SPECIES DIFFERENCES IN THE SENSITIVITY OF THREE IN VIVO GENOTOXICITY ASSAYS AND THE INFLUENCE OF ENZYME-INDUCING PRETREATMENTS

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by

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Declaration

I declare that this thesis contains no material which has previously been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge contains no material previously published by any person, except where due reference is made in the text.

The experimental work described herein was carried out from 1985 to 1988 in the Department of Clinical and Experimental Pharmacology, University of Adelaide. The author was a holder of a Commonwealth Postgraduate Research Grant during this period.

Results of this thesis have been presented to Meetings of the Australasian Society of Clinical and Experimental Pharmacologists in Melbourne (1986), Hobart (1987) and Adelaide (1988) and to the 10th International Congress of Pharmacology in Sydney (1987).

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree of Doctor of Philosophy.

Anthony P. Simula

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Abbreviations

AHH	Aryl Hydrocarbon Hydroxylase
BNF	β-Naphthoflavone
BrdU	5-Bromodeoxyuridine
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
CP	Cyclophosphamide
$CuSO_4$	Copper(II) Sulphate
DNA	Deoxyribonucleic Acid
DNP-GS	S-Dinitrophenylglutathione
7-ECOD	7-Ethoxycoumarin O-Deethylase
EDTA	Ethylenediaminetetra-acetic Acid
EH	Epoxide Hydrolase
EMS	Ethylmethanesulphonate
FCS	Foetal Calf Serum
FID	Flame Ionisation Detector
GLIM	Generalised Linear Interactive Modelling
GSH	Glutathione (reduced)
GST	Glutathione S-Transferase
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic Acid
i.p.	Intraperitoneally
IU	International Units
KCl	Potassium Chloride
3-MC	3-Methylcholanthrene
METEPA	Tris-(2-methyl-1-aziridinyl)-phosphine Oxide
$MgCl_2$	Magnesium Chloride
MMFO	Microsomal Mixed Function Oxygenase
MN	Micronucleus
MNNG	N-Methyl-N′-nitro-N-nitrosoguanidine

MNPCE	Micronucleated Polychromatic Erythrocyte
MNU	N-Methylnitrosourea
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
NADP	β-Nicotinamide Adenine Dinucleotide (oxidised)
NADPH	β-Nicotinamide Adenine Dinucleotide (reduced)
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
PAH	Polycyclic Aromatic Hydrocarbon
PB	Phenobarbital
PBS	Phosphate Buffered Saline
PCE	Polychromatic Erythrocyte
PM	Phosphoramide Mustard
PPO	2,5-Diphenyloxazole
PPO-OH	2-Phenyl-5-(p-hydroxyphenyl)oxazole
PPO-OHase	2,5-Diphenyloxazole Hydroxylase
RBC	Red Blood Cell
RI	Replicative Index
rpm	Revolutions per Minute
RT	Room Temperature
SCE	Sister Chromatid Exchange
sem	Standard Error of the Mean
SM	Sperm Morphology
SO	Styrene Oxide
SSC	Standard Saline Citrate
ST	Styrene
TEM	Triethylenemelamine
THIOTEPA	Triethylenethiophosphoramide
UV	Ultraviolet

Abstract

In vivo assays for genotoxicity have been used as tests for evaluating the carcinogenic potential of chemicals and as methods for estimating risk from occupational exposure. Three commonly assessed end-points are the formation of sister-chromatid exchanges (SCEs), micronuclei (MN) and abnormal sperm. Using a potent (CP) and a weak (ST) metabolically activated genotoxin, the sensitivity of the lymphocyte SCE, bone marrow MN and sperm morphology (SM) assays were assessed in the mouse and rat.

The rat was more sensitive than the mouse to the genotoxicity of CP in all three assays, while the opposite was the case for ST. Each assay in either species was able to detect both genotoxins. Of the three assays the SCE assay was the more responsive and the SM assay was the least responsive. Only with CP in the SCE assay was the threshold dose for detection of a genotoxic response below the threshold dose for the detection of any form of cytotoxicity in any of the three target tissues.

The effect of PB- or BNF-induced altered metabolism on CP and ST genotoxicity was also assessed in both species. PB potentiated the genotoxicity of CP in the mouse MN assay. The same inducer also influenced the timing of the maximal response to CP in the rat MN assay with no effect on the magnitude of the response. No other effects of PB or BNF were observed on the responses to either genotoxin, though they were able to alter the level of abnormal sperm in both species.

An attempt was made to determine the metabolic capacity of the target tissue of the in vivo assays and that of the liver, with and without enzyme inducers (phenobarbital, PB; beta-naphthoflavone, BNF). This allowed us to investigate the correlation between altered metabolism in the target tissues (or the liver) with the sensitivity of the target tissues to the metabolically activated compounds (CP and ST). Microsomal mixed function oxidase activity (MMFO), 7- ethoxycoumarin O-deethylase and 2,5-diphenyloxazole hydroxylase, was not detectable in the S9 mix of the target tissues. Epoxide hydrolase (EH) and glutathione S-transferase (GST)

were detectable in all tissues and furthermore, were inducible by PB or BNF in some of the target tissues.

PB was able to increase the hepatic activity of each enzyme in both species. PB also induced EH in rat testes and GST in mouse spleen. BNF induced PPO-OHase, 7-ECOD and EH in rat liver but only GST and EH in mouse liver. BNF also induced GST in mouse testes and spleen and EH in mouse and rat testes. Except for the potentiation of CP-induced MNPCEs in mice by PB, there did not appear to be any relationship between observed changes in metabolic enzyme activity with changes (or lack of changes) in the genotoxicity of CP and ST.

The study indicated that the three in vivo assays were insensitive in rodents, even in the presence of enzyme inducers, and may be equally insensitive as methods for monitoring exposure to man. More sensitive methods to monitor occupational exposure, such as quantitating DNA adducts, are discussed along with their role in future research into occupational monitoring and carcinogenicity testing.

Contents

Chapter 1

General Introduction

Quantitatively, approximately 35–40% of all cancers are thought to be due to exposure to chemicals through smoking, occupation and/or other environmental factors, although many scientists believe this figure to be much higher (Farber, 1987). Consequently, there has been a great need to identify and control these agents in order to reduce cancer incidence. Testing of compounds for carcinogenicity rely upon human epidemiological studies and prolonged life-time feeding studies in rodents, in which cancer incidence is determined histopathologically. With the current production rate of hundreds of new chemical substances annually, the task of carrying out long-term feeding studies becomes very difficult, if no impossible. This is due, in part, to their high costs, requirement of large animal numbers (500–1000 rodents per chemical) and the time involved (2–4 years per chemical)(Purchase & Ashby, 1982). Limitations associated with epidemiological studies include the fact that they are also extremely time consuming, not very sensitive and usually retrospective (Forni & Bertazzi, 1987).

1.1 Short-term Tests for the Detection of Genotoxic Carcinogens

It is now a widely accepted view that the carcinogenic process, for most chemically-induced tumors, involves an initial genetic change giving rise to heritably altered cells with an increased selective advantage over normal cells (Nowell, 1976). Recent evidence suggests that neoplastic development, at least in part, is the result of the abnormal activation of a small set of cellular genes (Stowers et al. 1987). These genes, often termed proto-oncogenes, are expressed during normal growth and developmental processes. The conversion of proto-oncogenes to activated oncogenes, by genetic alterations results in altered levels of expression of the normal protein product, or in normal or altered levels of expression of an abnormal protein. The na-

ture of these genetic changes include point and frameshift mutations, chromosomal rearrangements, gene amplification and aneuploidy (Barrett & Shelby, 1986). With the advent of techniques to detect such genetic alterations (or genotoxic events) in various whole animal, cellular or bacterial systems, short-term tests were developed in an attempt to screen chemicals for carcinogenic potential. The initial advantages of the tests, over the life-time feeding studies, are their simplicity, speed and economical use of biological materials.

In the early 70's, Ames developed a test based on the detection of mutations in bacteria, capable of recognising chemicals with mutagenic properties (Ames et al. 1971; Ames et al. 1973a; Ames et al. 1973b). The types of genetic damage detected by the different bacteria strains employed are point and frameshift mutations. Early work with the Ames test, found the correlation between carcinogenicity and mutagenicity in the test to be as high as 90% (Purchase et al. 1978; McCann et al. 1975). It was subsequently employed as a screening procedure for carcinogens and today is the most widely used in vitro genotoxicity assay. In the last few years it has become increasingly clear it is possible to select a group of chemicals in such a way as to produce excellent or poor results from a validation study. More recent assessment of the Ames test and the in vivo cancer bioassays, by the United States National Toxicology Program (NTP), has established a current view that the sensitivity of the former assay, to 130 carcinogens and equivocal carcinogens defined by the NTP, could be as low as 53% (Ashby & Purchase, 1988).

Conditions in vitro are very different from the whole animal (or in vivo) situation. Bacterial or mammalian cell assays are sometimes rendered more susceptible to genotoxins by various methods including switching off DNA repair, turning on error-prone DNA repair or by putting the cells in contact with metabolic activation systems often derived from livers of induced animals (Tassignon, 1985). Furthermore, the ability of a chemical, genotoxic in in vitro test systems, to induce a similar effect in vivo may depend on dispositional factors such as route of exposure, distribution, metabolism (activation/deactivation), excretion and cellular DNA-repair mechanisms. These factors may ultimately determine a chemical's species- and tissue-specific genotoxic potential (Ashby, 1983).

Many in vivo short-term tests have been available for as long as most in vitro tests but only over the last decade have they become more widely used as methods of genotoxicity testing. Some of the in vivo genotoxicity tests, able to detect various genotoxic endpoints include;

Mouse Spot Test	Russell and Majors, 1957.
Micronucleus Assay	Heddle, 1973.
Mouse Specific Locus Test	Russell, 1951.
Dominant Lethal	Bateman, 1966.
Sperm Morphology Assay	Wyrobek and Bruce, 1975.
Sister Chromatid Exchange Assay	Allen and Latt, 1976.

These assays are generally carried out in rodents such as rats, mice and hamsters. In vivo tests, like the micronucleus (MN) and sister chromatid exchange (SCE) assays, can be used as in vitro test, provided the target cells can be cultured. The MN and SCE tests are very commonly used as they are relatively simple procedures where a number of target tissues can be used, making possible the assessment of tissue differences in susceptibility to a genotoxic agent. The following tissues have been used in the micronucleus test: liver, bone marrow, germ cells and lymphocytes (Tates et al. 1983; Toppari et al. 1986; Schlegel et al. 1986; Schmid, 1975), while liver, bone marrow, germ cells, spleen-derived and peripheral blood lymphocytes have been used for the SCE test (Kligerman et al. 1985; Krishna et al. 1988; Schreck et al. 1979).

The development of many of the in vivo tests took place with the use of a single species. This lead to a situation where a species often became synonymous with a particular test with little or no development of other animal models. This has occurred, to varying degrees, with the Mouse Specific Locus, Mouse Spot, Micronucleus and the Sperm Morphology tests, where the mouse has been almost exclusively used. One of the difficulties associated with assessing genotoxic potential in vivo is that the response to genotoxic compounds may vary in different mammalian species.

Another area, in which in vivo tests for genotoxicity are finding an increased usefulness, is in biological monitoring. Where the target tissue is readily accessible (lymphocytes, sperm), the tests are being employed as a means of surveying human populations exposed to potentially genotoxic chemicals (Forni & Bertazzi, 1987), in order to prevent the occurrence of undesirable effects. Despite the increased application of cytogenetic monitoring of occupationally exposed populations, the recreation of such exposures in the laboratory has been difficult as exposure can often be to a mixture of chemicals, where complex interactions between the chemicals can occur. Some interactions may involve exposure-mediated changes in xenobiotic metabolism, altering the potential genotoxicity and cytotoxicity of other compounds also involved with the exposure. These interactions are difficult to isolate and examine in human studies.

1.2 Metabolism and Genotoxic Compounds

Many genotoxic compounds require activation to the toxic metabolite, by both Phase I, and to a limited extent, Phase II enzymes (Wright, 1980; Dipple et al. 1985). The reactive metabolites, which can take the form of nucleophiles or electrophiles, are then able to attack cellular macromolecules such as proteins and DNA. Variations in drug metabolism from one species to another may appear as qualitative differences in the actual pathways present and/or quantitative differences in the level of activity of pathways common to several species (Lorenz et al. 1984; Kato, 1979). These species differences may account for the marked species-specific toxicities often encountered, even in species as closely related as rats, mice and hamsters (Madle et al. 1986b; Tee et al. 1986). The distribution of various detoxifying enzymes, such as glutathione S-transferase, epoxide hydrolase and glucuronyl transferase, are as important as activating enzymes in determining species-specific toxicities (Oesch et al. 1977b; Bock et al. 1987). There also exist species differences in the activity and distribution of xenobiotic metabolising enzymes in various tissues, of which the liver

generally contains the highest concentration.

With the advent of more sensitive assays, Phase I and Phase II metabolic enzymes have been detected in a large number of other organs including the lung, kidney, placenta, testes, skin, spleen, bone marrow, pancreas and ovaries (Bend & Serabjit-Singh, 1984; Gram et al. 1986). Since many genotoxic carcinogens are able to induce tumors extrahepatically, tissue-specific differences in oxidative and conjugative enzymes (and the consequent balance between activating and deactivating pathways) can be major determinants in the principle site of action of many genotoxins, as activation would occur in close proximity to possible target tissues. This has been demonstrated by Gelboin et al. (1969), Kinoshita and Gelboin (1972) and Wattenberg and Leong (1968) where they showed a direct correlation between the susceptibility of certain tissues to polycyclic aromatic hydrocarbon (PAH)-induced tumors, the formation of PAH-DNA adducts within those tissues and their ability to metabolically activate polycyclic aromatic hydrocarbons.

There are hundreds of chemicals in our environment known to stimulate the activity of xenobiotic-metabolising enzymes (Conney, 1982). Exposure to these compounds may occur occupationally, iatrogenically or by self-administration (smoking, diet). These compounds have diverse structural and chemical characteristics including hypnotics and sedatives (phenobarbital, PB), polycyclic aromatic hydrocarbons (3-methylcholanthrene, 3-MC and β-naphthoflavone, BNF), insecticides, analgesics, antihistamines, anti-inflammatory drugs, hypoglycaemic agents, tranquillizers and various organic solvents. Studies concerned with the effects of compounds on drug-metabolising enzymes have dealt predominantly with the induction of the microsomal mixed-function oxidases (MMFO).

In mammals the MMFO system is made up of a number of isozymes of the cytochrome P-450 haemoprotein. The isozymes have differing and sometimes overlapping substrate specificities, each of which display differential inducibility profiles with various inducing agents. They are often grouped according to the class of compound they are induced by. There are at least four such classes of isozymes: the PB inducible, 3-MC inducible, steroid inducible and the pituitary hormone in-

ducible (Whitlock, 1986). Most of the studies investigating induction of xenobiotic metabolism have concentrated on the effects of PB and 3-MC (or BNF), as their respective isozymes are responsible for the biotransformation of many exogenous compounds. As well as Phase I metabolism, various Phase II enzymes are also inducible by different compounds. They include glutathione S-transferases, epoxide hydrolase and glucuronyl transferases (De Pierre et al. 1984; Burchell et al. 1984; Bock et al. 1987).

Enzyme induction may alter a compound's genotoxic potential in a number of ways. For a genotoxin not requiring activation and whose metabolism renders it non-genotoxic, induction of its biotransformation will reduce its DNA-damaging capacity. On the other hand, for a genotoxin requiring metabolic activation, where there is competition between activating and deactivating pathways, the extent to which the competing enzymes are induced will determine whether the balance changes in favour of increased or decreased toxicity. Species differences in the induction of xenobiotic metabolism has been demonstrated on a number of occasions (Litterst et al. 1977; Bilimoria & Ecobichon, 1980). Moreover, the induction profile of compounds like PB and 3-MC display tissue specificity. Burke and Orrenius (1979) showed 3-MC was able to induce 3,4-dimethylaminoazobenzene N-demethylation 7-fold in rat lung microsomes, while only detecting a 2-fold increase in rat liver microsomes. Similar results were also observed by Ciaccio and De Vera (1976), looking at aryl hydrocarbon hydroxylase (AHH) induction by benzo[a]pyrene in various tissues and by Guengerich and Mason (1979) measuring 7-ethoxycoumarin O-deethylase and AHH induction with PB and 3-MC. It is, therefore, conceivable that enzyme induction may alter the balance between activation and deactivation in a tissue-specific manner. This may lead to a shift in the primary site of insult of a particular genotoxin from one tissue to another. Ultimately, where the genotoxic event may be involved in tumour initiation and/or promotion, enzyme induction has the potential to alter the primary sites of tumour formation.

Short-term in vivo genotoxicity tests provide a convenient and apppropriate method with which to assess the ability of enzyme-inducing pretreatments to modify a chemical's genotoxic responses. The bone marrow MN, sperm morphology

(SM) and the splenocyte SCE tests were utilised in this study using PB and BNF pretreatment as methods of enzyme induction. Although 3-MC is a more potent inducer than BNF of aromatic hydrocarbon hydroxylases (Boobis et al. 1977), the latter was used in preference in this study, since 3-MC is carcinogenic and therefore a hazard when used on a continuous basis. Furthermore, 3-MC is found to be positive in the MN and SM assays (Wyrobek & Bruce, 1975; Jenssen & Ramel, 1980), although the in vivo genotoxicity of BNF is not known. This genotoxic property of 3-MC would make assessment of enzyme induction by this chemical, on the genotoxicity of test compounds, extremely difficult.

The sensitivity of these in vivo tests to metabolically activated genotoxic compounds is determined by a number of factors. Firstly, the ability of the target tissue to activate or deactivate the compound locally. Secondly, where the compound is not activated at the target tissue, the genotoxic metabolite may or may not be readily accessible to that tissue. Thirdly, the ability of the active metabolite, once in the target tissue, to induce the genotoxic endpoint being measured. This may be dependent on the efficiency with which the DNA repair mechanisms in that tissue are able to correct the damage before it is detected by the assay.

Studies are not available, which allow a comparison of the sensitivity of the three in vivo genotoxicity assays, although Madle et al. (1980a, 1980b) have compared species differences in the sensitivity of the SCE and MN tests in the rat and mouse. Assessing cyclophosphamide-induced genotoxicity, they found the mouse to be less sensitive than the rat in both test systems. Furthermore, the SCE assay gave more explicit responses than the MN test in both species. The same group also investigated aflatoxin B_1 mutagenicity in a similar manner and again found the rat to have greater sensitivity. In this case, comparison of the two test systems showed the compound to be a better inducer of micronuclei than SCE's. These studies suggest the sensitivity of in vivo assays may vary depending on the test compound being examined and its ability to produce the appropriate lesions responsible for the endpoints being measured. This study will also attempt to compare the sensitivity of the three in vivo assays and the effects of enzyme-inducing pretreatments on genotoxic responses in the rat and mouse.

1.3 In Vivo Genotoxicity Assays

1.3.1 Rodent Bone Marrow Micronucleus Assay

A micronucleus (MN), as the name implies, is a fragment of genetic material distinct from the main nucleus and usually no larger than $\frac{1}{4}$ the size of the main nucleus. Micronuclei are induced by agents that can cause chromosomal breakage or act as spindle poisons. The nature of the lesion(s) responsible for production of micronuclei is unclear. Evidence for a relationship between chromosomal breakage and carcinogenesis comes from studies with individuals suffering from diseases associated with increased susceptibility to chromosomal breakage, like Bloom's Syndrome, who also have a predisposition toward cancer (German, 1974). Compounds which are either carcinogenic or non-carcinogenic in rodent cancer bioassays, have been used to assess the predictivity of the MN assay for carcinogenicity of chemicals. The assay is found to have a high rate of correctly classing known non-carcinogens (specificity), while its ability to correctly class known carcinogens (sensitivity) is low. Consequently, it is commonly used as a confirmatory assay in chemical carcinogenicity testing.

Two independent investigators, Schmid (1975) and Heddle (1973), developed a protocol using rodent femoral bone marrow. In the bone marrow MN test, the polychromatic erythrocyte (PCE) population is assessed for micronucleus frequency. Although they are not the target cell, they represent a convenient stage, derived from the affected erythropoietic blast cells, at which to assess chromosomal damage. The test relies on the ability of the affected cells to undergo cellular division in vivo. In anaphase of the last mitosis of the erythroblast, any induced acentric chromatids or chromosomal fragments lag behind when the chromosomes move toward the spindle poles. At telophase of cell division any lagging elements are not integrated into the daughter nuclei and remain in the cytoplasm of the daughter cells as micronuclei. After the last mitosis, the erythroblasts expel their main nucleus and become PCEs,

with characteristic basophilic staining. For reasons not known, micronuclei present in the blast cells are not expelled along with the main nucleus. After a period of time, the PCEs lose their basophilic staining property and are then classed as normochromatic erythrocytes or red blood cells (RBC). By using a combination of a Romanowsky-type stain to distinguish between PCEs and RBCs and a DNA-specific stain, micronuclei can be visualised in PCEs and, with a lower frequency, in RBCs. This process is described diagrammatically in Figure 1.1. Although the micronucleus test has been conducted in a number of target cells from plants and animals, the mammalian method, using bone marrow, has become the standard system for this assay.

The time of sampling of bone marrow for assessment of genotoxicity, is critical to the sensitivity of the assay. Ideally, the chemical induction of micronuclei should only be assessed in PCEs derived from unequivocally exposed erythroblasts. The time to sample the bone marrow after treatment would be equivalent to the time taken from the last mitosis to the end of the PCE stage. This represents the time required for maximal accumulation of PCEs derived from exposed erythroblasts, which is the earliest time that a maximal response will appear. Various studies using mathematical models or ^{3}H-TdR incorporation, have estimated duration times ranging from 3–10 hrs for the time between the last cell division and nuclear expulsion and 10–33 hrs for the duration of the PCE stage (Tarbutt & Blackett, 1968; Mary et al. 1980; Cole et al. 1981; Jenssen & Ramel, 1978). Experimentally, the duration from the last mitosis to the end of the PCE stage, is observed to be between 24–30 hrs (Salamone & Heddle, 1983).

Several factors may influence the timing of the maximal response. Pharmacokinetic factors including the rate of absorption, metabolism (activation/deactivation) and transport to the target tissue may contribute to a delayed response. Since the assay depends on the ability of the affected cells to divide, any treatment which is cytotoxic will delay the cell cycle kinetics and consequently the timing of the maximal response. Hence the test is usually conducted with multiple sampling of the bone marrow after dosing to maximise the likelihood of detecting a genotoxic response. Salamone and Heddle (1983) compared the time of peak MN frequency

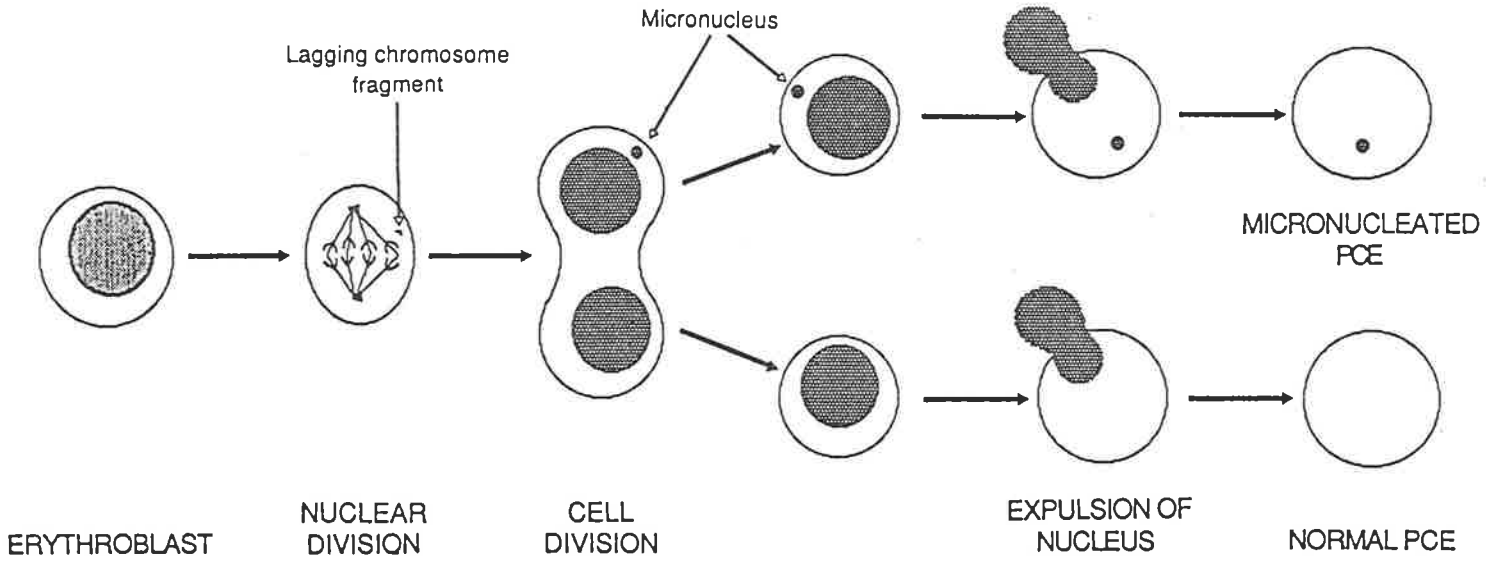


Figure 1.1: Formation of a micronucleated PCE from a DNA-damaged dividing erythroblast in femoral bone marrow.

for 15 different compounds and found it to range from 18–72 hrs post-dosing. They went on to suggest the interval between sampling times should not exceed the time from blast division to the end of the PCE stage (24–30 hrs) and sampling need not be carried out for longer than 96 hrs.

A number of studies have assessed numerous treatment regimens for this assay where one, two, three, four and five dosing-treatment protocols were adopted. Studies involving protocols of three or more treatments have been inconclusive. Salamone et al. (1980) demonstrated that some compounds when administered more than twice were toxic to the bone marrow, to the extent of interfering with the assay by severely suppressing the PCE formation. In some cases, the assay failed to detect the genotoxic activity of chemicals, known to induce micronuclei, when one- or two-dose protocols were employed. In other cases, comparing single-dose and five-dose regimens have shown the multi-dose regimen to produce a greater response than a single dose (Yamamoto & Kikuchi, 1981). Typically, when testing a compound's genotoxicity, the highest dose administered should be the maximum tolerated dose. MacGregor et al. (1987) proposed the following criteria for determining the highest dose: (1) It should cause a marked and significant increase in the micronucleus frequency in the target cell, (2) It should produce a significant suppression in the number of PCEs, or (3) It should cause compound-related signs of toxicity or significantly reduced survival.

The basal level of micronucleus frequency is very low in rats and mice, ranging from 1-3 micronucleated PCEs (MNPCEs) in 1000 PCEs. A statistically valid assessment of a chemical's genotoxic potential, would therefore require counting of at least 1000 PCEs per animal in order to establish a measurable baseline (MacGregor et al. 1987; Ashby & Mohammed, 1986). In addition to the frequency of micronucleated PCEs, the ratio of PCEs to RBCs can be determined. This ratio is usually approximately 1:1 and 2:3 in mice and rats, respectively. A chemically induced change of this ratio in favour of RBCs is an indication of cytotoxicity, causing impaired haemopoiesis.

The susceptibility of the bone marrow to a genotoxic chemical may be dependent on its ability to metabolically activate and/or deactivate the chemical locally. Unfortunately, there is a paucity of data on the metabolising capacity of the bone marrow. Dresner et al. (1981) investigated the activity of Phase I and Phase II in rat bone marrow. Using microsomal preparations, they were able to detect aminopyrene-N-demethylase activity which was inducible by 3-MC, while UDP-glucuronyl transferase was only detectable after 3-MC induction. Other investigators have detected glutathione S-transferase activity in mouse bone marrow (Adams et al. 1985) and 7-ethoxycoumarin O-deethylase and 3-MC-inducible benzo[a]pyrene hydroxylase in rabbit bone marrow (Andrews et al. 1976; Gollmer et al. 1984). These studies show that bone marrow does have the ability to metabolise xenobiotics by both Phase I and Phase II enzymes, albeit at a very low rate.

1.3.2 Rodent Sperm Morphology Assay

The Sperm Morphology (SM) assay is based on visually scoring the percentage of sperm with abnormal head shapes, in sperm smears from the epididymis or vas deferens after treatment. Although the test is applicable to any animal, most of the knowledge and data available has been derived from experiments in mice. In 1975, Wyrobek and Bruce proposed a system whereby the induction of abnormal sperm-head shapes could be used as an index of genotoxicity, after showing an increased production of abnormal sperm from mice exposed to mutagens and carcinogens. There are a number of lines of evidence supporting this original concept. Firstly, studies have indicated that sperm-head shape is genetically controlled by both autosomal and sex-linked genes (Krzanowska, 1976). Secondly, the level and types of sperm-head shape are characteristic of strains and the heritability of their dimensions in mice is very high (Illison, 1969). Thirdly, almost all germ-cell mutagens tested are able to induce sperm abnormalities in mice and this effect has been shown to be heritable (Topham, 1980a; Topham, 1980b).

It is not clear if or how a change in the proportion of abnormal sperm is related to carcinogenesis. Investigation of the ability of the sperm morphology test to assess a compound's carcinogenicity, has indicated it to have low sensitivity and very high specificity, when the results are compared to those of cancer bioassays (Wyrobek et al. 1983a; Purchase & Ashby, 1982). Like the MN test, a positive result in this assay may be helpful in assessing carcinogenic potential, while no conclusion can be drawn from a negative result.

One of the problems associated with this assay is the ease with which the basal level of abnormal sperm is altered by factors thought to be non-mutagenic. Komatsu et al. (1982) demonstrated an increased production of abnormal sperm with dietary restrictions. A reduction in food intake may be brought about through illness induced by the chemical being tested for genotoxicity. Such an effect would make interpretation of results at toxic dose levels difficult. A change in the animal's body temperature has also been demonstrated to increase the production of abnormal sperm in rodents (Cairnie & Leach, 1980). Factors such as infection, ischaemia, anaesthesia and endocrine dysfunction are also known to affect abnormal sperm production (Topham, 1983). A study by Sega and Owens (1978) showed EMS-induced dominant lethality at various stages of germ-cell development. They demonstrated that the stages of greatest sensitivity did not correlate with the timing of maximal DNA alkylation, but correlated with the timing of maximal protamine alkylation at cysteine-sulphydryls by EMS. As protamine disulphide bond formation is important in chromatin condensation in spermiogenesis. Impairment of this process, rather than a genotoxic event, may lead to abnormally-shaped sperm.

Two important factors, regarding the physiology and histology of the testis as a target organ, should be considered. Firstly, it has a tissue permeability barrier known as the blood testis-barrier (BTB), similar in structure to other barriers found in organs such as the brain and thymus (Neaves, 1977). Apart from its role in maintaining a special aqueous environment favourable to spermatogenesis, it is also thought to act as a protective screen against a variety of toxic agents. Okumura et al. (1975) demonstrated the ability of the BTB to restrict the access of certain xenobiotics into the testicular environment. A chemical's ability to cross this bar-

rier was found to depend on its molecular size and lipophilicity. This factor must be considered when assessing a chemical's genotoxicity to germ-cells. Secondly, the seminiferous tubules within the testes contain germ-cells at various stages of spermatogenesis as well as interstitial cells, all of which differ in their metabolic and DNA-damage repairing ability (Sega, 1982; Dixon & Lee, 1980). Therefore, germ-cells may vary in their sensitivity to genotoxic chemicals depending on their stage of development. Furthermore, it is possible to assess the susceptibility of the different stages of spermatogenesis to DNA damage and its persistence by sampling sperm at certain times after exposure to a chemical.

Spermatogenesis consists of 3 distinct phases. The first stage contains spermatogonia, which continually divide to give rise to spermatocytes and to renew their numbers. The second phase consists of primary and secondary spermatocytes, which undergo meiotic division to become spermatids. The last phase (spermiogenesis) involves the spermatids going through a complex cytological transformation developing into spermatozoa (Clermont, 1972). A number of studies have been conducted to estimate the time taken for a spermatogonial cell to mature into spermatozoa and eventually reach the vasa deferentia (Leblond & Clermont, 1952; Oakberg, 1956; MacMillan & Harrison, 1955). Based on these reports it is estimated that it would take an early spermatid, early primary spermatocyte and the earliest spermatogonium approximately 3, 5 and 7 and 5, 8 and 11 weeks to reach the vasa deferentia in the mouse and rat, respectively. Studies investigating the persistence of induced increases in abnormal are limited. Wyrobek and Bruce (1975), sampling sperm at 1, 4 and 10 weeks following completion of dosing over five consecutive days, found 3,4-benzpyrene, METEPA, THIOTEPA and mitomycin C induced changes that persisted for up to 10 weeks. Out of 25 chemicals they tested, the 4 week sample was more sensitive to induced abnormal sperm. Based on these results the sperm morphology test is usually conducted with a sampling time at 5 weeks from commencement of dosing.

With the advent of reproductive toxicity testing, there has been much research concerned with testicular metabolism of xenobiotics. Mukhtar et al. (1978) measured glutathione S-transferase, epoxide hydrolase, AHH and cytochrome P-450 con-

tent in rat testes. AHH had approximately 5% of the specific activity in liver while the Phase II enzymes were in the range of 10–50% of their hepatic counterparts. They also observed a differential distribution of these enzymes. Phase I enzymes were higher in the interstitial cells compared to germ-cells, while the opposite was true for phase II enzymes, suggesting a very high capacity to detoxify potentially toxic compounds. Oesch et al. (1977b), investigating species differences in the distribution and activity of epoxide hydrolase, found the microsomal activity to be twice as high in the testes of mice compared to liver. In contrast the activity in rat testes was only $\frac{1}{4}$ that of the liver. The inducibility of testicular xenobiotic-metabolising enzymes is variable. Lake et al. (1973) showed 3-MC induced benzpyrene hydroxylase by 50% in rats, while Mattison and Thorgeirsson (1978) found 3-MC was able to induce AHH by 200–300% in 3 out of 3 rat strains and 1 out of 2 mouse strains. Stripp et al. (1974) looking at hormone metabolism, observed no induction of testosterone hydroxylation or cytochrome P-450 content by PB or BNF.

1.3.3 Splenocyte Sister Chromatid Exchange Assay

Sister-chromatid exchanges (SCE), as defined by Latt et al. (1977), represent the interchange of DNA between replication products at apparently homologous loci. SCEs can be induced by physical agents such as ionizing and UV-radiation and many genotoxic chemicals. It is therefore reasonable to assume, they are the direct result of modification or damage to DNA.

Although a number of mechanisms for SCE formation have been considered, the exact mechanism is yet to be determined with DNA breakage and reunion during S-phase of the cell cycle being the most favoured. Shafer (1979) put forward a replication bypass model for SCE formation; bidirectional replication forks approach a segment of modified DNA, the resultant stress near the modified DNA causes breaks or incisions on opposite parental strands and the SCE is formed by incorrect rejoining of the opposite free ends. The modified DNA can take the form of crosslinks,

single-strand breaks or the site of some unrepaired or partially repaired DNA lesion. Painter (1980) suggested a similar mechanism whereby agents, which damage DNA in such a way as to block chain elongation, will often cause DNA in replication clusters to remain partially unreplicated for unusually long periods. In this situation the probability of a double-strand break at the junction between a replicated and partially replicated strand of DNA is increased. Incorrect recombination of the induced double-strand breaks at replication forks completes the chromatid exchange. This hypothesis may also explain the high number of spontaneous SCEs observed in cells from individuals with Bloom's syndrome, where it has been shown that the average rate of DNA replication-fork progression is about 30% slower than in normal cells (Chaganti et al. 1974; Hand & German, 1975).

Studies comparing the sensitivity of SCE induction with various other genotoxic endpoints, have found it to be as sensitive, if not more sensitive, than other tests for DNA damage. Perry and Evans (1975), when comparing the SCE test with induction of chromosomal aberrations, showed that in most of the 14 compounds tested the SCE test was more sensitive. Kaina (1985) using MNU and MNNG, found the SCE test to be more sensitive than mammalian cell mutation and chromosomal aberration tests. As a predictor of carcinogenicity, the SCE test correctly classes chemicals, which are rodent carcinogens or non-carcinogens according to cancer bioassays, approximately 50 and 100% of the time, respectively (Latt et al. 1981; Purchase & Ashby 1982). Popescu and coworkers (1984, 1985) showed a strong correlation between SCE induction and the morphological transformation of Syrian hamster foetal cells. Hence, they proposed that lesions involved in SCE formation may be important in the initiation of neoplastic development.

SCEs can be observed on chromosomes during metaphase of mitosis. The visualisation of the exchanges relies on the ability to differentially stain two sister-chromatids. The staining is based on a process which alters the DNA strand properties by incorporating base analogues (usually 5-bromodeoxyuridine, BrdU) followed by a procedure enabling the visual discrimination of the sister chromatids. The process requires the target cells undergo two cell cycles, provided either the first or both cycles are in the presence of the DNA base analogue. After that time one chromatid

will contain a greater proportion of the base analogue than its sister-chromatid. The cells are then arrested at metaphase of the cell cycle by spindle poisons such as colcemid, fixed, placed on slides and visualised by various means.

Kato (1974) originally used ^{3}H-thymidine incorporation for the first of two cell cycles followed by autoradiography to visualise the sister-chromatids. This procedure proved to be inadequate when assessing chemicals that produced a large number of SCEs, due to the low resolution obtained. The use of BrdU-incorporation over two cell cycles was later employed (Zakharov & Egolina, 1972), which gave greater resolving power for closely spaced SCEs. The differential appearance of the sister-chromatids is based on the inability of the more substituted chromatid to take up various dyes and hence stain less intensely than the less substituted chromatid (Figure 1.2).

Sister-chromatid differentiation can be carried out by a number of methods. The more commonly employed techniques consist of either staining with the DNA-binding fluorescent dye 33258 Hoechst and visualising by fluorescence microscopy (Latt, 1973) or following the staining with 33258 Hoechst, the chromosomes are exposed to UV light then stained with Giemsa and visualised with a light microscope (Perry & Wolff, 1974). The latter method, of which there exist many variants (Block, 1982), is preferred due to the permanent nature of the stain. The slides can be assessed and then stored for future reference.

The SCE test can be carried out entirely in vivo or by an in vivo/in vitro method. The former involves exposing the animal to the test compound, followed by administration of BrdU and subsequently colcemid (to arrest cells at metaphase). The animal is then sacrificed and the target tissue removed, the cells are fixed, placed on slides and stained (Allen et al. 1977). In the alternative method, animals are exposed to the compound, sacrificed after a predetermined period of time, the target tissue removed and the cells cultured in the presence of BrdU. Some important factors must be known before the former method can be used. Firstly, the target cells must be a continuously dividing population and a knowledge of the cell replication rate is required in order to obtain significant numbers of second division

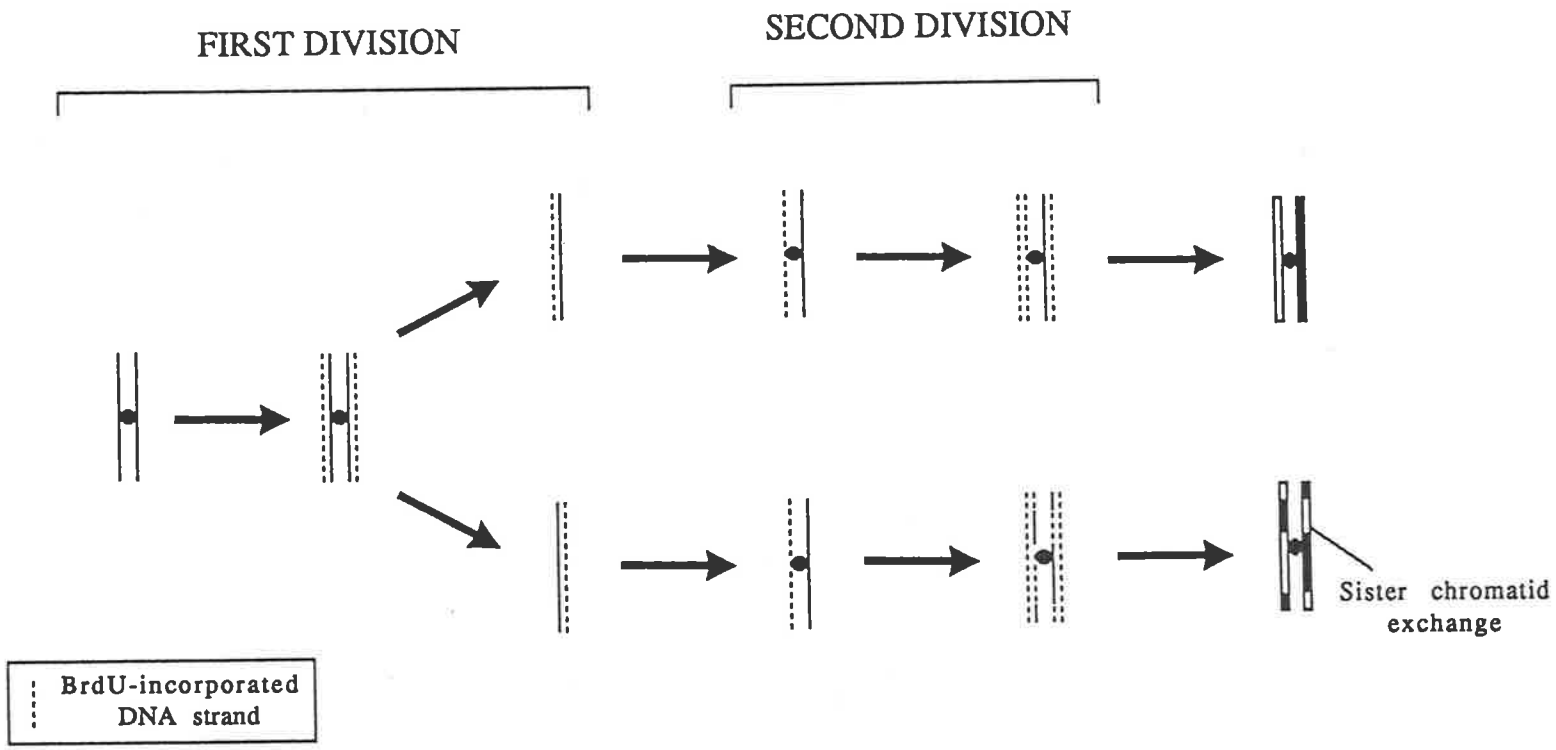


Figure 1.2: The differential staining of sister chromatids by the incorporation of BrdU into replicating DNA over two cell cycles.

metaphases. Secondly, sufficient BrdU must reach the target tissue to permit differential staining. Finally, the BrdU concentration must not severely inhibit cellular proliferation of the target tissue as this would reduce the number of second division metaphases (Schneider & Kram, 1982). Some non-dividing cell populations, such as lymphocytes, may be stimulated to divide ex vivo after in vivo exposure. For this reason, the in vivo/in vitro procedure of SCE analysis is less restrictive in the choice of target tissue and consequently is the more frequently used technique.

The baseline SCE frequency is consistently lower for in vivo than for in vivo/in vitro methods, nevertheless, they are found to be equally sensitive to genotoxic chemicals (Krishna et al. 1987; Kram et al. 1979). Kligerman et al. (1982) investigated the optimum culture conditions of lymphocytes for the assay and discovered the baseline SCE frequency to be dependent on such factors as the inoculum volume, cellular concentration and the BrdU concentration used. Traditionally, the peripheral blood lymphocytes are used as the target tissue since they are a slowly dividing cell population and hence may accumulate DNA damage, increasing their sensitivity to genotoxic compounds. More recently spleen-derived lymphocytes have been employed, which are also a slow dividing cell population. Spleen-derived lymphocytes were used in experiments described in this thesis in order to take advantage of the potentially greater yield of lymphocytes compared with the low yield of blood-derived lymphocytes from a small species like the mouse. As well as assessing genotoxicity by the induced SCE frequency of SCEs, the cytotoxicity of the test chemical can also be determined. A shift in the proportion of first, second and third division metaphases, distinguishable by the staining characteristics of the chromatids, in favour of first division metaphases indicates a reduced proliferating ability, suggestive of a cytotoxic effect of the test chemical.

Like the bone marrow there is little available data on the ability of the spleen to activate/deactivate genotoxins locally. A comprehensive study, estimating styrene monooxygenase and styrene oxide hydrolase activity in various tissues, found the spleen to have 13–15% the hepatic microsomal activity of these enzymes in both the rat and mouse (Cantoni et al. 1978). The ratio of hydrolase/monooxygenase activity in the spleen was 4.79 and 1.62 in the rat and mouse, respectively, suggesting a

greater capacity of the rat to detoxify potentially toxic reactive epoxides formed. In contrast, Oesch et al. (1977a) investigating the activity of benzo[a]pyrene-4,5-oxide hydrolase in different rat tissues showed the spleen to have only 2% of the microsomal activity of the liver. Schacter and Mason (1974) assessed the inducibility of MMFO activity and cytochrome P-450 content in rat spleens. PB induction was found to have no effect on monooxygenase activity, while 3-MC and 3,4-benzpyrene reduced this by 50 and 63%, respectively. Only 3,4-benzpyrene caused an increase in cytohrome P-450 content by 240%.

1.4 Cyclophosphamide and Styrene as Models of Metabolically Activated Genotoxic Compounds

In vivo short-term genotoxicity assays have two main applications in toxicology. Firstly, they are used to assess the genotoxicity of chemicals and secondly, they are used in the biological monitoring of occupational exposure to genotoxic chemicals. We were interested in assessing the sensitivity and variability of such assays in determining exposure of rodents to genotoxic chemicals by using suitable probe compounds. Cyclophosphamide (CP) and styrene (ST) are two examples of potentially genotoxic compounds, to which humans may be exposed occupationally. CP, a chemotherapeutic agent for the control of neoplasias, has been detected in the urine of nurses handling cytostatic drugs (Evelo et al. 1986). Styrene exposure of workers in the rubber, polymer and fibreglass industries has also been reported (Lof et al. 1986; Stock, 1983).

CP is a very potent genotoxic agent in both in vivo systems and in vitro systems, requiring metabolic activation by MMFO to a genotoxic metabolite (Hales, 1983). As it is a well characterised genotoxin, it is a useful tool in establishing that the assays are functioning correctly. For comparative purposes, ST will also be used as a test compound requiring metabolic activation to a genotoxic metabolite (Watabe

et al. 1978). Unlike CP, its ability to induce genotoxic responses in short-term tests is variable and consequently it is often described as an equivocal genotoxic agent. Since CP and ST are known to produce differential genotoxic effects in various target tissues (Madle et al. 1986a; Krishna et al. 1988; Salomaa et al. 1985; Conner et al. 1979; Sharief et al. 1986), they were suitable probes to determine whether induction of xenobiotic metabolism is able to alter the genotoxic profile of a compound, thereby, possibly changing the primary site for its detection.

1.5 Aims of the Study

In order to establish metabolic factors, which may influence the general utility of methods for monitoring occupational exposure to genotoxins, a controlled study was undertaken using suitable animal models by:

1. Establishing the relationship between the administered dose and the genotoxic response to CP and ST in 3 short-term in vivo assays, and if possible to determine a threshold dose.

2. Assessing species variation in the dose/response relationships in the target tissues.

3. Investigating whether the above genotoxic profiles may be altered in a species- and/or tissue-specific manner by pretreatment with broad-spectrum (PB) or specific (BNF) enzyme inducers.

4. Establishing whether any changes in genotoxicity in target tissues correlates with inducer-related changes in the activating and/or deactivating metabolic pathways.

Chapter 2

Materials and Methods

All chemicals purchased were of reagent (AR) or pharmaceutical grade quality. The sources of the chemicals used are outlined in appendix A. Male LACA Swiss mice (20–30 g) and male Porton rats (300–500 g), supplied by the University's Central Animal House, were used throughout the study. They were allowed food *ad libitum* and kept in a controlled environment with 12 hours light. Rats and mice were placed into appropriate cages and allowed to acclimatise for a minimum of 7 days prior to their use in all experiments. 4–12 animals were used per dose group, at each time point. All treatments were carried out between 8–10 a.m. of the dosing day to minimise any diurnal variation in metabolic enzyme activity and cellular glutathione (GSH) levels (White et al. 1987; Radzialowski & Bousquet, 1968; Davies et al. 1983). The dose volume for all compounds administered by i.p. route was 4 ml/kg body weight. CP and triethylenemelamine (TEM) were made up in normal saline for i.p. injections, while BNF and styrene were dissolved in peanut oil. All solutions were prepared daily or stored at 4^0 C for no longer than 24 hrs.

2.1 Procedures for the In Vivo Genotoxicity Assays

2.1.1 Bone Marrow Micronucleus Assay

2.1.1.1 Dosing Regimen

Treatment of the animals involved a single i.p. injection of the test compound or a comparable volume of the vehicle for control animals. Test compounds were assessed at a minimum of three dose levels, with the highest dose determined by the criteria set down by MacGregor et al. (1987) listed in section 1.3.1. Doses were lower by incremental factors of $\frac{3}{4} - \frac{1}{2}$ from the highest dose. Induction of the microsomal oxygenase system was effected by maintaining animals for 7 days on phenobarbital

(PB) in drinking water (1 mg/ml) or using a single i.p. injection of BNF (100 mg/kg) 48 hrs prior to dosing with the genotoxins. The PB and BNF pretreatment regimens employed in these experiments have been shown, both in our own laboratory and by other investigators, to be able to induce microsomal enzymes (Cooling, 1981; Boobis et al. 1977).

2.1.1.2 Harvesting and Preparation of Bone Marrow Smears

Femoral bone marrow was sampled from treated animals at 30 and 48 hrs post-dosing as recommended by Salamone and Heddle (1983), and in some experiments bone marrow was also sampled at 72 hrs. From freshly killed animals both femora were removed *in toto* by cutting through the pelvis and tibia. Each femur was freed of muscle by gently removing the distal epiphyseal portion together with the tibia. The proximal end of the femur was carefully cut to reveal a small opening to the marrow canal. Using a 26G needle, 0.2 ml of phosphate buffered saline (PBS) was used to perfuse each femur, collecting the perfusate into a test-tube. One drop of this cell suspension was applied to a coded slide and spread across the slide. Two slides were prepared per animal.

The slides were air-dried overnight, after which they were stained using a modified procedure of Schmid (1975): Fixed for 10 min. in anhydrous methanol; stained for 5 min. in undiluted Wright's solution (Wright's stain, 2.5 mg/ml in methanol); stained for 2 min. in Wright's solution diluted 1:4 in 55 mM phosphate buffer, pH 6.4; stained for 10 min. in Giemsa solution (7.6 mg/ml in 1:1 methanol/glycerine) diluted 1:19 with water; rinsed for 10 min. in distilled water and a further 30 min. in fresh distilled water; air-dried, cleared for 2 min. in a histological clearing agent (Histoclear, National Diagnostics, USA) and mounted with Depex mounting medium and a coverslip.

Coded slides were assessed in a blind fashion at 1000x magnification under oil-immersion using a KB-4 filter. The frequency of MNPCEs was determined by examining 1000 PCEs. The PCE/RBC ratio was determined from 500 total erythrocytes assessed.

2.1.2 Sperm Morphology Assay

2.1.2.1 Dosing Regimen

The procedure employed for these studies was in accordance with the recommendations of the report of the U.S. EPA Gene-Tox Program with modifications (Wyrobek et al. 1983a). Treatment of rats and mice with the test compounds required single i.p. injections on five consecutive days at three to four dose levels. There were additional groups for a negative and positive control. Control, PB and BNF pretreated animals, for each genotoxin, were received in a single batch and dosed at the same time (see Appendix E). The genotoxins were administered at 4 dose levels including positive and negative controls. The procedure for the pretreatment with the enzyme-inducers was a modification of that used for the MN assay; PB pretreatment commenced 7 days prior to dosing with the genotoxin, and was maintained over the 5-day dosing period. BNF pretreatment involved 3 i.p. doses at 48 hour intervals, with the first injection 48 hrs before genotoxin treatment was initiated.

2.1.2.2 Sperm Sampling and Evaluation

Animals were sacrificed at 3, 5 and 7 weeks and 5, 8 and 11 weeks from commencement of dosing for mice and rats, respectively. The vasa deferentia from each

animal were removed and their contents carefully expelled into 4 ml of PBS. Two drops of the sperm suspension were placed onto coded slides and air-dried overnight (two slides per animal). Sperm smears were stained as follows: 15 min. in 0.4% Harris's Haematoxylin; 1 min. in 0.1% Eosin Y; rinsed in absolute ethanol; cleared in Histoclear and mounted with a coverglass using Depex mounting medium. Sperm slides were examined in a blind fashion under a light microscope at 400x magnification using a KB-4 filter. For each animal 1000 sperm heads were assessed for morphological abnormalities (Figure 2.1).

2.1.3 Splenocyte Sister-Chromatid Exchange Assay

2.1.3.1 Dosing Regimen

In the pilot experiments animals were treated with a single i.p. injection of the test compounds at a dose which produced a significant increase in SCE frequency or was the highest dose possible without causing lethality. Two animals were sacrificed at 4, 8, 12, 24, 48, 72, and 96 hrs post-injection and a time/response profile was established to determine the timing of the maximal response.

Subsequent to the pilot study, animals were injected at various doses, the highest being that which produced either a marked significant increase in SCE frequency or compound-related signs of toxicity or mortality. Doses were lower by incremental factors of two. Control animals received an appropriate volume of the vehicle. The procedure for the treatment with enzyme inducers was the same as that used for the MN assay (Section 2.1.1.1). Animals were sacrificed at a time post-dosing, found to give a maximal response in the pilot studies.

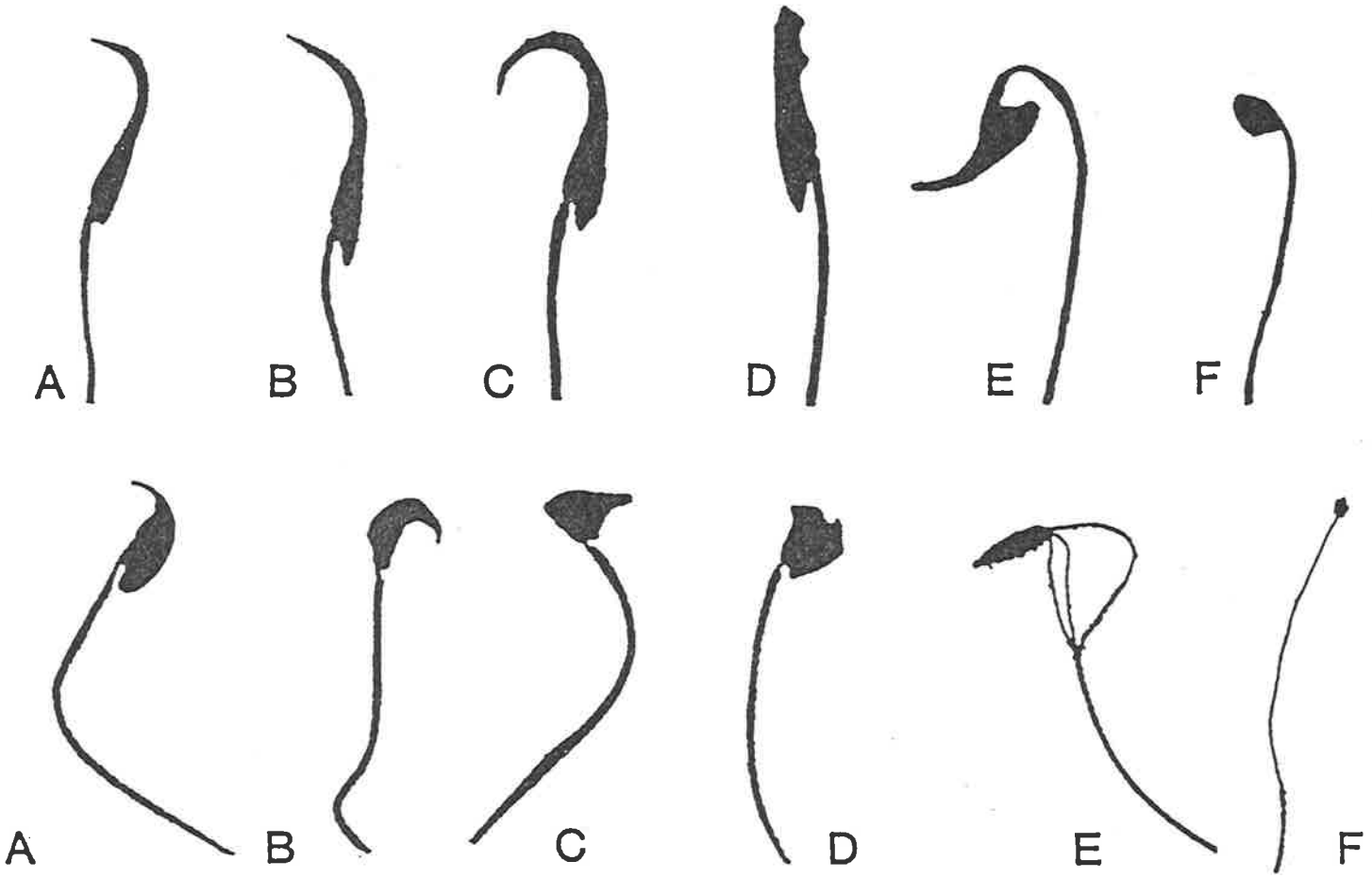


Figure 2.1: Normal (A) and abnormal (B-F) sperm head shapes frequently encounted in the rat (top) and mouse (bottom). Komatsu et al. (1982), Bruce et al. 1974) and Lock and Soares, (1980).

2.1.3.2 Splenocyte Isolation and Culture Conditions

The isolation and culture of splenocytes from exposed animals followed a modified procedure of Krishna et al. (1988). Spleens were removed from animals under aseptic conditions and were placed into a sterile petri-dish containing 5 ml sterile PBS. The spleen was mashed using surgical tweezers, the debris was removed and the resultant cell suspension was placed into a centrifuge tube. The suspension was carefully underlayed with ice-cold Ficoll-paque and centrifuged (1000 rpm for 10 min.), isolating the splenocytes at the interface of the two phases. The isolated cells were washed a further two times with sterile PBS followed by centrifugation at 1000 rpm for 5 min. After the last wash, cells were resuspended in complete medium to a volume of 1 ml. The complete medium consisted of RPMI 1640 supplemented with 24 mM $NaHCO_3$, 10 mM HEPES, 2 mM L-glutamine, 10% heat-inactivated FCS, 100 U/ml penicillin-G, 100 μg/ml streptomycin sulphate, 20 μM BrdU, 5 μg/ml concanavalin A and 5 μM 2-mercaptoethanol.

The number of viable cells isolated from each animal was determined by exclusion of the vital dye Trypan Blue. Isolated lymphocytes were continued through to culture only if cell viability was greater than 95%. An appropriate volume of the cell suspension was then aliquotted into complete media to give a final concentration of $3x10^6$ viable cells/ml in 1 ml total volume or $1x10^6$ viable cells/ml in 4 ml total volume for mouse or rat splenocytes, respectively. Cells were incubated in the dark at 37^0 in a 5% CO_2 environment. Colcemid (10 μM final concentration) was added at 40 and 62 hrs to mouse and rat splenocyte cultures, respectively, harvesting the cells 3 hrs later.

2.1.3.3 Cell Harvesting, Staining and Scoring

Harvested cells were centrifuged at 2000 rpm for 5 min., the supernatant was then removed and the pellet resuspended in 6 ml of 75 mM KCl for 15 min. at 37^0 C. The suspension was then centrifuged at 1000 rpm for 5 min., the supernatant was removed without disturbing the pellet, to which was added fresh fixative (3:1, methanol/acetic acid) for 30 min. Fixed cells were resuspended, washed a further two times in fresh fixative and stored overnight at 4^0 C. For slide preparation, cells were placed in fresh fixative and two drops of the suspension was applied to coded slides, flame-dried and left for 24 hrs prior to staining.

Slides were stained by a modified method of Perry and Wolff (1974): They were immersed in citrate buffer (40 mM trisodium citrate, 160 mM sodium dihydrogen-phosphate, pH 7.4) and exposed to UV-light of 254 and 336 nm at a distance of 5 cm for 8 min.; rinsed in distilled water and incubated for 30 min. at 60^0 in 2xSSC (0.3 M NaCl, 0.03 M trisodium citrate); stained for 4 min. in 6% Giemsa, pH 7.0. Slides were mounted with a coverglass using Depex mounting medium.

SCE frequency per animal was determined by analysing 20 metaphase spreads containing a minimum of 38 sister chromatids. The replicative index (RI), which is the frequency of first, second and third division metaphases, was determined in 100 metaphases. The RI was calculated as follows;

$$RI = \frac{1M_1 + 2M_2 + 3M_3}{100}$$

where M_1, M_2 and M_3 represent percentages of first, second and third division metaphases, respectively (Krishna et al. 1985).

2.2 Effects of PB and BNF on Various Metabolic Pathways in Liver, Testes, Spleen and Bone Marrow

2.2.1 Treatment of Animals

Rats and mice were treated with either PB in drinking water (1 mg/ml) and sacrificed at 7 and 12 days or BNF by i.p. injection (100 mg/ml/48 hrs) and sacrificed at 48, 72 and 120 hrs after the first injection. Control animals were treated with either 3 i.p. injections of peanut oil at 48 hour intervals and tissues sampled 24 hrs after the last dose or were untreated.

2.2.2 Tissue Preparation

All solutions for the enzyme assays were prepared fresh daily prior to their use. All tissues were prepared by the same procedure and all steps were carried out at 4^0 C prior to incubation. Animals were sacrificed (3 per treatment group) at the appropriate times and their liver, testes, spleen and both femora were removed. Bone marrow was collected by the same procedure used for the preparation of bone marrow smears in the MN test. Tissues were weighed and homogenised in 100 mM phosphate buffer, pH 7.4, to give a final tissue concentration of 80 mg/ml. In the case of mice, spleens and femoral bone marrow from a number of treated animals were pooled to give enough tissue for the enzyme assays. The homogenate was centrifuged at 9,000 g for 15 min. and the supernatant (S9) collected for determination of enzyme activities. The protein content of each tissue supernatant was determined by the colorimetric method of Lowry et al. (1951).

2.2.3 Assessment of Enzyme Activities

2.2.3.1 7-Ethoxycoumarin O-Deethylase Activity

The enzyme activity was determined using a modified method of Greenlee and Poland (1978). Reactions were carried out in duplicate in 10 ml flat-bottomed tubes in a total volume of 1 ml. The reaction mix contained 100 mM phosphate buffer, pH 7.4, 15 mM $MgCl_2$, 5 mM glucose-6-phosphate, 0.5 mM NADP, 2 IU/ml glucose-6-phosphate dehydrogenase and 50 μl of S9. The reaction was started, after a 2 min. preincubation at 37^0 C, by the addition of 25 μl of substrate (20 mM 7-ethoxycoumarin in 50% methanol) and incubated for 10 min. at 37^0 C with shaking. The reaction was stopped by the addition of 125 μl of 20% trichloroacetic acid. Blank tubes consisted of the complete reaction mix, to which was added 125 μl prior to the addition of substrate.

The reaction tubes were extracted with 2 ml of chloroform by vigorous shaking for 10 min. at room temperature (RT). A 1 ml portion of the organic phase was back-extracted into 2.5 ml of 10 mM NaOH/1 M NaCl with further shaking for 10 min. at RT. The fluorescence of the aqueous phase was measured at an excitation wavelength of 368 nm and an emission wavelength of 456 nm, using a Perkin Elmer LS-5 luminescence spectrometer (Perkin Elmer, Buckinghamshire, England). The amount of product formed was determined from the 7-hydroxycoumarin standard curve (Appendix B), which was carried through the same extraction procedure as the reaction tubes. The enzyme activity was expressed as nmol product/min./mg tissue protein.

2.2.3.2 2,5-Diphenyloxazole Hydroxylase (PPO-OHase) Activity

PPO-OHase has been used as a surrogate enzyme for assessing AHH activity (Philippides et al. 1983). The enzyme activity was determined using a modified procedure of Philippides et al. (1983), which measures the hydroxylation of 2,5-diphenyloxazole (PPO) to 2-phenyl-5-(p-hydroxyphenyl)-oxazole (PPO-OH). Duplicate reactions were carried out in 10 ml flat-bottomed tubes with a total volume of 1 ml. The reaction mix contained, 100 mM phosphate buffer, pH 7.4, 15 mM MgCl2, 5 mM glucose-6-phosphate, 0.5 mM NADP, 2 IU/ml glucose-6-phosphate dehydrogenase and 25 μl of S9. After a 2 min. preincubation at 37^0 C, the reaction was started by the addition of 25 μl of 2 mM PPO in acetone and incubated for a further 10 min. at 37^0 C with shaking. The reaction was quenched with 1 ml ice-cold acetone and the tubes were placed on ice. Blank tubes consisted of the reaction mix quenched prior to the addition of substrate.

The tubes were then extracted with 3 ml of n-hexane with shaking for 10 min. at RT. 1 ml of the organic phase was back-extracted into 2.5 ml of 1 M NaOH with shaking for 10 min. at RT. The fluorescence of the aqueous phase was determined at excitation and emission wavelengths of 355 and 505 nm, respectively, using a Perkin Elmer LS-5 luminescence spectrometer. The amount of product formed was read off the standard curve of authentic PPO-OH (Appendix B). Enzyme activity was expressed as nmol product/min./mg tissue protein.

2.2.3.3 Glutathione S-Transferase Activity

A modified procedure of Habig et al. (1974) was employed to assess the enzyme activity. Measurement of the enzyme activity involved monitoring the rate of change in absorbance of a reaction mix at 340 nm using a Unicam SP1800 (Pye-Unicam

Ltd., Cambridge, England), at 37^0 C against a blank cuvette containing the complete reaction mix without substrate.

The assay was conducted in 100 mM phosphate buffer, pH 6.5, containing enough reduced GSH to give a final concentration of 1 mM. To 825 μl of the GSH solution, prewarmed to 37^0 C, was added 100 μl of S9 diluted $\frac{1}{60}$ for liver or $\frac{1}{10}$ for all other tissues. 25 μl of 40 mM 1-chloro-2,4-dinitrobenzene in ethanol was added with rapid mixing and the change in absorbance over a maximum of 1 min. was recorded. The amount of product formed was determined from a standard curve of the conjugate, S-2,4-dinitrophenyl glutathione (DNP-GS) (Appendix B). Enzyme activity was expressed as nmol product/min./mg tissue protein.

2.2.3.4 Epoxide Hydrolase Activity

A gas chromatographic assay was developed to estimate the activity of epoxide hydrolase by measuring the conversion of styrene oxide to styrene glycol. The complete reaction mix (1 ml total volume) contained 100 mM phosphate buffer, pH 7.4 and 500 μl of S10 (or 100 μl of S9 for estimation in liver). After a 2 min. preincubation at 37^0 C, the reaction was started by adding 50 μl of substrate (40 mM styrene oxide in acetone) and incubated for 60 min. at 37^0 C. These conditions gave reaction rates that were linear with respect to time and tissue concentration (Appendix B). The reaction was stopped with 1 ml of ice-cold ethyl acetate containing 0.5 mM benzyl alcohol as the internal standard for the assay. The tube was then vortexed for 10 min. and the organic phase was collected after centrifugation at 3500 rpm for 5 min. A standard curve was prepared by adding known amounts of styrene glycol to quenched reaction tubes, which were also carried through the extraction procedure.

Samples were analysed gas chromatographically using a Varian Gas Chromatograph Model 3400, equipped with a flame-ionisation detector (FID), using a SGE

fused-silica capillary column (15 m x 0.22 mm ID) coated with BP-1 (nonpolar) stationary phase to a thickness of 0.25 microns. The gas chromatograph was coupled to a Varian In-built Data Handling System to integrate responses. 3 μl of the organic phase was injected at a column temperature of 60^0 C which was rapidly raised to 260^0 C at 50^0/min. followed by a 2 min. hold time. Injector and detector temperatures were 270^0 and 280^0 C, respectively. The flowrate of carrier gas (helium) was 1 ml/min. at a split ratio of 1:26.

A detection limit of 40 nmol/ml styrene glycol was found with the FID and the within-day and day-to-day reproducibility error was 2.8 and 4.1%, respectively. Enzyme activity was expressed as nmol product/min./mg tissue protein, which was determined from the standard curve of styrene glycol (Appendix B).

2.2.3.5 Estimation of Tissue Glutathione Content

Reduced GSH was determined by a modified procedure of Hissin and Hilf (1976). Rats and mice were sacrificed at 8 a.m. and the liver, testes, spleen and femoral bone marrow were collected and kept on ice. To 100 mg of each tissue, 400 μl of 25% metaphosphoric acid and 1.5 ml of phosphate-EDTA buffer (0.1 M sodium phosphate, 5 mM EDTA, pH 8.0) was added and homogenised at 4^0 C. For smaller quantities of tissue, additions of acid and buffer solutions were adjusted accordingly. The homogenate was centrifuged at 5,000 g at 4^0 C for 10 min. To 0.5 ml of the resultant supernatant was added 4.5 ml of phosphate-EDTA buffer and mixed. To a 100 μl aliquot of the diluted tissue supernatant was added a further 1.8 ml of buffer and 100 μl of o-phthalaldehyde solution (1 mg/ml in methanol) and mixed thoroughly. Following a 15 min. incubation, the fluorescence at 420 nm was determined with excitation at 350 nm on a Perkin Elmer LS-5 luminescence spectrometer. The level of tissue GSH was determined from the GSH standard curve in Appendix B.

2.2.3.6 Estimation of Protein Content in 9,000 g Supernatants

The protein content was determined by the method of Lowry et al. (1951). Solution A was prepared by mixing the following; 2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$ and 2% sodium tartrate in the proportions 100:1:1, respectively. Solution B was prepared by diluting Folin & Ciocalteau's Reagent 1:1 with distilled water. Bovine serum albumin (BSA) was used as the standard with a concentration range of 0-100 μg/ml.

The 9,000 g tissue supernatants, were diluted with 100 mM phosphate buffer, pH 7.4, to give a final concentration of 8 mg tissue/ml. To a 100 μl aliquot of the diluted supernatants (or standards) was added 5 ml of solution A and mixed. After standing at RT for exactly 10 min., 500 μl of solution B was added and mixed thoroughly. The solutions were incubated at RT for a further 30 min., after which their absorbance was read at 750 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. The tissue protein content was determined from the BSA standard curve (Appendix B).

2.3 Data Presentation and Statistical Analyses

The results of experiments were presented as the mean $\pm$ sem. The number of animals representing each data point was at least n=4, unless otherwise stated. Statistical analyses of the generated data were performed, using the GLIM System (Baker, 1985) to establish the significance of the results. For all analyses $p<0.05$ was set as the critical level of significance.

The genotoxicity data for the MN and SM assays followed a binomial distribution and was, therefore, analysed using Analysis of Deviance (see Appendix C for a

description of the analysis), examining the effects of and the interactions between the variables; dose of the genotoxin, time of sampling, species (where appropriate) and the presence of PB or BNF pretreatment on the extent of genotoxicity. The SCE data did not fit a binomial distribution. Hence, the data was analysed using a 2-Way ANOVA. In order to meet the required assumptions of the statistical procedure used to assess the effects of PB or BNF pretreatment on CP- and ST-induced SCEs, the data was transformed using the Angular Transformation, $X' = \arcsin\sqrt{X}$ as recommended by Gad and Weil (1986).

The cytotoxicity data from the SCE and MN experiments were analysed using Multifactorial ANOVA to assess the influence of species and time of sampling (where appropriate) and the presence of PB or BNF pretreatment on the cytotoxic potential of CP and ST. Where appropriate a logarithmic transformation of the data was used in order to meet the required assumptions of the statistical procedure. The results of these statistical analyses are presented in Appendix C.

Where the previous analyses indicated a significant dose-related effect, each dose/response curve was compared to its own control, using multiple-comparison testing. This included Tukey's Multiple-Comparison Test (Byrkit, 1987) for the data analysed using Multifactorial ANOVA and Asymptotic Tests (McCullagh & Nelder, 1983) for the binomial data. The multiple comparison tests were used to estimate a threshold dose for each compound in each assay system. Due to the large number of comparisons conducted, the power of such statistical analyses were reduced, compared to the ANOVA and Analysis of Deviance tests. Therefore they were only used to support the ANOVA or Analysis of Deviance analyses. Any significant effects detected by the multiple comparisons were indicated by an asterisk or a dagger (†) in the histograms or tables, respectively.

The enzyme activity data from Chapter 5, were analysed by a 1-way ANOVA, followed by Tukey's Multiple-Comparison Test, for any effect of PB or BNF on enzyme activity. The PB and BNF effects were compared directly with the untreated and peanut oil-treated controls, respectively. Significant effects were indicated by a dagger (†) for PB or a double-dagger (‡) for BNF pretreatment.

Chapter 3

The Genotoxicity of Cyclophosphamide in Three In Vivo Assays, in the Presence and Absence of PB and BNF Pretreatment.

3.1 Introduction

CP is a member of the nitrogen mustard class of alkylating compounds, used as a chemotherapeutic agent in neoplastic diseases. It is a known human carcinogen and has been implicated as the cause of a number of second tumours in cancer patients (Taylor & Wade, 1984). CP itself has weak alkylating ability and must be metabolically activated to exert its biological effect (Hales, 1983). The current knowledge on the major metabolic pathways of CP is summarised in Figure 3.1. The first step in the activation of CP is catalysed by the MMFO to form 4-hydroxy-CP, thought to exist in equilibrium with aldophosphamide. 4-Hydroxy-CP is then further metabolised to 4-keto-CP. The aldehyde tautomer can either be metabolised by aldehyde dehydrogenase to form carboxyphosphamide or may spontaneously break down to acrolein and phosphoramide mustard (PM). PM is further metabolised to nornitrogen mustard, however, it is not known whether this is spontaneous or enzymatic. Acrolein undergoes conjugation with GSH to a non-toxic metabolite.

The reported half-life for the clearance of CP in mice and rats has varied from 5–20 and 19–40 minutes, respectively (Torkelson et al. 1974; Garattini et al. 1974). A study, which used serum alkylating activity as a measure of total metabolite levels in serum, found their half-life to be 17 and 39 minutes in mice and rats, respectively. A more recent study investigating the kinetics of CP and its metabolites in mice (Struck & Alberts, 1984), observed half-lives of 13, 13, 37 and 24 minutes for CP, 4-hydroxy-CP, aldophosphamide and PM, respectively. The primary site of metabolism in both species is the liver. Activation of CP in the rat, has been measured in kidney and lung tissue, albeit at $\frac{1}{32}$ the rate of the liver (Torkelson et al. 1974). No such activity was detected in spleen, testes, adrenal cortex or muscle. Hemminki (1985) assessed the levels of DNA adducts in CP-treated mice, and found they were highest in lung, kidney and liver, suggesting that the major sites of CP activation are similar in the rat and mouse. Approximately 10% of a CP dose is renally excreted unchanged in mice, while figures reported for the rat range from 20-50% (Torkelson et al. 1974).

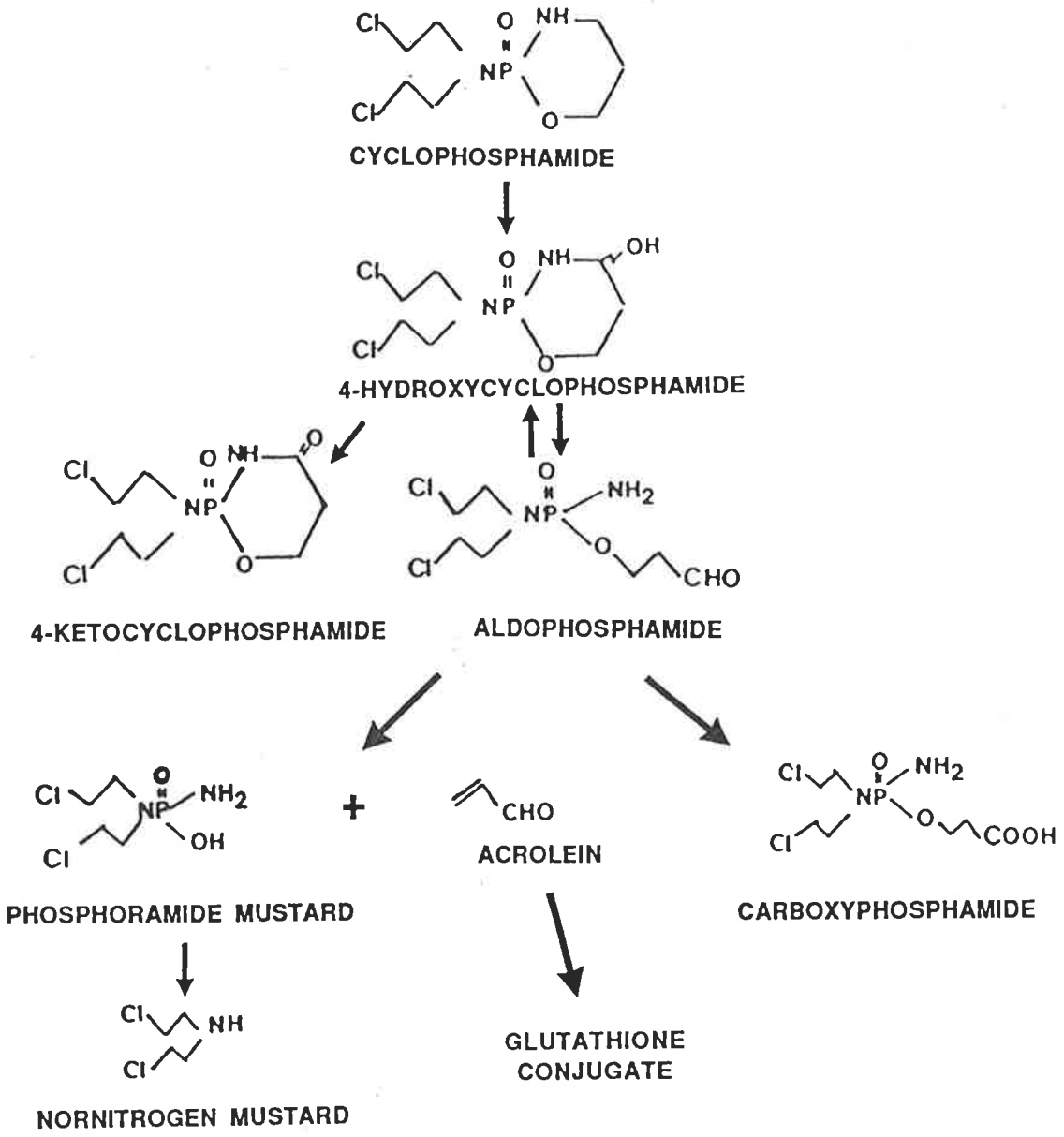


Figure 3.1: Major pathways for the metabolism of CP. Modified from Hemminki et al. (1987).

The effect of enzyme inducers on the oxidative metabolism of CP was assessed by measuring CP-induced depletion of NADPH in microsomal incubates (De Raat, 1977). Its metabolism was found to be inducible 5-6 fold by PB and aroclor 1254, whereas 3-MC had no effect, suggesting the involvement of PB- rather than 3-MC-inducible isozymes of cytochrome P-450. Similar results with PB induction were observed by Alberts et al. (1978) and Field et al. (1972). Garattini et al. (1974) have shown PB is also able to increase the clearance of CP-derived alkylating metabolites from mouse and rat plasma. As well as activating pathways, a possible deactivating pathway, catalysed by aldehyde dehydrogenase, is also inducible by PB in rats and mice (Dietrich et al. 1972).

A common characteristic of CP and other chemotherapeutic agents is their ability, under physiological conditions, to alkylate vital macromolecules such as DNA. In vitro studies, in which CP was incubated with DNA and a metabolising system, showed evidence of nornitrogen mustard adducts at the N^7-position of guanine, in the form of monoalkylated or crosslinked adducts (Colvin et al. 1976; Hemminki, 1985). CP, 4-hydroxy-CP, carboxyphosphamide, 4-keto-CP and nornitrogen mustard are poor alkylators of DNA at physiological pH (Ellenberger & Mohn, 1977) and as PM is a potent alkylator, it is believed to be the putative genotoxic metabolite. Acrolein is a poor alkylator of DNA but a good alkylator of protein and has been implicated as a mediator of the cytotoxicity associated with CP administr ation (Gurtoo et al. 1981; Crook et al. 1986). Domeyer and Sladek (1980) proposed that 4-hydroxy-CP and its tautomer serve as transport forms of the ultimate cytotoxic and genotoxic metabolites, as they could reach appreciable levels in the circulation of mice administered CP, while their breakdown products were not detectable. Their conclusions were later supported by Boyd et al. (1986), who used ^{31}P-NMR and cell perfusion techniques to show that 4-hydroxy-CP and aldophosphamide readily crossed cell membranes, while PM could not, due to it being almost completely ionised at physiological pH.

CP is a potent genotoxin able to elicit responses in various in vitro and in vivo short-term tests. Although Balbinder et al. (1981) found CP not to be mutagenic in the Ames test, Hales and Jain (1980a) showed it was weakly mutagenic in the same

system and were able to increase its mutagenicity by the addition of an S9 mix. In the rodent MN test CP is a potent clastogen in both rats and mice (Madle et al. 1986a), with the rat being more sensitive than the mouse at comparable doses. CP is also able to increase the frequency of micronuclei in developing rat spermatocytes (Tates et al. 1987).

CP is able to cross the blood-testis-barrier. It was detected in the seminal vesicle fluid of rats 10 min. after an IV bolus and was equilibrated with plasma levels within 30 min. (Hales et al. 1986). Bruce and Heddle (1979) and Wyrobek and Bruce (1975) demonstrated an ability of CP to increase the proportion of abnormal sperm after subchronic administration to mice of 50 mg/kg daily for 5 days. This dose was lethal to some of the mice. The maximal increase in sperm abnormalities was observed 4 weeks after dosing, corresponding to exposure of the primary spermatocyte. Vigil and Bustos-Obregon (1985), with a single i.p. dose of 200 mg/kg, also found the primary spermatocyte to be the most sensitive stage. In their study, effects on sperm morphology were observed as early as four days after dosing, in the form of abnormal flagellar. Studies investigating the heritability of CP-induced abnormal sperm are inconclusive. Male mice exposed to CP (20 and 80 mg/kg/d, over 5 days) were mated with unexposed females at 1, 5 and 8 weeks after dosing and their F_1 male progeny showed no increase in abnormal sperm (Topham, 1980a). In contrast, Sotomayor (1979) observed a significant elevation in deformed sperm in F_1 progeny sired from exposed males (200 mg/kg) and unexposed females, mated 3 weeks after dosing.

Comparing the SCE inducing ability of CP in different tissues, Krishna et al. (1988) and Dearfield et al. (1985), found the peripheral and spleen-derived lymphocytes were equally susceptible, whereas the bone marrow was less sensitive. Madle et al. (1986a) looking at CP-induced SCEs in rat and mouse bone marrow, observed the rat to be more sensitive. With regards to human exposure, Bochkov et al. (1986) demonstrated an elevated frequency of SCEs in peripheral lymphocytes of cancer patients after treatment with CP. Reports showing that occupational exposure to CP is associated with increased SCE levels are equivocal (Stucker et al. 1986; Barale et al. 1985; Norppa et al. 1980).

Studies looking at the effects of enzyme inducers on the genotoxicity of CP are limited. Hales and Jain (1980a, 1980b) were able to show increased mutagenicity in the Ames test in the presence of PB induction, while BNF produced a decrease in its mutagenicity. This supports the current knowledge of CP oxidation, which is believed to be mediated by PB-inducible rather than 3-MC-inducible isozymes of cytochrome P-450. Furthermore, the effect of BNF pretreatment is consistent with its reported ability to produce a decrease in the activity of PB-inducible isozymes (Guengerich et al. 1982). Schreck et al. (1982), investigating the response to PB pretreatment on CP-mediated increases in SCEs, found there to be no change in its genotoxic potential in spermatocytes and regenerating hepatocytes, even though P-450 activity (p-chloromethylaniline demethylase) was increased 6-fold.

The specific aim of the experiments reported in this chapter was to compare the dose/response relationships of CP in the micronucleus, sperm morphology and sister-chromatid exchange assays in the rat and mouse and to determine whether these would be altered by pretreatment with the microsomal enzyme inducers PB and BNF.

3.2 Materials & Methods

3.2.1 Micronucleus Assay

CP dissolved in 0.9% saline, was administered by a single i.p. injection to rats and mice, at doses of 0, 1.25, 2.5, 5, 10 and 20 mg/kg. Bone marrow was sampled at 30, 48 and 72 hrs post-dosing for control animals or 30 and 48 hrs for inducer pretreated groups. Enzyme-inducing pretreatments consisted of PB (1 mg/ml) in drinking water for 7 days or BNF (100 mg/kg in peanut oil) 48 hrs prior to CP dosing. Bone marrow smears prepared following the method in section 2.1.1.2, were air-dried immediately and left overnight before staining with Wright's stain and Giemsa. Slides were analysed in a blind fashion for the incidence of micronucleated PCEs (MNPCE) and the PCE/RBC ratio.

3.2.2 Sperm Morphology Assay

Rats and mice were given single i.p. daily doses of CP at 0, 5, 10, 20 and 40 mg/kg over 5 consecutive days. Positive control animals were treated with a single i.p. dose of triethylenemelamine (TEM, 1 mg/kg) on the first day of dosing. Mice were sacrificed at 3, 5 and 7 wks from commencement of dosing and rats at 5, 8 and 11 wks. Sperm smears prepared by the procedure in section 2.1.2.2, were analysed in a blind fashion for incidence of abnormal sperm heads. Pretreatment with enzyme inducers was similar to the MN test, with an extended dosing regimen. PB (1 mg/ml) was administered via the drinking water for 7 days prior to dosing and extended for the duration of the dosing period. BNF was injected i.p. at 100 mg/kg 48 hrs before the administration of CP followed by two further doses repeated at 48 hour intervals.

3.2.3 Splenocyte SCE Assay

The pilot studies involved a single i.p. dose of CP at 5 and 40 mg/kg in rats and mice, respectively. The optimum sampling time for each species, was chosen as the time at which the induced SCE frequency was maximal. This was then used as the sampling time in the main body of the study. In the main study, rats were given a single i.p. injection of CP at 0, 0.31, 0.62, 1.25 and 2.5 mg/kg and mice received doses of 0, 0.62, 1.25, 2.5 and 5 mg/kg. Splenocytes were isolated from treated animals at a time post-dosing, determined by the pilot studies. The enzyme-inducing treatments were the same as those for the MN test. The removal of the spleens, culturing and harvesting of cells, and the preparation and assessment of slides followed the method described in section 2.1.3.3, for both the pilot and main experiments.

3.3 Results

CP was found to be strongly genotoxic in 2 of the 3 in vivo assays. In the MN assay CP produced a significant, dose-related increase in the incidence of MNPCEs (χ^2=38.70, p<0.001, Appendix C) in both species, whose response differed between the two species (Figures 3.2). Although the Asymptotic tests indicated the threshold dose for CP-induced MNPCEs to be 2.5 mg/kg in both species, the level of the response was greater in the rat at comparable doses. There was also a significant interaction term involving time (χ^2=38.70, p<0.001), which indicated that the species-dependent response to CP varied significantly with the time of sampling. At all doses in the mouse, the response was greatest at 30 hrs and returned to baseline by 72 hrs. In the rat the timing of the maximal response varied in a dose-dependent manner. At low doses the genotoxic effect was greater at 30 hrs and as the dose was increased, the maximal response was more delayed. Like the mouse, the MNPCE frequency at 72 hrs had returned to baseline levels. Since a genotoxic effect of CP in both species was not observed at 72 hrs, experiments with PB or BNF pretreatment were conducted with 30 and 48 hrs samples only.

Analysis of the effects of PB or BNF on the genotoxic response in the MN assay, showed that dose, PB pretreatment, time of sampling and species were related in a complex manner (Appendix C). The interaction involving all 4 variables approached significance (χ^2=10.21, 0.05<p<0.10), while the 3-way interactions involving them were highly significant (Appendix C). That is, PB pretreatment significantly altered the response to CP in a species-specific manner (χ^2=16.15, p<0.01). Furthermore, the effect of PB on the response to CP was also influenced by the time of sampling (χ^2=24.02, p<0.001). In figure 3.3, the response to CP in mice was potentiated by PB, where the threshold dose for CP-induced MNPCEs in mice was lowered from 2.5 to 1.25 mg/kg and 10 to 2.5 mg/kg at 30 and 48 hrs, respectively. The effect of PB in the rat was restricted to the top doses, where the magnitude of the response was reduced at 48 hrs and increased at 30 hrs (Figure 3.4). In neither species was any effect of BNF pretreatment on the genotoxicity of CP detected (χ^2=2.72 and 3.69).

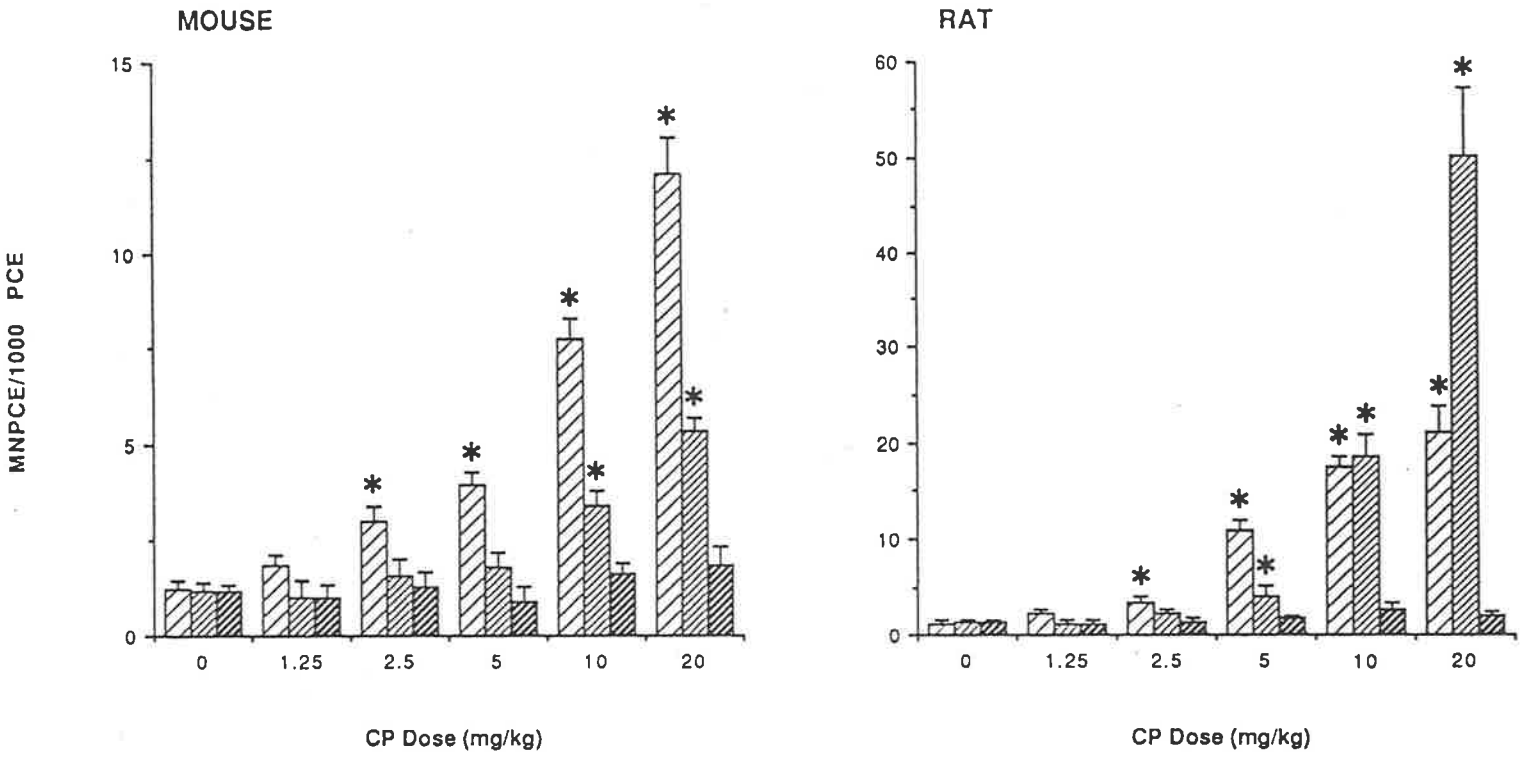


Figure 3.2: Effect of CP (0-20 mg/kg) on the frequency of MNPCEs in the mouse and rat, n=5-10 (mouse) and 4-10 (rat). * indicates significant difference from respective controls, $p<0.05$.

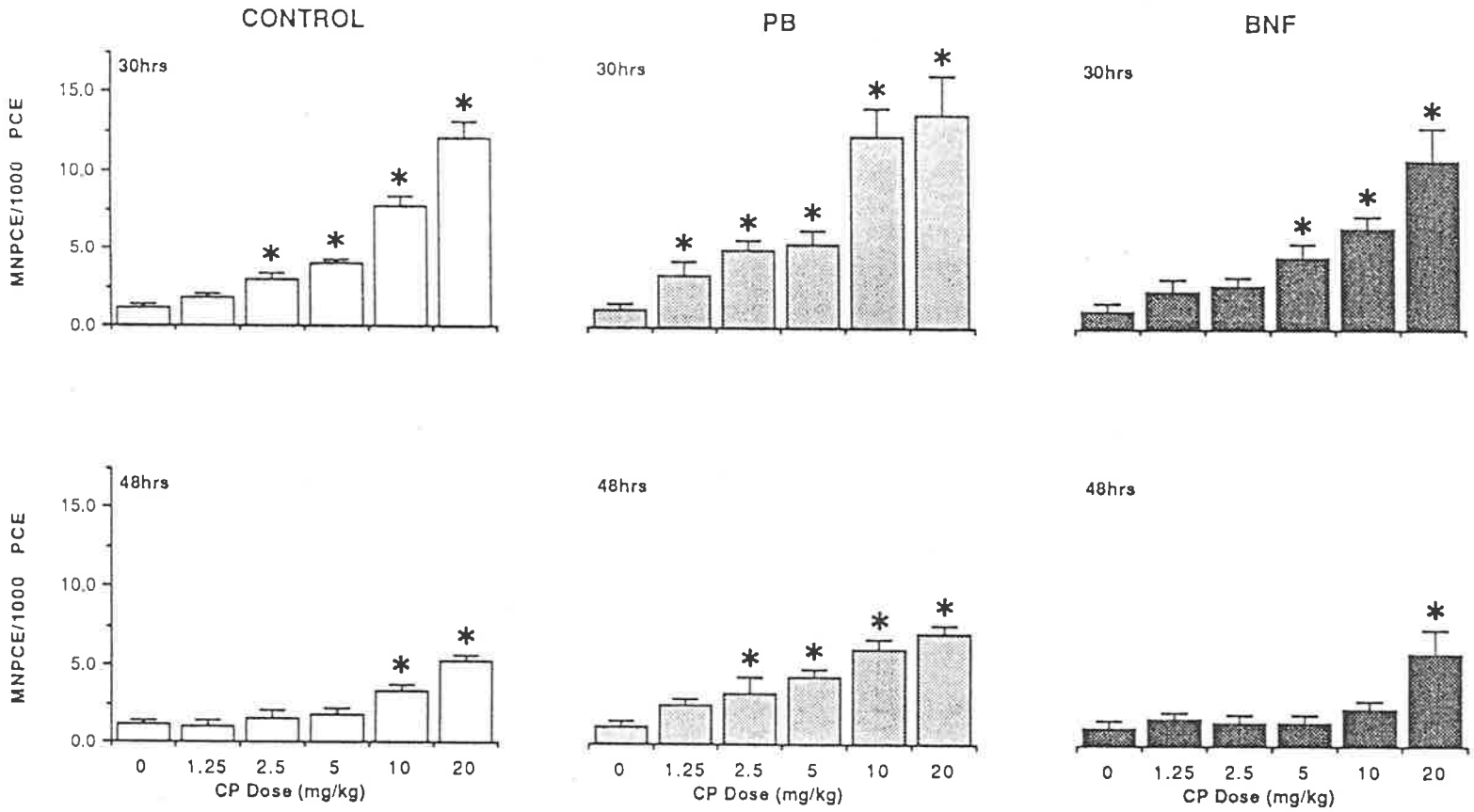


Figure 3.3: Effect of PB and BNF pretreatment on CP-induced MNPCEs in the mouse, n=5-10. * indicates significant difference from respective controls, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

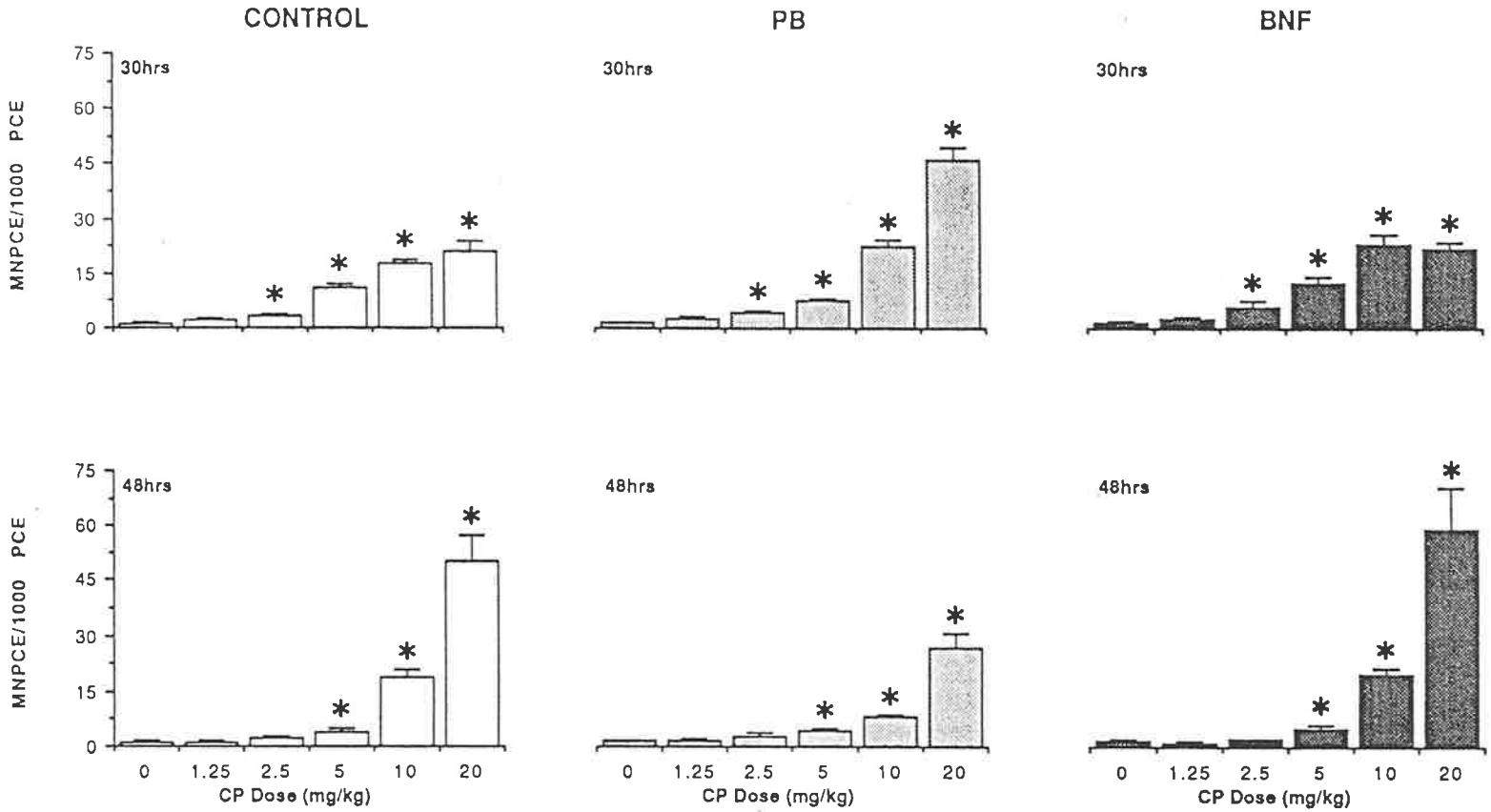


Figure 3.4: Effect of PB and BNF pretreatment on CP-induced MNPCEs in the rat. n=4-10. * indicates significant difference from respective controls, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

CP Dose (mg/kg)	PCE/RBC Ratio		
	30hrs	48hrs	72hrs
Cont.	1.00±0.01	1.00±0.01	1.00±0.01
1.25	1.06±0.03	1.05±0.04	1.00±0.05
2.5	1.09±0.04	1.09±0.07	1.00±0.05
5.0	1.11±0.03	1.03±0.05	0.87±0.05
10.0	1.07±0.10	0.90±0.07	0.86±0.05†
20.0	1.06±0.07	0.81±0.10	0.54±0.06†

Table 3.1: Effect of CP (0-20 mg/kg) on the PCE/RBC ratio in control mice, n=5-10. † indicates significant difference from respective controls, $p<0.05$.

CP Dose (mg/kg)	PCE/RBC Ratio			
	PB		BNF	
	30hrs	48hrs	30hrs	48hrs
Cont.	1.03±0.07	1.03±0.07	0.93±0.06	0.93±0.06
1.25	1.52±0.18	0.97±0.16	1.14±0.22	0.73±0.08
2.5	0.83±0.14	0.67±0.06	0.82±0.08	0.62±0.11
5.0	0.72±0.07	1.27±0.23	0.65±0.08	0.76±0.13
10.0	0.95±0.12	1.48±0.24	0.84±0.11	0.72±0.07
20.0	0.97±0.09	0.84±0.07	0.90±0.19	0.65±0.07

Table 3.2: Effect of CP (0-20 mg/kg) on the PCE/RBC ratio in PB and BNF pretreated mice, n=5-10.

CP Dose (mg/kg)	PCE/RBC Ratio		
	30hrs	48hrs	72hrs
Cont.	0.61±0.04	0.61±0.04	0.61±0.04
1.25	0.39±0.04	0.50±0.12	0.55±0.03
2.5	0.47±0.05	0.55±0.12	0.52±0.05
5.0	0.39±0.02†	0.24±0.15†	0.35±0.01†
10.0	0.32±0.03†	0.19±0.01†	0.26±0.02†
20.0	0.27±0.04†	0.11±0.02†	0.19±0.04†

Table 3.3: Effect of CP (0-20 mg/kg) on the PCE/RBC ratio in control rats, n=4-10. † indicates significant difference from respective controls, $p<0.05$.

CP Dose (mg/kg)	PCE/RBC Ratio			
	PB		BNF	
	30hrs	48hrs	30hrs	48hrs
Cont.	0.66±0.08	0.62±0.05	0.52±0.11	0.52±0.11
1.25	0.63±0.07	0.61±0.08	0.68±0.05	0.48±0.05
2.5	0.57±0.03	0.82±0.11	0.76±0.14	0.64±0.15
5.0	0.60±0.15	0.96±0.09	0.47±0.07	0.52±0.08
10.0	0.42±0.03	0.58±0.07	0.38±0.08	0.28±0.07†
20.0	0.33±0.05†	0.19±0.02†	0.30±0.03†	0.08±0.01†

Table 3.4: Effect of CP (0-20 mg/kg) on the PCE/RBC ratio in PB and BNF pretreated rats, n=4-10. † indicates significant difference from respective controls, $p<0.05$.

The PCE/RBC ratio, where a decrease indicates cytotoxicity, was significantly reduced by CP in both species ($F=2.77$, $p<0.01$), the effect of which was influenced by the species used and the time of sampling (Appendix C). Tukey's test detected a significant effect ($p<0.05$) of CP in rats down to 5 mg/kg at all time points (Table 3.3). In contrast, the response in the mouse was significant ($p<0.05$) at 10 and 20 mg/kg only after 72 hrs (Table 3.1).

PB and BNF pretreatments significantly altered the cytotoxicity of CP (Appendix C). Their effect was shown to be influenced significantly by the species used ($F=5.30$ (BNF), $F=4.25$ (PB), $p<0.01$) and the sampling time ($F=3.09$ (BNF), $F=6.97$ (PB), $p<0.01$). Tukey's test did not detect a significant ($p<0.05$) effect of PB or BNF on the cytotoxicity of CP in mice (Tables 3.1 and 3.2), although there appeared to be an inconsistent effect of PB and a consistent reduction in the cytotoxicity of CP with BNF. However, the same analysis in the rat, showed that both enzyme-inducing pretreatments reduced the cytotoxicity of CP (Tables 3.3 and 3.4). This effect appeared to be more pronounced with PB than BNF.

CP produced a significant dose-related increase in sperm abnormalities (Figures 3.5 and 3.6). Furthermore, the statistical analyses indicated that the effect of CP was influenced by the sampling time and the species ($\chi^2=65.07$, $p<0.001$), Appendix C). The interaction between dose and sampling time was illustrated by the responses in both species being greater at the intermediate sample time (5 wks, mouse); 8 wks, rat) with smaller responses at the last and first sample times, in decreasing order. The interaction involving species and dose was evident at the intermediate and last sample times, where the responses in the rat were greater than in the mouse at comparable doses. This was also supported by the Asymptotic tests, which showed a consistently lower threshold dose for CP in rats compared to mice.

PB and BNF were found to have no significant effect ($\chi^2=1.32$ and $\chi^2=6.4$, respectively) on CP-induced abnormal sperm in both species. However, they did have a significant additive effect on the level of abnormal sperm, in both species ($\chi^2=4.42$, $p<0.05$ (PB) and $\chi^2=7.34$, $p<0.01$ (BNF)). CP was lethal to 13 and 80% of rats at 20 and 40 mg/kg/d, respectively (Table 3.7). BNF increased, while PB

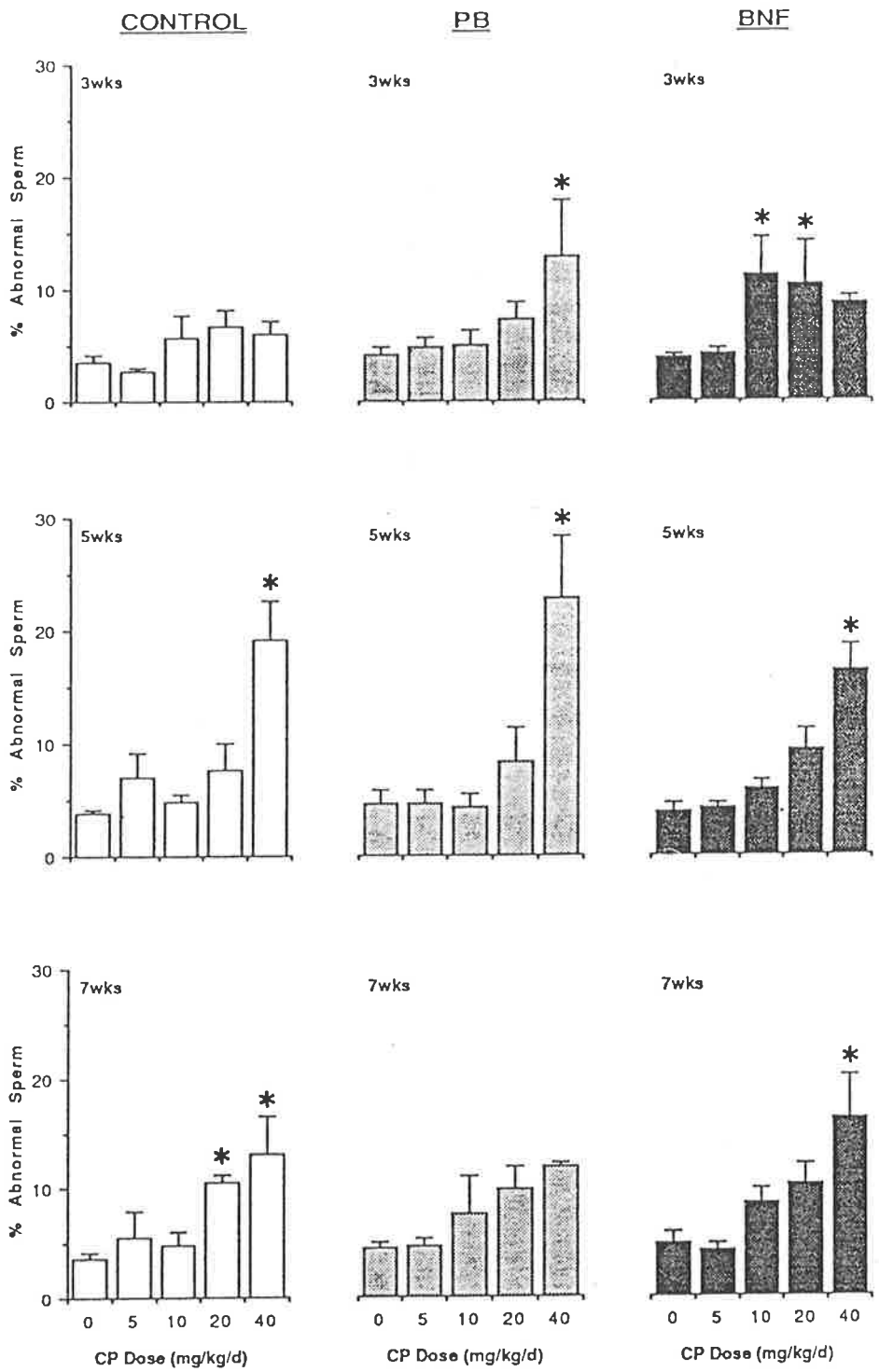


Figure 3.5: Percentage of abnormal sperm after treatment with CP (0-40 mg/kg/d) in control, PB and BNF pretreated mice, n=4-5. * indicates significant difference from respective controls, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

Figure 3.6: Percentage of abnormal sperm after treatment with CP (0-40 mg/kg/d) in control, PB and BNF pretreated rats. n=4-5, except in the following treatment groups;

40 mg/kg/d CP at 8 wks, n=3;

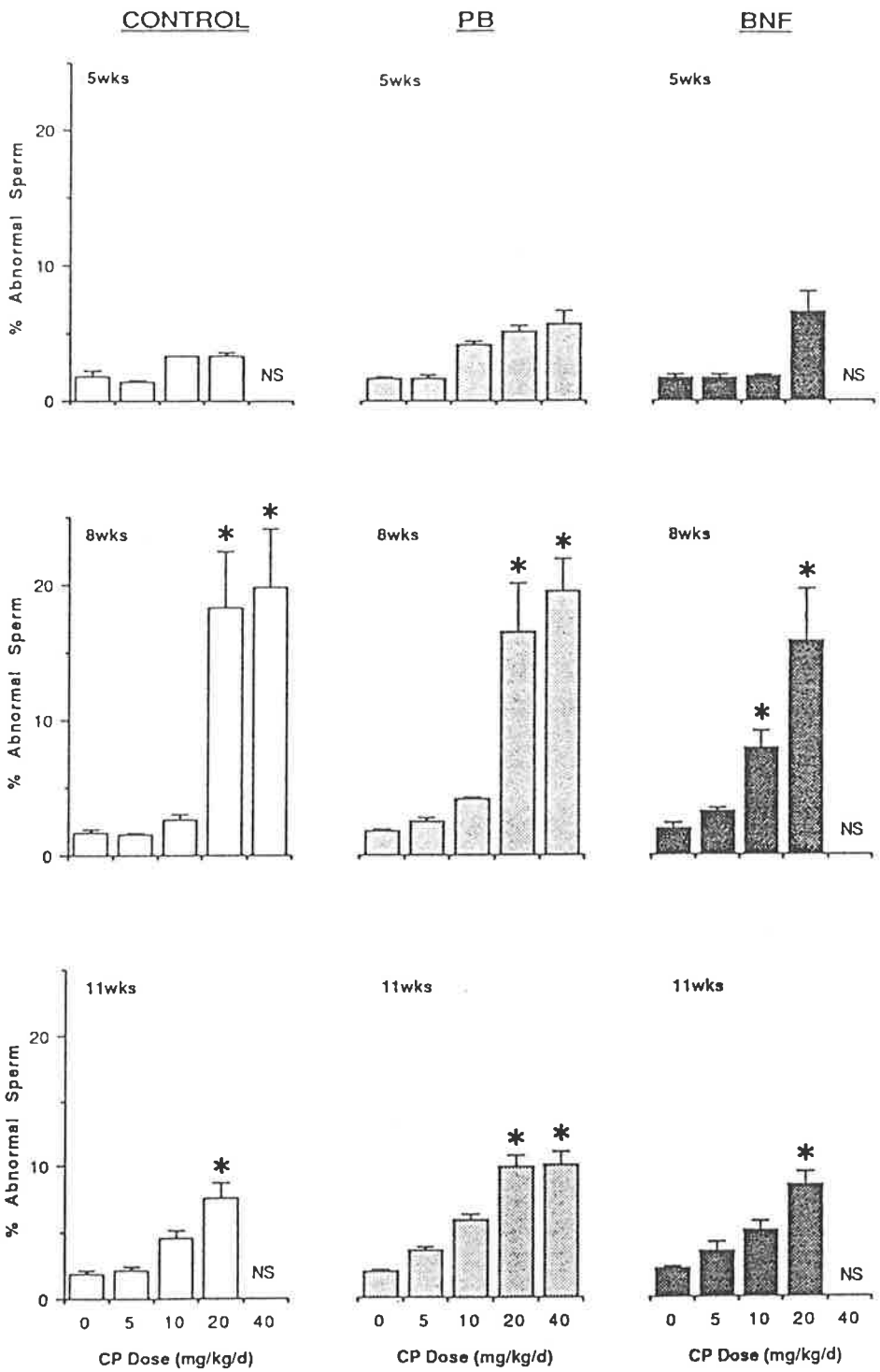
40 mg/kg/d CP plus PB at 5, 8 and 11 wks, n=3.

20 mg/kg/d CP plus BNF at 5, 8 and 11 wks, n=3.

* indicates significant difference from respective controls, $p<0.05$.

NS=No survivors.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.


CONTROL
PB
BNF
5wks
8wks
11wks
% Abnormal Sperm
0
10
20
NS
*
0 5 10 20 40
CP Dose (mg/kg/d)

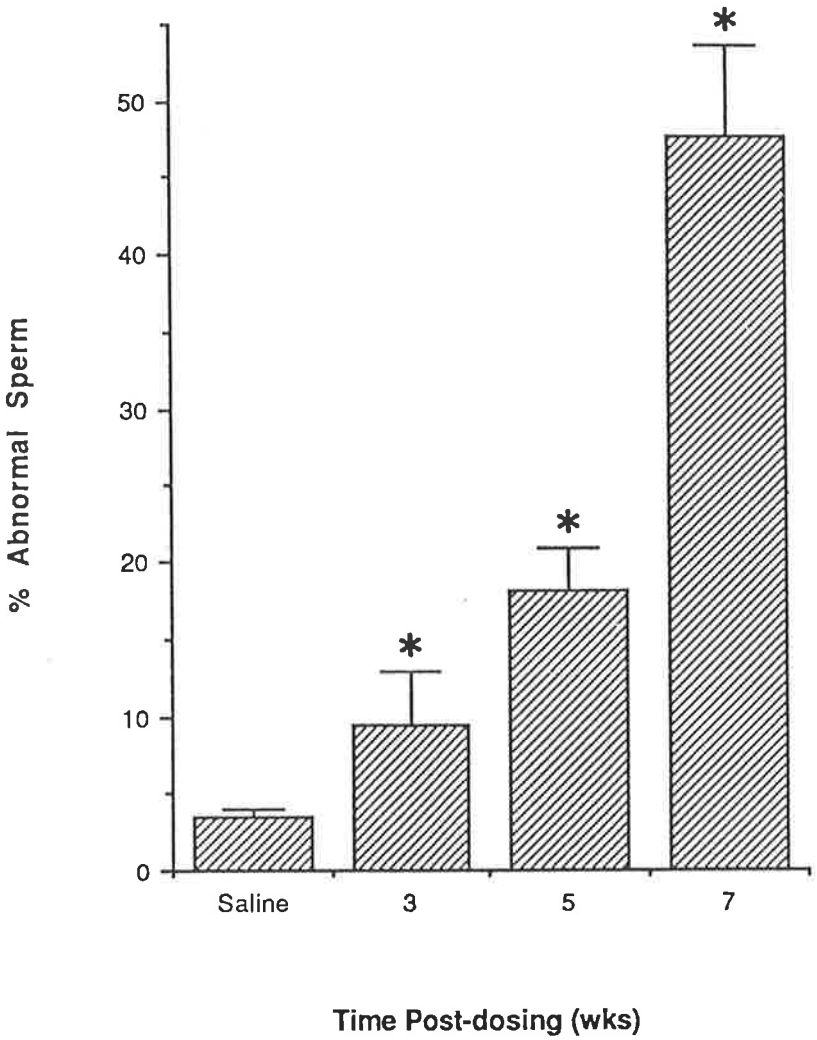


Figure 3.7: Effect of TEM (1/mg/kg) on the frequency of abnormal sperm in mice at 3, 5 and 7 wks (n=5). * indicates significant difference from respective controls, $p<0.05$.

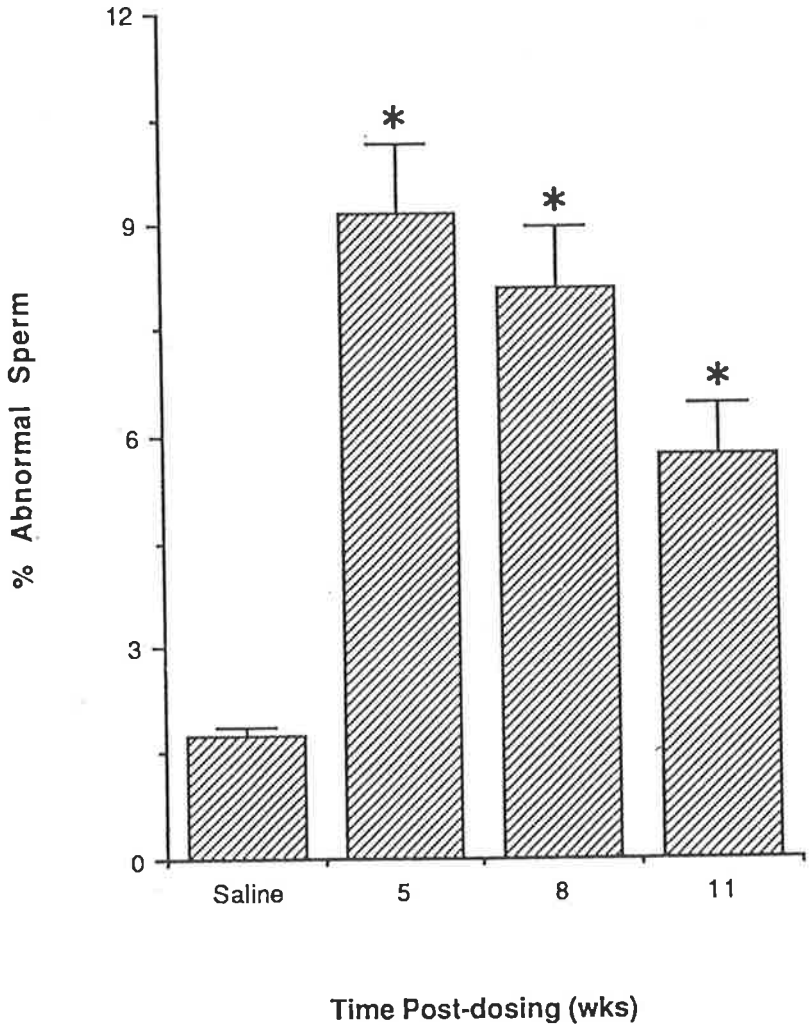


Figure 3.8: Effect of TEM (1/mg/kg) on the frequency of abnormal sperm in rats at 5, 8 and 11 wks (n=5). * indicates significant difference from respective controls, $p<0.05$.

reduced, the CP-induced mortality. In the mouse, CP-induced mortality was 7, 14 and 7% in control, PB and BNF pretreated animals, respectively (Table 3.7).

The positive control (TEM) produced a significant (χ^2=168.9, p<0.001) genotoxic response in both species (Figures 3.7 and 3.8). The magnitude of the response was significantly greater (p<0.001) in the mouse compared to the rat. Furthermore, the response varied with time of sampling in a species-specific manner, that is, the magnitude of the response in the mouse was greatest at the last time point and decreased toward the first time point, while the opposite was observed in the rat. The Asymptotic tests detected a significant (p<0.05) effect of TEM at all time points in both species.

In the pilot SCE experiments the maximal response to CP (40 mg/kg mouse, 5 mg/kg rat) was observed at 4 and 8 hrs after dosing in mice and rats, respectively (Figure 3.9). In the main experiments, where splenocytes were sampled at the optimum times of 4 (mouse) and 8 (rat) hrs after treatment, CP significantly increased the SCE frequency (F=798.34, p<0.001, mouse; F=337.69, p<0.001, rat) in a dose-related manner in both species (Figures 3.10 and 3.11). The genotoxicity of CP was not significantly modified by PB (F=1.33, mouse; F=0.70, rat) or BNF (F=0.09, mouse; F=0.85, rat). The response was greater in the rat than the mouse at comparable doses. This was illustrated by Tukey's test, where the limit of detection for a genotoxic response was 1.25 and 0.63 mg/kg in the mouse and rat, respectively. The ability of splenocytes to divide in culture (replicative index, RI) was not compromised by in vivo exposure to CP in the mouse (F=1.58), whereas a significant (F=15.15, p<0.001) effect on the RI was observed in the rat (Tables 3.5 and 3.6). Tukey's test was not able to discern at which dose of CP the cytotoxic effect was significant (p<0.05). In both species PB (F=0.18, mouse; F=3.56, rat) and BNF (F=0.09, mouse; F=0.51, rat) did not alter these responses.

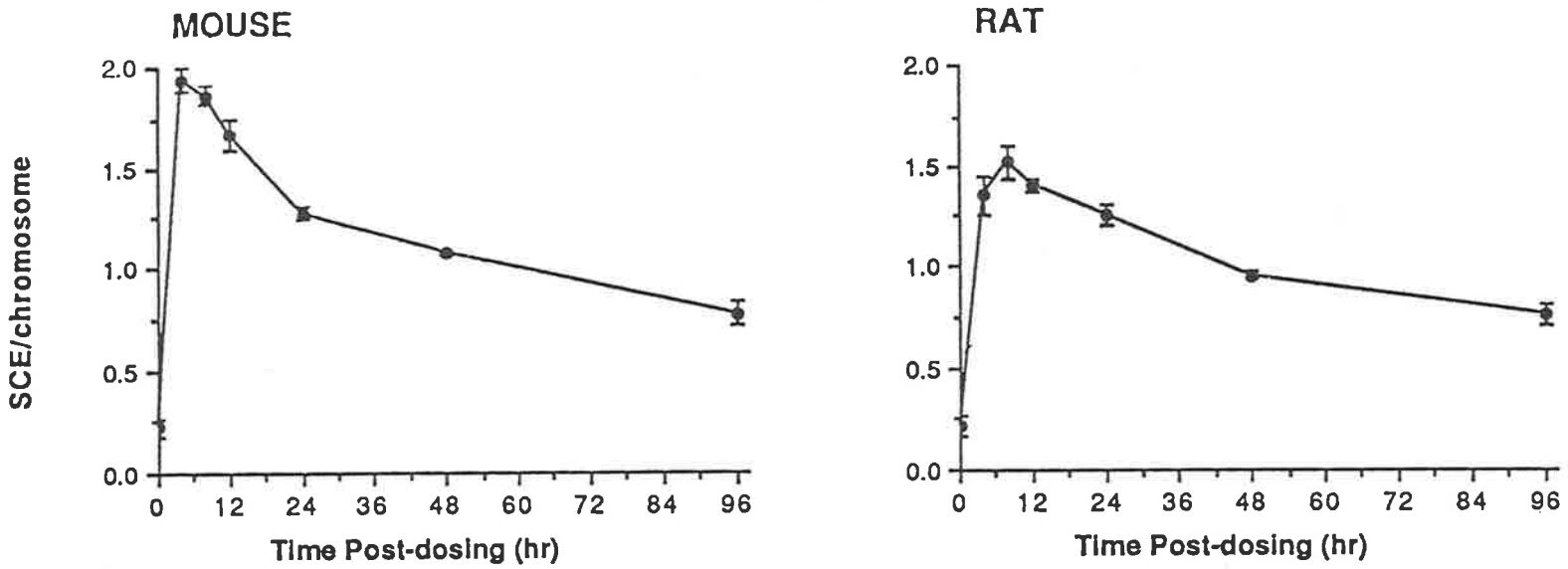


Figure 3.9: Frequency of SCEs in cultured splenocytes from rats and mice exposed to CP at 5 and 40 mg/kg, respectively. n=2 for both species.

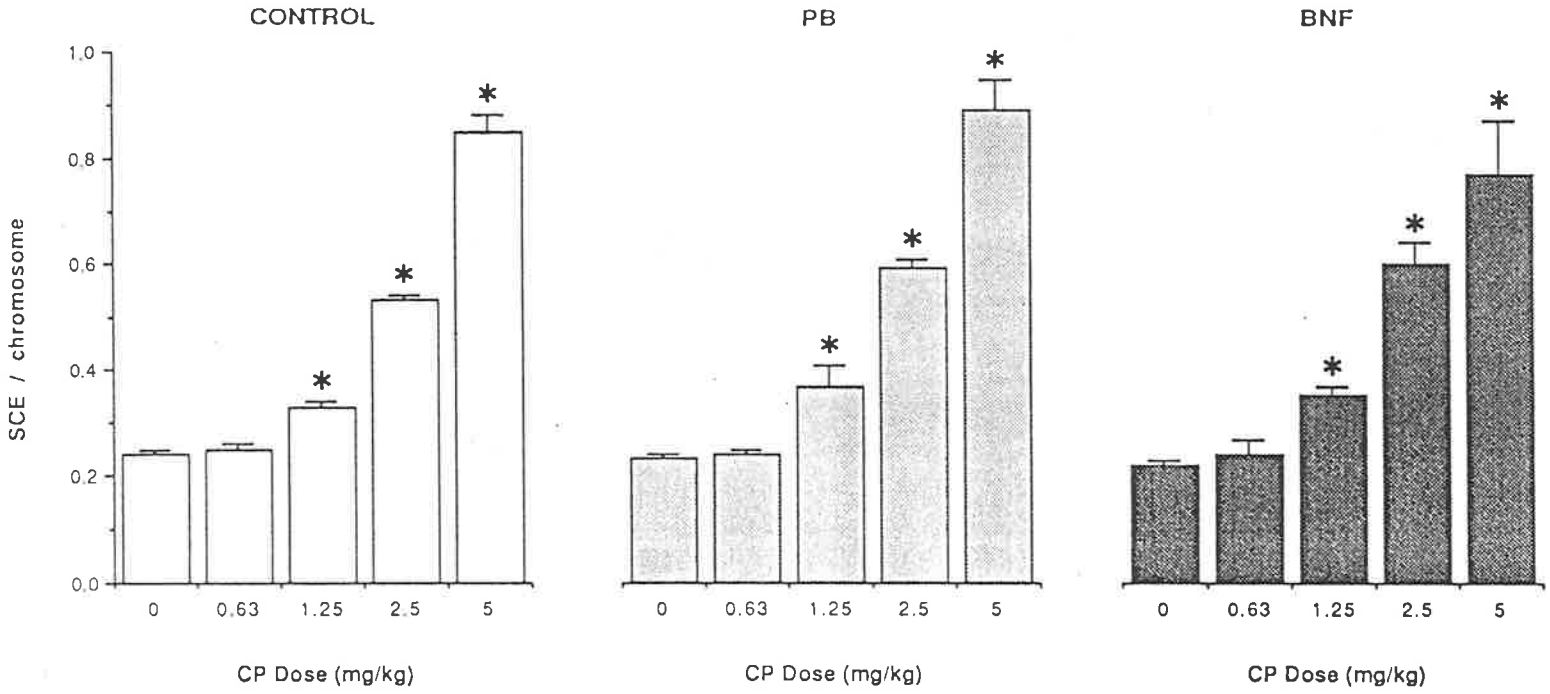


Figure 3.10: Effect of CP (0-5 mg/kg) on the SCE frequency in splenocytes from control, PB and BNF pretreated mice. n=5. * indicates significant difference from respective controls, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

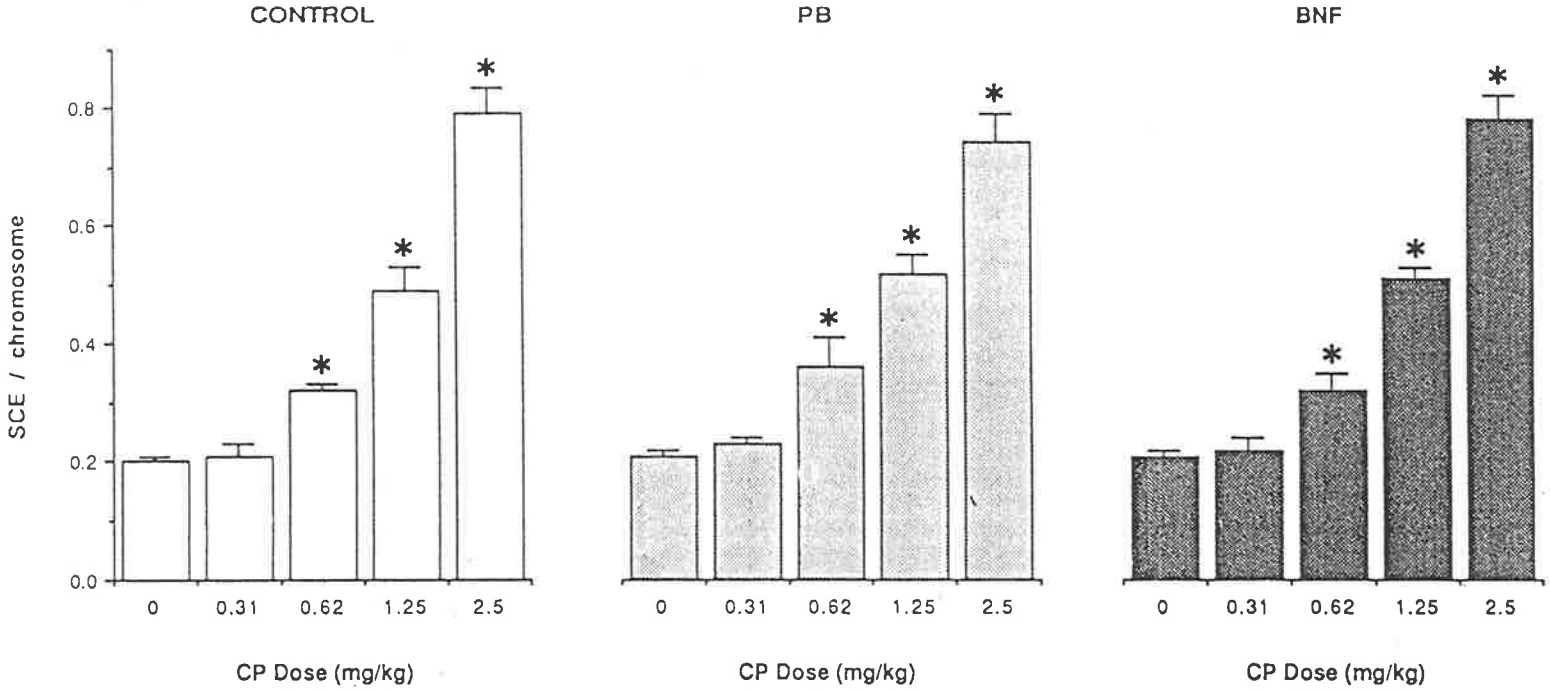


Figure 3.11: Effect of CP (0-2.5 mg/kg) on the SCE frequency in splenocytes from control, PB and BNF pretreated rats. n=5. * indicates significant difference from respective controls, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

CP Dose (mg/kg)	Replicative Index		
	Control	PB	BNF
Cont.	2.04±0.04	1.98±0.04	2.02±0.04
0.63	1.96±0.04	2.02±0.04	1.98±0.03
1.25	2.02±0.04	2.03±0.03	2.01±0.02
2.5	1.97±0.06	1.95±0.04	1.98±0.02
5.0	2.04±0.03	2.02±0.02	2.02±0.03

Table 3.5: Effect of CP on the replicative index (RI) of cultured splenocytes from control, PB and BNF pretreated mice, n=5.

CP Dose (mg/kg)	Replicative Index		
	Control	PB	BNF
Cont.	2.45±0.09	2.46±0.09	2.36±0.06
0.31	2.37±0.02	2.43±0.05	2.43±0.05
0.62	2.40±0.07	2.40±0.07	2.34±0.10
1.25	2.20±0.09	2.34±0.06	2.30±0.25
2.50	2.20±0.13	2.25±0.08	2.20±0.09

Table 3.6: Effect of CP on the replicative index (RI) of cultured splenocytes from control, PB and BNF pretreated rats, n=5.

Assay/Species	No. of deaths/No. treated			
	CP Dose	Control	PB	BNF
MN Assay				
Mouse	0–20 mg/kg	—	—	—
Rat	0–20 mg/kg	—	—	—
SM Assay				
Mouse	40 mg/kg/d	1/15	2/15	1/14
Rat	20 mg/kg/d	2/15	1/15	6/15
	40 mg/kg/d	12/15	6/15	15/15
SCE Assay				
Mouse	0–5 mg/kg	—	—	—
Rat	0–2.5 mg/kg	—	—	—

Table 3.7: CP-induced mortality in the 3 in vivo assays. For clarity, only doses at which mortality was observed are represented. With all other doses no mortality was observed.

3.4 Discussion

The genotoxicity of CP, as evidenced by the MN assay, was consistent with other investigators, who also found the rat to be more sensitive than the mouse, when given the same doses (Madle et al. 1986b). Although a greater proportion of CP is excreted unchanged in the rat compared to the mouse (Torkelson et al. 1974), the rat was more sensitive, which may be due to the higher peak serum level of CP attainable and the 3x slower rate of clearance of alkylating metabolites observed in rats compared to mice (Garattini et al. 1974).

The importance of multiple sampling for the MN assay was demonstrated in the rat, where at 30 hrs there was a disproportionately low level of CP-induced genotoxicity at higher doses and a disproportionately large increase at the same doses at 48 hrs. This apparent delay of the genotoxic response, with increasing doses, could be the result of two possible mechanisms. Firstly, a saturation of CP metabolism at higher doses may have produced the effect. This seems unlikely in view of the very short half-life and the level of drug excreted unchanged in rats (Torkelson et al. 1974). The alternative mechanism involves the cytotoxic property of CP in rats. The marked inhibition of haemopoiesis (Table 3.3) can delay progression of the damaged erythroblast to the PCE stage and hence delay the appearance of the MNPCEs (Section 1.3.1). These effects of CP in the rat have previously been observed by other investigators (Madle et al. 1986b; Goetz et al. 1975). A similar delay in the response to high doses of CP was not observed in the mouse, which is consistent with the relative lack of cytotoxicity at those doses.

The maximal response to CP appeared earlier in PB pretreated rats compared to controls. The concomitant reduction in CP-induced cytotoxicity with PB is consistent with the suggestion that the delayed response at higher doses was due to inhibition of bone marrow proliferation. The reduced cytotoxicity with PB may be due to an increased clearance of alkylating metabolites, which has been reported previously (Garattini et al. 1974). The possible increased clearance of the alkylating metabolites of CP, with PB pretreatment, may also have contributed to the reduced

lethality of CP in rats in the SM assay (Table 3.7). BNF produced a small reduction in cytotoxicity, but did not affect the timing of the response. Although PB altered the timing of the response in rats, the magnitude of the responses over the sampling period were comparable between the control and PB pretreated rats. Therefore, PB may not have changed the amount of genotoxic metabolite formed over that period of time.

The fact that the cytotoxic and genotoxic effects of CP were differentially altered by PB suggests that they may be due to separate mechanisms. This is supported by a number of investigators who propose that acrolein and PM are responsible for the cytotoxic and genotoxic effects of CP, respectively (Gurtoo et al. 1981; Crook et al. 1986; Hemminki, 1985). Notwithstanding, one should not discount the possibility that the genotoxic response to CP may be influenced in part, by the cytotoxic response or vice versa. The consistent potentiation, and the consequent reduction in the threshold dose of CP genotoxicity in mice with PB may be caused by an increased activation and/or decreased deactivation of CP. Like the rat, the lack of correlation between the effects of PB on CP-induced genotoxicity and cytotoxicity in the mouse, further supports the suggestion of different mediators for each event.

A faster rate of metabolism of CP in mice compared to rats (Torkelson et al. 1974) may account for the earlier expression of the maximal increase in SCE frequency in mice observed in the preliminary SCE experiments. The multiple-comparison tests indicated that the SCE assay is more sensitive than the MN assay to CP-induced genotoxicity. This may be due to tissue differences in the ability to locally activate and/or deactivate toxic metabolites or the ability of the tissues to repair DNA damage. Alternatively, any difference in assay sensitivity might simply be a function of differences in the ability of genotoxin-induced DNA damage to induce the endpoint being assessed. For example, a specific chemical-DNA adduct might induce SCEs, but not MNPCEs.

A number of extrinsic factors are known to be capable of influencing the incidence of abnormal sperm (Appendix E). Therefore, in order to determine whether the sensitivity of the animal models to genotoxins changed over time, and therefore

compromised comparison of the CP and ST experiments, a positive control was used to standardise the SM assay. Furthermore, since the test compounds (ST and CP) are thought to be only weak inducers of abnormal sperm production (Wyrobek and Bruce, 1975; Salomaa et al. 1985), the TEM data provided confidence that the assay was functioning properly. TEM significantly increased the proportion of abnormal sperm in both species and this effect was the same in the CP and ST experiments, which suggests that the animal model was consistent between the two experiments. The effect of enzyme inducers on TEM was not assessed as TEM does not require metabolic activation. CP is a potent inducer of MNPCEs and SCEs (Madle et al. 1986b) and has been used as a positive control in the both the MN and SCE assays. Therefore, it was not considered necessary to use a different chemical as a positive control with these two in vivo assays.

Compared to the MN and SCE assays, the SM assay was insensitive. CP was only able to produce an effect at lethal or near lethal doses in both species. In view of the susceptibility of the production of abnormal sperm to various non-mutagenic factors (Section 1.3.1), it is conceivable that the responses may be an indirect result of systemic toxicity induced by CP, rather than a true genotoxic effect. The MN assay results show that CP exerts toxicity at lower dose rates than those used in the SM assay, where there was significant bone marrow degeneration. Therefore, it is possible that animals treated with such high doses of CP over 5 days, may have become sufficiently anaemic for this to have affected normal sperm maturation. The proportion of abnormal sperm after CP exposure was greatest at 5 and 8 weeks in mice and rats, respectively, suggesting primary spermatocytes as the more sensitive stage of spermatogenesis to CP exposure in both species. Similar results, with respect to mice treated with CP, were also observed by Wyrobek and Bruce, (1975). The additive effect of PB and BNF on CP-induced sperm abnormalities indicates that the enzyme-inducing pretreatments are themselves altering the level of abnormal sperm production.

The inability of BNF to significantly modify the genotoxic potential of CP in the three in vivo assays is consistent with observations made by other investigators, where P-448 isozymes were not found to be involved in the oxidative metabolism

of CP (De Raat, 1977; Hales & Jain, 1980a; Hales & Jain, 1980b). Since BNF was able to alter the cytotoxicity of CP in rats suggests that it may have affected the clearance of the cytotoxic metabolites of CP.

The fact that, (A) PB pretreatment was able to alter the genotoxicity and cytotoxicity of CP in mice and rats, respectively, in the MN assay, and (B) the same pretreatment had no significant effect on CP genotoxicity in the other two systems, suggests that the bone marrow, in both species, may respond differently to enzyme-inducing pretreatments, from the other two target tissues. This tissue-specific effect of PB caused the bone marrow to be a more important site of CP-induced DNA damage in mice. In order to more thoroughly assess the concept of tissue- and species-specific induction profiles being responsible for these observations, an assessment of the xenobiotic metabolising capacity of the 3 target tissues and the liver, with and without PB or BNF pretreatments, was conducted in Chapter 5.

Chapter 4

The Genotoxicity of Styrene in Three In Vivo Assays, in the Presence and Absence of PB and BNF Pretreatment.

4.1 Introduction

Styrene (phenylethylene) is a commercially important chemical, widely used in the production of polymers, copolymers and reinforced plastics. ST is a suspected human carcinogen. According to a review by Valic, (1982), there have been case reports of neoplasias of the lymphatic and haemopoietic system amongst workers exposed to ST, while many similar studies found no such effects. Lifetime carcinogenicity testing of ST in rats and mice are also contradictory (Ponomarkov & Tomatis, 1978; Valic, 1982). Evidence for the carcinogenicity of styrene oxide (SO), the oxidative metabolite of ST, is less equivocal. It has been shown to be a direct acting carcinogen in rats and mice, of both sexes, producing squamous cell carcinomas or papillomas of the forestomach when given orally (Ponomarkov et al. 1984; Lijinsky, 1986).

The metabolism of ST is well characterised involving both Phase I and Phase II enzymes (Figure 4.1). The first step is the oxidation of a double bond, to form an epoxide at either the aliphatic side-chain (styrene-7,8-oxide, SO) or on the benzene ring (styrene-3,4-oxide). The formation of SO is predominantly mediated by cytochrome P450 dependent enzymes (Vainio et al. 1984). It is also formed by non-P450 dependent enzymes including oxyhaemoglobin and xanthine oxidase (Belvedere & Tursi, 1983; Ortiz de Montellano & Catalano, 1985), although their total contribution to styrene oxidation is very small. SO is the major product of oxidation and is further metabolised by two pathways. One pathway involving epoxide hydrolase, converts SO to styrene glycol. Styrene glycol can either form the glucuronide conjugate or is metabolised to mandelic acid, phenylglyoxylic acid, benzoic acid and hippuric acid. The alternate pathway for SO is the formation of glutathione (GSH) conjugates via glutathione S-transferases. These conjugates are further metabolised to mercapturates (Steele et al. 1981; Ryan & Bend, 1977). Previous work in our own laboratory (Stock, 1983) has shown a ST-related increase in urinary thioether output in rats after ST dosing (0.43–2.8 mmol/kg) and in occupationally exposed workers. Exposure to SO has also been shown to significantly reduce hepatic GSH levels in rats (Chakrabarti & Brodeur, 1981). Stock et al. (1986) proposed an alternative

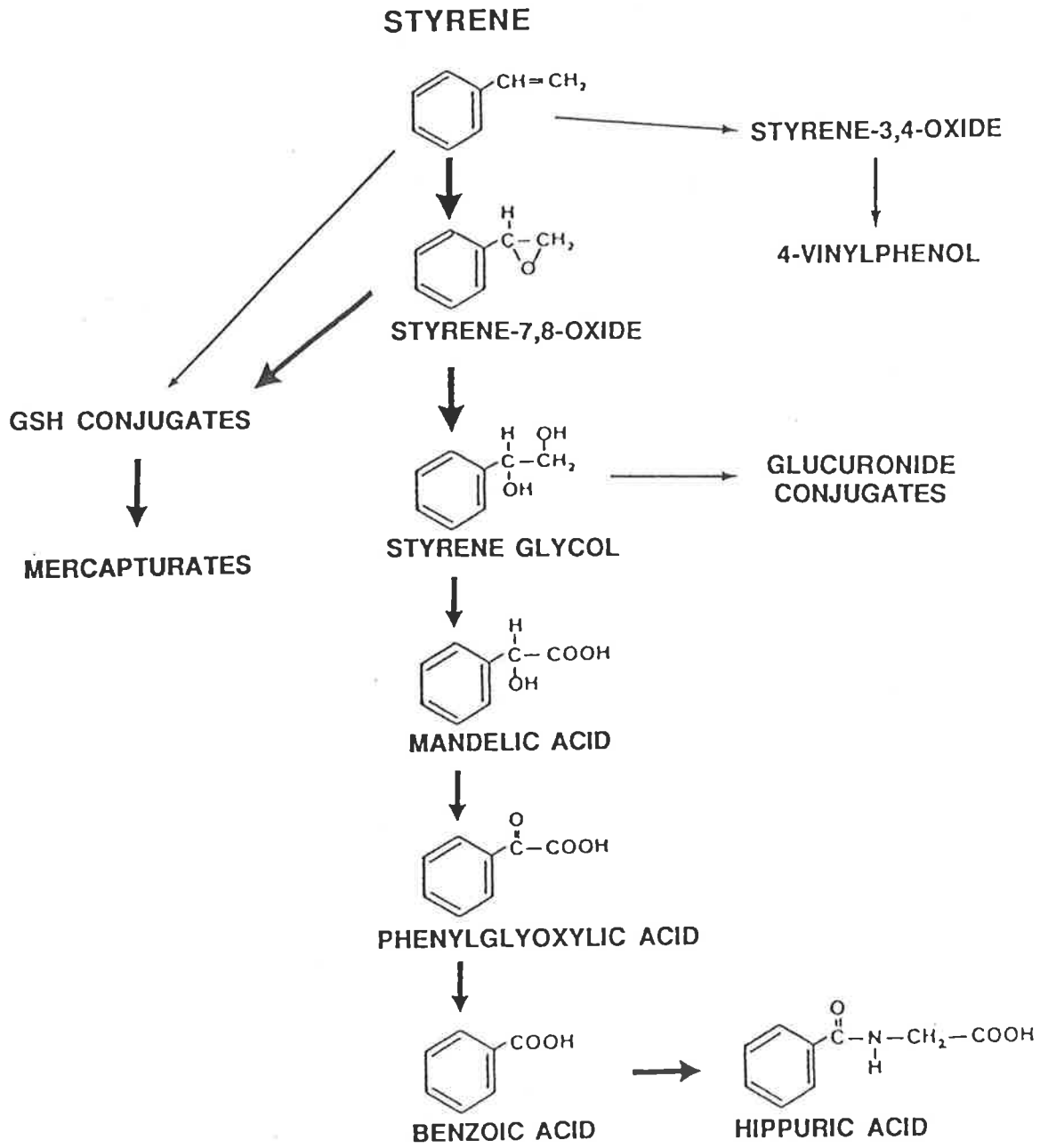


Figure 4.1: Major pathways for the metabolism of ST. Modified from Valic, (1982).

mechanism for the formation of ST-GSH conjugates mediated by prostaglandin-H-synthetase involving the production of glutathionyl radicals, rather than SO, as intermediates.

Withey and Collins (1977) investigated the pharmacokinetics of ST in rats and found the elimination rate to be dose dependent. At a dose of 1.337 mg/kg the kinetics could be described using a 2-compartment model with elimination half-lives of 4.68 and 26.6 hrs for the rapid and slow phases, respectively. When 9.359 mg/kg of ST was administered a 1-compartment model was observed with a half-life of 14.4 hrs. They suggested a saturation of ST metabolism occurring at high dose. Similar results were obtained with rats exposed to ST by inhalation (Ramsey & Young, 1978; Withey, 1978).

Rats pretreated with PB showed a 3-5 fold increase in the clearance of ST within 2 hrs after administration of ST. A similar stimulatory effect was not observed for the clearance of SO, styrene glycol, mandelic acid or phenylglyoxylic acid (Ohtsuji & Ikeda, 1971). Garattini et al. (1981), using rat liver microsomes and nuclear preparations, were able to show that styrene monooxygenase and epoxide hydrolase activities increased 3-fold with PB induction. In the same study BNF pretreatment had a similar inductive effect on styrene monooxygenase, while epoxide hydrolase was only induced 30-40%. Parkki et al. (1976) observed that ST (500 mg/kg/d over 3 or 6 days) was able to double the activity of hepatic microsomal p-nitroaniline O-demethylase, epoxide hydrolase and UDP-glucuronyl transferase. In the same study SO decreased the activity of benzo[a]pyrene hydroxylase and p-nitroaniline O-demethylase and P-450 content of liver, while styrene glycol had no effect on the activities of the enzymes assessed. Vainio et al. (1979) found inhalational exposure to ST (300 ppm, 6 hrs/day, 5 days/wk) enhanced the activities of both drug hydroxylating (ethoxycoumarin O-deethylase, cytochrome P450) and conjugating (epoxide hydrolase, UDP-glucuronyl transferase) enzymes in liver and kidneys of male rats. This indicated that ST was able to induce enzymes involved in its own metabolism. In contrast, unpublished data from our own laboratory (Stock, 1983) showed a lack of induction of thioether output in rats after chronic ST dosing (1.44 and 2.4 mmol/kg/day; 5 days/wk) for up to 3 wks.

A number of studies have assessed the ability of ST and SO to covalently bind to DNA. Nordqvist et al. (1985) found ST and SO to be equally effective as DNA-alkylating agents in vivo. The binding capacity of ST to DNA was saturable at higher doses, while its binding to haemoglobin was disproportionately higher at high doses. The covalent binding of SO to DNA is characterised by the alkylation at the C^7-, N^2- and O^6-positions of deoxyguanosine and the N^4-and O^2-positions of deoxycytosine as the main sites of covalent binding (Savela et al. 1986).

The ability of ST to induce genotoxic responses in in vitro and in vivo short-term tests has been variable. ST is mutagenic in the Ames test in the presence of a metabolising system (De Meester et al. 1977; Stoltz and Withey, 1977). Watabe et al. (1978) was only able to induce mutations in the Ames test when the microsomal system used was derived from a PB or 3MC-induced rodent liver. In the same study they showed that the addition of 3,3,3-trichloropropene oxide, an inhibitor of epoxide hydrolase, potentiated the mutagenicity of ST, implicating SO as the putative genotoxic metabolite. In in vitro mammalian cell culture systems, both ST (when metabolically activated) and SO, are potent inducers of chromosomal aberrations, micronuclei and SCEs. In contrast, the in vivo genotoxicity of ST is inconclusive. In the bone marrow MN test it was negative in Chinese hamsters at doses up to 1 g/kg (Pentilla et al. 1980), while giving a positive response in mice at 250 and 1000 mg/kg (Norppa, 1981).

Similar contradictory results have been reported with induction of SCEs by ST. Conner et al. (1979) demonstrated an increased number of SCEs in mouse bone marrow and regenerating liver after exposure by inhalation (87-220 ppm over 4 days), whereas, Sharief et al. (1986) could not show a similar effect of styrene in mouse bone marrow, up to 1000 mg/kg. Increases in the frequency of SCEs, in lymphocytes of humans occupationally exposed to low levels of ST, have been reported in two separate studies where the mean atmospheric concentrations of ST were 13 and 24 ppm (Hogstedt et al. 1983; Nordenson & Beckman, 1984). On the other hand Maki-Paakanen (1987), examining lymphocytes of workers exposed to comparable levels of ST (23 ppm), was not able to show a positive genotoxic response in the chromosomal aberration, MN or SCE assays. Investigation of the

genotoxicity of ST in germ cells is limited and only one study has involved the sperm morphology assay. Salomaa et al. (1985), exposing mice by inhalation (150 and 300 ppm; 6 hr/day; 5 days) or intraperitoneally (175, 350 and 700 mg/kg/d; 5 days), found no increase in the frequency of abnormal sperm at 3 and 5 weeks after dosing.

The aim of this chapter was to establish the dose/response relationships of styrene in the 3 in vivo assays (micronucleus, sperm morphology and sister-chromatid exchange assays) and to assess the effects of pretreatment with PB and BNF on its genotoxic potential. In addition a comparison between the rat and the mouse was conducted.

4.2 Materials & Methods

4.2.1 Micronucleus Assay

Mice and rats were given a single i.p. injection of ST (dissolved in peanut oil) at 0, 150, 300, 450, 600 mg/kg and 0, 300, 750, 1500, 3000 mg/kg, respectively. Bone marrow was sampled at 30, 48 and 72 hrs after dosing, from control animals and 30 and 48 hrs from inducer-pretreated animals. Induction of microsomal enzyme activity was affected using PB (1 mg/ml) in drinking water for 7 days or BNF (100 mg/kg in peanut oil) 48 hrs prior to dosing with ST. Bone marrow smears prepared following the procedure set out in section 2.1.1.2, were air-dried immediately and left overnight before staining with Wright's stain and Giemsa. Slides were assessed in a blind fashion for MNPCE frequency and cytotoxicity (PCE/RBC ratio).

4.2.2 Sperm Morphology Assay

Mice and rats were treated with ST by single daily i.p. injections over 5 days at 0, 50, 100, 200, 400 mg/kg and 0, 250, 500, 1000, 2000 mg/kg, respectively. Positive control animals were treated with a single i.p. injection of TEM (1 mg/kg) on the first day of dosing. Mice were sacrificed at 3, 5 and 7 wks from commencement of dosing

and rats at 5, 8 and 11 wks. Pretreatment with PB and BNF was similar to the MN test with the treatments extending for the length of the styrene dosing period. BNF pretreatment required a single i.p. dose of 100 mg/kg 48 hrs before treatment with ST followed by two further doses at 48 hour intervals. PB (1 mg/ml in drinking water) was commenced 7 days prior to dosing and maintained for the duration of the 5-day dosing period. Sperm smears prepared according to the procedure in section 2.1.2.2, were analysed in a blind fashion for the incidence of abnormal sperm heads.

4.2.3 Splenocyte SCE Assay

In the pilot studies mice and rats were given a single i.p. dose of ST at 450 and 1500 mg/kg, respectively. The timing of the maximal response was determined for each species and used as the sampling time for the main study. In the main study mice dosed at 0, 75, 150, 300, 450 mg/kg and rats dosed at 0, 375, 750, 1500, 3000 mg/kg, were sacrificed at the appropriate time post-dosing, determined by the pilot study. The enzyme-inducing treatments were the same as those for the MN test (Section 4.2.1). In the pilot and main studies, the removal of the spleens, culturing and harvesting of cells and the preparation and assessment of slides was performed according to the method described in sections 2.1.3.2 and 2.1.3.3.

4.3 Results

Styrene produced a weak but significant, dose-related increase (χ^2=11.89, $p<0.05$) in the incidence of MNPCEs in treated mice (Figure 4.2). At low doses the responses were similar in magnitude at 30 and 48 hrs, while at the top doses, which produced lethality in treated mice (Table 4.7), the responses were greater at 48 than 30 hrs post-dosing. The statistical analysis also indicated a significant additive effect (χ^2=32.29, $p<0.001$) due to time of sampling alone. That is, the dose/response curves for ST change in a parallel fashion from one time point to another. The Asymptotic test indicated a significant effect ($p<0.05$) at the top dose, at 48 hrs

only. In contrast, ST was not genotoxic in the rat (χ^2=1.92) up to lethal doses (Figure 4.2, Table 4.7).

Analysis of the effects of PB or BNF on ST-induced MNPCEs in mice (Figure 4.3), found no significant interaction (χ^2=1.66, PB; χ^2=8.84, BNF). A similar analysis in the rat (Figure 4.4) showed a significant effect of ST (χ^2=11.08, $p<0.05$) on the MNPCE frequency. Like the mouse the response to ST was not significantly altered by PB (χ^2=5.90) or BNF (χ^2=1.94). PB had a significant (χ^2=7.54, $p<0.01$) additive effect on the dose/response curve to ST, that is, PB itself significantly altered the MNPCE frequency.

In control mice, the vehicle alone (peanut oil) was not genotoxic, but did produce a fall in the PCE/RBC ratio at 30 hrs, which recovered by 48 hrs. Appendix D describes an experiment which confirmed this effect to be reproducible and significant ($p<0.05$). A similar effect was not observed in the rat. ST did not have a significant effect on haemopoiesis (F=1.86) in mice (Table 4.1), although the PCE/RBC dose/response curves did differ significantly (F=20.97, $p<0.05$) with time of sampling in a parallel manner. ST was significantly cytotoxic (F=2.18, $p<0.05$) to rat bone marrow, which was significantly influenced by the time of sampling (Table 4.3). The influence of sampling time on the appearance of the cytotoxic response to ST in rats, was also indicated by Tukey's test. The multiple-comparison test indicated a significant response down to 750 mg/kg at 72 hrs. No cytotoxicity was detected at 48 hrs, with only the top dose showing siginificant cytotoxicity at 30 hrs ($p<0.05$).

In the rat, there was a significant interaction (F=5.6, $p<0.001$) between BNF and the cytotoxicity of ST, while PB (F=1.44) had no effect (Tables 4.3 and reft-4.4). Tukey's test indicated that the top dose at 30 and 48 hrs was significantly cytotoxic ($p<0.05$) in the presence of BNF, compared to at 30 hrs only in control rats. PB significantly altered the PCE/RBC ratio (F=10.18, $p<0.001$) in treated mice (Tables 4.1 and 4.2), with no significant effect on ST-induced cytotoxicity (F=0.70). The analysis also indicated a significant interaction (F=8.28, $p<0.001$) between dose and time, which was not observed by the analysis of the data in table 4.1. BNF had no significant effect (F=1.75) in the same system.

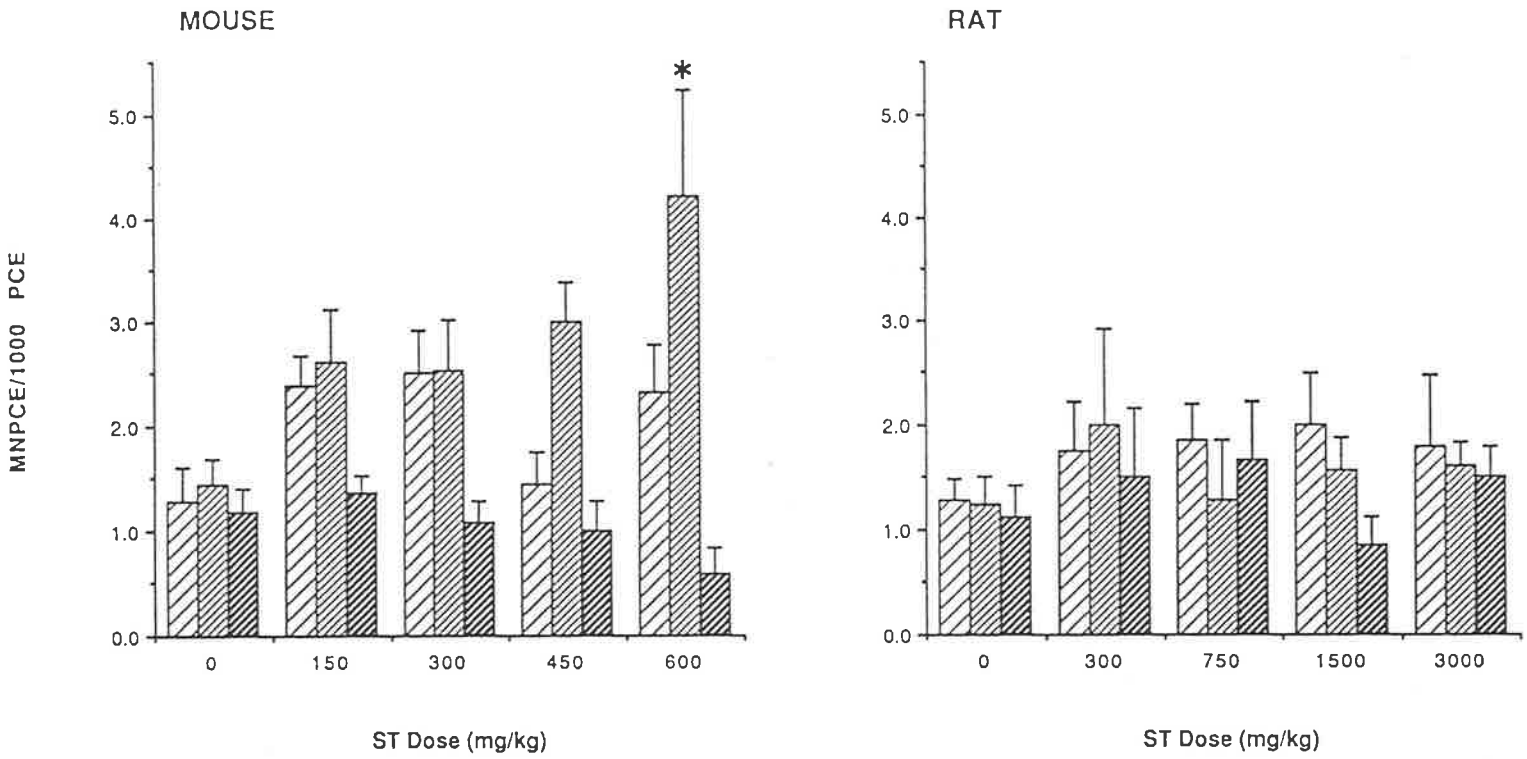


Figure 4.2: Effect of ST on the frequency of MNPCEs in the mouse (0-600 mg/kg) and rat (0-3000 mg/kg). n=5-10 (mouse) and n=4-8 (rat). * indicates a significant difference from the corresponding control group, $p<0.05$.

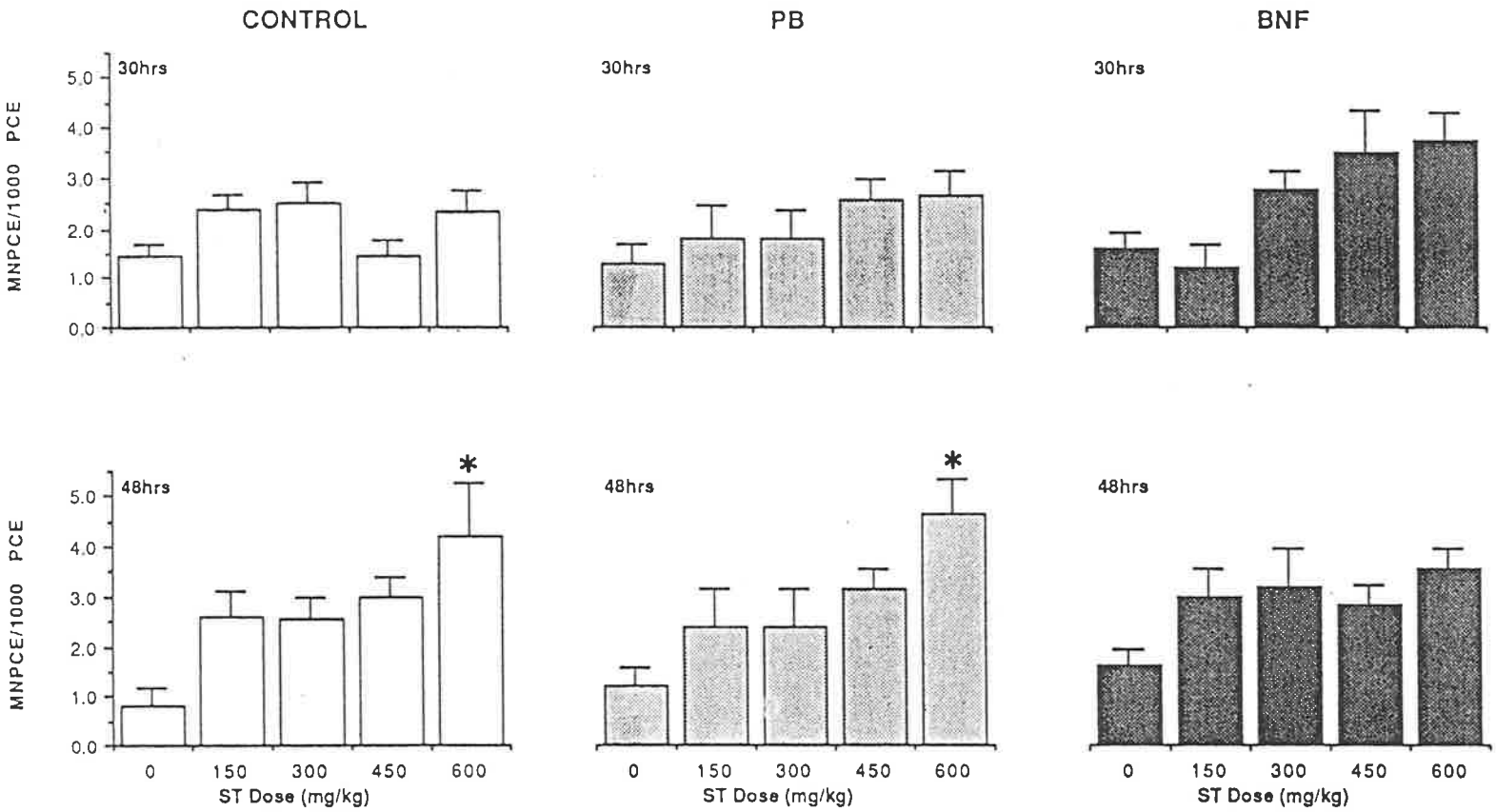


Figure 4.3: Effect of PB and BNF pretreatment on ST-induced MNPCEs in the mouse. n=5-10. * indicates a significant difference from the corresponding control group, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

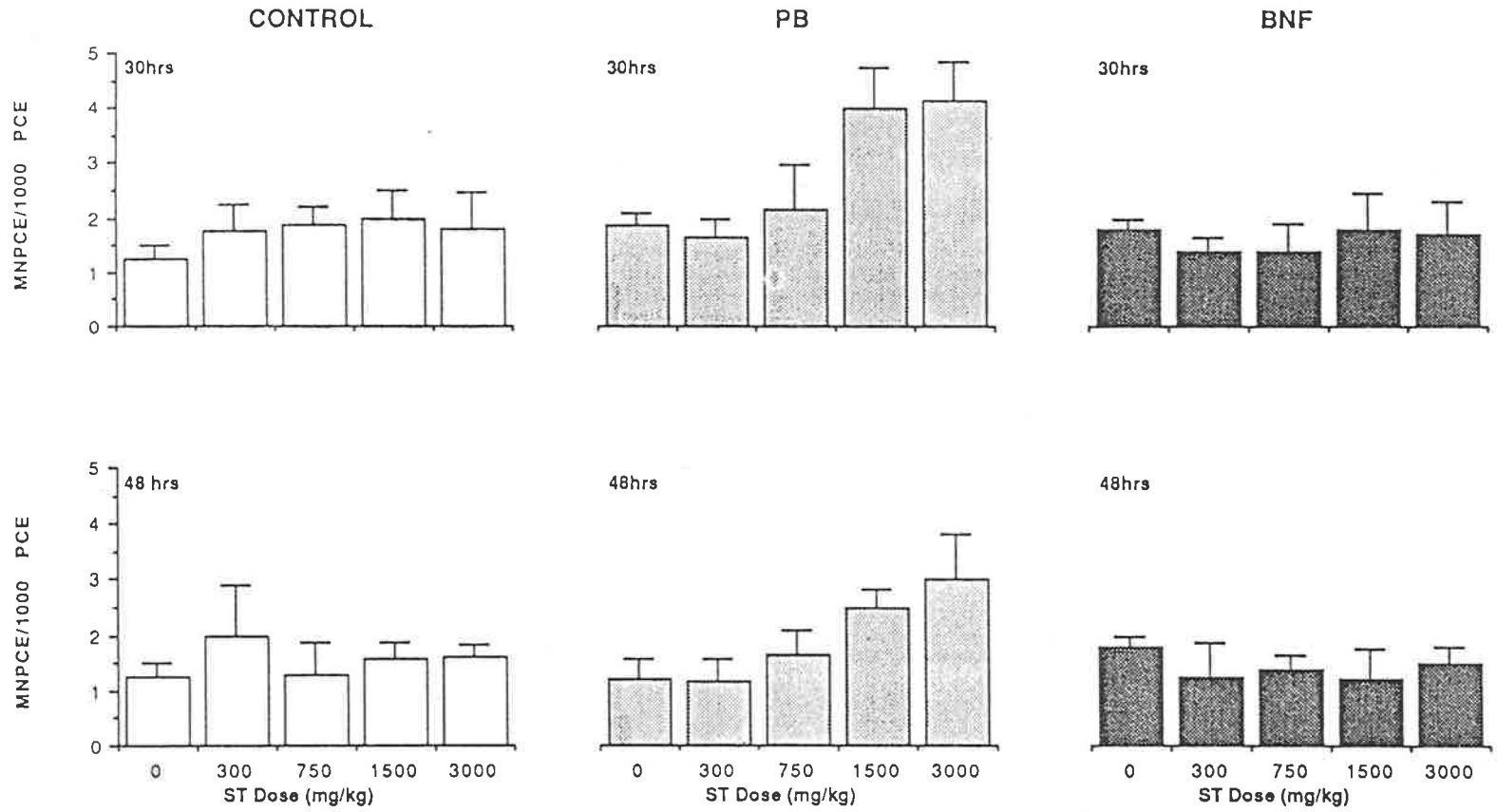


Figure 4.4: Effect of PB and BNF pretreatment on ST-induced MNPCEs in the rat. n=4-8.

ST Dose (mg/kg)	PCE/RBC Ratio		
	30hrs	48hrs	72hrs
Cont.	0.58±0.04	0.96±0.06	1.07±0.07
150	0.67±0.07	0.78±0.07	0.89±0.05
300	0.66±0.04	0.89±0.06	1.04±0.04
450	0.69±0.04	0.63±0.09	0.93±0.04
600	0.73±0.11	0.69±0.08	0.86±0.12

Table 4.1: Effect of ST (0-600 mg/kg) on the PCE/RBC ratio in control mice. n=4-10.

ST Dose (mg/kg)	PCE/RBC Ratio			
	PB		BNF	
	30hrs	48hrs	30hrs	48hrs
Cont.	0.58±0.03	1.09±0.15	0.67±0.07	0.90±0.15
150	0.80±0.05	1.06±0.06	0.79±0.11	0.95±0.09
300	0.90±0.05	0.83±0.11	0.56±0.05	0.69±0.12
450	0.86±0.10	0.65±0.07†	0.56±0.07	0.58±0.08
600	1.01±0.10†	0.73±0.05	0.77±0.11	0.72±0.16

Table 4.2: Effect of ST (0-600 mg/kg) on the PCE/RBC ratio in PB and BNF pretreated mice. n=5-10. † indicates a significant difference from the corresponding control group, $p<0.05$.

ST Dose (mg/kg)	PCE/RBC Ratio		
	30hrs	48hrs	72hrs
Cont.	0.70±0.05	0.70±0.05	0.70±0.04
300	0.72±0.03	0.53±0.08	0.74±0.05
750	0.61±0.08	0.75±0.07	0.37±0.05†
1500	0.48±0.03	0.63±0.09	0.42±0.09†
3000	0.42±0.09†	0.51±0.13	0.22±0.03†

Table 4.3: Effect of ST (0-3000 mg/kg) on the PCE/RBC ratio in control rats. n=4-8. † indicates a significant difference from the corresponding control group, $p<0.05$.

ST Dose (mg/kg)	PCE/RBC Ratio			
	PB		BNF	
	30hrs	48hrs	30hrs	48hrs
Cont.	0.60±0.06	0.60±0.06	0.72±0.03	0.73±0.03
300	0.68±0.06	0.68±0.07	0.96±0.15	1.01±0.08
750	0.96±0.16	0.65±0.16	0.59±0.05	0.56±0.12
1500	0.62±0.10	0.62±0.14	0.67±0.10	0.53±0.04
3000	0.52±0.03	0.53±0.14	0.21±0.03†	0.16±0.02†

Table 4.4: Effect of ST (0-3000 mg/kg) on the PCE/RBC ratio in PB and BNF pretreated rats. n=4-8. † indicates a significant difference from the corresponding control group, $p<0.05$.

Tukey's test was able to detect a significant decrease ($p<0.05$) in the PCE/RBC ratio in PB pretreated mice only. This was observed at 600 mg/kg at 30 hrs and at 450 mg/kg at 48 hrs. The data in tables 4.1 and 4.2 indicated a trend where the PCE/RBC ratio increased with increasing ST dose at 30 hrs, whereas it had a tendency to decrease with increasing ST dose at 48 hrs.

ST produced a significant increase ($\chi^2=110.70$, $p<0.001$) in the production of abnormal sperm in mice (Figure 4.5). There was no significant difference ($\chi^2=3.94$) in the responses between the sampling times. Analysis of the effects of PB and BNF (Appendix C), showed they did not significantly alter ST genotoxicity ($\chi^2=11.27$, PB; $\chi^2=14.74$, BNF). They were able to alter abnormal sperm production themselves, which was significantly dependent on the time of sampling ($\chi^2=27.24$, $p<0.001$, PB; $\chi^2=13.48$, $p<0.01$, BNF). ST was not lethal in control mice, but in the presence of PB and BNF pretreatment, the highest dose was lethal to 10 and 9% of treated mice, respectively (Table 4.7).

ST produced a genotoxic response in the rat SM assay ($\chi^2=132.27$), which was weaker than in the mouse (Figure 4.6). There was no statistically significant influence of PB ($\chi^2=7.75$) on the response to ST in the rat. Furthermore, it had no significant effect ($\chi^2=1.67$) of its own in the rat SM assay. Although BNF appeared to increase the genotoxic response to ST at the top doses, due to the limited number of survivors at the top dose (Table 4.7), their contribution to the overall analysis was small. Hence, the apparent interaction between BNF and dose was not found to be significant ($\chi^2=6.78$). On the other hand, BNF was found to significantly increase ($\chi^2=21.02$, $p<0.001$) the level of abnormal sperm. The Analysis of Deviance table (Appendix C) also indicated a significant effect ($\chi^2=40.07$, $p<0.001$) due to sampling time alone, that is, the dose response curves varied in a parallel manner.

ST was lethal to 14 of 15 treated rats at 2000 mg/kg/d in both control and PB pretreated animals (Table 4.7). BNF pretreatment reduced the mortality rate to 6 of 15. Four deaths had occurred within minutes of a ST injection. The four animals were immediately examined, where there was evidence of abdominal bleeding. Their deaths were therefore, attributed to injection artifact. Treatment with

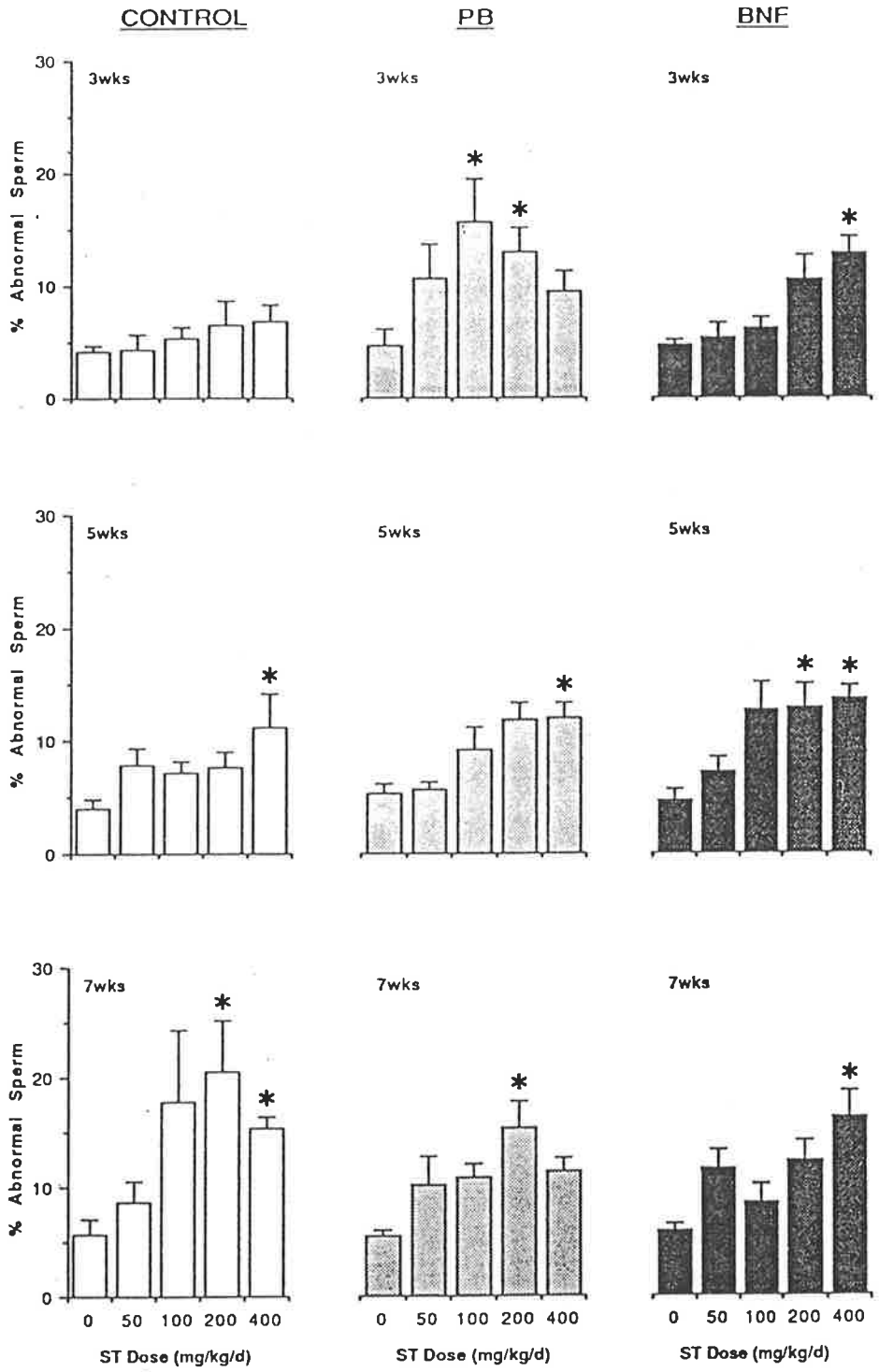


Figure 4.5: Percentage of abnormal sperm after treatment with ST (0-400 mg/kg/d) in control, PB and BNF pretreated mice. n=4-8. * indicates a significant difference from the corresponding control group, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

Figure 4.6: Percentage of abnormal sperm after treatment with ST (0-2000 mg/kg/d) in control, PB and BNF pretreated rats. n=4-5, except for the following groups;

2000 mg/kg/d ST alone at 8 wks, n = 1,

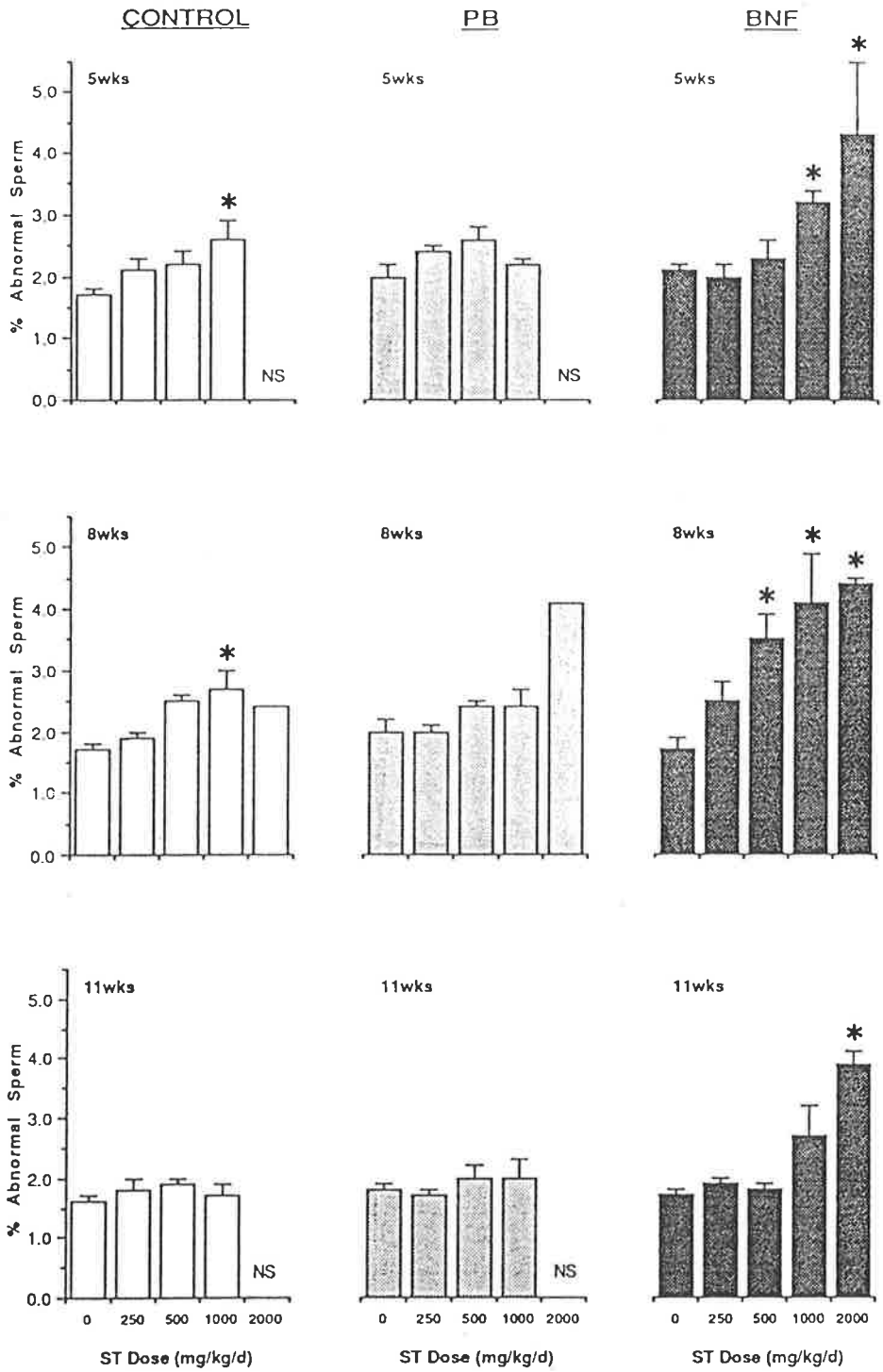
2000 mg/kg/d ST plus PB at 8 wks, n = 1,

2000 mg/kg/d ST plus BNF at 5, 8 and 11 wks, n=2.

* indicates a significant difference from respective control groups, $p<0.05$.

NS=No survivors.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.


CONTROL
PB
BNF
5wks
8wks
11wks
% Abnormal Sperm
5.0
4.0
3.0
2.0
1.0
0.0
NS
*
0
250
500
1000
2000
ST Dose (mg/kg/d)

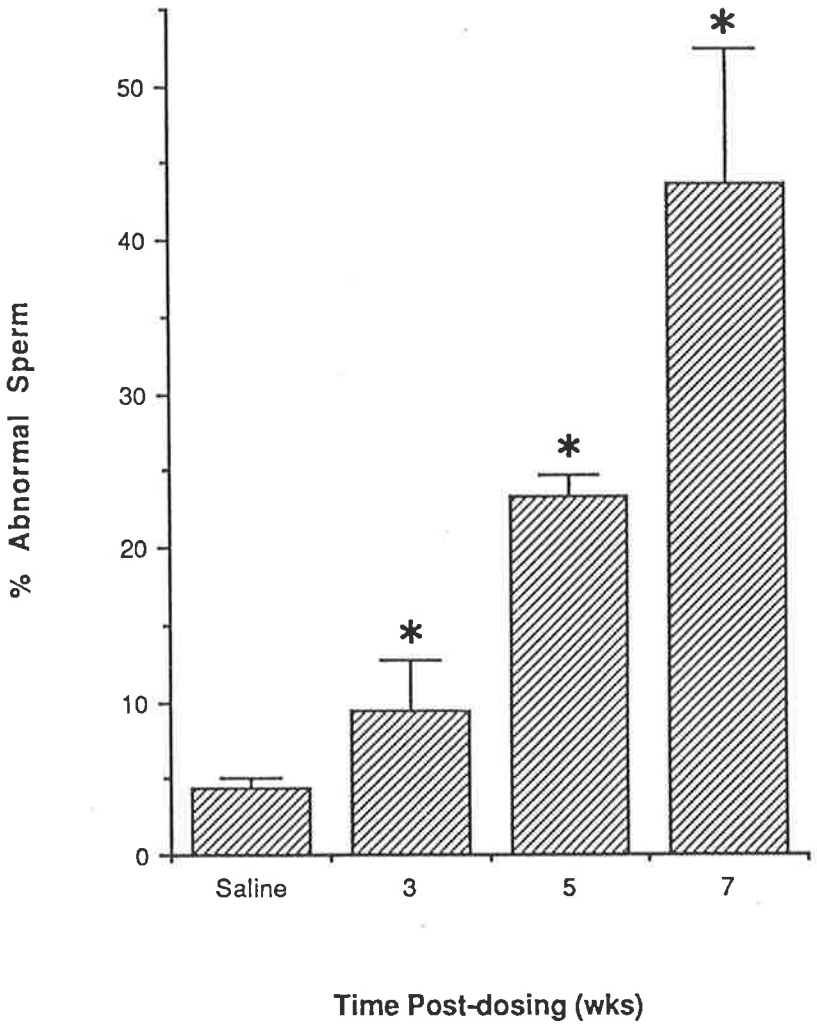


Figure 4.7: Effect of TEM (1 mg/kg) on the frequency of abnormal sperm in mice at 3, 5 and 7 wks (n=5). * indicates a significant difference from the corresponding control group, $p<0.05$.

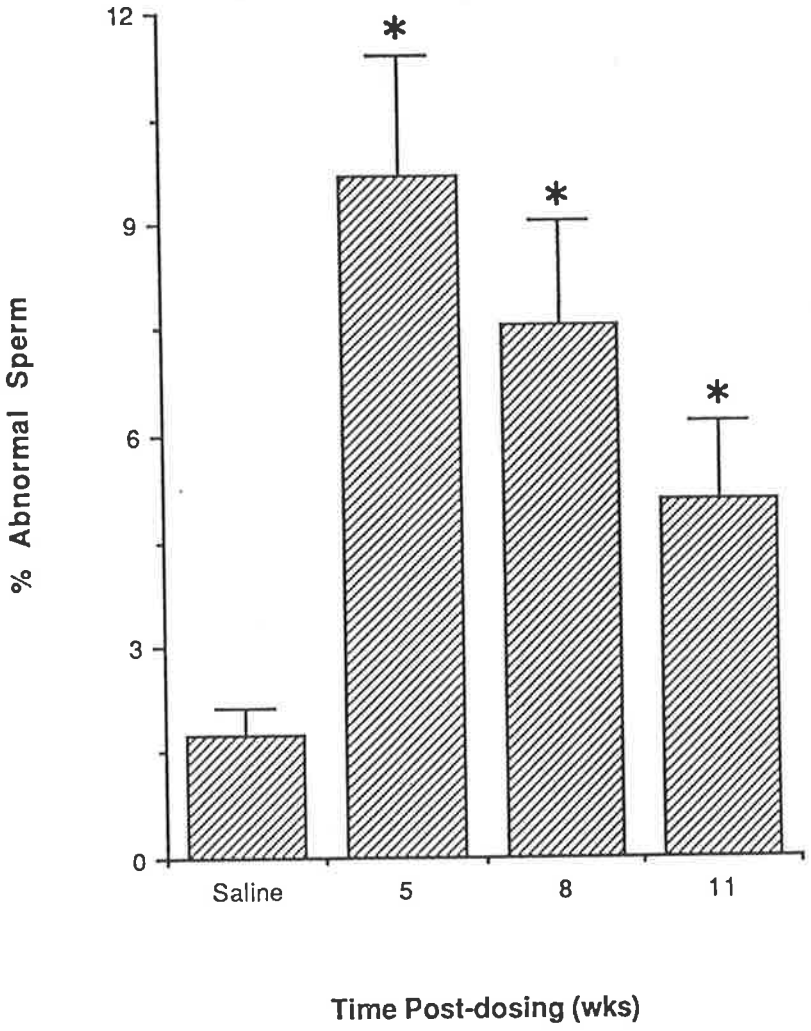


Figure 4.8: Effect of TEM (1 mg/kg) on the frequency of abnormal sperm in rats at 5, 8 and 11 wks (n=5). * indicates a significant difference from the corresponding control group, $p<0.05$.

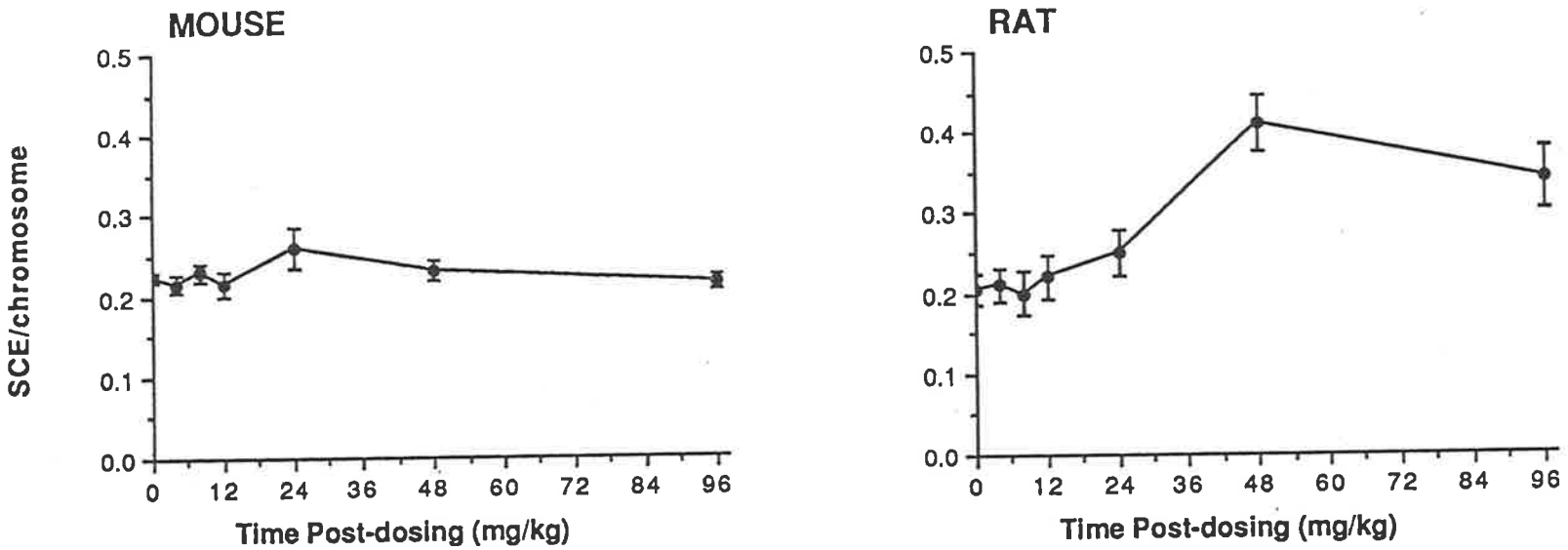


Figure 4.9: Frequency of SCEs in cultured splenocytes from mice and rats exposed to ST at 450 and 1500 mg/kg, respectively. n=2 for both species.

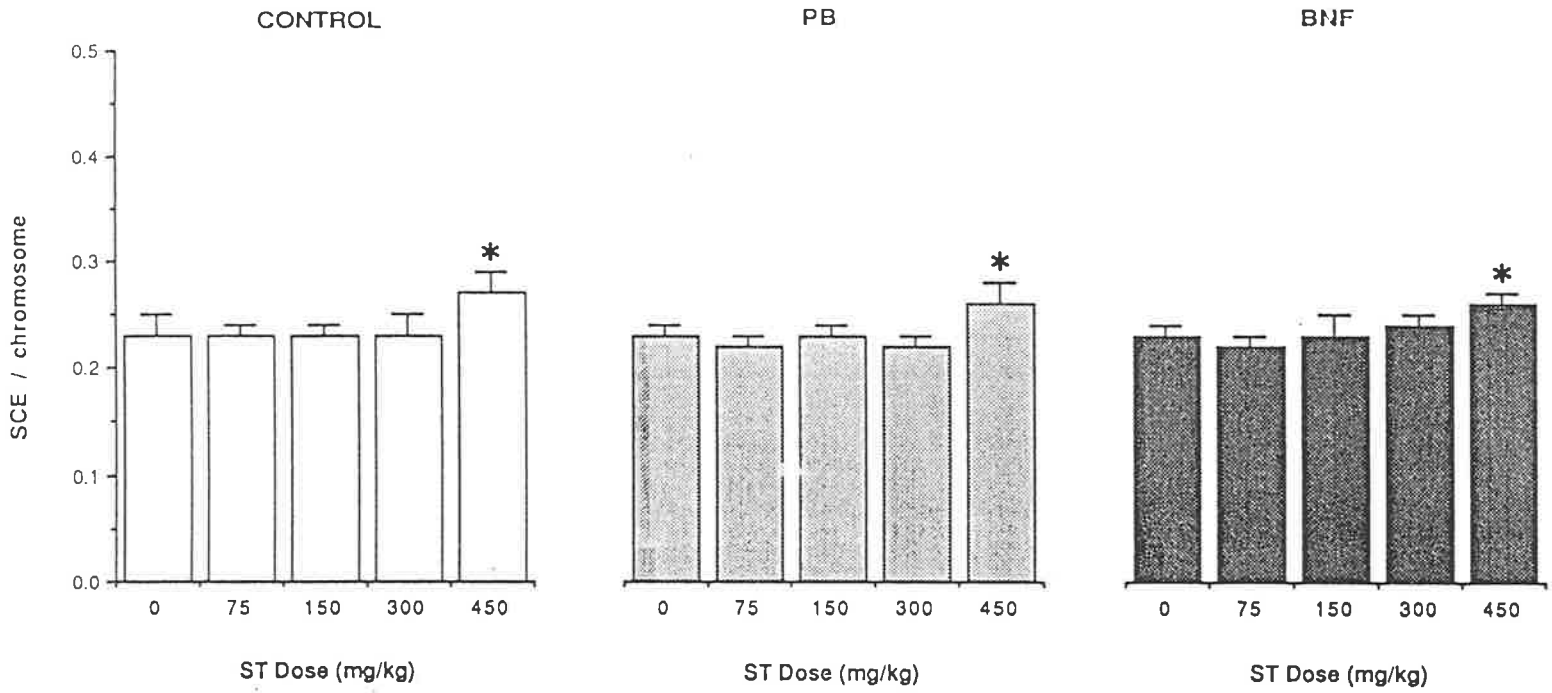


Figure 4.10: Effect of ST (0-450 mg/kg) on the SCE frequency in splenocytes from control, PB and BNF pretreated mice. n=4-5. * indicates a significant difference from the corresponding control group, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

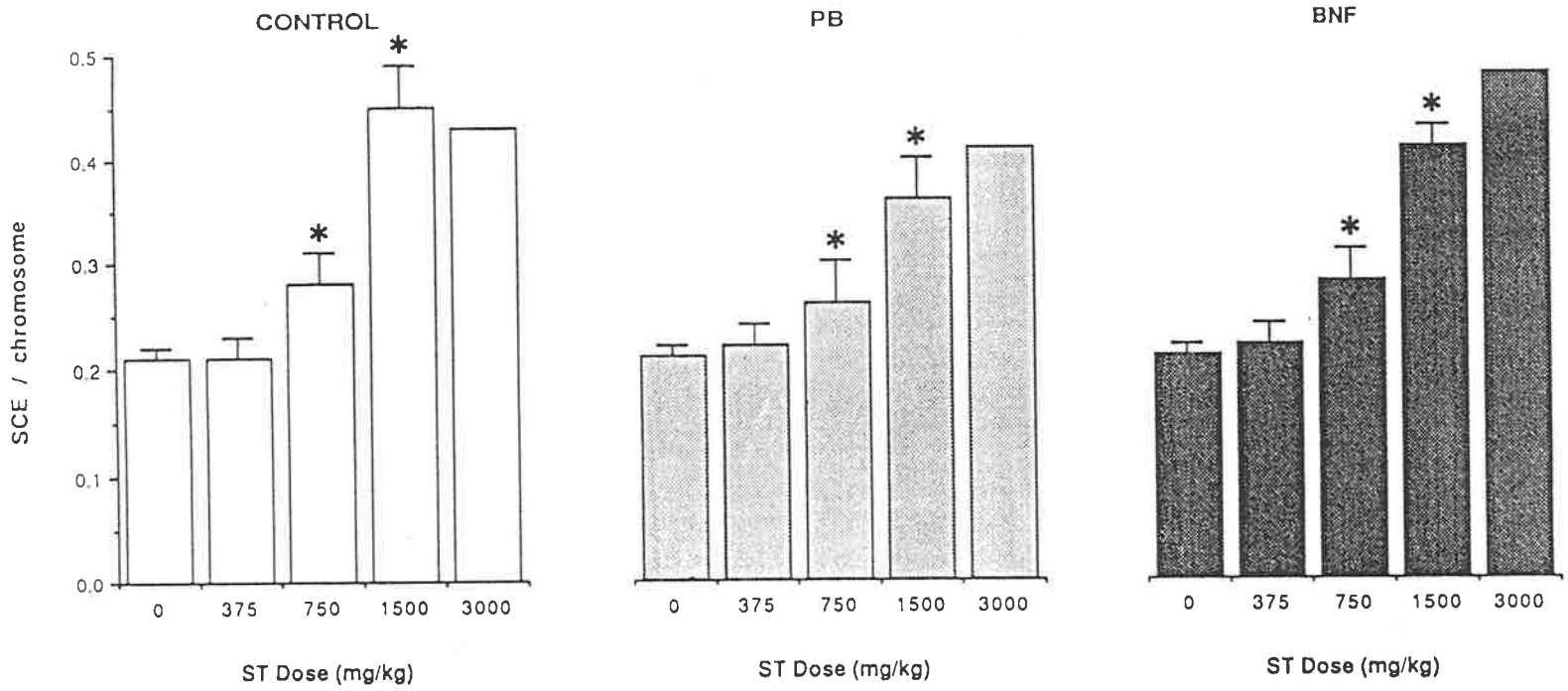


Figure 4.11: Effect of ST (0-3000 mg/kg) on the SCE frequency in splenocytes from control, PB and BNF pretreated rats. n=5, except at 3000 mg/kg in control, PB and BNF pretreated rats where n=1. * indicates a significant difference from the corresponding control group, $p<0.05$.

Asterisks indicate dose threshold for effects.
see p. 33-34 and Appendix C for details of statistical analysis.

ST Dose (mg/kg)	Replicative Index		
	Control	PB	BNF
Cont.	2.00±0.03	1.99±0.02	2.01±0.02
75	2.00±0.01	1.97±0.06	2.04±0.03
150	1.98±0.02	2.00±0.04	2.02±0.01
300	1.99±0.04	2.00±0.03	1.99±0.02
450	2.06±0.03	2.05±0.02	1.91±0.02

Table 4.5: Effect of ST on the replicative index (RI) of cultured splenocytes from control, PB and BNF pretreated mice, n=4-5.

ST Dose (mg/kg)	Replicative Index		
	Control	PB	BNF
Cont.	2.37±0.07	2.36±0.09	2.31±0.09
375	2.37±0.08	2.33±0.06	2.33±0.05
750	2.30±0.11	2.28±0.11	2.25±0.06
1500	2.32±0.03	2.34±0.11	2.20±0.12
3000	2.54	2.24	2.15

Table 4.6: Effect of ST on the replicative index (RI) of cultured splenocytes from control, PB and BNF pretreated rats. n=5, except at 3000 mg/kg in control, PB and BNF pretreated rats where n=1.

Assay/Species	No. of deaths/No. treated			
	ST Dose	Control	PB	BNF
MN Assay				
Mouse	450 mg/kg	2/23	1/21	—
	600 mg/kg	14/29	11/24	11/23
Rat	1500 mg/kg	2/22	1/13	—
	3000 mg/kg	14/33	11/24	9/23
SM Assay				
Mouse	200 mg/kg/d	—	1/14	—
	400 mg/kg/d	—	2/20	2/22
Rat	1000 mg/kg/d	—	1/15	—
	2000 mg/kg/d	14/15	14/15	9/15§
SCE Assay				
Mouse	450 mg/kg	—	—	1/5
Rat	3000 mg/kg	4/5	4/5	4/5

Table 4.7: ST-induced mortality in the 3 in vivo assays. For clarity, only doses at which mortality was observed are represented. With all other doses no mortality was observed. § indicates that 4 of the 9 deaths were due to injection artifact.

the positive control (TEM) produced a significant (χ^2=157.8, p<0.001) increase in abnormal sperm production in both species, which was remarkably consistent with the response to TEM observed in the previous chapter (Figures 3.7 and 3.8).

ST produced a small increase in SCE frequency in the preliminary experiments, which was maximal at 24 hrs (Figure 4.9). A more impressive response was observed in the rat reaching a maximum at 48 hrs (Figure 4.9). In the main experiments, ST induced a small but significant increase in the SCE frequency in the mouse (F=22.25, p<0.001), which was not significantly altered by PB (F=0.55) or BNF (F=0.73) pretreatment (Figure 4.10). Tukey's test indicated that only the top dose of ST was genotoxic to the mouse splenocytes. ST significantly (F=123.28, p<0.001) increased the SCE frequency in rat splenocytes (Figure 4.11), which was also not influenced by either enzyme-inducing pretreatments (F=1.95, PB; F=1.69, BNF). PB itself was able to significantly alter the level of SCEs in the cultured splenocytes in the rat (F=4.68, p<0.05). At the top dose of ST in rats, 4 out of 5 died in all pretreatment groups (Table 4.7).

ST was not cytotoxic to the splenocytes in the rat (F=0.45, Figure 4.5). PB (F=0.35) and BNF (F=0.45) had no significant effect on the cytotoxicity of ST in rat splenocytes. While PB did not alter the cytotoxicity of ST to mouse splenocytes (F=0.55), BNF was able to significantly influence the cytotoxic response to ST (F=4.69, p<0.01). Tukey's test was not able to establish which dose of ST was cytotoxic to the cultured splenocytes in mice.

4.4 Discussion

ST was weakly genotoxic in the MN assay in the mouse at dose levels approaching lethality. The weak response of ST in mice is consistent with the observations of Norppa (1981), who also found a weak positive response in mice at 250 and 1000 mg/kg. The apparently delayed appearance of the maximal response to ST to 48 hrs at the top doses, does not seem to be associated with any effect of ST on

haemopoiesis. The inhibition of bone marrow proliferation by the vehicle in the mouse, may be a more important factor influencing the timing of the maximal effect of ST in the mouse MN assay. Saturation of ST metabolism has been documented in rats exposed to much lower doses of ST (Withey and Collins, 1977; Ramsey and Young, 1978; Withey, 1978). This does not appear to be a factor in the delayed response, as PB and BNF, both known to induce ST and SO clearance (Garattini et al. 1981), did not alter the timing of the response to ST in mice.

The fact that ST was not found to be genotoxic in one analysis (Figure 4.2, χ^2=1.92), and then found to be genotoxic when the effects of PB and BNF were assessed statistically (Figure 4.4, χ^2=11.08, $p<0.05$), may be due to the very weak effect of ST in rats. That is, the response to ST was so small, an effect was only statistically verifiable when a larger number of treatment groups were analysed. This may have been the cause for the similar response observed with ST-induced cytotoxicity in the mouse MN assay.

The observed inhibition of haemopoiesis in mice at 30 hrs, was most likely due to the vehicle, as the PCE/RBC ratio decreased with increasing dose of ST (or decreasing dose of peanut oil). The dose-related decrease in the PCE/RBC ratio at 48 hrs may be due to a cytotoxic effect of ST. These observations were consistent regardless of the presence or absence of PB or BNF and were found to be significant only when the effects of PB and BNF were assessed (Appendix C). Appendix D describes an experiment, in which the genotoxicity and cytotoxicity of peanut, sunflower seed and olive oils were compared in the MN assay. While all 3 oils were not genotoxic, they each significantly reduced the PCE/RBC ratio. Peanut oil produced the weakest effect and its use as a vehicle for ST dosing was therefore maintained.

Exposure to ST in mice in the SM assay induced an increased production of abnormal sperm. The positive response to ST observed, is in contrast to work by Salomaa et al. (1985), who dosed mice with ST up to 700 mg/kg and found no significant increase in abnormal sperm production when sampling at 3 and 5 wks after dosing. The apparently larger response at 7 wks was not statistically verifiable, possibly due to the small sample sizes and the large variance within the treatment

groups. The weaker effect of ST in the rat compared to the mouse, is consistent with its weaker response in the MN assay. The ability of PB and BNF to alter the level of abnormal sperm in mice is consistent with the results of the previous chapter for the mouse SM assay. A similar effect was also observed in rats, but for BNF only.

In the SCE assay, both species were found to be susceptible to the genotoxic effects of ST. A weak, but significant response in the mouse was only detectable at 450 mg/kg. Higher doses were not administered, as the MN experiments revealed a 8 and 50% mortality rate at 450 and 600 mg/kg ST, respectively, in mice (Table 4.7). This result is not in agreement with Sharief et al. (1986), who failed to detect a significant increase in SCEs in the bone marrow of mice treated with a single i.p. dose of ST up to 1 g/kg. On the other hand, Conner et al. (1979) using inhalation as the route of exposure (565±15.8 ppm, 6 hr/day, 4 days), observed a significant increase in the frequency of SCEs in bone marrow cells. Although the threshold dose for the increase in SCE frequency in the rat was higher than the mouse, the responses in the rat were more pronounced.

The relative insensitivity of the rat compared to mice in the MN and SM assays, could be due to a greater ability of the rat to detoxify the genotoxic metabolite formed. This result is supported by the 3-fold higher ratio between epoxide hydrolase and mono-oxygenase activity in the rat liver compared to the mouse (Cantoni et al. 1978). The decrease in the PCE/RBC ratio observed in rats after ST exposure, suggests that a lack of accessibility of ST to bone marrow did not contribute to the insensitivity of that species in the MN assay.

ST itself can act as a CNS depressant (Valic, 1982), and this may have contributed to the mortality at higher doses. If exposure to BNF was able to increase the clearance of ST and its metabolites, you might expect effects on survival. This was observed in ST-treated rats in the SM assay. The lack of protection by BNF in the MN and SCE assays is difficult to interpret due to the different dosing regimens employed. The fact that BNF protected against the acute toxicity of ST in the SM assay, while having no effect on its genotoxicity, suggests that each effect may have been mediated by different mechanisms.

The fact that ST in rats was strongly genotoxic in the SCE assay but relatively weak in the SM and MN assays, indicates that no one assay is capable of assessing the full genotoxic potential of all chemicals. Therefore caution must be taken when interpreting genotoxicity data from results of a single assay. Activation and/or deactivation local to the target tissue can be an important factor in determining a tissue's susceptibility to metabolically activated genotoxic compounds (Kinoshita & Gelboin, 1972). Apart from the pharmacokinetic factors which can determine the sensitivity of the 3 in vivo assays, different DNA lesions could give rise to a restricted number of endpoints. For instance, agents which are known to produce DNA-damage (X-rays, bleomycin) can be very poor inducers of SCEs (Wolff, 1982).

Chapter 5

The Activity of Various Metabolic Enzymes in the Liver, Testes, Spleen and Bone Marrow of Rats and Mice, in the Presence and Absence of PB and BNF Pretreatment.

5.1 Introduction

Many genotoxic compounds require metabolic activation to exert their DNA-damaging effects. Where this activation is tissue specific, differential susceptibility to the DNA damaging properties of a chemical from one tissue to another, may exist. Enzyme induction can modify metabolic activity in a tissue- and species-specific manner (Burke & Orrenius, 1979; Ciaccio & De Vera, 1976; Guengerich & Mason, 1979), which may produce a shift in the primary site of action of a genotoxin.

The PB- and 3MC-inducible isozymes of cytochromes P450 are involved in the biotransformation of a large number of xenobiotics, hence PB and 3MC (or BNF) are often used as inducers of metabolic enzymes. PB induces cytochrome P450 activity by stimulating the rate of transcription of the genes coding for the PB-inducible isozymes. This causes an increased accumulation of the specific P450 haemoproteins (Omiecinski et al. 1985). The mechanism by which PB increases the rate of gene transcription is unknown. In contrast, the steps involved with 3MC-like induction is well characterised. The inducer binds to an intracellular protein, the inducer/receptor complex then interacts with a nuclear regulatory element to stimulate transcription of the specific P450 isozymes (Whitlock, 1986) This mechanism is thought to be distinct from that of PB, as it affects the activity of a restricted group of enzymes, often termed cytochrome P448.

Phase I enzymes such as ethoxycoumarin O-deethylase, antipyrine hydroxylase, ethylmorphine N-demethylase and benzo[a]pyrene hydroxylase have been used to assess the induction of P450 isozymes by PB or BNF (Greenlee & Poland, 1978; Bakke et al. 1974; Guengerich, 1988). The ability of PB and BNF to modify the activity of Phase II enzymes such as glutathione S-transferase (GST) and epoxide hydrolase (EH), usually associated with deactivation of toxic compounds, is variable. PB is a potent inducer of both enzymes. While BNF and BNF-like compounds are comparable inducers of GST, they have been shown to have little effect on EH (Seidegard & De Pierre, 1983; Kaplowitz et al. 1975; Glatt & Oesch, 1987; Salmona et al. 1976; Hammock & Ota, 1983).

The activity of certain metabolic pathways, like GST, are limited by the availability of cofactors (GSH) rather than enzyme kinetics. Although GSH can be found in large quantities in many tissues (Griffith & Meister, 1979), its availability for conjugation is readily reduced due to the low cellular levels of cysteine required for its synthesis. Consequently, many toxic metabolites, which undergo GSH conjugation and are able to deplete cellular GSH, may not produce toxicity until the GSH levels are reduced to a point where the metabolite can no longer be detoxified.

In order to estimate the activity of toxicologically important metabolic pathways involved in CP and ST biotransformation, the development of a large number of assays would have been required. Furthermore, to conduct all the necessary incubations a very large number of animals would have been needed. Therefore, the activities of various Phase I and Phase II enzymes were used as a non-specific measure of the metabolic capacity of the target tissues relative to the liver. As these estimates were an indirect assessment of the activity of enzymes required for the activation and deactivation of ST and CP, they were only used as a guide to determine the inducibility of the metabolism of the genotoxins in the liver and target tissues. Ethoxycoumarin O-deethylase (7-ECOD) and PPO-OHase activities were used as probes for P450 activity in the target tissues. EH and GST activities, using styrene oxide and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, respectively, and tissue GSH content were also estimated. GST and EH are important enzymes for detoxifying styrene, while GST is involved in CP metabolism.

The aim of the experiments reported in this chapter was to determine the metabolic capacity of the target tissues in rats and mice, relative to the liver and to assess the effects of pretreatment with PB and BNF on xenobiotic metabolism in the tissues.

5.2 Materials & Methods

Animals were given PB (1 mg/ml) in their drinking water and 3 animals per species were sacrificed after 7 and 12 days. Control animals for PB pretreatment were untreated. In the case of BNF, animals were given 3 i.p. injections at 100 mg/kg at 48 hour intervals. 3 rats and mice were sacrificed at 48, 72 and 120 hrs from the first dose. Control animals, for BNF pretreatment, were given 3 i.p. doses of peanut oil at 48 hour intervals and tissues were sampled 24 hrs after the last dose. The liver, testes, spleen and femoral bone marrow were removed at the time of sacrifice. The tissues were then prepared and the enzyme activities and GSH content were estimated by the method described in section 2.2. The data was analysed by a 1-Way ANOVA, followed by Tukey's Multiple-Comparison test as described in section 2.3.

5.3 Results

Activity of 7-ECOD and PPO-OHase was detectable in the liver (Tables 5.1 and 5.2), but not detectable in the testes, spleen or bone marrow with the techniques employed. GST and EH were measurable in all tissues assessed, with the highest activity in liver, followed by testes, marrow and spleen in descending order (Tables 5.3 and 5.4). The mouse had a higher basal hepatic activity than the rat for all four enzymes. The testicular activity of EH was also higher in mice than rats, while the GST activity was the same in both species. The splenic activity of EH and GST was higher in the mouse, while there was no difference between the species in femoral bone marrow activity.

PB pretreatment significantly induced hepatic PPO-OHase activity ($p<0.05$) in both rats and mice (Table 5.1). The magnitude of the induction was greater in the rat. Although BNF also induced the enzyme in the rat, its effect in the mouse was weak and significant at only one time point. PB significantly ($p<0.05$) induced 7-ECOD comparably in both species (Table 5.2). BNF induced the enzyme in the rat only, to a similar extent as the PB pretreatment. The induced levels of activity

Species	Hepatic PPO-OHase Activity (nmol product/min/mg protein)						
	Control	Peanut Oil	PB_1	PB_2	BNF_1	BNF_2	BNF_3
Mouse	0.48±0.08	0.50±0.06	0.80±0.02‡	0.65±0.03‡	0.65±0.15	0.80±0.14†	0.66±0.18
Rat	0.21±0.04	0.24±0.02	0.49±0.13‡	0.38±0.08‡	0.96±0.03†	0.84±0.18†	0.95±0.03†

Table 5.1: Activity of PPO-OHase in liver of mice and rats with and without pretreatment with PB and BNF, n=3. † or ‡ indicates a significant difference from the respective control group, $p<0.05$.

Control: Untreated.

Peanut Oil: 3 i.p. doses (4 ml/kg), sacrifice 24 hrs after the last dose.

PB1: PB (1 mg/ml) in drinking water for 7 days.

PB2: PB (1 mg/ml) in drinking water for 12 days.

BNF1: Single i.p. dose (100 mg/kg), sacrifice after 48 hrs.

BNF2: 2 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

BNF3: 3 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

Species	Hepatic 7-ECOD Activity (nmol product/min/mg protein)						
	Control	Peanut Oil	PB_1	PB_2	BNF_1	BNF_2	BNF_3
Mouse	0.36±0.05	0.39±0.02	2.67±0.56‡	2.82±1.56‡	0.54±0.13	0.60±0.10	0.45±0.11
Rat	0.18±0.09	0.24±0.05	1.16±0.37‡	0.89±0.22‡	0.92±0.03†	0.83±0.15†	0.89±0.01†

Table 5.2: Activity 7-ethoxycoumarin O-deethylase in liver of mice and rats with and without PB and BNF pretreatment, n=3. † or ‡ indicates a significant difference from the respective control group, $p<0.05$.

Control: Untreated.

Peanut Oil: 3 i.p. doses (4 ml/kg), sacrifice 24 hrs after the last dose.

PB1: PB (1 mg/ml) in drinking water for 7 days.

PB2: PB (1 mg/ml) in drinking water for 12 days.

BNF1: Single i.p. dose (100 mg/kg), sacrifice after 48 hrs.

BNF2: 2 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

BNF3: 3 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

GST Activity (nmol product/min/mg protein)							
	Control	Peanut Oil	PB_1	PB_2	BNF_1	BNF_2	BNF_3
Mouse							
Liver	1069±206	1059±129	3353±1519‡	3761±1455‡	1759±256†	1745±223†	1841±176†
Testes	396±46	383±54	503±27	491±15	289±26	588±119†	528±26†
Spleen	67±6	64±7	62±11	61±29	48±4	96±2†	69±4
Marrow	62±32	62±9	61±6	89±4	58±6	130±13†	119±18†
Rat							
Liver	796±94	844±55	1806±159‡	1625±232‡	973±89	963±50	978±158
Testes	420±21	427±23	401±34	476±39	314±20	551±34†	449±45
Spleen	38±1	37±5	33±5	36±7	33±5	43±5	37±3
Marrow	52±32	40±2	45±15	36±5	32±4	38±2	38±2

Table 5.3: Activity of glutathione S-transferase in liver, testes, spleen and bone marrow of mice and rats, with and without PB and BNF pretreatment, n=3. † or ‡ indicates a significant difference from the respective control group, $p<0.05$.

Control: Untreated.

Peanut Oil: 3 i.p. doses (4 ml/kg), sacrifice 24 hrs after the last dose.

PB1: PB (1 mg/ml) in drinking water for 7 days.

PB2: PB (1 mg/ml) in drinking water for 12 days.

BNF1: Single i.p. dose (100 mg/kg), sacrifice after 48 hrs.

BNF2: 2 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

BNF3: 3 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

EH Activity (nmol product/min/mg protein)							
	Control	Peanut Oil	PB_1	PB_2	BNF_1	BNF_2	BNF_3
Mouse							
Liver	2.18±0.30	2.33±0.22	3.19±0.21‡	2.87±0.53‡	3.32±0.52†	3.27±0.66†	2.91±0.18†
Testes	0.66±0.06	0.66±0.04	0.68±0.08	0.65±0.02	1.07±0.04†	1.18±0.25†	1.13±0.19†
Spleen	0.25±0.03	0.27±0.02	0.26±0.05	0.25±0.03	0.28±0.02	0.27±0.01	0.30±0.03
Marrow	0.45±0.05	0.44±0.05	0.55±0.05‡	0.55±0.01‡	0.53±0.06	0.56±0.14	0.70±0.04†
Rat							
Liver	1.66±0.26	1.73±0.29	8.06±0.59‡	8.61±1.39‡	2.87±0.44†	2.93±0.63†	3.11±0.22†
Testes	0.50±0.02	0.53±0.02	0.57±0.03‡	0.57±0.04‡	0.70±0.04†	0.69±0.09†	0.64±0.06†
Spleen	0.19±0.02	0.19±0.01	0.23±0.03	0.22±0.02	0.25±0.03†	0.22±0.02	0.24±0.03
Marrow	0.44±0.05	0.47±0.03	0.50±0.05	0.52±0.05	0.42±0.04	0.61±0.17	0.57±0.08

Table 5.4: Activity of epoxide hydrolase in liver, testes, spleen and bone marrow of mice and rats with and without PB and BNF pretreatment, n=3. † or ‡ indicates a significant difference from the respective control group, $p<0.05$.

Control: Untreated.

Peanut Oil: 3 i.p. doses (4 ml/kg), sacrifice 24 hrs after the last dose.

PB1: PB (1 mg/ml) in drinking water for 7 days.

PB2: PB (1 mg/ml) in drinking water for 12 days.

BNF1: Single i.p. dose (100 mg/kg), sacrifice after 48 hrs.

BNF2: 2 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

BNF3: 3 i.p. doses every 48 hrs, sacrifice 24 hrs after the last dose.

Species	GSH Concentration (nmol/mg tissue)			
	Liver	Testes	Spleen	Marrow
Mouse	3.63±0.70	2.94±0.08	1.27±0.09	1.94±0.12
Rat	3.51±0.10	2.10±0.11	0.87±0.10	1.54±0.07

Table 5.5: Glutathione content in liver, testes, spleen and bone marrow of rats and mice, n=3.

for both enzymes was maintained across the 5-day sampling period for both PB and BNF. The induction of PPO-OHase in mice and rats did drop with PB from day 7 to 12, but was still significantly higher than control mice.

GST was significantly induced by PB and BNF in mouse liver ($p<0.05$), while in rats only PB significantly raised hepatic GST activity (Table 5.3). GST activity in all three target tissues of both species was unaffected by PB. The enzyme was induced by BNF in mice in all 3 target tissues, after at least two doses of BNF. In the rat only the testicular activity was significantly affected by BNF, at the second time point only.

Hepatic EH activity was significantly induced ($p<0.05$) by PB and BNF pre-treatment during the 5-day sampling period in both species (Table 5.4). The effect of PB was greater in the rat than in the mouse, while BNF had a similar effect in both species. The enzyme was significantly induced by PB and BNF in rat testes but only by BNF in mouse testes. The effect of BNF on testicular EH activity was more pronounced in the mouse. There was no consistent induction of EH in the spleen of rats or mice, whereas the bone marrow activity was induced by BNF (after two doses) and PB in mice. Although the EH activity in rat bone marrow was raised at the last two time points with BNF and at both time points with PB, they were not found to be significantly different from their respective controls.

In both species the tissue GSH content of the 4 tissues in descending order was liver, testes, marrow and spleen (Table 5.5). The hepatic GSH content was similar for rats and mice, while in the 3 target tissues it was consistently higher in the mouse than the rat.

5.4 Discussion

Although there was very little effect on CP or ST genotoxicity by PB and BNF in the 3 in vivo assays, an assessment of the metabolic capacity of target tissues and liver, with and without enzyme induction, was undertaken nonetheless. Firstly, this

allowed us to establish that the enzyme-inducing pretreatment regimens were, in fact, inducing Phase I and Phase II enzyme activity and that this increased level of activity was maintained for the 5-day dosing period in the SM assay. Secondly, we were able to infer whether the changes, or lack of changes, may have been due to altered metabolism (activation/deactivation) or whether they were due to other as yet unknown mechanisms.

PB and BNF pretreatment regimens were able to produce a sustained induction level of hepatic activity, of all four enzymes in both species, over the 5 days. Using the techniques employed, the activity of the oxidative enzymes was not detectable in the three tissues, regarded as target tissues for the genotoxic responses assessed in Chapters 3 and 4. Weight for weight of microsomes, the 3 target tissues can have as little as 1% of hepatic MMFO activity (Lake et al. 1973; Gollmer et al. 1984; Mattison & Thorgiersson, 1978). Weight for weight of tissue protein, the 7-ECOD and PPO-OHase assays would be able to detect as little as 0.5-1.0% hepatic activity. The sensitivity of the assays used could be increased by using microsomal preparations, rather than S10 supernatants, but the microsomal content of the 3 tissues is very small such that a large number of animals would need to be used in order to conduct all the estimations of enzyme activities. On the other hand, EH and GST were measurable in the 3 target tissues, suggesting that they at least have a capacity to detoxify reactive metabolites. More importantly, they showed evidence of an effect due to inducing pretreatments.

The mouse generally had a higher activity of metabolic enzymes in the tissues of both uninduced and induced (except EH in rats with BNF), which is consistent with other studies comparing activity in various species (Glatt & Oesch, 1987; Lorenz et al. 1984). The induction of enzymes by PB and BNF showed tissue- and species-specificity. Firstly, BNF induced 7-ECOD in the rat liver while having no significant effect on that enzyme in mouse liver. Secondly, PB and BNF increased EH activity in rat liver and testes but did not alter its activity in bone marrow or spleen. These results are similar to other investigators who have also shown tissue differences in inducibility of enzymes (Burke & Orrenius, 1979; Ciaccio & De Vera, 1976; Guengerich & Mason, 1979).

The lack of any marked induction of PPO-OHase by BNF in mice, while producing a large increase in PPO-OHase activity in rats, suggests that this particular enzyme may not be an appropriate indicator of P-448 activity in this strain of mice. The fact that BNF was also a weak inducer of 7-ECOD in the mouse liver indicates that this lack of induction of MMFO by BNF in mouse liver may be due to the dose of BNF being too small. On the other hand, Boobis et al. (1977) were able to demonstrate a 3-fold induction of P-448 activity in mouse liver with a single dose of 100 mg/kg of BNF. This discrepancy may be due to the different strain of mouse used in this study.

The inability to measure PPO-OHase and 7-ECOD in the testes, spleen and bone marrow made any attempt to correlate PB- or BNF-induced changes in activating and deactivating pathways with changes in genotoxicity and cytotoxicity of ST and CP impossible. The potentiation of the CP-mediated increase in MNPCEs with PB is consistent with its induction of oxidative metabolism observed in the liver of mice (Tables 5.1 and 5.2). The lack of effect of PB in the rat in the MN assay, or its lack of effect in both species in the other two assays, is more difficult to reconcile with from these results. The activity and inducibility of possible detoxifying pathways, such as aldehyde dehydrogenase, which may reduce the formation of PM and acrolein from aldophosphamide, could explain the inability of PB to increase genotoxicity in the rat, while still increasing MMFO activity. Despite the very large changes in the activity of enzymes involved in ST and SO metabolism, ST genotoxicity was not affected by enzyme induction.

GSH content in the target tissues was highest in the testes, and therefore, may have a greater capacity to maintain detoxification of reactive metabolites by GSH conjugation than the other two target tissues. Moreover, Mukhtar et al. (1978) showed that spermatogonial cells had a high capacity to detoxify xenobiotics but a very low capacity to activate them. This may explain the fact that the doses required to elicit a response in the SM assay, for both genotoxins, were much higher than in the other in vivo assays.

Since the substrates used to assess enzyme activities were surrogates to determine the metabolism of CP and ST with and without induction, this indirect approach may account for the lack of correlation between the marked induction of enzymes and the relative lack of modification of genotoxicity. In order to properly investigate the influence of inducer-modified metabolism on in vivo genotoxicity, a more direct assessment of the changes in the metabolism of the substrates to potentially genotoxic and non-genotoxic metabolites, in both the liver and target tissues, is required. A useful approach would be to assess the pharmacokinetics of the parent drug and the relevant metabolites in the whole animal. This would have been a project in itself.

Chapter 6

General Conclusions

With the current awareness of the limitations of short-term in vitro assays for carcinogenicity (Ashby & Purchase, 1988), there has been a renewed interest in assessing the capabilities of in vivo short-term tests. There are two major reasons for this diverted interest to in vivo genotoxicity assays. Firstly, although early studies showed a high correlation between mutagenicity in the Ames test and bioassay carcinogenicity (McCann et al. 1975), a broader assessment has shown this correlation to be as low as 50% (Ashby & Purchase, 1988). Secondly, the carcinogenicity of a compound may ultimately depend on dispositional factors such as the route of exposure, distribution, metabolism and excretion. All these factors are more applicable to in vivo rather than in vitro systems.

The renewed interest in in vivo assays has encompassed both their use as tests for carcinogenicity and as methods for screening workers occupationally exposed to various potentially genotoxic chemicals. Occupational exposure often involves a number of chemicals at the same time (Forni & Bertazzi, 1987). Therefore there exists the potential for interactions between chemicals. One interaction of interest is that of chemical-induced altered metabolism affecting the potential toxicity of other chemicals. One of the advantages of the in vivo assays commonly employed is that the end-points being assessed can be derived from a number of different types of DNA damage. This allows an assessment of exposure to mixtures of chemicals.

A problem associated with occupational monitoring is the importance placed on a test result from an individual worker. Commonly used in vivo assays such as the SCE and MN assays, have a basal level of detectable genotoxicity. This basal level may be influenced by a number of factors, which may not involve occupational exposure. Therefore, in a cross-sectional study it is difficult to establish a causal relationship between occupational exposure and an apparently abnormal response in an in vivo genotoxicity test detected in an individual, without knowledge of the pre-exposure baseline. In a cross-sectional study, providing the sample size is sufficiently large, monitoring of a population makes it possible to establish a causal relationship between exposure and increased genotoxicity, for the cohort only. Hence, a longitudinal study, which monitors an individual over a period of time, would more readily detect a cause/effect relationship in that individual acting as

their own control.

This study was an attempt to compare and contrast the sensitivity of three commonly used in vivo genotoxicity assays and their ability to detect exposure to a relatively potent genotoxin (CP) and a relatively weak genotoxin (ST). Furthermore, the project aimed to assess response differences between rats and mice, the two most commonly used species for these tests, and to determine whether the sensitivity of the tests could be influenced by microsomal enzyme inducers, capable of altering the metabolic activation of the genotoxins.

Of the 3 assays, the SCE assay was found to be the most responsive. The SCE assay detected the potent genotoxin (CP) in both species. It detected the weak genotoxin (ST) in both species. It was less affected by chemically-induced cytotoxicity in target cells for the genotoxic responses. Its sensitivity was unaffected by enzyme inducers.

In contrast the SM was the least responsive. Although it was able to detect both the potent and the weak genotoxin, the doses required to produce a response were at least 2x (ST) to 40x (CP) compared to the SCE assay. Consequently, lethal or near lethal doses were required to produce a response for both genotoxins. The responses were also unaffected by enzyme-inducing pretreatments, though PB and BNF did have an effect of their own in both species.

The MN assay was intermediate in sensitivity. Like the other assays it was able to detect both potent and weak genotoxins. The doses required to produce a response were 2–4x those for the SCE assay. The target tissue (bone marrow) was very sensitive to the cytotoxic effects of the genotoxins. Except for the PB potentiation of CP-induced MNPCEs in mice, neither enzyme inducer altered the responses in both species.

The rat was found to be more sensitive than the mouse to the genotoxicity of CP in all three assays. This is in agreement with the results obtained by Madle et al. (1986a), who observed similar sensitivities with respect to the MN and SCE assays

in rats and mice. Moreover the disproportionately low response to 20 mg/kg of CP in rats in the MN assay was also observed by Madle et al. (1986a). This difference in species sensitivity illustrates the difficulty with choice of species to best assess a chemical's genotoxic potential. The response of CP-treated mice in the SM assay was consistent with Wyrobek and Bruce, (1975), who also required lethal doses of 50 mg/kg/day of CP to produce a significant response. Like the MN and SCE assays, the rat was more sensitive than the mouse to CP-induced abnormal sperm.

The species sensitivity was reversed in the case of ST in each assay. The results obtained for the MN assay agreed with those obtained by Norppa (1981) in mice, but not with Pentilla et al. (1980) in Chinese hamsters (Section 4.4). The lack of consistency between the results in the different studies may be due to the use of different species. As discussed in section 4.4, there was a disagreement between the positive response of ST in the SM assay in my study and the negative response reported by Salomaa et al. (1985). This may have been due to them not sampling sperm at 7 wks after dosing, given that the more pronounced effect of ST in my study was at 7 wks. The difference in the results obtained in the SCE assay in my study, compared with those of Sharief et al. (1986) and Conner et al. (1979), may have been due to the faact that different routes of exposure and different target tissues were involved.

The relative insensitivity of the SM assay suggests that it may not be an appropriate system to estimate risk from occupational exposure. A contrary position is taken by Wyrobek et al. (1982) and Wyrobek et al. (1983b), who propose that since the method of sampling and assessment is simple and non-invasive, it may be a useful technique for monitoring occupational exposure. Humans have been monitored for occupational exposure by this method. Some positive results have been found. For example, Lancranjan et al. (1975) found elevated levels of abnormal sperm in men exposed to lead and also showed a strong negative correlation between the level of abnormal sperm and fertility, and da Cunha et al. (1982) found elevated levels of abnormal sperm in cancer patients treated with AMSA (4′-(9-acridinylamino)methanesulfon-m-anisidide).

One of the major problems attending the use of this end-point, is that little is known of the exact mechanism by which genotoxic chemicals induce abnormal sperm production (Wyrobek et al. 1983a). Even less is understood of the differential sensitivity of spermatogenic stages to genotoxic chemicals. Experiments in the thesis using CP found it to have the greatest effect at the primary spermatocyte stage in both species (Figures 3.5 and 3.6). ST's response in both species did not vary significantly across the 3 time points (Figures 4.5 and 4.6), indicating that CP and ST may produce different DNA lesions. Until it is fully understood why one stage of spermatogenesis should be more sensitive than another, multiple sperm sampling as opposed to single-point sampling, would appear to be mandatory in order to maximise detection of a response. This approach would be difficult to adopt in humans as they are exposed more intermittently and usually over extended periods, rather than the controlled one-off exposures used in rodents.

One of the limitations of all three assays is their reliance on the ability of the affected cell-type to divide in order for any lesion produced to manifest itself in the form of a detectable end-point (MN, SCE or abnormal sperm). The situation may arise where a test compound is so cytotoxic that the affected cells die before the genotoxic end-point can be assessed. Marked bone marrow cytotoxicity is known to interfere with the MN assay (Salamone et al. 1980), hence it has been recommended that single- or double-dosing regimens be employed as standard practice in the MN assay (Salamone & Heddle, 1983). The importance of multiple-sampling protocols to allow for any delayed onset of response due to cytotoxicity was illustrated with CP in the rat. In this case the maximal response was observed at 48 hrs for 20 mg/kg of CP rather than 30 hrs as with the lower doses.

The greater sensitivity of the SCE assay may be due to the spleen-derived lymphocytes being a slowly dividing cell population (Krishna et al. 1988). Bone marrow cells are continually dividing and therefore, as target cells may only be exposed to a genotoxic metabolite for a limited length of time, particularly for slowly activated compounds. In contrast, splenocytes (and peripheral blood lymphocytes) can be exposed to genotoxic metabolites for greater lengths of time, which may lead to accumulated damage. This may explain the marked induction of SCEs by ST in

rats with very little induction of MNPCEs at comparable doses.

This can not explain the greater sensitivity of the SCE assay than the MN assay for CP in both species, for two reasons. Firstly, the timing of the maximal effect of CP in the SCE assay was less than the life-span of a dividing erythroblast in bone marrow ($\approx$10 hrs). Secondly, the very short half-life of CP in rodents, $<$ 40 min. (Torkelson et al. 1974). Other factors such as the disposition of the parent compound and its metabolites, the DNA repair characteristics of different tissues and the end-points being assessed may play a role in determining assay sensitivity.

The methods employed in Chapter 5 to establish the metabolic capacity of the liver, testis, spleen and bone marrow, were not sensitive enough to detect any MMFO activity in the latter three tissues. This may have been due to the use of an S9 fraction rather than microsomal preparations to determine enzyme activity. Nevertheless, GST and EH activities were detected in the S9 fractions from all tissues and these activities were inducible by PB or BNF. The mouse had a higher basal level than the rat of all four enzyme activities and this finding is consistent with other studies (Glatt & Oesch, 1987). Apart from PPO-OHase and 7-ECOD in mouse liver, both inducing pretreatments were able to produce a sustained induction of all four enzymes in the liver of both species, over the 5 days.

As a broad-spectrum inducer, PB was able to increase the hepatic activity of each enzyme in both species. Of the other tissues, PB only induced EH activity in rat testes and GST activity in mouse spleen. BNF induced the activity of PPO-OHase, 7-ECOD and EH in rat liver and GST and EH in mouse liver. BNF also induced EH activity in rat and mouse testes and GST in mouse testes and spleen.

The inability of BNF to induce PPO-OHase in the mouse liver is difficult to interpret, since the enzyme has been shown to be a good indicator of P-448 activity (Philippides et al. 1983). Moreover, the same dose of BNF (100 mg/kg) was able to induce benzo[a]pyrene hydroxylase, a putative indicator of P-448 enzyme activity, in mouse and rat liver by 3-fold (Boobis et al. 1977). A difference between mouse strains in their sensitivity to BNF pretreatment may account for this discrepancy. Since

PB was only able to produce a small induction of the same enzyme, an alternative explanation may be that the basal P-448 activity was already high as a result of exposure to some obscure environmental factor.

An effect of either enzyme inducer on the genotoxicity of CP or ST was only observed with PB, which potentiated CP-induced increased MNPCE frequency in mice. The subsequent decrease in the threshold dose of CP in the mouse MN assay, at 30 and 48 hrs, made that tissue a more important site of CP-induced genotoxicity. Another effect of PB induction was observed in the rat MN assay. PB caused the maximal response of CP to be detected at 30 rather than 48 hrs. This was thought to be due to the PB-mediated decrease in the cytotoxicity of CP to bone marrow. This effect further supports the need for multiple sampling in the MN assay as very different conclusions would have been drawn depending on the time of sampling. The only other consistent effect of PB and BNF was their ability to alter the level of abnormal sperm in both species.

It is clear from this study that the influences of altered metabolism on in vivo genotoxicity are complex and that the effects of PB and BNF may not be restricted to activating metabolic enzyme activity. Since surrogate substrates were used to assess possible changes in the biotransformation of CP and ST, a more direct determination of changes in their metabolism and clearance in the whole animal may be needed to fully understand the impact of altered metabolism on in vivo genotoxicity.

In a study, which also aimed to assess the effects of enzyme induction on genotoxin activation, Schreck and Latt (1980) reported a lack of correlation between PB and 3MC-induced changes in benzo[a]pyrene genotoxicity in the liver and bone marrow and its activation in the same tissues, even though they used a direct method to estimate the activation of the genotoxin. They concluded that detoxifying pathways, such as epoxide hydrolase, may be as important as activating pathways in determining the ultimate genotoxicity of a compound.

An assay for the assessment of risk to occupational exposure, in both humans subjects and animal models, should be one which is able to detect an effect on

DNA at an exposure level below that associated with systemic toxicity or other obvious adverse effects. The MN and SM assays employed in this study were not sensitive enough to do this in rodents, even in the presence of enzyme inducers, and presumably will be similarly insensitive in man. The SCE assay was able to detect a genotoxic response at doses an order of magnitude lower than those causing cytotoxicity, but only for the potent genotoxin. Since these assays are so insensitive, they may only be capable of detecting gross changes in genotoxic activity.

The use of lymphocytes as a target tissue has additional advantages. Firstly lymphocytes are easily isolated from blood in humans and rodents, therefore, a more direct comparison of the effects on the same target tissue can be made between rodents and humans. Secondly, as they seldom divide in vivo, they may be used to assess the long-term (or accumulated) effects of occupational exposure.

Compounds such as CP and ST can produce a range of different types of DNA adducts (Savela et al. 1986; Hemminki, 1987), which in turn, may have different implications for the integrity of the DNA. It has been shown that all adducts are not capable of producing SCEs as a single detectable end-point (Kaina et al. 1983; Day et al. 1987). Hence, it is conceivable that the three in vivo assays adopted in this study may have underestimated the effects of ST and CP on DNA. This may be a factor in their apparent insensitivity. A more direct approach, that of using highly sensitive immunochemical and other techniques to quantitate the levels of DNA adducts after exposure to specific chemicals, is gaining credence as a more suitable method of monitoring occupational exposure to genotoxins.

A number of techniques have been devised to detect DNA adducts, including HPLC, RIA, SFS (synchronous fluorescence spectrophotometry) and ^{32}P-postlabelling (Farmer et al. 1987). The major advantages of these systems is (1) their sensitivity, therefore, giving a more accurate estimate of the 'target dose' (i.e. the dose actually reaching the DNA), and (2) the potential, through mass spectrometry, to determine the structure of the adduct. The first three methods can detect as little as 1 adduct per 10^7 bases (Hsu et al. 1981; Rahn et al. 1982; Vahakangas et al. 1985). The ^{32}P-postlabelling method is the most sensitive (Randerath et al. 1985), able

to detect 1 adduct per 10^8–10^{10} bases, which is almost 1 adduct per cell ($1.2x10^{10}$ bases). Very high sensitivity is essential if the method is to be useful for detecting low levels of chemical exposure.

One of the difficulties associated with the HPLC, RIA and SFS methods is that they require a prior knowledge of the structure of the adducts formed. This information may not always be available. Though techniques, using polyclonal antibodies, have been developed able to detect a group of structurally related compounds. ^{32}P-postlabelling does not suffer from the same problem, as it only needs to quantitate the intensity of spots on an autoradiograph from ^{32}P, after ^{32}P-labelled bases have been separated chromatographically.

The use of these techniques to monitor occupationally exposed workers, is under active exploration by other workers. For example, they have been applied to detect DNA adducts resulting from exposure to such compounds as *cis*-diamminedichloroplatinum (Poirier et al. 1985), benzo[a]pyrene (Santella et al. 1985; Harris et al. 1985) and mitomycin C (Kato et al. 1988) and from cigarette smoking (Everson et al. 1986). The detection limits for adduct formation can be one to three orders of magnitude lower than is required to monitor exposures corresponding to those used in the low dose range for animal carcinogenicity studies. This poses a critical question: What significance can be attached to the finding of such adducts?

A review by Wogan and Gorelick, (1985) stated that the correlation between the level of DNA adducts produced by a compound and its carcinogenicity has not been shown to be consistent for all compounds. Most research has involved the assessment of total adduct formation, while an investigation of the correlation between specific adducts and carcinogenesis may provide more answers. Furthermore the persistence of adducts may also play an important role in determining a compound's carcinogenic potential. This was illustrated by Daniel and Joyce, (1984), who showed that although higher levels of DMBA adducts were formed in the rat liver (non-target organ) compared to the mammary gland (target organ), 70% of DMBA adducts were removed from liver DNA but essentially all DMBA adducts persisted in the mammary gland. Until the significance of the formation and persistence of specific

DNA adducts to carcinogenicity is determined, the usefulness of quantitating DNA adduct formation as a test for carcinogenic potential will be limited.

Monitoring of occupational exposure has not been restricted to effects on the genome. There exist equally sensitive GC-MS and RIA methods which can detect alkylation of protein at particular amino acids. Haemoglobin (Hb) has been the protein of choice, as it can be obtained very easily (Farmer et al. 1987; Neumann, 1984). The relevance of alkylation of protein to human risk is not known, although a correlation between protein and DNA adduct formation has been demonstrated for *trans*-4-dimethylaminostilbene (Neumann, 1980). Whether this correlation exist for some or all chemicals is not known.

This technique has successfully detected Hb adducts in animals exposed to as little as 35 μg/kg MMS (Murthy et al. 1984), 4-aminobiphenyl through exposure to cigarette smoke (Green et al. 1984), gasoline and diesel exhausts (Tornqvist et al. 1988) and humans exposed to ethylene oxide and propylene oxide (Farmer et al. 1986; Osterman-Golkar et al. 1984). An advantage of assessing Hb adducts over DNA adducts, which can be repaired, is that Hb adducts are longer-lived, hence the timing of blood is not critical. Moreover, accumulated exposure can be monitored.

One of the limitations of all forms of biomonitoring utilising accessible tissue (peripheral blood lymphocytes), is that they may not give a true indication of the effect of exposure on other less accessible tissues. For example, it has been shown that 2-acetylaminofluorene (2-AAF) will not produce detectable AAF-adducts in lymphocytes, even after very large doses, giving no indication of the extent of exposure to the target organ, the liver (Baan et al. 1985). Therefore research involving the use of animal models will always be necessary to provide such information.

Given the sensitivity of the techniques measuring DNA adducts, future research in animals investigating the effects of MMFO induction on in vivo genotoxicity may prove more fruitful than the use of more non-specific and less sensitive methods as SCE and MN formation. Firstly, quantitation of very low levels of DNA adducts would make it possible to detect smaller changes in the extent of DNA damage with

changes in metabolic capacity of various tissues. Secondly, with the potential to determine the structure of adducts, it may be possible to monitor changes in the level of different adducts with changes in MMFO activity and therefore, establish which adducts may be more toxicologically relevant. Thirdly, as a consequence of the first two points, the correlation between a tissue's capacity to activate and/or deactivate a chemical and its susceptibility to DNA damage can be assessed more accurately.

Appendix A

Sources of Drugs and Chemicals

All chemicals used during the course of these studies were of reagent (AR) or pharmaceutical grade quality. Suppliers, including representative lot numbers are set out below.

CHEMICAL	SOURCE	LOT No.
Benzyl alcohol	Ajax Chemicals	82712
5-Bromodeoxyuridine	Sigma	62F-0441
Bovine Serum Albumen	Sigma	80F-0630
1-Chloro-2,4-dinitro-benzene	Sigma	82F-0036
Colcemid	Calbiochem	201475
Concanavalin A	Sigma	113F-73501
Cyclophosphamide	Bristol Labs	126711
2,5-Diphenyloxazole	BDH	4792520G
S-2,4-Dinitrophenyl-glutathione	Gift from Dr. M. Whitehouse	

Eosin Y	BDH	
7-Ethoxycoumarin	Sigma	64F-3698
Ficoll-paque	Pharmacia	7133
Foetal Calf Serum	Commonwealth Serum	971.19-382-2
	Laboratories	971.19-342-2
Folin & Ciocalteau's Solution	Ajax Chemicals	107033
Giemsa	BDH	4643450G
Glucose-6-phosphate	Sigma	129C-3918
Glucose-6-phosphate dehydrogenase	Sigma	22F-8030
		64F-8065
l-Glutamine	Sigma	20F-0024
Glutathione (reduced)	Sigma	52F-0116
Harris's Haematoxylin	BDH	4525920G
HEPES	Sigma	28F-5627
7-Hydroxycoumarin	Sigma	58C-0536
2-Mercaptoethanol	Sigma	31F-0462
NADP	Sigma	88C-7610
β-Naphthoflavone	Sigma	106F-3695
Penicillin G	Sigma	109C-0248
Phenobarbital	FH Faulding	7085152
2-Phenyl-5-(p-hydroxy-phenyl)oxazole	Gift from Dr. J. Ahokas	
o-Phthalaldehyde	Sigma	28C-0017
RPMI 1640	Flow Laboratories	037169
Streptomycin sulphate	Sigma	48F-0402
Styrene	Ajax Chemicals	703617
Styrene glycol	Aldrich	08310LP
Styrene oxide	Aldrich	1215CJ
Triethylenemelamine	Polysciences	45772
Trypan Blue	Hopkins & Williams	11326
Wright's stain	Sigma	103F-3656

Appendix B

Standard Curves

Styrene Glycol Standard Curve

The activity of epoxide hydrolase was assessed by gas chromatographically measuring the formation of styrene glycol from styrene oxide, as described in section 2.2.3.4. A typical chromatogram is shown in Figure B.1a. The styrene glycol standard curve is displayed in Figure B.1b. The coefficient of variation (CV) was 2.1%.

S-2,4-dinitrophenyl glutathione (DNP-GS) Standard Curve

The glutathione conjugate of CDNB was measured spectrophotometrically by a modified procedure of Habig et al. (1974). The standard curve of DNP-GS is shown in Figure B.2. CV = 1.5%.

Standard Curve of Reduced Glutathione

The glutathione (GSH) standards were prepared fresh daily in phosphate-EDTA buffer, pH 8.0 and measured fluorometrically by the method of Hissin and Hilf (1976). The standard curve of GSH versus fluorescence of o-phthalaldehydederivatised GSH is represented in Figure B.3. CV = 2.7%.

7-Hydroxycoumarin Standard Curve

The 7-hydroxycoumarin (7-HC) standards were measured fluorometrically by the modified procedure of Greenlee and Poland (1978). The graph of fluorescence units versus 7-HC concentration is shown in Figure B.4. CV = 1.9%.

2-Phenyl-5-(p-hydroxyphenyl)oxazole (PPO-OH) Standard Curve

The PPO-OH standards were assayed fluorometrically following the method of Philippides et al. (1983). The standard curve is represented in Figure B.5. CV = 1.8%.

Bovine Serum Albumin (BSA) Standard Curve

The BSA standards were prepared fresh daily and the protein content was determined by the spectrophotometric method of Lowry et al. (1951). Figure B.6. CV = 2.5%.

Epoxide Hydrolase Activity Versus Incubation Time

Styrene oxide (2 mM) was incubated, following the procedure described in section 2.2.3.4, with a tissue concentration of 8 mg/ml over various times. The enzyme activity was found to be linear between 30 to 120 min. incubation time. Figure B.7.

Epoxide Hydrolase Activity Versus Tissue Concentration

Styrene oxide (2 mM) was incubated for 60 min. at 37^0 C with varying S10 concentrations (8-80 mg tissue/ml). Enzyme activity was linear over the tissue concentration range examined. Figure B.8.

Figure B.1a

A = BENZYL ALCOHOL (Internal Standard)

B = STYRENE OXIDE

C = STYRENE GLYCOL

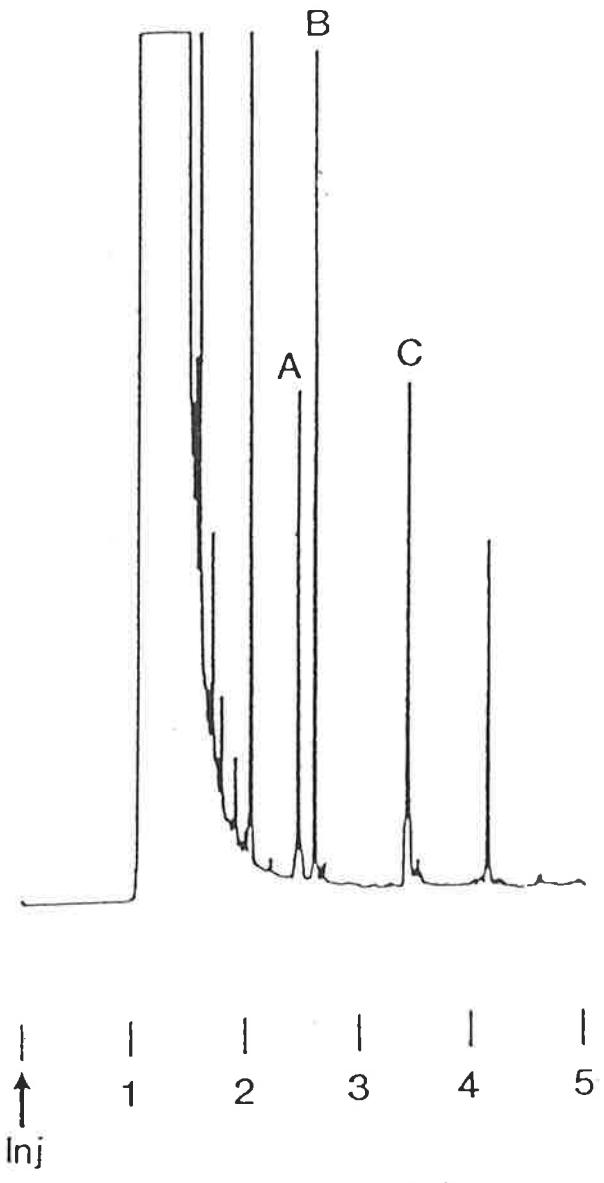


Figure B.1b: SG Standard Curve

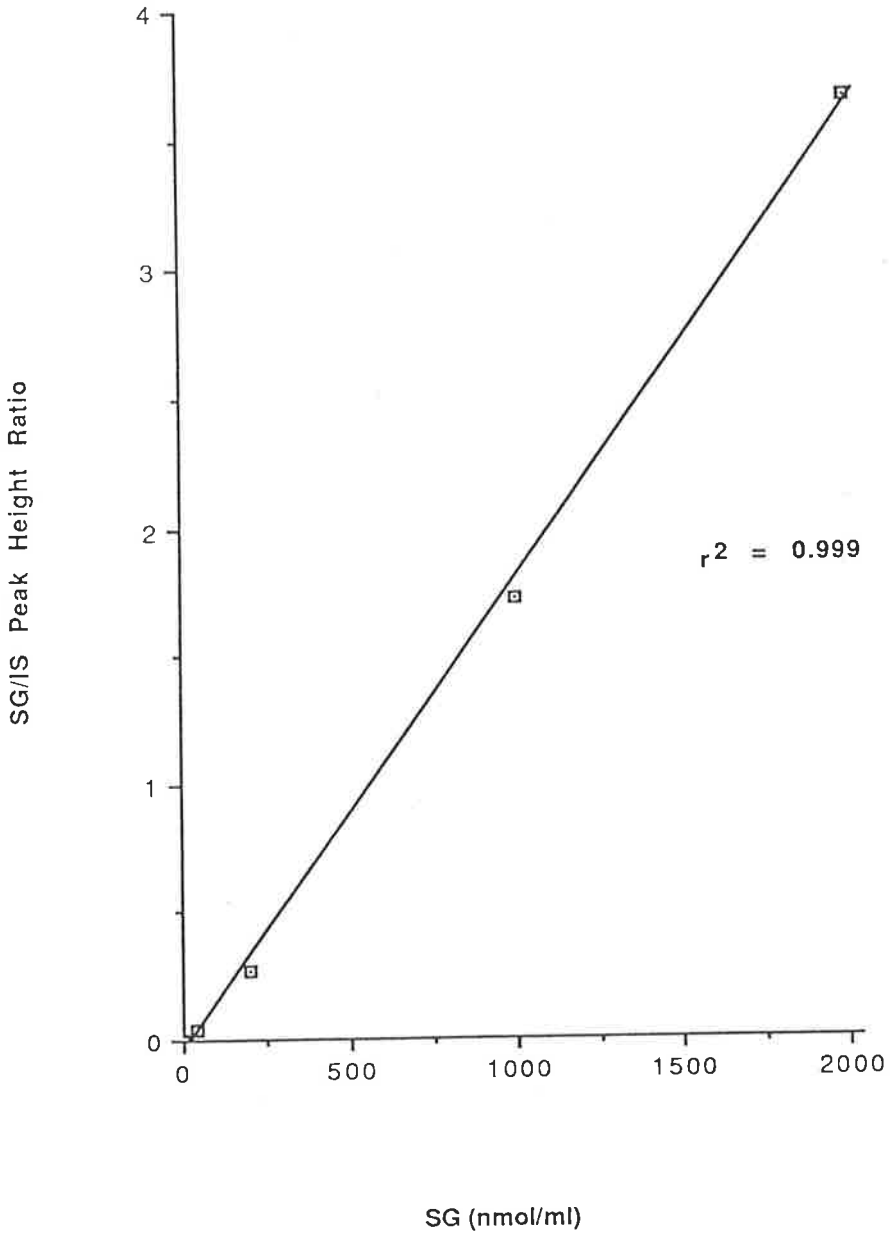


Figure B.2: DNP-GS Standard Curve

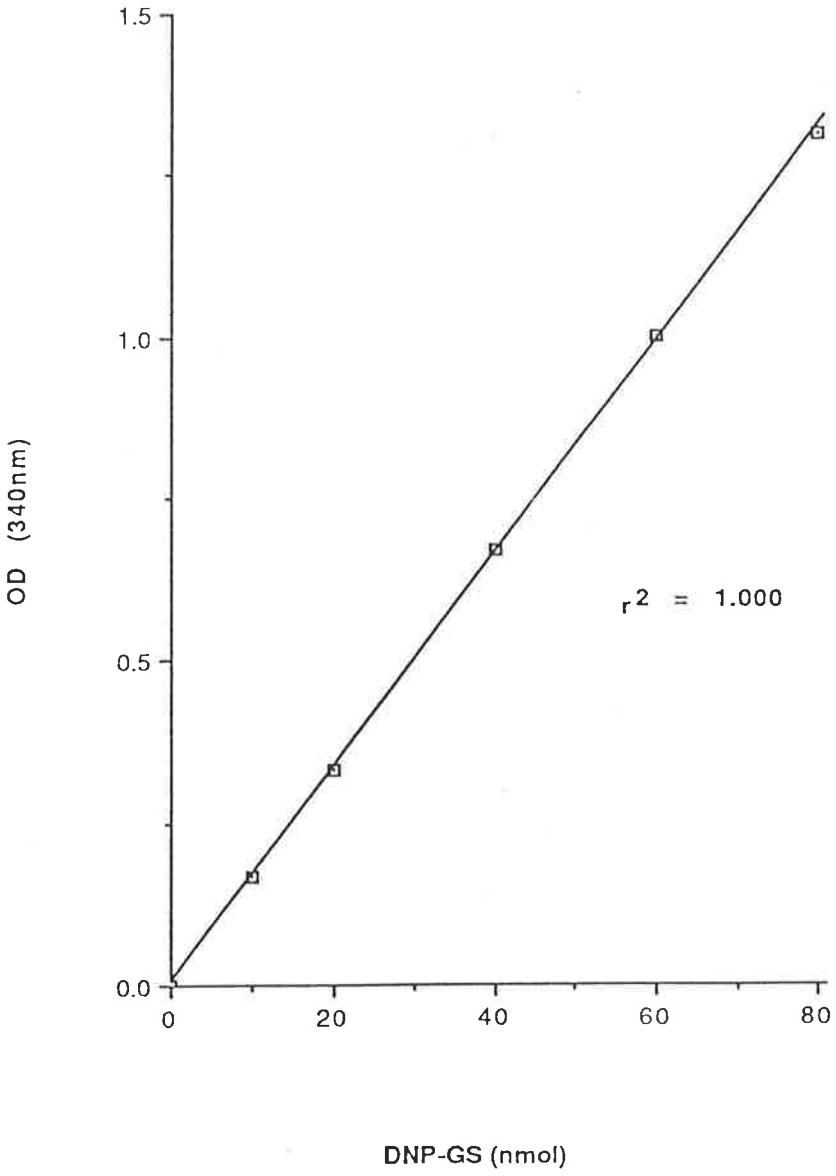


Figure B.3: GSH Standard Curve

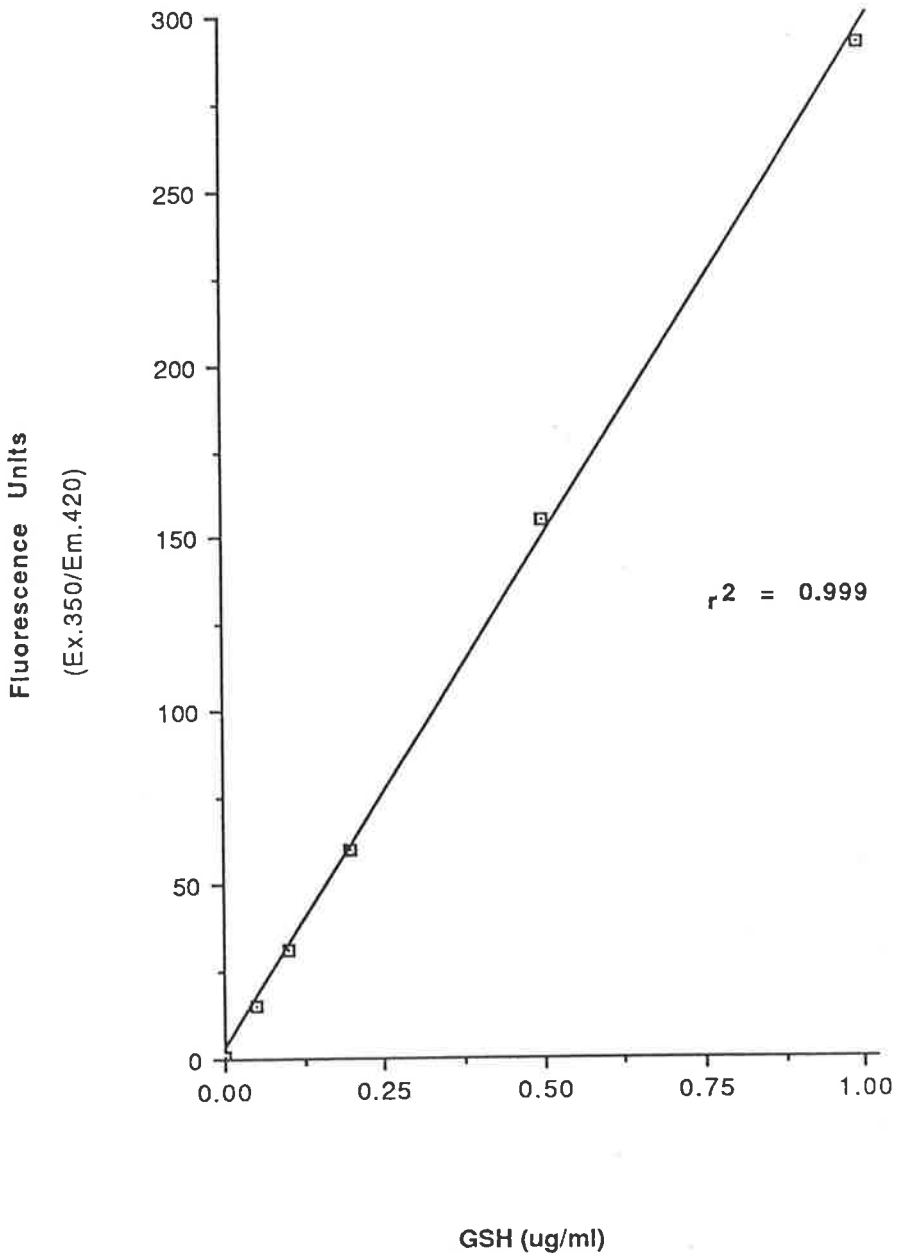


Figure B.4: 7-OH Coumarin Standard Curve

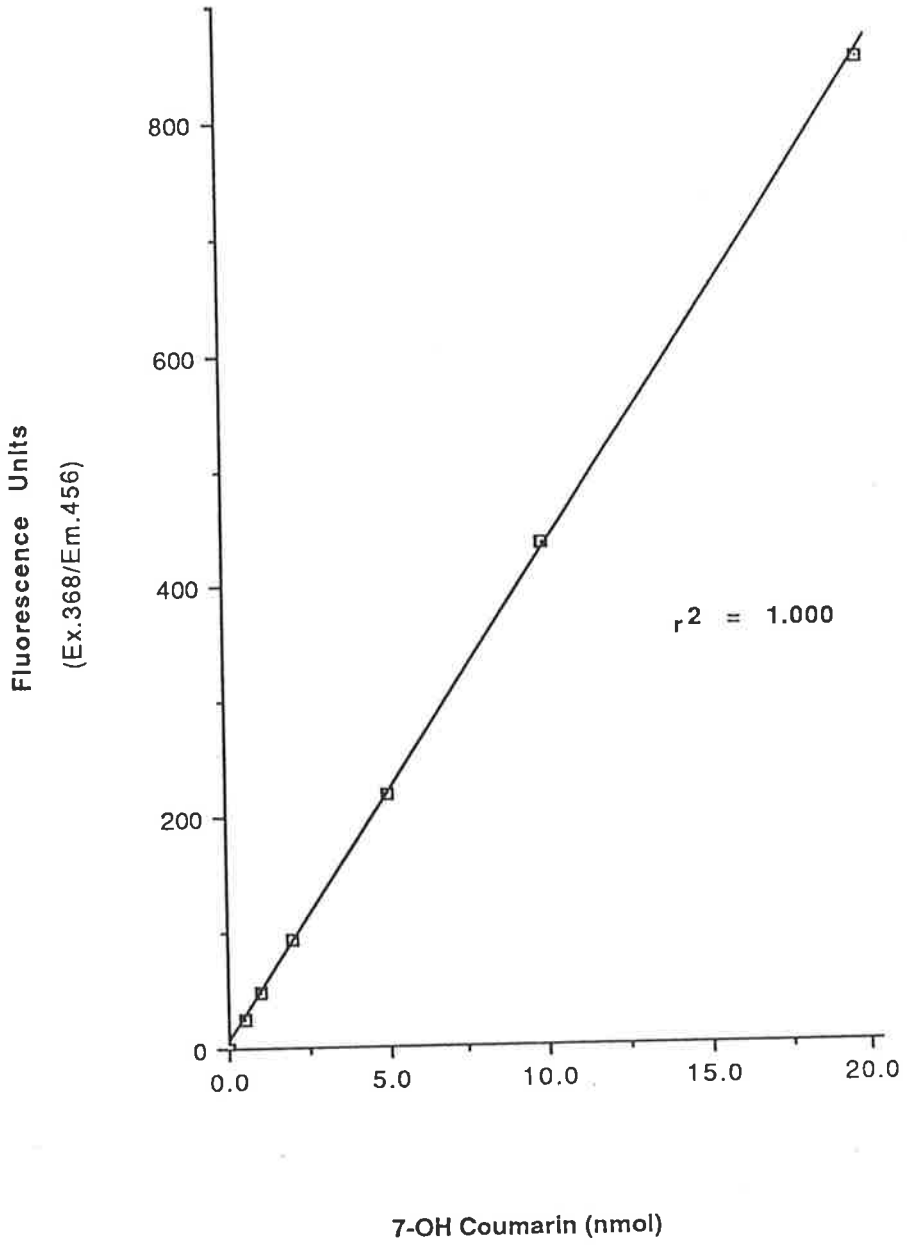


Figure B.5: PPO-OH Standard Curve

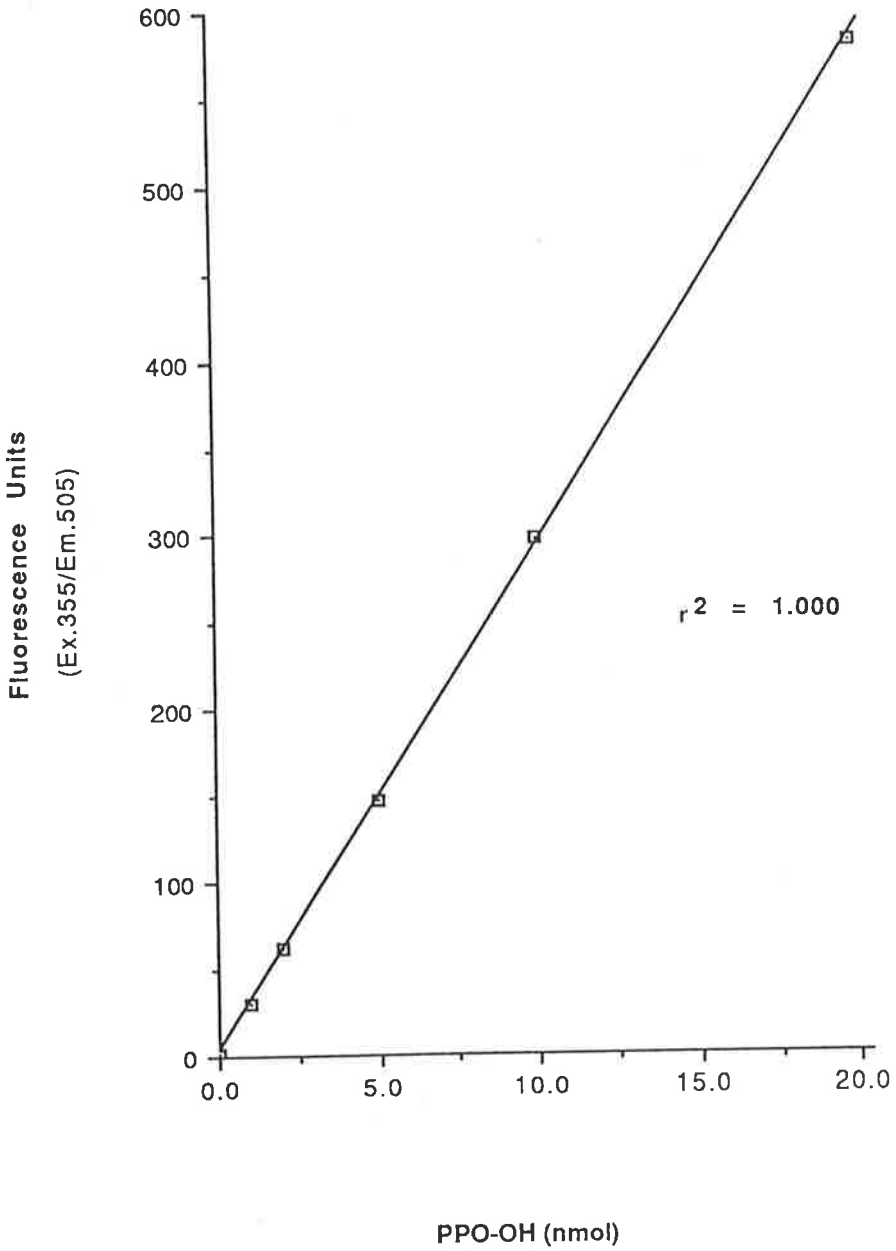


Figure B.6: BSA Standard Curve

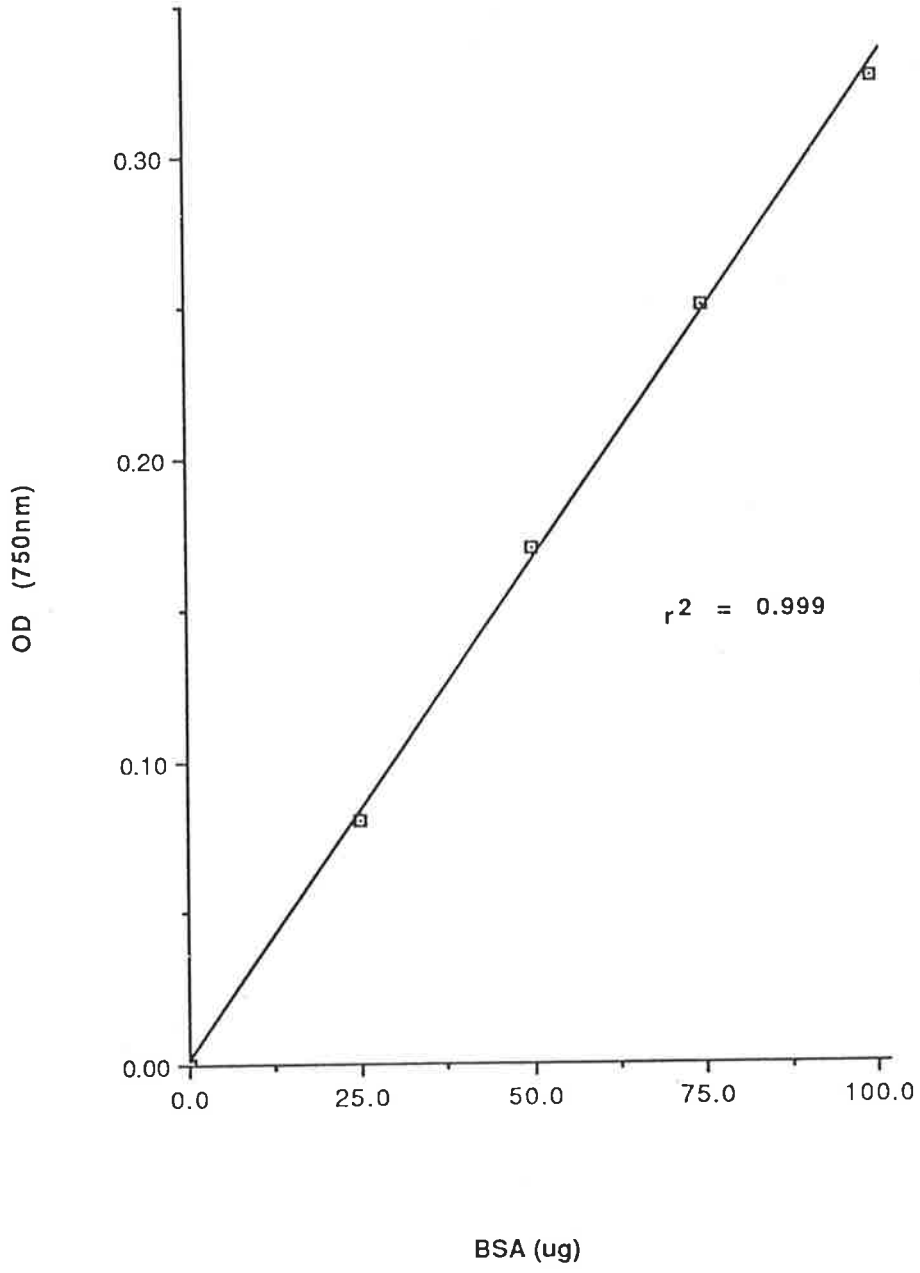


Figure B.7: Epoxide Hydrolase Activity Vs Incubation time.

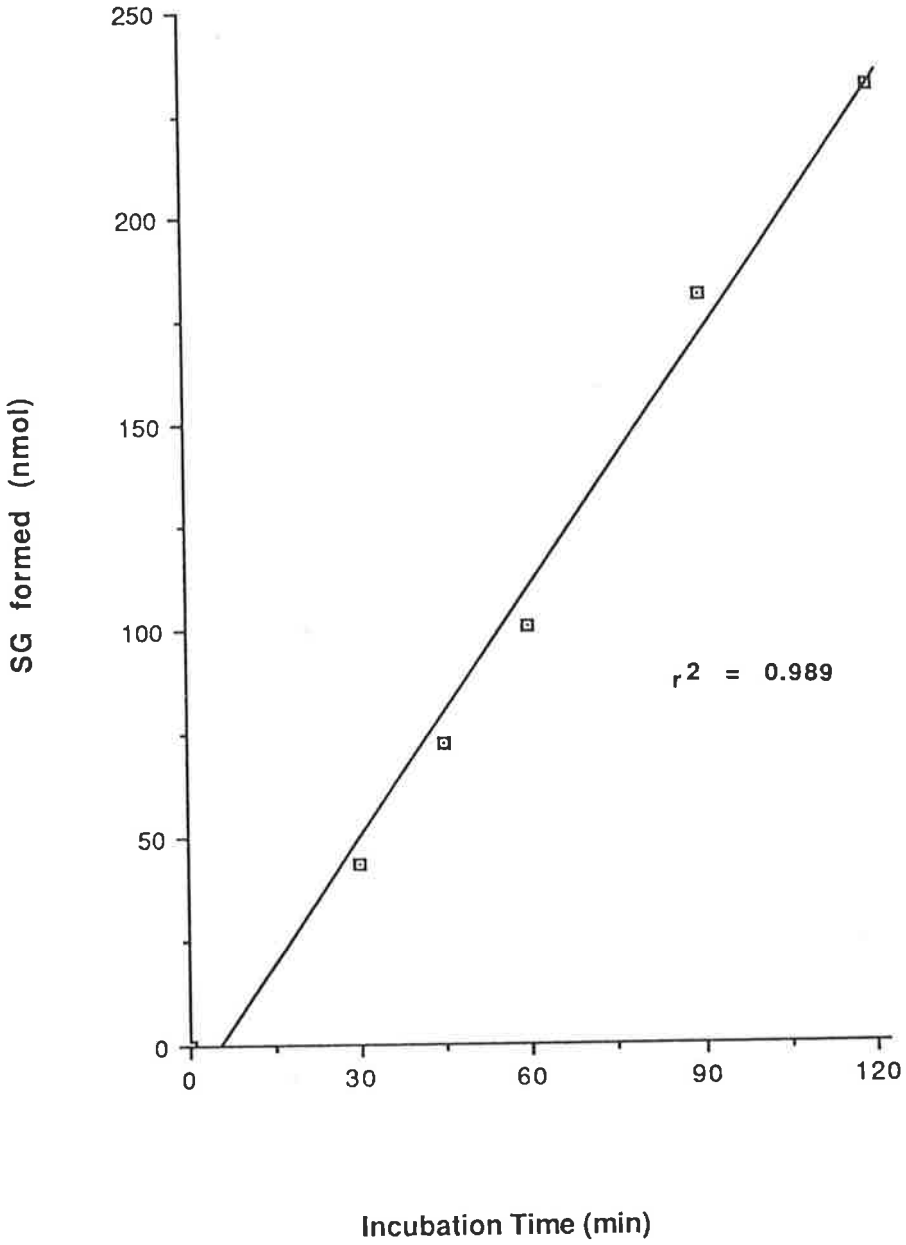
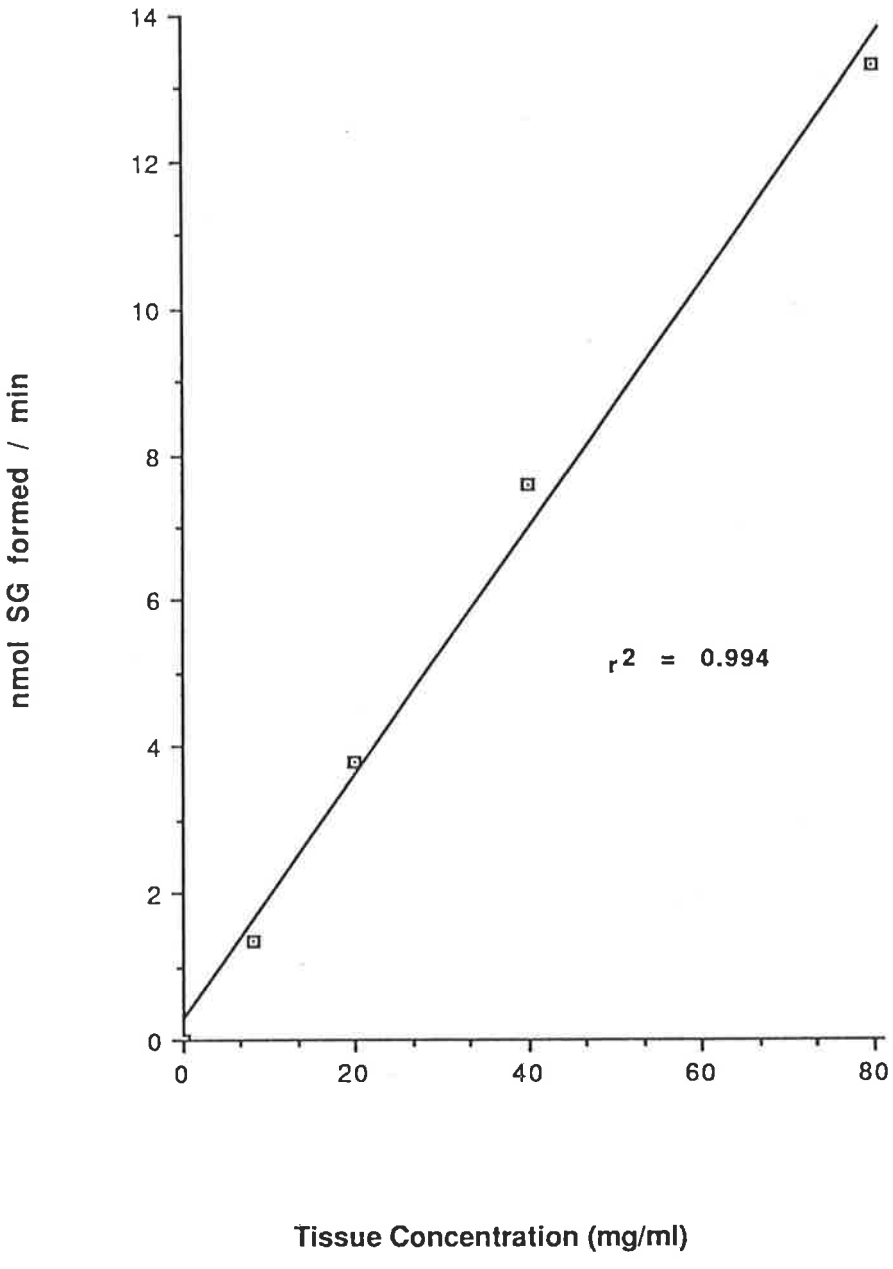


Figure B.8: Epoxide Hydrolase Activity Vs Tissue concentration.



Appendix C

Method of Statistical Analysis for the Binomial Data of the MN and SM Assays.

C.1 Analysis of Deviance

Analysis of variance forms the standard approach for the analysis of continuous data from designed experiments. Consider a simple 2-factor experiment, where the dosage of a drug is factor d (with 5 doses) and the time of sampling is factor t (with 3 time points). If Y_{ijk} is the k^{th} response to the drug at level i of d and level j of t, the usual statistical model is

$$Y_{ijk} = \mu + \delta_i + \tau_j + \gamma_{ij} + \varepsilon_{ijk} \tag{C.1}$$

where;

δ_i is the dose effect for level i,

τ_j is the time effect for level j,

γ_{ij} is the interaction effect for level i of dose and level j of time.

The term ε_{ijk} represents the unexplained variation. The usual assumptions are

$$\varepsilon_{ijk} \sim N(0, \sigma^2) \tag{C.2}$$

that is, the variation follows a normal distribution with zero mean and constant variance σ^2. These assumptions need to be checked in all analysis.

The additive model, equation C.1, may not always be applicable. Indeed often logarithms (or some other transformations) are required to allow an additive model with equation C.2 to be used. If equations C.1 and C.2 are reasonable, the mean or average Y_{ijk} is

$$E(Y_{ijk}) = \mu_{ijk} = \mu + \delta_i + \tau_j + \gamma_{ij} \tag{C.3}$$

and the mean is the appropriate characteristic of Y_{ijk} to model. In some of the experiments in this thesis, the response consists of a count of the frequency of abnormal cells in 1000 cells counted. The data was therefore discrete and not continuous. If we consider the factors above, and let Y_{ijk} be the number of abnormal cells, it follows a binomial distribution

$$Y_{ijk} \sim B_i(1000, p_{ij})$$

where p_{ij} is the probability a cell is abnormal. As indicated by the subscripts, this probability may depend on the dose level i and the time of sampling j. Notice that while for normally distributed data the mean is of interest, for the binomial it is the effect of the factors, δ and τ, on the probability which is of interest.

It can be shown that the natural function of p_{ij} is the logarithm of the odds of an abnormal cell, ie.

$$\eta_{ij} = \log \frac{p_{ij}}{1 - p_{ij}}$$

Thus we model η_{ij} as in equation C.3;

$$\eta_{ij} = \mu + \delta_i + \tau_j + \gamma_{ij} \tag{C.4}$$

and interpretation of results must, at least initially, be made in terms of the log of the odds. Note that

$$p_{ij} = \frac{e^{\eta_{ij}}}{1 + e^{\eta_{ij}}}$$

which ensures $0 \leq p_{ij} \leq 1$. This is how a probability should behave. Modelling p_{ij} and not η_{ij}, as in equation C.4, can lead to negative probabilities or probabilities greater than 1, which are difficult to interpret.

Two more differences between binomial and normal data, are that the variance of binomial data is a function of the mean and furthermore exact tests of hypotheses are not available. The first means the estimation process is iterative and the second means we rely on likelihood ratios or chi-square tests (similar to Pearson chi-square tests). As each model is fitted, a deviance and degrees of freedom are produced. The difference between two models is measured by the difference in deviance and the difference in degrees of freedom. The difference in deviance is approximately chi-square distributed on the difference in degrees of freedom if the null hypothesis is true. Thus many of the tables presented in section C.2 are simply these chi-square tests.

Finally, we note that to test for interactions, H_0: $\gamma_{ij} = 0$ in equation C.4 we fit

$$\mu + \delta_i + \tau_j + \gamma_{ij}, \text{ deviance} = D, \text{ d.f.} = d$$
$$\mu + \delta_i + \tau_j, \text{ deviance} = D_0, \text{ d.f.} = d_0$$

and $D_0 - D \approx \chi^2(d_0 - d)$ under H_0. If the interaction is not significant it is appropriate to test for $\delta_i = 0$ and $\tau_j = 0$. We do this by fitting

$$\left.\begin{array}{l} \mu + \delta_i + \tau_j \\ \mu + \tau_j \end{array}\right\} H_0 : \delta_i = 0$$

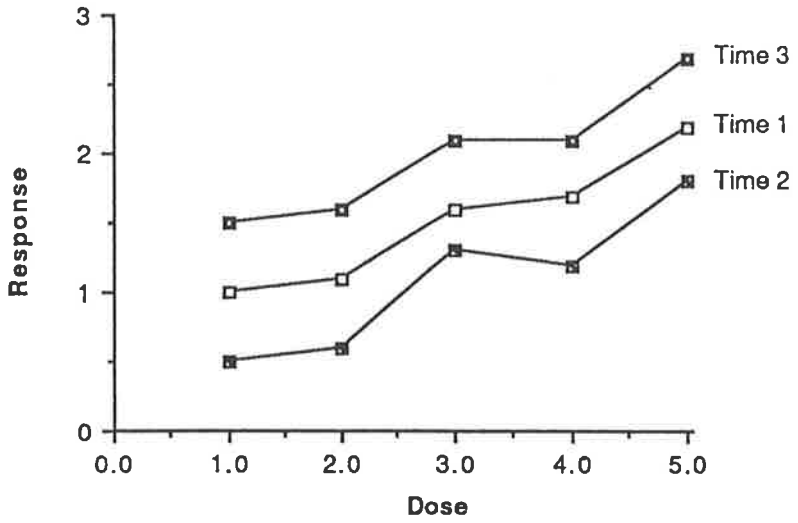
and

$$\left.\begin{array}{l} \mu + \delta_i + \tau_j \\ \mu + \delta_i \end{array}\right\} H_0 : \tau_j = 0$$

Leaving the μ factor present is necessary if we have a non-orthogonal design, that is, if the number of observations per treatment group are not equal. The following articles may be referred to for further clarification of the analysis of binomial data; Williams, (1975) and Williams, (1982).

C.1.1 Additive Effects

If two factors such as dose and time do not interact, so that $\gamma_{ij} = 0$ in equation C.1 or equation C.4, the effects of dose and time are additive. In the case of analysis of variance (Equation C.1), this implies that the average responses for different doses are parallel when examined for the time factor. Diagrammatically the situation is given in the following diagram:



This is also true of equation C.4, for binomial data but for the logarithm of the odds, not the proportion itself. Thus

$$\eta_{ij} = \log \frac{p_{ij}}{1 - p_{ij}} = \mu + \delta_i + \tau_j$$

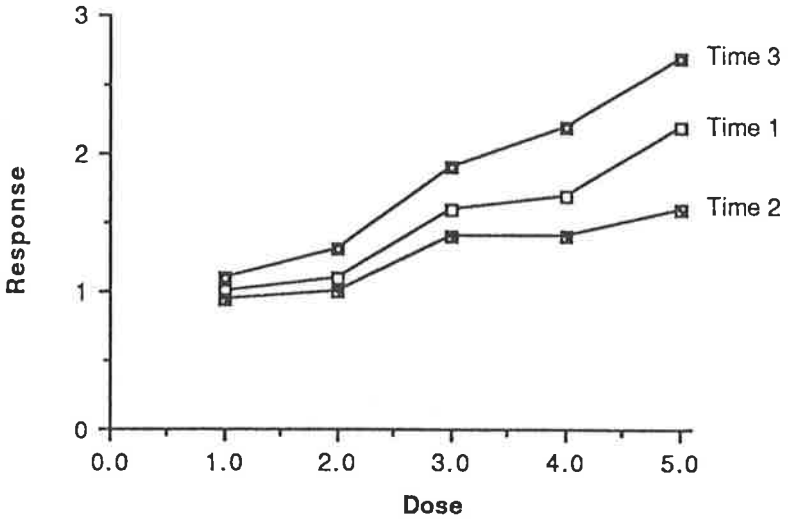
but,

$$p_{ij} = \frac{e^{\mu+\delta_i+\tau_j}}{1 + e^{\mu+\delta_i+\tau_j}}$$

and on the original scale the parallelism is lost. Notice, however that p_{ij} can be written as

$$p_{ij} = \frac{\mu^* \delta_i^* \tau_j^*}{1 + \mu^* \delta_i^* \tau_j^*}$$

where $\mu^* = e^{\mu}$, $\delta_i^* = e^{\delta_i}$, $\tau_j^* = e^{\tau_j}$ and thus there is a multiplicative relationship present in p_{ij}. This is much more difficult to represent in a table or graph of the original data, where there may appear to be an interaction as in the figure below. Thus these tables and graphs need to be examined very carefully.



C.2 Results of the Statistical Analyses of the Data from Chapters 3 & 4.

The results of the statistical analyses are presented in the following tables. The tables have a short-hand method of describing the presence of significant effects and the presence of interactions between 2 or more variables. For example, "Source" represents the source of the χ^2- or F-statistic. A "d.PB.t**" in the 'Source' column is interpreted as follows; there is a significant ($p<0.01$) dose-related effect due to the genotoxic compound, which is significantly influenced by the presence of PB pretreatment and the time, after dosing, at which the effect is assessed. The results of Tukey's Multiple-Comparison Test and the Asymptotic Test (Section 2.3) used for conducting the multiple comparisons are not represented in the following tables.

This legend contains symbols and abbreviations, which are used in the tables that summarise the results of the statistical analyses for Chapters 3 and 4:

d = dose of the genotoxic compound

t = time of sampling

s = species

PB = PB pretreatment

BNF = BNF pretreatment

$*$ = significant to $p<0.05$

$**$ = significant to $p<0.01$

$***$ = significant to $p<0.001$

C.2.1 Analyses of Data from Chapter 3.

- CP Genotoxicity Data from the MN Assay.

  1. Assessment of effects of CP dose, time of sampling and species used, on the MNPCE frequency:

Source	χ^2-statistic	d.f.
d.t.s	38.70***	10

  2. Assessment of the effects of CP dose, time of sampling, species and the presence of either PB or BNF pretreatment, on the MNPCE frequency:

Source	χ^2-statistic	d.f.
d.BNF.t.s	1.81	5
d.PB.t.s	10.21	5
d.BNF.t	3.69	5
d.PB.t	24.02***	5
d.BNF.s	2.72	5
d.PB.s	16.15**	5
d.t.s	41.55***	5
PB.t.s	23.80***	1
BNF.t.s	0.36	1

- CP Cytotoxicity Data from the MN Assay.

  1. Assessment of the effects of CP dose, time of sampling and species, on the PCE/RBC ratio:

Source	F-statistic	d.f.
d.t.s	2.77**	10;208

2. Assessment of the effects of CP dose, time of sampling, species and the presence of PB or BNF pretreatment, on the PCE/RBC ratio in bone marrow:

Source	F-statistic	d.f.
d.BNF.t.s	0.68	5;376
d.PB.t.s	0.59	5;376
d.BNF.t	3.09**	5;376
d.PB.t	6.97**	5;376
d.BNF.s	5.30**	5;376
d.PB.s	4.25**	5;376
d.t.s	6.20**	5;376
PB.t.s	3.22**	1;376
BNF.t.s	~0.00	1;376

- CP Genotoxicity Data from the SM Assay.

1. Assessment of the effects CP dose, time of sampling, species and the presence of PB or BNF pretreatment, on the frequency of abnormal sperm:

Source	χ^2-statistic	d.f.
d.BNF.t.s	16.42	14
d.PB.t.s	11.48	14
BNF.t.s	6.84	4
PB.t.s	3.48	4
d.BNF.s	4.93	3
d.PB.s	2.69	4
d.t.s	65.07***	12
PB.s	0.01	1
BNF.s	0.32	1
d.BNF	6.40	4
d.PB	1.32	4
PB	4.42*	1
BNF	7.34**	1

2. Assessment of the effect of TEM dose and time of sampling on the frequency of abnormal sperm in rats and mice:

Source	χ^2-statistic	d.f.
d.t.s	168.9***	2

- CP Genotoxicity Data from the SCE Assay.

1. Assessment of the effects of CP dose and the presence of PB or BNF pretreatment, on the SCE frequency in mouse splenocytes:

Source	F-statistic	d.f.
d.BNF	0.16	4;60
d.PB	1.19	4;60
d	798.34***	4;60
BNF	0.09	1;60
PB	1.33	1;60

2. Assessment of the effects of CP dose and the presence of PB or BNF pretreatment, on the SCE frequency in rat splenocytes:

Source	F-statistic	d.f.
d.BNF	0.27	4;60
d.PB	2.61	4;60
d	337.69***	4;60
BNF	0.85	1;60
PB	0.70	1;60

3. Assessment of the effects of CP dose and the presence of PB or BNF pretreatment, on the replicative index of cultured mouse splenocytes:

Source	F-statistic	d.f.
d.BNF	0.16	4;60
d.PB	0.83	4;60
d	1.58	4;60
BNF	0.09	1;60
PB	0.18	1;60

4. Assessment of the effects of CP dose and the presence of PB or BNF pretreatment, on the replicative index of cultured rat splenocytes:

Source	F-statistic	d.f.
d.BNF	0.39	4;60
d.PB	0.44	4;60
d	15.15***	4;60
BNF	0.51	1;60
PB	3.56	1;60

C.2.2 Analyses of Data from Chapter 4.

- ST Genotoxicity Data from the MN Assay

1. Assessment of the effects of ST dose and time of sampling on the incidence of MNPCEs in mouse bone marrow:

Source	χ^2-statistic	d.f.
d.t	10.19	8
d	11.89*	4
t	32.29***	2

2. Assessment of the effects of ST dose and time of sampling on the incidence of MNPCEs in rat bone marrow:

Source	χ^2-statistic	d.f.
d.t	2.21	8
d	1.92	4
t	1.28	2

3. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment, on the incidence of MNPCEs in mouse bone marrow:

Source	χ^2-statistic	d.f.
d.BNF.t	8.84	4
d.PB.t	1.66	4
d.PB	2.49	4
d.BNF	2.43	4
BNF.t	1.12	1
PB.t	2.49	1
d.t	3.32	4
d	41.42***	4
PB	0.03	1
BNF	1.30	1
t	4.52*	1

4. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment, on the incidence of MNPCEs in rat bone marrow:

Source	χ^2-statistic	d.f.
d.BNF.t	0.13	4
d.PB.t	0.12	4
d.PB	5.90	4
d.BNF	1.94	4
BNF.t	0.001	1
PB.t	0.65	1
d.t	0.90	4
d	11.08*	4
PB	7.54**	1
BNF	0.16	1
t	3.46	1

- ST Cytotoxicity Data from the MN Assay.

1. Assessment of the effects of ST dose and time of sampling on the PCE/RBC ratio in mouse bone marrow:

Source	F-statistic	d.f.
d.t	1.59	8;142
d	1.86	4;142
t	20.97***	2;142

2. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment on the PCE/RBC ratio in mouse bone marrow:

Source	F-statistic	d.f.
d.BNF.t	0.36	4;207
d.PB.t	1.51	4;207
BNF.t	0.03	1;207
PB.t	1.34	1;207
d.t	8.28***	4;207
d.BNF	1.75	4;207
d.PB	0.71	4;207
PB	10.18***	1;207
BNF	0.06	1;207

3. Assessment of the effects of ST dose and the time of sampling on the PCE/RBC ratio in rat bone marrow:

Source	F-statistic	d.f.
d.t	2.18*	8;73

4. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment on the PCE/RBC ratio in rat bone marrow:

Source	F-statistic	d.f.
d.BNF.t	1.13	4;145
d.PB.t	1.65	4;145
BNF.t	1.11	1;145
PB.t	2.12	1;145
d.t	0.27	4;145
d.BNF	5.64***	4;145
d.PB	1.44	4;145
PB	0.88	1;145
t	0.26	1;145

- ST Genotoxicity Data from the SM Assay.

1. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment on the frequency of abnormal sperm in mice:

Source	χ^2-statistic	d.f.
d.BNF.t	14.74	8
d.PB.t	11.27	8
BNF.t	13.48**	2
PB.t	27.24***	2
d.t	3.49	8
d.PB	1.91	4
d.BNF	2.99	4
d	110.7***	4

2. Assessment of the effects of ST dose, time of sampling and the presence of PB or BNF pretreatment on the frequency of abnormal sperm in rats:

Source	χ^2-statistic	d.f.
d.BNF.t	9.36	6
d.PB.t	4.06	6
BNF.t	1.73	2
PB.t	0.56	2
d.t	12.31	8
d.BNF	6.78	4
d.PB	7.75	4
t	40.07***	2
BNF	21.02***	1
PB	1.67	1
d	132.27***	4

3. Assessment of the effect of TEM dose and time of sampling on the frequency of abnormal sperm in mice and rats:

Source	χ^2-statistic	d.f.
d.t.s	157.8***	2

- ST Genotoxicity Data from the SCE Assay.

1. Assessment of the effects of ST dose and the presence of PB or BNF pretreatment on the SCE frequency in mouse splenocytes:

Source	F-statistic	d.f.
d.PB	0.55	4;59
d.BNF	0.73	4;59
d	22.25***	4;59
PB	2.83	1;59
BNF	2.38	1;59

2. Assessment of the effects of ST dose and the presence of PB or BNF pretreatment on the SCE frequency in rat splenocytes:

Source	F-statistic	d.f.
d.PB	1.95	4;48
d.BNF	1.69	4;48
d	123.28***	4;48
PB	4.68*	1;48
BNF	0.24	1;48

- ST Cytotoxicity Data from the SCE Assay.

1. Assessment of the effects of ST dose and the presence of PB or BNF pretreatment on the replicative index of cultured mouse splenocytes:

Source	F-statistic	d.f.
d.BNF	4.69**	4;59
d.PB	0.55	4;59
PB	0.18	1;59

2. Assessment of the effects of ST dose and the presence of PB and BNF pretreatment on the replicative index of cultured rat splenocytes:

Source	F-statistic	d.f.
d.PB	0.35	4;48
d.BNF	0.45	4;48
d	0.45	1;48
PB	0.26	1;48
BNF	2.38	1;48

Appendix D

The Influence of Various Vegetable Oils, as Vehicles in the MN Assay, on Bone Marrow Proliferation.

Introduction

Interpretation of genotoxicity data requires that the vehicle used to administer the test compound should have no effect of its own in the assay system employed. Preliminary experiments assessing ST's activity in the MN assay, found the vehicle being used was able to lower the PCE/RBC ratio. The vehicle used to deliver the ST was commercial grade peanut oil. A decrease in the PCE/RBC ratio was observed with peanut oil controls at the 30 hour sample, which returned to control levels at 48 and 72 hrs after dosing (Table 4.1). A similar response was not detected in rats dosed with peanut oil (Table 4.3). An experiment was therefore conducted to confirm this inhibitory effect of peanut oil on murine bone marrow proliferation. For comparison, two other commercially available oils (olive and sunflower seed) were also tested.

Materials and Methods

5 mice per group were given a single i.p. injection of each vegetable oil at a dose of 4 ml/kg. Femoral bone marrow was sampled at 30, 48 and 72 hrs post-dosing. Control mice were untreated. Bone marrow smears were prepared by the procedure described in section 2.1.1.2. The cytotoxicity data was analysed using a 1-Way ANOVA followed by Tukey's Multiple-Comparison Test to determine significance. Significant differences from the untreated controls ($p<0.05$) was indicated by a dagger (†) in Table D.1. The genotoxicity data was analysed by the Analysis of Deviance method described in Appendix C.

Results

None of the three oils significantly altered ($\chi^2=1.76$, d.f.=6) the frequency of MNPCEs in mice (Table D.2). In contrast, there was a significant reduction ($p<0.05$) in the PCE/RBC ratio, at 30 hrs with exposure to each oil (Table D.1). Bone marrow from mice exposed to peanut oil recovered by 48 hrs, while the effects of olive and sunflower oils persisted until 48 hrs ($p<0.05$). The effect on haemopoiesis was greater with olive oil. Peanut and sunflower seed oils produced similar inhibitory effects, although the duration of the response was longer with sunflower seed oil.

Discussion

The results demonstrated the ability of all 3 vegetable oils to inhibit haemopoiesis in mouse femoral bone marrow. The contribution of the differential composition of fatty acids in the three vegetable oils (Hilditch & Williams, 1964), to their differring cytotoxic responses is not known. Although interpretation of the cytotoxicity data would be made difficult by this effect, the lack of genotoxicity suggests they may still be useful as vehicles for the MN test, provided the extent of their cytotoxicity in known at all time points being assessed. Based on these results and the fact that preliminary experiments were conducted with peanut oil as the vehicle, its use was continued for the entire study.

Oils	PCE/RBC Ratio		
	30hrs	48hrs	72hrs
Control	1.01±0.04	1.00±0.03	1.02±0.03
Peanut	0.65±0.08†	0.92±0.06	1.00±0.06
Olive	0.55±0.09†	0.86±0.10†	0.99±0.09
Sunflower	0.64±0.05†	0.85±0.08†	0.97±0.06

Table D.1: The effect of peanut, olive and sunflower seed oil on the PCE/RBC ratio in mouse bone marrow, at 30, 48 and 72 hrs. † indicates a significant difference from the respective controls, $p<0.05$.

Oils	MNPCE/1000 PCE		
	30hrs	48hrs	72hrs
Control	1.00±0.32	1.00±0.32	1.20±0.49
Peanut	1.00±0.32	1.40±0.51	1.25±0.63
Olive	1.20±0.20	1.60±0.24	1.20±0.37
Sunflower	0.80±0.37	1.00±0.32	1.00±0.45

Table D.2: The effect of peanut, olive and sunflower seed oil on the frequency of MNPCEs in mouse bone marrow, at 30, 48 and 72 hrs.

Appendix E

The Susceptibility of the SM Assay to Changes in Sensitivity to the Genotoxicity of CP over Time.

Introduction

The susceptibility of the abnormal sperm frequency to factors thought to be non-mutagenic is well documented. They include youth (Krzanowska, 1981), dietary restrictions (Komatsu et al. 1982) and increased body temperatures (Cairnie and Leach, 1980). Since these factors alter the basal levels of the incidence of abnormal sperm, they may also modify the sensitivity of the assay to genotoxin-induced increases in sperm abnormalities. Preliminary experiments with the SM assay, conducted on CP-treated mice, displayed differing results over a one year period.

Materials and Methods

In two separate experiments, performed approximately one year apart, 5 mice per dose group were treated with CP at 0, 10, 20 and 40 mg/kg/d over 5 days. Sperm was sampled from the vasa deferentia at 5 weeks from commencement of dosing and

slides were prepared and assessed by the method described in section 2.1.2.2. The data was analysed by the Analysis of Deviance (Appendix C) for the influence of time on the dose/response curve to CP.

Results

The response of the mice to CP in the earlier experiment was a doubling of the percentage of abnormal sperm at the top dose (Figure E.1). When the experiment was repeated a year later, the mice were significantly ($p<0.01$) more sensitive to CP-induced sperm abnormalities. The mortality rate was 20% at the top dose on both occasions.

Discussion

Since the mortality rates were the same on both occasions an error in dosing is unlikely to have been the cause of the anomalous results. Unknown extrinsic factors may have induced the differential responses to CP. Although the mice used were an outbred strain, it was confirmed by the Adelaide University's Central Animal House that no new mice were introduced into the breeding stock over the last 10 years. Therefore, a change in the gene pool did not appear to be responsible for the unusual results. Interestingly, in the period between the two experiments the mice were transferred from a specific-pathogen free (SPF) environment to a non-SPF one. Whilst there is no evidence that the change in environment was the cause, it may have been a contributing factor.

As it was not possible to isolate the cause of the change in sensitivity over time, in order to eliminate the influence of the extrinsic factors, all mice and rats were received and acclimatised as a single batch. For each species treated with the particular genotoxin, control, PB and BNF pretreated groups were dosed at the same time, to ensure a valid comparison between induced and uninduced animals. The experiments of Chapters 3 and 4 were conducted between Nov. 1987-May 1988. One should note that the response to CP did not appear to change appreciably between Sept. 1986 and Nov. 1987. Furthermore, the responses to the positive

control (TEM) was remarkably consistent over that period of time (Figures 3.7, 3.8, 4.7 and 4.8).

These results illustrate the susceptibility of this assay system to extrinsic variables and, therefore, stresses the importance of the use of positive control groups to monitor any change in rodent sensitivity to chemical-induced abnormal sperm over time. Any change in the sensitivity of an animal model, to the positive control between experiments, would make any valid comparison of results between experiments impossible.

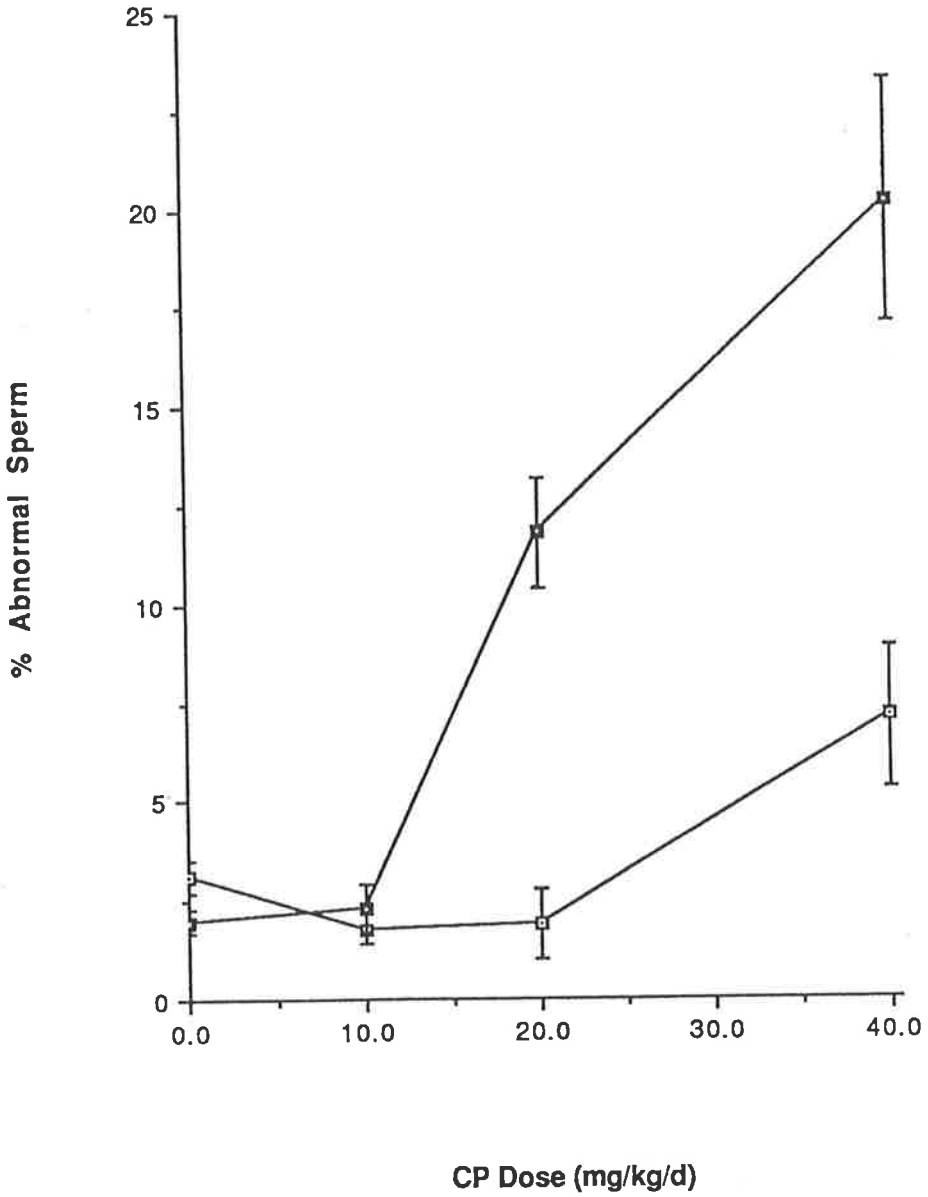


Figure E.1: Dose/response curves to CP in the mouse SM assay from 2 experiments conducted approximately 1 year apart (—▫— 21/10/85 and —▫— 29/9/86).

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