THE DEVELOPMENT OF FUNCTIONAL *IN VITRO* TOXICITY TESTS

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The Development of Functional *In Vitro* Toxicity Tests.

by

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Summary

In vitro toxicity tests which detect evidence of the formation of reactive metabolites have previously relied upon cell death as a toxicity end point. Therefore these tests determine cytotoxicity in terms of quantitative changes in specified cell functions.

In the studies involving the CaCO-2 cell model, there was no significant change in the transport of [^{3}H] L-proline by the cell after co-incubation with either dapsone or cyclophosphamide (50μM) and rat liver microsomal metabolite generating system. The pre incubation of the cells with N-ethylmalemide to inhibit Phase II sulphotransferase activity, prior to the microsomal incubations, resulted in cytotoxcity in all incubation groups.

Studies involving the L6 cell model showed that there was no significant effect in the cell signalling pathway producing the second messenger cAMP, after incubation with dapsone or cyclophosphamide (50μM) and the rat microsomal metabolite generating system. There was also no significant affect on the vasopressin stimulated production of the second messenger IP_3, after incubation with the hydroxylamine metabolite of dapsone, although there were some morphological changes observed with the cells at the highest concentration of dapsone hydroxylamine (100μM).

With the test involving the NG115-401L-C3 cell model, there was no significant changes in DNA synthesis in terms of [^{3}H] thymidine incorporation, after co-incubation with either phenytoin or cyclophosphamide (50μM) and the rat microsomal metabolite generating system.

In the one compartment erythrocyte studies, there were significant decreases in glutathione with cyclophosphamide (50μM) (0.44 ± 0.04 mM), sulphamethoxazole (50μM) (0.43 ± 0.08mM) and carbamazepine (50μM) (0.47 ± 0.034 mM), when co-incubated with the rat microsomal system, compared to the control (0.52 ± 0.07mM). There was no significant depletion in glutathione when the erythrocytes were co-incubated with phenytoin and the rat microsomal system. In the two compartment erythrocyte studies, there was a significant decrease in the erythrocyte glutathione with cyclophosphamide (50μM) (0.953 ± 0110mM) when co-incubated the rat microsomal system, compared to the control (1.124 ± 0.032mM). Differences were considered statistically significant for $p<0.05$, using the Student's two tailed 't' test with Bonferroni's correction. There was no significant depletion of glutathione with phenytoin, carbamazepine and sulphamethoxazole when co-incubated with the rat microsomal system, compared to the control.

Keywords : *In vitro*, cell lines, erythrocytes, metabolic activation, toxicity, transport systems, receptor mediated cell signalling, DNA synthesis, glutathione.

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ABBREVIATIONS

cAMP	Cyclic adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSO	Buthathionine sulfoxime
B.T.S	British Toxicology Society
CGRP	Calcitonin gene related protein
c.p.m.	Counts per minute
DEM	Diethyl maleate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
d.p.m.	Disintergrations per minute
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
FCS	Foetal calf serum
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane -sulphonic acid
HPLC	High performance liquid chromatography
IBMX	3-isobutyl-1-methyl xanthine
IP_3	Inositol trisphosphate
I.V.T.S.	In Vitro Toxicology Society
$NADP^+$	Nicotinamide adenine dinuceotide phosphate

NADPH	Reduced $NADP^+$
NEAA	Non essential amino acids
NEM	N-ethylmaleimide
p	Probability
P-450	cytochrome P-450 enzymes
PBS	Phosphate buffered saline
PBSG	Phosphate buffered saline with glucose (10mM)
r.p.m.	Revolutions per minute
S.D.	Standard deviation
TCA	Trichloroacetic acid
Tris	tris(hydroxymethyl)methylamine
uv	ultra violet
x	Mean

SECTION ONE

INTRODUCTION

1.1. FOREWORD

The use of animals to attain data in the name of science, has a history as long as science itself and considerable knowledge about biosystems has been derived via such usage. However, disatisfaction and debate about animal experimentation has increased steadily since the first documented cases in the mid 1800's. As Home Office figures show the medical and biological research or production procedures account for 91% of all animals used, considerable pressure has been exerted on the research establishments. These research groups have been publically pressured, by animal rights groups, to reduce or cease experiments and invest in alternative methods.

The great demands for advancement in medicine and drug therapies, has resulted in considerable achievements and greater scientific knowledge, but also increased pressure to further eradicate chronic or incurable illnesses. In addition, the very public disasters with the drugs thalidomide, benoxaprofen (Opren) and diethylstilboestrol, have lead to questions about the validity of animal testing. With thalidomide for example, its teratogenic effects were not initially seen in rats and other experimental animals, so the compound was considered suitable for use by pregnant women. However thalidomide did have serious effects when taken at a certain time during gestation and subsequent extensive research in rats, rabbits and monkeys confirmed these effects. Medical research groups and the pharmaceutical companies are constantly exhorted to discover new therapies quickly, but within the confines of the law and this conflicts with desired reductions in animal usage.

1.2. DRUG DEVELOPMENT AND ANIMAL TESTING

The passage of a new drug from initial concept to market is costly, time consuming and complex. It can take considerable time, from six to ten years and can cost in excess of 150 million pounds. The process has become even more difficult as novel drug entities become

more powerful, discrete and synthesized on the basis of structure-activity data. This prolongs drug research particularly with regards to drug safety, where considerable data must be provided for Legislatory committees such as the Committee for the Safety of Medicines, before a drug is referred for human trials and later before it is released on to the market.

Legislative committees in most countries require that a novel xenobiotic entity must be tested in two animal species, one rodent and one non rodent, prior to subsequent development and human studies. This will provide the basic pharmacological and toxicological data, used to assess the risk for human volunteers. However such legislation has not always been present and this was the case up until the 1970's, when testing procedures were reviewed after various incidents of serious drug toxicity. The most notably of these was the case of thalidomide in the 1960's, whose teratogenicity was not detected from the animal testing used and consequently lead to varying degrees of congenital limb defects in babies. These failures in predicting drug safety were one of several factors that have brought about a reduction in animal experiments.

Initially many experiments would be carried out, to produce the data required to fulfil legislative requirements. Now experiments are designed so that the data obtained from one procedure can fulfil several criteria at the same time, for example, an acute toxicity study at several dosages can also be used to give information on the metabolism and pharmacokinetics of the drug. This can reduce the overall number of studies performed and hence the number of animals. The costs involved in using animals, was another factor influencing reduction of their use. The requirements demanded by law, the Animals (Scientific Procedures) Act 1986 in the U.K., make the use of animals very expensive. Reduction of these overheads would significantly reduce the total research costs of a drug.

Data obtained from animal procedures, may not give a truly predictive scenario of toxicity of a drug when it is used by humans, due to species differences. This difference was clearly shown with the *in vivo* aromatic hydroxlation of aniline in females of various

species (Parke 1960). There are two routes of hydroxylation which produce the non carcinogenic 7-hydroxy metabolite or the carcinogenic N-hydroxy metabolite; monkeys and guinea pigs both produce the 7-hydroxy compound whereas humans, rats, rabbits, hamsters and dogs all produce the carcinogen. In addition, there may be differing dose responses, clearance times and overall response with a drug. Therefore predictive assessments have to be based on drug metabolism, pharmacokinetics and toxicity from several animal species. Even then, problems can still occur when testing in human volunteers, stopping development or even later resulting in product withdrawal.

The moral issue of man using animals in the name of science, has significantly reduced animal usage. For 150 years, there have been pressure groups publically denouncing experimentation and this has become more intense since the publication of 'Principles of humane experimental technique' in the early 1960"s. This highlighted concerns, described alternative experiments and advocated the implication of the three R's ; reduction, refinement and replacement. Public awareness has been further heightened, as a result of the actions of extreme animal rights groups against pharmaceutical companies, reasearch groups and their workers. Governments, particularly those in the European economic community, have further emphasized the need for reductions in animal numbers.

Reduction in animal usage continues, as further research into alternatives increases and with data from alternative procedures being considered along side animal data when preparing submissions to regulatory bodies.

1.3. ALTERNATIVES TO ANIMAL TESTING

The alternative techniques can be classified into one of several groups, that are listed overleaf.

1) Tissue culture.

2) Animal models.

3) *In vitro* techniques.

4) The use of physical and chemical methods
   (a) Computers.
   (b) Isotopes.
   (c) Refined analytical techniques.

5) Radioimmunoassays.

6) Plants and lower organisms.

There are many examples of the use of these techniques in research, some in combination with each other such as the Ames mutagenicity test (Ames *et al.* 1976 and Maron *et al.* 1983). This method involves the co-incubation of a microsomal *in vitro* system with the *Salmonella bacterium*. The use of refined analytical techniques is common place, often coupled with computers which manipulate the data produced in various software packages. The use of computers along with animal models in some areas, has almost replaced the use of animals in degree and other vocational courses. Many of the alternative techniques have not only reduced animal usage, but reduced total drug discovery time, as they become more sophisticated and efficient.

The use of tissue or cell culture and *in vitro* techniques, are alternatives of particular use in toxicology, as they have the capacity to show not only the metabolism of a potential toxic agent in animals, but also metabolism in humans.

1.4. TISSUE CULTURES

Tissue and cell culture was first used at the beginning of the century (Harrison 1907, Carrel 1912), to study animal cells free of systemic effects and initial work followed cell growth from primary explants of tissues, hence the term. The use of the technique has expanded rapidly since the 1950's, with the development being pushed by the demands of two research areas principally, cancer studies and virology. The term tissue culture encompasses two areas ; organ culture, where there is a three dimensional structure of non disaggregated cells and cell culture, which is dispersed cells obtained from an organ or tissue. Organ culture may retain some or all of the histological and metabolic features that the organ would possess *in vivo* and is widely used in pharmacology procedures. Cell culture comprises of four catagories ; freshly isolated cells of some tissue or organ, primary cultures, finite cell lines and continuous cell lines. Cells can be harvested using various disaggregation techniques which can be enzymatic, mechanical or chemical in nature. Virtually all types of cell can now be cultured, as knowledge of tissue and cell culture has increased; the major areas of interest are covered in Table 1.1. In *in vitro* toxicity testing, the majority of studies involve the use of cell cultures, although organ culture is used to a degree in *in vitro* metabolism as will be discussed later.

1.4.1. ADVANTAGES OF TISSUE CULTURE

The ability to control both the physiological and physiochemical environment of the culture is a major advantage. Maintenance of pH, temperature, osmotic pressure, O_2 and CO_2 tension can be precisely controlled and physiological conditions can be manipulated. The majority of cell lines require supplementation of the media to facilitate growth, usually with serum or other animal derived factors (Sato 1975). These are subject to variation between batch and often contain other substances such as hormones which can regulate cell processes. Many of the components of serum essential for cell growth have

being identified and the addition with these required elements, is resulting in serum free cell media (Barnes *et al.* 1987).

In samples taken from tissues, there are usually differences not only between individuals but also between samples from the same individual. After cell lines are sub-cultured once or twice, they take on a homogeneous or a uniform constitution (Augusti-Tocco and Sato 1962). The replicate samples produced after each subculture will be identical and so will reduce the need to use statistical analysis of variance with experimental data produced.

TABLE 1.1.

Major areas of interest in tissue culture

AREA	EXAMPLES
Intracellular activity	DNA transcription, protein synthesis, energy metabolism, drug metabolism.
Intracellular flux	RNA, hormones, metabolites, signal transduction, membrane trafficking.
Environmental interaction	Infection, drug action, ligand receptor interactions, carcinogenisis.
Cell-cell interaction	Embryonic induction, metabolic cooperation, cell proliferation, contact inhibition/density limitation of growth, paracrine control of growth and differentiation, matrix interaction, invasion.
Cell products	Product formation, exocytosis.
Genetics	Genetic analysis, genetic manipulation/intervention, transformation, immortalization.

(Information taken from Culture of animal cells, R. I. Freshney)

The characteristics of the cell line can be maintained by storing culture samples in liquid nitrogen and this also means different groups of workers can use the same cell line (Hay 1992). This is important in terms of validation of assays used in toxicity testing, as commonality must be maintained between research groups, for valid results.

There is the advantage of availability of cell culture when using continuous cell lines, as the constant proliferation results in plenty of material. This is of importance with assays, as it allows greater numbers of experiments, which are more statistically valid and for demonstrating the effects of a range of concentrations and conditions.

Tissue culture can be very economical for studies with reagents that are expensive or limited in amount. This is because less reagent is required for direct exposure in an *in vitro* system, compared to an *in vivo* injection where 90% of the compound will be lost due to excretion and tissue distribution. The compound and/or its metabolites can be selectively targetted at cell lines derived from various tissues, for example liver and kidney, to show specific tissue effects. In addition, any replicate and multivariable tests can be cheaper to screen than the animal equivalent.

Finally cultures can be derived from a unique tissue origin, which could be of particularly importance in drug safety testing. It is also possible to isolate cells from normal, diseased or dysfunctional tissues or organs (Gey and Gey 1936) for example, intestinal cells and intestinal carcinoma cells, to show any differences in the responses of the cells to a compound. This could be of use prior to clinical trials, as it could identify any possible risks in patients with particular conditions, for example, the elderly with impaired renal function.

1.4.2. DISADVANTAGES OF TISSUE CULTURE

The culturing of cells must be carried out aseptically, as contamination with bacteria, moulds or yeasts affects cell growth. In addition, the maintenance of the environment in which the cells are grown requires a level of expertise to mimic the conditions normally present in the complex environment of a multicellular animal. Using cell culture requires skill and understanding but also the employment of standard protocols, to ensure consistency in research work. This is not always seen to be the case and differences arise due to different techniques used by workers. This is a major problem in terms of validation as a test could be less robust due to these differences.

Producing the quantities of culture material required for test systems can also be a problematic. It is possible to produce an infinite amount of cells in culture but this is expensive in time, materials, costs and manpower. It is possible to reduce the need for large amounts of cells by scaling down the size of the assay, for example the use of microtitre plates. This reduces manipulation time and material costs and can allow the use of automated analysis techniques.

When the use of cell culture was advancing in the 1950's, it was noticed that there was irreversible loss of phenotypic characteristics which were typical of the original tissue. This has been extensively reported particularly with hepatocytes, in respect to the reduction or loss of specific liver functions (Sirica *et al.* 1980, Bissell and Guzelian 1980, Guegen-Guillouzo *et al.* 1983). This has been attributed to the selection or overgrowth of undifferentiated cells, for example, stromal cells and de-adaptation due to the absence of specific cell inducers. The loss of such properties results in difficulties in relating the cell line to the original tissue and is particularly important when these properties are used as markers of cell function, for example, alkaline phosphatase activity in epithelial cells. It is possible to reduce the loss of unique characteristics either by isolating the relevant cell line using selective serum free media, or by

maintaining the cells under defined culture conditions which may restore the lost characteristics (Hayashi and Sato 1976).

Loss of phenotype can also result for another reason, which is the instability of the cell line. This can be for one of two reasons, depending on the cell line type. First, with cultures of a heterogeneous nature, different rates of growth of cells and the absence of growth factors, can lead to instability between sub culturing and so differentiation within the culture (Freshney 1995, McKeenan *et al.* 1990). However, the major source of instability results from genetic changes in the cell line (Reed *et al.* 1986). Table 1.2. shows characteristics of the different types of cell lines and it shows that the more proliferative a cell line, the more unstable its chromosomal constitution. The cell lines with extended lives have aneuploid karotype, that is the cell nucleus does not contain the exact haploid number of chromosomes. This karotype is unstable as there may be chromosomal rearrangement and changes in the phenotypic properties of the cell line. This instability can be employed to initiate cell lines with changes in specific properties (Sato *et al.* 1970, Sanford *et al.* 1954), but it is generally a disadvantage, particularly when using the cell line in compound testing. To counteract problems associated with instability, samples of cell lines can be taken in the initial stages, mixed with a media/cryoprotectent solution and frozen by slow temperature reduction to -90 degrees centigrade (Hay 1992). Also the mixing of different stocks of cell cultures with the same line and passage, can overcome such problems.

The final disadvantage of using cell culture, is that it does not reflect the response seen in the multicellular system and so is not truly representative of the *in vivo* cell response. The toxin concentrations may not be uniform, as the concentrations studied *in vitro* may not be attainable in the *in vivo* system. The lack of cell-cell interactions and the systemic components of homeostatic regulation *in vivo*, such as hormones, affect cellular responses. In terms of toxicity testing, the disadvantage is clearly demonstrated, as a compound's toxicity depends partly on the adsorption, distribution, metabolism and excretion *in vivo* (Bedford 1995). An *in vitro* culture system may indicate toxicity

which is not reproduced *in vivo*. This is because the toxic compounds normally metabolised *in vivo* , may not be metabolised in the cell system and so cytotoxicity may be seen. Absorption *in vivo* may be slow, possibly due to tissue and protein binding, leading to low levels of the substance and no toxicity, Metabolism can convert the substance to a non toxic form or a metabolite which is more rapidly excreted, so reducing toxicity (Timbrell 1995).

TABLE 1.2.

Characteristics of different cell line types.

TYPE OF CELL LINE	CHARACTERISTICS
Freshly isolated cells	Cell life of hours, excellent retention of functions, no proliferation, diploid chromosome constitution.
Primary cell culture	Cell life of days, excellent or good retention of functions, limited or no proliferation, diploid chromosome constitution.
Finite cell lines	Cell life of months, variable retention of functions, limited proliferation, diploid or aneuploid chromosome constitution.
Continuous cell lines	Immortal, retain limited functions, unlimited proliferation, aneuploid chromosome constitution.

(Adapted from Reed *et al.* 1986)

The problems associated with cell culture can determine the manner in which it is used and this is particularly the case in toxicological testing. Toxicity tests require that data produced will correlate with *in vivo* data and give some idea of changes in cell functions. Therefore the cell system should use freshly isolated cells or primary cultures, which are more stable and retain tissue functions. But such cultures are labour intensive and more heterogeneous, so reducing reproducibility and increasing susceptibility to infections, as the source cannot be rendered totally sterile. The cells have a finite life

span and moreover do not show significant growth. Therefore, large numbers of animals could be required for multi drug testing, which obviously defeats the original purposes. Continuous cell cultures however, are less labour intensive, more homogeneous and can be obtained in a sterile condition, so maintaining asepsis in testing. Continuous growth allows an infinite number of experiments to be performed and deep freezing of samples means that studies can be repeated later with the same cell line. Most importantly, continuous cell lines do not need to be cultured from the beginning, as samples can be obtained from various sources, for example the American Type Culture Collection. However, these cells often do not compare to *in vivo* cells, with loss of functions, genetic instability and growth only being achieved by chemical or viral induction. Even cell line derived from a tissue neoplasm are not representative, regardless of retaining tissue phenotype and this is demonstrated by the work of many groups for example, resistance to oxidative cytolysis (O'Donnell-Tormey *et al.* 1985).

1.5. THE CELL - ORGANISATION AND FUNCTIONS

There are many types of cell in each organ or tissue, each type with its own characteristic functions which are closely adapted to their role in the organ. There are however, a number of common cell components which are described in Table 1.3. and common cell processes. These processes are of particular importance in understanding toxicity in the cell.

One common feature in cells, is that they will all grow and divide, though in a mature organism this should only be to replace old cells. With the exception of nerve and red blood cells, all cells can be stimulated to grow and form two new, identical daughter cell. The growth cycle begins with the G_1 (gap 1) phase, after which DNA replication is initiated and continues until two copies of the DNA exist. This period is known as S (synthetic) phase and is followed by another gap phase, G2. This is required before the cell can divide into the daughter cells, the period which is called the M or mitotic phase

(Stryer 1988). During growth, cells exhibit cellular activities which are absent in resting cells, the most apparent being the enhanced production of deoxynucleoside triphosphates for DNA synthesis.

DNA replication involves three phases; initiation, DNA chain synthesis and termination, with the synthesis phase being critical for mutagenic effects. Synthesis in mammals involves three different polymerases; α which is found in the nucleus, β present in the nucleus and cytoplasm and γ which is located principally in the mitochondria. α polymerase is the most important enzyme in DNA replication, and β polymerase is thought to be involved in DNA repair (Lindahl 1982). Inactivation of these enzymes can result in mutations, in addition to the direct action of compounds on DNA molecules. Mutations can result in alterations in the DNA, which results in the inability of the cell to synthesize vital proteins for example, the action of the DNA alkylating agent cyclophosphamide. The occurence of mutations is also considered to be the initial step in the process leading to the development of neoplasms or cancer. So some genotoxic agents may not be cytotoxic or cytostatic, but will increase cell growth.

Regulation of the intracellular environment is another common feature in cells and may be important in toxicity. There are cellular mechanisms, which can compensate for changes within the cell and within limits, the extracellular environment should not affect normal cell activity. Metabolic pathways in the cell can be regulated by feedback inhibition, which is where the concentration of the metabolic end product will regulate the activity of the initial enzyme of the pathway (Gerhart 1970). The passive diffusion of ions across the cell membrane changes the intracellular ionic content, which can influence water movement into the cell and so lead to expansion. If this is allowed to proceed, then cell lysis may occur, but there are mechanisms which can compensate for changes in ion concentration, for example, Na^+/K^+ ATP dependent pumps and Ca^{2+}ATPase which transports Ca^{2+}. The signal transduction pathways of the cell also influence cell regulation and this has been well documented, particularly with calcium ion homeostasis. The binding of neuropeptides such as bradykinin to their receptors, can

indirectly initiate the stimulation of inositol phospholipids production or the activation or inhibition of adenylate cyclase activity. The oxidative stress initiated by reactive metabolites, can affect all these regulation processes. It can inhibit the activity of enzymes and /or alter the oxidation states of the proteins. The depletion of the cytoprotectant glutathione due to oxidative stress has been reported to inhibit the phosphoinositide signal transduction pathway in cells (Orrenius *et al.* 1983). This results in an initial sharp elevation in intracellular calcium, followed by a decrease to a sustained, elevated level. If the membrane proteins responsible for calcium extrusion are inhibited or inoperative due to damage, this increase in calcium will lead to the activation of a variety of intracellular hydrolytic enzymes.

The production of energy is another common feature in cells and is required for movement, the active transport of molecules and for the synthesis of biomolecules such as protein. Adenosine triphosphate (ATP) is the central carrier of free energy and the energy is contained in the phosphoanhydride bonds. Oxidative phosphorylation is the process by which ATP is formed and this takes place in the mitochondria of cells. Therefore any changes in mitochondrial structure and functioning, will be reflected in the energy status of a cell (Erecinska and Wilson 1978).

All cells contain plasma membranes and usually membranes around the cell organelles, which are not connected to the plasma membrane, for example the mitochondria. With the organelles the membranes compartmentalise certain enzymes, which allows the concentration of the metabolites produced by the enzymic reactions and this results in increased enzymic rates. Toxicity can have a great impact on membranes, as well as the cell and its processes. Lipid peroxidation, which is the oxidative breakdown of polyunsaturated fatty acids by free radicals, can also compromise membrane integrity. This can cause an increase in membrane permeability (Richter 1987) and an increase in ionic and water movement. Also the membranes of the lysosomes in cells may degenerate due to lipid peroxidation, leading to lysosomal digestion of the cell contents.

TABLE 1.3.

The components of a typical cell and their functions.

COMPONENT	FUNCTIONS
Plasma membrane	Defines boundaries of cell and ensures retention of contents; Phospholipid bilayer serves as permeability barrier to most water soluble compounds; Integral proteins facing either side of membrane or traversing membrane may function as receptors, transport proteins or have enzymatic activity.
Nucleus	Controls cellular activities; produces all RNA and most protein molecules; Ribosomal RNA produced by nucleoli present in nucleus; Constructed of two membranes with numerous pores, through which ribosomes, mRNA, chromasomal proteins and enzymes are translocated.
Endoplasmic reticulum (ER)	Interconnected tubular membranes and cisternae that play a role in cellular transport; ribosomes when attached, produce proteins which are exported and glycosylated in ER lumen (rough ER); smooth ER synthesizes lipids and is responsible for cell detoxification
Golgi complex	Involved in protein sectretion with ER; synthesizes complex polysaccharides to modify glycoproteins.
Lysosomes	Vesicles containing inactive hydrolytic enzymes (primary lysosomes); Fuse with membrane vacuole to digest proteins, carbohydrates or lipids (secondary lysosomes).
Mitochondrion	Produces ATP and reduced co-enzymes via metabolism of sugars in citric acid cycle and catabolism by β oxidation of fatty acids.; ATP generated from energy release from oxidation of co-enymes by enyzme complexes.
Ribosomes	Responsible for protein synthesis by copying mRNA; mRNA transcribed from genes in nuclear DNA attaches to ribosomes; mRNA accepts tRNA with amino acid, according to base pairing rules; amino acid covalently attached to peptide chain.
Cytoplasm	Comprises of water and protein fluid through which cytoskeleton intracellular framework permeates; gives cells structural and functional organisation; structural elements are microtubules, microfilaments and intermediate filaments.

Finally, all cells must take in polysaccharides, lipids and amino acids in order survive and grow. The uptake of these basic units is via membrane proteins, that form a channel across the cell membrane. These transport systems are often active, requiring energy in order to facilitate uptake. Therefore, there are two areas were a toxic compound could have an effect on transport systems; by disrupting the energy supply or by inhibiting and/or changing the oxidation state of the protein structure (Roubel and Tappel 1966). Figure 1.1. shows the hypothetical sequence of events resultant from a cytotoxic insult. There is disruption of cell functions and if the cell injury is too severe, cell death will occur.

1.6. BIOTRANSFORMATION OF COMPOUNDS.

A toxin may enter a living system through a variety of routes. Absorption, coupled with various external and internal factors, determines the rate at which a compound is absorbed *in vivo*. However, when in the system, these compounds are usually biotransformed from a lipophilic, membrane penetrating species, to a water soluble, excretable metabolite. The different routes of entry of compound may lead to different biotransformation profiles due to many factors; for example, physiological nature of entry site, the pH and the biotransformation enzymes present. Principally, the organ the compound was distributed to, the total amount of the compound absorbed and the presence of any other compounds, will determine the metabolic profile seen.

The biotransformation of a compound, usually reduces the time it spends in the living system, as the process decreases lipophilicity, increases excretion and so decreases the compounds half life. Biotransformation may convert an initially toxic compound into a less toxic or completely non-toxic metabolite. However, it is often the case that increasing the polarity of a compound results in the production of reactive intermediates or metabolites, which are often more toxic (Figure 1.2.).

FIGURE 1.1.

Hypothetical sequence of events leading to a necrotic cell following cell injury.

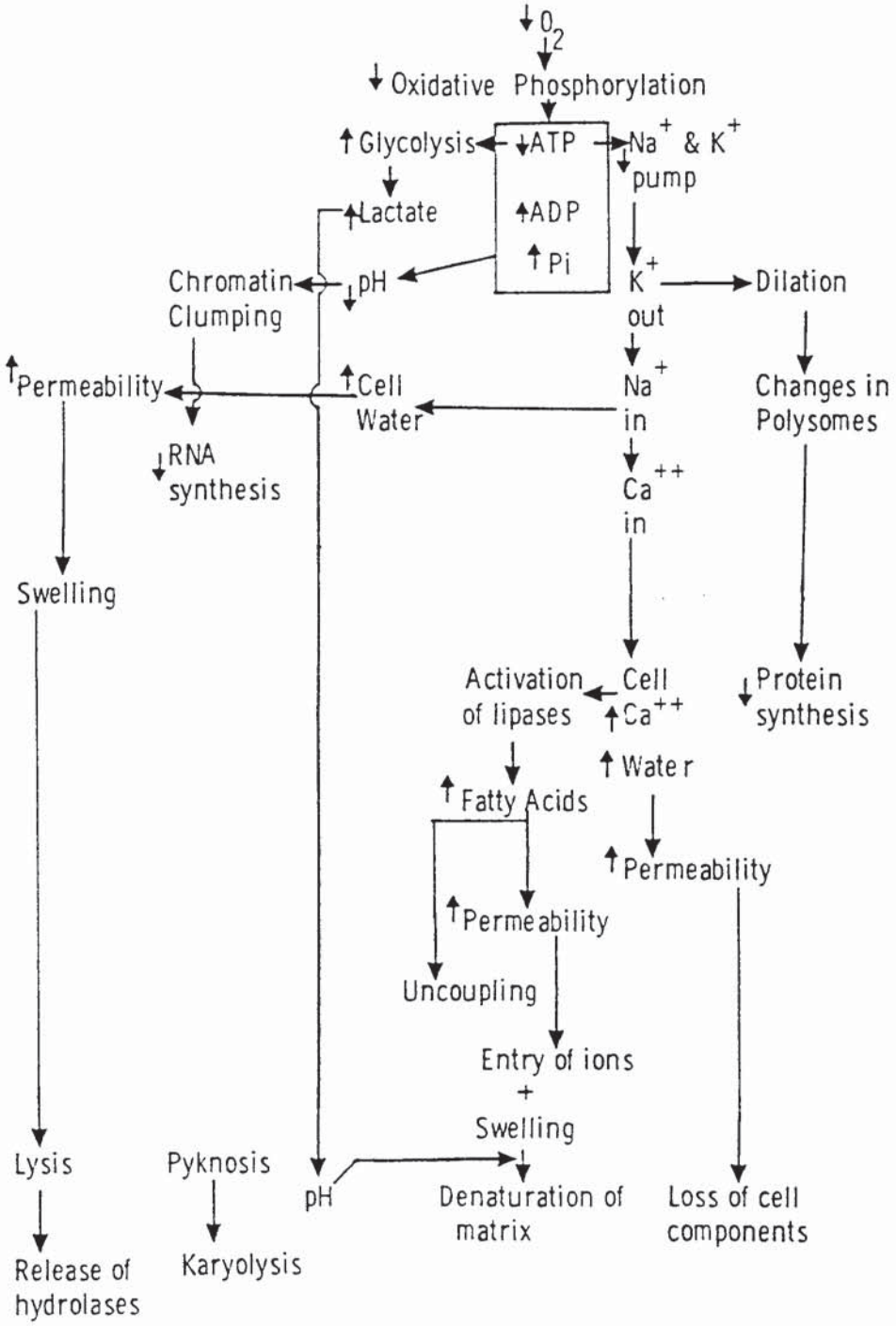
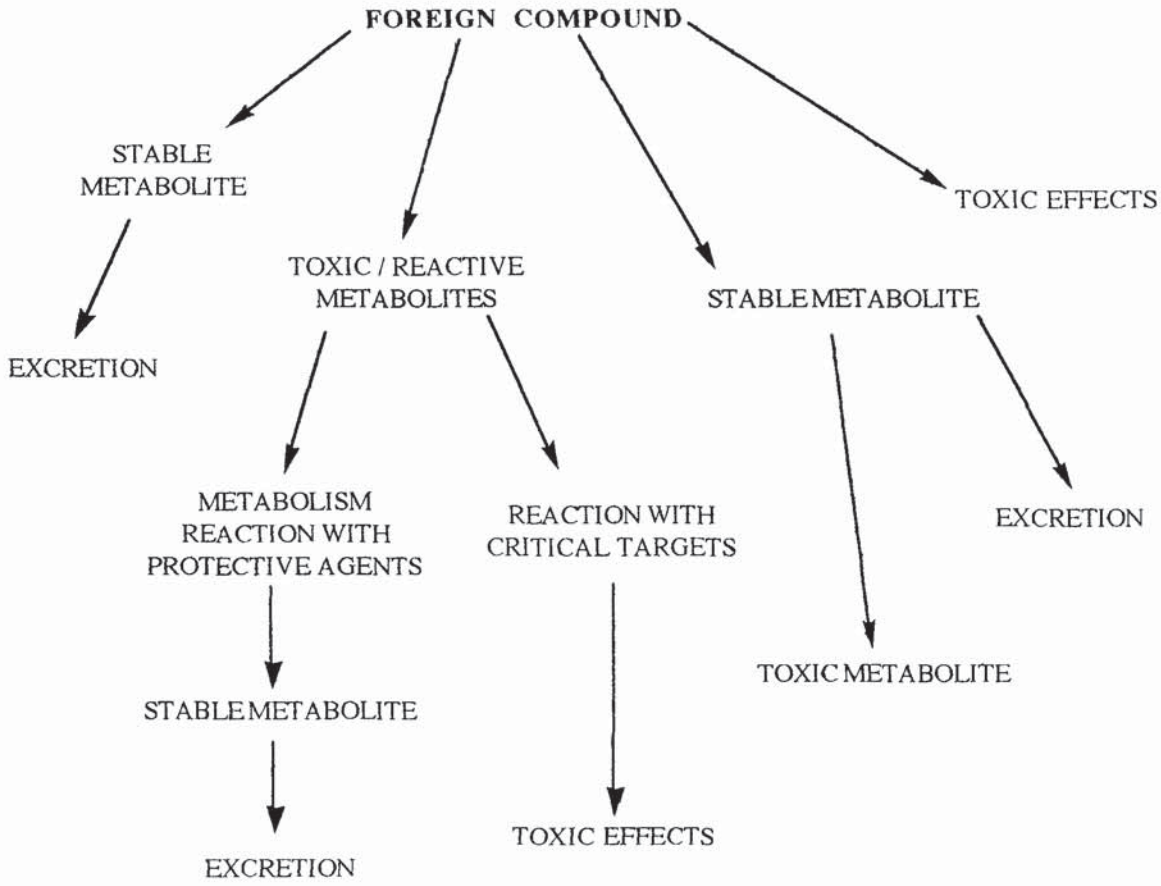


FIGURE 1.2.

The significance of metabolism for toxicity



(Adapted from Timbrell 1995)

The biotransformation of compounds is catalysed out by a wide array of enzymes and the chemical structure of a compound and/or its metabolites, to some extent determines the enzymes involved. Biotransformation is normally considered as two phases; the first, Phase I, is were the compound is modified by the conversion or addition of a group, to give a more polar molecule; these reactions are primarily oxidative. The second stage, Phase II, is generally where reactive metabolites are enzymatically conjugated with a highly water soluble substrate. This will give a polar compound, which is readily excretable from the body. Phase II biotransformation, particularly glucuronidation, can also occur directly with drugs for example, with dapsone or indomethacin.

As toxicity is frequently a result of the action of the reactive Phase I metabolites, several toxicity tests concentrate on this area. Many *in vitro* toxicity tests currently in use in drug safety research, rely on metabolism systems that involve the use of the post mitochondrial and microsomal fractions. These fractions contain the enzymes predominantly involved in the Phase I reactions and when used, should produce the highly reactive and cytotoxic metabolites.

1.6.1. PHASE I REACTIONS.

There are several types of reactions which can take place in Phase I metabolism , though the major reaction seen is oxidation.

The oxidation reactions are catalysed principally by the microsomal mono oxygenase enzymes in the smooth endoplasmic reticulum, although other oxidative enzymes are found in the mitochondrial or cytosolic fraction of the cell. The microsomal mono oxygenase enzymes can be classified in two ways, those containing FAD and those which are cytochrome P-450 dependent. Both types of enzymes can be found in various organs, but the P-450 dependant enzymes carry out the majority of the metabolic reactions. As the P-450 enzymes carry out the majority of reactions, most metabolising systems used

in *in vitro* tests tend to optimise the conditions and co factors, in favour of the cytochrome P-450 oxidation routes. This optimisation is easier for P-450 systems as the reactions all require oxygen, available from the air and the common co-factor NADPH, which can be added to the system as required.

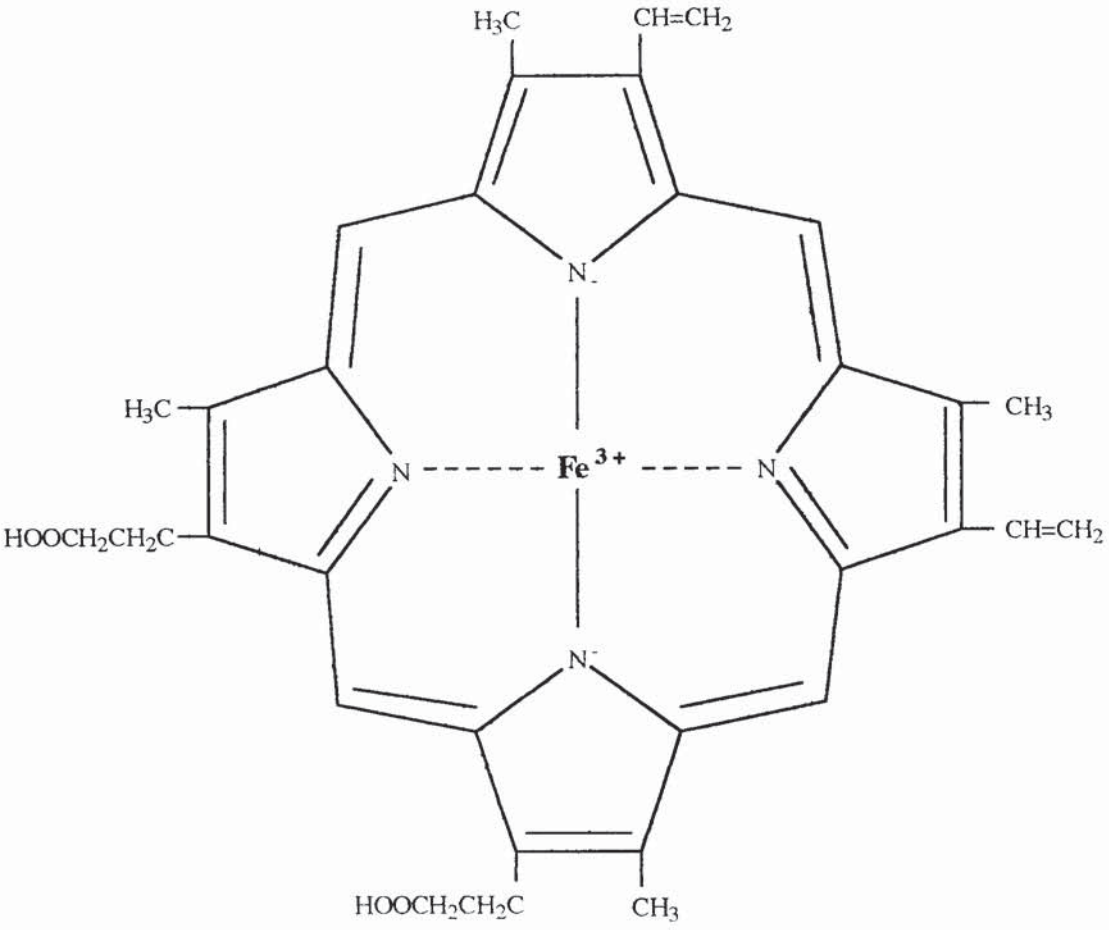
1.6.2. THE CYTOCHROME P-450 MONO OXYGENASE ENZYMES.

The cytochrome P-450 dependent enzymes are responsible for the majority of mono oxygenase, or mixed function oxidation reactions, so consequently are the most studied (Omura and Sato 1962). Cytochrome P-450's are haemoproteins (Sato *et al.* 1965) and are responsible for the terminal oxidase component in the electron carrier system of mono oxygenase reactions. The term cytochrome P-450 comes from the spectral absorbance maxima of 450nm exhibited when the cytochrome is reduced and complexed with carbon monoxide (Omura and Sato 1962). The active site of the haemoprotein contains iron protoporphyrin IX, which is bound in the molecule partly by hydrophobic forces (Figure 1.2.). The fifth ligand of this complex is a thiolate anion, provide by a cysteine residue and the sixth position may be occupied by an exchangable water molecule. When the central iron is reduced, oxygen can be bound in the sixth position (White and Coon 1982). The haem group is non covalently bound to the apoprotein, which confers the substrate specificity for each enzyme, by having a different structure. The P-450 family contains many different isozymes and more than 150 isoforms have been characterised (Porter and Coon 1991). The enzymes exhibit many differences, for example in molecular weight or amino acid composition and can show different substrate specificities. However, the mechanisms by which the P-450 catalysis the activation of oxygen and the subsequent oxidation of a substrate molecule appear very similar.

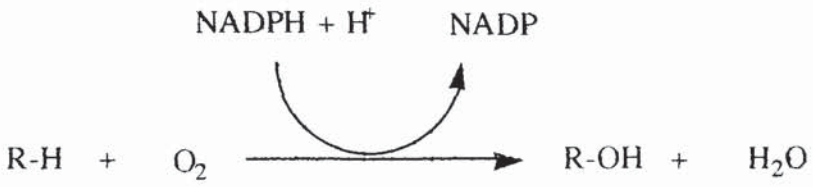
The haemoprotein serves as the substrate and oxygen locus in the mono oxygenase reaction, which can be empirically shown as

FIGURE 1.3.

Iron protoporphyrin IX



Adapted from Gibson and Skett (1986)



where one atom from a molecule of oxygen is incorporated into the compound (R-H), with the other being reduced to water. The mechanism of the cytochrome P-450 function has not been completely elucidated, but there is a generally recognised series of steps which are considered to take place (Figure 1.4.)

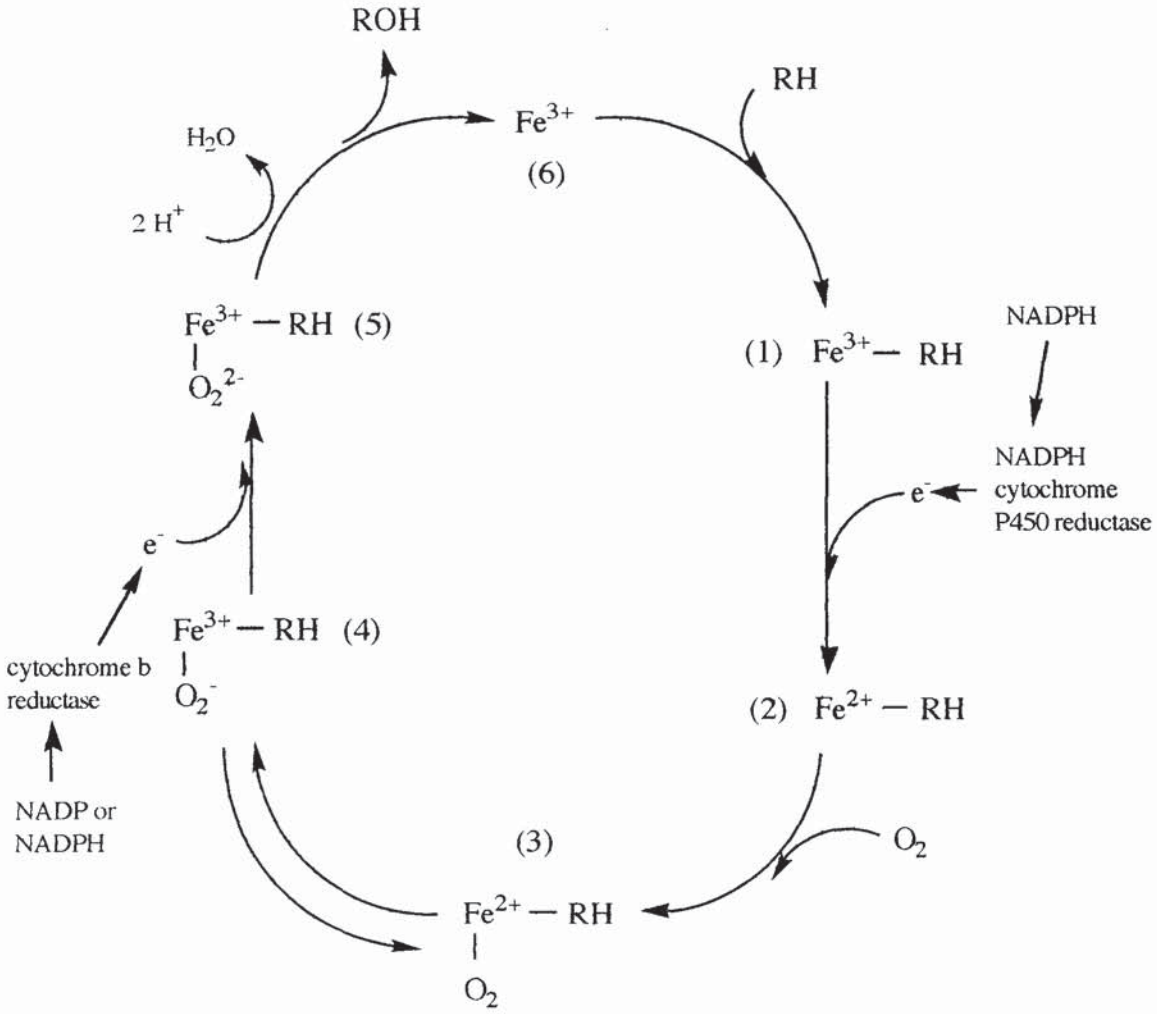
The substrate (RH), intially binds to the iron protophyrin IX area of the cytochrome P-450 (1) and there is an electron transfer to the compound, catalysed by NADPH reductase, giving a reduced cytochrome-substrate complex (2). Molecular oxygen is then incorporated into the complex, to give a ternary oxygenated complex (3 and 4). There is a second electron transfer to the ternary compound, which produces an iron peroxo species (5). This second electron may come from NADH of NADPH through cytochrome b reductase, but this has not be clearly established yet. There is cleavage of the oxygen-oxygen bond, which leaves an oxidized cytochrome-substrate complex and an atom of oxygen, which combines with two protons to give water. The next step, results in a two electron oxidation of the substrate, with the remaining oxygen is inserted into the substrate. The substrate is released and the ferric binding site is regenerated, for another catalytic cycle (6)(Gibson and Skett 1986).

1.6.3. PHASE II REACTIONS.

Phase II or conjugation reactions, involve a diverse group of enzymes, that conjugate compounds and/or Phase I metabolites with polar groups, generally producing a more hydrophilic species.

FIGURE 1.4.

The catalytic cycle of cytochrome P-450



Adapted from Gibson and Skett (1986).

These hydrophilic metabolites will then be excreted more readily in the bile or in urine. There are several types of conjugation reactions (Table 1.4.), but glutathione conjugation is the most important in terms of cytotoxicity.

TABLE 1.4.

Phase II conjugation reactions

REACTION	EXAMPLES OF COMPOUNDS INVOLVED
Sulphation	Steroids, heparin
Glucuronidation	Thyroxine, steroids
Glutathione conjugation	Paracetamol, naphthalene
Acetylation	Sulphanilamide, serotonin
Amino acid conjugation	Bile acids
Methylation	Noradrenaline, histamine

Glutathione is one of the most important factors in the cell's defence against oxidative stress. It can be conjugated with a wide array of reactive metabolites and is one of the systems involved in scavaging the free radicals generated by cell metabolism and exogenous biotransformation processes. Depletion of glutathione is known to be a critical factor in cytotoxicity, with its loss rapidly resulting in cell death (Orrenius 1985).

Inhibition of the enzymes involved in glutathione reduction, accelerates the disruption of intracellular calcium homeostasis, after the loss of signal transduction (Orrenius *et al.* 1983). In addition, complete loss of glutathione results in the initiation of lipid

peroxidation, which has profound effects on the cell plasma membrane (Richter 1987), organelle membranes, the cytoskeleton (Bellomo *et al.* 1988) and membrane and cellular proteins (Roubel and Tappel 1966). Therefore, glutathione and the enzymes involved in its production and maintenance, are frequently used as markers in evaluating toxicity towards cells.

1.7. *IN VITRO* BIOTRANSFORMATION SYSTEMS.

There are several types of biotransformation systems which can be employed in *in vitro* toxicity tests, each with their own advantages and disadvantages and they can be classed as either exogenous or endogenous.

Most exogenous systems used are derived from liver tissue, as this has the highest concentration and variety of the different metabolic enzymes, among metabolically active tissues. Ideally the systems should give the complete metabolic profile of a compound that would be seen in the human *in vivo* situation. However, in practice this cannot be achieved as human liver tissue is difficult to obtain, so rodent liver tissue is most often used. In addition, it is generally only possible to optimise incubation conditions for one type of metabolic route, usually oxidation. This may be inappropriate, especially when testing compounds with an unknown metabolic profile.

The types of exogenous supplement are :

(1) Whole liver homogenate.

(2) 9,000g supernatant - S9 or post mitochondrial supernatant.

(3) 15,000g supernatant - S15.

(4) 104,000g supernatant - microsomal fraction.

(5) Intact cells - usually hepatocytes.

(6) Precision cut liver slices.

(Benford 1995)

The liver homogenate and its supernatants, are the most commonly used supplements as they are easy to prepare and in the case of the supernatants, concentrate the Phase I cytochrome P-450 enzymes. They can be co-incubated with other cells (Shear and Spielberg 1985, Coleman *et al.* 1989) and the metabolites produced can exert a toxic effect on the cells. In addition, much of the Phase I activity can be retained with careful handling and preparation of the fraction. However, there are several disadvantages with these systems, which limit some *in vitro* tests. All of the fractions retain their enzymic activity for only a limited period of time, which makes them unsuitable for long incubation periods. Liver slices retain enzymic activity for approximately 24 hours, before rapid losses in enzyme function. Hepatocytes generally have a lifetime of 5 hours, before significant loss of activity and then liver homogenate and fractions, which have the shortest time of about two hours (Bedford 1995, Powis 1989, Strom *et al.* 1982) Liver slices and hepatocytes retain activity for a longer time period due to retention of cell structure, but they are more difficult to produce initially and so take more time to prepare. In addition, liver slices and hepatocytes have to be used on the day of preparation, as they cannot be preserved without loss of activity (Coundouris *et al.* 1993, Wishnies *et al.* 1991). Finally, all the exogenous supplements will exhibit heterogenicity in terms of amounts of the individual enzymes, which may be reflected in the range and amount of the metabolites produced. This may have an impact on the toxicity seen and the degree of damage caused in the cell.

The advent of genetic transcription has addressed one of the problems seen in using exogenous systems, that of known and reproducible metabolic profiling. Yeast cells with the genetic material for individual cytochrome P-450 enzymes can be grown and harvested to produce microsomal fractions expressing the particular P-450 enzyme. (Kelly 1994). Combinations of microsomal fractions can then be incubated in the same

manner as liver fractions, producing a compound's metabolic profile from those enzymes. However, as with microsomal liver fractions, this system only has a short life span and so cannot be used for long term incubations.

Endogenous systems, are generally primary cell cultures derived from metabolically active organs, such as the liver and kidney, or immortal cell lines, for example, the HepG2 human hepatoma cell line or LLC PK_1 kidney cell line (McDonald *et al.* 1994, Mertens *et al.* 1987. These cells can be grown alone or can be co-cultured with other cell lines to provide the metabolising component in a test. These cell lines have a virtual indefinate life span and the primary cultures can survive up to several weeks, without changes in the enzyme activity. In addition, the immortal cell cultures are homogeneous in nature, so consistent responses will be obtained with tests using these systems. These endogenous systems are therefore the system of choice for long term tests, however like the exogenous systems, there are several disadvantages.

Both immortal and primary cultures of cells, require a degree of expert knowledge and can be difficult to initiate and maintain. Primary cultures may not be homogeneous, so this may affect the metabolism of a compound and the response seen. Both primary and immortal cell lines will undergo or have already lost a number of the organ specific characteristics and this loss includes the drug metabolizing enzymes (Stewart *et al.* 1984). This loss can be addressed by alterations in media, substrate or by differentation of the cells by addition of a differentation inducing agent, for example dimethylsulphoxide. In addition, the co-culturing with other cells can minimize loss of functions for example, liver hepatocytes with liver epithelial cells (Begue *et al.* 1984). The eternal cell lines can lose aspects of their original phenotypic nature as time progresses, due to the unstable genotype and this too may lead to a loss in the biotransformation enzymatic activity. This can only be remedied by maintaining stocks of the cell or by mixing old and new cells, to maintain culture activity.

The problem of loss of enzyme activity has been addressed, again by the use of genetic transcription into cell lines (Gibson *et al.* 1995). The gene for the enzyme is introduced into the cell line as a plasmid or expression vector. The cell line is transformed and when the complementary DNA is incorporated into the genetic material of the cell, the cell expresses the relevant enzyme. These transgenic cells can then be used in co-incubation with other cell lines or may themselves be the target for the metabolites that they produce.

Currently the production of enzymes in transgenic cell lines, as with the yeast and bacterial transgenic cells, is transient due to instability in the cell's genetic material. However, it is hoped that the use of retroviruses on β lymphoblastoid cells, will enable a stable cell line that still expresses P-450 enzymes, to be produced.

1.8. *IN VITRO* TOXICITY TESTS.

In vitro toxicity tests have been of practical use for some years and are now accepted tools in the study of drug safety. The early literature reviews, proposed the use of tissues from animals and humans and the comparison of the responses elicited with a known toxic compound, with the *in vivo* response. The fields of genotoxicity and carcinogenicity produced the greatest number of tests initially, although tests for other areas were rapidly introduced.

Increasingly, the major role of metabolism in toxicity has been recognised. This factor was recognised in *in vitro* testing, with the inclusion of a metabolically active component in the *in vitro* tests. The *Salmonella* /microsome mutagenicity test (Ames *et al.* 1976, Maron and Ames 1983) has been the blueprint for most systems, with the target cells being co-incubated with the metabolising component. In the Ames test, this metabolism component is provided by S9 or microsomal liver fractions, although other tests use different metabolic systems such as hepatocytes (Acosta and Mitchell 1981).

In vitro tests have also been devised with a greater emphasis on the human tissue response, by combining human 'target' cells such as lymphocytes, with a metabolising system (Spielberg 1984, Shear and Spielberg 1985) or human epithelial cells with hepatocytes (Begue *et al.* 1984). Here, the viability of the lymphocytes is assessed by dye exclusion and cell death indicates the degree of toxicity. The 'mixed' *in vitro* tests, that is where target cells and the metabolite generating system are together in the same test tube, are of particular use when studying toxicity resulting from the action of short lived, reactive metabolites. Such metabolites and their intermediates are considered to be the agents responsible for the localised hepatotoxicity of drugs. Phenytoin for example, is documented as inducing hepatotoxicity, which is thought to be a result of a reactive arene oxide metabolite (Spielberg *et al.* 1981). Benzo[a]pyrene is also initially oxidized to a reactive epoxide and an *in vitro* toxicity test system, has shown the metabolite's effect on bacterial target cells (Glatt and Oesch 1977). In this system, the metabolite diffuses directly from the microsomes to the bacterial 'target' cells and not through the hydrophilic media.

This mixed system is unsuitable for assessing the ability of a metabolite to exert its effect at some distance from the site of formation. Several studies have attempted to assess this metabolite movement, using hepatocytes as the metabolising component. These include the use of glutathione transferase B to trap bromobenzene metabolites (Monks *et al.* 1984) and exogenous DNA to trap methylating metabolites of dimethylnitrosamine (Umbenauer and Pegg 1981). A multi-compartmental method has been developed which allows the study of metabolite movement and is based on the method devised by Spielberg (1984). In this test the target cells for example, lymphocytes or erythrocytes, are physically separated from the the metabolite activating system by a semi permeable membrane. The metabolites produced by the activating system must pass through the membrane to exert an effect on the target cells. This is similar to the process seen in the body, where metabolites must cross the cell membrane of the hepatocytes, before being transported away in the blood. This test system has been used in the study of dapsone and its

haematologically toxic N-hydroxylamine metabolite (Riley *et al.* 1990, Tingle *et al.* 1990, 1991). *In vivo*, the metabolite, which is not hepatotoxic, escapes from the liver in sufficient quantities to cause extensive haematological toxicity, particularly methaemoglobinemia. The two compartment system has been demonstrated to mimic this process and has been the basis of several human studies aimed at reducing dapsone toxicity (Coleman and Tingle 1992).

With these test systems, the metabolite toxicity can be studied as if the metabolite were at the site of biotransformation or in another compartment of the body. However, the Spielberg mixed test and the later two compartment test, show several of the negative aspects that can be seen in many of the current *in vitro* cytotoxicity tests.

The Spielberg mixed test uses freshly isolated peripheral mononucleocytes, because these demonstrate the genetic cytotoxic susceptibility, inherent in individuals or family groups, to certain drug therapies. However, freshly isolated cells are heterogeneous in nature, which leads to variable results and is a disadvantage for the system's further development as a standard cytotoxicity test. Mononucleocytes are also 'victim' cells, that is they do not have an extensively developed cellular defence system and so are more vunerable to toxic insult than for example, hepatocytes or gut epithelial cells. In addition, the actual experimental process of the isolation of these cells from whole blood, can affect the resistance of mononucleocytes to a cytotoxic agent. Different workers can produce different results, due to their methods used in the isolation of the cells and this can result in false positives particularily if the cells are damaged during isolation. Different workers can also introduce variability into results due to their cell counting techniques, particularly as the Spielberg and two compartment tests rely on dye exclusion in cells, to indicate cell death. Some workers may have difficulty in recognising non viable cells that have minimal dye inclusion. The neutral red assay (Parish and Mullbacher 1983) could be used, as the neutral red is only taken up by viable cells. Therefore any non viable cells will appear colourless within a red environment and be easier to identify and count.

Another negative aspect are the methods employed at present to determine cytotoxicity. There is considerable knowledge about the metabolism of a compound due to comprehensive analysis by HPLC and mass spectrometry or the analysis of metabolites conjugated with glucuronides, glutathione or activated sulphate. However, much is unclear about the mechanisms by which these metabolites exert their effect on cell functions and to what degree the damage will affect cell viability. There are a number of indices that are used in the evaluation of cytotoxicity, in terms of disruption of cell function.

The measurement of cellular metabolism, as an indicator of cell viability, has been used in many toxicity tests like the Spielberg test and dermal irritancy tests (Duffy and Flint 1987, Kao *et al.* 1983). The MTT test (Mosmann 1983) is the most commonly used assay, although there are other similar assays which use the same basic principle (Alley *et al.* 1988, Scudiero *et al.* 1988). The assay involves the cleavage of the tetrazolium salt MTT, by mitochondrial dehydrogenases, to produce a dark blue formazan product, which is then measured by colorimetry. However, fragments from lysed cells, for example, mitochondria and microsomes, can produce a positive result. Cell membrane integrity is another commonly measured parameter of cell viability and can be evaluated by measuring levels of leakage of either, lysosomal hexoaminidase (HEX) (Landegren 1984) or lactate dehydrogenase (modified from Korzeniewski and Callewaert 1983). Measurement of LDH is the most commonly used assay and the LDH is evaluated by its conversion of lactate and NAD, to pyruvate and NADH. The NADH then reduces a tetrazolium salt INT, to a red coloured product. However, LDH is a protein and so of considerable size, which makes the assay too insensitive. In addition, a number of toxic compounds are known to interfere with LDH activity, which could result in false positives.

Therefore cell toxicity systems must be established which include the incorporation of a metabolically active system, with a number of representative cells and which assess cell

damage, in terms of the degree of disruption of various cell functions. The tests must be sensitive, reproducible and functional capability must be measurable.

1.9. TEST COMPOUNDS.

To fully validate a new *in vitro* test system, a wide range of compounds with different modes of action and known toxicities, should be used. However when designing the tests covered in this thesis, a small selection of drugs were used, which have been well documented to be bioactivated to toxic derivatives both *in vivo* and *in vitro*. Preliminary work done for this thesis (Section 3) using the Spielberg mixed test (Spielberg 1984) showed the cytotoxicity of the panel of drugs involved.

Cyclophosphamide (N,N-bis(b-chloroethyl)-N',O-propylenephosphoric acid ester diamide) is a DNA alkylating agent used in the treatment of malignant disease (Figure 1.5.). Cyclophosphamide is a pro-drug, requiring cytochrome P-450 mediated activation (Cox *et al.* 1975, Acosta and Mitchell 1981, Hales 1982). It causes a number of side effects *in vivo*, principally in suppression of generative cells, such as in bone marrow and in gamete production. The metabolic activation results in the production of 4-hydroxy intermediates and ultimately the two major toxic metabolites, phosphoramide mustard and acrolein (Figure 1.6.). The toxicity of these metabolites, particularly acrolein, has been well documented, both *in vivo* (Subramaniam *et al.* 1994) and *in vitro* (Acosta and Mitchell 1981, Horner *et al.* 1985, Wildenauer *et al.* 1982). It is widely used as a test compound, for validating novel *in vitro* tests, concerned with the study metabolically activated toxic agents (Wiebkin *et al.* 1978). It is also a control chemical in most mutagenicity and carcinogenicity tests.

Carbamazepine (5-carbamyl-5H-dibenz[b,f]azepine) (Figure 1.6.) is principally used in the treatment of most forms of epilepsy, although it can also be used in the treatment of several other neural disorders (British National Formulary 1986). It is metabolized to

an epoxide intermediate by the cytochrome P-450 oxidation pathway and this reactive epoxide is then hydroxylated. Hepatotoxicity has been reported with carbamazepine and the metabolites generated by the action of epoxidation, have been demonstrated to be protein reactive and cytotoxic (Pirmohamed *et al.* 1992). This cytotoxicity is inhibited in a concentration dependent manner, by microsomal epoxide hydrolase. In addition, carbamazepine and oxcarbazepine-10,11-dihydro-10-carbamazepine have been reported to induce rat liver microsomal enzymes (Wagner and Schmid 1987). Reported *in vivo* side effects include hepatitis and jaundice, neurological effects for example, ataxia and haematological disorders.

Phenytoin (5,5-diphenylhydantoin) (Figure 1.6.) is used in the treatment of most forms of epilepsy, other neurological conditions and in ventricular arrythmias (British National Formulary 1986). It undergoes aromatic hydroxylation and methylation, which are mediated by cytochrome P-450 and can also undergo heterocyclic ring cleavage. The P-450 hydroxylation of phenytoin has demonstrated the evidence of genetic polymorphism and phenytoin has also been identified as a P-450 enzyme inducer in experimental animals (Gibson and Skett 1986). It is proposed that the *in vivo* toxicity seen, particularly the hepatotoxic effects, are the result of a reactive arene oxide intermediate which covalently binds to macromolecules (Spielberg *et al.* 1981). This arene oxide intermediate has also been implicated in other *in vivo* side effects such as birth defects (Martz *et al.* 1977) and gingival hyperplasia (Rao and Wortel 1980). Phenytoin has also been identified to have an inhibitory effect on DNA synthesis in human marrow cells (Taguchi *et al.* 1977). *In vitro* studies have also looked at these effects for example, on teratogenesis with micromass cultures (Flint 1987) and DNA synthesis (Taguchi *et al.* 1977). Other reported *in vivo* adverse reactions include a number of neurological, dermatological side and haematological effects.

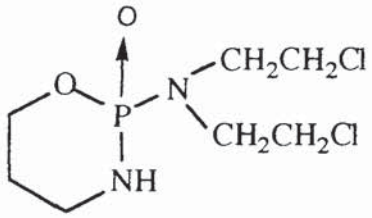
Sulphamethoxazole (4-amino-N-(5 methyl-3-isoxazolyl)-benzenesulphonamide) (Figure 1.6.) is used in conjunction with trimethoprim in the treatment of various infections and in high doses to treat *Pneumocystis carinii* pneumonia. The major

metabolite of sulphamethoxazole is the N4 -acetyl derivative, produced by the N-acetylation of the parent compound in the hepatocyte cytosol. Cytochrome P-450 catalyses the N-hydroxylation of the parent drug and the acetyl metabolite. The hydroxylamine metabolite has been reported as the cytotoxic component, in studies involving peripheral mononucleocytes (Shear and Spielberg 1985, Carr *et al.* 1993) and adherent cell lines (Riley *et al.* 1993). There are various *in vivo* effects reported which include gastro-intestinal disturbances, dermatological and haematological effects.

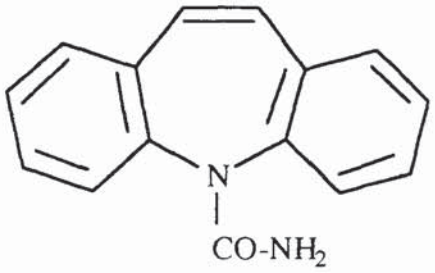
Dapsone (4,4' diaminodiphenyl sulphone) (Figure 1.6.) is an agent primarily used in the treatment of leprosy, malaria, *Pneumocystitis carinii* pneumonia, as well as dermatitis herpetiformis. Dapsone is metabolized in the liver by two routes, N-hydroxylation and N-acetylation, with N-hydroxylation being the major route of metabolism in man (Israili *et al.* 1973). N-hydroxylation is cytochrome P-450 mediated and results in production of the haematoxic hydroxylamine metabolite (Grossman and Jollow 1988). Dapsone is also metabolised by direct conjugation with glucuronic acid, producing the N-glucuronide product. The *in vivo* adverse reactions of dapsone include gastro intestinal disturbances, allergic reactions, hepatitis and haematological effects. *In vitro* studies have demonstrated the haematological effects of dapsone, in co-incubation tests (Coleman *et al.* 1989) and two compartment models (Tingle *et al.* 1990, 1992).

FIGURE 1.5.

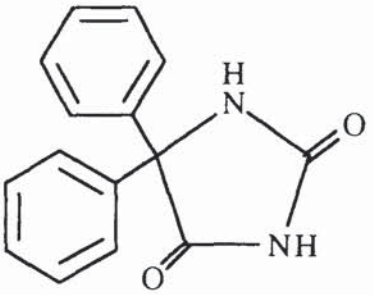
Structures of test compounds.



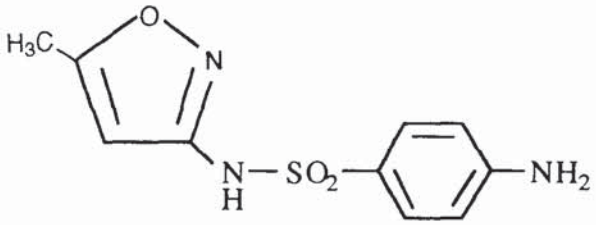
CYCLOPHOSPHAMIDE



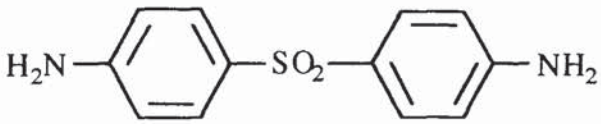
CARBAMAZEPINE



PHENYTOIN



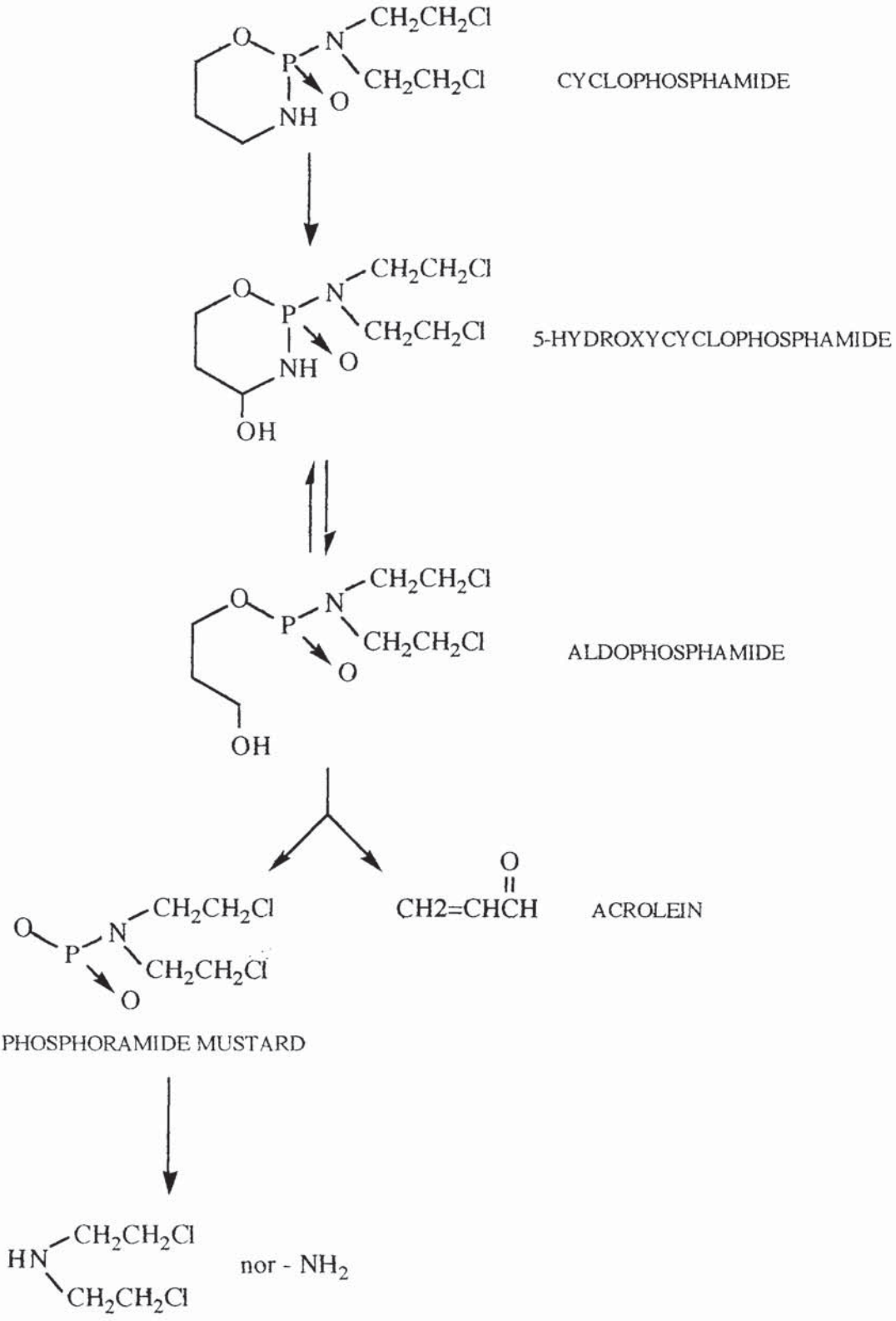
SULPHAMETHOXAZOLE



DAPSONE

FIGURE 1.6.

Metabolic activation pathway of cyclophosphamide.



1.10. RATIONALE AND OBJECTIVES.

The objective of this thesis, was to develop novel *in vitro* toxicity tests, which would evaluate toxicity in terms of quantitative changes in cell function, rather than cell death. The tests were co-incubation systems, comprising target cells and a microsomal metabolite activating system.

In all the tests, the microsomal system was derived from rat liver and was used with NADPH, to generate the toxic metabolites of the test compounds detailed in section 1.9. The test compounds were chosen based on the data derived from preliminary work, which is detailed in Section 3 and research material from other workers. As the microsomal fraction is being used, the metabolism will produce Phase I metabolites of the drug. In addition, the use of NADPH in the system, provided the reducing power for cytochrome P-450 reactions, which are predominantely oxidative.

There are four 'target' cell models which were used in these tests; the CACO-2 human adenocarcinoma cell line, the L6 rat thigh muscle cell line, the NG115-401L-C3 murine neural glioma cell line and freshly isolated human erythrocytes. These target cells were chosen on the basis of documented research involving their cell functions and in the case of erythrocytes, research evidence of cytotoxicity from the drugs to be used. The CACO-2 cell line, was used to demonstrate the effects of toxicity on energy dependent transport systems, in this case, the L-proline transport system. The L6 cell line, was used to study two receptor mediated cell signalling pathways, the phosphoinositide and adenylate cyclase cascades. The second messengers produced in these pathways, cyclic AMP and inositol triphosphate, were determined, to evaluate any changes in the cell signalling response after toxicity. The NG115-401L-C3 cell line was used to study the effects of metabolites on receptor mediated mitogenesis. This was evaluated by the measurement of DNA synthesis, which is determined by [^{3}H] thymidine incorporation into the DNA. In addition, all the cell lines were used to study the effect of the metabolites on the intracellular glutathione levels.

The freshly isolated human erythrocytes were used in two types of toxicity test. The first involved the erythrocytes being incubated in direct contact with the metabolites, that were generated by the microsomes. This system provided data on the immediate interaction of highly reactive, unstable metabolites with the erythrocytes. The second test involved the use of a two compartment system, where the erythrocytes were separated from the microsomal component by a semi permeable membrane. This provided data on the ability of the metabolites generated to exert an effect after diffusing some distance from the site of formation and passing through a semi-permeable membrane.

In addition to identifying any toxic effects, these tests may also provide some knowledge into the structure-toxicity of chemical groups involved, in terms of the cell processes identified. Toxicity shown during individual cellular events may shed light on the mechanisms of systemic toxicity seen with these test compounds.

SECTION 2

METHODS

2.1 CELL CULTURE

2.1.1 MEDIA

CACO-2 cell line

DMEM supplemented with 10%($^{V}/_{V}$) FCS

1% ($^{V}/_{V}$) NEAA

1% ($^{V}/_{V}$) Glutamine 200 mM (100x)

1% ($^{V}/_{V}$) Antibiotic/antimycotic solution (100x)

L6 cell line

DMEM supplemented with 5% ($^{V}/_{V}$) FCS

1% ($^{V}/_{V}$) Glutamine 200 mM (100 x)

1% ($^{V}/_{V}$) Antibiotic/antimycotic solution (100x)

NG115-401L-C3 cell line

DMEM supplemented with 5% ($^{V}/_{V}$) FCS

1% ($^{V}/_{V}$) Glutamine 200 mM (100x)

1% ($^{V}/_{V}$) Antibiotic/antimycotic solution (100x)

Media changed on cells every 48-72 hours.

2.1.2. INCUBATION CONDITIONS

All cells incubated at 37 degrees centigrade. CACO-2 cell line was in a humid atmosphere of 10% CO_2 in air and the other cell lines were in a humid atmosphere of 5% CO_2 in air.

2.1.3. SUB CULTURING OF CELL LINES

CACO-2 and NG115-401L-C3 cells were sub-cultured when the monolayers were confluent and the L6 cells were sub-cultured when the monolayers were 70% confluent. The L6 were sub-cultured at the point of 70% confluence to avoid any chances of cell differentiation and also cell death.

The media was aspirated from cells and the monolayer was washed with sterile phosphate buffered saline, which was then aspirated off. Trypsin-EDTA (0.1%) was added to detach the cell monolayer and when the cells were free, the trypsin's action was stopped by addition of media. The cell suspension was then gently triturated to ensure the monolayer was completely dissociated and the cell suspension added to flasks containing fresh media.

If cells were to be plated out, the cell concentration and cell viability were evaluated before adding media. 30 μl of 3% ($^{W}/_{V}$) Trypan blue solution was added to 100 μl of cell suspension and the resulting solution applied to a haemocytometer. Viable cells appeared as clear with well defined membranes whilst non viable cells appear blue Viability was calculated in the following manner :-

$$\% \text{ viability} = \frac{\text{number of viable cells}}{\text{total number of cells}} \times 100$$

The number of cells was evaluated as follows :-

No. of cells = Total number of viable cells/ml x volume of cell suspension

Cells were plated in 6 well plates at a concentration of 2 x 10^5 cells/ml and total well volume of 3ml, with the exception of the NG115-401L-C3 cells, which were plated out at $1x10^4$ cells/ml.

2.2. ISOLATION OF ERYTHROCYTES

Throughout the isolation, procedures were performed on ice wherever possible. The whole blood was washed with an equal volume of phosphate buffered saline with 10 mM glucose (PBSG) and then centrifuged at 2500 rpm. for 5 mins. The supernatant was aspirated away and the packed erythrocytes were washed a further two times as before. After the final wash, the packed erythrocytes were resuspended with PBS-G to give a 50% haematocrit.

2.3. ISOLATION OF MONONUCLEAR LYMPHOCYTES

Initial isolation work was performed under aseptic conditions, in a laminar air flow cabinet.

Approximately 25 ml of whole blood was obtained from a healthy volunteer, by venupuncture and diluted 1:1 with 0.9 % ($^{w}/_{v}$) sodium chloride solution. 5 ml aliquots of Lymphoprep^{R} (sodium metrizoate/Ficoll) were transferred to 30 ml volume universal tubes and approximately 7 ml of the diluted blood was gently layered on top of the Lymphoprep. The samples were cetrifuged at 2500 rpm. for 10 mins. to separate the blood components. The lymphocytes appeared as a buffy layer just above the pellet of erythrocytes. The lymphocytes were removed gently using a glass pasteur pipette and transferred to plastic tear bottomed centrifuge tubes. The lymphocytes were diluted 1:1

with 50 mM phosphate buffer then centrifuged at 1000 rpm. for approximately 4 mins. The supernatant was removed and the lymphocytes were resuspended with 3 ml. of HEPES buffer.

Cell viability was determined by trypan blue exclusion and then visual examination using a haemocytometer slide. 100 ml of the cell suspension was mixed with 20 ml of 3 % ($^{w}/_{v}$) trypan blue solution and 30 ml of this suspension was transferred to the haemocytometer slide. The total number of cells per ml. and the total number of dead cells per ml. were evaluated, along with the total volume of lymphocyte suspension.

% viability and total number of cells were calculated using the formula shown in 2.1.3.

2.4. PREPARATION OF LIVER MICROSOMES

Where possible, all procedures were carried out on ice, to maintain enzyme levels in the microsomes. In addition, the buffer solutions and equipment were pre-cooled to 4 degrees centigrade.

The animals were killed by cervical dislocation, the livers removed and immediately washed in ice cold Tris buffer. The livers were then weighed, finely chopped and transferred to a glass homogenising vessel with three times the volume of Tris buffer. The liver homogenate was then prepared using the Camlab Homogeniser. The homogenate was transferred to Beckmann polycarbonate centrifuge tubes and the microsomes isolated by differential centrifugation using the Beckmann L8-60M ultracentrifuge. The initial centrifugation was at 13,000 rpm. (9000 g) for 20 mins. and this removed the large cellular fractions. The supernatant produced was then centrifuged at 42,000 rpm. (105000 g) for 1 hour, to separate soluble cytosolic fractions from the microsomal fraction. The microsomal pellet produced, was resuspended in Tris buffer by gentle hand homogenation and the resulting homogenate centrifuged at 42,000 rpm. for 1 hour. This procedure was performed to remove any haemoglobin still present. The microsomal pellet

produced was gently hand homogenised then stored as 1ml pellets covered by 0.5ml of phosphate buffer at -70 degrees centigrade. The protein content of the microsomal suspension was determined according to the method of Lowry *et al.* (1951).

2.5. MICROSOMAL INCUBATIONS WITH CELL LINES

All incubations were carried out at 37 degrees centigrade using six well plates containing cell monolayers and incubation conditions of a humid atmosphere containing 5% CO_2 in air. Incubations were carried out for 1 hour.

The incubates (final volume 2 ml) were prepared as follows:-

CONTROLS		
CELL	Cell media to	2 ml
DRUG	Drug solution	20 µl
	Cell media to	2 ml
DRUG AND	Drug solution	20 µl
MICROSOMES	Microsomal equivalent of 4mg of protein	
	Cell media	2 ml
METABOLITE GENERATING	Drug solution	20 µl
INCUBATION	Microsomal equivalent of 4 mg of protein	
	NADPH (1 mM)	200 µl
	Cell media to	2 ml

The microsomal suspensions and the NADPH solution were both stored on ice before use and the NADPH solution was made up before prior to addition to the incubate, to retain the substrate activity.

2.6. MICROSOMAL INCUBATIONS WITH ERYTHROCYTES

2.6.1. ONE COMPARTMENT (STILL) INCUBATIONS

All incubations were performed in groups of between n=3 and n=6, in a water bath set at 37 degrees centigrade and with gentle agitation. Incubates were set up with one of the following metabolite generating systems:-

SYSTEM ONE

CONTROLS		
ERYTHROCYTES	50% haematocrit	500µl
	Phosphate buffered saline	to 1ml
	with 10 mM glucose	
DRUG	Drug solution	20µl
	50% haematocrit	500µl
	Phosphate buffered saline	to 1ml
	with 10 mM glucose	
DRUG AND	Drug solution	20µl
MICROSOMES	50% haematocrit	500µl
	Microsomal equivalent of 2mg of protein	
	Phosphate buffered saline	to 1ml
	with 10 mM glucose	

METABOLITE GENERATING	Drug solution	20μl
INCUBATION	50% haematocrit	500μl
	Microsomal equivalent of 2mg of protein	
	NADPH (1 mM)	100μl
	Phosphate buffered saline	to 1ml
	with 10 mmol glucose	
SYSTEM TWO		
CONTROLS		
ERYTHROCYTES	50% haematocrit	1ml
	Phosphate buffered saline	to 2ml
	with 10 mM glucose	
DRUG	Drug solution	20μl
	50% haematocrit	1ml
	Phosphate buffered saline	to 2ml
	with 10 mM glucose	
DRUG AND	Drug solution	20μl
MICROSOMES	50% haematocrit	1ml
	Microsomal equivalent of 4 mg of protein	
	Phosphate buffered saline	to 2ml
	with 10 mM glucose	

METABOLITE GENERATING INCUBATION	Drug solution	20μl
	50% haematocrit	1ml
	Microsomal equivalent of 4 mg of protein	
	NADPH Generating system	1ml
	Phosphate buffered saline with 10 mM glucose	to 2ml

NADPH generating system (1mM in incubation) was prepared as follows:-

Glucose-6-phosphate	56.42mg
NADP	15.30mg
Magnesium Chloride, 100 mM	4ml
Glucose-6-phosphate dehydrogenase	40 units
Phosphate buffer	to 10ml

All incubations were carried out for 1 hour then samples were analysed to establish glutathione levels in the erythrocytes.

2.6.2. TWO COMPARTMENT (DIANORM) INCUBATION

All incubations were performed in groups of five, in a water bath at 37 degrees centigrade with the dianorm speed set at 7 rpm. The semi permeable membrane separating the two compartments of the dianorm discs was prepared by soaking in distilled water, then in phosphate buffered saline with 10 mM glucose.

The incubates were set up as follows:-

COMPARTMENT ONE (MALE)	50% haematocrit	500μl

COMPARTMENT TWO (FEMALE)

CONTROLS

ERYTHROCYTE	Phosphate buffered saline with 10 mM glucose	500µl
DRUG	Drug solution	20µl
	Phosphate buffered saline with 10 mM glucose	to 500µl
DRUG AND MICROSOMES	Drug solution	20µl
	Microsomal equivalent of 2 mg of protein	
	Phosphate buffered saline with 10 mM glucose	to 500µl
METABOLITE GENERATING INCUBATION	Drug solution	20µl
	Microsomal equivalent of 2 mg of protein	
	NADPH (1 mM)	100µl
	Phosphate buffered saline with 10 mM glucose	to 500µl

All incubations were carried out for 1 hour then samples were analysed to establish glutathione levels in the erythrocytes.

2.6.3. PRE-INCUBATION WITH ERYTHROCYTES WITH DIETHYL MALEATE PRIOR TO ONE COMPARTMENT INCUBATIONS

Erythrocytes were isolated as described in 2.2 and then pre-incubated for 30 minutes with diethly maleate (50 μM). The cells were then washed as described in 2.2 and the one compartment incubations set up as described in 2.6.1.

2.7. MICROSOMAL INCUBATIONS WITH MONONUCLEAR LYMPHOCYTES

All incubations were performed in groups of n=4, in a water bath set at 37 degrees centigrade and with gentle agitation for two hours. Incubates were set up with the following metabolite generating system:-

CONTROLS		
LYMPHOCYTES	@ 7 x 10^5 lymphocytes	Variable volume
	HEPES buffer	to 1ml
DRUG	Drug solution	10μl
	@ 7 x 10^5 lymphocytes	Variable volume
	HEPES buffer	to 1ml
DRUG AND	Drug solution	10μl
MICROSOMES	@ 7 x 10^5 lymphocytes	Variable volume
	Microsomal equivalent of 2mg of protein	
	HEPES buffer	to 1ml

METABOLITE GENERATING	Drug solution	10μl
INCUBATION	@ 7 x 10^5 lymphocytes	Variable volume
	Microsomal equivalent of 2mg of protein	
	NADPH (1 mM)	100μl
	HEPES buffer	to 1ml

After incubation, the samples were centrifuged at 100 rpm. for 5 mins., the supernatants removed and the lymphocytes resuspended in 1 ml of HEPES buffer. The lymphocytes were then incubated in the water bath at 37 degrees centigrade for a further 16-18 hours.

Cell viability and the total number of lymphocytes were determined as described in 2.3.

2.8. PROLINE UPTAKE ASSAY WITH CACO-2 CELL LINE

Cells plated out were incubated at 37 degrees centigrade in humid atmosphere containing 10% CO_2 in air for seven days, feeding every 48 hours with fresh media.

2.8.1. PROLINE UPTAKE IN PRESENCE OF EXCESS PROLINE

All solutions used with cell monolayers were pre-warmed for 30 minutes in a water bath at 37 degrees centigrade. The incubation media to be used in the assay was prepared as follows :-

Bovine serum albumin	0.5 g
Hanks balanced salt solution to	500 ml

The incubation media was buffered to pH 7.4 with 25mM HEPES solution.

The cell monolayers were washed with the incubation media (2ml) for 15 minutes, incubating at 37 degrees centigrade in a humid atmosphere of 10% CO2 in air

The proline solutions were prepared as follows :-

L-Proline (100 mM)	0.0115 g
Hanks balanced salt solution + 0.1% BSA pH 7.4	to1ml
[^{3}H] L-Proline (1 mCi/ml)	1µl
Hanks balanced salt solution + 0.1% BSA pH 7.4	to 1ml

The volume of the solutions were altered according to the number of wells in the assay

Washing solutions were aspirated from wells and to each well the [^{3}H] proline solution (1ml) was added. To half the wells, the incubation media was added (1ml) and the Hanks balanced salt solution with BSA and 100 mM L-Proline (1ml) was added to the remaining wells. The plates were incubated for 20 minutes in a humid atmosphere containing 10% CO_2 in air at 37 degrees centigrade.

Well solutions were collected and transferred to scintillation vials containing Optiphase Hi-safe 3 (10ml). The monolayers were washed three times with ice cold phosphate buffered saline with 0.05% (w/v) sodium azide (1ml). The monolayers were then solubilized by shaking with 1% (v/v) Triton X-100 (2ml) for 15 minutes. The solubilized monolayers were collected and transferred to scintillation vials containing Optiphase Hi-safe 3 (10ml). Vials were shaken well then counted for 2 minutes using the Packard C 2200 Liquid scintillation counter.

2.8.2. PROLINE UPTAKE AFTER MICROSOMAL INCUBATION

After the microsomal incubations, the monolayers were washed with phosphate buffered saline (2ml) then washed with Hanks balanced salt solution with BSA pH7.4 as in 2.8.1.

Washing solutions were aspirated from wells and to each well the [^{3}H] proline solution (1ml) was added and then Hanks balanced salt solution + BSA solution was added (1ml). The monolayer solutions and monolayers were collected as described in 2.8.1.

2.8.3. PRE-INCUBATION WITH PHENOL TRANSFERASE BLOCKER

Cell monolayers were pre-incubated with N-ethylmalamide (6.5 nM) in Hanks balanced salt solution for 15 minutes in a humid atmosphere of 10% CO_2 in air. The monolayers were then washed with phosphate buffered saline (2ml) and the microsomal incubations performed.

Proline uptake in the monolayers was carried out as described in 2.8.2.

2.9. CYCLIC AMP ASSAY WITH L6 CELL LINE

Cells plated out were incubated at 37 degrees centigrade in a humid atmosphere with 5% CO_2 in air for 5 days.

The cell monolayers were washed with physiological salt buffer (1ml) for 20 minutes incubating at 37 degrees centigrade in a humid atmosphere of 5% CO_2 in air. The drug solutions (10µl of 10^{-3}M) were added and incubated with the cell monolayers for 5 minutes at 37 degrees centigrade as before. The well contents were aspirated and ice cold Tris/EDTA (1ml) was added to each well. The plates were left on ice for 2 mins then placed in a water bath at 100 degrees centigrade for 5 minutes. The plates were then returned to ice, to cool to room temperature. The well solutions were collected and transferred to centrifuge tubes then centrifuged at 12,000 rpm for 5 minutes. Aliquots (50 µl) of the supernatants were transferred to centrifuge tubes. To each sample, [^{3}H] cyclic AMP (50 µl) was added and cyclic AMP binding protein (100 µl). The samples were then shaken well and stored at 0-4 degrees centigrade for 19 hours. Charcoal

slurry (100 μl) was added to each sample, with shaking and then the samples were centrifuged for 5 minutes at 12,000 rpm. Aliquots (185 μl) of the supernatants were transferred to scintillation vials with 4ml of Optiphase Hi safe II liquid scintillant.

The vials were shaken well to mix the contents and then counted for 5 minutes using the Packard C2200 Liquid scintillation counter.

2.9.1. CYCLIC AMP ASSAY AFTER MICROSOMAL INCUBATION

After the microsomal incubations, the monolayers were washed with phosphate buffered saline (2 ml) and the assay carried out on the cell monolayers as described in 2.9., except that the monolayers were not incubated with any drug solutions. In addition, one plate was not subjected to the microsomal incubation and this was the control plate. The assay was carried out on the control plate as described in 2.9., with drug solutions being incubated with the cell monolayers in four wells and no drug solutions in two wells. This gives both a positive and negative control response for comparisons.

2.10. GLUTATHIONE ASSAY

Determination of glutathione levels in samples uses the method described by Anderson (1985).

During the assay, samples and glutathione standards were kept on ice wherever possible, until addition of acid, to maintain glutathione levels.

Glutathione standard solutions were prepared from 5 mM stock solution (77 mg in 100 ml GSH buffer) over the concentration range 0 to 5 mM. All standard samples were prepared in triplicate and the control (0 mM) standard was prepared using GSH buffer alone.

2.10.1. GLUTATHIONE ASSAY WITH ERYTHROCYTES

The erythrocytes were washed with phosphate buffered saline and 10 mM glucose (PBS-G) and then centrifuged at 2500 rpm. for 5 minutes. The supernatant was aspirated and the packed erythrocytes were washed a further two times as before. The erythrocytes were resuspended with an equal volume of PBS-G. Aliquots (50 μl) were taken from each sample/standard solution and transferred to centrifuge tubes. 10 mM HCl (100 μl) was added to the samples, mixed by shaking, and the samples were stored at -20 degrees centigrade for 15 to 20 minutes. The samples were thawed at room temperature and centrifuged for 5 minutes at 13,000 rpm. Aliquots (100 μl) of the supernatants were transferred to centrifuge tubes and 5-sulphasalacylic acid solution (50 μl) was added. The samples were then centrifuged for 5 minutes at 13,000 rpm.

GSH buffer (700 μl) and DTNB solution (100 μl) were mixed in vials then pre-warmed for 15 minutes in a water bath set at 30 degrees centigrade. Aliquots (50 μl) of the sample supernatants were added to the buffer/DTNB solutions. Absorbance of the solutions was determined against a blank (GSH buffer) using the Cecil CE 594 Double beam UV spectrophotometer set at 412 nm. A calibration curve was constructed from the GSH standard solutions and from this, the glutathione content of the erythrocyte samples was evaluated

2.10.2. GLUTATHIONE ASSAY WITH CELL LINES

All cell lines were assayed for glutathione content and cells were plated out at 2×10^5 cells/ml. Cells were incubated under the conditions described in 2.1.2.

After the microsomal incubations, the monolayers were washed with phosphate buffered saline (2 ml). The phosphate buffered saline was aspirated to waste and the monolayers detached by the addition of trypsin-EDTA solution. The trypsin's action was stopped by

addition of ice cold media (4 ml) and the cell suspensions were transferred to centrifuge tubes. The cell suspensions were centrifuged for 5 minutes at 1000 rpm then placed in ice. The supernatants were aspirated to waste and aliquots (50 μl) of the cell pellets were transferred to centrifuge tubes on ice.

The cell samples were then analysed using the glutathione method described in 2.10.1.

2.11. THYMIDINE ASSAY WITH NG115-401L-C3 CELL LINE

All solutions used with cell monolayers were pre-warmed for 30 minutes in a water bath at 37 degrees centigrade.

After one hour of plating out the cells, the media was replaced with Foetal calf serum free Dulbecco's modified Eagle medium and the cells incubated at 37 degrees centigrade in a humid atmosphere of 5% CO_2 in air, for 24 hours.

The incubation media used in the assay was prepared as follows:

[^{3}H] Thymidine (1 mCi/ml)	1 μl
DMEM	1 ml

The volume of solution was altered according to the number of wells in the assay

The media was aspirated from the wells and replaced with the [^{3}H] thymidine media (1ml/well). The cell monolayers were then stimulated by addition of Angiotensin III solution (10μl of10^{-4} M) and incubated for 24 hours at 37 degrees centigrade in a humid atmosphere of 5% CO_2 in air.

The media was aspirated from the wells and the cell monolayers were washed twice with ice cold Kreb's buffer (1 ml). Ice cold trichloracetic acid (5%) solution (1 ml) was added to each well and the plates were stored at 0 to 4 degrees centigrade for 20 minutes.

The well solutions were transferred to scintillation vials with 4 ml of Optiphase Hi-safe 3.

Each well was then washed twice with ice cold absolute alcohol (1 ml) and the plates were left to air dry for 30 minutes. The Na_2CO_3/NaOH solution (500 µl) was added to each well and the solutions were transferred to scintillation vials with 4 ml of Optiphase Hi-safe 3. Vials were shaken well then counted for 5 minutes using the Packard C 2200 Liquid scintillation counter.

2.11.1. THYMIDINE ASSAY AFTER MICROSOMAL INCUBATIONS

The microsomal incubations were performed after the cells had been grown for 24 hours. After the incubations, the cell monolayers were washed with phosphate buffered saline (2ml). The cell monolayers were then assayed as described in section 2.11.

2.12. INOSITOL ASSAY WITH L6 CELLS

Cells plated out were incubated at 37 degrees centigrade in a humid atmosphere with 5% CO_2 in air for 5 days.

The inositol incubation media was prepared as follows:

[^{3}H] Inositol (1 mCi/ml)	1 µl
DMEM	1 ml

The volume of solution was altered according to the number of wells in the assay.

The media was aspirated from the wells and was replaced with [^{3}H] inositol media (1ml). The plates were then incubated for 24 hours at 37 degrees centigrade in a humid atmosphere of 5% CO_2 in air.

The [^{3}H] inositol media was aspirated to waste and the monolayers washed with Kreb's and BSA buffer for 30 minutes incubating at 37 degrees centigrade in a humid atmosphere of 5% CO_2 in air. The buffer was aspirated from the wells and the monolayers washed with the Kreb's buffer with BSA and LiCl (1 ml) for 10 minutes, incubating as before.

The incubates were set up as follows

CONTROLS

UNSTIMULATED	1 ml DMEM (No Vasopressin)
STIMULATED	1 ml DMEM (Vasopressin added)

DRUG INCUBATIONS

Dapsone hydroxylamine (in acetone)	10 µl
Media to	1 ml

The dapsone hydroxylamine solutions used gave concentrations in the wells from 10 to 100 µM

The cell monolayers were stimulated with 1 mM vasopressin (10 µl) incubating for 30 minutes at 37 degrees centigrade in a humid atmosphere of 5% CO2 in air.

The well solutions were aspirated to waste and ice cold 20 % TCA/EDTA (2 mM) was added to the wells (1 ml). The plates were placed on ice for 15 minutes, then the well solutions were transferred to centrifuge tubes. The samples were centrifuged for 5 minutes at 13,000 rpm, to remove protein. The supernatants were transferred to vials containing a 1:1 freon:trioctylamine mixture (5 ml) and shaken well. This neutralises the trichloracetic acid in the supernatants. Aliquots (500 µl) of the aqueous layers were removed and transferred to centrifuge tubes prior to the separation of the inositol phosphate by ion exchange.

The Dowex resin for the columns was initially soaked in an excess of distilled water for 2 hours. The supernatant was removed from the resin and replaced with more distilled

water. The dowex was allowed to settle and the process was repeated a further three times to remove undersize particles. The resin was adjusted with more distilled water to give a 1:1 Dowex/distilled water mixture then stirred continously. Dowex resin (1 ml) was poured into preplugged columns and after settling, the columns were washed with distilled water (4 ml) to ensure flow through the columns.

Distilled water (4 ml) then the sample solutions (500 μl) were run through the column. The column was washed twice with distilled water (4 ml) and then washed with NH_4HCO_2 (1 M)/HCO_2H (0.1 M). The formate fractions were collected in scintillation vials and Optiphase Hi-safe III (10 ml) was added to the vials. The vials were shaken well then counted for 5 minutes using the Packard C 2200 Liquid scintillation counter.

2.13. MEASUREMENT OF HAEMOGLOBIN, REDUCED HAEMOGLOBIN AND METHAEMOGLOBIN

Haemoglobin in g/dL and reduced haemoglobin and methaemoglobin as a percentage were all measured using a CO-Oximeter (Instrumentation Laboratories, Warrington). After the incubations, the blood samples were washed with ice cold PBS-G as detailed in the glutathione assay (2.10.1.) and then the samples were analysed by the CO-Oximeter.

2.14. STATISTICAL METHODS

All statistical analysis was by Student's 't' test and all data are represented as mean + S.D. (n-1). Statical calculations performed using Apple Macintosh Statistics software package. Where more than one comparison was made with the same data, Bonferroni's correction was used. The accepatable level of significance (p) was reduced to 0.05/n where n is the number of tests.

SECTION 3

PRELIMINARY STUDIES

3.1. INTRODUCTION

The principle objective of this thesis, is to develop toxicity tests that will ultimately predict the toxicity of experimental compounds in humans. However the test must be demonstrated to be valid for the purpose, by evaluating it with a selection of known cytotoxic agents. The cytotoxic agents that were to be used during this thesis experimental work would be determined on the basis of; availability of the compound, published and unpublished research relating to the compound and its cytotoxicity and finally evaluation of the compound in a known cytotoxicity assay.

The preliminary studies were initiated with compounds readily available within the Toxicology Group at Aston and that were already test compounds in on-going cytotoxicity work within the group. N-methylformamide and 2-nitropropane were being shown to be cytotoxic in rat hepatocytes (Toxicology Group communication) and the toxicity of dapsone within an *in vitro* system had been shown (Tingle *et al.* 1990). Cyclophosphamide is a widely used test compound and a control agent in mutagenicity and carcinogenicity tests. Propranolol, phenytoin and sulpamethoxazole were chosen on the basis that the compounds had already been used in the *in vitro* system that was to be used, that is the lymphocyte assay (Spielberg 1984) and had shown positive results (Spielberg 1984, Shear and Spielberg 1985, Tingle-Unpublished communication).

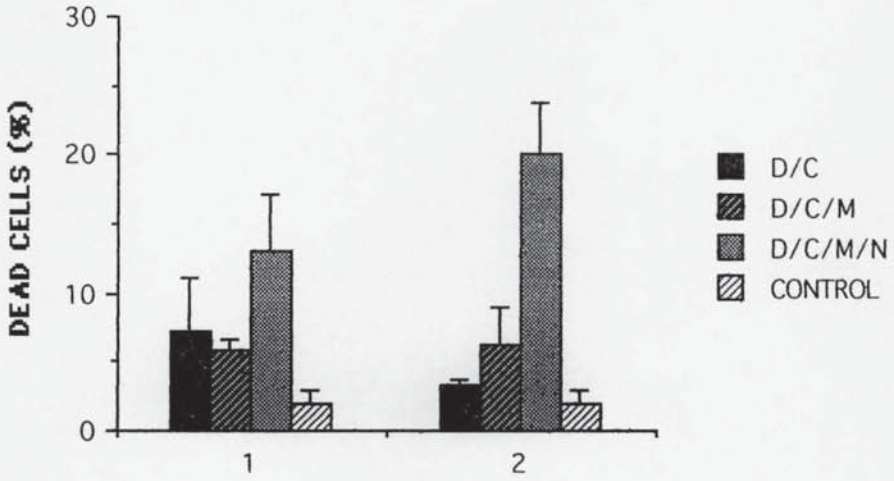
The lymphocyte assay was chosen as it was a relatively easy to perform, well documented and had already been used with some of the test compounds. It incoporates a metabolising element, which is also relatively easy to produce and already used as a predictor of toxicity in humans. In addition, it is a generally robust and quick assay, giving good reproducible results (Spielberg 1984, Shear and Spielberg 1985).

3.2. RESULTS

The most significant results were seen with cyclophosphamide and dapsone (Figure 3.2.1.) compared with the control. Cyclophosphamide resulted in 20 + 3.74 % cell death and dapsone 13 + 4.16% cell death. N-methylformamide was also shown to be cytotoxic at the concentrations used, that is 5, 10 and 20 mM (Figure 3.2.2.), the most significant being at 5 mM which showed 6 + 2.45 % cell death. At 10 and 20 mM, cell death was 3.5 + 1 % and 3.25 + 0.95 % respectively. Propranol was cytotoxic when compared to the control, but sulphamethoxazole showed no significant cytotoxicity compared with the control (Figure 3.2.3.).

FIGURE 3.2.1.

Lymphocyte assay - % non viable cells after one hour incubation with cyclophosphamide or dapsone and rat microsomes.



1 = Incubations with dapsone (50 μM)

2 = Incubations with cyclophosphamide (50 μM)

CONTROL = Cells and HEPES buffer

D/C = Lymphocytes, drug and HEPES buffer

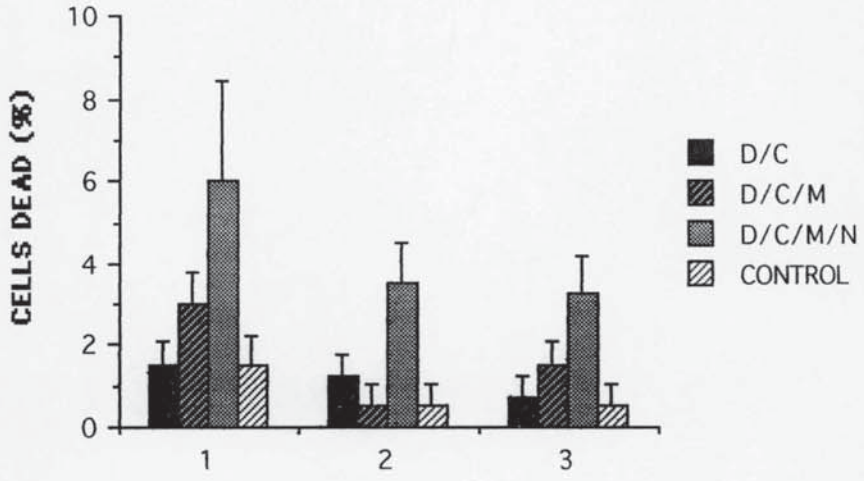
D/C/M = Lymphocytes, drug, rat microsomes (2mg/ml) and HEPES buffer.

D/C/M/N = Lymphocytes, drug,rat microsomes, NADPH (1 mM) and HEPES buffer

n = 4 for each incubation group with data represented as mean + S.D. (n-1)

FIGURE 3.2.2.

Lymphocyte assay - % non viable cells after one hour incubation with 5, 10 and 20 mM N-methylformamide and rat microsomes.



1 = Incubations with N-methylformamide (5 mM)

2 = Incubations with N-methylformamide (10 mM)

2 = Incubations with N-methylformamide (20 mM)

CONTROL = Cells and HEPES buffer

D/C = Lymphocytes, drug and HEPES buffer

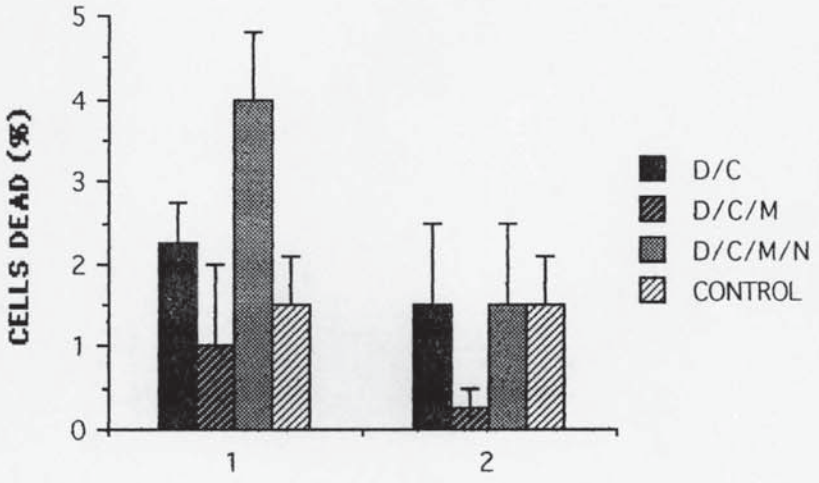
D/C/M = Lymphocytes, drug, rat microsomes (2mg/ml) and HEPES buffer.

D/C/M/N = Lymphocytes, drug,rat microsomes, NADPH (1 mM) and HEPES buffer

n = 4 for each incubation group with data represented as mean + S.D. (n-1)

FIGURE 3.2.3.

Lymphocyte assay - % non viable cells after one hour incubation with propranolol and sulphamethoxazole and rat microsomes.



1 = Incubations with propranolol (50 μM)

2 = Incubations with sulphamethoxazole (50 μM)

CONTROL = Cells and HEPES buffer

D/C = Lymphocytes, drug and HEPES buffer

D/C/M = Lymphocytes, drug, rat microsomes (2mg/ml) and HEPES buffer.

D/C/M/N = Lymphocytes, drug,rat microsomes, NADPH (1 mM) and HEPES buffer

n = 4 for each incubation group with data represented as mean + S.D. (n-1)

3.3. DISCUSSION

The cytotoxicity demonstrated with cyclophosphamide and dapsone in the microsomal metabolite generating systems, concurred with that seen in other similar studies (Wildenauer *et al.* 1992, Coleman *et al.* 1989). Therefore based on these results and the published literature, the use of cyclophosphamide and dapsone in further work would continue.

There appeared to be significant cytotoxicity with N-methylformamide at several concentrations, which coupled with the evidence of cytotoxicity in primary cultures of hepatocytes (Group Communication), would make the compound suitable for inclusion in a panel. However, N-methylformamide is a volatile compound and the open conditions of the incubations, particularly with the cell lines which employ large surface areas in the wells, will have resulted in a potential loss of compound due to volatilization and so reduction of cytotoxicity due to the loss. Although a closed system and N-methylformamide could be used for some of the experiments, cell line work could not use a closed system as all the cells require an oxygenated environment. Based on this fact, there would be no inclusion of N-methylformamide into the panel of compounds.

There was evidence of cytotoxicity in the lymphocytes with propranolol, but although it was significant compared with the control lymphocytes, it appeared not be significant compared with the drug and cells control. Although the compound is known to be acutely toxic, its effects are reported to be specific, by its action on ion channels and membrane excitability (Gulden and Seibert 1996). These effects were not under direct in the thesis so propranolol was omitted from the panel of compounds.

Although sulphamethoxazole was not observed to be cytotoxic in the assay performed, it has been shown to be cytotoxic in this system (Shear and Speilberg 1985) and is cited as having considerable haematological side effects (British National Formulary 1986) as well as a cytotoxic effect with lymphocytes from HIV patients (Carr *et al.* 1993). Carr *et*

al. demonstrated that the hydroxylamine metabolite of sulphamethoxazole was responsible for cytotoxicity, so it may have been possible that the causative metabolite had not been produced in sufficient quantity to exert its cytotoxic effect. Therefore as the research from other groups supported cytoxicity with this compound and the study of cytotoxicity in the haemic system formed a large basis of the experimental work, sulphamethoxazole was included.

In addition, further research into compounds exhibiting cytotoxicity, particularly with mononuclear lymphocytes (Spielberg 1984) and the haemic system, identified that phenytoin and carbamazepine would both be suitable candidates for inclusion into the test compound panel. Both compounds have highly reactive epoxide metabolites formed after Phase 1 metabolism, which have been identified as causative agents in cytotoxicity (Spielberg 1984, Pirohamed *et al.* 1992, Panesar *et al.* 1996).

SECTION 4

CACO-2 STUDIES

4.1. INTRODUCTION

The digestive tract is the first barrier to absorption of an oral dose of a compound and is thus the first site of action for potential toxicity. The absorption of orally ingested xenobiotics tends to be predominantly via the small intestine. This is due to the greater surface area compared with the stomach and the higher pH, which may suppress the ionization and increase the lipophilicity of a compound. Therefore a potential toxicity assay could be one that looks at the effects of toxic compounds on the cellular functions of a small intestine model culture.

Human adenocarcinoma cell lines have been extensively used in studies requiring *in vitro* models of the human gastrointestinal epithelium, due to the poor viability of primary cultured epithelial cells (Moyer 1983). The adenomacarcinoma cell lines demonstrate many of the properties characteristic of differentiated intestinal epithelial cells, such as a well defined brush border (Rousset 1986, Pinto *et al.* 1983, Neutra *et al.* 1989), which makes such cell lines applicable for studies on intestinal functions.

The Caco-2 cell line (Fogh *et al.* 1977) is an effective model, as under standard culture conditions, it demonstrates highly differentiated properties of intestinal epithelial (Zweibaum *et al.* 1989). The columnar cells have tight cell junctions and well developed apical microvilli when confluent (Pinto *et al.* 1983) and express numerous enterocytic enzymes such as alkaline phosphatase, lactase and sucrase-isomaltase (Pinto *et al.* 1983, Hauri *et al.* 1985, Zweibaum *et al.* 1983, Hidalgo *et al.* 1989). The Caco-2 model, resembles foetal intestinal cells in nature, expressing hydrolases with the molecular forms typical of such cells (Hauri *et al.* 1985) and secreting apolipoproteins and alphafoetoprotein (Traber *et al.* 1987).

The Caco-2 model has been extensively used in studies on transport pathways, for lipoproteins (Traber *et al.* 1987) and newly synthesized membrane proteins (Hughson *et al.* 1990). It has also been used to study transepithelial transport , for example, with

bile acids (Wilson *et al.* 1990, Hidalgo *et al.* 1990), hexose (Riley *et al.* 1991), cobalamin (Dix *et al.* 1987) and proline (Nicklin *et al.* 1992). In addition, there have been studies which have examined transport and permeability in the Caco-2 model (Hidalgo *et al.* 1989), in terms of the absorption and transepithelial transport of drugs (Wilson *et al.* 1990, Hu and Borchardt 1990). From these studies, it has been established that most of the transport employs the use of energy dependent carrier systems, which require ATP to function (Riley *et al.* 1991, Nicklin *et al.* 1992). It has also been demonstrated that these systems, like the expression of brush border enzymes, may be regulated by the cell under certain conditions (Riley *et al.* 1991).

It is known that the small intestine is also a site of metabolism *in vivo* and many intestinal cell lines have been demonstrated to retain many of the metabolic enzymes such as the LS174T model (Fang *et al.* 1982). The Caco-2 cell line has been reported to possess Phase II conjugative enzymes responsible for glucuronidation and sulfation and possibly Phase I enzymes (Baranczyk-Kuzma *et al.* 1991).

This high degree of differentiation in the Caco-2 model, gives a considerable number of reproducible cell functions, which could be used as markers in a cytotoxicity assay. In these studies, the uptake of the amino acid L-proline was examined, after co-incubation with a rat microsomal system. Uptake of proline in the Caco-2 model is by two paths, a passive system which contributes about one third of the observed flux and an active component which accounts for two thirds of the total. The active carrier is saturable, sodium dependent and significantly less efficient when protein synthesis, Na^+/K^+-ATPase or cellular metabolism are inhibited (Nicklin *et al.* 1992). Therefore, it was proposed that these cells could be used to demonstrate any toxic impact on the cell, as proline uptake might decrease compared to the control incubation, during toxic pressure.

4.2. RESULTS

The initial study, which was carried out prior to the introduction of the rat microsomal system, established that the CACO-2 cells responded as previously reported. After the 20 minute incubation period, either in the presence or absence of L-proline (50 mM), the monolayer solutions contained radioactivity of 16,485 $\pm$ 4,466 cpm and 36,360 $\pm$ 13,566 cpm.respectively. The monolayer donor solutions contained radioactivity of 875,838 $\pm$ 22,027 cpm. and 860,989 $\pm$ 45,718 cpm. The data is represented graphically in Figures 4.2.1. and 4.2.2.

Once the uptake of L-proline was established, studies involving the potential toxic drug solutions (50 μM) mixed with the rat microsome system (4 mg protein equivalent), were carried out. Controls were employed which would show the response of the monolayers when incubated with media alone, media and the drug solution and finally media, drug solution and the microsomes. The [^{3}H] L-proline radioactivity in the monolayers and donor solutions, was measured as disintegrations per minutes (dpm.), as well as counts per minute (cpm.). The dpm. values more accurately reflect the actual radioactive content of the samples. (Figures 4.2.3., 4.2.4., 4.2.5. and 4.2.6.). The was no significant reduction in the uptake of proline by the monolayers, after incubation with either cyclophosphamide or dapsone and the rat microsomes, compared with the controls.

It was demonstrated that the Caco-2 model, expresses the Phase II enzyme phenol sulphotransferase (Baranczyk-Kuzma *et al.* 1991) and expresses the Phase II enzymes UDP glucuronyltransferase, N-acetyltransferase and glutathione S transferase (Prueksaritanont *et al.* 1996). It is possible that the reactive metabolite phosphoramide mustard produced in the incubations, was further metabolised by phenol sulphotransferase, to the conjugated less reactive species. This was investigated by blocking the action of the enzyme with an inhibitor, N-ethylmaleimide, prior to any microsomal incubations (Baranczyk-Kuzma *et al.* 1991). N-ethylmaleimide reacts with the sulfhydryl groups of the enzyme. The monolayers were incubated with N-

ethylmaleimide (6.5 nM) for 15 minutes, then the incubations and proline assay were performed as before. (Figures 4.2.7., 4.2.8., 4.2.9. and 4.2.10.)

N-ethylmaleimide was itself cytotoxic in this *in vitro* system, with a significant difference in [^{3}H] proline uptake, when comparing the media only control groups. The addition of N-ethylmaleimide, decreased proline uptake in the media only control by 24% $\pm$ 6%. The cytotoxicity of N-ethylmaleimide was also seen in the decreased uptake in the other incubations, particularly those with cyclophosphamide. There was however, no difference in uptake with the incubations containing the microsome generated metabolites of cyclophosphamide or dapsone. The cytotoxic effect of N-ethylmaleimide was also observed during the cell solubilization step of the proline assay. The monolayers that had been exposed to N-ethylmaleimide, solubilised much more rapidly than those which were untreated.

FIGURE 4.2.1.

[^{3}H] L-Proline present in cell monolayer solutions after incubation after 20 minute incubation with or without excess L-proline

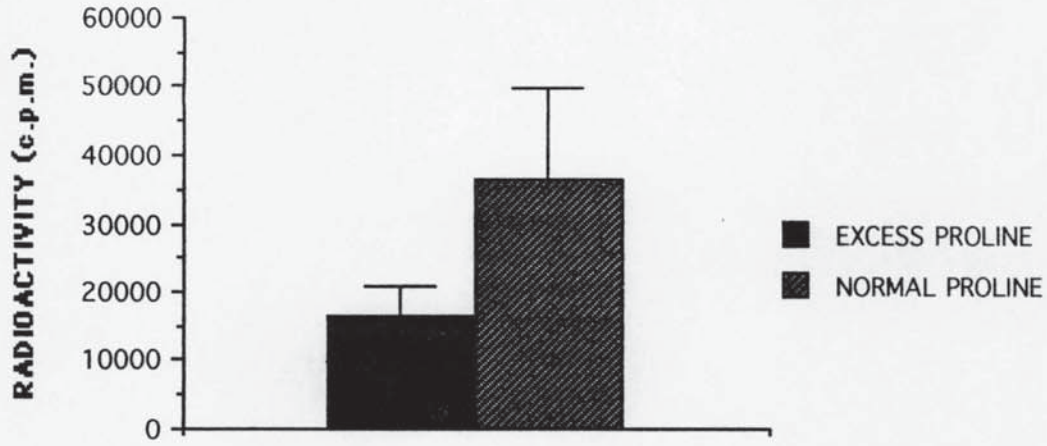
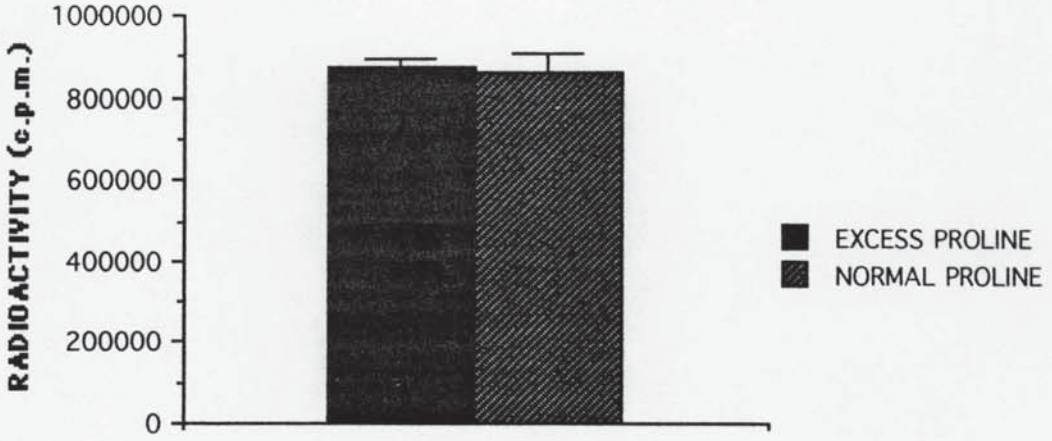


FIGURE 4.2.2.

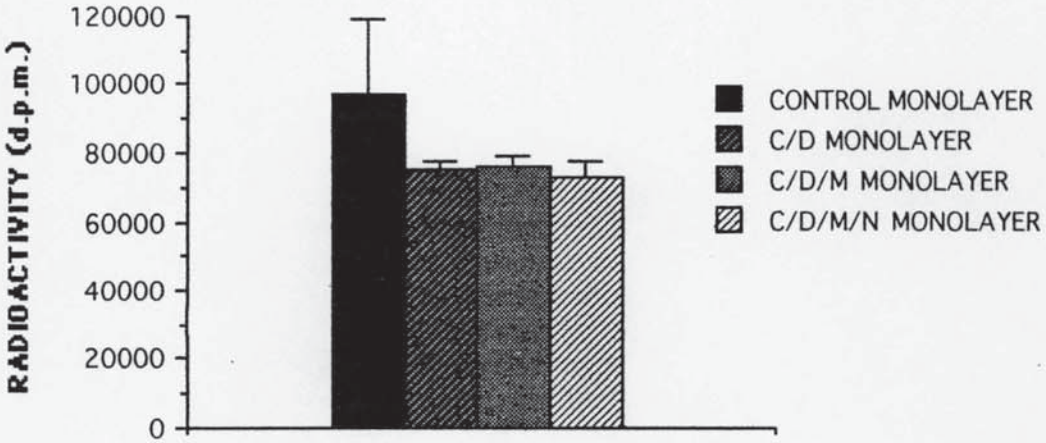
[^{3}H] L-Proline present in monolayer donor solutions after incubation with or without excess L-proline.



n = 6 for each group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 4.2.3.

[^{3}H] L-Proline present in monolayer solutions after one hour incubation with cyclophosphamide, rat microsomes and NADPH.



CONTROL = media alone.

C/D = cyclophosphamide (50 μM) and media.

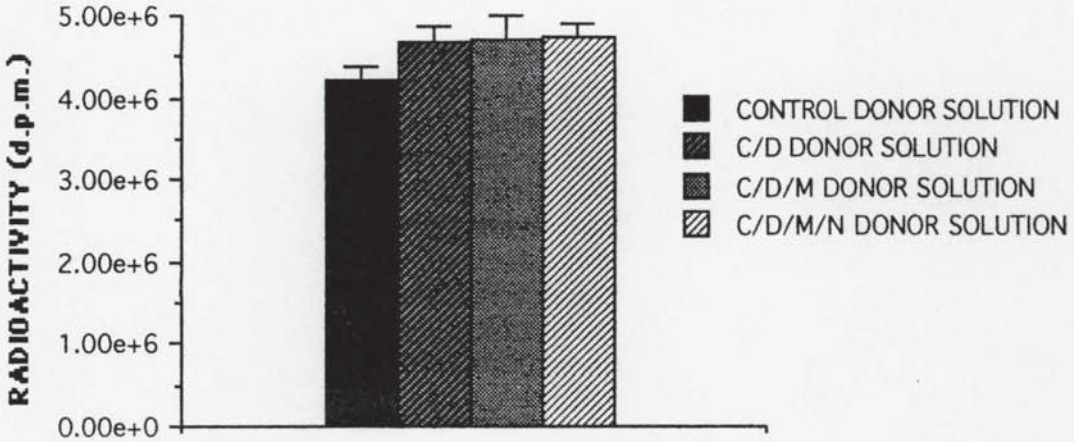
C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = cyclophosphamide, rat microsomes, NADPH (1 mM) solution and media.

n = 6 for each group with data represented as mean ± S.D. (n-1).

FIGURE 4.2.4.

[^{3}H] L-Proline present in monolayer donor solutions after one hour incubation with cyclophosphamide, rat microsomes and NADPH.



CONTROL = media alone

C/D = cyclophosphamide (50 μM) and media

C/D/M = cyclophosphamide, rat microsomes (4 mg) and media

C/D/M/N = cyclophosphamide, rat microsomes, NADPH (1 mM) solution and media

n = 6 for each group with data represented as mean ± S.D. (n-1).

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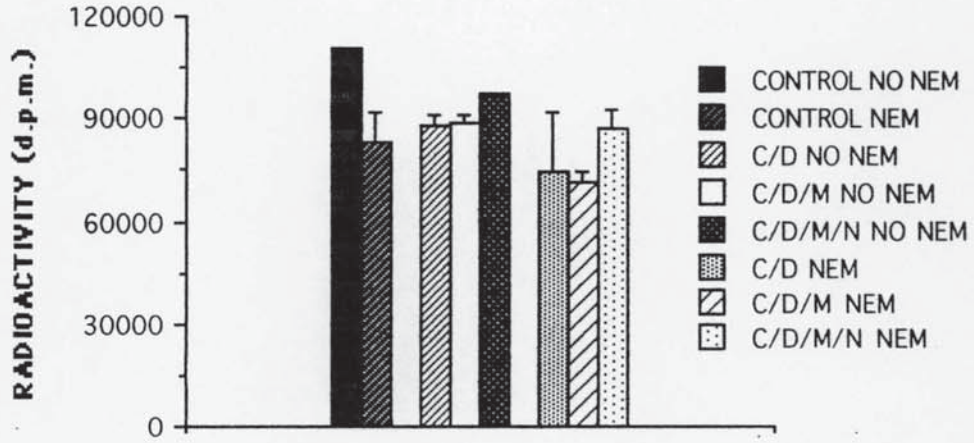
FIGURE 4.2.7.

[^{3}H] L-Proline present in monolayer solutions after one hour incubation with dapsone, rat microsomes and NADPH - pre-incubation with 6.5 nM N-ethylmaleimide.



NEM = N - ethylmaleimide

CONTROL = media alone

C/D = dapsone (50 μM) and media

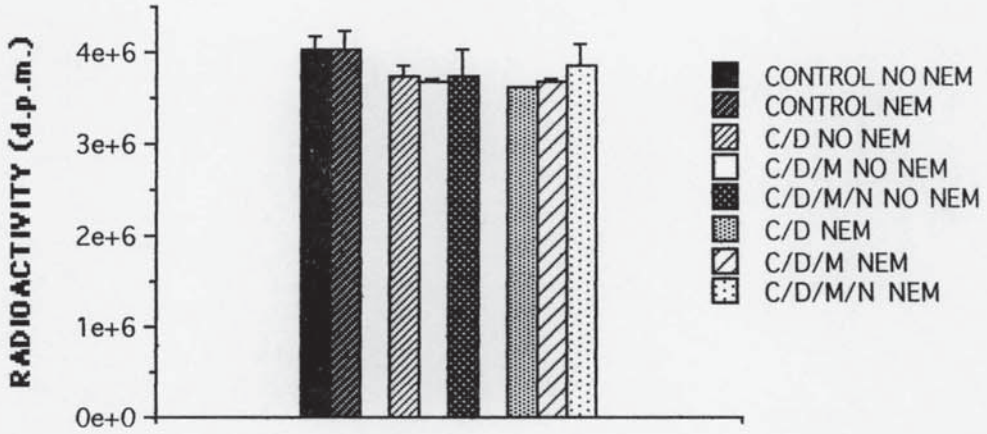
C/D/M = dapsone, rat microsomes (4 mg) and media

C/D/M/N = dapsone, rat microsomes, NADPH (1 mM) solution and media

n = 3 for each group with data represented as mean ± S.D. (n-1).

FIGURE 4.2.8.

[^{3}H] L-Proline present in donor solutions after one hour incubation with dapsone, rat microsomes and NADPH - pre-incubation with 6.5 nM N-ethylmaleimide.



NEM = N-ethylmaleimide

CONTROL = media alone

C/D = dapsone (50 μM) and media

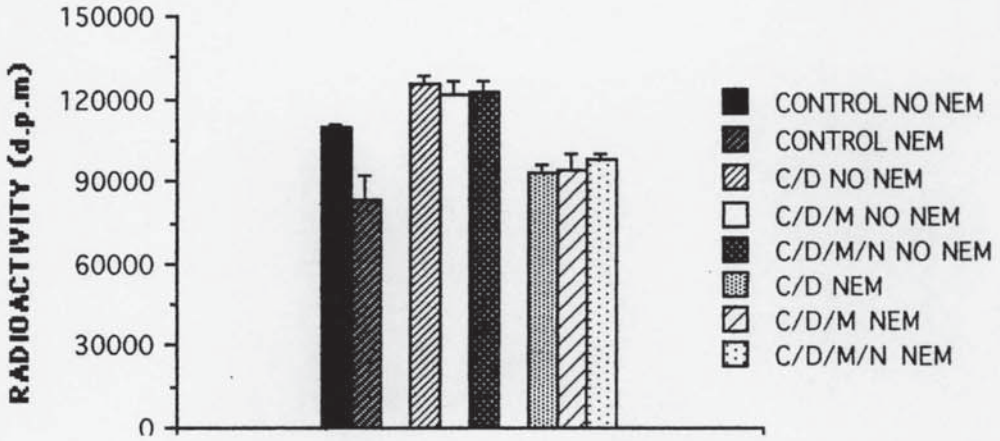
C/D/M = dapsone, rat microsomes (4 mg) and media

C/D/M/N = dapsone, rat microsomes, NADPH (1 mM) solution and media

n = 3 for each group with data represented as mean ± S.D. (n-1).

FIGURE 4.2.9.

[^{3}H] L-Proline present in monolayer solutions after one hour incubation with cyclophosphamide, rat microsomes and NADPH - pre-incubation with 6.5nM N-ethylmaleimide.



NEM = N-ethylmaleimide

CONTROL = media alone

C/D = cyclophosphamide (50 μM) and media

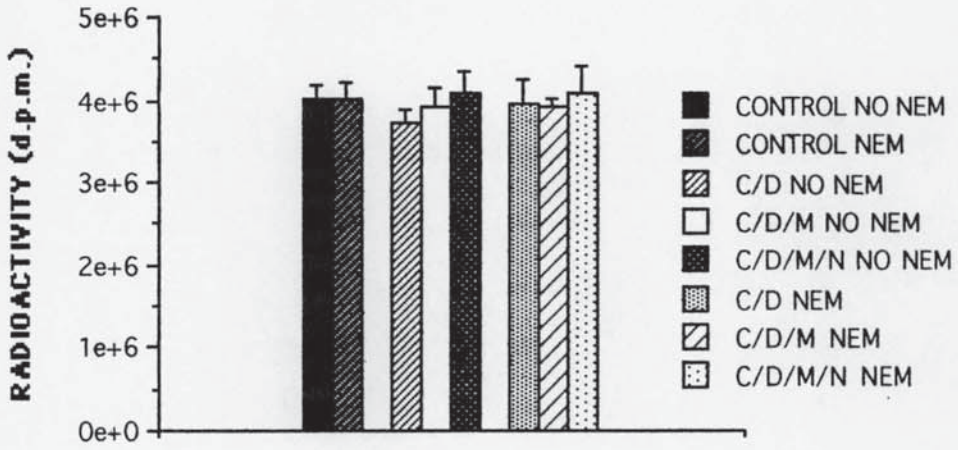
C/D/M = cyclophosphamide, rat microsomes (4 mg) and media

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media

n = 3 for each group with data represented as mean ± S.D. (n-1).

FIGURE 4.2.10.

[^{3}H] L - Proline present in donor solutions after one hour incubation with cyclophosphamide, rat microsomes and NADPH - pre-incubation with 6.5 nM N-ethylmaleimide.



NEM = N-ethylmaleimide

CONTROL = media alone

C/D = cyclophosphamide (50 μM) and media

C/D/M = cyclophosphamide, rat microsomes (4 mg) and media

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media

n = 3 for each group with data represented as mean ± S.D. (n-1).

4.3. DISCUSSION.

It is known that the metabolites of cyclophosphamide, acrolein and phosphoramide mustard, are potent cytotoxic agents both *in vivo* and *in vitro* (Subramaniam *et al.* 1994, Alberts *et al.* 1984). There are also reports of cytotoxicity with a human colon tumor cell line (Fang *et al.* 1982), therefore it might be anticipated that there would have been drug related cytotoxicity in the CACO-2 cells.

Cell toxicokinetics are similar to *in vivo* toxicokinetics, in that there are several factors that can influence the concentration of a compound, in a culture test system. The absorption, distribution, biotransformation and elimination of toxic substances all have an influence, upon the actual concentration of a toxin at the site of damage. In addition, the process is dynamic, so as the compound is transformed, its chemical nature changes and so does its absorption, distribution or elimination. Therefore the apparent lack of cytotoxicity in the CACO-2 model, could be due to an insufficient initial concentration of the reactive metabolites in contact with the plasma membrane. However, studies carried out previously, have shown evidence of cytotoxicity with lymphocytes at this concentration, with both dapsone and cyclophosphamide (Section 3). Further biotransformation maybe responsible for the lack of cytotoxicity, as there may have been conjugation reactions by the Phase II enzymes phenol sulfotransferase and UDP glucuronyl transferase. These conjugation reactions may be producing highly water soluble compounds, which were not able to penetrate the plasma membrane. In addition, previous studies have shown there can be little or no biotransformation of the parent compound, due to the effect of toxic metaolites produced initially and this is possible with the cyclophosphamide incubations, as acrolein is known to affect P-450 enzyme activity and expression (Gurtoo *et al.* 1981, LeBlanc *et al.* 1990). However as has been mentioned, the previous studies with lymphocytes indicated that there is biotransformation taking place, to a sufficient degree to produce the cytotoxic metabolites

(Section 3). This confirmed reported studies that used lymphocytes (Spielberg *et al.* 1984) and erythrocytes with a rabbit microsomal system (Wildenauer *et al.* 1982).

Binding to proteins is a well recognised factor in the distribution of a compound, both *in vivo* and *in vitro* (Timbrell 1982). It is possible that the metabolites preferentially bind to proteins in the incubation system, either on the cell matrix or in the media for example with the microsomes. The binding of metabolites of cyclophosphamide to microsomes, has been described by Wildenauer *et al.* (1982). It has been shown however, that this is unlikely to be an issue as there was cytotoxicity observed with mononuclear lymphocytes (Section 3) and erythrocytes (Section 7). It may have been that there was no effect due to no metabolite production with the microsomal system. But this too was proven not to be the case, as the experimental work with the concurrent cell line and mononuclear lymphocyte assays showed. There was a positive result with the mononuclear lymphocytes, but no cytotoxicity observed with the cell line.

The conjugation of the reactive metabolites with glutathione, is one reason for the absence of cytotoxicity. This is an important factor in defence against cytotoxicity, as glutathione is present in high concentrations in most cells, mostly as the reduced form (GSH). The effects of loss of intracellular glutathione protection have been described by several groups, especially with relation to reactive cytotoxic intermediates (Orrenius 1985, Peters *et al.* 1990, Lunel-Orsini *et al.* 1995). This cytoprotection is more evident in proliferative cell lines, than in non proliferative cells such as those freshly isolated from a host. This is due to high concentrations of not only glutathione, but also the enzymes involved in glutathione production and conjugation. This is seen with the CACO-2 cells which have high glutathione S-transferase activities, comparable with that seen *in vivo* with human small intestines (Prueksaritanont *et al.* 1996). These facts have also been reported, in a study involving a cyclophosphamide resistant medulloblastoma cell line, which had elevated levels of glutathione and increased glutathione S-transferase activity (Colvin *et al.* 1993). The nature and activity of glutathione S-transferases has been shown to increase in CACO-2 cells, as the monolayer ages (Peters *et al.* 1989) and

has been identified as having a role in resistance to other cytotoxins (Peters *et al.* 1992). These factors are a result of the secondary role glutathione plays in the cell, that of electron donor in the synthesis pathway for deoxyribose units. These are sub-units which form part of DNA and as there is an almost constant production of DNA, there is a constant requirement for glutathione. Therefore any toxic entity will be trapped and conjugated quickly before it exerts any effect, any intracellular reduction of glutathione being adjusted quickly. There maybe a reduction in colony growth, due to the possible affect on DNA synthesis, but this will not be immediate as the cell division cycle can take several hours under normal conditions.

One factor that also influences the cytotoxic response, is the time that the initial concentration of toxin is in contact with the cells. It is this factor, coupled with the high intracellular glutathione levels, that may have resulted in the negative response. It has been reported that cytotoxicity due to reactive metabolites occurs, not only when glutathione levels are reduced by pre-treatment with buthionine sulphoxime (BSO) (Riley *et al.* 1993), but also if the reactive metabolites are incubated with the cell model for long periods of time. The constant oxidative stress resulting from the reactive metabolites, depletes the glutathione to the extent that the cell is compromised and the reactive metabolites affect the plasma membrane (Richter 1987), enzymes and receptors (Roubal and Tappel 1966), as well as the cell cytoskeleton (Bellomo *et al.* 1988). The proline uptake studies were carried out for one hour and it is possible that this time period was too short to significantly deplete the glutathione reserves of the cells. Other groups have generally reported incubation time periods of at least 3 hours, before evaluating changes in cell function (Waterfield *et al* 1995, Kilgour *et al* 1995). However the use of the exogenous metabolite generating system, the rat microsomes, caused microbial contamination within the cells environment, which made long term incubation unfeasable. It is possible to produce sterile microsomal preparations, however there were not the facilities available for this during the research period. This problem of sterility with the microsomes also precluded an extended growth period, as after the

incubations, it was seen that even extensive washing of the cells and environment could not remove the contamination. This contamination resulted in the cells tending to die one day after the incubation due to the microbial infection.

To use the proline uptake system for toxicity testing, would require several changes to the system. The incubation with the reactive metabolites should be carried out for longer, to significantly deplete the intracellular glutathione, which is not possible with the current metabolite generating system. There are several alternatives which could be employed, freshly isolated hepatocytes, liver slices, primary hepatocyte cultures or hepatoma derived cell lines. Freshly isolated hepatocytes have been commonly used in *in vitro* assays (Paine 1990, LeBot *et al.* 1988, Waterfield *et al.* 1995), but rapidly lose their ability to produce P-450 enzymes after isolation. Liver slices have a longer lifespan, up to 24 hours and have been used for *in vitro* toxicology (Smith *et al.* 1985). However, both hepatocytes and liver slices are less suitable not only because of the life span, but also the sample heterogeneity which will introduce variation between tests. Primary hepatocyte cultures and cell lines have a longer lifespan, more homogeneous sample nature and have been used extensively (Hall *et al.* 1993, Waterfield *et al.* 1995, Hall *et al.* 1991). The use of such models has the disadvantage that there is considerable loss of liver specific properties, particularly the expression of Phase I and II enzymes. Regardless of this, these have been the metabolic systems chosen for a number of *in vitro* tests. The colon adenocarcinoma cell lines could be used to metabolise compounds themselves, but again the metabolic capabilities of the cells are one of the properties frequently lost. The use of cell lines and yeasts, transfected with the genetic material responsible for cyctochrome P-450 expression could represent a suitable alternative to the use of exogenous liver fractions, and the use of such systems has already been demonstrated (Gibson *et al.* 1995).

The concentrations of the test compounds could be increased, as it has been shown that there can be variability in response between the *in vivo* and *in vitro* systems and with different types of cells *in vitro* (Waterfield *et al.* 1996, Richold 1989).

The way in which the monolayers are grown and incubated is another area which could be changed. In all the uptake and transport papers, the monolayers are grown on supports either semi permeable filters or plastic supports. This exposes the basal and apical surfaces of the cells and mimics the intestinal cell structure. The system would be mimicking the physiology of the cell lines origins, in such a experimental set up and would give transport and toxicity profiles more like the *in vivo* situation. In addition, the co-incubation of the CACO-2 model with a 'feeder' endothelial cell line, could possibly have produced a response, as such this has been described as improving cell to cell transport of compounds and cell to cell signalling (Freshney 1995).

The only cytotoxicity seen in these studies was as a result of the inhibition of the phenol sulfatransferase activity. This could be because the inhibition of phenol sulfatransferase, not only as affects a detoxification pathway, but also the synthesis and possible transport pathway for steroids in the cell. The maintenance of steroid production and transport is essential for the production of a viable plasma membrane (Chen *et al.* 1978, Coleman *et al.* 1981) and it has been described that changes in membrane permeability, are indicators of cytotoxicity (Richter 1987). Another factor to consider is that the N-ethylmaleimimde not only inhibited the phenol sulfatransferase, but also depleted the cellular glutathione by reacting with the sulfhydryl group. This depletion could have been so severe that it brought about cell death, as has been described in other research (Orrenius 1985).

In conclusion, there was no significant affect observed on the uptake of L-proline, after incubation with cyclophosphamide or dapsone and the rat microsomal system. The likely reasons for this are firstly, an insufficient concentration of reactive metabolite at the site of damage, which may have been due to glutathione conjugation. Finally, it is likely that the incubation period was too short for the metabolites to have an effect. Changes in the metabolising component used could allow longer incubation times, which would reduce the cytoprotective action of glutathione and affect the transport process. Changes in the

cell culture methods, for example, use of serum free media or addition of hormones, could also affect the results making the model more like the physiological system seen *in vivo*.

SECTION 5

L6 STUDIES

5.1. INTRODUCTION.

The homeostasis of energy in cells in most living systems has been identified as important in the maintenance of cell viability (Redegeld *et al.* 1988). The free energy can be obtained from the oxidation of polysaccharides, lipids and proteins or by trapping *uv* light energy. The central carrier of free energy, for most biological processes is adenosine triphosphate (ATP), with the energy being stored in the phosphoanhydride bonds.

ATP is involved in a variety of cell processes, such as active transport pathways, biosynthesis and movement. This involvement has been demonstrated in two particular cell processes, the adenylate cyclase and the phosphoinositide cascades (Stryer 1988). These pathways are the result of transmembrane signalling and lead to a cellular response such as growth, secretion, metabolism or contraction. The response is elicited by the actions of the second messengers, cyclic adenosine monophosphate (cAMP) and inositol 1,4,5 trisphosphate (IP_3) (Figure 5.1.1.). The action of adenylate cyclase on ATP results in the production of cAMP and this is a result of hormone or agonist action on the receptor in the cell membrane. cAMP is important in cell growth, also the uptake and breakdown of glycogen in the cell and the production of cAMP can lead to changes in protein production, particularly enzymes involved in the production of energy from sources other than glucose. Similarly in the production of the phosphoinositides, ATP is required for the eventual synthesis of inositol trisphosphate (IP_3). IP_3 production opens the calcium channels of the cell and so its levels will affect calcium homeostasis in the cell.

It is known that muscle cells require high levels of ATP for contraction (Eisenberg *et al.* 1985). Therefore a cell line derived from a muscle source should retain the need for high levels of ATP and so be relevant to the study of the effects of toxins on ATP and ATP driven systems. The L6 cell line is derived fron rat thigh muscle and immortalized by treatment with 3-methylcholantrene (Sen *et al.* 1993). L6 myocytes have been extensively used in, *in vitro* studies of the various cell processes which involve ATP. The carrier mediated

active transport of hexose and glucose has been actively studied, particularly with respect to insulin stimulation (Sarabia *et al.* 1990, Cheung et al. 1984, Klip et al. 1984). Myogenesis, the growth of myocytes and cAMP dependent protein kinase have also been investigated with the cell line (Lorimer *et al.* 1989, Martelly *et al.* 1989). Stimulation of the adenylate cyclase and phosphoinositide systems have also been studied, using both hormonal and xenobiotic agonists (Ewton *et al.* 1984, Zhu *et al.* 1991).

The L6 cell line has also been used in an *in vitro* toxicity system, which studied the irritancy potential of several parental antibiotics (Laska *et al.* 1990). This demonstrated, that this cell line could be used in a cytotoxicity assay , evaluating toxicity resulting from intramuscular adminstration. The L6 model has also been used in investigations with the cytotoxic hydroxylamine metabolite of dapsone (Smith 1995). This work showed that the production of phosphoinositides was affected and there was extensive cytotoxicity, following incubation with dapsone hydroxylamine.

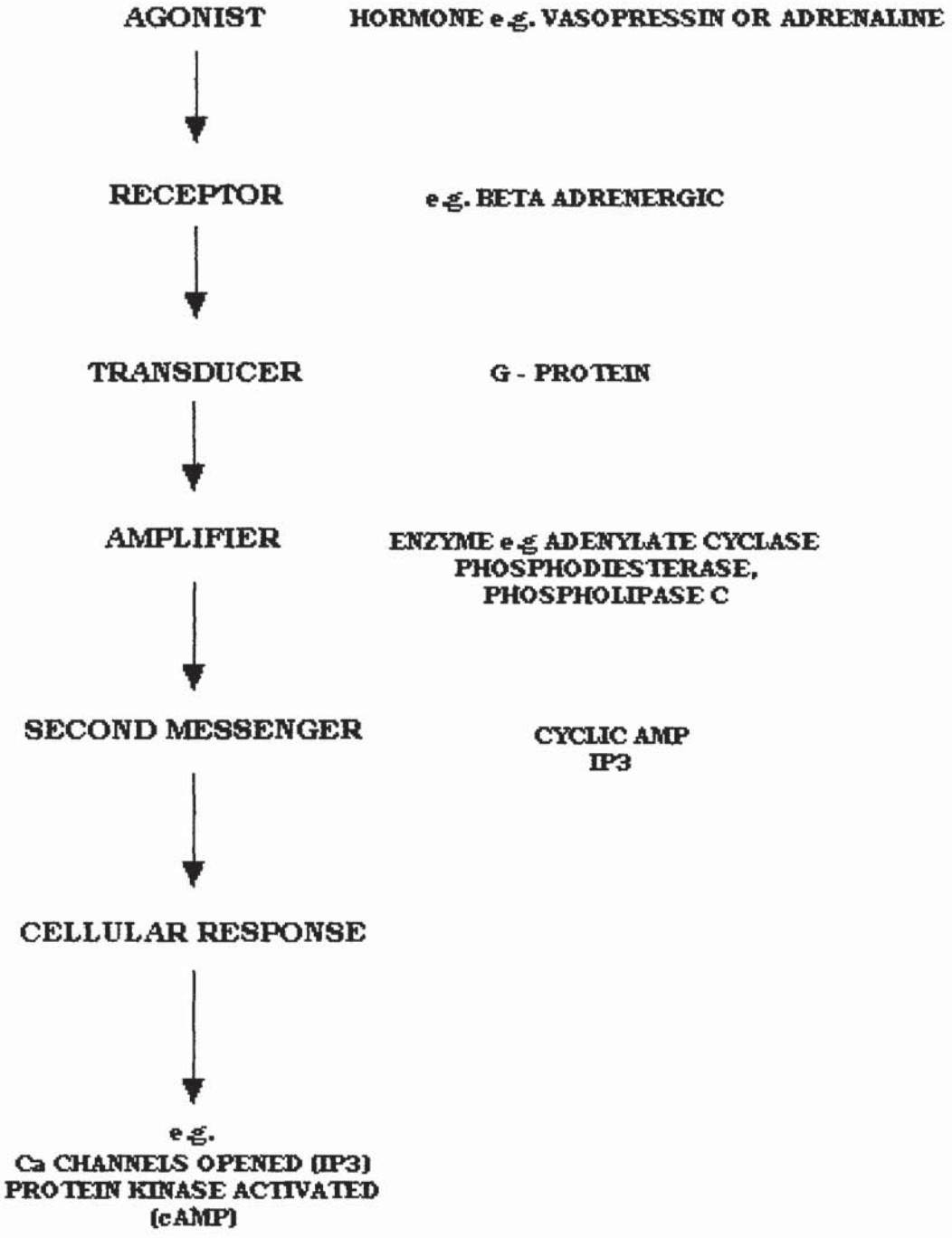
Although it is possible to evaluate cytotoxicity by measuring depletion of ATP, this may not reflect the true extent of cellular toxicity. It has been postulated that in free radical attack, ATP levels are not grossly affected initially, unlike the plasma membrane and membrane proteins (Richards *et al.* 1988). Thus, the production of the second messengers which followed receptor stimulation were proposed as markers for cytotoxicity. The measurement of cAMP formation after receptor stimulation, has been described in assays involving other cell lines which respond in a similar manner to L6 myocytes (Hanley 1987). The evaluation of cAMP relies on competition between [^{3}H] cAMP and endogenous cAMP, for cAMP binding protein. Therefore any increase in cAMP, will result in a decrease in the radioactivity measured in a sample. Similarly, the inositol phosphates, for example inositol(1,4,5)P_3 and (1,3,4)P_3, produced as a result of receptor stimulation, have been evaluated (Poyner *et al.* 1990). This was achieved by incorporation of [^{3}H] inositol into the phosphoinositides and these compounds evaluated using chloroform/methanol/10mM EDTA, 0.1M HCl extraction and anion exchange HPLC

(Jackson *et al.* 1987). In NG115-401L-C3 cells stimulation was through receptors, with bradykinin (Jackson *et al.* 1987, Hanley 1987).

The cAMP production in the L6 model, is stimulated by isoprenaline through beta adrenergic receptors. In addition, another receptor stimulated response was produced by using calcitonin gene related peptide (CGRP). A control response, which is elicited by stimulation of adenylate cyclase rather than the receptor, was observed by using forskolin. The phosphoinositide system was stimulated, using the anti diuretic hormone vasopressin.

FIGURE 5.1.1.

Sequence of events in second messenger cascade pathways



(Adapted from Challis 1994)

5.2. RESULTS

5.2.1. cAMP STUDIES

Control observations were carried out, prior to performing the metabolising incubation that would generate the cytotoxic metabolite(s), to establish that the cells reacted as previously reported (Figure 5.2.1.). The cells did respond in the manner previously described with other cell lines (Hanley 1987) and there was a decrease in radioactivity in all groups, which is consistent with increased cAMP formation.

The L6 cells were then incubated with the rat microsome system (4 mg), NADPH (1 mM) and either cyclophosphamide or dapsone (50 μM), for one hour. The cAMP assay was then repeated with these cell monolayers, along with control incubations (Figures 5.2.2 and 5.2.3).

There were no significant changes in cAMP formation following incubation with dapsone or cyclophosphamide, in the presence of rat microsome and NADPH, compared to the non stimulated control. There was no increase in cAMP, which would indicate the metabolites are not receptor agonists or adenylate stimulators. In addition, the addition of the positive control with forskolin (10μM) after the microsomal incubations, also showed that there were no significant changes in cAMP, apart from that elicited by forskolin (Figure 5.2.4.).

The concurrent incubations of L6 cells and mononuclear lymphocytes showed that there was a significant increase in cell death with the lymphocytes, but no significant changes in cAMP levels in the L6 cells (Figures 5.2.5. and 5.2.6.)

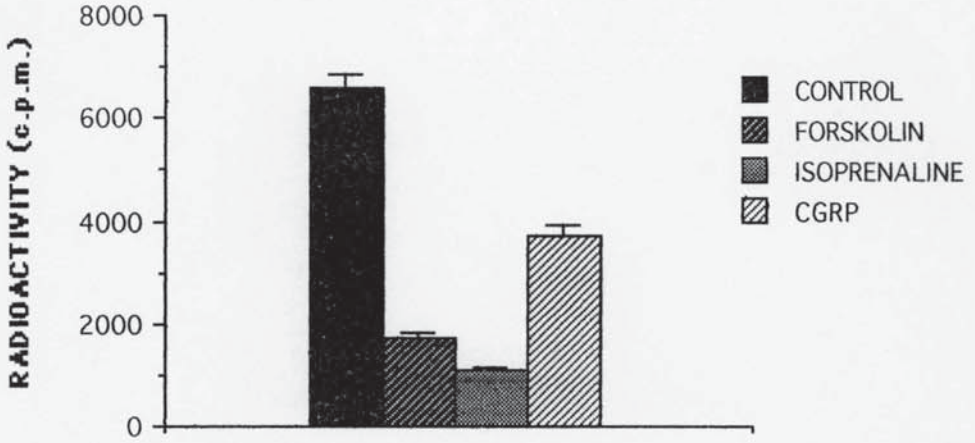
5.2.2 INOSITOL TRISPHOSPHATE (IP_3) STUDIES

Previous work (Smith 1995) had identified that dapsone hydroxylamine was not cytotoxic at 10 μM, but was cytotoxic at 20 - 100 μM (final concentration). These studies therefore evaluated the concentration at which the metabolite began to affect the I P_3 production. The concentration previously seen to be the most cytotoxic (100 μM) was included as a positive cytotoxicity control. In addition, a control of cells and media alone was added. The initial study looked at IP_3 production, after incubation with the metabolite and stimulation with vasopressin. (Figure 5.2.7.). In addition, after incubation with the hydroxylamine, the phosphoinositide pathway was stimulated using sodium fluoride. This stimulates the production of IP_3 by another non receptor pathway and would show if any inhibition was due to receptor blocking (Figure 5.2.8.).

The production of IP_3 was not affected by dapsone hydroxylamine, even at the 100 μM concentration, compared to the control (hydroxylamine free). The NaF stimulation study, also showed no significant decrease in IP_3 production after incubation with the hydroxylamine. Microscopic examination of the monolayers involved in the 100 μM concentration incubations, showed that there were some changes in cell morphology. There was evidence of slight vacuolation in the cytosol, which is an indicator of damage to the plasma membrane. However the cytotoxicity observed, was not as severe as described with the previous study (Smith 1995), in which there was 100 % irreversible cytotoxicity.

FIGURE 5.2.1.

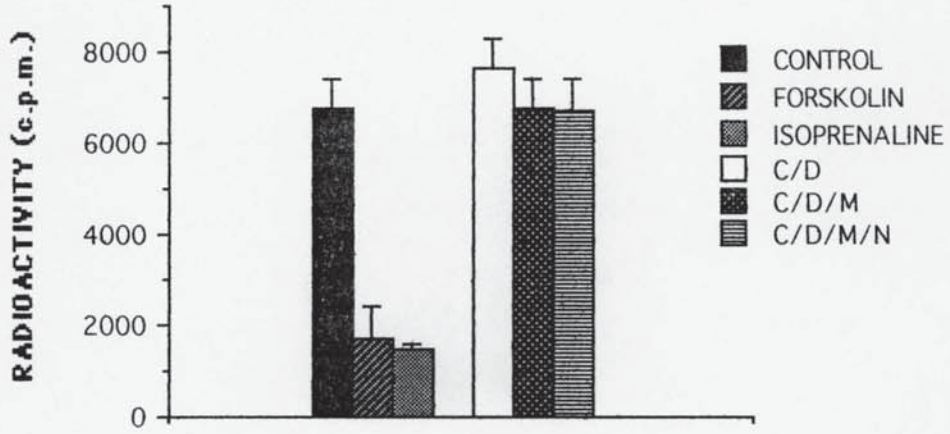
[^{3}H] cAMP content in cell supernatants after incubation with Isoprenaline, Forskolin or Calcitonin gene related peptide (CGRP).



n= 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1)

FIGURE 5.2.2.

[^{3}H] cAMP content in cell supernatants after pre-incubation with dapsone, rat microsomes and NADPH for one hour.



CONTROL = media alone.

C/D = dapsone (50 μM) and media.

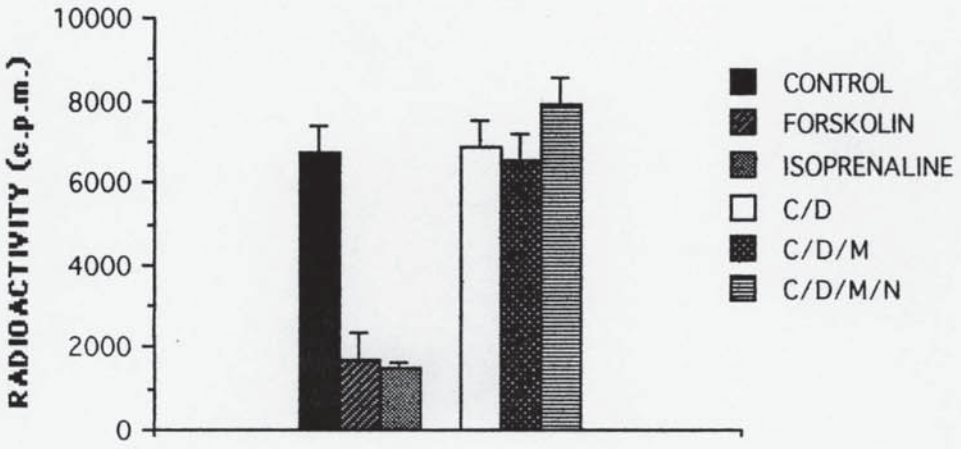
C/D/M = dapsone, rat microsomes (4 mg) and media.

C/D/M/N = dapsone, rat microsomes, NADPH solution (1 mM) and media.

n= 3 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 5.2.3.

[^{3}H] cAMP content in cell supernatants after pre-incubation with cyclophosphamide, rat microsomes and NADPH for one hour.



CONTROL = media alone.

C/D = cyclophosphamide (50 μM) and media.

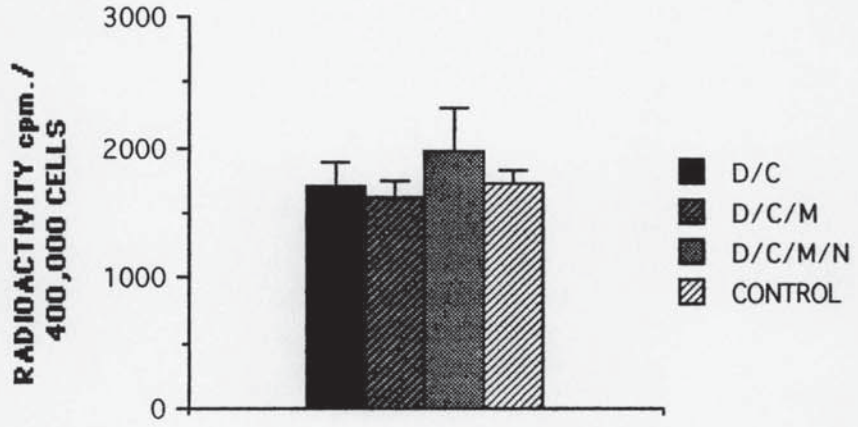
C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media.

n= 3 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 5.2.4.

[^{3}H] cAMP content in cell supernatants after pre-incubation with cyclophosphamide and rat microsomes for one hour - Stimulation with forskolin post incubation



CONTROL = media alone.

C/D = cyclophosphamide (50 mM) and media.

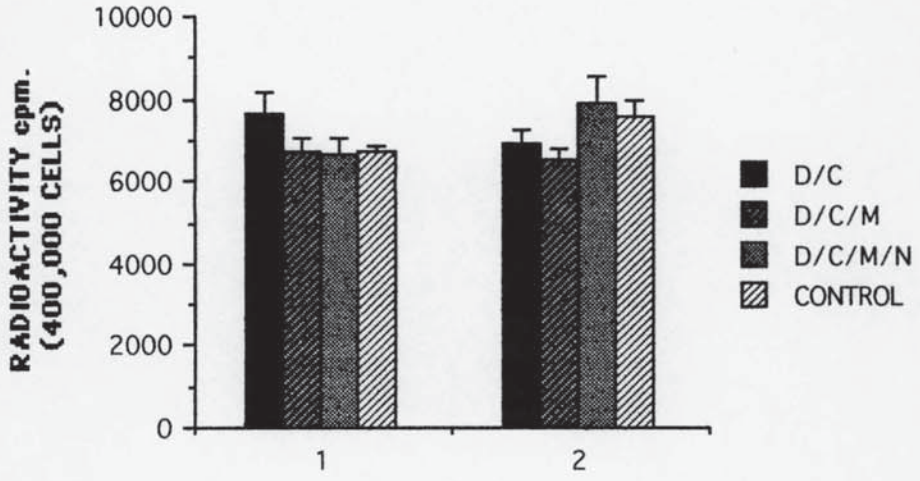
C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media

n= 6 for each incubation group with data represented as mean + S.D. (n-1).

FIGURE 5.2.5.

[^{3}H] cAMP content in L6 cell supernatants after pre-incubation with cyclophosphamide or dapsone, rat microsomes and NADPH for one hour - concurrent with MNL assay



CONTROL = media alone.

1 = Dapsone (50 mM)

2 = Cyclophosphamide (50 mM)

C/D = drug and media.

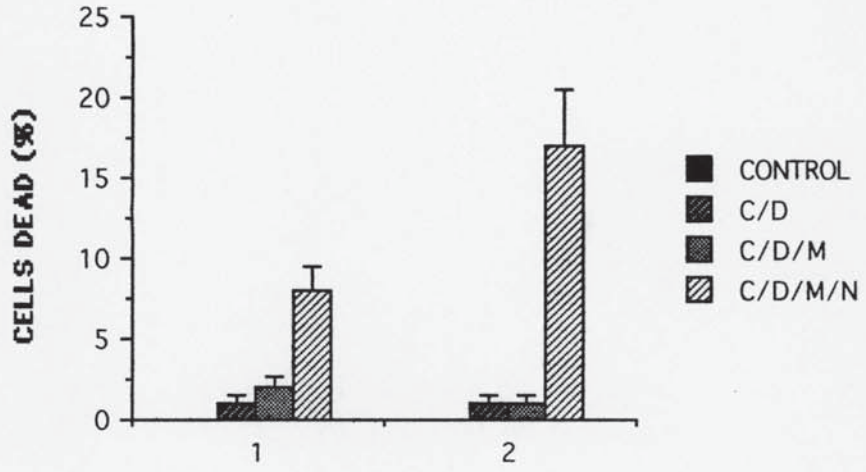
C/D/M = drug, rat microsomes (4 mg) and media.

C/D/M/N = drug, rat microsomes, NADPH solution (1 mM) and media.

n= 3 for each incubation group with data represented as mean + S.D. (n-1).

FIGURE 5.2.6.

Lymphocyte assay - % non viable cells after one hour incubation with cyclophosphamide or dapsone, rat microsomes and NADPH - concurrent with cAMP assay in L6 cells.



CONTROL = media alone.

1 = Dapsone (50 mM)

2 = Cyclophosphamide (50 mM)

C/D = drug and media.

C/D/M = drug, rat microsomes (4 mg) and media.

C/D/M/N = drug, rat microsomes, NADPH solution (1 mM) and media.

n= 3 for each incubation group with data represented as mean + S.D. (n-1)

FIGURE 5.2.7.

[^{3}H] Inositol phosphates in formate fractions after one hour incubation with dapsone hydroxylamine - Vasopressin stimulated.

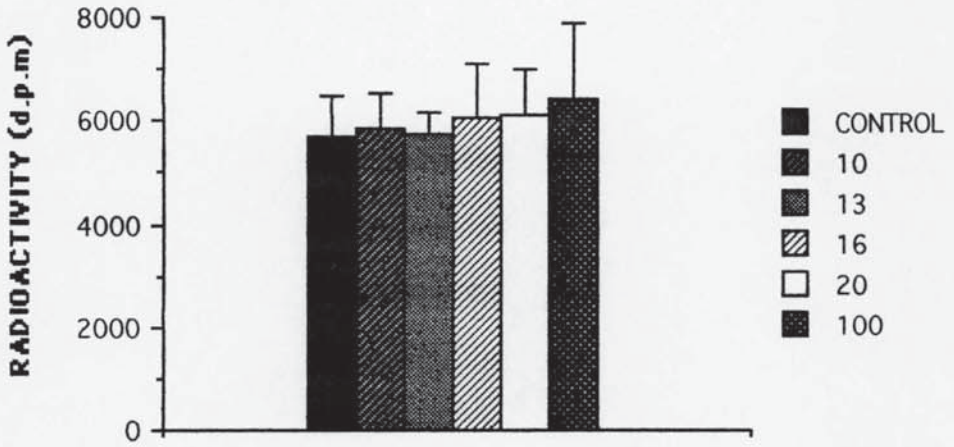
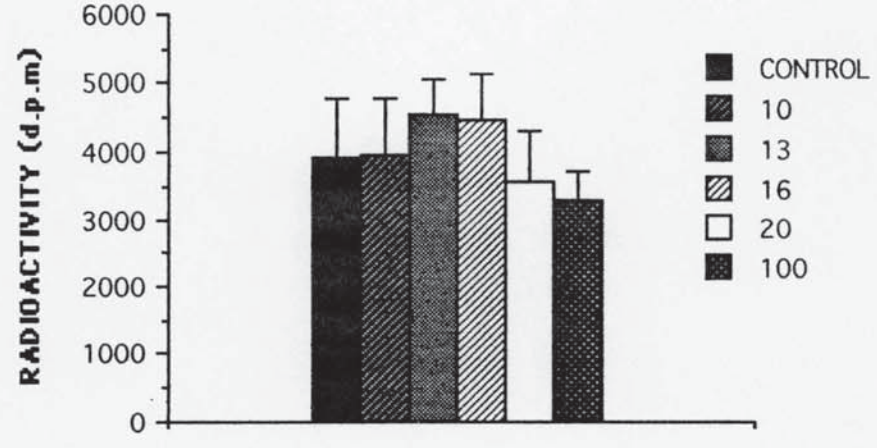


FIGURE 5.2.8.

[^{3}H] Inositol phosphates in formate fractions after one hour incubation with dapsone hydroxylamine - NaF stimulated.



CONCENTRATIONS = 10, 13, 16, 20 and 100 μM in final solution.

n= 3 for each incubation group with data represented as mean ± S.D. (n-1).

5.3. DISCUSSION

The metabolites of dapsone and cyclophosphamide have been demonstrated to be cytotoxic, both *in vitro* and *in vivo* (Coleman *et al.* 1989, Coleman *et al.* 1991, Subramaniam *et al.* 1994, Alberts *et al.* 1984). There is also evidence of cytotoxicity in cardiac muscle cells, with the cyclophosphamide metabolite, 4-hydroperoxycyclophosphamide (Levine *et al.* 1993). With the reported cytotoxicity of dapsone hydroxylamine (Smith 1995), it was expected that cAMP and IP_3 formation in the L6 model would be compromised. In addition, it was anticipated that the threshold toxicity concentration for dapsone hydroxylamine, would be established. However, there was little evidence of cytotoxicity in the studies, particularly with respect to second messenger production.

There could be several reasons for the lack of cytotoxicity, in the L6 model. The concentration of the metabolites at the cell surface is one factor and this is influenced by the toxicokinetics in the cell system. The Phase I biotransformation of the pro-drugs, may result in non-toxic species or insufficient concentrations of active metabolites. The major Phase I metabolite of dapsone is the N-oxidation product and this is not considered responsible for cytotoxicity, dapsone hydroxylamine is considered responsible (Coleman *et al.* 1990). There have been reports of cytotoxic metabolites of cyclophosphamide, affecting cytochrome P-450 expression and activities (Gurtoo *et al.* 1981, LeBlanc *et al.* 1990). However, studies carried out with the metabolite generating system and lymphocytes, have shown that there is cytotoxicity at this concentration. Similarly, *in vitro* toxicity tests involving microsomal or S9 fraction and either lymphocytes or erythrocytes, have also shown that there was cytotoxicity with cells incubated with the generated metabolites (Spielberg *et al.* 1984, Kugler *et al.* 1987, Sbrana *et al.* 1984, Wildenauer *et al.* 1982).

It is possible in this model, that again, toxic metabolites were conjugated with glutathione. Glutathione (GSH) is an important cytoprotectant and particularly in skeletal muscle, as it manages the oxidative stress resulting from exercise (Lew *et al.* 1985,

1991, Sen *et al.* 1992). The L6 cell line has been observed to contain high levels of total intracellular glutathione and a particularly efficient glutathione metabolism system (Sen *et al.* 1993). In addition, proliferative cells like the L6 cell line, have been shown to have elevated levels of glutathione and enzymes involved with glutathione, although there can be up to 200 fold difference between various cell lines (Wolf *et al.* 1987). There are also differences in GSH levels and in GST expression when looking at confluent cells and cells in logarithmic growth or drug sensitive and drug resistant cell lines. This is due to the other role of glutathione, that of the electron donor in deoxyribose synthesis. Therefore the toxic metabolites will have to deplete a large intracellular GSH pool and irreparably damage the GSH metabolism enzymes, before significant cytotoxicity will occur. Measurements of glutathione in the L6 cell line are lower than that of erythrocytes, that is 0.2 mM compared with approximately 0.6 mM (Section 6) which would appear contradict the presence of a large intracellular pool. However there is no depletion of glutathione seen with the cyclophosphamide metabolite generating system in the L6 cells, as there is with the erythrocytes. It may also be that the cell line is actively transporting glutathione extracellularly to conjugate with metabolites. With cell lines, cytotoxicity can cause an increase in the enzymes responsible for drug metabolism (Freshney 1995), so although intracellularly there maybe lower levels of GSH, extracellularly levels maybe high.

In chapter 4, it was noted that the amount of time that reactive metabolites are present at the site of damage, was another factor to be considered. Studies involving erythrocytes, which are reported in this thesis (Section 6), did exhibit toxicity after an incubation period of one hour. But the studies with the L6 model, did not show significant cytotoxicity and like the CACO-2 studies, may be due to the insignificant depletion of glutathione during the incubations.

To use the L6 test for observing cytotoxicity, several changes would have to be made and several further studies carried out, before starting to validate the test. The metabolite incubation time should be extended and the use of a microsomal metabolite generating

system reviewed. The ideal system would be endogenous for example, cultured primary hepatocytes or hepatocyte cell lines and are used in other *in vitro* tests (Hall *et al.* 1991, 1993, Waterfield *et al.* 1995). The manner in which the cells are grown and incubated, is another area which could affect the possibility of cytotoxicity. Skeletal muscle cells grow in bundles in the body, so the mimicking of this *in vitro* may give more representative results. The cells could be induced to reaggregate, by growing on cellophane for example (Halbert *et al.* 1971) or by constantly rotating cells (Atterwill 1987). Alternatively cells could be grown in a plastic filament culture system which mimics muscle fibres. Co-incubation of various cell lines with a 'feeder' layer of epithelial cells, may also give a more representative response, as the epithelial cells encourge cell-cell signalling. Cell-cell signalling helps in maintaining the cell phenotype, regulation of growth and importantly cascade interactions for example, the phosphoinositide pathway (Freshney 1995)

In conclusion, no significant irreversible cytotoxicity occured with the studies involving L6 cells. The likely reasons for this are; an insufficient concentration of reactive metabolite, due to glutathione conjugation and too short a metabolite incubation period. Changes in the metabolising system used, could allow longer incubation times and so reduce the cytoprotective action of glutathione. Changes in the cell culturing procedures, to mimic skeletal muscle structure, could further improve the test and so yield results which may correlate better with *in vivo* studies.

SECTION 6

GLUTATHIONE STUDIES

6.1. INTRODUCTION

The eventual fate of a xenobiotic, regardless of its route of absorption, will be its transfer to the circulatory system, for transport to the liver. Often there will be binding of the compound to circulatory proteins or to the cells in the circulation, making the system a distribution compartment in the body. Then, after metabolism in the liver, the metabolites of the chemical may also be further transported around the circulatory system, exerting their effects on tissues. Once again, the circulatory system can represent a tissue binding compartment for such moieties, as well as a transport system. Any toxicity from either the parent compound or the metabolites, can often be detected in this system at a very early stage. Consequently considerable work has shown the cytotoxic potential of a number of compounds, using cells derived from the circulatory system (Spielberg 1984, Coleman *et al.* 1991, Palmen and Evelo 1993)

The circulatory system might be regarded as three compartments; the generative bone marrow, the distributive vascular/lymphatic system and the peripheral organs (Luke and Betton 1987). It is possible to look at the effects of toxic compounds in cells from all of these compartments, as there are cell culture systems available from each area. However the distribution compartment is the immediate acceptor of a compound in the haemic system and so often primarily affected. The toxicity could affect haemostasis for example, by prolonging clotting time or could change the cellular constitution of the blood. Such toxicity could also affect erythrocytes and their ability to transport oxygen (Peisach *et al.* 1975).

Toxic compounds can compromise erythrocytes in several ways, by affecting membrane integrity, the haemoglobin or the cytoprotective systems of the cell. Membrane damage can involve lipid peroxidation, membrane protein cross linking or a combination of both of these events. Lipid peroxidation is a free radical generated process, where the plasma membrane integrity is compromised, by conversion of polyunsaturated fatty acids in the membrane, to lipid radicals. The free radical maybe formed by a xenobiotic spontaneously

or as a result of metabolism. Peroxidation increases membrane permeability, which can allow an influx of the toxic agents, water and ions like Ca^{2+}. The peroxidation also affects the protein structures in the membrane, with the lipid free radicals reacting with proteins, causing further radical generation and membrane cross-linking. There are changes in the activity of enzymes utilized in cell defence, so this will affect the cells' defensive capabilities. The effects on various enzymes, particularly glutathione S-transferase, superoxide dismutase and glucose-6-phosphate dehydrogenase, have been noted in several papers investigating erythrocyte toxicity (Ansari *et al.* 1987, Trieff *et al.* 1993). The protein pumps essential for the active transport of Na^{+} and Ca^{2+} out of the cell may also be affected (Jacob *et al.* 1968, Maridonneau *et al.* 1983). This coupled with increased membrane permeability, results in changes in ionic and water movement in the cell and consequently cell swelling. In general, the damage is also seen in many types of cells and in membrane bound organelles such as the endoplasmic reticulum , which will affect a variety of cellular functions particularly detoxification. Toxic compounds affect the haemoglobin, by oxidizing the haem group and producing methaemoglobin. This can be further oxidized producing reversible and irreversible haemichromes, which represent the oxidation states of the altered iron-porphyrin complexes (Peisach *et al.* 1975). Irreversible haemichromes are unstable and following denaturation, will precipitate in the cell membrane as Heinz bodies, so decreasing cell flexibility.

The peroxidation of the erythrocyte membrane is not unexpected, as it has a high content of polyunsaturated fatty acids, which give it the flexibilty required in circulation. However the red cell is protected by several antioxidation mechanisms, which require vigorous challenge or inhibition by reactive compounds, before membrane and haemoglobin damage is seen (Table 6.1.1.). This cytoprotection involves enzymes such as catalase and superoxide diamutase and reducing compounds. Of all these reducing compounds, glutathione and its concentration, both in the cell and at the membrane, is of major importance in defence against toxicity. Depletion of intracellular glutathione has

been demonstrated to lead to cell lysis in erythrocytes and other mammalian cells (Kosower *et al.* 1981, 1982, Orrenius 1985).

TABLE 6.1.1.

Principal Pathways, Enzymes, and Reducing Compounds Utilized in Erythrocyte Oxidative Damage.

PATHWAYS	Glycolytic (GLY)
	Hexose Monophosphate System (HMS)
ENZYMES	Superoxide dismutase
	Catalase
	Glutathione peroxidase (HMS)
	Methaemoglobin reductases
REDUCING COMPOUNDS	NADH (GLY)
	NADPH (HMS)
	GSH (HMS)
	Ascorbic acid
	Vitamin E

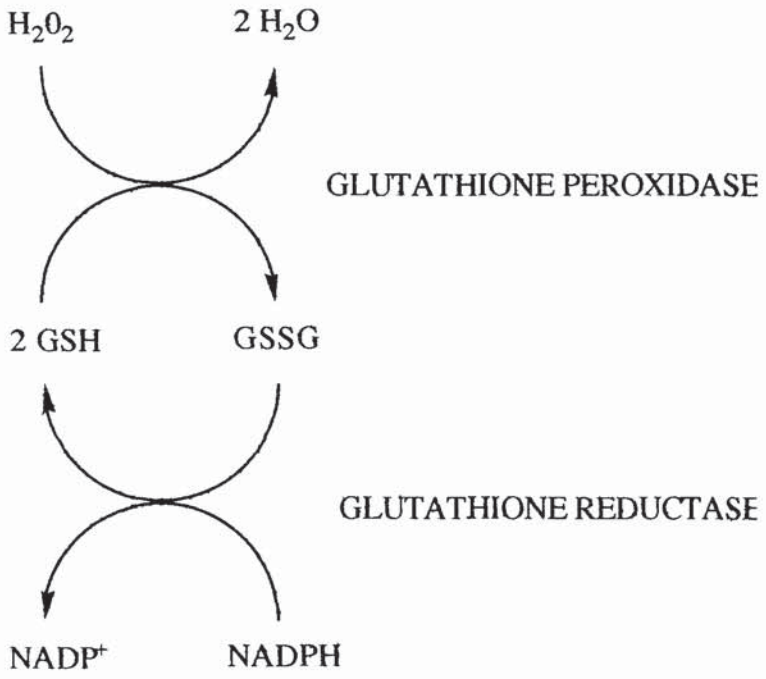
Glutathione or L-γ-glutamyl-L-cysteinyl-glycine, is the key compound in the cytoprotective γ-glutamyl cycle (Meister 1983, Deneke *et al.* 1989, Sen *et al.* 1992). In the cell, nearly all glutathione exists in the reduced form (GSH), with remainder present as the disulphide (GSSG). The by-products of aerobic life, for example, hydrogen peroxide and organic peroxides, are normally the compounds detoxified by the glutathione redox system (Figure 6.1.1.). This redox system requires NADPH, which in the red cell, must come from the hexose monophosphate pathway, as it lacks mitochondria. Therefore,

the production of glutathione in the γ-glutamyl cycle is influenced not only by the feedback inhibition with glutathione, but also by the hexose monophosphate system.

The measurement of glutathione and the activity of the γ-glutamyl enzymes, can provide indications of toxicity, both with erythrocytes and other cells. Consequently, the use of these functions has be described with many *in vitro* toxicity tests (Tew *et al.* 1986, Bump *et al.* 1983, Ansari *et al.* 1987, Ciccoli *et al.* 1994). In these studies, total glutathione in erythrocytes and the cell lines, was determined using the method described by Anderson (1985). In addition, the total haemoglobin, methaemoglobin and reduced haemoglobin of the erythrocytes were determined, to give further information on the cellular changes, after incubation with the drugs, rat microsomes (2 mg/ml) and NADPH (1 mM). Cyclophosphamide, phenytoin, carbamazepine and sulphamethoxazole were used, all of which have been documented as producing toxic effects against erythrocytes (Section 1.9.).

FIGURE 6.1.1.

Detoxification of hydrogen peroxide by the glutathione redox cycle.



6.2. RESULTS

The initial studies involved the use of an NADPH regenerating system, with the rat microsomes (2 mg) and cyclophosphamide (50 μM) (Figure 6.2.1.). There was a significant decrease in glutathione content in the erythrocytes incubated with cyclophosphamide, rat microsomes and NADPH regenerating system compared to all the other incubation groups. The metabolite generating group (0.320 ± 0.031 mM) showed the greatest decrease, when compared to the drug and microsome control (1.242 ± 0.079mM); $P < 0.05$. There was also a significant decrease with the metabolite generating group when compared to the drug control (0.917 ± 0.150 mM) and the erythrocyte only control (0.911 ± 0.033 mM). Differences were considered statistically significant for $p<0.05$ using the Student's two tailed 't' test. The samples from metabolite generating group, also showed appreciable haemolysis after centrifugation which could have contributed to the glutathione depletion. Therefore the experiment was repeated, to establish if the haemolysis seen was an isolated event. The procedure was identical, except that a regenerating system and microsome control group was included (Figures 6.2.2., 6.2.3., 6.2.4. and 6.2.5.). In addition, all the incubations were performed with phenytoin as the drug solution (Figures 6.2.6., 6.2.7., 6.2.8. and 6.2.9.).

There was a significant decrease in erythrocyte glutathione in the cyclophosphamide, rat microsomes and NADPH regenerating incubation (0.189 ± 0.041 mM), compared to the erythrocyte only control (0.445 ± 0.071 mM). In addition, there was a significant decrease compared to the drug control (0.521 ± 0.083 mM) and the erythrocyte only control (0.420 ± 0.047 mM). However the NADPH regenerating system control incubation (0.265 ± 0.023 mM), also showed a significant decrease in glutathione, compared to the erythrocyte only control. Differences were considered statistically significant for $p<0.05$ using the Student's two tailed 't' test. The samples from the regenerating system control and the metabolite generating incubations, showed haemolysis after centrifugation, to the same degree seen in the previous experiment. This

haemolysis was confirmed, when the amount of total haemoglobin was determined in all samples. There was significantly less haemoglobin in the regenerating system control (4.32 $\pm$ 0.46 g/dL) and metabolite generating incubation (3.50 $\pm$ 0.35 g/dL) samples, compared to the erythrocyte control (7.90 $\pm$ 0.38 g/dL); $P < 0.05$. In addition, there was an increase in reduced haemoglobin with the regenerating system control (16.05 $\pm$ 5.02%) and metabolite generating incubation (7.92 $\pm$ 4.06 %) samples, compared to the erythrocyte control (0.56 $\pm$ 0.17 %). There was also a significant increase in methaemoglobin in the regenerating system control (3.50 $\pm$ 0.22 %) and metabolite generating incubation (5.65 $\pm$ 0.58 %) samples, compared to the erythrocyte control (0.30 $\pm$ 0.21 %). Again, differences were considered statistically significant for $p<0.05$ using the Student's two tailed 't' test.

With the phenytoin incubations, there was no significant difference in the glutathione content of the metabolite generating incubation samples, compared to the erythrocyte control. There was haemolysis in the metabolite generating incubation samples, as seen in the cyclophosphamide incubations and this is shown in decrease the total haemoglobin content of the metabolite generating samples (4.98 $\pm$ 0.17 g/dL), compared to the erythrocyte control (7.96 $\pm$ 0.38 g/dL). There was a significant increase in reduced haemoglobin in the metabolite generating incubations (23.62 $\pm$ 0.99 %) samples, compared to the erythrocyte control (0.56 $\pm$ 0.17 %). There was also a significant increase in methaemoglobin in the metabolite generating incubations (4.90 $\pm$ 0.08 %) samples, compared to the erythrocyte control (0.30 $\pm$ 0.21 %). Differences were considered statistically significant for $p<0.05$ using the Student's two tailed 't' test.

As the results indicated that the NADPH system was responsible, in some part, for the cytotoxicity seen in the erythrocytes, further experiments were carried out involving the NADPH generating system. Components of the NADPH regenerating system were individually incubated with erythrocytes, to establish the causitive agent(s). In addition, microsomal incubations involving the regenerating system or NADPH solution (1 mM), were also carried out, with or without the addition of catalase (50 units) (Table 6.2.1.).

There was a significant decrease in glutathione in the erythrocyte, rat microsomes (2mg/ml) and NADPH solution incubations (0.182 $\pm$ 0.054 mM), compared to the erythrocyte only control (0.358 $\pm$ 0.048 mM). In addition, there were decreases with the erythrocyte, rat microsomes and NADPH regenerating system with catalase (50 units) incubations (0.195 $\pm$ 0.046 mM) and the erythrocytes and NADP incubations (0.186 $\pm$ 0.013 mM), compared to the erythrocyte control. When the total haemoglobin was evaluated, only the erythrocytes and NADP incubations (6.33 $\pm$ 0.42 g/dL) showed a significant decrease, compared to the erythrocyte control (8.10 $\pm$ 0.20 g/dL).

There were significant increases in reduced haemoglobin, with the erythrocytes, rat microsomes and NADPH regenerating system, both with (25.30 $\pm$ 2.67 %) and without (18.20 $\pm$ 4.12 %) catalase, compared to the erythrocyte control (0.80 $\pm$ 0.10 %). The erythrocyte, rat microsomes and NADPH incubations also showed increases, both with (2.73 $\pm$ 0.81 %) and without (2.00 $\pm$ 0.87 %) catalase, compared with the erythrocyte control. The erythrocyte and NADPH regenerating system incubations (2.00 $\pm$ 0.10 %) also showed increases in reduced haemoglobin, compared to the erythrocyte control incubations.

There were significant increases in methaemoglobin in the erythrocyte, microsomes and regenerating system incubations, both with (4.67 $\pm$ 0.67 %) and without (4.73 $\pm$ 0.21%) catalase, compared with the erythrocyte control (0.37 $\pm$ 0.15 %). There were also significant increases in the erythrocyte, microsomes and NADPH solution incubations, both with (1.20 $\pm$ 0.10 %) and without (1.10 $\pm$ 0.10 %) catalase, compared with the erythrocyte control.

In all the parameters, differences were considered statistically significant for $p<0.05$ using the Student's two tailed 't' test.

Based on these results, the subsequent microsomal incubation experiments were carried out using the NADPH solution, as it demonstrated less cytotoxicity to the erythrocytes.

The incubations were carried out as before, with cyclophosphamide, phenytoin, carbamazepine and sulphamethoxazole (50 μM) (Figures 6.2.10., 6.2.11., 6.2.12. and 6.2.13).

There was a significant decrease in glutathione levels with the incubations containing the erythrocytes, rat microsomes, NADPH solution and the solutions of cyclophosphamide, carbamazepine and sulphamethoxazole. The metabolite generating incubation containing cyclophosphamide (0.44 ± 0.04 mM) was the most statistically significant compared to the erythrocyte, microsomes and NADPH control (0.52 ± 0.07 mM). The incubations with sulphamethoxazole (0.43 ± 0.08 mM) showed the next most significant reduction and then finally the incubations with carbamazepine (0.47 ± 0.034 mM). There was no significant decrease in the glutathione content of erythrocytes incubated with rat microsomes, NADPH solution and phenytoin. Differences were considered statistically significant for $p<0.05$, using the Student's two tailed 't' test with Bonferroni's correction. There was no significant decrease in total haemoglobin with any of the metabolite generating incubations, compared to the microsomes and NADPH control. In addition, there were no increases in the percentage of reduced haemoglobin, with any of the metabolite generating incubations. There were significant increases in methaemoglobin, with the sulphamethoxazole (2.44 ± 0.15 %), cyclophosphamide (1.56 ± 0.42 %), and phenytoin (1.30 ± 0.23 %) metabolite generating incubations, compared to the microsomes and NADPH control (0.60 ± 0.25 %). There was no significant difference seen with the carbamazepine metabolite generating incubations.

Once cytotoxicity towards erythrocytes had been established, in the one compartment incubations, further experiments would establish the capacity of the reactive metabolites to exert the toxic effects, when separated from the 'target' erythrocytes by a semi permeable membrane. Incubations were carried out using a two compartment system, where the erythrocytes were separated by a semi permeable membrane, from the metabolite generating system of drug solutions, rat microsomes and NADPH solutions. The

drug solutions previously used were involved in these experiments, with the exception of carbamazepine (Figures 6.2.14. to 6.2.25.).

There was a significant decrease in glutathione in the erythrocytes incubated with cyclophosphamide (50 μM), rat microsomes (2 mg/ml) and NADPH (1 mM)(0.953 ± 0.110 mM), compared with the microsomes and NADPH erythrocyte control (1.124 ± 0.032 mM). Differences were considered statistically significant for $p<0.05$, using the Student's two tailed 't' test. There were no significant changes in the total haemoglobin content, of the erthyrocytes incubated with the cyclophosphamide, rat microsomes and NADPH solution, compared to the microsomes and NADPH erythrocyte control. In addition, there were no significant changes in the percentage of reduced haemoglobin or the percentage of methaemoglobin in the erythrocytes, compared to the control.

In the erythrocytes incubated with phenytoin (50 μM), rat microsomes (2 mg/ml) and NADPH (1 mM), there were no significant changes in glutathione levels after incubation, compared to the microsome and NADPH erythrocyte control. There were also no changes in total haemoglobin, reduced haemoglobin or methaemoglobin, compared to the control.

The erythrocytes incubated with sulphamethoxazole (50 μM), rat microsomes (2mg/ml) and NADPH (1mM), showed no significant decrease in glutathione, compared to the microsome and NADPH erythrocyte control. There was also no change in the total haemoglobin content or the percentage of reduced haemoglobin in the erythrocytes, compared to the control. There was a significant increase in the percentage of methaemoglobin in the erythrocytes incubated with sulphamethoxazole, rat microsomes and NADPH (2.16 ± 0.44 %), compared to the microsomes and NADPH control (0.62 ± 0.13 %).

There was a significant reduction in the erythrocytes that were pre-treated with diethyl maleate (50 μM) before the cyclophosphamide, rat microsomes and NADPH incubation (0.321 ± 0.028). In addition, there was a significant difference between the DEM treated control blood (0.381 ± 0.029) and untreated control blood (0.561 ± 0.021)(Figure

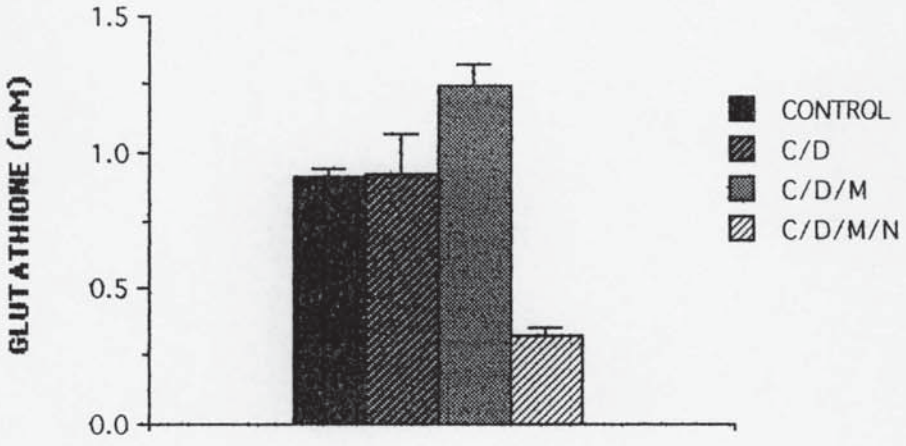
6.2.26.). Differences were considered statistically significant for $p<0.05$, using the Student's two tailed 't' test.

The incubations with cyclophosphamide, rat microsomes and NADPH were repeated, with cultures cell lines as the target cells. The cell lines used were those involved in other studies in this thesis, the CACO-2, L6 and NG115-401L-C3 cell cultures. The incubations were carried out as before and the glutathione determined using the method of Anderson (1985) (Figures 6.2.27., 6.2.28. and 6.2.29.).

There was no significant reduction in the glutathione content of the CACO-2 or L6 cells, after incubation with cyclophosphamide (50 μM), rat microsomes (2 mg/ml) and NADPH solution (1mM), compared with the cells alone control. In the NG115-401L-C3 experiment, there was a significant reduction in the glutathione content of the cells that had been incubated with cyclophosphamide solution only (1.795 ± 0.148 mM), compared to the cells only control (2.205 ± 0.219 mM).

FIGURE 6.2.1.

Glutathione content of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50% Haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

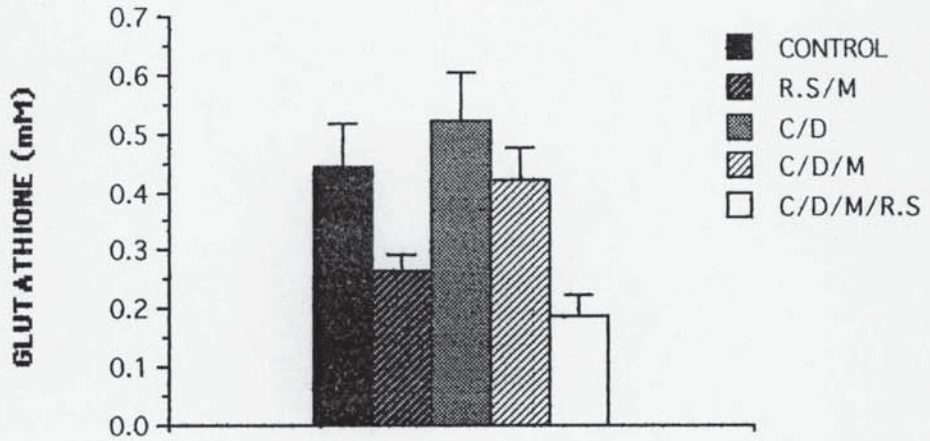
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH regenerating system (1 mM) and buffer.

n = 4 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.2.

Glutathione content (mM) of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50% Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

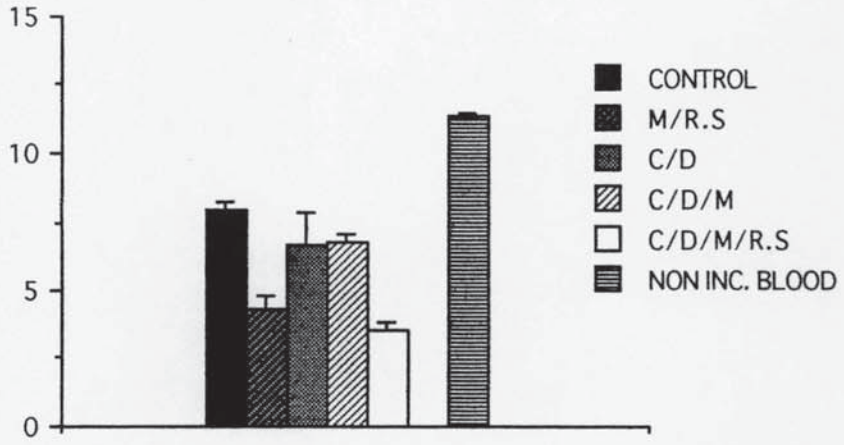
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes and buffer.

C/D/M/R.S. = Erythrocytes, cyclophosphamide, rat microsomes, NADPH regenerating system (1 mM) and buffer.

n = 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.3.

Total haemoglobin (g/dL) content of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50% Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

C/D/M = Erythrocytes, cyclophosphamide, rat microsomes and buffer.

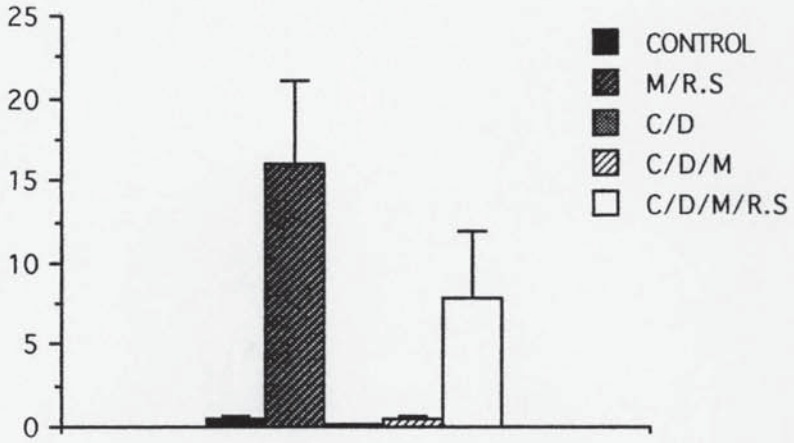
C/D/M/R.S. = Erythrocytes, cyclophosphamide, rat microsomes, NADPH regenerating system (1 mM) and buffer.

NON INC. BLOOD = Fresh blood which has not been incubated or sample processed.

n = 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.4.

Reduced haemoglobin (%) in erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50 % Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

C/D/M = Erythrocytes, cyclophosphamide, rat microsomes and buffer.

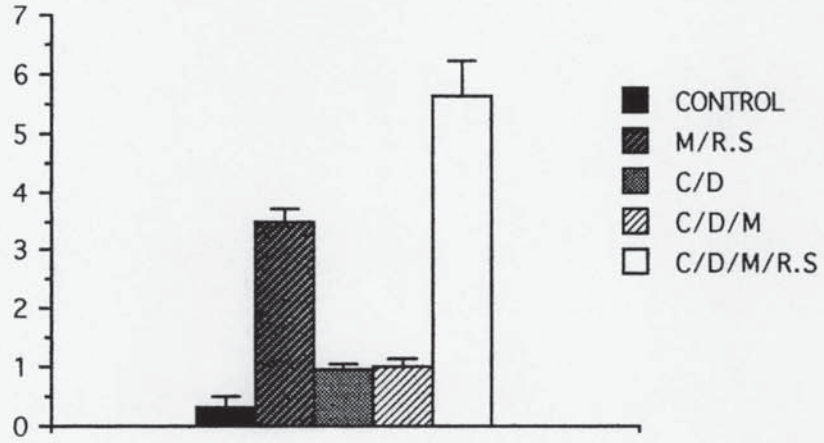
C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH regenerating system (1 mM) and buffer.

NON INC. BLOOD = Fresh blood which has not been incubated or sample processed.

n = 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.5.

Methaemoglobin (%) in erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50% Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

C/D/M = Erythrocytes, cyclophosphamide, rat microsomes and buffer.

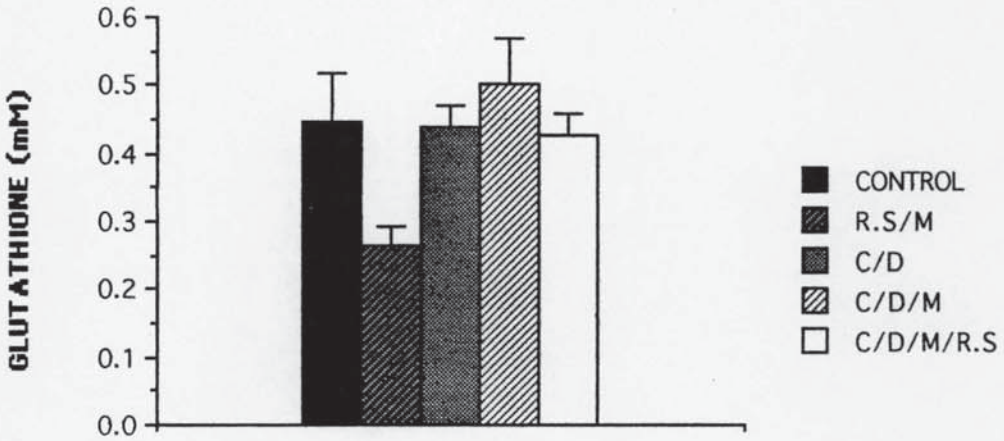
C/D/M/R.S. = Erythrocytes, cyclophosphamide, rat microsomes, NADPH regenerating system (1 mM) and buffer.

NON INC. BLOOD = Fresh blood which has not been incubated or sample processed.

n = 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1)

FIGURE 6.2.6.

Glutathione content (mM) of erythrocytes after one hour incubation with phenytoin, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50 % Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, phenytoin (50 μM) and buffer.

C/D/M = Erythrocytes, phenytoin, rat microsomes and buffer.

C/D/M/N = Erythrocytes, phenytoin, rat microsomes, NADPH regenerating system (1mM) and buffer.

n = 4 for each incubation group with data represented as mean ± S.D. (n-1).

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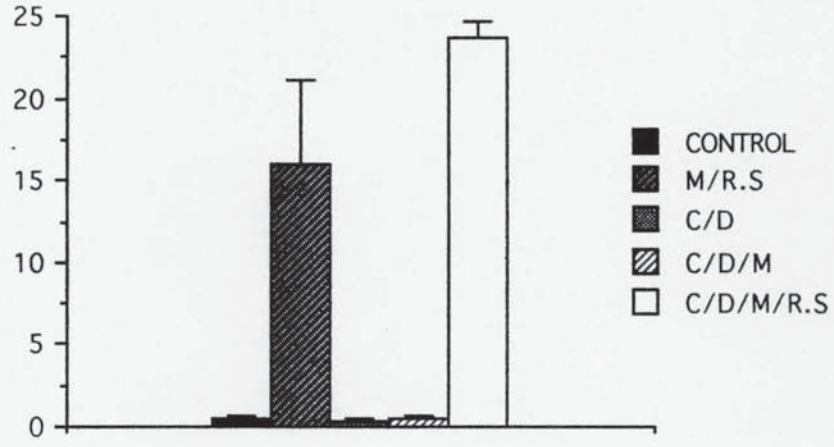
FIGURE 6.2.8.

Reduced haemoglobin (%) in erythrocytes after one hour incubation with phenytoin, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50 % Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2 mg) and buffer.

C/D = Erythrocytes, phenytoin (50 μM) and buffer.

C/D/M = Erythrocytes, phenytoin, rat microsomes and buffer.

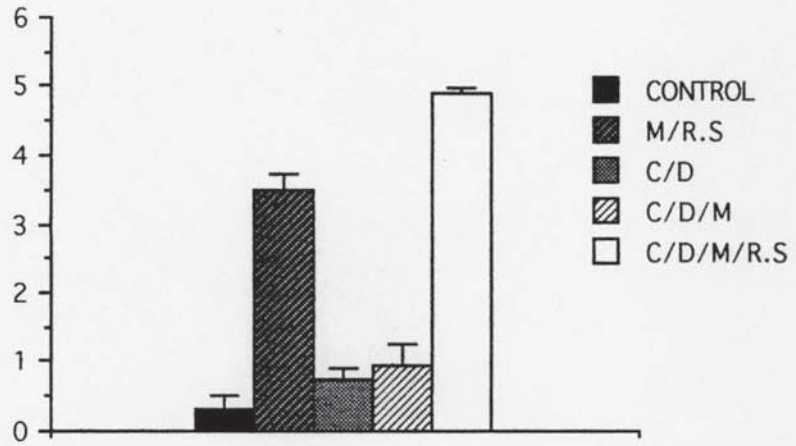
C/D/M/N = Erythrocytes, phenytoin, rat microsomes, NADPH regenerating system (1mM) and buffer.

NON INC. BLOOD = Fresh blood which has not been incubated or sample processed.

n = 4 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.9.

Methaemoglobin (%) in erythrocytes after one hour incubation with phenytoin, rat microsomes and NADPH regenerating system.



CONTROL = Erythrocytes (50 % Haematocrit) and PBSG buffer.

R.S./M = Erythrocytes, NADPH regenerating system (1 mM), rat microsomes (2mg/ml) and buffer.

C/D = Erythrocytes, phenytoin (50 μM) and buffer.

C/D/M = Erythrocytes, phenytoin, rat microsomes and buffer.

C/D/M/R.S. = Erythrocytes, phenytoin, rat microsomes, NADPH regenerating system (1mM) and buffer.

NON INC. BLOOD = Fresh blood which has not been incubated or sample processed.

n = 4 for each incubation group with data represented as mean ± S.D. (n-1).

TABLE 6.2.1.

Glutathione content, Total haemoglobin (THb), Reduced haemoglobin (RHb) and Methaemoglobin in erythrocytes after one hour incubation with rat microsomes and components of NADPH regenerating system.

	GLUTATHIONE (mM)	THb (g/dL)	RHb (%)	MetHb (%)
CONTROL (x)	0.358	8.10	0.80	0.37
S.D.	0.048	0.20	0.10	0.15
B/M/R.S. (x)	0.277	7.63	18.20 *	4.73 *
S.D.	0.102	0.21	4.12	0.21
B/M/N (x)	0.182 *	6.57	2.00 *	1.20 *
S.D.	0.054	1.83	0.87	0.10
B/M/R.S/C (x)	0.195 *	7.10	25.30 *	4.67 *
S.D.	0.046	0.92	2.67	0.67
B/M/N/C (x)	0.285	7.17	2.73 *	1.10 *
S.D.	0.058	0.72	0.81	0.10
B/MgCl2 (x)	0.312	6.86	0.57	0.37
S.D.	0.025	1.04	0.15	0.21
B/NADP (x)	0.186 *	6.33 *	0.80	0.23
S.D.	0.013	0.42	0	0.05
B/G-6-P (x)	0.308	8.07	1.30	0.10
S.D.	0.095	0.51	0.20	0.05
B/G-6-P-D (x)	0.356	8.40	0.43	0.43
S.D.	0.003	0.40	0.15	0.35
B/R.S. (x)	0.350	7.67	2.00 *	0.40
S.D.	0.033	0.40	0.10	0.10

n = 3 for each incubation group with data represented as mean (x) and S.D. (n-1).

TABLE 6.2.1. (cont'd)

* = Statistically significant result ($p<0.05$), when compared with erythrocyte only control, using Student's 't' test.

CONTROL = Erythrocytes (50% haematocrit) and PBSG buffer.

B/M/R.S. = Erythrocytes, rat microsomes (2 mg/ml), NADPH regenerating system (1mM) and buffer.

B/M/N = Erythrocytes, rat microsomes, NADPH solution (1mM) and buffer.

B/M/R.S./C = Erythrocytes, rat microsomes, NADPH regenerating system, catalase solution (50 units).and buffer

B/M/N/C = Erythrocytes, rat microsomes, NADPH solution, catalase solution and buffer.

B/$MgCl_2$ = Erythrocytes, magnesium chloride solution (50 mM) and buffer.

B/NADP = Erythrocytes, NADP solution (10 mM) and buffer.

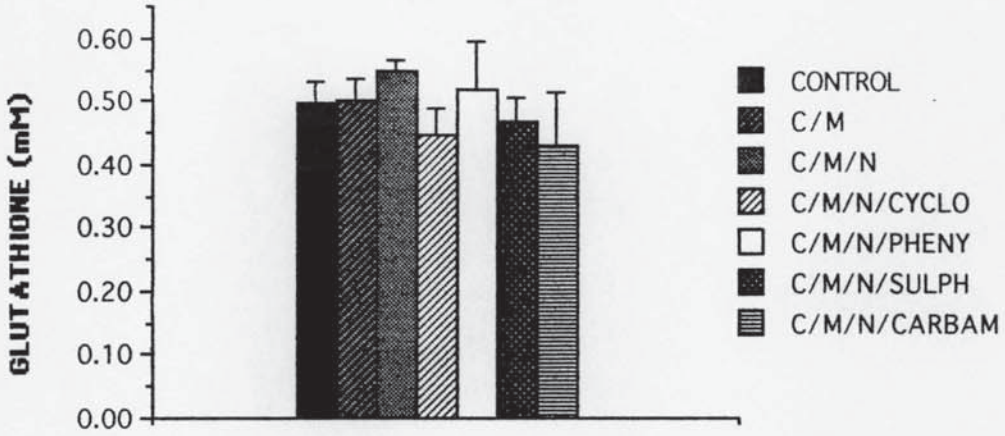
B/G-6-P = Erythrocytes, glucose-6-phosphate solution (20 mM) and buffer.

B/G-6-P-D = Erythrocytes, glucose-6-phosphate dehydrogenase solution (4 units) and buffer.

B/R.S. = Erythrocytes, NADPH regenerating system and buffer.

FIGURE 6.2.10.

Glutathione content (mM) of erythrocytes after one hour incubation with rat microsomes, drug solutions and NADPH solution.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/M = Erythrocytes, rat microsomes (2 mg) and buffer.

C/M/N = Erythrocytes, rat microsomes, NADPH solution (1 mM) and buffer.

C/M/N/CYCLO = Erythrocytes, rat microsomes, NADPH solution, cyclophosphamide solution (50 μM) and buffer.

C/M/N/PHENY = Erythrocytes, rat microsomes, NADPH solution, phenytoin solution (50μM) and buffer.

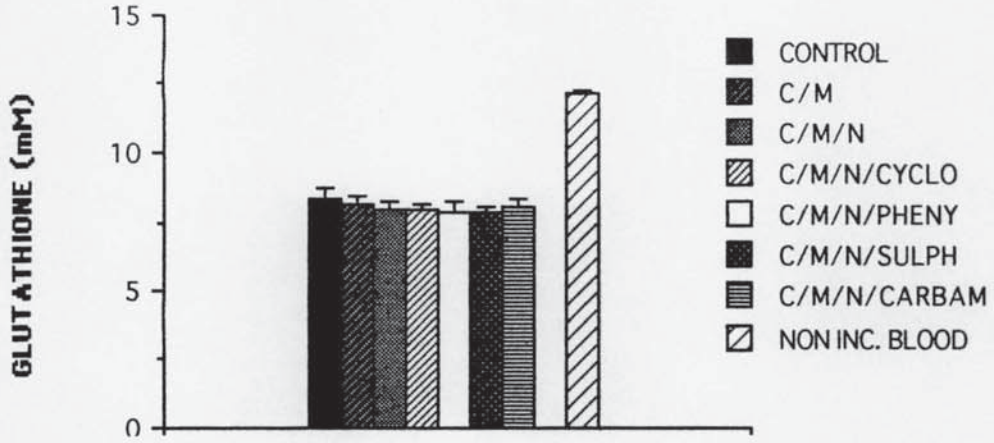
C/M/N/SULPH = Erythrocytes, rat microsomes, NADPH solution, sulphamethoxazole solution (50 μM) and buffer.

C/M/N/CARBAM = Erythrocytes, rat microsomes, NADPH solution, carbamazepine solution (50 μM) and buffer.

n = 5 for each incubation group with data represented as mean $\pm$ S.D. (n-1)

FIGURE 6.2.11.

Total haemoglobin content (g/dL) of erythrocytes after one hour incubation with rat microsomes, drug solutions and NADPH solution.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/M = Erythrocytes, rat microsomes (2 mg) and buffer.

C/M/N = Erythrocytes, rat microsomes, NADPH solution (1 mM) and buffer.

C/M/N/CYCLO = Erythrocytes, rat microsomes, NADPH solution, cyclophosphamide solution (50 μM) and buffer.

C/M/N/PHENY = Erythrocytes, rat microsomes, NADPH solution, phenytoin solution (50μM) and buffer.

C/M/N/SULPH = Erythrocytes, rat microsomes, NADPH solution, sulphamethoxazole solution (50 μM) and buffer.

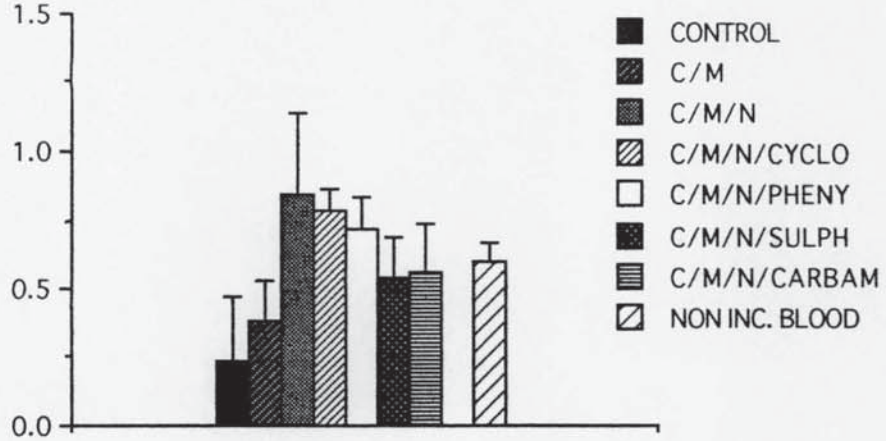
C/M/N/CARBAM = Erythrocytes, rat microsomes, NADPH solution, carbamazepine solution (50 μM) and buffer.

NON INC. BLOOD = Fresh blood not incubated or sample processed

n = 5 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.12.

Reduced haemoglobin (%) in erythrocytes after one hour incubation with rat microsomes, drug solutions and NADPH solution.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/M = Erythrocytes, rat microsomes (2 mg) and buffer.

C/M/N = Erythrocytes, rat midrosomes, NADPH solution (1 mM) and buffer.

C/M/N/CYCLO = Erythrocytes, rat microsomes, NADPH solution, cyclophosphamide solution (50 μM) and buffer.

C/M/N/PHENY = Erythrocytes, rat microsomes, NADPH solution, phenytoin solution (50μM) and buffer.

C/M/N/SULPH = Erythrocytes, rat microsomes, NADPH solution, sulphamethoxazole solution (50 μM) and buffer.

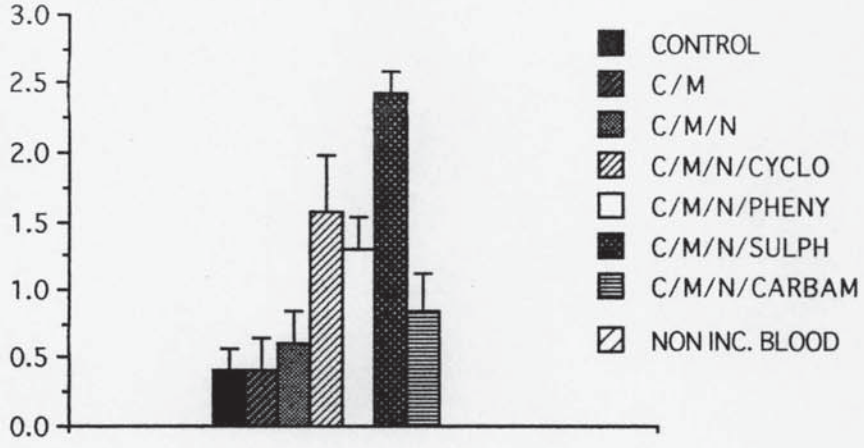
C/M/N/CARBAM = Erythrocytes, rat microsomes, NADPH solution, carbamazepine solution (50 μM) and buffer.

NON INC. BLOOD = Fresh blood not incubated or sample processed

n = 5 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.13.

Methaemoglobin (%) in erythrocytes after one hour incubation with rat microsomes, drug solutions and NADPH solution.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/M = Erythrocytes, rat microsomes (2 mg) and buffer.

C/M/N = Erythrocytes, rat midrosomes, NADPH solution (1 mM) and buffer.

C/M/N/CYCLO = Erythrocytes, rat microsomes, NADPH solution, cyclophosphamide solution (50 μM) and buffer.

C/M/N/PHENY = Erythrocytes, rat microsomes, NADPH solution, phenytoin solution (50μM) and buffer.

C/M/N/SULPH = Erythrocytes, rat microsomes, NADPH solution, sulphamethoxazole solution (50 μM) and buffer.

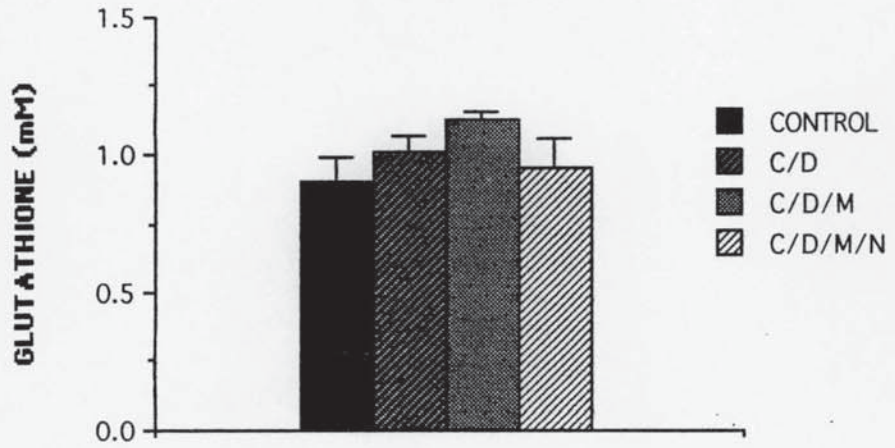
C/M/N/CARBAM = Erythrocytes, rat microsomes, NADPH solution, carbamazepine solution (50 μM) and buffer.

NON INC. BLOOD = Fresh blood not incubated or sample processed

n = 5 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.14.

Glutathione content (mM) of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

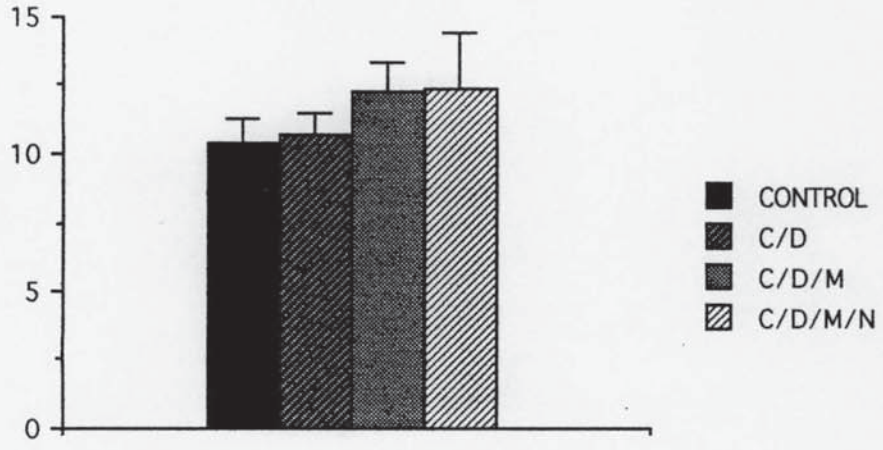
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.15.

Total haemoglobin content (g/dL) of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

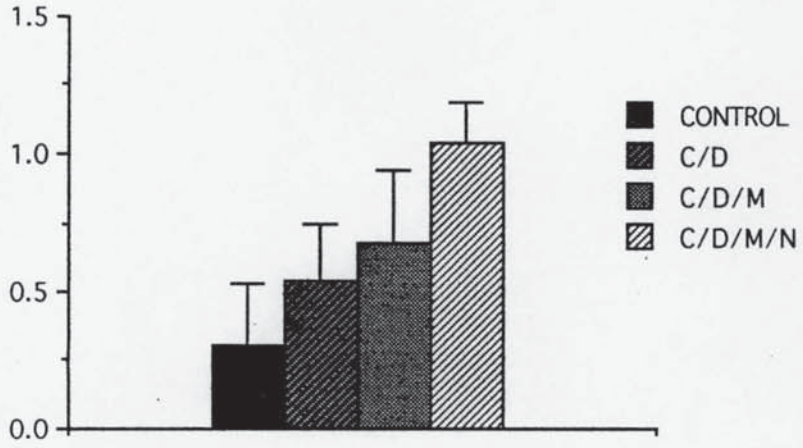
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.16.

Reduced haemoglobin (%) in erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

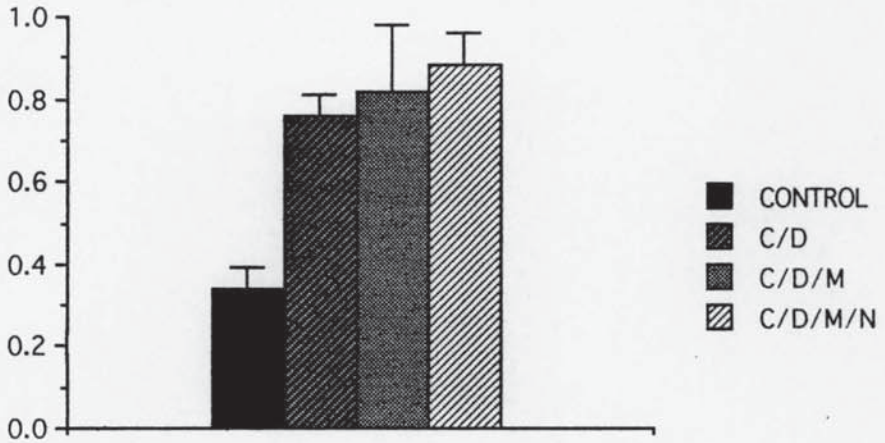
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.17.

Methaemoglobin (%) in erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

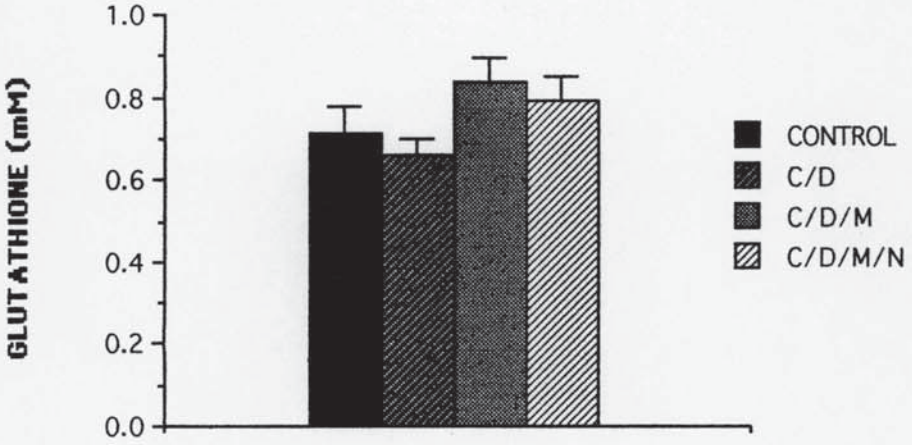
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.18.

Glutathione content (mM) of erythrocytes after one hour incubation with phenytoin, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, phenytoin (50 μM) and buffer.

C/D/M = Erythrocytes, phenytoin, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, phenytoin, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

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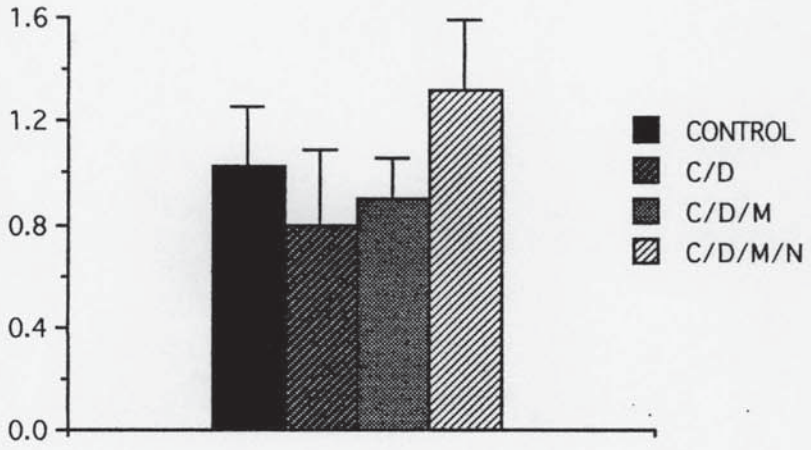
FIGURE 6.2.24.

Reduced haemoglobin (%) in erythrocytes after one hour incubation with sulphamethoxazole, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, sulphamethoxazole (50 μM) and buffer.

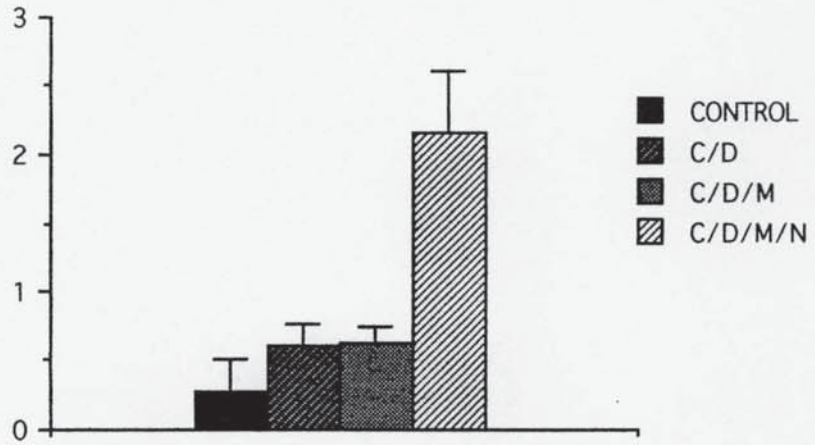
C/D/M = Erythrocytes, sulphamethoxazole, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, sulphamethoxazole, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.25.

Methaemoglobin (%) in erythrocytes after one hour incubation with sulphamethoxazole, rat microsomes and NADPH solution - Dianorm study.



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, sulphamethoxazole (50 μM) and buffer.

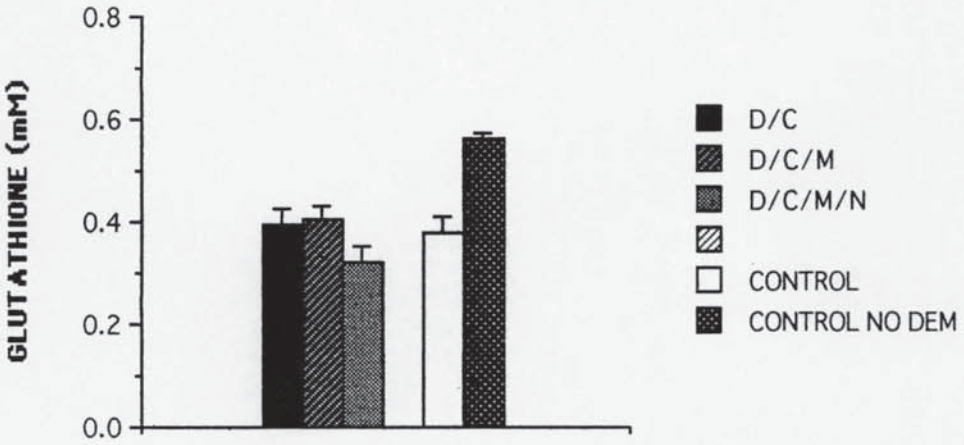
C/D/M = Erythrocytes, sulphamethoxazole, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, sulphamethoxazole, rat microsomes, NADPH solution (1 mM) and buffer.

n = 5 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 6.2.26.

Glutathione content (mM) of erythrocytes after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution - pre-incubation with diethyl maleate (DEM)



CONTROL = Erythrocytes (50 % haematocrit) and PBSG buffer.

C/D = Erythrocytes, cyclophosphamide (50 μM) and buffer.

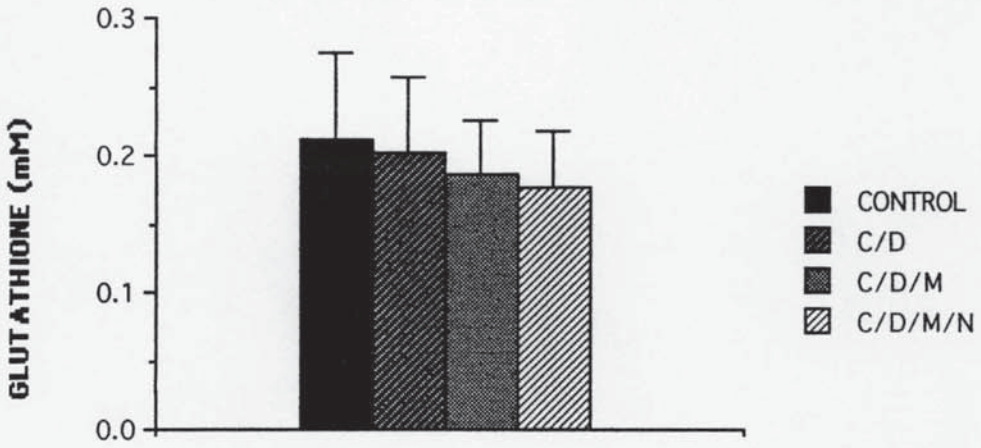
C/D/M = Erythrocytes, cyclophosphamide, rat microsomes (2 mg) and buffer.

C/D/M/N = Erythrocytes, cyclophosphamide, rat microsomes, NADPH solution (1 mM) and buffer.

n = 4 for each incubation group with data represented as mean $\pm$ S.D. (n-1).

FIGURE 6.2.27.

Glutathione content (mM) of L6 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution.



CONTROL = Cells and media alone.

C/D = Cells, cyclophosphamide (50 μM) and media.

C/D/M = Cells, cyclophosphamide, rat microsomes (4 mg) and media.

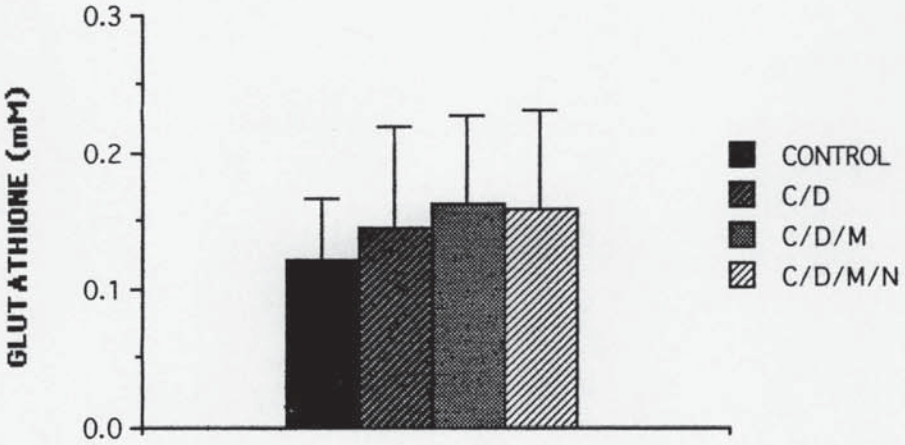
C/D/M/N = Cells, cyclophosphamide, rat microsomes, NADPH (1 mM) and media.

Data derived from 3 experiments where n = 6 in each experiment.

Data represented as mean ± S.D. (n-1).

FIGURE 6.2.28.

Glutathione content (mM) of CACO-2 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution.



CONTROL = Cells and media alone.

C/D = Cells, cyclophosphamide (50 μM) and media.

C/D/M = Cells, cyclophosphamide, rat microsomes (4 mg) and media.

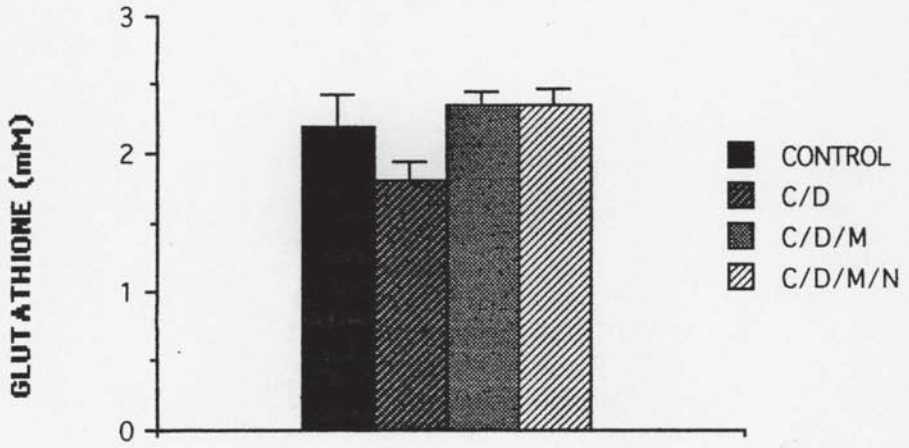
C/D/M/N = Cells, cyclophosphamide, rat microsomes, NADPH (1 mM) and media.

Data derived from 4 experiments where n = 6 in each experiment.

Data represented as mean ± S.D. (n-1).

FIGURE 6.2.29.

Glutathione content (mM) of NG115-401L-C3 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH solution.



CONTROL = Cells and media alone.

C/D = Cells, cyclophosphamide (50 μM) and media.

C/D/M = Cells, cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = Cells, cyclophosphamide, rat microsomes, NADPH (1 mM) and media.

n = 6 in each experiment with data represented as mean ± S.D. (n-1).

6.3. DISCUSSION.

The erythrocyte cytotoxicity demonstrated in the rat microsome and NADPH incubations, concurred with that seen in other studies involving cyclophosphamide and similar metabolising systems (Wildenauer *et al.* 1992, Palmen *et al.* 1993). The toxicity seen with carbamazepine and sulphamethoxazole metabolites was anticipated, as there was evidence of cytotoxicity with microsomal systems and other cells (Shear and Spielberg 1985, Pirmohamed *et al.*1992). However, the cytotoxic metabolites of cyclophosphamide were expected to deplete glutathione in the cell lines, based on the erythrocyte results and on the reports from other studies (Crook *et al.* 1986, Institoris *et al.* 1993).

In the studies using the NADPH regenerating system, there was considerable glutathione depletion seen in the erythrocytes and haemolysis, which generally resulted in considerable haemoglobin loss. Even without the presence of any metabolites, there were considerable changes in the haemoglobin, with increases in the amounts of reduced haemoglobin and methaemoglobin. The $NADP^+$ species, on investigation, appeared to be the causative agent and would appear to cause oxidative damage, with lipid peroxidation of the erythrocyte membrane. The highly electrophilic nature of the $NADP^+$ could remove an electron from a variety of sources, resulting in the generation of a free radical. This radical would then interact with the polyunsaturated fatty acids,with lipid, superoxide and peroxide radicals being formed. The superoxide and peroxide radical formation is seen in the cell, as a by product of cell metabolism and therefore there are mechanisms in the erythrocyte to scavenge these radicals, for example, superoxide dismutase and glutathione peroxidase. But in the incubation, the concentrations of radicals generated would have be too great, due to the high initial concentration of $NADP^+$ from the addition of the NADPH generating system. Glutathione plays a central role in defence against lipid peroxidation (Trotta *et al.* 1982) and it has been shown that peroxidation does not occur, until all cellular glutathione has been oxidized (Trotta *et al.* 1983). Therefore the $NADP^+$ may initially deplete glutathione, then promote the lipid peroxidation. The haemolysis seen

with the samples, indicates the membrane damage normally associated with peroxidation. In addition, the haemolysis could have potentiated the oxidative damage seen, as the iron from haemoglobin can initiate further lipid peroxidation and this has been demonstrated in mouse erythrocytes (Ciccoli *et al.* 1994).

The studies showed increases in reduced haemoglobin and methaemoglobin in the erythrocytes. These increases are further indicators of the process of oxidative damage, with the changes in the oxidation-reduction state of the haemoglobin and in the iron of the haem molecule. The reduction of the haemoglobin will probably have originated as a result of interaction of hydrogen radicals from the lipid peroxidation, with the haemoglobin. Haemoglobin can accept hydrogen ions *in vivo* in its capacity as a pH buffer, so this effect is reversible and would not impact on the oxygen transport ability. The methaemoglobin where the iron has been oxidized from 2^+ to 3^+, is a result of the action of the superoxide species. This species is generated in the oxidation - reduction cycles of the cell and in the drug metabolising systems used in *in vitro* tests. Normally the production of methaemoglobin is reversed by the action of methaemoglobin reductase, but haemolysis greatly reduces methaemoglobin reductase activity. The increase of methaemoglobin *in vivo* would have serious consequences in terms of impairment of oxygen transport. In addition, the increase in methaemoglobin in this *in vitro* system, could have potentiated the lipid peroxidation, as it has been demonstrated to catalyse the reaction at low levels (Trotta *et al.* 1981).

It is known that high concentrations of hydrogen peroxide can compromise the erythrocyte's defence mechanisms (Dodge *et al.* 1967) and so induce lipid peroxidation. Therefore, addition of catalase should have had an effect on the degree of glutathione depletion and lipid peroxidation, by converting the extracellular peroxide and reducing its effect. This was seen in the incubations with the NADPH solution, but not with the NADPH regenerating system, so indicating that hydrogen peroxide may not be a major factor in the cytotoxicity seen.

The effect of $NADP^+$ from the regenerating system would never normally be seen in an *in vitro* toxicity test such as this. This is because the use of a regenerating system normally includes the addition of glutathione in the system, which would react with the high initial concetration of $NADP^+$ and eliminate its potential cytotoxicity. However in these studies, the presence of glutathione was the marker of cell functioning, so addition of exogenous glutathione may have confused the end results.

The studies involving the metabolite generation with the rat microsomes and NADPH solution, showed that the glutathione depletion seen was a result of the metabolites and not of the NADPH solution in the microsomal system. The depletion may be attributed to glutathione mediated protection from the superoxide species and other reduced oxygen products, generated by the microsomal system. In addition, the detoxication of the reactive metabolites by glutathione conjugation, will significantly deplete the intracellular thiol levels. The reactive nature of the metabolites of cyclophosphamide and sulphamethoxazole was probably responsible for the slight increases in the percentage of methaemoglobin, seen in the erythrocytes after incubation. The absence of significant glutathione depletion with the metabolites of phenytoin, was unexpected as microsomal P-450 action is considered to produce a unstable, highly reactive arene oxide intermediate (Spielberg *et al.* 1981). This has been proposed to be cytotoxic, as well as altering lymphocyte functioning and inducing immunological reactions. The action of any epoxide hydrolase present in the incubations, could explain the absence of a response, as this would detoxify arene oxide metabolites. However if this were the case, there would have also been a reduction in glutathione depletion with carbamazepine, as this also has a reactive epoxide intermediate (Panesar *et al.* 1995). There may have been glutathione depletion, although data variability meant that there was no significant decrease

The two compartment studies showed that the toxicity mediated by the metabolites, appeared to be significantly attenuated when the erythrocytes were not incubated in a one compartment system with the microsomes. This would imply that generally, the reactive

metabolites are so short lived, that they are unable to diffuse through the permable membrane and exert an effect. This would explain the incidences of *in vivo* hepatotoxicity, seen with the reactive arene oxide metabolites . There was a decrease in glutathione with the cyclophosphamide microsomal incubations, which could be due to the action of acrolein. This metabolite is known to cause heart damage *in vivo*, which would suggest it is a reactive metabolite with a longer life and capable of exerting an effect outside of the liver.

The *in vitro* test with erythrocytes has evaluated the impact of cytotoxins on cell function. However, there are a number of areas in the system which have to be addressed before further work should be carried out. Firstly, the test does not have a positive glutathione control, against which any depletion seen can be assessed. This positive control could be achieved by using buthionine sulfoxime, which depletes glutathione without toxicity, by selectively inhibiting γ-glutamylcysteine synthetase (Deneke and Fanburg 1989). The incubations were carried out in a water bath at body temperature, but during the incubation period the erythrocytes were not oxygenated in the same manner as in the body. Although the tubes were not completely closed, the conditions may have been too oxygen deficient, which would have had an impact on the cell defences. Anoxia is known to cause cell necrosis and it is documented that glutathione depletion of cells *in vitro*, is not so great when the cells are in aerated or hyperoxic conditions. Therefore the aeration of the samples may give different results, which would correlate better with any *in vivo* toxic events reported. Finally, the glutathione detemination by spectroscopy, may not have been the most sensitive method to determine the intracellular glutathione and probably contributed to the deviation between results. The erythrocytes taken from a variety of donors, may not be homogeneous in response, as most freshly isolated cells exhibit variable responses, but the use of a HPLC method will have given more sensitive sample analyses and so possibly reduced differences. An alternative method for determination of glutathione, could have been utilised, such as that of Peters *et al.* (1990), which measures the esters of glutathione with electrochemical detection. This

use of HPLC may be particularly beneficial in glutathione analysis with samples from cell lines, where the concentration of glutathione is not as high as that seen in erythrocyte samples.

The absence of any glutathione depletion in the cell lines, when incubated with a microsomal system, would appear to reinforce the arguement detailed in the other sections of the thesis, for the lack of disruption to other cell functions. It also concurs with the results seen in other *in vitro* test systems, particularly those looking at cyclophosphamide cytotoxicity (Crook *et al.* 1986, Peters *et al.* 1990). There was a decrease with the NG115-401L-C3 samples incubated with cyclophosphamide alone, compared to the cell only control. However, this result is likely to be an anomaly and may be due to an overall smaller sample number, compared to the other cell lines and low, variant glutathione readings throughout. The results of all the cell line studies also show the differences in response between the different cell types, with the freshly isolated cells demonstrating glutathione depletion and no effect seen in the immortal cell lines. These cell lines were incubated in an aerated environment, so this could have reduced the GSH depletion, as has been discussed. But as been mentioned in the dicussions of the other cell line studies, these immortal cell lines have a high intracellular glutathione pool and elevated rates of synthesis, which confers greater resistance to toxicity.

In conclusion, there was evidence that the $NADP^+$ in the NADPH regenerating system. depleted glutathione and may have induced lipid peroxidation. This possible evidence for lipid peroxidation is based on the haemolysis exhibited by samples including $NADP^+$, coupled with increases in reduced haemoglobin and methaemoglobin. The use of a NADPH solution also depleted glutathione and induced lipid peroxidation but not to the same degree. Incubations of cyclophosphamide, carbamazepine and sulphamethoxazole with rat microsomes and NADPH demonstrated significant decrease in glutathione, but no evidence of peroxidation. There was no significant glutathione depletion with phenytoin under the same conditions, although there was evidence of methaemoglobin formation. The separation of the erythrocytes from the metabolising element, showed the evidence for the

metabolites being too short lived to diffuse through a membrane and exert effects. The exception was with the cyclophosphamide incubations, where significant glutathione depletion was detected. The co-incubation of cyclophosphamide and the rat microsomal system with three cell lines, did not result in any significant glutathione depletion in the cell monolayer samples.

SECTION 7

NG115-401L-C3 STUDIES

7.1. INTRODUCTION

DNA damage by toxic agents and the results of such damage in a cell, is a widely studied area. In fact the area of genotoxicity, is recognized as having rapidly expanded the use of *in vitro* methods in toxicity overall. Consequently many of the established *in vitro* tests for example, the Salmonella microsome assay or Ames test (Ames *et al.* 1976, Maron *et al.* 1983), evaluate the genotoxic effects of chemicals.

DNA provides the information which leads to the production of proteins and this is encoded in the unique sequence of nitrogenous bases in the molecule. Damage to the DNA molecule can result in bases being altered or lost and phosphodiester bonds in the backbone being broken. In addition, covalent binding of a reactive chemical to the DNA can result in cross linking of the two strands or links between bases of one strand. These reactions can result in cell mutation and chromosome deletion, translocation or even duplication. In the cell, the damage can be manifested as cell proliferation, which *in vivo*, can result in the occurance of neoplasms . This demonstrates therefore, the importance of testing novel entities for potential genotoxicity. The proliferative effect can also be harnessed, in differentiating cell types and producing eternal cell lines, which have the phenotypic properties of their origins.

In an *in vitro* culture system, the effects of toxic agents can be observed by looking for mutations, as in the Ames test, or looking at the chromosomes for evidence of ploidy and sister chromatid exchanges (SCE) (Danford 1984, Perry *et al.* 1975). There are DNA repair systems in living cells which can recognise DNA damage and can remove damaged areas. The excised area can then be replaced with undamaged nucleotides,using the opposite strand as a template and the new DNA rejoined to the existing strand. These repair systems can be monitored by measuring the uptake of labelled nucleotides with autoradiography or scintillation (Williams 1976, Martin et al. 1978). In addition, monitoring some of the basic components and functions of the cell can illustrate DNA damage. The production of proteins and nucleic acids can be inhibited, which can result in

cytotoxicity, due to the lack of essential proteins and amino acids. Synthesis can also be increased, as a result of second messenger action, after hormonal receptor stimulation (Benton *et al.* 1989).

The NG115-401L-C3 cell line is a MAS-transfected neural murine cell line (Jackson *et al.* 1987). Though it is a hybrid of mouse neuroblastoma and rat glioma, it is phenotypically a clonal mouse cell type and has been shown to synthesize and store acetylcholine. This cell line has been used to study adenylate cyclase and phosphodinositide pathways (Hanley 1987, Jackson *et al.* 1987). It has also been used to show increases DNA synthesis, after stimulation of the phosphoinoditide cascade by angiotesin III (Jackson *et al.* 1987). The cell line has also been used in *in vitro* studies with a tumour promotor, thapsigargin (Jackson *et al.* 1988).

DNA synthesis was stimulated by angiotensin III, after the NG115-401L-C3 cells had been incubated with the metabolites of cyclophosphamide and phenytoin. The DNA synthesis was evaluated by [^{3}H] thymidine uptake and incorporation in the cells. In addition, the uptake and incorporation of [^{3}H] thymidine was evaluated in the cells in the absence of angiotensin III.

7.2. RESULTS.

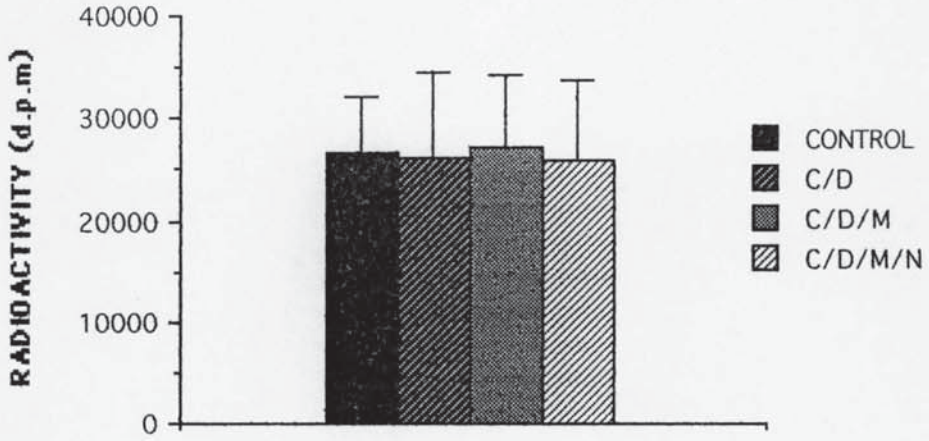
There was no significant difference in [^{3}H] thymidine uptake and incorporation, compared to the control, in any of the cell monolayers incubated with rat microsomes (2mg/ml), NADPH (1 mM) and cyclophosphamide (50 μM)(Figures 7.2.1. and 7.2.2.). The incubations with phenytoin (50 μM), rat microsomes, and NADPH, also showed no change in [^{3}H] thymidine uptake and incorporation (Figures 7.2.3. and 7.2.4.)

The uptake of [^{3}H] thymidine after the microsomal incubation with cyclophosphamide, but without the stimulation by angiotensin III, also showed no significant difference, compared to the control, in any of the incubation groups (Figures 7.2.5. and 7.2.6.).

Microscopic examination of the cell monolayers after the microsomal incubations, showed no evidence of any morphological changes in the cells, compared to the control monolayers. There were areas in the monolayers which had been incubated with microsomes, NADPH and the drug solutions, where the monolayer appeared to be disrupted. But these areas were where the incubation components had been introduced into the well and so cell loss was a result of physical stress on the cells, from the pipetting method used.

FIGURE 7.2.1.

[^{3}H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH - Acid insoluble (DNA) fraction.



CONTROL = media only.

C/D = cyclophosphamide (50 μM) and media.

C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

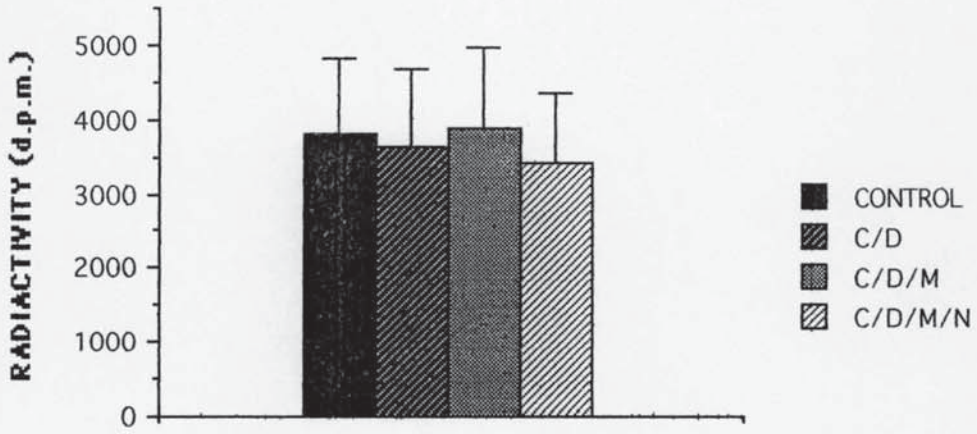
C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media.

5 assays carried out with n = 12 for each incubation group.

Data represented as mean ± S.D. (n-1).

FIGURE 7.2.2.

[^{3}H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH - Acid soluble (cytosolic) fraction.



CONTROL = media only.

C/D = cyclophosphamide (50 μM) and media.

C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

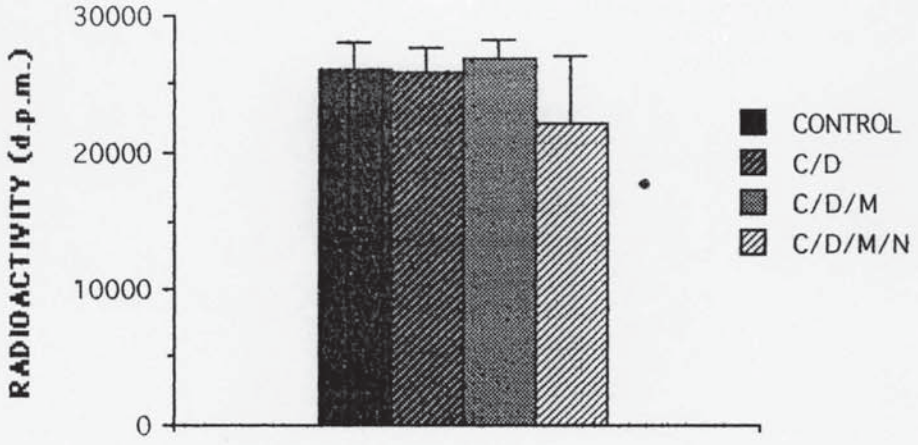
C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media.

5 assays carried out with n = 12 for each incubation group.

Data represented as mean ± S.D. (n-1).

FIGURE 7.2.3.

[^{3}H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with phenytoin, rat microsomes and NADPH - Acid insoluble (DNA) fraction.



CONTROL = media only.

C/D = phenytoin (50 μM) and media.

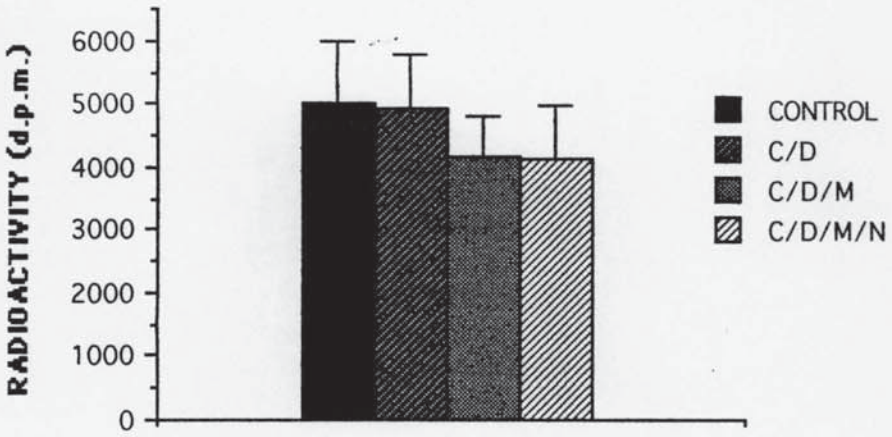
C/D/M = phenytoin, rat microsomes (4 mg) and media.

C/D/M/N = phenytoin, rat microsomes, NADPH solution (1 mM) and media.

n = 12 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 7.2.4.

[^{3}H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with phenytoin, rat microsomes and NADPH - Acid soluble (cytosolic) fraction.



CONTROL = media only.

C/D = phenytoin (50 μM) and media.

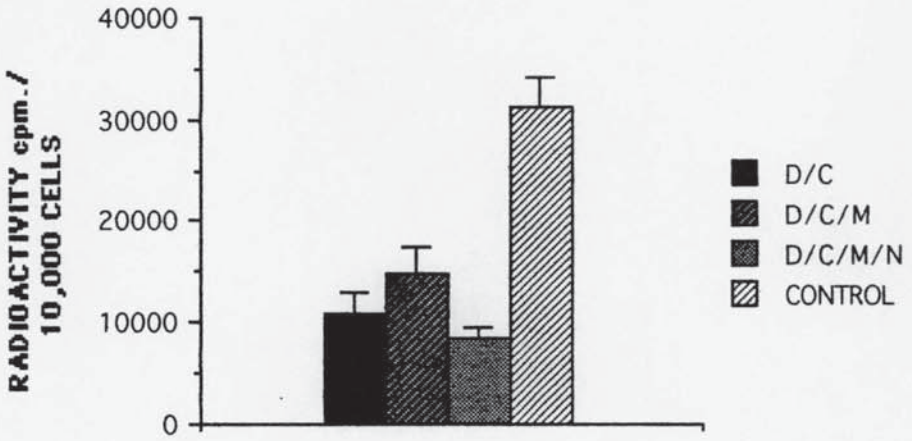
C/D/M = phenytoin, rat microsomes (4 mg) and media.

C/D/M/N = phenytoin, rat microsomes, NADPH solution (1 mM) and media.

n = 12 for each incubation group with data represented as mean ± S.D. (n-1).

FIGURE 7.2.5.

[3H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH and no stimulation with Angiotensin III - Acid insoluble (DNA) fraction.



CONTROL = media only.

C/D = cyclophosphamide (50 μM) and media.

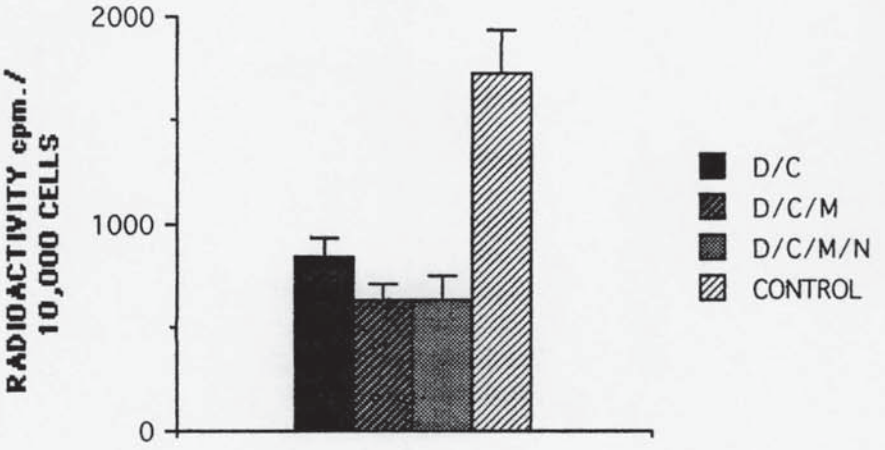
C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media.

n = 12 for each incubation group and data represented as mean ± S.D. (n-1).

FIGURE 7.2.6.

[^{3}H] Thymidine incorporation in NG115-401L-C3 cells after one hour incubation with cyclophosphamide, rat microsomes and NADPH and no stimulation with Angiotensin III - Acid soluble (cytosolic) fraction.



CONTROL = media only.

C/D = cyclophosphamide (50 mM) and media.

C/D/M = cyclophosphamide, rat microsomes (4 mg) and media.

C/D/M/N = cyclophosphamide, rat microsomes, NADPH solution (1 mM) and media.

n = 12 for each incubation group and data represented as mean + S.D. (n-1).

7.3. DISCUSSION

Cyclophosphamide and phenytoin metabolites are documented as being genotoxic agents, particularly in terms of inhibition of DNA production (R.T.E.C.S., 1985-86). Therefore, it was expected that there would be a inhibition in DNA synthesis, after incubation with the metabolites of each drug, which would ultimately lead to cytotoxicity.

The absence of any significant effects on DNA synthesis is probably a result of several factors. The concentration of drug at the site of damage, was probably too low to result in significant genotoxicity. To inhibit production of DNA, there must be inhibition of the polymerases which are responsible for synthesis. In the mammalian DNA synthesis pathway, α polymerase is the most important of the three polymerases and this is situated in the nucleus. Therefore any cytotoxic metabolites must pass through the plasma membrane, cytoplasm and nuclear membrane, before inhibiting α polymerase. As previous studies in this thesis have postulated (Sections 4 and 4), glutathione conjugation seems traps the reactive metabolites produced before even significant membrane damage can occur, so reducing the chance of inhibition of DNA synthesis. It is possible that some effect not immediately detectable, would have arrested synthesis and with this in mind, another assay could have been carried out perhaps 24 hours later. This would have allowed for several periods of growth in the culture system and may have illustrated such inhibitory effects. The evidence of a lag period before DNA related cytotoxicity was seen, has been demonstrated in an *in vitro* assay with cyclophosphamide, rat hepatocytes and fibroblasts (Acosta and Mitchell 1981).

Another reason for a lack of inhibition was, as has been discussed, the implication of glutathione conjugation as a cytoprotective mechanism. The NG115-401L-C3 cell has in previous studies, been shown to have a particularly high intracellular glutathione content and this will result in the cell model being more resistant to cytotoxicity. The depletion of the glutathione, by either a chemical such as buthionine sulphoxinine or by higher concentrations of metabolites, would probably result in inhibition of DNA synthesis.

However in the case of a higher concentration of reactive metabolites, cytotoxicity due to membrane disruption, membrane protein denaturation and loss of cell regulation mechanisms, will probably result before DNA inhibition.

The short incubation period of the assay, will also have been a factor in the apparent lack of effect. Other similar assay systems have incubation times of 3 hours to 24 hours (Horner *et al.* 1985, Acosta and Mitchell 1981) and both showed positive effects. This extension of incubation time, will allow a greater concentration of the reactive metabolites to be produced, but will also allow the reduction of glutathione. However, this would be unfeasible with this system, due the unsterile microsomal solutions. In addition, both the microsomes and the NADPH solution have very little activity after one hour, so there would be no further significant increase in the levels of metabolites

There was evidence of slight disadherrence in the monolayers which were incubated with the drug, rat microsomes and NADPH solution. It is probable that this is a result of the physical stress caused by several introductions of liquid with a pipette. Although the liquids were dispensed slowly down the side of the well, it would appear that the pressure exerted by the forced ejection was enough to remove the cells. Every effort had been taken to minimise stress on these cells, however this event could be a factor in the results. There was a lowering of the cell population of the metabolite generating incubation, but an similar ^{3}H thymidine incorporation compared to the control and can be interpreted in two ways. It could be that the number of cells lost was negligible overall and there was no change in DNA synthesis. However, the loss could have been significant, which would imply that there was a change in DNA synthesis as a result of incubation with the drug metabolites. This could have been investigated by, determining the total protein content of the monolayers from each group and then incorporating this as a factor in the determination of the final results. However, a change in the methods used in culturing and incubating the cell monolayers could avoid the physical stress involved. The growth of the monolayers on a membrane assembly, which would then be inserted into the incubation system would avoid physical disadherrence and cell loss. The use of a semi permeable

membrane system would also allow the co-incubation of a feeder cell line with the NG115-401-C3 model. The feeder cells would enable the improvement of cell to cell communication in the model cell line and give a more representative response to the cytotoxic metabolites. This could be particularly important in terms of cell-cell and cross cell transport of metabolites and the ability of a genotoxic to inhibit DNA production

In summary, there was no apparent inhibition or change in DNA synthesis, after incubation with the reactive metabolites of cyclophosphamide and phenytoin. The cytoprotective effect of glutathione and the high concentration of intracellular glutathione in this cell model, probably significantly reduce the toxicity of the metabolites. In addition, the concentration of drug solution used and the incubation time with rat microsomes and NADPH, significantly affected the outcome of the results. Addressing these factors, will probably result in a significant change in the results seen. In addition, there was disruption of the cell monolayer, which was probably not a chemical effect, but a result of methods used in the introduction of incubation components.

SECTION 8

GENERAL DISCUSSION

In vitro methods are now accepted as valid tools in toxicity testing, as they contribute information about mechanisms of toxicity at a cellular and molecular level. The results presented in this work illustrate this, but also highlight the problems encountered with *in vitro* methods. The work also shows that good method development is vital and of importance if a test is to be considered for validation.

There are many factors that will affect the final response of a cell to a potential toxin, but the most important are the concentration of toxin and its duration of action. Concentration can be affected *in vitro*, by many of the same factors that can affect *in vivo* studies. Protein binding to the media serum and microsomes is well documented (Roy *et al.* 1990, Pirmohamed *et al.* 1992, Wildenauer *et al.* 1982) and can affect the toxic impact of metabolites on cells. The protein binding can be avoided by using serum free media during incubations, as was the case in much of the work performed, but total serum free culturing would been inadvisable as it can bring about phenotypic changes. With the microsomes, there is also a possibility of the drugs causing inactivation of the P-450 enzymes, for example, cyclophosphamide can alter metabolism, due to acrolein mediated P-450 inactivation (Leblanc *et al.* 1990, Gurtoo *et al.*1981). Inactivation of P-450 activity tends to occur only with high levels of acrolein and is unlikely to be a limiting factor at the concentration range used here. There is also the possibility of binding with the plastics involved, although plastics are used with very low binding properties. The adsorption of drugs and the metabolites generated from these drugs onto the plastics, is a possibility as both plastics and the drug solutions are hydrophobic in nature.

It may be that there was insufficient production of the toxic species produced by the microsomal system and so low levels of toxicity. This is a result of either too little pro-drug or insufficient time being allowed for the generation of a cytotoxic level of metabolites. The solution would be to perform the incubations with a range of concentrations, rather than just the concentration at which the erythrocyte toxicity was seen. This would result in the threshold concentration being established for all the cells

used and could allow the study of the disruption of the cell functions. In addition, changing the length of incubation times, will allow the metabolite concentrations to accumulate and so increase the toxicity seen. This is seen to be the case in the metabolic activation of cyclophosphamide by primary cultures of rat hepatocytes (Acosta and Mitchell 1981). In assays reported at the B.T.S./I.V.T.S. conference (1995), *in vitro* assays with cell lines were incubated for at least several hours before evaluation of cell function, but more routinely for 24 to 48 hours. Incubation times of this length, would be impractical with the microsomal system used, as P-450 activity decreases rapidly after about 2 hours.

The influence of the cytoprotective mechanisms in the cell, appear to be a central factor in the toxic impact of metabolites, after the metabolite concentration and incubation time. The cellular content of glutathione and the activity of the enzymes in the γ-glutamyl cycle, would appear to be particularly important especially in the cell lines.

In the erythrocyte incubations, the depletion of glutathione was demonstrated not only.with the drug metabolites, but also with the electrophilic $NADP^+$. The incubations with $NADP^+$, also demonstrated the probable outcome of prolonged glutathione depletion in the erythrocyte. The extensive haemolysis and considerable increases in methaemoglobin and reduced haemoglobin, are factors associated with the occurence of lipid peroxidation. Several groups have demonstrated effects on glutathione in erythrocytes after microsomal incubation with cyclophosphamide (Palmen et al. 1993, Wildenauer *et al.* 1992). However, Ciccoli *et al.* (1994) describe changes like those above in murine erythrocytes and cite the cause of toxicity as lipid peroxidation. The peroxidation of the membrane has been reported to occur in erythrocytes, only after considerable oxidative challenge or when cytoprotective enzymes are inhibited (Stocks *et al.* 1971). In addition, peroxidation will take place when there is depletion of intracellular and membrane glutathione (Trotta *et al.* 1983). The effects on the enzyme systems and on the cytoskeleton of the cell were not established, but oxidative damage and lipid peroxidation are known to affect both of these cell features.

The results have shown that glutathione depletion was apparent in the erythrocytes co-incubated with the metabolites of cyclophosphamide, carbamazepine and sulphamethoxazole in the one compartment system. The effect of the cyclosphophamide metabolites could again be seen to a much lesser degree with the dianorm experiments. This is similar to responses reported with *in vivo* studies, although the cytotoxicity was apparently greater than in *in vivo* studies. Subramaniam *et al.* (1994), report that the metabolites of cyclophosphamide reduce the activities of various enzymes involved in cytoprotection, in erythrocytes of breast cancer patients treated with cyclophosphamide, methotrexate and 5-fluorouracil. In addition, acrolein is demonstrated to increase the rate of glutathione synthesis in liver cells, although does not change the cytosolic glutathione levels (Warholm *et al* 1984). The dianorm studies also demonstrated that the metabolites were probably too short lived to diffuse through the semi permeable membrane, due to the lack of glutathione depletion observed with the other drug incubations.

The cell lines used in these studies, demonstrated no evidence of glutathione depletion, despite being incubated with the microsome/drug system for the same period of time. Other work has reported that the cytotoxic effects are seen with various cell lines, but only after prolonged glutathione depletion (Institoris *et al* 1993, Dethlefsen.*et al* 1986, Chen *et al* 1995). This differing of response seen in the non proliferative and proliferative cells is well documented and is the problem which is behind the lack of correlation between data obtained from *in vitro* tests with cell lines and *in vivo* responses. This has been demonstrated well by J. Timbrell's group at the University of London, reporting the bichemical effects of hydrazine *in vivo* and *in vitro* (Timbrell *et al.* 1995). Toxicity was seen *in vitro* , but at concentrations 10 to 100 times greater than that in the *in vivo* studies.

This phenomenon occurs, not so much because the cell lines contain a nucleus and so are capable of renewing cell defences, but because the immortal cells are constantly growing,

whereas the erythrocyte has a finite life span. Rapid proliferation requires rapid synthesis of all cell contents, particularly proteins and DNA. Glutathione is important in the cell not only as a cytoprotectant, but also as an electron donor in the synthesis of deoxyribose units, a precursor of DNA. Therefore, the cell lines will probably have greater resources in resisting cytotoxicity, not only due to a greater intracellular pool of glutathione, but also due to the increased activity of relevant enzymes. This is reported to be the case in a cyclophosphamide resistant medulloblastoma cell line, which has elevated levels of glutathione and increased activity of glutathione-s-transferase (Colvin *et al.* 1993).

The lack of response to toxicity in the cell lines used, may be accounted for in the cells The occurence of 'multi drug resistance' is a event commonly seen in cancer cells, where resistance to the toxicity of different antimetabolic drugs is seen. This is a result of over production and stimulation of a membrane protein, results in a 30 fold decrease in cell sensitivity to toxins. The presence of 'pump-proteins' like this, which expel toxins as a protective mechanism, usually requires many periods of exposure of the cells, to subtoxic doses of the agent concerned. Another possibility, is that the cell lines have the capacity to further metabolise the toxic species not just with glutathione conjugation, but also other Phase II reactions like sulphation or methylation. This was suggested with the CaCO2 cell line and is well reported with other colon carcinoma derived cell lines (Moskwa *et al.* 1985) and hepatocyte cell lines. The best way to ascertain this, would be to analyse the final incubation solutions for the products of Phase II reactions, for example, glutathione-metabolite compounds. Inhibiting the Phase II reactions is not advisable, as this may be affect cell functioning as was seen in the CACO-2 studies.

There are many aspects of the studies, which if changed, could lead to a different response or more precision in the final data. The use of a range of drug concentrations and incubation times would affect the toxicity seen in these *in vitro* tests, but these changes could introduce more problems. With the cell lines, long term exposure to metabolites would probably deplete the intracellular glutathione and then lead to further functional

damage. However, long term incubations were thought to be unsuitable for several reasons. Firstly, the microsomes were not sufficiently sterile, which would affect the cell viability due to the introduction of bacteria, viruses and yeasts. Secondly, the activity of the microsomal suspension and the NADPH solution is known to decrease markedly after approximately 20 minutes, so the system would be ineffective for a long incubation. This could be overcome by the continuous addition of microsomal supension and NADPH, but this would compromise sterility even further. The use of short term assays are more practical, when considering the eventual use of the assays, that of screening new compounds. However changes would be necessary, in order to develop any assay further.and there are various options which can be employed.

Hepatocytes are frequently used in in vitro incubations, but these also have a limited life span and like microsomes and S9 liver fractions, they have differing levels of activity according to variation. It is possible to produce microsomes from genetically transfected yeast cells, which express specific cytochrome P-450. The microsomes can be then combined or used on their own, to produce a known metabolic profile for a drug. The use of these yeasts for metabolism studies, was considered as a replacement for rat microsomes in the tests. However, like the other exogenous metabolism systems, these microsomes have a limited period of activity and would not be suitable for long term incubations. Another option, is the co-incubation with a metabolically active cell line, for example, HepG2 cells (Hall *et al.* 1991). The problem with such cell lines, is that they often do not express all the P 450 enzymes normally seen in hepatocytes and so may not show the 'full metabolic picture' and toxic events. However, with the advent of gene transcription, cell lines have been cultured which can express the P-450's, whose genetic material has been encoded within the nuclear material. The use of such cell lines in *in vitro* assays, has been described previously (Gibson 1995).

The cell line used in a test and the way in which it is grown, is also important. In the studies, the cells were all grown such that the monolayers adhered to the bottom of the wells and only the apical surface was exposed to the potential toxic agent. Work by

Freshney (1995), indicates that monolayers should be grown in a system that gives greater exposure to other cell surfaces. Growth on membranes or other solid supports like polyacrylamide beads is one option or growth in capillary system, were cells adhere to the lumen of the fibres and are pefused with media. The use of 'feeder' epithelial cells with the cell line was also recommended as cell-cell signalling is maintained, which can have various consequences, for example, in perpetuation of enzyme expression and response to toxicity. It has been seen that there is a difference in response between non-proliferative and proliferative cells. But there also maybe differences in response to toxicity, between cell lines of different origin. This results from specific mechanisms inherent in the cell from to its original source. Based on this, all of the cell lines used should have been subjected to all of the function assays used, to establish the impact of toxic metabolites on the same functions in different types of cell. In addition, the ability of metabolites to disrupt cell functions may not be immediately apparent. Therefore, further incubation of the cell cultures, before assessing the functions, may show such pertubation of the cell systems. This also allows the growth of the colony and the protein content to be assessed, which will indicate any cytostatic effect.

The object of this thesis was to develop *in vitro* tests where changes in cell function, rather than cell death, were the indices of cytotoxicity. The biochemical markers chosen, with the exception of inositol phospholipid expression, are commonly used by many workers, as can be seen in many publications (Kilgour *et al.* 1995, Timbrell *et al* 1995). However, most studies appear to employ several methods of determining cell functionality, some specific to the type of cells used. In addition, reported studies also assess growth rates and cell viability, by membrane exclusion methods or LDH leakage. Therefore, with the work described in this thesis, more assessment of the different aspects of cell functionality and investigations into growth and cell viability after exposure, may have given evidence of toxicity in the cells.

Finally it is known that, *in vivo*, the toxic effects of drugs are lessened by the influence of systemic glutathione and this is demonstrated in the cases of patients with low systemic

glutathione (Van der Ven *et al.* 1991). With the cell lines, there is a loss of many of the factors that can affect a tissues response to a toxin, in the host system. Hormones, growth factors and second messengers can all have an effect and though can be introduced into the media of the cells, will not necessarily be that seen in a physiological response. The homogenicity of the cells and lack of three dimensional structure, is also a factor to be noted. The use of reaggregates to mimic 3D effects can be employed and the heterogenicity can be mimicked by growing different cells of the same organ in a capillary arrangement.

If toxicity were seen in all the cell systems, after these changes were made then further experiment would have to be performed, to give more data to statistically interpret. The studies would have to be performed several times in one day and over several days. The experiments would also have to repeated by several workers, all to establish the robustness of the test. The battery of test compounds would have to be increased, all with known cytotoxic potential but different pharmacological action. These validation steps would be necessary, before the test could be used routinely as a adjunct to drug safety testing. However, these tests are unlikely at the present time to replace animal testing, as the correlation with results from *in vivo* testing is poor. There is still a requirement to study the fate of a drug *in vivo* and the toxicity arising, as many of the mechanisms of toxicity *in vivo* are still unexplained. The validity of *in vitro* toxicity tests should not be diminished, as there are still areas of cell functioning and its disruption by compounds, that have not been established.

The central finding of the research, was that the practicality of using cell lines was outweighed by the relevance of the results obtained. The immortal cell lines may be proliferative and the assays using cell lines robust and capable of producing reproducible results. However, the cell lines were unrepresentative, showing no apparent vunerability to toxicity and not correlating with *in vivo* studies. The primary cells such as erythrocytes, did show vunerability to toxic metabolites and the results correlated well with *in vivo* studies. Assays involving primary cultured cells are limited by the

non-proliferative nature of such cells, in addition to the poor reproducibility and robustness often seen.

There is a continuing imperative for the development and validation of novel toxicity tests. The studies described in this thesis indicate that the type of cell and assay used, must be carefully choosen, so that the results obtained for *in vitro* tests are of the closest relevance to *in vivo* tests.

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APPENDIX 1

MATERIALS

1. CELL LINES

CACO-2 cell lines donated by Dr. P. Nicklin (Ciba-Geigy, Horsham, Sussex, U.K.). L6 and NG115-401L-C3 cell lines donated by Dr. D. Poyner, (Pharmacology Research Group, Aston University, Birmingham, U.K.).

2. ANIMALS

Male Sprague Dawley rats (approximately 300g in weight) were used for microsome preparation. The animals were supplied with food and water *ad libitum* and were supplied by Charles River (Portage, Michagan, U.S.A.).

3. DRUGS

Cyclophosphamide, propranolol, sulphamethoxazole and phenytoin were all purchased from Sigma Chemical Co. (Poole, U.K.)

Dapsone was purchased from Sigma Chemical Co. (Poole, U.K.)

Dapsone hydroxylamine was supplied by Jacobus Pharmaceuticals (Princetown, New Jersey, U.S.A.).

4. RADIOISOTOPES

^{3}H Proline (Activity 629 GBq/mmol.,14 Ci/mmol),

^{3}H Thymidine (Activity 962 Gbq/mmol, 26 Ci/mmol),

^{3}H cAMP (Activity)

^{3}H Inositol (Activity 666 GBq/mmol, 18 Ci/mmol).

All were purchased from Amersham International (Amersham, Surrey, U.K.).

5. BLOOD

Whole blood was obtained from healthy volunteers within the Department of Pharmaceutical and Biological Sciences, Aston University.

6. MATERIALS FOR CELL CULTURE.

DMEM, HBSS, Glutamine 200mM (100 x strength), FCS, NEAA (100 x strength) and Antibiotic/Antimycotic solution (100 x strength) were all purchased from Life Sciences U.K. Ltd.(formerly Gibco).

7 MATERIALS FOR MICROSOME PREPARATION

(i) BUFFERS

Phosphate buffer : potassium dihydrogen orthophosphate and di-sodium hydrogen orthophosphate were purchased from BDH Chemicals Ltd. (Poole, U.K.)

Tris KCl buffer : Tris base and potassium chloride were purchased from Fisons p.l.c (Loughborough, U.K.)

(ii) CO FACTORS

NADP (sodium salt),

NADPH (tetra-sodium salt),

Glucose-6-phosphate (mono-sodium salt)

Glucose-6-phophate dehydrogenase (Type VII from Baker's yeast)
were all purchased from Sigma Chemical Co. (Poole, U.K.)

NADPH (tetra sodium salt) was also purchased from Fluka Chemicals (Gillingham, U.K.)

Magnesium chloride (6 H_2O) was ourchased from Fisons p.l.c (Loughborough, U.K.)

(iii) LOWRY PROTEIN ASSAY

Bovine Serum Albumin (fraction V) and Folin's and Ciocalteau's Phenol Reagent were purchased from Sigma Chemical Co. (Poole, U.K.)

Copper sulphate (5 H_2O), Potassium sodium tartrate and sodium bicarbonate were all purchased from Fisons p.l.c. (Loughborough, U.K.)

8. MATERIALS FOR PROLINE ASSAY

Bovine Serum Albumin, Sodium azide, Proline and N-ethylmalamide were all purchased from Sigma Chemical Co. (Poole, U.K.)

N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES) was purchased from BDH Chemicals Ltd. (Poole, U.K.)

Triton X-100 and Optiphase Hi-safe 3 liquid scintillant were purchased from Fisons p.l.c. (Loughborough, U.K.)

Phosphate buffered saline tablets were purchased from Oxoid

9. MATERIALS FOR CYCLIC AMP ASSAY

(i) KREB'S BUFFER

Sodium chloride

Potassium chloride

Calcium chloride 1M solution

Sodium hydrogen carbonate

Magnesium sulphate (7 H_2O)

were all purchased from Fisons p.l.c. (Loughborough, U.K.)

Potassium di hydrogen orthophosphate was purchased from BDH Chemicals Ltd. (Poole, U.K.) and dextrose was purchased from Sigma Chemical Co. (Poole, U.K.)

(ii) OTHER MATERIALS

Bovine serum albumin, Charcoal, 3-isobutyl-1-methyl xanthine (IBMX), cAMP binding protein, forskolin, isoprenaline and CGRP were all purchased from Sigma Chemical Co.

Tris base and ethylene di-amine tetra acetate (EDTA) were purchased from Fisons p.l.c. (Loughborough, U.K.)

HEPES was purchased from BDH Chemicals Ltd. (Poole, U.K.)

10. MATERIALS FOR GLUTATHIONE ASSAY

(i) GLUTATHIONE BUFFER

Tri-sodium orthophosphate

Ethylene di-amine tetra acetate (EDTA)

were both purchased from Fisons p.l.c. (Loughborough, U.K.)

(ii) OTHER MATERIALS

5-Sulphasalacylic acid, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and glutathione (reduced form) were purchased from Sigma Chemical Co. (Poole, U.K.)

11. MATERIALS FOR THYMIDINE ASSAY

Angiotensin III (Acetate salt, 99% pure) and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (Poole, U.K.)

Ethanol (absolute) and sodium bicarbonate were purchased from Fisons p.l.c. (Loughborough, U.K.)

12. MATERIALS FOR INOSITOL ASSAY

Bovine serum albumin, trichloroacetic acid (TCA), ammonium formate, formic acid and (Arg^8) vasopressin (acetate salt, 98% pure) were purchased from Sigma Chemical Co. (Poole, U.K.)

Ethylene di-amine tetra acetate (EDTA) and Freon were purchased from Fisons p.l.c. (Loughborough, U.K.)

Trioctylamine was purchased from Fluka Chemicals (Gillingham, U.K.)

Lithium chloride was purchased from May and Baker (Dagenham, Essex, U.K.)

Dowex resin was donated by Dr. D. Poyner (Pharmacology Research Group, Aston University)

13. MISCELLANEOUS

Sodium hydroxide (Convol ampoule 10M strength) was purchased from BDH Chemicals Ltd. (Poole, U.K.)

Hydrochloric acid (Volumetric standard ampoule 10M), methanol and acetone were purchased from Fisons p.l.c (Loughborough, U.K.)

Dimethylsulphoxide (DMSO) was purchaesd from Sigma Chemical Co. (Poole, U.K.)

APPENDIX II

FORMULAE OF SOLUTIONS

HEPES BUFFER

HEPES	0.894 g
NaCl	1.828 g
KCl	0.112 g
$MgSO_4$	0.074 g
NaH_2PO_4	0.039 g
$CaCl_2$	0.037 g
Glucose	0.045 g
Distilled water	to 250 ml

Buffer adjusted to pH 7.4 with 1 M NaOH.

TRIS BUFFER (50 mM) $\pm$ KCl (0.154 M)

Tris buffer	6.055 g
KCl	11.500 g
Distilled water	to 1 litre

Buffer adjusted to pH 7.4 with 1 M HCl.

PHOSPHATE BUFFER (50 mM)

SOLUTION 1

Potassium di-hydrogen orthophosphate	1.36g
Distilled water	to 1 litre

SOLUTION 2

Di-sodium hydrogen orthophosphate	3.54 g
Distilled water	to 1 litre

Solution 1 was added to solution 2 until a pH of 7.4 was attained.

PHYSIOLOGICAL SALT SOLUTION

Bovine serum albumin	3 g
3-isobutyl-1-methyl xanthine (2mM)	
HEPES (20 mM)	4.7662 g
Kreb's buffer to	1 litre

KREB'S BUFFER

Sodium chloride	13.84 g
Potassium chloride	0.708 g
Calcium chloride (1 M)	2.5 ml
Potassium di hydrogen orthophosphate	0.324 g
Sodium hydrogen carbonate	4.200 g
Magnesium sulphate (7 H_2O)	0.580 g
Dextrose	4.200 g

TRIS/EDTA SOLUTION

Tris base (20 mM)	2.422 g
EDTA (5 mM)	1.901 g
Distilled water to	1 litre

CHARCOAL SLURRY

Charcoal	50 g
Bovine serum albumin	3 g
HEPES (20 mM)	4.7662 g
EDTA (5 mM)	1.901g
Distilled water to	1 litre

GSH BUFFER

tri-sodium orthophosphate (143mM)	23.4692g
EDTA (6.3mM)	2.3953g
Distilled water to	1 litre

The pH was adjusted to 7.4 using 10 M HCl

DTNB SOLUTION

DTNB (6mM)	0.2378g
GSH buffer to	100ml

5-SULPHASALACYLIC ACID SOLUTION

5-sulphasalacyclic acid (6mM)	0.2378g
Distilled water to	100ml

TRICHLOROACETIC ACID SOLUTION (5%)

Trichloroacetic acid	25g
Distilled water to	500ml

DI SODIUM CARBONATE IN SODIUM HYDROXIDE

Na_2CO_3 (2%)	10g
Sodium hydroxide solution (0.1M) to	500ml

KREB'S AND BSA(0.3%)

Bovine serum albumin	1.5g
Kreb's buffer to	500ml

KREB'S, BSA (0.3%) AND LiCl (10mM)

Bovine serum albumin	1.5g
Lithium chloride	0.2125g
Kreb's buffer to	500ml

20% TCA/EDTA (2 mM)

Trichloroacetic acid	100g
EDTA (2 mM)	0.7604g
Distilled water to	500ml

NH_4HCO_2 (1 M)/HCO_2H (0.1 M)

Ammonium formate	31.500g
Formic acid	2.300g
Distilled water to	500ml