 T.l.c. analysis of bile (1-2 h) and urine (0-24 h) following i.d. infusion of (^{14}C)-labelled N-acetyl-S-(2,4-dinitrophenyl)cysteine (1.2 μmole/kg, 0.75 μCi)

Rf[a]	% Total ^{14}C in Bile	Urine
0.42	4.6 ± 0.8	9.2 ± 2.7
0.50	2.0 ± 0.4	8.9 ± 3.2
0.55	n.d.[b]	15.2 ± 5.5
0.62	86.4 ± 2.5	47.1 ± 9.5

(a) Solvent system A was used

(b) n.d. = not detected

Values are mean ± S.D. (n=3)

appeared to co-chromatograph on t.l.c. with standard S-(2,4-dinitrophenyl)cysteine, although it was not possible to demonstrate this on h.p.l.c. The presence of a number of other metabolites derived from the infused dinitrophenyl mercapturic acid in bile and urine was observed.

4.2 v Intraperitoneal administration of S-(2,4-dinitrophenyl)glutathione

In a further experiment, (^{14}C)-labelled S-(2,4-dinitrophenyl)glutathione was administered by i.p. injection (4.3 μmole/kg, 0.3 μCi) to a group of bile duct-cannulated rats and excretion of radioactivity in bile and urine followed (Table 4.10). A greater excretion in bile and urine over 24 h was observed following i.p. (91.9±11.5% of the dose) as compared with i.d. administration (63.8±10.1% of the dose; Table 4.6). Uptake and biliary excretion of radioactivity following i.p. administration was rapid, with most (80%) of the radioactivity excreted in bile collected for 1 h.

Analyses of bile and urine collected for 1 h and 24 h, respectively, following i.p. administration of (^{14}C)-S-(2,4-dinitrophenyl)glutathione (Table 4.11) showed a similar profile to that seen following i.d.

TABLE 4.11 T.l.c. analyses of bile (0-1 h) and urine (0-24 h) following i.p. administration of (^{14}C)-S-(2,4-dinitrophenyl)glutathione (4.3 μmole/kg, 0.3 μCi)

Rf[a]	% Total ^{14}C in Bile	% Total ^{14}C in Urine
0.43	35.0 ± 0.8	n.d.
0.49	7.7 ± 0.8	n.d.
0.56	n.d.[b]	22.2 ± 1.0
0.61	53.2 ± 3.0	55.4 ± 4.1

(a) Solvent system A was used

(b) n.d. = not detected

Values are mean ± S.D. (n=3)

TABLE 4.10 Excretion of radioactivity following i.p. administration of (^{14}C)-S-(2,4-dinitrophenyl) glutathione (4.3 μmole/kg, 0.3 μCi)

Route	Time (h)	% Dose excreted
Bile	0 - 1	30.3 ± 4.5
	1 - 2	5.7 ± 1.4
	2 - 3	1.2 ± 1.0
	3 - 4	0.8 ± 0.8
	4 - 5	0.1 ± 0.1
	5 - 6	0.1 ± 0.1
	6 - 24	0.0
Urine	0 - 24	53.6 ± 8.5
Total	0 - 24	91.9 ± 11.5

Values are mean ± S.D. (n = 3)

infusion (cf. Table 4.7). However, following i.p. administration there were greater amounts of the glutathione conjugate and lesser amounts of the mercapturic acid in bile when compared with the same metabolites in bile following i.d. infusion (Table 4.11 cf. Table 4.7). Differences in the relative quantities of biliary metabolites obtained when S-(2,4-dinitrophenyl)glutathione was administered intraduodenally and intraperitoneally may have reflected the metabolic form in which the glutathione conjugate was presented to the liver.

In all the samples of urine analysed from the experiments involving 1-chloro-2,4-dinitrobenzene, S-(2,4-dinitrophenyl)glutathione or N-acetyl-S-(2,4-dinitrophenyl)cysteine, whether given by i.v., i.d. or i.p. administration, a metabolite remained unidentified but was the most significant of the minor metabolites identified in urine on t.l.c. (see Discussion).

4.2 vi Enterohepatic recycling of 1-chloro-2,4-dinitrobenzene metabolites in antibiotic-treated rats

To investigate the role of the intestinal microflora in the enterohepatic circulation of dinitrophenyl glutathione and N-acetylcysteine conjugates, these conjugates in bile were infused intraduodenally into a group of bile duct-cannulated rats which had been pretreated with antibiotics and the excretion compared with control-treated rats. For this purpose, bile was collected for 1 h from rats administered (^{14}C)-1-chloro-2,4-dinitrobenzene (5 μmole/kg, 3 μCi, i.v.). This bile contained approximately equal amounts of the glutathione and N-acetylcysteine conjugates (see Table 4.4) and rats received the biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene at a dose

of 1.1 μmole/kg (0.66 μCi).

Excretion in antibiotic- and carrier-treated rats is shown in Table 4.12. No significant differences ($p<0.05$) were found between excretion in bile and urine over 24 h when antibiotic-treated rats were compared with carrier-treated. Examination by t.l.c. (solvent system A) of bile and urine collected 1-2 h and 0-24 h, respectively, showed no significant differences in the presence of the major metabolites in bile and urine. Thus, N-acetyl-S-(2,4-dinitrophenyl) cysteine represented 79.0±2.1% and 78.3±0.7% of the biliary radioactivity and 50.8±3.9% and 48.6±2.3% of the urinary radioactivity in antibiotic- and carrier-treated rats, respectively. Similarly, S-(2,4-dinitrophenyl)glutathione accounted for 7.1±1.9% and 8.1±0.8% of the biliary radioactivity in antibiotic- and carrier-treated rats, respectively. It was found difficult to observe differences between antibiotic- and control-treated rats in the relative amounts of minor metabolites, especially in urine, because of the low amounts of radioactivity which could be chromatographed.

4.3 DISCUSSION

1-Chloro-2,4-dinitrobenzene has been used for a number of years as a substrate for the assay of glutathione S-transferases (Habig et al., 1974a) and the formation of a glutathione conjugate catalysed by these enzymes is responsible for the depletion of cellular glutathione caused by 1-chloro-2,4-dinitrobenzene seen in liver (Wahlländer and Sies, 1979), skin (Summer and Göggelmann, 1980) and erythrocytes (Awasthi et al., 1981). The metabolism of 1-chloro-2,4-dinitrobenzene and excretion in bile and urine has been investigated in the rat.

TABLE 4.12 Excretion of radioactivity following i.d. infusion of biliary metabolites of (^{14}C)-1-chloro-2,4-dinitrobenzene (1.1 μmole/kg, 0.66 μCi) in carrier-and antibiotic-treated rats

Route	Time (h)	% Dose excreted in	
		Carrier-treated	Antibiotic-treated
Bile	0 - 1	4.5 ± 1.2	2.8 ± 0.5
	1 - 2	13.4 ± 2.3	7.9 ± 0.5
	2 - 3	5.9 ± 1.8	5.3 ± 0.5
	3 - 4	3.2 ± 0.6	3.5 ± 0.8
	4 - 5	1.8 ± 0.4	2.8 ± 0.5
	5 - 6	1.0 ± 0.4	2.3 ± 0.3
	6 - 24	3.1 ± 1.0	1.9 ± 1.2
Urine	0 - 24	36.1 ± 4.4	42.4 ± 5.6
Total	0 - 24	69.0 ± 3.6	69.1 ± 6.9

Values are mean ± S.D. (n = 3)

1-Chloro-2,4-dinitrobenzene underwent glutathione conjugation, following i.v. administration (5 μmole/kg, 3 μCi), and the glutathione conjugate formed, S-(2,4-dinitrophenyl)glutathione, was identified as a major biliary metabolite (Table 4.4). This is in agreement with the findings of Wahlländer and Sies (1979), who reported the biliary excretion of S-(2,4-dinitrophenyl)glutathione, following perfusion of the isolated rat liver with 1-chloro-2,4-dinitrobenzene. Metabolism of the glutathione conjugate was presumably the source of the other major metabolite of 1-chloro-2,4-dinitrobenzene found in rat bile, N-acetyl-S-(2,4-dinitrophenyl)cysteine (dinitrophenyl mercapturic acid). The mercapturic acid was also found as the major urinary metabolite of 1-chloro-2,4-dinitrobenzene in bile duct-cannulated rats (Table 4.4). N-Acetyl-S-(2,4-dinitrophenyl)cysteine was identified as the major urinary metabolite (60% of the administered dose) of 1-bromo-2,4-dinitrobenzene, when administered by i.p. injection to non-bile duct-cannulated rats at a dose of 50 mg/kg (Chen and Dorough, 1980). The same mercapturic acid would be expected to be formed from either 1-chloro- or 1-bromo-2,4-dinitrobenzene, since glutathione conjugation occurs by replacement of the halide atoms with glutathione (Habig et al., 1974a).

Hence, the major biliary metabolites of 1-chloro-2,4-dinitrobenzene following i.v. administration have been identified as S-(2,4-dinitrophenyl)glutathione and N-acetyl-S-(2,4-dinitrophenyl)cysteine. This metabolic disposition differs from that seen with naphthalene (Chapter 3), since the major biliary metabolite of naphthalene is the corresponding glutathione conjugate and no mercapturic acid was identified in bile following i.v. administration. Differences between the two substrates in terms of their metabolism and disposition will be discussed further in Chapter 7.

The biliary metabolites of 1-chloro-2,4-dinitrobenzene underwent enterohepatic circulation (Table 4.3). Since the major biliary metabolites are the glutathione and mercapturic acid derivatives, the enterohepatic circulation of each of these conjugates was investigated by i.d. infusion of the purified (^{14}C)-labelled compounds. Radioactivity derived from these conjugates was absorbed from the intestine and excreted in bile and urine, following i.d. infusion of 4.3 μmole/kg, or 1.2 μmole/kg of the glutathione of N-acetylcysteine conjugates, respectively (Tables 4.6 and 4.8). The major metabolite excreted following i.d. infusion of either conjugate was the mercapturic acid derivative (Tables 4.7 and 4.9). The infused glutathione conjugate required metabolism to the mercapturic acid, but presumably the infused mercapturic acid was absorbed and excreted directly since it is difficult to envisage a process of cleavage and resynthesis of the mercapturic acid on enterohepatic circulation. Excretion in urine of the mercapturic acids of propachlor (Bakke et al., 1981a) and 2-acetamido-4-(chloromethyl)thiazole (Rafter and Bakke, 1982) following absorption from the gut have been reported.

The absorption and excretion in bile of materials derived from i.d. infusion of (^{14}C)-labelled S-(2,4-dinitrophenyl)glutathione was similar to that observed for the glutathione conjugate of naphthalene (see Chapter 3). Thus, peak concentrations of radioactivity were found in bile samples collected 1-3 h following infusion (Table 4.6). Absorption of S-(2,4-dinitrophenyl)glutathione from the intestine may occur without metabolism, since this conjugate was identified in bile following i.d. infusion of biliary metabolites of 1-chloro-2,4-dinitrobenzene (Table 4.5) or following i.d. infusion of S-(2,4-dinitrophenyl) glutathione (Table 4.7). This was similar to the enterohepatic recycling of S-(1,2-dihydrohydroxynaphthyl)glutathione (Chapter 3).

Intraduodenal infusion of N-acetyl-S-(2,4-dinitrophenyl)cysteine led to the excretion of a number of compounds in bile and urine other than the mercapturic acid (Table 4.9), indicating that some metabolism of the mercapturic acid occurred. Until recently, it was believed that mercapturic acids were the end point of the metabolism of glutathione conjugates and were excreted without further metabolism. However, studies have indicated that N-deacetylation of the mercapturic acids of some xenobiotics may occur in liver and kidney tissue (Suzuki and Tateishi, 1981; see Section 1.5 i). Also, recent studies have indicated that cysteine conjugates of certain xenobiotics can undergo further metabolism by cleavage of the βC-S linkage to form thiols and methylthio compounds (Bakke et al., 1981a; Rafter and Bakke, 1982; Section 1.5 ii). This type of metabolism may be proposed to occur during enterohepatic recycling of dinitrophenyl mercapturic acid. However, it is believed that gut bacteria are principally responsible for C-S lyase activity (Bakke et al., 1980) and antibiotic-pretreatment of rats did not significantly alter the enterohepatic recycling of either N-acetyl-S-(2,4-dinitrophenyl)cysteine or S-(2,4-dinitrophenyl)glutathione (Table 4.12). A relatively minor effect of the elimination of gut bacteria by antibiotic treatment on the metabolism of dinitrophenyl conjugates could not be eliminated in the experiments reported here (Section 4.2 vi). In addition, a cysteine conjugate β-lyase has been isolated from rat liver (Tateishi et al., 1978), which may be involved in further metabolism of dinitrophenyl conjugates. However, although S-(2,4-dinitrophenyl)cysteine is regarded as the usual substrate for the assay of this enzyme (Tateishi and Shimizu, 1980), the enzyme shows no activity with S-(2,4-dinitrophenyl)glutathione nor N-acetyl-S-(2,4-dinitrophenyl) cysteine (Section 1.5 ii). If metabolism of either the glutathione or N-acetylcysteine conjugates to S-(2,4-dinitrophenyl)cysteine occurs

in vivo, then the β-lyase of liver or, indeed bacteria, may play a role in the metabolism of 1-chloro-2,4-dinitrobenzene and may account for the presence of other dinitrophenyl derivatives observed in bile and urine in experiments reported in this Chapter.

1-Chloro-2,4-dinitrobenzene has not only the capacity for nucleophilic replacement of chlorine by glutathione, but also may be subject to reduction of one or both of the aromatic nitro groups. Liver and, especially, intestinal microflora are known to contain active nitroreductases (Hewick, 1982). Indeed, intestinal microflora have been shown to play a large role in the in vivo metabolism of dinitrotoluenes to reactive metabolites. Dinitrotoluene is an important intermediate in the production of toluenediisocyanate used in the manufacture of polyurethane foams, coatings and elastomers and technical grade dinitrotoluene is 75.8% 2,4-dinitrotoluene, 19.5% 2,6-dinitrotoluene and 4.7% other isomers (Long and Rickert, 1982). Both 2,4- and 2,6-dinitrotoluene induce liver tumours when fed to rats and nitro reduction processes catalysed by intestinal microflora are believed to be important in the expression of the genotoxic responses (Rickert et al., 1981; Long and Rickert, 1982; Mirsalis et al., 1982). Thus, axenic rats excreted only 0.1-0.2 times as much of an oral dose of 2,4-dinitrotoluene as the reduced derivatives, 2-amino-4-nitrobenzoic acid and 4-(N-acetyl)-amino-2-nitrobenzoic acid. This was concomitant with a decrease in the covalent binding of metabolites derived from 2,4-dinitrotoluene in axenic when compared wtih conventional rats (Rickert et al., 1981). Similarly, extensive dinitrotoluene-induced DNA repair was shown in rats having the normal complement of gut flora, but not in rats which have no gut flora (Mirsalis et al., 1982). Hence, aromatic nitroreduction of 1-chloro-2,4-dinitrobenzene may be important toxicologically. Metabolites of

1-chloro-2,4-dinitrobenzene containing reduced nitro groups may account for some of the compounds present in bile and urine which remain unidentified.

Reduction of nitro groups has been shown to occur for a number of chloromononitrobenzenes (Bray *et al.*, 1957, 1958), where the products are the related chloroanilines. Moreover, thioether formation and nitro reduction may be proposed to occur together for 1-chloro-2,4-dinitrobenzene, although no aminomercapturic acids have been reported as rat urinary metabolites of chloromononitrobenzenes (Betts *et al.*, 1955, 1957; Bray *et al.*, 1958).

Following i.p. administration of *S*-(2,4-dinitrophenyl)glutathione a much greater proportion of the dose was excreted in bile and urine (91.5±11.5% of the dose), than was seen following i.d. infusion of the same dose of this compound (63.8±10.1% of the dose) (Tables 4.6 and 4.10). Analysis by t.l.c. of bile collected for 1 h following i.p. administration of *S*-(2,4-dinitrophenyl)glutathione, indicated that less metabolism of the glutathione conjugate had occurred, when compared with the metabolite profile in bile following i.d. infusion of *S*-(2,4-dinitrophenyl)glutathione (Tables 4.7 and 4.11). The main differences between these two routes of administration is that following i.d. infusion, metabolites have to pass through intestinal tissue to get into the portal blood system whereas this structure is bypassed following i.p. administration. Differences in the metabolic profile of *S*-(2,4-dinitrophenyl)glutathione following i.d. and i.p. administration may reflect the metabolic forms in which this compound is delivered to the liver and may indicate that metabolism of the conjugate can occur in the intestine (see Chapter 7).

CHAPTER FIVE

GLUTATHIONE CONJUGATION AND ENTEROHEPATIC CIRCULATION IN THE METABOLISM OF BROMSULPHTHALEIN

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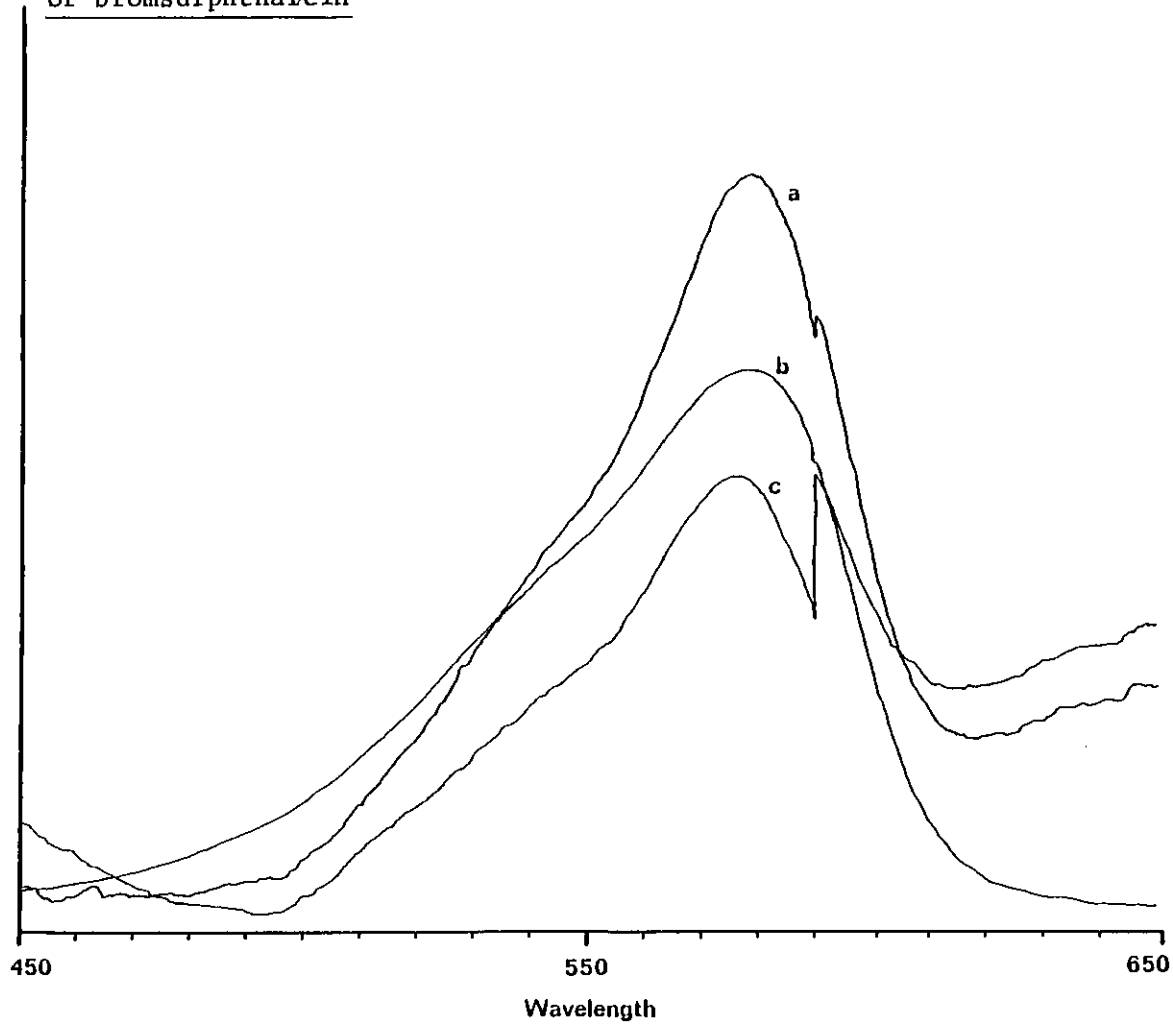
5.1 MATERIALS AND METHODS

5.1 i Chemicals

Bromsulphthalein (sulphobromophthalein, BSP; Na^+ salt) was purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset. The glutathione conjugate of BSP (BSP-GSH) was synthesised as described by Whelan et al. (1970) by reaction of BSP with GSH at pH 10. The final purple-tinged solid (yield 43%, m.p. 211°C, decomp.) was analysed by t.l.c. (solvent systems C, Rf 0.47 and D, Rf 0.12) and found to be 95.5% pure, following quantitation of dye on the chromatograms as described below. The major contaminant was a more polar component, believed to be a diglutathione conjugate of BSP (Javitt et al., 1960; Whelan et al., 1970). The glutathione conjugate was further purified by paper chromatography on Whatman 3MM paper in solvent system C. The conjugate was eluted from the paper into water and lyophilised.
The absorption spectra of BSP and BSP-GSH between 450-650 nm is shown in Figures 5.1a and b, respectively. Both compounds absorbed maximally at a wavelength of 578 nm. The molar extinction coefficient of BSP at 578 nm was calculated to be 6.6×10^4 $cm^2 mol^{-1}$ and those of BSP-GSH and metabolites at the same wavelength were assumed to be identical (Combes, 1965; Whelan et al., 1970). Attempts to analyse the glutathione conjugate of BSP by FAB mass spectrometry (positive ion mode) were unsuccessful. The cysteine conjugate of BSP (BSP-Cys) was synthesised essentially as the glutathione conjugate, except cysteine replaced GSH. The product was analysed by t.l.c. (solvent system C, Rf 0.66) and gave positive reactions with ninhydrin and potassium dichromate/silver nitrate spray reagents (see Chapter 2). The conjugate was further purified by p.c. (solvent system C) and its absorption spectrum found to be identical to that of BSP and BSP-GSH (Figure 5.1c).

FIGURE 5.1 Absorption spectra of (a) bromsulphthalein and (b) the glutathione and (c) the cysteine conjugates of bromsulphthalein



5.1 ii Administration of compounds

BSP and BSP-GSH were administered by i.v. injection to bile duct-cannulated rats. BSP (11.7 µmole/kg) or BSP-GSH (7.5 µmole/kg) were administered to rats in 0.3 ml water. Rats were maintained under anaesthesia throughout the experiments and bile collected on ice at various times up to 4 h, when the animals were killed and urine taken from the bladder.

For enterohepatic circulation experiments, BSP or BSP-GSH (both 9.5 µmole/kg), were dissolved in bile (1 ml) and infused intraduodenally over 1 h into bile duct-cannulated rats. The rats were allowed to recover from anaesthesia and restrained as described in Chapter 2. Bile, urine and faeces were collected for 24 h and the intestines removed from animals when the rats were killed.

5.1 iii Analysis of excreta

BSP-related dye was measured in bile by diluting samples (2-1000-fold v/v) with a reagent as described by Schulze et al. (1979), consisting of sodium chloride (100 ml of a 0.9% solution) and sodium hydroxide (5 ml of a 10% solution). The resulting alkaline solutions were measured for absorbance at 578 nm using a dual beam spectrophotometer (Cecil CE595) against a blank of the alkaline sodium chloride reagent. Absorbance readings of blanks obtained by dilution of bile with appropriate volumes of water were subtracted from measurements in the presence of alkaline sodium chloride. Absorption at 578 nm of bile collected prior to the administration of BSP or BSP-GSH and diluted with alkaline sodium chloride differed by approximately 0.001 absorbance units when compared with the same

bile diluted with water.

Bile samples in which a precipitate had formed were centrifuged (12000 r.p.m., 5 min) and the supernatants analysed.

Urine samples were centrifuged (10000 r.p.m., 5 min) and the pellet obtained washed with water (5 ml). This was centrifuged again and the combined supernatants analysed following 2-fold (v/v) dilution with alkaline sodium chloride as described above.

Dye present in intestines following i.d. infusion of BSP and BSP-GSH was measured by an adaption of the method of Whelan and Combes (1971) for hepatic BSP measurement. Intestines were washed with water (20 ml) and the washings, together with collected faecal samples, were homogenised (Ultra-Turrax; half max. speed, 3 x 15 sec). This homogenate was centrifuged (10000 r.p.m., 10 min), the supernatant removed and the volume recorded. 10 ml 70% (v/v) Methanol/water was then added to the pellet and mixed well. A further 10 ml 100% methanol was added (giving a suspension of approximately 70% methanol) and, following mixing, centrifuged as described above. The supernatant was removed and the volume noted. Washing of the pellet was repeated with 20 ml 30% (v/v) methanol/water and centrifuged. Absorbance measurements were made at 578 nm following 5- or 10-fold (v/v) dilution of supernatants with alkaline sodium chloride. Absorbances of blanks, consisting of water in place of alkaline sodium chloride, were subtracted from measurements in the presence of alkaline sodium chloride. Experiments in which BSP and BSP-GSH were added to the intestinal washes of control rats showed that the recovery of dye by the above procedure was 89.6% and 97.2%, respectively. Dye measurements in the intestinal washings of the enterohepatic cir-

culation experiments were adjusted for these recoveries. Concentrations of the amount of dye in samples were calculated by use of the molar extinction coefficient for BSP at 578 nm. Standard curves for absorbances at 578 nm constructed for BSP and BSP-GSH were linear for concentrations to 10 μM.

Samples of bile (0.001-0.002 ml) were analysed by t.l.c. in solvent systems C and D. BSP and its metabolites were visualised on t.l.c. by viewing under short-wave U.V. light (see Chapter 2) or by exposure to ammonia vapour, when BSP and its metabolites were blue-purple. Quantitation of compounds from t.l.c. of bile (solvent system C) was carried out by scraping 0.5 cm wide strips of silica from chromatograms into alkaline sodium chloride (1 ml) followed by vortexing and centrifugation (12000 r.p.m., 5 min). The absorbances of the supernatants were then measured at 578 nm. Recoveries by this method were found to be 95.0±6.8(n=12)% of the material applied to the chromatograms.

5.2 RESULTS

5.2 i Biliary excretion of bromsulphthalein and its glutathione conjugate

BSP was extensively excreted in bile following i.v. administration to rats (Table 5.1). Following a dose of 11.7 μmole/kg, 81.0±5.8% of the dose was excreted in bile in 4 h. Most (approximately 90%) of this excretion occurred in the first 30 min following BSP administration, demonstrating rapid elimination of BSP in bile. No dye could be measured in the 4 h urine taken from the bladder of these animals (<0.1% of the dose).

TABLE 5.1 Cumulative excretion of dye following i.v. administration of bromsulphthalein (11.7 μmole/kg)

Route	Time (min)	Dye excreted (μmole)
Bile	15	1.31 ± 0.28
	30	1.96 ± 0.29
	45	2.05 ± 0.28
	60	2.09 ± 0.28
	90	2.14 ± 0.28
	120	2.16 ± 0.28
	180	2.18 ± 0.27
	240	2.21 ± 0.25
Urine	240	n.d.

Values are mean ± S.D. (n = 3)

n.d. = not detected (limit 0.0035 μmole)

TABLE 5.2 Cumulative excretion of dye following i.v. administration of bromsulphthalein glutathione conjugate (7.5 μmole/kg)

Route	Time (min)	Dye excreted (μmole)
Bile	15	1.50 ± 0.10
	30	1.93 ± 0.08
	45	2.04 ± 0.08
	60	2.09 ± 0.08
	90	2.13 ± 0.09
	120	2.16 ± 0.09
	180	2.16 ± 0.08
	240	2.19 ± 0.10
Urine	240	n.d.

Values are mean ± S.D. (n = 3)

n.d. = none detected (limit 0.0035 μmole)

The glutathione conjugate of bromsulphthalein (BSP-GSH) was entirely eliminated in bile, following i.v. administration to rats (Table 5.2). 99.7±3.8% of the dose was recovered in bile collected 0-4 h following administration of BSP-GSH (7.5 μmole/kg). As with BSP, biliary excretion was rapid, most (approximately 88%) of the excreted dye appeared in the first 30 min following administration of the conjugate. Dye was undetected (<0.1% of the dose) in the urine of animals administered BSP-GSH.

5.2 ii Analyses of bile

Bile samples containing the highest concentrations of BSP-related dye (0-15 and 15-30 min) following i.v. administration of BSP and BSP-GSH, were analysed by t.l.c. in solvent system C. Following administration of BSP, bile contained unchanged (free) BSP, together with larger amounts of conjugated BSP (Table 5.3). The conjugated

TABLE 5.3 T.l.c. analyses* of bile following i.v. administration of bromsulphthalein (11.7 μmole/kg)

Compound identified	% Total dye in bile 0-15 min	15-30 min
BSP	24.4 ± 0.6	19.7 ± 4.6
BSP-Cys	16.9 ± 9.0	21.4 ± 8.4
BSP-GSH	52.7 ± 9.6	50.2 ± 8.4

* Solvent system C was used
Values are mean ± S.D. (n=3)

forms of BSP were identified as the glutathione and cysteine conjugates by co-chromatography with standards. Conjugated BSP accounted

for 69.5±1.8% and 71.5±1.7% of the total dye in 0-15 and 15-30 min bile samples, respectively. The major form of BSP in bile was the glutathione conjugate. Bile samples collected 15-30 min after administration of BSP contained less BSP and BSP-GSH and relatively more of the cysteine conjugate when compared with bile samples collected 0-15 min (Table 5.3). This was consistent with conjugation of BSP and GSH and metabolism of the glutathione conjugate to the cysteine conjugate before biliary excretion.

Following i.v. administration of BSP-GSH, no free BSP was detected in bile (Table 5.4). The major metabolite in bile samples collected 0-15 and 15-30 min after dosing was unchanged BSP-GSH. The only

TABLE 5.4 T.l.c. analyses* of bile following i.v. administration of bromsulphthalein-glutathione (7.5 μmole/kg)

Compound identified	% Total dye in bile 0-15 min	15-30 min
BSP-Cys	22.4 ± 3.5	38.1 ± 6.5
BSP-GSH	70.4 ± 4.8	52.7 ± 6.5

* Solvent system C was used
Values are mean ± S.D. (n=3)

other metabolite detected in these bile samples was the cysteine conjugate. As in bile from rats given BSP (vide supra), the concentration of BSP-GSH was less in bile collected 15-30 min than in 0-15 min bile samples, and the concentration of the cysteine conjugate was correspondingly increased (Table 5.4).

The presence of the cysteine conjugate in bile samples following

i.v. administration of BSP or BSP-GSH was due to metabolism of BSP-GSH in liver and not breakdown of BSP-GSH in bile itself following excretion, since control experiments in which BSP-GSH was added to bile and incubated at 37°C for 1 h showed no detectable breakdown of the glutathione conjugate (see Chapter 6).

5.2 iii Enterohepatic recycling of bromsulphthalein and its glutathione conjugate

Since BSP and BSP-GSH were the major forms in which BSP was excreted in bile, the enterohepatic circulation of these compounds was investigated. For this purpose, BSP and BSP-GSH were infused intraduodenally into bile duct-cannulated rats and bile and urine analysed for dye. Following i.d. infusion of BSP and BSP-GSH (9.5 μmole/kg), excretion in bile collected for 24 h was found to be very low, accounting for 1.1±0.6% and 1.8±1.2% of the dose of BSP and BSP-GSH, respectively. No dye could be detected in urine (0-24 h), representing <1.0% of the infused dose, for both forms of the dye. The remainder of the dye, 89.8±8.6% of the infused BSP and 88.9±2.8% of the infused BSP-GSH, was recovered in the intestinal washings. Thus, it appeared that neither BSP nor BSP-GSH, which represented the major forms of BSP in bile following i.v. administration, could be absorbed from the intestinal tract and undergo enterohepatic circulation in the rat.

5.3 DISCUSSION

Bromsulphthalein (BSP) was included in investigations designed to obtain information with respect to the enterohepatic disposition of glutathione derivatives because of the well-studied observations that this dye is handled almost entirely by the liver and is excreted

in bile, primarily as a glutathione conjugate. This hepatobiliary disposition was re-investigated in the rat, together with the metabolism of the glutathione derivative following intravenous administration.

BSP was found to be almost entirely eliminated (approximately 81% of the dose in 4 h) in bile of rats following i.v. administration (Table 5.1). This is in agreement with the published results of other workers relating to the elimination of BSP in rat bile (Mendeloff et al., 1949; Krebs, 1959; Klaassen et al., 1981). These findings are also in accordance with its use as an index of normal hepatic function in humans (Rosenthal and White, 1925).

The excretion of BSP in rat urine was found to be undectable (<0.1% of the dose) at the dose levels used (11.7 μmole/kg), again in agreement with published data (Rosenthal and White, 1925). Urinary excretion of BSP in man and experimental species is regarded as a function of liver damage and fairly high BSP levels have been reported in the urine of patients with severe hepatic disorders (Ingelfinger et al., 1948).

BSP was excreted in bile as a mixture of free (nonconjugated) BSP and BSP conjugated with glutathione and cysteine. The major metabolite in the 0-30 min bile samples analysed by t.l.c. was the glutathione conjugate, representing approximately 50% of the biliary BSP-material (Table 5.3). Other workers have found a similar preponderance of the glutathione conjugate in bile following BSP administration in rats (Krebs and Brauer, 1958; Combes, 1959), dogs (Javitt et al., 1960), and man (Monroe and Kittinger, 1961).

When BSP-GSH was administered intravenously (7.5 μmole/kg), all of the

dose was recovered in bile collected for 4 h (Table 5.2). No urinary excretion of the dye was detected (<0.1% of the dose). Some workers have found that BSP is removed from the blood stream by the liver at a greater rate than BSP-GSH, but biliary excretion of the glutathione conjugate is greater than that of BSP (Yokota et al., 1981). This is because conjugation with glutathione facilitates the transport of BSP into bile but is not necessary for hepatic uptake (Combes, 1965; Whelan et al., 1970). It was not possible to discern any differences in the biliary excretion of dye following i.v. administration of BSP and BSP-GSH with the dose levels used in the experiments reported here (Tables 5.1 and 5.2).

Following i.v. administration (7.5 μmole/kg), BSP-GSH was excreted unchanged in bile, together with a metabolite, identified by t.l.c. as the cysteine conjugate (Table 5.4). No cysteinylglycine derivative of BSP could be resolved from the cysteine conjugate band on t.l.c. using solvent systems C and D. Other workers investigating BSP metabolites in bile have identified 2, and occasionally 3, BSP conjugate bands on t.l.c. (Combes, 1959; Combes and Stakelum, 1961; Whelan et al., 1970). These have been tentatively identified as the glutathione conjugate of BSP, by co-chromatography with the synthesised standard, and the cysteine and cysteinylglycine conjugates of BSP, by reaction with ninhydrin and consideration of their t.l.c. mobilities relative to BSP-GSH and BSP (Javitt et al., 1960; Combes, 1965; Whelan et al., 1970). Provided that the t.l.c. used for the analyses of bile reported in Section 5.1 iii was capable of separating the cysteine and cysteinylglycine derivatives of BSP, it appears that the cysteinylglycine derivative was not present in bile collected for 30 min following i.v. administration of BSP or BSP-GSH. Quantitative and qualitative differences in the forms of BSP in bile may be effects of dose,

the cysteinylglycine conjugate only detectable at higher doses of BSP. Indeed in the studies of Whelan et al. (1970) and Combes (1965) in the rat, doses of BSP of 120-180 μmole/kg were used, rather than 11.7 and 7.5 μmole/kg (Tables 5.1 and 5.2). As was observed with metabolites of BSP present in bile after i.v. administration of BSP (Table 5.3), the cysteine conjugate of BSP was present in a proportionally greater amount in bile samples collected 15-30 min when compared with 0-15 min bile samples following i.v. administration of BSP-GSH (Table 5.4). Metabolism of BSP-GSH to the cysteine derivative is catalysed primarily by γ-glutamyltransferase (see Chapter 1). The formation of the cysteine conjugate of BSP excreted in bile following i.v. administration of BSP-GSH may result from γ-glutamyltransferase and aminopeptidase activity present in liver on the luminal surface of cells lining the biliary tract (Tate, 1980), rather than in other tissues (see Chapter 7).

Hence, following i.v. administration of bromsulphthalein to rats, the dye has been shown to be extensively excreted in bile as a mixture of free and conjugated metabolites. The major conjugated form of bromsulphthalein in rat bile was the glutathione derivative (BSP-GSH). The fate of this conjugate, together with the parent drug, in the gastrointestinal tract was examined by infusion into the duodena of bile duct-cannulated rats and bile and urine were assayed for the presence of the dye. Intraduodenal infusion of BSP and BSP-GSH (9.5 μmole/kg), led to little absorption and excretion of dye in bile and urine (1.1% and 1.8% of the dose of BSP and BSP-GSH, respectively). These findings, together with the identification of most of the dose in the intestine, indicated a lack of bioavailability of the biliary metabolites from the intestine (i.e. low enterohepatic circulation). A low enterohepatic recycling of BSP and BSP-GSH is perhaps not

surprising, since the high polar nature of these compounds (see Chapter 6) makes them unsuitable candidates for intestinal absorption, which tends to favour lipophilic compounds (Schanker, 1971). The low bioavailability of the biliary metabolites of bromsulphthalein in the rat in relation to lipid solubility will be discussed more fully in Chapter 7 (Section 7.4).

CHAPTER SIX

IN VITRO METABOLISM OF GLUTATHIONE CONJUGATES

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6.1 MATERIALS AND METHODS

6.1 i Chemicals

Hepes, (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid), bovine serum albumin (Fraction V), protease (type VII) EGTA and EDTA were obtained from Sigma Chemical Co. Ltd. Collagenase (from Clostridium histolyticum) and L-γ-glutamyl-3-carboxy-4-nitroanilide (γ-GCNA) were obtained from Boehringer GmbH, Mannheim, W. Germany.

Medium for cell preparations was a modified Hanks buffer, pH 7.4 (NaCl 8.0 g, KCl 0.4 g, $MgSO_4 7H_2O$ 0.2 g, $NaHPO_4.12H_2O$ 1.2 g, KH_2PO_4 0.06 g and $NaHCO_3$ 2.1 g in 1 litre of water) with additions as described. Medium for all incubations was a Krebs-Henseleit buffer, pH 7.4, containing 25 mM Hepes and, for intestinal cells, 5 mM glucose. Trypan blue (Sigma) was used as a 0.16% (w/v) solution in Krebs-Henseleit buffer, pH 7.4, containing Hepes (25 mM).

All solutions for cell isolation and incubations were bubbled with carbogen gas mixtrue (95% O_2, 5% CO_2) and preheated to 37°C before use. The same gas mixture was used during the perfusion of liver and kidneys and for incubations with isolated cells.

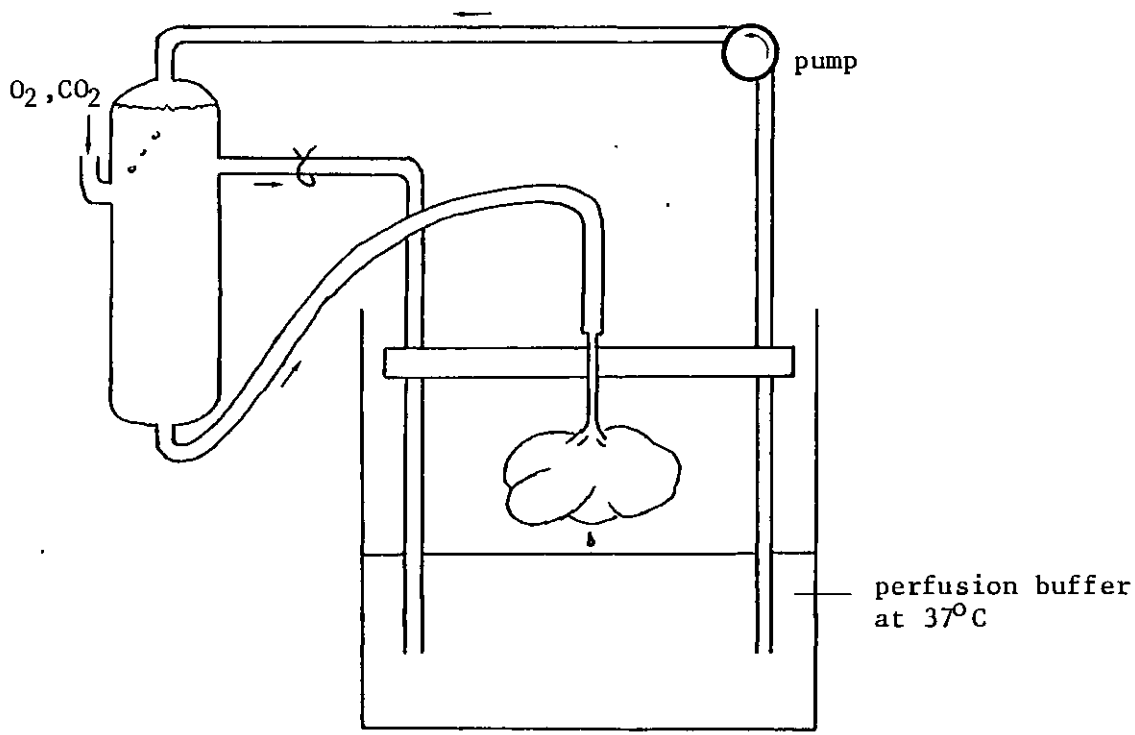
6.1 ii Isolation of liver cells

Isolation of rat hepatocytes was performed essentially by the method of Moldéus et al. (1978b) in which the liver is perfused with collagenase after removal of Ca^{2+} by prior perfusion with a chelator.

Rats were anaesthetised with sodium pentobarbitone (40 mg/kg, i.p.) and the peritoneal cavity opened by a midventral incision. Heparin (500 IU) was injected into the caval vein. A ligature was placed around the portal vein and the vein cannulated towards the liver using a metal cannula, the cannula being secured with a second ligature. To avoid air embolism, perfusion solution was allowed to drip from the cannula before insertion into the vein. Perfusion of the liver was started *in situ* using a modified Hanks buffer, pH 7.4, containing 0.5 mM EGTA, maintained at 37°C.

An adequate perfusion of the liver was observed if the liver cleared immediately and completely. The liver was removed by carefully cutting around it and placed in the perfusion buffer, so that the perfusate could be collected and recirculated (Figure 6.1). After approximately 5 min perfusion, the perfusion assembly was removed from the beaker and transferred to another beaker containing a modified Hanks buffer containing 0.075% (w/v) collagenase and 4 mM CaCl. This buffer was recirculated for approximately 10 min, after which time the liver appeared pale and swollen. The liver was removed from the perfusion apparatus and placed in Krebs-Henseleit buffer, pH 7.4, containing 25 mM Hepes. The capsula was cut and the cells dispersed using a pair of forceps and gently mixed by passing up and down a 10 ml pipette. The cells were filtered through gauze (folded twice) to remove connective tissue and clumps of cells. The cells were allowed to settle at ambient temperature for approximately 5 min and the excess supernatant removed by aspiration. Cells were counted in an improved Neubauer chamber (Scientific Supplies Ltd.) and viability assessed by the ability of the cells to exclude trypan blue. Yields were approximately 150 x 10^6 cells/liver and the cells were found to be 90-95% viable.

FIGURE 6.1 Diagram of perfusion system used for the isolation of rat hepatocytes



6.1 iii Isolation of kidney cells

Isolated rat kidney cells were prepared by the method of Jones *et al.* (1979b), using a recirculating perfusion system similar to that used for hepatocyte isolation.

Rats were anaesthetised with sodium pentobarbitone (40 mg/kg, i.p.) and heparin (1000 IU) was injected into the femoral vein. The peritoneal cavity was opened by a mid-ventral incision and the aorta freed below and above the renal arteries. The coeliac arteries were ligated to avoid leakage of perfusion fluid. Ligatures were placed around the aorta below the renal arteries and around the aorta as high in the abdomen as possible. The aorta was cannulated below the upper ligature using a metal cannula and secured with a new ligature. To avoid air embolism, perfusion fluid was allowed to drip from the cannula during insertion.

Perfusion was started with kidneys *in situ* using a modified Hanks buffer, pH 7.4, containing 12.5 mM Hepes and 0.5 mM EGTA, maintained at 37°C. The flow rate was adjusted to a pump pressure of approximately 30 cm water and within about one minute, both kidneys became pale. The kidneys were excised from the rat and transferred to a beaker from which the perfusate was withdrawn to provide a recirculating system. After approximately 5 min perfusion, the perfusion assembly was transferred to another beaker of modified Hanks buffer, pH 7.4, containing $CaCl_2$ (4 mM) and collagenase (0.075%, w/v). This solution was circulated through the kidneys under constant pressure for approximately 15 min. At the end of this time, the kidneys were removed from the perfusion apparatus, fat and connective tissue were removed and the kidney cells were gently dispersed in Krebs-Henseleit buffer,

pH 7.4, containing 25 mM Hepes. The dispersed cells were filtered through nylon mesh (pore size 100μ diam.; Henry Simon Ltd., Stockport, Cheshire) and allowed to settle for 5 min at ambient temperature, before the removal of excess medium by aspiration. The number of cells was estimated using a modified Neubauer chamber and viability assessed by the ability of cells to exclude trypan blue. Typically, the yield of cells from two kidneys was approximately 15 x 10^6 cells, and the cells were found to be 85-90% viable on isolation.

6.1 iv Isolation of small intestinal cells

Rat small intestinal cells were isolated by incubation of intestinal tissue in a buffer containing protease and EDTA similar to the method of Dawson and Bridges (1979) for guinea pig small intestinal epithelial cells.

Rats were killed by cervical dislocation and the small intestine flushed with ice-cold 0.9% (w/v) sodium chloride. The upper 60 cm of intestine was removed from the rat and cleared of excess connective tissue. Three 20 cm lengths of intestine were then in turn everted over a metal rod, removed from the rod and ligated. Each length of intestine was filled to slight distension with a calcium- and magnesium-free Krebs-Henseleit buffer, pH 7.4, containing 5 mM glucose 20 mM Hepes and 0.5% (w/v) bovine serum albumin (Krebs-CMF). The other end was tied and the lengths of intestine were placed in a 250 ml conical flask containing 40 ml of Krebs-CMF buffer with the addition of protease (10 units/ml) and EDTA (1 mM). The flask was incubated with shaking at 37°C for 20 min. The lengths of intestine were then transferred to another flask containing 40 ml of Krebs-CMF buffer, without enzyme or EDTA, and incubation continued for a further 10 min.

The first incubation with protease and EDTA contained a large amount of mucus and was not used for the harvesting of cells. The cells from the second flask were filtered through gauze (folded twice) and the gauze washed with enzyme-free buffer. The filtrate was centrifuged at approximately 500 r.p.m. for 5 min and the pellet obtained washed with a modified Krebs-Henseleit buffer, pH 7.4, containing 25 mM Hepes and 5 mM glucose. Cells were resuspended at a concentration of approximately 20 x 10^6 cells/ml. Cells were counted in an improved Neubauer chamber and viability assessed by the ability of the cells to exclude trypan blue. The total yield from a 60 cm length of small intestine was approximately 60 x 10^6 cells. The cells were found to be 90-95% viable.

6.1 v Cell incubations

Isolated cells were routinely used in incubations within 30 min of preparation. Incubations were carried out in Krebs-Henseleit buffer, pH 7.4, containing Hepes (25 mM) and, with intestinal cells, glucose (5 mM). Incubations were performed at 37 °C in shaking (100 cycles/min) 25 ml round-bottom flasks, under a carbogen atmosphere. (^{14}C)-Labelled S-(2,4-dinitrophenyl)glutathione (sp. act. 0.37 µCi/µmole) and S-(1,2-dihydrohydroxynaphthyl)glutathione (sp. act. 0.62 Ci/ mole) dissolved in Krebs-Henseleit buffer, pH 7.4, were added (0.1-0.3 µCi, 0.1-0.5 mM) to isolated cell suspensions (2 ml) and incubated, in duplicate, for 60 min.

The concentration of isolated cells in incubations was approximately 2 x 10^6 cells/ml for liver and small intestine cells and approximately 1 x 10^6 cells/ml for kidney cells. Samples (0.25 ml) were removed at 30 min and 60 min and centrifuged (12000 r.p.m., 5 sec). The super-

natants were removed, the cell pellet washed with Krebs-Henseleit buffer (2 x 0.05 ml) and 0.02 ml samples of the combined supernatants were measured for radioactivity. Samples (0.02-0.1 ml) of supernatants were analysed for metabolites by t.l.c. (solvent system A) or by h.p.l.c. (solvent system I). Water (0.5 ml) was added to lyse the cells in the pellet obtained after centrifugation of incubation samples, and this was then transferred to scintillation vials for measurement of radioactivity. The viability of liver, small intestine and kidney cells, as judged by their abilities to exclude trypan blue, decreased to approximately 90%, 55% and 57%, respectively over 1 h under the incubation conditions used for the metabolism studies.

6.1 vi Assay for γ-glutamyltransferase activity of isolated cells

γ-Glutamyltransferase activity of isolated cells was measured using γ-glutamyl-3-carboxy-4-nitroanilide (γ-GCNA) and glycylglycine as a γ-glutamyl acceptor. The reaction was measured by recording the absorbance due to the production of 2-nitro-5-aminobenzoate (Grafström et al., 1980). Incubations were carried out in Krebs-Henseleit buffer, pH 7.4, containing 25 mM Hepes, with 5 mM γ-GCNA and 50 mM glycylglycine. Hydrolysis of γ-GCNA was recorded continuously at 405 nm in a 1 cm cuvette maintained at 37°C in a Pye Unicam SP1800 dual beam spectrophotometer and using a Unicam AR25 linear recorder. Cells which had been kept on ice were preincubated for 2 min at 37°C in the cuvette before the addition of γ-GCNA. Absorbance changes were measured against a blank cuvette containing cells, but no γ-GCNA. The concentration of the cells in the cuvettes varied between 0.05 and 0.3 x 10^6 cells/ml. γ-Glutamyltransferase activity of the cells was calculated as nmole 2-nitro-5-aminobenzoate formed/min/(10^6 cells), following measurement of the rate of change of absorbance

at 405 nm with respect to time, and calculation using the molar extinction coefficient for 2-nitro-5-aminobenzoate at 405 nm (9490 $M^{-1}cm^{-1}$) (Grafström et al., 1980). The change in absorbance due to the non-enzymic breakdown of γ-GCNA, found to be 2.2±0.9(n=4) nmole/min, was subtracted from rates obtained with cells.

6.1 vii Incubation of glutathione conjugates with bile and small intestinal washings

To assess the stability of the glutathione conjugates of naphthalene, 1-chloro-2,4-dinitrobenzene and BSP in bile and the gastrointestinal tract, incubations of these conjugates in freshly collected rat bile and in duodenal washings were set up as follows.

Bile: The glutathione conjugates (0.2-0.5 mM) were incubated, in duplicate, in bile (0.5 ml) which was collected for 1 h on ice from anaesthetised rats. Following incubation, at 37°C for 1 h, samples (0.2-0.05 ml) of the incubations were analysed by t.l.c. (solvent systems A or C) or h.p.l.c. (solvent system I) and compared with control incubations in which bile was replaced by water. Metabolites were identified by co-chromatography with standards and quantitated by liquid scintillation counting (naphthalene and 1-chloro-2,4-dinitrobenzene) or by absorbance measurements (BSP) as previously described (Materials and Methods, Chapters 3, 4 and 5).

Duodenal washings: Rats were killed by cervical dislocation and a 10 cm length of intestine from the position of entry of the bile duct into the duodenum was removed and washed with 10 ml 0.9% (w/v) saline. The glutathione conjugates (in 0.1 ml water) were then incubated (0.2-0.5 mM) with 0.4 ml of the suspension of duodenal washings at

37 °C for 1 h. After this time, the incubations were centrifuged (12000 r.p.m., 1 min) and the supernatants analysed as described for incubations with bile.

6.1 viii Measurement of partition ratios

The partition ratios of substrates used in the in vivo experiments, together with their major biliary metabolites, were measured between octan-1-ol and 0.1 M phosphate buffer, pH 7.4, a model system for both biological lipid and aqueous phases (Leo et al., 1971; Tate, 1971). The method of measurement of the partition ratios for the (^{14}C)-labelled compounds was similar to that described by Hirom et al. (1974).

Because of their very low solubility in phosphate buffer, (^{14}C)-naphthalene and (^{14}C)-1-chloro-2,4-dinitrobenzene were added to tubes in a small amount (0.005 ml) of ethanol. Other (^{14}C)-labelled compounds were added in 0.1 M phosphate buffer, pH 7.4. Volumes were made up to 1 ml with 0.1 M phosphate buffer, pH 7.4 and 1 ml octan-1-ol added. The tubes were then inverted 100 times by hand, followed by centrifugation (3000 r.p.m., 15 min) to break up any emulsions which had formed. Samples (0.02-0.05 ml) from both the upper organic layer and the aqueous layer were measured for radioactivity by liquid scintillation counting as described (Section 2.3 i). All determinations were performed in duplicate.

For the measurement of the partition ratio of BSP and its glutathione S-conjugate, a slightly different method of determination was used. BSP or BSP-GSH were dissolved in 1 ml 0.1 M phosphate buffer, pH 7.4, and partitioned with 1 ml octan-1-ol, as described above. The octan-1-ol

phase was removed to another tube and 1 ml 0.1 M sodium hydroxide was added. The samples were inverted 100 times by hand, centrifuged and a sample (0.1 ml) of the lower aqueous layer, together with a sample of the aqueous layer from the first partition, was taken for determination of dye concentration by measurement of absorbance at 578 nm, as described in Chapter 5. Determinations were carried out in triplicate. The recovery of dye in the phosphate buffer and sodium hydroxide layers represented 100% of the original dye added.

0.1 M Phosphate buffer (pH 7.4) and 0.1 M sodium hydroxide used for the determination of partition ratios were equilibrated with octan-1-ol for at least one week before use. The partition ratio (P) for each compound was calculated by division of the amount (d.p.m. or absorbance at 578 nm) of the compound in the octan-1-ol layer by the amount in the phosphate buffer layer.

6.2 RESULTS

6.2 i Metabolism of glutathione S-conjugates by isolated cells

Isolated rat liver, kidney and small intestine cells were prepared by enzymic breakdown of tissues and used to investigate the ability of these tissues to metabolise the glutathione S-conjugates of 1-chloro-2,4-dinitrobenzene i.e. S-(2,4-dinitrophenyl)glutathione, and naphthalene, i.e. S-(1,2-dihydrohydroxynaphthyl)glutathione. Table 6.1 shows that both of these glutathione conjugates were metabolised to different extents by isolated renal and small intestinal cells. The values in Table 6.1 when expressed as rates of metabolism in nmoles glutathione S-conjugate hydrolysed/min/10^6 cells were, for the dinitrophenyl conjugate, 11.1$\pm$3.1, 0.10$\pm$0.06, and undetected for kidney,

TABLE 6.1 Metabolism of glutathione S-conjugates by isolated rat liver, small intestine and kidney cells [a]

Tissue	Glutathione conjugate of 1-Chloro-2,4-dinitrobenzene	Naphthalene
Liver	n.d. [b]	2.1 ± 0.6
Small intestine	0.5 ± 0.2	10.2 ± 4.1
Kidney	91.6 ± 4.1	93.9 ± 3.2

(a) Metabolism expressed as % glutathione conjugate metabolised/ 30 min/(10^6 cells). Values are mean ± S.D. of duplicate determination of incubation with two different cell preparations

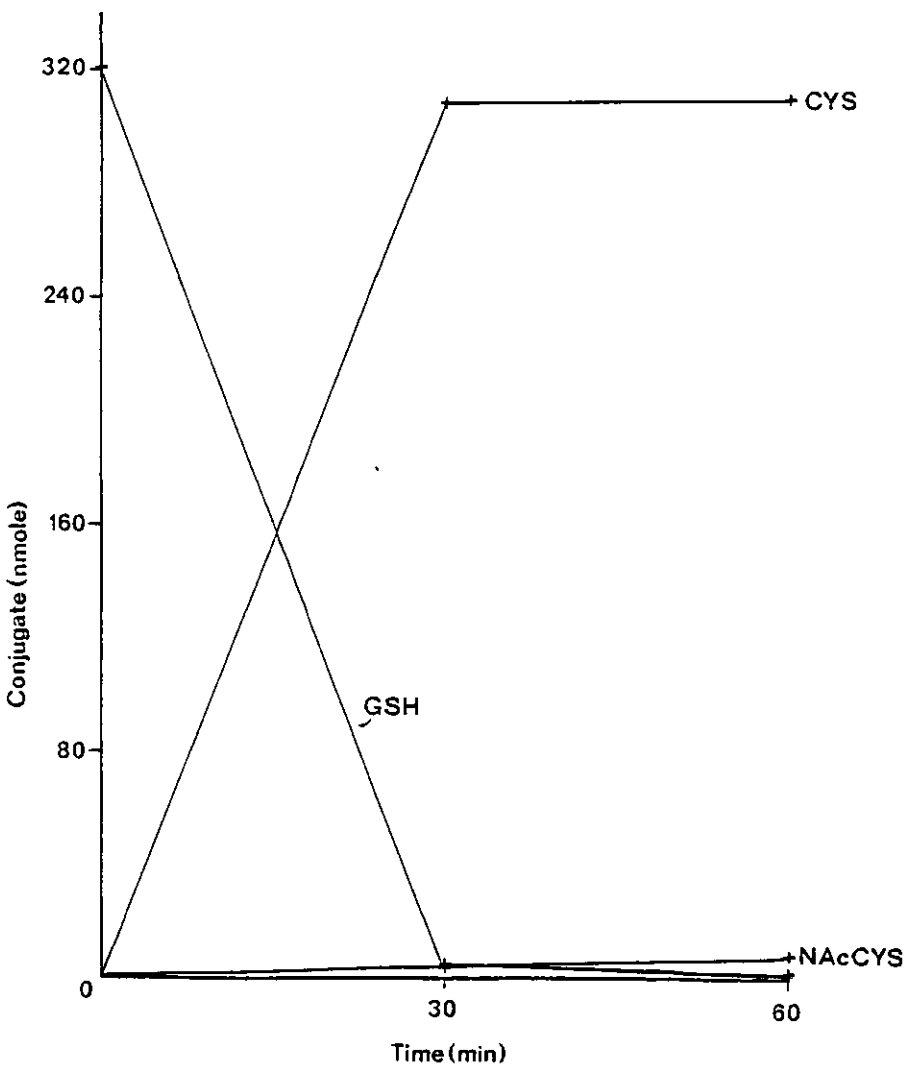
(b) n.d. = none detected

small intestine and liver cells, respectively. The rates for the metabolism of the naphthyl S-conjugate by the cells were 5.2±0.1, 0.21±0.07 and 0.09±0.02 nmole glutathione S-conjugate hydrolysed/min/(10^6 cells). Kidney cells were found to be the most active in the metabolism of both types of glutathione conjugate, with intestinal cells showing higher activities than hepatocytes.

The glutathione conjugate of naphthalene was metabolised by all three cell types to the corresponding cysteine derivative. Metabolism was particularly high in kidney cells (Figure 6.2). No cysteinylglycine derivative of naphthalene could be detected in any of the incubations. On incubation with kidney cells, the cysteine conjugate of naphthalene formed by metabolism of the glutathione derivative, was slowly acetylated to N-acetyl-S-(1,2-dihydrohydroxynaphthyl)cysteine (pre-mercapturic acid, Figure 6.2). No pre-mercapturic acid could be identified in either hepatocyte or intestinal cell incubations of the naphthyl glutathione conjugate. Levels of radioactivity associated with the cell pellets from centrifugation of samples of the incubations with naphthylglutathione conjugate were less than 1% of the radioactivity in the incubations.

S-(2,4-Dinitrophenyl)glutathione was metabolised in a manner similar to naphthyl glutathione since the kidney showed muchhigher metabolism of the conjugate than was seen in intestinal cells, whilst metabolism by hepatocytes was undetectable (Table 6.1). The major products of kidney cell metabolism of S-(2,4-dinitrophenyl)glutathione were S-(2,4-dinitrophenyl)cysteine and compounds of an intense yellow colour, which migrated with high Rf values (>0.7) when analysed by t.l.c. (solvent system A). This latter material was derived from the cysteine conjugate, since incubation of standard S-(2,4-dinitrophenyl)

FIGURE 6.2 Metabolism of S-(1,2-dihydrohydroxynaphthyl)glutathione by isolated rat kidney cells. GSH, CYS and NAcCYS represent the glutathione, cysteine and N-acetylcysteine derivatives respectively



cysteine (but not the glutathione nor N-acetyl cysteine derivatives) in Krebs-Hepes buffer, pH 7.4, at 37°C for 30 min resulted in the formation of compounds which co-chromatographed (t.l.c., solvent system A and h.p.l.c., solvent system I) with the non-polar materials. S-(2,4-Dinitrophenyl)cysteine is very labile due to intramolecular rearrangements (Tateishi and Shimizu, 1980) and this conjugate produced in the kidney cell metabolism of S-(2,4-dinitrophenyl)glutathione was hydrolysed under the incubation conditions used. No evidence was found for the production of the N-acetylcysteine derivative of 1-chloro-2,4-dinitrobenzene in cell incubations and this may have been a consequence of the instability of S-(2,4-dinitrophenyl) cysteine.

Following incubations of S-(2,4-dinitrophenyl)glutathione with kidney cells, approximately 15% of the radioactivity present in the incubations was found associated with the cell pellet after centrifugation of samples of the incubations. Whether this radioactivity reflected any form of covalent interaction of radiolabelled metabolites in the cells is unknown, since exhaustive washings of the cellular material was not performed. Radioactivity associated with the cellular pellets following centrifugation of samples of incubations of (^{14}C)-S-(2,4-dinitrophenyl)glutathione with isolated hepatocytes and small intestinal cells, was found to be approximately 1% of the radioactivity added to the incubations.

6.2 ii γ-Glutamyltransferase activities of isolated cells

Isolated kidney, liver and intestine cells were assayed for γ-glutamyl transferase activities using γ-glutamyl carboxynitroanilide as substrate. As is shown in Table 6.2, a very high activity of this enzyme

TABLE 6.2 γ-Glutamyltransferase activity of isolated rat kidney, liver and small intestine cells

Tissue	Activity [a]	
Kidney	2923 ± 309	(100) [b]
Liver	10.9 ± 2.6	(0.4)
Small intestine	4.7 ± 1.9	(0.2)

(a) Activity expressed as nmole 2-nitro-5-aminobenzoate formed/min/(10^6 cells). Values are mean ± S.D. of duplicate determinations on two different cell preparations.

(b) Figures in brackets are activity of γ-glutamyltransferase expressed as % of the activity in kidney cells.

was found in kidney cells when compared with liver and small intestine cells. Liver cells had twice the activity of γ-glutamyltransferase which was observed in small intestine cells, although determination of the activity of the latter was made difficult by large amounts of mucus associated with the cells, which interfered with absorbance measurements. The high activity of γ-glutamyltransferase measured in kidney cells was compatible with the metabolism of naphthyl and dinitrophenyl glutathione conjugates seen in kidney cell incubations (Table 6.1).

6.2 iii Metabolism of glutathione conjugates in bile and duodenal washings

The glutathione conjugates of naphthalene and 1-chloro-2,4-dinitrobenzene undergo enterohepatic circulation (see Chapters 3 and 4). Metabolism of these conjugates during enterohepatic recycling could occur not only at a cellular level but also in bile or in the intestinal tract. The stability of these conjugates together with that of the glutathione conjugate of bromsulphthalein (BSP), was assessed by incubation in bile and in duodenal contents (Table 6.3). The glutathione conjugates of both naphthalene and 1-chloro-2,4-dinitrobenzene were metabolised in bile at a rate of approximately 20 nmoles/h/ml bile. The product in each incubation was the corresponding cysteine conjugate, indicative of γ-glutamyltransferase and aminopeptidase activities. In addition, S-(1,2-dihydrohydroxynaphthyl)glutathione was observed to undergo 78% hydrolysis to the cysteine conjugate in bile kept at -20°C for approximately one year.

Following incubation of the glutathione conjugate of naphthalene in duodenal contents some metabolism (approximately 5 nmoles/h/ml contents) of S-(1,2-dihydrohydroxynaphthyl)glutathione to the cysteine

TABLE 6.3 Hydrolysis of glutathione S-conjugates in bile and duodenal contents

Glutathione conjugate of	Hydrolysis[a] in	
	Bile	Duodenal contents
Bromsulphthalein	n.d.[b]	n.d.
1-Chloro-2,4-dinitrobenzene	18.9 ± 2.6	n.d.
Naphthalene	24.4 ± 1.2	4.9 ± 1.1

(a) Values are expressed as nmole glutathione conjugate hydrolysed/h/ml bile or/ml duodenal contents, and are mean ± S.D. (n = 2)

(b) n.d. = none detected

conjugate was observed. No metabolism of S-(2,4-dinitrophenyl)glutathione could be detected. Since the total volume of duodenal washings was 10 ml (see Section 6.1 vii), total metabolism of S-(1,2-dihydro-hydroxynaphthyl)glutathione to the corresponding cysteine conjugate on incubation in duodenal contents, represented approximately 50 nmoles conjugate hydrolysed/h.

No metabolism of the glutathione conjugate of BSP could be detected on incubation in bile or in duodenal contents.

6.2 iv Partition ratios

Relative lipid-solubilities of compounds were determined by measuring their partition ratios (P) between octan-1-ol and 0.1 M phosphate buffer, pH 7.4 and are given in Table 6.4, together with the logarithm of these ratios (log P).

The relative lipid solubilities (as measured by the partition ratios) of 1-chloro-2,4-dinitrobenzene (log P=+1.13) and bromsulphthalein (log P=-1.07), which form glutathione conjugates by direct replacement of a halide atom by glutathione, were decreased approximately 2000 and 40-fold, respectively, by glutathione conjugation. The relative lipid solubility of naphthalene (log P=+2.93; cf. 3.01, Rogers and Cammarata, 1969), a compound which requires metabolic activation by a microsomal oxidase system to produce an epoxide metabolite prior to glutathione conjugation, was decreased approximately 200000-fold.

6.3 DISCUSSION

The glutathione conjugates of 1-chloro-2,4-dinitrobenzene and naphthalene

TABLE 6.4 Partition ratios (P) between octan-1-ol and 0.1 M phosphate buffer, pH 7.4, of naphthalene, 1-chloro-2,4-dinitrobenzene and bromsulphthalein with their principal biliary metabolites

Compound	P	Log P
Naphthalene	847.3	+ 2.93
1-Chloro-2,4-dinitrobenzene	13.66	+ 1.13
Bromsulphthalein	0.0852	- 1.07
DNP-MA(a)	0.0785	- 1.11
DNP-GSH	0.0075	- 2.12
Naphthyl-GSH	0.0051	- 2.29
BSP-GSH	0.0022	- 2.66

(a) DNP-MA = N-acetyl-S-(2,4-dinitrophenyl) cysteine,
DNP-GSH = S-(2,4-dinitrophenyl) glutathione,
Naphthyl-GSH = S-(1,2-dihydro-1-hydroxy-2-naphthyl) glutathione,
BSP-GSH = glutathione conjugate of bromsulphthalein

were metabolised by isolated cells to different extents dependent upon cell type (Table 6.1). Kidney cells were much more active than liver and small intestine cells at hydrolysing the glutathione conjugates to their corresponding cysteine derivatives. Results obtained with isolated cell incubations of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione (Table 6.1 and Figure 6.2) agreed with observations of the metabolism of other glutathione conjugates by isolated cells. Kidney cells have been found to be the most active cell type tested in the hydrolysis of the glutathione conjugate of paracetamol and leukotriene C_3 (Jones et al., 1979b; Ormstad et al., 1982). The glutathione conjugate of paracetamol is also hydrolysed by small intestinal cells (Grafström et al., 1979a) and, at a very slow rate, by hepatocytes (Moldéus, 1978). All of these three cell types form the cysteine conjugate from a paracetamol glutathione conjugate. Kidney cells and, at a slower rate, small intestinal cells, N-acetylate the cysteine conjugate of paracetamol to the corresponding mercapturic acid (Jones et al., 1979b; Grafström et al., 1979a). Some N-acetylation of S-(1,2-dihydrohydroxynaphthyl) cysteine was observed in kidney cell incubations (Figure 6.2), although no mercapturic acid could be identified on incubation with small intestinal cells. It may be important to note that the method of preparation of small intestinal cells was not the same as used by Grafström et al. (1979b) and differences in the rate and type of metabolism seen in the study reported in this thesis and that of Grafström et al. may be a reflection of the type of cells obtained by the two methods used.

The cysteinylglycine derivatives of naphthalene and 1-chloro-2,4-dinitrobenzene were not observed in any of the cell incubations. This may reflect the sampling times employed and the high activity of

associated aminopeptidases catalysing the hydrolysis of cysteinylglycine conjugates to the corresponding cysteine derivatives. The cysteinylglycine conjugate of paracetamol is reported to have been observed from renal metabolism of the glutathione conjugate, although it was only found in very early (<5 min) samples from the incubation (Jones et al., 1979a). Thus, S-(1,2-dihydrohydroxynaphthyl)glutathione and S-(2,4-dinitrophenyl)glutathione were metabolised to the corresponding cysteine conjugates by isolated rat small intestine and kidney cells. The cysteine conjugate of naphthalene was slowly acetylated to N-acetyl-S-(1,2-dihydrohydroxynaphthyl)cysteine by isolated kidney cells. Metabolism by isolated hepatocytes was only detected in incubations with S-(1,2-dihydrohydroxynaphthyl)glutathione and this was only at a much slower rate relative to kidney and small intestine cells.

1-Chloro-2,4-dinitrobenzene and naphthalene are both known to possess mutagenic and toxic properties (Summer and Göggelmann, 1980; van Heyningen and Pirie, 1967). It is unknown which metabolites of these two compounds are responsible for the toxic responses, but for naphthalene, oxidative processes are believed to play a significant part (see Horning et al., 1980). In the case of 1-chloro-2,4-dinitrobenzene, reduction of nitro groups is likely to be important, but it is interesting to note that radioactivity was found to be associated with cellular material following metabolism of (^{14}C)-1-chloro-2,4-dinitrobenzene by isolated kidney, but not small intestine or liver, cells (Section 6.2 i). This was probably due to the chemical instability of S-(2,4-dinitrophenyl)cysteine in the medium used for cell incubations and may be important with respect to 1-chloro-2,4-dinitrobenzene toxicity.

γ-Glutamyltransferase is the first enzyme involved in the pathway of degradation of glutathione conjugates to mercapturic acids (see Section 1.4 ii). The activity of this enzyme in the isolated cell preparations used for the studies of the metabolism of glutathione conjugates correlated fairly well with the extent of metabolism observed. Thus, kidney cells showed the greatest metabolism of the glutathione conjugates of naphthalene and 1-chloro-2,4-dinitrobenzene, and had the highest γ-glutamyltransferase activity when compared with small intestine and liver cells (Table 6.2). The γ-glutamyltransferase activity of small intestine cells was lower than that measured in hepatocytes, although metabolism of the glutathione conjugates by the former was greater. The γ-glutamyltransferase activity of small intestinal cells has been reported as being 60-70 times higher than that of hepatocytes (Grafström et al., 1980). Problems were encountered in the accurate measurement of the γ-glutamyltransferase activity of isolated small intestine cells due to the presence of mucus, a normal product of healthy, functioning intestinal cells, which interfered with absorbance measurements. This may have led to the erroneous measurement of low levels of γ-glutamyltransferase activities for small intestinal cells.

Metabolism of glutathione conjugates during enterohepatic circulation may not only occur at a tissue level but also may occur in bile or in the intestinal lumen, either chemically or enzymically, following excretion. S-(2,4-Dinitrophenyl)glutathione and S-(1,2-dihydro-hydroxynaphthyl)glutathione were metabolised at a rate of approximately 20 nmole/h/ml bile (Table 6.3), to the corresponding cysteine conjugates. No metabolism of the glutathione conjugate of BSP could be detected. Relatively high levels of γ-glutamyltransferase are demonstrable in bile (Rosalki, 1975) and may be involved in the hydrolysis of gluta-

thione conjugates excreted in bile. The large number of non-specific extracellular aminopeptidases present in pancreatic secretions and in the gut may participate in the further hydrolysis of cysteinylglycine compounds to cysteine derivatives.

S-(1,2-Dihydrohydroxynaphthyl)glutathione was hydrolysed to a small extent (5 nmole/h/ml of washings) on incubation with duodenal contents. No metabolism of S-(2,4-dinitrophenyl)glutathione nor the glutathione conjugate of bromsulphthalein was detected. Metabolism of the naphthyl glutathione conjugate may have resulted from γ-glutamyltransferase present in bile (vide supra) or from small intestinal cells shed from the intestinal wall (Renwick, 1982).

The evidence from incubations of glutathione conjugates in bile and duodenal contents, indicated that the conjugates are relatively stable in these media, at least up to 1 h following the biliary excretion of these metabolites.

Glutathione conjugation leads to an increase in the water solubility of xenobiotics, as judged by their partition ratios between octan-1-ol and phosphate buffer, pH 7.4 (Table 6.4). This is in keeping with the maxim that conjugation of xenobiotics leads to an increase in water-solubility compared with their parent compounds (Williams, 1959). It may be noted that glutathione conjugation of 1-chloro-2,4-dinitrobenzene and bromsulphthalein, two compounds which do not require Phase I metabolism to react with glutathione, resulted in a decrease in their relative lipid solubilities (as measured by their partition ratios between phosphate buffer, pH 7.4, and octan-1-ol) approximately 200- and 40-fold, respectively. The relative lipid solubility of naphthalene, which requires metabolic bioactivation to an epoxide

before it is sufficiently reactive towards glutathione (Jerina et al., 1970), was decreased approximately 200000-fold. These limited results help demonstrate that Phase I and Phase II metabolic reactions (Williams, 1959) can combine to markedly alter the physical characteristics of xenobiotics with a view to excretion from the organism.

CHAPTER SEVEN

GENERAL DISCUSSION

CONTENTS

CHAPTER SEVEN

FIGURE TITLE

7.1 Biliary excretion of glutathione conjugates

It is generally accepted that, following i.v. administration of mercapturic acid precursors to rats, the initial stage of mercapturic acid formation i.e. glutathione conjugation, occurs principally in the liver (Chasseaud, 1976). This is probably a consequence of the presence of high levels of glutathione and drug-metabolizing enzymes, including glutathione S-transferases, in liver (see Chapter 1), together with the high blood flow to the liver. Glutathione conjugates formed in liver may be released at low rates into the systemic circulation, although this release may become more significant at higher rates of conjugate formation (Barnhart and Combes, 1976; Whallander and Sies, 1979). Because of their high polarity and relatively large molecular weight, glutathione conjugates have the requisite physicochemical properties for biliary excretion and are generally found in bile in high concentrations (see Section 1.7 i).

The fact that the rat excreted S-(2,4-dinitrophenyl)glutathione, S-(1,2-dihydrohydroxynaphthyl)glutathione and the glutathione conjugate of bromsulphthalein in bile following i.v. administration of the parent compounds is not unexpected since the rat has been shown to possess a molecular weight threshold of 325±50 for the biliary excretion of organic anions (Millburn et al., 1967; Section 1.7). Conjugates of molecular weight of about 350 or more tend to be excreted in bile, whilst compounds of about 300 or less tend to undergo urinary excretion (Hirom et al., 1972a). The molecular weights of the glutathione conjugates in the studies reported in this thesis are 473, 451 and 1063 and would, therefore, be expected to undergo excretion in bile, rather in urine. Indeed, no glutathione conjugates were found in urine following i.v. administration of 1-chloro-2,4-dinitrobenzene,

naphthalene or bromsulphthalein to bile duct-cannulated or convential rats. In addition, the low relative lipid solubilities of the glutathione conjugates, as measured by their partition ratios (see Table 6.4), would tend to favour their biliary excretion, since the presence of a strong polar group, which allows molecules to exist at physiological pH as water-soluble cations or anions, has been shown to be a requirement for extensive biliary excretion (Smith, 1973).

Interestingly, the molecular weight of N-acetyl-S-(2,4-dinitrophenyl) cysteine in 329 and this compound was excreted by the rat in both bile and urine following i.v. administration of 1-chloro-2,4-dinitrobenzene (see Table 5.4).

7.2 Sites of metabolism of glutathione conjugates during enterohepatic circulation

Metabolism of glutathione conjugates to mercapturic acids requires the sequential participation of three enzymes, γ-glutamyltransferase, aminopeptidase(s) and N-acetyltransferase (see Section 1.4). Metabolism may occur in one or more of the following sites:-

a) In bile.
b) In the gastrointestinal tract by host enzymes.
c) In the gastrointestinal tract by gut microflora.
d) In intestinal cells prior to or during absorption.
e) In tissues, essentially liver and kidney, following absorption.

Since γ-glutamyltransferase is the only known enzyme capable of catalysing the hydrolysis of the γ-glutamyl linkage of glutathione

and glutathione S-conjugates and, hence, catalyse the first step of mercapturic acid formation, consideration of a primary site of glutathione conjugate metabolism must be accompanied by a consideration of the activity of γ-glutamyltransferase at these sites.

Results obtained with the incubation of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione in bile suggested that the hydrolysis of glutathione conjugates occurs at a low rate in bile (Table 6.3). This is presumably a result of γ-glutamyltransferase activity, reported to be present in bile (Rosalki, 1975) and further hydrolysis of the formed cysteinylglycine derivatives by peptidases. At the rates observed for dinitrophenyl and dihydrohydroxynaphthyl glutathione conjugates in *in vitro* incubations, hydrolysis of these glutathione conjugates in bile during enterohepatic recycling would only represent approximately 5% of the glutathione conjugates excreted in bile, following i.v. administration of the parent compounds (naphthalene 30 μmole/kg, 1-chloro-2,4-dinitrobenzene, 5 μmole/kg). Metabolism of S-(1,2-dihydrohydroxynaphthyl)glutathione, but not S-(2,4-dinitrophenyl)glutathione, was observed in the incubations of these compounds with rat duodenal contents (Table 6.3). The specific hydrolytic activity was less than that measured in incubations with bile, but since the total volume of washings was 10-fold greater than that of bile, total hydrolysis was 2.5 times greater in washings when compared with bile. This hydrolysis may result from γ-glutamyl-transferase present in bile (*vide supra*) or from intestinal epithelial cells which are shed from the mucosal lining (Renwick, 1982), with subsequent peptidase hydrolysis. Again, metabolism of the glutathione conjugate of naphthalene in duodenal contents only accounted for the hydrolysis of approximately 5% of the glutathione conjugate excreted in bile, after i.v. administration of naphthalene (30 μmole/kg).

Non-enzymatic hydrolysis of glutathione conjugates as a function of pH is unlikely to occur during the enterohepatic recycling of glutathione conjugates, since the pH of bile and, consequently, duodenal contents is approximately 7-8 (Renwick, 1982). S-(2,4-Dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione were stable at this pH in incubations of up to 1 h at 37°C. Glutathione is hydrolysed under much harsher conditions of acid and base (Wieland, 1954).

The involvement of intestinal microflora in the enterohepatic recycling of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxy-naphthyl)glutathione was investigated by measurement of the excretion of radioactivity in bile and urine following i.d. infusion of these (^{14}C)-labelled conjugates into rats which had been pretreated with antibiotics and compared with control rats. Antibiotic pretreatment of rats did not significantly affect the enterohepatic circulation of the glutathione conjugates (Tables 3.11 and 4.12). The antibiotic regime used in the experiments with S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione has been shown to cause a significant decrease in the enterohepatic recycling of glucuronic acid conjugates of a number of xenobiotics (Parker et al., 1980; Huckle et al., 1981).

The enterohepatic circulation of glutathione conjugates in the rat, therefore, differs from that of the other major type of xenobiotic conjugate found in bile, namely, β-glucuronides. For these latter compounds, hydrolysis to the aglycone catalysed by gut microfloral β-glucuronidase has been shown to be a prerequisite for the absorption and recycling of metabolites and suppression of the intestinal microflora by oral antibiotics led to a decrease in the enterohepatic circulation of a range of xenobiotics (Illing, 1982). The profile

of excretion in bile of radioactivity derived from intraduodenally infused radiolabelled β-glucuronide conjugates differed from that seen following i.d. infusion of radiolabelled glutathione conjugates. With the former conjugates, peak concentrations of radioactivity occurred in bile at a later time point (approximately 7 h) following i.d. infusion of the conjugate (Parker et al., 1980), than was seen in bile following i.d. infusion of glutathione conjugates (1-3 h; Tables 3.4 and 4.6). The longer time period for absorption and excretion of radioactivity following administration of xenobiotic β-glucuronides into the intestinal lumen of rats is believed to be due to the time required for the β-glucuronides to pass down the gut to the large intestine and for hydrolysis to the aglycone catalysed by the microflora present there (Colburn et al., 1979). A more direct absorption of glutathione conjugates from the small intestine is suggested by a different and earlier biliary excretion profile and the presence of glutathione conjugates in the bile of rats infused intraduodenally with the same glutathione conjugates (see later).

Recently, reports have appeared in the literature showing the presence of an enzyme which catalyses the hydrolysis of the C-S linkage of thioether compounds (Tateishi and Shimizmu, 1980; See Section 1.5 ii). This enzyme, designated cysteine conjugate β-lyase (C-S lyase), has been shown to be present in rat liver (Tateishi et al., 1978) and intestinal microflora (Suzuki et al., 1982) and was active only with aromatic cysteine conjugates of xenobiotics. No activity was observed with mercapturic acids or glutathione conjugates nor with alkylcysteine conjugates (Tateishi and Shimizu, 1980). Involvement of gut microfloral β-lyase has been implicated in the metabolism and enterohepatic recycling of the thioether metabolites of propachlor (Bakke et al., 1980; Bakke et al., 1981a), caffeine (Rafter and Nilsson, 1981)

and S-acetamido-4-(chloromethyl)thiazole (Rafter and Bakke, 1982). The product of β-lyase action on cysteine conjugates is the corresponding thiol compound, which is then methylated in a reaction catalysed by S-methyltransferase using S-adenosyl-L-methionine (Weisiger and Jakoby, 1980). Further oxidation of thiomethyl (-SMe) compounds yields methylsulphinyl (-SOMe) and methylsulphonyl ($-SO_2Me$) derivatives which are excreted in urine. These derivatives have been identified in the urine of rats administered naphthalene (Stillwell et al., 1978a), and their production is decreased in rats treated with neomycin (Stillwell et al., 1978b). However, the amounts of these types of compounds in the urine of rats administered high amounts of naphthalene (100 mg/kg, i.p.) was only <1% of the dose (Stillwell et al., 1978a) and thus represented only a minor pathway of naphthalene metabolism. Intestinal microfloral C-S lyase is probably important in the metabolism of compounds excreted in bile as cysteine conjugates and has little involvement in the metabolism of glutathione and N-acetylcysteine conjugates, which are the major biliary metabolites of naphthalene and 1-chloro-2,4-dinitrobenzene (Chapters 3 and 4). Both the hepatic and bacterial forms of the enzyme may be important in the further metabolism of cysteine conjugates of xenobiotics formed from glutathione and N-acetylcysteine conjugates following enterohepatic circulation (see Chapter 1).

It was decided to investigate the tissue metabolism of glutathione conjugates by using freshly isolated cells, an experimental model which serves as an intermediate between studies with solubilized enzymes or isolated organelle fractions, on the one hand, and studies with whole animal or isolated or in situ perfused tissues on the other.

Isolated small intestinal cells metabolised S-(2,4-dinitrophenyl)

glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione to the corresponding cysteine conjugates in incubations at 37°C for 1 h (see Table 6.1). Metabolism was not as extensive as that seen in isolated kidney cells. Studies using the glutathione conjugate of paracetamol showed similar hydrolysis by intestinal cells, to the corresponding cysteine conjugate (Grafström et al., 1979a). γ-Glutamyltransferase and aminopeptidases are membrane-bound enzymes which act on extracellular glutathione and cysteinylglycine conjugates, respectively (Tate, 1980; Sections 1.4 ii and 1.4 iii). Metabolism of glutathione and of glutathione conjugates by these enzymes in the lumen of the intestine and kidney tubules, led to the uptake of the formed cysteine conjugates by intestinal and renal cells, respectively (Grafström et al., 1979a; Wendel et al., 1978; Ormstad et al., 1982). The above evidence with isolated small intestinal cells indicates that hydrolysis of glutathione conjugates to cysteine derivatives by successive action of γ-glutamyltransferase and aminopeptidase(s) in the intestine during or prior to uptake could play a part in the enterohepatic recycling of glutathione conjugates (see later).

Absorption of glutathione conjugates without metabolism in the gastrointestinal tract would lead to their appearance in the portal blood system and transport to the liver. Isolated hepatocytes showed negligible or undetectable levels of metabolism of S-(1,2-dihydrohydroxynaphthyl)glutathione and S-(2,4-dinitrophenyl)glutathione (Table 6.1), results which correlated well with the low levels of γ-glutamyltransferase measured in these cells (Table 6.2). Very low metabolism of the glutathione conjugate of paracetamol to the cysteine derivative was observed in hepatocyte incubations (Moldéus, 1978). Measurement of γ-glutamyltransferase activity of hepatocytes has been reported by other workers (Grafström et al., 1980) to be very low

relative to other tissues. These results indicate that the liver has a low ability to metabolise extracellular glutathione conjugates, principally due to the presence of low amounts of γ-glutamyltransferase. When radiolabelled S-carbamidomethyl glutathione was administered intravenously to mice, radioactivity accumulated primarily in kidney tissue (Inoue et al., 1982). Similarly, studies using glutathione in the rat (Hahn et al., 1978) and paracetamol glutathione conjugate in kidney cell incubations (Moldéus et al., 1978a) indicated that the kidney was the major site of metabolism of glutathione. Metabolism of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl) glutathione by isolated rat kidney cells led to the rapid formation of the corresponding cysteine derivatives (Figure 6.2). The major metabolite which accumulated in the kidneys of mice given S-carbamidomethyl glutathione was identified as the cysteine derivative (Inoue et al., 1982) and rat kidney cells rapidly hydrolysed the glutathione conjugate of paracetamol to the cysteine derivative (Moldéus et al., 1978a).

Evidence for the major site of N-acetylation of cysteine conjugates to form mercapturic acids is difficult to determine from the studies with S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl) glutathione reported here. Isolated kidney cells metabolised S-(1,2-dihydrohydroxynaphthyl)cysteine, formed from the glutathione conjugate to the N-acetylcysteine derivative (pre-mercapturic acid) at a slow rate (Figure 6.2). Inoue et al. (1982) in in vivo studies using S-carbamidomethyl conjugates, believed the major site of N-acetylation to be the liver, the mercapturic acid being formed following release of the cysteine conjugate from the kidney into the systemic circulation. Although the specific activity of N-acetyltransferase of the rat liver microsomal fraction is one-half of that in the kidney microsomal fraction, total enzymic activity is about 3 times

higher in liver than in kidney (Green and Elce, 1975). Experiments with *in vitro* incubations of liver homogenates or slices indicated that liver has the capacity to form mercapturic acids from cysteine conjugates, but not glutathione conjugates (Bray *et al.*, 1959a; Booth *et al.*, 1960). Thus, metabolism of cysteine conjugates to mercapturic acids in rat liver probably occurs at a high rate. However, this is dependent upon a supply of the cysteine derivatives and, since little metabolism of glutathione conjugates by liver γ-glutamyltransferase occurs, the formation of cysteine derivatives in the kidney or intestine would be consequently more important. This metabolic cooperation of the liver, kidney and intestine will be discussed further in Section 7.5.

No cysteinylglycine conjugates derived from *S*-(2,4-dinitrophenyl) glutathione or from *S*-(1,2-dihydrohydroxynaphthyl)glutathione were identified in any of the *in vivo* studies or *in vitro* incubations. This may be a consequence of the sampling times employed (since cysteinylglycine derivatives are primary products of the action of γ-glutamyltransferase on glutathione conjugates), the chemical instability of these derivatives or maybe a reflection of the physical association of γ-glutamyltransferase and aminopeptidase enzymes in the cell membranes (Okajima *et al.*, 1981). Cysteinylglycine derivatives of xenobiotics have been identified in bile as a result of the administraion of high doses of the parent xenobiotic (e.g. naphthalene, Boyland *et al.*, 1961) and in *in vitro* incubations as a result of the inhibition of aminopeptidase activity (Ormstad *et al.*, 1982; Chapters 3 and 4).

7.3 Uptake of glutathione conjugates from the intestine

The biliary excretion of radioactivity following the i.d. infusion of

(^{14}C)-S-(2,4-dinitrophenyl)glutathione and (^{14}C)-S-(1,2-dihydrohydroxy-naphthyl)glutathione showed rapid uptake and excretion. Maximal concentrations of radioactivity were found in bile samples which were collected 1-3 h following the i.d. infusion of these conjugates (Tables 3.12 and 4.6). As was mentioned earlier (Section 7.2), the profile of biliary excretion of metabolites following i.d. infusion of the glutathione conjugates differed from that following infusion of β-glucuronides. This may be a reflection of two different mechanisms which operate for the two types of conjugates. β-Glucuronides require gut microbial action, principally in the large intestine, for the hydrolysis to aglycones which are then absorbed. The relative lipid solubilities of the glutathione conjugates studied, as measured by their partition ratios between octan-1-ol and phosphate buffer, pH 7.4, (Table 6.4) suggests that they are too hydrophilic to favour absorption from the intestine, since highly lipophilic unionised compounds tend to be absorbed more readily from the intestine than more polar ionised conjugates (Schanker, 1971). When the glutathione conjugate of paracetamol was instilled in the intestinal lumen of *in situ* intestinal segments, rapid metabolism of the glutathione conjugate occurred, which resulted in the appearance of the cysteine conjugate in portal blood (Grafström *et al.*, 1979a). Formation of this latter metabolite occurred through metabolism of the glutathione conjugate by superficially located γ-glutamyltransferase and aminopeptidase(s) in the brush border of the villous tip cells before or during absorption. These studies indicated at least for paracetamol that metabolism of glutathione conjugates to cysteine conjugates is a prerequisite of absorption, since no glutathione conjugate was found in the portal blood after instillation of the latter in the intestinal lumen. Isolated small intestinal cells have been shown to metabolize S-(2,4-dinitrophenyl) glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione to the

corresponding cysteine derivatives and, following i.d. infusion of these conjugates into rats, metabolites (principally mercapturic acids) derived from the glutathione conjugates were identified in bile and urine. It is not possible to ascertain from these studies the exact nature of the metabolites of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione which were absorbed from the intestine without direct sampling of portal blood. Further work with *in situ* closed intestinal segments would help to clarify this point.

Despite the evidence cited above for the metabolism of glutathione conjugates to cysteine derivatives prior to or during absorption of glutathione conjugates from the small intestine, evidence was found following i.d. infusion of S-(2,4-dinitrophenyl)glutathione and S-(1,2-dihydrohydroxynaphthyl)glutathione for the direct uptake of the glutathione conjugates without metabolism. The respective glutathione conjugates were identified in the bile of rats following i.d. infusion of the naphthyl and dinitrophenyl glutathione conjugates (Tables 3.8 and 4.7). It is difficult to envisage a process of resynthesis of the glutathione conjugates from the cysteine derivatives or for hydrolysis of the linkage between cysteine and the xenobiotic moiety, followed by reconjugation with glutathione. No such processes have been reported in the literature. Another explanation is that the glutathione conjugates may be absorbed from the intestine directly, without metabolism. Absorption from the intestinal lumen without metabolism has been reported for ß-glucuronic acid conjugates of other compounds (Bock and Winne, 1975; Grafström *et al.*, 1979a).

7.4 Lack of an enterohepatic circulation of bromsulphthalein glutathione conjugate (BSP-GSH)

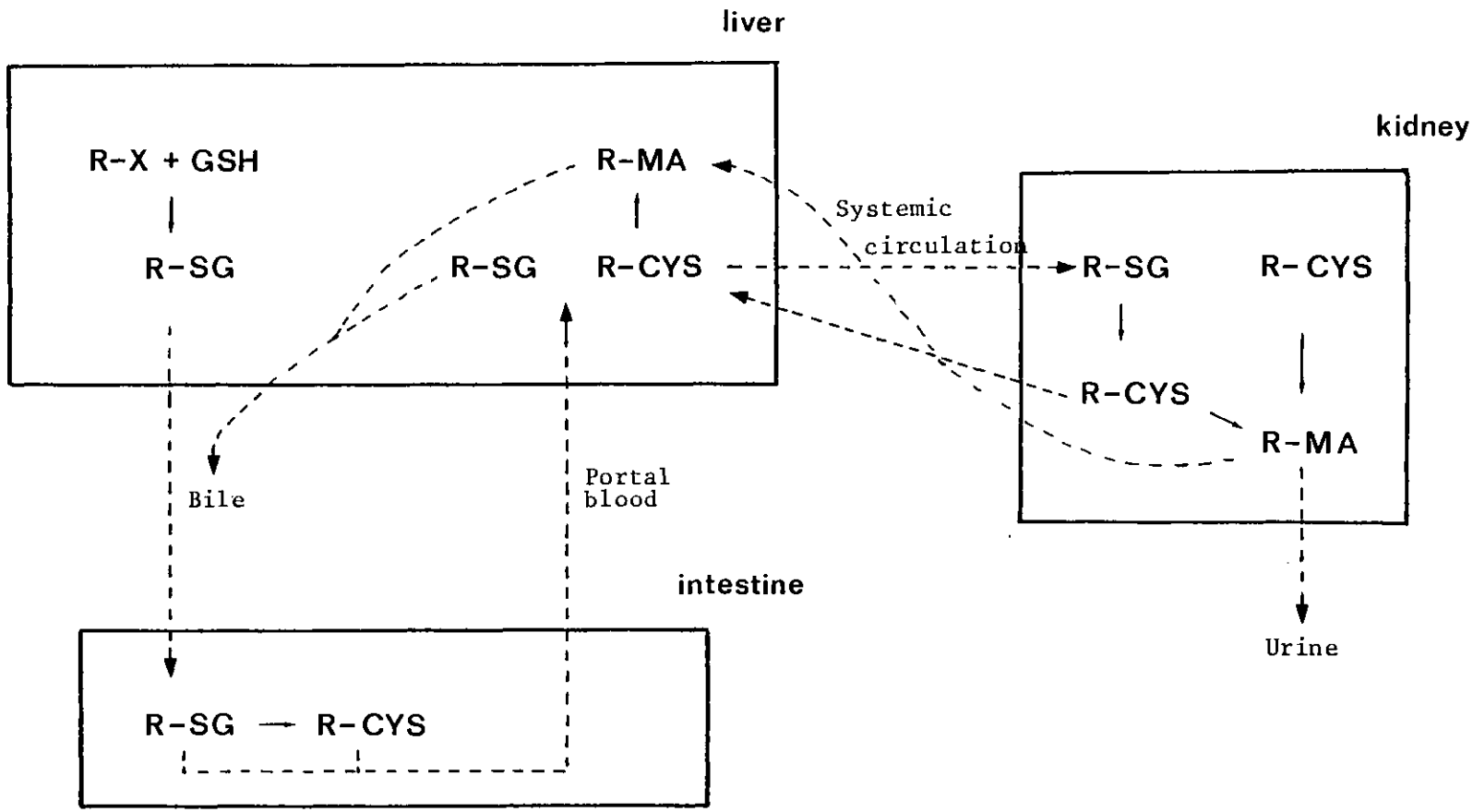
Following i.d. infusion of the glutathione conjugate of bromsulphthalein only 1-2% of the infused dose could be identified in 0-24 h bile (Section 5.3). The remainder of the infused dose was recovered from the intestine. BSP-GSH was not significantly hydrolysed on incubations in bile, duodenal washings (Table 6.3) or, in preliminary experiments, with isolated small intestine cells. The relative lipid solubility of BSP-GSH, as judged by its partition ratio between octan-1-ol and phosphate buffer, pH 7.4, is very high even when compared with other glutathione conjugates. This fact alone may be the reason why BSP-GSH does not undergo significant enterohepatic recycling. Similarly, free BSP, excreted in bile, was not significantly absorbed from the gastrointestinal tract and did not undergo enterohepatic recycling. Again, BSP has a fairly high relative lipid solubility, as judged by its partition ratio, and this may be responsible for its poor absorption from the intestine. A number of other highly polar anionic compounds excreted in bile but which do not undergo enterohepatic circulation include chromoglycate, indocyanine green and the fluoresceins (Smith and Millburn, 1975).

7.5 Correlation of biliary glutathione conjugates with urinary mercapturic acids

A direct relationship between glutathione conjugates of xenobiotics excreted in bile and urinary mercapturic acids has been demonstrated in the rat. The overall disposition is a consequence of the metabolic cooperation of liver, intestine and kidney and a convenient term for this relationship is enterohepatorenal disposition. It has been

well established that glutathione conjugation is the first step in the pathway of formation of urinary mercapturic acids by a number of *in vivo* and *in vitro* studies (see Chapter 1). The biliary excretion of a number of xenobiotics as glutathione conjugates is well known and an even larger number of xenobiotics have been identified as mercapturic acids in urine. The relationships between these two forms of excretion has been demonstrated for 1-chloro-2,4-dinitrobenzene and for naphthalene. Results obtained with these xenobiotics, together with other studies in the literature, can be formulated into a general scheme of entero-hepatorenal disposition of xenobiotics which undergo reaction with glutathione as outlined in Figure 7.1. A xenobiotic (R-X), possessing a sufficiently electrophilic centre undergoes attack by glutathione, usually enzymic principally in the liver, to form a glutathione conjugate (R-SG). This conjugate is rapidly excreted in bile and enters the enteric pool. Metabolism of the glutathione conjugate by γ-glutamyltransferase and aminopeptidase(s) present on the luminal surface of small intestinal epithelial cells (Cornell and Meister, 1976) results in the formation of the corresponding cysteine conjugate (R-CYS), which is absorbed (Grafström *et al.*, 1979a). Direct absorption of the glutathione conjugate without metabolism can also occur (Section 7.3). The glutathione and cysteine conjugates present in portal blood are transported to the liver. The liver has a limited capability to take up glutathione and, possibly, cysteine conjugates from the portal blood. Liver shows a rapid disposition of glutathione conjugates into bile (Barnhart and Combes, 1976) and glutathione conjugates absorbed from the portal system are excreted into bile. One would expect the metabolism of the glutathione conjugates by hepatic γ-glutamyltransferase to be low, since the activity of this enzyme in hepatocytes is very low (Grafström *et al.*, 1980; Table 6.2) and little metabolism of glutathione conjugates occurs in hepatocytes incubations (Moldéus, 1978; Table 6.1).

FIGURE 7.1 Schematic representation of metabolic co-operation between liver, intestine and kidney in the metabolism and excretion of glutathione S-conjugates



Glutathione and cysteine conjugates not taken up by the liver would pass into the systemic circulation (these metabolites exhibit a poor "first pass effect" or presystemic hepatic elimination) and be transported to the kidneys, a major site of extrahepatic thioether metabolism (Wendel et al., 1978; Inoue et al., 1982). Glutathione and cysteine conjugates are secreted into the tubular lumen of the kidneys. The glutathione conjugates are hydrolysed to the cysteine derivatives by γ-glutamyltransferase and aminopeptidase(s) present in the brush border of the plasma membrane of tubular epithelial cells (Tate, 1980; Section 7.2). The cysteine conjugates (produced by hydrolysis of the glutathione conjugates and present as a result of direct secretion) are reabsorbed by renal cells (Ormstad et al., 1982). Metabolism of the cysteine conjugates by N-acetyltransferase present in the microsomal fraction of renal cells is responsible for the formation of the N-acetylcysteine derivatives of the xenobiotic (Figure 5.2), or mercapturic acids (R-MA), which are excreted in urine. The cysteine conjugates may be released into the systemic circulation and return to the liver (Inoue et al., 1982). The release of mercapturic acids into the systemic blood from the kidney may also occur. N-Acetylation of the cysteine conjugate in liver is responsible for the mercapturic acid excretion in bile which passes into the enteric pool, raising the possibility of further intestinal metabolism and secondary enterohepatic recycling. Metabolism of glutathione, cysteine or N-acetylcysteine conjugates by further oxidative (Horning et al., 1980), or hydrolysis (Tateishi et al., 1978) processes in the gastrointestinal tract or liver or kidney may result in the formation of a number of further metabolites excreted in both bile and urine. The overall effect of the metabolic cooperation of liver, intestine and kidney is the urinary disposition of xenobiotics as mercapturic acids following conjugation with glutathione and enterohepatic circulation of glutathione conjugates.

7.6 Concluding remarks

Following the administration of xenobiotics to animals, the main exogenous conjugated species found in bile are derivatives formed between the xenobiotics or their metabolites and glucuronic acid or glutathione. The fate of xenobiotic conjugates containing glucuronic acid which are excreted in bile, had been established by a number of early studies and the enterohepatic recycling of metabolites derived from these β-glucuronides was well known (see Section 1.9 i). The situation with respect to glutathione conjugates was less clear and only circumstantial evidence indicated the presence of enterohepatic recycling of metabolites derived from these compounds. Results presented in this thesis demonstrate that an enterohepatic circulation of metabolites derived from glutathione conjugates of xenobiotics does exist, at least for the aromatic hydrocarbons, naphthalene and 1-chloro-2,4-dinitrobenzene. No enterohepatic circulation of metabolites was observed in the rat with the glutathione conjugate of another compound examined, bromsulphthalein. This indicated that there appeared to be certain requirements for the enterohepatic recycling of metabolites derived from glutathione S-conjugates, although these may have been the factors known to be associated with absorption of compounds from the intestinal tract (see Section 7.4).

Essential differences appeared to exist in the enterohepatic circulation of glucuronic acid and glutathione derivatives of xenobiotics. With the former type of compound, intestinal microfloral enzyme activity is a specific requirement for the hydrolysis of the glucuronic acid moiety, leading to the absorption of the aglycone product. However, with the enterohepatic recycling of glutathione S-conjugates there appeared to be little or no direct involvement of intestinal

microflora. Instead, metabolism which occurred in the intestinal tract, was catalysed by enzymes in the epithelial cells lining the gastro-intestinal tract. This metabolism appeared to occur in conjunction with the direct absorption of unchanged glutathione S-conjugates, which were metabolised at other sites in the body. The main site of primary metabolism was identified in isolated cell incubations (see Chapter 6) to be principally the kidney with the small intestine and liver of less significance.

The net result of the enterohepatic recycling and further metabolism of glutathione S-conjugates excreted in bile has been shown in the rat to be the formation of mercapturic acids which are excreted in urine. This work has demonstrated a direct relationship between the hepatobiliary elimination of glutathione derivatives of naphthalene and 1-chloro-2,4-dinitrobenzene and the excretion of the corresponding mercapturic acids by the kidney. Whether or not similar relationships exist for the large number of xenobiotics which are known to be excreted in the urine of treated animals as mercapturic acids is unknown, but is obviously important with respect to a better understanding of the metabolism and disposition of such compounds. Glutathione forms derivatives with a number of reactive electrophiles (Chasseaud, 1979), which may also be those that bind to DNA, RNA and/or proteins. Enterohepatic recycling and the further metabolism of glutathione S-conjugates of potentially carcinogenic and mutagenic compounds may be important with respect to the persistence and toxicity of these compounds. For example, the carcinogens benzo(a)pyrene and aflatoxin B_1 form glutathione conjugates and have been demonstrated in the bile of animals administered these compounds (see Section 1.7 i).

Further work is obviously necessary in this area. It is also important to ascertain fully the specific sites of the metabolic reactions involved in the enterohepatic circulation of glutathione S-conjugates. Results described in this thesis indicate that the kidney, especially, and the intestine, but not the liver are the important sites in the primary metabolism of glutathione. This is principally a function of the relative activity of γ-glutamyltransferase in these tissues (see Chapter 6). However, since the metabolic conversion of glutathione S-conjugates to mercapturic acids is a three-step pathway, other tissues may be involved. Investigation of, for example, the metabolic fate of cysteine S-conjugates in various cell types should be undertaken. Metabolism of these compounds should be viewed not only with respect to N-acetylation to form mercapturic acids, but also in the light of hydrolysis, methylation or oxidation reactions and the possible formation of reactive metabolites.

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BILIARY EXCRETION AND ENTEROHEPATIC RECYCLING OF GLUTATHIONE CONJUGATES

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A number of drugs are known to undergo glutathione conjugation and subsequent excretion in bile (Chasseaud, 1979). The fate of these conjugates in the gastro-intestinal tract is poorly understood, but it appears that they can undergo reabsorption and metabolism, before being excreted in urine as mercapturic acids and other metabolites (Larsen & Bakke, 1981). We have investigated the fate in the GI tract of three glutathione-conjugated xenobiotics.

Bile duct-cannulated male Wistar rats were dosed i.v. with bromsulphthalein (BSP, 12 μmol/kg), [^{14}C]-1-chloro-2,4-dinitrobenzene (CDNB, 5 μmol/kg) or [^{14}C]-naphthalene (30 μmol/kg). The biliary metabolites were analysed by t.l.c. The extent of biliary excretion of these compounds and the proportion of biliary metabolites as glutathione conjugates are shown in Table 1(a).

Table 1 Excretion of metabolites of BSP, CDNB and naphthalene (Figures are Av. ± S.D.(n). N.D. = not detected)

a) Intravenous administration of parent compound

Compound	Time (h)	% Dose excreted in Bile	Urine	% Biliary metabolites as glutathione conj.
BSP	4	81.0± 5.8(3)	N.D. (3)	52.7±9.6(3)
CDNB	24	35.5± 5.3(3)	50.6±10.0(3)	40.0±2.6(3)
Naphthalene	6	63.9±11.6(7)	13.5±10.5(5)	76.0±2.6(3)

b) Intraduodenal infusion of glutathione conjugate

Glutathione conj. of	Time (h)	% Dose excreted in Bile	Urine	% Urinary metabolites as mercapturic acid
BSP	24	N.D. (3)	N.D. (3)	-
CDNB	24	28.5±6.3(3)	35.5±11.1(3)	59.8±5.2(3)
naphthalene	24	37.7±2.6(3)	44.5± 2.5(3)	81.6±4.0(3)

Glutathione conjugates of BSP, [^{14}C]-CDNB and [^{14}C]-naphthalene were infused intraduodenally into a further group of bile duct-cannulated rats. The re-excretion of the infused doses of these glutathione conjugates is shown in Table 1(b). In the case of BSP, no metabolites were detected in the bile or urine indicating the absence of enterohepatic circulation which may be due to the highly polar nature of BSP. The glutathione conjugates of CDNB and naphthalene, however, underwent metabolism during enterohepatic recycling and appeared in the bile and urine predominately as the corresponding mercapturic acids. Enterohepatic recycling of glutathione conjugates is thus important in the overall disposition and excretion of drugs as mercapturic acids in urine.

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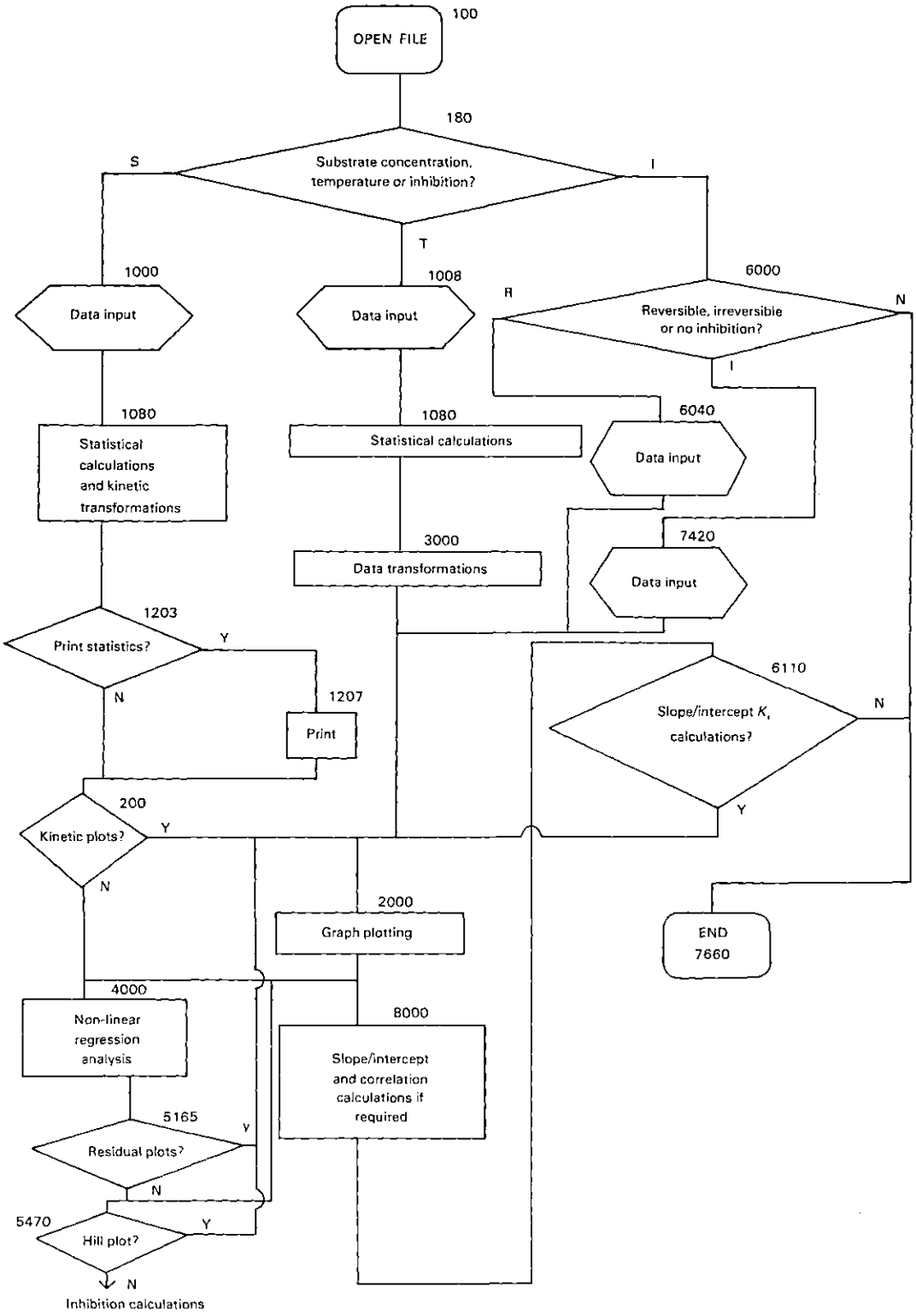

Fig. 1. *Block diagram of the complete enzyme-kinetics program, simplified to show the major routes for data transformations, printing and plotting (the numbers refer to program lines)*

desk-top microcomputer with 16Kbyte memory, and subsequently stored on tape or disc. It has been used in our laboratory during kinetic experiments on several enzymes, and provides useful information on how the experiments are progressing: whether more data are needed at any one substrate concentration, temperature, etc., or whether a cluster of data around certain areas would reveal inflexions or turning points. It should be particularly useful to the laboratory without access to any large computers, and gives complete kinetic information in systems where the Michaelis–Menten equation is a valid approximation, and in studies on metabolic fluxes using, for example, non-invasive nuclear magnetic resonance.

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The enterohepatic circulation of a glutathione conjugate of naphthalene in the rat

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The significance of biliary secretion and intestinal metabolism of glutathione conjugates of xenobiotics has received relatively little attention to date (see Chasseaud, 1979). A recent study has, however, shown that the glutathione and related thioether conjugates of the herbicide Propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] undergo metabolism in the gastrointestinal tract, leading to reabsorption and eventual excretion in the urine (Bakke *et al.*, 1980). Glutathione conjugates are major metabolites of both carcinogenic and non-carcinogenic polycyclic aromatic hydrocarbons and in the present study we demonstrate that naphthalene undergoes enterohepatic circulation in the rat after the biliary excretion of a glutathione conjugate.

Male Wistar rats (200–250g) were given [1-^{14}C]naphthalene (30μmol/kg) by intravenous injection. 90$\pm$7.1% (mean$\pm$S.D., $n=3$) of the dose was recovered in 5 days in the urine, mostly during the first 24h (80$\pm$10%, $n=3$), with the remainder excreted in the faeces (11$\pm$3.8%, $n=3$).

T.l.c. of the urine revealed four peaks of ^{14}C radioactivity, the major component (43% of urinary ^{14}C) being *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine, a 'pre-mercapturic acid' conjugate.

Administration of [^{14}C]naphthalene to bile-duct-cannulated rats resulted in 63$\pm$13% ($n=6$) of the dose appearing in the bile in 6h with 13.5$\pm$10.5% ($n=5$) in the urine. T.l.c. of bile revealed four peaks of ^{14}C radioactivity. The major biliary metabolite (76% of ^{14}C) was *S*-(1,2-dihydro-2-hydroxy-1-naphthyl)glutathione, but no 'pre-mercapturic acid' conjugate was detected.

The high level of biliary excretion observed in bile-duct-cannulated rats compared with the low faecal excretion in intact rats suggests that enterohepatic circulation is occurring. To investigate this further, bile taken from rats dosed with [^{14}C]naphthalene was infused intraduodenally into a further group of bile-duct-cannulated rats. Over a 6h period, approx. 70% of the infused dose was absorbed and re-excreted, 24$\pm$0.7% ($n=3$) in the bile, and 45$\pm$6.6% ($n=3$) in the urine. Examination of the metabolites present in these fluids showed that 82% of the urinary radioactivity was *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine, which would thus appear to have originated from the intraduodenally infused glutathione conjugate. The bile contained some of this 'pre-mercapturic acid' conjugate (20% of ^{14}C), but the major component (49% of ^{14}C) was an unknown compound of a more polar nature than any other metabolite, as indicated by its R_F value in the t.l.c. systems used.

Thus it appears that the major metabolite of naphthalene in the rat is a glutathione conjugate secreted in the bile. Further metabolism of this conjugate during enterohepatic recycling seems to be the origin of a major proportion of the *N*-acetyl-*S*-(1,2-dihydro-2-hydroxy-1-naphthyl)cysteine excreted predominantly in the urine. This enterohepatic circulation of the glutathione conjugate may also explain the origin of other minor metabolites of naphthalene such as reported by Stillwell *et al.* (1978).

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Role of γ-glutamyl transferase and glutathione in amino acid uptake by isolated hepatocytes in culture

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The role of the γ-glutamyl cycle as a mechanism for amino acid uptake by cells was proposed by Meister (1973). Although the enzymes are present in several tissues, direct evidence of the functioning of the cycle in amino acid translocation was required. A major problem was that γ-glutamyltransferase (EC 2.3.2.2) must react with intracellular glutathione (GSH) and extracellular amino acids. Using isolated hepatocytes in culture, we provide evidence for the role of the cycle in amino acid uptake by hepatocytes.

Isolated hepatocytes were cultured as described by Wanson *et al.* (1977), and γ-glutamyltransferase activity was measured as described by Tate & Meister (1974). Results are means$\pm$S.D. for the numbers of observations in parentheses.

Isolated hepatocytes in culture for 3 days (controls) had a γ-glutamyltransferase activity of 4.3$\pm$1.0nmol of *p*-nitroaniline/min per mg of protein. However when they were cultured in the presence of 3μM-dexamethasone, the activity rose to 9.2$\pm$1.9nmol/min per mg, i.e. 213% of the controls.

Dexamethasone also increased the amino acid uptake by hepatocytes in culture. Thus uptake of 2mM-[U-^{14}C]serine was 191$\pm$41(3)μmol/h per mg of protein and rose to 281$\pm$94(4)μmol/h per mg in hepatocytes cultured in the presence of 3μM-dexamethasone. However, when γ-glutamyltransferase activity was inhibited by 20mM-serine-borate, amino acid uptake by liver cells was impaired. Thus, in hepatocytes incubated with 3μM-dexamethasone uptake of 1mM-[U-^{14}C]glutamine was 85$\pm$10μmol/h per mg of protein. However, when they were incubated with 3μM-dexamethasone and serine-borate, uptake of 1mM-[*U*-^{14}C]glutamine was 62$\pm$5 μmol/h per mg.

Another important metabolite of the γ-glutamyl cycle is GSH. When intracellular GSH concentration was lowered to a value of 16% of controls by incubation (10min) with 10μM-diethyl