A MECHANISTIC INVESTIGATION OF THE PREDISPOSING FACTORS FOR IDIOSYNCRATIC DRUG REACTIONS

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CONTENTS

A MECHANISTIC INVESTIGATION OF THE PREDISPOSING FACTORS FOR IDIOSYNCRATIC DRUG REACTIONS

PhD Abstract

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The balance between bioactivation of a drug to its chemically reactive metabolite and its detoxication is an important determinant of individual susceptibility to idiosyncratic drug toxicity. This concept has been investigated using two widely used drugs, carbamazepine (CBZ) and sulphasalazine (SPS). Both drugs have been reported to cause such toxicity, which can affect multiple organ systems to a variable degree and with variable severity.

Eight patients clinically hypersensitive to CBZ were identified. Using a cytotoxicity assay, mononuclear leucocytes from these patients showed significantly ($p<0.001$) higher *in vitro* sensitivity ($7.9 \pm 0.8\%$) to oxidative metabolites of CBZ generated by a murine microsomal system than control subjects ($2.6 \pm 0.3\%$), suggesting that the assay could be used for diagnosis of hypersensitivity. The chemical specificity of the *in vitro* system was verified by showing no difference in cellular sensitivity between patients and controls on exposure to phenytoin, oxcarbazepine, dapsone hydroxylamine and amodiaquine quinoneimine. Taken together with the ability of human livers to bioactivate CBZ, the results are suggestive of a defect in cellular detoxication in patients affected by toxicity.

Two further CBZ-hypersensitive patients (one with hepatotoxicity and one with toxic epidermal necrolysis) were studied during the acute phase of the reaction. Cells from both patients again showed higher *in vitro* sensitivity than controls to oxidative metabolites of CBZ. On immunoblot analysis, serum from the patient with hepatotoxicity recognised a 94kDa protein band on human liver microsomes, but none out of 24 control sera recognised this band. No bands were recognised by the patient serum on human kidney microsomes or on microsomes from mouse or rat liver. Immunohistochemical analysis of the lesional skin taken from the patient with toxic epidermal necrolysis showed infiltration with various specific T-cell subsets, including $CD8^+$ cells, consistent with the involvement of cellular immunity in the pathogenesis of the adverse reaction.

The *in vitro* formation of the cytotoxic, protein-reactive and the stable 10,11-epoxide metabolites of CBZ by both human and mouse liver microsomes was inhibited by ketoconazole (10-250μM), indicating that these pathways are catalysed by the cytochrome P450 enzymes. Exogenous microsomal epoxide hydrolase (mEH), but not cytosolic epoxide hydrolase, caused a concentration-dependent inhibition of cytotoxicity reaching a maximum of 60% at 100 units mEH. Covalent binding was also reduced by 60% by 100 units mEH. Glutathione, but not ascorbic acid, also reduced cytotoxicity and covalent binding of CBZ, while neither compound affected the formation of the stable 10,11-epoxide. Taken collectively, these results indicate that the reactive intermediate formed from CBZ is an unstable arene oxide.

Enzyme induction studies with hepatic microsomes prepared from male CBA/ca mice showed that the formation of the cytotoxic, protein-reactive and stable metabolites of CBZ is catalysed by phenobarbitone- and dexamethasone-inducible forms of cytochrome P450, but not ß-naphthoflavone inducible forms. Gestodene, an

inhibitor of cytochrome P450 3A (CYP3A), inhibited the bioactivation of CBZ. Immunoblotting of the microsomes with anti-CYP3A antibodies revealed the presence of a 52kDa protein band in all the microsomes, the intensity of the bands being highest with the phenobarbitone and dexamethasone microsomes. 6ß-hydroxycortisol formation was highest with the dexamethasone and phenobarbitone microsomes and was inhibited by gestodene. These data suggest that the bioactivation of CBZ is dependent on the CYP3A subfamily.

Sulphasalazine (SPS) consists of two moieties, sulphapyridine and 5-aminosalicylic acid, linked by an azo bond. Of the metabolites of SPS investigated, only sulphapyridine was bioactivated by human liver microsomes in the presence of NADPH to a metabolite which caused methaemoglobinaemia and mononuclear leucocyte cell death. Sulphapyridine (100μM) was converted *in vitro* to a metabolite (conversion 6.8 $\pm$ 0.3%), the retention time of which on HPLC corresponded to synthetic sulphapyridine hydroxylamine. The bioactivation of sulphapyridine was inhibited by ketoconazole. In the absence of a metabolising system, only the hydroxylamine caused a concentration-dependent (10-500μM) increase in methaemoglobinaemia (2.9%-24.4%) and cytotoxicity (5.4%-51.4%), whereas SPS, sulphapyridine, 5-hydroxy sulphapyridine and 5-aminosalicylic acid had no effect. Thus, *N*-oxidation products of sulphapyridine may play a role in the haematological toxicity which has been reported with SPS.

A clinical study was performed in patients with rheumatoid arthritis to determine whether the haematological toxicity of SPS could be reduced by cimetidine (400mg three times daily), an inhibitor of human cytochrome P450 enzymes. All patients on entry into the study had elevated methaemoglobin levels (3.5 $\pm$ 0.7%), but administration of cimetidine did not result in a significant decrease in methaemoglobinaemia (2.9 $\pm$ 0.4%). In addition, cimetidine had no effect on the disease activity index, other haematological variables or sulphapyridine pharmacokinetics. These results suggest that cimetidine does not inhibit the P450 enzymes responsible for the *N*-hydroxylation of sulphapyridine *in vivo*.

In summary, the findings of these studies suggest that an imbalance between bioactivation and detoxication resulting from either enhancement of the former and/or reduction of the latter, is an important determinant of individual susceptibility to toxicity with CBZ and SPS. The use of human tissues, particularly from patients affected by idiosyncratic toxicity, in the *in vitro* methods described in this thesis, may help in evaluating the mechanisms of idiosyncratic drug reactions, with the aim of being able to predict individual susceptibility and develop newer compounds which are less liable to cause such reactions.

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ABBREVIATIONS

°C	degree(s) centigrade
cm	centimetre(s)
CYP	cytochrome P450
h	hours
HPLC	high performance liquid chromatography
HSA	human serum albumin
i.p.	intra-peritoneally
kg	kilogram
l	litre
M	molar
mCi	millicurie
min	minute(s)
mg	milligram(s)
ml	millilitre
mM	millimolar
MNL	mononuclear leucocytes
NADPH	reduced nicotinamide dinucleotide phosphate
nm	nanometre(s)
PBS	phosphate buffered saline
rev	revolutions
sec	second(s)
S.D.	standard deviation
SEM	standard error of mean
TCPO	trichloro-propene oxide
μCi	microcurie
μM	micromolar
v/v	volume by volume
w/v	weight by volume

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Introduction

Adverse drug reactions represent a large clinical problem accounting for a great deal of morbidity and occasional mortality. Idiosyncratic drug reactions can be a particular problem largely because of their unpredictability and severity. Drug metabolism by forming chemically reactive metabolites has been postulated to play a pivotal role in the pathogenesis of such reactions. Therefore, in this thesis, the role of metabolism, and in particular, the balance between metabolic activation and metabolic detoxication, has been investigated using two drugs, carbamazepine and sulphasalazine. These two compounds have been chosen because they have well-documented idiosyncratic toxicity of relatively high frequency and they are widely used and thus patients with and without adverse drug reactions taking these drugs can be identified and investigated using relevant *in vitro* techniques to determine unique susceptibility factors. The known pharmacology and toxicity of these two drugs is detailed later in this chapter. Initially, however, an overview is given of adverse drug reactions followed by a discussion of pathways of drug metabolism and how an imbalance in such pathways can lead to various forms of drug toxicity.

1.2. Adverse drug reactions

1.2.1. Epidemiology

Incidence estimates of adverse drug reactions between different studies are widely variable largely because of differences in definition, detection and reporting of the reactions (Lawson, 1991). Thus, the reported incidence varies from 1% to 28% (MacDonald and MacKay, 1964; Miller, 1974), with most studies reporting an incidence of 10-20% (Seidl *et al.*, 1965; Smith *et al.*, 1966; Ogilvie and Ruedy, 1967;

Gardner and Watson, 1970). Drug-related deaths occurring as a result of either therapeutic errors or because of unexpected and unpredictable drug effects, range from 0.01% (for surgical inpatients) to 0.1% (for medical inpatients; Lawson, 1991).

Apart from the obvious cost of drug toxicity causing patient morbidity and mortality, adverse drug reactions also represent an unnecessary burden on already overstretched medical and national resources. Mach (1975) has attempted to estimate the cost by dividing it into direct and indirect costs; direct costs include the cost of treatment, hospitalisation and prevention and detection of adverse drug reactions, while indirect costs include the lost contribution of the patient to the gross national product. Since about 1 in 20 beds in the UK and 1 in 7 beds in the USA are occupied by patients who have had adverse drug reactions (D'Arcy, 1986), the economic cost of drug toxicity is enormous, estimated to be about $5 billion per year in the USA (D'Arcy, 1986).

1.2.2. Classification

Many classifications of adverse drug reactions have been proposed (Rang and Dale, 1987), but perhaps the most useful clinically is the classification proposed by Rawlins and Thompson (1977) which was later extended (Grahaeme-Smith and Aronson, 1992), where adverse drug reactions are divided into four basic types :-

Type A: These reactions are predictable from the known pharmacology of the drug and are dose-dependent and host-independent. They account for 75% of all adverse drug reactions, and although they are usually mild, by virtue of their common occurrence, they account for a great deal of morbidity. Such reactions should be anticipated, and can often be eliminated by dose

reduction. Typical examples include hypoglycaemia with oral hypoglycaemics and hypotension with anti-hypertensives.

Type B (idiosyncratic): These reactions are unpredictable from the known pharmacology of the drug, show no simple dose-response relationship, and are apparently host-dependent. They tend to be more severe than type A reactions and account for the majority of drug-associated fatalities. They account for 10% (Uetrecht, 1992) of all adverse drug reactions.

Type C: Reactions associated with long-term therapy, examples of which include benzodiazepine dependence and analgesic nephropathy. These reactions are well described and can be anticipated.

Type D: Delayed effects such as carcinogenicity and teratogenicity. It is thought that such toxicities are precluded by the extensive programme of preclinical mutagenicity and carcinogenicity studies that a new chemical entity must undergo before a product licence is granted.

The type B or idiosyncratic drug reactions have resulted in the withdrawal of several potentially useful drugs (table 1.1). The mechanisms of these reactions, in general, are poorly understood. Part of the reason for this is that they cannot be reproduced in animal models, and thus investigation has been dependent on retrospective clinical analysis (Park *et al.*, 1992). At present, new chemical entities cannot be evaluated for their ability to cause idiosyncratic reactions, and thus, such reactions are often not detected until the postmarketing surveillance phase of drug development. This is not only wasteful in terms of commercial investment but could also be considered an unacceptable risk for patients participating in clinical studies. Thus, investigations into the mechanisms of idiosyncratic drug reactions is essential

in order to gain a better understanding of the chemical and cellular basis of drug toxicity with the aim of preventing such idiosyncratic reactions.

Table 1.1

Drugs which have been withdrawn as a result of idiosyncratic drug reactions.

Drug	Adverse reaction
Alcofenac	Hypersensitivity reactions
Althesin	Anaphylaxis
Benoxaprofen	Hepatotoxicity, nephrotoxicity
Fenclofenac	Toxic epidermal necrolysis
Glafenine	Anaphylaxis
Ibufenac	Hepatotoxicity
Nomifensine	Haemolytic anaemia, hepatotoxicity
Practolol	Oculo-mucocutaneous syndrome
Temofloxacin	Haemolytic anaemia, anaphylaxis
Tienilic acid	Hepatotoxicity
Zimeldine	Guillain-Barre syndrome
Zomepirac	Anaphylaxis

Adapted from Park et al., (1992).

1.3. The role of drug metabolism in adverse drug reactions

The primary function of the drug metabolising enzymes in the body is to facilitate the excretion of xenobiotics and endobiotics from the body by converting essentially lipid-soluble, non-polar compounds to more polar, less lipid-soluble

compounds with a smaller volume of distribution (Woolf and Jordan, 1987; Alvares and Pratt, 1990). Metabolism usually renders the compound therapeutically inactive, although in some cases, the metabolites themselves may be pharmacologically active (Woolf and Jordan, 1987), and thus have to undergo further metabolism to inactivate them and enhance their excretion. Drug metabolism can also lead to the formation of toxic, chemically reactive metabolites which have been implicated in various forms of drug toxicity (see section 1.3.3). Advantage has also been taken of the drug-metabolising enzymes by administration of inactive compounds (i.e. prodrugs) which are converted by these enzymes to therapeutically active compounds (Garattini, 1985).

Williams (1959) has provided a useful classification of drug metabolism into two phases, termed phase I and phase II reactions (table 1.2).

1.3.1. Phase I metabolic pathways

Phase I reactions consist of oxidative, reductive and hydrolytic reactions (table 1.2) usually converting the parent drug to a more polar metabolite. The oxidative and reductive reactions alter and create new functional groups, while the hydrolytic reactions cleave esters and amides to release functional groups (Woolf and Jordan, 1987; Alvares and Pratt, 1990), allowing the drug to undergo phase II or conjugation reactions.

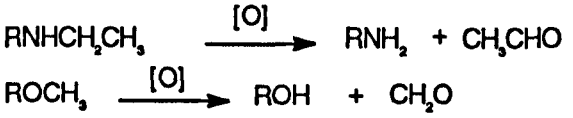
The enzymes responsible for phase I reactions are located mainly in the endoplasmic reticulum, i.e the microsomal fraction, with some enzymes such as alcohol and aldehyde dehydrogenase, being located in the cytosol (table 1.3.; Jakoby, 1980).

Table 1.2 Drug Biotransformation Reactions

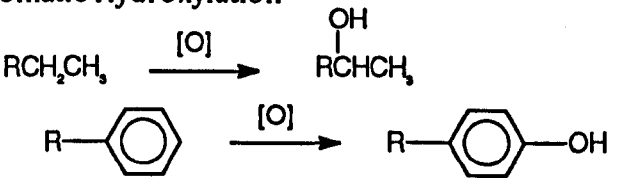
Phase I reactions

I. Oxidative Reactions

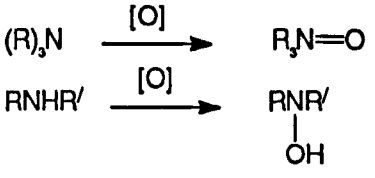
(1) N- and O-Dealkylation



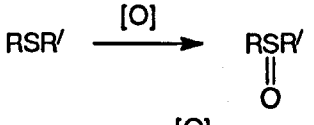
(2) Side Chain (Aliphatic) and Aromatic Hydroxylation



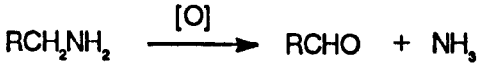
(3) N-Oxidation and N-Hydroxylation



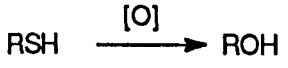
(4) Sulphoxide Formation



(5) Deamination of Amines

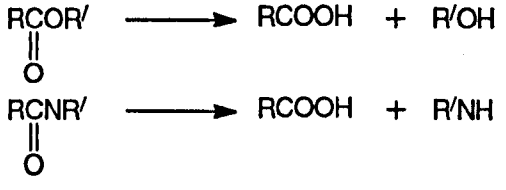


(6) Desulphuration

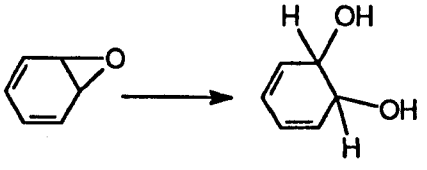


II. Hydrolysis Reactions

(1) Hydrolysis of Esters and Amides

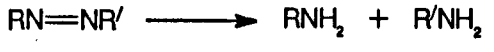


(2) Hydrolysis of Arene Oxides



III. Reductions

(1) Azo Reduction



(2) Nitro Reduction

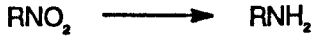
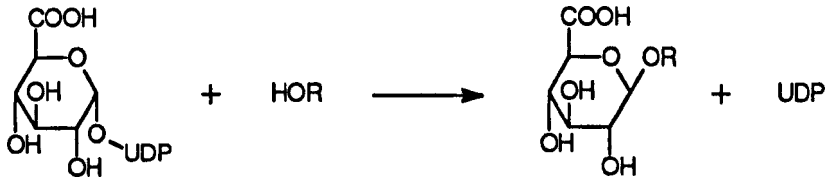


Table 1.2 Drug Biotransformation Reactions (continued)

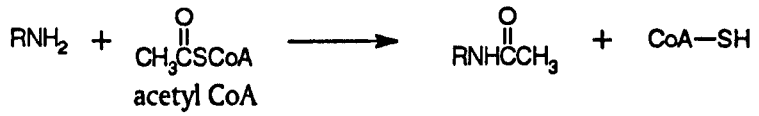
Phase II reactions

I. Glucuronide reactions

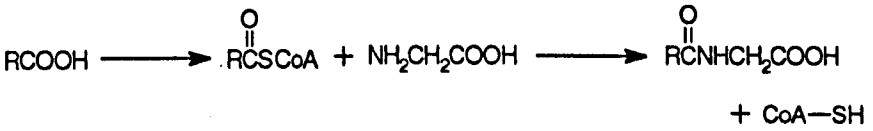


II. Other Conjugation Reactions

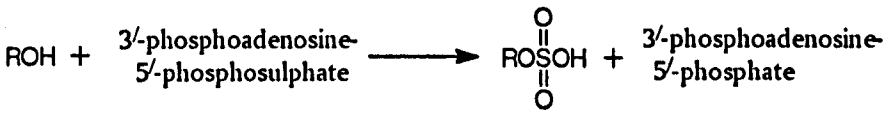
(1) Acetylation



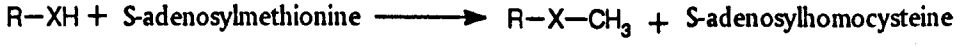
(2) Conjugation with Glycine



(3) Conjugation with Sulphate



(4) O-, S-, andN-Methylation



(X = O, S, N)

Table 1.3

The subcellular localisations of the major drug metabolising enzymes.

Microsomes	Cytosol	Mitochondria
Cytochrome P450	Glutathione S-transferases	Monoamine oxidase
NADPH-cytochrome P450 reductase	Epoxide hydrolase	Glutathione peroxidase
UDP glucuronosyl transferases	Aldehyde and ketone reductase	Aldehyde dehydrogenase
Glutathione S-transferase	Catechol *O*-methyl transferase	Cytochrome P450
Epoxide hydrolase	Aldehyde dehydrogenase	
Flavin-containing monooxygenase	*N*-acetyl transferases	
Xanthine and aldehyde oxidase	Sulphotransferases	
Carboxyl esterases		
Aldehyde dehydrogenase		

Adapted from Jakoby, (1980).

Of the microsomal drug metabolising enzymes, the most important are the cytochrome P450 enzymes, which comprise about 95% of the phase I enzymes (Burchell *et al.*, 1991). These enzymes are present mainly in the liver, particularly within the centrilobular region (Pessayre and Larrey, 1988), although they have also been located in extra-hepatic tissues such as the kidney, lungs, gut and skin (Woolf and Jordan, 1987).

The cytochrome P450 enzymes are a superfamily of haemoprotein enzymes, encoded by multiple genes (Gonzalez *et al.*, 1986; Gonzalez, 1989). At least 10 gene families are known to exist in all mammals (Nebert *et al.*, 1991), resulting in isozymic forms of the enzymes which have diverse, but often overlapping substrate specificities (Forrester *et al.*, 1990). Indeed, it is no exaggeration to state that P450 is the most versatile biological catalyst known (Porter and Coon, 1991). Since over 150 isoforms of the enzymes have been characterised in recent years, many of which catalyse multiple reactions, a nomenclature system based on structural homology rather than the usual method based on catalytic activities, has been devised by Nebert and colleagues (1989) which has recently been updated (Nebert *et al.*, 1991). The individual genes are named with the prefix *CYP* (*C*ytochrome *P*450) followed by an Arabic numeral designating gene family, a letter designating subfamily and another Arabic numeral designating the gene number. Thus, *CYP1A1* and *CYP1A2* are individual genes 1 and 2 in family 1, subfamily A. A protein within one subfamily exhibits <40% amino acid similarity with genes in other subfamilies. P450s within the same subfamily display at least 59% similarity (Nebert *et al.*, 1991). In man, six families of steroidogenic P450s (*CYP7, CYP11, CYP17, CYP19, CYP21* and *CYP26*) which carry out highly specific reactions in steroid or bile acid synthesis, and four families

(*CYP1, CYP2, CYP3* and *CYP4*) which are responsible for metabolism of foreign chemicals (Gonzalez *et al.*, 1991) have been identified. The latter group of P450 enzymes is listed in table 1.4 together with their representative substrates.

With the cytochrome P450 reactions, the initial binding of the drug substrate to the oxidised P450 [Fe^{3+}] is followed by a complex series of events (figure 1.1) finally leading to the formation of the oxidised metabolite and water together with the regeneration of the oxidised P450 (Benet and Scheiner, 1985; Porter and Coon,1991). The reactions that have been demonstrated include hydroxylation, dealkylation, epoxidation, peroxygenation, deamination, desulphuration, and dehalogenation, as well as reduction (Porter and Coon, 1991).

Another family of enzymes which are important in phase I metabolism are the epoxide hydrolases. The cytochrome P450-mediated metabolism of xenobiotics can occasionally lead to the formation of epoxides (Jerina and Daly, 1974), some of which may be highly reactive electrophiles. The epoxide hydrolases can catalyse the conversion of such epoxides to dihydrodiols (Jerina and Daly, 1974; Guenthner, 1990), which are usually much less reactive than their epoxide precursors. At least four different catalytically and immunochemically distinguishable epoxide hydrolases exist (figure 1.2), three of which are of relatively minor toxicological significance (Guenthner, 1990). The microsomal epoxide hydrolase is regarded as an essential endogenous means of protection against reactive epoxide intermediates (Seidegard and DePierre, 1983; Guenthner, 1990). It catalyses the hydrolysis of arene oxides and other cis-disubstituted or monosubstituted oxiranes. It has been found in all investigated human tissues including liver, lung, kidney, skin, placenta and circulating lymphocytes (Cantoni *et al.*, 1978; Pacifici and Rane, 1983; Seidegard *et al.*, 1984;

Table 1.4

Listing of the human P450s together with their representative substrates, inducers and inhibitors.

Name	Substrates*	Inducers#	Inhibitors
CYP1A1	Polycyclic aromatic hydrocarbons	Polycyclic aromatic hydrocarbons	
CYP1A2	Aromatic amines, Aflatoxin B_1, Caffeine, Phenacetin, Theophylline		Furafylline, Ciprofloxacin, Enoxacin
CYP2B7	Aflatoxin B_1, Ethoxycoumarin	Phenobarbitone	
CYP2C8	Tolbutamide	Phenobarbitone Rifampicin	Sulphaphenazole
CYP2C9	Tolbutamide, Hexobarbital		
CYP2D6	Debrisoquine, Sparteine, Perhexiline, Metoprolol Dextromethorphan		Quinidine
CYP2E1	Benzene, Paracetamol, Nitrosamines, Chlorzoxazone	Acetone, Alcohol, Isoniazid	Disulfiram, Dihydrocapsaicin
CYP3A3	Aflatoxin B_1, Cyclosporin, Erythromycin, Oestrogen, Nifedipine, Testosterone	Glucocorticoids, Rifampicin, Phenytoin, Phenobarbitone	Gestodene, Troleandomycin
CYP3A4			
CYP3A5	Cyclosporin, Nifedipine, Testosterone		
CYP3A6	Aflatoxin B_1, Dehydroepiandrosterone 3-sulphate		
CYP4B1	Testosterone, Nifedipine	Clofibrate	

Adapted from Gonzalez, (1989); Gonzalez et al. (1991); Nebert *et al.* (1991).

* This is only a partial list of substrates for each P450 form.

\# The list of inducers has been determined by either a study of functional activity in man or has been deduced from animal studies.

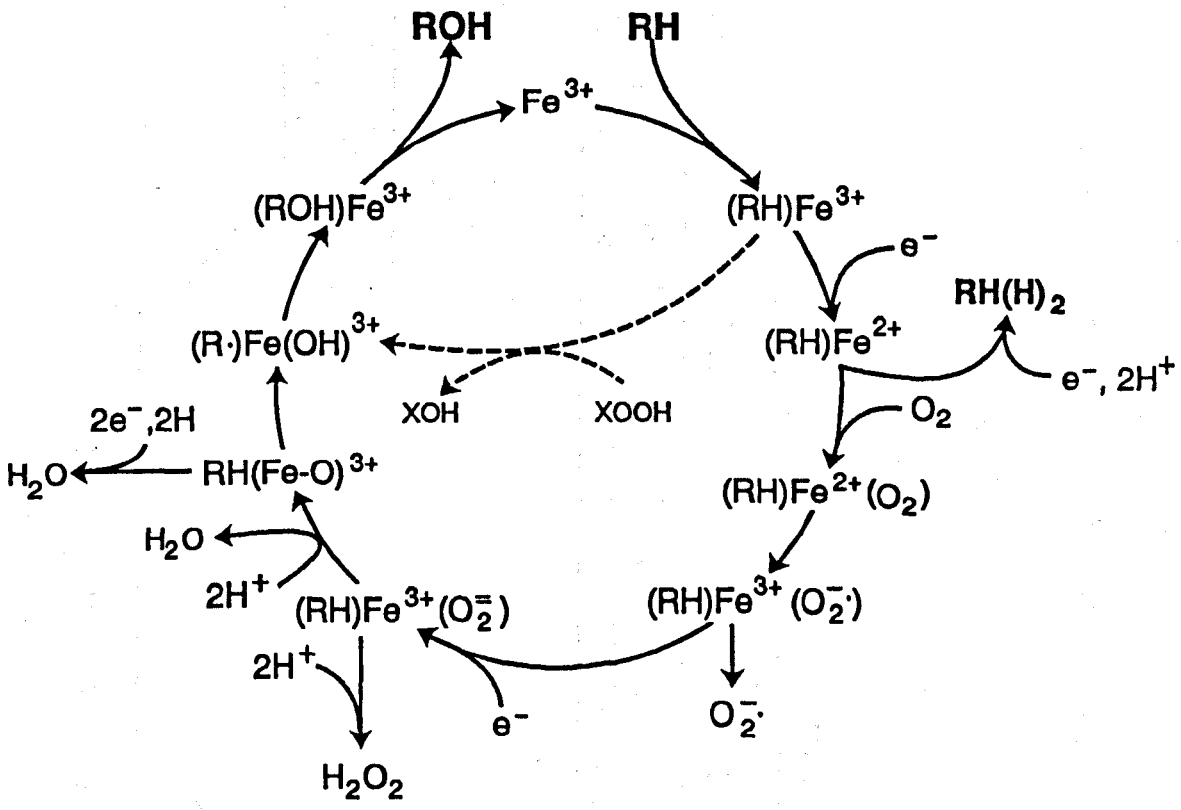


Figure 1.1. Scheme for mechanism of action of cytochrome P450.
Fe represents the haem iron atom at the active site, *RH* the substrate, *RH(H)2* a reduction product, *ROH* a monooxygenation product, and *XOOH* a peroxy compound that can serve as an alternative oxygen donor.
(adapted from Porter and Coon, 1991)

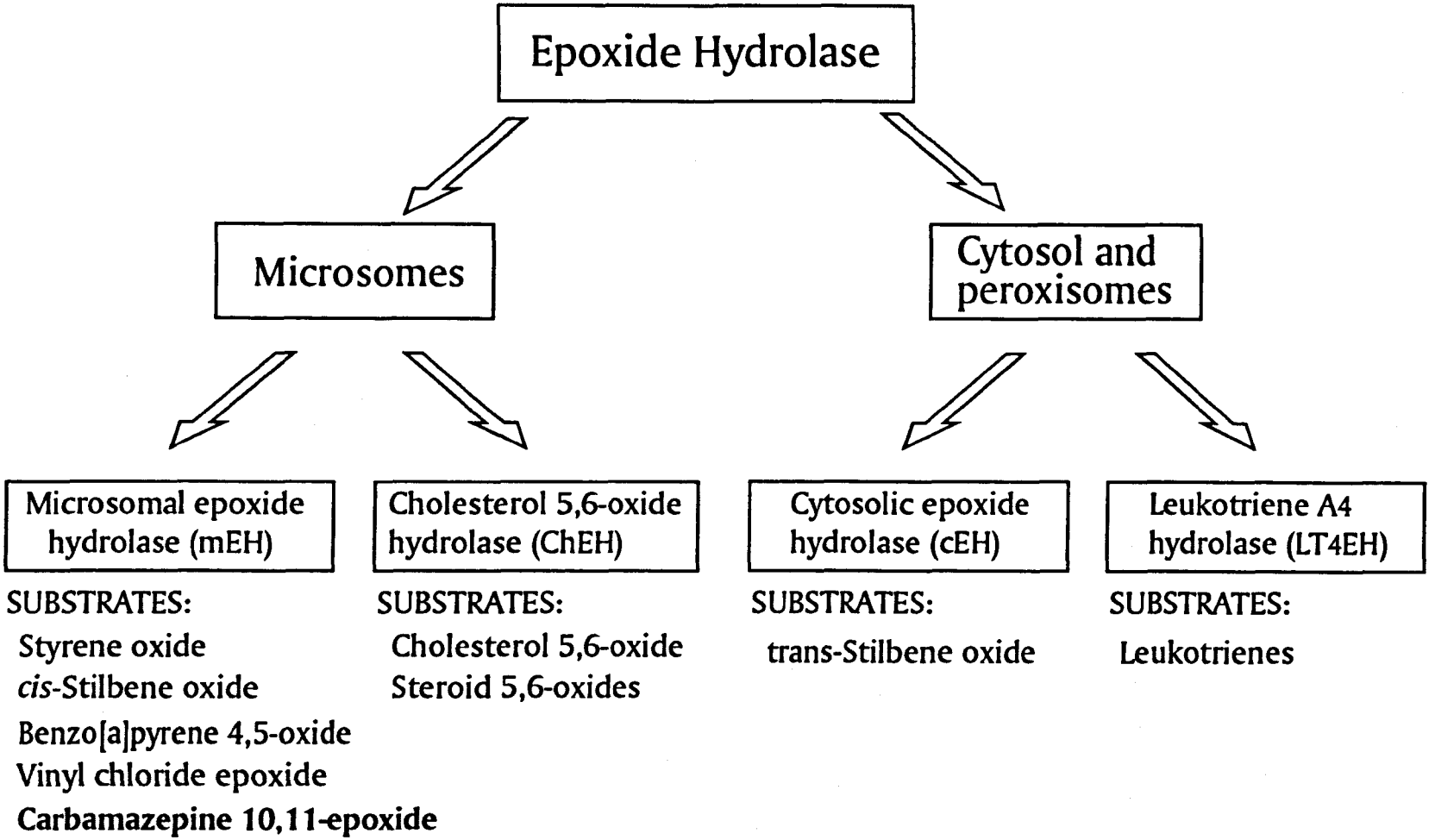


Figure 1.2. The epoxide hydrolase enzymes together with some of their endogenous and exogenous substrates.

Guenthner and Karnezis, 1986; Pacifici *et al.*, 1988; Pertruzzelli *et al.*, 1988; Eaton and Stapleton, 1989), the highest levels being found in the liver (Guenthner *et al.*, 1992).

1.3.2. Phase II metabolic pathways

The phase II metabolic pathways are conjugation reactions which involve the addition of endogenous moieties such as glucuronic acid, sulphate and glutathione (table 1.2) resulting in hydrophilic products which can be more readily excreted by the body (Alvares and Pratt, 1990). A drug may undergo sequential phase I and phase II reactions before excretion, or alternatively (and indeed in the majority of cases), it may be conjugated without having to undergo functionalisation (Tephly and Burchell, 1990).

Quantitatively, the most important phase II pathway is glucuronidation, catalysed by the UDP glucuronosyltransferases, which like the P450 enzymes, form a superfamily of enzymes, encoded by multiple genes (Burchell *et al.*, 1991). To date, over 20 different enzymes are known to exist in mammals (Tephly *et al.*, 1989; Burchell *et al.*, 1991), the nomenclature of which is confusing. However, recently, Burchell and colleagues (1991) have devised a nomenclature system based on evolutionary divergence which should, at least partially, resolve the confusion.

Conjugation with glutathione is a major endogenous protective system against chemically reactive metabolites (Reed, 1986). Glutathione, a nucleophile present in millimolar concentrations in most cells (Reed, 1990), reacts with various types of electrophilic metabolites resulting in their detoxication. Conjugation with glutathione can occur either spontaneously or may be catalysed by the glutathione transferases (Deleve and Kaplowitz, 1990), a family of isozymes (table 1.5) with

unique but overlapping substrate specificities (Jakoby, 1978; Boyer, 1989). The spontaneous reactions is particularly likely to occur with the so-called 'soft' electrophiles, while enzymatic catalysis is more common with the 'hard' electrophiles (Coles, 1985). However, enzymatic catalysis by the glutathione transferases assumes dominance when glutathione concentrations are low, even with soft electrophiles (Coles *et al.*, 1988).

Table 1.5

Classification of glutathione S-transferases.

Class	pI	Class-distinguishing substrate
Alpha	Alkaline (>8.0)	Organic hydroperoxides
Mu	Neutral (7-8)	Epoxides
Pi	Acidic (<7.0)	Ethacrynic acid

Adapted from Boyer, 1989.

1.3.3. Drug metabolism and drug toxicity

Drug metabolism can normally be considered a detoxication process. However, in certain circumstances, the metabolic pathways can result in the formation of toxic, chemically reactive metabolites (figure 1.3; Guengerich and Liebler, 1985; Park, 1986; Parke, 1987) which, by binding irreversibly to cellular macromolecules such as nucleic acids and proteins, can lead to various forms of

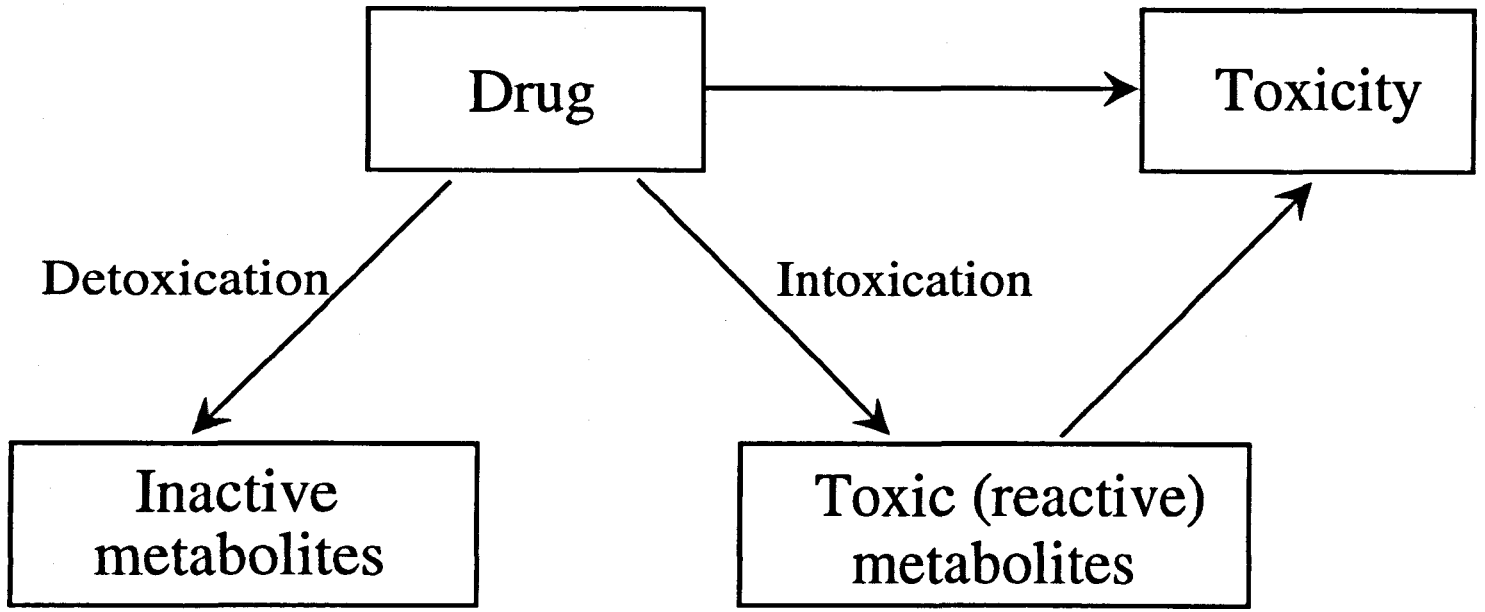


Figure 1.3. The role of drug metabolism in the formation of chemically reactive metabolites.

toxicity including carcinogenicity, teratogenicity, cellular necrosis and hypersensitivity (figure 1.4; Park, 1986; Park and Kitteringham, 1987). The site of toxicity of the chemically reactive metabolite is determined by several factors including the half-life of the toxic metabolite, the nature of the macromolecule to which the metabolite binds and the immunogenicity of the resulting drug-macromolecule conjugate. Fortunately, in the majority of individuals, bioactivation of a drug to its chemically reactive metabolite is balanced by its detoxication resulting in harmless excretion of the metabolite from the body (Bock *et al.*, 1987; Park and Kitteringham, 1990a). Thus, it has been postulated that only individuals who have an imbalance between activation and detoxication will develop such unpredictable drug toxicity.

Most attention has focused on the role of the cytochrome P450 enzymes in the generation of toxic metabolites (Guengerich and Liebler, 1985; Park, 1986; Parke, 1987). However, other drug metabolising enzymes, even those with a primary detoxication role such as glucuronidation and epoxide hydrolase, can bioactivate compounds (Guengerich, 1992b). Conjugation with glutathione can also bioactivate compounds; for example, dibromoethane, a highly toxic (Storer and Connolly, 1983), mutagenic (Rannug, 1980) and carcinogenic (Huff, 1983) compound, undergoes enzymatic conjugation with glutathione forming a 2-bromoether, which is then transformed to a reactive species, possibly an episulfonium ion (Rannug *et al.*, 1978; Rannug, 1980).

1.3.3.1 Direct toxicity caused by chemically reactive metabolites

The chemical interaction of a chemically reactive metabolite with essential cellular macromolecules, through either covalent bond formation (Watkins, 1990) or

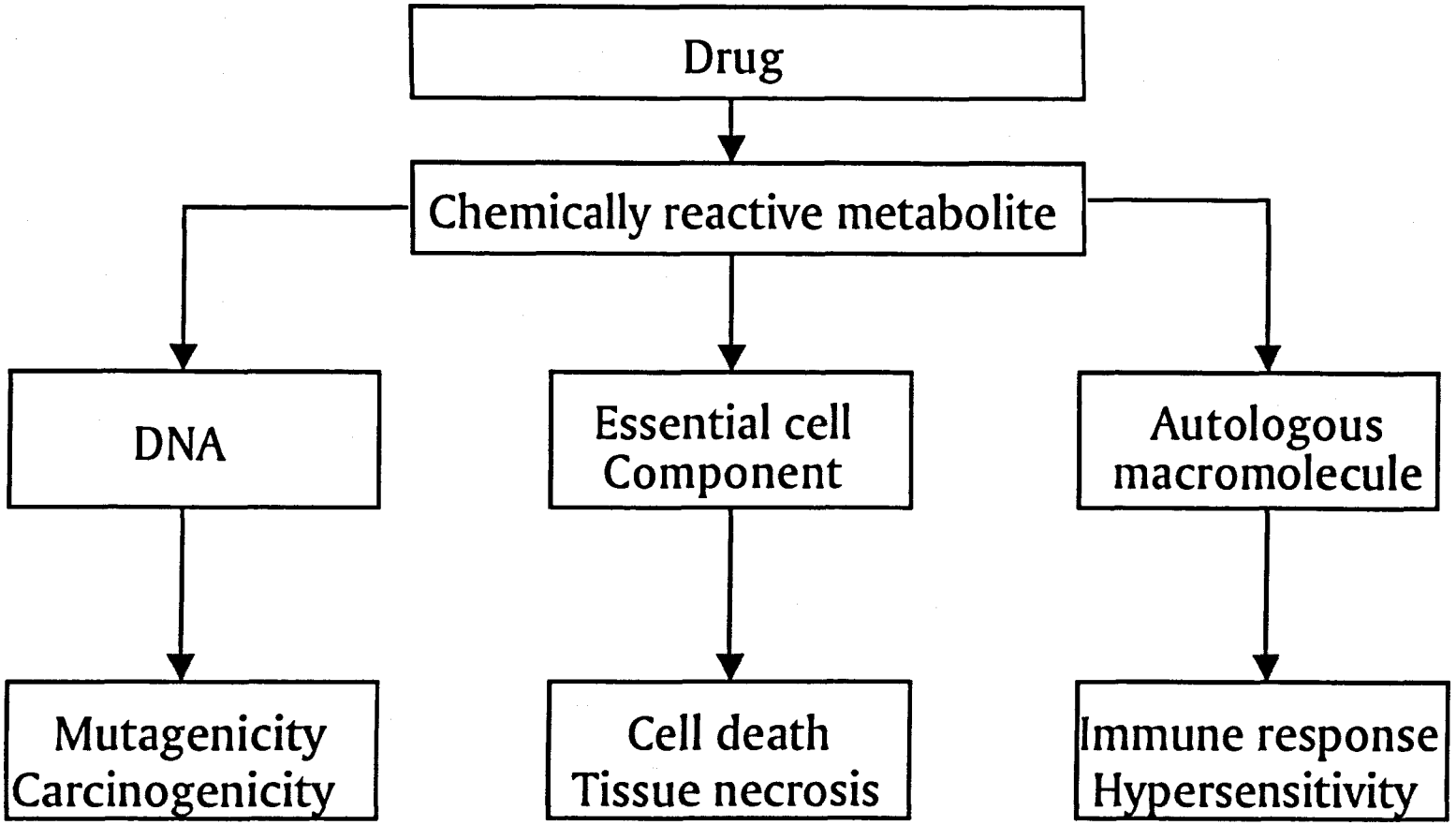


Figure 1.4. The relationship between the formation of chemically reactive metabolites and the different forms of drug toxicity. Adapted from Park (1986).

free radical formation (Williams and Burk, 1990), can result in loss of cellular viability leading to tissue or organ necrosis (Park, 1986; Parke, 1987; Pessayre and Larrey, 1988). The toxic response to the initial challenge and to any subsequent re-challenge is often quite rapid, occurring within a few days, and is not accompanied by symptoms suggestive of an immunological response (Pessayre and Larrey, 1988). The site of toxicity is dependent upon the site of generation of the toxic metabolite. Since the liver is the main site of drug metabolism in the body, this mechanism has been implicated in causing hepatotoxicity with drugs such as paracetamol and isoniazid.

Paracetamol, when taken in overdosage, is the commonest cause of hepatic failure in the UK, accounting for 40% of all cases (Eddleston, 1987). In therapeutic doses, paracetamol is metabolised by conjugation to form paracetamol glucuronide and sulphate (Nelson, 1990). A smaller proportion (5-10%) is oxidised by cytochrome P450 (thought to be CYP2E1 and CYP1A2; Raucy *et al.*, 1989) to a chemically reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine, which is normally detoxified by glutathione conjugation and then excreted as cysteine and mercapturate conjugates (Figure 1.5; Prescott, 1983). However, after overdosage (10-15g), a larger proportion of paracetamol is oxidised to the reactive metabolite because of saturation of the sulphation pathway (Meredith *et al.*, 1986). This leads to depletion of the liver glutathione stores resulting in inadequate detoxication of the reactive metabolite and subsequent binding to hepatic proteins, with resultant necrosis (Jollow *et al.*, 1973; Mitchell *et al.*, 1973a, b; Potter *et al.*, 1973). Thus, *N*-acetyl cysteine, which is used in the treatment of paracetamol overdosage acts by increasing intracellular glutathione levels, restoring the detoxication of the reactive metabolite (Meredith *et*

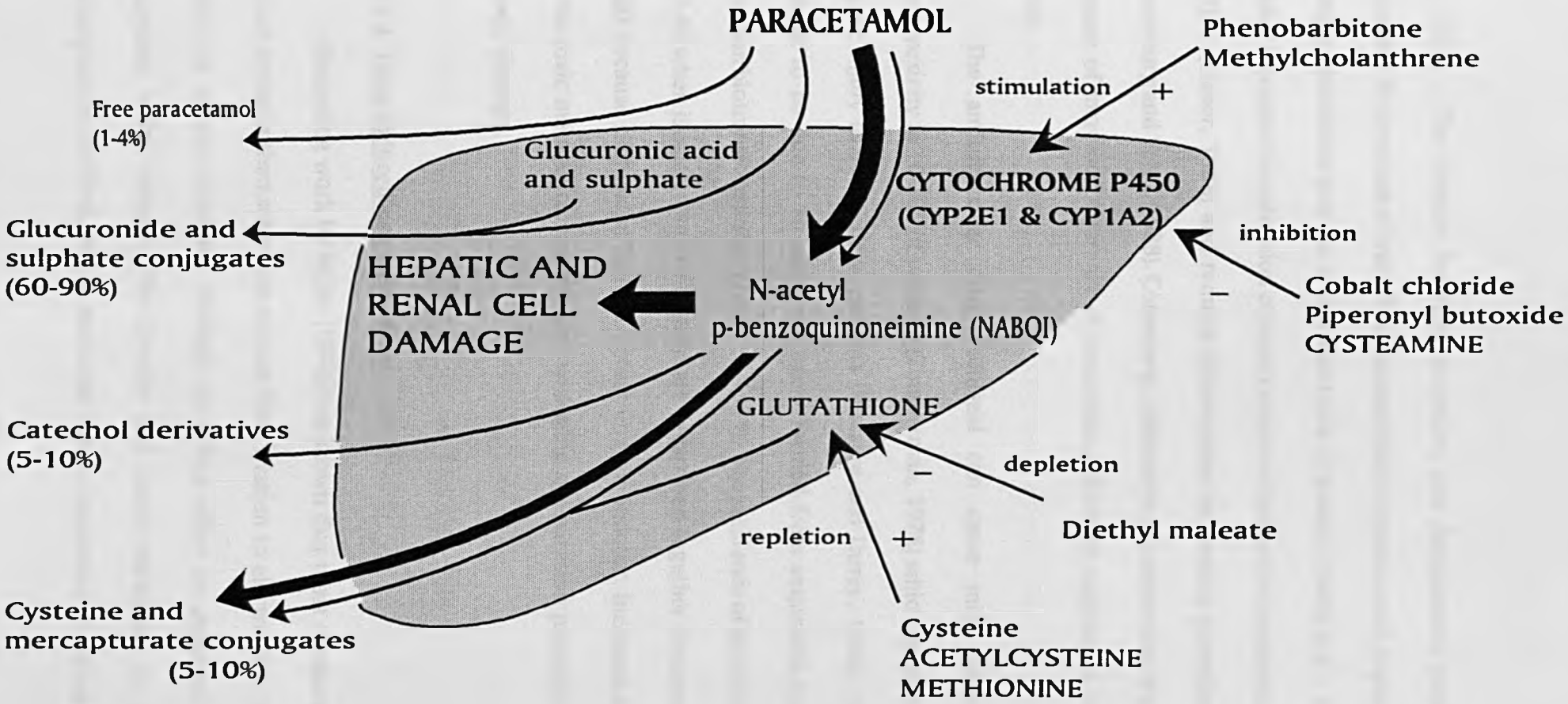


Figure 1.5 The metabolism and mechanism of hepatotoxicity of paracetamol. Adapted from Eddleston, (1987).

al., 1986). The balance between activation and detoxication seems to be an important determinant of individual susceptibility to paracetamol hepatotoxicity. For example, alcoholics may be at increased risk of toxicity (Seeff *et al.*, 1986) because alcohol increases bioactivation of paracetamol to its reactive metabolite by inducing CYP2E1 (Lieber, 1988) and reduces detoxication by causing glutathione depletion (Lauterburg and Velez, 1988). Conversely, children have a lower risk of hepatotoxicity because of increased clearance of paracetamol through sulphation (Lieh-Lai *et al.*, 1984).

The antitubercular drug, isoniazid can cause mild, often subclinical, hepatotoxicity in 10-20% of patients (Zimmerman, 1978) which progresses to severe hepatic injury in about 1% of patients (Pessayre and Larrey, 1988). The toxicity is thought to be due to an alkylating agent formed from sequential acetylation and oxidation biotransformations (Timbrell, 1983). The incidence of hepatitis is increased to 5-8% when isoniazid and rifampicin are prescribed together (Pessayre and Larrey, 1988) because rifampicin, being a potent enzyme inducer, increases the formation of the toxic metabolite, thereby overwhelming detoxication processes resulting in hepatic damage.

1.3.3.2 Drug-induced carcinogenicity

Pioneering work by Miller (1970) has shown that most chemical carcinogens are not active in themselves but require bioactivation to electrophilic intermediates which can induce neoplastic changes by acting either as genotoxic carcinogens (initiators), which damage DNA directly and cause mutations, or as epigenetic carcinogens (promoters), which accelerate the accumulation of critical spontaneous

mutations (Lutz and Maier, 1988; Boyd and Barrett, 1990). As with other forms of drug toxicity, the same enzymes which are responsible for detoxication can also bioactivate chemicals to their carcinogenic metabolites (Guengerich, 1992b). In this respect, one of the most widely studied chemicals is benzo(a)pyrene (Conney, 1982; Dipple, 1983) which undergoes oxidative metabolism to several epoxides. The 7,8-epoxide is hydrolysed by microsomal epoxide hydrolase to the dihydrodiol. However, this compound undergoes a further oxidation step to form the ultimate carcinogen, the diol-epoxide, which is far more reactive than any of the epoxides formed initially, and furthermore, is no longer a substrate for the epoxide hydrolase.

With drugs, carcinogenesis has fortunately not been a major problem to date. This is probably due to the extensive preclinical mutagenicity and carcinogenicity testing which a new compound has to undergo thereby identifying possible carcinogens at an early stage in their development. Perhaps the most widely studied aspect of drug-induced cancer in humans, and certainly the most widely publicised, has been the relative risk associated with the use of the combined oral contraceptive. The major studies looking at the association between the oral contraceptive pill and breast cancer have produced contradictory data (Romieu *et al.*, 1990), and in those studies where a positive association has been demonstrated, the relative risk has been modest (Anon, 1990; Romieu *et al.*, 1990). Thus, no revision of prescribing criteria has been recommended (CSM, 1988) and it is generally agreed that further larger and more carefully designed studies are required.

1.3.3.3 Drug teratogenicity

Drugs are thought to cause about 1-5% of all congenital abnormalities (Ruddon, 1990). It is only since the thalidomide disaster that all drugs have to be screened for their teratogenic potential. This usually involves administration of the drug during the period of embryogenesis in two species (usually rat or mouse and the rabbit), and subsequent examination of the foetus for developmental defects (Sullivan and McElhatton, 1986). Drugs are also administered during the third trimester to assess effects on foetal growth (Sullivan and McElhatton, 1986). Although such extensive teratogenicity testing may identify some teratogens, it is well known that the absence of such defects in animal species does not guarantee that the drug will be non-teratogenic in man (Sullivan and McElhatton, 1986), and thus, constant pharmacovigilance is necessary to identify any teratogenic effects as soon as possible after the introduction of the drug.

The effect of a drug on the foetus depends on the stage of pregnancy at which it is administered. Thus, administration during the period of organogenesis, which in man is usually between the third week to the third month of pregnancy, will result in major organ malformations (Sullivan and McElhatton, 1986; Ruddon, 1990), while drug exposure in late pregnancy can result in foetal growth retardation or microcephaly and mental retardation (Sullivan and McElhatton, 1986; Ruddon, 1990).

Many compounds including thalidomide (Newman, 1985), alcohol (Jones, 1988), phenytoin (Hanson and Smith, 1975; Hanson *et al.*, 1976), and androgens (Ruddon, 1990) are known to be teratogenic in man. Only a minority of foetuses exposed in utero to a known teratogen will develop malformations (Buehler *et al.*,

1990) indicating that host factors are an important determinant of individual susceptibility. For example, heavy consumption of alcohol by the mother leads to the foetal alcohol syndrome in 30-50% of the offspring (Jones, 1988), while with thalidomide the risk of foetal malformations has been estimated to be between 10% and 50% (Newman, 1985).

For some drugs such as androgens which cause masculinisation of the female foetus, teratogenesis is a direct extension of their known pharmacological actions (Ruddon, 1990). However, for the majority of drugs, the mechanism of teratogenesis is unknown. It has been proposed that drug bioactivation to chemically reactive intermediates may be important in the pathogenesis of drug teratogenicity in such cases (Juchau, 1989), the foetal malformations occur because of covalent binding of the chemically reactive metabolite to foetal nuclear material (Juchau, 1989).

Bioactivation of a drug to a chemically reactive metabolite may occur either in the mother or in the foetus. The former mechanism presupposes that the reactive metabolite would be stable enough to pass from maternal liver into the blood stream and across the placenta before coming into contact with the embryonic cells. However, this is unlikely for the majority of toxic metabolites as these are, by definition, unstable and thus have very short half lives. Embryonic bioactivation of a drug is an alternative possibility (Juchau, 1989) which has been strengthened by the increasing realisation that the foetus contains drug metabolising enzymes at an early stage in its development (Krauer and Dayer, 1991), the function of which, as in post-natal life, is to detoxify foreign compounds, but in certain circumstances, could also result in the formation of toxic, chemically reactive intermediates.

Predisposition to drug teratogenicity may depend on the balance between

bioactivation and detoxication, with either enhanced bioactivation and/or reduced detoxication (Lindhout, 1992), leading to persistence of the chemically reactive metabolite, and hence foetal malformations. In this respect, one of the most comprehensively studied drugs is phenytoin, a widely used anticonvulsant. It has been estimated that 1 in 500 pregnancies are exposed to phenytoin (Hanson, 1986). Of these 5-10% will develop severe malformations such as microcephaly and congenital heart disease, while up to one-third will develop minor malformations such digital hypoplasia (Hanson and Smith, 1975; Hanson, 1986; Kelly, 1987). Animal experiments using a mouse model have shown that phenytoin is metabolised to a reactive intermediate, with strains having the lowest epoxide hydrolase activity having the highest incidence of malformations (Finnell *et al.*, 1992), indicating that the reactive intermediate is an arene oxide. In man Strickler *et al* (1985) have suggested that predisposition to the foetal hydantoin syndrome is due to a genetically determined deficiency of epoxide hydrolase. More recently, Buehler *et al* (1990) have shown that children born with this syndrome may have a quantitative deficiency of microsomal epoxide hydrolase. The rate of foetal malformations increases with concomitant prescription of another enzyme inducing anticonvulsant such as phenobarbitone presumably because of enhancement in the bioactivation of phenytoin (Kaneko *et al.*, 1988). Conversely, concurrent administration of stiripentol, a cytochrome P450 inhibitor, with phenytoin reduces the malformation rate (Finnell *et al.*, 1992). Inhibition of detoxication enzymes increases malformation rate; this is clearly seen when valproate, an inhibitor of epoxide hydrolase (Kerr *et al.*, 1989), is co-prescribed with phenytoin (Kaneko *et al.*, 1988).

1.3.3.4 Drug hypersensitivity

Immunologically mediated or hypersensitivity reactions are thought to account for between 3-25% of all adverse drug reactions (Pohl *et al.*, 1988). Clinically, such reactions occur about a week after first exposure, indicating that a period of sensitisation is required (Pohl *et al.*, 1988; Pohl, 1990). On reexposure, however, the reaction occurs much sooner than on first exposure, suggesting the presence of specifically sensitised cells (Pohl *et al.*, 1988). The manifestations can be quite diverse affecting any organ system either in isolation or as part of a multi-organ syndrome (Patterson and Anderson, 1982; Matthews, 1984; Ownby, 1987). In addition, such reactions are often accompanied by so-called hypersensitivity manifestations such as skin rash, fever, arthralgia and eosinophilia (Pohl *et al.*, 1988; Shear *et al.*, 1988). The clinical picture is also dependent on the patient to some extent since the same drug can produce entirely different toxic manifestations in different patients.

It is well established that low molecular weight compounds (<1000Da), i.e drugs, cannot function as immunogens *per se*, but can initiate immune reactions only after covalent interaction with a macromolecular carrier, such as a protein, thereby acting as an hapten (Ownby, 1987; Park *et al.*, 1987). The covalent linkage is required for antigen processing by either macrophages or B-lymphocytes, while antigen presentation and the resulting immune reaction involves a complex interaction between the major histocompatibility antigens and T- and B-lymphocytes (Pohl *et al.*, 1988; Coleman, 1990).

A drug may form drug-carrier conjugates by three different general mechanisms (Pohl, 1990). First, drugs which are intrinsically chemically reactive can react directly with proteins (Park *et al.*, 1987; Park and Kitteringham, 1990b), for

example, penicillin (Sogn, 1984), captopril (Coleman *et al.*, 1986) and penicillamine (Joyce *et al.*, 1991). Secondly, and perhaps most commonly (Pohl *et al.*, 1988), drugs which are not intrinsically reactive are activated through biotransformation into chemically reactive metabolites which then react with proteins (Park *et al.*, 1987; Park and Kitteringham, 1990b). The most widely studied example of a drug causing hypersensitivity through bioactivation is with halothane (Pohl *et al.*, 1989). Finally, photoactivation in the skin is another mechanism by which drugs might be activated into reactive species that can form drug-carrier conjugates (Pohl *et al.*, 1988).

The drug-carrier conjugate can act as an immunogen and elicit a specific humoral (antibody) response, a specific cellular (T lymphocyte) response, or both responses (Coleman, 1990; Pohl, 1990). The immune response can be directed against either the drug (haptenic epitopes), the carrier protein (autoantigenic determinants) or the neoantigen created by the combination of the drug and the protein (new antigenic determinants) (figure 1.6; Pohl *et al.*, 1988). Tissue damage following an immune response is usually mediated through four general immunological mechanisms of hypersensitivity (figure 1.7; Coombes and Gell, 1968).

Although an immunological aetiology has been suggested for many adverse drug reactions, the molecular events underlying such reactions are poorly understood, the involvement of the immune system being surmised from clinical observations (Park *et al.*, 1992). Direct evidence of immune involvement depends on the demonstration of either a specific cellular and/or a humoral immune response. In this respect, halothane hepatitis can be regarded as a model for immune-mediated toxicity.

Halothane is known to cause two forms of hepatic injury, the first form

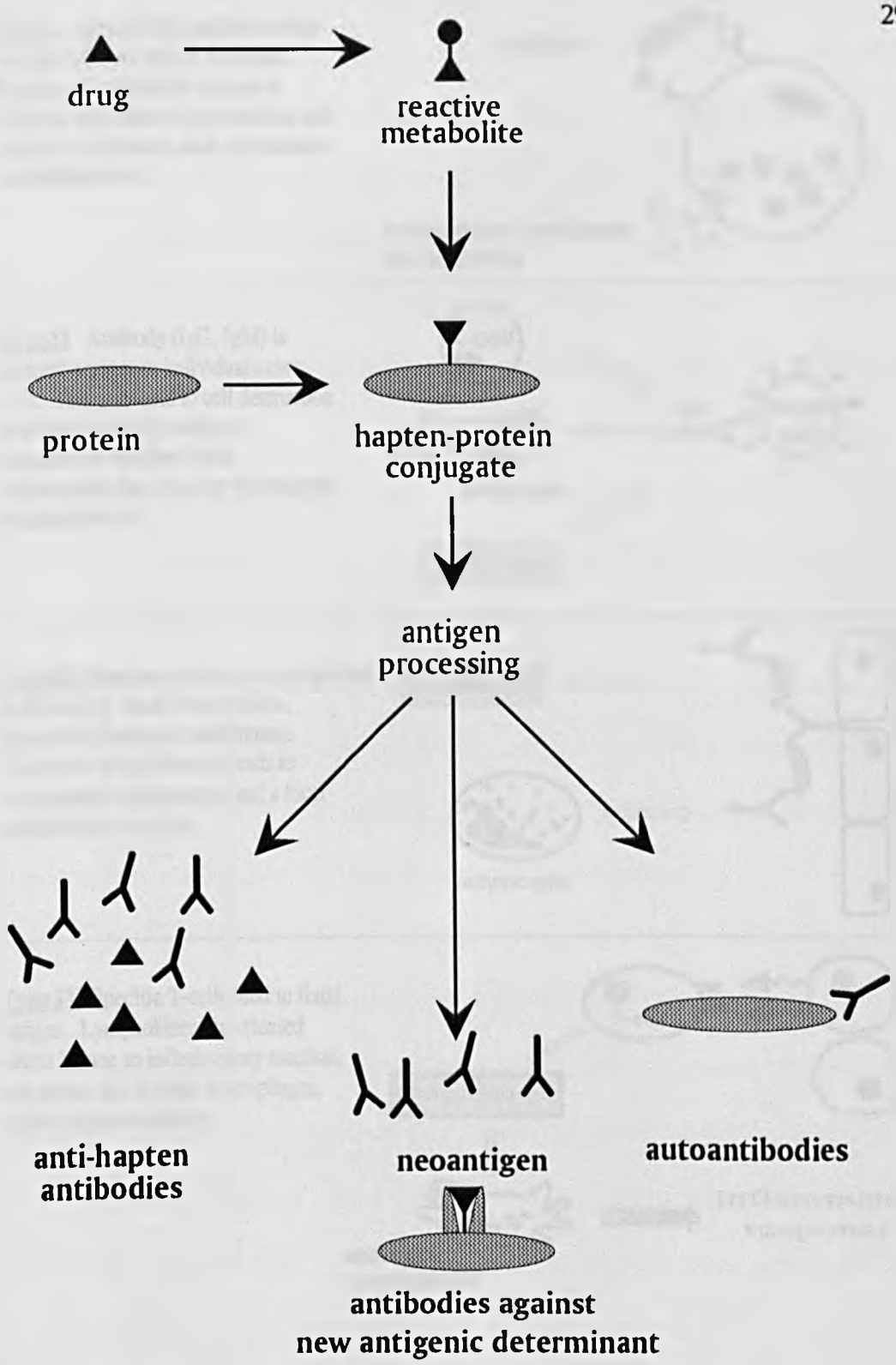
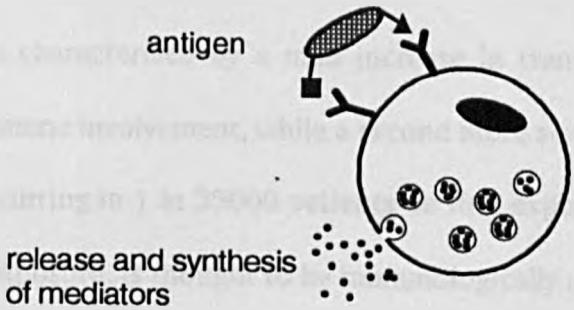
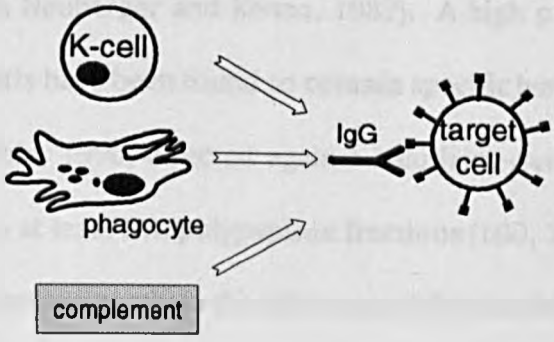


Figure 1.6. The specificity of the immune response following the formation of the hapten-protein conjugate.

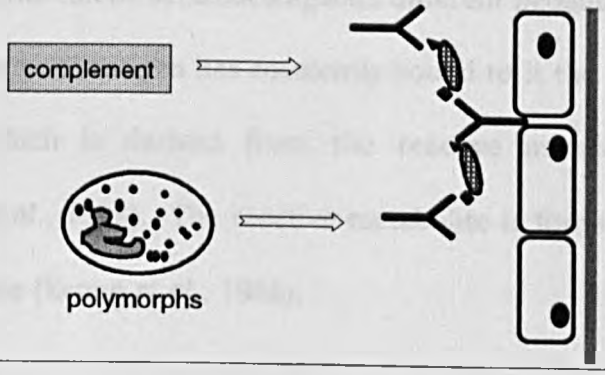
Type I Specific IgE antibodies bind to mast cells via their F receptors. Binding of multivalent antigen to adjacent IgE induces degranulation and release of mediators, such as histamine and leukotrienes.



Type II Antibody (IgG, IgM) is directed against an individual's own cells. This may lead to cell destruction by killer T-cells (K-cells) or complement-mediated lysis. Alternatively the cells may be removed by phagocytosis.



Type III Immune complexes are deposited in tissue (e.g. small blood vessels, glomerular basement membranes). Activation of complement leads to recruitment of polymorphs and a local inflammatory response.



Type IV Specific T-cells bind to fixed antigen. Lymphokines are released which induce an inflammatory reaction, and attract and activate macrophages, which release mediators.

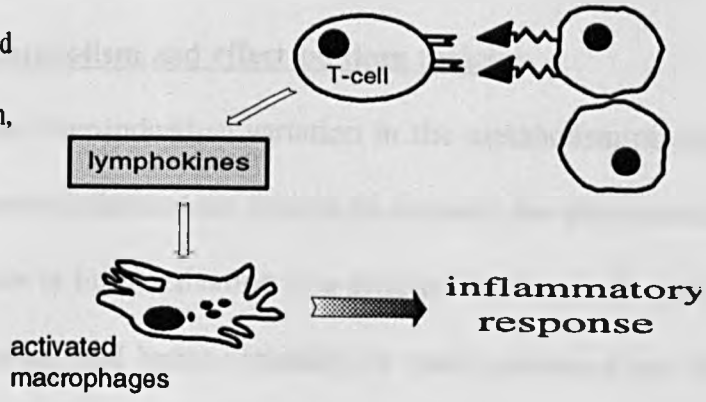


Figure 1.7. Mechanisms of drug hypersensitivity. Adapted from Coombes and Gell (1968).

occurring in 20% of patients is characterised by a mild increase in transaminase levels, but has no features of immune involvement, while a second more severe form (termed halothane hepatitis) occurring in 1 in 35000 patients on first exposure and 1 in 3700 patients on multiple exposure, is thought to be immunologically mediated (National Halothane Study, 1966; Neuberger and Kenna, 1987). A high proportion of patients with halothane hepatitis have been found to contain specific lymphocytes and antibodies in their blood (Pohl, 1990) directed against halothane-derived liver neoantigens which correspond to at least five polypeptide fractions (100, 76, 59, 57 and 54 kDa) that are expressed predominantly in the microsomal fraction of the liver (Pohl *et al.*, 1989). Although patients can be sensitised against different neoantigens (Neuberger and Kenna, 1988), each neoantigen has covalently bound to it the same trifluoroacetyl (TFA) hapten, which is derived from the reactive metabolite, trifluoroacetyl chloride (Kenna *et al.*, 1988). The reactive metabolite is formed by oxidative metabolism of halothane (Kenna *et al.*, 1988).

1.3.4. Variation in drug metabolism and effect on drug toxicity

There is considerable inter-individual variation in the metabolism of drugs; both genetic and environmental factors are known to account for this variation. Drug metabolism plays a role in both activation of a drug to its chemically reactive metabolite and its detoxication, and hence variability in these processes may alter individual susceptibility to adverse drug reactions by altering the rate and/or route of drug elimination and thus, the balance between activation and detoxication.

1.3.4.1 Genetically determined variation in drug metabolism

Genetic variation in drug metabolism is usually due to a mutant allele causing either a quantitative enzyme deficiency or a qualitative change in gene expression (i.e. a structural abnormality with altered substrate specificity and/or affinity; Ayesh and Smith, 1989). Genetic polymorphisms in various types of drug biotransformations have been described including those in drug oxidation, hydrolysis, acetylation and glutathione conjugation.

N-acetylation of xenobiotics was the first polymorphism to be described (Price Evans and White, 1964), the general population having a bimodal distribution of slow and fast acetylators (Price Evans, 1989). Recent studies have shown that there are two distinct loci for N-acetyltransferase, one of which codes for the polymorphic enzyme (NAT-2), while the other is a monomorphic form (NAT-1) which does not vary in the population (Hickman and Sim, 1991). About 60% of the British population are slow acetylators (Price Evans, 1989). A large number of drugs undergo N-acetylation (Price Evans, 1989), and in general, in slow acetylators, drugs are eliminated more slowly with higher plasma levels of the parent drug and/or phase I oxidative metabolites which can predispose to drug toxicity (Park, 1986). This can be clearly illustrated with respect to procainamide and hydralazine, two drugs known to cause systemic lupus erythematosus (SLE). Woosley and colleagues (1978) have shown that although both fast and slow acetylators develop SLE with procainamide, the rate of development of antinuclear antibodies and the lupus syndrome was significantly higher in slow acetylators than in fast acetylators. With hydralazine, lupus occurs almost exclusively in slow acetylators (Perry *et al.*, 1970; Batchelor *et al.*, 1980; Timbrell *et al.*, 1984; Russell *et al.*, 1987). With both drugs, acetylation may be

acting in competition with the oxidative pathway, and thus, slow acetylators will have a greater proportion of the parent drug undergoing oxidative biotransformation to chemically reactive intermediates (Hein and Weber, 1989) which could form protein- and nucleic acid-adducts resulting in SLE. With procainamide, the protective effect of N-acetylation has been further confirmed by antiarrhythmic therapy with N-acetylprocainamide which does not induce SLE (Lahita *et al.*, 1979; Roden *et al.*, 1980).

With the debrisoquine oxidation polymorphism, two distinct phenotypes, termed extensive (EM) and poor metaboliser (PM) can be distinguished by their ability to hydroxylate debrisoquine (Jacqz *et al.*, 1986). The PM phenotype, present in about 8% of the British population (Price Evans *et al.*, 1980), is characterised by a deficiency in the cytochrome P450 enzyme CYP2D6 (Gonzalez and Meyer, 1991), which at the molecular level has been shown to be due to at least four types of gene mutation (Gonzalez and Meyer, 1991). CYP2D6 is known to metabolise at least 20 commonly used drugs (Jacqz *et al.*, 1986). A deficiency of this enzyme has been documented to lead to three types of drug toxicity: first, poor metabolism of a drug will diminish its first pass metabolism, increase bioavailability and therefore, result in an exaggerated pharmacological response, for example, hypotension with debrisoquine (Park, 1986; Eichelbaum and Gross, 1990). Secondly, diminished metabolism can prolong the half-life of a drug resulting in its accumulation and consequent toxicity, for example, neuropathy (Shah *et al.*, 1982) and hepatotoxicity (Morgan *et al.*, 1984) with perhexiline was found to be much more common in PM. Thirdly, the deficiency of the usual metabolic pathway can alter the route of metabolism of a drug, changing it from a detoxication pathway to a bioactivation

pathway causing the formation of toxic metabolites, as for example seen with phenacetin-induced methaemoglobinaemia (Eichelbaum and Gross, 1990). Conversely, where activation of a drug depends on CYP2D6, PM will be protected since they cannot form the toxic metabolite, although this is largely theoretical. Some of the adverse reactions associated with the PM phenotype are listed in table 1.6.

Table 1.6.

The association between the debrisoquine poor metaboliser phenotype and drug toxicity.

Drug	Adverse Reaction
Debrisoquine	Hypotension
Metoprolol	Excessive β-blockade
Nortryptiline	Confusion
Perhexiline	Neuropathy, hepatotoxicity
Phenacetin	Methaemoglobinaemia
Phenformin	Lactic acidosis

Adapted from Park, (1986).

Inter-individual variation in the cellular detoxication of chemically reactive metabolites produced from drugs may also be an important factor in determining predisposition to idiosyncratic toxicity (Park and Kitteringham, 1990a). In this respect, peripheral blood lymphocytes provide a useful, easily accessible source of human cells to assess variability in detoxication of reactive species (Spielberg, 1984). Lymphocytes from patients with and without idiosyncratic adverse drug reactions can

be incubated with the suspect drug and a drug metabolising system (i.e. microsomes and NADPH) to assess inter-individual variability in *in vitro* chemical sensitivity (Spielberg, 1980, 1984). Indeed, using this *in vitro* system, it has been shown that lymphocytes from patients hypersensitive to the aromatic anticonvulsants (Spielberg *et al.*, 1981; Shear *et al.*, 1988), amineptine (Larrey *et al.*, 1989), sorbinil (Spielberg *et al.*, 1991) and sulphonamides (Reider *et al.*, 1989) all exhibit higher *in vitro* chemical sensitivity when co-incubated with the respective drug, than appropriate controls, suggestive of a defect in cellular detoxification.

Lymphocytes also serve as a useful marker for one form of glutathione transferase, GST μ, which is expressed polymorphically in both liver and lymphocytes (Seidegard *et al.*, 1988); 50% of individuals do not express this enzyme and appear to be at greater risk of cigarette smoke-induced lung cancer than the corresponding positive phenotype (Seidegard *et al.*, 1990). This polymorphism, in isolation, has so far not been shown to be important in predisposing to idiosyncratic drug toxicity (Park *et al.*, 1992), although it is possible that it may act as a contributory risk factor producing a multifactorial predisposition to drug toxicity.

1.3.4.2 Environmental factors affecting drug metabolism

The relative importance of environmental factors in determining variation in drug metabolism is difficult to define precisely, and often cannot be separated from genetic factors, since the magnitude of any change produced by the environment is to some extent determined by heredity (Mucklow, 1988). Any environmentally produced change in drug metabolism is usually brought about by induction or inhibition of the cytochrome P450 enzymes, which results in an increase or decrease,

respectively, in the basal level of enzyme activity (Mucklow, 1988). Thus, age, pregnancy, diet, alcohol, tobacco, foreign chemicals, and disease (either hepatic or extra-hepatic) have been shown to alter the rate or capacity of drug metabolism. Additionally, concurrently prescribed drugs may induce the P450 enzymes (see table 1.4) increasing the formation of chemically reactive metabolites thereby overwhelming detoxication and resulting in drug toxicity (Park and Kitteringham, 1990a).

1.4. Immunologic responsiveness and susceptibility to drug toxicity

There is inter-individual variability in the ability to mount an immune response to a given antigen, such that in some cases the generation of a drug-protein conjugate does not necessarily result in an immune-mediated idiosyncratic reaction. For example, administration of halothane will result in the generation of the antigen in all those exposed (Kenna *et al.*, 1988; Neuberger and Kenna, 1988). However, only susceptible individuals seem to be able to mount an immune response to the antigen (Kenna *et al.*, 1988). Similarly, with penicillin, there is little inter-individual variation in circulating penicilloyl antigens in patients given high doses of penicillin (Lafaye and Lapresie, 1988). However, several studies have shown that there is a genetic restriction of immune responsiveness to the penicilloyl antigen (Adkinson and Wheeler, 1983). Thus, in a prospective study it has been shown that 62% of patients on high dose penicillin therapy for at least ten days had no detectable serological response to the major penicillin antigenic determinant (Adkinson and Wheeler, 1983).

Furthermore, the induction of an antibody response does not necessarily

equate with tissue damage since many patients with anti-drug antibodies remain asymptomatic (Park *et al.*, 1992), and thus individual risk factors may be important in the translation of an immune response into tissue damage. Adkinson and Wheeler (1983) have tried to define the factors which may be important with respect to penicillin allergy. First, the ability to respond serologically, as discussed; secondly, the half-life of IgE antibodies which ranged from 10 days to >1000 days; thirdly, age since penicillin-induced anaphylactic reactions are apparently less frequent in children than adults; fourthly, the route of administration is important because allergic reactions are less frequent after oral than parenteral administration; and finally, an ill-defined factor which relates to the ability to release chemical mediators from mast cells, a process which is regulated by a myriad of physiological and pathological factors.

The ability to mount an immune response is to some extent under genetic control (Park *et al.*, 1992). Such inter-individual variability in immune responsiveness may be determined by the major histocompatibility complex (MHC) expressed on accessible cells. The MHC genes are, in fact, the most polymorphic genes known in higher vertebrates (Weatherall, 1991). Such a high level of polymorphism could theoretically result in individuals who are more efficient at recognising certain epitopes than others with the result that only these individuals are more likely to mount a vigorous immune response to drug related antigens. Thus, natural selection which maintained a large variety of MHC proteins in the population may, inadvertently, produce a subpopulation of individuals who are more likely to develop drug hypersensitivity (Park *et al.*, 1992).

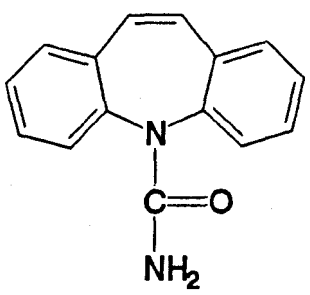
Investigation into the empirical relationship between some forms of drug

toxicity and HLA phenotype has identified some positive associations (Park *et al.*, 1992). However, such associations can only be used to estimate the relative risk of developing the adverse reaction and do not predict individual susceptibility. For example, Batchelor *et al.* (1980) investigated 26 patients with SLE induced by hydralazine, 25 of whom were slow acetylators. Although the frequency of HLA-DR4 (73%) was significantly higher in the hydralazine SLE group than in a group of patients treated with hydralazine without developing SLE or in a group of patients with idiopathic SLE, 25% of the control group were also positive.

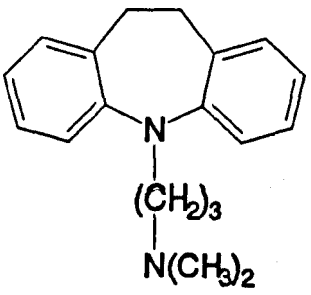
1.5. The drugs studied

1.5.1. Carbamazepine

Carbamazepine (5-carbamyl-5H-dibenz[*b,f*]azepine; figure 1.8) was synthesised by Schindler in 1953, and has been most widely used as an anticonvulsant, being effective for most seizure types, except absence seizures (Eadie and Tyrer, 1989). In addition, carbamazepine is the treatment of choice for trigeminal neuralgia (Blom, 1962) and has also been tried with some success in other pain syndromes including glossopharyngeal neuralgia (Ekbom and Westerberg, 1966), painful crises in Fabry's



Carbamazepine



Imipramine

Figure 1.8. A comparison of the structure of imipramine and its structural congener, carbamazepine

disease (Shibasaki *et al.*, 1973), tabetic lightning pains (Ekbom, 1972) and painful diabetic peripheral neuropathy (Chakrabarti and Samantaray, 1976). It is chemically similar to imipramine (figure 1.8), and thus, has been used to treat depression, particularly bipolar depression (Post *et al.*, 1983). More recently, carbamazepine has been successfully used in the treatment of high-frequency diaphragmatic flutter (Vantrappen *et al.*, 1992).

1.5.1.1 Pharmacodynamics

As stated above, carbamazepine has been most widely used as an anticonvulsant. It is effective in suppressing seizure activity in various animal models, including those with spontaneous seizures (Meldrum *et al.*, 1975; Eadie and Tyrer, 1989) and in animals with evoked (either electrically or chemically) seizures (Eadie and Tyrer, 1989). However, the mechanisms responsible for these effects of carbamazepine are not clearly understood (Rall and Schleifer, 1985), although it is thought to act on neuronal sodium channels. However, various other neuronal and biochemical effects of carbamazepine have been elicited (table 1.7), which may, either individually or collectively, be important in its anticonvulsant actions.

1.5.1.2 Disposition and metabolism

The oral absorption of carbamazepine is slow, the absorption half-time being slower when the drug is given as tablets (1.72h) rather than as a solution (0.29h) (Levy *et al.*, 1975; Graves *et al.*, 1985), and when larger doses are administered (Gerardin *et al.*, 1976). Peak plasma concentrations are achieved in 4-8h after oral

ingestion but may be delayed by as much as 24h (Eadie and Tyrer, 1989). Carbamazepine has an oral bioavailability of 58-85%, there being no difference when the drug is given in tablet and in syrup form (Eadie and Tyrer, 1989).

TABLE 1.7.

Known actions of carbamazepine which may contribute to its antiepileptic effects.

Action	Reference
Facilitation of inhibitory inputs to spinal trigeminal neurones in the cat	*Fromm and Killian, 1967*
Increased rate of firing of noradrenergic neurones in the locus ceruleus of the rat	*Olpe and Jones, 1983*
Binding to and upregulation of adenosine receptors	*Marangos et al., 1983, 1985*
Binding to benzodiazepine receptors	*Marangos et al., 1983*
Dose-dependent fall in sodium and potassium conductances in squid giant exon	*Schauf et al., 1974*
Inhibition of calcium-calmodulin regulated protein phosphorylation	*De Lorenzo, 1984*
Increased acetylcholine concentrations in the striatum	*Consolo et al., 1976*
Increased dopamine release	*Barros et al., 1986*
Inhibition of depolarisation-induced rises in both cAMP and cGMP levels	*Ferrendelli and Kinscherf, 1979*
Fall in serum folate levels	*Reizenstein and Lund, 1973*

The apparent volume of distribution of carbamazepine is about 1l/kg (Bertilsson and Tomson, 1986). The drug distributes into all tissues and organs without any preferential affinity for particular organs. In plasma, carbamazepine

binds to albumin and α_1-acid glycoprotein, the plasma protein binding varying from 60-81% in different studies (Eadie and Tyrer, 1989). Pregnant women and neonates have lower binding capacities, 30-31% and 47-52%, respectively.

The *in vivo* metabolism of carbamazepine is extensive and complex, with over 30 metabolites having been identified (Lertratanangkoon and Horning, 1982). Only 2% of a carbamazepine dose is excreted unchanged in the urine (Eadie and Tyrer, 1989). Figure 1.9 shows the major pathways of biotransformation of carbamazepine in humans. The most important pathway is the formation of the stable carbamazepine-10,11-epoxide (Eichelbaum *et al.*, 1985), which itself is an anticonvulsant (Morselli *et al.*, 1975). The carbamazepine-10,11-epoxide is converted by epoxide hydrolase to the trans-carbamazepine diol which is then excreted in the urine as its glucuronic acid conjugate (Eichelbaum *et al.*, 1985). Single dose studies in volunteers have shown that 21.5 ± 5.6% of the initial dose was excreted in the urine as the trans-carbamazepine diol. Direct N-glucuronidation of the carbamoyl side-chain of carbamazepine and hydroxylation in the aromatic rings are also major pathways (figure 1.9; Beyer and Spiteller, 1992).

Carbamazepine is known to be an enzyme inducer capable of inducing its own metabolism (autoinduction; Bertilsson *et al.*, 1980; Eichelbaum *et al.*, 1985) and of concomitantly administered drugs such as warfarin and oral contraceptives (heteroinduction; Hansen *et al.*, 1971). In addition, the concomitant administration of drugs such as phenobarbitone and/or phenytoin can also induce the metabolism of carbamazepine (Eichelbaum *et al.*, 1979). During both auto- and hetero-induction of carbamazepine, there is an increase in the percentage of the carbamazepine dose excreted as the diol (figure 1.10) suggesting that it is the epoxide-diol pathway which

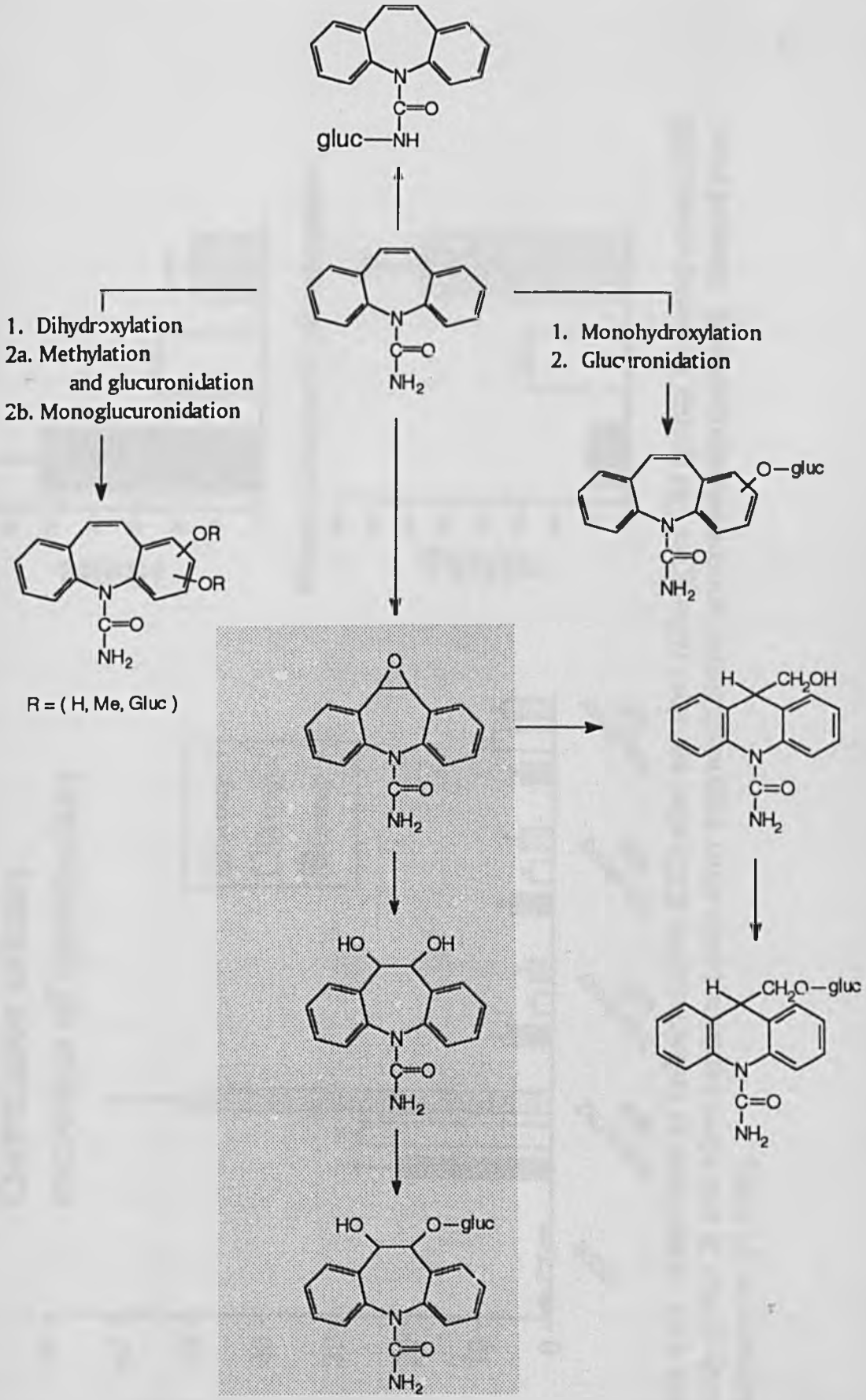


Figure 1.9. Major pathways of metabolism of carbamazepine. The shaded area represents the epoxide-diol pathway which is the major route of metabolism and is inducible.

(adapted from Beyer and Spiteller, 1992)

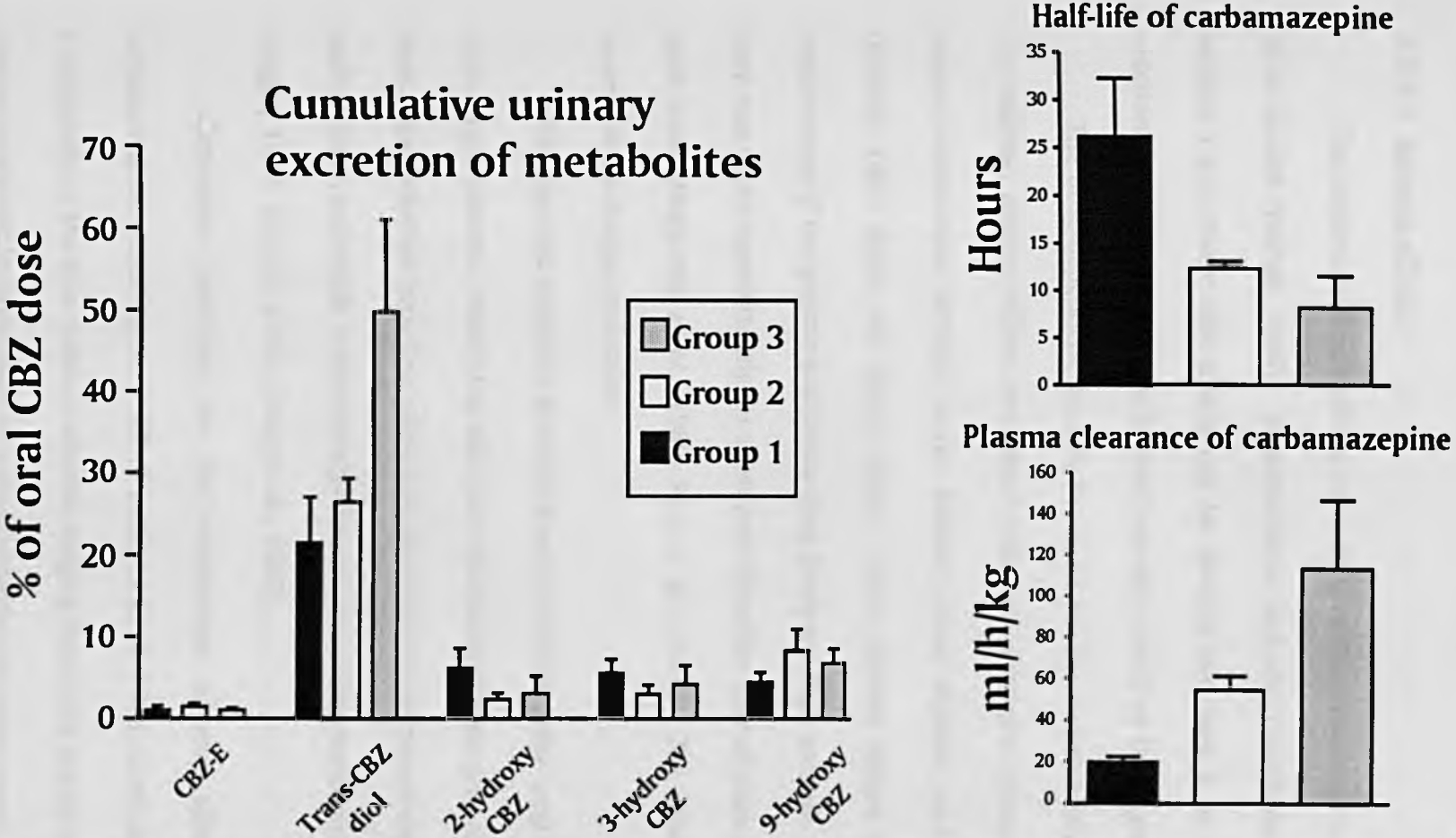


Figure 1.10. Disposition of carbamazepine (CBZ) after one dose (200mg) of CBZ (group 1), during chronic CBZ therapy (group 2) and when combined with other enzyme-inducing anticonvulsants (group 3). Adapted from Eichelbaum *et al* (1985).

is induced (Tybring *et al.*, 1981; Eichelbaum *et al.*, 1985; Kudriakova *et al.*, 1992).

1.5.1.3 Adverse effects

The incidence of side effects associated with carbamazepine therapy ranges from 33-50% (Pellock, 1987). In accordance with the classification described in section 1.2.2., these side effects can be divided into type A or dose-dependent reactions, type B or idiosyncratic reactions and type D or teratogenic reactions.

The dose-dependent (type A) reactions are the commonest, but fortunately the mildest, adverse effects associated with carbamazepine therapy; they include nausea, drowsiness, vertigo, ataxia, blurred vision, diplopia and slurred speech (Pellock, 1987; Eadie and Tyrer, 1989). These adverse effects may be present continuously if the plasma and tissue drug levels are high enough, or alternatively, they may occur intermittently for short periods at the time of peak drug levels after each dose (Hoppener *et al.*, 1980; Riva *et al.*, 1984). The adverse effects are reversible on dosage reduction.

Idiosyncratic reactions associated with carbamazepine tend mainly to affect three organ systems, namely the skin, liver and haematologic system (Pellock, 1987). Such organ-selective toxicity is often accompanied by hypersensitivity manifestations such as fever, arthralgia, lymphadenopathy and circulating eosinophilia (Dreifuss and Langer, 1987; Pellock, 1987; Shear *et al.*, 1988).

Cutaneous reactions are the commonest adverse effects caused by carbamazepine occurring in 3-16% of patients (Crill, 1973; Chadwick *et al.*, 1984). The severity of the skin rashes is variable ranging from mild erythematous eruptions (not accompanied by hypersensitivity manifestations) to more severe, life-threatening

reactions such as toxic epidermal necrolysis (Sakellariou *et al.*, 1991). Severe cutaneous reactions such as Stevens-Johnson syndrome, toxic epidermal necrolysis, exfoliative dermatitis and erythema multiforme are less common than the mild reactions, the number reported to the Food and Drug Administration (FDA) in the USA and Ciba-Geigy Pharmaceuticals representing 7.8%, 0.5%, 2.5% and 3.0%, respectively, of the total number of cutaneous reactions reported (Pellock, 1987).

Mild, usually transient elevation of liver enzymes, which may be due to hepatic enzyme induction, is common occurring in 5-10% of patients on carbamazepine (Pellock, 1987). More severe hepatic dysfunction, i.e. at least a 2- to 3-fold increase in hepatic enzymes, is much less common, and is often accompanied by hypersensitivity manifestations which may be mistaken for biliary tract infection (Dreifuss and Langer, 1987). Both children and adults are susceptible to carbamazepine hepatotoxicity, the severity being variable, resulting in death in 25% of cases (Horowitz *et al.*, 1988). Histologically, the majority of cases show hepatocellular necrosis (Dreifuss and Langer, 1987), although granulomatous changes (Levy *et al.*, 1981) and cholangitis (Larrey *et al.*, 1987) have also been reported.

The most common haematologic side effect associated with carbamazepine therapy is thrombocytopenia (Sobotka *et al.*, 1990). Minor decreases in the platelet count occur in approximately 2% of patients (Sobotka *et al.*, 1990). Kornberg and Kobrin (1982) have identified carbamazepine-dependent antiplatelet antibodies in a patient with carbamazepine-induced thrombocytopenia suggestive of an immune-mediated mechanism.

Mild leucopenia, i.e. a WBC count of $<4000/mm^3$, has been reported in 12% of children and 7% of adults (Sobotka *et al.*, 1990) treated with carbamazepine. This

is usually seen within the first 3 months of treatment but does not require drug withdrawal, the WBC count returning to normal despite continued carbamazepine therapy (Sobotka *et al.*, 1990). More severe reductions in WBC count, and in particular a reduction of granulocytes (agranulocytosis) is less common with an incidence of 1.4 per million patients (Pellock, 1987). In some cases, the reduction in granulocytes is accompanied by anaemia and thrombocytopenia (pancytopenia), which in the most severe cases leads to aplastic anaemia (incidence 0.5 : 100000 per year), a rare condition with a high mortality (Hart and Easton, 1982). Pellock (1987) has indicated that 2.2 deaths per million exposures to carbamazepine were associated with aplastic anaemia and agranulocytosis.

Carbamazepine idiosyncratic toxicity is thought to be immune mediated (Dreifuss and Langer, 1987; Shear *et al.*, 1988), but this is largely based on clinical manifestations. Direct evidence for an immune mechanism is limited, with several studies showing the presence of reactive T-lymphocytes using the lymphocyte transformation test (Zakrzewska and Ivanyi, 1988), although this test can be unreliable (Houwerzijl *et al.*, 1977). It has been postulated by Spielberg and co-workers that the unique susceptibility of certain individuals may be due to the inability to detoxify a toxic metabolite produced from carbamazepine secondary to a deficiency of cellular detoxification, possibly epoxide hydrolase (Shear *et al.*, 1988). In any instance, it can be clearly seen that these idiosyncratic adverse effects bear no relationship to either the primary or secondary pharmacology of the drug (section 1.5.1.1 and table 1.7).

The third type of adverse reactions associated with carbamazepine are the type D or teratogenic reactions. Early studies with carbamazepine suggested that

it did not cause teratogenicity and it was therefore the drug of first choice in epileptic women of child bearing age (Anon, 1991). However, Lindhout and co-workers (1984) first showed that carbamazepine was teratogenic particularly when it was given together with sodium valproate, the malformation rate reaching as high as 58%. In this study, it was found that the carbamazepine-10,11-epoxide levels were elevated, and thus it was suggested that concomitant administration of valproate, an inhibitor of epoxide hydrolase (Kerr *et al.*, 1989), increased the foetal burden of epoxides overwhelming its detoxication capacity resulting in malformations. However, it is known that carbamazepine-10,11-epoxide is a stable compound which is not cytotoxic (Kaneko *et al.*, 1988; Shear *et al.*, 1988), and thus it is possible a chemically reactive epoxide may be the toxic species (Kaneko *et al.*, 1988; Lindhout, 1992). More recently, Jones *et al* (1989), have studied 35 children exposed to carbamazepine prenatally and found an impressive frequency of craniofacial defects (11%), fingernail hypoplasia (26%) and developmental delay (20%). Rosa (1991) has also shown that in utero exposure to carbamazepine is associated with a 1% risk of spina bifida, which is about 14 times the expected rate (Anon, 1991).

1.5.2. Sulphasalazine

Sulphasalazine was introduced for the treatment of rheumatoid arthritis by Svartz (1942). However, the early studies which were often uncontrolled, failed to show any benefit of sulphasalazine in rheumatoid arthritis (Pullar, 1989), and thus, sulphasalazine fell into disuse for the treatment of rheumatoid arthritis for 30 years. In the mid-1970s, sulphasalazine was again investigated for the treatment of

rheumatoid arthritis. These studies showed sulphasalazine to be effective producing laboratory and clinical improvement by 6 weeks (McConkey *et al.*, 1978, 1980). Its effectiveness in rheumatoid arthritis has since been confirmed by other studies (Pullar, 1989), and indeed, comparative studies have shown sulphasalazine to be as effective as gold (Pullar *et al.*, 1983) and D-penicillamine (Neumann *et al.*, 1983).

The other main indication of sulphasalazine is in the treatment of inflammatory bowel disease, where it is used for both symptomatic improvement and maintaining remission (Riley and Turnberg, 1990). It is highly effective, inducing and maintaining remission in over 60% of patients (Dronfield and Langman, 1978; Klotz *et al.*, 1980). Recently, its use in inflammatory bowel disease has been declining because of the introduction of 5-aminosalicylic acid derivatives (Riley and Turnberg, 1990).

Sulphasalazine consists of two moieties, sulphapyridine and 5-aminosalicylic acid, linked by an azo bond (Das and Dubin, 1976; Klotz, 1985). In rheumatoid arthritis, sulphapyridine is thought to be the active moiety (Pullar *et al.*, 1985b; Neumann *et al.*, 1986), while in inflammatory bowel disease, it is the 5-aminosalicylic acid which is active (Azad Khan *et al.*, 1977; Dew *et al.*, 1982).

The mode of action of sulphasalazine in both conditions has not been clearly elucidated. Suggested explanations for the antirheumatic activity of sulphasalazine or sulphapyridine, or both, include an antibacterial effect in the colon (Neumann *et al.*, 1987), immunosuppressive activity (Sheldon *et al.*, 1987), interference with prostaglandin metabolism (Hoult and Moore, 1980) or an effect on neutrophil function by inhibition of superoxide generation (Kanerud *et al.*, 1990). 5-Aminosalicylic acid is known to inhibit both prostaglandin and leukotriene formation

(Lauritsen *et al.*, 1986), and is also known to act as a radical scavenger (Williams and Hallett, 1989; Ahnfelt-Ronne *et al.*, 1990); both mechanisms have been proposed for its antiinflammatory activity in inflammatory bowel disease.

1.5.2.1 Disposition and metabolism

Sulphasalazine (figure 1.11) is absorbed in the small intestine, being detectable in the serum after 1.5h and reaching peak concentrations after 3-5h (Pullar, 1989). Its elimination half-life varies from 2.0-16.8h and its volume of distribution is small (<10l) (Klotz, 1985). About 70% of an ingested dose of sulphasalazine reaches the caecum unchanged with a further 14% reaching the caecum as sulphapyridine (Das *et al.*, 1974). Usually less than 10% of an ingested dose of sulphasalazine is excreted unchanged in the bile or urine (Das *et al.*, 1974).

In the colon, sulphasalazine undergoes azo-reduction by bacteria to yield sulphapyridine and 5-aminosalicylic acid (Peppercorn and Goldman, 1972). Sulphapyridine is almost completely absorbed (60-80%; Schroder and Campbell, 1972), appearing in the blood 3-6h after a dose of sulphasalazine (Azad Khan *et al.*, 1982) and reaching maximum serum concentrations in 10-30h (Azad Khan *et al.*, 1982). The elimination of sulphapyridine is accomplished via the hepatic route, the major metabolite being N-acetyl sulphapyridine (Figure 1.11; Pullar, 1989). This pathway displays genetic polymorphism, the slow and fast acetylators comprising 60% and 40% of the population respectively (Price Evans and White, 1964; Das and Dubin, 1976). Thus, the elimination half-life of sulphapyridine in slow and fast acetylators is 15-21h and 6-11h, respectively (Fischer and Klotz, 1980; Taggart *et al.*, 1987). Sulphapyridine also undergoes ring hydroxylation (figure 1.11), a pathway which

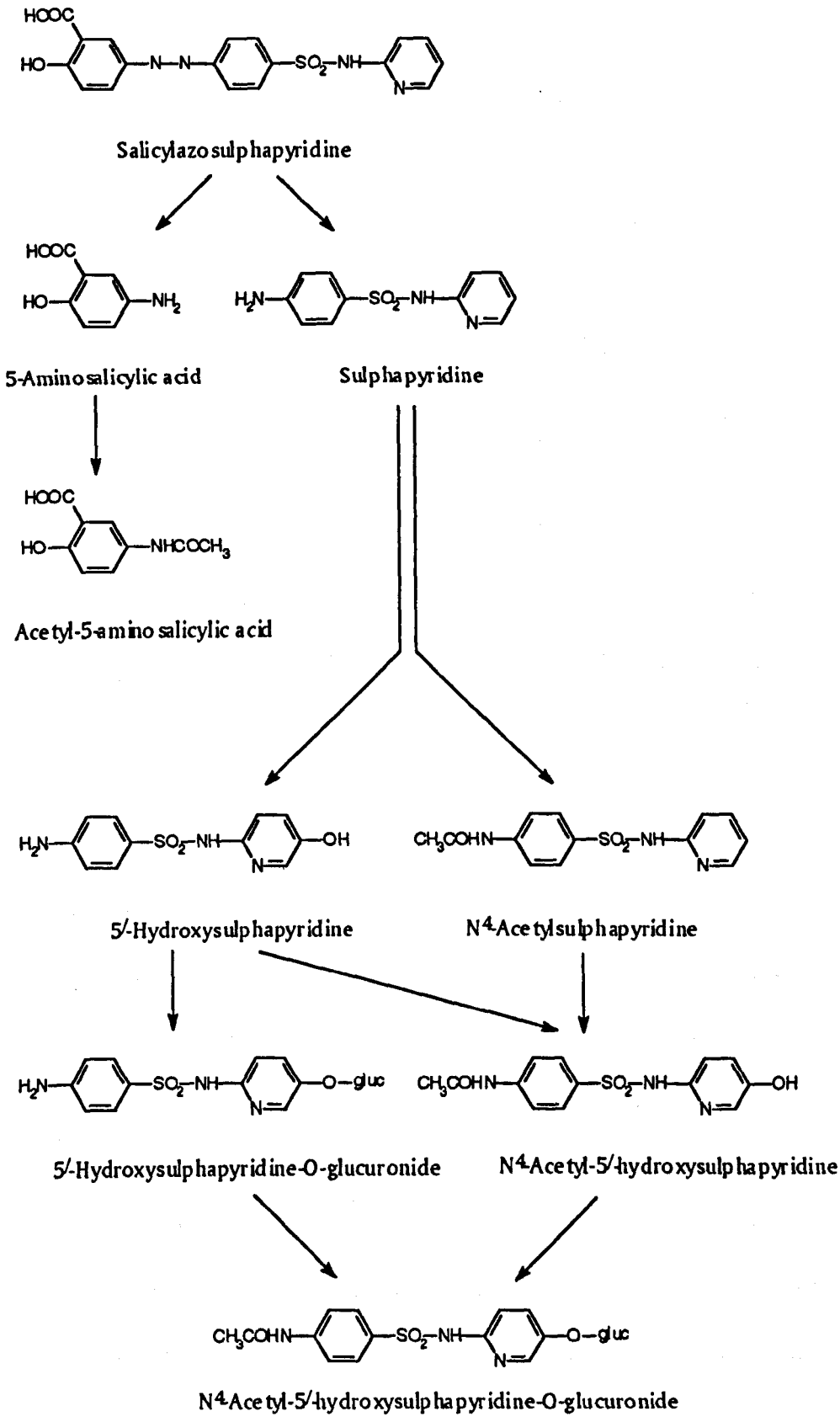


Figure 1.11. Metabolism of sulphasalazine in man.

(adapted from Das and Dubin, 1976)

shows unimodal distribution (Klotz, 1985); this is followed by conjugation with glucuronic acid and/or acetylation (Klotz, 1985).

In contrast to sulphapyridine, 5-aminosalicylic acid is minimally absorbed, the large majority of it remaining in the bowel lumen and being excreted in the faeces (Pullar, 1989). Only about 22% of an ingested dose of sulphasalazine is recoverable in the urine as 5-aminosalicylic acid or its metabolites and only very low concentrations are detectable in the plasma (Azad Khan *et al.*, 1982). 5-Aminosalicylic acid is acetylated to acetyl-5-aminosalicylic acid (figure 1.11) in the gut wall and liver (Klotz, 1985); unlike the acetylation of sulphapyridine, the acetylation of 5-aminosalicylic acid does not exhibit genetic polymorphism (Fischer and Klotz, 1980; Klotz, 1985) presumably because it is metabolised by the monomorphic (NAT1) form of N-acetyltransferase.

1.5.2.2 Adverse effects

The side effects of sulphasalazine can be divided into type A or type B reactions. Adverse reactions can occur in up to 50% of patients taking sulphasalazine, although the majority are minor (Riley and Turnberg, 1990), discontinuation being required in only about 10% of cases (Riley and Turnberg, 1990).

The commonest dose-dependent side effects associated with sulphasalazine are nausea and vomiting which are often associated with abdominal pain and dizziness (Das and Dubin, 1976; Pullar, 1989; Riley and Turnberg, 1990). Dose-related haematological abnormalities such as macrocytosis, reticulocytosis and folate deficiency have also been reported with sulphasalazine (Riley and Turnberg, 1990). The correlation of the above side effects with the acetylator status and plasma

sulphapyridine levels have implicated the sulphapyridine moiety as the major culprit of sulphasalazine toxicity (Das *et al.*, 1973). Sulphasalazine is also known to cause oligospermia and poor sperm motility which is more common in slow acetylators (Riley and Turnberg, 1990). Animal studies have shown the sulphapyridine moiety to be responsible for seminal abnormalities (O'Morain *et al.*, 1984).

The commonest idiosyncratic adverse effect associated with sulphasalazine is cutaneous toxicity, variable in severity from erythematous eruptions (Riley and Turnberg, 1990) to the more serious reactions such as erythema multiforme (Cameron *et al.*, 1976) and toxic epidermal necrolysis (Strom, 1969).

Transient leucopenia can occur in up to 10% of patients (Amos and Bax, 1988) on sulphasalazine, although, in the majority of cases, this tends to improve despite continuation of the drug. Neutropenia, which does require drug withdrawal, is less common occurring in approximately 1% of patients (Pullar, 1989). Drug-induced hepatitis occurs in 0.25% of patients and reverses on stopping the drug (Pullar, 1989). Transient dyspnoea occurs in 0.25% of patients and usually improves on drug withdrawal (Pullar, 1989). In some cases, however, infiltrative lung disease may occur (Thomas *et al.*, 1974).

There is no evidence of teratogenesis with sulphasalazine following either maternal or paternal use of the drug (Willoughby and Truelove, 1980; Mogadam *et al.*, 1981).

1.6. Aims of the work

Chemically reactive metabolites have been postulated to cause idiosyncratic adverse reactions with many drugs, although for most compounds, there is little direct evidence to support this. Predisposition to such reactions seems to be due to an imbalance between bioactivation of the drug to its chemically reactive metabolite and its detoxication, with individuals who have either enhanced activation and/or reduced detoxication developing the adverse reaction.

The aim of the studies was to determine at a functional, biochemical, chemical and clinical level the importance of this balance between bioactivation and detoxication in predisposing to idiosyncratic drug toxicity. This clearly necessitates identification and investigation of patients who have had idiosyncratic drug reactions. For this reason, carbamazepine, a widely used drug with well-documented toxicity was used as the model compound. Thus, the initial investigation of hypersensitive patients (using cells *ex vivo*) was to determine whether activation or detoxication of the drug was the most important predisposing factor. The mechanism of toxicity is often difficult to investigate since this has to be done at the time the patient presents, i.e. acutely, which is often not possible. Two patients identified during the course of these studies presented acutely and have been investigated to determine the immune mechanisms involved in carbamazepine hypersensitivity. The subsequent aims of the studies were to evaluate how this balance may be perturbed in the clinical situation. This was undertaken by using *in vitro* techniques which allow chemical perturbation of the system to determine first the factors affecting detoxication and the possible nature of any detoxication deficiency and secondly the effect of enhancing bioactivation of the drug by using model inducers of the

cytochrome P450 enzymes.

For the last part of these studies, a second drug, sulphasalazine, again a widely used compound with well documented toxicity which seems to have a different biochemical basis than carbamazepine, was also investigated again with a view to evaluating the importance of the balance between activation and detoxication. In contrast to carbamazepine, the initial studies were carried out *in vitro* to identify reactive metabolite responsible for toxicity and the factors affecting its formation. Subsequently, a patient study was undertaken with the aim of favourably altering the balance between bioactivation and detoxication by concurrently administering a cytochrome P450 inhibitor, cimetidine.

CHAPTER 2

CARBAMAZEPINE IDIOSYNCRATIC TOXICITY : ASSESSMENT OF CLINICAL AND *IN VITRO* CHEMICAL CROSS-REACTIVITY WITH PHENYTOIN AND OXCARBAZEPINE

2.1. Introduction

Carbamazepine is widely used in the treatment of epilepsy, neuralgic pain syndromes and more recently, affective disorders.

It has been estimated that between 33% and 50% of patients on carbamazepine develop side effects (Pellock, 1987). Most of the side effects are mild and dose-dependent (type A reactions). Cutaneous reactions to carbamazepine are amongst the commonest adverse effects, estimated to occur in 3-16% of patients (Crill, 1973; Chadwick *et al.*, 1984). It has been suggested that the milder eruptions, which are relatively common, may be related to the initial carbamazepine dosage and, thus the serum concentrations (Chadwick *et al.*, 1984). The more severe reactions, such as Stevens-Johnson syndrome, however, are independent of drug dosage and unpredictable from the known pharmacology of the drug (type B or idiosyncratic reactions; Shear *et al.*, 1988). Other idiosyncratic reactions reported with carbamazepine include aplastic anaemia (Gerson *et al.*, 1983), agranulocytosis (Pellock, 1987), hepatitis (Horowitz *et al.*, 1988), pneumonitis (Stephan *et al.*, 1978) and pseudolymphoma (Shuttleworth *et al.*, 1984) - these may occur either alone or in various combinations. In addition, they are often accompanied by hypersensitivity manifestations such as fever, arthralgia and circulating eosinophilia.

The pathogenesis of these idiosyncratic reactions is not fully understood. Carbamazepine is extensively metabolised (Lertratanangkoon and Horning, 1982), and thus it has been postulated that phase I metabolism of carbamazepine may lead to the formation of chemically reactive metabolites (Shear *et al.*, 1988; Riley *et al.*, 1989), which could cause toxicity either directly or indirectly by initiating an immune response (Park, 1986). The latter mechanism, i.e. immune-mediated, is thought to

be more likely in view of the symptomatology accompanying carbamazepine idiosyncratic toxicity (Dreifuss and Langer, 1987).

Idiosyncratic reactions associated with carbamazepine are relatively rare, with an estimated incidence of 1:5000-10000 (Shear *et al.*, 1988). However, this must be regarded as a crude estimate mostly based on retrospective clinical analysis. Part of the reason for this is that the diagnosis of idiosyncratic toxicity can be difficult as there are no operational diagnostic criteria, and thus diagnosis depends on the suspicions of the attending physician. Furthermore, the pattern of toxicity seen with carbamazepine may be difficult to distinguish from non-drug induced diseases such as lymphoma (Shuttleworth *et al.*, 1984). Therefore, missed diagnoses or misdiagnosis can lead to underestimation of the true incidence of an adverse drug reaction. Overall, the availability of an *in vitro* assay would be a great advance facilitating not only diagnosis of idiosyncratic toxicity, but also fostering better patient management.

To this end, Spielberg (1980, 1984) has developed an *in vitro* assay (figure 2.1) which tries to recreate the *in vivo* generation of oxidative drug metabolites by incorporating a drug metabolite generating system (microsomes and NADPH), and their subsequent detoxication by the assessment of the viability of co-incubated mononuclear leucocytes (MNL). MNL are used in this assay for four main reasons (Spielberg, 1984): first, they are easily available and can be easily isolated from whole blood; secondly, they contain detoxication enzymes such as epoxide hydrolase (Seidegard *et al.*, 1984) and glutathione transferase μ (Seidegard *et al.*, 1987); thirdly, any genotypic abnormality in these detoxication mechanisms will be phenotypically expressed in these cells, and finally, they cannot bioactivate drugs by themselves.

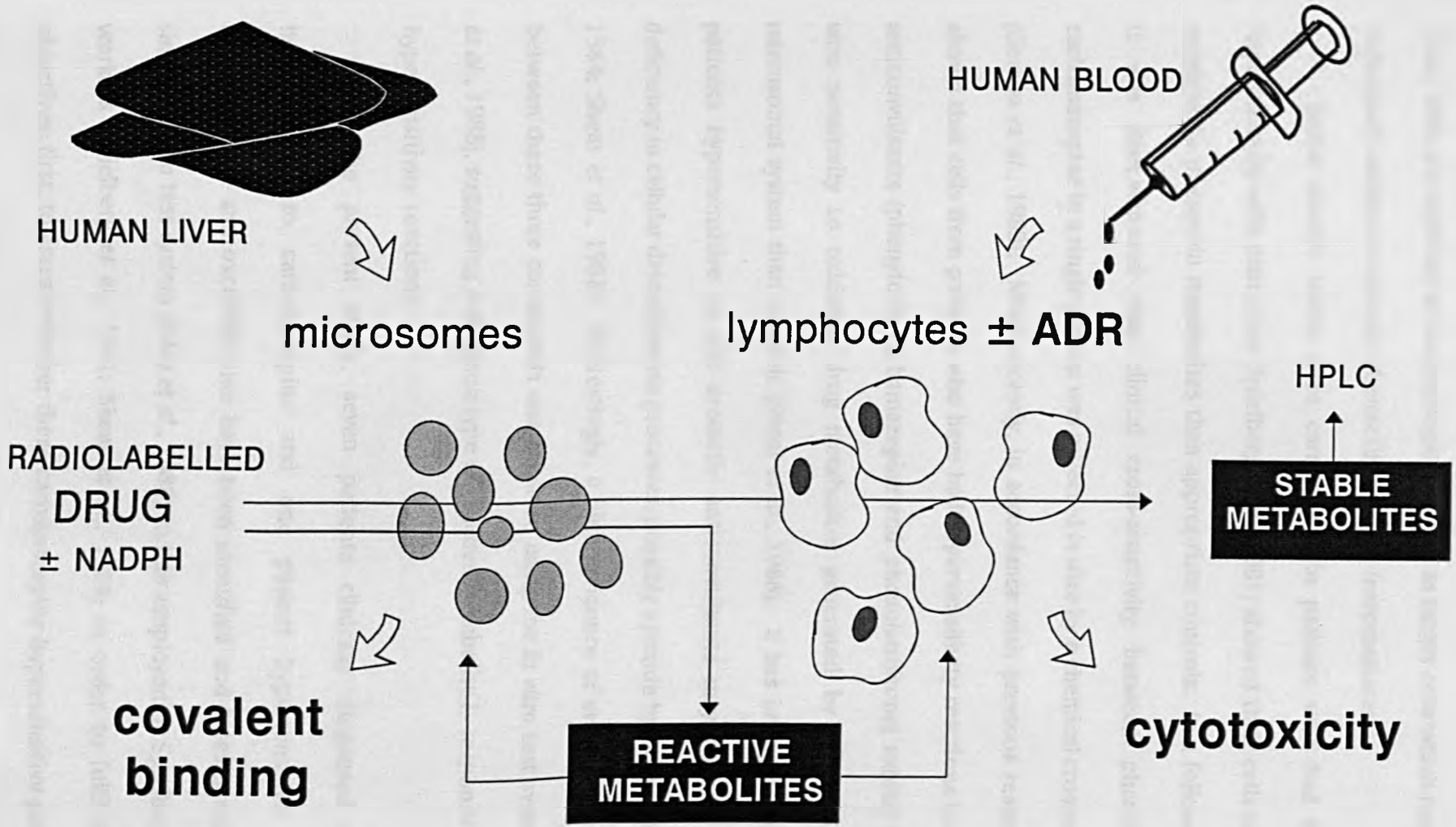


Figure 2.1. Schematic representation of the *in vitro* cytotoxicity assay first described by Spielberg (1980), as modified by Riley *et al.* (1988).

Thus, MNL are used not as immunologic cells, but as target cells which represent an individual's ability to detoxify chemically reactive intermediates.

Initial studies which were carried out in patients who had developed hepatotoxicity with phenytoin (Spielberg *et al.*, 1981) showed their cells were more sensitive to phenytoin metabolites than appropriate controls. In a follow-up study it was demonstrated that clinical cross-sensitivity between phenytoin and carbamazepine in a single patient was reflected *in vitro* by a chemical cross-sensitivity (Gerson *et al.*, 1983). More recently, in accordance with previous results, it was shown that cells from patients who have had hypersensitivity reactions to aromatic anticonvulsants (phenytoin, carbamazepine and phenobarbitone) exhibit higher *in vitro* sensitivity to oxidative drug metabolites generated by a murine hepatic microsomal system than controls (Shear *et al.*, 1988). It has been postulated that patients hypersensitive to the aromatic anticonvulsants may have a heritable deficiency in cellular detoxification processes, possibly epoxide hydrolase (Spielberg, 1984; Shear *et al.*, 1988). Interestingly, a high incidence of cross-reactivity (80%) between these three compounds was reported using the *in vitro* test system (Shear *et al.*, 1988), suggesting a common type of reactive metabolite is responsible for the hypersensitivity reactions.

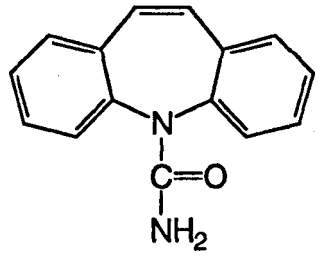
In the present study, seven patients clinically diagnosed as being hypersensitive to carbamazepine and one patient hypersensitive to both carbamazepine and oxcarbazepine have been identified and investigated using a similar *in vitro* test system (Riley *et al.*, 1988) to that employed by Spielberg and co-workers (Spielberg *et al.*, 1981; Shear *et al.*, 1988) in order to fulfil four main objectives: first, to assess whether these carbamazepine-hypersensitive patients can

be differentiated by *in vitro* analysis, from "control subjects" (comprising patients on chronic carbamazepine therapy without adverse effects and normal healthy volunteers who have never been exposed to anticonvulsants); secondly, to assess *in vitro* chemical cross-reactivity and whether this corresponds to the presence or absence of clinical cross-reactivity, two compounds functionally and structurally related to carbamazepine, phenytoin and oxcarbazepine (figure 2.2) have been used; thirdly, to exclude a non-specific *in vitro* chemical sensitivity of the hypersensitive patient cells, they have been challenged with two toxic metabolites unrelated to carbamazepine, namely dapsone hydroxylamine and amodiaquine quinoneimine; and finally, all previous work with carbamazepine has used induced murine microsomes to generate the toxic metabolite (Shear *et al.*, 1988; Riley *et al.*, 1989) and therefore, to investigate the importance of the balance between activation and detoxication in predisposing an individual to idiosyncratic drug toxicity, the ability of 10 human livers to bioactivate carbamazepine to a cytotoxic metabolite, has been determined.

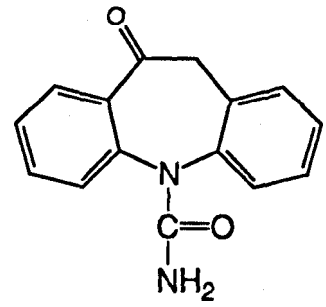
2.2. Methods

2.2.1. Carbamazepine-hypersensitive patients

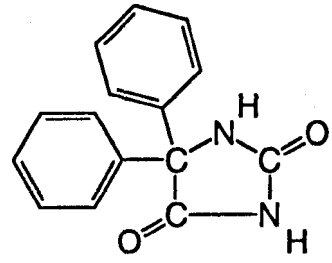
The study was performed in 8 patients (mean age 39 years): 7 were hypersensitive to carbamazepine, while one patient (patient 8) was sensitive to both carbamazepine and oxcarbazepine. Their clinical features are summarised in table 2.1. The mean time to the occurrence of the reaction following the initiation of carbamazepine therapy was 25 days (range 7-42 days). Two patients (patients 3 and 4) had positive rechallenges to carbamazepine: patient 3 was deliberately rechallenged because of poor control of epilepsy and developed a rash and fever



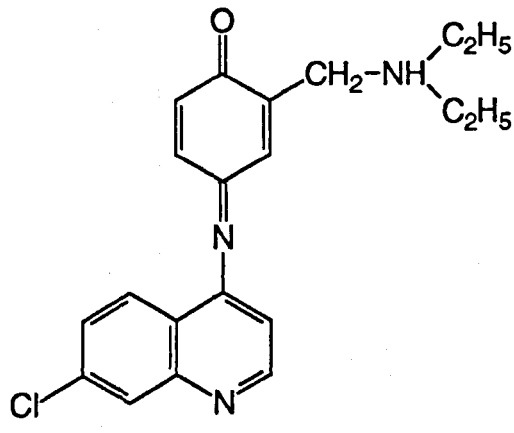
carbamazepine



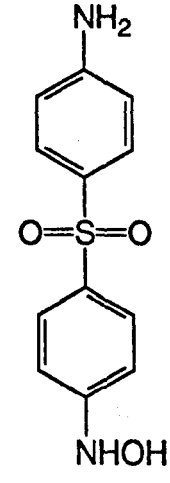
oxcarbazepine



phenytoin



amodiaquine quinoneimine



dapsone N-hydroxylamine

Figure 2.2. Structures of compounds used in this study

Table 2.1.

A summary of the clinical features of hypersensitive patients. Patients 1-7 were hypersensitive to carbamazepine only, while patient 8 was hypersensitive to both carbamazepine and oxcarbazepine.

Patient number	*Sex*	*Present age (years)*	*Age at time of reaction (years)*	*Time to onset of reaction*	*Rechallenge*	*Major manifestation*	*Other manifestations*	*Present treatment*
1	Male	18	18	3 weeks	-	Exfoliative dermatitis	Eosinophilia fever	Phenytoin
2	Male	16	8	5 weeks	-	Rash pneumonitis	Eosinophilia fever splenomegaly	Phenytoin Valproate
3	Female	21	13	6 weeks	Yes	Rash hepatospleno-megaly	Fever eosinophilia lymphadenopathy	Phenytoin Primidone
4	Male	46	42	4 weeks	Yes	Rash	Fever	Valproate
5	Female	46	45	1 week	-	Stevens-Johnson syndrome	Fever Abnormal liver function tests	-
6	Female	78	78	4 weeks	-	Stevens-Johnson syndrome	Fever Abnormal liver function tests	Phenytoin Valproate
7	Male	16	11	2 weeks	-	Rash	Fever	Valproate Phenytoin Phenobarbitone
8	Female	76	75	4 weeks	Exposure to oxcarbazepine	Rash	Fever	Valproate

within 24h. Patient 4 was a schizophrenic with epilepsy who was inadvertently rechallenged on 2 occasions by separate general practitioners, both times requiring hospitalisation with a widespread erythematous rash and fever.

Patient 8 was prescribed carbamazepine for phantom limb pain and within 4 weeks developed an erythematous rash. This resolved after the carbamazepine was withdrawn. Subsequently, oxcarbazepine was started several weeks later for the same indication and the patient re-developed a rash within a few days.

All patients (except patients 7 and 8) required hospitalisation during the acute phase and had relevant investigations to exclude other causes. In all patients, withdrawal of carbamazepine (and oxcarbazepine in patient 8) resulted in clinical improvement.

2.2.2. Control subjects used for comparison with the hypersensitive patients

Two groups of controls (mean age 32 years; range 21-74; 3 females, 14 males) were used for experiments with the anticonvulsants: the first group (n=5) were patients on chronic carbamazepine therapy without adverse effects. The second group (n=12) were normal, healthy volunteers never exposed to any anticonvulsants. There was no difference in the *in vitro* responses of these two control groups to any of the compounds tested. Thus, the two control groups will hereafter be referred to as "control subjects".

Only normal healthy volunteers were used as controls for experiments with dapsone hydroxylamine (n=22; mean age 26 years) and amodiaquine quinoneimine (n=9; mean age 27 years). These are also referred to as control subjects.

2.2.3. Subjects used for experiments with human liver microsomes

Mononuclear leucocytes isolated from normal, healthy male volunteers (n=7, mean age 28 years) were used for experiments with human liver microsomes. All volunteers gave their informed consent.

All patients and control subjects gave their informed consent. The study was approved by the local ethical committee.

2.2.4. Chemicals

5,5-diphenyl hydantoin (phenytoin), carbamazepine, 1,1,1-trichloro-2-propene oxide (TCPO) and human serum albumin (HSA, fraction V) were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Oxcarbazepine was a gift from Ciba-Geigy Pharmaceuticals (Horsham, Surrey, UK). Reduced nicotinamide adenine dinucleotide phosphate (NADPH; tetrasodium salt) was obtained from BDH Chemicals Ltd. (Poole, Dorset, UK). All solvents were of h.p.l.c. grade and were products of Fisons plc (Loughborough, Leics., UK).

Dapsone hydroxylamine was synthesised by Dr. F. Hussain (University of Liverpool) using the method of Uetrecht *et al* (1988). Amodiaquine quinoneimine was synthesised by Prof. B.K. Park and characterised as described by Maggs *et al.* (1988).

2.2.5. Preparation of human and murine liver microsomes

Microsomes were prepared from histologically normal livers obtained from 10 kidney transplant donors (table 2.2). Informed consent for removal of liver samples was obtained from donors' relatives and ethical approval was granted by the

Table 2.2.

Details of donors of human liver samples

Liver	*Age*	*Sex*	*Cause of death*	*Drugs*	*Histology*	*Cytochrome P450 (nmol/mg protein)*
I	40	F	Road traffic accident	Clomipramine Procyclidine Flupenthixol Sulpiride	Normal	0.32
II	60	M	Subarachnoid haemorrhage	None	Normal	0.24
III	18	M	Head injury	None	Normal	0.47
IV	22	M	Road traffic accident	None	Mild fatty change	0.36
V	55	M	Subarachnoid haemorrhage	None	Normal	0.18
VI	49	F	Road traffic accident	None	Normal	0.22
VII	27	M	Head injury	None	Normal	0.56
VIII	29	M	Head injury	None	Normal	0.44
IX	66	F	Brain haemorrhage	None	Normal	0.48
X	8	M	Road traffic accident	Phenytoin (7 days)	Non-specific inflammatory infiltrate	0.5

Mersey Regional Ethics Committee. The livers were removed from the donor immediately after cessation of life support and quickly cooled on ice. They were subsequently divided into portions (10-20g) and frozen in liquid nitrogen within 1h of removal. The samples were stored at -80°C until required for use.

For preparation of human liver microsomes, a liver sample was thawed, finely chopped with scissors in ice-cold sodium phosphate buffer (0.067M; pH 7.4) containing 1.15% KCl and homogenised initially by using an Ultra-Turrax homogeniser (3 x 30 sec passes) and subsequently by a motor-driven Potter-Elvehjem teflon pestle (3 passes). Throughout the homogenisation procedure, the samples were kept on ice in order to prevent overheating. Human liver microsomes were prepared from the liver homogenate by a differential ultracentrifugation procedure using an MSE Europa 55M or Prepsin ultracentrifuge. Initially, the liver homogenate was centrifuged at 10,000*g* for 22 min at 4°C to remove cellular debris (nuclear and cell membranes, and mitochondria). The resulting supernatant was centrifuged at 105,000*g* for 65 min at 4°C to sediment the microsomes. The microsomal pellet was then resuspended in phosphate buffer containing 1.15% KCl (10% v/v) and re-centrifuged at 105,000*g* for 65 min at 4°C. The supernatant was discarded and the microsomal pellet stored at -80°C until required.

Groups of 6 male CBA/ca mice (25-30g) were pretreated with phenobarbitone (60mg/kg body weight/day, i.p. in 0.9% w/v saline for 3 days). Following the final set of injections, the mice were starved for 24h, and then killed by cervical dislocation, their livers being removed into ice-cold sodium phosphate buffer (0.067M, pH 7.4) containing 1.15% KCl. After rinsing, weighing and removal of the gall bladders and extraneous tissue, the livers were roughly chopped with a pair of scissors,

homogenised and microsomes prepared by the same centrifugation procedure used to obtain washed human liver microsomes. The microsomes were stored at -80°C until use. The microsomal protein content was determined by the method of Lowry *et al* (1951).

Concentrated suspensions of the human and induced mouse microsomes (*ca.* 10mg microsomal protein/ml) in 0.067M phosphate buffer (pH 7.4) were prepared for incubation with MNL. Aliquots of the microsomal suspension were stored at -80°C. It has been shown that microsomes stored at this concentration and temperature are stable for long periods of time (von Bahr *et al.*, 1980).

2.2.6. Determination of the cytochrome P450 content of the hepatic microsomes

The cytochrome P450 content of the hepatic microsomal suspensions was measured by the method of Omura and Sato (1964). Microsomal suspensions were diluted in 0.067M phosphate buffer (pH 7.4) to approximately 1mg/ml. The diluted sample (2ml) was added to both matched sample and reference cuvettes and baseline determined spectrophotometrically between 400 and 500 nm in a CE 505 dual beam spectrophotometer (Cecil Instruments, Cambridge, UK). A few grains of solid sodium dithionite were added to both sample and reference cuvettes with gentle mixing, and the sample cuvette only was bubbled with carbon monoxide (1 bubble/sec) in a fume cupboard for 1 min. Both the sample and reference cuvettes were then re-scanned from 400 to 500 nm. Quantitative determination of the haemoprotein was then made using an extinction coefficient for the wavelength couple 450-490 nm of 91 mM/cm.

2.2.7. Isolation of human mononuclear leucocytes

To prevent contamination by micro-organisms, peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinised venous blood from patients and control subjects in a Class II Biohazard Cabinet with a vertical laminar air flow (Gelaire BSB 4A, Flow Laboratories, Italy). All the distilled water used to make up the buffers had been autoclaved, and the buffers were filter sterilised prior to use with a 0.22μm pore size disposable membrane filter (Millipore, UK). In addition, the buffers were stored either irradiated under ultra-violet light or at 4°C. A further precaution against contamination was that buffers were stored for no longer than 7 days.

The blood was diluted (1:1, v/v) with phosphate-buffered saline (PBS; NaCl 8g/l, KCl 0.2g/l, Na_2HPO_4 1.15g/l, KH_2PO_4 0.2g/l; pH 7.2). Aliquots (12ml) were layered onto Lymphoprep (9ml; Nyegard UK Ltd, Birmingham) and centrifuged at 1,900 rev/min (650*g*) for 25 min (figure 2.3) in a Centaur 2 centrifuge (MSE, Crawley, Sussex, UK). It was found that 25 min was the optimum time for the first centrifugation step, a shorter time period leading to inadequate separation from, and therefore increased contamination by, erythrocytes, and a reduced yield of MNL. The interface layer was harvested with a Pasteur pipette, the suspension of cells diluted with PBS (1:1, v/v) and the cells sedimented by centrifugation (1,400 rev/min, 347*g*, for 10 min). They were gently resuspended in PBS and sedimented at a low speed (1,100 rev/min, 214*g*, for 8 min) to reduce contamination by platelets; this procedure was repeated once. Erythrocyte contamination of MNL was removed by lysis with ammonium chloride (0.85% for 5 min) following a washing phase in RPMI 1640 medium. The MNL were finally resuspended in 15mM 4-(2-hydroxyethyl)-1-

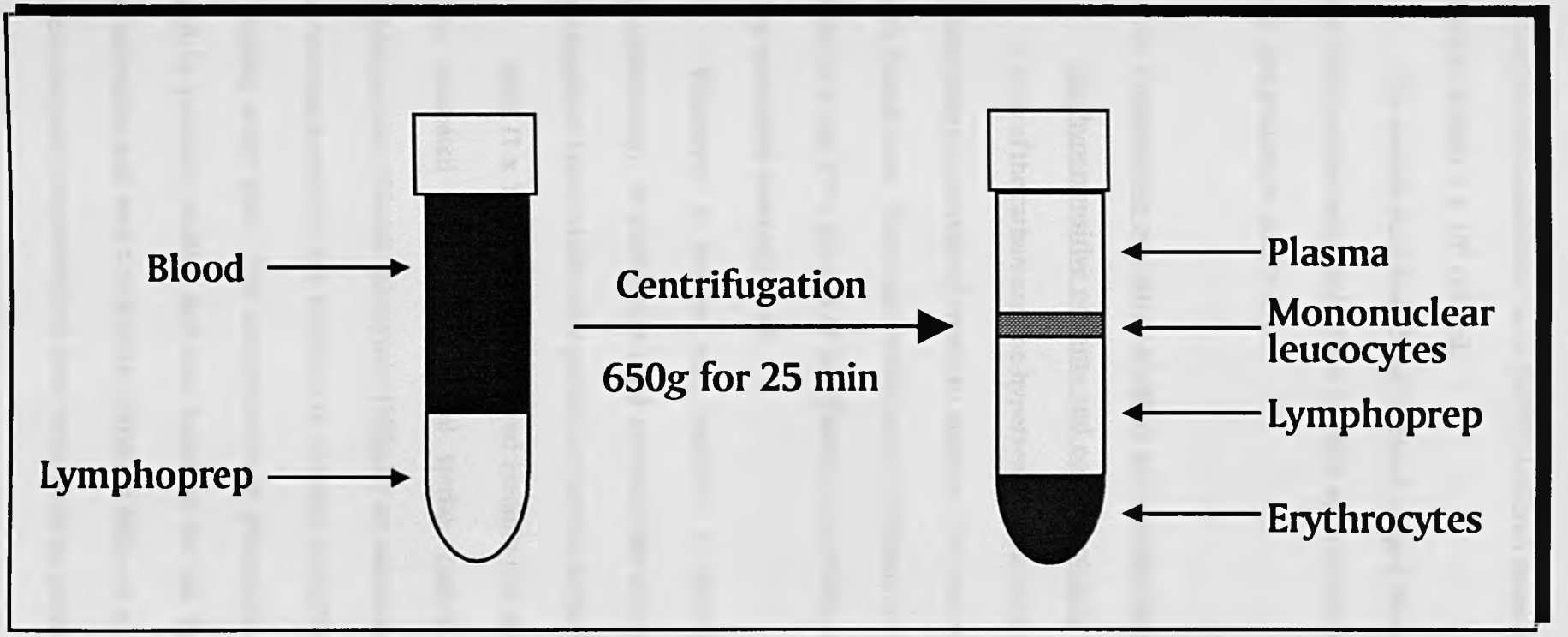


Figure 2.3. Diagrammatic representation of the method used for isolation of mononuclear leucocytes by centrifugation over a density gradient (lymphoprep)

piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium (Spielberg, 1980) to obtain 1 x 10^6 cells/ml.

The overall yield from 50ml of blood ranged from 35 x 10^6 to 85 x 10^6 cells depending on the individual. Their viability upon isolation, as determined by trypan blue dye exclusion, was >95%.

2.2.8. Comparative evaluation of direct and metabolism-dependent cytotoxicity in the hypersensitive patients and control subjects

Four of the carbamazepine-hypersensitive patients and 8 control subjects were tested twice (at intervals of weeks to months). The rest of the patients and controls were tested once. The mean coefficient of variation for intrapatient reproducibility of the test was 27%. Figures of 22% (Farrell *et al.*, 1985) and 23% (Larrey *et al.*, 1989) have previously been reported.

Whenever a patient was studied, 2 control subjects were tested simultaneously. In addition, a blind protocol was adopted such that none of the investigators knew which were patient or control subject lymphocytes.

MNL (1 x 10^6) in HEPES-buffered medium (1.0 ml; pH 7.4; Spielberg, 1980) were incubated under air in 10ml sterile, conical polystyrene tubes with carbamazepine (50μM), phenytoin (150μM) or oxcarbazepine (50μM) and 0.5mg microsomal protein in the presence or absence of NADPH (1mM) for 2h at 37°C in a shaking water bath. The concentration of phenytoin chosen (150μM) had been used in previous studies and was found to be the optimum concentration for bioactivation and was not directly cytotoxic (Riley *et al.*, 1988). A wide range of carbamazepine concentrations have been used in previous studies (Gerson *et al.*,

1983; Shear *et al.*, 1988; Riley *et al.*, 1989); for this study, a concentration of 50μM was used because it was not directly cytotoxic and approximated to *in vivo* concentrations. Similarly, with oxcarbazepine, an equimolar concentration (50μM) which was not directly cytotoxic was used. The total incubation volume was 1ml. The drugs were added in 10μl of h.p.l.c.-grade methanol, which, as a 1% solution (v/v) was non-toxic. Incubation of cells with methanol in the absence of drug provided the baseline cell death values.

In some experiments, the MNL were pretreated with the epoxide hydrolase inhibitor, TCPO (30μM for 10 min), to characterise further the cytotoxic metabolite. This concentration of TCPO has previously been shown not to alter cell viability (Riley *et al.*, 1989).

The direct toxicity of amodiaquine quinoneimine and dapsone hydroxylamine was determined by incubating these compounds with 1 x 10^6 MNL for 2h at 37°C in the absence of microsomes (final incubation volume 1ml). The concentration of amodiaquine quinoneimine used was 10μM which has previously been shown to be cytotoxic (Maggs and Park, 1988). With dapsone hydroxylamine, initial experiments using 10μM, 100μM and 500μM were conducted with cells from healthy volunteers (n=4) to determine the concentration to be used in the patient study. Amodiaquine quinoneimine and dapsone hydroxylamine were added in 10μl of methanol and acetone, respectively, which as 1% solutions (v/v), were non-toxic.

After 2h, the cells were sedimented by centrifugation at low speed (1,100 rev/min, 214*g*, for 8 min) and resuspended in 1ml of drug-free medium (HEPES-buffered medium containing 5mg/ml HSA). Incubations were continued for 16h at 37°C in tubes with loosely placed caps. The cells were then resuspended and

aliquots were taken for the determination of cytotoxicity.

2.2.9. Determination of cytotoxicity by trypan blue dye exclusion

Cell viability (% excluding dye) was assessed using 100µl aliquots of the cell suspensions by mixing it with a solution of trypan blue (dye content > 50%; Aldrich Chemical Co., Gillingham, Dorset, UK) in phosphate-buffered saline to give a final concentration of 0.2%. The cells were examined after the addition of trypan blue on an Improved Neubauer haemocytometer under a Model Wilovert II microscope (Will Wetzlar GmbH, Nauborn, FRG) using a x40 (low power) objective and phase contrast microsocopy. Trypan blue was added to the cell aliquot immediately prior to examination under the microscope, and counting with all samples was carried out within 1 min of mixing, since trypan blue itself was cytotoxic when left incubated with cells for longer than 5 min. At least 200 cells were counted, the number of dead cells (which were stained blue) being expressed as a percentage of cells not excluding dye. To avoid counting the same cells twice, cells on or touching the top and left border lines of a square were considered to be in that square, and thus counted, while cells on or touching the bottom and right border lines were excluded.

2.2.10. Determination of metabolism-dependent cytotoxicity of carbamazepine with human liver microsomes

The method used to determine the bioactivation of carbamazepine (50µM) by human liver microsomes was the same as that used for bioactivation with phenobarbitone-induced mouse microsomes (outlined above), except that a range of protein concentrations (0.5mg - 2mg/incubation) were used with 5 human livers, and

2mg protein/incubation was used for the other 5 human livers.

2.2.11. Statistical analysis

Each assay was set up in quadruplicate and the mean percentage of dead MNL was determined. The results are expressed as mean ± SEM. Statistical analysis has been performed by one way analysis of variance, accepting $p<0.05$ as significant. To analyse the difference between the hypersensitive-patient and control groups, the increase in cell death above the baseline, i.e. cell death in the presence of drug and the full metabolising system minus cell death in the absence of drug, was determined; the difference was then analysed by one way analysis of variance.

2.3. Results

2.3.1. Bioactivation of carbamazepine to a cytotoxic metabolite by human liver microsomes

The cytochrome P450 content of the individual human livers used is shown in table 2.2, the mean (± SEM) for the 10 human livers being 0.38 ± 0.04 nmol/mg protein.

In order to determine the optimum concentration of human liver microsomes required to bioactivate carbamazepine (50μM), in initial experiments with 5 human livers, a range of protein concentrations (0.5mg, 1.0mg, 1.5mg and 2mg per incubation) were used. This showed that the bioactivation of carbamazepine increased with increasing protein concentration (Figure 2.4), reaching statistical significance at protein concentrations of 1.0mg ($p<0.05$), 1.5mg ($p<0.005$) and

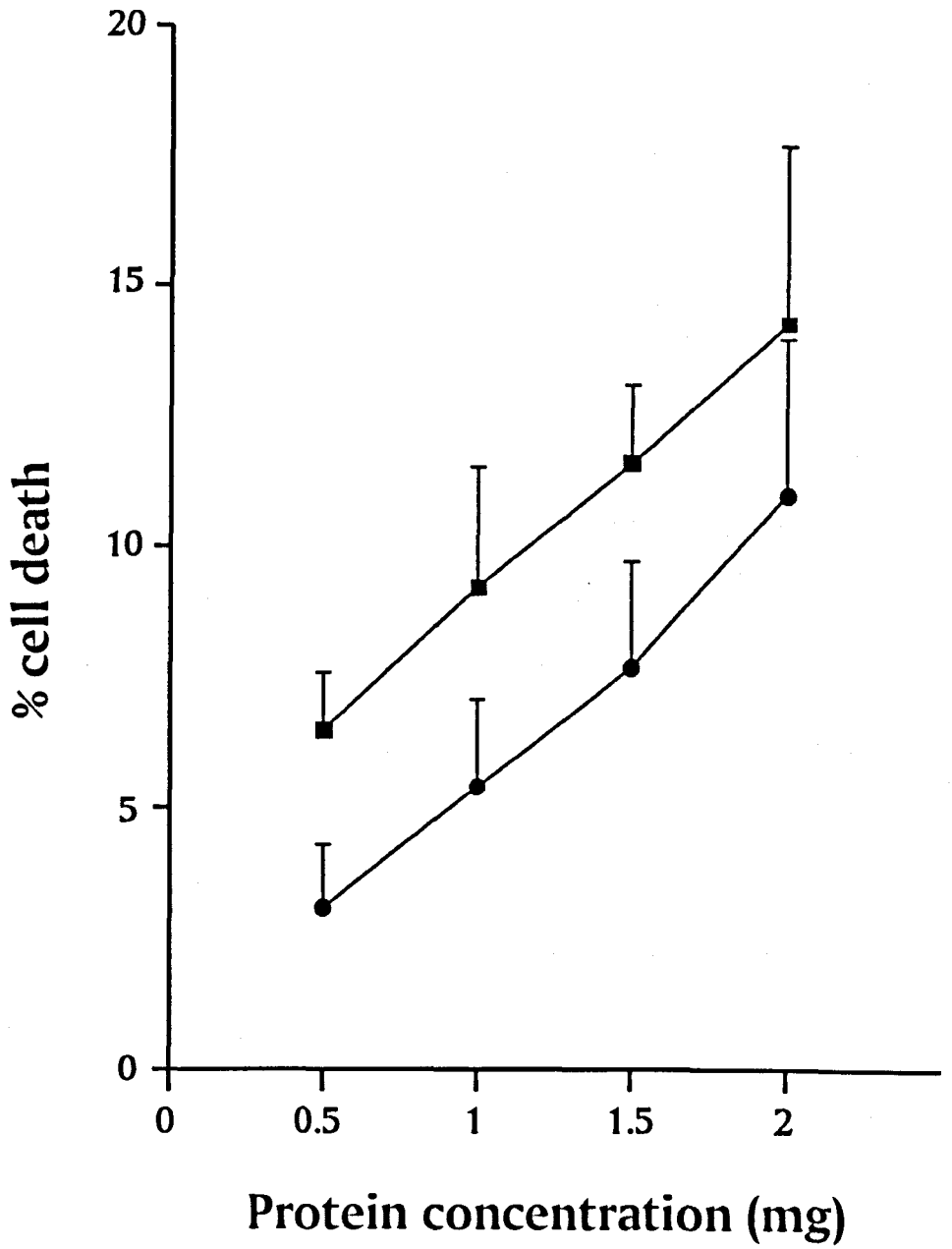


Figure 2.4. The effect of increasing the protein concentration of human liver microsomes on the cytotoxicity of carbamazepine (50uM) in the presence of NADPH (●), and after preincubation with trichloropropene oxide (■). The results are represented as increase in cell death above the baseline and represent the mean ± SEM of five experiments with different human livers (performed in quadruplicate).

2.0mg ($p=0.005$) per incubation. Preincubation of cells with TCPO (30μM) resulted in a non-significant increase in cytotoxicity at all protein concentrations (figure 2.4).

At a protein concentration of 2mg per incubation, carbamazepine was bioactivated to a cytotoxic metabolite by 9 of the 10 human livers tested (figure 2.5), the mean cell death value (for the 10 samples) increasing from 7.2 ± 0.8% (baseline) to 16.4 ± 2.1% in the presence of NADPH ($p=0.002$).

2.3.2. Comparison of the metabolism-dependent cytotoxicity of carbamazepine, phenytoin and oxcarbazepine in hypersensitive patients and control subjects

The cytochrome P450 content of the phenobarbitone pretreated mouse liver microsomes was 1.2 ± 0.1 nmol/mg protein ($n=3$ sets of pooled microsomes prepared from groups of six mice), confirming induction.

The baseline cell death values, i.e. in the absence of drug, for the carbamazepine-hypersensitive patients and control subjects were 7.0 ± 0.6% ($n=8$) and 5.7 ± 0.5% ($n=17$) respectively.

Although a significant increase in cell death (in the presence of microsomes and NADPH) was seen with the 3 anticonvulsants in both the hypersensitive patients and control subjects, the mean increase in cytotoxicity above the baseline in the presence of carbamazepine was significantly ($p<0.001$) greater in the carbamazepine-hypersensitive patients than in the control subjects (figure 2.6). In contrast, with both phenytoin (figure 2.7) and oxcarbazepine (figure 2.8), there was no significant difference in the cell death values of the two groups, with overlap in the individual values. Patient 8 who was hypersensitive to both carbamazepine and oxcarbazepine had high cell death values with both these drugs (figures 2.6 and 2.8) compared to

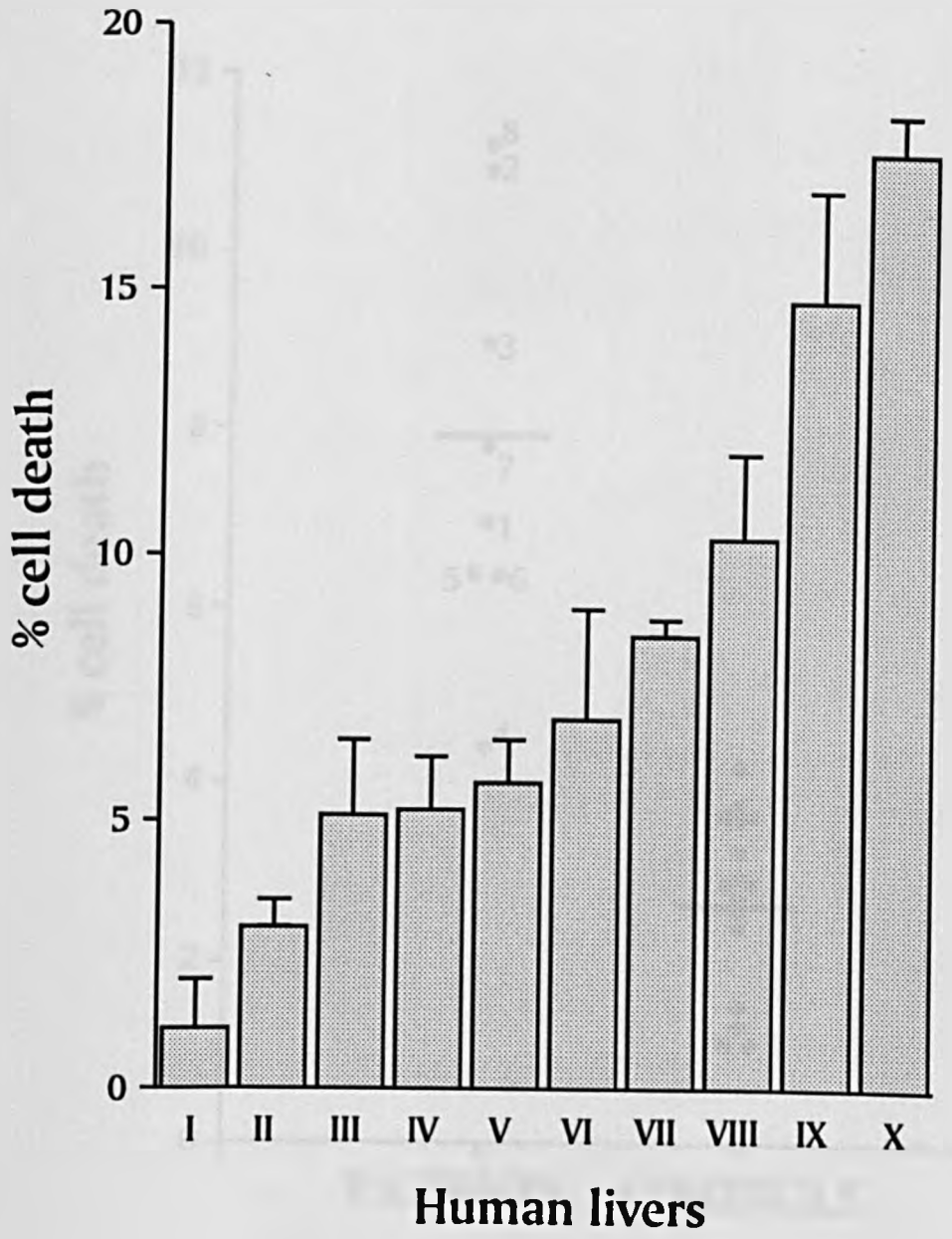


Figure 2.5. Bioactivation of carbamazepine to a cytotoxic metabolite by individual human livers, numbered as listed in table 2.2. The results are shown as increase in cytotoxicity above the baseline cell death values. All human livers, apart from liver I, significantly bioactivated carbamazepine ($p<0.05$) to a cytotoxic species.

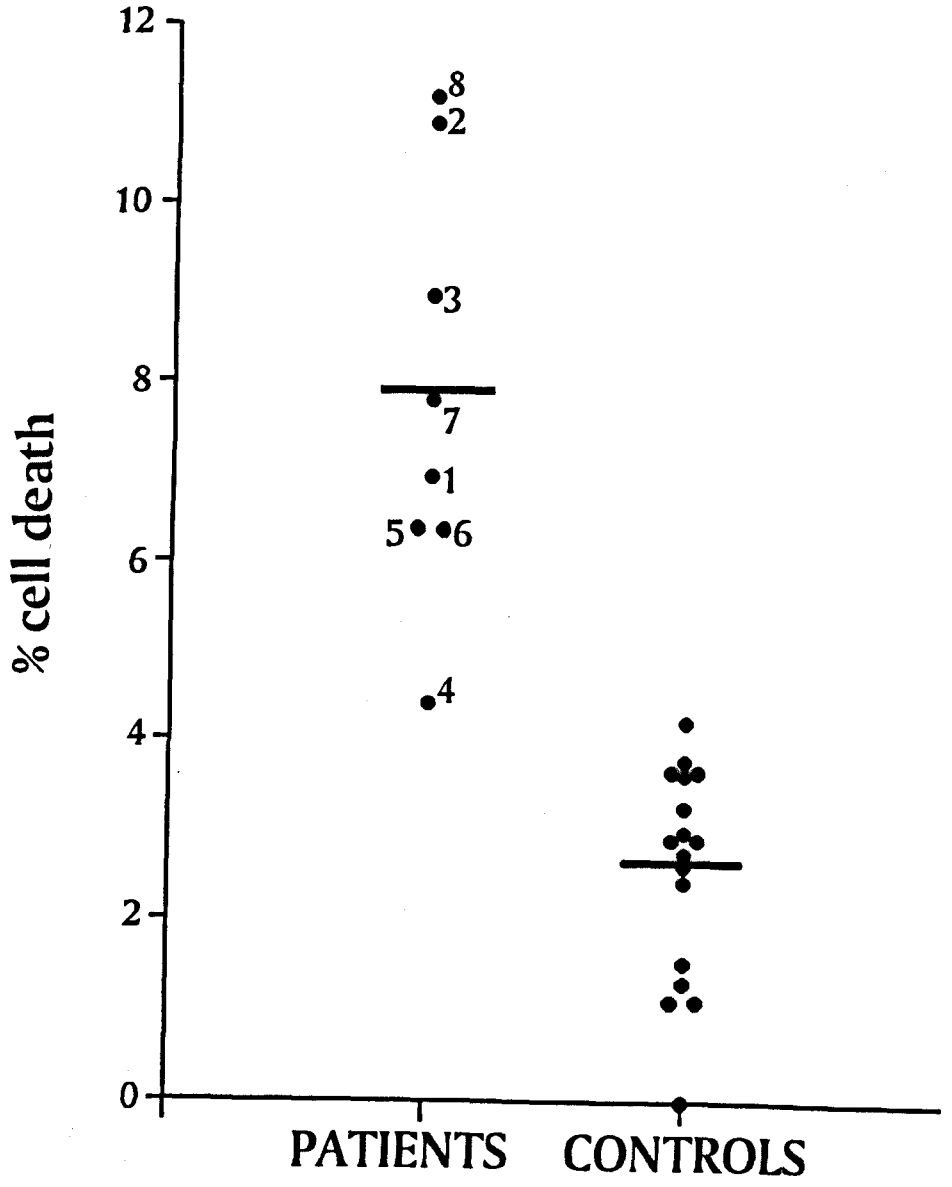


Figure 2.6. A comparison of the effect of carbamazepine in the presence of microsomes and NADPH in carbamazepine-hypersensitive patients and control subjects. The results for each individual are expressed as the increase in cell death above the baseline, i.e. cell death in the presence of carbamazepine, microsomes and NADPH minus cell death in the absence of carbamazepine. The patients are individually numbered as listed in table 2.1. The horizontal bars indicate the mean values.

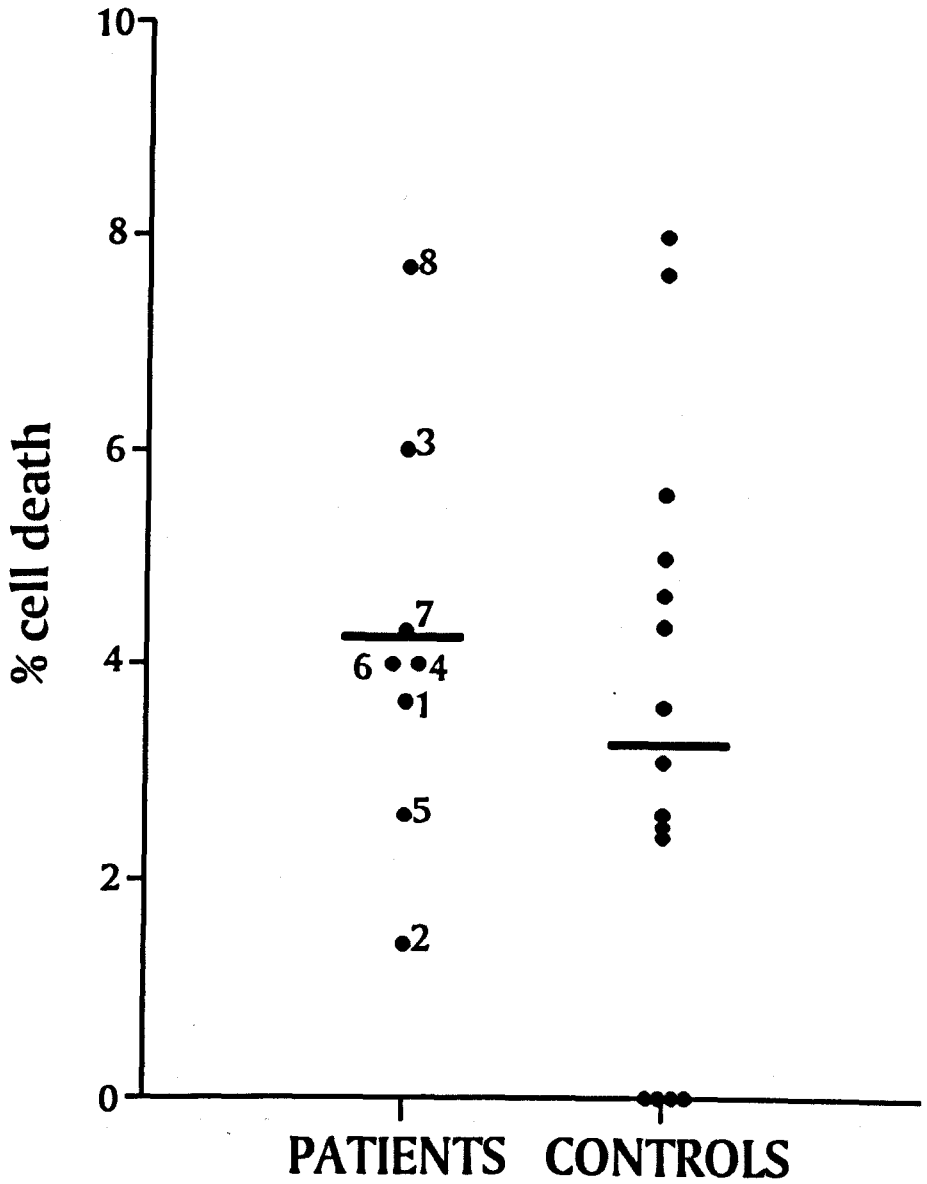


Figure 2.7. A comparison of the effect of metabolism-dependent phenytoin cytotoxicity in carbamazepine-hypersensitive patients and control subjects. The results are again expressed as increase in cell death above the baseline. The horizontal bars indicate mean values.

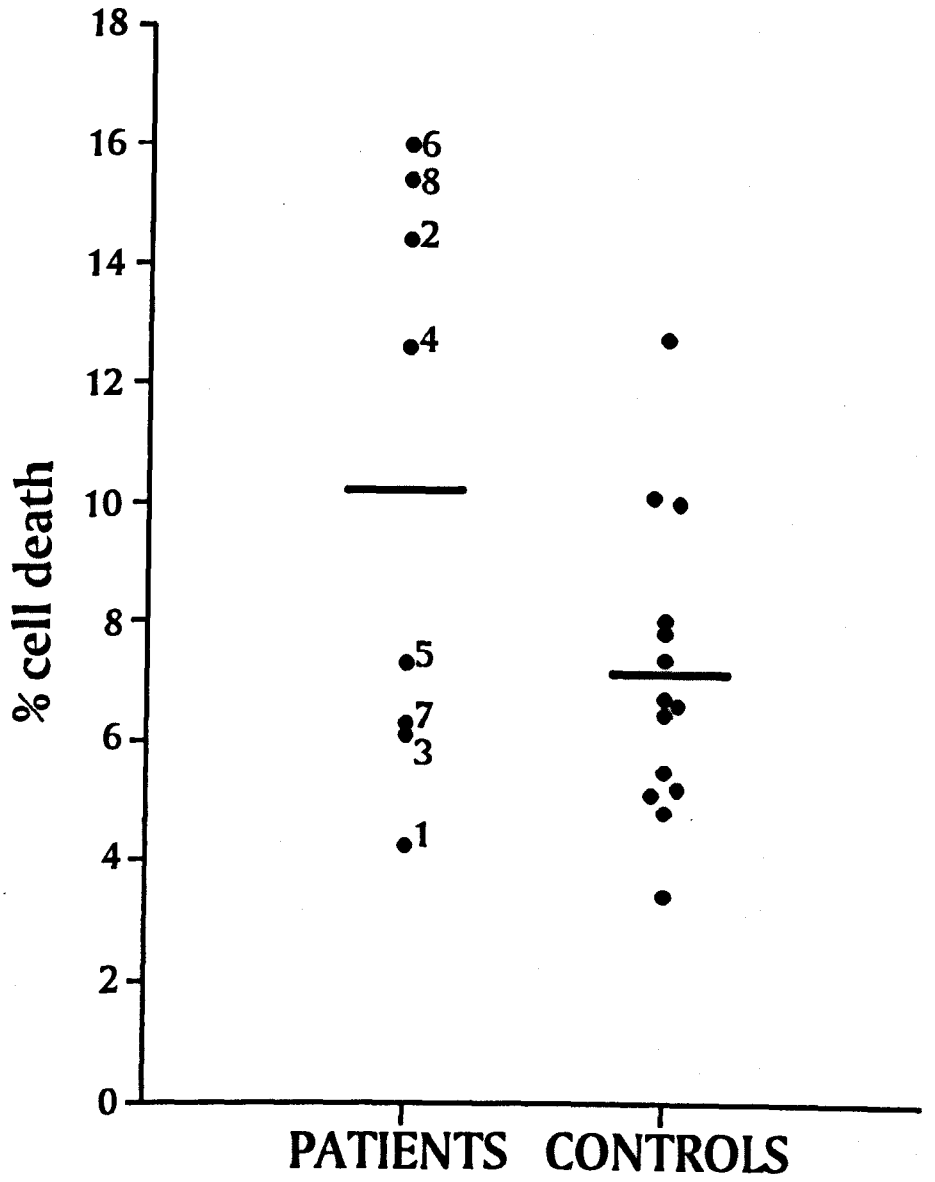


Figure 2.8. A comparison of the effect of metabolism-dependent oxcarbazepine cytotoxicity in carbamazepine hypersensitive patients and controls. The results are expressed as increase in the cell death above the baseline. The horizontal bars indicate the mean values.

the controls, while the cell death with phenytoin (figure 2.7) was the same as that with control subjects.

With carbamazepine (50μM), preincubation with TCPO, increased metabolism-dependent cytotoxicity in control subjects (figure 2.9) but not in patients (table 2.3). TCPO increased the cytotoxicity of phenytoin (150μM) in both groups but had no effect on the metabolism-dependent cytotoxicity of oxcarbazepine (50μM; table 2.3).

2.3.3. Comparison of the direct cytotoxicity of dapsone hydroxylamine and amodiaquine quinoneimine in the hypersensitive patients and control subjects

In order to determine the concentration of dapsone hydroxylamine to use in the patient study, initial experiments using cells from volunteers (n=4) were conducted. These showed there was a concentration-dependent increase in cytotoxicity (8.2 ± 0.7%, 8.3 ± 0.8%, 24.9 ± 4.0% and 45.6 ± 5.7% at 0μM, 10μM, 100μM and 500μM of dapsone hydroxylamine, respectively) reaching significance only at concentrations above 100μM ($p<0.01$). Thus, for future experiments a concentration of 100μM dapsone hydroxylamine was used. Amodiaquine quinoneimine was used at a concentration of 10μM which has previously been shown to be cytotoxic (Maggs and Park, 1988).

Incubation of MNL from carbamazepine-hypersensitive patients and control subjects with dapsone hydroxylamine and amodiaquine quinoneimine resulted in a significant increase in cytotoxicity above the baseline in both patients ($p<0.005$ and $p<0.01$ for dapsone hydroxylamine and amodiaquine quinoneimine, respectively) and

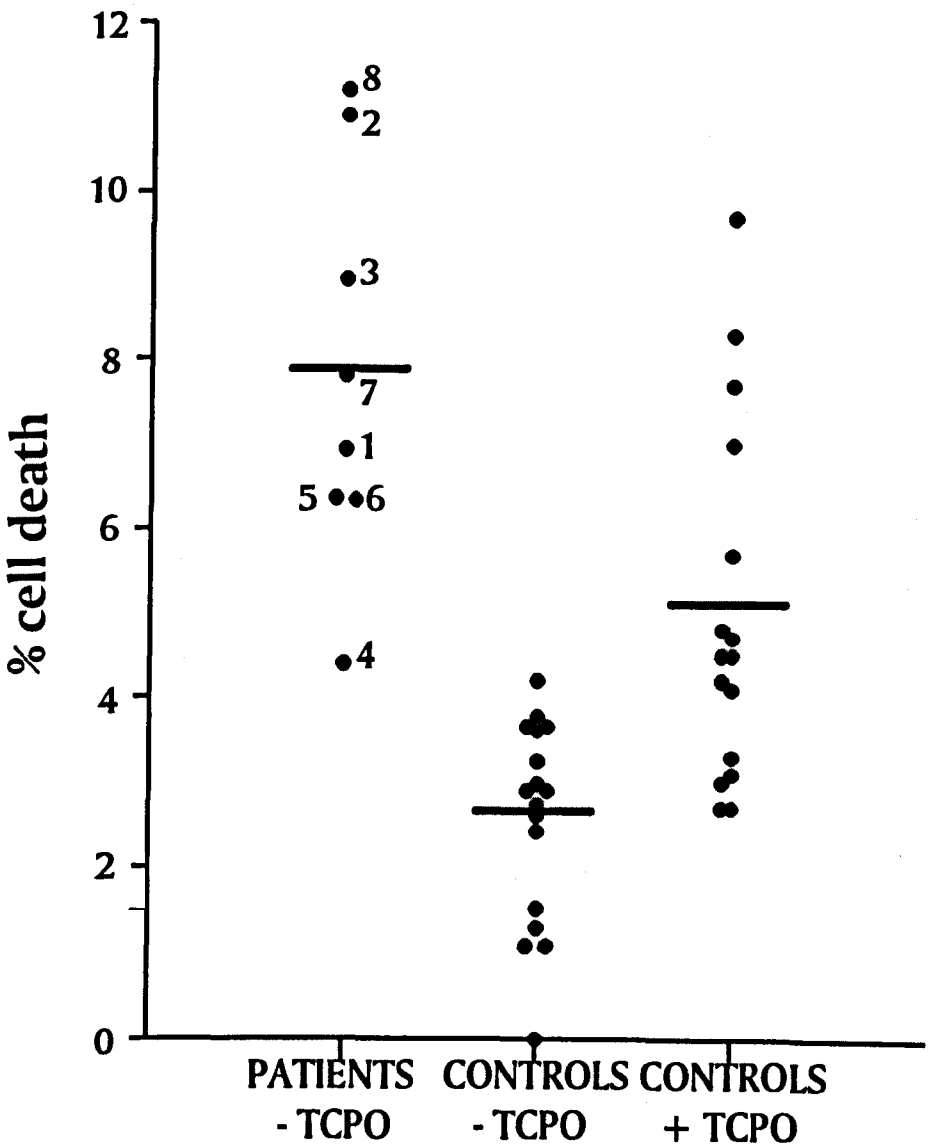


Figure 2.9. The effect of preincubation of trichloropropene oxide (TCPO) on the metabolism-dependent cytotoxicity of carbamazepine in control subjects. For comparison, cytotoxicity using cells from carbamazepine hypersensitive pateints in the absence of TCPO is shown. The results are expressed as the increase in cell death above the baseline, with the horizontal bars indicating mean values.

Table 2.3

The effect of preincubation with trichloropropene oxide (TCPO) on the metabolism (NADPH)-dependent lymphocyte cytotoxicity of carbamazepine (50μM), phenytoin (150μM) and oxcarbazepine (50μM) with phenobarbitone-induced mouse microsomes in carbamazepine-hypersensitive patients and control subjects

	CARBAMAZEPINE-HYPERSENSITIVE PATIENTS			*CONTROL SUBJECTS*		
		-TCPO	*+TCPO*		*-TCPO*	*+TCPO*
Drug	*n*	*(% cell death)*	*(% cell death)*	*n*	*(% cell death)*	*(% cell death)*
Carbamazepine	8	14.8 ± 1.0	15.5 ± 2.0	16	8.2 ± 0.5	10.8 ± 0.8*
Phenytoin	8	10.4 ± 1.1	15.5 ± 1.6*	10	9.5 ± 0.7	12.6 ± 1.2*
Oxcarbazepine	8	17.6 ± 1.7	16.3 ± 2.8	13	12.7 ± 1.0	12.7 ± 1.2

The results are expressed as mean ± SEM (performed in quadruplicate). Statistical analysis has been performed within the same group comparing incubations with and without TCPO (trichloropropene oxide): * $p<0.05$.

controls ($p<0.001$ and $p<0.005$ for dapsone hydroxylamine and amodiaquine quinoneimine, respectively; figure 2.10). However, there was no significant difference in the cell sensitivity of patients and controls to dapsone hydroxylamine and amodiaquine quinoneimine.

2.4. Discussion

On the basis of clinical manifestations, idiosyncratic reactions to carbamazepine are thought to be immunologically mediated. The patients in our series (table 2.1) satisfy the clinical criteria for hypersensitivity (Pohl *et al.*, 1988; Shear *et al.*, 1988) with the adverse reaction occurring after a mean of 25 days (range 8-42 days) and associated with a fever and rash in all patients. In addition, two of our patients had positive rechallenges to carbamazepine, with the reaction typically occurring much sooner on the second and subsequent re-exposures.

The unique susceptibility of certain patients to developing such idiosyncratic reactions may be due to a critical imbalance between the activation of carbamazepine to its postulated reactive epoxide metabolite and its detoxification. The evidence that a chemically reactive epoxide metabolite may be responsible for carbamazepine toxicity is based on the effect of TCPO, an epoxide hydrolase inhibitor, which increases *in vitro* cytotoxicity (Riley *et al.*, 1989). Thus, it has been suggested that a deficiency of the enzyme epoxide hydrolase (Gerson *et al.*, 1983; Shear *et al.*, 1988; Riley *et al.*, 1989) would lead to inadequate detoxification of the toxic metabolite, and hence the adverse reaction. The *in vivo* metabolism of carbamazepine is complex, with multiple epoxides being formed (Lertratanangkoon and Horning, 1982). However, only the 10,11-epoxide has been isolated, although

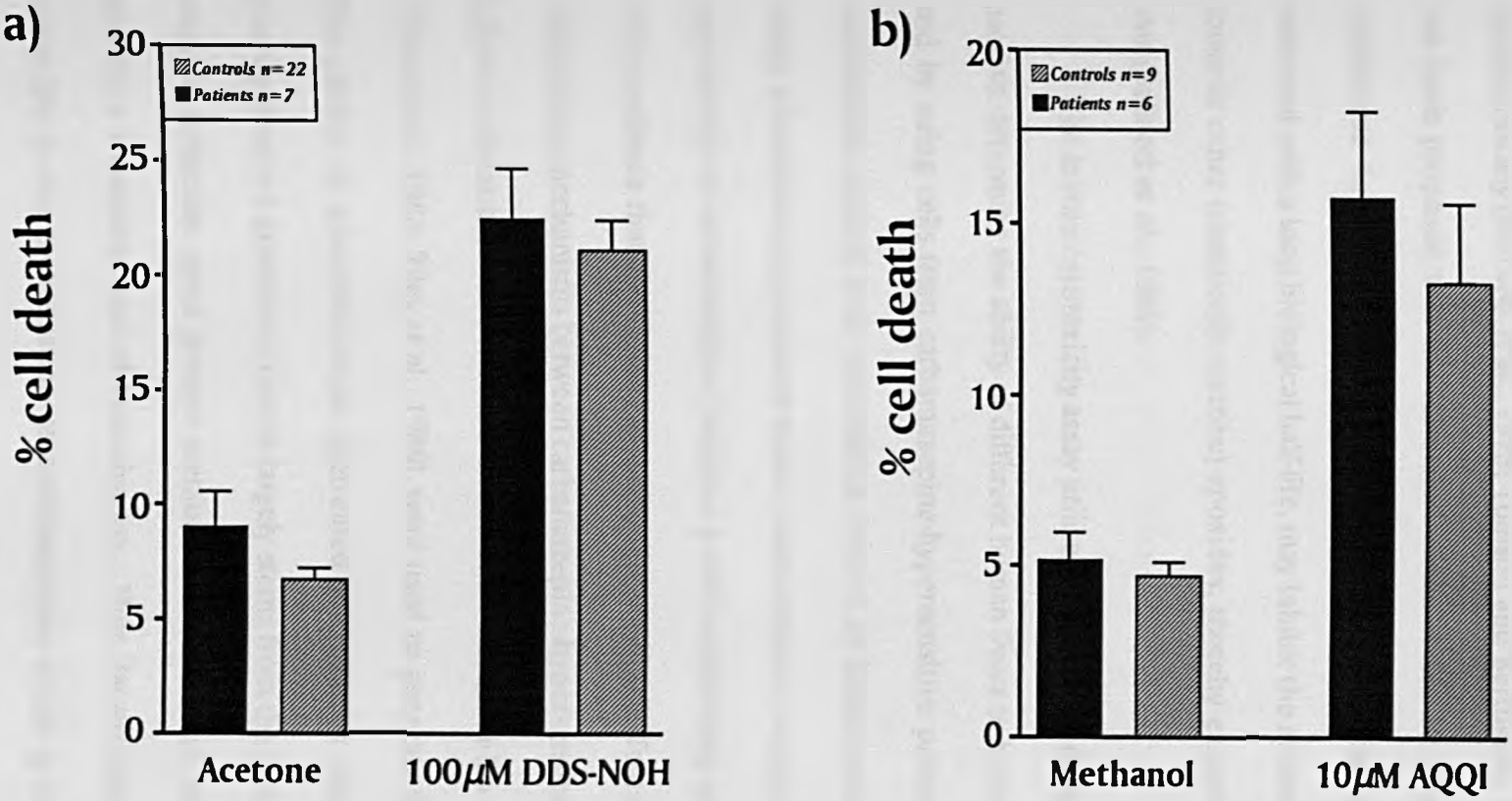


Figure 2.10. Direct cytotoxicity of (a) dapsone hydroxylamine (DDS-NOH) and (b) amodiaquine quinoneimine (AQQI) in carbamazepine hypersensitive patients and controls.

this is not the metabolite which is directly responsible for idiosyncratic toxicity since it is stable, non-cytotoxic (Shear *et al.*, 1988; Riley *et al.*, 1989), possesses pharmacological activity similar to carbamazepine, and indeed, has been used therapeutically (Morselli *et al.*, 1975; Tomson and Bertilsson, 1984). Interestingly, it has been proposed that the formation of the stable 10,11-epoxide may indirectly predispose to idiosyncratic toxicity with carbamazepine since this metabolite, endowed with a long biological half-life, may inhibit the activity of epoxide hydrolase towards other (chemically reactive) epoxides, thereby exacerbating their toxicity *in vivo* (Bellucci *et al.*, 1989).

The *in vitro* cytotoxicity assay utilised in this study (Riley *et al.*, 1988) has been used to determine the ability of different human livers to bioactivate carbamazepine, and by using cells from carbamazepine-hypersensitive patients assess cell defence mechanisms against toxic metabolites formed by bioactivation. In the presence of either phenobarbitone-induced mouse microsomes or human liver microsomes, the cytotoxicity of carbamazepine required a full metabolising system, consistent with the hypothesis that the toxicity is metabolite-mediated. To assess the differences in cell defence mechanisms between carbamazepine-hypersensitive patients and control subjects, phenobarbitone-induced mouse microsomes, in line with previous studies (Shear *et al.*, 1988; Riley *et al.*, 1989), were used to generate the toxic metabolite(s). The choice of phenobarbitone pretreated microsomes rather than human liver microsomes as a generating system largely stems from the fact that cytochrome P450 enzyme induction gives greater metabolic turnover, and more importantly, they provide a consistent level of bioactivation. Thus, by excluding the inter-individual variability in the bioactivation of carbamazepine which is clearly seen in the ten

human livers used in the study (figure 2.5), the only variable left in the *in vitro* test system is the variability in detoxication mechanisms between the cells obtained from different individuals. Therefore, using such a system, cells from the carbamazepine-hypersensitive patients were significantly more susceptible to the cytotoxic metabolite than the cells from control subjects (figure 2.6) - this is in accordance with the findings of Shear *et al.* (1988). The *in vitro* susceptibility to the carbamazepine metabolite was apparently independent of the time of the adverse drug reaction, as only two patients were tested acutely, while the rest were all investigated retrospectively. The importance of detoxification processes (or lack of them) in predisposing individuals to carbamazepine toxicity is further emphasised by the experiments with the human livers, which showed that they were capable of bioactivating carbamazepine to cytotoxic metabolites. In addition, enzyme induction by carbamazepine which would occur with continuous therapy, may enhance the conversion of carbamazepine to the toxic metabolite, thus further exacerbating the imbalance between activation and detoxification in predisposed individuals.

Phenytoin has a similar side-effect profile to carbamazepine (Pellock, 1987). It has been suggested that phase I metabolism of phenytoin can lead to the formation of the electrophilic 3,4-epoxide, which can either rearrange to the stable metabolite, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, or become covalently bound to protein (Spielberg *et al.*, 1981; Riley *et al.*, 1988), and thus it is possible that predisposition to carbamazepine and phenytoin idiosyncratic toxicity may have a similar biochemical basis (Gerson *et al.*, 1983; Shear *et al.*, 1988). In accordance with this, clinical cross-sensitivity between carbamazepine and phenytoin has been reported (Gerson *et al.*, 1983; Reents *et al.*, 1989), although the exact incidence is

unknown. Alternatively, however, hypersensitivity to one of these compounds does not necessarily predict hypersensitivity to the other, and this is clearly seen in the present study where five of the carbamazepine-hypersensitive patients were on chronic phenytoin therapy without adverse effects and one patient (patient 4) had previously been treated with phenytoin without any problems. Exposure of these patients' cells to phenytoin *in vitro* in the presence of microsomes and NADPH resulted in cell death values comparable to those of the control subjects (figure 2.7); thus, the lack of chemical cross-reactivity *in vitro* corresponds to the lack of clinical cross-reactivity seen in our group of carbamazepine-hypersensitive patients. The lack of *in vitro* chemical cross-reactivity between phenytoin and carbamazepine in our group of 8 patients contrasts with the findings of Shear *et al.*, (1988), who reported an 80% incidence of *in vitro* chemical cross-reactivity between the aromatic anticonvulsants. The variability in the incidence of clinical cross-reactivity and, *in vitro* chemical cross-reactivity, between different groups of patients may be a reflection of the variation in the enzyme structure which could occur as a result of genetic heterogeneity of the epoxide hydrolase gene. Genetic heterogeneity, which has been demonstrated for adverse drug reactions associated with the debrisoquine/sparteine oxidation polymorphism (Brosen, 1990), could in theory, lead to clinical heterogeneity among anticonvulsant hypersensitive patients with subsets of patients being hypersensitive to one or more of the aromatic anticonvulsants.

Oxcarbazepine, a keto-analogue of carbamazepine, is widely used in Scandinavia. It is of comparable efficacy to carbamazepine (Reinikainen *et al.*, 1987) but is said to cause fewer allergic reactions than carbamazepine (Dam *et al.*, 1989). A clinical study in carbamazepine-hypersensitive patients showed that only about a

quarter of these patients cross-reacted with oxcarbazepine (Jenson *et al.*, 1986). In our study, exposure of MNL from carbamazepine-hypersensitive patients and control subjects to oxcarbazepine (and its oxidative metabolites) *in vitro* did not result in a significant difference in the mean values of the two groups (figure 2.8), although significant cytotoxicity was observed with cells from both groups. However, patient 8 who was sensitive to both carbamazepine and oxcarbazepine had high cell death values to both these compounds (figures 2.6 and 2.8).

The effect of preincubation of the patient and control cells with TCPO varied according to the drug used in the *in vitro* cytotoxicity assay. In accordance with previous studies (Spielberg *et al.*, 1981; Riley *et al.*, 1988), preincubation with TCPO in the presence of phenytoin increased cytotoxicity in both patient and control cells (table 2.3) confirming that cytotoxicity may be dependent on the formation of an epoxide metabolite. In contrast, with carbamazepine, preincubation with TCPO significantly ($p<0.05$) increased cytotoxicity in the control subjects but had no effect in the hypersensitive patient group (figure 2.9 and table 2.3) - this may reflect a lack of inhibitable enzyme in the patient group. TCPO had no effect on the cytotoxicity of oxcarbazepine with both patient and control cells (table 2.3). Possible reasons for this may be that the chemically reactive metabolite formed from oxcarbazepine may not be an epoxide, or it may be an epoxide which is a weak substrate for epoxide hydrolase. Alternatively, it is known that inhibition of epoxide hydrolase by TCPO is highly substrate-dependent (Luo *et al.*, 1992), and thus the use of oxcarbazepine may have rendered the enzyme uninhibitable by TCPO.

Cells from the carbamazepine-hypersensitive patients were also challenged with the toxic drug metabolites (dapsone hydroxylamine and amodiaquine

quinoneimine) which are structurally and functionally unrelated to anticonvulsants, and more importantly, are not detoxified by the epoxide hydrolase enzymes. Dapsone hydroxylamine is thought to be responsible for many of the adverse effects associated with chronic dapsone therapy (Uetrecht *et al.*, 1988; Coleman *et al.*, 1989), while amodiaquine quinoneimine has been implicated in causing amodiaquine-induced hepatotoxicity and agranulocytosis (Maggs *et al.*,1988). The rationale behind this was to exclude non-specific chemical sensitivity of patient cells and to define further the specificity of the *in vitro* test system. Perhaps the best example of enzyme-mediated cell sensitivity is seen with erythrocytes deficient in glucose-6-phosphate dehydrogenase, where a wide range of chemically unrelated compounds can lead to red cell haemolysis (Beutler, 1991). However, the data presented here clearly shows that although there was an increase in cytotoxicity above the baseline with both compounds, there was no significant difference in the sensitivity of the patient and the control groups (figure 2.10), reinforcing not only the chemical specificity of this *in vitro* test system, but also the unique sensitivity of the carbamazepine-hypersensitive patient cells.

It has been suggested by Shear *et al.* (1988) that *in vitro* cytotoxicity assays could be used to prospectively individualise drug therapy. The results obtained with carbamazepine in the present study are consistent with the hypothesis that a chemically reactive metabolite is being produced and that cells from the hypersensitive patients are more sensitive to the metabolite. However, with the patient cells there was only a two-fold increase in cytotoxicity above the baseline, while with control cells, the cytotoxicity increased above the baseline by a factor of 1.4, and thus overall, the difference in cell death between patients and controls was

small suggesting that the test system lacks sensitivity. It has been postulated that the chemically reactive metabolite formed from carbamazepine is an epoxide, although this is based largely on circumstantial evidence. Elucidation of the chemical nature of the carbamazepine reactive metabolite would help not only in developing a more sensitive assay system but would also assist in identifying the reason for unique susceptibility of the hypersensitive patient. The former point is clearly illustrated by studies carried out by Spielberg and co-workers in patients with sulphonamide hypersensitivity (Shear and Spielberg, 1985; Reider *et al.*, 1989). As with the anticonvulsants, cells from patients clinically diagnosed as being sulphonamide hypersensitive were more sensitive *in vitro* to oxidative metabolites of sulphadiazine generated by a murine microsomal system (Shear and Spielberg, 1985). However, identification of the reactive metabolite as being the hydroxylamine, its subsequent synthesis and incorporation into the *in vitro* system (in the absence of a generating system) not only allowed *in vitro* identification of the hypersensitive patients but also resulted in increased sensitivity with a greater margin in cell death values between the patients and control subjects (Reider *et al.*, 1989).

Shear *et al.* (1988) also showed a high *in vitro* chemical cross-reactivity (80%) between the aromatic anticonvulsants. In the present study, the lack of clinical cross-sensitivity between carbamazepine and phenytoin was reflected by a lack of *in vitro* chemical cross-reactivity. Oxcarbazepine, which shows a 25% clinical cross-sensitivity rate with carbamazepine (Jenson *et al.*, 1986), was also included in the study to determine *in vitro* chemical cross-reactivity. However, this was not possible because oxcarbazepine showed metabolism-dependent cytotoxicity with both patient and control cells, and furthermore, there was a great deal of variability in the cell

death values to oxcarbazepine in the carbamazepine-hypersensitive patient group making it very difficult to predict with confidence which patients might cross-react with oxcarbazepine. Further studies to determine the mechanism of bioactivation of oxcarbazepine and the nature of the reactive metabolite formed are essential before a recommendation could be made as to the use of this assay to predict sensitivity and cross-sensitivity. In addition, assuming that reactive epoxides are formed from all the aromatic anticonvulsants, a more sensitive and direct measure of the relevant detoxication mechanisms, such as epoxide hydrolase, would be essential to predict clinical sensitivity to a drug and clinical cross-sensitivity between related and unrelated drugs.

In summary, the *in vitro* cytotoxicity assay which has been described shows a high degree of chemical specificity and does help in differentiating carbamazepine-hypersensitive patients and control subjects (healthy volunteers and patients on chronic carbamazepine therapy without adverse effects). Although it could be used for retrospective diagnosis of hypersensitive patients, and thus help define the chemical mechanism of drug toxicity, its lack of sensitivity will limit its use as a predictive test for drug hypersensitivity. All subjects may be capable of bioactivating carbamazepine to a toxic metabolite, and the critical factor in predisposing patients to hypersensitivity may be a deficiency in cellular detoxication, namely a deficiency of epoxide hydrolase. Further research is, therefore, required to establish the biochemical and genetic basis, so that patients who are particularly sensitive to serious adverse drug reactions to anticonvulsants can be easily identified.

CHAPTER 3

INVESTIGATION OF TWO PATIENTS WITH SEVERE CARBAMAZEPINE-INDUCED IDIOSYNCRATIC TOXICITY : EVIDENCE FOR THE IMMUNE BASIS OF THE ADVERSE REACTION

3.1. Introduction

The adaptive immune system is a highly versatile and efficient system capable of recognising and mounting a specific immune response to a multitude of antigens. Many idiosyncratic drug (Type B) reactions are labelled as allergic or hypersensitive largely because the clinical manifestations resemble those seen in the various types (I-IV) of classical hypersensitivity (Chapter 1, figure 1.7), or involve some form of immunological disruption (Park *et al.*, 1987; Coleman,JW, 1990). However, there is often very little laboratory evidence to support an immune-mediated mechanism. Carbamazepine is no exception to this, where immune system involvement has been assumed on the basis of clinical manifestations (Dreifuss and Langer, 1987). This can be clearly seen in the patients described in chapter 2 where several clinical features are present indicating an immune-mediated reaction. First, the reaction occurred between 1-6 weeks after the start of carbamazepine, indicating that a period of sensitisation was required. Secondly, rechallenge in 2 patients resulted in the recurrence of the reaction much more quickly than on primary exposure and finally, so-called hypersensitivity manifestations such as fever and eosinophilia accompanied the organ-directed toxicity. Some evidence for specific cell mediated immunity has been provided in some studies of carbamazepine-hypersensitive patients by showing proliferation of their lymphocytes *in vitro* on exposure to the drug (Virolainen, 1971; Houwerzijl *et al.*, 1977; Zakrzewska and Ivanyi, 1988), although this can be unreliable producing false negatives (Houwerzijl *et al.*, 1977; Merk *et al.*, 1988), presumably because the relevant "antigen" is not generated *in vitro*.

The current understanding of drug hypersensitivity is based largely on the hapten hypothesis (figure 3.1; Park *et al.*, 1987). The drug, being of low molecular

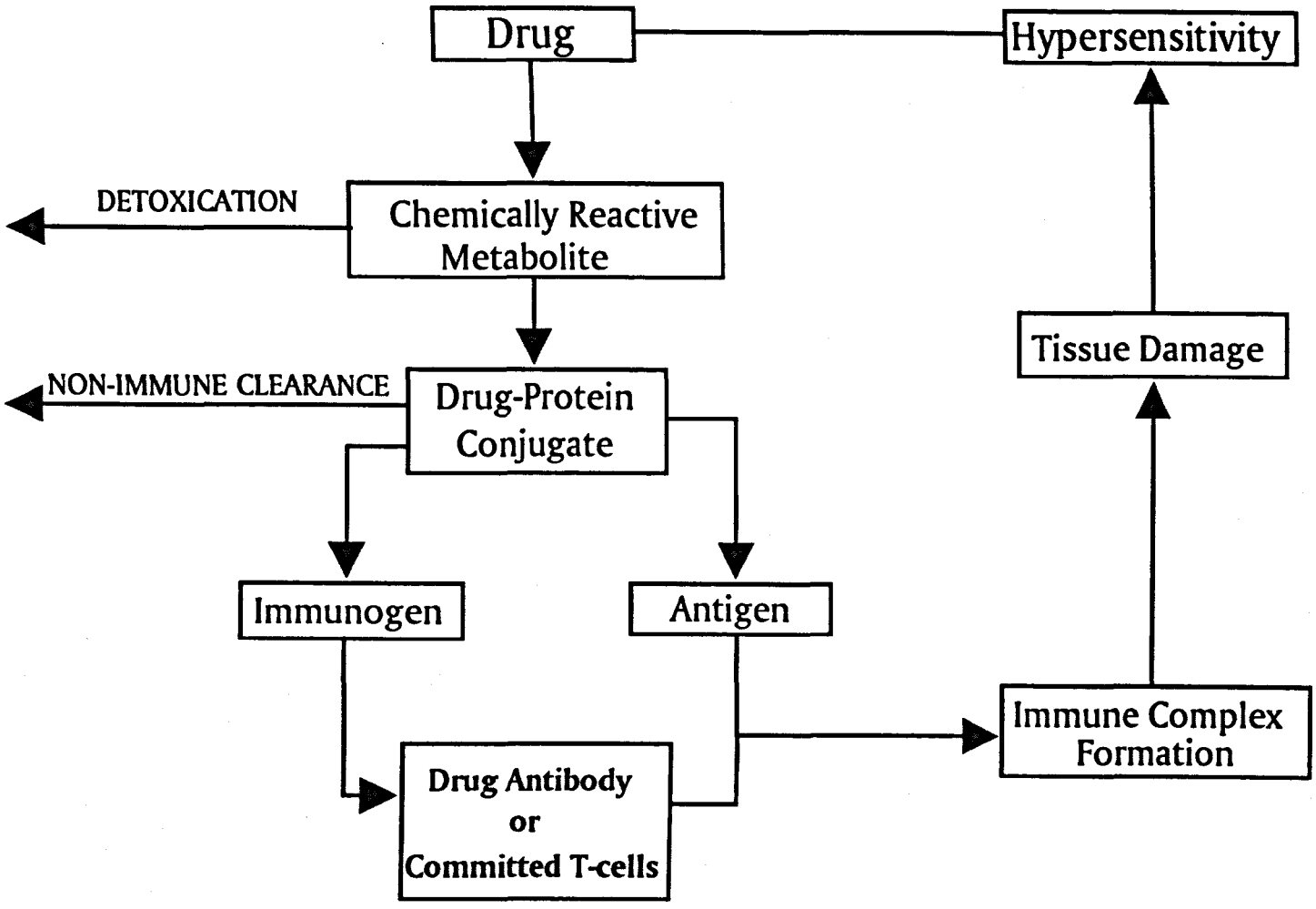


Figure 3.1. The hapten hypothesis of drug hypersensitivity

weight, is not immunogenic *per se*, but only becomes so when it becomes covalently bound to a macromolecular carrier such as a protein (Park *et al.*, 1987). The hypothesis is based on studies carried out on penicillin, which can react directly with proteins, resulting in an immune response in some individuals (Park *et al.*, 1987). However, most drugs, including carbamazepine, are not intrinsically chemically reactive, and have to be bioactivated by drug metabolising enzymes to form chemically reactive species which can then bind to proteins, thereby acting as haptens (Park and Kitteringham, 1990b). The resulting immune response often involves both the cellular and humoral fractions of the immune system, being directed against the drug itself or the neoantigen created by interaction of the drug with the macromolecule (chapter 1). Alternatively, native determinants may no longer be recognised as "self" as a result of chemical modification of adjacent structures leading to "bypass" of immune suppression (Allison *et al.*, 1971), and hence resulting in an autoimmune response (Pohl *et al.*, 1988; Coleman, 1990).

The laboratory diagnosis of allergic reactions to most drugs, particularly in those which have to undergo bioactivation to from drug-macromolecule conjugates, has been hampered mainly because the nature of the drug-derived antigen is unknown, and thus relevant test antigens cannot be synthesised for use in laboratory tests such as RAST, ELISA and lymphocyte transformation (Park *et al.*, 1987; Coleman, 1990). A further problem is that patients with adverse drug reactions are often identified retrospectively when the acute phase of the reaction has settled. Investigation during the acute phase may be particularly important to detect anti-drug antibodies and for histological examination of accessible tissues affected by the drug toxicity, for example the skin.

Two avenues which can be pursued in order to provide evidence of the immunological nature of an adverse drug reaction, and which also overcomes to some extent our ignorance of the chemical nature of the drug-derived antigen, are first, the demonstration of autoantibody formation, and secondly, immunohistochemical examination of affected tissues using specific monoclonal antibodies directed against specific lymphocyte subsets (figure 3.2).

Drug-induced autoimmunity has been well described occurring with drugs such as hydralazine, procainamide and α-methyl dopa (Parker, 1982). Autoantibodies detected with such reactions are often non-organ specific, for example antinuclear antibodies (Homberg *et al.*, 1985), and can be demonstrated by techniques such as immunofluorescence (Homberg *et al.*, 1985). More recently, the technique of immunoblotting (also termed Western blotting) has been used to detect drug-induced organ-specific autoantibodies, particularly in cases of drug-induced hepatotoxicity (Beaune *et al.*, 1987; Bourdi *et al.*, 1990). Western blotting is a powerful technique that combines the resolving power of electrophoresis with the specificity, sensitivity and simplicity of solid-phase immunoassays. In this technique, a complex mixture of proteins is separated by electrophoresis according to molecular weight, transferred to a membrane support (termed blotting) to produce a replica of the protein separation pattern from the gel, and probed with antibodies (either purified antibodies or those present in serum of hypersensitive patients) to identify any antigens to which the antibody might bind. A schematic representation of the immunoblot method which might be used to detect specific autoantibodies is shown in figure 3.3.

Histological examination of an organ affected by an idiosyncratic drug

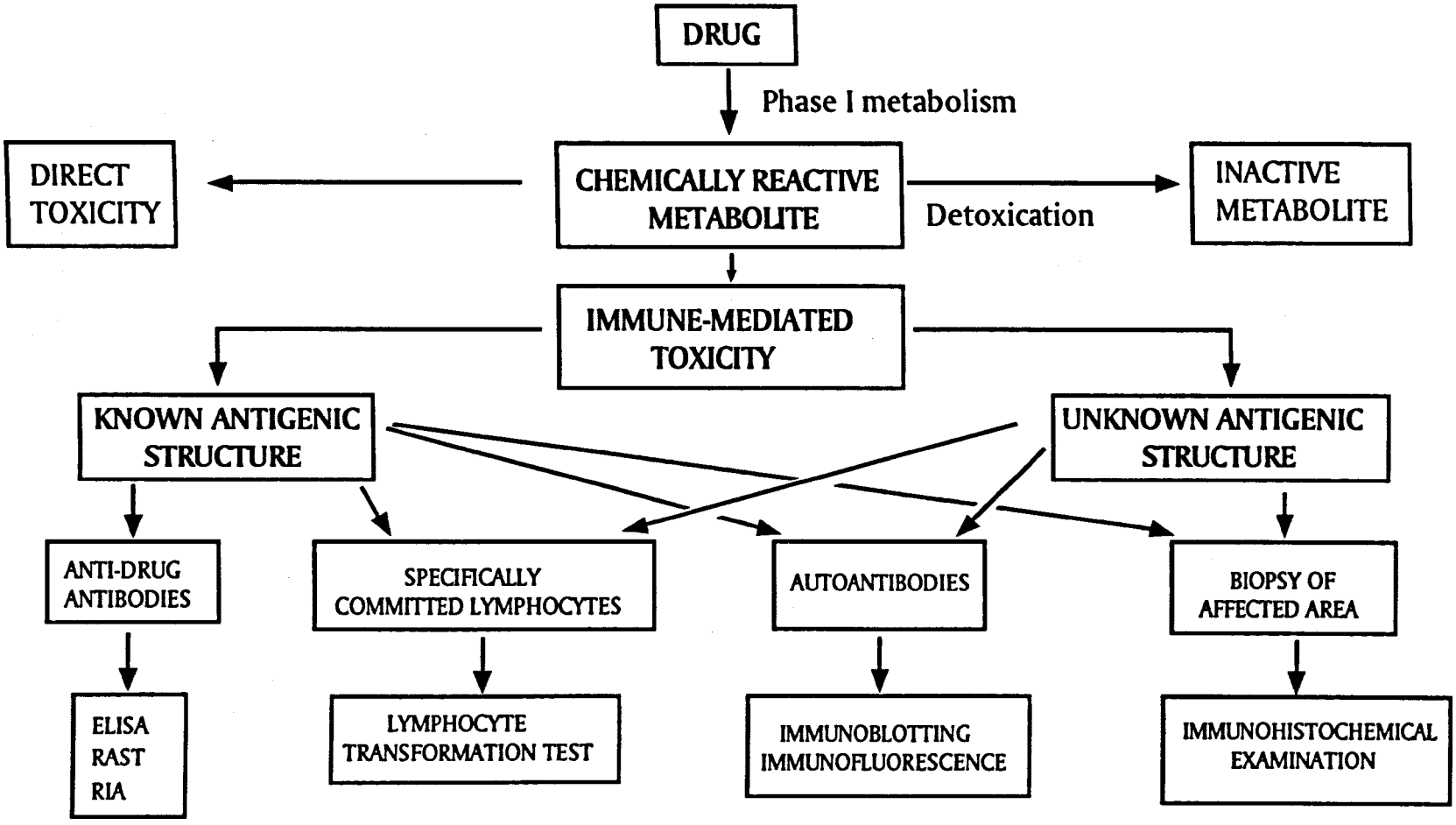


Figure 3.2. Schematic representation of investigations which can be undertaken to investigate the immunological basis of an adverse drug reaction.

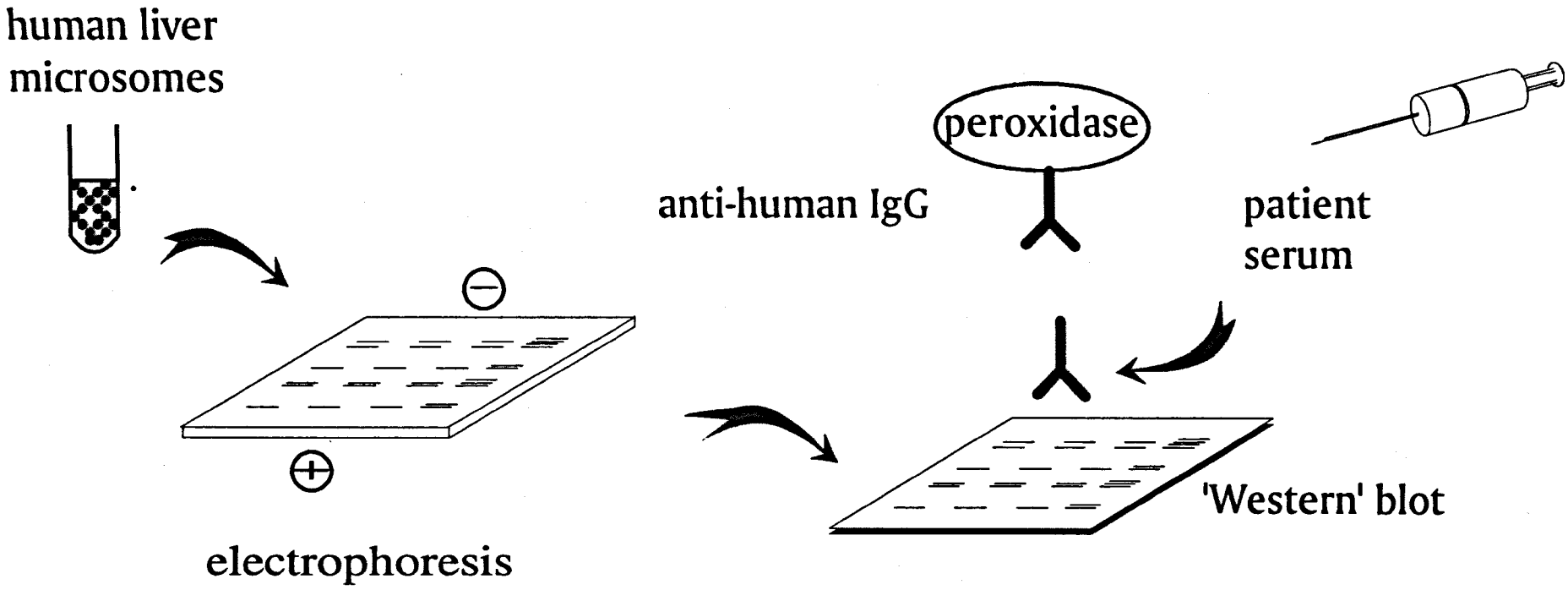


Figure 3.3. Schematic representation of immuno- (Western) blotting and its use in identifying specific autoantibodies.

reaction often shows infiltration by white blood cells, the presence of macrophages indicating inflammation, while eosinophilic infiltration is often used as evidence of the allergic nature of a reaction. However, such conventional histological techniques only provide limited information. The development of immunohistologic techniques using specific monoclonal antibodies can provide an insight into the functional characteristics and specificity of infiltrating T- and B-lymphocytes (Frew and O'Hehir, 1992), and by using a knowledge of their specific function, their possible role in the immunopathogenesis of the adverse drug reaction can be deduced.

In this chapter, two patients who presented with idiosyncratic reactions to carbamazepine have been investigated during the acute phase of their illness in order to determine whether the immune system is involved in the pathogenesis of the adverse reaction. The first patient who had severe hepatotoxicity (patient SR) has been investigated for the presence of a circulating autoantibody to hepatic proteins, while the second patient who had toxic epidermal necrolysis (patient JS), has been investigated by immunohistochemical techniques using biopsies of affected skin. In addition, lymphocytes from both patients have been examined to determine whether their cells are more sensitive to toxic metabolites of carbamazepine generated *in situ* by a microsomal system than controls, as was found in the patients described in chapter 2.

3.2. Description of the carbamazepine hypersensitive patients

Patient SR

A 16 year old boy was started on carbamazepine (400mg/day) for recent onset

generalised epilepsy. He was not on any other drugs. Four weeks after the start of therapy, he developed a fever, followed by a generalised rash (after 5 weeks) and jaundice (6 weeks).

On admission to hospital, the patient had a temperature of 39°C, and had a generalised erythematous, desquamating rash. He was icteric and had palpable cervical lymph nodes and liver. Laboratory investigations showed a mild leucocytosis (11.3 x 10^9/l) with an eosinophilia (39%) and atypical lymphocytes on a peripheral blood film. The liver function tests were as follows: total serum bilirubin 45μmol/l (normal range (NR) 2-17), alkaline phosphatase 617 IU/l (NR 35-130), alanine aminotransferase 328 IU/l (NR 7-45), and gamma-glutamyl transferase 310 IU/l (NR 0-65). Immunological screening, including anti-nuclear antibody, anti-mitochondrial antibody, anti-smooth muscle antibody and anti-reticulin antibody were all negative. Tests for EB virus (including glandular fever slide test) and hepatitis viruses were negative, while cytomegalovirus antibodies were weakly positive.

Carbamazepine-induced hepatotoxicity was diagnosed and the carbamazepine withdrawn. The patients clinical condition and abnormal laboratory investigations improved over the subsequent 6 weeks, the abnormal physical signs disappearing in the same order in which they appeared. No specific therapy was required.

Patient JS

A 19 year old female who had been an epileptic since the age of 12 years was started on carbamazepine (600mg daily) because of poor control of her epilepsy. She had previously been treated with sodium valproate 400 mg b.d. which was continued. She was also taking an oral contraceptive (Norinyl™) for dysmenorrhoea.

Fourteen days after the start of carbamazepine the patient developed a rash which began as a pruritic erythematous maculopapular eruption of erythema multiforme spreading up the body from the legs. After 2 days vesico-pustules appeared on the face and trunk with erosion of the lips. By 4 days large bullae and sheets of toxic epidermal necrolysis were present on the limbs and trunk. This was accompanied by fever (40°C), malaise, retrosternal soreness and cough, oral ulceration and conjunctival injection.

The patient was admitted to hospital at 48 hours. Carbamazepine was discontinued and oral prednisolone 120 mg daily commenced. After 48 hours (4 days from onset) as there was extension of the rash with vesicle formation and systemic symptoms, plasmapheresis was commenced. Five daily exchanges of 3 litres were performed with 6 bags of fresh frozen plasma and 3 of purified protein fraction. The activity of the toxic epidermal necrolysis subsided during the plasmapheresis but it became active again when it was discontinued. Therefore gammaglobulin infusion (Sandoglobulin) 0.4g/kg/day, was commenced and continued for 5 days. During this time the fever and malaise settled and extensive re-epithelialization occurred. During the peak of the process and while plasmapheresis was in progress, there was a transient fall in the leucocyte count down to 2.1 x 10^9/l and elevation of alanine aminotransferase (peak level 222U/l) but not of bilirubin or gamma-glutamyl transferase. The C-reactive protein, an acute phase reactant, was measured simultaneously in the serum and blister fluid on day 4 and was found to be elevated in the former (58mg/l; NR <13mg/l) but not in the latter (0mg/l). In contrast, interleukin-6, another acute phase reactant, showed the opposite response, the plasma level being 21.9U/ml while the blister fluid level on the same day was

505U/ml.

All the above investigations carried out in these two patients were done as part of their routine clinical care and were performed by the Departments of Haematology, Chemical Pathology, Immunology and Virology at the Royal Liverpool University Hospital.

3.3. Methods

3.3.1. Materials

Glycine, sodium dodecylsulphate (SDS), tris(hydroxymethyl)methylamine (tris), casein hammersten grade, triton X 100, glycerol, bromophenol blue, Coomassie blue R250 and ammonium persulphate were obtained from BDH (Liverpool, Merseyside). Acrylamide, N,N-methylene-bis-acrylamide, and unstained molecular weight markers were purchased from Biorad (California, USA). Thimerosal and 4-chloro-1-naphthol were obtained from Sigma (Poole, Dorset). The reagents required for determination of *in vitro* cytotoxicity were obtained as outlined in chapter 2, section 2.2.1. All other reagents were of analytical grade.

3.3.2. Antisera used for immunoblotting to identify circulating autoantibodies

Serum from patient SR was obtained on three occasions: at the time of the adverse reaction, 2 months and 6 months after the reaction. Serum was also obtained from patient JS during the acute phase.

Sera for comparison (hereafter called 'control sera') were obtained from 24 individuals, as follows: 12 normal, healthy volunteers who had never been exposed

to carbamazepine, three patients on chronic carbamazepine therapy without adverse effects, six patients with hepatitis induced by drugs other than carbamazepine (three due to lofepramine, one due to sulphasalazine, one due to phenytoin, and one due to norfloxacin). In addition, sera from three other patients with carbamazepine-induced toxicity were also used for comparison; in two of these patients, the sera were collected during the acute phase, although they did not have hepatic manifestations of toxicity (one with Stevens-Johnson syndrome and one with aplastic anaemia), while in the other patient who had carbamazepine-induced hepatitis (full clinical details of severity of the reaction were unavailable), the serum was collected one year after the occurrence of his reaction.

3.3.3. Preparation of human and animal microsomes and liver cytosol

Washed microsomes were prepared from nine human livers as described in section 2.2.5. Donor details are listed in table 3.1. In addition, microsomes were also prepared from one human kidney using the same method used to prepare human liver microsomes (section 2.2.5.). Clinical details of the kidney donor were unavailable. Ethical approval for use of human tissue was granted and informed consent obtained from donors' relatives.

Microsomes were also prepared from the livers of two male Wistar rats, two male CBA/ca mice and six phenobarbitone-treated (60mg/kg body weight per day i.p. in 0.9% w/v saline for 3 days) male CBA/ca mice. Prior to the preparation of microsomes from the animal livers, the animals had been fasted for 24h, and then killed by cervical dislocation, their livers dissected out, and the microsomes prepared as described previously (section 2.2.5.).

Table 3.1.

Details of human liver donors from which microsomes were prepared for the investigation of anti-hepatic antibodies by immunoblotting

Age	*Sex*	*Cause of death*	*Drugs*	*Histology*
46	F	Subarachnoid haemorrhage	Phenytoin, Phenobarbitone	Normal
27	M	Head injury	None	Normal
35	M	Subarachnoid haemorrhage	None	Normal
45	M	Road traffic accident	None	Normal
49	F	Road traffic accident	None	Normal
21	M	Herpes simplex encephalitis	Acyclovir, dopamine, heparin	Normal
27	M	Head injury	None	Normal
18	M	Head injury	None	Normal
66	F	Brain haemorrhage	None	Normal

The supernatant after the first 100,000g centrifugation step while preparing both human and animal microsomes was used as cytosol. The protein content was determined by the method of Lowry *et al.* (1951). The microsomes and cytosol were stored at -80°C until required for use.

The cytochrome P450 content of the microsomes prepared from phenobarbitone-pretreated mice which were used in the *in vitro* cytotoxicity assay was measured according to the method of Omura and Sato (1964), as described in section 2.2.6. The P450 content of the pooled microsomes prepared from the livers of six animals was 1.2nmol/mg protein.

3.3.4. Isolation of mononuclear leucocytes from human blood

Mononuclear leucocytes (MNL) were isolated from blood freshly drawn from the two patients (SR and JS) and four healthy male volunteers who acted as controls by centrifugation through a density gradient as described in section 2.2.7. The viability of the cells upon isolation was >95%. The blood from patients SR and JS was obtained 6 and 4 months, respectively, after their adverse reactions.

3.3.5. Determination of metabolism-dependent cytotoxicity of carbamazepine

As decribed in the preceding chapter, phenobarbitone-induced mouse microsomes were again used as a generating system to obtain a consistent and sufficient metabolic turnover of carbamazepine, so that the only variable in the system was the difference between cells obtained from different individuals.

A similar protocol to that adopted in chapter 2 (section 2.2.8.) was used in that two controls were tested at the same time as the patients, and a blind protocol was adopted.

The MNL (1×10^6/ml) were incubated with carbamazepine (50μM) and phenobarbitone-induced mouse liver microsomes (0.5mg) in the presence or absence of NADPH (1mM) for 2h in HEPES-buffered medium (final volume 1ml) at 37°C. The drug was added in 10μl of methanol, which as a 1% (v/v) solution, was non-toxic. Following the 2h incubation, the cells were recovered and resuspended in drug-free medium as previously described (section 2.2.8.). After 16h, the cytotoxicity was determined by trypan blue dye exclusion by counting at least 200 cells, a failure to exclude dye being taken as an index of cell death (section 2.2.9). The incubations were performed in quadruplicate and the results are presented as the increase in cell

death above the baseline.

3.3.6. SDS-PAGE electrophoresis and immunoblotting of microsomal and cytosolic proteins

Electrophoresis can be performed under continuous conditions where the same buffer ions are present in the gel and the electrode solutions, or under discontinuous conditions, in which the buffer ions in the gel and electrode solution differ (Hayes and Stockman, 1989). The advantage of the latter system is that it gives a better resolution of the final protein separation, and thus this system has been used in the present chapter. In a discontinuous buffer system, the stacking gel, which concentrates (or stacks) proteins into a very sharp zone, is cast on top of a resolving gel, which then unstacks the proteins, the final migration being dependent on their size and charge (figure 3.4).

Preparation of polyacrylamide resolving and stacking gels

The resolving gel was prepared by mixing a solution containing 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide (21ml), tris-HCl buffer (22.5ml, 1.5M tris, 1M HCl, pH 8.7) and distilled water (46.5ml) with aliquots of 20% (w/v) SDS (450µl), N N N',N'-tetramethylethylenediamine (TEMED, 45µl) and 10% (w/v) ammonium persulphate (450µl) to initiate polymerisation. This was immediately applied to the top of the gel moulds (prepared by placing a glass and ceramic plate together, separated by a pair of spacers) in the casting stand to a level approximately 3cm from the top. The resolving gels were allowed to set for at least 1h at 20°C, resulting in a resolving gel with a final concentration of 7% polyacrylamide. After

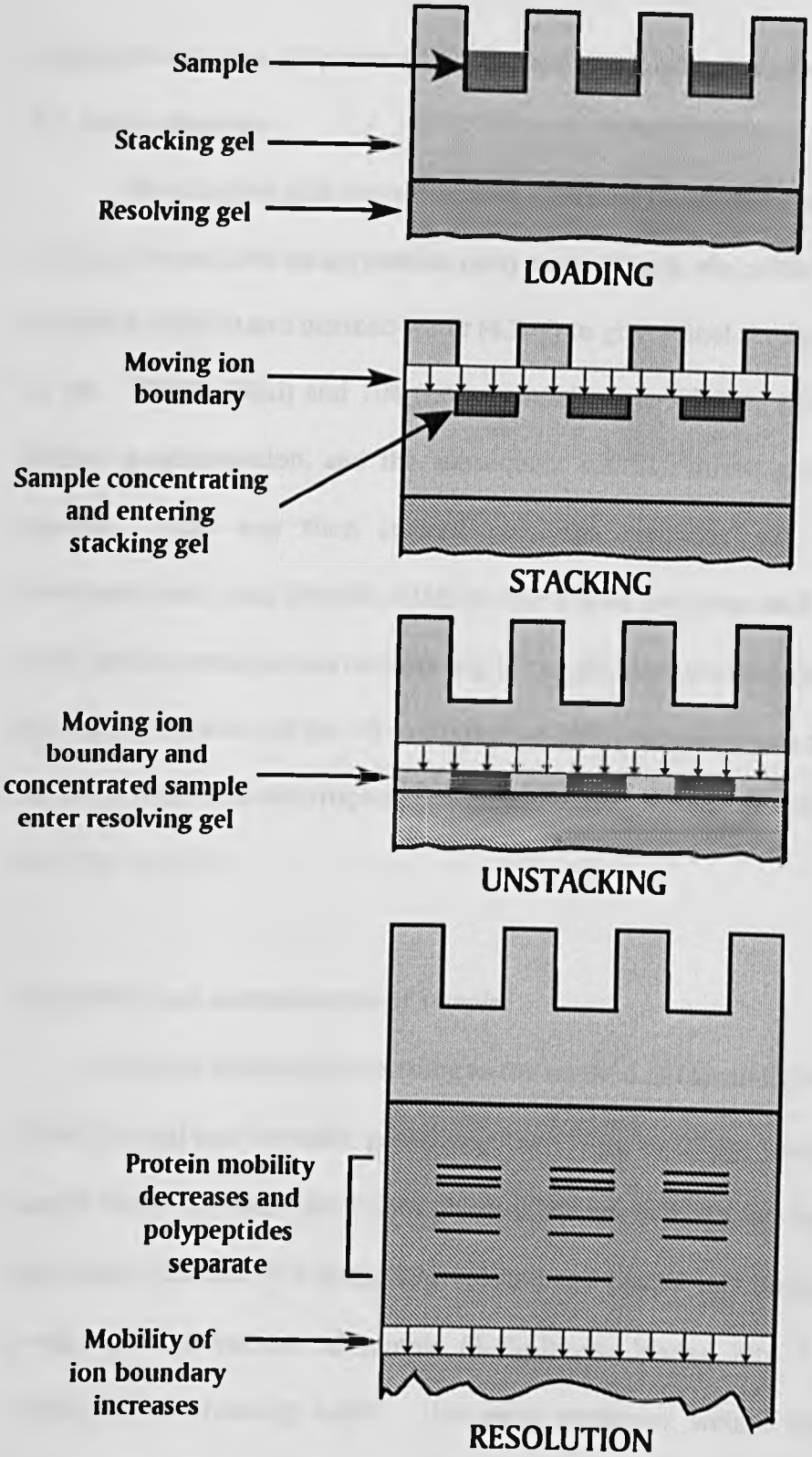


Figure 3.4. SDS-PAGE electrophoresis of proteins using a discontinuous buffer system. The stacking gel concentrates the proteins (stacks), which are then unstacked as they enter the resolving gel, their subsequent migration being dependent on their size and charge. Adapted from Hayes and Stockman (1989).

polymerisation, the gel plates were wrapped in cellophane and kept at 4°C until used for electrophoresis.

The stacking gels were prepared by making a solution containing 30% (w/v) acrylamide and 0.8% bis-acrylamide (w/v) stock (1.3ml), tris buffer (3.7ml, 0.25M tris, 0.2% SDS, pH 6.8) and distilled water (4.7ml) to give a final acrylamide concentration of 4%. TEMED (30µl) and 10% (w/v) ammonium persulphate (50µl) were added to initiate polymerisation, and the subsequent solution mixed gently with a Pasteur pipette. This was then layered over the resolving gel, supported in an electrophoresis tank (Hoeffer, USA) to the top of the plate and either a 10 or 15 track plastic comb pushed into the top of the gel plate to create wells. The stacking gel was allowed to set for 15 to 30 min at 20°C, the comb was then removed and the wells filled with electrophoretic running buffer (25mM tris, 192mM glycine, 0.1% w/v SDS, pH 8.3).

Preparation and electrophoresis of samples

This was performed according to the method of Laemmli (1970). The samples of microsomal and cytosolic proteins (either 10µg or 25µg) were diluted 1:1 with sample buffer (0.13mM tris, 2.2M glycerol, 8% w/v SDS, 1% w/v bromophenol blue) containing dithiothreitol (6mg, 39µmols/ml) and placed in a boiling water bath for 5 min. A fixed volume of sample (5-15µl) was loaded into a well filled with electrophoretic running buffer. Unstained molecular weight markers were also included on each gel, and comprised myosin (200 kDa), ß-galactosidase (117 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa). Once the samples had been loaded into the wells, the compartment immediately

behind the plate (approximately 20ml) and the base of the tank (to a depth of about 2cm) were filled with buffer to complete the circuit between the electrodes.

A current of 30mA/gel was then applied at a constant temperature of 10°C maintained with a cooling unit (Neslab Instruments, USA), until the samples had reached approximately 1cm from the bottom of the resolving gel (approximately 1h).

Electrophoretic transfer of proteins from the gel to nitrocellulose membrane filters

The separated proteins were transferred from the gel to nitrocellulose membrane filters (NTC) electrophoretically using the method of Towbin *et al.*(1979).

A tray filled with transfer buffer (16mM tris, 120mM glycine, 20% v/v methanol, pH 8.3) was used to keep all the components wet and prevent air bubbles. Filter paper (Whatman, Merseyside), NTC (Schleicher and Schuell, mean pore size 0.45μm) and supporting sponge were first soaked in transfer buffer. A transfer sandwich was then prepared by layering the resolving gel between these components in the following order: supporting sponge, filter paper, resolving gel, NTC, filter paper and finally another supporting sponge. Any air bubbles were removed by firmly smoothing with a glass rod. The transfer sandwich was then clamped and placed in a transfer electrophoresis unit filled with transfer buffer, placing the NTC side closest to the anode and the resolving gel closest to the cathode within the sandwich. The proteins were then transferred at 90mA overnight or 150mA for 2h at 4°C.

Following transfer, the NTC was used for either antibody overlay (see below) or was stained for protein with 1% (w/v) amido black in 45% (v/v) methanol and 10% (v/v) glacial acetic acid for 2 min, and then repeatedly washed with 70% methanol and

2% glacial acetic acid to remove non-specific dye.

Immunoblotting of the nitrocellulose blot

Prior to blotting with antibody, non-specific binding sites were blocked with casein buffer (2.5% (w/v) casein, 154mM NaCl, 10mM tris, 0.5mM thimerosal; pH 7.6) for 1h at room temperature. The blots were then incubated with sera from the hypersensitive patient or controls diluted 1:20, 1:50 or 1:100 in casein washing buffer (casein 0.5% (w/v), 0.02% (w/v) thimerosal, 154mM NaCl, 5mM tris; pH 7.6) for 3h, followed by successive washes with casein washing buffer for 10 min, casein buffer containing 0.1% (w/v) SDS and 0.5% (w/v) triton X 100 for 5 min (to remove non-specifically bound plasma proteins and antibodies) and then twice for 10 min each in casein washing buffer again. The second antibody, horseradish peroxidase conjugated goat anti-human IgG (diluted 1:250 in casein washing buffer; Tissue Culture Services, Bucks., England) was then incubated with blots overnight at room temperature. This was followed by successive washes with casein washing buffer (4 x 10 min) and tris-saline buffer (50mM tris, 0.2mM NaCl; pH 7.4; 4 x 10 min). Sites of antibody binding were visualised by incubation of the blots with 3.4mM 4-chloro-1-naphthol in 17% (v/v) methanol with 0.05% (v/v) H_2O_2 in tris-saline buffer (5-10ml). The reaction was terminated by washing the NTC under tap water for 5 min and the blots dried between filter paper overnight. The apparent molecular mass of any bands detected was determined by comparison of their electrophoretic mobilities with mobilities of marker proteins of known molecular mass.

To determine whether any protein band recognised by the patient sera was dependent on carbamazepine, human liver microsomes were preincubated with

carbamazepine (50μM) in the presence and absence of NADPH (1mM) at 37°C for 2h prior to electrophoresis and immunoblotting with the patient sera. In addition, the effect of preincubation of patient sera with free carbamazepine (2mM) was also investigated prior to immunoblotting.

3.3.7. Histological and immunohistochemical studies of skin biopsies obtained from patient JS

This was performed by Prof. Friedmann, Department of Dermatology, Liverpool University. Biopsy specimens were obtained from the cutaneous lesions of blistering erythema multiforme from patient JS. The biopsies were snap frozen in isopentane chilled with liquid nitrogen, mounted in OCT embedding compound (Miles Laboratories, UK), stored at -70°C and processed for immunohistochemical examination. Cryostat tissue sections (5μ) were air-dried and fixed in ice cold acetone for 10 min before being stained with a panel of antibodies (Table 3.2) that identified leucocyte types, adhesion molecules and various cytokines. In most cases, binding of primary antibodies was visualized with a commercial biotinylated secondary antibody followed by streptavidin-peroxidase complex (Zymed Histostain-SP, Cambridge Bioscience, Cambridge, U.K.). Endogenous peroxidase was quenched by incubation of sections in fresh 3% (v/v) hydrogen peroxide in methanol before addition of aminoethyl carbazole (AEC) as chromogen. Binding of antibodies against CD3, CD4, CD8, TNFα, and interleukin 1-α (IL-1α) was visualized best with a peroxidase-anti-peroxidase method: A peroxidase-conjugated secondary anti-immunoglobulin of appropriate species specificity (Dakopatts, High Wycombe, U.K.) was followed by complexes of peroxidase/monoclonal anti-peroxidase (Dakopatts).

Table 3.2.

Antibodies used to identify human inflammatory constituents

Antigen	Monoclonal	[1]Antibody/Clone	[#]	Antigen Distribution
CD1a	NA 1/34	(Serotec)	S	Langerhans cells (LC)
CD1b	4.A7.6	(Serotec)	A	Human dermal dendritic cells
CD3	T3-4B5	(Dako)	S	All normal human T cells
CD4	MT310	(Dako)	S	Human T helper/inducer cells
CD8	DK25	(Dako)	S	Hu. T cytotoxic/suppressor cells
CD45RO	UCHL-1	(Dako)	S	Memory T cells
CD25	ACT-1	(Dako)	S	Specifically activated T cells
CD14/LeuM3	M1/P9	(Becton Dickinson)	Ig	Human macrophage
CD56				Natural killer cells
ICAM-1	6.5B5*		S	Endothelial cells
ELAM-1	1.2B6*		S	Endothelial cells
VCAM-1	1.4C3*		S	Endothelium and dermal dendocytes
HLA-DR				LC, activated T cells
Polyclonal Antibodies (Rabbit Hyperimmune sera)				
IL-1α	LP-710	(Beckton Dickinson)		Interleukin 1α
TNF-α	JID9[+]			Macrophages, T cells

[1]All monoclonal antibodies were from mice
#Form of antibody: S = supernatant; A = ascites; Ig = purified Ig
*Antibodies were the gift of Dr. D.O. Haskard, Dept. of Medicine, Hammersmith Hospital, London
[+]Donated by Dr. P.J. McLaughlin, Dept. of Immunology, Liverpool University

They were developed with diaminobenzidine (Sigma, Poole, Dorset) and 1% hydrogen peroxide. Sections were counterstained with haemalum and mounted in glycerol and then examined by light microsocopy.

3.4. Results

3.4.1. Metabolism-dependent cytotoxicity of carbamazepine in the presence of phenobarbitone-induced mouse microsomes

The increase in cell death above the baseline for MNL from the carbamazepine-hypersensitive patients SR and JS was greater than for the four controls (figure 3.5a). Taken with the results presented in chapter 2, these data indicate that patients SR and JS may be categorized within a group of carbamazepine-hypersensitive patients whose lymphocytes are more susceptible to carbamazepine metabolites *in vitro* than lymphocytes from appropriate controls (figure 3.5b).

3.4.2. Immunoblot analysis of microsomal and cytosolic proteins

The serum from patient SR (up to the maximum dilution tested 1:100) recognised a protein band with an apparent molecular mass of 94kDa on all human liver microsomes (n=9) used but none of the control sera recognised this band (figure 3.6). Serum from the patient was taken on 3 occasions: all three sera recognised the same band with no apparent diminution in intensity (figure 3.6). The band was not recognised by either the patient or control sera in human kidney microsomes, mouse and rat liver microsomes, or cytosol from human and mouse liver. The serum from patient JS did not recognise any bands in any of the protein

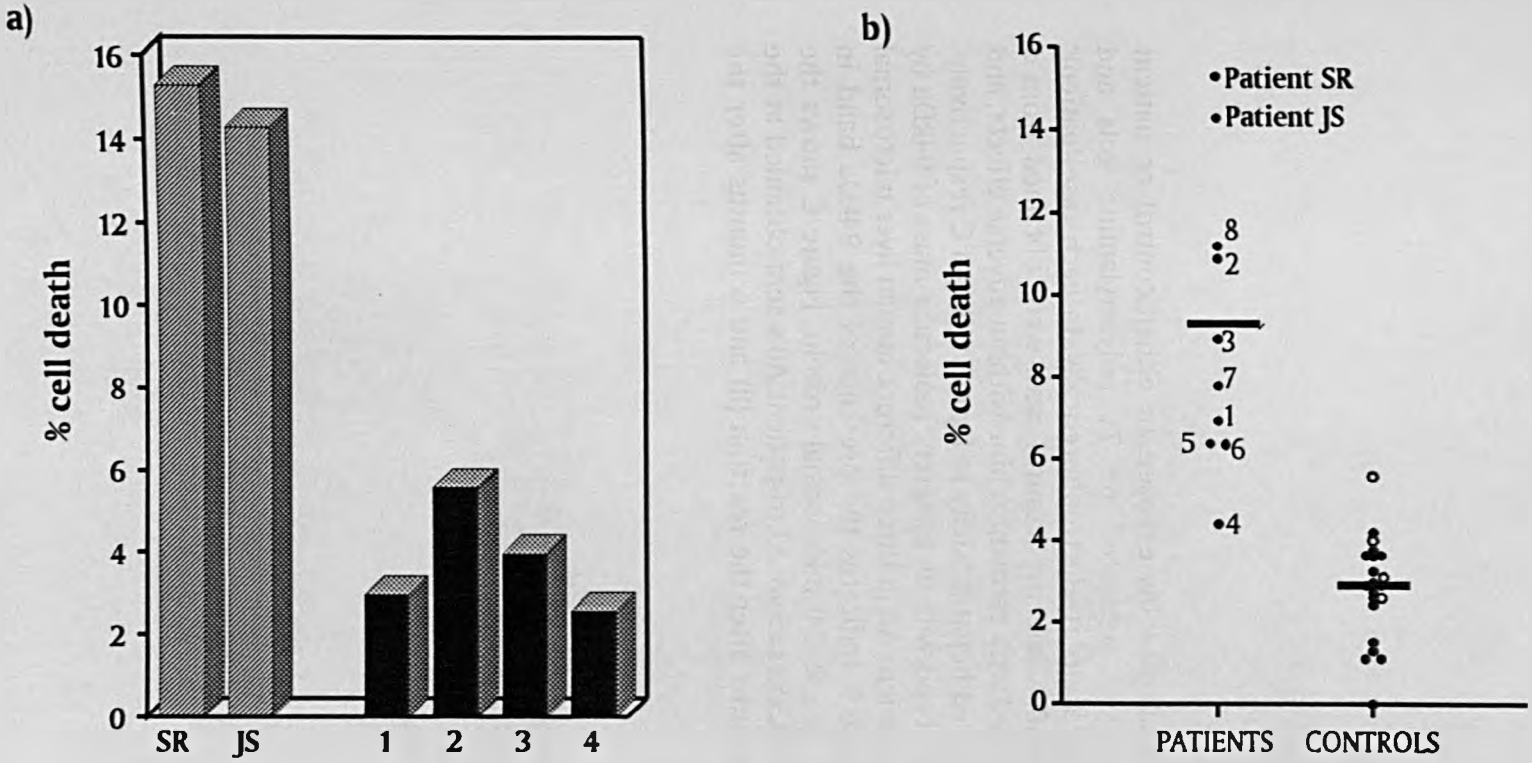
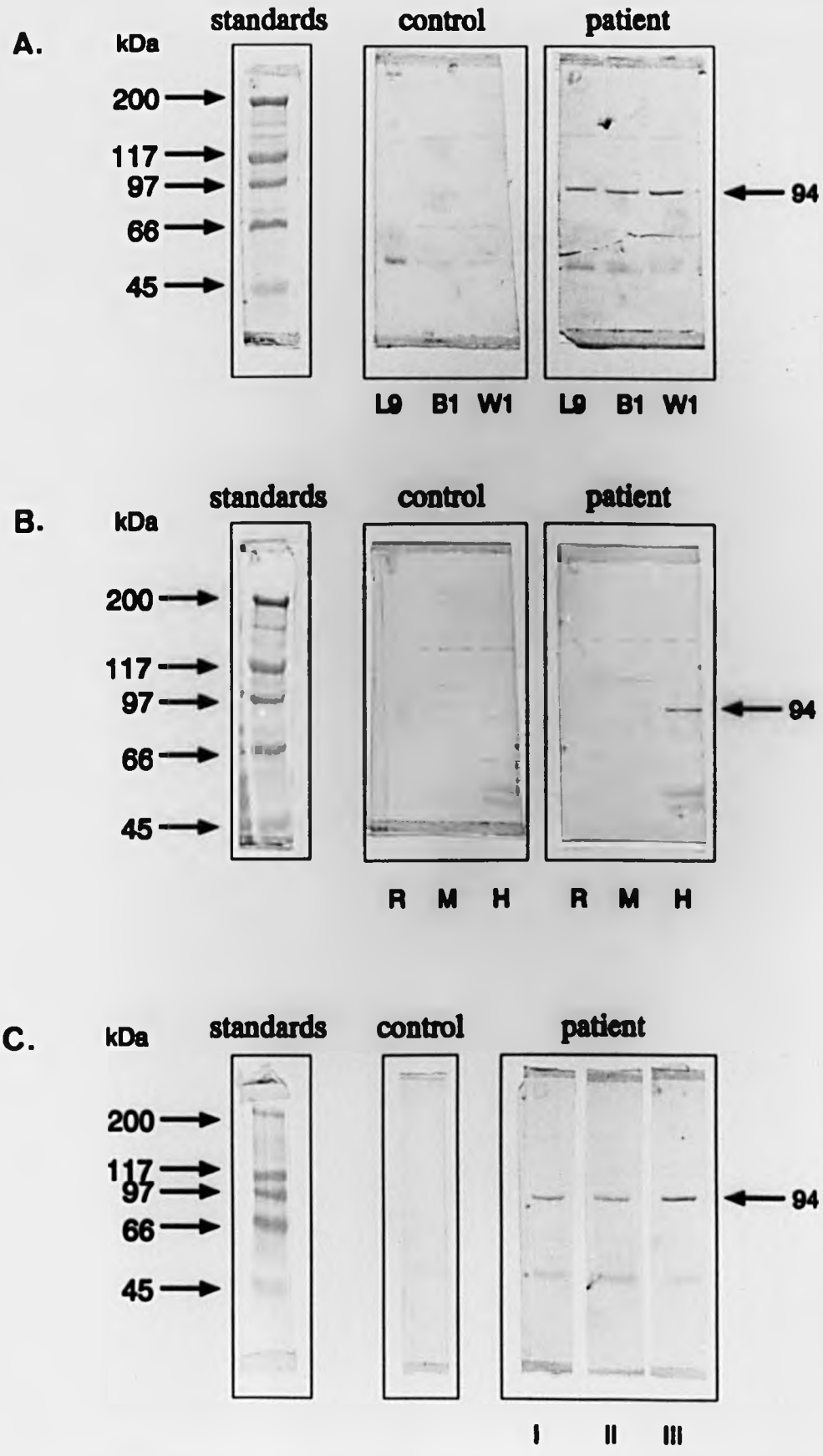


Figure 3.4. A comparison of the metabolism-dependent cytotoxicity of carbamazepine (50μM) in carbamazepine-hypersensitive patients and controls. Graph (a) shows the increase in cell death above the baseline in patients SR and JS and 4 controls, while graph (b) is a comparison of cytotoxicity between the patients and controls described in this and the preceding chapter. The baseline cell death values for patients SR and JS were 8.6 $\pm$ 1.2% and 5.7 $\pm$ 0.6%, respectively. The baseline cytotoxicity of the controls was as follows: 8.6 $\pm$ 0.9%, 12.4 $\pm$ 1.4%, 5.5 $\pm$ 0.3% and 10.6 $\pm$ 0.5% for controls 1, 2, 3 and 4, respectively.

Figure 3.6.

Immunoblots of liver microsomal proteins exposed to either control or patient serum. Microsomal proteins were separated on 7% polyacrylamide gels and transferred to the nitrocellulose support electrophoretically. In each case 'patient' refers to serum from patient AB, while the 'control' sera were obtained from a normal volunteer, a patient treated with carbamazepine without adverse effects, and a patient with lofepramine-induced hepatotoxicity in figure A, B and C respectively. Figure A shows recognition of a band with an apparent molecular mass of 94kDa by the serum (dilution 1:20) from patient AB in three different human liver microsomal samples (L9, B1 and W1). Figure B indicates the presence of the 94kDa band in human (H), but not rat (R) or mouse (M) microsomal protein. Figure C shows the same band in human liver microsomes exposed to patient AB's sera obtained at the height of the reaction (I), 2 months after the reaction (II) and 6 months after the reaction (III).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samples studied.

Preincubation of the human liver microsomes with carbamazepine (50μM; in the presence or absence of NADPH) at 37°C for 2h prior to immunoblotting did not affect the recognition of the 94kDA protein band by serum from patient SR. In addition, preincubation of free carbamazepine (2 mM) with serum from patient SR did not inhibit antibody binding to the 94kDa band.

3.4.3. Immunohistochemical findings on skin biopsies from patient JS

Dermis

There was a predominantly perivascular infiltrate composed of T lymphocytes ($CD3^+$, $CD45RO^+$) and macrophages ($CD14^+$). The T cells were mainly $CD4^+$ although about one third were $CD8^+$. About 10% of the T cells were stained for CD25, the low affinity interleukin-2 receptor. Significant numbers of $CD1a^+$ Langerhans cells were present in the superficial dermis. Cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1), E-selectin (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were present on dermal microvascular endothelial cells.

Epidermis

There were areas in which keratinocytes were vacuolated and damaged. These areas were depleted or devoid of $CD1a^+$ Langerhans cells (figure 3.7a) but contained many $CD14^+$ macrophages (figure 3.7b). In addition, T lymphocytes were present, with $CD8^+$ cells outnumbering $CD4^+$ cells by 4 to 1 (figure 3.7c). Approximately 25% of the T cells expressed CD25. In and around the areas of

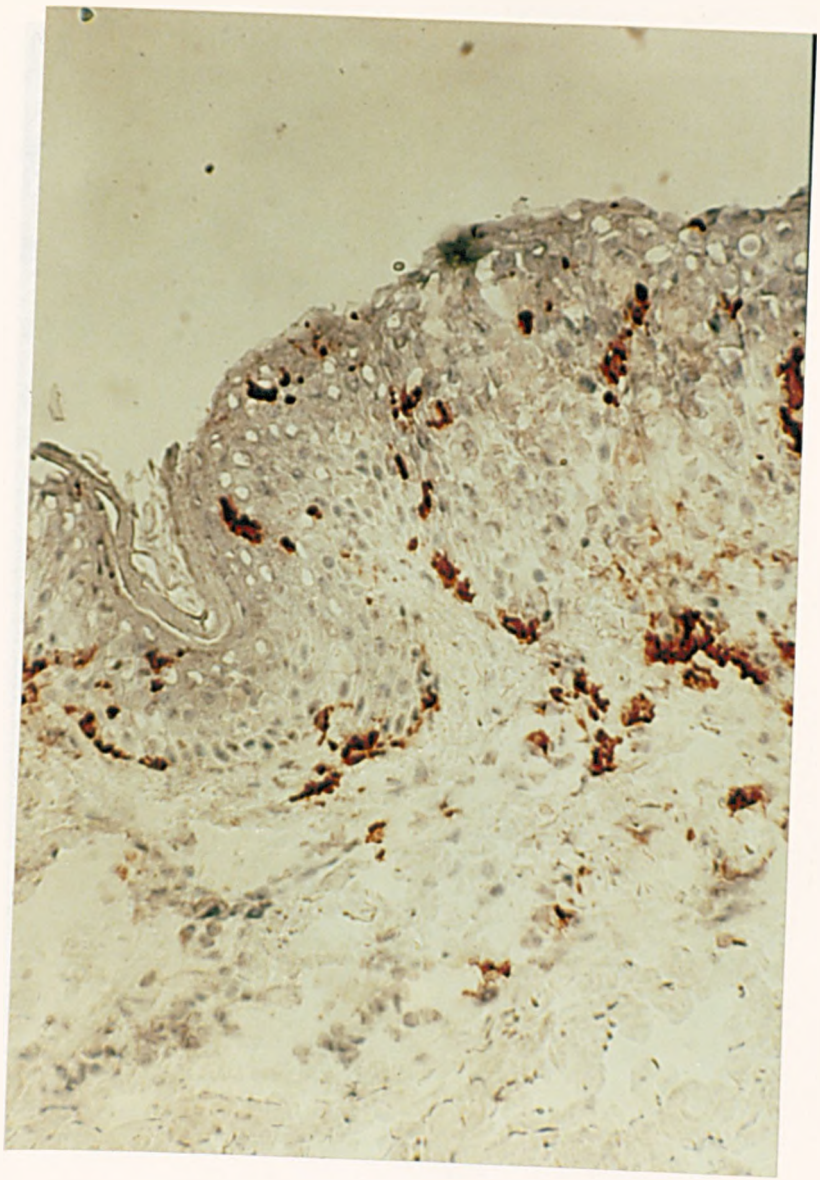


Figure 3.7a. Photograph showing a section of lesional skin taken from patient JS who developed toxic epidermal necrolysis with carbamazepine therapy. The skin section was stained with a monoclonal antibody directed against $CD1a^+$ (Langerhans) cells.

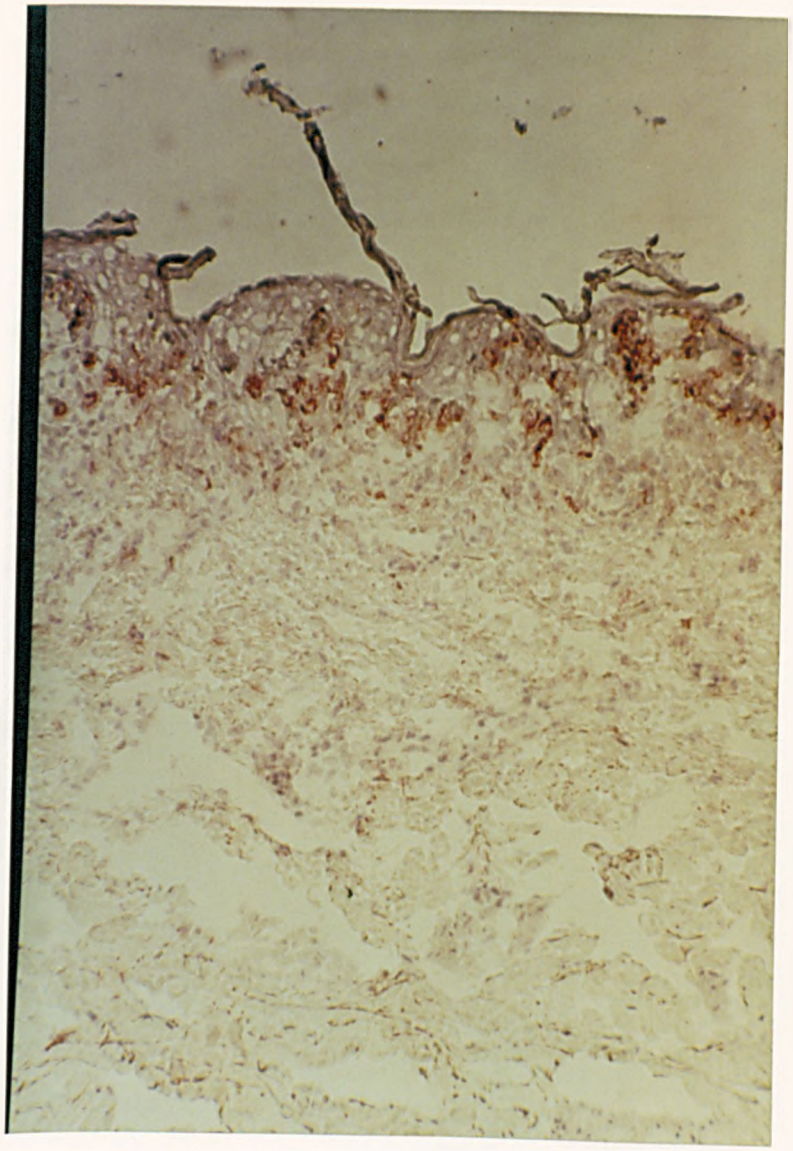


Figure 3.7b. Photograph showing a section of lesional skin taken from patient JS who developed toxic epidermal necrolysis with carbamazepine therapy. The skin section was stained with a monoclonal antibody directed against $CD14^+$ cells (macrophages).

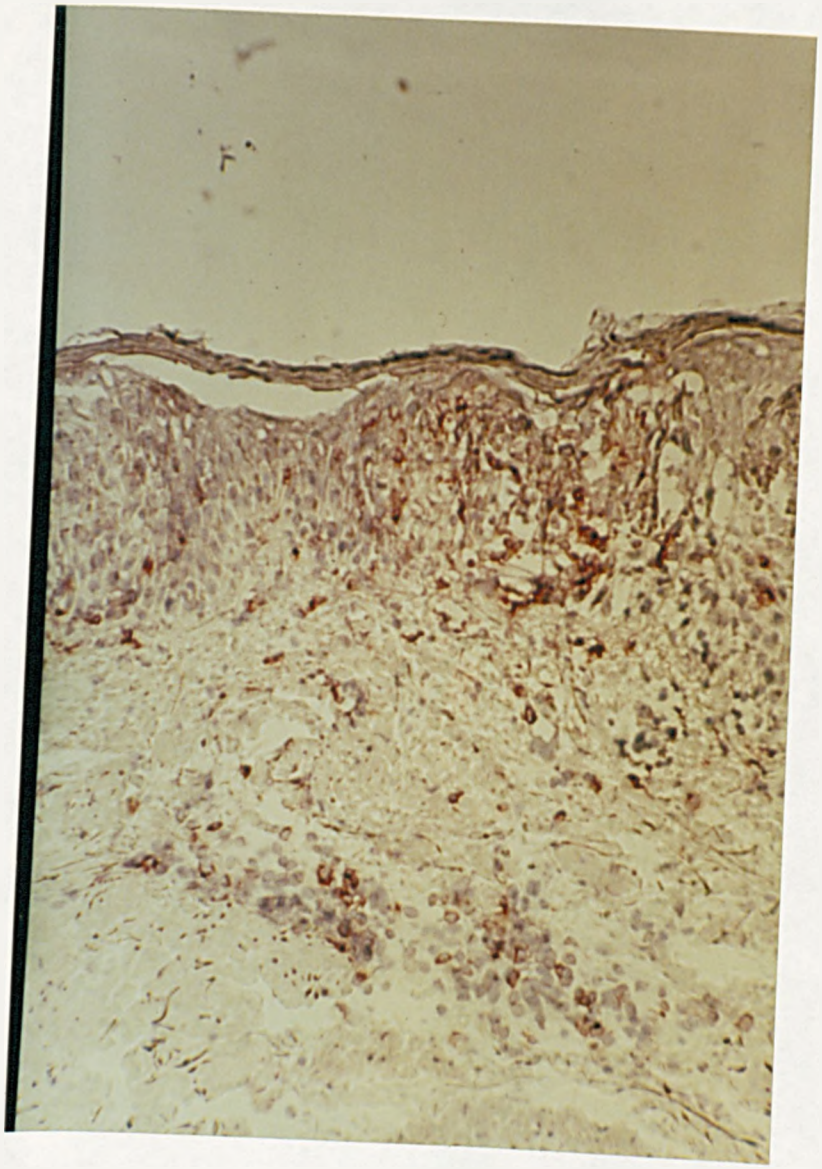


Figure 3.7c. Photograph showing a section of lesional skin taken from patient JS who developed toxic epidermal necrolysis with carbamazepine therapy. The skin section was stained with a monoclonal antibody directed against $CD8^+$ (T cytotoxic/supressor) cells.

damaged keratinocytes, focal areas stained strongly for ICAM-1 and also TNF-α. Keratinocytes also expressed moderate amounts of HLA-DR.

3.5. Discussion

The two patients described in this chapter fit the diagnostic criteria for carbamazepine-induced idiosyncratic toxicity. In both patients the reaction started soon after primary exposure to carbamazepine (at a relatively low dose), in the absence of any other factors known to cause either hepatic injury or toxic epidermal necrolysis. In addition, the reactions improved once the carbamazepine was withdrawn, although in patient JS, further intensive medical treatment was required to ensure recovery. Consistent with the findings of Shear *et al.*, (1988) and the results of the *in vitro* cytotoxicity assay presented in chapter 2 in eight patients with carbamazepine-induced idiosyncratic toxicity, the cells from these two patients also showed enhanced *in vitro* chemical sensitivity to oxidative metabolites of carbamazepine generated *in situ*, than four controls (figure 3.4), providing further supportive evidence for the clinical diagnosis of carbamazepine-induced hepatotoxicity and toxic epidermal necrolysis. Although these results again suggest that predisposed patients may have a deficiency of detoxication, they do not provide any evidence as to the actual mechanism of the toxic reaction.

It is possible that the formation of the chemically reactive metabolite from carbamazepine could lead to toxicity directly by interfering with the function of essential cellular proteins. Alternatively, the reactive species could bind to macromolecules and by acting as an hapten, initiate an immune response. The

clinical and experimental data presented here suggest that the latter mechanism is responsible for carbamazepine toxicity. Furthermore, the ability of the immune system to recognise small quantities of hapten (<1nmol), mount a specific humoral and/or cellular response and subsequently amplify that response (Park and Coleman, 1988) also provides a rationale of how drug bioactivation, usually a minor metabolic pathway (<5%; Park *et al.*, 1987), producing small quantities of the toxic metabolite, can lead to reactions of such severity as those seen in patients SR and JS.

The clinical signs in patients SR and JS developed, respectively, four and two weeks after the start of carbamazepine, which suggests a period of immune sensitisation was required. Patient SR did have hypersensitivity manifestations of rash, fever and eosinophilia, while in patient JS, the skin was the major organ involved and was accompanied by fever but not eosinophilia. Clearly, the presence of such associated manifestations does point towards an immune aetiology, although their absence does not exclude immune system involvement (Watkins, 1991).

Two types of autoantibodies have been described in drug-induced hepatotoxicity, non-organ specific antibodies and organ-specific antibodies (Homberg *et al.*, 1985). Homberg and co-workers (1985) have shown that the non-organ specific antibodies such as antinuclear, antimitochondrial and anti-smooth muscle antibodies were present in 70% of cases of hepatitis caused by a heterogeneous group of drugs including clometacin, fenofibrate, oxyphenisatin and papaverine. Carbamazepine has also been shown to induce antinuclear antibody formation (Alarcon-Segovia *et al.*, 1972), although this was in patients without adverse effects, and thus the significance of this was unclear.

More specific autoantibodies have been described with three drugs associated

with hepatitis, including tienilic acid (anti-LKM2 antibodies), dihydralazine (anti-LM antibodies) and halothane (anti-LKM1; Watkins, 1991). The antibody described in patient SR clearly falls into this group, and is the first example of such an antibody described with carbamazepine toxicity. In addition, it also supports the clinical evidence that the hepatotoxicity caused by carbamazepine was immune-mediated. The autoantibodies found in patients with hepatitis secondary to tienilic acid (Beaune *et al.*, 1987) and dihydralazine (Bourdi *et al.*, 1990) are directed against the cytochrome P450 enzymes (CYP2C and CYP1A2 in humans, respectively) that normally hydroxylate the drug. In contrast, however, the antibody found in patient SR is directed towards a 94kDa protein, which is above the molecular weights of the known cytochrome P450 enzymes. The nature of the protein is not known; it appears to reside in the endoplasmic reticulum, although the possibility of contamination with other subcellular fractions, such as cell membrane, cannot be excluded. However, this is unlikely since the protein band was observed only in human liver microsomes and not in human kidney microsomes or microsomes prepared from rat or mouse liver.

The reason why an antibody should be directed against an intracellular protein is not clear. It is unlikely to be due to the protein being liberated as a result of cellular lysis, since six other patients with hepatitis induced by drugs other than carbamazepine who all had elevated transaminases at the time the blood was taken, did not demonstrate the presence of this autoantibody. It is possible that a reactive metabolite of carbamazepine, which was inadequately detoxified as demonstrated by the results of the *in vitro* cytotoxicity assay, can bind to this protein, behave as an hapten and elicit an immune response against the normal part of the carrier protein.

Such a mechanism has been postulated for the occurrence of anti-cytochrome P450 antibodies due to tienilic acid (Beaune *et al.*, 1987) and dihydralazine (Bourdi *et al.*, 1990). To explain such a series of events, a hypothesis termed "T cell bypass" has been invoked (Homberg *et al.*, 1985; Mackay, 1985): normally, nonresponsiveness to "self" constituents is maintained because there is T cell tolerance to a carrier moiety even though B cells may be responsive to a hapten moiety. However, if a self hapten were to become attached to a foreign moiety as a carrier, e.g. a drug, then T cells could be stimulated and T cell help provided to autoreactive B cells. Alternatively, an autoantibody may be produced because of structural similarity between the drug and autoantigen (Homberg *et al.*, 1985), a mechanism akin to that proposed for the occurrence of autoantibodies directed against CYP2D6 in autoimmune hepatitis, where homology between a part of the epitope on the P450 molecule and several microorganisms has been identified (Gueguen *et al.*, 1991). However, this is unlikely in this case since the antigen-antibody reaction could not be inhibited by prior incubation of the microsomes or serum with carbamazepine.

Most cases of toxic epidermal necrolysis are thought to be drug-induced (Guillaume *et al.*, 1987). Three groups of drugs, the antibacterials (penicillin and sulphonamides), non-steroidal antiinflammatory drugs and aromatic anticonvulsants, have been most frequently implicated to cause toxic epidermal necrolysis (Park *et al.*, 1992). Histopathologic examination of lesional skin provides limited information; it usually reveals a mononuclear infiltrate with areas of necrosis and dermoepidermal separation (Breathnach *et al.*, 1982). Recently, immunohistologic techniques which are more informative have been applied to study the pathogenesis of toxic epidermal necrolysis by determining the lymphocyte subsets present *in situ* (Miyauchi *et al.*,

1991; Villada *et al.*, 1992). These studies have suggested that cell mediated immunity is important in the pathogenesis of toxic epidermal necrolysis.

Immunohistochemical examination of lesional skin from patient JS showed a number of aspects of inflammation. In the dermis, endothelial cells expressed cell adhesion molecules ICAM-1, E-selectin and VCAM-1, which are all important in the recruitment of cellular inflammatory infiltrates. However, these reactions are seen in both allergic and irritant dermatitis and do not themselves indicate that the inflammation is immune in nature. More interestingly, there was an infiltration by $CD14^+$ macrophages both in the dermis and in the epidermis. This has been an inconsistent finding in previous studies (Villada *et al.*, 1992), largely because of methodologic problems. However, the presence of these cells particularly in areas of epidermal cell damage suggests their involvement in the pathogenetic process.

Infiltration by T-lymphocytes ($CD3^+$) has been shown before by many studies (Miyauchi *et al.*, 1991; Villada *et al.*, 1992), although this by itself does not prove that the T cell is an active participant in the inflammatory process. However, subtyping of these T lymphocytes in the lesional skin of patient JS clearly shows several features consistent with the presence of activated T cells and their involvement in the pathogenesis of the epidermal necrosis.

First, in the dermis the ratio of $CD4^+$ to $CD8^+$ cells was 2:1, a ratio seen in peripheral blood. However, in the epidermis, the ratio was reversed with most of the cells being of the $CD8^+$ cytotoxic/suppressor phenotype. This has also recently been reported by Miyauchi *et al.* (1991). In most immune/allergic processes involving the epidermis, the predominant cell which enters the epidermis is the $CD4^+$ cell (Scheynius *et al.*, 1984; McMillan *et al.*, 1985). One of the exceptions and one which

is undoubtedly immune in nature is graft versus host disease (GVHD). In this disease, $CD8^+$ cytotoxic T cells enter the epidermis and damage and kill epidermal keratinocytes because they express surface proteins including class I MHC determinants that are recognised as "non-self" by the T lymphocytes (Paller *et al.*, 1988). Experimental (Billingham and Streilein, 1968) and clinical (Peck *et al.*, 1972; Saurat, 1981) data strongly suggest that toxic epidermal necrolysis and GVHD are mediated by similar immunological and pathogenic events.

Secondly, a high proportion, up to 25%, of the intra-epidermal T cells expressed CD25. This is the light chain of the receptor for interleukin 2 which is expressed by T cells that have been activated by specific interaction with their target antigen (O'Garra, 1989).

Thirdly, there was a perivascular dermal infiltrate by $CD45RO^+$. These are memory T cells, the presence of which would be consistent, but not conclusive, of T cell participation in the inflammatory process (Frew and O'Hehir, 1992).

Fourthly, HLA-DR molecules which are normally expressed only on B lymphocytes, Langerhans cells, macrophages, and activated T lymphocytes (Villada *et al.*, 1992), were found to be aberrantly expressed on keratinocyte cell surfaces. This has been observed in many inflammatory skin disorders (Lampert, 1984), and more recently in drug-induced toxic epidermal necrolysis (Villada *et al.*, 1992). Such aberrant expression only seems to occur in the presence of activated T cells (Lampert, 1984), presumably reflecting interferon gamma secretion by the activated lymphocytes (Villada *et al.*, 1992).

Clearly, the features described in the two patients show involvement of the immune system, but do not absolutely prove a cause and effect relationship. Ideally,

larger numbers of patients with such toxicities should be investigated to determine whether these are common features. However, in view of the relative rarity and incorrect identification of these reactions, this is extremely difficult to achieve. In the absence of this, in order to explain all the features described in the two patients, a sequence of events may be postulated. On exposure to carbamazepine, reactive intermediates were generated which were inadequately detoxified as suggested by the results of the *in vitro* cytotoxicity assay. In patient SR, the reactive metabolite acted as an hapten, altered the carrier protein such that it was no longer recognised as self resulting in loss of suppressor T cell activity and hence autoantibody formation. In patient JS, the reactive metabolite by acting as an hapten altered epidermal cell surface proteins including MHC class I molecules, resulting in activation of the immune system with the generation of cytotoxic $CD8^+$ cells, with consequent attack on and damage of epidermal cells, as occurs in GVHD. Further investigations are obviously required to determine whether this hypothesis is correct. In addition, the elucidation of the structure of the carbamazepine antigen would enable more direct immunological tests to be performed which would assist greatly in determining the immune processes involved in carbamazepine idiosyncratic toxicity, and more specifically, the cellular and tissue sites of drug hapten formation.

CHAPTER 4

INVESTIGATION INTO THE FORMATION OF CYTOTOXIC, PROTEIN REACTIVE AND STABLE METABOLITES FROM CARBAMAZEPINE *IN VITRO*

4.1. Introduction

The primary function of phase I oxidative metabolic pathways is the conversion of lipophilic, non-polar compounds to hydrophilic, polar compounds to facilitate excretion from the body. However, phase I metabolism can also lead to the formation of chemically reactive metabolites (Park, 1986; Parke, 1987) which, if not adequately detoxified, can react by covalent linkage with cellular or autologous macromolecules resulting in either necrosis or secondary immune reactions (Park *et al.*, 1987). Therefore, the balance between activation to a chemically reactive metabolite and its detoxication may be a critical determinant of the likelihood of developing idiosyncratic toxicity with a compound.

Chemically reactive species vary widely in their stability, and hence their reactivity, which is also an important factor in determining not only whether toxicity ensues, but also the form of toxicity, i.e. direct toxicity, carcinogenicity or hypersensitivity (Gillette *et al.*, 1984). There seems to be a wide spectrum of stability of chemically reactive metabolites (table 4.1), at one end being the ultra-short lived metabolites which never leave their site of production and therefore cause little toxicity, while at the opposite end of the spectrum, are metabolites which have a long half-life and can pass from their site of formation to cause toxicity in distant tissues (Gillette *et al.*, 1984). The chemical identification of a reactive species thus depends on its stability, the more stable, long-lived metabolites being identified by analytical techniques such as HPLC and mass spectrometry. A typical example of such a metabolite is dapsone hydroxylamine, which can be identified in urine (Coleman, *et al.*, 1990). However, the majority of electrophilic metabolites are too unstable to be identified analytically, and therefore, indirect methods have to be

TABLE 4.1.

Characteristics and toxic manifestations of the different types of chemically reactive metabolites which can be formed from xenobiotics.

Functional category	*Characteristics*
Ultra short-lived metabolite	Never leave site of formation either decomposing to form a stable metabolite or binding to enzyme and inactivating it ("suicide inhibitor").
Short-lived metabolite	Metabolite never leaves cell, forming either a stable metabolite or undergoing covalent binding. Toxicity therefore is cell-selective.
Intermediate-lived metabolite	Metabolite leaves cells but not organ; therefore, toxicity is organ-selective.
Long-lived metabolite	Metabolite enters virtually all cells in the body, and therefore can lead to distant toxicity.
Ultra long-lived metabolite	Metabolite is excreted in urine and bile. Toxicity may be preferentially localised to kidney, urinary bladder and intestine because of concentration of the metabolite.

Adapted from Gillette et al. (1984).

used for their formation and identification. Identification depends on trapping the reactive metabolite formed *in vitro* by co-incubation with nucleophiles such as glutathione to form stable conjugates which may then be isolated and their structure resolved. However, this can be extremely difficult, particularly when metabolic conversion *in vitro* to the reactive metabolite is low. Indirect assessment of the formation of the toxic metabolite *in vitro* can be undertaken using metabolism-dependent cytotoxicity and irreversible protein binding as end-points. The former method has been described in chapters 2 and 3. Covalent binding of a radiolabelled compound to protein *in vitro* also provides a useful index of the formation of a

chemically reactive metabolite, although discrepancies are often observed between irreversible binding and cytotoxicity (Devalia *et al.*, 1982; Riley *et al.*, 1988). With some drugs, such as paracetamol, a relationship has been observed between the degree of covalent binding and severity of toxicity (Jollow *et al.*, 1973; Potter *et al.*, 1973). However, such a relationship does not always exist, which leads to a large difference in the bioactivation observed *in vitro* and *in vivo*. For example, 17α-ethinyl oestradiol shows extensive *in vitro* covalent binding (Kitteringham *et al.*, 1988), but *in vivo* the bioactivation is largely precluded by competing phase II metabolic pathways including methylation, glucuronidation and sulphation (Maggs *et al.*, 1983). Hence, the balance between activation and detoxication of a compound seems highly important in determining *in vivo* toxicity.

However, despite these reservations, these two techniques are useful indices for the potential of a drug to form chemically reactive metabolites in the presence of drug metabolising enzymes. Furthermore, by using diagnostic inhibitors, nucleophiles and anti-oxidants, a knowledge of the mechanisms involved in bioactivation of the drug and detoxication of its chemically reactive metabolite, and hence possible reasons for the unique predisposition of certain patients to drug toxicity can be ascertained. Thus, carbamazepine, which causes well-documented idiosyncratic toxicity (chapter 1) has clearly been shown to undergo metabolism-dependent cytotoxicity with both human and mouse liver microsomes (chapter 2). It has also been shown that cells from patients clinically diagnosed as being hypersensitive to carbamazepine are more sensitive to this cytotoxic metabolite *in vitro* than cells from control subjects, indicating a detoxication deficiency in the patients (chapters 2 and 3). The chemically reactive metabolite responsible for

carbamazepine idiosyncratic toxicity has been postulated to be an arene oxide on the basis of two lines of evidence: first, there is *in vitro* and clinical cross-reactivity between phenytoin and carbamazepine (Gerson *et al.*, 1983; Shear *et al.*, 1988; Reents *et al.*, 1989) suggesting that they have common biochemical basis for their toxicity. Phenytoin has been postulated to form a reactive 3,4-epoxide (Spielberg *et al.*, 1981), although this metabolite has never been isolated and chemically characterised. Secondly, the metabolism-dependent cytotoxicity of carbamazepine can be enhanced by trichloropropene oxide (TCPO; Riley *et al.*, 1989), an inhibitor of epoxide hydrolase. However, deduction of the possible nature of the toxic metabolite from this evidence is complicated by the fact that TCPO is also known to deplete glutathione (Larrey *et al.*, 1989) and inhibit cytochrome P450 (Ivanetich *et al.*, 1982). In addition, assuming that a chemically reactive epoxide is formed from carbamazepine, it is not known which form of epoxide hydrolase would be the detoxifying enzyme. TCPO has been classified as a selective inhibitor of the microsomal epoxide hydrolase (Guenthner, 1986); however, recent evidence suggests that TCPO can also inhibit the cytosolic form of the enzyme, the selectivity of the inhibition being dependent on the substrate (Luo *et al.*, 1992).

In view of the above considerations, in this *in vitro* study, we have investigated the characteristics of the stable, cytotoxic and protein-reactive metabolites formed from carbamazepine in the presence of human and mouse liver microsomes, and factors which may affect the formation of these metabolites.

4.2. Methods

4.2.1. Chemicals

Carbamazepine, 1,1,1-trichloropropene-2,3-oxide(TCPO), reduced glutathione, N-acetylcysteine, ascorbic acid, ketoconazole and human serum albumin (HSA; fraction V) were purchased from Sigma Chemical Co. (Poole, U.K.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH; tetrasodium salt) was obtained from BDH Chemicals Ltd (Poole, U.K.). [10,11-^{14}C]-carbamazepine (radiochemical purity 99%) and carbamazepine 10,11-epoxide were gifts from Ciba-Geigy Pharmaceuticals (Basle, Switzerland). All solvents were of HPLC grade and were products of Fisons plc (Loughborough, U.K.).

4.2.2. Purification of microsomal and cytosolic epoxide hydrolases

Both microsomal epoxide hydrolase and cytosolic epoxide hydrolase were purified from guinea pig liver by Dr Thomas Guenthner, The University of Illinois at Chicago (USA).

Microsomal epoxide hydrolase was purified to homogeneity by the method of Lu *et al.* (1975), and was dialysed in 10mM sodium phosphate buffer (pH 7.4). Cytosolic epoxide hydrolase was prepared from guinea pig liver (Qato *et al.*, 1990) and the semi-purified product was dialysed in 10mM tris buffer and 0.1mM EDTA (pH 8.0). The activities of these enzymes were determined using styrene oxide (Lu *et al.*, 1975) and *trans*-stilbene oxide (Qato *et al.*, 1990) as substrates for microsomal epoxide hydrolase and cytosolic epoxide hydrolase, respectively, and are expressed as units, where one unit converts one nanomole of substrate to the diol product per minute.

4.2.3. Preparation of human and murine hepatic microsomes

Washed human liver microsomes were prepared from six livers (table 4.2) obtained from kidney transplant donors using the method described in section 2.2.5. Ethical approval was granted by the local ethical committee and consent was obtained from donors' relatives.

A group of six male CBA/ca mice were treated with phenobarbitone once daily for three days (60mg/kg body weight per day) by i.p. injection (0.15ml) in 0.9% w/v saline. After the final set of injections, the mice were fasted for 24h. The animals were then killed by cervical dislocation, their livers dissected out and washed microsomes prepared by the same centrifugation procedure used to obtain washed human liver microsomes (section 2.2.5.).

The microsomal pellets obtained from human and mouse livers were stored at -80°C until used. The microsomal protein content was estimated by the method of Lowry *et al.*, (1951). Microsomal suspensions were prepared (either 10 or 20mg/ml) in 0.067M phosphate buffer (pH 7.4) for determination of the metabolism-dependent cytotoxicity and irreversible binding of carbamazepine immediately prior to use and were maintained at 4°C throughout.

The cytochrome P450 content was measured in diluted samples (1mg/ml in phosphate buffer, pH 7.4) of the microsomal suspensions by the method of Omura and Sato (1964) as described in section 2.2.6. The cytochrome P450 content of the pooled liver microsomes prepared from six phenobarbitone-pretreated mice (on three separate occasions) was 1.4 ± 0.3 nmol/mg protein. The mean cytochrome P450 content of the six human livers used in the study 0.4 ± 0.06 nmol/mg protein; the individual values are listed in table 4.2.

TABLE 4.2.

Details of the human liver donors used in the study of the formation of cytotoxic, protein-reactive and stable metabolites from carbamazepine.

Liver	*Age*	*Sex*	*Cause of death*	*Drugs*	*Histology*	*Cytochrome P450 (nmol/mg protein)*
L1	66	F	Brain haemorrhage	None	Normal	0.50
L2	60	M	Subarachnoid haemorrhage	None	Normal	0.24
L3	29	M	Head injury	None	Normal	0.54
L4	46	F	Cardiac arrest	None	Mild fatty change	0.37
L5	56	M	Head injuries	None	Mild fatty change	0.26
L6	27	M	Head injuries	None	Normal	0.60

4.2.4. Isolation of human mononuclear leucocytes from peripheral blood

Peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinised venous blood (30-50ml) from four healthy male volunteers (age range 22-37 years) as previously described in section 2.2.7. The MNL were suspended in 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium to obtain 1 x 10^6 cells/ml.Their viability upon isolation as determined by trypan blue dye exclusion was >95%.

4.2.5. Isolation of T- and B-lymphocytes

T- and B-lymphocytes were isolated by a process of negative selection using magnetic monosized polymer microspheres (Dynabeads M-450, Dynal (UK), Ltd., Wirral, U.K.) coated with monoclonal antibodies specific for the B-cell CD19 and T-cell CD2 antigens, respectively, using a modification of the method described by Vartdal *et al.*, (1987).

Mononuclear leucocytes were isolated from 100ml fresh heparinised venous blood obtained from three volunteers (age range 28-36 years) by a modification of the density gradient centrifugation method described in section 2.2.7. Initially, the blood was diluted 1:1 with phosphate-buffered saline (PBS) and aliquots (12ml) were layered onto Lymphoprep (9ml) and centrifuged for 25 min at 1,900 rev/min (650*g*). The interface layer was harvested using a Pasteur pipette, diluted 1:1 (v/v) with PBS and the cells sedimented by centrifugation (1,400 rev/min, 346*g*, 10 min). The cells were resuspended in 10ml of PBS and sedimented at low speed (1,100 rev/min, 214*g*, 8 min). The number of cells obtained was then estimated by counting under a Improved Neubauer haemocytometer and the cells were again sedimented (1,100

rev/min for 8 min) and resuspended in PBS to obtain 1 x 10^7 cells/ml.

Initial experiments were performed to determine the amount of microspheres to be used to obtain the optimal yield of the lymphocytes subsets. Although the manufacturer recommends a ratio of 10:1 microspheres : cells, it was found that by using a diluted (1 x 10^7 cells/ml) suspension of previously isolated MNL, a 1:1 ratio resulted in an equivalent yield of cells to that when a higher ratio was used. In addition, all isolations were performed at room temperature as opposed to manufacturers recommendations of 4°C, because the negatively selected cell populations were to be used for the *in vitro* cytotoxicity assay, and room temperature allows any macrophages present in the initial isolate to phagocytose the magnetic beads and thus be removed as positively selected cells improving the purity of the negatively selected cell populations.

Thus, the isolated MNL were mixed with antibody-coated microspheres, corresponding to a ratio of approximately 1:1, for 30 min at room temperature by tilting and rotating. After 30 min the mixture was diluted 1:5 (v/v) with PBS and immediate magnetic separation carried out by placing the tube on a six-tube magnetic particle concentrator (MPC-6, Dynal (UK) Ltd., Wirral, U.K.) for 1-3 min. The microsphere-free fluid phase was aspirated into a universal container. The rossetted cells, i.e. the positively selected cells, were then resuspended in 3 ml PBS and magnetic separation repeated. The rossetted cells were discarded, while the negatively selected cell populations were resuspended in HEPES-buffered medium to obtain approximately 1 x 10^6 cells/ml. The cells upon isolation were of > 95% viability as determined by trypan blue dye exclusion. The purity of the negatively selected cells was determined by Mr I. Crosby, Department of Immunology, The

Royal Liverpool University Hospital and was found to be 95% and 90%, for the T- and B-cells, respectively.

4.2.6. Determination of the metabolism-dependent cytotoxicity of carbamazepine

Isolated MNL (1 x 10^6) in HEPES-buffered balanced salt medium (1ml) were incubated with carbamazepine (50μM) and murine or human liver microsomes (0.5mg - 2.0mg microsomal protein/incubation) in the presence or absence of NADPH (1mM) for 2h at 37°C (section 2.2.8.). The concentration of carbamazepine used was not directly cytotoxic, and the drug was added in 10μl HPLC grade methanol, which, as a 1% solution (v/v) was non-toxic. All incubations were carried out in quadruplicate.

In some incubations, with phenobarbitone-induced mouse microsomes, varying concentrations of microsomal epoxide hydrolase and cytosolic epoxide hydrolase were added, with control incubations receiving an equivalent volume of the dialysing buffer. In addition, in other experiments, the effect of pre-incubation (10 min) of cells with glutathione (500μM), *N*-acetyl cysteine (50μM) and ascorbic acid (100μM) was assessed. The concentrations chosen had previously been shown to decrease the metabolism-dependent cytotoxicity of dapsone (Coleman *et al.*, 1989).

After 2h, the cells were sedimented and resuspended in a drug-free medium (HEPES buffered medium containing 5mg/ml HSA). Incubations were continued for 16h at 37°C and aliquots removed in order to determine cell viability by trypan blue dye exclusion (0.2% trypan blue) as previously described (section 2.2.9.), a failure to exclude dye being taken as an index of cell death.

Experiments with the separated T- and B-lymphocyte subpopulations were carried out with phenobarbitone-induced mouse microsomes (0.5mg/ml) in the same

manner as above, except that the final incubation volume was 0.5ml rather than 1ml. The drug was added in 5µl of methanol to maintain a final solvent concentration of 1% (v/v). Initial experiments had shown that the metabolism-dependent cytotoxicity of carbamazepine was not altered by reducing the final incubation volume to 0.5ml (4.5 ± 0.4% and 4.3 ± 0.3% at incubation volumes of 1ml and 0.5ml, respectively).

4.2.7. Metabolism of carbamazepine by human and murine hepatic microsomes

[^{14}C]-carbamazepine (50µM, 0.15µCi) was incubated with murine (1mg protein) or human (2mg protein) hepatic microsomes and 1 x 10^6 MNL in HEPES-buffered medium (pH 7.4; final incubation volume 1ml). Radiolabelled carbamazepine (0.15µCi) was added in 20µl of methanol, the solvent then being evaporated under N_2, and unlabelled carbamazepine added (in 10µl of methanol) to ensure the final concentration of carbamazepine was 50µM. The final concentration of methanol in the incubations, as in the *in vitro* cytotoxicity assay, was 1% (v/v). The incubations were carried out in 10ml glass bottles under air in a shaking water bath for 2h at 37°C. The reactions were initiated by the addition of NADPH (1mM) which was omitted in control incubations.

In order to determine the factors affecting the bioactivation of carbamazepine some incubations also contained TCPO (30µM), microsomal epoxide hydrolase (100units), glutathione (500µM), N-acetyl cysteine (50µM), ascorbic acid (100µM) or ketoconazole (10-250µM). The concentrations chosen were equivalent to those used in the *in vitro* cytotoxicity assay.

The reactions were terminated by the addition of 3ml acetonitrile and left overnight at -20°C to precipitate the protein. Following this, the incubations were

centrifuged (2000*g* for 10 min) in a Beckman J-6B centrifuge and the supernatants removed for analysis of stable metabolites by radiometric HPLC.

4.2.8. Determination of irreversible binding of radiolabelled carbamazepine to microsomal protein

Radiolabelled material irreversibly bound to precipitated protein was measured after removal of unbound drug by exhaustive solvent extraction. Methanol (5ml) was added to the protein pellet and mixed thoroughly initially by vortexing and then on a rotary mixer for 20 min. The tubes were then centrifuged (2000*g* for 10 min) to precipitate the protein. The protein precipitate was then successively extracted with methanol (5ml) again and 70% (v/v) methanol (5ml x 2) using the same procedure. After each extraction, the radioactivity in the supernatant was determined by liquid scintillation spectroscopy to ensure complete removal of non-covalently bound material.

Following the final extraction procedure, the protein was dissolved in 1M NaOH at 60°C overnight. 1ml and 2ml of NaOH were used to dissolve the murine and human microsomes, respectively, as it was found that the human microsomes could not be dissolved with just 1ml NaOH.

To determine the amount of radiolabelled carbamazepine irreversibly bound to protein, aliquots (800μl) of the solubilised protein were taken for quantification of radioactivity by mixing with 12ml liquid scintillation fluid (Aqua Luma Plus) and counted in a Beckman LS1801 liquid scintillation counter with automatic external standardisation for 4 min. Protein loss in the extraction procedure was accounted for by measuring the protein content in another aliquot (100μl) of the solubilised

protein by the method of Lowry *et al.* (1951). Irreversible binding of radiolabelled carbamazepine is expressed as a percentage of the initial radioactivity bound to the incubated microsomal protein.

4.2.9. Analysis of the microsomal metabolism of carbamazepine to the stable 10,11-epoxide by radiometric HPLC

The supernatants from the mouse or human microsomal incubations with [^{14}C]-carbamazepine were analysed for unchanged carbamazepine and its 10,11-epoxide essentially as described by Regnaud *et al.* (1988) using an HPLC (LKB, Bromma, Sweden) linked to a radiometric detector (Flo-one Beta, Canberra, Packard). The volume of the supernatant was reduced to *ca.* 1ml by vacuum centrifugation (Univap, Uniscience Ltd., London, UK) at 40°C for 40 min. The resulting solution was filtered through a 0.45μm filter (Millipore, U.K.) and 50μl was injected onto an C_{18} Ultratech 50DS column (20cm; HPLC Technology, Macclesfield, Cheshire). The solvent system (30% acetonitrile : 10% methanol : 60% 0.005M tetrabutyl ammonium chloride in water) was delivered at a flow rate of 1.2ml/min and the absorbance of the eluant was monitored at 230nm. Carbamazepine-10,11-epoxide and carbamazepine were identified by comparison of their retention times (6.3 min and 11.9 min for carbamazepine-10,11-epoxide and carbamazepine, respectively) with those of co-injected authentic compounds (figure 4.1). No other metabolites were detected on the chromatogram. Radioactivity was monitored throughout the run and peaks were integrated and expressed as percentage radioactivity eluting from the column. Integration of radioactivity over the full 15 min run time showed that >95% of injected radioactivity was recovered.

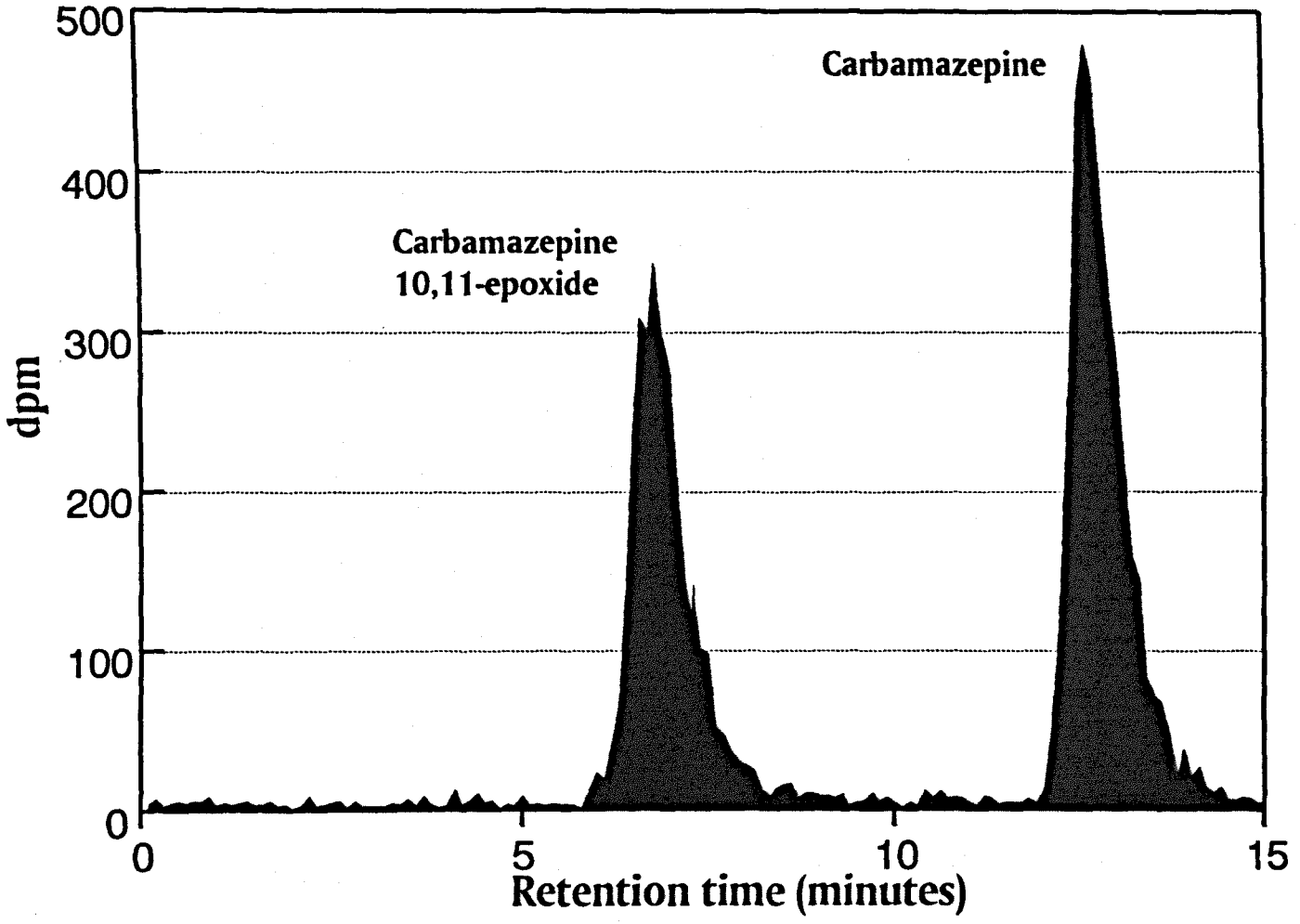


Figure 4.1. Radiometric HPLC chromatogram showing the metabolism of carbamazepine to the stable 10,11-epoxide by phenobarbitone-induced mouse liver microsomes.

4.2.10. Statistical analysis

Statistical analyses were performed by one-way analysis of variance accepting $p \leq 0.05$ as significant. Data are presented as mean ± SEM.

4.3. Results

4.3.1. Bioactivation of carbamazepine to a protein-reactive metabolite by human liver microsomes

In chapter 2, it was shown that human livers bioactivated carbamazepine (50μM) to a cytotoxic metabolite. In the present study, experiments with 6 human livers showed that all of them were capable of forming protein-reactive metabolites with the addition of NADPH (table 4.3), the mean covalent binding (n=6 livers) increasing from 0.08 ± 0.01% (without NADPH) to 0.27 ± 0.09% (with NADPH; $p \leq 0.05$). The addition of TCPO (30μM; n=3 livers) resulted in a non-significant increase in covalent binding (0.18 ± 0.01% *vs.* 0.22 ± 0.02%; N.S.).

In accordance with a previous study (Tybring *et al.*, 1981), carbamazepine was also converted *in vitro* to the stable metabolite, carbamazepine-10,11-epoxide, by all human liver microsomes (table 4.3). No other stable metabolites, including the dihydrodiol, were identified on the chromatogram.

TABLE 4.3.

The NADPH-dependent conversion of carbamazepine (50μM) to protein-reactive and stable (carbamazepine-10,11-epoxide) metabolites by human liver microsomes (2mg).

Liver	*Irreversible binding (%)*	*Carbamazepine-10,11-epoxide formation (%)*
L1	0.15	7.7
L2	0.10	4.3
L3	0.10	8.8
L4	0.10	5.9
L5	0.11	3.7
L6	0.58	8.5

Values represent the means of triplicate determinations and have been determined by subtracting the result obtained in incubations without NADPH from incubations containing NADPH.

4.3.2. Effect of ketoconazole on the formation of metabolites from carbamazepine

Ketoconazole, a cytochrome P450 inhibitor (Sheets and Mason, 1984), reduced irreversible binding and stable metabolite formation with both phenobarbitone-induced mouse microsomes (1mg) and human liver microsomes (2mg; table 4.4). As with human microsomes, no other stable metabolites, apart from the 10,11-epoxide, were identified on the chromatogram.

With phenobarbitone-induced mouse microsomes (1mg), ketoconazole also inhibited the metabolism-dependent cytotoxicity of carbamazepine (figure 4.2), although the maximum inhibition of carbamazepine-cytotoxicity was limited by cytotoxicity of ketoconazole (10μM) itself (figure 4.2), thus precluding the use of higher ketoconazole concentrations.

TABLE 4.4.

The effect of ketoconazole on the metabolism-dependent irreversible binding of radiolabelled carbamazepine (50μM) and stable (carbamazepine-10,11-epoxide) metabolite formation in the presence of either phenobarbitone (PB)-induced mouse (1mg) or human (2mg) liver microsomes

Ketoconazole concentration (μM)	PB MOUSE LIVER MICROSOMES		HUMAN LIVER MICROSOMES	
	Covalent binding (%)	*Carbamazepine-10,11-epoxide formation (%)*	*Covalent binding (%)*	*Carbamazepine-10,11-epoxide formation (%)*
0	0.37 ± 0.01	23.1 ± 1.3	0.7 ± 0.02	8.6 ± 0.1
10	0.16 ± 0.03*	8.9 ± 1.1*	0.32 ± 0.06*	2.4 ± 0.3*
100	0.05 ± 0.00*	2.8 ± 0.06*	0.25 ± 0.01*	0.5 ± 0.1*
250	Not determined		0.20 ± 0.01*	0.2 ± 0.1*

The results represent the mean ± SEM of triplicate incubations performed with pooled mouse liver microsomes or microsomes prepared from one human liver (L6). Statistical analysis performed by comparing incubations with and without added ketoconazole: * $p \leq 0.001$

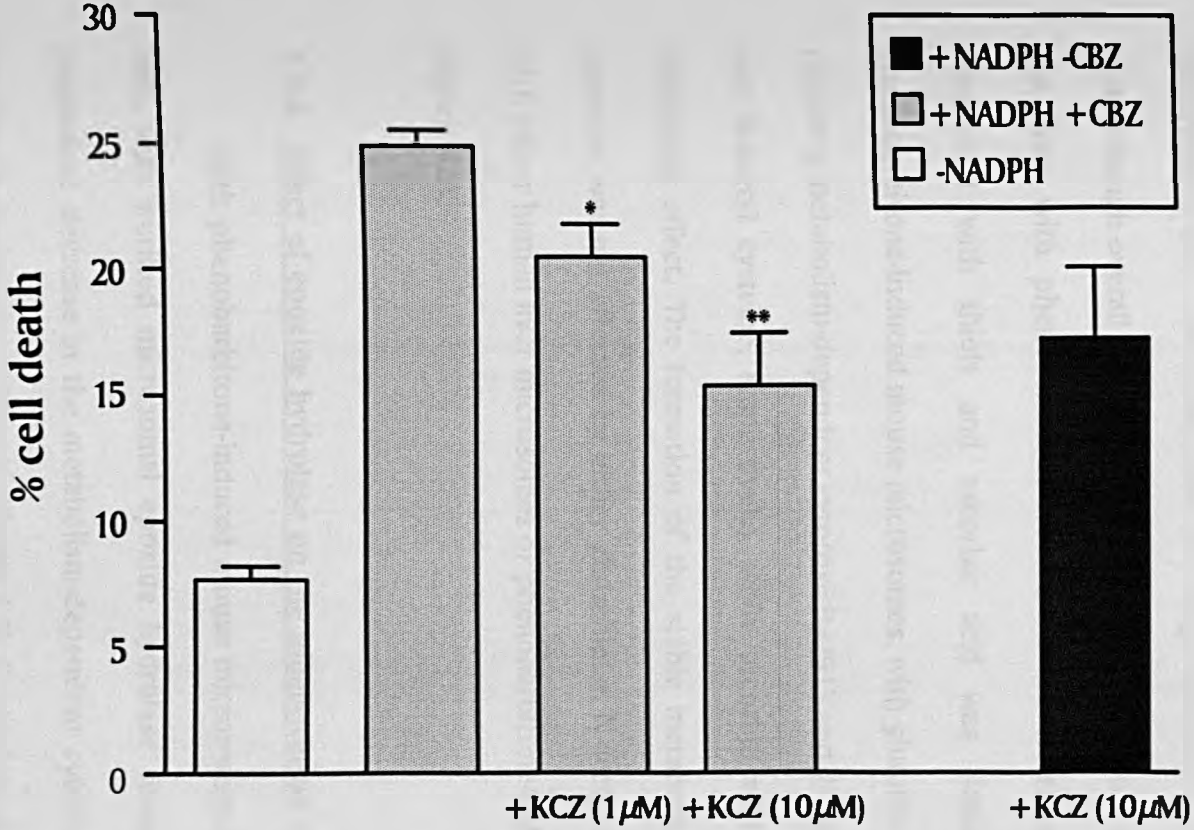


Figure 4.2. The effect of ketoconazole (KCZ; 1μM and 10μM) on the bioactivation of carbamazepine (CBZ; 50μM) to a cytotoxic metabolite by phenobarbitone-induced mouse liver microsomes (1mg) either in the presence or absence of NADPH (1mM). The cytotoxicity of ketoconazole (10μM; in the absence of carbamazepine) is also shown. The results represent the mean $\pm$ SEM of quadruplicate incubations. The control cell death value in the absence of drug was 6.6 $\pm$ 0.9%. The inhibition of carbamazepine cytotoxicity has been analysed by comparing incubations with and without ketoconazole (in the presence of NADPH): * $p < 0.05$, ** $p < 0.005$.

4.3.3. Effect of thiols and ascorbic acid on metabolite formation from carbamazepine

Inclusion of glutathione (500μM) significantly reduced metabolism-dependent cytotoxicity and protein-reactivity of carbamazepine, while ascorbic acid (100μM) had no effect (table 4.5). N-acetylcysteine (50μM) reduced metabolism-dependent cytotoxicity but not irreversible binding of radiolabelled compound (table 4.5). Experiments with one human liver (L1; 2mg; incubations in quadruplicate) showed that although overall bioactivation of carbamazepine to a cytotoxic metabolite was less than with phenobarbitone-induced mouse microsomes, the effect of co-incubation with thiols and ascorbic acid was similar to that seen with phenobarbitone-induced mouse microsomes, with glutathione and N-acetyl cysteine reducing metabolism-dependent cytotoxicity (91% and 71% inhibition for glutathione and N-acetyl cysteine, respectively), while ascorbic acid (4% inhibition) had no significant effect. The formation of the stable metabolite, carbamazepine-10,11-epoxide, was not affected by either glutathione, N-acetyl cysteine or ascorbic acid with either human liver microsomes or phenobarbitone-induced mouse microsomes (figure 4.3).

4.3.4. Effect of epoxide hydrolase on the bioactivation of carbamazepine

With phenobarbitone-induced mouse microsomes (0.5mg), co-incubation of MNL with purified microsomal epoxide hydrolase resulted in a concentration-dependent decrease in the metabolism-dependent cytotoxicity of carbamazepine, reaching a maximum at 100 units (figure 4.4). Control incubations containing human serum albumin (HSA) at an equivalent protein concentration to that of microsomal

TABLE 4.5.

The effect of glutathione (500μM), *N*-acetylcysteine (50μM) and ascorbic acid (100μM) on the metabolism-dependent cytotoxicity and irreversible binding of carbamazepine (50μM) with either phenobarbitone-induced (PB) mouse (1mg) or human (2mg) liver microsomes.

Compound	PB MOUSE LIVER MICROSOMES		HUMAN LIVER MICROSOMES
	Cytotoxicity % inhibition	*Covalent binding % inhibition*	*Covalent binding % inhibition*
Glutathione	59 ± 3%*	63 ± 2%*	51 ± 8%*
N-acetylcysteine	33 ± 2%*	28 ± 13%	4 ± 9%
Ascorbic Acid	0.7 ± 21%	12 ± 17%	-11 ± 13%

Results for phenobarbitone-induced mouse liver microsomes represent the mean ± SEM for 3 experiments with pooled microsomes (incubations in triplicate or quadruplicate) while for human liver microsomes, the results are the mean ± SEM for 4 human livers (incubations in triplicate). Statistical analysis performed by comparing degree of inhibition obtained by co-incubation with the above compounds with incubations without any added compounds: * $p \leq 0.001$.

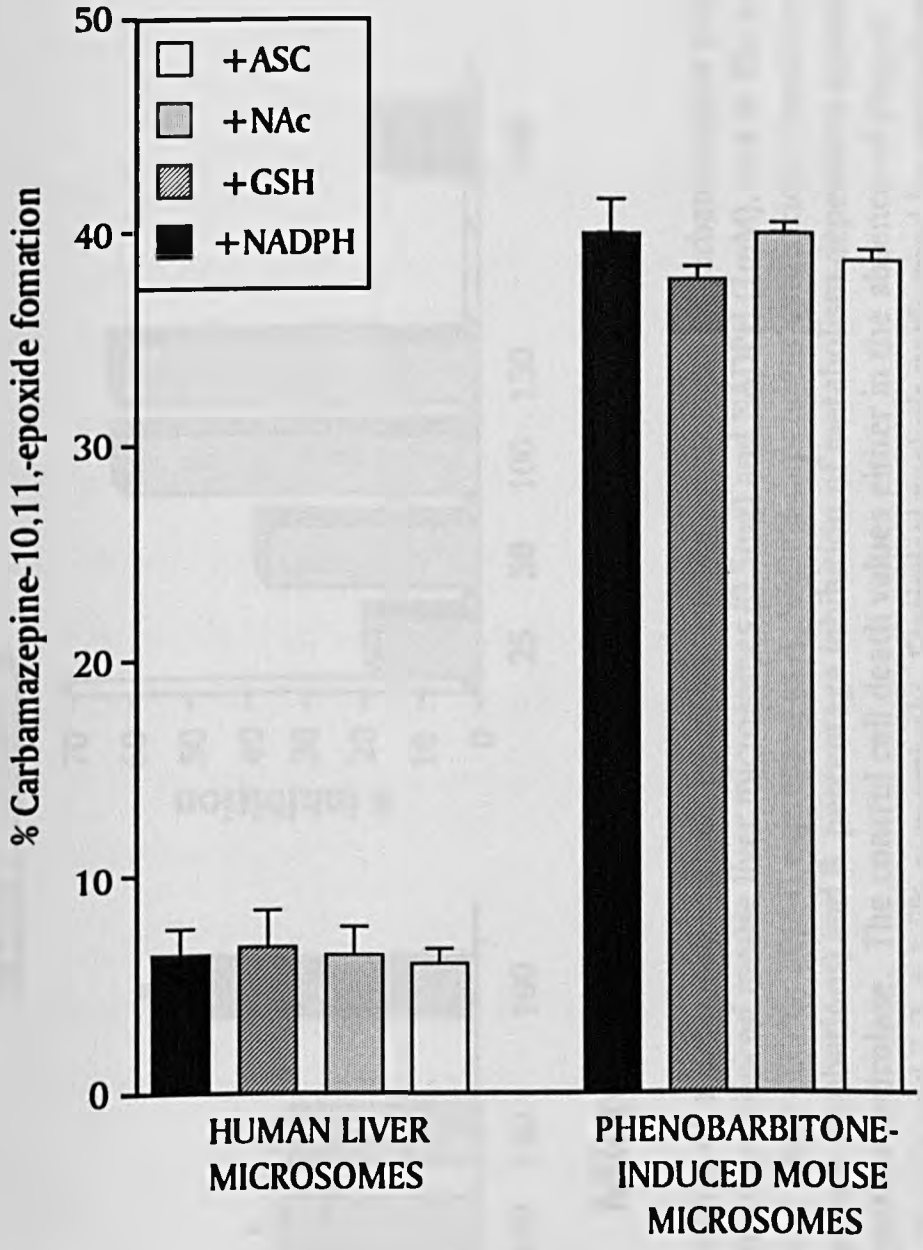


Figure 4.3. The effect of glutathione (GSH), N-acetylcysteine (NAc) and ascorbic acid (ASC) on the formation of carbamazepine-10,11-epoxide from carbamazepine in the presence of human and phenobarbtione-induced mouse liver microsomes. The results for the mouse liver microsomes represent the mean ± SEM for three experiments (incubations in triplicate), while for human microsomes the results are the mean ± SEM for four human livers (incubations in triplicate).

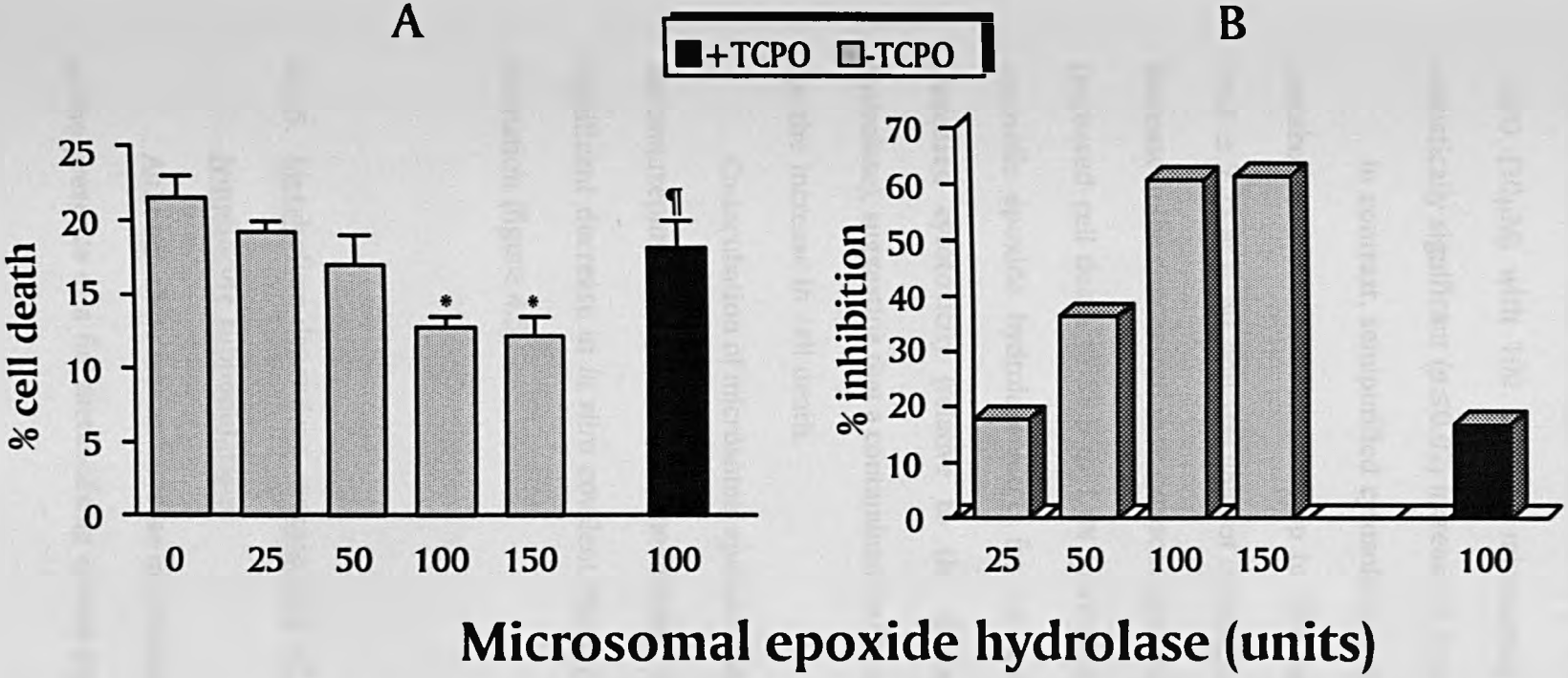


Figure 4.4. The effect of addition of microsomal epoxide hydrolase on the cytotoxicity of carbamazepine (50μM) in the presence of phenobarbitone-induced mouse liver microsomes (0.5mg) and NADPH (1mM), and in the presence or absence of trichloropropene oxide (TCPO; 30μM) expressed as A. NADPH-dependent cytotoxicity (results represent the mean ± SEM of quadruplicate incubations) and B. percentage inhibition of metabolism-dependent cytotoxicity by addition of microsomal epoxide hydrolase. The control cell death values either in the absence of drug or absence of NADPH were 7.2 ± 0.5% and 7.3 ± 1.5%, respectively. Statistical analysis performed by comparing incubations with and without microsomal epoxide hydrolase : * $p < 0.005$, and for incubations containing 100 units microsomal epoxide hydrolase, with and without TCPO : ¶ $p < 0.02$.

epoxide hydrolase (75.4μg and 150.8μg at 50U and 100U of microsomal epoxide hydrolase, respectively) did not reduce cytotoxicity (22.6 ± 1.7%, 21.5 ± 1.8% and 21.5 ± 0.4% at 0μg, 75.4μg, and 150.8μg of HSA, respectively). Co-incubation of TCPO (30μM) with 100 units of microsomal epoxide hydrolase resulted in a statistically significant ($p \leq 0.02$) increase in cytotoxicity (figure 4.4).

In contrast, semipurified cytosolic epoxide hydrolase caused no decrease in metabolism-dependent cell death up to 100 units (29.9 ± 1.5%, 32.9 ± 2.1% and 31.8 ± 2.2% at 0, 50, and 100 units of cytosolic epoxide hydrolase, respectively). An increase in the concentration of cytosolic epoxide hydrolase to 200 units significantly increased cell death to 39.9 ± 1.6% ($p=0.003$). However, use of heat-inactivated cytosolic epoxide hydrolase (60°C for 10 min) at a similar concentration also increased cytotoxicity (relative to the incubations without cytosolic epoxide hydrolase), suggesting that a contaminant in the enzyme preparation was responsible for the increase in cell death.

Co-incubation of microsomal epoxide hydrolase (100 units) with radiolabelled carbamazepine and phenobarbitone-induced mouse microsomes resulted in a significant decrease in *in vitro* covalent binding and carbamazepine-10,11-epoxide formation (figure 4.5).

4.3.5. Metabolism-dependent cytotoxicity of carbamazepine with T- and B-lymphocyte subpopulations

Although there was an increase in cell death of both the T- and B-lymphocytes in the presence of a full metabolising system (figure 4.6), there was no significant

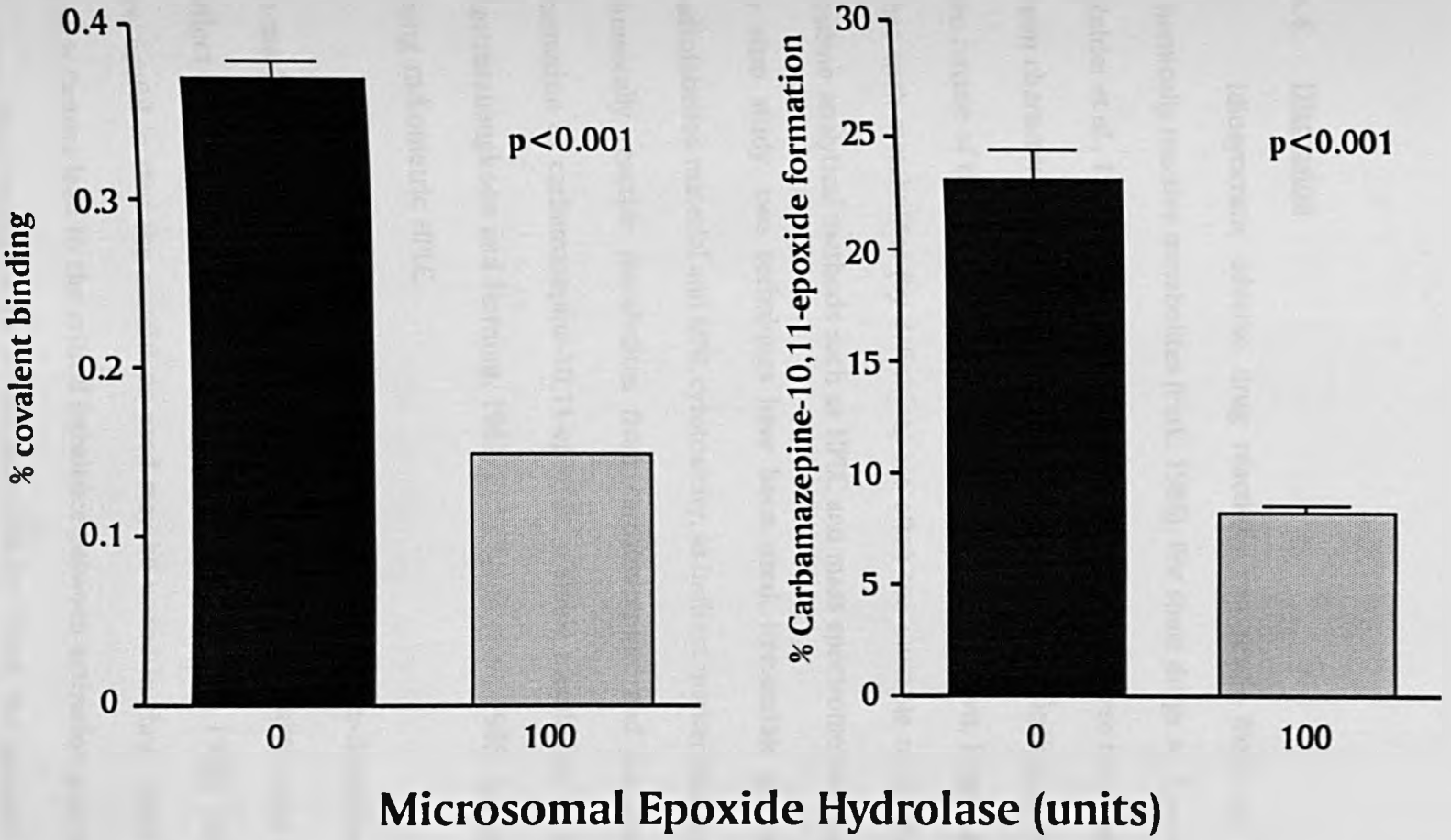


Figure 4.5. The effect of 100 units microsomal epoxide hydrolase on the formation of the protein-reactive and stable (carbamazepine-10,11-epoxide) metabolites from carbamazepine in the presence of induced mouse microsomes. The results represent the mean ± SEM of triplicate incubations.

difference in the sensitivity of the T- and B-cell subpopulations to the carbamazepine metabolite.

4.4. Discussion

Idiosyncratic adverse drug reactions can result from the formation of chemically reactive metabolites (Park, 1986). For some drugs such as sulphonamides (Reider *et al.*, 1989) and dapsone (Coleman *et al.*, 1989), these toxic metabolites have been characterised. However, for a wide variety of drugs, including carbamazepine, the nature of the chemically reactive metabolite is unknown, largely due to the fact that such metabolites (by definition) are often too unstable to be characterised by routine analytical methods such as HPLC and mass spectrometry. Therefore, in this *in vitro* study, two techniques have been used, irreversible protein binding of radiolabelled material and MNL cytotoxicity, as indirect markers for the formation of chemically reactive metabolites from carbamazepine, and for comparison, the formation of carbamazepine-10,11-epoxide, a stable metabolite of carbamazepine (Lertratanangkoon and Horning, 1982; Eichelbaum *et al.*, 1985) has been measured using radiometric HPLC.

Although the use of covalent binding and metabolism-dependent cytotoxicity as markers for the formation of chemically reactive metabolites may not accurately reflect the *in vivo* toxicology of a drug (Kitteringham *et al.*, 1988), these techniques are useful in that the conditions used may be varied in three ways to determine which factors lead to the critical imbalance between activation and detoxication *in vivo* and thus predispose to idiosyncratic toxicity. First, the generating system, i.e

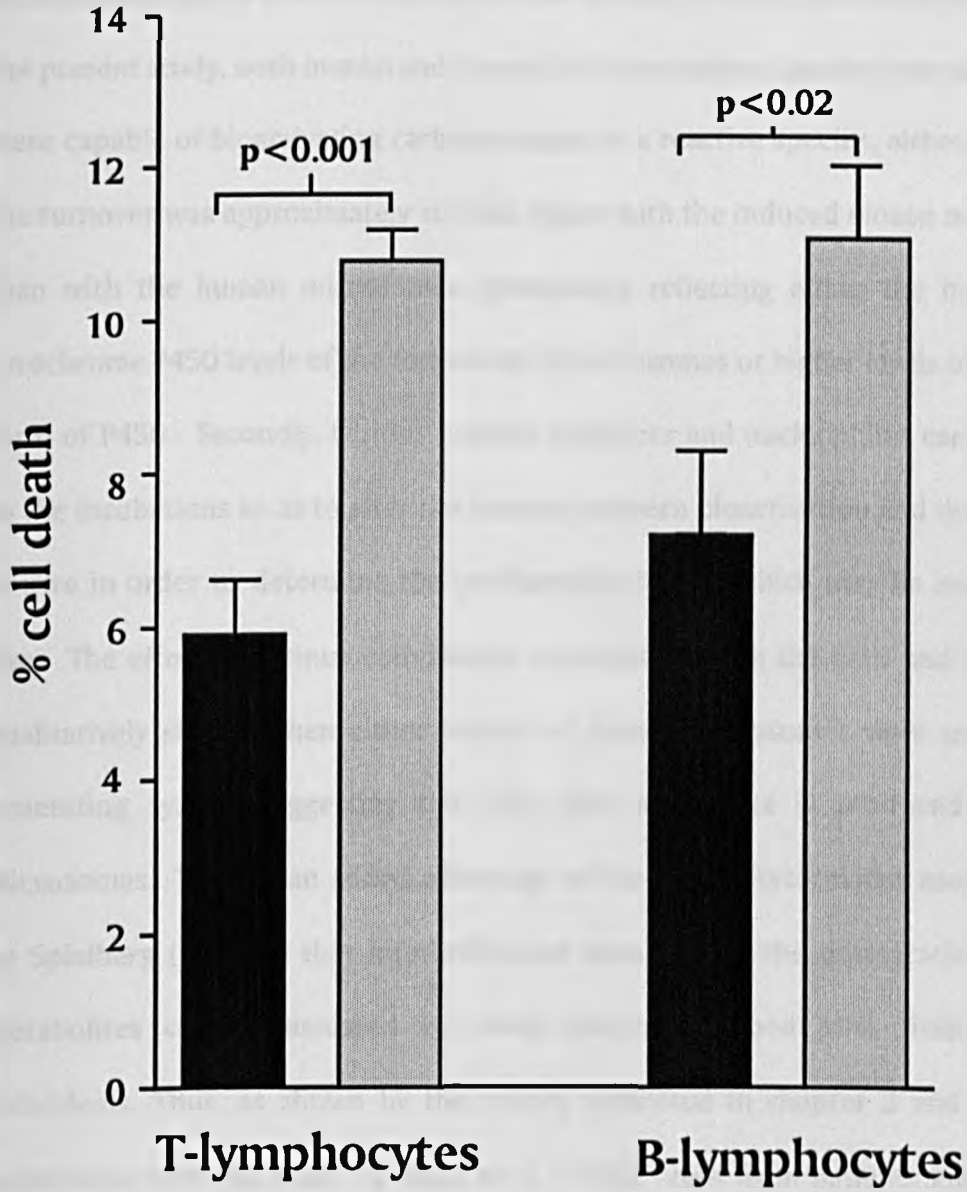


Figure 4.6. A comparison of the sensitivity of separated T- and B-lymphocyte subpopulations to the cytotoxic metabolite of carbamazepine generated *in vitro* by a murine microsomal system (0.5mg). Results represent the means ± SEM of three separate experiments (incubations in quadruplicate). Statistical analysis within the same group performed by comparing incubations with (□) and without (■) NADPH, with significance values as marked on the graph. Analysis of any difference in the sensitivity of the two cell types performed by comparing the NADPH-dependent cytotoxicity of the two types (no significant difference).

microsomes, can be varied to determine the factors affecting bioactivation. Thus, in the present study, both human and phenobarbitone-induced mouse liver microsomes were capable of bioactivating carbamazepine to a reactive species, although overall the turnover was approximately six-fold higher with the induced mouse microsomes than with the human microsomes, presumably reflecting either the higher total cytochrome P450 levels of the former set of microsomes or higher levels of a specific form of P450. Secondly, various enzyme inhibitors and nucleophiles can be added to the incubations so as to alter the balance between bioactivation and detoxication *in vitro* in order to determine the predisposing factors which may be important *in vivo*. The effect of various compounds co-incubated with the cells and drug were qualitatively similar when either murine or human microsomes were used as the generating system suggesting that the same metabolite is produced by these microsomes. Thirdly, an added advantage of the *in vitro* cytotoxicity assay devised by Spielberg (1980) is that inter-individual variability in the detoxication of drug metabolites can be assessed by using peripheral blood MNL from different individuals. Thus, as shown by the results presented in chapter 2 and 3, and in accordance with the study by Shear *et al.* (1988), cells from patients known to be hypersensitive to carbamazepine show greater *in vitro* sensitivity to oxidative metabolites of carbamazepine, than those from appropriate controls. In chapter 2 it was shown that human livers could bioactivate carbamazepine to a cytotoxic metabolite; in the present study, bioactivation to a protein-reactive metabolite has also been shown (table 4.3) further reinforcing that the carbamazepine-hypersensitive patients have a deficiency in cellular detoxication.

Based largely on the observation that TCPO enhances the *in vitro* cytotoxicity

of carbamazepine (Riley *et al.*, 1989), it has been suggested that the hypersensitive patients have a deficiency of epoxide hydrolase (Shear *et al.*, 1988). However, further evidence is needed to substantiate this hypothesis since TCPO has multiple actions (Ivanetich *et al.*, 1982; Larrey *et al.*, 1989) and at least two forms of epoxide hydrolase, with different substrate specificities (Guenthner, 1990), exist. Of the two major forms of epoxide hydrolase, the microsomal enzyme (microsomal epoxide hydrolase) is located mainly in the endoplasmic reticulum and catalyses the hydrolysis of xenobiotic epoxides (including arene oxides; Guenthner, 1990), while the cytosolic form (cytosolic epoxide hydrolase), located both in cytosol and peroxisomes, catalyses the hydrolysis of *trans*-substituted styrene oxides (Guenthner, 1990). Microsomal EH is in fact known to hydrolyse CBZ-10,11-epoxide to its major urinary metabolite, carbamazepine-dihydrodiol (Bellucci *et al.*, 1987). Indeed, in accordance with this, it was found that the net recovery of carbamazepine-10,11-epoxide *in vitro* was reduced by the addition of exogenous microsomal epoxide hydrolase (figure 4.5). Furthermore, microsomal epoxide hydrolase, but not cytosolic epoxide hydrolase, also reduced the metabolism-dependent cytotoxicity of carbamazepine (figure 4.4), providing further evidence that the toxic metabolite is an arene oxide. The reduction in cytotoxicity is unlikely to be due to the addition of exogenous protein since equivalent amounts of HSA did not affect cytotoxicity and co-incubation of microsomal epoxide hydrolase with TCPO reduced the inhibition in cytotoxicity. A similar reduction in irreversible binding of radiolabelled carbamazepine with microsomal epoxide hydrolase (figure 4.5) suggests that the protein reactive metabolite may be the same metabolite as that responsible for cytotoxicity. Therefore, these results indicate that any deficiency of epoxide

hydrolase in the carbamazepine-hypersensitive patients may be of the microsomal, rather than the cytosolic form of the enzyme.

In the *in vitro* test systems used in the present study, it is quite possible that more than one type of chemically reactive metabolite may be formed from carbamazepine, although only one may be responsible for toxicity *in vivo*. Although reactive species may vary in their stability (and hence their half-lives) this may not be distinguished *in vitro* since all of them may be capable of interacting with protein resulting in cell death and/or covalent binding. It can be deduced from the chemical structure of carbamazepine and from the known metabolic profile (Lertratanangkoon and Horning, 1982), that more than one reactive epoxide may be formed, not all of which may be responsible for toxicity. A parallel may be drawn here with the model hepatotoxin, bromobenzene. Cytochrome P450-mediated bioactivation of bromobenzene leads to the formation of two reactive epoxide intermediates, the 2,3- and 3,4-epoxides. However, only the 3,4-epoxide which binds to histidine residues on protein is hepatotoxic, while the 2,3-epoxide which binds selectively to cysteine residues is thought not to be hepatotoxic (Lau *et al.*, 1980; Lau and Monks, 1988). In addition, although several epoxide metabolites may be formed from carbamazepine, not all of them may be substrates for microsomal epoxide hydrolase. This may be a reason why only a maximum of 60% inhibition of the metabolism-dependent cytotoxicity of carbamazepine was obtained by co-incubation with microsomal epoxide hydrolase. Similarly, covalent binding was also only reduced by 60% with 100 units of microsomal epoxide hydrolase. However, the amount of the stable 10,11-epoxide detected by the addition of microsomal epoxide hydrolase was also reduced to a similar extent, suggesting it may not be possible to completely

hydrolyse epoxides (stable or otherwise) using these *in vitro* conditions. Clearly, measurement of dihydrodiol formation may have helped in determining the reason for incomplete inhibition of the toxic end-points, but using the HPLC system described, this metabolite was not detectable.

The glutathione redox cycle is an important protective mechanism for the cell (Reed, 1986, 1990). Reduced glutathione acts as a nucleophilic "scavenger" conjugating, either nonenzymatically or enzymatically, with different types of electrophiles, including arene oxides, leading to their detoxication (Moldeus and Quanguan, 1987; Reed, 1990). Thus, a subphysiological concentration of reduced glutathione inhibited both metabolism-dependent cytotoxicity and protein-reactivity (table 4.5), but had no effect on the formation of the stable 10,11-epoxide. The inhibition of irreversible binding *in vitro* by glutathione supports the concept that the irreversible binding observed with radiolabelled carbamazepine in this study represents covalently bound drug metabolites and not non-extractable metabolites. A method of chemically identifying reactive intermediates is by trapping them off as glutathione adducts. However, co-incubation of carbamazepine with glutathione, although reducing the toxic end-points, did not result in any extra peaks on the chromatogram in the presence of either phenobarbitone-induced mouse or human liver microsomes, presumably because the *in vitro* metabolic turnover was inadequate to result in the formation of an identifiable glutathione conjugate. Such a problem in the *in vitro* identification of the epoxides formed from naphthalene has been overcome by using a crude mixture of glutathione transferases together with glutathione which greatly increased the formation, and thus helped the subsequent chemical characterisation, of the glutathione conjugate (Buckpitt *et al.*, 1984). This

was not performed in the present study, although clearly this represents a useful approach which may aid in the identification of the chemically reactive metabolite(s) formed from carbamazepine.

Further evidence that the chemically reactive metabolite formed from carbamazepine is an arene oxide is provided by the lack of effect of ascorbic acid on the *in vitro* toxicity of carbamazepine (table 4.5). This concentration of ascorbic acid has previously been shown to reduce the cytotoxicity associated with dapsone (Coleman *et al.*, 1989), a drug whose chemically reactive metabolite is known to be other than an epoxide metabolite. Interestingly, N-acetyl cysteine reduced metabolism-dependent cytotoxicity but had a minimal effect on covalent binding to microsomal protein (table 4.5) suggesting that protection for cells may have been afforded by virtue of its ability to enter cells and subsequently serve as a precursor for glutathione formation (Reed, 1990).

The *in vitro* cytotoxicity assay devised by Spielberg (1980) uses MNL as target cells. This is a mixed cell population consisting of mainly lymphocytes (T- and B-cells) and monocytes (Seidegard *et al.*, 1984). In this study, we have separated the T- and B-lymphocytes, obtaining high purity sub-populations, and found that there is no difference in the sensitivity of the isolated cells, presumably reflecting an equivalent capacity for the detoxification of the chemically reactive metabolite of carbamazepine.

The role of glutathione in protecting against idiosyncratic toxicity with carbamazepine *in vivo* is unclear; however, as has been suggested for detoxication of *trans*-β-ethylstyrene 7,8-oxide by cytosolic epoxide hydrolase (Kramer *et al.*, 1991), the concentration of the chemically reactive metabolite may be important with

microsomal epoxide hydrolase being more important than the glutathione transferases at low concentrations. Another possibility raised by the detoxication of the chemically reactive metabolite of carbamazepine by both microsomal epoxide hydrolase (figure 4.4) and glutathione (table 4.5) is that some carbamazepine-hypersensitive patients may have more than one pharmacogenetic disorder, i.e. a double genetic polymorphism. Glutathione transferase μ, like microsomal epoxide hydrolase, is an enzyme known to detoxify certain epoxides (Mannervik and Danielson, 1988). However, it is known to be polymorphically expressed in the liver and lymphocytes (Seidegard *et al.*, 1988), being absent in approximately 50% of the population (Seidegard *et al.*, 1988; Boyer, 1989). Therefore, by virtue of the fact that it is absent in such a large proportion of individuals, glutathione transferase μ cannot be a sole predisposing factor for carbamazepine idiosyncratic toxicity, although it may act as a contributory factor producing a multifactorial predisposition to toxicity.

The epoxidation of carbamazepine to the stable 10,11-epoxide is cytochrome P450 dependent (Eichelbaum *et al.*, 1985). Consistent with this, the formation of carbamazepine-10,11-epoxide was inhibited by ketoconazole (table 4.4), a cytochrome P450 inhibitor (Sheets and Mason, 1984). In addition, the formation of the stable epoxide was higher with the use of induced mouse microsomes than with (uninduced) human liver microsomes (figure 4.3), again indicating that P450 enzymes are responsible for the epoxidation of carbamazepine. Our results also indicate that the formation of the chemically reactive metabolite of carbamazepine is also cytochrome P450 dependent, since both cytotoxicity and protein reactivity were affected in a similar manner to that seen with the stable epoxide. Carbamazepine is well known to cause both auto- and hetero-induction of the cytochrome P450

enzymes (Bertilsson *et al.*, 1980; Eichelbaum *et al.*, 1985), and thus, will to some extent induce the formation of its own reactive epoxide metabolite. However, carbamazepine is also known to induce the epoxide hydrolase system (Tybring *et al.*, 1981; Regnaud *et al.*, 1988), but several studies have found that the inducibility of the carbamazepine-epoxidase pathway is higher compared with the hydration (i.e. epoxide to dihydrodiol) pathway (Rane *et al.*, 1976; Tybring *et al.*, 1981; Eichelbaum *et al.*, 1985), suggesting that enzyme induction (either autoinduction or induction by other concurrently administered anti-epileptics such as phenytoin and phenobarbitone) may serve as a contributory risk factor by further altering the balance between activation and detoxication in certain patients.

In summary, our results indicate that carbamazepine is bioactivated by the cytochrome P450 enzymes to a chemically reactive metabolite, thought to be an arene oxide, which is detoxified by microsomal epoxide hydrolase and glutathione. The unique predisposition of patients to develop idiosyncratic toxicity with carbamazepine may be due to a deficiency in cellular detoxication, predominantly microsomal epoxide hydrolase, with enzyme induction serving as a contributory risk factor (figure 4.7). The inadequate detoxication of the chemically reactive metabolite may lead to covalent binding with subsequent toxicity either directly or indirectly by initiating an immune response. The latter is thought more likely for carbamazepine in view the accompanying symptomatology (chapter 2; Dreifuss and Langer, 1987; Shear *et al.*, 1988), the demonstration of reactive (Houwerzijl *et al.*, 1977; Zakrzewska and Ivanyi, 1988) and tissue-infiltrating (chapter 3) T-lymphocytes and the demonstration of a specific autoantibody in a hypersensitive patient (chapter 3). Further studies are required to charaterise the chemical nature of the reactive

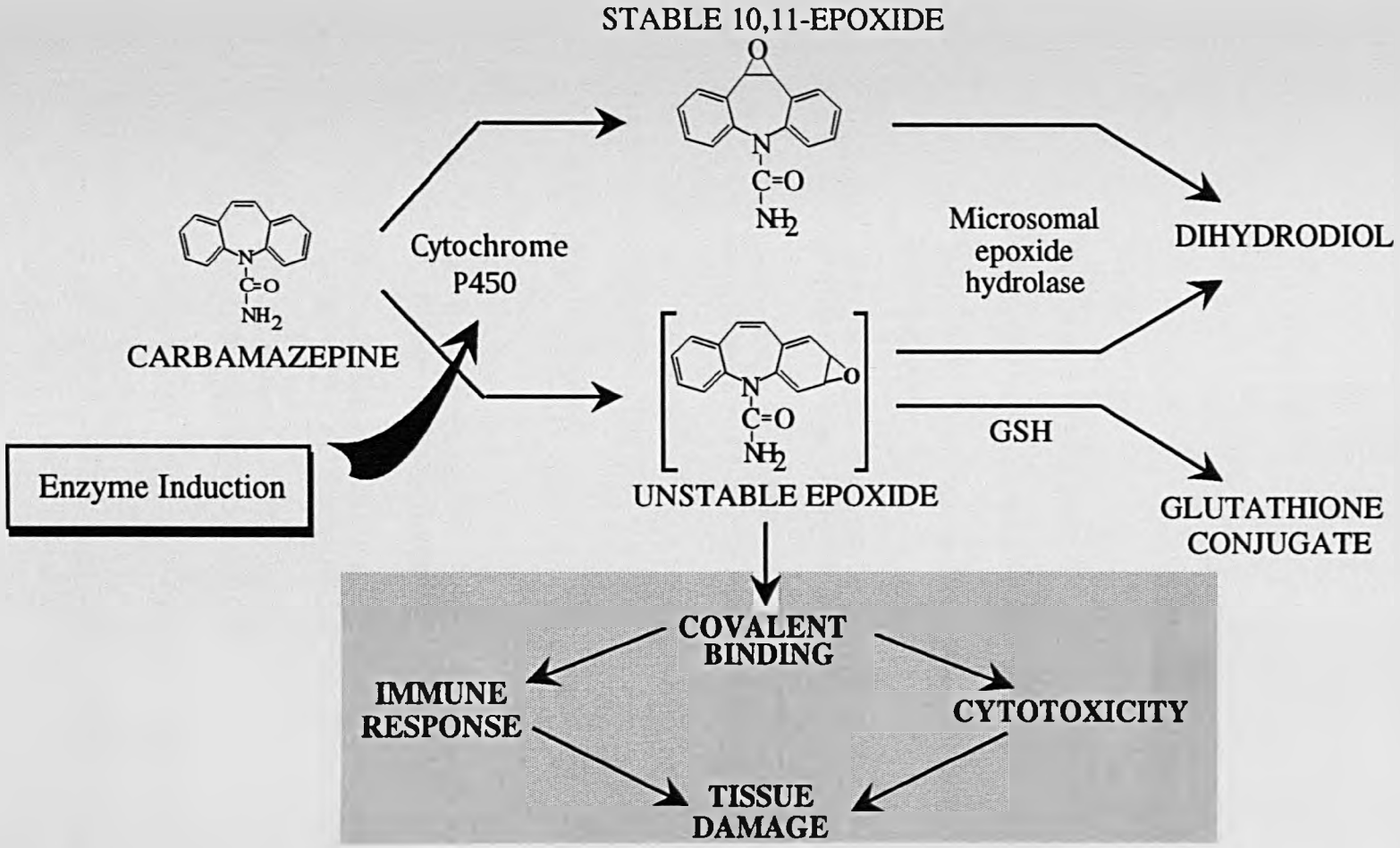


Figure 4.7. Schematic representation of the importance of the balance between activation of carbamazepine to a chemically reactive epoxide metabolite and its detoxication in predisposing to idiosyncratic toxicity.

metabolite formed from carbamazepine and to demonstrate any enzyme deficiency in patients and the possible long-term consequences of such a deficiency.

CHAPTER 5

THE EFFECT OF ENZYME INDUCTION ON THE CYTOCHROME P450-MEDIATED BIOACTIVATION OF CARBAMAZEPINE BY MOUSE LIVER MICROSOMES

5.1. Introduction

Phase I metabolic reactions can lead to bioactivation of a drug causing the formation of a chemically reactive intermediate (Park, 1986). The toxicity of such a metabolite is normally precluded by detoxication pathways resulting in excretion of the harmless metabolite. Thus, the balance between activation and detoxication seems to be an important determinant of individual susceptibility to idiosyncratic toxicity.

An imbalance between the bioactivation and detoxication of a compound may be due to either deficient detoxication and/or enhanced bioactivation. For carbamazepine, a drug well-known to cause idiosyncratic toxicity (Pellock, 1987), it has been shown in the preceding chapters that a cytotoxic and protein-reactive, i.e. chemically reactive, metabolite is formed in the presence of both murine and human hepatic microsomes, with cells from hypersensitive patients showing higher *in vitro* chemical sensitivity to such metabolites of carbamazepine than controls, suggestive of a deficiency in detoxication. Furthermore, the metabolism-dependent cytotoxicity of carbamazepine was enhanced by trichloropropene oxide, an inhibitor of epoxide hydrolase (chapter 2) and mitigated by exogenous microsomal epoxide hydrolase (chapter 4), indicating that the toxic metabolite is an arene oxide and that affected patients may have a deficiency of this detoxication enzyme. Such a deficiency may be genetically determined as has been postulated in patients known to be phenytoin hypersensitive (Spielberg *et al.*, 1981). On the other hand, a relative deficiency may be caused by an enhancement of bioactivation such that the detoxication processes are overwhelmed (Park and Kitteringham, 1990a). In chapter 4, it was shown that bioactivation of carbamazepine was catalysed by a cytochrome P450 enzyme, and

thus induction of the P450 enzymes could also be a possible risk factor in predisposing to idiosyncratic toxicity with carbamazepine. Indeed, the bioactivation of carbamazepine was much greater with induced mouse microsomes than with human microsomes (chapter 4), although this could have been due to a species difference in the isozyme profile and thus the metabolism of carbamazepine.

It has been proposed that the toxicological consequences of P450 enzyme induction may be good, bad or unimportant (Conney, 1982) depending upon the forms of cytochrome P450 which are induced, the chemical environment of the organism and the genetic constitution of the individual. If the model hepatotoxins, bromobenzene and paracetamol are taken as examples, it has been clearly shown that enzyme induction with phenobarbitone increases the hepatotoxicity of both compounds (Mitchell *et al.*, 1973a), while, in contrast, induction with 3-methylcholanthrene increases the liver necrosis caused by paracetamol but not by bromobenzene (Mitchell *et al.*, 1973a; Zampaglione *et al.*, 1973). Enzyme induction caused by chronic alcohol intake also increases the likelihood of paracetamol hepatotoxicity (Lieber, 1988).

In the clinical situation, one group of patients who are chronically exposed to enzyme inducing drugs are epileptic patients. Three out of the four most commonly used anticonvulsants (phenytoin, phenobarbitone, carbamazepine) are potent enzyme inducers (Park and Breckenridge, 1981; Eadie and Tyrer, 1989; Brodie, 1990), known to enhance the metabolism of many other concurrently administered drugs such as warfarin and oral contraceptives (Hansen *et al.*, 1971; Park and Breckenridge, 1981), as well as inducing their own metabolism (Bertilsson *et al.*, 1980; Eichelbaum *et al.*, 1985). The toxicological consequences of such P450 enzyme

induction can be illustrated with reference to hepatotoxicity caused by paracetamol and sodium valproate.

There have been several reports suggesting that patients on chronic anticonvulsant therapy are at an increased risk of developing hepatotoxicity with paracetamol overdosage (Wright and Prescott, 1973; Minton *et al.*, 1988; McClements *et al.*, 1990). This has also been observed in animal studies (Mitchell *et al.*, 1973a). More recently, a retrospective study of patients on long-term anticonvulsant therapy who had taken an overdose of paracetamol, were shown to have a significantly higher mortality than patients not on antiepileptic drugs (Bray *et al.*, 1992). It was postulated that anticonvulsant-mediated induction of CYP1A2 and/or CYP2E1 may have enhanced the formation of the reactive metabolite of paracetamol, N-acetyl-p-benzoquinoneimine, increasing the severity of the hepatic injury (Bray *et al.*, 1992).

Sodium valproate can cause an idiosyncratic form of hepatic failure similar to that seen in Reyes syndrome. Overall, the incidence is about 1 in 10000 (Dreifuss and Langer, 1987). However, the incidence is much higher (1 in 500) in children under the age of two, particularly when they are on multiple (enzyme-inducing) anticonvulsants (Dreifuss and Langer, 1987). Again, it has been postulated that enzyme induction increases the formation of the toxic metabolite of valproate, 2-n-propyl-4-pentenoic acid (Rettie *et al.*, 1987), and indeed, to support this hypothesis, Rettie *et al.* (1987) have shown that the production of the metabolite is cytochrome P450 dependent and inducible by phenobarbitone.

Therefore, it is possible that enzyme induction could increase the bioactivation of carbamazepine, overwhelming detoxication processes and leading to toxicity in certain individuals. Indeed, carbamazepine is an autoinducer (Bertilsson

et al., 1980), and may therefore, increase the formation of its own reactive metabolite. In addition, at least 10% of epileptics are on multiple antiepileptic drugs (Brodie, 1990), which may have additive effects on the bioactivation of carbamazepine.

In order to provide a sufficient and consistent level of metabolic conversion, phenobarbitone-induced mouse microsomes have been used in the preceding chapters and in previous studies (Gerson *et al.*, 1983; Shear *et al.*, 1988; Riley *et al.*, 1989) to investigate the mechanisms of carbamazepine hypersensitivity. Furthermore, a mouse model has also been used to investigate the mechanisms of anticonvulsant teratogenicity, particularly with phenytoin (Finnell, 1991). Therefore, in this study in order to elicit the factors affecting carbamazepine bioactivation, the mouse has been used as an animal model to investigate the effects of three different types of model enzyme inducing agents, phenobarbitone, dexamethasone and β-naphthoflavone.

5.2. Methods

5.2.1. Chemicals

Carbamazepine, human serum albumin (HSA, fraction V), phenobarbitone, dexamethasone, β-naphthoflavone, NADPH (tetrasodium salt), glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (Poole, U.K.). [10,11-^{14}C]carbamazepine (radiochemical purity 99%) and carbamazepine-10,11-epoxide were gifts from Ciba-Geigy Pharmaceuticals (Basle, Switzerland). [1,2,6,7-^{3}H]Cortisol (radiochemical purity 98%) was obtained from

Amersham International while 6ß-hydroxycortisol was synthesised by Dr. J.H.K. Yeung (Chinese University of Hong Kong). All solvents were of HPLC grade and were products of Fisons plc (Loughborough, UK).

5.2.2. Treatment of animals and preparation of mouse liver microsomes

All animals used were male CBA/ca mice (25-30g), housed in plastic cages on hardwood chip bedding, in an animal room with controlled temperature (22-24°C) and photoperiod (12h light, 12h dark cycles). The mice were provided with tap water and pelleted food (Labsure Rodent Breeder Diet, Rank Hovis McDougall, Poole, UK) *ad libitum*.

Groups of male CBA/ca mice (25-30g) were induced with phenobarbitone, dexamethasone and β-naphthoflavone (table 5.1), the control mice (n=6 per group) receiving equivalent volumes (0.15ml) of vehicle only. As per standard protocols, saline and corn oil were used as vehicles for phenobarbitone and β-naphthoflavone, respectively (Riley *et al.*, 1990b). With dexamethasone, initial experiments were carried out with dexamethasone phosphate dissolved in saline; however, this failed to cause cytochrome P450 induction. Thus, dexamethasone base at a dose of 100mg/kg which was found to be optimal for enzyme induction was used. Dexamethasone base was insoluble in saline, but was found to be soluble in a mixture of polyethylene glycol and saline (75:25, v/v), the polyethylene glycol being added first followed by the slow addition of saline while continually mixing. Following the final set of injections, the mice were fasted for 24h and then killed by cervical dislocation. The livers were dissected out and microsomes prepared by differential centrifugation as described in section 2.2. Pooled microsomes from each

Table 5.1.

Protocols used for treatment of male CBA/ca mice with the model enzyme inducers, phenobarbitone, dexamethasone and β-naphthoflavone

Compound	*Number of mice*	*Dose*	*Route of administration*	*Vehicle*	*Duration*
Phenobarbitone	6	60mg/kg/day	intraperitoneally	Saline	3 days
Dexamethasone	6	100mg/kg/day	intraperitoneally	Polyethylene glycol/saline (75:25 v/v)	3 days
β-naphthoflavone	6	60mg/kg/day	intraperitoneally	Corn oil	3 days

group of animals were then stored at -80°C until used. Protein content was estimated by the method of Lowry *et al.* (1951). Microsomal suspensions were prepared (20mg/ml) in 0.067M phosphate buffer (pH 7.4) for the experiments immediately prior to use and were maintained at 4°C throughout. The cytochrome P450 content was measured in diluted samples (1mg/ml in phosphate buffer, pH 7.4) of the pooled microsomal suspensions by the method of Omura and Sato (1964) as described in section 2.2.6.

5.2.3. Isolation of human mononuclear leucocytes from peripheral blood

Peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinised venous blood (30ml) as described previously in section 2.2.7. Their viability upon isolation as determined by trypan blue dye exclusion was >95%. To eliminate inter-individual variability in detoxication enzymes, MNL used in these experiments were isolated from the same male donor (aged 30 years).

5.2.4. Determination of the metabolism-dependent cytotoxicity of carbamazepine

Isolated MNL (1 x 10^6) in HEPES-buffered balanced salt medium (1ml) (Spielberg, 1980) were incubated with carbamazepine (50μM; dissolved in methanol, 1% (v/v) final concentration) and either induced or control murine liver microsomes (1mg) in the presence or absence of NADPH (1mM) for 2h at 37°C (section 2.2.8.). The concentration of carbamazepine used was not directly cytotoxic. After 2h, the cells were sedimented and resuspended in drug-free medium (HEPES-buffered medium containing 5mg/ml HSA). After a further 16h incubation at 37°C (section 2.2.8.), cell viability was determined by trypan blue dye exclusion as described in

section 2.2.9. At least 200 cells were counted, the cell death being expressed as a percentage of cells not excluding the dye. All incubations were performed in quadruplicate.

5.2.5. Determination of the metabolism of carbamazepine to protein-reactive and stable metabolites by murine hepatic microsomes

Irreversible binding of radiolabelled material was determined by the method described in section 4.2.8. except that the MNL were omitted from the incubations. [^{14}C]Carbamazepine (50μM; 0.15μCi) was incubated with the different sets of murine microsomes (1mg protein) in HEPES-buffered medium (pH 7.4; final volume 1ml), the reaction being initiated by the addition of NADPH (1mM, omitted from control incubations) and terminated after 2h at 37°C by the addition of 3ml acetonitrile. The incubations were left overnight at -20°C to precipitate the protein. Irreversible binding of the radiolabelled material to the precipitated protein was determined after removal of unbound drug by exhaustive solvent extraction of the protein as described in section 4.2.9. The protein was solubilised in 1M NaOH (1ml) at 60°C overnight and aliquots were taken for quantification of radioactivity and protein estimation (section 4.2.8.). All incubations were performed in quadruplicate. Irreversible binding of [^{14}C]carbamazepine is expressed as a percentage of the initial radioactivity bound to the incubated microsomal protein.

The effect of gestodene, an inhibitor of CYP3A (Guengerich, 1990a, b), on the covalent binding of carbamazepine was determined by the addition of gestodene (10-250μM; dissolved in 10μl methanol) to the above incubations prior to the initiation of the reaction by NADPH. Methanol (10μl) was added to control incubations

without gestodene so that the final concentration of methanol in these incubations was 2% (v/v).

The supernatants from the incubations with [^{14}C]carbamazepine were analysed for unchanged carbamazepine and its 10,11-epoxide by radiometric HPLC using the method of Regnaud *et al.* (1988) as described in section 4.2.9. The radioactivity was monitored throughout the run and the peaks were integrated and expressed as the percentage of radioactivity eluting from the column.

5.2.6. Determination of the *in vitro* metabolism of cortisol to 6ß hydroxycortisol by murine hepatic microsomes

The effect of the different model enzyme inducing agents on CYP3A activity was determined by measuring the *in vitro* conversion of cortisol to 6β hydroxycortisol (Abel *et al.*, 1992). Hepatic microsomes (1mg protein) from control or induced mice were incubated with [^{3}H]cortisol (0.1μCi) and cortisol (1μM) in 0.067M phosphate buffer (pH 7.4; final incubation volume 0.5ml) for 2h at 37°C in silanised 10ml glass tubes. The reaction was initiated by the addition of a NADPH-regenerating system (10mM $MgCl_2$, 10mM glucose-6-phosphate, 5mM NADP and 2 units glucose-6-phosphate dehydrogenase). The individual components of the NADPH-regenerating system had initially been mixed together and warmed at 37°C for 10 min prior to their addition to the microsomal incubations. The effect of inhibition of CYP3A on 6β hydroxylation of cortisol was determined by the addition of gestodene (50μM) to some incubations prior to the addition of the NADPH-regenerating system. The cortisol and gestodene were both added in methanol; these were added to the tubes first, the solvent then being evaporated under nitrogen. After 2h, the reaction was

terminated by cooling in crushed ice and followed by extraction with ethyl acetate (2ml). After mixing on a rotary mixer for 5 min, the tubes were centrifuged for 5 min at 2000*g* and the organic layer was transferred to a 10ml silanised glass tube. The aqueous layer was extracted again with ethyl acetate (2ml), which was then aspirated and pooled with the organic layer from the first extraction. The organic phase (4ml total volume) was evaporated under nitrogen and the samples reconstituted in 100µl methanol.

HPLC analysis was performed on 25µl aliquots of the sample using a reversed phase C_8 column (Spherisorb, 5µm, 25cm x 4.6mm i.d.; HPLC Technology, Macclesfield, U.K.). The solvent system (75% 0.4mM ammonium phosphate buffer : 25% acetonitrile) was delivered at a flow rate of 0.7ml/min (Altex, Anachem, Luton, U.K.) and the absorbance of the eluant was monitored at 220nm (Kratos Spectraflow 773) for 50 min. Cortisol and 6ß-hydroxycortisol were identified by co-injection of authentic compounds. Quantification of cortisol and 6ß-hydroxycortisol was accomplished by collecting 1ml fractions of eluant to which 4ml of scintillant were added before determination of radioactivity using liquid scintillation spectroscopy for 4 min. 6ß-hydroxycortisol was expressed as percentage of the total radioactivity eluting from the column.

5.2.7. SDS-PAGE electrophoresis and immunoblotting of the murine microsomal proteins

Essentially, the same procedure described in section 3.3.6. was used for electrophoresis and immunoblotting of the murine microsomes except that the

resolving gel had a final polyacrylamide concentration of 10%. This was prepared by mixing a solution containing 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide (30ml), tris-HCl buffer (22.5ml, 1.5M tris, 1M HCl, pH 8.7) and distilled water (37.5ml) with aliquots of 20% (w/v) SDS (450µl), TEMED (45µl) and 10% (w/v) ammonium persulphate (450µl). The stacking gel was prepared as described in section 3.3.6. and had a final acrylamide concentration of 4%.

The murine hepatic microsomes (50µg) were diluted 1:1 with sample buffer containing dithiothreitol and boiled for 5 min (section 3.3.6.). The samples (10µl) were loaded into the wells and a current of 30mA/gel applied for approximately 1h (Laemmli, 1970). Unstained low molecular weight markers were also included on each gel, and comprised phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soya bean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The separated proteins were transferred to nitrocellulose electrophoretically, which was then used for either protein staining or taken for antibody overlay according to the method of Towbin and Gordon (1984), as described in section 3.3.6.. Following blocking of non-specific binding sites with casein buffer (2.5% (w/v) casein), 154mM NaCl, 10mM tris, 0.5mM thimerosal; pH 7.6), the nitrocellulose was incubated with either polyclonal antibodies to rat CYP3A (1:2500 dilution, Oxygene, Dallas) or mouse monoclonal antibodies to CYP1A2 (1:2500 dilution; supplied by Dr. R. J. Riley, Fisons plc, Loughborough, UK) for 3h. Following three successive washes (section 3.3.6.) a horseradish peroxidase-labelled second antibody (dilution 1:5000; either goat anti-rabbit IgG or goat anti-mouse IgG) was incubated with the blots overnight at room temperature to reveal the immunoreactive polypeptides. The sites of antibody binding were visualised by

enhanced chemiluminescence (ECL; Amersham International, Amersham, UK), a technique which is more sensitive than the chloro-naphthol method used in section 3.3.6.. In this technique, the peroxidase-labelled second antibodies catalyse the oxidation of luminol which in the presence of a chemical enhancer produces a sustained light emission which can be readily detected on film. Thus, after incubation with the peroxidase-labelled second antibodies, the blots were washed with casein washing buffer (3 x 10 min) followed by incubation with the ECL detection reagent for 1 min and exposure to film (Hyperfilm-ECL, Amersham International, UK) for 30 sec. The relative intensities of the bands in the different sets of microsomes were quantified by laser densitometry (LKB Ultroscan XL, Bromma, Sweden) and integration of the absorbance peak associated with each band.

5.2.8. Statistical analysis

All the results are presented as mean ± SEM. Statistical analysis was performed by ANOVA, accepting $p<0.05$ as significant. Correlation coefficients were calculated by linear regression analysis.

5.3. Results

5.3.1. The effect of pre-treatment of mice with model enzyme inducers on the microsomal cytochrome P450 content

The total cytochrome P450 content of the murine microsomes increased (when compared with microsomes prepared from control animals) after pretreament with both phenobarbitone (1.3 *vs.* 1.0 nmol/mg protein) and dexamethasone (1.6 *vs.*

1.0 nmol/mg protein), but not with β-naphthoflavone pretreatment (0.6 *vs.* 0.5 nmol/mg protein). However, immunoblotting of the microsomes using monoclonal antibodies directed against CYP1A2 revealed the presence of a protein band with an apparent molecular mass of 53kDa, the most intense band being seen with microsomes prepared from mice pretreated with β-naphthoflavone, indicating induction of this isozyme by β-naphthoflavone (Figure 5.1).

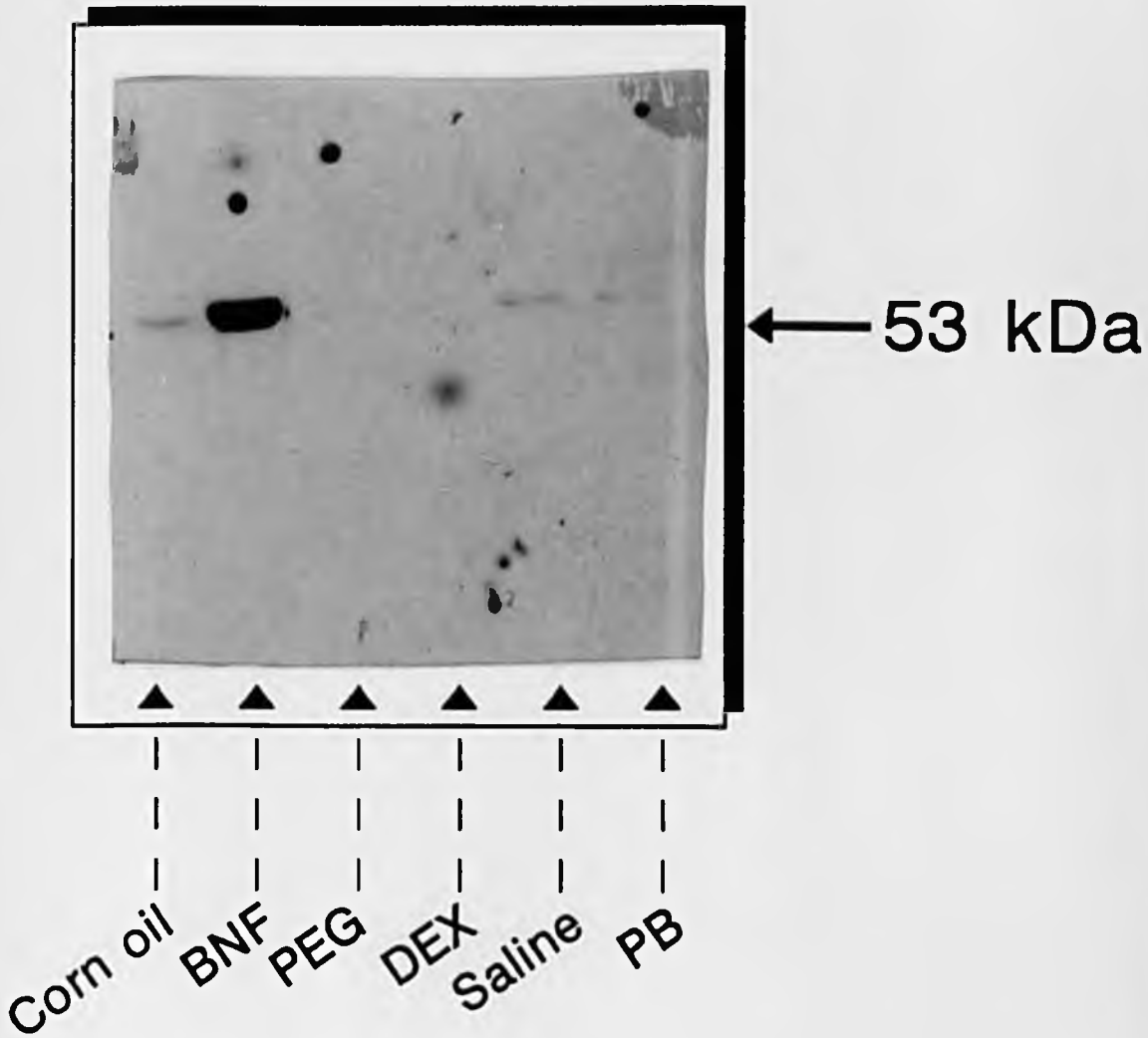
5.3.2. Effect of enzyme induction on the formation of cytotoxic, protein-reactive and stable metabolites of carbamazepine

Enzyme induction with both phenobarbitone and dexamethasone increased the bioactivation of carbamazepine to cytotoxic (figure 5.2) and protein-reactive species (figure 5.3) when compared with their respective control microsomes. In contrast, β-naphthoflavone pre-treatment did not increase the bioactivation of carbamazepine relative to the corn oil (control) pre-treated mice (figures 5.2 and 5.3).

The metabolic conversion of carbamazepine to the stable 10,11-epoxide was increased (relative to the respective control microsomes) after induction with dexamethasone (38.0 ± 1.3% *vs.* 19.8 ± 0.8%; $p<0.001$) and phenobarbitone (33.8 ± 1.1% *vs.* 18.1 ± 0.9%; $p<0.001$) but not β-naphthoflavone (5.0 ± 0.4% *vs.* 5.6 ± 0.5%; N.S.).

Figure 5.1.

Immunoblots of murine liver microsomal proteins exposed to monoclonal antibodies to CYP1A2 (provided by Dr. R.J. Riley, Fisons, Loughborough). Microsomal protein (50μg) prepared from mice pretreated with corn oil, ß-naphthoflavone (BNF), Polyethylene glycol (PEG), dexamethasone (DEX), saline and phenobarbitone (PB) were separated on a 10% polyacrylamide gel and transferred to the nitrocellulose support electrophoretically. Immunoblotting was carried out as described in the methods section, with the sites of antibody binding being visualised by enhanced chemiluminescence. The indicated size (53kDa) was determined by interpolation of the mobility of molecular weight standards.


53 kDa
Corn oil
BNF
PEG
DEX
Saline
PB

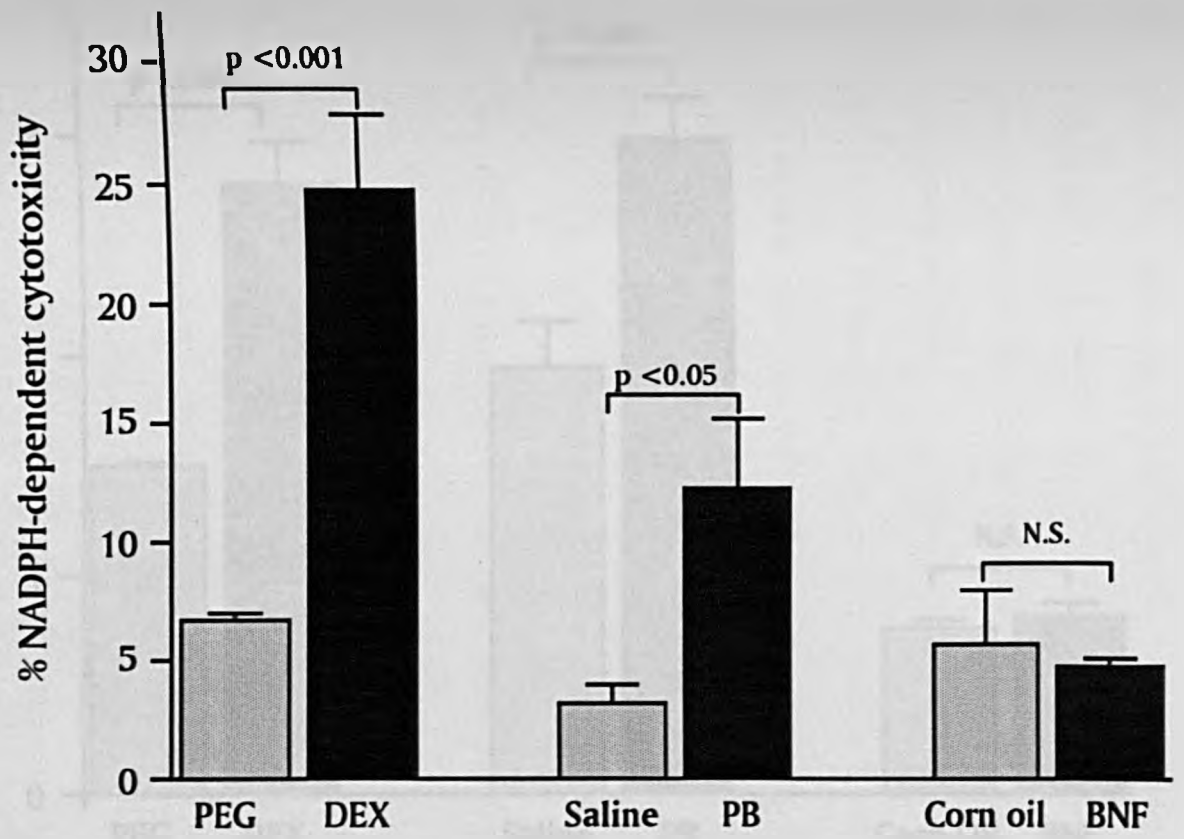


Figure 5.2. The effect of induction with dexamethasone (DEX), phenobarbitone (PB) and ß-naphthoflavone (BNF) on the metabolism (NADPH)-dependent cytotoxicity of carbamazepine (50μM). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by ANOVA comapring the induced microsomes with their respective controls : polyethylene glycol (PEG), saline and corn oil for dexamethasone, phenobarbitone and ß-naphthoflavone microsomes, respectively.

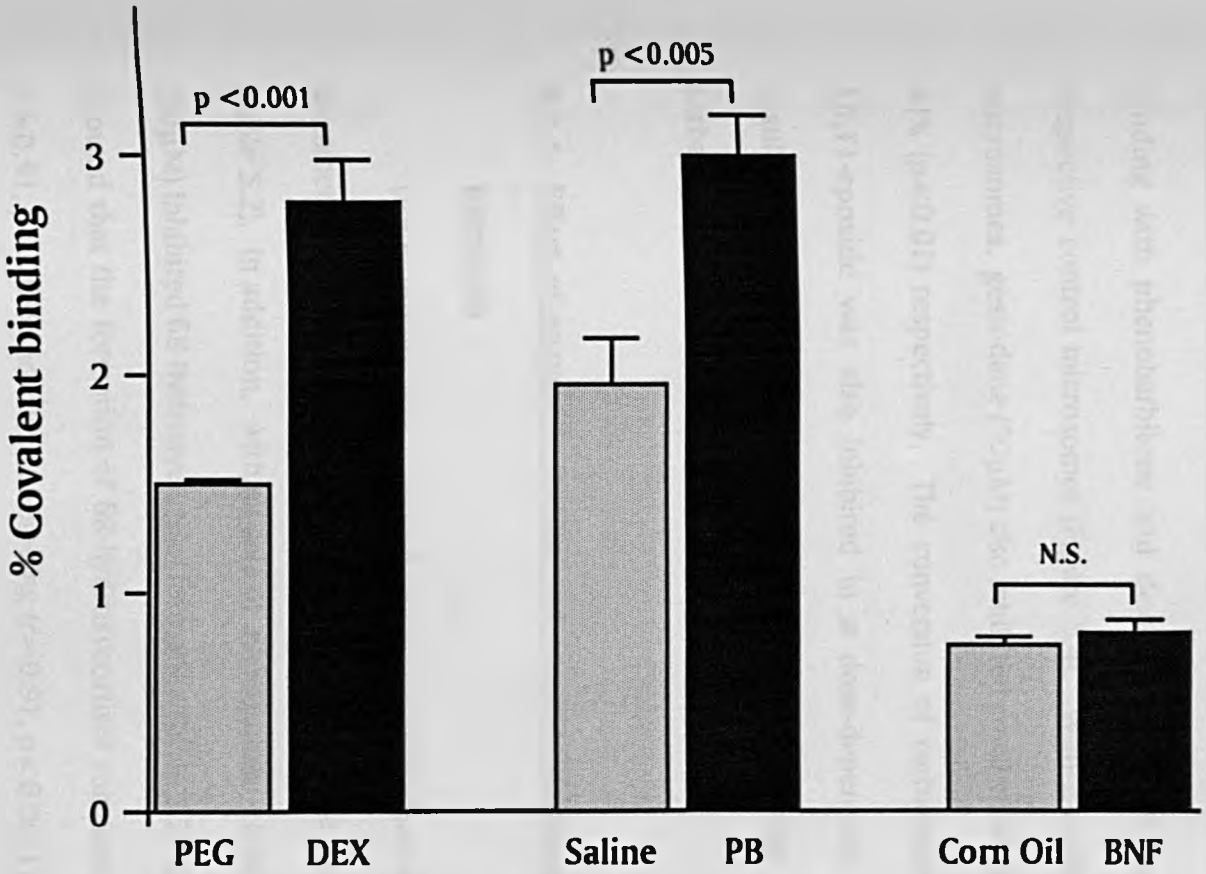


Figure 5.3. The effect of induction with dexamethasone (DEX), phenobarbitone (PB), and ß-naphthoflavone (BNF) on the irreversible binding of radiolabelled carbamazepine (0.15μCi; 50μM). The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by ANOVA comparing the induced microsomes with their respective controls: polyethylene glycol (PEG), saline and corn oil for dexamethasone, phenobarbitone and ß-naphthoflavone microsomes, respectively.

5.3.3. Effect of gestodene on the formation of protein-reactive and stable metabolites of carbamazepine

Gestodene, a specific inhibitor of CYP3A (Guengerich, 1990b), could not be used as an inhibitor of carbamazepine bioactivation in the lymphocyte cytotoxicity assay because it was cytotoxic itself. However, co-incubation of gestodene with radiolabelled carbamazepine resulted in a dose-dependent inhibition of covalent binding with phenobarbitone and dexamethasone microsomes, and with their respective control microsomes (Figure 5.4). With β-naphthoflavone and corn oil microsomes, gestodene (50μM) also inhibited covalent binding by 30% ($p<0.05$) and 41% ($p<0.01$) respectively. The conversion of carbamazepine to carbamazepine-10,11-epoxide was also inhibited in a dose-dependent manner by gestodene, resulting in a highly significant correlation between covalent binding and carbamazepine-10,11-epoxide formation (figure 5.5).

5.3.4. Effect of enzyme induction and enzyme inhibition on 6ß-hydroxycortisol formation

Use of microsomes prepared from mice pretreated with phenobarbitone and dexamethasone, but not β-naphthoflavone, increased the 6ß-hydroxylation of cortisol (table 5.2). In addition, with all sets of microsomes, co-incubation with gestodene (50μM) inhibited 6ß-hydroxycortisol formation (table 5.2). Linear regression analysis showed that the formation of 6ß-hydroxycortisol correlated with both cytotoxicity ($r=0.91$, $p<0.01$) and covalent binding ($r=0.91$, $p<0.001$) of carbamazepine (figure 5.6).

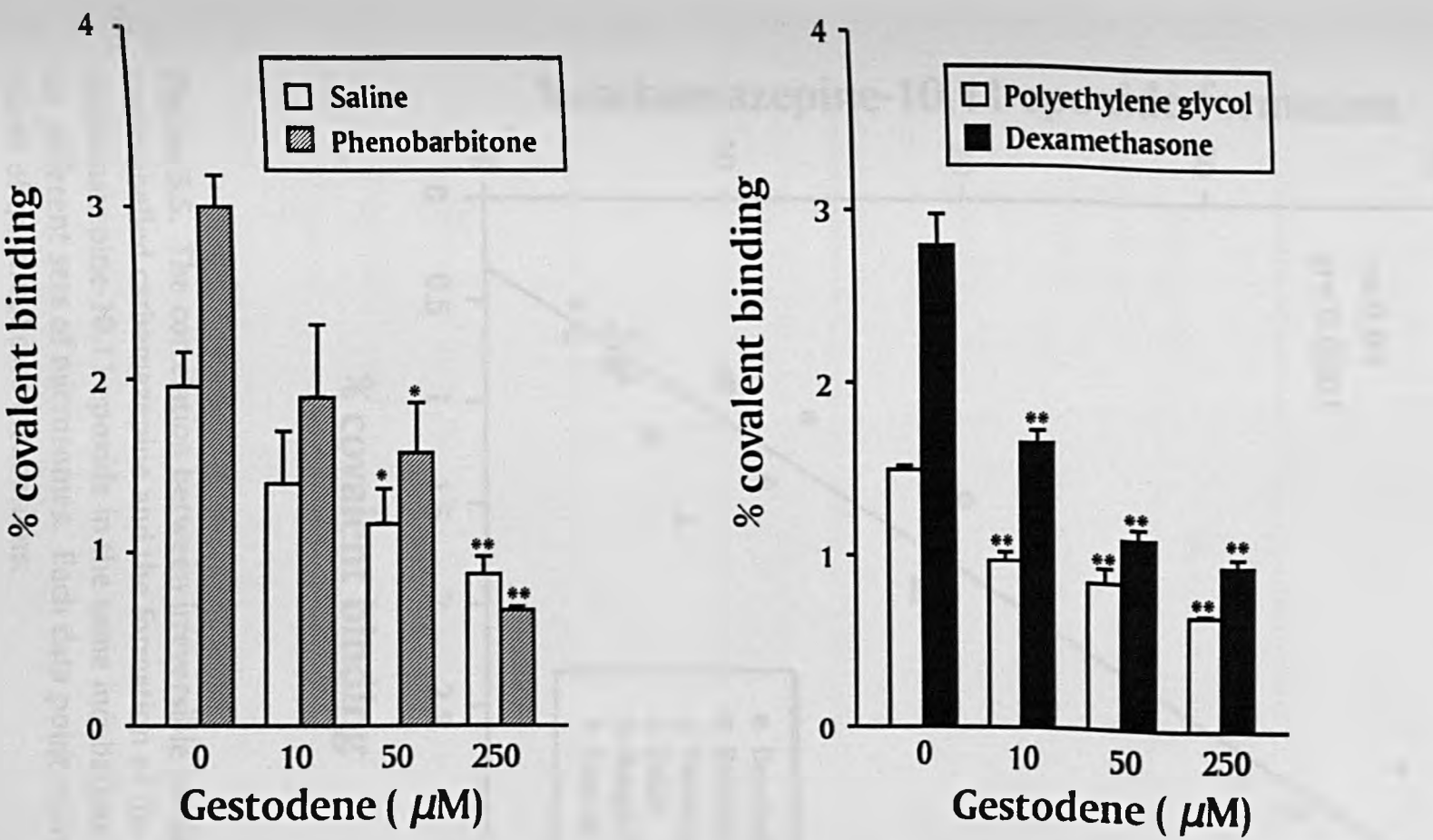


Figure 5.4. The effect of gestodene (10, 50 and 250 μM) on the bioactivation of radiolabelled carbamazepine to a protein-reactive metabolite in the presence of hepatic microsomes prepared from mice pretreated with phenobarbitone or its vehicle (saline) and dexamethasone or its vehicle (polyethylene glycol). The results represent the mean of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without gestodene : * $p < 0.05$, ** $p < 0.001$.

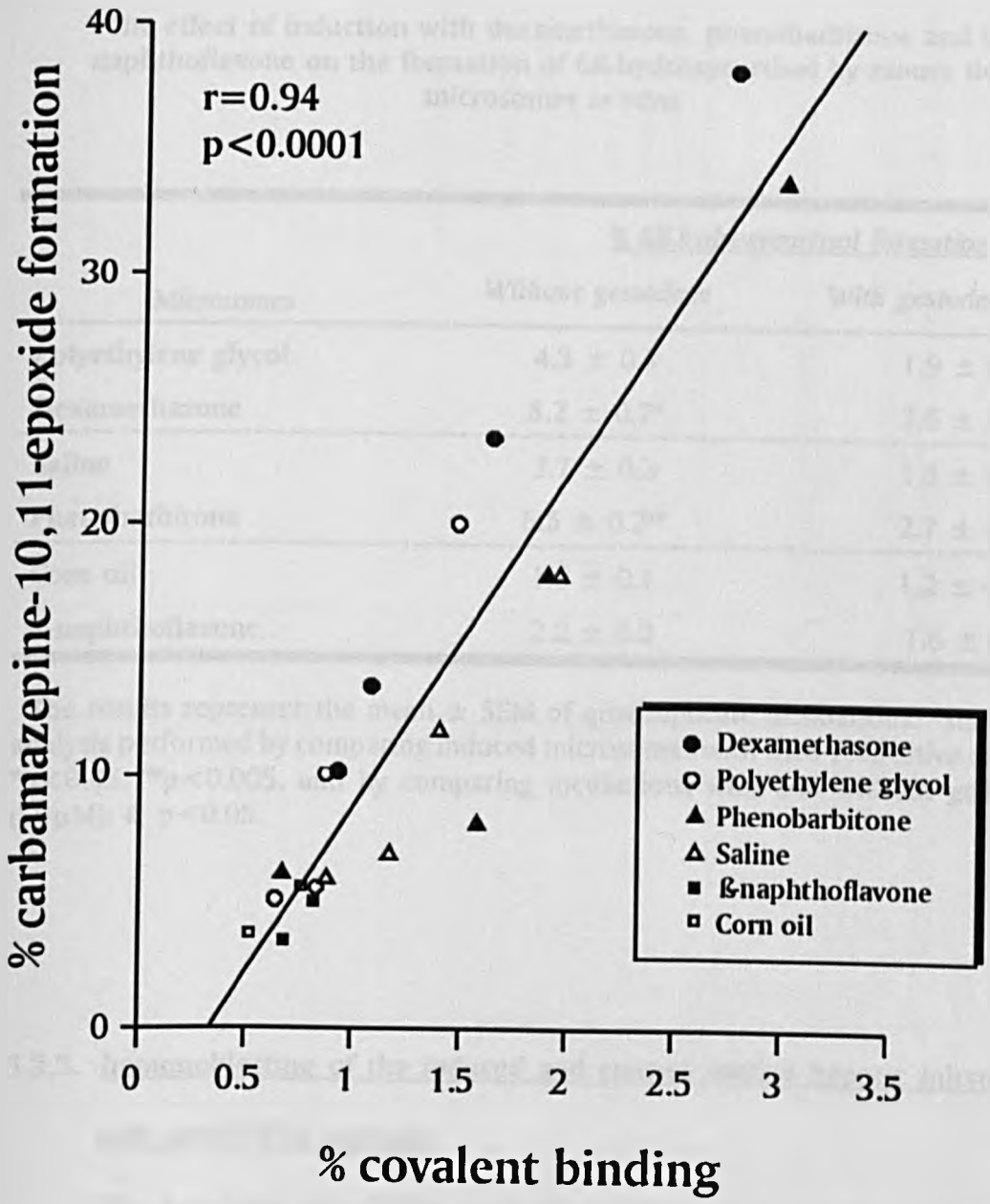


Figure 5.5. The correlation between irreversible binding of radiolabelled carbamazepine and the formation of the stable carbamazepine-10,11-epoxide in the same incubations with the six different sets of microsomes. Each data point represents the mean of quadruplicate incubations.

Table 5.2.

The effect of induction with dexamethasone, phenobarbitone and ß-naphthoflavone on the formation of 6ß-hydroxycortisol by mouse liver microsomes *in vitro*

	% 6ß-hydroxycortisol formation	
Microsomes	*Without gestodene*	*With gestodene (50 µM)*
Polyethylene glycol	4.3 ± 0.9	1.9 ± 0.2#
Dexamethasone	8.2 ± 0.7*	2.6 ± 0.5#
Saline	2.7 ± 0.3	1.8 ± 0.2#
Phenobarbitone	6.5 ± 0.2**	2.7 ± 0.7#
Corn oil	1.6 ± 0.1	1.2 ± 0.1#
ß-naphthoflavone	2.2 ± 0.2	1.6 ± 0.1

The results represent the mean ± SEM of quadruplicate incubations. Statistical analysis performed by comparing induced microsomes with their respective controls: *$p<0.05$, **$p<0.005$, and by comparing incubations with and without gestodene (50µM): # $p<0.05$.

5.3.5. Immunoblotting of the induced and control murine hepatic microsomes with anti-CYP3A antibody

The polyclonal anti-CYP3A antibodies recognised a protein band with an apparent molecular mass of 52kDa in all the murine hepatic microsomes. However, the relative intensity of the bands, as determined by laser densitometry, was variable between the microsomes, being highest with the dexamethasone and phenobarbitone pre-treated microsomes (Figure 5.7). A cross-reacting band of lower molecular weight was seen with the dexamethasone-pretreated microsomes (figure

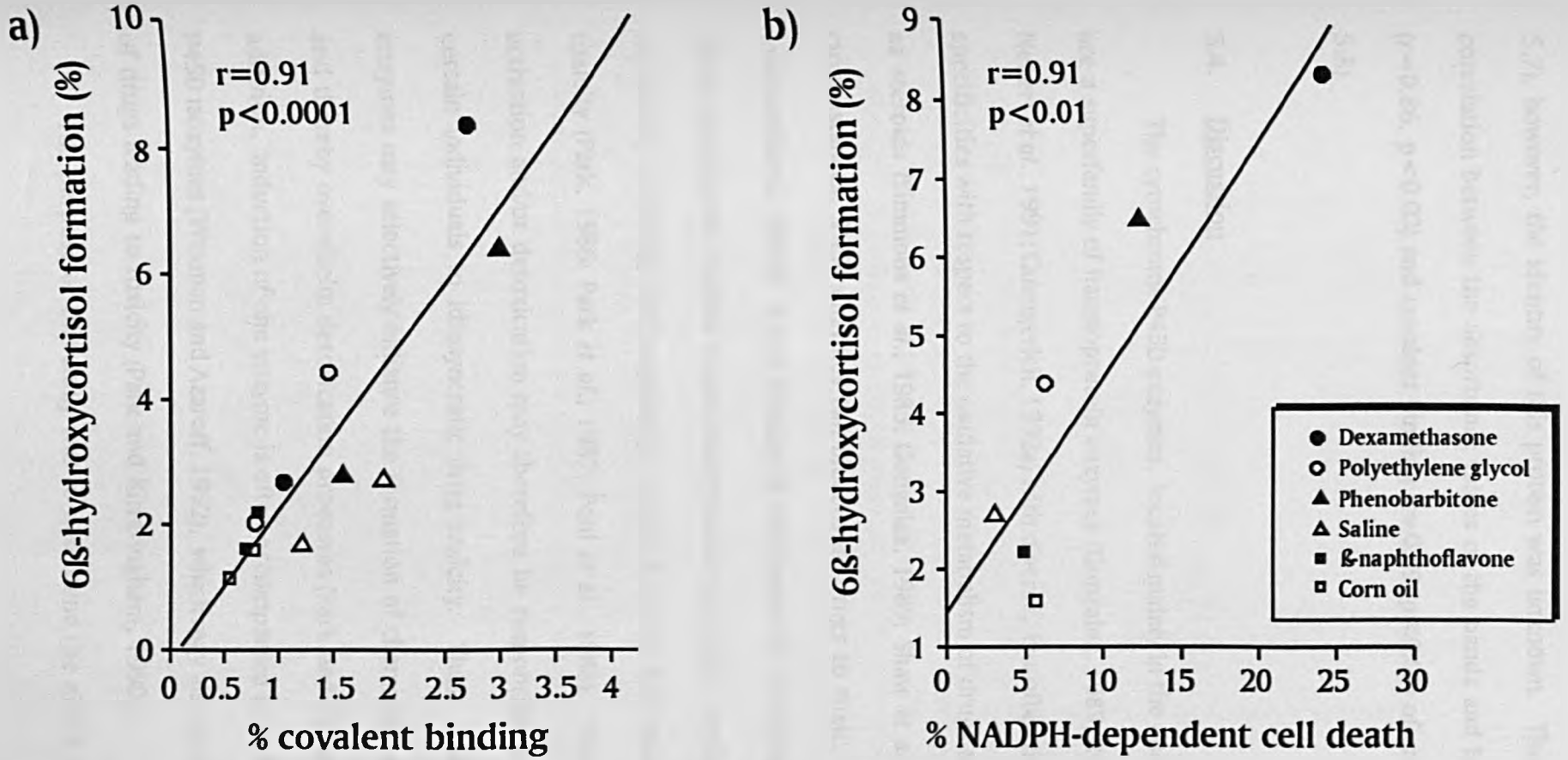


Figure 5.6. The correlation between the formation of 6ß-hydroxycortisol and (a) covalent binding and (b) metabolism-dependent cytotoxicity of carbamazepine with the six different sets of microsomes. Each data point represents the mean of quadruplicate incubations.

5.7), however, the identity of this protein was unknown. There was a significant correlation between the absorbance values of the bands and both the cytotoxicity ($r=0.86$, $p<0.03$) and covalent binding ($r=0.89$, $p<0.02$) of carbamazepine (figure 5.8).

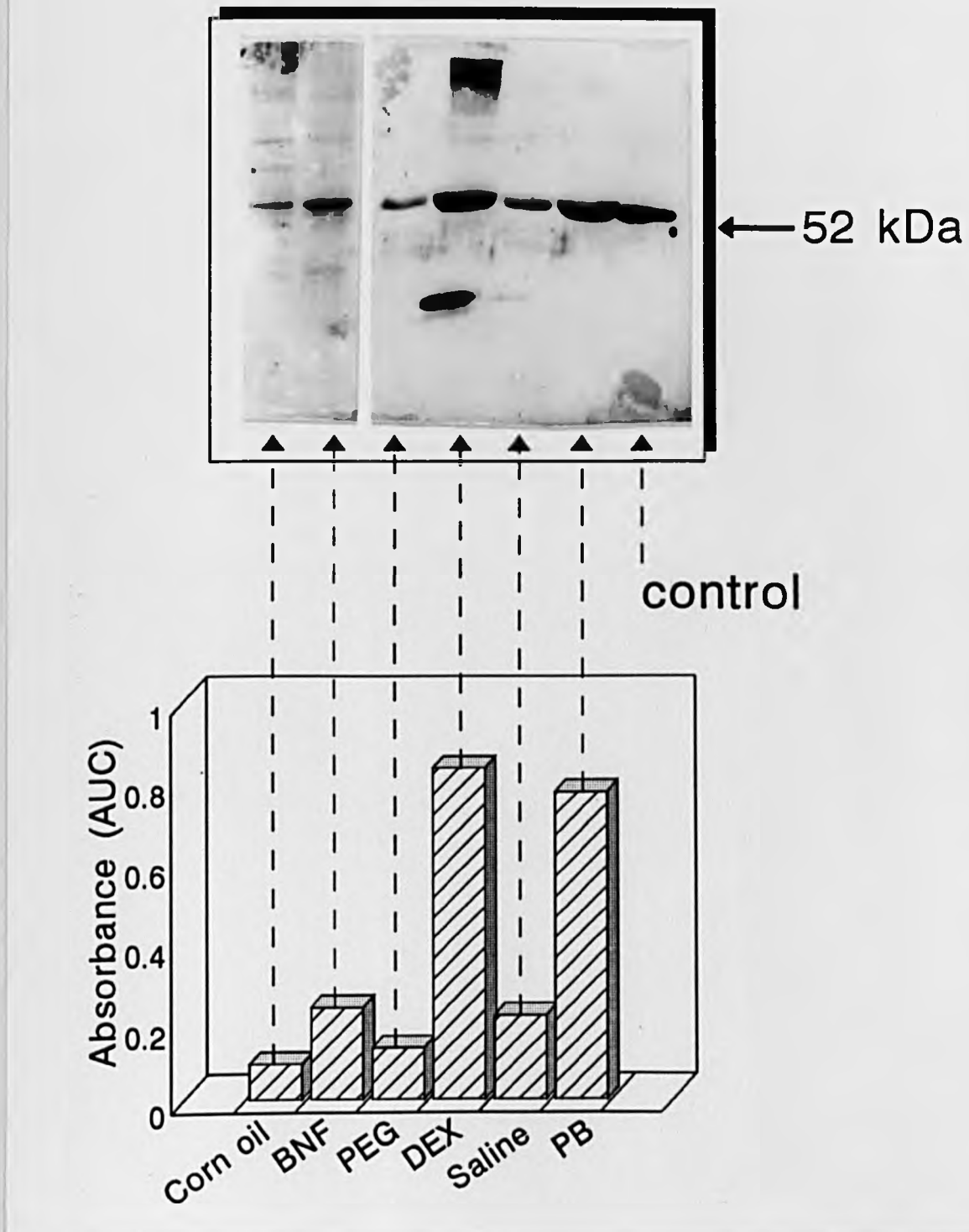
5.4. Discussion

The cytochrome P450 enzymes, located mainly in the endoplasmic reticulum, are a superfamily of haemoprotein enzymes (Gonzalez, 1989; Ryan and Levin, 1990; Nebert *et al.*, 1991; Guengerich, 1992a) with diverse, but often overlapping substrate specificities with respect to the oxidative metabolism of drugs and endobiotics such as steroids (Simmons *et al.*, 1985; Gonzalez, 1989; Shaw *et al.*, 1989). In certain circumstances, P450 enzymes can bioactivate drugs to toxic, chemically reactive intermediates, which, if not adequately inactivated by detoxication processes such as the glutathione enzyme system and epoxide hydrolase, can lead to various forms of toxicity including carcinogenicity, tissue necrosis and immune-mediated drug toxicity (Park, 1986; Park *et al.*, 1987; Pohl *et al.*, 1988). Factors affecting either activation and/or detoxication may therefore be responsible for predisposition of certain individuals to idiosyncratic drug toxicity. Thus, induction of the P450 enzymes may selectively enhance the formation of chemically reactive metabolites, and thereby overwhelm detoxication processes (Park and Kitteringham, 1990a). In addition, induction of one enzyme is often accompanied by a decrease in the other P450 isozymes (Waxman and Azaroff, 1992), which may also perturb the metabolism of drugs leading to toxicity (Park and Kitteringham, 1990a).

The purpose of this study was to determine the effect of enzyme induction

Figure 5.7.

Immunoblots of murine liver microsomal proteins exposed to polyclonal antibodies raised against rat CYP3A (Oxygene, Dallas, USA). Microsomal protein (50μg) prepared from mice pretreated with corn oil, ß-naphthoflavone (BNF), Polyethylene glycol (PEG), dexamethasone (DEX), saline and phenobarbitone (PB) were separated on a 10% polyacrylamide gel and transferred to the nitrocellulose support electrophoretically. Immunoblotting was carried out as described in the methods section, with the sites of antibody binding being visualised by enhanced chemiluminescence. The "control" refers to microsomes prepared from rats pretreated with pregnenolone-16α-carbonitrile (supplied by the manufacturer as a positive control). The BNF and corn oil microsomes were run on a separate gel from the other four sets of microsomes; the "control" microsomes were run on both gels, the relative intensities of the bands on the two gels being similar (0.95 and 1.0 absorbance units as assessed by laser densitometry). The indicated size (52kDa) was determined by interpolation of the mobility of molecular weight standards. The bottom half of the figure is a graphical representation of the relative intensities of the bands with the different microsomes, as determined by laser densitometry.


52 kDa
control
Absorbance (AUC)
0
0.2
0.4
0.6
0.8
1
Corn oil
BNF
PEG
DEX
Saline
PB

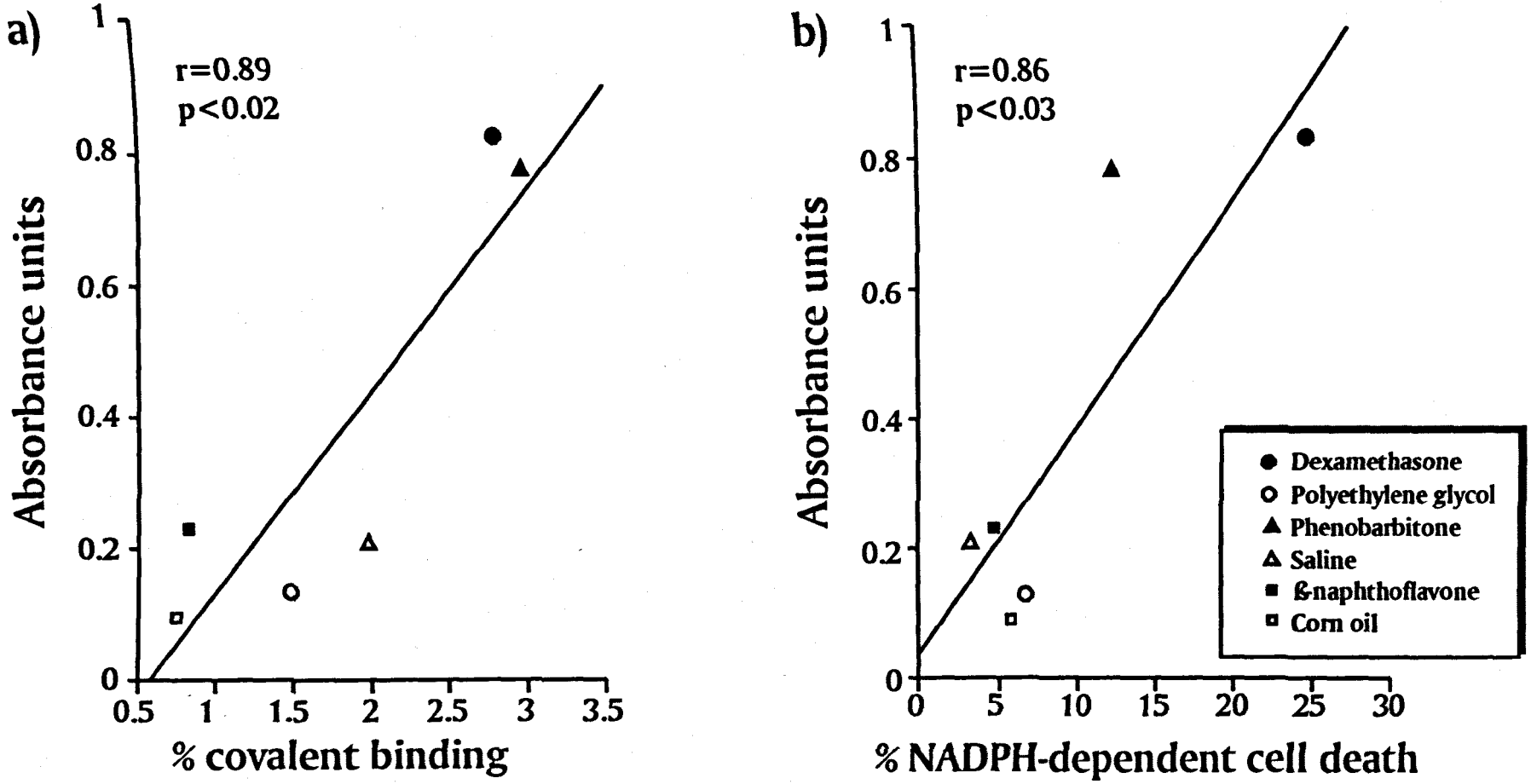


Figure 5.8. Correlation between the intensity of the protein bands (as measured by laser densitometry and expressed as absorbance units) on the nitrocellulose blots after incubation with antibody directed against CYP3A and (a) the covalent binding and (b) metabolism-dependent cytotoxicity of carbamazepine.

on the bioactivation of carbamazepine, using two indirect markers for the formation of toxic metabolites, namely cytotoxicity and covalent binding. Thus, it was found that enzyme induction with phenobarbitone and dexamethasone, but not β-naphthoflavone, increased the formation of stable and toxic metabolites of carbamazepine (figures 5.2 and 5.3) relative to their respective control microsomes, which had been treated with vehicle only. Such treatment of control animals was considered to be important because vehicles such as corn oil can themselves have an effect on the P450 isozyme profile (Yoo *et al.*, 1990). The three model enzyme inducing agents used in this study are selective enzyme inducers in that phenobarbitone is an inducer of CYP2B, CYP2C and CYP3A (Waxman and Azaroff, 1992) while dexamethasone and β-naphthoflavone are inducers of CYP3A (Watkins *et al.*, 1985; Wrighton *et al.*, 1985b; Waxman and Azaroff, 1992) and CYP1A (Okey, 1990), respectively. Pretreatment with phenobarbitone and dexamethasone increased the total cytochrome P450 content, while β-naphthoflavone pretreatment did not increase the total P450 content despite specific induction of CYP1A2 which was demonstrated by immunoblotting of the microsomes with an anti-CYP1A2 monoclonal antibody. Indeed, the total P450 content of the microsomes prepared from ß-naphthoflavone and corn oil treated animals was lower than that of the saline and polyethylene glycol (i.e. control) treated animals. This may be due to the fact that both ß-naphthoflavone (Okey, 1990) and corn oil (Yoo *et al.*, 1990) although inducing specific (but minor) P450 isozymes, often lead to a decrease in the other P450 families (Waxman and Azaroff, 1992). The increase in the bioactivation of carbamazepine observed after induction with dexamethasone and phenobarbitone would suggest that the CYP3A family is involved in its bioactivation. In accordance

with this, immunoblotting performed with antibodies raised against rat CYP3A (which have been shown to cross-react with the mouse orthologue; Wrighton *et al.*, 1985b) not only recognised a protein in all the mouse liver microsomes (figure 5.7) but also the relative intensities of these bands were highest with microsomes prepared from mice pre-treated with phenobarbitone and dexamethasone (figure 5.7) showing an excellent correlation with the two parameters of carbamazepine bioactivation.

Supportive evidence for the involvement of CYP3A in carbamazepine bioactivation was provided by measurement of 6ß-hydroxycortisol formation with the same microsomes used to determine carbamazepine bioactivation and by the effect of gestodene. Cortisol 6ß-hydroxylase activity, which has been used as an index of of CYP3A for human studies both *in vitro* (Ged *et al.*, 1989; Abel *et al.*, 1992) and *in vivo* (Park and Ohnhaus, 1983; Ged *et al.*, 1989; Ohnhaus *et al.*, 1989), was increased by induction of CYP3A with phenobarbitone and dexamethasone (table 5.2), and showed an excellent correlation with both the cytotoxicity and covalent binding of carbamazepine. Cortisol 6ß-hydroxylase activity has not been measured *in vitro* with mouse liver microsomes but the effect of induction combined with the inhibition of its formation with gestodene, an inhibitor of CYP3A (Guengerich, 1990b), suggest that it can also be used as an indicator of induction of CYP3A in the mouse.

Gestodene, a specific mechanism-based inactivator of CYP3A (Guengerich, 1990b), inhibited irreversible binding of radiolabelled carbamazepine (and the formation of carbamazepine-10,11-epoxide) in a dose-dependent manner (figure 5.4). Gestodene has been found to be an effective inhibitor of human CYP3A activity *in vitro*, but only after pre-incubation of human liver microsomes with gestodene in the

presence of NADPH (Guengerich, 1990b; Fleming *et al.*, 1992). However, with induced and untreated mouse liver microsomes, we found that pre-incubation with gestodene was not necessary for significant (but not total) inhibition of CYP3A, as reflected by the results observed not only with covalent binding of carbamazepine (figure 5.4) but also with 6ß-hydroxycortisol formation (table 5.2). This may be due to higher relative CYP3A activity in the mouse microsomes compared with human microsomes prepared from the liver bank (Eberhardt *et al.*, 1991). In chapter 4 it was shown that ketoconazole, like gestodene, can also inhibit carbamazepine bioactivation. Although ketoconazole can inhibit multiple P450 isozymes at higher concentrations (Maurice *et al.*, 1992), at concentrations less than 5μM it is a relatively selective inhibitor of CYP3A (Maurice *et al.*, 1992). Thus, in agreement with the findings of this study, it was possible to inhibit carbamazepine bioactivation by 1μM ketoconazole (chapter 4), consistent with the involvement of CYP3A.

Using the techniques outlined in this chapter, the specific isozyme(s) of the CYP3A family responsible for carbamazepine bioactivation cannot be stated. Further techniques including immunoinhibition and use of cell lines expressing specific CYP3A isozymes would be required for precise identification. In addition, the use of an inhibitor of CYP3A which is more specific then gestodene, for example cannabidiol, would also help in isozyme identification. Bornheim and Correia (1990) have shown that cannabidiol inactivates CYP3A isozymes which are either constitutively expressed in mouse liver or those that are inducible by phenobarbitone pretreatment. However, it does not inhibit CYP3A isozymes that are steroid inducible.

How do these results with mouse microsomes relate to man? It is well known that there are cross-species differences in the metabolism of many compounds (Smith, 1991), and therefore it is not always possible to extrapolate directly from data obtained in animals to man (Gonzalez *et al.*, 1991). However, the CYP3A family is highly conserved in mammalian species (Wrighton *et al.*, 1985b; Ciaccio and Halpert, 1989; Smith, 1991) and immunochemical similarity has been observed between the human orthologue and the mouse CYP3A enzyme (Bornheim and Correia, 1991) suggesting that in man carbamazepine may also be metabolised by the CYP3A enzyme. In support of this, Kerr *et al* (1991) have recently shown that the epoxidation of carbamazepine to the stable 10,11-epoxide in man is mediated by CYP3A4. Our results in the mouse are in accordance with this in that the formation of carbamazepine-10,11-epoxide was enhanced by induction with both phenobarbitone and dexamethasone but not β-naphthoflavone, and reduced by co-incubation with gestodene, resulting in an excellent correlation between carbamazepine-10,11-epoxide formation and covalent binding (figure 5.5), suggesting that the epoxidation of carbamazepine to both the stable 10,11-epoxide and the putative toxic, chemically reactive epoxide is mediated by the same P450 isozyme. In addition, drugs such as erythromycin and verapamil, which are both metabolised by CYP3A (Wrighton *et al.*, 1985b, 1989; Kroemer *et al.*, 1991, 1992), have been reported to inhibit the metabolism of carbamazepine resulting in elevated serum carbamazepine concentrations (Macphee *et al.*, 1986; Brodie, 1990) suggesting that competitive inhibition of this enzyme may be the basis for the interaction.

CYP3A is the major P450 enzyme in human liver (Shaw *et al.*, 1989; Wrighton *et al.*, 1989), although a 26-fold variation in levels has been demonstrated between

different individuals (Shaw *et al.*, 1989). It can be induced by glucocorticoids (Wrighton *et al.*, 1985b), macrolide antibiotics (Wrighton *et al.*, 1985a), and importantly, with regard to carbamazepine, by anticonvulsants such as phenytoin (Werk *et al.*, 1964; Shaw *et al.*, 1989) and phenobarbitone (Burstein and Klaiber, 1965; Shaw *et al.*, 1989; Waxman and Azaroff, 1992). Indeed, carbamazepine is an autoinducer (Brodie, 1990; Kudriakova *et al.*, 1992) and has been shown to increase urinary 6ß-hydroxycortisol (Park and Breckenridge, 1981), suggesting induction of CYP3A. Thus, urinary 6ß-hydroxycortisol in patients on carbamazepine could be used not only to determine the induction of CYP3A, but also as an indirect marker for the bioactivation of carbamazepine. Concomitant administration of carbamazepine with either phenytoin and/or phenobarbitone has been reported to reduce the efficacy of carbamazepine by enhancing its metabolism and thus reducing serum (and tissue) levels of the therapeutically active parent compound (Brodie, 1990). Interestingly, with phenytoin, the interaction with carbamazepine is associated with a rise in plasma carbamazepine-10,11-epoxide levels relative to the decreased carbamazepine levels (Dam *et al.*, 1975; Westenberg *et al.*, 1978; Brodie *et al.*, 1983). Therefore, enzyme induction may also increase the bioactivation of carbamazepine to the toxic epoxide leading to idiosyncratic toxicity in certain individuals, particularly in those who have low activity of microsomal epoxide hydrolase, which may be either genetically determined (Shear *et al.*, 1988) or by concurrent administration of anticonvulsants such as valproic acid (Kerr *et al.*, 1989; Kerr and Levy, 1990), valpromide (Kerr *et al.*, 1989; Kerr and Levy, 1990) or progabide (Kutt *et al.*, 1984) which are known to be inhibitors of this enzyme.

The present study has concentrated on the effect of enhancing bioactivation

by using specific inducers of the phase I enzymes. However, it is known that induction of the phase I metabolic (i.e. bioactivation) pathways is often accompanied by induction of phase II (detoxication) pathways (Bock *et al.*, 1987), thus maintaining the balance between bioactivation and detoxication. Therefore. it is not surprising that the detoxication pathway, i.e. microsomal epoxide hydrolase, can also be induced by the aromatic anticonvulsants (carbamazepine, phenytoin, phenobarbitone); however, several studies have shown that the epoxidation of carbamazepine is induced to a greater extent than the hydration pathway (Rane *et al.*, 1976; Eichelbaum *et al.*, 1985; Kudriakova *et al.*, 1992), thus creating (or further exacerbating) the imbalance between bioactivation and detoxication.

Carbamazepine, in addition to causing idiosyncratic toxicity, has more recently been implicated to cause foetal malformations (Jones *et al.*, 1989; Anon, 1991; Rosa, 1991), although it is difficult to establish its teratogenic potential compared to phenytoin largely because the data pertaining to teratogenicity is retrospective. Based on the effect of concurrent administration of sodium valproate, an epoxide hydrolase inhibitor (Lindhout *et al.*, 1984; Kaneko *et al.*, 1988), in increasing the rate of teratogenicity associated with carbamazepine, it has been postulated that a chemically reactive arene oxide is responsible (Lindhout *et al.*, 1984; Kaneko *et al.*, 1988). However, further research in affected patients and using animal models is required to determine the pathogenesis of carbamazepine teratogenicity. With phenytoin, a mouse model has been used to determine the effects of enzyme induction and enzyme inhibition of both the activation and detoxication pathways on the frequency of foetal hydantoin syndrome (Finnell, 1991; Finnell *et al.*, 1992). The data presented in this study would suggest that it may also be possible to use

a mouse model to investigate the mechanism of carbamazepine teratogenicity.

In conclusion, the results of this study suggest that bioactivation of carbamazepine in the mouse is dependent on CYP3A, induction and inhibition of this enzyme increasing and reducing bioactivation, respectively. Furthermore, given the high degree of conservation across species among the CYP3A sub-family of enzymes, the mouse provides a useful model for investigating the effects of enzyme induction not only on carbamazepine idiosyncratic toxicity, but also on the teratogenicity of carbamazepine. In man, inducers of the orthologous form of this enzyme, most notably the aromatic anticonvulsants, may create an imbalance between bioactivation and detoxication of carbamazepine resulting in idiosyncratic toxicity.

CHAPTER 6

DIRECT AND METABOLISM-DEPENDENT TOXICITY OF SULPHASALAZINE AND ITS PRINCIPAL METABOLITES TOWARDS HUMAN ERYTHROCYTES AND LEUCOCYTES

6.1. Introduction

The risk associated with the use of a particular drug obviously has to be balanced against the benefit that might ensue from that treatment. Thus, use of a drug associated with a high incidence of toxicity in a relatively benign illness would not be acceptable. On the other hand, treatment with such a drug would be justified for a disease which ran a potentially fatal course if untreated, particularly when no other suitable drugs were available. Such was the case when sulphonamides were introduced as antibacterials in 1936. Although clearly effective against previously untreatable infections, it was soon realised that a large number of patients, later estimated to be between 1-3% (Koch Weser *et al.*, 1971; Anon, 1985; Shear *et al.*, 1986), developed idiosyncratic drug reactions (Van Dyke, 1943). Similar to the idiosyncratic toxicity described with anticonvulsants in the preceding chapters, adverse reactions with the sulphonamides can involve nearly every organ system, often in multiple fashion and to a varying degree (Weinstein *et al.*, 1960). The commonest systems to be affected are the skin, haematological system and the liver (Reider *et al.*, 1989). Again, as with the anticonvulsants, an immune aetiology has been proposed largely on the basis of accompanying hypersensitivity manifestations (Reider *et al.*, 1989), such as fever, eosinophilia and atypical circulating lymphocytes. With the advent of newer and safer antibiotics such as the penicillins, the use of sulphonamides declined, although more recently, they have been extensively used for treatment of opportunistic infections seen in AIDS, where it might be argued that the benefit of treatment exceeds the risk.

One sulphonamide which has remained in extensive use (but not for overt infections) since its introduction in the early 1940s, and indeed has found newer

indications is sulphapyridine, which is used in chemical linkage with 5-amino salicylic acid, as sulphasalazine. It is used in the treatment of rheumatoid arthritis (Pullar, 1989) where it is of comparable efficacy to other disease-modifying agents, such as sodium aurothiomalate (Pullar *et al.*, 1983) and D-penicillamine (Neumann *et al.*, 1983). It is also used for the treatment of inflammatory bowel disease (Riley and Turnberg, 1990), although in this condition, it is now being superseded by 5-aminosalicylate derivatives (Riley and Turnberg, 1990). It is also currently being investigated for its possible therapeutic potential in multiple sclerosis (Proseigel *et al.*, 1989). Particularly for the treatment of rheumatoid arthritis, a similar argument may be applied, in that although the drug has appreciable toxicity (see below), it has a better benefit-risk ratio when compared to the other disease modifying agents which are available (Pullar, 1989).

Sulphasalazine consists of two moieties: sulphapyridine and 5-amino salicylic acid, which are linked by an azo bond. Sulphasalazine reaches the colon unchanged, where bacteria cleave the azo link, liberating sulphapyridine which is absorbed and 5-amino salicylic acid which is excreted in the faeces with minimal systemic absorption (Das and Dubin, 1976; Bondesen *et al.*, 1988; Riley and Turnberg, 1990). Subsequently, sulphapyridine undergoes metabolism through hydroxylation and acetylation, to 5-hydroxy sulphapyridine and N^4-acetyl sulphapyridine, respectively (figure 1.11, chapter 1). These metabolites are then excreted in the urine predominantly as their glucuronic acid conjugates (Das and Dubin, 1976).

A wide range of toxic effects has been reported with sulphasalazine including skin rashes (Strom, 1969; Cameron *et al.*, 1976), agranulocytosis (Amos and Bax, 1988), hepatotoxicity (Farr *et al.*, 1985), neurotoxicity (Wallace, 1970) and lung

disorders (Thomas *et al.*, 1974). The moiety responsible for this toxicity is thought to be sulphapyridine as the side effects associated with sulphasalazine are very similar to those caused by sulphonamides (Das *et al.*, 1973), and minimal amounts of 5-amino salicylic acid are absorbed and metabolised (Bondesen *et al.*, 1988). However, it is not known whether sulphapyridine itself or one of its metabolites which is responsible.

Sulphasalazine can also cause intravascular haemolysis (Das *et al.*, 1973; Mechanick, 1985), particularly in glucose-6-phosphate dehydrogenase deficient patients (Pounder *et al.*, 1975), an adverse effect also seen with other sulphonamide compounds (Beutler, 1978). However, unlike the other sulphonamides, sulphasalazine can also cause methaemoglobinaemia, of variable severity, in up to 40% of patients (Pounder *et al.*, 1975). Interestingly, such a side-effect profile is also seen with dapsone, a compound structurally similar to sulphapyridine (figure 6.1), which is classified as a sulphone (Uetrecht *et al.*, 1988; Coleman *et al.*, 1989; Uetrecht, 1992).

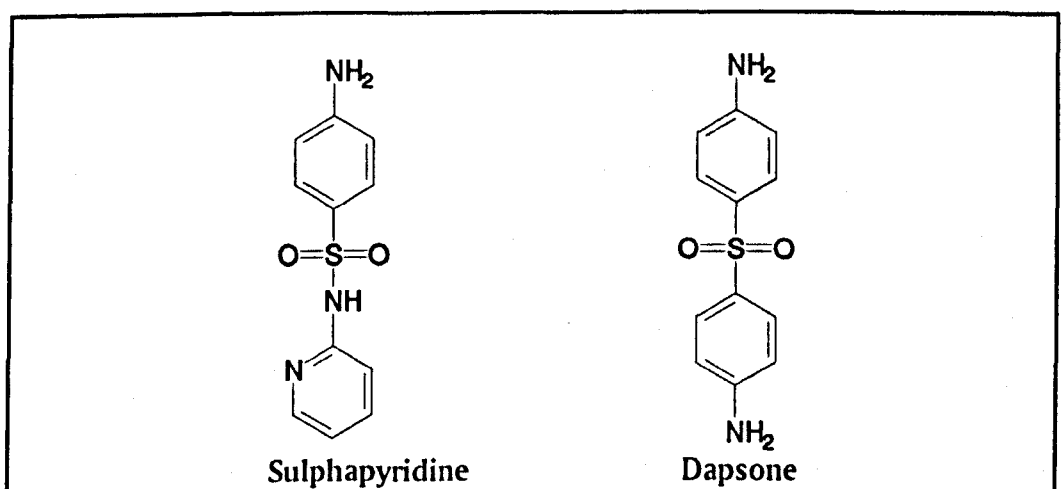


Figure 6.1. Comparison of the structures of sulphapyridine and dapsone

It is also interesting to note that apart from the red cell toxicity, the range and pattern of Type B adverse effects seen with sulphasalazine are very similar to those associated with carbamazepine (see preceding chapters), despite the fact that these two drugs are functionally and chemically unrelated. Such a similarity could only be explained by invoking an immune-mediated mechanism for the adverse drug reactions associated with the two drugs. Thus, for carbamazepine evidence has been presented for the formation of a chemically reactive metabolite which by acting as an hapten initiates an immune reaction (chapters 2-5). For sulphasalazine, the formation of a chemically reactive metabolite has not been shown.

Recent evidence suggests that the toxicity of sulphadiazine (Shear and Spielberg, 1985; Shear *et al.*, 1986), sulphamethoxazole (Reider *et al.*, 1989) and dapsone (Uetrecht *et al.*, 1988; Coleman *et al.*, 1989) is mediated by their respective hydroxylamine metabolites. Hydroxylamines may also be involved in the toxicity of drugs such as procainamide (Rubin *et al.*, 1987) and several aromatic amines (Radomski, 1979). No such metabolite of either sulphasalazine or its two components, sulphapyridine and 5-amino salicylic acid, has yet been identified.

Therefore, in this study, in order to determine the biochemical basis of sulphasalazine-mediated toxicity, erythrocytes and leucocytes have been used as target cells with methaemoglobin formation and cytotoxicity, respectively, as the defined toxic end-points. The latter has been investigated by using techniques described in the preceding chapters, while the former has been investigated using a novel two-compartment system, termed the Dianorm apparatus (figure 6.2). This was originally developed for equilibrium dialysis but has been modified (Riley *et al.*, 1990a) and successfully used for determining *in vitro* cytotoxicity (Riley *et al.*, 1990a) and methaemoglobin formation (Tingle *et al.*, 1990) from dapsone.

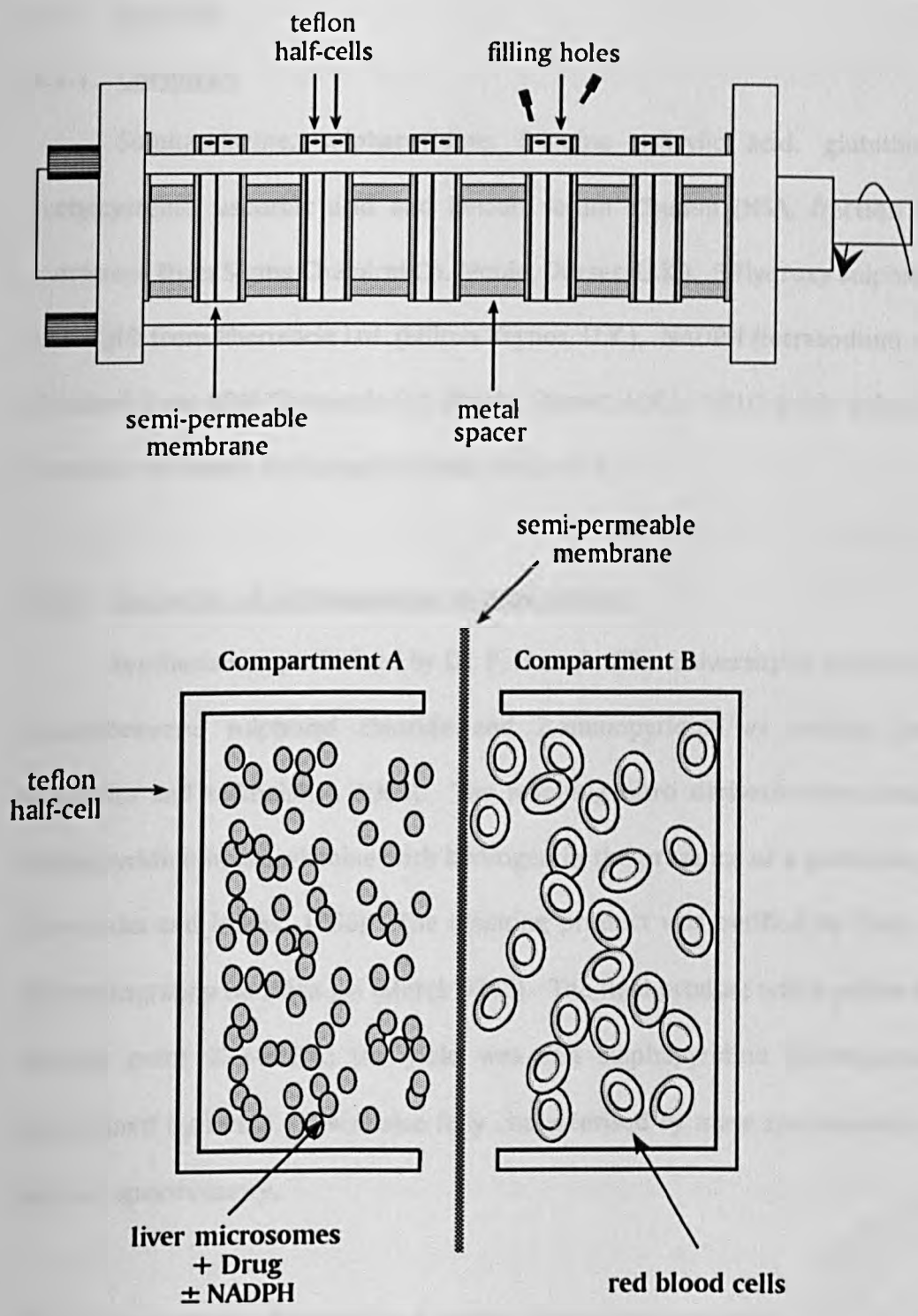


Figure 6.2. The dianorm apparatus shown as 5 pairs of half cells stacked in a rack prior to incubation in a water bath, and in cross-section.

6.2. Methods

6.2.1. Chemicals

Sulphasalazine, sulphapyridine, 5-amino salicylic acid, glutathione, N-acetylcysteine, ascorbic acid and human serum albumin (HSA, fraction V) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 5-Hydroxy sulphapyridine was a gift from Pharmacia Ltd. (Milton Keynes, U.K.). NADPH (tetrasodium salt) was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). HPLC grade solvents were products of Fisons plc (Loughborough, Leics. U.K.).

6.2.2. Synthesis of sulphapyridine hydroxylamine

Synthesis was performed by Dr. F. Hussain (The University of Liverpool) using p-nitrobenzene sulphonyl chloride and 2-aminopyridine as starting materials (Fel'dman and Mikhailova, 1965). The resulting nitro derivative was reduced to sulphapyridine hydroxylamine with hydrogen in the presence of a platinum catalyst (Grossman and Jollow, 1988). The resulting product was purified by flash column chromatography on silica gel (Merck 9385). The final product was a yellow solid of melting point 232-233°C; the yield was 96% sulphapyridine hydroxylamine (as determined by HPLC). It was also fully characterised by mass spectrometry, n.m.r. and i.r. spectrometry.

6.2.3. Preparation of human and murine hepatic microsomes

Histologically normal livers were obtained from six kidney transplant donors (table 6.1). Ethical approval was granted and informed consent obtained from donors' relatives. Livers from donors were cooled to 0°C, divided into 10-20g

portions and frozen in liquid nitrogen within 1h of removal and stored at -80°C until required for use. Washed human liver microsomes were prepared by differential centrifugation as described in section 2.2.5..

Six male CBA/ca mice (25-50g) were administered phenobarbitone once daily for three days (60 mg/kg) by i.p. injections (0.15 ml) in 0.9% (w/v) saline. Another group of six mice received i.p. injections (0.2 ml) of β-naphthoflavone suspended in corn oil at a dose of 75 mg/kg for 3 days. After the final set of injections, the mice were fasted for 24h and then killed by cervical dislocation. The livers were removed and pooled; the microsomes were prepared by the same centrifugation procedure used to obtain washed human liver microsomes (section 2.2.5.).

The protein content of the liver microsomes was measured by the method of Lowry *et al.* (1951). The microsomes were either used immediately or stored at -80°C as an intact pellet. Microsomal suspensions (in 0.067M phosphate buffer, pH 7.4) were prepared at 10mg/ml immediately prior to use and were maintained at 4°C throughout.

The cytochrome P450 content was measured by the method of Omura and Sato (1964) as described in section 2.2.6. The cytochrome P450 content of the pooled microsomes prepared from phenobarbitone pretreated mice was 1.3nmol/mg protein, while the level for the ß-naphthoflavone pretreated mice was 0.8nmol/mg protein. The mean cytochrome P450 content of the six human livers used in the study was 0.42 ± 0.06 nmol/mg protein; the individual values are listed in table 6.1.

Table 6.1.

Details of the human liver donors used in the present study.

Liver	*Age*	*Sex*	*Cause of death*	*Drugs*	*Histology*	*Cytochrome P450 (nmol/mg protein)*
1	46	F	Subarachnoid haemorrhage	Phenobarbitone Phenytoin	Normal	0.60
2	29	M	Head injury	None	Normal	0.48
3	27	M	Head injury	None	Normal	0.21
4	8	M	Head injury	None	Non-specific inflammatory infiltrate	0.50
5	40	F	Road traffic accident	Clomipramine Procyclidine Flupenthixol Sulpiride	Normal	0.50
6	18	M	Head injury	None	Normal	0.47

6.2.4. Isolation of peripheral blood leucocytes

Isolation of mononuclear leucocytes

Peripheral blood mononuclear leucocytes (MNL) were isolated from fresh heparinised venous blood from six healthy male volunteers (aged 22-36 years) as described in section 2.2.7. Their viability upon isolation as determined by trypan blue dye exclusion, was > 95%. The MNL were suspended in 15 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)-buffered balanced salt medium to obtain 1 x 10^6 cells/ml.

Isolation of T- and B-lymphocytes

MNL were isolated from whole blood (100ml) as described in section 2.2.7. T- and B-lymphocytes subpopulations were then separated using magnetic monosized polymer microspheres (Dynabeads M-450, Dynal (UK), Ltd., Wirral, U.K.) coated with monoclonal antibodies specific for the B-cell CD19 and T-cell CD2 antigens, respectively, as described in section 4.2.5. The negatively selected cell populations were resuspended in HEPES-buffered medium to obtain approximately 1 x 10^6 cells/ml. The cells upon isolation were of > 95% viability, as determined by trypan blue dye exclusion. The purity of the negatively selected cells was determined by Mr Ian Crosby (Department of Immunology, The Royal Liverpool University Hospital) and was found to be 95% and 90%, for the T- and B-cells, respectively.

Isolation of neutrophils

Peripheral blood neutrophils were isolated on a discontinuous density gradient of ficoll-hypaque (Histopaque; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.)

according to the method of Ringertz *et al.* (1982), with minor modifications. The histopaque 1077 (5ml; density 1.077 g/ml) was carefully layered over histopaque 1119 (5ml; density 1.119 g/ml). Aliquots (10ml) of freshly drawn heparinised venous blood which had been diluted 1:1 (v/v) with phosphate buffered saline (PBS; NaCl 8g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l; pH 7.2) were then carefully layered over the histopaque 1077 (figure 6.3) and centrifuged at 2,300 revs/min (700g) for 30 min in a Centaur 2 centrifuge (MSE, Crawley, Sussex, UK). The band containing granulocytes (figure 6.3) was harvested using a sterile Pasteur pipette and mixed 1:1 with PBS and centrifuged at 347g for 10 min. The resulting cell pellet was heavily contaminated by erythrocytes which were removed by lysis with NH_4Cl (0.85% for 5 min) following a washing phase in RPMI 1640 medium. Following erythrocyte lysis, PBS (10ml) was added and the mixture centrifuged at 214g for 8 min. Finally, the neutrophils were resuspended in HEPES-buffered medium to obtain approximately 1×10^6 cells/ml. The purity of the neutrophils which was checked using a Coulter Counter (Department of Haematology, The Royal Liverpool University Hospital) was found to be > 95%. The viability of the neutrophils as determined by trypan blue dye exclusion was >98%.

6.2.5. Determination of the direct and metabolism-dependent cytotoxicity of sulphasalazine and its metabolites

Direct cytotoxicity was determined by incubating MNL (1×10^6 cells/ml) in HEPES-buffered medium with varying concentrations of sulphasalazine and its principal metabolites, including sulphapyridine hydroxylamine, in polystyrene tubes at 37°C for 2h in a shaking water bath. The drugs were added in 10µl

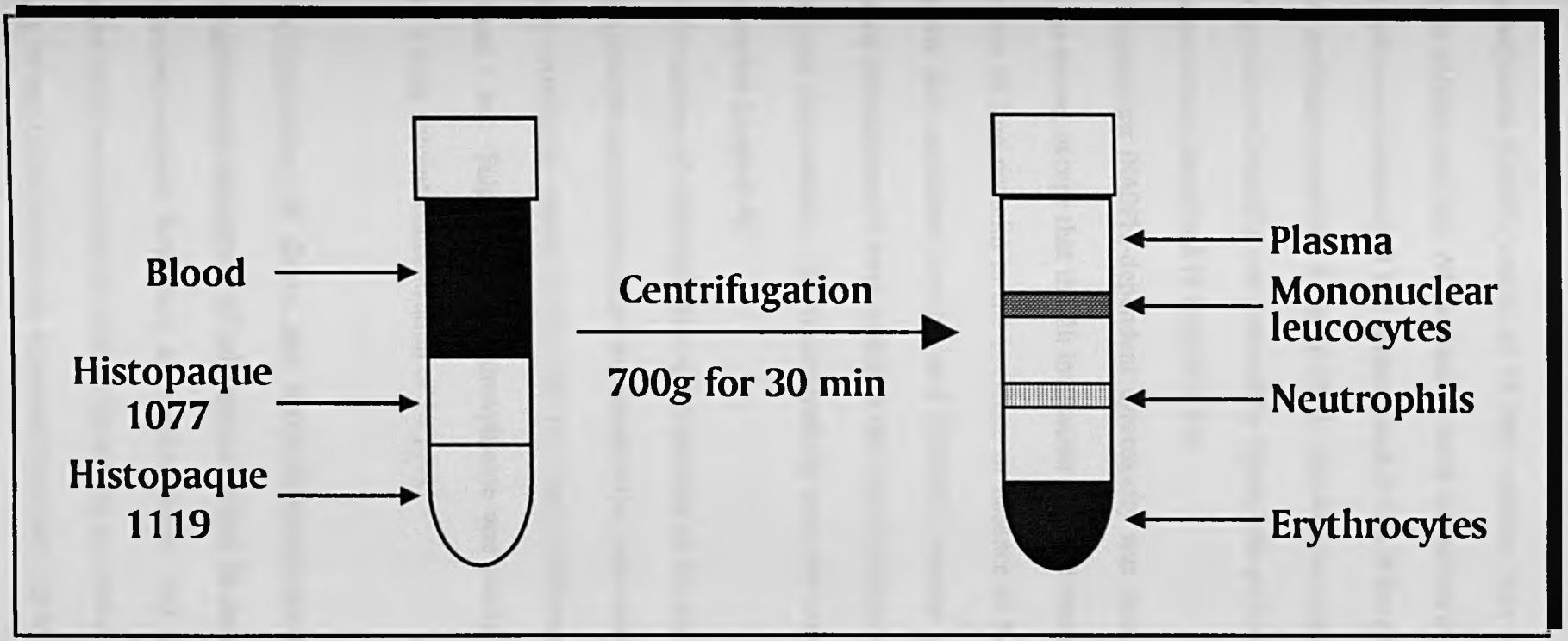


Figure 6.3. Diagrammatic representation of the method used for isolation of neutrophils by centrifugation over a discontinuous density gradient (Histopaque; density 1.077 g/ml and 1.119 g/ml)

dimethylsulfoxide (DMSO), which, as 1% (v/v) solution, was non-toxic. The total incubation volume was 1ml. All incubations were carried out in quadruplicate. After 2h, the cells were sedimented and resuspended in 1ml of drug-free medium (HEPES-buffered medium containing 5 mg/ml HSA). Incubations were continued for 16h, following which cell viability was assessed by trypan blue exclusion (0.2% (w/v) trypan blue) as previously described in section 2.2.9.

Metabolism (NADPH)-dependent cytotoxicity was determined in the same manner as above, except that the 2h incubation included murine or human hepatic microsomes (0.5mg protein) in the presence or absence of NADPH (1mM). Some incubations also contained ascorbic acid (100μM), N-acetyl cysteine (50μM) and glutathione (500μM) which were added to the cells 10 min prior to the addition of the drug and microsomes. The concentrations were the same as those used for carbamazepine (chapter 4).

The toxicity of sulphapyridine hydroxylamine to the separated neutrophil, T- and B-lymphocyte suspensions was determined in the same manner as that described for direct cytotoxicity above, except that the final incubation volume was 0.5 ml rather than 1 ml. Sulphapyridine hydroxylamine was added in 5μl of DMSO to maintain a final solvent concentration of 1% (v/v).

6.2.6. Determination of direct and metabolism-dependent red cell toxicity (methaemoglobinaemia) of sulphasalazine and its metabolites

Methaemoglobin formation by sulphasalazine and its metabolites was determined using an equilibrium dialysis (Dianorm) apparatus (Tingle *et al.*, 1990), consisting of two 0.5ml teflon cells separated from each other by a semi-permeable

membrane (figure 6.2) made of cellulose with a molecular weight cut-off equivalent to 10 kDa (Diachema, Munich, F.R.G.). Incubation mixtures are introduced into the compartments via holes in the cells which are then occluded with special pegs. The bicompartmental cells are held in a motor driven rotating rack which is immersed in a heated water bath kept at 37°C. Prior to their use in the Dianorm, the semi-permeable membranes were rinsed in phosphate buffered saline (3 x 10 min washes) at 37°C.

To investigate the role of metabolism in methaemoglobin formation, varying concentrations (100μM - 2mM) of the compounds in DMSO (5μl) and human liver microsomes (liver 1; 2mg protein) were placed in compartment A. Compartment B contained 500μl human RBC washed three times with PBS and then resuspended to approximately 50% haematocrit. Metabolism was initiated by the addition of NADPH (2mM; omitted from controls) to compartment A and conducted for 1h at 37°C with constant rotation (8 rev/min). The final volume in each half-cell was 500 μl. The ability of the compounds to cause methaemoglobin formation directly, i.e. in the absence of a microsomal system, was examined in a similar manner, except that the microsomes and NADPH were omitted from compartment A. The compounds were added in DMSO (5μl), the volume being made up to 500μl by PBS.

The effect of reduced glutathione (500μM), ascorbic acid (100μM), N-acetylcysteine (50μM) and the cytochrome P450 inhibitor, ketoconazole (100μM; Sheets and Mason, 1984), on metabolism-dependent methaemoglobinaemia was investigated by co-incubating these compounds with the test drug and microsomal system in compartment A, as described above.

After the 1h incubation, the RBC were expelled from compartment B and the

methaemoglobin level relative to the total haemoglobin was measured by the method of Harrison and Jollow (1986).

6.2.7. Spectrophotometric determination of methaemoglobin formation

Methaemoglobin content was measured in 100µl aliquots of the erythrocytes expelled from the Dianorm apparatus after the 1h incubation. The blood samples (100µl) were haemolysed by addition of 5ml of phosphate buffer (KH_2PO_4 2.77g/l, Na_2HPO_4 2.89g/l; pH 7.8) containing 0.05% triton X-100. The haemolysed solution was divided into four 1.25ml aliquots; one drop of aqueous 10% (w/v) KCN was added to aliquots No.2 and No.4 and one drop of aqueous 20% (w/v) $K_3Fe(CN)_6$ was added to aliquots No.3 and No.4. After mixing, the absorbance of each aliquot was measured at 635nm in a single beam spectrophotometer (Cecil 272 UV spectrophotometer, Cambridge, UK), yielding A_1, A_2, A_3, and A_4. The percentage of the total methaemoglobin present as methaemoglobin was then calculated:

$$\% \text{ methaemoglobin} = \frac{A_1 - A_2}{A_3 - A_4} \times 100$$

6.2.8. Metabolism of sulphapyridine by human liver microsomes

Sulphapyridine (100µM) was incubated with human liver microsomes (0.5 mg protein) and 1 x 10^6 MNL in HEPES-buffered medium (pH 7.4; final incubation volume 1ml). Sulphapyridine was added in DMSO, the final concentration of which was 1% (v/v). Ascorbic acid (2mM) was also added to the incubations to prevent autooxidation of any sulphapyridine hydroxylamine formed to nitrososulphapyridine.

The incubations were carried out in polystyrene tubes under air in a shaking water bath at 37°C, the reactions being started by the addition of NADPH (1mM) which was omitted in control incubations. The reactions were terminated by the addition of 1 ml methanol to each tube. The tubes were left at -20°C overnight and then centrifuged (650*g* for 10 min) to precipitate all the protein.

Aliquots (50 μl) of the methanol extract were analysed by HPLC. Sulphapyridine hydroxylamine, sulphapyridine and 5-hydroxy sulphapyridine were identified by comparison of their retention times with those of co-injected authentic compounds. Chromatography was performed on a Spectra-Physics chromatograph using a method based on that of Fischer and Klotz (1978). Separation was achieved using a μ Bondapak C_{18} column (30 cm x 0.39 cm i.d., 10μM, Waters Assoc., Hartford, Cheshire, U.K.). The mobile phase consisted of acetonitrile: 0.1M sodium acetate (ph 4.7) buffer (12:88 v/v) flowing at 1.5 ml/min. Eluate was monitored at 254 nm. Under these conditions, the retention times of sulphapyridine hydroxylamine, sulphapyridine and 5-hydroxy sulphapyridine were 4.6, 6.5 and 7.1 min., respectively.

6.2.9. Determination of the half-life of sulphapyridine hydroxylamine

The half-life of sulphapyridine hydroxylamine was determined by repeated sampling over 2h of an aqueous 100μg/ml solution (0.067M phosphate buffer; pH 7.4) of the metabolite which was maintained at 37°C. The samples were analysed by HPLC (using the method described above) immediately on collection. For comparison, the half-life of dapsone hydroxylamine, a structurally related compound, was determined in the same manner with the samples being analysed by HPLC using

the method of Uetrecht *et al.*, (1988). The solvent mobile phase consisted of water : acetonitrile : acetic acid : triethylamine (80:20:1:0.05, v/v). The flow rate was 1.2 ml/min and the eluate was monitored at 254 nm.

6.2.10. Statistical analysis

Statistical analysis was performed by the use of one way analysis of variance accepting $p < 0.05$ as significant. Data are presented as mean ± SEM.

6.3. Results

6.3.1. Methaemoglobin formation from sulphasalazine and its stable metabolites

Metabolism (NADPH)-dependent methaemoglobin formation (with human liver microsomes) which was investigated in the two-compartment system was observed only with sulphapyridine but not with sulphasalazine, 5-amino salicylic acid or 5-hydroxy sulphapyridine (table 6.2). Incubation of sulphapyridine across a concentration range (100μM - 2mM) with microsomes (2 mg) and NADPH (2mM), resulted in a concentration-dependent increase in methaemoglobin formation (figure 6.4).

Ascorbic acid (100μM), glutathione (500μM) and N-acetyl cysteine (50μM) did not reduce metabolism-dependent methaemoglobin formation (11.4 ± 1.3%, 10.7 ± 0.9%, and 14.5 ± 3.6%, respectively) compared with control, i.e. in the presence of NADPH (2mM) only, (10.4 ± 0.7%). In contrast, ketoconazole (100μM) significantly reduced methaemoglobin formation (3.8 ± 0.2%; $p < 0.001$).

Table 6.2.

Metabolism (NADPH)-dependent methaemoglobin (MetHb) formation from sulphasalazine (2 mM) and its principal metabolites with human liver microsomes (liver 1) after co-incubation with RBC at 37°C for 1h

Compound	*-NADPH % MetHb*	*+NADPH % MetHb*
Sulphasalazine	2.2 ± 0.4	2.0 ± 0.4
Sulphapyridine	2.4 ± 0.7*	8.6 ± 0.7*
5-hydroxy sulphapyridine	3.7 ± 0.1	3.2 ± 0.3
5-aminosalicylic acid	2.4 ± 0.3	1.8 ± 0.1

Results represent the mean ± SEM with each incubation performed in triplicate. The control methaemoglobin values (in the absence of drug) were 3.4 ± 0.4%, 2.7 ± 0.8%, 3.9 ± 0.3% and 3.4 ± 0.4% for sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-aminosalicylic acid, respectively.

Statistical analysis performed by comparing the difference between -NADPH and +NADPH incubations for the same drug: * $p < 0.05$.

In the absence of a metabolising system, only sulphapyridine hydroxylamine caused a concentration-dependent increase in methaemoglobin formation; the other compounds did not show any detectable red cell toxicity (figure 6.5).

To investigate the stability of sulphapyridine hydroxylamine, the compound was incubated with erythrocytes (in the absence of microsomes) in 10 ml polystyrene tubes (total volume: 500 µl) for 1h at 37°C in a shaking water bath. This resulted in significantly higher methaemoglobin levels when directly compared with incubations carried out in the two-compartment system ($p < 0.001$ at all concentrations; figure 6.6).

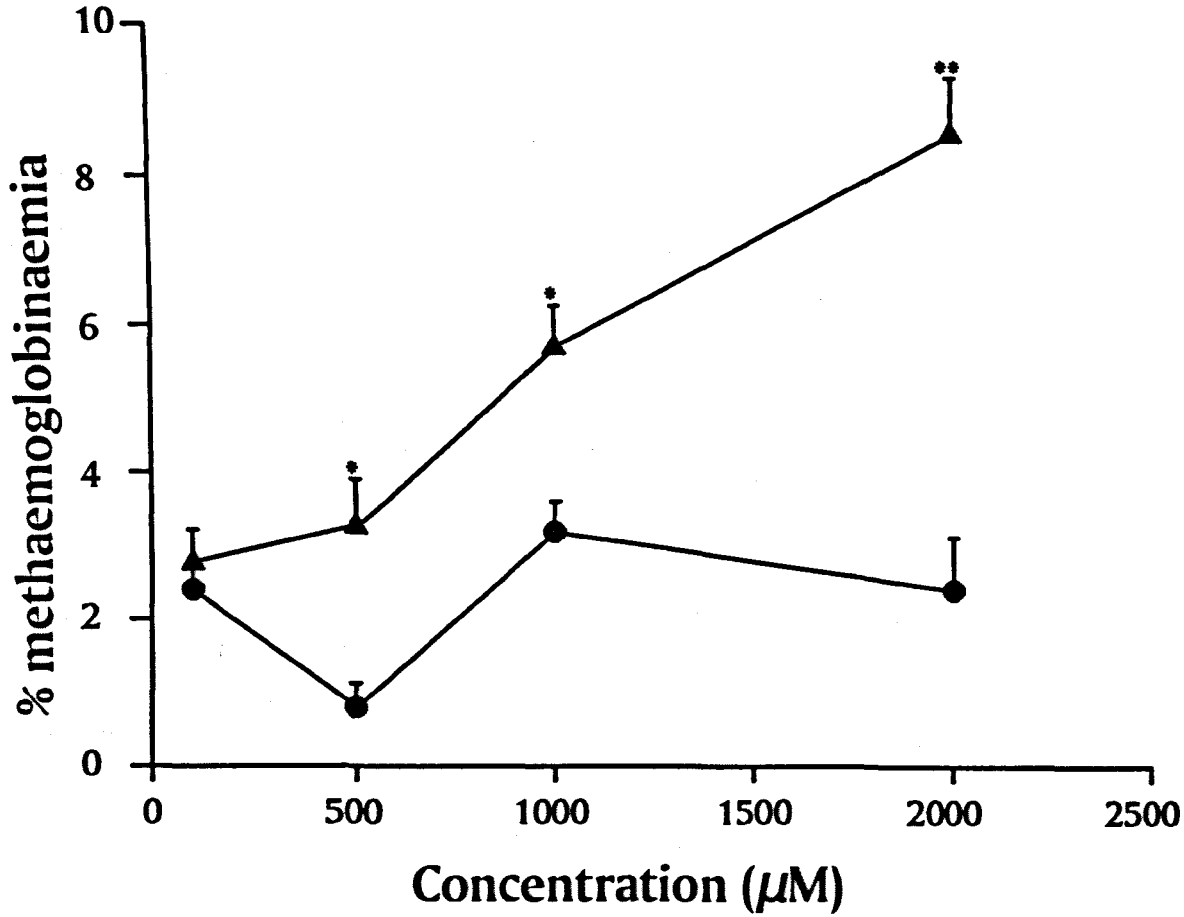


Figure 6.4. Effect of varying concentrations of sulphapyridine on methaemoglobin formation in the absence (●) and presence (▲) of NADPH (2mM) and human liver microsomes (Liver 1). Each point represents the mean ± SEM of triplicate incubations. The control methaemoglobin value (in the absence of drug) was 2.7 ± 0.8%. Significant differences between the -NADPH and +NADPH incubations: * $p<0.05$, ** $p<0.005$

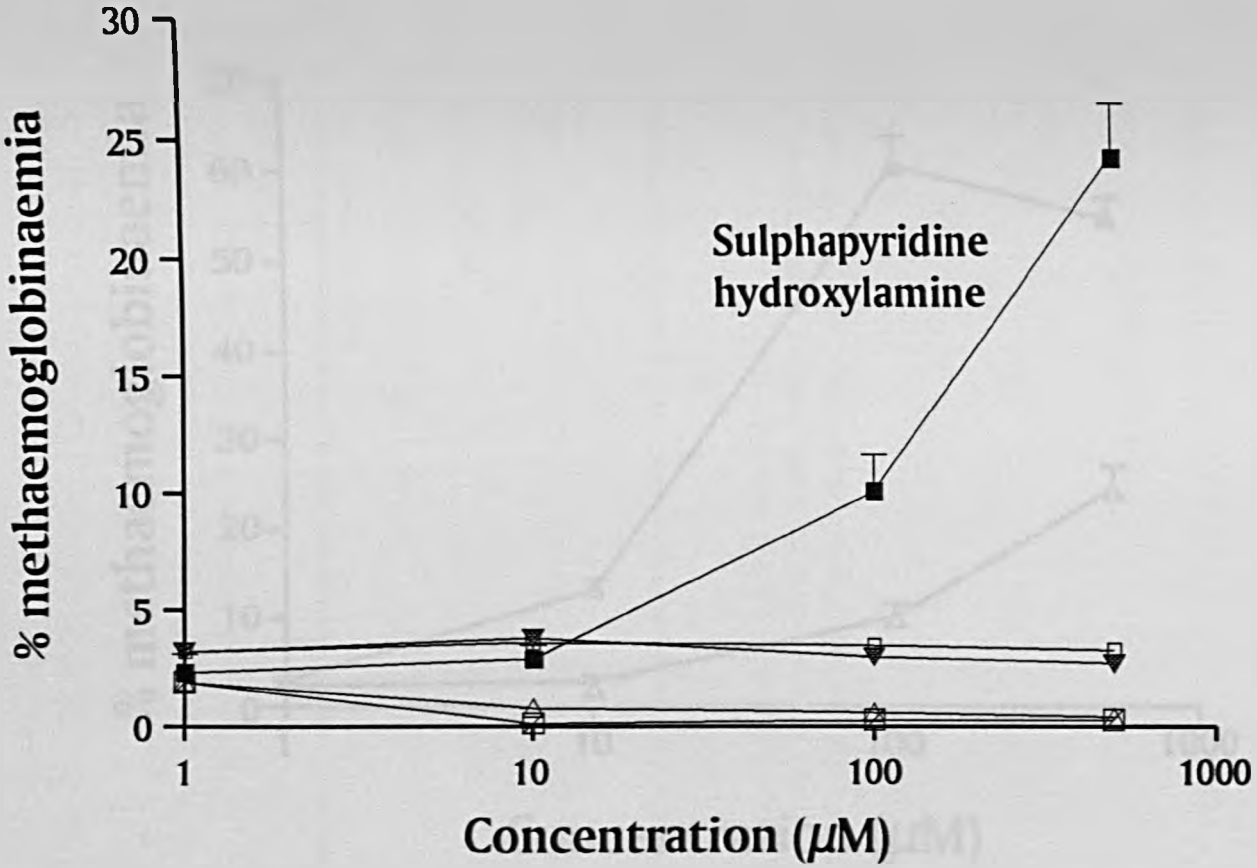


Figure 6.5. The ability of varying concentrations of sulphasalazine (▨), sulphapyridine (△), 5-hydroxy sulphapyridine (▫), 5-aminosalicylic acid (▼) and sulphapyridine hydroxylamine (■) to cause methaemoglobinaemia in the absence of a microsomal system. Each point represents the mean of triplicate determinations (error bars omitted for clarity), except for sulphapyridine hydroxylamine where each point represents the results of two separate experiments (performed in triplicate).

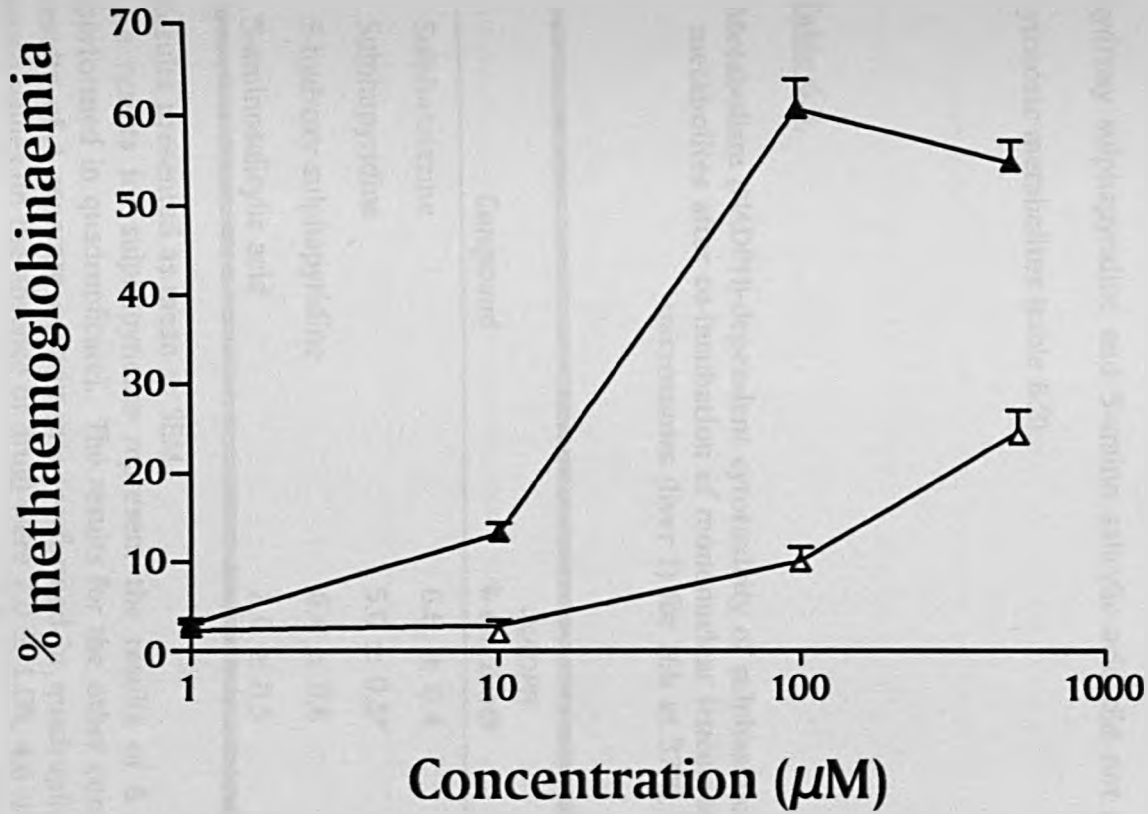


Figure 6.6. A comparison of the ability of sulphapyridine hydroxylamine to cause methaemoglobinaemia in the bicompartmental system (△) and in 10ml polystyrene tubes (▲). Each point represents the mean ± SEM of triplicate (bicompartmental system) or quadruplicate (polystyrene tubes) determinations.

6.3.2. Bioactivation of sulphasalazine and its stable metabolites to cytotoxic products

Incubation of sulphapyridine with MNL at a concentration of 100μM in the presence of human liver microsomes and NADPH resulted in a small, but significant degree of bioactivation ($p<0.05$) to a cytotoxic metabolite, while sulphasalazine, 5-hydroxy sulphapyridine and 5-amino salicylic acid did not undergo activation to cytotoxic metabolites (table 6.3).

Table 6.3.

Metabolism (NADPH)-dependent cytotoxicity of sulphasalazine (100 μM) and its metabolites after co-incubation of mononuclear leucocytes with human liver microsomes (liver 1) for 16h at 37°C.

Compound	*-NADPH % cell death*	*+NADPH % cell death*
Sulphasalazine	6.6. ± 0.4	6.3 ± 0.4
Sulphapyridine	5.0 ± 0.5*	8.3 ± 0.7*
5-hydroxy sulphapyridine	6.4 ± 0.6	8.1 ± 0.5
5-aminosalicylic acid	8.0 ± 0.5	7.9 ± 0.3

Results presented as mean ± SEM.
The results for sulphapyridine represent the results of 6 separate experiments (performed in quadruplicate). The results for the other compounds represent the results of 4 separate experiments (performed in quadruplicate). The control cell death values (in the absence of drug) were 7.0 ± 1.0%, 4.6 ± 0.3%, 7.0 ± 1.0% and 8.7 ± 1.2% for sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-amino salicylic acid, respectively.

Difference between -NADPH and +NADPH incubations: * $P < 0.005$

There was a significant ($p<0.05$) bioactivation of sulphapyridine (100μM) by four of the six different liver samples tested (figure 6.7). Overall, the difference between the mean data for these six samples (i.e. the diference between incubations with and without NADPH) just reached statistical significance (table 6.4). In contrast to metabolism-dependent methaemoglobinaemia, increasing the concentration of sulphapyridine (100μM - 2mM) did not increase cytotoxicity (figure 6.8).

NADPH-dependent cytotoxicity of sulphapyridine was greatest with phenobarbitone-induced mouse microsomes, whereas β-naphthoflavone induced mouse microsomes did not bioactivate sulphapyridine to a significant degree (table 6.4).

Table 6.4.

Variability in the bioactivation of sulphapyridine (100 μM) to a cytotoxic metabolite in the presence of human and mouse liver microsomes

Incubation microsomes	*-NADPH %*	*+NADPH %*	*p value (ANOVA)*
Human liver microsomes	3.2 ± 0.8	6.6 ± 1.3	0.05
Phenobarbitone-induced mouse microsomes	5.0 ± 0.8	12.1 ± 0.4	<0.001
β-naphthoflavone-induced mouse microsomes	3.7 ± 0.4	4.5 ± 0.6	N.S.

For human liver microsomes, the results represent the mean ± SEM for 6 human livers (incubations in quadruplicate for each human liver). For mouse liver microsomes, the results represent the mean ± SEM for quadruplicate incubations with pooled microsomes. The control cell death values (in the absence of drug) were 3.3 ± 0.6% (human liver microsomes), 5.3 ± 0.7% (phenobarbitone-induced mouse microsomes) and 2.5 ± 0.3% (β-naphthoflavone induced mouse microsomes). N.S.-not significant.

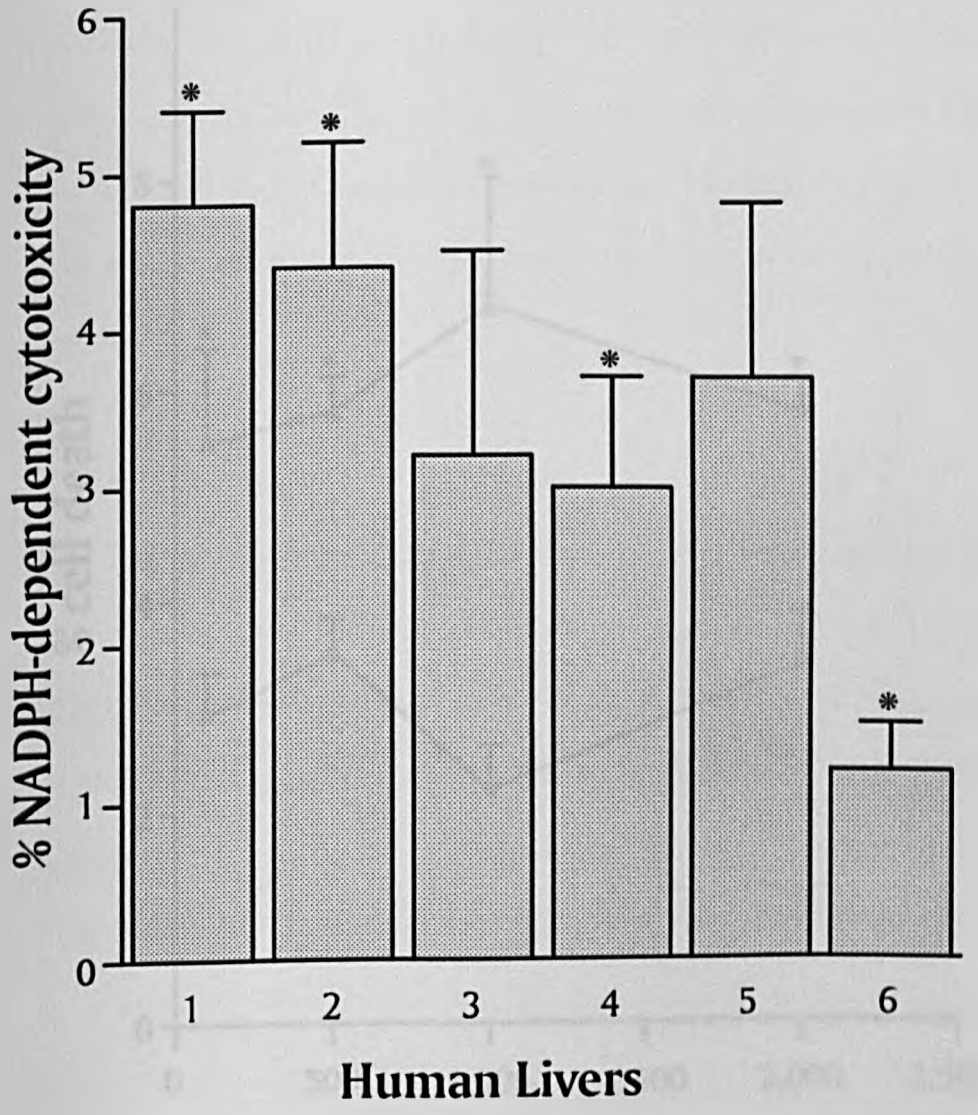


Figure 6.7. The metabolism (NADPH)-dependent cytotoxicity of sulphapyridine (100μM) with six different human livers as described in table 6.1. Statistical analysis performed by comparing incubations with and without NADPH: *$p<0.05$. The cell death values in the absence of NADPH were 6.0 $\pm$ 0.6%, 4.2 $\pm$ 0.8%, 4.8 $\pm$ 0.5%, 1.6 $\pm$ 0.7%, 1.8 $\pm$ 0.5% and 0.9 $\pm$ 0.2% for human livers 1, 2, 3, 4, 5 and 6, respectively.

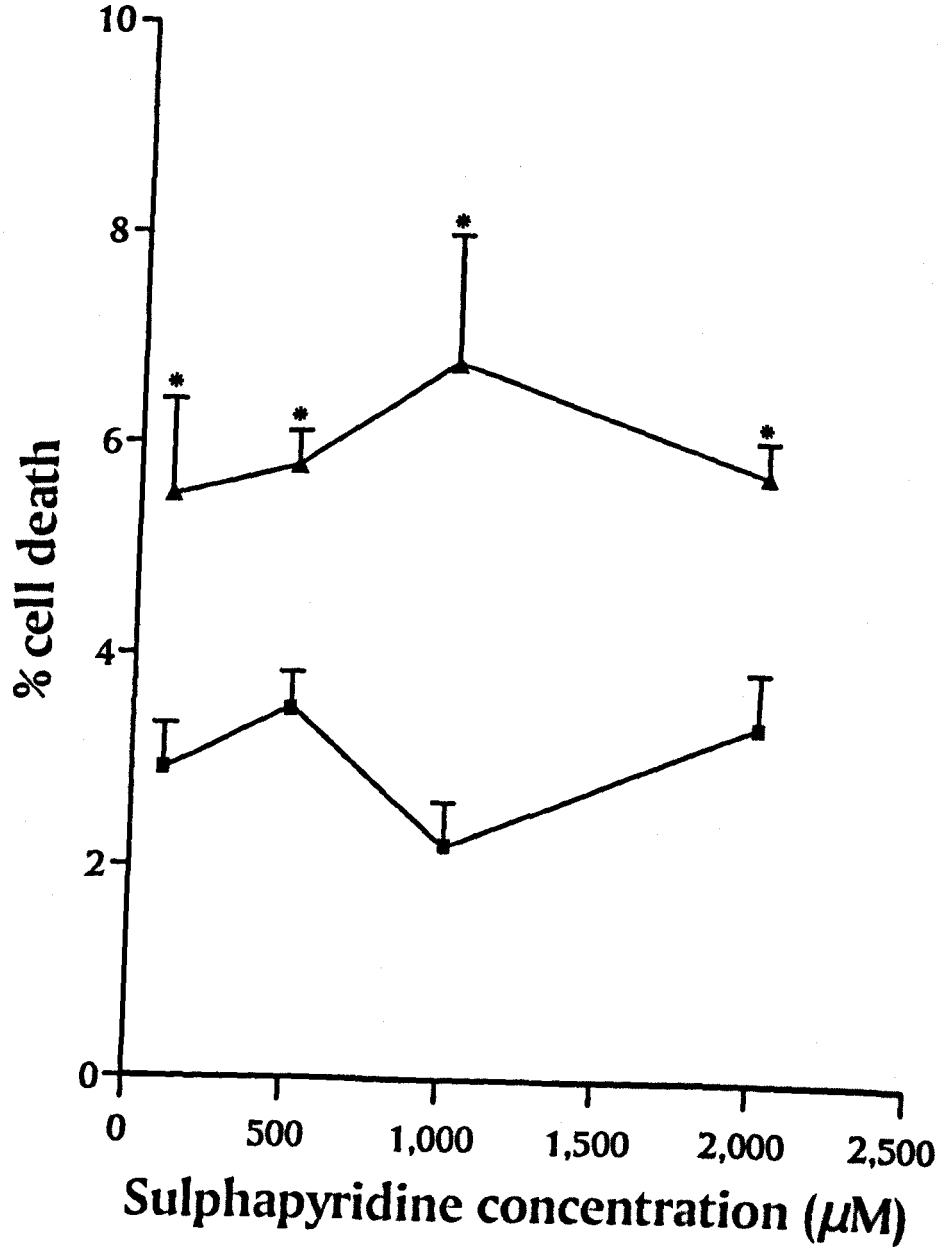


Figure 6.8. The effect of increasing sulphapyridine concentration on cytotoxicity with human liver microsomes (Liver 1) in the absence (■) and presence (▲) of NADPH (1mM). The results represent the mean ±SEM of quadruplicate incubations. Statistical analysis performed by comparing incubations with and without NADPH: * $p<0.05$. NADPH-dependent cytotoxicity was used to determine any differences in cell death at different sulphapyridine concentrations with no statistically significant difference being found.

Metabolism-dependent cytotoxicity of sulphapyridine with human liver microsomes (liver 1) was significantly reduced by the addition of ascorbic acid, glutathione and N-acetyl cysteine (at the same concentrations used for the methaemoglobin assay; figure 6.9). The concentrations chosen have been shown to reduce the metabolism-dependent cytotoxicity of dapsone (Coleman *et al.*, 1989) and were shown in chapter 4 (apart from ascorbic acid) to reduce carbamazepine cytotoxicity.

6.3.3. Direct cytotoxicity of sulphasalazine and its stable metabolites

Incubation of sulphapyridine hydroxylamine with MNL in the absence of a metabolising system resulted in a concentration-dependent (10μM-500μM) increase in cytotoxicity (LC_{50} value of 475μM), while sulphasalazine, sulphapyridine, 5-hydroxy sulphapyridine and 5-amino salicylic acid did not significantly increase the cytotoxicity above background values (figure 6.10).

Sulphapyridine hydroxylamine also produced a concentration-dependent increase in cytotoxicity of separated T- and B-lymphocyte subpopulations; however, there was no significant difference in sensitivity between the two cell types (figure 6.11). In contrast, neutrophils were relatively insensitive to sulphapyridine hydroxylamine, with cell death not exceeding background values even at the highest concentration (500μM) tested (7.8 ± 1.5% vs. 6.3 ± 1.8%; figure 6.11).

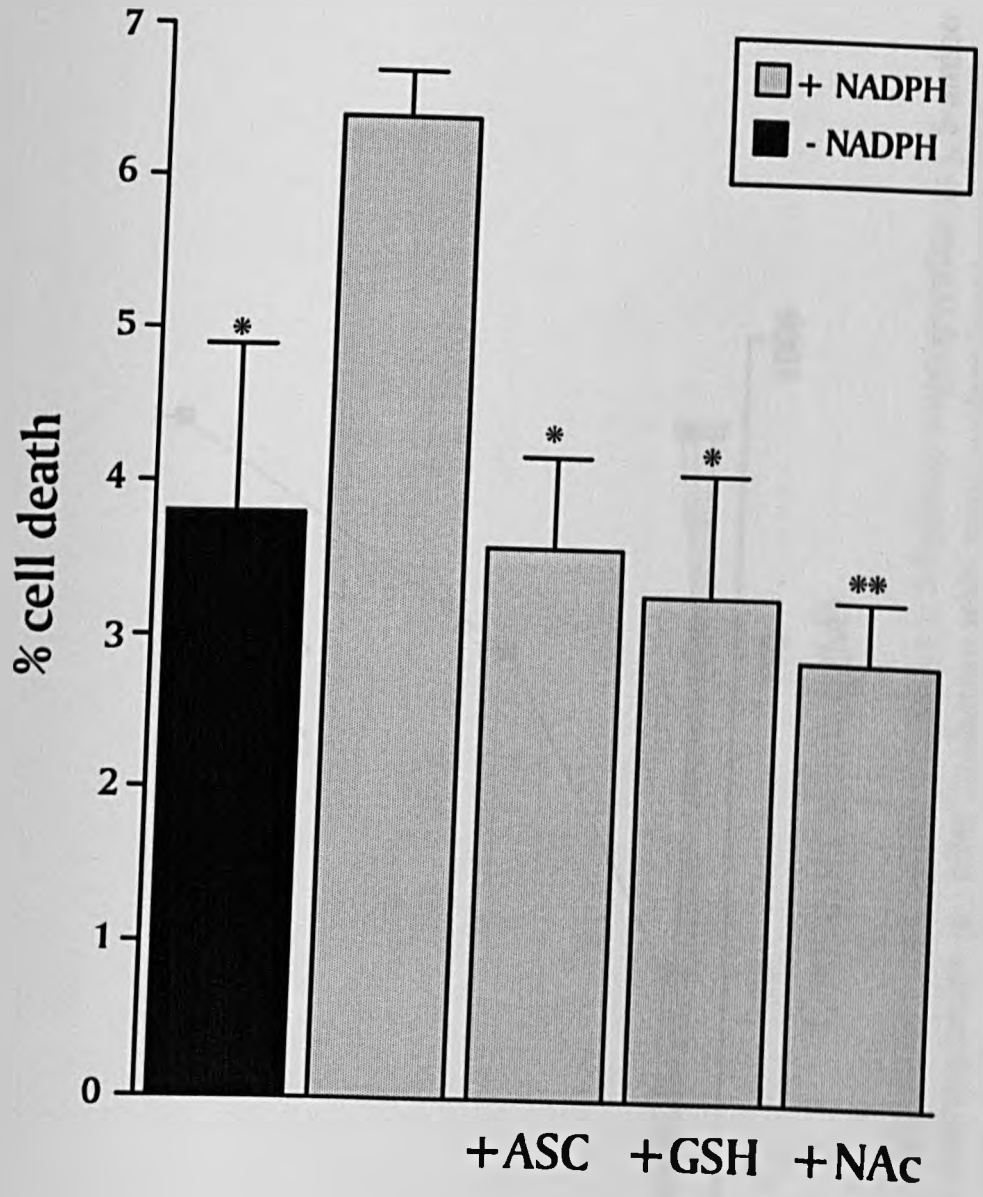


Figure 6.9. Effect of various compounds on metabolism (NADPH)-dependent bioactivation of sulphapyridine (100μM) to a cytotoxic metabolite in the presence of human liver microsomes (liver 1). Results represent the mean $\pm$ SEM of quadruplicate incubations. The control cell death (in the absence of drug) was 3.2 $\pm$ 0.3%. ASC : ascorbic acid (100μM); GSH : glutathione (500μM); NAc : N-acetylcysteine (50μM). Significant difference from incubations containing NADPH (1mM) only: *$p<0.05$, **$p<0.005$.

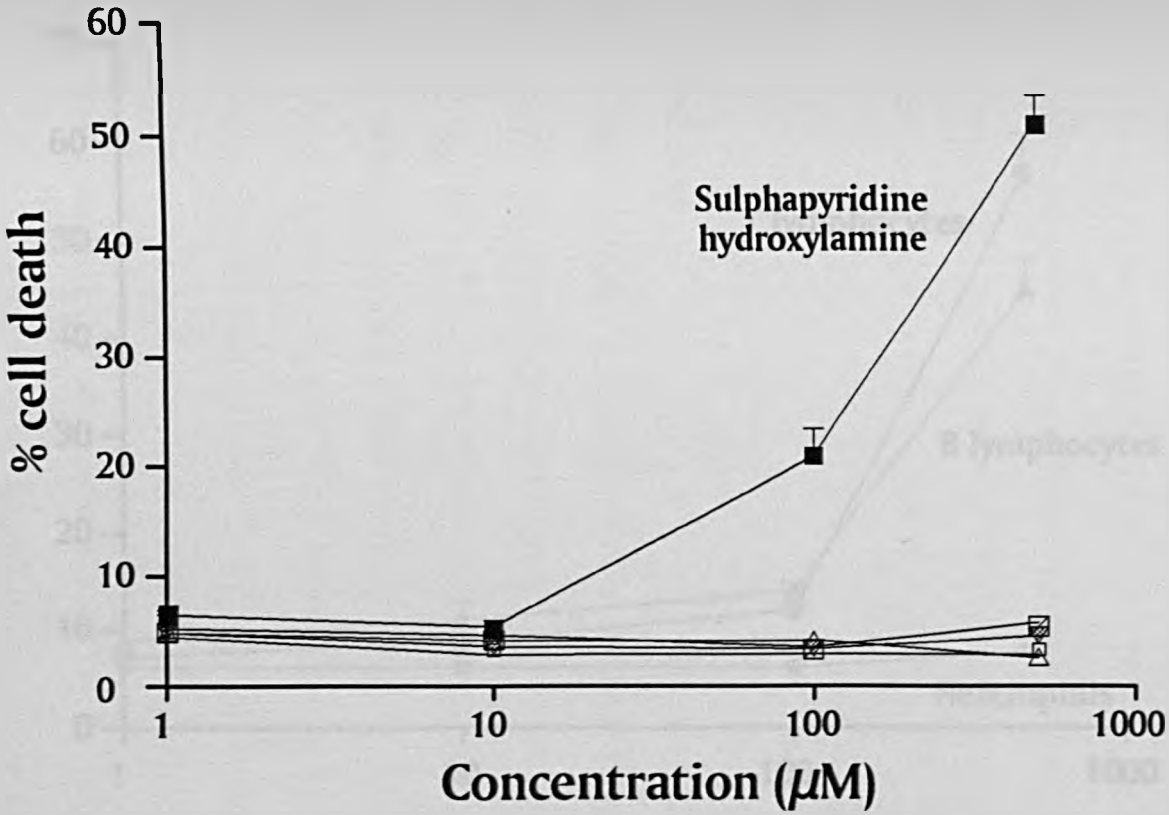


Figure 6.10. Direct cytotoxicity of sulphasalazine (▨), sulphapyridine (△), 5-hydroxy sulphapyridine (▫), 5-amino salicylic acid (▼) and sulphapyridine hydroxylamine (■) after incubation with mononuclear leucocytes for 16h at 37°C. Each point represents the mean of quadruplicate determinations (error bars omitted for clarity), except for sulphapyridine hydroxylamine where each point represents the mean ± SEM of four separate experiments (performed in quadruplicate)

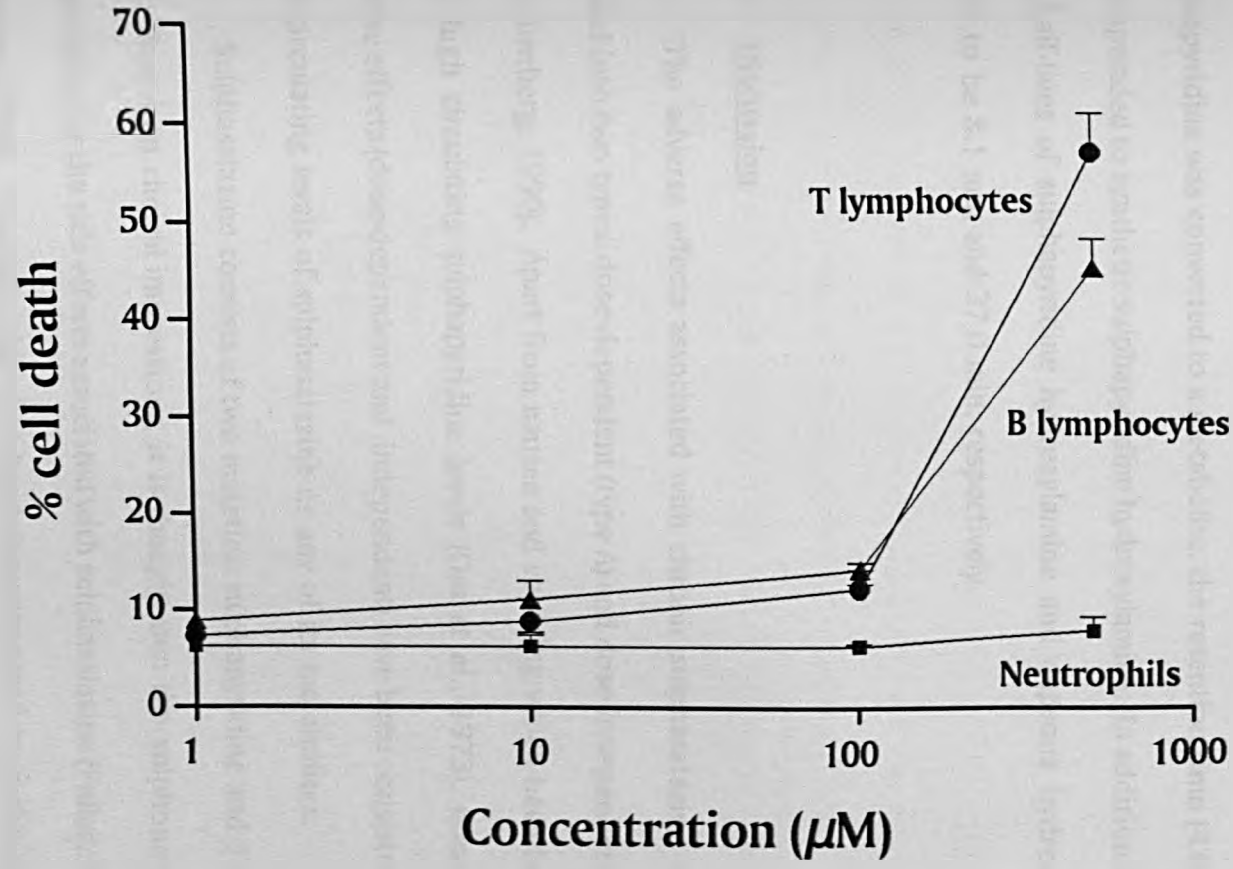


Figure 6.11. Cell selective cytotoxicity of varying concentrations of sulphapyridine hydroxylamine after incubation with T-lymphocytes, B-lymphocytes and neutrophils. Each point represents the mean ± SEM of quadruplicate incubations.

6.3.4. Metabolism of sulphapyridine by human liver microsomes to sulphapyridine hydroxylamine

Chromatographic analysis of the human liver microsomal incubations containing sulphapyridine (100μM) and NADPH (1mM) in the presence of ascorbic acid (2mM) revealed that 6.8 ± 0.3% (n = 6 microsomal incubations) of sulphapyridine was converted to a metabolite, the retention time (4.6 min) of which corresponded to synthetic sulphapyridine hydroxylamine. In additional experiments, the half-lives of sulphapyridine hydroxylamine and dapsone hydroxylamine were found to be 8.1 min and 37.0 min, respectively.

6.4. Discussion

The adverse effects associated with chronic sulphasalazine therapy can be divided into two types: dose-dependent (type A) and dose-independent (type B; Riley and Turnberg, 1990). Apart from nausea and vomiting which have been associated with high circulating sulphapyridine levels (Das *et al.*, 1973), none of the other adverse effects (dose-dependent and -independent) have been consistently correlated with circulating levels of sulphasalazine or any of its metabolites.

Sulphasalazine consists of two moieties: sulphapyridine and 5-amino salicylic acid. Based on clinical impressions, it is thought that the sulphonamide moiety is responsible for the side effects associated with sulphasalazine (Pullar, 1989; Riley and Turnberg, 1990). Recently, this view has been supported by a clinical study which showed that patients who had previously experienced severe side effects or allergic reactions with sulphasalazine were able to tolerate 5-amino salicylic acid (Mulder *et*

al., 1988).

In the present study, in order to investigate which component of sulphasalazine is responsible for the reported toxicity, we have used human cells as targets to assess the toxicity of sulphasalazine and its metabolites in the presence of human liver microsomes. Methaemoglobin formation and cytotoxicity have been used as the toxic end-points to provide an indirect marker for the *in situ* formation of reactive metabolites. The results indicate that only sulphapyridine was bioactivated by human liver microsomes in the presence of NADPH to a metabolite which caused both methaemoglobinaemia and cytotoxicity (tables 6.2 and 6.3). Chromatographic analysis of the microsomal incubation supernatant showed that sulphapyridine was converted to a metabolite, the retention time of which corresponded to synthetic sulphapyridine hydroxylamine. Moreover, the cellular toxicity of this metabolite was confirmed by using synthetic sulphapyridine hydroxylamine which caused a concentration dependent increase in methaemoglobin formation (figure 6.5) and cytotoxicity (figure 6.10). This metabolite, to the best of our knowledge, has not previously been identified in either *in vivo* or *in vitro* studies involving sulphasalazine. It is highly likely that this metabolite (or its further oxidation product, the nitroso compound) is responsible for many of the adverse effects associated with sulphasalazine. This is in agreement with the proposed mechanisms of toxicity of other sulphonamides (Shear and Spielberg, 1985; Reider *et al.*, 1989), dapsone (Uetrecht *et al.*, 1988; Coleman *et al.*, 1989) and procainamide (Uetrecht, 1985); the hydroxylamines of the latter two compounds have, as with sulphapyridine hydroxylamine in the present study, been shown to cause cytotoxicity (Rubin *et al.*, 1987; Coleman *et al.*, 1989) and methaemoglobin formation (Roberts *et al.*, 1989;

Tingle *et al.*, 1990).

The metabolism-dependent cytotoxicity observed with sulphapyridine in the presence of human liver microsomes is low (table 6.3); this is partly due to the low *in vitro* conversion of sulphapyridine to the hydroxylamine (6.8%), and thus the use of activating microsomes with an increased cytochrome P450 content, i.e. phenobarbitone-induced mouse microsomes, results in increased cytotoxicity (table 6.4). However, this cannot be the full explanation as studies with the structurally related compound dapsone (Coleman *et al.*, 1989; Tingle *et al.*, 1990) have shown that it causes a greater degree of metabolism-dependent cytotoxicity (and methaemoglobinaemia) than sulphapyridine, despite an *in vitro* conversion to the reactive metabolite which is chemically equivalent to that of sulphapyridine. This apparent discrepancy can be accounted for by the much shorter half-life of sulphapyridine hydroxylamine (8 min) compared with that of dapsone hydroxylamine (37 min). The functional significance of the instability of sulphapyridine hydroxylamine is further illustrated in figure 6.6, which shows that the ability of sulphapyridine hydroxylamine to cause methaemoglobinaemia is significantly greater when the metabolite and red blood cell are in direct contact (20% methaemoglobinaemia at 22μM) than when they are initially separated by a semi-permeable membrane in the bicompartmental system (20% methaemoglobinaemia at 384μM). Thus, with MNL, it is possible that the majority of the hydroxylamine of sulphapyridine formed by microsomes oxidises to a stable non-toxic product before it reaches the cell. In addition, co-incubation of the MNL with reducing agents such as ascorbic acid, glutathione and N-acetylcysteine reduces cytotoxicity (figure 6.8) by preventing the auto-oxidation of the hydroxylamine to the ultimately toxic nitroso

species. This effect of the reducing agents is not seen with erythrocytes, however, because the hydroxylamine, as with other toxic arylamines (Kiese, 1966; Eyer, 1983, 1988), is readily taken up by the red blood cell to undergo rapid redox cycling to the nitroso species and methaemoglobin (figure 6.12).

How does the *in vitro* toxicity demonstrated in this study relate to the side effects seen with sulphasalazine in patients? While the conversion of sulphapyridine to the hydroxylamine *in vitro* is low, the amount of sulphapyridine hydroxylamine formed *in vivo* is unknown, but is currently under investigation. It is unlikely that the hydroxylamine would be detectable in the circulation because of its instability and rapid uptake by the red blood cells, but its formation could be estimated by the measurement of conjugates in the urine. The ability of sulphasalazine to cause adverse effects may in part be a reflection of the high doses (up to 3g/day) of the drug which have to be given in order to achieve therapeutic concentrations of sulphapyridine (Pullar *et al.*, 1985a); this may result in high local concentrations of the hydroxylamine. Thus, Pounder and colleagues (1975) have shown that red cell contraction was related to the dose of sulphasalazine. However, other forms of red cell toxicity, including methaemoglobinaemia, were not shown to be dose-related (Pounder *et al.*, 1975). This contrasts with the results of the present *in vitro* study which show a concentration-dependent increase in methaemoglobin formation, both with sulphapyridine (in the presence of a metabolising system) and its reactive metabolite, sulphapyridine hydroxylamine. Clearly, *in vivo* it may be more difficult to show such a relationship because of the confounding effects of competing phase I and phase II metabolic pathways, the latter (N-acetylation) being by far the most important metabolic pathway for sulphapyridine (Das and Dubin, 1976; Klotz, 1985).

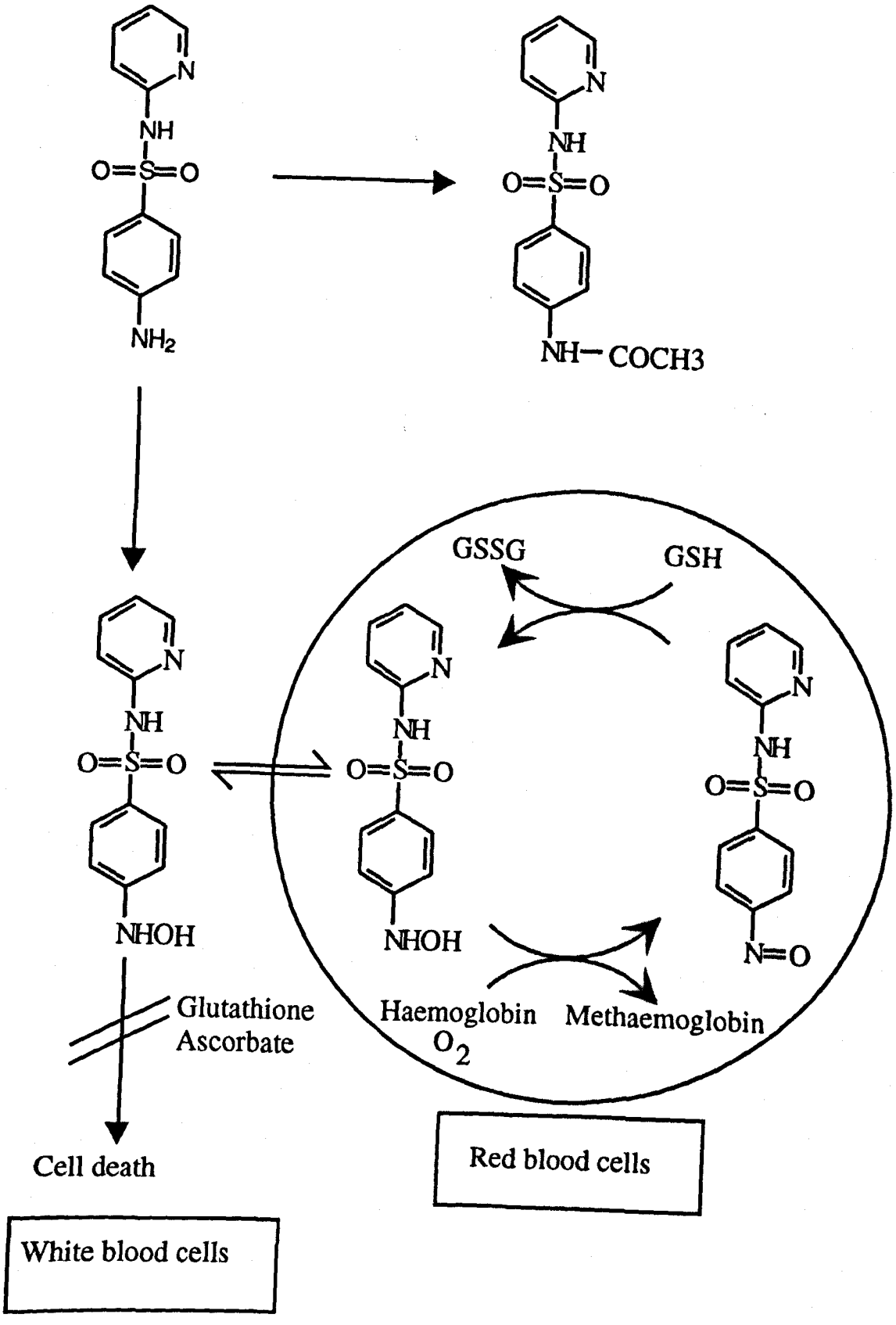


Figure 6.12. Toxicological consequences of the metabolism of sulphapyridine.

The idiosyncratic reactions associated with sulphasalazine are not dose-related (Pullar, 1989; Riley and Turnberg, 1990). As with the anticonvulsants (chapters 2 and 3; Shear *et al.*, 1988), individual susceptibility to the adverse effects of sulphasalazine may be due to inadequate detoxification of its reactive metabolite. Indeed, it has been shown that a heritable deficiency in cellular detoxification may be responsible for predisposing individuals to sulphonamide hypersensitivity and that such patients could be identified by *in vitro* challenge of their lymphocytes to the reactive metabolite (Reider *et al.*, 1989). However, the nature of the deficiency has not been elucidated. For carbamazepine (chapter 4) and phenytoin (Spielberg *et al.*, 1981), indirect evidence suggests that the reactive species is an arene oxide and thus affected patients may have a deficiency of the enzyme microsomal epoxide hydrolase. However, the reactive metabolite formed from sulphapyridine and other sulphonamides is chemically distinct from arene oxides, and in addition, no *in vitro* chemical cross-sensitivity was found when cells from anticonvulsant hypersensitive patients were exposed to hydroxylamines (chapter 2; Shear *et al.*, 1986), suggesting that susceptibility to sulphonamide hypersensitivity may be due to deficiency of a detoxication pathway which is different from that encountered in anticonvulsant hypersensitive patients. There are several lines of evidence which suggest that the glutathione cycle may be important in determining susceptibility to sulphonamide hypersensitivity. First, it is known that cells from patients deficient in glutathione synthetase show higher *in vitro* sensitivity than cells from normal volunteers when exposed to oxidative metabolites of sulphonamides (Shear and Spielberg, 1985). Secondly, as shown with sulphapyridine in the present study (figure 6.9) and with sulphadiazine (Shear and Spielberg, 1985), *in vitro* toxicity can be reduced by

exogenous glutathione and N-acetylcysteine. Thirdly, patients with AIDS have been shown to have a higher incidence of hypersensitivity to sulphamethoxazole (prescribed with trimethoprim) than the general population which has been attributed to a systemic and intracellular deficiency of glutathione observed in these group of patients (van der Ven *et al.*, 1991). However, further research is still required to determine the exact nature of the defect associated with glutathione metabolism.

Sulphasalazine causes neutropenia in approximately 1% of patients (Pullar, 1989). Our data suggest that this is unlikely to be due to direct toxicity of the sulphapyridine hydroxylamine as the peripheral blood neutrophil was relatively insensitive to this compound *in vitro*. The reason for the insensitivity of the neutrophils is unclear, but could be related to better self-defence mechanisms when compared to non-phagocytic white blood cells. Immune-mediated destruction of neutrophils might be the possible mechanism; in fact, formation of leukoagglutinins has been demonstrated in patients treated with sulphasalazine (Evans and Ford, 1958). It is known that activated neutrophils, because of their content of myeloperoxidase and ability to generate oxygen radicals, can activate drugs such as dapsone (Uetrecht *et al.*, 1988) and procainamide (Uetrecht, 1989) to highly reactive metabolites, which by adsorption on to the cell surface could lead to neoantigen formation and thus, an immune response.

The other component of sulphasalazine, 5-amino salicylic acid is present in low concentrations in the blood (Riley and Turnberg, 1990). Clinically, it has not been considered to be the toxic moiety of sulphasalazine. In our *in vitro* study, 5-amino salicylic acid did not show either direct or metabolism-dependent cellular

toxicity in keeping with the clinical impression. However, more recently, with the increasing use of 5-amino salicylic acid derivatives in inflammatory bowel disease (Riley and Turnberg, 1990), a small number of patients have been reported to have suffered adverse effects. These include peripheral neuropathy (Woodward, 1989) and pancreatitis (Deprez *et al.*, 1989). The mechanism of these side effects is unknown.

In summary, the results of this *in vitro* study suggest that N-oxidation products of sulphapyridine may play a role in the haematological toxicity which has been reported with sulphasalazine. The hydroxylamines of other sulphonamides (Reider *et al.*, 1989) and dapsone (Uetrecht *et al.*, 1988) have been implicated in the idiosyncratic reactions associated with these drugs, and thus, sulphapyridine hydroxylamine may also be important in the idiosyncratic reactions reported with sulphasalazine. It is therefore important to determine the role of such metabolites in the *in vivo* toxicity of sulphasalazine, define factors which may influence N-hydroxylation and the further metabolism of sulphapyridine hydroxylamine, and identify the nature of the detoxication defect which influences unique susceptibility to idiosyncratic toxicity.

CHAPTER 7

AN INVESTIGATION INTO THE EFFECT OF CONCURRENT ADMINISTRATION OF CIMETIDINE ON SULPHASALAZINE-MEDIATED HAEMATOLOGICAL TOXICITY IN PATIENTS WITH RHEUMATOID ARTHRITIS

7.1 Introduction

In general, drug-drug interactions can be divided into pharmacokinetic and pharmacodynamic interactions, the former affecting the processes of drug absorption, distribution, metabolism and excretion. The most important interactions, in a clinical context, are those which involve drugs with a low therapeutic index. Interactions which involve drug metabolism are usually due to either induction or inhibition of the cytochrome P450 enzymes. Enzyme induction may lead to enhanced metabolism of co-administered drugs reducing plasma levels with consequent loss of therapeutic efficacy. Thus, carbamazepine is a potent enzyme inducer capable of inducing its own metabolism and of other co-administered drugs such as warfarin and oral contraceptives (Pippenger, 1987). Enzyme induction may also selectively enhance the formation of chemically reactive metabolites as has been discussed in chapter 5.

Enzyme inhibition, on the other hand, can lead to accumulation of co-administered drugs which may result in overt dose-dependent toxicity (Park and Breckenridge, 1981). One of the most widely prescribed drugs in clinical practice (Gerber *et al.*, 1985), which is also a potent inhibitor of oxidative metabolism in man, is cimetidine (Rendic *et al.*, 1979; Gerber *et al.*, 1985; Nazario, 1986). Since it was first approved for general use in 1977, there has been a steady stream of reports of adverse drug interactions when cimetidine is prescribed concurrently with other medications (Gerber *et al.*, 1985), some of which are listed in table 7.1. Cimetidine is an imidazole derivative (figure 7.1), and as such it has been found to undergo direct binding to both the substrate and O_2 binding sites of cytochrome P450, reducing interaction between P450 and other substrates (Wilkinson and Hetnarski, 1972; Somogyi and Gugler, 1982), which is thought, at least partly, to account for

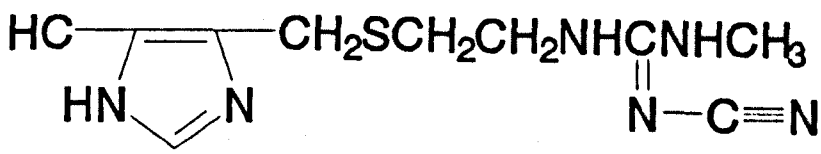


Figure 7.1. The structure of cimetidine

its inhibitory properties (Nazario, 1986).

Although the use of cimetidine is most frequently associated with unwanted adverse drug interactions, it is also possible that use of its enzyme inhibitory properties could be made to either enhance the efficacy of concurrently administered drugs or to reduce their toxicity by selective inhibition of metabolic pathways known to cause the formation of toxic metabolites. In this respect, Dorr and Alberts (1982) have shown in a mouse model that cimetidine enhanced the antitumour activity of cyclophosphamide, there being a dose-dependent prolongation in survival following intraperitoneal instillation of leukaemic cells. This was postulated to be secondary to microsomal enzyme inhibition. Cimetidine has also been shown to interact with paracetamol (Gerber *et al.*, 1985), and thus it has been suggested that it might be a useful adjunct to N-acetylcysteine treatment for paracetamol overdosage (Gerber *et al.*, 1985), although this may be impractical since cimetidine would have to be given before paracetamol (Sherlock, 1986).

More recently, it has been shown that the haematological toxicity of dapsone which is mediated by its hydroxylamine metabolite (Coleman *et al.*, 1989; Tingle *et al.*, 1990), can be reduced by concurrent administration of cimetidine in both healthy volunteers (Coleman *et al.*, 1990) and in patients on chronic dapsone therapy for

Table 7.1.

A list of some of the drugs whose metabolism is known to be inhibited by cimetidine

Drug	*Possible clinical significance*
Caffeine	May enhance cardiovascular and CNS side effects
Carbamazepine	Dose-dependent carbamazepine toxicity
Chlordiazepoxide	Increase sedation and sleepiness
Diazepam	Increase sedation and sleepiness
Ethanol	Enhance alcohol intoxication
Imipramine	Enhanced anticholinergic side effects
Lignocaine	Lignocaine-induced lightheadedness and paraesthesia
Nifedipine	Increased antihypertensive effect
Phenytoin	Phenytoin toxicity
Propranolol	Enhanced ß-blockade
Quinidine	Ectopy and bradycardia
Theophylline	Theophylline toxicity, e.g. arrhythmias and convulsions
Verapamil	Unknown
Warfarin	Increased anticoagulant effect

Adapted from Nazario, 1986.

dermatitis herpetiformis (Coleman *et al.*, 1992a). The mechanism of this beneficial interaction has been shown to be due to a selective inhibition of the N-hydroxylation of dapsone (Coleman *et al.*, 1990, 1992a, b).

In chapter 6, it was shown that sulphapyridine, which is one of the moieties of sulphasalazine, was metabolised by a cytochrome P450 dependent pathway to a

hydroxylamine, which *in vitro* was shown to cause dose-dependent cytotoxicity and methaemoglobinaemia. This metabolite may be responsible for the *in vivo* haematological toxicity which has been reported with sulphasalazine therapy. In addition, as has been postulated with other sulphonamides (Reider *et al.*, 1989), the hydroxylamine metabolite may also be important in causing idiosyncratic reactions, the affected individuals having a deficiency in cellular detoxication (Reider *et al.*, 1989), the nature of which is unclear. Therefore, as has been shown with dapsone, concurrent administration of cimetidine may inhibit the N-hydroxylation of sulphapyridine, and thus by favourably altering the balance between bioactivation and detoxication, reduce the toxicity associated with sulphasalazine, improving the benefit-risk ratio in patients on chronic therapy. However, no studies have been undertaken to determine whether there is any interaction between sulphasalazine and cimetidine. Interestingly, the concurrent administration of sulphasalazine and cimetidine may be quite frequent in current clinical practice since patients with rheumatoid arthritis have a three to four fold higher incidence of peptic ulceration than the general population, which may be due to a combination of disease manifestation and adverse effects of drug therapy, in particular non-steroidal anti-inflammatory drugs (Farah *et al.*, 1988). Thus, investigation into whether there is an interaction between sulphasalazine and cimetidine, beneficial or otherwise, is also important because of this.

Therefore, the aim of this study was determine whether there was any interaction between cimetidine and sulphasalazine, and its potential consequences. The study was performed in patients with rheumatoid arthritis, a group which is likely to be exposed to both drugs. The kinetics of sulphapyridine were determined

before and after the start of cimetidine. In addition, since sulphasalazine causes haematological toxicity (Pounder *et al.*, 1975), several haematological parameters were monitored throughout the duration of the study. Finally, since the mode of action of sulphasalazine in rheumatoid arthritis is not known, disease activity was monitored to ensure that cimetidine did not interfere with its mechanism of action.

7.2. Methods

7.2.1. Patients

The study was approved by the local ethical committee. Patients with rheumatoid arthritis were recruited from the rheumatology clinic at the Royal Liverpool University Hospital. All patients gave written informed consent after a full explanation of the purpose of the study and risks involved.

Nine patients with rheumatoid arthritis (1 male; mean age 56 years, range 39-74 years) on sulphasalazine (mean dose 1.2 g/day; range 0.5-2.0 g/day) were started on cimetidine 400mg three times daily. This group of patients will hereafter be referred to as group I. Assessment (see below) was carried out on two occasions (one week apart) prior to the start of cimetidine, and subsequently at 4, 8, 12, 16 and 18 weeks after the start of cimetidine. Five other patients (1 male; mean age 55 years, range 34-77 years) who were also on sulphasalazine (mean dose 1.6g/day; range 1.0-2.0 g/day), hereafter referred to as group II, were also recruited to the study, but were not started on cimetidine. The clinical parameters derived from group II served as a control group for group I. They were assessed and followed up in the same manner as the patients in group I. One patient in group I had to withdraw from the study after 12 weeks because of a long holiday abroad - her

results up to 12 weeks are included in the analysis.

All the patients, apart from two (one in each group) were also on the following non-steroidal anti-inflammatory drugs: diclofenac (7 patients), indomethacin (4 patients), naproxen (3 patients), ibuprofen (1 patient), aspirin (1 patient) and tolmetin (1 patient). One patient in group II was also on prednisolone 2.5mg on alternate days. Prior to the start of the study, none of the patients were on drugs known to be cytochrome P450 enzyme inhibitors.

7.2.2. Assessment of patients

Assessment in all patients was carried out before midday prior to the first dose of sulphasalazine, the last dose of sulphasalazine having been taken 12h beforehand. At each assessment venous blood was taken for haematological monitoring and measurement of plasma levels of the drug and its metabolites. The plasma was stored at -20°C until analysed. Patients were also asked to collect urine for 24 hours prior to their visits; the total volume of urine was measured and an aliquot frozen until analysed.

Each assessment also included a clinical examination to determine the activity of the rheumatoid disease. As rheumatoid arthritis is a multi-faceted disease with the various facets becoming variably active in different cases, a multivariate analysis first described by Mallya and Mace (1981) comprising morning stiffness, pain scale, grip strength, articular index, haemoglobin and erythrocyte sedimentation rate (ESR) was used to determine the index of disease activity. Morning stiffness was recorded in minutes. The pain scale, a measure of the severity of the joint pain, was determined using a visual analogue scale, i.e. a 10cm line with the worst possible

pain being marked on one end and no pain on the other end, the severity of the pain being determined by asking the patient to put a cross on the line which was then measured in centimetres. The grip strength was measured with an anaeroid manometer attached to a rubber cuff which was inflated to 20mmHg; three readings were taken from each hand and the mean of six was taken as the grip strength. The articular index was calculated in accordance with the method of Ritchie *et al.* (1968). This involves examination of all joints known to be commonly affected by rheumatoid arthritis (figure 7.2). Tenderness of the joints was elicited by firm pressure over the joint margin, except in the case of the cervical spine, the hip joints, the talo-calcaneal and the mid-tarsal joints, where tenderness was elicited by passive movement of the joint. The tenderness of each joint was graded as follows, a total score (the articular index) being obtained by the addition of the scores of each of the joints examined:

Grade 0 - patient had no tenderness

Grade +1 - patient complained of pain

Grade +2 - patient complained of pain and winced

Grade +3 - patient complained of pain, winced and withdrew.

The haemoglobin and ESR were measured by a Coulter Counter and the Westegren's method, respectively. Each of the six features was divided into four arbitrary grades (table 7.2.) and the total of all grades was then divided by six to obtain the mean which was then used as an index of disease activity.

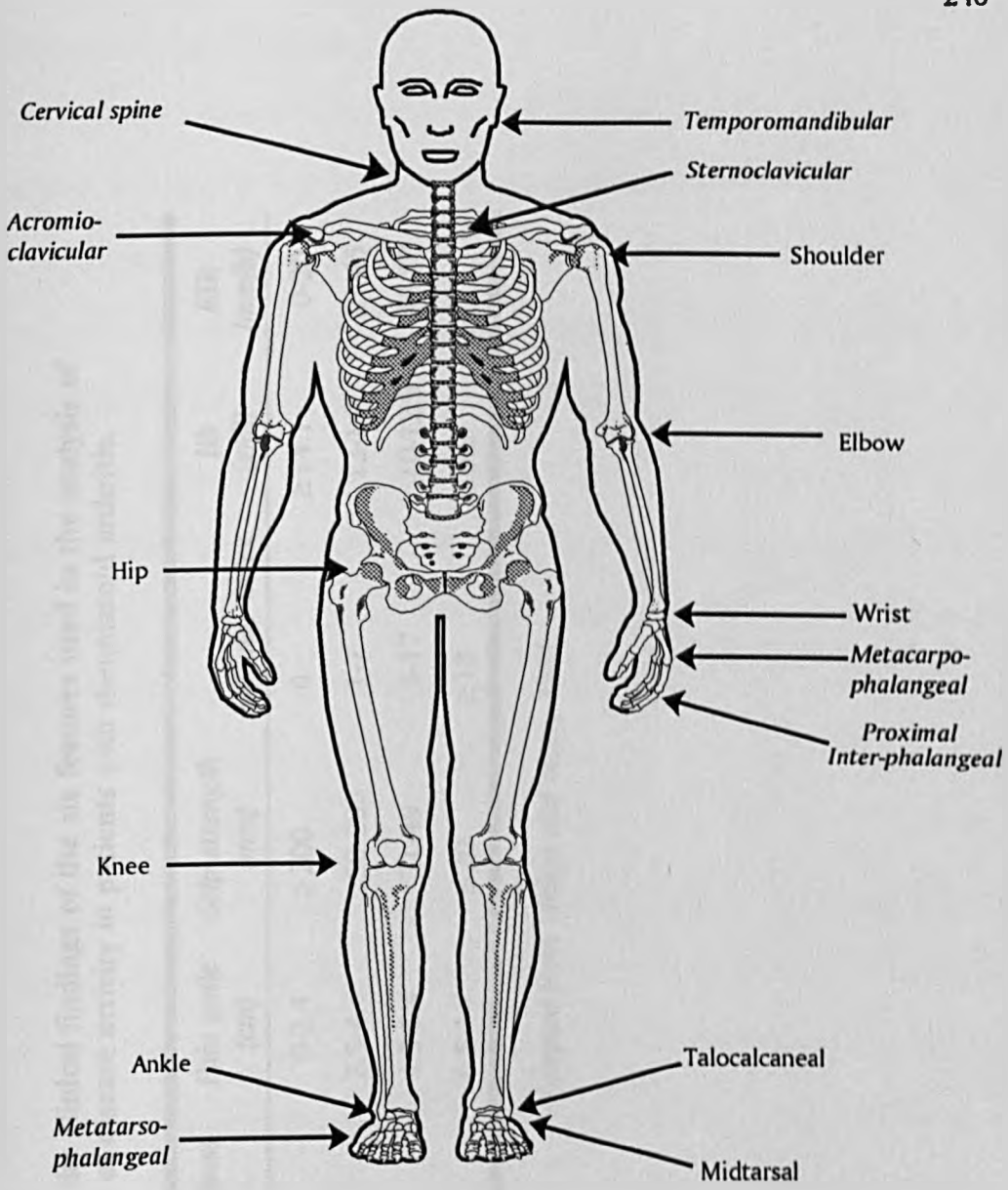


Figure 7.2. The joints examined to determine the articular index according to the method of Ritchie *et al* (1968). All joints were examined on both sides of the body, with the italicised joints being treated as single units. Tenderness was determined in all the joints by palpation (except for hip and cervical spine were tenderness was determined by movement), and graded as stated in the text to obtain a cumulative score (the articular index).

Table 7.2.

The grading of the clinical findings of the six features used in the analysis of the index of disease activity in patients with rheumatoid arthritis.

Grade	*Morning stiffness (min)*	*Pain scale (cm)*	*Grip strength (mm)*	*Articular index*	*Hb (g/dl)*	*ESR (mm/h)*
1	<10	0-2.4	>200	0	≥14.1	0-20
2	10-30	2.5-4.4	50-200	1-7	13-14	21-45
3	31-120	4.5-6.4	21-49	8-17	10-12.9	46-80
4	>120	6.5-10	≤20	≥18	≤9.9	≥81

Adapted from Mallya and Mace, 1981.

7.2.3. Haematological monitoring of patients

Methaemoglobinaemia was measured in 100µl aliquots of heparinised blood obtained from patients on the day of their assessment using the spectrophotometric method of Harrison and Jollow (1986) as described in section 6.2.6.

The rest of the haematological monitoring was performed by the Department of Haematology, The Royal Liverpool University Hospital. Full blood count (FBC) was estimated using a Coulter Counter. Reticulocytes were stained supravitally using brilliant cresyl blue and Heinz Body estimation, by counting at least 300 cells, was carried out using 0.5% methyl violet. The blood film was assessed by Dr. D. Galvani (Department of Haematology, The University of Liverpool) who was unaware of whether the patients were in group I or group II.

7.2.4. Chromatographic analysis of plasma and urine samples from patients

Analysis of the plasma and urine samples was performed by Dr. M.D. Coleman (Department of Pharmacology and Therapeutics, The University of Liverpool). Plasma samples (0.2ml) were extracted twice with ethyl acetate (800µl). The extracts were combined and evaporated under nitrogen gas. The samples were reconstituted and analysed by an HPLC method based on that of Fischer & Klotz (1978) as described in section 6.2.7. Separation was achieved using a µBondapak C_{18} column (30cm x 0.39cm i.d., 10µm pore size, Waters Associates, Hartford, Cheshire, U.K.). The mobile phase consisted of 0.1M sodium acetate buffer : acetonitrile : methanol (86:9:5) containing 0.005% triethylamine flowing at 1ml/min. The eluate was monitored by UV spectroscopy at 245nm. Under these conditions the retention times of sulphapyridine hydroxylamine, sulphapyridine, 5-hydroxy sulphapyridine and *N*-acetyl sulphapyridine

were 6.0, 8.3, 10.1, and 12.4 min, respectively. Urine samples were analysed by direct injection of 5µl onto the HPLC column.

7.2.5. Hydrolysis of patient urine samples

Urine samples (100µl) were incubated with glucurase^{R} (100µl; Sigma, Poole, U.K) along with 10µl of a 50mM solution of ascorbic acid to give a final concentration of 5mM. In a pilot study, incubations were set up for time 0, 1, 4, 8, 16 and 24 hours in order to ascertain the optimum incubation time which provided maximum liberation of compound. An incubation time of 1h was shown to be optimal for liberation of 5-hydroxy sulphapyridine. Incubation for up to 24h did not cause any increase in sulphapyridine or N-acetyl sulphapyridine levels. The incubation conditions had previously been shown to prevent autooxidation of sulphapyridine hydroxylamine when incubations were spiked with a known concentration (1µg/ml) of the compound.

7.2.6. Statistical analysis

Statistical analysis was carried out by the use of paired or unpaired Student's t-test, and the Mann-Whitney test, as appropriate. The acetylator phenotype was measured according to a standard method (Gelber *et al.*, 1971), i.e the ratio of the acetylated derivative to the parent drug at a 3 hour plasma time point. Fast acetylator status was accepted to be a ratio of greater than 0.3. Statistical analysis of urine was achieved by comparison of data derived from time 0 (no cimetidine) with each subsequent time point (collected in the presence of cimetidine).

7.3. Results

7.3.1. The effect of administration of cimetidine on the disease activity index and haematological parameters

The activity of the disease and several haematological parameters were monitored in both groups of patients at entry into the study (week 0) and at four-weekly intervals until the end of the study. There was no difference in the activity of the disease or in haematological parameters between the two groups during the course of the study (table 7.3). In addition, disease activity and the haematological parameters in the patients in group I did not change after the addition of cimetidine.

Table 7.3.

The index of disease activity and haematological parameters in patients on sulphasalazine and cimetidine (group I) and patients on sulphasalazine only (group II) at the beginning (week 0) and end of the study (week 18).

Parameter	*Group I*		*Group II*	
	Week 0	*Week 18*	*Week 0*	*Week 18*
Index of disease activity (IDA)	2.2 (1.7 - 3.2)	2.0 (1.5 - 3.3)	2.2 (1.7 - 2.6)	2.0 (1.8 - 3.2)
Haemoglobin (g/l)	11.6 ± 0.9	11.5 ± 1.2	11.6 ± 1.6	12.1 ± 1.3
Mean corpuscular volume (fl)	87.9 ± 9.6	88.3 ± 10.8	83.9 ± 9.0	84.6 ± 8.8
Mean corpuscular haemoglobin concentration (%)	33.7 ± 0.9	33.6 ± 0.9	33.8 ± 1.1	33.4 ± 1.2
Reticulocyte count (x 10^9/l)	62 ± 22	61 ± 29	91 ± 32	76 ± 35
White cell count (x 10^9/l)	6.2 ± 2.2	5.4 ± 1.4	6.9 ± 1.3	6.3 ± 2.0
Platelet count (x 10^9/l)	349 ± 99	354 ± 114	327 ± 115	337 ± 64

Results are presented as mean ± S.D., except for IDA which are presented as median (range). Statistical analysis was performed by paired or unpaired t-test (as appropriate) for all parameters except for IDA which was analysed by the Mann-Whitney test.

No patient was found to have Heinz bodies. All patients also had their blood film examined at four-weekly intervals to determine the percentage of malformed cells, which in a previous study had been reported to occur in 70% of patients (Azad Khan *et al.*, 1980) on sulphasalazine. Although these were detected in all the patients at some point in the study, their presence was inconsistent and when detected, they were present in small numbers (<1%), and thus could not be used as a marker for the haematological toxicity of sulphasalazine.

7.3.2. Plasma disposition of sulphapyridine and its metabolites in patients

The plasma disposition of sulphapyridine and its acetylated metabolite was determined in patient group I, which resolved into five slow and four fast acetylators. There were no significant differences between the plasma concentrations measured before and after the commencement of cimetidine administration (figure 7.3) in both the slow and fast acetylators. Overall, the plasma sulphapyridine concentrations were significantly lower ($p<0.05$) in the fast acetylators (figure 7.3A) compared with the slow acetylators (figure 7.3B). The only other detectable metabolite in plasma was the N-acetyl sulphapyridine, the levels being significantly higher ($p<0.05$) in fast acetylators compared with slow acetylators. Neither the 5-hydroxy nor the N-hydroxylated metabolite could be detected in plasma.

7.3.3. Methaemoglobin levels in patients before and after the initiation of cimetidine therapy

The normal methaemoglobin level in healthy individuals not on any medication is quoted as being below 1% (Eastham, 1985). At entry into the study, all

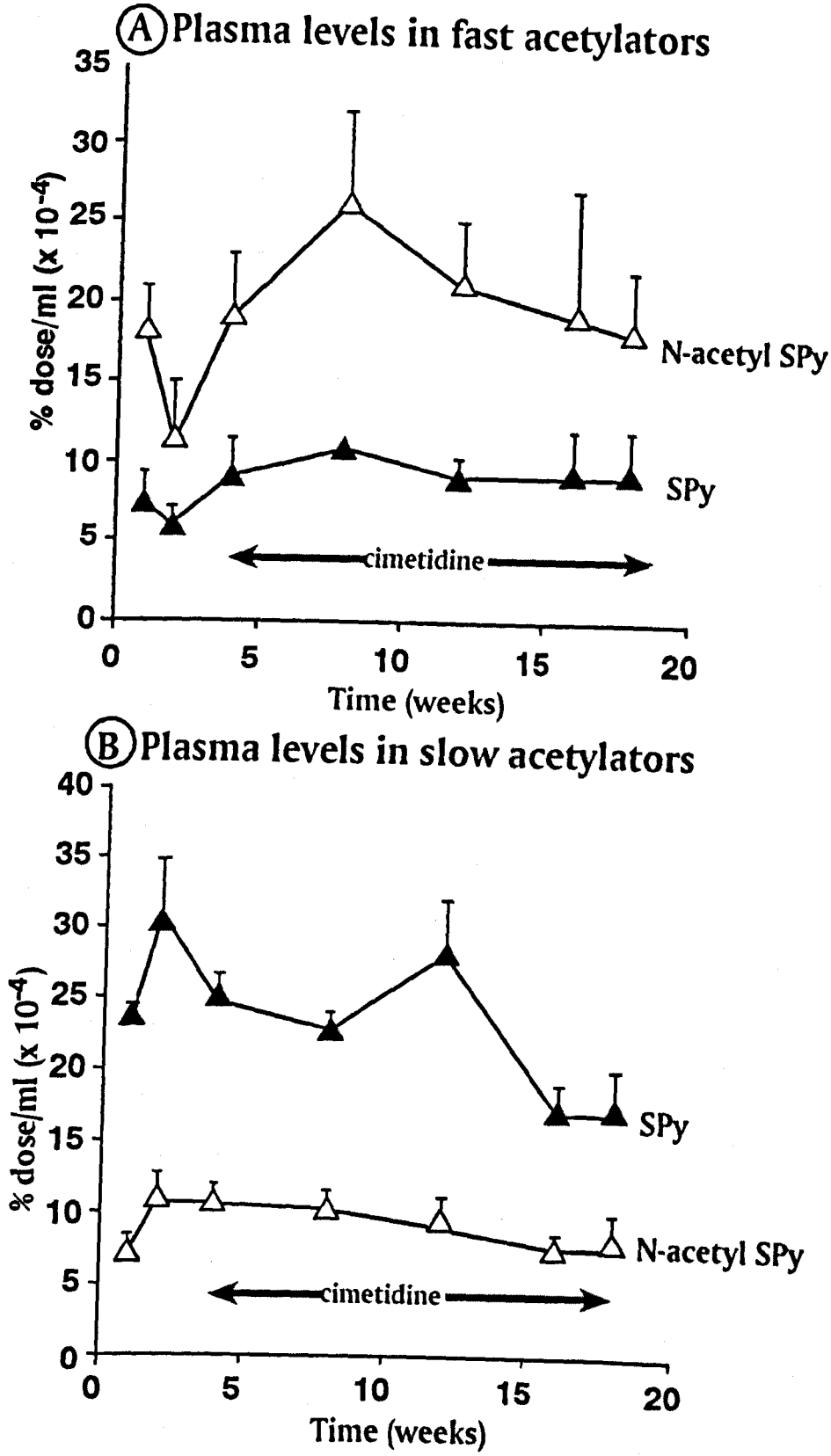


Figure 7.3. Sulphapyridine (SPy; ▲) and N-acetyl sulphapyridine (△) concentrations in patients receiving sulphasalazine plotted against time for A: fast acetylators (n=4) and B: slow acetylators (n=5) before and after the concurrent administration of cimetidine. Each data point represent the mean ± SEM.

of the 14 patients on sulphasalazine had methaemoglobin levels higher than 1% (mean ± S.D. methaemoglobin level for 14 patients was 3.7 ± 0.8%). There was no significant difference in the background methaemoglobin levels between the slow acetylators (n=7; 3.8 ± 0.85%) and fast acetylators (n=7; 3.6 ± 0.67%).

With patients in group I (n=9), the initiation of cimetidine therapy resulted in a small, but statistically non-significant decrease in the mean methaemoglobin levels from 3.5 ± 0.7% at week 0 to 2.9 ± 0.4% at week 18.

7.3.4. Urinary elimination of sulphapyridine and its metabolites in patients

HPLC analysis of patient urines before and after hydrolysis revealed no significant differences in either parent drug or acetylated metabolite concentrations. However, relatively mild hydrolysis (1 hr) revealed the presence of a 5-hydroxy metabolite, which was not detectable in urine prior to hydrolysis. Cimetidine therapy did not significantly change the urinary excretion of the 5-hydroxy sulphapyridine (figure 7.4). In comparison with control there was also no significant difference in the urinary excretion of either sulphapyridine or acetylsulphapyridine (figure 7.5) in the presence of cimetidine. Overall, elimination of the N-acetyl sulphapyridine was greater within the fast acetylators. However, the differences at specific time points were not always significant due to the variability in the excretion of the metabolite (figure 7.5). Fast acetylators excreted significantly less parent drug compared with slow acetylators (figure 7.5). Overall, less than 40% of the dose could be accounted for as either parent drug, 5-hydroxy sulphapyridine or acetylated derivatives. N-hydroxy sulphapyridine was intermittently detectable at low levels although interfering peaks in the urine made accurate quantification of N-hydroxy sulphapyridine impossible.

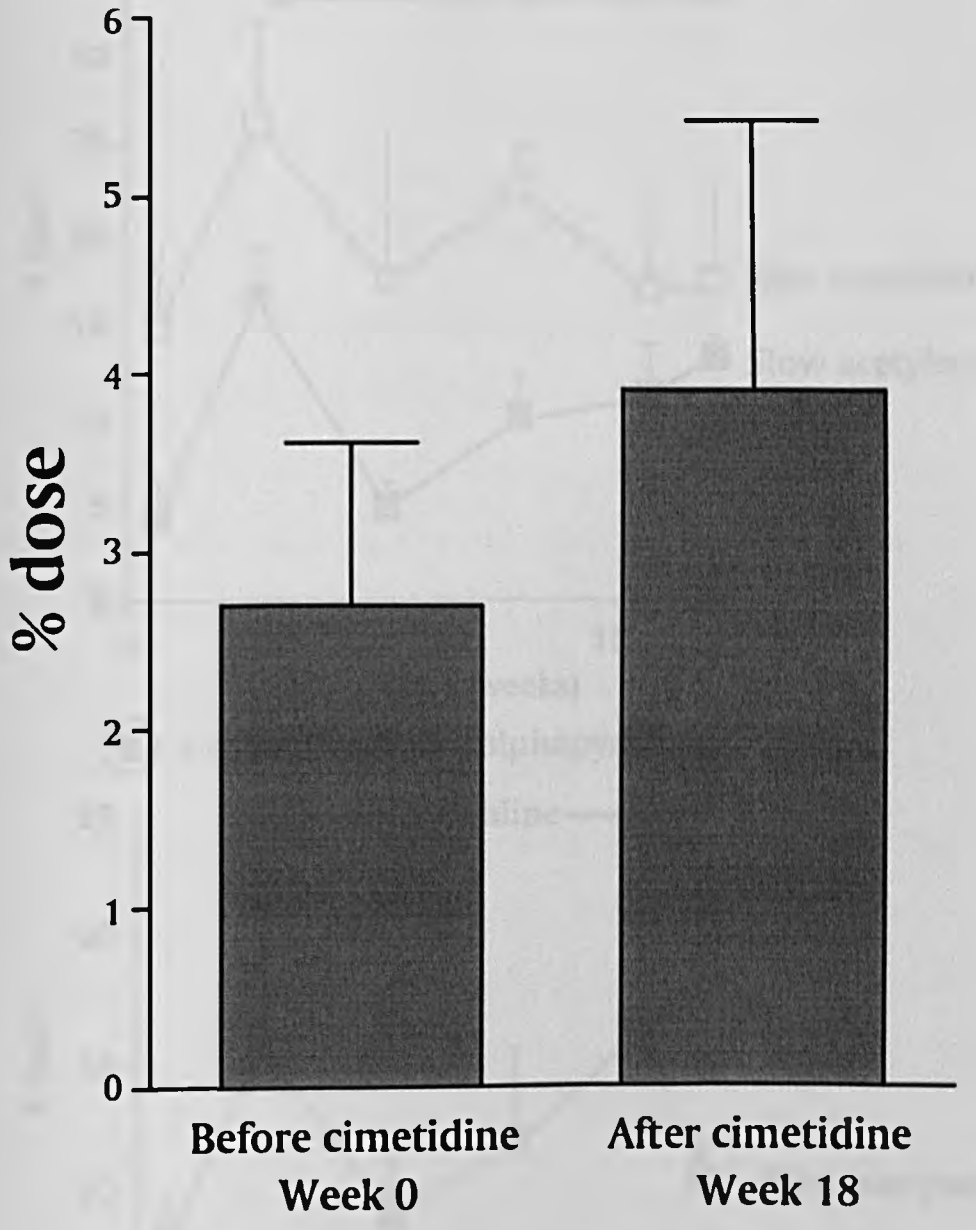


Figure 7.4. Urinary excretion of 5-hydroxy sulphapyridine in patients (n=9) expressed as percentage of total oral dose before and 18 weeks after the start of cimetidine (400mg t.d.s.) therapy. The results are presented as mean ± SEM.

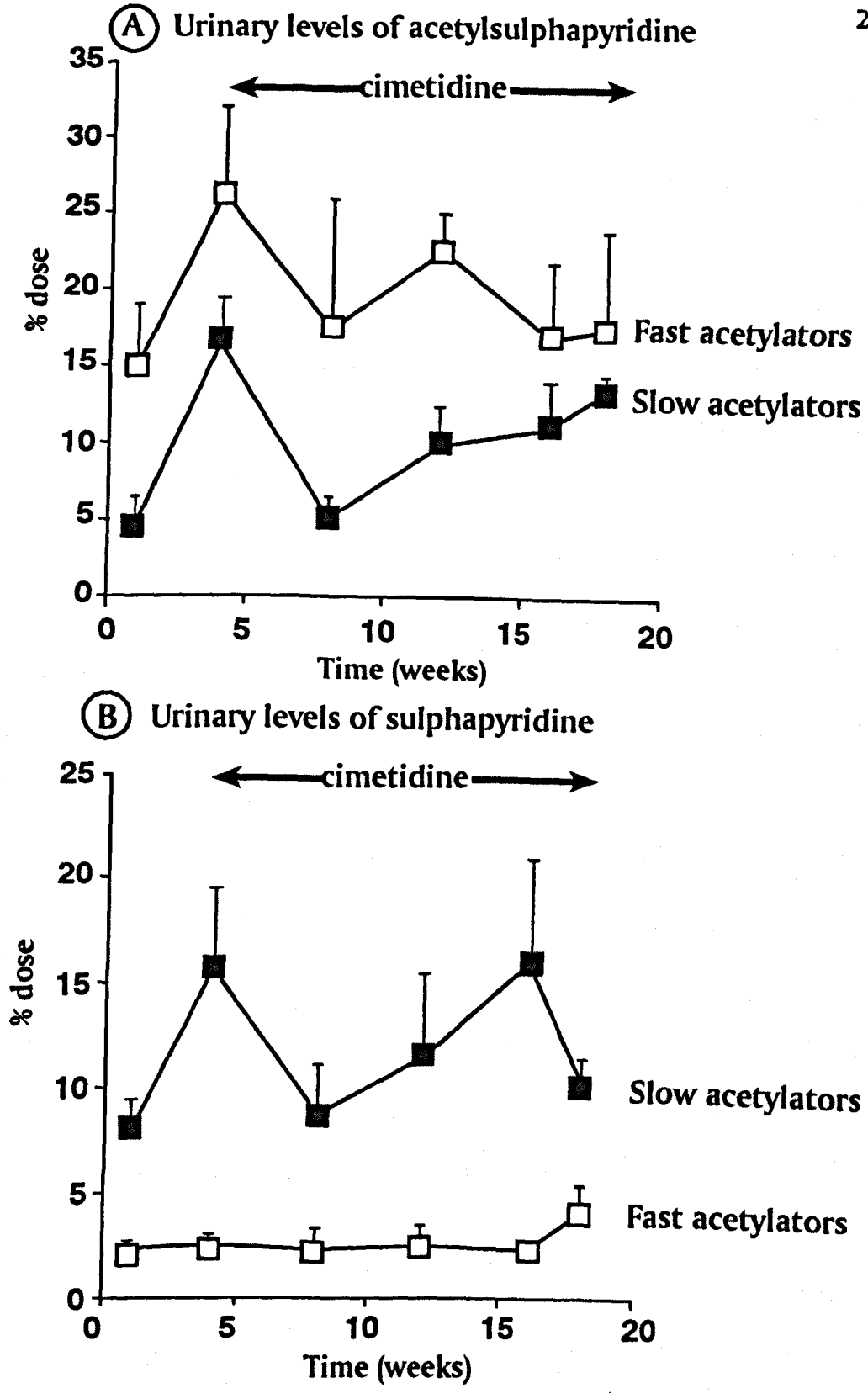


Figure 7.5. Percentage of dose of sulphasalazine excreted in urine as A, N-acetylsulphapyridine, and B, sulphapyridine in fast acetylators (□ n=4) and slow acetylators (■ n=5) in patients receiving sulphasalazine before and after cimetidine administration. Each data point represent the mean ± SEM.

7.4. Discussion

As with carbamazepine (chapters 2-5), predisposition to developing some of the adverse effects reported with sulphasalazine may be due to an imbalance between bioactivation of the sulphapyridine moiety to its chemically reactive metabolite and its cellular detoxication (chapter 6). The detoxication processes thought to protect against sulphonamide toxicity have not been clearly established, but may involve a defect in the glutathione cycle (Shear and Spielberg, 1985; Shear *et al.*, 1986). In order to favourably alter the balance between bioactivation and detoxication, it may theoretically be possible to inhibit the former metabolic pathway, and thus reduce the formation of the chemically reactive metabolite. Hence, the H_2 antagonist, cimetidine, a potent inhibitor of oxidative drug metabolism (Rendic *et al.*, 1979; Gerber *et al.*, 1985; Nazario, 1986), was administered to patients with rheumatoid arthritis on chronic sulphasalazine therapy with the aim of evaluating whether there was any interaction, beneficial or adverse, between these two drugs. This combination of drugs is quite likely to be encountered anyway in patients with rheumatoid arthritis in view of the higher incidence of peptic ulceration associated with this disease (Farah *et al.*, 1988). The design of the study was such that patients would be evaluated before and after the start of cimetidine, and thus each patient acted as a control for themselves when determining the effect of concurrent administration of cimetidine. However, a second group (group II) of patients who did not receive cimetidine was also studied primarily to act as a control group for group I with respect to the haematological parameters which were monitored during the study in order to exclude any effect of the disease on these parameters.

The azo linkage between sulphapyridine and 5-amino salicylic acid, the two moieties of sulphasalazine, is reduced and cleaved by colonic bacteria. Subsequently, 5-amino salicylic acid is largely excreted unchanged in the urine (Klotz, 1985; Bondesen *et al.*, 1988; Riley and Turnberg, 1990), with very low concentrations detectable in the plasma (Pullar, 1989). In contrast, sulphapyridine is almost completely absorbed and undergoes extensive metabolism (Das and Dubin, 1976; Klotz, 1985; Riley and Turnberg, 1990). N-acetylation, the major metabolic pathway of sulphapyridine, is under genetic control, and thus, as has been shown in the present study and by several previous studies (Das *et al.*, 1973; Goldstein *et al.*, 1979; Taggart *et al.*, 1992), slow acetylators have a lower steady-state concentration of the acetylated metabolite than fast acetylators. Sulphapyridine also undergoes ring hydroxylation to give 5-hydroxy sulphapyridine which is excreted in the urine as its glucuronic acid conjugate (Das and Dubin, 1976; Klotz, 1985). In addition, it was shown in chapter 6, that sulphapyridine can also be N-hydroxylated to form the reactive metabolite, sulphapyridine hydroxylamine.

Thus, since only sulphapyridine (and not 5-amino salicylic acid) is known to undergo oxidative metabolism, any potential effect of cimetidine would be on the sulphapyridine moiety. However, pharmacokinetic analysis revealed that there was no overall significant change in the kinetics of sulphapyridine upon administration of cimetidine with the urinary elimination of the major oxidative metabolite, 5-hydroxy sulphapyridine also being unchanged (figure 7.4). In addition, cimetidine did not affect the acetylation pathway.

Sulphasalazine can cause a wide variety of adverse effects, the haematological system being one of the most commonly affected - indeed, monthly monitoring of

the blood count is recommended in patients started on sulphasalazine (Pullar, 1989). The haematological adverse effects include leucopenia (Amos and Bax, 1988) and red cell haemolysis (Das *et al.*, 1973; Pullar, 1989; Riley and Turnberg, 1990). Although the latter is uncommon, pre-haemolytic changes such as Heinz bodies (Azad Khan *et al.*, 1980) and crenated red cells are common, the latter occurring in up to 70% of patients (Azad Khan *et al.*, 1980). In addition, macrocytosis (Farr *et al.*, 1989), reticulocytosis (Riley and Turnberg, 1990) and methaemoglobinaemia (in over 40% of patients; Pounder *et al.*, 1975) have been reported to occur in patients on chronic sulphasalazine therapy. In chapter 6 it was shown that sulphapyridine was converted by human liver microsomes *in vitro* to a toxic metabolite, sulphapyridine hydroxylamine, which may be responsible for the haematological toxicity seen in clinical practice. The hydroxylamine is unstable under physiological conditions (half-life 8.1 min) and is therefore difficult to quantify in biological fluids by conventional chromatographic techniques. However, the hydroxylamine, as with other haemotoxic arylamines such as dapsone (Tingle *et al.*, 1990), may be rapidly taken up by red cells to undergo redox cycling to the nitroso species and produce methaemoglobin (chapter 6). Thus, methaemoglobin formation could be used as an indirect marker for the formation of sulphapyridine hydroxylamine. Indeed, all our patients had abnormal methaemoglobin levels (>1%) but administration of cimetidine did not produce a significant reduction. Moreover, there was no perturbation of the haematological parameters monitored (table 7.3.), and thus the lack of effect of cimetidine on sulphapyridine kinetics was reflected by an absence of any effect on the pharmacodynamic parameters which were measured in this study. The absence of any interaction with sulphapyridine contrasts markedly with the effect of

cimetidine on the haematological toxicity of dapsone, where it has been shown that the formation of the hydroxylamine metabolite can be inhibited *in vitro* and *in vivo* with a consequent reduction in methaemoglobin formation (Coleman *et al.*, 1990, 1992a; Tingle *et al.*, 1990).

A higher dose of cimetidine (1200mg/day) than that therapeutically recommended (800mg/day) was used in this study because enzyme inhibition with cimetidine is a dose-dependent phenomenon (Feely *et al.*, 1984; Nazario, 1986). Therefore, despite the small numbers of patients in this study, the demonstrated lack of interaction of sulphasalazine with the higher dose of cimetidine makes it highly unlikely that there would be an interaction between sulphasalazine and therapeutic doses of cimetidine.

The lack of interaction between cimetidine and sulphapyridine may be due to the fact that the particular cytochrome P450 isozyme responsible for the oxidative metabolism of sulphapyridine is not inhibited by cimetidine. Cimetidine is a relatively selective inhibitor of the P450 enzymes and does not inhibit the metabolism of ibuprofen (Forsyth *et al.*, 1988; Evans *et al.*, 1989), tolbutamide (Adebayo and Coker, 1988), bupivicaine (Pihlasamaki *et al.*, 1988), mexiletine (Brockmeyer *et al.*, 1989), metronidazole (Loft *et al.*, 1988) and sulphamethoxazole (Rogers *et al.*, 1980). As can be seen from table 7.1., cimetidine can inhibit the metabolism of theophylline and nifedipine, which are known substrates for CYP1A and CYP3A, respectively (Gonzalez *et al.*, 1991). The inhibition of warfarin metabolism by cimetidine in man is stereoselective, the metabolism of S warfarin not being inhibitable by cimetidine (Choonara *et al.*, 1985). Recent evidence suggests that S warfarin is metabolised by CYP2C9 (O'Reilly *et al.*, 1992); therefore, this evidence taken together with the lack

of effect of cimetidine on tolbutamide metabolism (Adebayo and Coker, 1988), another CYP2C9 substrate (Gonzalez *et al.*, 1991), suggests that this P450 isozyme cannot be inhibited by cimetidine in man. Thus, it is possible that the oxidative metabolism of sulphapyridine to the 5-hydroxy and the N-hydroxy metabolites is dependent on CYP2C9, although clearly further investigation is required to establish this.

An alternative (or possibly contributory) reason for the lack of interaction may be that the fraction of sulphapyridine that undergoes oxidative metabolism is relatively low, most of the drug undergoing N-acetylation, a pathway known not to be affected by cimetidine (Wright *et al.*, 1984). Therefore, acetylator phenotype will dictate the rate of clearance of sulphapyridine itself (figures 7.3 and 7.4). This is in contrast to dapsone, where more than 50% of the total dose is excreted in the urine as the hydroxylamine metabolite (Gordon *et al.*, 1979).

A large limitation of the study was that the sample size was small, and this was compounded by the predominant effect of N-acetylation, which meant that the fast and slow acetylators had to be analysed separately with respect to the effect of cimetidine, thus reducing sample size even further. Therefore, the possibility of a type 2 statistical error cannot be excluded. However, the primary aim of the study was to determine whether there was any interaction with cimetidine, with the patients being assessed at four-weekly intervals up to 18 weeks, and in that time, no effect of cimetidine on methaemoglobinaemia (an indirect marker for the formation of the hydroxylamine) or on sulphapyridine kinetics was observed, indicating that there was no interaction. Clearly, a study involving larger numbers of patients would have been preferable, but unfortunately, this was not possible because of a limited

population from which the sample could be drawn.

In conclusion, no evidence for a clinically important interaction between sulphasalazine and cimetidine was found. Clearly, a similar approach could be pursued for sulphasalazine using other enzyme inhibitors, and indeed for other drugs associated with toxicity, in order to reduce bioactivation, and hence improve the balance between bioactivation and detoxication. However, this does require the availability of an effective and safe enzyme inhibitor, which is not always possible. An alternative but parallel approach is to determine the nature of the detoxication deficiency in individuals affected by drug toxicity, which would not only allow prospective identification of susceptible individuals but also lead to the development of analogues which would bypass the detoxication defect, thus preventing the toxicity.

CHAPTER 8

FINAL DISCUSSION

8.1. Overview

Idiosyncratic drug reactions, by definition, affect only a minority of individuals exposed to the drug. The susceptibility factors predisposing certain individuals are poorly understood, as is the pathogenesis of the toxicity. This is partly due to the fact that such reactions cannot be reproduced in animal models. In addition, rechallenge with the offending drug is often not acceptable, particularly given the serious nature of some of these reactions. Therefore, investigation of affected patients depends on the development of *in vitro* methods, whereby samples from the patient can be utilised *ex vivo*, to identify susceptibility factors for the adverse drug reaction. Allied to this are investigations which can be performed using human tissues, although not necessarily from hypersensitive patients, to determine at a chemical, biochemical, functional and clinical level the pathogenesis of the idiosyncratic drug reactions.

Therefore, such an approach has been adopted to investigate the mechanisms of idiosyncratic toxicity associated with two widely used drugs, carbamazepine and sulphasalazine, in the studies described in this thesis. Both drugs are known to cause toxicity which can affect multiple organ systems to a variable degree and with variable severity.

Collectively, the data presented in the studies described in this thesis are consistent with previous hypotheses regarding idiosyncratic drug toxicity, i.e. the metabolising enzymes can convert drugs to chemically reactive intermediates which then cause toxicity either directly or indirectly by initiating an immune response (figure 8.1; Park, 1986). Thus, for carbamazepine, it can be postulated that a chemically reactive arene oxide is formed by the cytochrome P450 isozyme, CYP3A,

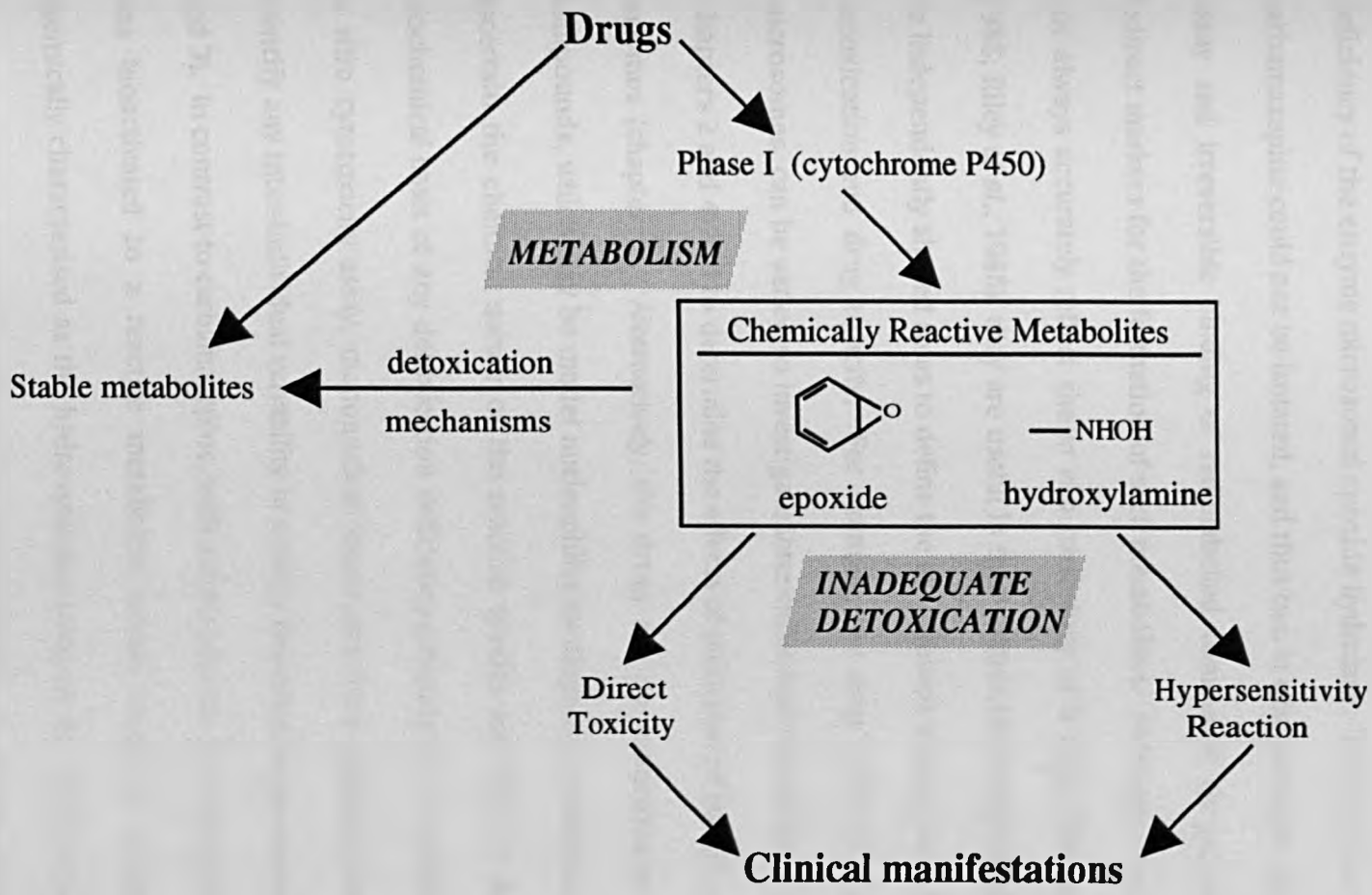


Figure 8.1. Schematic representation of the role of drug metabolism in mediating the idiosyncratic toxicity associated with drugs such as carbamazepine and sulphasalazine.

which then causes toxicity by acting as an hapten and initiating an immune response which can involve the humoral and/or the cellular fractions of the immune system. Furthermore, predisposition to idiosyncratic toxicity seems to be secondary to a deficiency of the enzyme microsomal epoxide hydrolase. The reactive metabolite of carbamazepine could not be isolated, and thus two *in vitro* methods, the cytotoxicity assay and irreversible binding of radiolabelled compound to protein, served as indirect markers for the formation of such metabolites. Although these methods do not always accurately reflect the *in vivo* toxicology of a drug (Kitteringham *et al.*, 1988; Riley *et al.*, 1988), they are useful in that various biochemical parameters can be independently altered so as to define the mechanisms of drug bioactivation, drug detoxication and drug toxicity. For example, the drug metabolising system, i.e microsomes, can be varied to investigate inter-individual variability in bioactivation (chapters 2 and 4) and to determine the effects of induction of the cytochrome P450 enzymes (chapter 5). Alternatively, the drugs can be co-incubated with various compounds, which may be model nucleophiles or diagnostic inhibitors, in order to ascertain the chemical nature of the reactive species and also to determine the biochemical basis of any detoxication deficiency (chapter 4). In addition, with the *in vitro* cytotoxicity assay, mononuclear leucocytes from patients can be used to identify any inter-individual variability in cellular detoxication processes (chapters 2 and 3). In contrast to carbamazepine, with sulphasalazine, its sulphapyridine moiety was bioactivated to a reactive metabolite, which could be isolated and was chemically characterised as the hydroxylamine (chapter 6). This metabolite (or its further oxidation product) seems to be responsible for the haematological toxicity associated with sulphasalazine and may also be responsible for the multi-system

idiosyncratic reactions which have been reported with this drug.

8.2. Carbamazepine: the nature of the detoxication deficiency

In chapters 2 and 3, a total of ten patients with a clinical diagnosis of carbamazepine-hypersensitivity were identified. In agreement with the study by Shear *et al.* (1988) mononuclear leucocytes from these patients showed significantly higher *in vitro* sensitivity to oxidative metabolites of carbamazepine generated *in situ* than controls which comprised cells from healthy volunteers and patients on carbamazepine therapy without adverse effects, suggestive of a detoxication deficiency in the affected patients.

A clue to the nature of the detoxication deficiency was provided by the effect of TCPO, an epoxide hydrolase inhibitor (Spielberg *et al.*, 1981), which increased the metabolism-dependent cytotoxicity of carbamazepine when pre-incubated with cells from control subjects, but had no effect with the cells from hypersensitive patients, indicating that the affected patients may have a different form of epoxide hydrolase. However, in view of the limitations of using TCPO as a diagnostic inhibitor (Ivanetich *et al.*, 1982; Larrey *et al.*, 1989), discussed in chapter 4, the effect of purified microsomal and cytosolic epoxide hydrolases on the *in vitro* metabolism-dependent toxicity of carbamazepine was investigated. Microsomal epoxide hydrolase reduced the cytotoxicity and covalent binding of carbamazepine, while cytosolic epoxide hydrolase had no effect. Clearly, these data provide indirect evidence of the possible nature of the detoxication deficiency in predisposed patients but does not provide absolute proof. The ideal situation would obviously be the direct demonstration of

an enzyme deficiency in the patients affected by carbamazepine toxicity. However, two major problems have to be overcome to be able to achieve this. First, the identification of a better substrate for microsomal epoxide hydrolase as the currently known substrates are all relatively unstable epoxides which undergo an appreciable degree of spontaneous hydrolysis (Guenthner, 1990). Second, the enzyme has to be measurable in accessible tissues, peripheral blood cells being the obvious choice. However, the activity of the enzyme within mononuclear leucocytes towards cis-stilbene oxide is approximately 1000-fold lower than that observed in human liver (Seidegard *et al.*, 1984). In addition, as with many other genetically determined enzymatic disorders (Ayesh and Smith, 1989), the enzyme deficiency may be qualitative rather than quantitative, in which case substrate affinity may be altered which would require the determination of enzyme kinetics to demonstrate the deficiency, a task which may not be practical given the low activity of microsomal epoxide hydrolase in the mononuclear leucocytes and the lack of more suitable substrates.

The use of mononuclear leucocytes in the *in vitro* cytotoxicity assay is based on the fact that they are easily available and contain detoxication enzymes (Spielberg, 1980, 1984). Thus, when investigating carbamazepine idiosyncratic toxicity, these cells are being used as peripheral markers for enzymes (microsomal epoxide hydrolase and glutathione transferase μ) known to detoxify epoxides. Seidegard *et al.* (1987) have shown that glutathione transferase μ in lymphocytes is identical to the enzyme found in the liver, but since the enzyme is polymorphically expressed, being absent (Seidegard *et al.*, 1985, 1987) in at least 50% of individuals, it cannot be the sole predisposing factor for toxicity mediated by drugs known to

form reactive epoxides. For microsomal epoxide hydrolase, it has been assumed that the lymphocyte and liver enzymes are the same (Spielberg, 1984). In support of this hypothesis, studies over the last two decades have failed to show any enzyme heterogeneity (Guenthner, 1990), and in addition, several workers have suggested that there is only one gene for microsomal epoxide hydrolase (Skoda *et al.*, 1988), located on chromosome 1. However, in the rat, a more recent study (Honscha *et al.*, 1991) has shown that at least three different mRNA forms, which differed in the 5' regulatory region, can be isolated from different tissues indicative of alternative splicing of the initial gene transcript and regulation of expression by different (tissue-specific) promoters. Given the high degree of homology between the rat and human enzymes (Skoda *et al.*, 1988), it is possible that a similar situation exists in humans, although further research is needed to determine whether there is such tissue-specific expression in man and its importance in determining individual susceptibility to adverse drug reactions.

A further point of interest is the wide variation in the clinical presentation of the toxicity between the different patients described in chapters 2 and 3. There is clinical variability not only of the severity of the reaction but also of the organs affected. For example, two of the most severely affected patients had completely different presentations, the major manifestation being hepatotoxicity in patient SR and toxic epidermal necrolysis in patient JS. The reason for organ-selective toxicity in these cases is unknown, but could be due a tissue-specific imbalance between drug bioactivation and detoxication of any metabolite(s). In this respect, tissue-specific differences in expression of microsomal epoxide hydrolase may be an important determinant of which organ is affected by which drug.

Although the studies with the hypersensitive patient cells suggest a detoxication deficiency as the prime determinant of individual susceptibility, it is also possible that inter-individual variability in bioactivation of carbamazepine may also be a contributory risk factor. Thus, in chapters 2 and 4, it was shown that human livers were capable of bioactivating carbamazepine to a cytotoxic and protein-reactive metabolite. In chapter 5, a mouse model was used to investigate the effect of induction of the cytochrome P450 enzymes on carbamazepine bioactivation. The data suggest that induction of the CYP3A sub-family, the major P450 form present in man (Shaw *et al.*, 1989) which is inducible by anticonvulsants, can increase bioactivation, which in certain individuals may result in the detoxication mechanisms being overwhelmed, with consequent toxicity. The individuals affected may be those with low levels (or low activity) of epoxide hydrolase which may be the result of the normal variation in levels which is seen in the population (Mertes *et al.*, 1985), or alternatively, secondary to the co-administration of other anticonvulsants such as sodium valproate or valpromide which are known to be inhibitors of epoxide hydrolase (Kerr *et al.*, 1989; Kerr and Levy, 1990).

Predisposition to idiosyncratic toxicity with carbamazepine, therefore, seems to be due to a local or systemic imbalance between bioactivation and detoxication. The data presented in two patients studied during the acute phase of the reaction (chapter 3) suggest that the toxicity of the inadequately detoxified chemically reactive metabolite is immune mediated. However, the presence of a specific circulating autoantibody in the patient with the hepatotoxicity and the presence of infiltrating T-lymphocyte subsets in the lesional skin of the patient with the toxic epidermal necrolysis represent indirect evidence for the immune basis of

carbamazepine idiosyncratic toxicity. It could be argued that these may be epiphenomena secondary to direct toxic damage from the chemically reactive metabolite, although this does seem unlikely given the accompanying symptomatology in patients described in this thesis (chapter 2 and 3) and in other studies (Dreifuss and Langer, 1987; Shear *et al.*, 1988), and by the presence of specifically reactive circulating T-lymphocytes in affected patients (Virolainen, 1971; Houwerzijl *et al.*, 1977; Zakrzewska and Ivanyi, 1988). Identification of the structure of the antigen formed from carbamazepine would allow more direct immunological tests to be performed, for example the demonstration of anti-drug antibodies and/or specifically committed lymphocytes by ELISA and lymphocyte transformation test, respectively, in order investigate further the immune basis of carbamazepine toxicity.

8.3. Sulphasalazine: the biochemical basis of toxicity

Clinically, the toxicity of sulphasalazine is very similar to that seen with carbamazepine. Given the differences in the structures of these two drugs, the similarity in the adverse effect profile can only be explained by invoking an immune mechanism for the adverse reactions, which is certainly supported by the accompanying symptomatology (Reider *et al.*, 1989). Therefore, in chapter 6, the biochemical and chemical bases of sulphasalazine-mediated toxicity have been investigated using erythrocytes and mononuclear leucocytes as target cells. Of the two moieties of sulphasalazine, only sulphapyridine was bioactivated to a metabolite, later identified as sulphapyridine hydroxylamine, which caused both cytotoxicity and methaemoglobinaemia. Moreover, the cellular toxicity of the hydroxylamine was

confirmed by co-incubating it with erythrocytes and mononuclear leucocytes in the absence of a metabolising system. Use of a bicompartmental chamber showed that the hydroxylamine was able to traverse a semi-permeable membrane and cause toxicity on the opposite side of the membrane. Taken together with estimation of the half-life to be 8 min, this would suggest that *in vivo*, the metabolite may be able to cause toxicity distant from the site where it is formed and would be categorised as long-lived according to the classification of Gillette *et al.* (1984).

As with carbamazepine, the balance between bioactivation of sulphapyridine to the hydroxylamine and its detoxication may an important determinant of individual susceptibility. The conversion of sulphapyridine to the hydroxylamine was inhibited by ketoconazole (chapter 6) indicating that it was a cytochrome P450-dependent process. In view of this, a study was undertaken in patients with rheumatoid arthritis (chapter 7) to determine whether the balance between bioactivation and detoxication could be altered *in vivo* by reducing the cytochrome P450-dependent formation of the hydroxylamine. There are many P450 inhibitors in current clinical use (Park and Kitteringham, 1989, 1990a), but most of them are selective, and thus since the P450 isozyme responsible for bioactivating sulphapyridine was unknown, a relatively non-selective inhibitor needed to be used. It was felt that ketoconazole could not be used as an inhibitor in patients since it also has well-documented toxicity, in particular causing hepatic injury (Lewis *et al.*, 1984). Therefore, cimetidine, a widely used relatively safe drug was chosen as the inhibitor, particularly since it has been reported to interact with a large number of drugs (table 7.1; Gerber *et al.*, 1985; Nazario, 1986), and has also been shown to reduce the toxicity associated with dapsone (Coleman *et al.*, 1990, 1992a), a

compound structurally related to sulphapyridine with a similar side effect profile. Furthermore, cimetidine does not affect acetylation and glucuronidation pathways (Gerber *et al.*, 1985), which in the case of sulphapyridine can be considered as detoxication pathways. However, concurrent administration of cimetidine with sulphasalazine did not interfere with sulphapyridine kinetics or affect the haematological parameters which were used as the end-points of toxicity, possibly because the metabolism of sulphapyridine is dependent on a P450 isozyme which cannot be inhibited by cimetidine. Clearly, such an approach is valuable and could be used for other drugs to favourably alter the balance between bioactivation and detoxication, although perhaps future studies should determine not only that bioactivation is P450 dependent but also which P450 isozyme is responsible for bioactivation so that more selective inhibitors can be used.

Two of the major detoxication processes (for chemically reactive metabolites) in the body are the hydrolysis of epoxides by the hydrolase enzymes and conjugation or interaction with glutathione catalysed by specific transferases. The former, as has been discussed, may be an important predisposing factor for carbamazepine idiosyncratic toxicity, while the latter has been implicated in sulphonamide toxicity (Shear and Spielberg, 1985; Shear *et al.*, 1986). A recent study, using sulphamethoxazole hydroxylamine has shown that conjugation of the reactive metabolite with glutathione is not a major elimination pathway, the glutathione functioning primarily as a reductant to prevent oxidation of the hydroxylamine to the more reactive nitroso-compound (Cribb *et al.*, 1991). In accordance with this (chapter 6), exogenous glutathione reduced sulphapyridine cytotoxicity but had no effect on methaemoglobin formation, the protection of the white cells resulting from

stabilisation of the hydroxylamine, while with the red cells, stabilisation of the hydroxylamine allows it to be taken up into the cell, where it undergoes rapid redox cycling to the nitroso species and methaemoglobin. A defect of glutathione homeostasis, possibly resulting from abnormal activity of the enzyme glutathione reductase has been suggested as a predisposing factor (Cribb *et al.*, 1991), although this requires further study.

8.4. Areas for further research

The areas where further research is required have been indicated in the relevant chapters and in this chapter. With both drugs, determination of the predisposing factor for idiosyncratic toxicity is of paramount importance. For carbamazepine, chemical identification of the reactive metabolite would obviously help in defining whether a deficiency of microsomal epoxide hydrolase was responsible. Given the limitations of the chemical assays, the short-lived nature of the reactive species and the possibility that multiple epoxides could be formed from the drug, a molecular approach may be more appropriate to identify an abnormal (mutant) enzyme. Similarly, for sulphasalazine, a molecular approach may help in determining any defect of glutathione metabolism which leads to sensitivity to the hydroxylamine metabolite. Further studies are also needed to distinguish the adverse effects which are due to the sulphonamide moiety of sulphasalazine and those which are due to 5-aminosalicylic acid, especially in view of the increasing use of the latter in inflammatory bowel disease. Toxicity has been reported with 5-aminosalicylic acid, although the mechanism remains obscure and clearly needs further investigation.

8.5. Conclusion

The studies presented in this thesis have largely used *in vitro* methods to define the mechanisms of idiosyncratic toxicity associated with two drugs, carbamazepine and sulphasalazine. Collectively, the data suggests that the balance between bioactivation of the drug to its chemically reactive metabolites and its detoxication, which is dependent on both genetic and environmental factors, is an important determinant of individual susceptibility (figure 8.2). A knowledge of the factors affecting bioactivation and detoxication may help in improving drug safety by allowing prospective individualisation of drug therapy, the individuals susceptible to toxicity having been identified and treated with alternative drugs. Such an approach may not be feasible in all cases, especially where there is multifactorial predisposition to toxicity. An alternative approach involves chemical modification of existing drugs in such a way that the therapeutic efficacy is retained but metabolism is altered so that the drug either does not undergo bioactivation or is detoxified by an alternative pathway. In addition, a knowledge of the mechanism of idiosyncratic toxicity may also help in the development of new chemical entities which are less likely to cause such toxicity. As indicated, further research is still required in several areas before these goals can be achieved not only with respect to carbamazepine and sulphasalazine, but also with other drugs.

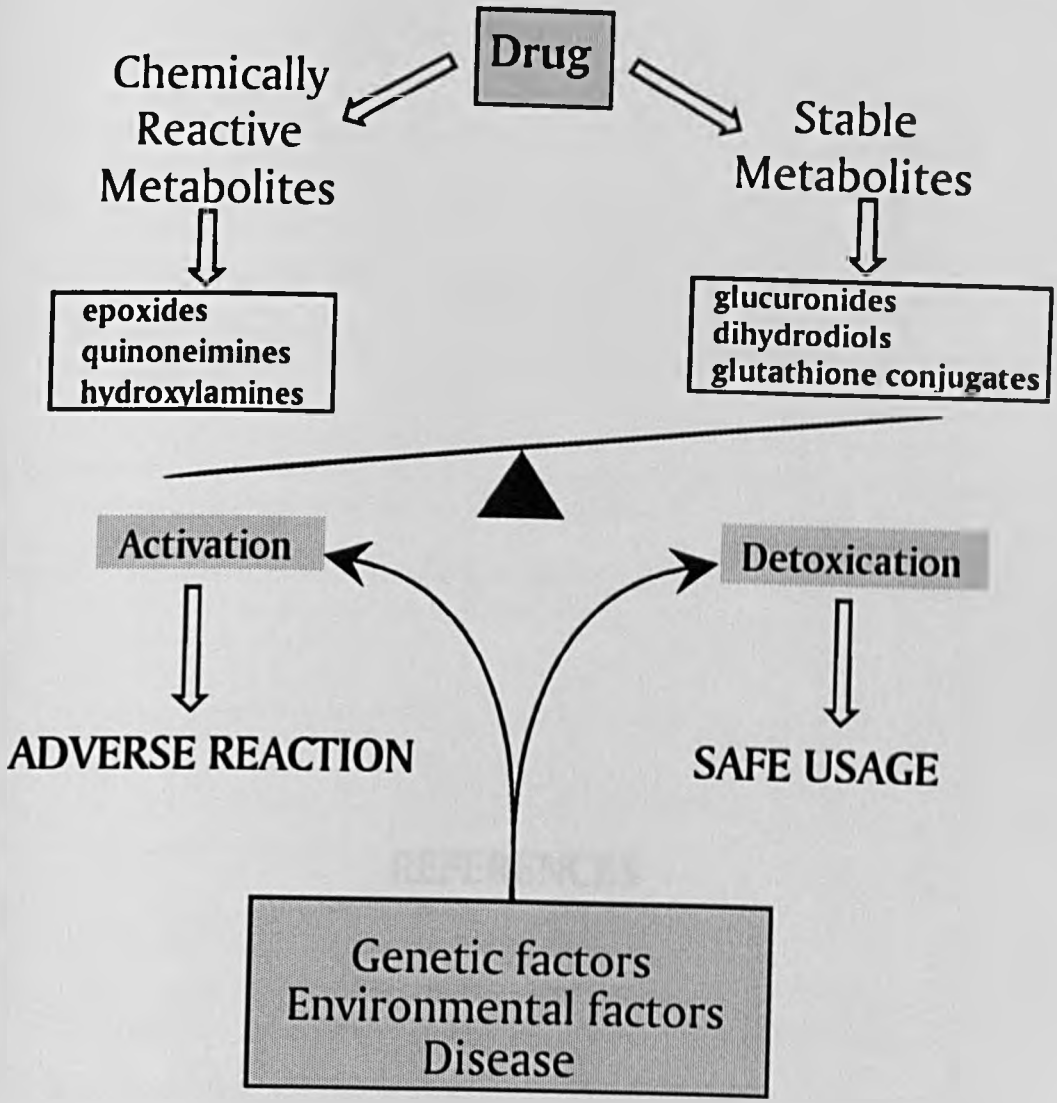


Figure 8.2. Scheme depicting the role of chemically reactive metabolites in the initiation of adverse drug reactions. The balance between activation and detoxication is seen as pivotal in determining susceptibility of an individual to an adverse reaction. This balance can be affected by both external factors, such as disease and concomittant drug therapy, and internal factors, such as an inherited deficiency in drug metabolism.

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