Mechanisms of *m*-dinitrobenzene-induced selective neurotoxicity and the roles of brain glutathione

A thesis submitted for the Degree of Doctor of Philosophy
at the University of Leicester

By

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December 1996

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Preface

The translation of the word 'doctor' from English to Chinese gives two distinct entities. One is used for medical professionals while the other for the Doctor of Philosophy. Having been called a 'doctor' for many years, I became very enthusiastic in reading for a Doctor of Philosophy to make myself the other doctor. The desire to do so is not only for an extra title and another degree, but also for the purpose of being trained to a higher scientific standard, by following all the standard processes of PhD training in United Kingdom, a country which has made such a strong impact on modern science and technology.

From experience I found that one attraction of scientific research is that a new phenomenon always turns up before the previous phenomenon has been fully understood. This may be what has been referred to as the 'challenge' which keeps scientists interested and motivated. In the past three years, I enjoyed very much doing the proposed research programmes however at the same time, I also had the opportunity of enjoying exciting unexpected findings and looking into these interesting phenomena to a reasonable depth. I feel clearly that I am so small in the front of nature that it is impossible to look at everything in which I am interested, however small a specific research area is.

Writing up this thesis is more or less telling the scientific stories that happened in this wonderful MRC laboratory during the past three years. This thesis is written and submitted for a Degree of Doctor of Philosophy, however I have no intention of implying that the project has been completed, simply because the more research is carried out, the more interesting questions turn up. Some pieces of work are quite pilot and preliminary although hopefully innovative.

CONTENTS

ABSTRACT

m-Dinitrobenzene (*m*-DNB) is a neurotoxin producing selective damage in the brain stem. The mechanisms of the selective neurotoxicity induced by this compound have been investigated in this project. *In vitro* studies demonstrated that brain has considerable nitroreductive capacity towards *m*-DNB. *m*-Nitroaniline was the main metabolite produced, attaining up to 66% of the original concentration of *m*-DNB while nitrosonitrobenzene was also detectable in brain slices. Glutathione (GSH) and ascorbic acid were able to chemically reduce nitrosonitrobenzene. The results indicate that *in situ* reduction of *m*-DNB may play an important role in the *m*-DNB-induced neurotoxicity.

Both GSH and ascorbic acid were lower in normal brain stem. Striatum had the highest level of GSH while hippocampus had the highest level of ascorbic acid. In contrast, free malondialdehyde (MDA) was higher in the brain stem than the other areas. Brain stem areas were more sensitive to the BSO-induced GSH depletion since GSH half life in those areas were shorter than in other brain areas. These results indicate a lower antioxidant capacity and a higher spontaneous oxidative stress in brain stem. This may partially explain the susceptibility of brain stem to neurotoxic xenobiotics.

In order to test the hypothesis that GSH status in brain plays an important role in the selective neurotoxicity of *m*-DNB, the susceptibility of the rat to intoxication was studied in 6 month old animals that show naturally lower GSH levels in brain and also in young adult animals treated with BSO to deplete brain GSH. Control adult animals developed brain stem lesions after 4 doses of *m*-DNB (4×10mg/kg); but 6 month old animals showed these changes after 3 doses, while BSO-treated animals had brain stem lesions after only 1 dose of DNB. This demonstrated that brain GSH status is likely to be an important factor in determining regional sensitivity to gliovascular damage induced by *m*-DNB.

Selective GSH depletion in rat brain caused generalised seizures and running fits which could be prevented by maintaining brain GSH levels with co-administration of γ-glutamylcysteine (γ-GC) into the brain. These observations suggest a primary involvement of GSH in modulating excitability of the central nervous system (CNS). A close parallel between developmental changes of hippocampal GSH and cysteine suggests that cysteine may be a rate limiting factor for brain GSH synthesis during the early period of life.

ABBREVIATIONS

AA:	Ascorbic acid
AF64A:	Ethylcholine aziridinium
ANOVA:	Analysis of variance
BHA:	Butylated hydroxyanisole
BHT:	Butylated hydroxytoluene
BSO:	*L*-buthionine-S,R-sulfoximine
CNS:	Central nervous system
CSF:	Cerebrospinal fluid
DEPA:	Diethylenetriaminepentaacetic acid
m-DNB:	*m*-Dinitrobenzene, *1,3*-Dinitrobenzene
o-DNB:	*o*-Dinitrobenzene
p-DNB	*p*-Dinitrobenzene
DTNB:	5,5-Dithio-bis-2-nitrobenzoic acid
DTT:	Dithiothreitol
EGF:	Endothelial growth factor
EI:	Electron Ionisation
EM:	Electronic Microscopy
ESR:	Electron Spin Resonance
FMO:	Flavin-containing monooxygenases
GC-MS:	Gas chromatography-mass spectrometry
γ-GCS:	γ-Glutamylcysteine synthetase
γ-Glu-Glu:	γ-Glutamylglutamine
GSH RI:	GSH Redox Index
GSH:	Glutathione (reduced)
GSNO:	S-nitrosoglutathione
GSSG:	Oxidised glutathione
GST:	Glutathione S-transferases
γ-GT:	γ-Glutamyl transferase
HPLC:	High performance liquid chromatography
i.c.v.:	Intracerebroventricular

i.p.:	Intraperitoneal
iNOS:	Calcium independent inducible nitric oxide synthase
MAO:	Monoamine oxidase
MDA:	Malondialdehyde
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS:	Mass Spectrometry
MSO:	Methionine sulfoximine
NADP(H)	β-Nicotinamide adenine dinucleotide phosphate (reduced)
NMDA:	*N*-methyl-*D*-aspartate
nNOS:	Calcium-dependent neuronal constitutive NOS
NO:	Nitric oxide
NOS:	NO synthase
3-NP:	3-Nitropropionic acid
6-OHDA:	6-Hydroxydopamine
OTC:	*L*-2-oxo-4-thiazolidinecarboxylic acid
PCA:	Perchloric acid
PDA:	Photodiode Array
Pr-SSG:	Protein glutathione mixed disulfide
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
SPS:	Sleep-promoting substance
TF:	Tissue factor

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ACKNOWLEDGEMENTS

I would like to express my deepest and cordial gratitude to Dr. David Ray for his kindness in helping me to register for a PhD, and of course for his responsible and friendly supervision during the past three years. From David, I have learnt a great deal in all aspects of scientific research.

I am grateful to all my colleagues in the Neurotoxicology Section for their great help in analytical, experimental and pathological work. My thanks are due to Mr. Neil Bennett, Mr. Tim Lister, Mr. Chris Nolan, Dr. Janice Holton, Miss Lisa Brown, Mr Adrian Davies and Mrs Qing Zhang. I have enjoyed studying and working in this laboratory with its pleasant and effective environment.

I very much appreciate the suggestions and discussions offered by Dr. Paul Glynn, Dr. Ian White, Dr Sian Thomas and Professor John Cavanagh. Dr White and Dr Alan Waterfall kindly provided the technical details of their HPLC methods for GSH and ascorbic acid plus free MDA measurements respectively. Mr John Lamb offered help in Mass Spectrometry and Dr. Ellis provided nitrosonitrobenzene standard. Their help is highly appreciated.

I would like to take this opportunity to thank my wife Qing for her understanding and full support that make my overtime working possible. Plus, I enjoyed being frequently asked some curious questions by my son Kun, who makes me feel important and great in doing science although I couldn't really answer all the questions. My great appreciation also goes to my parents and all my friends who have offered me support, encouragement, and help.

CHAPTER 1
GENERAL INTRODUCTION

Neurotoxicology in general

Neurotoxicity has been defined as any adverse effect on the structure and function of the nervous system following exposure to a chemical agent. In this sense the structural aspects (neuromorphology and neuropathology) and the functional aspects (neurophysiology and neurobehavior) of neural alterations are critical for the assessment of neurotoxicity. Biochemical and molecular events underlying the toxic effects can provide clues to the mechanisms of action of the toxicants involved (Chang, 1995). There is widespread recognition that numerous chemicals of diverse composition have the capacity in sufficient doses to perturb the structural and/or functional integrity of the human nervous system. Chemicals with neurotoxic potential are recognised as the natural products of bacteria, fungi, plants and animals, as components of foods, food additives and fragrances, and as contaminants of air, food, and water. Alcohol, drugs of abuse, and certain therapeutic agents commonly elicit undesired neurological and/or behavioural changes (Spencer and Schaumburg, 1980). Neurotoxic potential is reported for more than half of the 1,400 active pesticide ingredients (mostly organophosphates and carbamates) registered in the USA (Moses, 1984). About one third of the approximately 600 chemicals for which the American Conference of Governmental Industrial Hygienists has adopted Threshold Limit Values is known to affect the nervous system (Anger, 1984).

As an applied science, neurotoxicology finds its advance dependent on the development of methodology and progress in fundamental understanding of basic sciences and other applied sciences, e.g. neuroscience, physiology, biochemistry, chemistry, molecular biology, pharmacology, toxicology, clinical sciences and even computer technology.

Functional or cellular selectivity of neurotoxicity

The biological effects of the xenobiotics are usually target selective. Selectivity can be beneficial; for example, the control of target pests at commercial doses too low to threaten non-target organisms is a useful agrochemical property and a large therapeutic

ratio is necessary for the safe use of clinical pharmaceuticals. On the other hand however, selective toxicity can lead to serious problems in the use of drugs, industrial compounds and pesticides. The following is a brief summary on the selective actions of neurotoxic compounds.

Compounds that alter electrical impulse conduction

The electrical properties of axons and their ability to transmit information depend on the maintenance of differential concentrations of potassium and sodium ions in the extracellular and intracellular environment. Interference with the function of sodium ion channels by blockade (tetrodotoxin, saxitoxin) or persistent activation (ciguatoxin, batrachotoxin, pyrethroids) alters the signalling properties of nerve fibres (Spencer, 1989).

Compounds that disrupt neurotransmitter function

In theory, chemicals may interfere with neurotransmitter function at many levels, including the synthesis of the transmitters or related enzymes in the neuronal soma, their transport anterogradely along the axon to the terminal, their packaging and release from the synaptic membrane, and the enzymatic breakdown or re-uptake of excess transmitter from the synaptic cleft. Anticholinesterase agents, such as many organophosphorus and carbamate pesticides, are typical examples of chemicals acting on the breakdown of neurotransmitter.

Compounds that disrupt myelin continuity

Normal impulse conduction in myelinated nerve fibres is also interrupted by agents that interfere with the myelin sheath. Diphtheria toxin inhibits the synthesis of myelin-specific proteins in myelinating (Schwann) cells. Experimental Schwann cell damage can be induced by lead salts and ethidium bromide. The myelin sheath is the primary target of the detergent lysolecithin, and intramyelinic edema precedes myelin loss after exposure to hexachlorophene, triethyltin or the salicylanilides, all three of which uncouple oxidative phosphorylation (Cammer, 1980).

Compounds that disrupt axons

Retrograde degeneration of the distal extremities of long axons in spinal cord and peripheral nerves is the common pathological hallmark of a number of chemical intoxications that cause sensory or sensorimotor neuropathies, such as organophosphates and arsenic & thallium salts. The ultrastructural changes include the dissolution of microtubules (vinca alkaloids), the accumulation of intermediate neurofilaments

(acrylamide, gamma diketones), or an increase in the prominence of tubulovesicular components of the endoplasmic reticulum (organophosphates) (Spencer and Schaumburg, 1980).

Compounds that disrupt neurones

Neurones can be killed either by interfering with specialised nerve cell receptors or by disrupting intracellular processes common to other cells. The latter theoretically would include perturbations of transcription, translation, post-translational modification, mitochondria, and other critical cellular organelles or functions.

Some neurotoxic compounds exploit the specialised properties of neurones. Examples include toxins active on neurones utilising dopamine (methamphetamine, 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) and serotonin or noradrenaline (5,7-dihydroxytryptamine) as neurotransmitters (reviewed by Spencer, 1989). Another typical example of neurotoxic agents causing neuronal death would be excitotoxins which mimic the excitatory neurotransmitter action of glutamate, causing repetitive depolarisation of the target neurone, failure of membrane-pumping systems, uncontrolled influx of ions or water, and death of the nerve cell (Olney, 1979). Excitotoxicity has become of great interest in recent years.

Neurotoxins targeting glial and vascular cells

Damage to the gliovascular cells leads to alterations in the permeability of the blood brain barrier (BBB), and then causes the passage of macromolecular substances across the cerebral endothelium and alterations in specific transport systems. Generalised changes in BBB permeability invariably lead to brain oedema which is defined as an increase in the brain content of water, sodium and plasma constituents. Haemorrhage is the other common manifestation of gliovascular lesions.

Acute exposure to high doses of two herbicides (2,4-dichlorophenoxyacetic acid and methyl-4-chlorophenoxyacetic acid) has been linked to neurotoxic effects in man and experimental animals including stiffness of the extremities, lack of coordination, lethargy, stupor, coma and even death (Desi et al., 1962). Both of these herbicides cause a reversible increase in the BBB permeability to Evans blue-bound albumin and IgG in the cerebrum and medulla oblongata of rats after subcutaneous administration. These changes were accompanied by increased numbers of vesicles in endothelial cells and swelling of the basement membrane (Hervonen et al., 1982; Tyynela et al., 1986). 1,3-

bis[2-chloroethyl]-1-nitosourea (BCNU), an anticancer agent, induces haemorrhagic lesions in the cerebral hemisphere ipsilateral to an intracarotid injection in dogs (Omojola et al., 1982) and in rats (Nagahiro et al., 1991). Multifocal necrosis of blood vessels, mainly arterioles, is accompanied in severe lesions by generalised oedema and necrosis of the brain parenchyma in the intoxication of BCNU. A direct action of BCNU and other nitrosoureas on astroglia and oligodendroglia has been demonstrated as well as its action on the vasculature in the form of haemorrhagic foci (Szczech, 1987). Acute haemorrhagic leucoencephalopathy is a dominant feature in patients with trypanosomiasis when treated with melarsoprol and other organic arsenicals. This type of encephalopathy shows fibrinoid necrosis in the walls of small blood vessels, ring and ball haemorrhages and varying degrees of infiltration by polymorphs, scattered throughout the brainstem (Adams et al., 1986). The pathogenesis of this condition remains unclear.

In addition, thiamine deficiency, nicotinamide analogues, chlorosugar and some nitro-aromatic compounds can cause brain damage with vacuolated lesions of gliovascular origin (Cavanagh, 1993).

Regional selectivity of neurotoxicity

Besides the cellular and functional selectivity, neurotoxicologists face another challenge in understanding the regional selectivity caused by some neurotoxins. Cytotoxic chemicals which cross the blood-brain barrier give rise to various patterns of neurotoxicity. Organometallic compounds provide graphic examples. Methylmercury primarily affects the cerebellum and calcarine cortex; trimethyltin damages the limbic system, particularly the hippocampus, and organolead compounds produce diffuse neuronal damage in the cerebral cortex, brain stem and spinal cord (Chang, 1987). The molecular mechanisms underlying these changes have not been elucidated. 3-Nitropropionic acid (3-NPA) causes selective neuronal damages in striatum (Hamilton and Gould, 1987) and hippocampus (Ray, et al. unpublished results, personal communications). Penitrem has been found to selectively attack cerebellar Purkinje and granule cells (Cavanagh et al., personal communications).

The selective damage in the brain stem by some neurotoxins is of great interest because the vacuolated lesions of gliovascular origin are common to many pathological

situations. Brain stem lesions can be induced by an antibiotic agent, metronidazole (Rogulja et al., 1973) and some nitro-aromatic and heterocyclic compounds, e.g. mononitrobenzene (Morgan et al., 1985), *m*-dinitrobenzene (*m*-DNB) (Philbert et al., 1987; Ray et al., 1992), misonidazole (Griffin et al., 1980). Analogous lesions can also be caused by 6-aminonicotinamide (Schneider and Cervos-Navarro, 1974) and 6-chloro-6-deoxyglucose (Jacobs and Ford, 1981), a putative antifertility agent. Similar lesions are seen in acute thiamine deficiency in rodents and man (Collins et al., 1970; Watanabe et al., 1981a,b). It has been speculated that what these chemicals may have in common is a potential either to disrupt energy supply in the brain or to drain the energy reserves used to fuel the brain's activities (Cavanagh, 1988; 1993). The initial cellular changes responsible for the spongiform lesions of acute thiamine deficiency (Wernicke's encephalopathy) are not understood, but there is involvement of a number of enzymes which require thiamine pyrophosphate as a cofactor, namely the pyruvate dehydrogenase complex, α-ketoglutarate dehydrogenase and transketolase of the pentose phosphate pathway (Butterworth et al., 1985). There is difficulty in producing acute thiamine deficiency in experimental animals but, in contrast, some analogous chemicals-induced lesions, such as *m*-DNB neurotoxicity are much easier to produce. *m*-DNB intoxication, with the involvement of vascular damage in the initiation of the symmetrical brain stem lesions, may be a very useful and more convenient model in study of brain stem lesions.

Oxidative stress in central nervous system

The brain and nervous system may be especially prone to damage by free radicals for a number of reasons (Halliwell and Gutteridge, 1985; LeBel and Bondy, 1991). First, the membrane lipids are very rich in polyunsaturated fatty acid side chains, which are especially sensitive to free radical attack. Second, the brain is poor in catalase activity and has only moderate amounts of superoxide dismutase (SOD) and glutathione peroxidase (Cohen, 1988). Third, several areas of the human brain (e.g., the globus pallidus and substantia nigra) are rich in iron (Youdim, 1988a,b), yet CSF has no significant iron-binding capacity because its content of transferrin is very low (Gruener et al., 1991).

Oxidative stress has been suggested to be relevant to a variety of neurological disorders (Ogawa, 1994; Beal, 1995; Dawson and Dawson, 1996), including ischemia, trauma (Braughler, 1989; Traystman et al., 1991; Yamada, 1994), and neurodegenerative diseases (Evens, 1993; Jenner, 1996), such as Parkinson's disease (Dexter et al, 1989; Jenner et al., 1992; Jenner, 1994; Owen et al., 1996; Jenner and Olanow, 1996) and Alzheimer's disease (Subbarao et al, 1990; Lyras et al., 1997). There is also increasing evidence of oxidative injury induced by Alzheimer's beta-amyloid peptide (Harris et al., 1995; Richardson and Zhou, 1996; Zhang et al., 1996).

A large number of neurotoxins can cause oxidative stress in the CNS. Toluene can induce the formation of reactive oxygen species (ROS) in hippocampus and striatum (Mattia et al., 1993). 3-nitropropionic acid, a plant mycotoxin causing selective striatal lesions, was reported to induce oxidative stress following free radical generation (Schulz et al., 1996). Ethylcholine aziridinium (AF64A), a toxic analogue of choline that disrupts high affinity choline transport and produces a persistent cholinergic hypofunction, can cause significant oxidative stress in brain (Gulyaeva et al., 1996). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) can cause oxidative stress and disturb antioxidant systems in striatum and brain stem (Desole et al., 1995). Oxidative stress may be responsible for glutamate-mediated excitotoxicity, in which NO is involved (Dawson and Dawson, 1996). Production of ROS by N-methyl-D-aspartate (NMDA) has been demonstrated in cultured murine cortical neurones. This production can be blocked by inhibitors of mitochondrial electron transport, rotenone or antimycin (Dugan et al., 1995). Several metal ions and organo metals can cause oxidative stress in brain, e.g. iron (Braughler et al., 1986), manganese (Soliman et al., 1995), aluminium (Julka and Gill, 1996), and methylmercury (Yee and Choi, 1996). Finally, *m*-DNB, which has been studied and reported in this thesis, has been suggested to induce brain stem lesions via oxidative stress (Ray et al., 1994; Romero et al., 1995; Romero et al., 1996).

The attempts to unify the diffuse discipline of neurotoxicology have led to the concept of final common pathways that characterise frequently occurring cellular responses to disruption of homeostasis resulting from exposure to xenobiotic agents (Bondy and Lebel, 1992). Such final common pathways in neurotoxicology may include features such as elevation of intracellular levels of free ionic calcium, disruption of

membrane function, failure of oxidative phosphorylation and induction of critical protooncogenes. Oxygen radicals seem to be mediators of a final common pathway in several mechanisms of neurotoxicity as discussed above. This means that it is important to distinguish between an early and causal oxidative stress, and a later and secondary oxidative process when considering pathogenesis.

Metabolic capacity of brain towards xenobiotics

It has long been appreciated that, in addition to being a major target for toxicological manifestations of exposure to a variety of chemicals, the liver is quantitatively the major organ involved in the *in vivo* metabolic disposal of most xenobiotics. However, especially during the past two decades, the importance of extrahepatic metabolism has been increasingly recognised, particularly with respect to its potential role in target organ and target cell toxicity.

A role for environmental toxins in the etiopathogenesis of certain neurodegenerative disorders has been suggested (Calne, et al., 1986). It was hypothesised that differential distribution of xenobiotic-metabolising enzymes in neural tissues may be a determinant of specific cell and/or regional toxicities (Mesnil et al., 1984; Ravindranath and Anandatheerthavarada, 1989; Minn et al., 1991; Lowndes et al., 1995). In addition to being a locus of pharmacologically significant metabolism of certain therapeutic agents, central nervous system cells may similarly be a potential locus of toxicologically significant *in situ* metabolism of certain compounds. Generally speaking, the *in situ* metabolism of foreign compounds in brain is mainly determined by three factors: firstly the access of the substrate to brain and the metabolising cells; secondly the existence of specific enzyme(s) and the affinity of the substrate to the enzyme(s); and thirdly the availability of essential cofactors such as NADPH and GSH.

The primary structural modification of the foreign compound is termed phase I metabolism, while the conjugations of the parent xenobiotics or its metabolite with endogenous substrates, such as glucuronic acid, glutathione or sulphate, are the so-called phase II reactions of drug metabolism. A number of drug-metabolising enzymes involved in both phase I and II metabolism have been found in the brain (Minn et al., 1991; Ravindranath and Boyd, 1995).

Monoamine oxidase

Monoamine oxidase (MAO) is a mitochondrial enzyme involved in the oxidative deamination of neurotransmitter biogenic amines such as serotonin, dopamine, norepinephrine and epinephrine, leading to the formation of respective aldehydes, ammonia and hydrogen peroxide. MAO, present in astroglial cells and in certain serotonergic and histamine-containing neurones, is of special interest due to its involvement in the metabolism of MPTP, an agent producing Parkinson's disease-like symptoms in humans (Davis et al., 1979) and non-human primates (Burns et al., 1983), with selective loss of dopaminergic neurones in the nigrostriatal pathway.

Flavin-containing monooxygenase

Flavin-containing monooxygenases (FMO) comprise a distinct subgroup of NADPH- and oxygen-dependent oxygenases which monooxygenate compounds containing soft nucleophiles such as nitrogen, phosphorus and sulphur (Ziegler, 1988). Several psychoactive drugs such as imipramine, chlorpromazine and trifluoperazine were found to be substrates for purified FMO (Ziegler, 1993; Hines et al., 1994). Immunohistochemical localisation of FMO in rat brain has been carried out using an antibody to rabbit lung FMO (Bhamre et al., 1993). The FMO enzyme was found predominantly in the neuronal cell bodies in hippocampus, brain stem, thalamus, and hypothalamus, and in the Purkinje cells of the cerebellum, but not in glial and vascular elements. A similar FMO distribution pattern was observed in human brain (Bhamre et al., 1995).

Cytochrome P450 and associated monooxygenases

The cytochrome P450 and associated monooxygenase systems are generally considered the most important class of drug-metabolising enzymes in the body. The microsomal P450 monooxygenase enzyme system has two important major components:

1. The heme protein, cytochrome P450.
2. the flavoprotein, NADPH cytochrome P450 reductase.

The mitochondrial P450 monooxygenase system consists of the heme protein and an FAD-containing flavoprotein (adrenodoxin) and the iron-sulphur protein ferredoxin. This system uses primarily NADPH as an electron source and the electrons are transferred through adrenodoxin to P450 (Ravindranath and Boyd, 1995).

The presence of NADPH cytochrome P450 reductase immunoreactivity in the rat and monkey brain preferably in neurones was demonstrated by use of antiserum to rat liver NADPH cytochrome P450 reductase (Haglung et al., 1984; Ravindranath et al., 1990; Ghersi-Egea et al., 1993; Ghersi-Egea et al., 1994). The existence of multiple forms of cytochrome P450 in rat brain has been reported by a number of groups (Sasame et al., 1977; Kapitulnik et al, 1987; Naslund et al., 1988; Warner et al., 1988; Iscan et al., 1990; Anandatheerthavarada et al., 1990; Volk et al., 1991; Ghersi-Egea et al., 1993; Schilter and Omiecinski, 1993; Lowndes et al., 1994; Bhagwat et al., 1995), although there is a slight inconsistency in the regional and cellular distribution of P450 among these reports. Generally speaking P450 exists in both neurones and glial cells; it exists in all brain areas but with higher level in brainstem (Ravindranath and Boyd, 1995). P-450 and NADPH cytochrome P-450 reductase seem to be co-localised in the brain (Ravindranath et al., 1990). There is only one report on the subcellular localisation of P-450 in the brain which showed its main localisation to be mitochondrial (Walther et al., 1986). The intense immunostaining for P450 observed in the brainstem coupled with the low levels of the antioxidant glutathione known to be present in the brainstem may render this region particularly vulnerable to damage through bioactivation of xenobiotics.

Glutathione S-transferase

Glutathione S-transferases (GSTs) belong to a supergene family of enzymes. In the rodent the isoforms of GST have been broadly grouped into classes, designated α, μ and π (Pickett and Lu, 1989). These three classes vary in their subunit composition (homodimers and heterodimers) and in affinities for different substrates, although they overlap in specificities. The GSTs are found in relative abundance in mammalian liver, testis, adrenal glands, intestine and kidney where their role in phase II conjugation of endogenous and xenobiotic metabolites has been extensively investigated (Sies, 1988). Localisation of specific classes of GSTs in certain cell types in brain has been demonstrated and documented (Lowndes et al., 1994): α-GST in neuronal nuclei in the neocortex, cerebellum, hippocampus and brainstem (Carder et al., 1990; Johnson et al., 1993; Philbert et al., 1995), μ-GST in astrocytes (Cammer et al., 1989; Cammer and

Zhang, 1992a; Philbert et al., 1995), π-GST in oligodendrocytes (Cammer et al., 1989; Cammer and Zhang, 1992b; Philbert et al., 1995).

γ-Glutamyl transferase

γ-Glutamyl transferase (γ-GT) is a membrane-bound enzyme which catalyses cleavage of the γ-glutamyl bond of glutathione and transfers the γ-glutamyl moiety to other amino acids or peptides. This reaction helps in the recycling of the glutamyl residue after the leakage of glutathione conjugate. γ-GT ranging from 74 kD to 234 kD in molecular weight have been reported in rat brain (Reyes and Barela, 1980). It exists mainly in non-neuronal elements of the central nervous system, particularly ependymal cells and choroid plexus (Sessa et al., 1976; Philbert et al., 1995).

Toxicities of *m*-dinitrobenzene

m-Dinitrobenzene (*m*-DNB) is a nitroaromatic compound widely used in the synthesis of dyes, plastics and explosives. Exposure to this compound results in acute toxicity and methaemoglobinemia in both humans (Beritic, 1956; Isihara et al., 1976) and laboratory animals (Watanabe et al., 1976). In humans, *m*-DNB exposure through the working environment can result in methaemoglobinaemia, testicular atrophy, and diverse neurological sy mptoms, such as headache, confusion, vertigo, nausea, loss of cognition, hyperalgesia, paraesthesia and polyneuritis (Von Burg, 1989). When *m*-DNB (1% suspension in corn oil) was given to rats orally, the LD50 was 83 mg/kg and the compound was equally toxic in both sexes (Cody et al., 1981). *m*-DNB at a dose of 10 and 20 mg/kg produced 6.6 and 10.2 g of methaemoglobin per 100 ml blood (Kiese, 1947). It was suggested that *m*-nitrosonitrobenzene or *m*-nitrophenylhydroxylamine, the active metabolites from *m*-DNB reduction, enter the erythrocyte and thus induce methaemoglobin *in vivo* (Kiese, 1974). After the exposure, the divalent iron in haemoglobin is oxidised to trivalent form which results in the inability of haemoglobin to carry molecular oxygen. *In vitro* in isolated erythrocytes from rats, Rhesus monkeys and humans, all three isomers *o*- *m*- and *p*-DNB induced methaemoglobinemia . However *m*-DNB did not conjugate with glutathione in the erythrocytes while *o*- and *p*-DNB did. With different species, *m*-DNB induced higher level of methaemoglobin in rat erythrocytes than in monkey and human erythrocytes (Cossum and Rickert, 1987). Without the formation of adduct, these isomers of DNB were found to induce a direct

conversion of deoxyhaemoglobin to methaemoglobin, but no conversion of oxyhaemoglobin (Vasquez et al., 1995). This implies that the reaction of DNBs with haemoglobin may require direct access to the heme and/or that the reaction is initiated by oxidation of the heme, which occures more readily in the deoxy state.

m-DNB causes testicular toxicity in rats (Cody, et al., 1981; Linder et al., 1986; 1990; Hess et al., 1988; Obasaju et al., 1991) and mouse (Evenson et al., 1989). Ultrastructural analyses of the testicular lesions have suggested that the Sertoli cell was the primary target for *m*-DNB toxicity and that germ cell damage occurred subsequent to Sertoli cell toxicity (Foster et al., 1986; Blackburn et al., 1988). The testicular toxicity was found only at particular stages of spermatogenesis, with one report indicating damage only to seminiferous tubules in stages VIII to XI (Blackburn et al., 1988) and another finding lesions in stages VII through XIII (Hess et al., 1988). Species differences in susceptibility to *m*-DNB-induced testicular toxicity have been noted, with the mouse showing less damage than the rat (Evenson et al., 1989). The hamster was resistant to the testicular effects of *m*-DNB, showing no evidence of testicular damage at dose levels at which there was marked damage in the rat (Obasaju et al., 1991).

It has been proposed that differences in *m*-DNB metabolism as well as pharmacokinetics may provide at least a partial explanation for species differences in toxicity (Obasaju et al., 1991). In the hamster the rate of *m*-DNB elimination was slower and blood levels of metabolites were lower than in the rat (McEuen and Miller, 1991). Older rats (75 and 120 day old) were found more susceptible to *m*-DNB-induced testicular damage than 30 day old rats. In those older animals the *m*-DNB half lives (3.29 and 4.53 h respectively) are much longer than in young animals (1.91 h) (Brown et al., 1994).

In experimental intoxication with *m*-DNB, symmetrical morphological changes in the brain stem associated with a variable degree of ataxia have been reported in rats (Philbert et al., 1987). The symmetrical damage was characterised by spongy lesions of astrocytic vacuolation and perivascular oedema, followed by neuronal involvement (Philbert, et al., 1987, Ray, et al., 1992). The vascular involvement was indicated by petechial haemorrhages and focal leakage of injected horseradish peroxidase tracer in the inferior colliculi, cerebellar roof, vestibular and superior olivary nuclei with astrocyte swelling and retraction seen at 12 h after the last dose of a three dose regimen

(Romero, et al., 1991). In time order, the increase in blood flow and methaemoglobinemia occurred prior to morphological changes, and the blood flow increase has been demonstrated to show a close association with those regions expected to develop lesions. Haemorrhages and astroglial damage occurred afterwards, which were then followed by secondary neuronal damage.

Working hypotheses and experimental strategies

The neurotoxicity of *m*-DNB may not be a very serious concern in occupational medicine, but elucidation of the mechanism underlying its neurotoxicity will surely enhance understanding of other similar brain stem lesions, such as those caused by acute thiamine deficiency and the other analogously selective brain stem lesions mentioned earlier.

The mechanism underlying the selectivity of *m*-DNB-induced brain stem lesions is unclear, but what all the damaged areas have in common are a high metabolic demand and a low level of glutathione (GSH) (Cavanagh, 1993). Intoxication with *m*-DNB leads to increased cerebral glucose utilisation which, together with stimulation of lactate production prior to vascular damage or lesion development, were suggested to be pre-cytotoxic effects (Romero et al., 1995). Metabolic stimulation does not, of itself, lead to neurotoxicity. However neuronal activity and its metabolic consequences, both up and down, modulate DNB neurotoxicity (Ray et al., 1992; 1996). *In vitro* reduced glutathione(GSH) depletion and oxidative stress have been demonstrated in *m*-DNB intoxicated astrocytic cultures (Romero et al., 1995). Pretreatment of rats with large doses of vitamin E protected against *m*-DNB neurotoxicity (Lowndes et al., 1994). Therefore, it has been suggested that free radical-induced oxidative stress might be responsible for the brain stem lesions caused by *m*-DNB (Ray et al., 1994; Romero et al., 1995; Romero et al., 1996).

This PhD project has been proposed to research two mechanisms:

1. Biochemical mechanism of *m*-dinitrobenzene (DNB) neurotoxicity, with emphasis on the oxidative stress;

2. Mechanism underlying the selectivity of DNB neurotoxicity, with emphasis on endogenous antioxidants, especially GSH.

These mechanisms have been studied by two approaches. One approach is to investigate the metabolic fate of DNB in the brain *in situ*; Brain slices have been used to test the metabolism of DNB in brain tissue. This *in vitro* testing system has an advantage over *in vivo* study since it excludes possible metabolic contributions from liver and other organs. The other approach is to look at the role of GSH in DNB induced neurotoxicity by manipulating brain GSH levels and then assessing the alterations of brain susceptibility.

In the course of investigating these mechanisms, some interesting phenomena relating to brain GSH were observed in the animal model of brain GSH depletion. Considerable research effort was made to study these phenomena further. These studies, in turn, not only improved our understanding of the involvement of GSH in brain susceptibility, but also provided evidence about additional functions of GSH in the central nervous system.

CHAPTER 2

METABOLISM OF *M*-DINITROBENZENE

(LITERATURE REVIEW 1)

As mentioned in chapter 1, *m*-Dinitrobenzene (*m*-DNB) is a nitroaromatic compound which is a potent inducer of methaemoglobinaemia (Beritic, 1956; Isihara et al., 1976; Watanabe et al., 1976) and a testicular toxicant (Cody, et al., 1981; Linder et al., 1986, 1990; Obasaju et al., 1991; Evenson et al., 1989). It is also a neurotoxin causing selective brain stem lesions in experimental animals (Philbert et al., 1987; Ray et al., 1992). The lesions in brain stem are characterised by astrocyte swelling and retraction, vascular dysfunction and bioenergetic disturbance in *m*-DNB intoxicated rats (Romero et al., 1991; 1995). However, the mechanism underlying *m*-DNB-induced brain stem lesions as well as the testicular toxicity is unclear. Comprehension of *m*-DNB metabolism, particularly the metabolic fate of *m*-DNB and its metabolites in brain is important to the understanding of the mechanism of its neurotoxicity. This review summarises the available information in respect of the metabolism of DNB, including absorption, distribution, catabolism and excretion. The results derived from this research project are not included in the review because they will be shown and discussed in the later chapters.

Absorption and excretion of *m*-DNB after oral administration

m-DNB can be absorbed by the skin (Isihara et al., 1976) and gastrointestinal tract (Beritic, 1956; Parke, 1961; Philbert, 1987). In a bioavailability study in F344 rats, Philbert (1988) measured *m*-DNB and its two metabolites, *3*-nitroaniline and *3*-nitroacetanilide in blood, urea and faeces using gas chromatography. By orally giving ^{14}C-*1,3*-dinitrobenzene (3.5 μCi/rat) together with 10 mg /kg non-radiolabled *m*-DNB to rats, the author found that 70% (51% contributed by faeces, 19% by urine), 96% (75% by faeces, 21% by urine) and approximately 100% (80% by faeces and 22% by urine) of dose radioactivity were excreted at 24, 48, and 72 h respectively in conventional rats, while the rate of excretion of radioactivity in germ free rats was much slower. Urine seemed to be the main route of excretion in germ free rats but faeces seemed to be the main route in conventional rats. Comparison of the data derived from

germ free rats with that from conventional rats indicates that intestinal microflora could markedly reduce the absorption of *m*-DNB. The major portion of radioactivity excreted from faeces in conventional rat indicates that most of the radiolabel originating form the oral dose could be retained in gastrointestinal tract due to existence of microflora and then excreted. The differences in absorption properties of gut wall between germ free and conventional rats might be another reason for the difference in *m*-DNB absorption.

Parke (1961) studied the metabolism of *m*-DNB in rabbits by giving doses of *m*-DNB and *m*-dinitro *[^{14}C]* benzene by gavage at a dose of 50-100 mg/kg. 65-93% of the dose was excreted in the urine during the first two days and only 1-5% in the faeces. These results were markedly different from Philbert's results derived from conventional rat (Philbert, 1988), although the total excretion percentage of the administrated dose at 48 h is quite close in these two studies. Comparable with Philbert's (1988) results, Nystrom and Richert (1987) reported that 81.3% and 7.6% of dose were excreted in the faeces and urine respectively over the 48 h period in rats after an oral dose of 25 mg/kg in rats. Not all the excreted DNB in faeces is necessarily that left by non-absorption, part of it may come from biliary excretion. This is supported by Brown et al. (1994), who reported that 53-68% of an i.p. dose of *m*-DNB to rats was excreted in urine over 48 h, while 17-30% was in faeces.

Distribution and pharmacokinetics of *m*-DNB in experimental animals

A great difference in tissue distribution was observed by Philbert (1988) with a decreasing order of radiolabel in blood, plasma, pancreas, stomach, liver, kidney and other organs or tissues in conventional rat at 24 h after a single dose of *m*-DNB. In germ free rats, the decreasing order was liver, kidney, white fat, brown fat, pancreas, sciatic nerve, whole blood and lung. However it should be pointed out that the measurement of radiolabel concentration did not exclude any contribution from metabolites. Therefore the distribution differences described above should be carefully interpreted.

Compared with other tissues, central nervous system attained a relatively lower concentration after the rat received oral dose of 25 mg/kg *m*-DNB spiked with *[14C]1,3*-dinitrobenzene and no significant difference in *m*-DNB concentration was observed among spinal cord, cerebellum, cortex and brain stem (Philbert, 1988). Xu and Ray (1996, personal communication) also showed no significant differences between brain

areas. These results indicated that selective brain stem damage is unlikely to be due to differential tissue uptake of *m*-DNB.

In conventional rats, *m*-DNB concentration in the blood arrived at a peak value of 3.5 μg/ml at 2 h after oral administration of 25 mg/kg body weight, then sharply declined to 0.4 μg/ml at 24 h. In contrast, a higher peak concentration of *m*-DNB (7.0 μg/ml) in the blood of germ-free rats was at 3 h, then steadily declined to 3.5 μg/ml at 18 h with the same dose (Philbert, 1988).

Brown et al. (1994) studied the pharmacokinetics of *m*-DNB in 31, 75, and 120 day old male Sprague-Dawley rats and found that half lives of *m*-DNB in blood were 1.91, 3.29, and 4.53 h respectively in these three groups of rats, a difference which is statistically significant. The elimination of *m*-DNB from blood was characterised by an initially rapid decrease in blood levels followed by a slower elimination phase (McEuen and Miller, 1991; Brown et al., 1994) after single i.p. administration of *m*-DNB. One of the main metabolites, nitroaniline, reached its peak concentration in blood at 1 h in 31 and 75 day old rats, but 2 h in 120 day old rats. A similar pattern was also seen for another two metabolites, nitroacetanilide and diacetamidobenzene (Brown et al., 1994). Most recently, Xu and Ray (1996, personal communication) conducted a comprehensive kinetic study and observed the pharmacokinetics of *m*-DNB and its metabolites in various tissues, especially in different brain areas in rat; their results were consistent with those above and, they also demonstrated the importance of both concentration threshold and time threshold in determining the onset of DNB neurotoxicity.

Metabolic degradation of *m*-DNB in *in vivo* and *in vitro*

m-Nitroaniline (Belaborodova, 1945) and *m*-nitrophenylhydroxylamine (Lipschitz, 1920) were observed in rabbit tissues after doing with *m*-DNB. In addition to these early studies, Parke (1961) made an investigation in rabbits. After oral administration of *m*-dinitro[*14C*]benzene at the dose of 2-5 μCi per rabbit, together with 50-100 mg/kg non-radiolabelled *m*-DNB, urine was collected over two days for detecting metabolites. The principal metabolites in the urine were *2,4*-diaminophenol (31% of dose), *m*-phenylenediamine (21%), *m*-nitrophenol (14%), *2*-amino-*4*-nitrophenol (14%), and *3*-nitroaniline (14%). Traces of unchanged *m*-DNB (0.7%), *2,4*-dinitrophenol (0.1%), *4*-amino-*2*-nitrophenol (0.8%), *m*-nitrophenylhydroxylamine

(0.8%), *m*-nitrosonitrobenzene (0.25%) and *3,3'*-dinitoazozybenzene (0.3%) were also detected. As mentioned before, *3*-nitroaniline and *3*-nitroacetanilide were seen in the blood of rat with peak concentrations at 4 h after an oral dose of 25 mg/kg body weight (Philbert, 1987). Nystrom and Rickert (1987) detected eight metabolites in the sulfatase-treated urine from male F344 rats which had received an oral dose of *m*-DNB (25mg/kg). Among those metabolites, four were identified as *3*-nitroaniline, *4*-acetamidophenol, *3*-aminoacetanilide, and *1,3*-diacetamidobenzene. In a separate study, *3*-nitroaniline, *1,3*-diacetamidobenzene, *2,4*-diacetamidophenol, *4*-nitro-*2*-acetamidophenol, and *2*-nitro-*4*-acetamidophenol were detected in rat urine after i.p. doing with 25 mg/kg *m*-DNB (Brown et al., 1994).

In addition to these *in vivo* studies, dinitrobenzene metabolism has also been investigated *in vitro* using isolated erythrocytes (Cossum and Rickert,1987), hepatocytes (Cossum and Rickert, 1985) and testicular cells (Foster et al., 1987; Brown and Miller, 1991). Reduction of *m*-DNB to *m*-nitrophenylhydroxylamine has been demonstrated *in vitro* in rabbit muscle (Comel, 1931) and blood (Lipschitz, 1948). Cossum and Rickert (1987) isolated erythrocytes from rat, monkey and human, and incubated them with three isomers of dinitrobenzene (*o*-DNB, *p*-DNB, and *m*-DNB) at 37 ^{o}C for 120 min open to the air. The erythrocytes from the three sources were able to metabolise *o*-DNB and *p*-DNB to *s*-(*-2*-nitrophenyl)glutathione (37% of added *o*-DNB) and s-(*-4*-nitrophenyl)glutathione (6% of added *p*-DNB) respectively as well as 4-nitrophenylhydroxylamine (10% of added *p*-DNB) as a metabolite of *p*-DNB. Surprisingly *m*-DNB could not be metabolised by these erythrocytes. A further product of *s*-(*-2*-nitrophenyl)glutathione conjugate, *s*-(*2*-nitrophenyl)-*N*-acetylcysteine, was found to be the major metabolite in the urine of male Fischer-344 rats after an oral dose of *o*-DNB (Holtzman et al., 1981), which confirmed its conjugation with glutathione. Only 2% of the added *m*-DNB became covalently bound to erythrocyte macromolecules in 30 min in marked contrast to 24% of added *o*-DNB and 40% of added *p*-DNB bound.

The metabolism of the three isomers of dinitrobenzene has also been studied in isolated rat hepatocytes (Cossum and Rickert, 1985). The major metabolite formed during incubation of *m*-DNB with hepatocytes was nitroaniline which represented 74% of the total radioactivity after a 30 min incubation. No conjugation of *m*-DNB with glutathione was observed in the incubation with microsomes or cytosol. Compared with

the metabolic rates of *o*-DNB and *p*-DNB in terms of reduction, that of *m*-DNB was 3-5 times slower. Reduction of *m*-DNB to *3*-nitroaniline occurred in microsome incubations but not in cytosol, which means that the microsome is a major metabolic site for *m*-DNB metabolism in the hepatocytes. Incubation of a larger amounts of microsomal protein and a higher substrate concentration of *m*-DNB led to formation of nitrosonitrobenzene within 5 min. The metabolism of *m*-DNB in microsomes was found to be NADPH-dependent, which suggests the involvement of NADPH-cytochrome P-450 reductase in the metabolism. However it cannot be excluded that in intact cells mitochondrial enzymes may also play a role because Abou-Khallil et al (1985) have shown that *p*-DNB could be aerobically reduced to *p*-nitroaniline in an exclusively NADH-dependent manner in mitochondria.

Similar to the metabolism in hepatocytes, *m*-DNB can be metabolised to 3-nitroaniline, 3-nitrophenylhydroxylamine, and 3-nitrosonitrobenzene by rat testicular subcellular fractions (Ellis and Foster, 1992).

It is likely that *m*-DNB is mainly metabolised in the liver, but little is known about its extra-hepatic metabolism except for that in blood and testis. Because of its neurotoxicity, it is very important to know what is the fate of *m*-DNB or its metabolites in the brain. Romero et al. (1994) reported that *m*-DNB could intoxicate primary cultured astrocytes and alter glutathione metabolism. These result imply that *m*-DNB could possibly be metabolised by astrocytes and thus disturb some important biomolecules (e.g. glutathione). The metabolic fate of *m*-DNB in brain has been investigated and reported in this thesis (Chapter 5).

Further degradation of *m*-DNB metabolites

Some of the DNB metabolites can be further degraded if conditions permit. The following is a summary of some information on the further metabolism of 2,4-dinitrophenol and 3-nitroaniline, which may be of help in understanding the comprehensive metabolic pathway.

2,4-Dinitrophenol

Dinitrophenol is regarded as an inhibitor of phosphorylation in various enzyme systems and has been shown to affect the normal metabolism of tissues *in vitro* (Parker, 1952). Magne et al (1932) reported that the excretion products in the urine from man and dog were unchanged dinitrophenol and *2*-amino-*4*-nitrophenol after exposure to *2,4*-

dinitrophenol. The latter metabolite was demonstrated to be the main product when *2,4*-dinitrophenol was acted on by a bacterial succinic dehydrogenase system. By incubating liver homogenate, Parker (1952) found that *2,4*-nitrophenol was converted to two metabolites, *4*-amino-*2*-nitrophenol and *2*-amino-*4*-nitrophenol. Furthermore, by incubating these two compounds with liver homogenate, Parker (1952) also found that *4*-amino-*2*-nitrophenol rapidly disappeared. This explains why *2*-amino-*4*-nitrophenol appears in urine as a predominant reductive product of *2,4*-dinitrophenol and also explains why the presence of these two isomers in urine was different in rats treated with *m*-DNB (Parke, 1961).

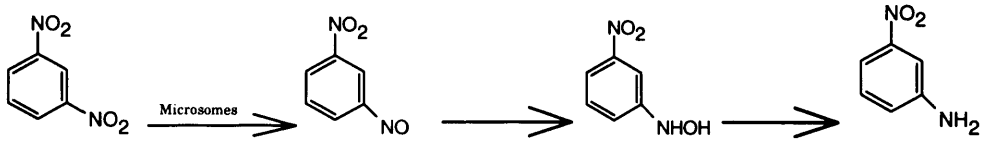
Reductive activities of tissues are different towards *2,4*-dinitrophenol. In rat the relative reduction activity of tissues is, in decreasing order: liver (100%), kidney (60%), spleen (59%), intrascapular fat (47%), heart (29%), muscle (16%), brain (3%) and blood (0%). In rabbit, the tissue activity in decreasing order is: liver (100%), kidney (41%), heart (3%), spleen, brain and intrascapular fat (0%). Obviously, brain has very little if any reductive activity towards *2,4*-dinitrophenol.

Nitroaniline

As one of the major metabolites, further metabolism of *3*-nitroaniline has not been specifically studied. However, metabolism of its isomer, *4*-nitroaniline has been investigated by several authors (Mate, 1967, Smith and Gorrod, 1978, Anderson et al, 1984). In both *in vivo* and *in vitro* studies, *2*-hydroxy-*4*-nitroaniline was demonstrated to be a major oxidative metabolite and was found to be more toxic. This metabolic step occurred in liver microsomes, required NADPH, and could be inhibited by a cytochrome P-450 inhibitor.

Possible metabolic pathway of *m*-DNB

Cossum and Rickert (1985) proposed the following metabolic pathway of *m*-DNB based on their research on rat hepatocytes:



m-DNB *3*-nitrosonitrobenzene *3*-nitrophenylhydroxylamine *3*-nitroaniline

Obviously, this scheme does not cover all the metabolites observed by various authors. In a review article, Rickert (1987) made another suggestion, which was more comprehensive but still left out some important information. Based our study, and taking into account all updated data as well as Rickert's (1987) suggestions, a comprehensive pathways has been proposed and is shown in Chapter 5.

Metabolism of *m*-DNB and possible free radical generation

It is well known that superoxide and nitro anion radicals can be generated in the nitroreduction process, under the action of xanthine oxidase and NADPH-supported microsomal reductases, e.g., NADPH- cytochrome P-450 reductase (Mason and Holtzman, 1975). Nitroreductase activity was demonstrated in hepatic microsomes (Fourts and Brodie, 1957), which is either NADH or NADPH dependent, inhibited by oxygen but markedly stimulated by flavines (Gillette 1971, Weisburger and Weisburger, 1971). This enzyme system has been demonstrated to exhibit little substrate specificity. Generally speaking, the reduction entails electron transfer from the enzyme system to the nitrobenzene moiety with ultimate formation of amine (Mason and Holtzman, 1975).

$$Ph\text{-}NO_2 \rightarrow Ph\text{-}NO_2^{\cdot -} \rightarrow Ph\text{-}NO \rightarrow Ph\text{-}NH_2$$

The first intermediate in the reduction is the nitrobenzene anion radical since microsomal flavines and cytochromes are assumed to be one-electron donors (Iyanagi and Yamazaki, 1969). This nitrobenzene anion radical has been demonstrated by electron spin resonance (ESR) when microsomes, mitochondria or a flavine-containing model system was anaerobically incubated with an appropriate electron-donating cofactor and nitrobenzene (Mason , 1974, Peterson et al, 1979). The nitrobenzene anion radical could only last for a few seconds, before then receiving another electron to become the corresponding nitroso compound. Subsequent, further reduction produces phenylhydroxylamine which accumulates during the formation of the amine product (Kato et al., 1969).

Mason (1975) found that without heme-containing compounds, the anion radical could still be produced. So it was suggested that the formation of nitroaromatic anion radicals may be mediated through flavine, not essentially cytochrome P-450.

In the presence of oxygen the first product of reduction, nitroaromatic anion free radical, can be oxidised and regenerated to parent nitro compound while the molecular oxygen receives the single electron from the nitro anion radical to form superoxide (Mason, 1975), as shown below.

$$RNO_2^- + O_2 \rightarrow RNO_2 + O_2^-$$

The generation of free radicals by nitroreduction does not necessarily require enzyme involvement. Rao et al. (1987) reported that nitro anion radicals could be formed by nitroreduction with ascorbate, and superoxide anion radical and hydrogen peroxide could also be produced by reaction of these radicals with oxygen.

Therefore at least two types of free radicals, nitro anion and superoxide can possibly be produced in the metabolism of *m*-DNB. It has been suggested that *m*-DNB-induced brain stem damage might be due to oxidative stress induced by free radicals (Romero et al., 1991, 1995; Ray et al., 1994). If this suggestion is true, the next question to answer is where the free radicals come from. It is unlikely that free radicals are generated in liver and then transferred to brain. Therefore the *in situ* metabolism of *m*-DNB and its metabolic fate in brain is worthwhile investigating.

CHAPTER 3
METABOLISM AND FUNCTIONS OF GLUTATHIONE IN THE CENTRAL NERVOUS SYSTEM
(*LITERATURE REVIEW 2*)

Glutathione, a tripeptide, is widely distributed in human and animal tissues, plants and microorganisms (Meister, 1988). Present at 0.1-10 mM, GSH is the most prevalent cellular thiol and the most abundant low molecular weight peptide. In many cells GSH accounts for more than 90% of the total nonprotein sulphur.

Glutathione (GSH) has been studied for over 100 years (Meister, 1989) and it is well known that glutathione functions in redox system as an endogenous antioxidant and also functions in the phase I and II metabolism of xenobiotics as a co-factor or conjugation reagent (Meister, 1983). The toxicological implications of GSH have been briefly summarised in the general discussion chapter and have also been reviewed by Reed (1990) and Smith et al. (1996). After we observed unexpected phenomena in BSO-treated rats we started to pay attention to alternative functions of GSH in brain. Brain GSH has received increasing interest due to its possible involvement in neurodegeneration and neuronal activity modulation. However the latest literature review specifically on GSH in the CNS is that of Orlowski and Karkowsky (1976) two decades ago. I have therefore made this review which includes experimental evidence derived from this thesis.

GSH metabolism and its biological importance

GSH has two distinct characteristic structural features: a γ-glutamyl bond and a sulphhydryl group, which lay the basis for the following general biological functions.

1. GSH protects cells from the toxic effects of reactive oxygen species and other free radicals.
2. GSH is an important component of the system that uses reduced pyridine nucleotide to provide the cell with it reducing properties; this promotes intracellular formation of cysteine from cystine and the thiol forms of proteins.

3. GSH functions in catalysis, metabolism, and transport. It participates in reactions involving the synthesis of proteins and nucleic acids.
4. GSH forms conjugates with a variety of compounds of endogenous and exogenous origin and is a cofactor for various enzymes, such as glyoxylase.
5. GSH serves as a storage and transport form of cysteine moieties and protects thiol group in proteins.
6. GSH may be a neuropeptide modulating excitability of the central nervous system.
7. GSH may also be involved in the maintenance of cerebrovascular function.

A large number of enzymes are involved in GSH metabolism and functions. They can be summarised as following according to their function.

GSH synthesis: γ-glutamylcysteine synthetase (γ-GCS) (Reaction 1) and GSH synthetase (Reaction 2).

L-Glutamate + L-cysteine + ATP ⟶ L-γ-glutamyl-L-cysteine + ADP + Pi **(1)**

L-γ-Glutamyl-L-cysteine + glycine + ATP ⟶ Glutathione + ADP + Pi **(2)**

GSH degradation: γ-glutamyltranspeptidase (γ-GT) (Reaction 3), dipeptidases (Reaction 4), γ-glutamylcyclotransferase (Reaction 5).

Glutathione + amino acid ⟶ cysteinyl-glycine + γ-glutamyl-amino acid **(3)**

Cysteinyl-glycine ⟶ cysteine + glycine **(4)**

γ-Glutamyl-amino acid ⟶ 5-oxoproline + amino acid **(5)**

GSH-GSSG cycle: GSH peroxidase (Reaction 6), glutathione disulphide (GSSG) reductase (Reaction 7).

$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$ **(6)**

$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+$ **(7)**

GSH conjugation: GSH S-transferases (Reaction 8).

GSH + eletrophilic substance X ⟶ γ-Glu-(Cys-X)-Gly **(8)**

Regional distribution of GSH in brain

GSH is unevenly distributed across brain regions, highest in striatum and hippocampus followed by cerebral cortex, cerebellum and thalamus; lowest in brain stem (Pileblad et al, 1990; Adams et al., 1991; Shivakamar et al, 1992; this thesis). This distribution profile is mainly based on the measurement of GSH levels in microdissectied tissues. Cellular GSH distribution as well as regional distribution in brain has been successfully shown by qualitative histochemical staining with mercury orange and *o*-phthaldialdehyde (Slivka et al., 1987; Philbert et al., 1991). This will be discussed later.

Why do striatum and cortex have higher levels of GSH than brain stem? There may be a functional reason which requires this. The other question is: what is the biochemical mechanism to maintain the different levels in those regions? If striatum requires the highest GSH level for a certain function, should it be more functionally susceptible to GSH depletion than all other brain areas?

Using intracerebroventricular or intrathecal administration of *L*-buthionine-S,R-sulphoximine (BSO), one research group noticed that the maximal depletion was in brain stem (Shivakamar et al, 1992), while an group reported a different turnover of GSH between substantia nigra and striatum (Pileblad E. et al, 1990). Our study showed dramatic heterogeneity in GSH half lives across brain areas, being much faster in brain stem than in other areas.

According to their level of BSO-induced GSH depletion, brain areas can be divided into sensitive and resistant areas. There are two possibilities in explaining why cortex and other areas show lesser depletion by BSO than that in brain stem. Firstly they may have a bigger mitochondrial GSH pool which is less affected by BSO and has a slow turnover; secondly, an alternative pathway of GSH synthesis (from cysteinylglycine to GSH) may be more active than that in brain stem. In other word, GSH synthesis by the pathway of r-GC synthetase might account for a relatively smaller proportion of its total synthesis than in brain stem. The greater depletion in brain stem may also indicate greater susceptibility to oxidative stress.

GSH half life is 5 hours in primary cultured astrocytes which was evaluated after those cells were treated with BSO (Devesa, et al 1993). Since GSH exists mainly in glial

cells, turnover in astrocytic cultures may represent the *in vivo* situation to a certain extent.

Cellular and subcellular distributions of GSH in the brain

As pointed out by Smith et al. (1996), despite the extensive evidence implicating the depletion and/or oxidation of glutathione in a wide variety of human and experimental toxicities, critical examination of such studies frequently reveals that injury is not simply related to glutathione status. This is because GSH is compartmentalised at several levels and the compartmentation appears to exert considerable influence on the relationships between glutathione depletion or oxidation and the onset of injury.

Generally speaking, neuronal cells do not contain appreciable amounts of GSH except for cerebellar granule cells. The primary location of GSH is in the non-neuronal components of the neuropil and white matter tracts (Philbert et al., 1991). The enzymes associated with GSH functions seem to be co-localised with the tripeptide. Glutathione peroxidase and glutathione-S-transferases (GSTs) have both been observed in astrocyte foot processes, oligodendrocytes, ependymal cells, subventricular zone cells and tanycytes (Ushijima et al., 1986; Abramovitz et al., 1988; Cammer et al., 1989). γ-Glutamyl transpeptidase immunoreactivity was confined to non-neuronal elements in the CNS (Philbert et al., 1995).

GSH appears to be synthesised primarily, if not exclusively, in the cytoplasmic compartment of cells, yet is distributed and utilised in many other compartments, including the nucleus, mitochondrial matrix, endoplasmic reticulum and extracellular spaces (Meister 1991). Recently, the subcellular distributions of GSH and its related enzymes in cultured cerebellar astrocytes and cerebellar granule cells were studied (Huang and Philbert, 1995). Cytosolic GSH levels and cytosolic activities of glutathione reductase, glutathione peroxidase and glutathione-S-transferase in astrocytes were 57, 153, 245, and 92% higher than those found in granule cells respectively. Subcellular distributions of GSH are not the same in different cell populations. Even though astrocytes contain markedly higher whole intracellular and cytosolic GSH levels, their mitochondrial GSH pool is smaller than that of granule cells. The mitochondrial GSH contents in astrocytes and granule cells account for 2.4% and 4.7% of the respective

total cellular GSH, which is much smaller than those reported for isolated hepatocytes (10-15%) (Reed, 1990).

It seems impossible to completely deplete brain GSH. The reason for this is not clear because the multiple-pool theory (Jain et al., 1991) cannot satisfactorily explain this. If a second or a third pool was a slow turnover one, it should still be depleted provided they inter-communicate. However it appears that this is not the case (Pileblad and Magnusson, 1989), as a total dose of 5 × 3.2 mg BSO over a period of 12 days could not completely deplete all GSH. There must be a mechanism to maintain GSH which is not affected by inhibition of γ-GCS with BSO. One possibility is that if GSH is broken down to form cysteinylglycine, the product can be used for the re-synthesis of GSH to some extent though this may not be a major route of GSH synthesis.

Age dependent change of brain GSH levels

Brain GSH is low in neonates (Jefauconnier et al, 1976; Bien et al., 1990; Kudo et al., 1990; Glockner and Kretzschmar, 1991; Vina et al., 1995), reaches adult levels by 36 days in F344 rats, and decreases with aging (Benzi et al., 1988; Ravindranath et al., 1989; Iantomasi, 1993). This profile is also confirmed in this thesis.

With a histochemistry study in rats using mercury orange, Beiswanger et al (1995) reported that both neuronal and glial progenitor cells stain uniformly positive for GSH at embryonic day 13 and 17, but neurones lost GSH staining at postnatal day 5.

This age-dependent profile of brain GSH may due to highly regulated GSH synthesis. Even if as we think, the increase of brain GSH in development is because of an increasing requirement, it would be most unlikely that the decrease of brain GSH in the course of ageing is due to a lower demand. Two possible factors may be linked to the regulation of brain GSH synthesis: growth factors and bioavailability of cysteine.

Recently, epidermal growth factor (EGF) has been found to increase intracellular levels of GSH and cysteine in vascular endothelial cell cultures (Yang and Hu et al., unpublished data). Interestingly they also increased intracellular cysteine levels. These results suggest that growth factors increase the cellular uptake of cysteine, in addition to their action on the synthetic enzymes such as γ-GCS (Pan and Perez-Polo, 1993). Cysteine uptake has been suggested to be a rate-limiting step in GSH synthesis (Bannai, 1986; Griffith,1987). Our study (see chapter 7) also revealed that brain cysteine does not

reach the adult level until brain GSH reaches at the adult level. This suggests that cysteine may be the rate-limiting factor for GSH synthesis in neonatal brain. Cysteine is not an essential amino acid in healthy adults because adequate amounts of cysteine can be provided by transsulphuration of methionine. However it may be an essential amino acid for infants, especially for premature or low body weight infants (Sturman et al., 1970; Zlotkin and Anderson, 1982; Vana et al., 1995).

Even though GSH levels are low in the early stages of life, neonates are not necessarily suffering from oxidative stress due to the low level of brain GSH despite brain metabolic rate being somewhat higher than adult values (Mcllwain, ----). This may indicate that in the adult, GSH may has other functions than as an antioxidant.

Manipulation of brain GSH levels

One of the useful approaches to assess the functions of a cellular component like GSH is to deplete it and then assess the consequences. The understanding of the biological functions of GSH has been greatly promoted by the studies in which relevant enzymes were selectively inhibited. BSO, an irreversible specific inhibitor of γ-glutamylcysteine synthetase (γ-GCS) (Griffith and Meister, 1979), has been widely used for depleting GSH and proved itself a powerful tool in the elucidation of GSH functions (Martensson et al., 1991; Jain et al., 1991; Pileblad & Magnusson, 1989; this thesis).

BSO inactivates the enzyme in the presence of ATP. It is phosphorylated on the sulfoximine nitrogen atom; the amino sulfoximine phosphates bind tightly but noncovalently to γ-GCS, thus inactivating the enzyme (Griffith and Meister, 1979; 1982). BSO can not pass through the blood brain barrier, so selective depletion of brain GSH has to be achieved by intracerebroventricular administration of BSO. Systemic administration of BSO to pre-weaning animals has been tried by a number of authors (Jain et al., 1991; Heales et al., 1995; Heales et al., 1996), but this model of brain GSH depletion is complicated by the effects of concurrent.

If depleting GSH helps us in understanding its biological roles, repletion of GSH would be of pharmaceutical importance. The effort has been made to increase tissue GSH levels. Administration of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), two synthetic phenolic antioxidants, can increase GSH levels in various organs by 1.5-3.0 fold (Jaeschke and Wendel, 1985; 1986). Eaton and Hamel

(1993) found that BHA could increase γ-GCS activity *in vivo*, which may explain the elevated GSH levels by BHA treatment. This is supported by a finding that BHA significantly increases hepatic messenger RNA for γ-GCS large subunit (GCS-LS mRNA) (Borroz et al., 1994). The elevation of GCS-LS mRNA was suggested to be triggered by the formation of GSH conjugates.

Administration of cysteine may increase cellular levels of GSH, but even moderate doses of L-cysteine are toxic (Olney and Ho, 1970; Olney et al, 1971; Lund et al., 1981; Olney et al., 1990). An ideal intracellular cysteine delivery system would consist of a cysteine derivative (in which the thiol is masked) that is readily transported and effectively converted to cysteine. *L*-2-oxo-4-thiazolidinecarboxylic acid (OTC) is a typical example which has been widely used to increase tissue GSH levels. Significant increase in GSH concentration (145-170%) was achieved in the substantia nigra and brain stem in rats with intracerebroventricular (i.c.v.) injection of γ-GC and OTC but not GSH monoethyl ester (Pileblad and Magnusson, 1992). However the limitation of the dosing route is obvious. In addition, caution should be taken on the potential toxicity of γ-GC, since breakdown of γ-GC may produce excess cysteine which is neurotoxic.

EGF can protect vascular endothelial cells from oxidative stress induced by hydrogen peroxide by stimulating intracellular GSH levels and increasing intracellular cysteine levels (Yang and Hu et al., unpublished data). A similar effect has also been reported for nerve growth factor (NGF) which can protect pheochromocytoma PC12 cells from oxidative stress by increasing intracellular GSH levels, an event which may be due to increased activity of γ-GCS (Pan and Perez-Polo, 1993). This indicates that the growth factor can increase cellular uptake of cysteine from the medium and thus increase cellular GSH levels.

Brain GSH and ascorbic acid

GSH and ascorbic acid have been found to share the workload in the antioxidant system (Meister, 1994). Adverse effects of GSH deficiency can be prevented by administration of ascorbate in newborn rats (Martensson and Meister, 1991), mice (Martensson and Meister, 1992) and guinea pigs (Jain et al., 1992) which do not synthesise ascorbate. On the other hand, the scurvy induced in guinea pig by ascorbate-deficient diet can be prevented by the supplement of GSH monoethyl ester (Martensson

et al., 1993). This may be attributed to the GSH-dependent reduction of dehydroascorbate by both enzymatic (Christine et al., 1956; Hughes, 1964; Bigley et al., 1981; Rose, 1989; Wells et al., 1992) or non-enzymatic modes (Winkler, 1992) as GSH functions in the reduction of many other cell components. Oxidation of ascorbate leads to the formation of dehydroascorbate, which is irreversibly degraded if not quickly reduced back to ascorbate.

Our study revealed that ascorbic acid has a similar distribution pattern across brain regions to that of glutathione, which is lower in brain stem and higher in the other brain regions showing a decreasing gradient from rostral to caudal. This distribution profile is consistent with the data reported by Rice et al. (1995). Rice et al. (1995) also reported higher levels of ascorbic acid, although not glutathione, in the CNS of anoxia-tolerant reptiles than those in anoxia-intolerant species.

Although there is significant overlap in the functions of GSH and ascorbate in the destruction of reactive oxygen compounds, GSH has functions that are not served by ascorbate (see the beginning of this review), and ascorbate performs functions that are not efficiently carried out by GSH. It is probable that there are critical essential minimum levels of both ascorbate and of GSH.

Incidentally the sharing of antioxidation function may not be restricted to GSH and ascorbic acid. An increase in heme oxygenase-1 (HO-1) mRNA as well as protein in rat brain has been found in rats 5 h after treatment with the GSH depleter, diethyl maleate (DEM) (Ewing and Maines, 1993). These changes are also seen in systemically BSO-treated neonatal rats (Ewing and Maines, 1993). HO-1 has been identified as HSP 32 (Shibahara et al., 1987) which degrades heme with production of bile pigments. The bile pigments biliverdin and bilirubin themselves have been added to the list of cellular constituents with potent antioxidant activity (Stocker et al., 1987). The reciprocal relationship between brain GSH content and HO-1 mRNA levels following GSH depletion suggests compensatory change in the endogenous antioxidant system.

GSH and Nitric oxide

Besides its roles as a potent vasodilator and inhibitor of platelet aggregation, nitric oxide (NO) can act as a neurotoxin under conditions of excessive production, which suggests a role for nitric oxide in neurodegenerative diseases (Schulz et al.,

1995). NO is involved in excitotoxicity since activation of excitatory amino acid receptors leads to activation of neuronal nitric oxide synthase via an increase in intracellular calcium concentration. In addition, NO may inhibit key enzymes of energy metabolism, damage DNA, deplete intracellular GSH and react with superoxide to form peroxynitrite, a highly reactive substance causing oxidative stress (Schulz et al., 1995; Lupechio et al. 1996). Oxidation of vitamin E, ascorbic acid, and thiols by peroxynitrite has been demonstrated in rat brain synaptosomes (Vatassery, 1996). Nitric oxide (NO) was found to inactivate glutathione peroxidase, an inhibition which can be blocked by the NO synthase (NOS) inhibitor, N-methyl-L-arginine (Asahi, 1995). This may explain NO-induced accumulation of peroxides. NO can deplete intracellular GSH levels through the formation of intracellular S-nitrosoglutathione (Clancy et al., 1994). Therefore prolonged NO exposure might deplete antioxidants and facilitate the oxidation of dopamine and thereby cause neurotoxicity (Cook et al., 1996).

NO reacts with GSH to generate S-nitrosoglutathione (GSNO) under aerobic conditions but generating nitrous oxide and GSSG under anaerobic conditions. GSNO then reacts with GSH to further form nitrous oxide and GSSG. In the process of these reactions, the nitroxyl anion may be an intermediate which then generates peroxynitrite under aerobic conditions (Hogg et al., 1996). Ascorbic acid can decompose GSNO to form GSH and nitrous oxide (KashibaIwatsuki et al., 1996).

NOS requires NADPH and tetrahydrobiopterin (H4biopterin) to convert L-arginine to L-citrulline. GSH has been described as a cofactor for purified macrophage calcium independent inducible nitric oxide synthase (iNOS) (Stuehr et al., 1990). This is supported by the finding that GSH can increase the activity of NOS and also enhance the stability of the enzyme, actions which may be also due to the reductive protection of NOS protein thiols by GSH (Hofmann and Schkidt, 1995). However *in vitro*, GSH inhibits purified preparations of calcium-dependent neuronal constitutive NOS (nNOS) (Giovenelli et al., 1991). Recently, Heales et al. reported that GSH depletion is accompanied by increased neuronal nitric oxide synthase activity (Heales et al., 1996).

GSH and mitochondrial functions

In the previous section, the existence of GSH in mitochondria has been mentioned. Mitochondrial GSH is mainly imported from cytosol by a system that

contains a high-affinity transporter (Griffith and Meister, 1985; Martensson et al., 1990). GSH deficiency has been found to lead to myofiber degeneration in skeletal muscle (Martensson and Meister, 1989), damage to type 2-cell lamellar bodies and capillary endothelial cells in the lung (Martensson et al., 1989), and epithelial cell damage to jejunum and colon (Martensson et al., 1990) in adult mice and to lens epithelial cell degeneration and cataract formation in newborn mice (Calvin et al., 1986; Martensson et al., 1989) and rats (Martensson et al., 1989). These effects were associated with mitochondrial swelling with vacuolisation and rupture of cristae and mitochondrial membranes as seen by electron microscopy, as well as a decreased citrate synthase activity. One of the primary effects of GSH deficiency may be mitochondrial damage, although respiratory enzyme function seems not to be altered following brain GSH depletion (Seaton et al., 1996).

Mitochondrial GSH may be important in regulating inner membrane permeability by maintaining intramitochondrial thiols in the reduced state (Kosower and Kosower, 1983; Beatrice et al., 1984). Certain proteins are highly sensitive to changes in the cellular thiol status, e.g. Ca^{2+}-dependent ATPases (Bellomo et al., 1983), which serve as membrane-bound Ca^{2+} pumps to maintain cytoplasmic Ca^{2+} at low levels. Interference with Ca^{2+} homeostasis and increased levels of cytoplasmic-free Ca^{2+} are believed to participate in cell injury (Bellomo and Orrenius, 1985). Homeostasis of Ca^{2+} and thiols in the mitochondria is believed to be closely linked, either directly (Beatrice et al., 1984) or through the pyridine nucleotides (Lehninger et al., 1978).

Jain et al. (1991) showed enlargement and reduction in density of mitochondria in brain after BSO treatment. Citrate synthase activity (Jain et al, 1991, Heales et al, 1995), a mitochondrial matrix marker enzyme, and the activity of cytochrome C oxidase (Heales et al, 1995) were found to be significantly decreased by BSO treatment. Brain mitochondria are suggested to be more susceptible to oxidative stress since they are the sites of endogenous H_2O_2 generation. Mitochondria was found to be a sensitive target of some neurotoxicants such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (DiMonte et al., 1992) and butyl hydroperoxide (Ravindranath and Reed, 1990). After exposure to butyl hydroperoxide, GSH levels in the mitochondria isolated from rat brain decreased rapidly and irreversibly with the formation of protein-SS-glutathione mixed disulfides, while liver mitochondria could partially reverse their formation (Ravindranath and

Reed, 1990). The mitochondrial localisation of GSH and GSH-related enzymes in neural cells (Huang and Philbert, 1995) may provide very important defences against toxic oxygen species in the CNS.

Glutathione and neurodegenerative diseases

As mentioned in the general introduction chapter, oxidative stress has been suggested to be involved in a number of neurodegenerative diseases. Free radical toxicity is normally prevented by a range of antioxidants and protective enzymes. Levels of catalase and glutathione peroxidase are normal or moderately reduced (Ambani et al., 1975; Kish et al, 1985; Marttila et al., 1988). No change was observed in the levels of ascorbic acid (Riederer et al., 1989) and alpha-tocopherol (Dexter et al. 1992). However a decrease in total glutathione and GSH content in the substantia nigra of patients dying from Parkinson's disease was reported in 1982 (Perry et al. 1982) and then confirmed by Riederer and colleagues (1989) who reported a 50% decrease in total GSH level. Sian et al. (1994a) reported a selective decrease of GSH but no change in GSSG in substantia nigra of Parkinson's patients, a phenomenon which may be due to efflux of GSH from glia promoted by a correspondingly selective elevation of γ-glutamyltranspeptidase activity in the substantia nigra (Sian et al., 1994b). Most recently, Pearce et al. (1997) reported alterations in the distribution of GSH in the substantia nigrain Parkinson's disease. Mercury orange staining showed depletion of GSH in substantia nigra and a significant loss of neuronal GSH in surviving nigral neurons in Parkinson's disease. However, long term brain GSH depletion does not cause nigrostriatal pathway degeneration ((Toffa et al., 1997). This suggests that the loss of GSH alone does not lead to nitrostriatal damage in PD, but may render substantia nigra more susceptible to destruction by endogenous or exogenous toxins.

GSH is increased in the globus pallidus coupled with a decrease of GSSG in the patients with multiple system atrophy (Sian et al., 1994a), while glutathione peroxidase activity was increased in the lateral globus pallidus and caudate nucleus (Sian et al., 1994b). In Huntington's disease, GSSG is decreased in the caudate nucleus (Sian et al., 1994a). Despite profound nigral cell loss in the substantia nigra in Parkinson's disease, multiple system atrophy, and progressive supranuclear palsy, the level of GSH in the substantia nigra was depleted only in Parkinson's disease. In addition, a significant

increase of GSH level was reported in hippocampus and midbrain from the patients with Alzheimer's disease (Adams et al., 1991). These paradoxical increase in GSH may represent 'end stage' conditions in which neuronal loss may have removed the oxidative stress seen in the developing period of these diseases (except for Parkinson's disease), and glial proliferation has increased the GSH levels. Therefore the situation in the developed stage may be distinct from the developing stage of these diseases.

Glutathione, NMDA receptor and CNS excitability

Decreased GSH has been found to be associated with focal seizures (Berl et al., 1959; Hiramatsu and Mori, 1981). Furthermore, the glutathione levels were lower in the occipital cortex of genetically epileptic (tg/tg) mice compared with controls (Abbott et al., 1990). Genetic defects in GSH synthesis are linked to mental retardation and serious neurologic selequilae (Meister, 1978).

GSH is known to bind at specific sites in the CNS (Ogita and Yoneda, 1987; Guo et al., 1992; Lanius et al., 1994). The binding leads to an increase of inositol-1,4,5-triphosphate in astrocytes *in vitro* (Guo et al., 1992). An increasing number of reports support the theory that GSH modulates the functions of *N*-methyl-*D*-aspartate (NMDA) receptors and participates in cellular calcium homeostasis (Gilbert et al., 1991; Sucher and Lipton, 1991; Levy et al., 1991; Leslie et al., 1992; Janaky et al, 1993; Janaky et al., 1994; Ogita et al., 1995).

Receptors for excitatory amino acids (EAAs), including L-glutamic acid, are divided into two major categories according to their signal transduction systems. A metabotropic type of the EAA receptors is linded to hydrolysis of particular membrane phospholipids or formation of intracellualr cyclic AMP, whereas the ionotropic type is coupled to ion channels permeable to particular cations. Ionotropic EAA receptors are further classified on the basis of sensitivity to excitation by the exogenous agonist NMDA. The ionotropic receptors insensitive to NMDA are distinguishable from each other based on diferential preference to the other exogenous agonist DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) or kainic acid (KA) (reviewed by Ogita et al., 1995 and the references cited). A number of pharmacological sites modulate the activity of the NMDA subtype of the excitatory amino acid (EAA) receptor (MacDonald and Nowak, 1990), among which the redox modulatory site (Aizenman et

al, 1989; Levy et al, 1990) is of particular interest. It acts as a gain control for current flux through NMDA receptor-operated channels and can affect the degree of neurotoxicity produced by excessive NMDA receptor activation (Sucher et al., 1989; Levy et al., 1990). The redox modulatory site is affected by sulphhydryl reducing and oxidising reagents, indicating the existence of a disulphide(s) on the NMDA receptor-channel complex (Aizenman et al., 1989; Lazarewicz et al,. 1989). Reducing agents such as dithiothreitol (DTT) break disulfide bonds and enhance the activity of the NMDA receptor, whereas oxidising agents such as 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) form disulfide bonds from free thiol groups and decrease NMDA receptor function (Aizenman et al., 1989). Dithiothreitol (DTT), a xenobiotic which protects sulphhydryl groups, elicits epileptiform firing in hippocampal neurons (Tolliver and Pellmar, 1987) and aids glutamate in killing cultured retinal ganglion cells (Levy et al., 1990).

The specificity of this redox site has been further investigated in recombinant NMDA receptors by Kohr et al. (1994), who found that DTT rapidly potentiates L-glutamate-activated whole-cell currents in cells expressing NR1-NR2A channels, but had less effect in cells expressing the NR1-NR2B, -NR2C and -NR2D subunits. In contrast, GSH mimicked the DTT effects only in the NR1-NR2A receptor.

Intracerebroventricular injections of NMDA, glutamate and kainate (Pollard et al., 1994; Saija et al., 1994; Tsirka et al., 1995) can induce convulsions in animals. Kainate-induced convulsions may be a little more complicated since kainate not only acts on the NMDA receptor directly, but also inhibits γ-GCS (McBean et al., 1995), a rate-limiting enzyme in GSH synthesis. Intravenous administration of GSH prevents kainate-induced excitotoxicity in rat brain, but shows no protection against the convulsions (Saija et al., 1994). This may be because GSH has a poor permeability into the normal brain (Meister and Anderson, 1983), but may enter after seizures become established as these cause opening of the blood brain barrier (Saija et al., 1992) which may enable protection against the consequent pathological damage.

In this study, brain GSH depletion in adult rats by BSO appeared to be associated with running fits and seizures which could be prevented by maintaining GSH levels by co-administration of γ-GC. At 5 h, when the onset of the seizures occurred after BSO treatment, GSH was significantly decreased while glutamate, aspartate, and

cysteine had not yet been changed. These results demonstrate that GSH is active in the modulation of CNS excitability.

The precise mechanism whereby GSH is involved in the regulation of the NMDA receptor and other EAA receptors as well as excitability is not yet clear. Whether GSH modulates NMDA receptor by its chemically specific effects or just by general redox effects needs to be established, although the later possibility is less likely. If the action of GSH on NMDA receptor is mediated solely by redox modulation, then the reduced and oxidised forms might be expected to yield opposite influences. However GSH and GSSG were found to have a similar protective effect on NMDA receptor-mediated neurotoxicity in rat retinal ganglion cells (Levy et al., 1991). Lack of protection of animals by ascorbate against BSO-induce seizures (this thesis) and the dissociation of GSH and DTT actions on NR1-NR2B, -NR2C and -NR2D subunits would also support a GSH-specific effect.

In rat cortical neurones (Sucher and Lipton, 1991) and rat forebrain neurones (Gilbert et al., 1991), GSSG but not GSH, inhibited NMDA-induced increase of intracellular calcium. GSSG also inhibited NMDA-evoked whole cell currents in rat forebrain neurones (Sucher and Lipton, 1991). These effects were suggested to be due to its action on the redox modulatory site of NMDA receptor-channel complex because the inhibition by GSSG could not be further enhanced by additional treatment of 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), an oxidising agent and vice versa (Sucher and Lipton, 1991).

In the cells preparations from whole rat brain, both GSH and GSSG were demonstrated to produce concentration-dependent increase in intracellular calcium level in 100 seconds, a effect which is similar to that induced by NMDA and other agonists at the NMDA receptor (Leslie et al., 1992; Weaver et al., 1993). The response to both agents was prevented or reversed by either competitive or noncompetitive antagonists of NMDA receptor-mediated calcium entry. In rat cerebellar granule cells GSH and GSSH did not markedly affect the basal influx of calcium into cells during 5 min incubation. (Janaky et al., 1993). GSH slightly activated the gluatmate- and NMDA-induced influx while GSSG was inhibitory, but the enhancement of calcium entry by DTT and cysteine was attenuated by GSH and GSSG (Janaky et al., 1993). In addition, GSH and GSSG have been reported to attenuate the release of GABA evoked by excitatory amino acids,

which was suggested to be a consequence of sustained activation of the NMDA receptor-governed ionophores containing functional thiol groups (Janaky et al., 1994).

Due to its redox property, glutathione can act on the redox regulatory binding site on NMDA receptors and concurrently it may also act on the co-agonist recognition sites for glycine and glutamate. The latter action would displace glycine and glutamate from their binding sites and thus display an inhibitory effect. Therefore as to GSSG, its actions on the redox sites and recognition site of NMDA receptors produce a similarly inhibitory effect on the functions of NMDA receptors. For GSH, it causes slight activation by reducing disulfide bonds in the NMDA receptor, but can also bind to the recognition sites in the receptors as a γ-glutamyl compound, displace glutamate and hence diminish the nmber of accessible thiol groups. Some inconsistencies amongst the results on the actions of glutathione reported by various authors may be due to the variations in cells and experimental conditions because those observations were 'compromised effects' of dual action of GSH. All the studies on the effects of glutathione on NMDA receptors were carried out *in vitro* in brain cells by manipulating extracellular glutathione levels. Intracellular glutathione may have some hidden effects, especially when the dissociated cells have intracellular glutathione, such as cerebellar granule cells and glial cells.

The predominant form of excitotoxic injury observed after a variety of neurologic insults is mediated by activation of NMDA receptors. These insults include hypoxia/ischemia, trauma, epilepsy, and possibly a variety of neurodegenerative diseases including HIV-associated dementia (Choi, 1988; Meldrum and Garthwaite, 1989; Lipton, 1991; Lipton et al., 1991). Thus, a non-toxic agent that down-regulates NMDA receptor activity at the redox modulatory site could be of considerable importance in combating these maladies. Oxidising agents, which decrease NMDA-activated ionic current (Reynolds et al., 1990; Sucher et al., 1990), have been shown to decrease NMDA receptor-mediated neurotoxicity (Levy et al., 1990; Aizemnan et al., 1990). Oxidised glutathione, an endogenous agent, not only down-regulates the redox modulatory site but has also been shown to be safe to use in animal experiments, even at millimolar concentrations (Martensson et al., 1989; 1990).

Brain glutathione and behaviour

Oxidised glutathione (GSSG) has been identified as a physiological sleep regulator called sleep-promoting substance (SPS) (Komoda et al., 1990), which was originally extracted from the brainstem of 24-h sleep-deprived rats. It was found capable of inducing both slow wave sleep and paradoxical sleep when intracerebroventicularly infused in unrestrained rats (Inoue et al., 1984; Honda et al., 1994). These effects of GSSG were suggested to be relevant to its inhibitory modulation on glutamatergic neurotransmission in the brain (Honda et al. 1994). Pal and Dandiya (1994) reported the correlation of depletion of GSH with depression. Appreciable depletion of cortical GSH was observed in the mice with depression induced by inescapable foot shock stress. Significant increase in cortical GSH level has been demonstrated by either preshock and postshock administration of antidepressants, e.g. imipramine, maprotiline, fluvoxamine, alprazolam (Pal and Dandiya, 1994).

Transport of glutathione.

Inter-organ circulation of GSH is supported by the finding of low plasma GSH levels in the renal vein and of relatively high levels in the hepatic vein (Griffith and Meister, 1979; Meister, 1988). Plasma GSH arises largely from the liver. The cells that have high transpeptidase activity (e.g. kidney) utilise plasma GSH. Mitochondria lack the enzymes required for GSH synthesis and mitochodrial GSH seems to come from the cytoplasm by transport (Meister, 1988). Depletion of GSH by giving BSO in mice and rats leads to rapid loss of liver cytoplasmic GSH, but to a much lesser loss effect on mitochondrial GSH. The epithelial cells of the choroid plexus, which are the major sites of cerebrospinal fluid formation, function in the blood-cerebrospinal fluid barrier (Katzman, 1981; Pardridge, 1983). There is some analogy between the secretion of the cerebrospinal fluid by the choroid plexus and the secretion of urine by the renal tubule; this is reflected by structural and physiological similarities (Pollay, 1974), and also by the presence in the choroid plexus and kidney of high levels of the enzymes involved in the synthesis and degradation of glutathione (Tate et al., 1973). The significance of the presence in the brain endothelium of high amounts of γ-glutamyl transpeptidase (γ-GT) (Orlowski et al., 1974), an enzyme that provides cysteine to cells by GSH breakdown, is still not clear. Inhibition of this enzyme with AT-125 (i.c.v.) leads to a substantial increase (about 170%) in the GSH level of cerebrospinal fluid (CSF) (Anderson et al.,

1989). This indicates a role of γ-GT in re-cycling of intracellular GSH from brain tissue. Administration of L-2-oxo-4-thiazolidinecarboxylic acid (OTC) at a dose which did not increase brain glutathione levels (Anderson and Meister, 1989), lead to a significant increase (about 40%) in the GSH level of cerebrospinal fluid (Anderson et al., 1989).

GSH may cross the blood-brain barrier by a saturable low affinity transport process (Kannan et al., 1990). This transport is more active in young, developing rats (Kannan et al., 1992). Breakdown and resynthesis of GSH as the mechanism of apparent GSH uptake is unlikely because more than 87% of injected radio-labelled cysteine taken up at the BBB remained unchanged with negligible incorporation into GSH (Kannan et al., 1992).

Inborn errors of GSH synthesis

Patients with severe GSH synthetase deficiency associated with 5-oxoprolinuria have been recognised in several countries (Larsson, 1988). All of them showed markedly decreased erythrocyte GSH synthetase activity levels and also decreased erythrocyte GSH levels. Most of them exhibit an increased rate of hemolysis and some extent of CNS involvement including mental retardation and motor dysfunction, while metabolic acidosis due to accumulation of the acid, 5-oxoproline, is usually the first symptom. Two of the patients were found to exhibit an apparently increased susceptibility to bacterial infections.

An inborn deficiency of γ-glutamylcysteine systhetase has also been reported in human subjects (Konrad et al., 1972; Richards et al., 1974). These patients exhibit a syndrome of hemolytic anaemia, spinocerebellar degeneration, peripheral neuropathy, myopathy and aminoaciduria associated with GSH deficiency and developed muscular weakness, ataxia and decreased vibratory and position sensation in both upper and lower extremities in their thirties.

CHAPTER 4
GENERAL MATERIALS AND METHODS

Animals and housing

Male Fischer-344 rats (6-8 weeks, 200-220 g) were mainly used, while 6, 12, and 18 month old rats were used for the study on age dependent GSH changes and 6, 12, 24 & 36 day old rats for the observations on the developmental change of brain GSH and cysteine. Experimental animals (except for the neonates) were allowed two weeks to recover from transport before the start of experiments.

Rats were allowed free access to RM1 maintenance diet (SDS Ltd.) and given water *ad lib.* throughout the experiments. Soaked soft diet was provided when it was thought that the animal may have difficulty in climbing for food. All rats were housed in plastic-sided cages and maintained on a 12 h light/dark cycle and at a room temperature of 20-22 ^{o}C. All procedures conformed with the ethical guidelines of the U.K. Home Office Regulations for Experimentation on Laboratory Animals.

Rat models of DNB neurotoxicity

Two types of dosing schedules in producing a rat model of DNB neurotoxicity were used. Schedule 1 was three doses of 10 mg/kg DNB given at 0, 4 and 24 h. This model produces a rapid onset of damage for biochemical time course assessment. Brain stem lesions could be seen at 12 h after the last dose of DNB and were fully developed at 24 h. Schedule 2 was a daily dose model of 7.5 mg/kg given on four consecutive days, producing a slower but more reproducible onset of damage for morphological assessment. This was believed to be more appropriate for the susceptibility test. *m*-DNB (Sigma) was dissolved in DMSO (10mg or 7.5 mg/ml) and given by intraperitoneal injection at a does volume of 1ml/kg. The solution was freshly prepared and kept in the dark.

Observations of clinical signs of neurotoxicity and behaviour changes

General health checking

The rats were examined daily after each experiment started for appearance of neurological signs and behavioural changes as well as general health condition. Body weight was recorded daily throughout the experiment.

Temperature control

When rats were anaesthetised for intracerebroventricular administration, rectal temperature was monitored and maintained using a rectal probe thermistor connected to an electronic thermometer and a heat blanket (Comark Electronics Ltd., Littlehampton, Sussex, U.K.).

Ataxia scores

Rats were assessed daily for the severity of ataxia and muscle weakness according to a 10 point severity scale (Ray et al., 1996): 1 = body sways when walking; 2 = legs extended or splayed when walking; 3 = slow to pick up hind legs when walking; 4 = clear signs of hind limb weakness; 5 = occasionally drags a leg when walking; 6 = hind legs splayed out behind body; 7 = some additional forelimb weakness; 8 = cannot lift 350 g with forelimbs; 9 = difficult to right body; and 10 = unable to right body.

Seizure observation and behaviour monitoring

After administration of BSO, behaviour of animals in the seizure study was under close observation and also continuously monitored by video camera for 24 hours.

In vitro metabolic studies on tissue slices

Brain and liver slices preparation and incubation

After the male Fischer 344 rats were killed by cervical dislocation, the brains were removed and immersed in ice-cold normal saline immediately. After the cerebellum was removed, the forebrain and brain stem were dissected and sliced to a thickness of 400 μm by a McIlwain tissue chopper. The liver was removed and sliced to the same thickness. Tissue slices totalling 100 mg were incubated in conical flasks containing 5 ml of Krebs-Henseleit buffer, pH 7.4, gassed with 95% O_2 and 5% CO_2 for 120 min. The buffer was composed of 124 mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 1.2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 5 mM D-glucose.

The slices were treated in one of three ways. Solvent control was made by incubating 100 mg slices in 0.4% ethanol (solvent for *m*-DNB, 20μl in 5 ml buffer).

Reagent control was made by incubating 100 mg heat-inactivated slices in 5 ml buffer with 0.2 mM *m*-DNB and 0.4% ethanol. Test slices (100 mg) were incubated in 5 ml buffer with 0.2 mM *m*-DNB and 0.4% ethanol. The experiment was repeated using tissues from four animals.

Sampling and HPLC sample preparation for DNB measurement

A volume of 20 μl incubation solution was removed from the control tissue incubation every 15 min for monitoring glucose consumption. Reagent control and test incubations were sampled every 30 min for glucose measurement. Glucose concentration was measured with the hexokinase ultraviolet method, using standard diagnostic kits from Sigma (St. Louis, USA). After 2 h of incubation, the culturing mixture was centrifuged at 1500 g for 10 min at 4 ^{o}C and 0.5 ml supernatant taken and mixed with an equal volume of methanol. After a further centrifugation at 6500 g for 5 minutes 50 μl of the supernatant was applied to the HPLC. The tissue residue was homogenised in 1 ml methanol with glass homogeniser, centrifuged twice at 6500 g for 5 min, and 50 μl of supernatant injected into the HPLC.

Detection and identification of m-DNB and its metabolites

A Waters liquid chromatography pump and a UV detector (Waters, 486) set at a wavelength of 254 nm were used. The separation was carried out on a C18 reverse phase column (Spherisorb 5 ODS2, 250×4.6 mm) and a pre-column (30×4.6 mm). The gradient mobile phase consisted of water and acetonitrile. The initial condition was 95% water and 5% acetonitrile for 1 min, then a step increase to 50% acetonitrile for 20 min (gradient curve 1 was chosen). A flow rate of 1.0 ml/min was used throughout.

Metabolite standards commercially available (Sigma) are *m*-nitroaniline, *2*-amino-*4*-nitrophenol, *4*-amino-*2*-nitrophenol, *2,4*-diaminophenol, *1,3*-diaminobenzene, and *2,4*-dinitrophenol. Nitrosonitrobenzene was a gift from Dr. Martin Ellis (Zeneca Pharmaceuticals, Macclesfield, UK). The optimal gradient mobile phase conditions as mentioned above enabled the discrimination of five relevant compounds, *m*-DNB, *m*-nitroaniline, nitrosonitrobenzene, *4*-amino-*2*-nitrophenol and *2*-amino-*4*-nitrophenol. Detection recovery for these compounds were between 96-102%. The rest of the metabolites could not be detected with this system.

For peak identification, a Photodiode Array (PDA) detector (Waters) was connected to the HPLC column to scan the spectra of each peak component within the

range of 220-280 nm wavelength. In addition, fractions were also collected after HPLC column separation, vacuum dried, re-dissolved in acetonitrile and analysed by gas chromatography- mass spectrometry (GC-MS) which was performed on a V.G. Autospec Ultima-Q (V.G. Organic, Manchester) instrument in Electron Ionisation (EI) mode. A 'D.B. 5' GC column with 0.25 mm internal diameter and 30 m length was used. Mass spectra were acquired for DNB and the metabolites in HPLC fractions for comparison with those obtained from authentic standards (Sigma).

Implantation of intracerebroventricular (i.c.v.) injection cannula guides

Male F344 rats were prepared for intraventricular injection by implantation of a sterile supradural guide tube over the right lateral ventricle 7-10 days before injection using a slight modification of the method of Ray et al. (1996).

Under isoflurane anaesthesia, a 0.35-mm-diameter (30 gauge) stainless steel needle was advanced into the right lateral ventricle 1.5 mm lateral and 0.9 mm caudal to bregma using a stereotaxic device. The needle was previously fitted with a 5-mm-long, 0.6-mm-diameter (23-gauge) outer guide tube held 4 mm from the tip by a fixed stop. Pressure in the needle was maintained at 20 cm saline to prevent blockage, and penetration of the lateral ventricle was confirmed by the sudden fall in back pressure. When the needle was in place, the outer guide tube remained just above the surface of the dura. Three 1.2-mm diameter (12BA gauge) stainless steel screws were then threaded into the skull around the guide tube and the whole assembly firmly fixed in place with dental acrylic (Simplex Rapid, Austenal Dental Products Ltd., Harrow, England). Once the acrylic was set, the needle was withdrawn from the ventricle, leaving the outer guide tube in place. The guide tube was then temporarily closed with a 5-mm-long stainless steel stylet, and the rat allowed to recover.

i.c.v. Administration of BSO and rat model of brain GSH depletion

Preparatory to making the injections, the rats were anaesthetised with isoflurane, body temperature was maintained with a Homeothermic Blanket (Harvard Apparatus Ltd, UK). The stylet was then removed and a sterile injection needle (identical to that used during implantation) manually inserted into the implanted guide tube. The needle was connected by a length of sterile Portex PE10 tubing (International Market Supplies,

Congleton, Cheshire, England) to a saline filled microliter syringe. The injection was made by displacement of the injectant with saline and mixing between the two fluids prevented by a 1-μl air gap. A delay of 2 minutes was allowed after the end of the injection before the needle was withdrawn and the stylet then replaced. The rat was then allowed to recover and placed in its home cage, where it was closely observed.

BSO, dissolved in distilled water and pH adjusted to 7.4 by sodium hydrogen carbonate. Injection volume was 40 μl containing 3.2 mg BSO for each rat per injection. Injection was accomplished in 20 min at a constant rate of 2μl/min, which matches the rate of CSF turnover. For some rats, three doses of BSO were given on alternate days.

Microdissection of brain regions for the biochemical analysis

Brains and livers were removed and immersed in the ice-cold normal saline immediately after the rats were killed by cervical dislocation. Eleven brain coronal slices 2 mm thick were cut at the same time with a set of blades when the whole brain was put in a pre-made paraffin mould which held the brain in shape. Microdissection was performed on ice under a microscope to obtaining tissues from eleven representative areas: medulla; vestibular, cerebellar roof and cochlear nuclei; inferior and superior colliculi; hippocampus; striatum; cerebellum; thalamus; and cerebral cortex. Figure 4.1 demonstrates the coronal brain slices and the microdissection. Microdissection and sample preparation required two people to work together to minimise delay. While one was performing the microdissection, the other was making homogenates. Preparation took about 15-20 min for each brain. Stability of measured compounds was tested by comparing an immediate measurement with one delayed for 30 min (GSH is 1.05± 0.10 μmol/g in 30 min delay samples compared with 1.11±0.07 μmol/g in immediately treated samples, n=4, $p > 0.05$). It was noticed that GSH is very stable once tissue was homogenised in PCA. If the homogenate was stored in 0-4 ^{o}C, no significant change was seen over 24 h.

HPLC measurement for brain GSH and relevant amino acids

Derivatisation

GSH measurement by an HPLC fluorescence method was undertaken following Martin and White's method (Martin and White, 1991) with some modifications in sample preparation and mobile phase gradient conditions. Once the tissue from a

specific region was dissected, homogenate (10%, w/v) was made rapidly in 0.6 M perchloric acid solution using a glass homogeniser. After centrifugation (× 6500 g for 10 min), supernatant of tissue homogenate was used for derivatisation with dansyl chloride. To 50-100 µl supernatant was added PCA solution, to make up to 0.4 ml, and 0.1 ml iodoacetic acid was then added. Approximately 0.18 ml of lithium hydroxide (2M) was used to bring the pH of the mixture to 8.5-9.0 which was ensured by a colour change from pink to violet of the indicator dye, cresol red. After 30 min in dark, 0.4 ml dansyl chloride solution (1mg/ml in acetonitrile) was added, the reaction mixture returned to the dark and dansylated for 1 hour. Afterwards, 0.5 ml chloroform was used to extract the unreacted dansylchloride and acetonitrile to diminish the fluorescence interference and increase sensitivity.

HPLC conditions

The dansylated samples were applied to a Waters HPLC system controlled by Millennium (V 2010) software. The system consisted of an HPLC pump (Waters 600), a fluorecence detector (Waters 470), an autosampler (Waters 717) and a LiChromsper C18 column (15cm×4.6mm) connected to a compatible precolumn (Merck, Germany). The gradient mobile phase was comprised of two solutions. Solution A is methanol and water (4:1) and solution B consisted of 900 ml solution A and 100 ml 24% acetic acid solution containing 54.4g sodium acetate dissolved. The sample injection volume of 20 µl was applied to HPLC. Coefficient of Variation (CV) for the measurement of GSH and GSSG is 0.75 and 1.80 respectively.

Reagent preparation

1. Stock solution of iodoacetic acid (20 mM) is made in water.
2. γ-Glu-Glu (0.4 mM) is prepared in 50 mM HEPES buffer, pH 7.4 containing 2 mM diethylenetriaminepentaacetic acid (DEPA).
3. PCA-DEPA-boric acid solution (PCA solution): 5% (v/v) perchloride acid is made in water, containing DEPA (2 mM), boric acid (0.2 M) and cresol red indicator (5 mg/l).
4. 2 M lithium hydroxide is made in water.
5. Dansyl chloride is made 1mg/1ml in acetonitrile.
6. Deionised water is used throughout.

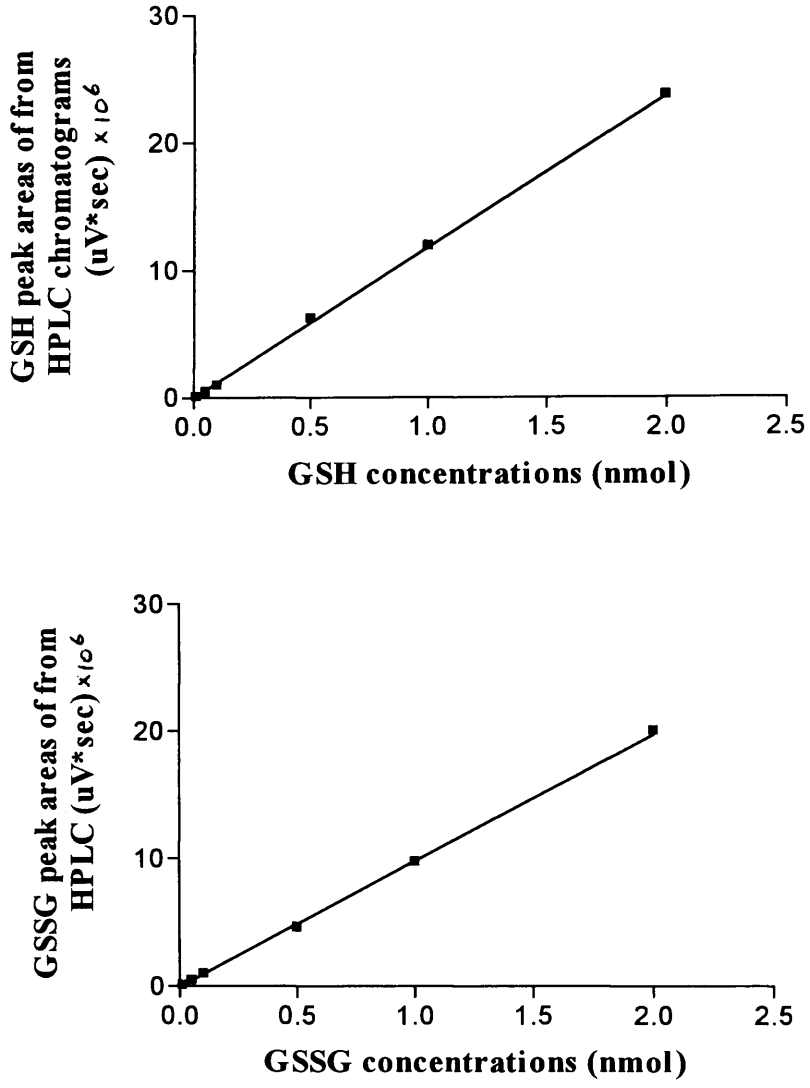


Figure 4.2, Calibration curves for GSH and GSSG measurements

HPLC method for the determination of ascorbic acid and free MDA

The homogenates (10%, w/v) for free MDA and ascorbic acid measurement were rapidly made in a solution of acetonitrile and water (1:1) after microdissection of brain slices. After centrifugation at 10,000g for 10 min, the supernatant of homogenate was immediately used for the measurement of ascorbic acid and free MDA by an HPLC method with minor modifications (Waterfall et al., 1995). Briefly a C18 Ultrasphere reverse phase column (25cm×4.6mm, Beckman, USA) was connected with a Waters HPLC pump and a UV detector set at a wavelength of 267 nm (Waters 486). Sample of 10 µl was injected via Waters 717 auto-sampler. The mobile phase comprised acetonitrile (12.5%), water (87.5%), containing 4.3 mM disodium hydrogen phosphate and 1.07 mM myristyltrimethylammonium bromide, pH adjusted to 7.4 with phosphoric acid. A flow rate of 1.5 ml/min was used throughout. The complete HPLC system was controlled by Millennium (V2010) software. Peak identification was made by comparing the retention time and spiking standard with the biological samples. MDA standard was prepared from malonaldehyde bis(diethylacetal) 98% solution (Aldrich) following the procedure of Csallany et al. (1984) and Cini et al. (1994).

Pathological examination

Perfusion fixation

The perfusion fixation technique used for obtaining the best preservation of tissue, free from known artefacts, was essentially as described by Brown and Brierley (1968). Animals were deeply anaesthetised with halothane, the rib cage opened and 500 I.U. of heparin injected into the left ventricle of the heart. The descending aorta was clamped with curved artery forceps and a narrow metal cannula was inserted through the left ventricle into the root of the ascending aorta. This was secured with a cotton ligature previously softened by soaking in water. The right auricle was then incised and physiological saline was perfused through to wash out the remaining blood from the circulation for 30-60 sec. or until the perfusate was colourless. This was followed by perfusion with fixative at a pressure of 100-120 mmHg for 12 min. The head was removed and stored in fixative until the brain tissue was removed.

The fixative solution was formalin 10% (v/v)-acetic acid 2% (v/v). The excised brain was immersed in fresh fixative. The step serial sections of brain were paraffin embedded, stained with H&E, and examined by light microscopy.

Paraffin sections

Each brain was sliced coronally into five blocks (4-5 mm thick), dehydrated in graded alcohol, cleared in chloroform and embedded in paraffin wax. The composite brain blocks were then step-serially sectioned (1 in 20) at 7 μm. Paraffin sections were dewaxed in xylene and serially hydrated through graded alcohol to water. They were then stained in Harris's haematoxylin and washed in tap water for 5 min until the sections were stained blue. Following differentiation in 1% acid-alcohol (1% HCl in 70% ethyl alcohol) for 5 sec, the sections were washed in tap water until they were blue again. The sections were then stained in 1% Eosin Y for 1 min and washed in tap water for 5 min. Finally they were dehydrated through graded alcohols, cleared in xylene, and mounted in distrene tricresylphosphate xylene (DPX).

Neuropathological assessment

Neuropathological assessment of paraffin sections was made according to the severity of tissue damage and vacuolation found and graded into four categories: normal (0 or -), slight (1 or +), moderate (2 or ++), and severe (3 or +++).

Statistical analysis

All data were stored and analysed with Microsoft Excel and Minitab. Analysis of variance (ANOVA), student's t test and Chi Square test were used for corresponding types of data.

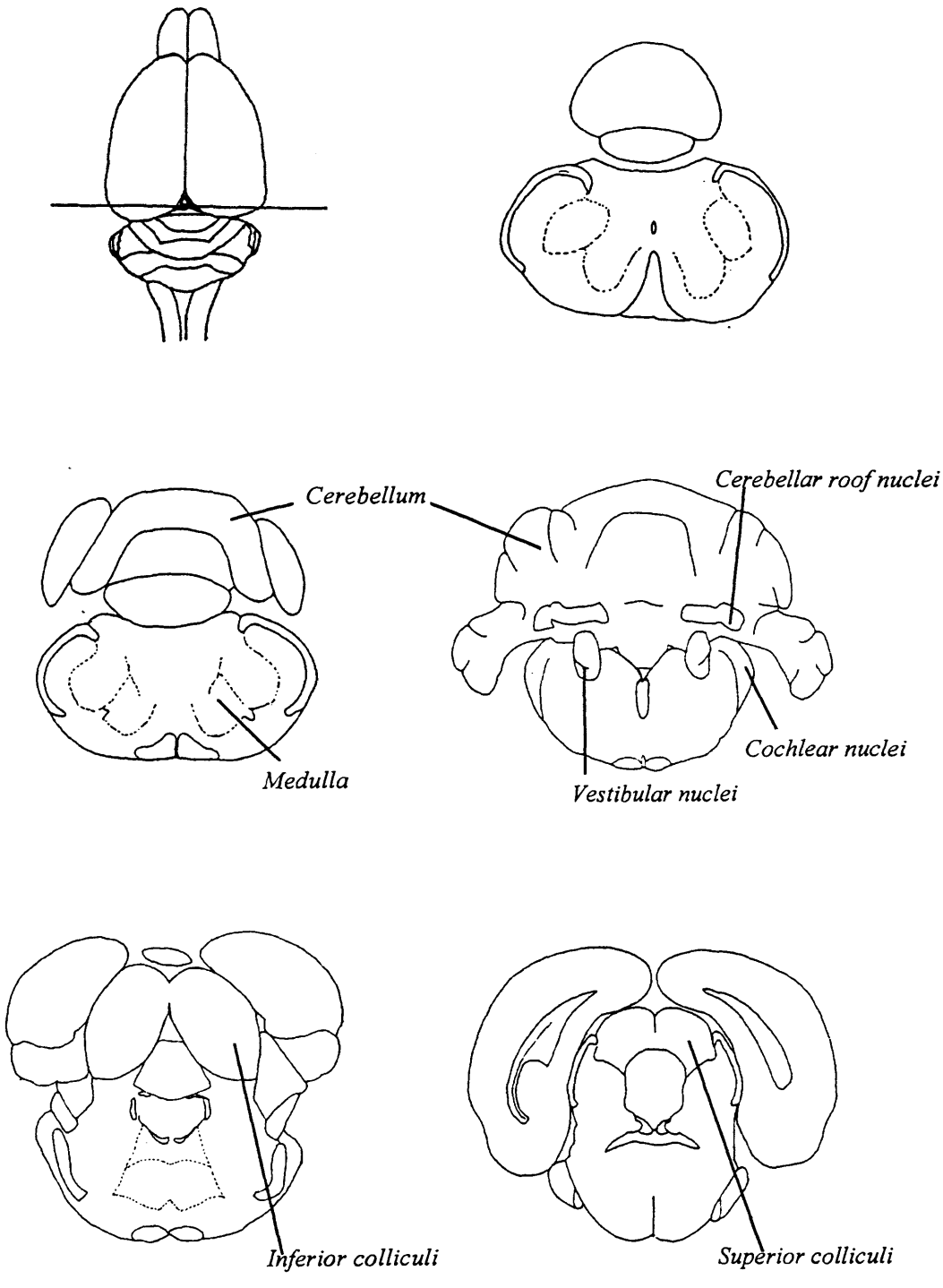


Figure 4.1a, Illustration of coronal sections of rat brain used in microdissection of specific regions.

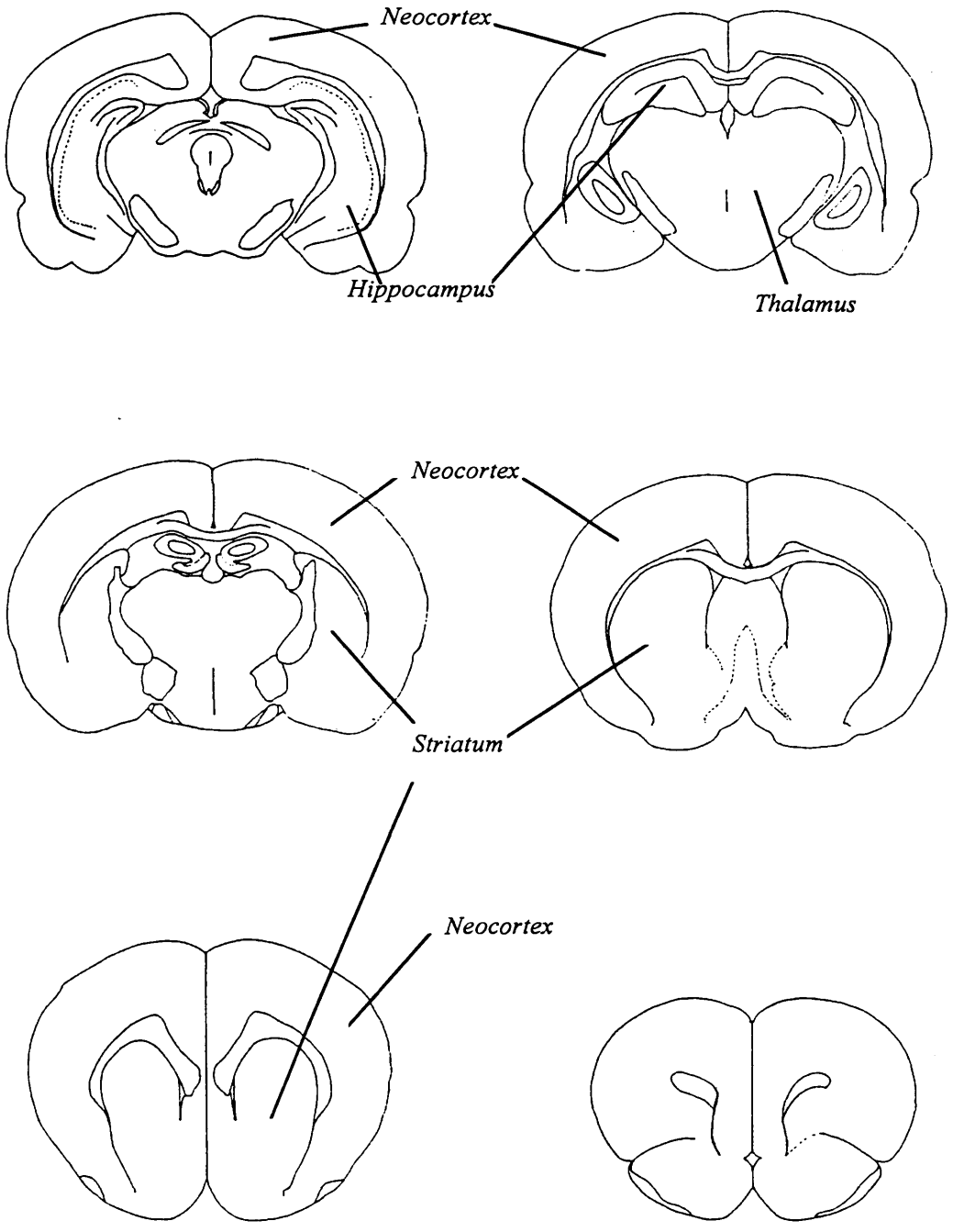


Figure 4.1b, Illustration of coronal sections of rat brain used in microdissection of specific regions.

CHAPTER 5

METABOLISM OF *M*-DINITROBENZENE BY BRAIN: *IN VITRO* STUDIES

Summary *m*-Dinitrobenzene (*m*-DNB) is a neurotoxin producing selective brain lesions, but the *in situ* metabolic fate of *m*-DNB in brain is unknown. In this study, nitroreductive capacity of brain towards *m*-dinitrobenzene (*m*-DNB) has been investigated. Tissue slices from F344 rat brain stem, forebrain, and liver were separately incubated with 0.2 mM *m*-DNB. *m*-DNB and its metabolites were detected by HPLC, and identified by either HPLC or Mass Spectrometry (MS). All three tissues showed metabolic activity towards *m*-DNB. Metabolic disposal of *m*-DNB was 1.05±0.11 μmol/g wet weight/h in liver, 0.49±0.05 in brain stem, and 0.44±0.05 in forebrain (mean±SD, n=4). *m*-Nitroaniline was found to be the main metabolite produced by both brain and liver slices, representing 57-66% of the disposal of *m*-DNB. Liver slices also produced 2(or 4)-amino-4(or 2)-nitrophenol, which was not detected in brain slices. We detected nitrosonitrobenzene in the slices from both parts of brain, but not in liver slices. The glucose consumption of brain slices from both areas was significantly increased in the presence of *m*-DNB: by 26% in the brain stem ($p<0.001$) and by 17.9% in forebrain ($p<0.01$). This may be considered a pre-cytotoxic effect. The results demonstrate that brain has considerable nitroreductive capacity towards *m*-DNB, and that the *in situ* reduction of *m*-DNB may be responsible for its neurotoxicity.

INTRODUCTION

m-DNB is an industrial intermediate used for synthesis of certain dyes, plastics and explosives. It induces methaemoglobinaemia (Beritic,1956; Facchini and Griffiths, 1981; Cody et al., 1981), neurotoxicity (Philbert et al., 1987; Ray et al., 1992) and testicular toxicity (Cody et al., 1981; Cave and Foster, 1990). Systemic metabolism of *m*-DNB has been studied *in vivo* in the rat (Nystrom and Rickert, 1987; McEuen and Miller, 1991), rabbit (Parke, 1961), guinea pig (Parke, 1955) and hamster (McEuen and Miller, 1991) and as well as *in vitro* in hepatocytes (Cossum and Rickert, 1985), testicular cells (Foster, 1987) and erythrocytes (Cossum and Rickert, 1987).

In vivo studies showed that *m*-DNB could be metabolised to the following products: *m*-nitroaniline (Nystrom and Rickert, 1987; McEuen and Miller, 1991; Parke, 1961), *2,4*-diaminophenol (Parke, 1961), *2*-amino-*4*-nitrophenol (Parke, 1961), *1,3*-diacetamidobenzene (Nystrom and Rickert, 1987; McEuen and Miller, 1991), *3*-nitroacetanilide (McEuen and Miller, 1991), *2,4*-diacetamidophenol (McEuen and Miller, 1991), *4*-acetamidophenol (Nystrom and Rickert, 1987; McEuen and Miller, 1991) and *3*-acetamidophenol (Nystrom and Rickert, 1987). *m-N*itroaniline and two unidentified metabolites were reported in *m*-DNB treated hepatocytes *in vitro* (Cossum and Rickert, 1985). Reduction of *m*-DNB has been demonstrated in rat testicular cell cultures (Foster et al., 1987), but not in the erythrocytes from rats, rhesus monkeys and humans (Cossum and Rickert, 1987).

The metabolic fate of *m*-DNB in brain tissue is however unknown. Due to the putative role of bioactivation in its neurotoxicity (Romero et al., 1995), the *in situ* metabolism of *m*-DNB in brain is of particular interest. In this study, brain slices were chosen to assess their metabolic capacity towards *m*-DNB, since this would enable us to preserve cellular differentiation and exclude the contribution from any other tissues e.g. liver. Liver slices were also used for the purpose of comparison. Because forebrain and brain stem are respectively resistant and sensitive towards *m*-DNB toxicity (Philbert et al., 1987), their metabolic capacities were assessed separately.

MATERIALS AND METHODS

Experimental treatment of tissue slices

Preparation of tissue slices and incubation conditions have been described in chapter 4 (General materials and methods).

The slices were treated in one of three ways. Solvent controls were made by incubating 100 mg slices in 0.4% ethanol (20µl in 5 ml buffer). Reagent controls were made by incubating 100 mg heat-inactivated slices in 5 ml buffer with 0.2 mM *m*-DNB and 0.4% ethanol. Test slices (100 mg) were incubated in 5 ml buffer with 0.2 mM *m*-DNB and 0.4% ethanol. The experiments were repeated using tissues from four animals.

Sampling and HPLC sample preparation

An aliquot of 20 μl incubation solution was removed from the control tissue incubation every 15 min for monitoring glucose consumption. Reagent control and test incubations were sampled every 30 min for glucose measurement. Glucose concentration was measured with the hexokinase ultraviolet method, using standard diagnostic kits from Sigma (St. Louis, USA). After 2 h, the incubation culturing mixture was centrifuged at 1500 g for 10 min at 4 ^{o}C, 0.5 ml supernatant was taken and, after mixing with an equal volume of methanol, 50 µl was injected into the HPLC. The tissue residue was homogenised in 1 ml methanol with a glass homogeniser, centrifuged twice at 6500 g for 5 min, and 50 µl of the supernatant injected into the HPLC (see details in the General Materials and Methods). Glucose utilisation was calculated from the slope in plotting glucose concentrations against time.

Detection and identification of *m*-DNB and its metabolites

A reverse phase HPLC system with a UV detector set at a wavelength of 254 nm was used for detecting *m*-DNB and its metabolites. A photodiode array (PDA) detector (Waters) and GC-MS were used for peak identification (please see details in the Chapter 4)

Chemical reactivity tests

All tests were carried out at room temperature and in Krebs-Henseleit buffer (pH7.4). Concentrations of 0.1-1mM were used for all reagents such as glutathione (GSH), ascorbic acid (AA), nitrosonitrobenzene, *m*-DNB, *m*-nitroaniline, and hydrogen peroxide (H_2O_2). GSH, AA or H2O2 was incubated with nitrosonitrobenzene, *m*-DNB or *m*-nitroaniline, then solution was analysed with the above mentioned HPLC method.

Statistics

Student's t-test was used to assess significance of the difference between groups. ANOVA was used for the comparison of glucose consumption across different time points.

RESULTS

Tissue capacity for metabolising m-DNB

Comparison of test chromatograms with tissue and reagent controls discriminated *m*-DNB and its metabolites in the tested slices from those substances originally existing in tissue (Figure 5.1, 5.2, 5.3). The slices from both brain stem and forebrain, as well as liver slices, were found capable of metabolising *m*-DNB. No metabolism occurred in heat-inactivated tissue.

Identification of metabolites and comparison of metabolic patterns

Figure 5.4 summarises the chromatograms of testing slices from the three types of tissue. Peaks 2 and 3 have been identified by GC-MS as nitroaniline (Figure 5.5) and dinitrobenzene (Figure 5.6). Peaks 1 and 4 have been chromatographically identified to be amino-nitrophenol and nitrosonitrobenzene, based on the comparison of their retention times (spiking with standard) and UV spectra (PDA confirmation) with standards. As shown in Table 5.1, the metabolic activity of liver towards *m*-DNB is 2.1-2.4 times that of brain slices. *m*-Nitroaniline was the main metabolite of *m*-DNB both in brain and liver slices, accounting for 57%-66% of the disappearance of *m*-DNB. Liver slices produced *2*-(or *4*)-amino-*4*(or *2*)-nitrophenol, which was not detected in brain slices. Unexpectedly, nitrosonitrobenezene was found in brain slices, but not in liver slices (peak 4 in Figure 5.4).

Effects of m-DNB on glucose consumption

The glucose consumption by brain and liver slices was monitored during the course of 120 min incubation, to assess the viability of slices. The glucose concentration in the brain slice incubation medium decreased in a linear manner during this time (data not shown), which indicated the maintenance of metabolic activity during the incubation period. *m*-DNB was found to increase the glucose consumption of brain slices by 17.9-26.4% (Table 5.2).

Chemical reactivity of some endogenous substances with m-DNB and its metabolites

The detection of nitrosonitrobenzene is rather surprising, since it is chemically unstable, and so we tested for potential chemical reactivity of nitrosonitrobenzene with

glutathione and ascorbic acid in order to address the possible importance of these endogenous substances in the further reduction of nitrosonitrobenzene. GSH in neutral aqueous solution was found to convert nitrosonitrobenzene to *m*-nitroaniline while ascorbic acid was found to convert it to amino-nitrophenol. Nitroaniline did not react with ascorbic acid under these conditions, however, indicating that the generation of amino-nitrophenol from the reaction of nitrosonitrobenzene and ascorbic acid is not via *m*-nitroaniline. None of these endogenous substances could chemically convert *m*-DNB. All these reactions are summarised in Figure 5.7. The recyling reaction of nitrosonitrobenzene to *m*-dinitrobenzene is also non-catalytic in nature, since our studies indicate that *m*-dinitrobenzene can be generated by direct reaction of nitrosonitrobenzene with hydrogen peroxide.

DISCUSSION

Extra-hepatic metabolism of xenobiotics is an area of current research interest. As Krishna and Klotz have reviewed, a number of extrahepatic organs or tissues have metabolic capacity, such as the mucosa of gastrointestinal tract, kidney, lung, brain, and skin (Krishna and Klotz, 1994). Metabolic activity of brain towards xenobiotics is also attracting increasing attention due to its neuropharmacological and neurotoxicological importance (Mesnil et al., 1984; Ravindranath and Boyd, 1995). Drug metabolising enzymes enriched in blood-brain interfaces and circumventricular organs have been proposed to act as an enzymatic barrier for the brain (Ghersi-Egea et al., 1993; Ghersi-Egea et al., 1994). However, these metabolic enzymes may function in both detoxification and activation of xenobiotics.

The reductive metabolism of *m*-DNB by brain slices shown in this study provides evidence for an intrinsic reductive metabolic capacity of brain towards xenobiotics. *In vivo* studies have shown that *m*-DNB and some of its metabolites can be detected in brain tissue (Xu et al, 1996), but such studies have not determined the origin of these metabolites. This investigation, by use of brain slices, clearly shows that the metabolites are produced by brain. The relevance of the *in situ* nitroreduction to neurotoxicity is that it could represent bioactivation with formation of nitro anion radicals (Mason and Holtzman, 1975) which cause oxidative stress to target cells (Romero et al., 1994; Ray et al., 1994).

Although the main metabolite of *m*-DNB in brain tissue is *m*-nitroaniline, the reduction is likely to be stepwise as suggested for the *in vivo* situation (Nystrom and Rickert, 1987; McEuen and Miller, 1991), according to the presence of the nitroso intermediate. In liver slices, besides the *m*-nitroaniline observed as the main metabolite, 2(or4)-amino-4(or2)-nitrophenol were also detected (these two isomers could not be separated by our HPLC system). This indicates the occurrence of hydroxylation, in which aniline hydroxylase might be involved. However, this hydroxylation product could not be detected in brain slices. This might be due to the lack of aniline hydroxylase in brain tissue (Ravindranath et al.,1989). The lack of production of amino-nitrophenol by brain also indicates the reaction of nitrosonitrobenzene with glutathione, rather than ascorbic acid, possibly due to compartmentation of metabolism.

The results from liver slices are consistent with those from isolated hepatocytes (Cossum and Rickert, 1985), in which *m*-nitroaniline was found to be the main metabolite of *m*-DNB. Cossum and Rickert reported two unknown metabolites (Cossum and Rickert, 1985), one of which might be amino-nitrophenol as identified in our study. Taking all available data about the metabolism of DNB which includes our results and those cited, the main metabolic pathways may be summarised as shown in Fig. 5.7.

In the chemical reactivity tests, neither ascorbate or glutathione could chemically reduce *m*-DNB. Therefore, the very first step of DNB reduction to form nitrosonitrobenzene is probably enzymatic, but the further reduction can be accomplished non-enzymatically by some endogeneous substances such as GSH and ascorbic acid (AA). Reduction of *m*-DNB was demonstrated in both brain slices and liver slices under aerobic conditions maintained by gassing with 95% O2 and 5% CO2. Nitroreductases have been divided into oxygen sensitive and oxygen-insensitive nitroreductase, both of which have been reported in mammalian systems (Peterson et al., 1979; Miwa et al., 1982). The oxygen sensitive nitroreductase initiates 1-electron reduction forming a nitro anion radical, which then reacts with oxygen to form superoxide and parent nitro compound (Mason and Holtzman, 1975; Peterson et al., 1979). However, Holtzman and his colleagues demonstrated that nitroreduction with formation of nitro anion radical and superoxide could be achieved even in the presence of oxygen (Holtzman et al., 1981). The present study could not provide direct evidence as to which enzymes were responsible for the metabolism, but the likely candidate to catalyze this reductive pathway might be one or more of the nitroreductases with low substrate specificity found in brain. Since reduction of *m*-DNB in microsomal incubation is NADPH-dependent (Cossum and Rickert 1985), we would suggest that in brain slices, nitroreductases such as NADPH-cytochrome P450 reductase, which has been observed in brain (Ghersi-Egea et al., 1993; Ghersi-Egea et al., 1994; Holtzman et al., 1981; Ravindranath et al., 1990), are responsible for the reduction of *m*-DNB. Even though cytochrome P450 exists in rat brain in multiple forms (Ghersi-Egea et al., 1993; Ray et al., 1994; Sasame et al., 1977; Kapitulnik et al, 1987; Naslund et al., 1988; Warner et al., 1988; Bhagwat et al., 1995; Iscan et al., 1990; Volk et al., 1991; Schilter and Omiecinski, 1993), it may not be directly involved in the nitroreduction of *m*-DNB, because the reduction in microsomes was unaffected by the presence of P-450 inhibitors

such as SKF525A, metyrapone, or carbon monoxide (Cossum and Rickert, 1985; Rickert, 1987). In addition, xanthine oxidase (Tatsumi et al., 1978) and/or NADH-dependent nitro reductase (Köchli et al., 1980) may also contribute to the reduction in brain. By use of specific inhibitors of certain proposed enzymes, it may be possible to clarify which enzyme(s) are involved in the metabolism. Furthermore by incubating DNB with cultures of different cell types from the central nervous system, it will become clear which cell types contribute to this metabolism.

The *m*-DNB treated brain slices showed a significantly higher glucose consumption than control slices. This is considered a pre-cytotoxic effect which has been also observed at sub-cytotoxic conditions in primary cultures of glial cells and also *in vivo* (Romero et al., 1995; Ray et al., 1994).

As shown in the proposed pathway (Fig. 5.7), nitrosonitrobenzene is an intermediate in *m*-DNB reduction. Theoretically it should exist both in brain and liver slices. Its presence in brain slices but not in liver slices indicates that the capacity of metabolising nitrosonitrobenzene in brain, enzymatic, non-enzymatic or both, may be lower than that in liver. Accumulation of this locally produced metabolite in brain may contribute to the neurotoxicity of *m*-DNB since nitrosonitrobenzene has been demonstrated to be more toxic than m-DNB towards testicular cells *in vitro* (Cave and Foster, 1990; Cossum et al., 1989). Brain stem is a selective target of *m*-DNB neurotoxicity (Ray et al., 1992), but the lack of a gross metabolic difference between forebrain and brain stem indicates that the selectivity of the toxicity is due to other factors, such as functional activity (Cavanagh, 1993) and heterogeneity of glutathione metabolism (Hu et al., 1996a). A detoxifying action of GSH in removing nitrosonitrobenzene may partially explain why brain GSH depletion increases the neurotoxicity of *m*-DNB (Hu et al., 1996b).

Table 5.1, *m*-DNB utilisation and production of metabolites by brain and liver slices

(μmol/g/h)

	m-DNB	*m*-nitroaniline	Aminonitrophenol	Nitrosonitrobenzene
Brain stem	0.49±0.05*	0.28±0.04*	undetectable**	0.17±0.05
Forebrain	0.44±0.05*	0.29±0.04*	undetectable**	0.19±0.04
Liver	1.05±0.11	0.65±0.06	0.28±0.03	undetectable**

Tissue slices were incubated with Krebs-Henseleit buffer containing 0.2 mM *m*-DNB at 37°C for 2 h. Data show the consumption of DNB and the production of metabolites determined with HPLC. Results presented are means±SD, n=4. * $P < 0.01$, compared with that of liver. ** The detection limit is 0.02 μmol/g/h.

Table 5.2, Glucose utilisation of brain slices with or without treatment with 0.2mM *m*-DNB (μmol/min/g tissue)

	Control	*m*-DNB treated	% increase
Brain stem	0.54±0.13	0.67±0.14*	26.4
Forebrain	0.67±0.16	0.79±0.17*	17.9

Results presented are means±SD, n=4. * $p < 0.05$ compared with control.

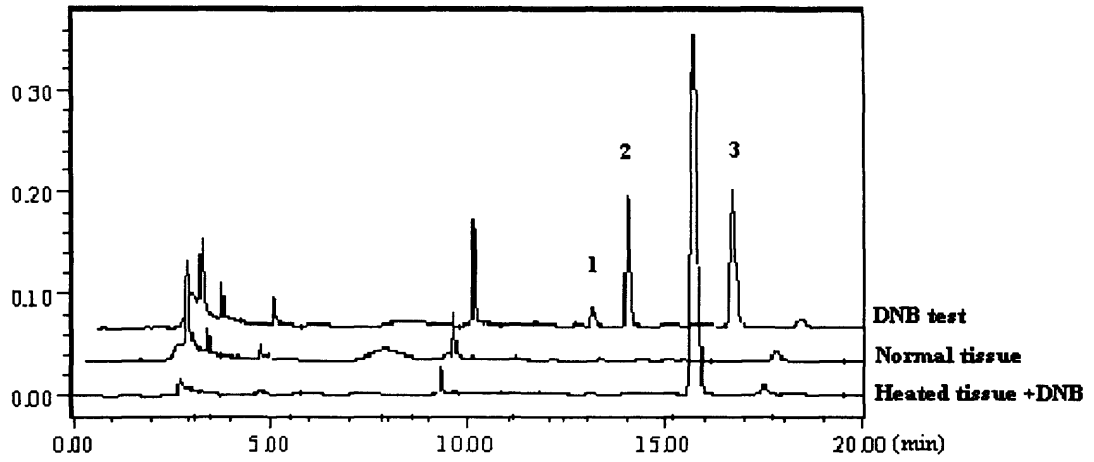


Figure 5.1 HPLC chromatograms of the extracts from liver slices. Peaks 1, 2, and 3 were recognised as DNB relevant substances by excluding those peaks shown on the normal tissue and those on the heated tissue except for the DNB peak.

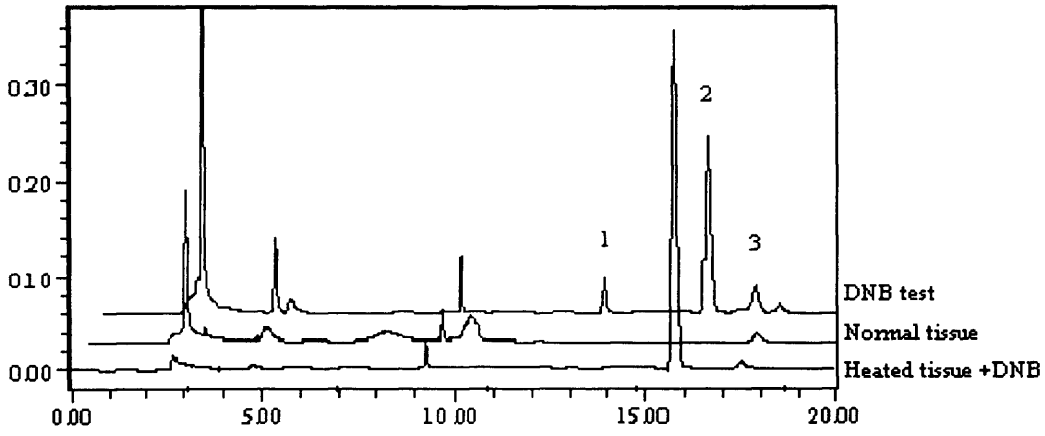


Figure 5.2 HPLC chromatograms of the extracts from brain stem. Peaks 1, 2, and 3 were recognised as DNB relevant substances by excluding those peaks shown on the normal tissue and those on the heated tissue except for the DNB peak.

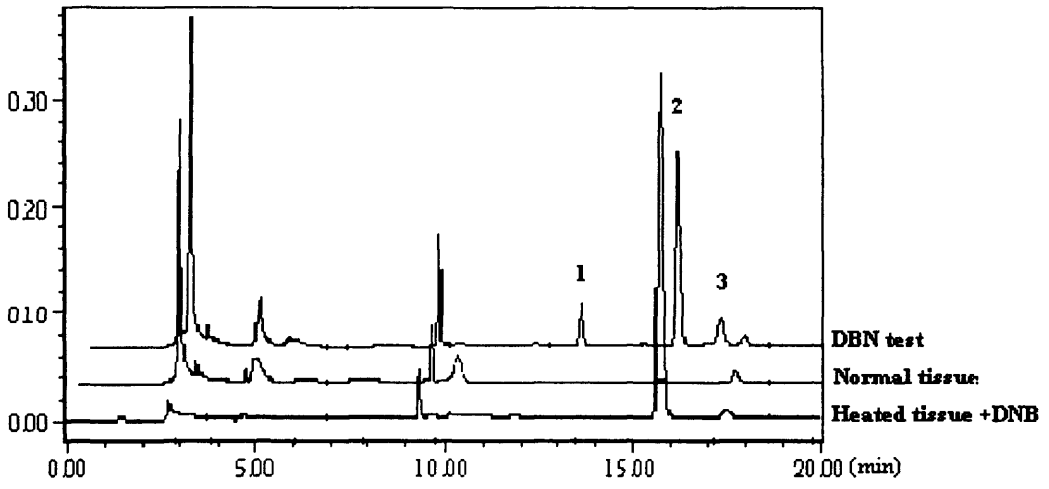


Figure 5.3 HPLC chromatograms of the extracts from forebrain. Peak 1, 2, and 3 were recognised as DNB relevant substances by excluding those peaks shown on the normal tissue and those on the heated tissue except for the DNB peak.

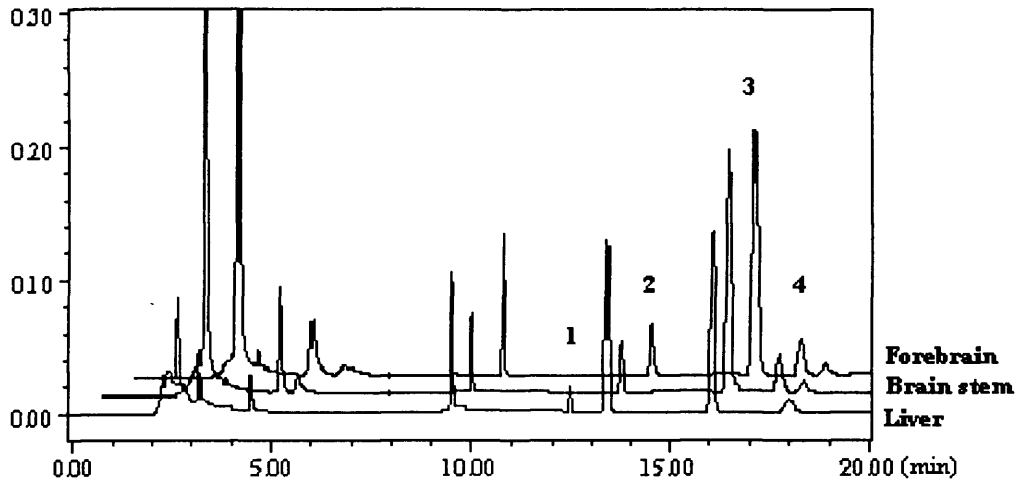


FIGURE 5.4 HPLC analysis of *m*-DNB and its metabolites in homogenates of forebrain, brain stem, and liver slices incubated with 0.2 mM *m*-DNB containing media for 2 h. HPLC was performed as described in *Materials and Methods*. Peak 1 is amino-nitrophenol; Peak 2 is *m*-nitroaniline; Peak 3 is *m*-DNB; Peak 4 is nitrosonitrobenzene. All the peaks before peak 1 and after peak 4 were seen in solvent control slices. Wavelength was set at 254 nm for the UV detection.

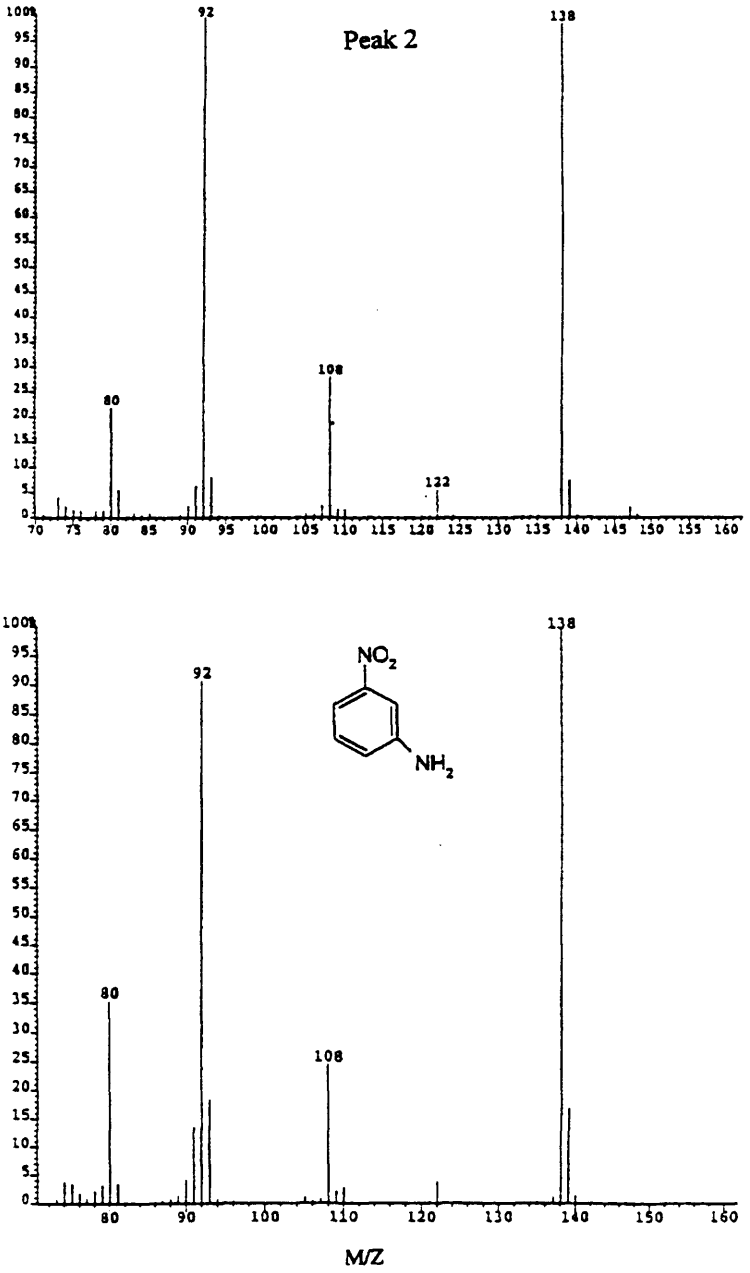


FIGURE 5. 5, Mass spectra of authentic standard m-nitroaniline (bottom) and m-DNB metabolite peak 2 (top). HPLC fraction preparation and GC-MS scanning were performed as described in Materials and Methods.

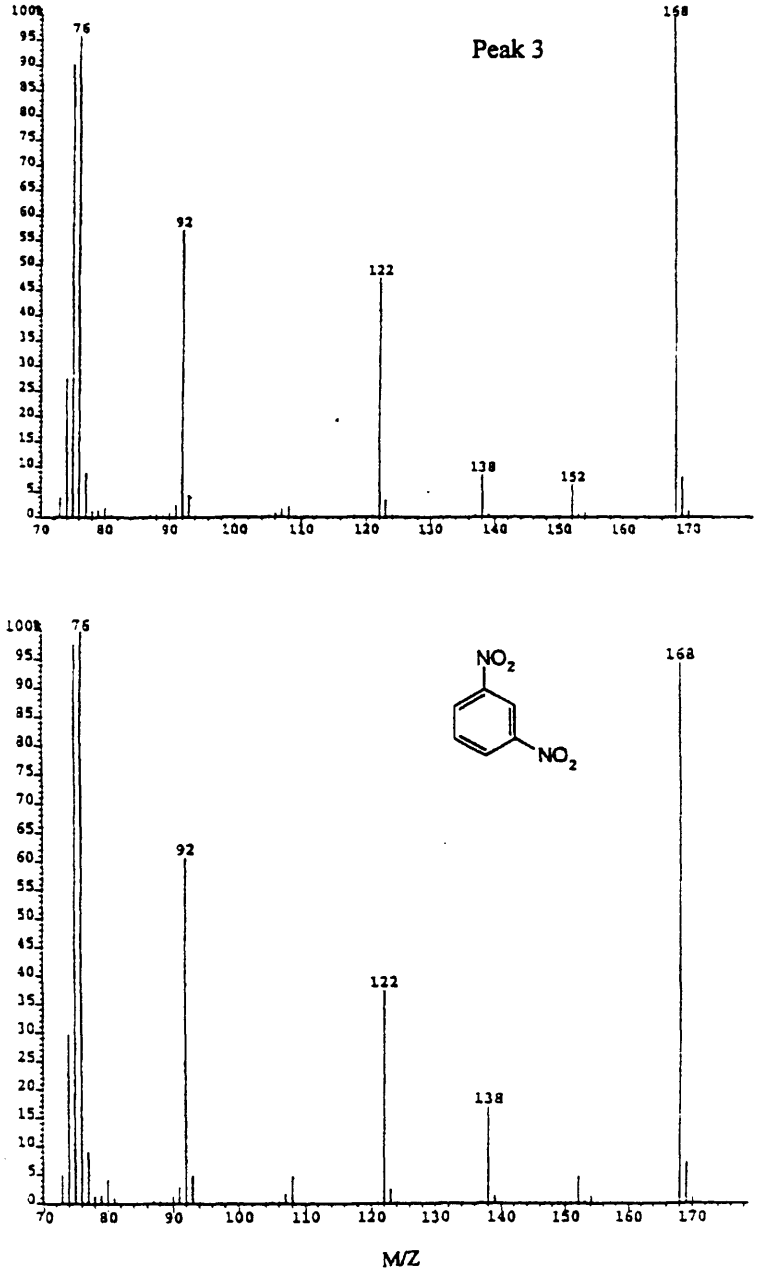


FIGURE 5. 6, Mass spectra of authentic standard m-DNB (bottom) and HPLC chromatogram peak 3 (top). HPLC fraction preparation and GC-MS scanning were performed as described in Materials and Methods.

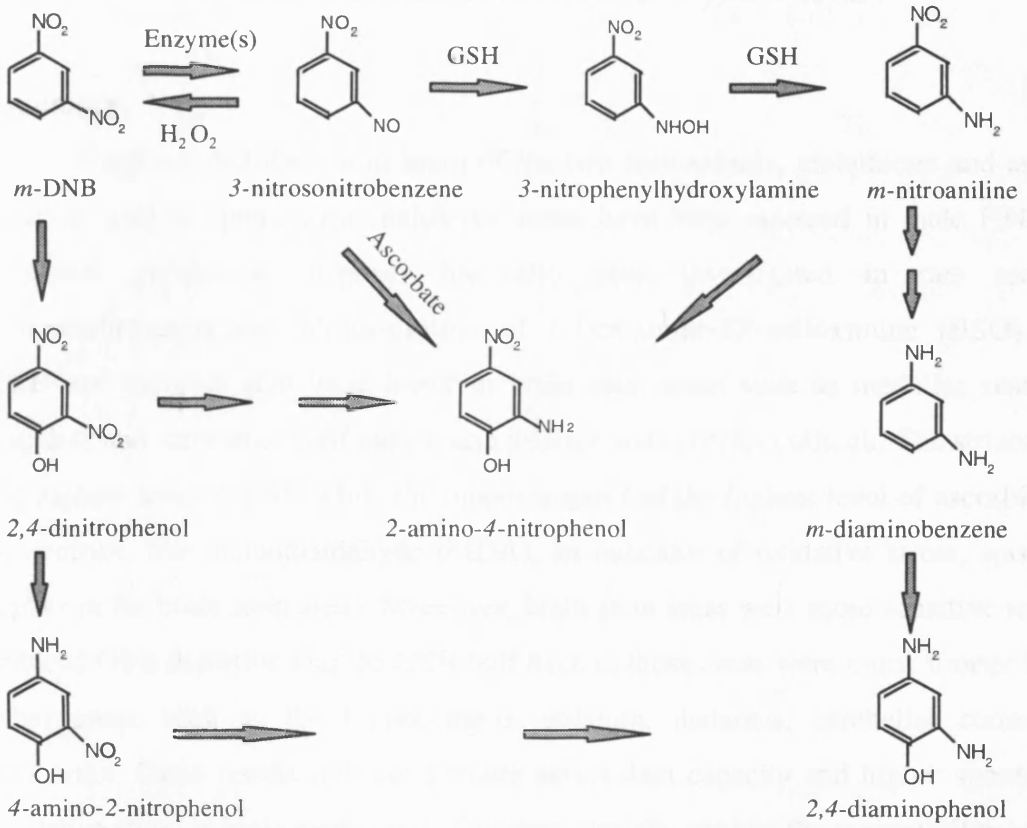


FIGURE 5.7. Proposed metabolic pathway of *m*-DNB deduced from our data and those authors mentioned in the *introduction*. Both reduction and oxidation occurred in liver while only reduction of *m*-DNB could be demonstrated in brain.

CHAPTER 6
HETEROGENEITY IN REGIONAL DISTRIBUTION OF GLUTATHIONE, ASCORBIC ACID AND ENDOGENOUS LIPID PEROXIDATION IN RAT BRAIN

Summary

Regional distribution in brain of the two antioxidants, glutathione and ascorbic acid, as well as spontaneous oxidative stress have been assessed in male F344 rats. Regional glutathione turnover has also been investigated in rats receiving intracerebroventricular administration of *L*-buthionine-*SR*-sulfoximine (BSO). Both GSH and ascorbic acid were lower in brain stem areas such as medulla; vestibular, cochlear and cerebellar roof nuclei; and inferior and superior colliculi. The striatum had the highest level of GSH while the hippocampus had the highest level of ascorbic acid. In contrast, free malondialdehyde (MDA), an indicator of oxidative stress, was much higher in the brain stem areas. Moreover, brain stem areas were more sensitive to BSO-induced GSH depletion and the GSH half lives in those areas were much shorter than in other areas, such as the hippocampus, striatum, thalamus, cerebellar cortex, and neocortex. These results indicate a lower antioxidant capacity and higher spontaneous oxidative stress in brain stem areas. This may partially explain the susceptibility of brain stem to neurotoxic xenobiotics.

Introduction

Brain consumes proportionately more oxygen than other organs, and may be especially prone to oxidative stress, a property which has been suggested to be relevant to a number of CNS disorders (Halliwell, 1992). Lipid peroxidation is one of the main consequences of oxidative stress. It occurs in the brain after acidosis (Waterfall et al., 1996), ischemia and trauma (Braughler, 1989; Traystman et al., 1991; Yamada, 1994), and neurodegenerative diseases (Evans, 1993; Jenner, 1996), such as Parkinson's (Dexter et al, 1989; Jenner et al., 1992; Jenner, 1994) and Alzheimer's (Subbarao et al, 1990) diseases. There is increasing evidence of oxidative injury induced by Alzheimer's beta-amyloid peptide (Harris et al., 1995; Richardson and Zhou, 1996; Zhang et al., 1996). Lipid peroxidation and protein oxidation lead to the alteration of membrane

fluidity, receptor function and ion channel function, and ultimately cell death (Schroeder 1984; Halliwell and Gutteridge, 1985; Bondy et al, 1990; Benzi et al., 1991; Viani et al., 1991; Joseph and Roth, 1992).

GSH is an important endogenous tripeptide in biological systems, functioning to protect cells from oxidative stress by scavenging free radicals and peroxides under the action of glutathione peroxidase. It also participates in the elimination of endogenous substances and detoxification of xenobiotics by conjugation with their metabolic intermediates (Meister and Anderson, 1983). Existing in the brain at millimolar concentrations, GSH is non-uniformly distributed across brain regions (Bien et al., 1990; Philbert et al., 1991). However, the significance and the mechanism underlying the heterogeneous distribution is unknown.

A review of ascorbic acid in the brain has recently been published by Grunewald (1993). The scorbate/dehydroascorbate redox couple also provides a powerful antioxidant buffering system. As a co-factor it functions in hydroxylation reactions (Hu et al., 1997). Ascorbate inhibits binding of substrates to a number of receptors binding, including the N-methyl-D-aspartate, adrenergic, serotonergic and dopaminergic receptors (Kaylaap et al., 1981; Jones et al., 1986; Todd and Bauer, 1988; Majewska et al., 1990).

Because of the functional variety and structural differences across brain regions, it may come as no surprise that brain regions are biochemically heterogeneous. Previous studies showed that brain stem is more susceptible to *m*-dinitrobenzene-induced neurotoxicity (Philbert et al, 1987; Ray et al, 1992). Brain GSH depletion increases brain's susceptibility to *m*-dinitrobenzene neurotoxicity (Hu et al., 1996). *In vitro* studies have linked the loss of cellular GSH with susceptibility of neurones to the oxidant peroxynitrite (Bolanos et al., 1995). Clinically, the early and selective loss of GSH (Jenner et al., 1992) as well as the alteration in GSH related enzymes (Sian et al., 1994) have been observed in the substantia nigra of patients suffering from Parkinson's disease. This led to the thought that antioxidant capacity may be one of the important factors determining tissue susceptibility. Therefore, this study was made to look into the distribution profiles of the two main antioxidants, GSH and ascorbic acid, together with spontaneous formation of free malondialdehyde (MDA), an end product of lipid

peroxidation in normal rats. By use of the i.c.v. administration of BSO to deplete brain GSH, regional heterogeneity in GSH turnover across brain has also been demonstrated.

METHODS AND MATERIALS

Experimental

Twenty young (2-3 months) adult F344 male rats were implanted with i.c.v. injection cannula guides following the method of Ray et al. (1996). The animals were allowed to recover from the surgery for 7-10 days before dosing. Sixteen rats were given a single dose of BSO i.c.v. (3.2mg), while the other four were treated with normal saline via the same route for control. BSO (Sigma, USA) dissolved in distilled water and pH adjusted to 7.4 by sodium hydrogen carbonate, was injected directly into right lateral ventricle through the pre-implanted guide with a fixed length 30 gauge needle. The injection volume was 40 µl, containing 3.2 mg BSO per rat. Injection was accomplished over 20 min at the rate of 2µl/min with a microdrive syringe. Animals were killed by cervical dislocation at 5, 15, 24, and 48 h, while 4 normal saline-treated animals were used as control (0 h). Another eight unimplanted rats were used to measure regional brain ascorbic acid and MDA levels, four of which had received DNB (3×10 mg/kg/day ip).

Brain was removed immediately post mortem, cut into 11 slices each 2 mm thick, and microdissected under a microscope to obtain tissues from eleven areas: medulla; vestibular, cochlear and cerebellar roof nuclei; inferior and superior colliculi; hippocampus; striatum; cerebellar cortex; thalamus; and cerebral cortex. Tissue homogenates (10% w/v) for GSH measurement were made in perchloric acid solution (0.6 M) by glass micro homogeniser. The homogenates (10% w/v) for free MDA and ascorbic acid measurement were made in a solution of acetonitrile and water (1:1).

HPLC measurement of brain GSH

An HPLC fluorescence method (Martin and White, 1991) was used to measure GSH with some modifications in sample preparation and mobile phase gradient conditions. Technical details have been described in the chapter 4.

HPLC method for the determination of ascorbic acid and free MDA

After centrifuging at 10,000g for 10 min, the supernatant of the homogenate was immediately used for the measurement of ascorbic acid and free MDA by an HPLC method with minor modifications (Waterfall et al., 1995). Briefly a C18 Ultrasphere reverse phase column (15×4.6 cm, Beckman, USA) was connected with a Waters HPLC pump and a UV detector set at a wavelength of 267 nm (Waters 486). 10 µl samples were injected via a Waters 717 auto-sampler. The mobile phase was prepared following the description in the above mentioned reference (Waterfall et al., 1995). A flow rate of 1.5 ml/min was used throughout. The HPLC system was controlled by Millennium (V2010) software. Peak identification was made by comparison of the retention times on spiking standard into test samples. MDA standard was prepared from 1,1,3,3-tetraethoxypropane following the procedure of Csallany et al. (1984) and Cini et al. (1994).

RESULTS

Heterogeneity of GSH and ascorbic acid distribution in the brain

GSH is unevenly distributed across brain areas (Figure 6.1). Striatum has the highest GSH concentration, followed by hippocampus, neocortex, cerebellar cortex and thalamus. Brain stem areas have relatively low GSH levels while medulla has the lowest. Interestingly, ascorbic acid has a very similar distribution pattern to GSH, although hippocampus has the highest level of ascorbic acid and cochlear nuclei the lowest (Figure 6.1). Striatum GSH was 50% higher than that in the medula ($p<0.01$), while hippocampal ascorbic acid was 225% higher than in the cochlear nuclei ($p<0.01$).

Heterogeneity of spontaneous lipid peroxidation across the brain areas

Spontaneous lipid peroxidation, assessed as MDA concentrations however although always low, was detectable in all eleven dissected brain regions (Figure 6.2). Regional heterogeneity was obvious. MDA is much higher in brain stem areas, including medulla; vestibular, cerebellar roof and cochlear nuclei; inferior and superior colliculi. Cerebellum has a relatively higher MDA concentration, while hippocampus, striatum, neocortex and thalamus have a much lower MDA level. The MDA level in the cochlear nuclei was 9.2 times higher than that in neocortex ($p< 0.01$).

Heterogeneity of regional sensitivity to BSO induced GSH depletion

As shown in Figure 6.3, the depletion of GSH by BSO was heterogeneous across the eleven brain areas assessed. Those brain areas could be classified into two broad categories according to the degree of GSH depletion seen at 24 hours after administration of BSO. One category, which is brain stem areas with low initial GSH levels, showed greater depletion of GSH by 68-73% in response to BSO. This includes the medulla; vestibular, cochlear and cerebellar roof nuclei; and the inferior and superior colliculi. The other category includes cerebral cortex, striatum, hippocampus, thalamus and cerebellum which exhibit relatively higher initial GSH levels, and showed a lesser GSH depletion of 36-46%.

Heterogeneity of GSH depletion half lives across the brain areas

GSH half lives in different brain areas were calculated from the semi-logarithmic representation of the data. These half lives are based on the disappearance of GSH after the inhibition of GSH synthesis with BSO. The half lives of GSH in brain stem areas were about three to four times shorter than in other brain regions (Table 6.1). This difference was far greater than the difference in absolute GSH levels in the same brain areas. It was also noted that the decline in GSH began to reverse in brain stem 48 h after administration of BSO while continuing to decrease in other regions (Figure 6.4).

Effects of DNB on brain antioxidants and lipid peroxidation

No change was seen in the ascorbic acid (Table 6.2) and free MDA levels (Table 6.3) in brain after rats were treated with *m*-DNB at a dosage which normally causes brain stem lesions. No effect of *m*-DNB on brain GSH levels was noted (see Chapter 8).

DISCUSSION

The regional distribution pattern of GSH across brain derived from this study is similar to those reported elsewhere. Measurements of brain ascorbic acid levels are also in similar ranges to those reported previously (Kinuta et al., 1989; Zhang et al., 1993). Ascorbic acid concentration in the CNS is thought to be highly regulated. The distribution pattern of ascorbic acid in brain observed in this study is in agreement with previously reported partial pattern of hippocampus > cortex > striatum in gerbil brain (Zhang et al., 1993). The similarity in GSH and ascorbic acid distribution patterns across brain areas supports the hypothesis that ascorbate and GSH have actions in common and can support each other (Martensson and Meister, 1991; Meister, 1994). GSH depletion decreases the ascorbic acid level, and the adverse effects of GSH deficiency in new-born rats can be prevented by a supplement of ascorbate (Martenson and Meister, 1991, Jain et al., 1992; Martensson and Meister, 1992). Similarly, GSH ester supplement to guinea pigs delayed the onset of scurvy induced by an ascorbate-deficient diet (Martensson et al., 1993). These observations may be explained by the GSH-dependent reduction of dehydroascorbate by both enzymatic (Wells et al., 1992) and non-enzymatic modes (Winkler, 1992).

Regional differences in spontaneous lipid peroxidation (indicated by the basal free MDA levels) were demonstrated in this study, despite the very low free MDA

concentrations (almost negligible in some brain areas). These low levels in some brain areas are close to the report of Cini et al. (1994). The free MDA levels in brain measured with this HPLC method are much lower than those assessed with the thiobarbituric reaction method (Gupta et al., 1991; Hu and Chen, 1992), due to the specificity of the measurement and less artificial effects. The central nervous system may be especially prone to radical damage (Halliwell and Gutteridge, 1985; Labela and Bondy, 1991), because of its richness in iron and polyunsaturated fatty acids as well as insufficient activity of catalase, SOD and GSH peroxidase (Halliwell, 1992). A number of free radical species are produced in brain during normal metabolism, e.g. $NO^{\bullet}$, $O2^{\bullet -}$, $^{\bullet}OH$ (Halliwell, 1992).

In this study no measure was made of brain alpha-tocopherol, another important antioxidant, but the available data is not consistent in terms of brain distribution. In human subjects, the medulla had a higher level of alpha-tocopherol than any other region, while caudate nucleus had the lowest level which is about half the level in the medulla (Adams et al., 1991), but in dog it seems otherwise (Pillai et al., 1993). Similar to the cellular distribution of GSH, alpha-tocopherol concentration is higher in astrocytes than in neurones (Makar et al., 1994).

GSH synthesis takes two steps, of which the first is the combination of glutamic acid and cysteine under the action of γ-glutamylcysteine synthetase (γ-GCS); the second step is the addition of glycine to the γ-GC under the action of glutathione synthetase. BSO, a potent, irreversible and specific inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979a), has been widely used for depleting GSH. Due to its poor passage across the blood-brain barrier, systemic administration of BSO to adult rodents produced no effects on brain GSH (Griffith and Meister, 1979b). Local administration (i.c.v.) of BSO into brain, proved effective in depleting brain GSH (Pileblad and Magnusson, 1988; 1989). This provides a useful tool to study the metabolism and biological importance of GSH in the CNS.

Brain GSH mainly exists in non-neuronal cells with the exception of cerebellar granule neurones (Philbert et al., 1991). GSH half life in cultured astrocytes was reported as 5 h (Devesa et al, 1993); however, GSH half life in whole brain is 70 h, much longer than in liver or kidney (Douglas and Mortensen, 1956). The depletion of whole brain GSH showed single order exponential kinetics over 48 hours, even though

GSH may be compartmentalised into different GSH pools with possibly different turnovers (Jain et al, 1991), i.e. in cytoplasm, nucleus, mitochondria, and endoplasmic reticulum (Smith et al., 1996). The mitochondrial GSH pool in astrocytes is much smaller than the cytosolic pool (Huang and Philbert, 1995). The measurement of the half life in this study is based on GSH disappearance in whole tissue and is thus an apparent, or overall, half life. This could reflect: 1) insensitivity to small pools (i.e. actually not monoexponential, but second component is too small to be seen); 2) rapid re-distribution between pools which was faster than the overall rate of depletion. The greater depletion of GSH in brain stem observed in this study is consistent with a report on mice (Shivakumar and Ravindranath, 1992). Our kinetic study, as far as we are aware, gave the most comprehensive regional turnovers, and showed that the greater GSH depletion seen in brain stem is due to a faster turnover.

Together with the slightly lower level of GSH and ascorbic acid plus much higher level of MDA, the disproportionately faster GSH turnover in brain stem may reflect a greater oxidative stress and a lower antioxidant capacity; but there is no evidence for the latter in the cases of superoxide dismutase and catalase (Pardo et al., 1995). Alternatively it may reflect additional, non-oxidative demands on the variety of GSH functions in the brain stem. It is interesting to note that haemorrhages after BSO treatment were seen in the area of fastest turnover (Hu et al., 1996; and the chapter 8), suggesting that this measure correlates with vulnerability. The higher vulnerability is supported by a demonstration that brain GSH depletion increases brain susceptibility towards *m*-dinitrobenzene, a neurotoxin leading selective lesions in brain stem (Hu et al., 1996). However DNB treatment does not significantly alter the absolute levels of brain GSH (chapter 8) and ascorbic acid, nor cause significant elevation of free MDA.

Finally, the even distribution of BSO in bilateral ventricles was confirmed by the equal depletion of GSH in both side hippocampus. The possibility of uneven distribution of BSO causing the differential depletion of GSH is unlikely because hippocampus is close to the ventricle but has less depletion.

Table 6.1. GSH half lives (T1/2) in brain regions (mean ± SE)

GSH depletion sensitive areas	T1/2, h	GSH depletion resistant areas	T1/2, h
Medulla	10.4 ±3.4	Hippocampus	35.0 ±1.5
Vestibular nuclei	11.2 ±1.8	Striatum	33.0 ±1.6
Cerebellar nuclei	12.9 ±1.1	Cerebellar cortex	40.1 ±1.1
Cochlear nuclei	10.1 ±1.0	Neocortex	43.1 ±1.2
Inferior colliculi	13.4 ±1.1	Thalamus	34.6 ±1.1
Superior colliculi	13.1 ±1.2		

Table 6.2. Effects of *m*-dinitrobenzene on brain ascorbic acid concentrations (μmol/g)

Areas	Control (n=4)	DNB-treated (n=4)	t-test
Medulla	0.87±0.17	0.65±0.16	n.s.
Vestibular nuclei	0.87±0.13	0.93±0.26	n.s.
Cerebellar roof nuclei	0.95±0.22	0.80±0.16	n.s.
Cochlear nuclei	0.83±0.08	0.84±0.16	n.s.
Inferior colliculi	1.20±0.23	1.29±0.17	n.s.
Superior colliculi	1.60±0.23	1.65±0.12	n.s.
Hippocampus	2.70±0.17	2.96±0.18	n.s.
Striatum	1.92±0.17	1.96± 0.25	n.s.
Cerebellar cortex	2.18±0.09	2.03±0.42	n.s.
Neocortex	2.41±0.28	2.72±0.96	n.s.
Thalamus	1.30±0.16	1.14±0.06	n.s.

The difference amongst control brain areas, tested with ANOVA analysis, is highly significant ($p < 0.01$); n.s. not significant between control and DNB-treated groups.

Table 6.3. Effects of *m*-dinitrobenzene on brain MDA concentrations

Areas	Control (n=4)	DNB-treated (n=4)	t-test
Medulla	25.83±9.82	23.24±10.71	n.s.
Vestibular nuclei	18.66±5.49	31.15±11.53	n.s.
Cerebellar roof nuclei	26.84±12.69	21.88±7.58	n.s.
Cochlear nuclei	31.50±11.59	40.08±5.29	n.s.
Inferior colliculi	26.99±11.51	32.73±7.29	n.s.
Superior colliculi	13.23±5.95	25.20±9.56	n.s.
Hippocampus	0.73±0.27	1.51±1.21	n.s.
Striatum	0.81±0.17	1.33±1.04	n.s.
Cerebellar cortex	3.08±2.04	4.85±3.34	n.s.
Neocortex	0.56±0.17	1.68±1.58	n.s.
Thalamus	0.48±0.50	3.24±2.47	n.s.

The difference amongst control brain areas, tested with ANOVA analysis, is highly significant ($p < 0.01$); n.s. not significant between control and DNB-treated groups.

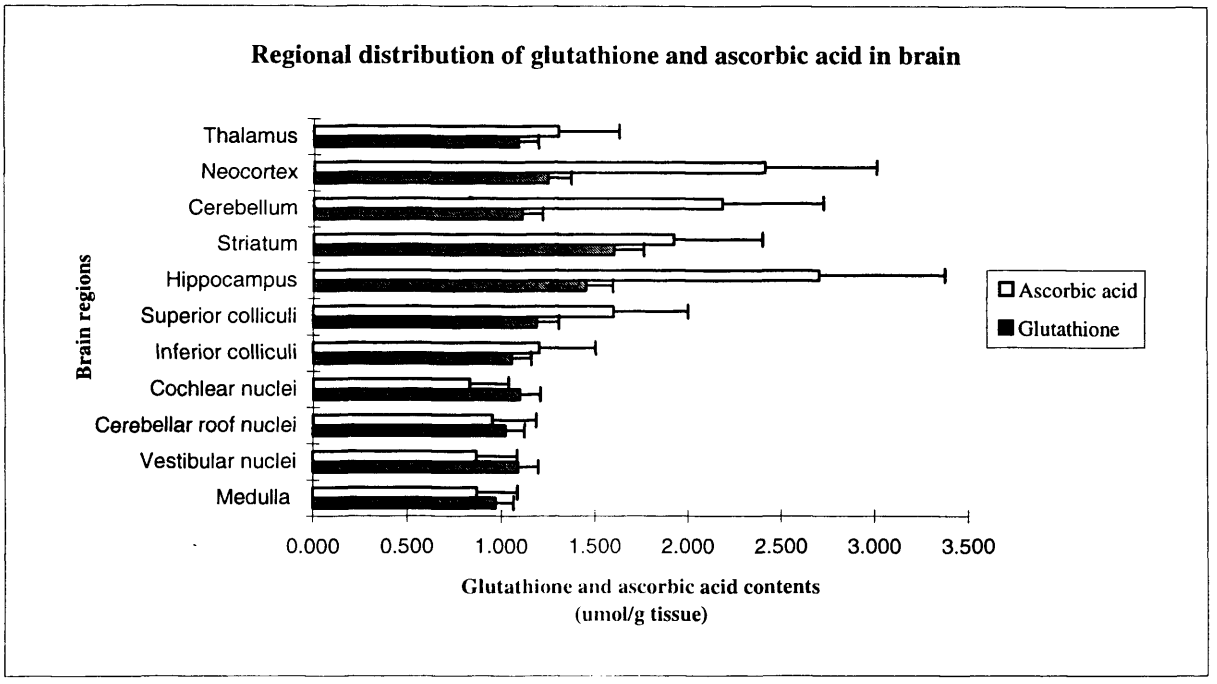


Figure 6.1, Regional distribution of glutathione and ascorbic acid in brain. The regional difference is statistically significant for both glutathione and ascorbic acid ($p < 0.01$,with ANOVA).

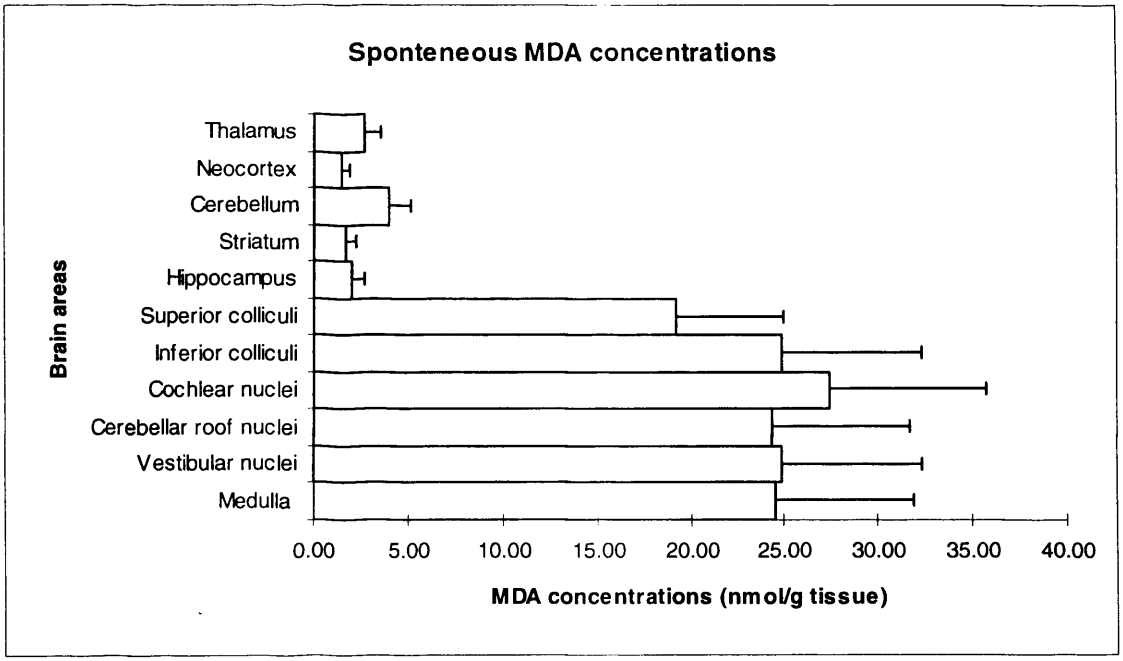


Figure 6.2, Regional distribution of endogenous free MDA in brain. The regional difference is statistically significant ($p < 0.01$, with ANOVA).

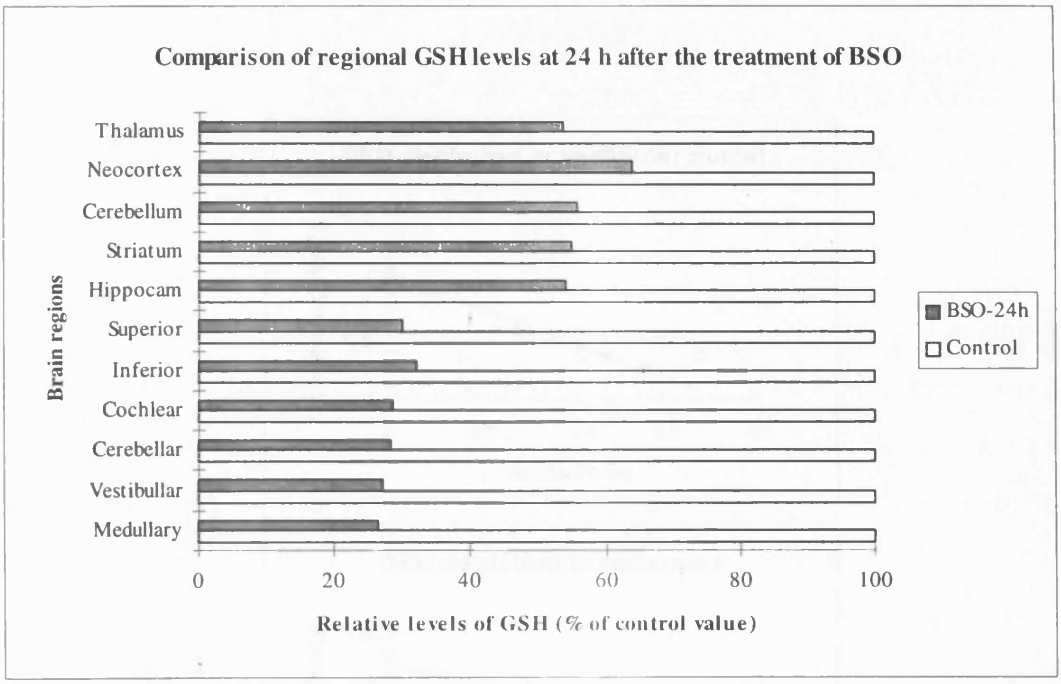


Figure 6.3, Comparison of rat brain GSH levels in BSO treated rats (24 h) with those in control 2-3 month old rats. GSH levels checked in 4 normal animals with the same neurosurgery followed by i.c.v. administration of equal volume of normal saline, were found not significantly different compared with non-operation control rats. Therefore the control values were derived from both non-operation and operated control (9 rats in total). The relative level of specific regional GSH in BSO-treated rats was represented as a percentage of the control level in the same region.

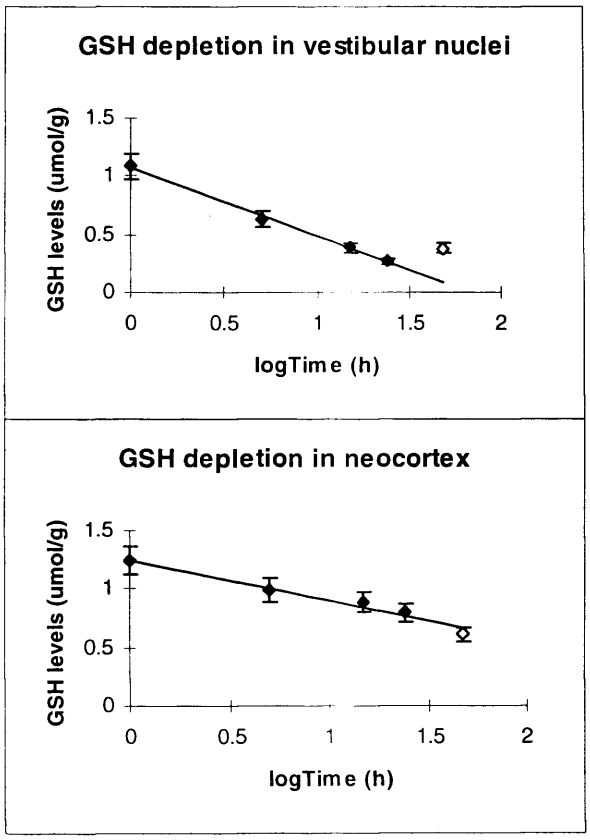


Figure 6.4, Semi-logarithmic plot of GSH levels with time points after BSO treatment. The plots showed a difference in the decline trend of GSH levels at 48 h, reversing in vestibular nuclei while continuing to decrease in neocortex.

CHAPTER 7
AGE-DEPENDENT CHANGES OF BRAIN GSH AND THE REGULATION OF GSH SYNTHESIS

Summary

This is a relatively short chapter illustrating the brain GSH levels in different aged rats. This work was done to confirm lower brain GSH levels in aged animals and to help select appropriate age animals for testing the possible alteration of susceptibility to DNB with age. The GSH levels in hippocampus of 6 d rats was only about half that of young adult rats, then increase sharply until the age of 36 d when GSH levels reach the highest plateau adult levels which lasted until at least 2 months. Brain GSH was decreased significantly in all areas studied at the age of 6 months and thereafter. The developmental changes in hippocampal GSH and cysteine showed an interesting pattern which indicate that cysteine may be a rate limiting factor in brain GSH synthesis during the early period of life.

Introduction

GSH has been suggested to be involved in DNB-induced neurotoxicity (Ray et al., 1994; Romero et al., 1995). This is supported by the *in vitro* results reported in Chapter 5. A lower level of brain GSH has been reported in aged rats (Ravindranath et al., 1989). Thus it would be interesting to assess the susceptibility of aged rats towards DNB-induced neurotoxicity. Prior to the tests on susceptibility, the levels of brain GSH were monitored in experimental rats at different ages. Some interesting related results such as developmental changes in brain cysteine turned up along with the expected GSH results. These led to further thinking about the regulation of brain GSH synthesis through the whole life span, and this is also discussed.

Materials and methods

Animals and brain sampling

After cervical dislocation, the brains were immediately removed from 2-3 month, 6 month, 12 month, and 18 month male rats and dissected into eleven brain areas

as described in Chapter 4. Hippocampus was removed from 6, 12, 24, and 36 day old F344 gender mixed rats (four for each age) after culling by the same method.

HPLC measurement of GSH, GSSG and cysteine

GSH, GSSG and cysteine were measured with the HPLC method which has been fully described in the Chapter 4.

Results

Developmental changes in hippocampal GSH and cysteine

Developmental changes in hippocampal GSH and cysteine levels are illustrated in Figure 7.1. GSH levels increased from 0.73±0.05 μmol/g at day 6 to 1.42±0.09 μmol/g at day 36, remained at this level for 1-2 months, then declined to an level of 1.17±0.05 μmol/g at 6 months. Interestingly, within the neonatal period , brain cysteine is similarly very low, and reached the adult level only after 2-3 months (Figure 7.1).

Age-related changes in brain regional GSH levels

A significant decrease in GSH level was seen in all brain areas of 6 month old and older rats compared with the values in 2-3 month old rats (Table 7.1). The decrease was pronounced in 6 month old animals, but little change occurred afterwards.

Age-related changes in brain regional GSSG levels

At 6 month no change in GSSG level was seen in brain stem areas except for inferior colliculi showing a decrease, while GSSG levels decreased in the other brain areas, i.e. hippocampus, striatum, cerebellar cortex, neocortex, and thalamus. By 12 months, A significant decrease was shown in all brain areas (Table 7.2). Although both GSH and GSSH decreased during ageing, the lack of a decrease of GSSG in brain stem areas of 6 month old rats made the ratio of GSSG/GSH in these areas higher than in other brain areas (Table 7.3).

Age-related changes in brain regional cysteine levels

From 3 months onwards, brain cysteine showed less change than GSH and GSSG. No change was seen in 6 month old rats except for a significant decrease of cysteine in cerebellar nuclei. This decrease was observed in hippocampus and neocortex as well as in cerebellar nuclei in 12 month rats (Table 7.4).

Discussion

Age related profile of brain GSH

The age related profile of brain GSH observed in this study is very well matched with other reports (Bien et al., 1990; Iantomasi, 1993). The content of GSH in rat brain shows a significant increase during the last prenatal phase followed by a significant decrease after birth (Bien et al., 1990). Therefore brain GSH is low in neonates (Jefauconnier et al, 1976; Bien et al., 1990; Kudo et al., 1990; Glockner and Kretzschmar, 1991; Vina et al., 1995), reaches adult levels by 36 days in F344 rats, and decreases with aging (Benzi et al., 1988; Ravindranath et al., 1989; Iantomasi, 1993). There seems to be no gender difference in brain GSH levels (Bien et al., 1990). The brain GSH values measured in this study with the HPLC method are consistent with those cited above, but contrast with the higher GSH levels reported by Shivakumar et al. (1991) who used an enzymatic recycling method (Tietz, 1969). There is also a contradictory report of increased GSH in various brain areas of mouse (Hussain et al., 1995). It is difficult to give a clear explanation for this but the GSH was measured with a less specific method (Ellman, 1959).

GSH and development

Much attention has been paid to the role of GSH in the two extreme phases of life, since the changes in brain GSH levels occur in both the early and final stages of life. BSO treatment to preweanling mice caused retarded growth, severe cataracts and untidy fur (Calvin and Grosshans, 1985). In rat embryonic cultures, glutathione depletion by BSO caused growth retardation and malformations of embryos, the abnormalities including blebs of the maxillary or nasal processes, prosencephalon or forelimb buds; small or misshapen heads, small prosencephalons and swollen hind brains, and tail defects (Slott and Hales, 1987).

GSH and aging

Several reports (Chen et al., 1989; Mizuno and Ohta, 1986; Farooqui et al., 1987; Ravindranath et al., 1989) were in favour of the free radical theory of aging (Harman , 1981), according to which aging may be due, at least in part, to a decrease of the antioxidant capacity and to the destructive effects of free radicals in brain. This theory has recently been criticised since the levels of free-radical reaction products in the brain do not increase with age (Cand and Verditti, 1989; Barja et al., 1990). Thus the derease in some antioxidant systems and in related enzymes may represent down-regulation rather than a cause of further free radical damage. However, a recent report

on gerbils showed significantly higher oxidative stress in three studied brain regions (hippocampus, cortex, and striatum) in middle-aged (6 month old) and old gerbils (20-24 month old) compared with young animals (3 month old) (Zhang et al., 1993). In their study, the oxidative stress was assessed using the formation of 2,3-dihydroxybenzoic acid from salicylate.

Regulation of brain GSH synthesis

Brain cysteine does not reach the adult level until brain GSH arrives at its adult level. This suggests that cysteine may be a rate-limiting factor for GSH synthesis in neonatal brain, because it is likely that all available cysteine is used for GSH synthesis. This observation is consistent with the suggestion that cysteine uptake is a rate-limiting step in GSH synthesis (Bannai, 1986; Griffith,1987).

When human lung carcinoma cells, which use cystine as the sulphur source for the synthesis of GSH, were treated with BSO cystine uptake by the cells was inhibited by up to 75% (Brodie and Reed, 1985). It is believed that this inhibition is due to a decreased need of cystine for the GSH synthesis which is inhibited by BSO, although the uptake inhibition may also be an additional direct effect of BSO (Brodie and Reed, 1985).

Aged animal used in toxicological studies

The decrease of GSH with age is itself of toxicological importance. Aged rats have been found to be more susceptible to DNB-induced testicular toxicity (Brown et al., 1994). After the confirmation that brain GSH was decreased in 6 month old and older F344 male rats, 6 month old rats were used for testing their susceptibility to *m*-DNB induced neurotoxicity (Chapter 8).

Table 7.1, Regional GSH levels in rats of different ages (μmol/g wet tissue)

Brain areas	2-3 month old (n=5)	6 month old (n=4)	12 month old (n=4)	18 month old (n=4)
Medulla	0.97±0.13	0.76±0.06*	0.75±0.05*	0.69±0.06**
Vestibular nuclei	1.09±0.17	0.84±0.10*	0.79±0.04*	0.71±0.08**
Cerebellar nuclei	1.02±0.16	0.81±0.09*	0.69±0.06**	0.75±0.04**
Cochlear nuclei	1.09±0.12	0.78±0.03***	0.75±0.08***	0.74±0.15***
Inferior colliculi	1.05±0.13	0.78±0.09**	0.78±0.03**	0.82±0.06**
Superior colliculi	1.18±0.15	0.95±0.12*	0.93±0.03*	0.93±0.15*
Hippocampus	1.45±0.08	1.17±0.05***	1.08±0.05***	1.06±0.05***
Striatum	1.60±0.09	1.25±0.04***	1.19±0.03**	1.21±0.05**
Cerebellar cortex	1.10±0.14	0.93±0.08*	0.88±0.03*	0.92±0.07*
Neocortex	1.24±0.09	1.04±0.07**	0.93±0.07*	0.96±0.10*
Thalamus	1.08±0.16	0.90±0.07*	0.80±0.02*	0.82±0.05*

The data are presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with GSH levels in 2-3 month old animals.

Table 7.2, Regional GSSG levels in rats of different ages (nmol/g wet tissue)

Brain areas	2-3 month old (n=5)	6 month old (n=4)	12 month old (n=4)	18 month old (n=4)
Medulla	29±4	28±9	22±4*	22±9
Vestibular nuclei	23±4	26±5	19±3*	20±7
Cerebellar nuclei	23±5	26±6	15±5*	23±3
Cochlear nuclei	34±6	35±5	20±6*	31±10
Inferior colliculi	25±4	16±3**	15±6*	22±9
Superior colliculi	29±2	25±7	19±6**	26±2*
Hippocampus	32±9	21±3*	16±6*	19±3*
Striatum	32±5	20±5**	13±2***	17±4*
Cerebellar cortex	20±3	12±1**	9±2**	15±4*
Neocortex	24±7	16±4*	11±3*	15±4*
Thalamus	24±8	12±2*	10±5*	13±5*

The data are presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with GSSG levels in 2-3 month old animals.

Table 7.3, Regional ratio of GSSG/GSH in rats of different ages (as a % of GSH)

Brain areas	2-3 month old (n=5)	6 month old (n=4)	12 month old (n=4)	18 month old (n=4)
Medulla	3.0±0.4	3.7±1.2	2.9±0.5	3.2±1.1
Vestibular nuclei	2.2±0.1	3.1±0.7	2.4±0.3	2.9±1.0
Cerebellar nuclei	2.2±0.4	3.2±0.3	2.2±0.5	3.0±0.3
Cochlear nuclei	3.0±0.6	4.5±0.6	2.7±1.0	4.4±1.7
Inferior colliculi	2.3±0.1	2.0±0.3	2.0±0.8	2.8±1.2
Superior colliculi	2.5±0.4	2.6±0.9	2.1±0.7	2.8±0.4
Hippocampus	2.2±0.6	1.8±0.2	1.5±0.5	1.8±0.3
Striatum	2.0±0.2	1.6±0.4	1.1±0.2	1.4±0.4
Cerebellar cortex	2.0±0.4	1.3±0.2	1.1±0.2	1.6±0.5
Neocortex	2.0±0.4	1.5±0.3	1.2±0.3	1.6±0.5
Thalamus	2.3±0.8	1.4±0.2	1.3±0.5	1.6±0.6

The data are presented as mean ± SD. The regional difference is statistically significant ($p < 0.01$, ANOVA) in 6, 12, and 18 month old animals.

Table 7.4 Regional cysteine levels in rats of different ages (μmol/g wet tissue)

Brain areas	2-3 month old (n=5)	6 month old (n=4)	12 month old (n=4)	18 month old (n=4)
Medulla	0.31±0.06	0.26±0.12	0.22±0.11	0.23±0.03*
Vestibular nuclei	0.25±0.04	0.19±0.07	0.22±0.08	0.23±0.06
Cerebellar nuclei	0.52±0.04	0.31±0.13**	0.35±0.18*	0.28±0.06***
Cochlear nuclei	0.33±0.05	0.37±0.16	0.37±0.21	0.23±0.04**
Inferior colliculi	0.30±0.05	0.29±0.10	0.29±0.14	0.28±0.04
Superior colliculi	0.28±0.04	0.26±0.09	0.30±0.15	0.32±0.09
Hippocampus	0.28±0.07	0.22±0.05	0.19±0.06*	0.20±0.02*
Striatum	0.23±0.07	0.29±0.10	0.18±0.07	0.21±0.02
Cerebellar cortex	0.36±0.06	0.38±0.15	0.32±0.09	0.27±0.05*
Neocortex	0.30±0.06	0.29±0.08	0.20±0.05*	0.17±0.02**
Thalamus	0.24±0.08	0.27±0.09	0.28±0.09	0.30±0.06

The data is presented as mean ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with cysteine levels in 2-3 month old animals.

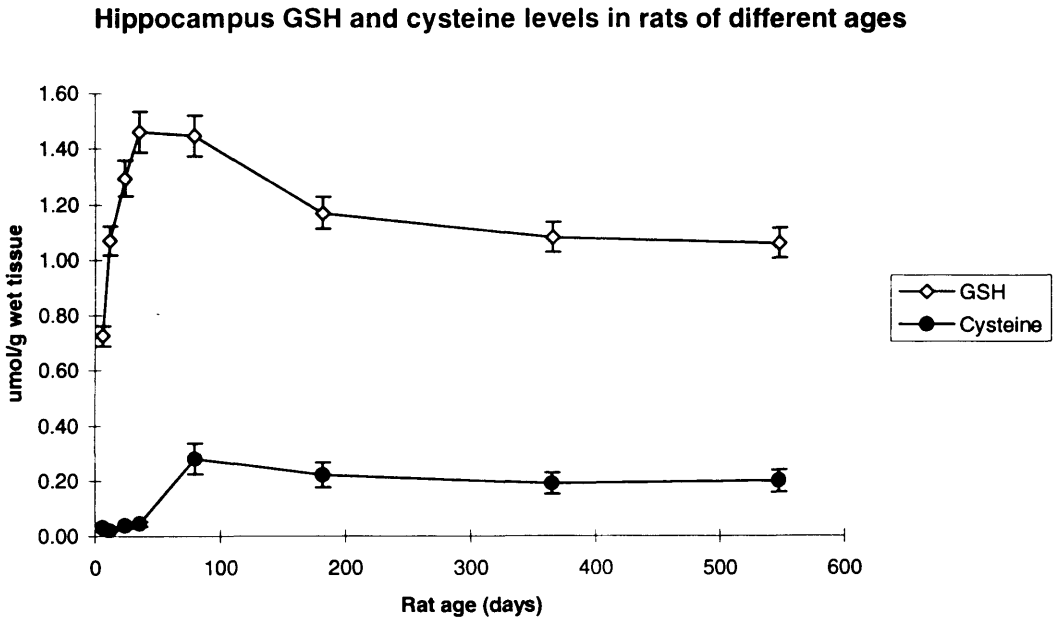


Figure 7.1, Developmental and age-related changes of hippocampal GSH and cysteine level.

CHAPTER 8

BRAIN GLUTATHIONE STATUS AND *M*-DINITROBENZENE NEUROTOXICITY

SUMMARY To test the hypothesis that glutathione (GSH) status in brain tissue plays an important role in the selective neurotoxicity of *m*-dinitrobenzene (DNB), the sensitivity to intoxication of three groups of male F344 rats were studied and correlated with brain tissue GSH levels. In Group I were young 2-3 month old rats with "normal" GSH levels and in Group II were rats of the same age whose brain GSH levels had been reduced by i.c.v. injections of BSO, an inhibitor of γ-glutamylcysteine synthetase. In Group III were 6 month old rats that normally show GSH levels reduced by 16-29% compared with younger animals. All three Groups were subjected to a 3 or 4 dose schedule of dosing with DNB (7.5 mg/kg/day i.p.) and killed 1 day after the last dose of DNB. It was found that whereas Group I animals developed ataxia and brain stem lesions after 4 doses, Group III animals showed these changes after 3 doses while Group II animals had brain stem lesions after only 2 doses of DNB. The timing of the onset of these changes correlated closely with the degree of reduction of brain tissue levels of GSH, this being greatest in those animals dosed i.c.v. with BSO. This demonstrates that GSH status in brain tissue is an important factor in determining regional sensitivity to gliovascular damage from this agent.

INTRODUCTION

m-Dinitrobenzene (DNB) is a neurotoxicant causing glio-vascular brain lesions (Philbert et al., 1987; Romero et al., 1991; Ray et al., 1992). The lesions are selectively localised in brain stem nuclei (particularly the inferior colliculi, vestibular, cochlear, and cerebellar roof nuclei) and show primary endothelial and glial cell damage, followed by secondary neuronal damage. A marked increase in blood flow, petechial haemorrhages, increased permeability of blood vessels and astrocytic swelling are the main early changes in the DNB-intoxicated rat brain (Romero et al., 1991). Biochemically, a significant increase in local cerebral glucose uptake and lactic acid formation occurs both *in vivo* (Ray et al., 1994) and in primary astrocyte cultures (Romero et al., 1995).

Also GSH is significantly decreased in astrocytic cultures 2 h after exposure to sub-cytotoxic levels of DNB (Romero et al., 1995). Therefore it was suggested that the neurotoxicity of DNB may be mediated by oxidative stress in which GSH is involved (Romero et al., 1991, 1995). This hypothesis has been supported by the finding that DNB can be locally metabolised to *m*-nitroaniline in brain tissue (Hu and Ray, 1995) with generation of nitrosonitrobenzene as an intermediate (Hu et al., 1996), indicating that free radicals may also be locally generated in brain by this means. Nitrosonitrobenzene has been demonstrated to be more toxic than DNB to testicular cells *in vitro* (Cave and Foster, 1990).

GSH is well known to be important in the cellular defence against oxidative stress as well as functioning in phase 1 and phase 2 reactions by reduction of, or conjugation with, with xenobiotics and endogenous substances (Meister and Anderson, 1983; Hu et al., 1997). The present study was designed to evaluate the effects of GSH depletion on DNB neurotoxicity. Six month old rats were also chosen to test DNB toxicity as a model of naturally low brain GSH, since brain GSH decreases in aged animals (Benzi et al., 1988; Ravindranath et al., 1989; Iantomasi, 1993). In a preliminary study brain GSH levels were measured in rats at 6, 12 and 18 months old, all of which showed a naturally low level of brain GSH. The extent of this depletion was similar to that reported by others (Benzi et al., 1988; Ravindranath et al., 1989). Therefore six month old rats were chosen for this study. BSO, a specific inhibitor of γ-glutamylcysteine synthetase, was administered by intracereberoventricular (i.c.v.) injection to produce brain GSH depletion in young adult rats (Pileblad and Magnusson, 1988; 1989). The effect of diminished GSH on DNB neurotoxicity was thus examined in both 6 month old animals and in animals with BSO-induced brain GSH depletion.

MATERIALS AND METHODS

Implantation of i.c.v. injection cannula guide and BSO injection

6-8 week old male F344 strain rats were implanted with a cannula guide (Atlas of Paxinos & Watson) for injection into the lateral ventricle by the method of Ray et al.

(1996). The animals were allowed to recover from surgery for 7-10 days before dosing. Full details is in Chapter 4.

BSO was dissolved in distilled water, pH adjusted to 7.4 by sodium hydrogen carbonate, filtered through a 0.2 μm sterile syringe filter (Acrodisc, Gelman Sciences, USA) and was injected directly into the ventricle at an injection volume of 40 μl containing 3.2 mg BSO per rat. A microdrive syringe was used to achieve an injection rate of 2 μl/min, which matches natural CSF turnover. A single dose of BSO was given on 3 alternate days to deplete GSH and maintain the expected low level for the period required by the 4 dose model of DNB toxicity. Solvent controls were injected with 40 ul of 0.9% sodium chloride.

Models of DNB neurotoxicity

Intraventricular saline injected 6-8 week old (Group I), intraventricular BSO-injected 6-8 week old (Group II), and 6 month old (Group III) male F344 rats were allowed free access to food and water and kept in plastic cages. Two DNB neurotoxicity models were used. Model 1 was three doses of 10 mg/kg DNB given at 0, 4 and 24 h (Ray et al., 1992). This is a model producing rapid onset of damage for biochemical time course assessment. Model 2 was a daily dose model of 7.5 mg/kg given on four consecutive days, producing exactly the same kind of lesions to the that of model one, but with an onset of damage at day 4, rather than day 1, and (most importantly) much less inter-animal variability in lesion severity. Because of this more reproducible damage, the second model was used for the most of this study. However because of the prolonged dosing period, and hence possible overlap between acute and reactive responses known to be produced by DNB (Romero et al., 1995), the time course of the effect of DNB on GSH in normal rats was still investigated with the more acute Model 1. In both cases DNB was dissolved in DMSO and given by intraperitoneal injection at the volume of 1ml/kg.

Tissue preparation and HPLC measurement of brain GSH

Rats were killed by cervical dislocation. Brain and liver were removed immediately. The brain was cut into eleven coronal slices (2 mm thick). Microdissection was performed to obtain tissues from the following eleven representative areas:

medulla; vestibular, cerebellar roof and cochlear nuclei; inferior and superior colliculi; hippocampus; striatum; cerebellum; neocortex; and thalamus. Homogenates (5% or 10%, w/v) were made in 0.6 M perchloric acid (PCA) with a hand operated glass homogeniser.

GSH measurement was by a fluorimetric HPLC method (Martin and White, 1991) with slight modifications in sample preparation and mobile phase gradient conditions. The technical details have been described in the Chapter 4. GSH measurements were carried out 2, 8, 15, 24 hours after the last dose of DNB for the DNB time course study, and 24 hours after the first and last dose of BSO for the BSO study. No GSH measurements were made in the combined BSO and DNB study since all tissue was used for histology.

Ataxia scores

Rats were assessed daily for the severity of ataxia and muscle weakness according to a 10 point severity scale (Ray et al., 1996). See Chapter 4 for details.

Pathological examination

Animals were perfused through the ascending aorta with normal saline followed by formalin-acetic acid (10% and 2%, v/v) under terminal halothane anaesthesia. Step serial sections (1:15, 10 μm) of paraffin embedded blocks were stained with H & E.

RESULTS

Glutathione concentration in brain and its depletion

As shown in Table 1, GSH is non-uniformly distributed across brain regions in untreated 2-3 month old rats. The concentration is highest in striatum, followed by hippocampus, neocortex, cerebellum, thalamus, and lowest in brain stem nuclei ($p < 0.001$ by ANOVA).

BSO effectively depleted GSH in all brain regions (Table 1), however the extent of depletion differed in individual regions. In brain stem regions GSH was depleted by 68-73% 24 h after the first dose of BSO (full data were displayed in Chapter 6) and by

84-90% after the third dose of BSO. In the other regions GSH depletion was less marked, being 36-48 % after the first dose and 63-83% after the third dose of BSO. In contrast to GSH depletion by BSO, GSH was decreased more or less equally (16-29 %) in all regions of aged rats.

Regional GSH concentration was measured in the three-dose DNB model rats at 2, 8, 15, and 24 hours after the final dose of DNB. The data presented in the Table 1 is for 24 h, when the lesions are fully developed. No significant change of GSH and GSSG was observed at any time point. The same measurement has also been done at 2 and 5 hours after a single dose of DNB (20mg/kg) and similarly no significant change was seen in brain GSH and GSSG in either sensitive areas or resistant areas.

Most of the BSO-treated animals had brief episodic running fits and epileptiform seizures 5-20 h after the first (but not subsequent) administration of BSO. No morphological change was observed in brains 24 h after a single BSO treatment (four rats were investigated). However, mild haemorrhages, without astrocytic or neuronal damage, were found in 2 out of 5 rats receiving 3 doses of BSO (Figure 2).

DNB intoxication

In the DNB toxicity study, experiments were carried out with the four daily dose model. No ataxia was seen in any of the three groups after the first or second dose of DNB. After the third dose of DNB, ataxia developed in the 6 month old and BSO treated groups, but not in the young adults which only developed ataxia after the fourth dose of DNB (Table 2).

Consistent with the previous reports (Philbert et al., 1987, Ray et al., 1992), lesions were seen in a number of brain stem areas (Table 3). In young adult animals, pathological changes (not including haemorrhage) could only be seen after the fourth daily dose of DNB, while these changes were present after the third dose of DNB in the 6 months old group (4 out of 4 rats) and were found even after the first or second dose in some animals from the BSO treated group (2 out of 4 rats) (Figure 3a; 3b). Lesions were also found in the hypothalamus in one animal from the BSO treated group after the fourth dose of DNB, while no hypothalamic lesions were observed in the young adult animals given DNB alone, nor seen in previous studies (Philbert et al., 1987, Ray et al., 1992).

DISCUSSION

An increased susceptibility of rats to DNB neurotoxicity was associated with both age-related and BSO-induced brain GSH depletion. BSO-induced depletion was checked at 24 hours after the last of 3 doses of BSO, but it has been reported (Pileblad and Magnusson, 1989) that BSO greatly depletes brain GSH from 24 h onwards if injections of BSO are on every second day. This suggests that GSH would have been maintained at a low level throughout DNB pathogenesis in our experiments, as the BSO was given from 48 h before the first dose of DNB and continued throughout DNB intoxication. The resulting increase in susceptibility to DNB neurotoxicity is consistent with an *in vitro* study by Cave and Foster (1990) of testicular toxicity, which demonstrated that GSH depletion with diethyl maleate enhanced toxicity of DNB and nitrosonitrobenzene in Sertoli-germ cell co-cultures. Although 3 doses of BSO alone induced haemorrhages in some of our animals, the enhancement of the astrocytic and neuronal lesions caused by DNB was clearly not an additive effect, since BSO alone caused no such lesions. Although BSO is a specific inhibitor of γ-glutamylcysteine synthetase, it is of course possible that the enhancement of DNB neurotoicity was brought about by a mechanism other than GSH depletion. A related question has been discussed by Beiswanger et al. (1993) with regard to the increased severity of the acrylamide induced cerebellar granule cell lesions that is produced by prior depletion of glutathione with styrene oxide, since depletion of glutathione with other agents did not produce an equivalent effect.

An increase in DNB neurotoxicity was also observed in 6 month old rats, which were found to have significantly lower brain GSH than young adult rats. It may be argued that GSH depletion might be only one of several age-related changes contributing to the increased susceptibility to DNB in older animals. However the results from young rats treated with BSO suggest that GSH depletion may be an important factor in this increased susceptibility. Brown et al. (1994) reported an age-related increase in the testicular toxicity of DNB in rats, associated with a longer DNB half life of 4.5 h in blood in 120 day old rats compared with 3.3 h in 75 day old rats. However, these authors suggested that the metabolic difference was insufficient to adequately explain the increase in testicular damage in older rats (Brown et al., 1994). Brain GSH decrease with age has been reported previously (Benzi et al., 1988;

Ravindranath et al., 1989) however, strain variations may exist as the decrease we observed started at a younger age than that previously reported.

These experiments suggest that the level of brain GSH may be crucial in determining the susceptibility to DNB. It could be speculated that depletion of brain GSH may altere the antioxidative status and molecular defence system of the DNB-sensitive brain areas. Vitamin E, an alternative antioxidant, has been shown to protect against DNB toxicity (Lowndes et al., 1994). Recent *in vitro* results (Hu et al, 1996) show that nitrosonitrobenzene, a DNB metabolite more toxic to testis (Cave and Foster, 1990), can be further reduced to nitroaniline by GSH in a non-enzymatic reaction. This suggests that GSH could affect DNB toxicity in at least two ways. One is as an antioxidant, and the other is its involvement in the detoxification of the intermediate. Although GSH depletion clearly enhances DNB toxicity and DNB depletes GSH *in vitro* (Ray et al., 1994), no significant fall in brain GSH was seen during the development of DNB-induced lesions. This is not surprising because only less than 3% of brain GSH would be consumed if GSH was mainly involved in a covalent reaction with nitrosonitrobenzene, which is present in brain at about 10^{-5} M during intoxication. Even though GSH plays a protective role in DNB neurotoxicity, it is clear that it can not provide complete protection. This may indicate that only a minor compartment of total cellular GSH has immediate access to the generated nitrosonitrobenzene. Once GSH is depleted, especially if γ-glutamylcysteine synthetase is inhibited, its defensive ability will be greatly diminished.

Mitochondrial dysfunction may be another important factor responsible for the increased sensitivity following GSH depletion. Jain et al. (1991) have clearly shown the enlargement and reduction in density of mitochondria in brain after BSO treatment. Citrate synthase activity (Jain et al, 1991, Heales et al, 1995), a mitochondrial matrix marker enzyme, and the activity of cytochrome C oxidase (Heales et al, 1995) were found to be significantly decreased by BSO treatment. This may affect DNB metabolism in cells, and prolong the life span of toxic intermediates, such as nitrosonitrobenzene. Energetic disturbance has been reported in association with DNB toxicity (Romero et al., 1995). Moreover, increased or decreased neuronal activity and its metabolic consequences modulate DNB neurotoxicity to enhance or diminish histological lesions (Ray et al, 1992, Cavanagh, 1993). Although increases or decreases in metabolic

demand may affect antioxidative capacity by leaving less or more reduced equivalents for antioxidant functions, the exact manner in which they interact is not yet understood.

One of the most challenging concerns in neurotoxicology is the mechanism underlying selective toxicity. Glial cells and vascular endothelial cells seem to be the selective target of DNB (Philbert et al., 1987, Romero et al., 1991). This may be partially explained by their enrichment in non- specific nitroreductases such as xanthine oxidase (Betz 1985). We observed that GSH levels were lower in brain stem areas compared with other areas (Table 1), which is consistent with other reports (Benzi et al., 1988; Ravindranath et al., 1989; Iantomasi, 1993, Philbert et al., 1991). In addition to this distribution difference, the sensitivity towards GSH depletion is higher in brain stem than other areas. This is a reflection of higher GSH turnover in brain stem (Hu et al., 1996). Even though it is too early to conclude that the low level of GSH is the dominant reason for higher sensitivity of these areas to DNB, this study does provide substantial evidence of the involvement of GSH in determining susceptibility. In the BSO-treated rats a hypothalamic lesion was found after the third dose of DNB. This area was not damaged in young adult rats, even after the fourth dose of DNB. This observation means that depletion of GSH may not only increase the sensitivity of the sensitive areas, but also caused an originally insensitive area to become sensitive. This raises the question whether other resistant areas e.g. hippocampus or cortex would become sensitive with adequate GSH depletion. This is an extremely difficult question to answer *in vivo*, since more severe brain stem lesions would prove fatal (as in our GSH depleted groups given 4 doses of DNB) before less sensitive areas became involved, unless the GSH in insensitive areas could be selectively depleted without affecting the originally sensitive areas.

Table 8.1 Brain regional GSH levels in rats with different treatment (μmol/g)

Regions	Group I (6-8 week old)	Group II (3 dose BSO treated)	Group III (six month old)	DNB treated (6-8 week old)
	(n=5)	(n=4)	(n=4)	(n=6)
Medulla	0.97±0.13	0.10±0.04***	0.76±0.06*	0.90±0.08
Vestibular nuclei	1.09±0.17	0.09±0.01***	0.84±0.10*	0.98±0.20
Cerebellar nuclei	1.02±0.16	0.09±0.01***	0.81±0.09*	0.91±0.22
Cochlear nuclei	1.09±0.12	0.11±0.01***	0.78±0.03***	0.98±0.14
Inferior colliculi	1.05±0.13	0.15±0.03***	0.78±0.09**	0.98±0.14
Superior colliculi	1.18±0.15	0.19±0.06***	0.95±0.12*	1.23±0.18
Hippocampus	1.45±0.08	0.42±0.11***	1.17±0.05***	1.45±0.12
Striatum	1.60±0.09	0.33±0.11***	1.25±0.04***	1.60±0.11
Cerebellar cortex	1.10±0.14	0.26±0.02***	0.93±0.08*	1.11±0.09
Neocortex	1.24±0.09	0.46±0.09***	1.04±0.07**	1.21±0.16
Thalamus	1.08±0.16	0.18±0.06***	0.90±0.07*	1.15±0.14

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with values of the same regions from Group I rats.

DNB treatment: 3 × 10 mg / kg at 0, 4, and 24h, GSH was measured at 48 h.

BSO treatment: 3 × 3.2 mg / kg at 0, 48, and 96h, GSH was measured at 120 h.

Table 8.2 Mean ataxia scores (out of 10) in different groups of rats after DNB intoxication

No. of DNB doses	1 (n=12)	2 (n=12)	3 (n=8)	4 (n=4)
2-3 months old	0	0	0	4.3
6 months old	0	0	4.5 *	no survival
BSO treated	0	0	3.8 *	no survival

* $P < 0.01$, compared with the that of 2-3 months old rats after the third dose of DNB, by Wilcoson unpaired test.

Table 8.3, Comparison of the pathological changes† in brain stem areas of DNB treated rats with different status

Group	Group I				Group II‡			Group III	
DNB dose	1	2	3	4	1	2	3	2	3
Cerebellar roof nuclei	0/4	0/4	0/4	3/4	2/4*	2/4*	4/4*	0/5	4/4*
Vestibular nuclei	0/4	0/4	0/4	3/4	0/4	0/4	4/4*	0/5	4/4*
Cochlear nuclei	0/4	0/4	0/4	2/4	0/4	1/4	3/4*	0/5	0/4
Hypothalamus	0/4	0/4	0/4	0/4	0/4	0/4	1/4	0/5	0/4

† The figure is presented as number of rats with pathological changes / number of all rats observed. * $P < 0.05$ compared with that of young adult rats receiving same number of doses of DNB, by Chi square test. ‡ The number of BSO doses were 2, 2 and 3 for the 1, 2, and 3 dose DNB animals in Group II. (Group I were 6-8 week old rats given intraventricular saline. Group II were 6-8 week old rats given intraventricular BSO. Group III were normal 6 month old rats). The number of BSO doses were 2, 2 and 3 for the 1, 2 and 3 dose DNB animals.

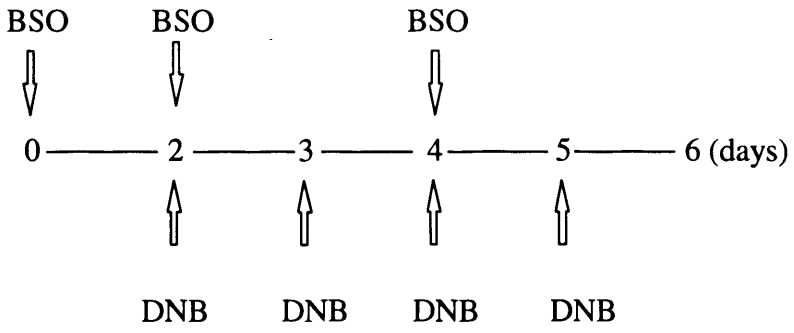


Figure 8.1 The dosing schedule for evaluating DNB neurotoxicity in BSO treated rats. BSO was administered by i.c.v. and DNB was given by i.p. injection. Animals were killed 24 h after the scheduled final dose of DNB.

Figure 8.2 Coronal H&E section through the brainstem of a rat given 3 doses of 3.2 mg BSO i.c.v. on alternate days and killed 24 hours after the third dose, showing a haemorrhage (H) with compaction of surrounding neuropil (➡) adjacent to the fourth ventricle (V). Magnification × 150.

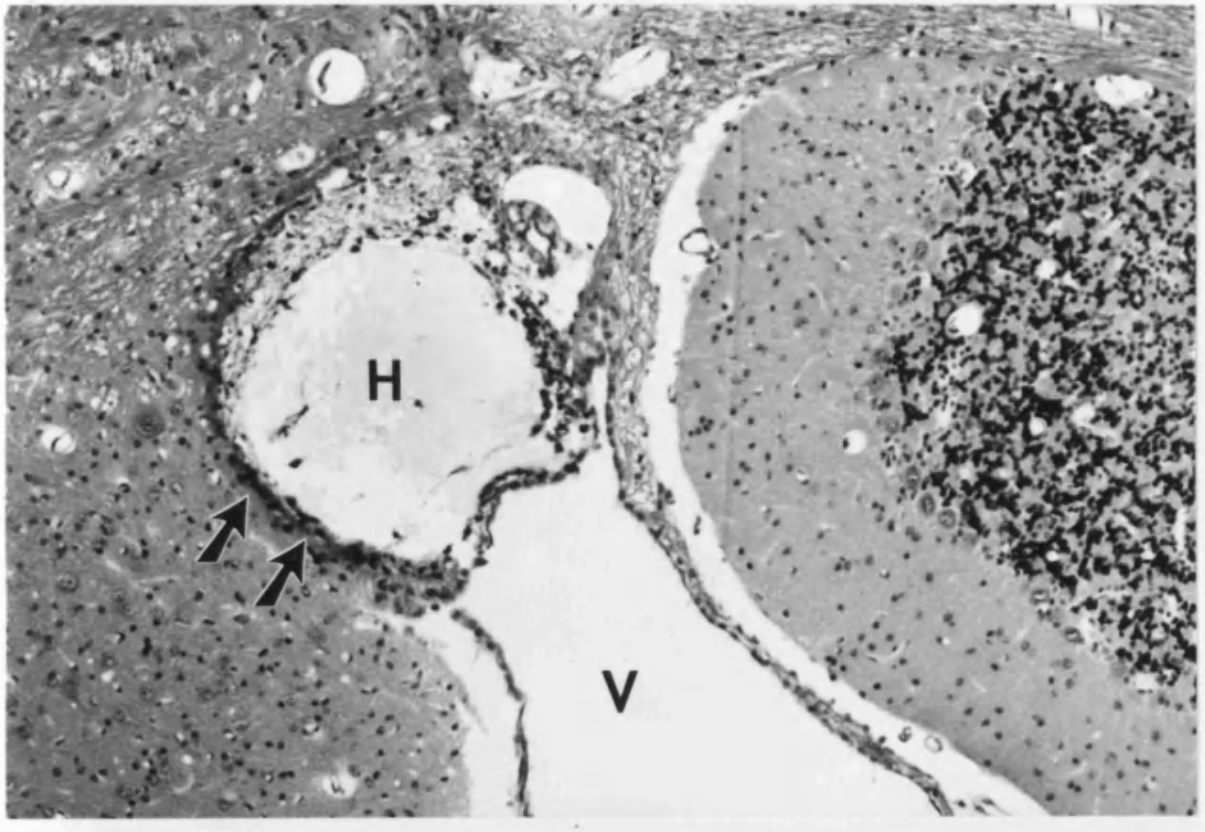
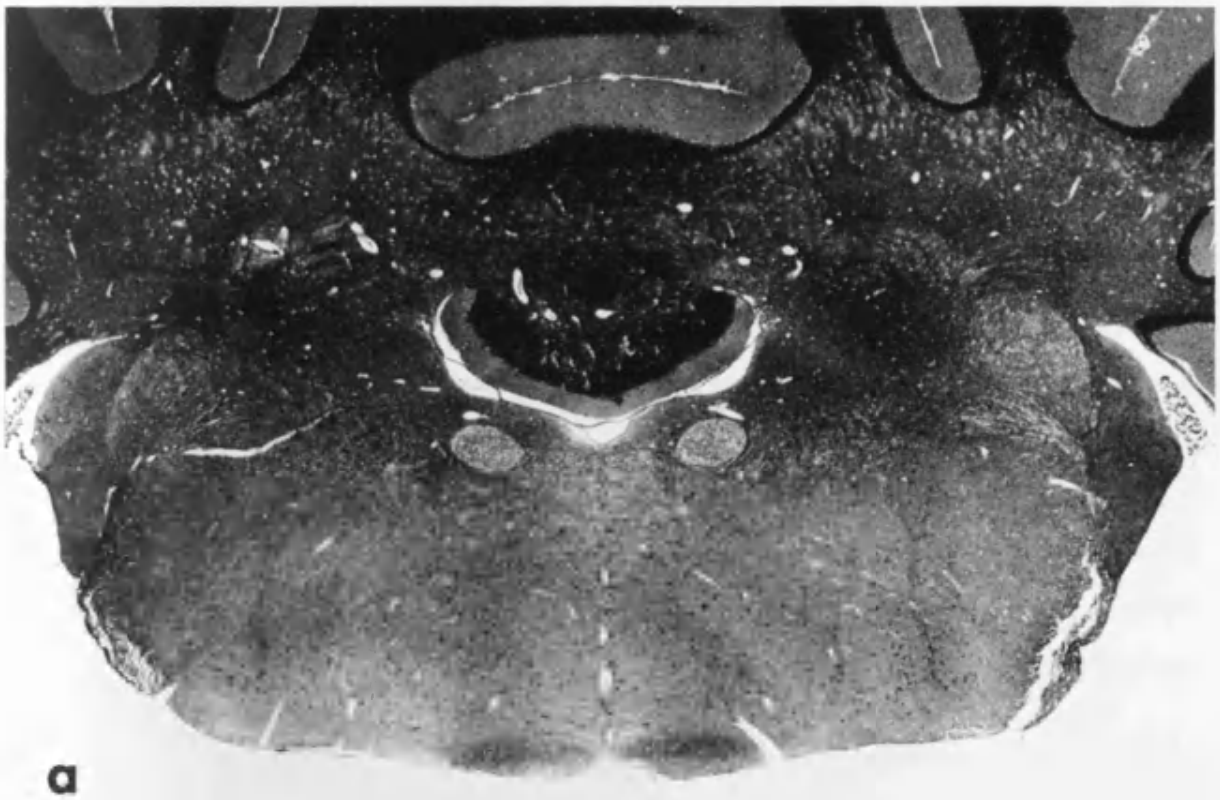
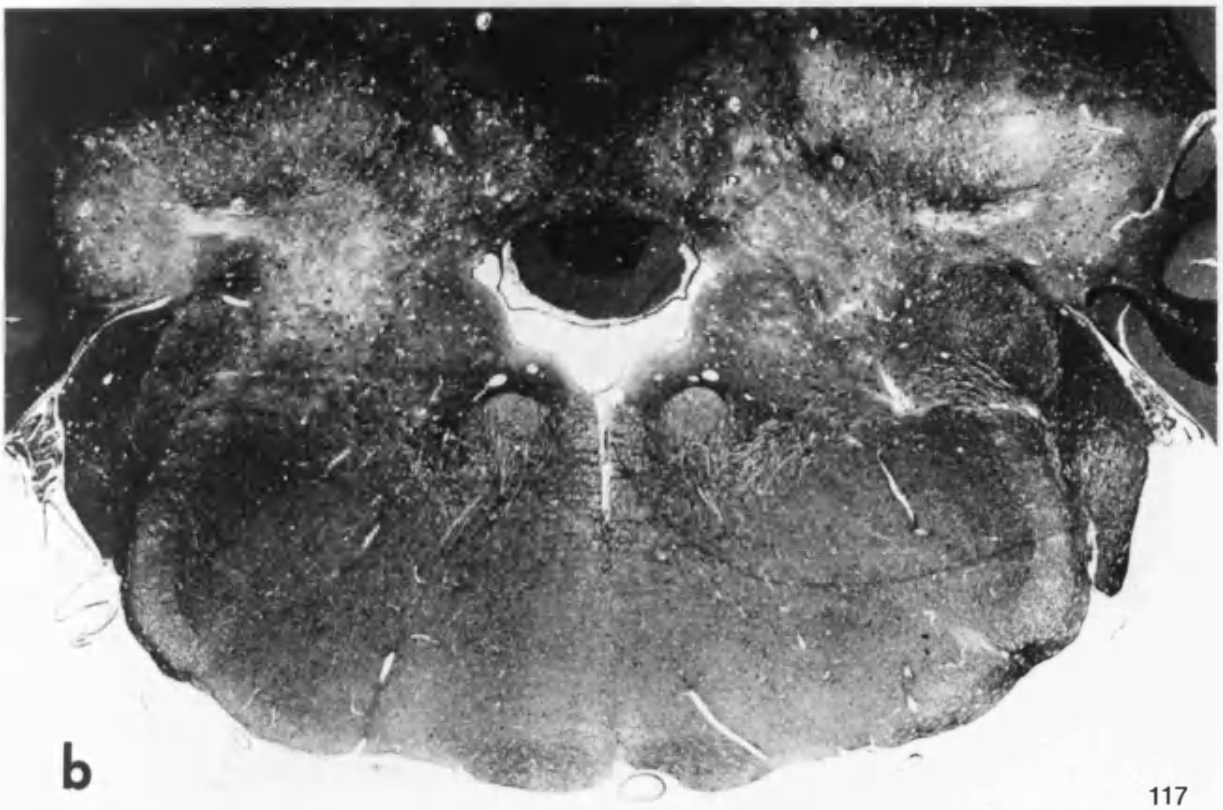


Figure 8.3 Coronal H&E sections through the brainstem of rats given 3 daily doses of 7.5 mg/kg DNB and killed 24 h after the final dose. DNB alone produced no visible damage (a), whereas animals with depleted glutathione developed severe vacuolar lesions (b) typical of DNB in the cerebellar roof, vestibular and cochlear nuclei.





CHAPTER 9

BRAIN GLUTATHIONE IN THE MODULATION OF THE EXCITABILITY OF CENTRAL NERVOUS SYSTEM

Summary Following selective inhibition of brain glutathione (GSH) synthesis by i.c.v. administration of BSO, we found that GSH depletion caused generalised seizures in rats between 5-20 h, which could be prevented by maintaining brain GSH levels with co-administration of γ-glutamylcysteine (γ-GC) by the same route. At 5 h when the seizures started, brain GSH was depleted to 61-80% of predose concentrations depending on region, but concentrations of brain GSSG (except for that in medulla), aspartic acid, glutamic acid, and cysteine were unchanged. Only by 48 h (when GSH was depleted to 35-50%) was a decrease of GSSG, increase of aspartic acid and glutamic acid, but no change of cysteine observed. These observations suggest a primary involvement of GSH in modulating excitability of the central nervous system. The greatest depletion of GSH was seen in brain stem areas. The results may provide an alternative explanation of methionine sulfoximine-induced convulsions in experimental animals, and have implications for the understanding of the evolution of spontaneous seizures such as neonatal seizure.

Introduction

Glutathione (GSH), a tripeptide consisting of glycine, cysteine, and glutamic acid, has an important biological role in the degradation of endogenous substances and xenobiotics by conjugation with their metabolic intermediates. It also protects cells from oxidative stress by detoxifying free radicals and peroxides under the action of glutathione peroxidase (Meister and Anderson, 1983). Present in the brain at millimolar concentrations, GSH may also have a more direct but poorly characterised involvement in central nervous system functions (Orlowski and Karkowsky, 1976). There is increasing interest in the possible function of GSH as a neuromodulator acting at specific binding sites in the CNS (Ogita and Yoneda, 1987; Guo et al., 1992; Lanius et al., 1994). Patients with inherited GSH synthetase deficiency exhibit mental retardation, spastic tetraparesis and signs of cerebellar damage, while patients with γ-

glutamylcysteine synthetase (γ-GCS) deficiency exhibit signs of spinocerebellar degeneration, absent lower limb reflexes and ataxia (Orlowski and Karkowsky, 1976).

GSH synthesis occurs in two steps; the first is the conjugation of glutamic acid and cysteine under the action of γ-GCS, and the second is the further addition of glycine under the action of glutathione synthetase. *L*-buthionine-*SR*-sulfoximine (BSO), a potent and specific inhibitor of γ-GCS (Griffith and Meister, 1979a), has been widely used for depleting tissue GSH. However, due to its poor passage across the blood-brain barrier, systemic administration of BSO to adult rodents produces no effect on brain GSH (Griffith and Meister, 1979b). Local administration of BSO into the cerebral ventricles (i.c.v.), however, proves effective in depleting brain GSH (Pileblad and Magnusson, 1988; 1989). This provides a useful way to look into the biological importance of GSH in the CNS.

In our previous study, we noticed that rats with brain GSH depletion induced by BSO developed seizures (Hu et al., 1996; Chapter 8), but BSO-induced seizures have not yet been fully described. BSO is not generally regarded as causing seizures (Griffith, 1982), though occasional and minor seizures have been mentioned as a dose-limiting effect in BSO-treated rats by some authors (Pileblad and Magnusson, 1989; Jain et al., 1991), but not others (Martensson et al, 1991; Heales et al, 1995; Heales et al, 1996). This study was designed to investigate the BSO-induced seizures in detail and investigate the neurochemical basis for the onset of hyperexcitation.

METHODS AND MATERIALS

Implantation of i.c.v. injection cannula guide and administration of BSO and γ-GC

Male F344 rats (6-8 week old, weighting 200-220 g) were implanted with a cannula guide to allow injections into the lateral ventricle (Ray et al. 1996) and allowed to recover for 7-10 days before dosing.

BSO (Sigma, USA), BSO plus γ-GC or BSO plus ascorbic acid, dissolved in distilled water and adjusted to pH 7.4 with sodium hydrogen carbonate, was injected directly into the right lateral ventricle through the inplanted injection guide with a fixed length 30 gauge needle. The injection volume was 40 μl containing 3.2 mg BSO per rat

(approximately 1.6 mg/g brain). Injection was accomplished over 20 min at a rate of 2μl/min, using a microdrive syringe. The dosage for γ-GC was 2.0 mg/injection, and 1mg/injection for ascorbic acid.

Tissue preparation for biochemical analysis

Rats were killed by cervical dislocation and the brains immediately removed. Whole brain was cut into 11 slices of 2 mm thickness. Microdissection was performed on ice under a microscope to obtain tissues from the following eleven representative areas: medulla; vestibular, cerebellar roof & cochlear nuclei; inferior & superior colliculi; hippocampus; striatum; cerebellar cortex; thalamus; and cerebral cortex. Homogenates (5% or 10% w/v) were made in 0.6 M perchloric acid (PCA) solution using a glass microhomogeniser.

HPLC measurement of brain GSH and relevant amino acids

An HPLC fluorescence method was used to measure GSH and other relevant substances. The technical details have been described in the General Materials and Methods (please see Chapter 4).

Since the concentration of glutamic acid was two orders of magnitude greater than GSSG, the signal was too large to be quantified from the same sample. Consequently a second, smaller sample was analysed for glutamic acid from two representative areas: vestibular nuclei and hippocampus.

Pathological examination

Four rats were terminally anaesthetised and perfused through the ascending aorta with normal saline followed by formalin-acetic acid (10%/2%, v/v), at 24 hours after the administration of BSO. The brain was removed, sliced and processed into paraffin wax. Step serial sections (10μm, 1 in 15) were cut and stained with haematoxylin and eosin and examined by light microscopy.

Seizure observation and behavioural monitoring

After the administration of BSO, animal behaviour was under close observation and continuously monitored via a video camera for 24 hours, and daily observations

made thereafter. All animal experiments were conducted under U.K. requirements for humane treatment.

Statistics

ANOVA was used to test the variation among the brain regions at 0 h. Student's t-test was used to test the difference between 0h and later time points. The Chi-square test was used to examine the difference in the incidence of seizures across groups.

RESULTS

Effects of BSO treatment on rat excitability

After the administration of BSO, 15 of 16 treated rats developed generalised seizures and running fits starting at 5 hours and continuing intermittently for up to 20 hours (Table 9.1). Typically 3-5 episodes occurred over 5-8 h post-dose, each episode lasting 5-10 seconds, usually of a sudden onset but sometimes preceded (and followed) by facial myoclonus. The running fits showed vigorous continuous uncontrolled running or jumping movements which threw the body about. The movements continued even if the rat was held such that the legs were not in contact with a surface. Rats were unreactive to external stimuli during the fits. The fits always terminated with a tonic extensor spasm lasting about 5 seconds. Recovery of reactivity to sensory stimuli was then gradual over 2-3 minutes. No histopathological alteration was observed in the brains of rats killed at 24 h after BSO administration.

To clarify whether the seizures were caused by GSH depletion alone or by some other unknown effect of BSO, we co-administered i.c.v. BSO with γ-GC, to provide a substrate to compensate for the inhibitory effect of BSO and thus maintain brain GSH levels (Pileblad and Magnusson, 1992). This fully prevented the seizures (Table 9.1). Ascorbic acid, another antioxidant, gave no protection against seizures in BSO-treated rats. It is also noteworthy that no further hyperexcitation was observed after 20 h, despite continuing GSH depletion, nor at any time after a second or a third dose of BSO given at two day intervals which depleted GSH to10-20 % of predose values.

Neurochemical changes after BSO treatment

In order to understand more about the neurochemical basis of this excitability, brain GSH, GSSG, aspartic acid, glutamic acid and cysteine were measured in BSO treated animals at 5, 15, and 48 h. As expected, depletion of GSH was seen after the i.c.v. administration of BSO (Table 9.2). Depletion was statistically significant at 5 h, the earliest measurement. GSH was clearly non-uniformly distributed across brain areas and the depletion was also heterogeneous across the eleven brain areas, being greater in the brain stem and relatively less in forebrain and cerebellar cortex (Table 9.2). The low

GSH level was maintained for at least 48 h after a single administration of BSO (Table 9.2).

GSSG, which accounts for 1-2% of GSH in normal brain samples, was significantly depleted in all areas at 48 h, with a somewhat earlier depletion in the medulla and inferior colliculi (Table 9.3). GSSH/GSH ratios were not significantly changed.

The concentration of glutamic acid in controls was higher in the hippocampus than in the vestibular nuclei (Table 9.4). In these two areas, the glutamic acid concentration was unchanged at 5 and 15 h, but elevated by 48 h, the vestibular nuclei showing a larger increase (+43 %) than the hippocampus (+18 %).

Aspartic acid concentrations in brain were also unchanged at 5 and 15 h, but significantly elevated in four brain stem areas, (i.e. medulla; vestibular, cerebellar roof, and cochlear nuclei) at 48 h (Table 9.5).

Normal cysteine contents ranged from 0.07±0.01 μmol/g (in striatum) to 0.14±0.03 μmol/g (in cerebellar cortex). Cysteine, a precursor for GSH synthesis, was not altered in any area except for an increase of 56% in medulla at 48 h ($p < 0.05$).

An unidentified HPLC peak in the analytical chromatogram (Figure 9.1) gave a signal that was 50 times higher in cerebellum than that in neocortex and somewhat greater in brain stem areas (Table 9.6). This substance was markedly increased after the BSO treatment from as early as 5 hours (Table 9.6).

DISCUSSION

This study demonstrates that acute depletion of brain GSH is closely associated with running fits and seizures. This conclusion is supported by the association between BSO-induced GSH depletion and seizure occurence, and also by the protective effect of γ-GC when co-administered with BSO. A depressed GSH level has been observed in epileptic foci of some experimental seizure models (Berl et al., 1959; Hiramatsu and Mori, 1981) and the brain of genetically epileptic mice (Abbott et al., 1990), but it could be argued that this might have been secondary to seizure activity. Systemic administration of BSO to new-born rats (which lack the blood brain barrier) causes signs of neurological disturbance, including lethargy, intermittent tremors and minor fits (Jain et al., 1991). These might however have been attributable to the complex effects of whole body GSH depletion. Occasional convulsions after i.c.v. administration of BSO have been mentioned by others (Pileblad and Magnusson, 1989), but not much comment has been made on the phenomenon. This may be due to others having missed the effect due to its delayed and transient nature. Our findings now provide direct evidence that GSH may act as a neuromodulator regulating neuronal excitability and convulsive threshold. Others have suggested a further role for GSH in other behaviour patterns such as depression (Pal and Dandiya, 1994) and sleep (Honda et al., 1994). Even though glutamic acid and aspartic acid are two well known excitatory amino acids, no change was observed in their concentrations until after the seizure period suggesting that these changes were of a secondary nature.

GSH is known to bind at specific sites in the CNS (Ogita and Yoneda, 1987; Guo et al., 1992; Lanius et al., 1994). The binding leads to an increase of inositol-1,4,5-triphosphate in astrocytes *in vitro* (Guo et al., 1992). An increasing number of reports support the theory that GSH modulates the functions of *N*-methyl-*D*-aspartate (NMDA) receptors and participates in cellular calcium homeostasis (Gilbert et al., 1991; Sucher and Lipton, 1991; Levy et al., 1991; Leslie et al., 1992; Janaky et al, 1993; Janaky et al., 1994; Ogita et al., 1995). It has been suggested that the NMDA receptor has a number of binding sites, one of which is a redox regulator (Aizenman et al, 1989; Levy et al, 1990). The specificity of this redox site has been further investigated in recombinant NMDA receptors by Kohr et al. (1994), who found that a reducing agent, dithiothreitol (DTT), rapidly potentiated L-glutamate-activated whole-cell currents in cells expressing

NR1-NR2a channels, but had less effect in cells expressing the NR1-NR2B, -NR2C, and -NR2D subunits. In contrast, GSH mimicked the DTT effects only in the NR1-NR2A receptor.

Intracerebroventricular injections of NMDA, glutamate and kainate (Pollard et al., 1994; Saija et al., 1994; Tsirka et al., 1995) can induce convulsions in animals. Kainate-induced convulsions may be a little more complicated since kainate not only acts on the NMDA receptor directly, but also inhibits γ-GCS (McBean et al., 1995), a rate-limiting enzyme in GSH synthesis. Intravenous administration of GSH prevents kainate-induced excitotoxicity in rat brain, but shows no protection against the convulsions (Saija et al., 1994). This may be because GSH has a poor permeability into the normal brain (Meister and Anderson, 1983), but could enter after seizures become established as these cause opening of the blood brain barrier(Saija et al., 1992) which may enable protection against the consequent pathological damage.

The precise mechanism whereby GSH is involved in the regulation of the NMDA receptor and excitability is not yet clear. Whether GSH modulates NMDA by its chemically specific effects or just by general redox effects needs to be established, although the lack of protection of our animals by ascorbate would support a GSH-specific effect. Some authors suggest its role as an endogenous agonist at the N-methyl-D-aspartate recognition domain (Ogita et al., 1995), activating the NMDA receptor followed by calcium entry into neurons (Leslie et al., 1992; Weaver et al., 1993), or activating the glutamate and NMDA-induced influx of calcium (Janaky et al., 1993). On the other hand, GSH has been reported to inhibit the binding of glutamate in rat brain (Ogiat et al., 1987) and inhibits NMDA responses in neurones (Majewska et al., 1989; Gilbert et al., 1991). In addition, GSH and GSSG have been reported to attenuate the release of GABA evoked by excitatory amino acids, which was suggested to be a consequence of sustained activation of the NMDA receptor-governed ionophores containing functional thiol groups (Janaky et al., 1994). GSH has been described as a cofactor for purified macrophage calcium independent inducible nitric oxide synthase (iNOS) (Stuehr et al., 1990). *In vitro*, GSH inhibits purified preparations of calcium-dependent neuronal constitutive NOS (nNOS) (Giovenelli et al., 1991). Most recently, Heales et al. reported that GSH depletion is accompanied by increased neuronal nitric oxide synthase activity (Heales et al., 1996) as well as impaired mitochondrial function

and decreased N-acetyl aspartate concentration (Heales et al., 1995). However mediated, our observations make it clear that depletion of GSH itself dramatically alters excitability over an extended time period.

The lack of further hyperexcitability after 20 h, despite continuing depletion of GSH, and the lack of further seizures on giving a second injection of BSO suggests that an adaptation or substitution mechanism may compensate for GSH depletion in terms of excitability. However this is unlikely to involve an increase in brain ascorbic acid, as this did not protect BSO treated rats in this study, even though ascorbic acid has been reported to protect cortical neurones in culture from the toxic effects of NMDA by action at the NMDA receptor (Bell et al., 1996). It is possible that following acute loss of brain GSH there is some redistribution between cellular or subcellular compartments that limits excitation.

All the studies on the relations of GSH with NMDA may have mainly addressed the action of extracellular GSH but intracellular GSH may regulate excitability by some other mechanisms, such as controlling free radical concentration or affecting the active re-uptake of excitatory neurotransmitter from synapses.

The incidence of epilepsy is much higher in the first year than any other age in children (Camfield et al., 1996). Bearing in mind that the brain GSH level is relatively low during the neonatal period (Jefauconnier et al, 1976; Bien et al., 1990), our results suggest that low GSH levels may play a role in the evolution of spontaneous seizures in very young children (Painter and Gaus, 1991). Our findings may also provide a new interpretation to the action of methionine sulfoximine (MSO) which has been extensively used to produce an experimental seizure model. MSO inhibits both glutamine synthetase and γ-GCS (see Meister, 1978), but while glutamine synthetase inhibition (Sellinger and Weiler, 1963; Lamar and Sellinger, 1965; Ronzio, 1969) and consequential hyperammonaemia (Tews and Stone, 1964; Folbergrova et al., 1969; Subbalakshmi and Murthy, 1984) have attracted much research attention, γ-GCS inhibition and its consequential GSH depletion by MSO has been largely ignored.

Most of the other changes in brain amino acids seen in the present study appeared to be secondary to GSH depletion or seizures. In contrast to this, there were marked and early increases in the level of an as yet unidentified HPLC peak in a number of brain areas. Unfortunately it is not yet possible to identify this peak but, from its

chromatographic behaviour, it should contain an amine group since the dansylation is directed at amine groups. It could be an uncommon amino acid, peptide, or other kind of amine containing substance. No naturally occurring amino acids, catecholamine, or GSH related dipeptides matched its chromatographic properties. The identity of this peak is to be investigated.

Table 9.1, Effect of γ-GC and ascorbic acid on BSO-induced seizures

Treatments	n	No. of rats having seizures
BSO	16	15
BSO+γ-GC	4	0*
BSO+Ascorbic acid	3	3

* $p < 0.01$ compared with that of BSO treatment alone.

Table 9.2, Effects of single BSO administration on brain GSH concentration (μmol/g wet tissue)

Hours after BSO	0 h (control)	5 h	15 h	48 h
Medulla	0.81±0.14	0.49±0.05***	0.35±0.03***	0.35±0.09***
Vestibular nuclei	0.91±0.09	0.63±0.06***	0.39±0.02***	0.38±0.08***
Cerebellar roof nuclei	0.84±0.15	0.65±0.05**	0.44±0.02***	0.29±0.03***
Cochlear nuclei	0.89±0.13	0.56±0.09**	0.34±0.03**	0.35±0.09***
Inferior colliculi	1.06±0.05	0.68±0.05***	0.50±0.04***	0.51±0.11***
Superior colliculi	1.16±0.06	0.76±0.09***	0.57±0.04***	0.57±0.18***
Hippocampus	1.50±0.08	1.15±0.11***	1.02±0.15**	0.66±0.04***
Striatum	1.56±0.13	1.19±0.13**	1.00±0.05***	0.67±0.08***
Cerebellar cortex	1.06±0.12	0.85±0.10**	0.75±0.07**	0.53±0.05***
Neocortex	1.31±0.03	0.99±0.06***	0.89±0.09***	0.61±0.04***
Thalamus	1.09±0.11	0.85±0.08**	0.66±0.08**	0.53±0.12***

Data are presented as mean±SD, n=4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with that at 0 h. ANOVA for non-hemogeneous distribution of GSH across regions at 0h: $p < 0.001$.

Table 9.3, Effects of BSO on GSSG concentration in brain (nmol/g wet tissue)

Hours after BSO	0 h (control)	5 h	15 h	48 h
Medulla	23±5	14±4*	16±6*	10±7*
Vestibular nuclei	23±6	18±6	15±6	12±5*
Cerebellar roof nuclei	18±6	17±2	14±3	9±3*
Cochlear nuclei	19±5	17±3	19±4	13±2*
Inferior colliculi	20±7	14±3	11±3*	11±5*
Superior colliculi	24±9	17±6	21±6	11±8*
Hippocampus	18±9	15±3	13±6	8±4*
Striatum	15±7	14±3	13±1	6±4*
Cerebellar cortex	13±4	11±1	10±5	5±4*
Neocortex	18±9	15±2	11±2	6±4*
Thalamus	14±4	11±3	12±5	7±3*

Data are presented as mean±SD, n=4. * $p < 0.05$, compared with that at 0 h. ANOVA for non-homogeneity of GSH distribution at 0 h: $p > 0.05$.

Table 9.4, Effects of BSO on glutamic acid concentration in brain (μmol/g wet tissue)

Hours after BSO	0 h (control)	5 h	15 h	48 h
Vestibular nuclei	3.69±0.34	3.55±0.21	4.09±1.04	5.26±0.44**
Hippocampus	4.66±0.13	4.46±0.45	4.31±0.28	5.49±0.65*

Data are presented as mean±SD, n=4. * $p < 0.05$, ** $p < 0.01$ compared with that at 0 h. *t*-Test for the comparison of vestibular nuclei with hippocampus at 0 h: $p < 0.01$.

Table 9.5, Effects of BSO on aspartic acid concentration in brain (μmol/g wet tissue)

Hours after BSO	0 h (control)	5 h	15 h	48 h
Medulla	1.11±0.31	1.12±0.23	1.95±0.58	2.25±0.38**
Vestibular nuclei	1.25±0.31	1.30±0.30	1.32±0.52	2.45±0.28**
Cerebellar roof nuclei	0.86±0.44	0.94±0.28	0.62±0.13	1.43±0.30*
Cochlear nuclei	0.72±0.23	0.90±0.21	0.82±0.26	1.80±0.31**
Inferior colliculi	1.42±0.10	1.14±0.31	1.40±1.21	1.86±0.54
Superior colliculi	1.26±0.21	1.24±0.07	1.43±0.91	1.74±0.39
Hippocampus	0.80±0.31	0.79±0.25	0.98±0.97	0.82±0.25
Striatum	0.83±0.11	0.86±0.16	0.95±0.84	0.76±0.24
Cerebellar cortex	0.94±0.14	0.94±0.22	0.96±0.17	1.13±0.30
Neocortex	1.36±0.15	1.18±0.27	1.61±0.93	1.34±0.45
Thalamus	1.12±0.08	0.97±0.20	1.07±0.44	1.27±0.43

Data are presented as mean±SD, n=4. * $p < 0.05$, ** $p < 0.01$ compared with that at 0 h. ANOVA for non-homogeneous distribution of aspartic acid at 0 h: $p < 0.05$.

Table 9.6, Effects of BSO on the unidentified substance in brain (HPLC peak area, arbitrary units)

Hours after BSO	0 h (control)	5 h	15 h	48 h
Medulla	1.75±1.05	2.85±0.59	2.33±0.25	4.30±0.95**
Vestibular nuclei	2.38±1.11	3.43±0.39	3.59±0.16	5.27±1.01**
Cerebellar roof nuclei	8.66±1.63	10.96±0.31*	12.47±2.28*	18.49±1.51***
Cochlear nuclei	4.09±1.02	6.95±0.83**	6.9±1.04*	8.78±1.79**
Inferior colliculi	0.45±0.04	2.39±0.60**	2.53±1.03*	3.42±2.04*
Superior colliculi	0.25±0.08	1.28±0.15***	2.84±1.68*	3.62±2.27*
Hippocampus	0.18±0.02	1.42±0.07***	1.17±0.66*	2.66±0.07**
Striatum	0.46±0.17	1.83±0.30***	1.97±0.27**	2.15±0.30**
Cerebellar cortex	8.59±0.52	12.30±0.93**	14.83±3.31*	19.41±2.44**
Neocortex	0.16±0.04	0.69±0.08***	0.98±0.14*	2.88±0.38**
Thalamus	0.31±0.15	2.04±0.33***	1.98±0.36*	2.83±0.42*

Data are presented as mean±SD, n=4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with that at 0 h. ANOVA for the data at 0h: $p < 0.001$.

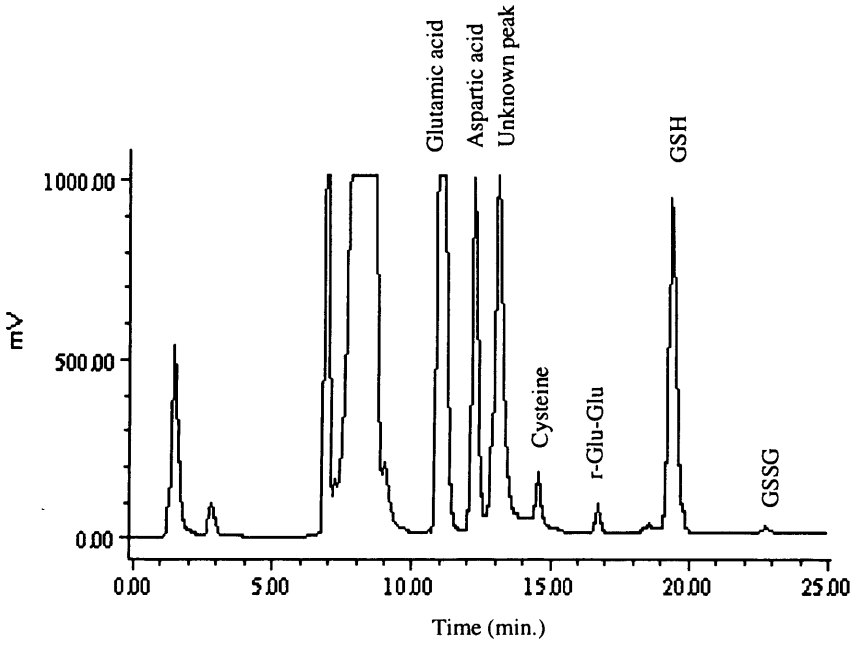


Figure 9.1, A HPLC chromatogram showing GSH and relevant amino acids in cerebellum and also showing the unknown peak which is very high in this area. HPLC conditions have been described in the General Materials and Methods (Chapter 4).

CHAPTER 10
GENERAL DISCUSSION

Is oxidative stress responsible for *m*-DNB-induced neurotoxicity?

Nitroreduction can generate at least two types of free radicals; nitro anion free radical and superoxide (Mason and Holtzman, 1975; Ghersi-Egea and Livertoux, 1992). Nitroreduction of *m*-DNB has been demonstrated *in situ* in brain (Chapter 5). Therefore oxidative stress induced by these free radicals is bound to occur during the pathogenesis of the neurotoxicity caused by *m*-DNB. However this conclusion does not exclude any other alternative mechanisms for the pathogenesis, nor claim its priority in explaining the brain stem lesions induced by *m*-DNB.

It has to be admitted that the attempt to find direct evidence of oxidative stress *in vivo* failed, although a decrease of GSH was reported *in vitro* (Romero et al., 1995). When lesions occurred in the brain stem, there was no change in the levels of GSH, ascorbic acid and free MDA which is an indicator of lipid peroxidation. This may be because, as Halliwell (1992) pointed out, damage caused by oxidative stress need not necessarily involve lipid peroxidation (Halliwell, 1987; Orrenius et al., 1989; Cochrane, 1991). Hence the failure to find lipid peroxides in injured nervous tissue does not rule out the occurrence of oxidative damage. Damage to DNA (nuclear and mitrochondrial) and proteins may be of equal, or even greater, importance *in vivo* (Halliwell, 1987).

Bioactivation of *m*-DNB in brain *in situ* and neurotoxicity

There has been increasing interest in the extrahepatic metabolism of toxic compounds, although liver is quantitatively the major organ involved in the *in vivo* metabolic disposal of most xenobiotics. Containing most of the essential metabolic enzymes acting on xenobiotics, brain is well endowed with metabolic machinery capable of mediating a considerable diversity of biotransformations of drugs and other environmental chemicals (Mesnil et al., 1984; Ravindranath and Boyd, 1995). Such biotransformations may lead either to less toxic or more toxic products, thus conceivably contributing to local toxicological processes in certain regions of the brain or specific cells. In the case of *m*-DNB-induced neurotoxicity the locally produced active intermediate(s) may be crucial to the pathogenesis, although these intermediates

may also be transported from other metabolic organs e.g. liver. As discussed in the previous section, the in situ metabolism produces at least two types of free radicals. The detection of nitrosonitrobenzene not only evidenced the process of nitroreduction, but also indicates that it might be generated over a relatively long period *in vivo* under certain circumstances. This intermediate has been proven more toxic than its parent compound *m*-DNB in terms of testicular toxicity (Cave and Foster, 1990; Cossum et al., 1989), though the manner of its action has yet to be established.

Recent advances on DNB metabolism have been made on three fronts in this laboratory. Cellular studies show that the metabolic activity towards *m*-DNB exists not only in astrocytic and endothelial cultures (Xu et al., personal communications), but also in cultured cerebellar granule neurons and neuroblastoma cells (Hu et al., unpublished data). Subcellular studies on liver tissue revealed that the activity was mainly in microsomes, less in mitochondria, and not in the cytosolic fraction (Hu et al., unpublished data). Finally and most excitingly, NADPH dependent cytochrome P-450 reductase has been demonstrated to be able to reduce *m*-DNB (Hu et al., unpublished data) although the actual enzymatic nature is still under study.

Involvement of brain GSH in *m*-DNB-induced neurotoxicity

An involvement of GSH in *m*-DNB-induced neurotoxicity has been demonstrated (Chapter 8). GSH may act in three different ways: 1) as a catalyst or scavenger of toxic intermediates such as nitrosonitrobenzene (Chapter 5; Ellis et al., 1992); 2) as a scavenger of free radicals in antioxidation; 3) protecting the important enzymes in nuclear, mitochondrial or biological membrane by maintaining the reducing status of thiol groups in those enzymes. It is a little surprising but understandable that the brain regional GSH level did not change even when lesions occurred, because less than 3% of brain GSH could be consumed if GSH was mainly involved in a 1:1 covalent reaction with nitrosonitrobenzene, which is present in brain only at about 10^{-5} M during intoxication. The other two isomers of DNB, 1,2-DNB and 1,4-DNB, are not neurotoxic whereas 1,3-DNB (*m*-DNB) is, and moreover, it is the only one which is not conjugated with glutathione (Cossum and Richert, 1985).

In addition to its involvement in DNB-induced neurotoxicity, GSH depletion has been recently found to sensitise to some other types of neurotoxicities. Depletion of rat

brain GSH by i.c.v. administration of BSO potentiates the toxicity of 6-hydroxydopamine (6-OHDA) to the nigrostriatal pathway as judged by striatal dopamine content and the number of tyrosine hydroxylase immunoreactive cells in substantia nigra (Seaton et al., 1996). Gabbay et al. (1996) reported that depletion of GSH by BSO enhance the apoptosis in human neuronal cells induced by dopamine. Furthermore, this apoptosis can be blocked by GSH, but not alpha-tocopherol and ascorbic acid (Gabbay et al., 1996).

Although GSH depletion clearly enhances DNB toxicity (Chapter 8) and DNB depletes GSH *in vitro* (Ray et al., 1994), no significant fall in brain GSH was seen during the development of *m*-DNB-induced lesions (Chapter 8). This is in contrast to the results derived from an *in vitro* study on astrocytic cultrues which showed a significant decrease after *m*-DNB treatment (Romero et al., 1995). There are at least three possible reasons for the difference in observations: 1) multiple cell types were involved when brain GSH was measured in the *in vivo* study, compared with the single cell population of astrocytes which are target cells; 2) the speed of GSH synthesis and the reduction of GSSG may be different *in vitro* compared with *in vivo*; 3) the dose of DNB needed to produce cytotoxicity was higher *in vitro*.

Even though GSH plays a protective role in DNB neurotoxicity, it is clear that it can not provide complete protection, since normal undepleted levels could not prevent the brain stem lesions induced by *m*-DNB. This may indicate that only a minor compartment of total cellular GSH has immediate access to the generated nitrosonitrobenzene. This also raised the interesting issue of the importance of the relationship between cellular localisation of bioactivation of neurotoxic agents and cellular distribution of GSH.

An emerging area of research inquiry is the pathological implications of loss of mitochondrial function and the role of protein thiol in the maintenance of cellular integrity. GSH has important cellular relationships to protein thiols (Smith et al., 1996). Little is known about the molecular events that influence the status of protein thiols within the cell and, more particularly within the mitochondria and nucleus. Improvement of our knowledge in those aspects would help in understanding what is occurring during the *m*-DNB intoxication.

The ratio of GSSG/GSH may not necessarily provide much useful information on the redox status in all circumstances. GSSG is naturally about 1-3% of the GSH level, and its measurement is easily compromised during sample handling and preparation. Therefore if calculated as an absolute value, a small change in GSH will give rise a many fold change of GSSG, and thus dramatically alter the ratio of GSSG/GSH. One may argue that the ratio is very sensitive because of this amplification, but it should be born in mind that change in GSSG may not be specifically related to the real status of *in vivo* oxidative stress. Further to the above mentioned ratio, GSH redox index (GSH RI) is thought to be a comprehensive marker of the relationship among the compounds of the GSH-system determined by the formula: = {[GSH] + 2[GSSG]}/ {2[GSSG]×100}(Benzi et al., 1988; Iantomasi et al., 1993). However this has not yet been widely accepted as an indicator for oxidative status, since GSSG does not necessarily increase significantly when GSH decreases under oxidative stress.

Glutathione (GSH) in toxicology

The importance and metabolism of GSH in the central nervous system have been reviewed in Chapter 3. Here, the general implications of GSH in toxicology are discussed. GSH is present in high concentrations in most living cells and participates in a variety of vital cellular reactions (Meister and Anderson, 1983; also the review in Chapter 3). In particular, GSH protects cells from potentially toxic electrophiles formed during the metabolism of xenobiotics. This protection is achieved through either conjugating the toxic chemicals or scavenging active free radicals. GSH appears to be synthesised primarily, if not exclusively, in the cytoplasmic compartment in cells, yet exists and is utilised in various other compartments, including the nucleus, mitochondrial matrix, endoplasmic reticulum and extracellular spaces (Meister, 1991). The existence of GSH in all these compartments is of toxicological importance. GSH depletion to 20-30% of total glutathione levels can impair the cell's defence against the actions of toxic compounds and may lead to cell injury and death (Reed and Fariss, 1984).

GSH associated with the nuclear pellet accounts for about 5-10% of total cellular GSH, a ratio which is approximately that of the nuclear volume to the total cell volume. The toxicological significance of GSH in the nucleus is proposed to be in maintaining

the redox status of critical protein sulphydryls, thereby affecting the efficiency of DNA repair enzymes (reviewed by Smith et al., 1996).

Mitochondrial GSH appears to be of exceptional interest in terms of toxicology. Because of their lack of catalase (Neubert et al., 1962), mitochondria rely on GSH peroxidase to detoxify hydroperoxides (Chance et al., 1979). Endogenous oxidative stress is a consequence of aerobic metabolism, which, in eukaryotes, occurs mostly in the mitochondria. Reduction of oxygen in the respiratory chain involves the formation of toxic oxygen intermediates. About 2-5% of mitochondrial O_2 consumption generates hydrogen peroxide (Chance et al., 1979). In addition, GSH also functions in the maintenance of the reduced status of essential sulphydryl groups in numerous mitochondrial proteins, such as dehydrogenases, ATPases, and transport proteins (Smith et al., 1996).

Some neurotoxic compounds can induce a decrease of GSH level in brain. Chronic administration of disulfiram to rats was found to decrease brain GSH level and GSH reductase activity, and increase the oxidised glutathione (GSSG) level (Nagendra et al., 1994). 2-Chloropropionic acid depletes GSH in cerebellum but has no effect on the GSSG level, an action which has been suggested to render granule cells of the cerebellum more vulnerable to oxidative free radical damage (Wyatt et al., 1996). Intraperitoneal injection of toluene to rat decreased GSH levels in brain as well as in liver (Mattia et al., 1993). Chronic administration of haloperidol, an antipsychotic drug increased lipid peroxidation and decreased GSH levels in brain (Shivakumar and Ravindranath, 1993). Single subcutaneous administration of haloperidol, also caused a significant decrease of GSH levels at 4 h in rat cortex, striatum and midbrain. In some cases, the depleted GSH was recoverable as protein/glutathione mixed disulphide (Pr-SSG) but not necessarily GSSG (Shivakumar and Ravindranath, 1992). During oxidative stress when the NADPH levels are not sufficient to reduce all of the formed GSSG to GSH (through GSH reductase), the GSSG is able to diffuse out of cells. Excessive Pr-SSG and Pr-SS-Pr can lead to membrane damage and dysfunction of affected enzymes or receptors. In hepatocytes subjected to oxidative stress induced by menadione, the formation of Pr-SSG accounts for only 15% of the depleted GSH (Dimonte et al., 1984). This is contrast with the 90% of depleted GSH converted to Pr-

SSG in the brain under the oxidative stress caused by haloperidol (Shivakumar and Ravindranath, 1992).

However it should be mentioned that there is also accumulating evidence indicating that GSH conjugation plays an important role in the formation of toxic metabolites from a variety of chemicals, although it is unlikely to be related to DNB-induced neurotoxicity. This special action has been termed as *'GSH conjugation mediated toxicities'* (Monks et al., 1990). Monks et al (1990) proposed four types of GSH-dependent bioactivation: (1) toxic GSH conjugates may be formed from vicinal dihaloalkanes via formation of electrophilic sulphur; (2) cysteine conjugate beta-lyase-dependent bioactivation is involved in the selective nephrotoxicity of haloalkanes; (3) GSH conjugates of hydroquinones and isothiocyanates may serve as transport and targeting metabolites; and (4) GSH-dependent reactions may be involved in the release of toxic agents from precursor organic thiocyanates and nitrosoguanidines (e.g. N-methyl-N'-nitro-N-nitrosoguanidine).

Regional and cellular selectivity of *m*-DNB neurotoxicity and brain antioxidants

The neurotoxicity induced by *m*-DNB presents two kinds of selectivity; regional and cellular. Brain lesions are localised in brain stem and the primary cellular targets are glial cells. *In vivo* pharmacokinetic studies showed a fairly homogeneous distribution of *m*-DNB in the brain (Xu et al., 1996). There is no variation in the metabolic activity towards *m*-DNB between brain stem and forebrain, the toxicity-sensitive area and toxicity-resistant areas respectively (Chapter 5). Thus the knowledge of pharmacokinetic nature of *m*-DNB does improve our understanding of the molecular mechanism of its toxicity, but provides no clue as to its selectivity.

We may need to find the basis of the selectivity of neurotoxicity from the physiological and/or biochemcial nature of the brain stem. The two main water soluble endogenous antioxidants, GSH and ascorbic acid, are naturally lower in brain stem compared with the other brain areas. Coincidentally the basal level of free MDA is higher in brain stem. Furthermore GSH turnover is much faster in brain stem than the other regions, a phenomenon which may reflect a higher demand of the reducing substance or/and reflect a compensation for the lower absolute level. All this information suggests that brain stem may have a lower capacity antioxidant defence

system relative to a higher metabolic demond according to a relatively higher resting rates of glucose utilisation (Schwartz and Sharp, 1978). These factors together may contribute to the susceptibility of brain stem to the neurotoxicity induced by *m*-DNB. Again, the above mentioned antioxidants have characteristic reducing properties, and thus are chemically reactive (Chapter 5). Their actions may not be limited to antioxidation.

Coupling of oxidative stress with bioenergetic disorder in DNB neurotoxicity

Disturbance of energy metabolism has been reported in association with DNB toxicity (Romero et al., 1995). Both increased or decreased neuronal activity and its metabolic consequences modulate DNB neurotoxicity to correspondingly enhance or diminish histological lesions (Ray et al, 1992, Cavanagh, 1993). If oxidative stress and bioenergetic disorder both truly occur in the neurotoxicity induced by *m*-DNB, are they separate events or linked with each other? It is highly possible that these two events are coupled in *m*-DNB-induced neurotoxicity.

Firstly, if the metabolism of *m*-DNB can occur in mitochondria as well as in microsomes (this has been confirmed by recent study, unpublished data), free radicals could well be generated in the mitochondria. This direct free radical attack can cause mitochondrial dysfunction by affecting membranes, enzymes or mitochondrial DNA, though whether or not mitochondria is one of the main intracellular targets for *m*-DNB needs to be established..

Secondly, oxidative stress increases the cellular demand on reducing equivalent NADPH which is produced via the pentose phosphate shunt pathway, thus activating the activity of this pathway and correspondingly limiting the glycolysis responsible for producing ATP. Exposure of primary astrocytic cultures to H_2O_2 was found to cause stimulation of the pentose phosphate pathway (PPP) activity from 7% to 67% (Ben-Yoseph et al., 1996). This means that the majority of the available glucose will be utilised via the PPP to produce NADPH under conditions of oxidative stress. As a result, the capacity for glycolysis could be greatly diminished and ATP production decreased. This clearly links the oxidative stress and consequential bioenergetic disorder, e.g. energy depletion which in turn affects ionic homeostasis and causes cell damage.

Evidence for novel functions of GSH in the CNS

Aside from its conventionally recognised functions, GSH has been suggested to be a neuropeptide modulating some important cellular events such as Ca2+ homeostasis and NMDA receptor function (Chapter 3). This study provided convincing *in vivo* evidence of its action in modulating CNS excitability, although the exact mechanism underlying this modulation is not clear. The modulation seems to be far more complex than a simple steady state concentration effect because the seizures no longer appeared from 20 hours after the BSO treatment although brain GSH levels were still low. This is a very curious phenomenon which is difficult to explain from current work; but clearly implies that the rate of change of GSH is more important than absolute concentration.

This study also revealed that sustained depletion of brain GSH induced by 3 doses of BSO (3.2mg/rat on alternate days) caused vascular damage and haemorrhage in the cerebellar roof nuclei, one of the areas showing greatest GSH depletion (Chapter 8). This suggests a function of GSH in maintaining cerebrovascular function, an idea supported by a previous report that GSH depletion caused damage to capillary endothelial cells in lung (Martensson et al., 1989). Bearing in mind that brain GSH levels are lower during early development and also ageing, I would tentatively speculate that low GSH might be a contributing factor to the occurrence of both spontaneous neonatal haemorrhages and cerebral haemorrhages in the elderly.

Conclusions

1. *m*-DNB can be reductively metabolised in brain *in situ*. This metabolism provides a basis for the formation of toxic intermediate(s) and free radicals within target cells.

2. Brain GSH is involved in the neurotoxicity induced by *m*-DNB because depletion of brain GSH increases the brain's susceptibility to this neurotoxin. The action of GSH is no doubt based on its reducing property, but may not be solely due to its antioxidant nature.

3. The levels of GSH and ascorbic acid are lower, but the spontaneous oxidative stress is higher in brain stem compared with other brain areas. This may explain the the susceptibility of brain stem to the neurotoxicity of *m*-DNB.

4. A greater depletion of GSH was shown in brain stem areas at 24 h after i.c.v. administration of BSO. This was due to faster GSH turnover in these areas.

5. Brain GSH levels are lower in the two extreme ends of life, during development and aging. Cysteine may be a limiting factor for GSH synthesis in the developing brain.

6. Brain GSH depletion is associated with excitability and seizures in rats. GSH may be involved in the modulation of excitability of CNS.

Future work

1. DNB metabolism and intracellular GSH distribution

Our previous studies have demonstrated that DNB can be locally metabolised in the brain with the production of nitroaniline and nitrosonitrobenzene. Brain glutathione (GSH) was found to react with nitrosonitrobenzene non-enzymatically to produce nitroaniline. Brain stem lesions induced by DNB despite the presence of GSH at millimolar concentrations in gross tissue assays suggest that the cellular compartment where degradation of DNB occurs may have little GSH available to convert nitrosonitrobenzene. Therefore, we need answers for the following questions:

(1) Which types of cells in CNS have the capacity to metabolise DNB? Metabolism of DNB in a single cell population has been recently tested in four types of cells in this laboratory. These are astrocytes, cerebral vascular endothelial cells (Xu et al., personal communication), cerebellar granule cells and neuroblastoma cells (unpublished data).

(2) Where does DNB metabolism occur in the cells? Preliminary results have shown that microsomes have highest activity, mitochondria have considerable activity while the cytosol has no detectable activity (unpublished data).

(3) Which enzyme(s) or enzyme system(s) is or are responsible for DNB metabolism? Preliminary qualitative study on the purified recombinant cytochrome P450 reductase (provided by Prof. Gordon Roberts and Dr. Sandeep Modi) has shown a putative activity of the enzyme towards *m*-DNB (unpublished data).

(4) How much GSH exists in the compartment where the DNB degradation occurs? If the metabolism of DNB occurs mainly in microsomes and mitochondria, it

would be interesting to know the GSH status in these intracellular compartments, although it is difficult to look at the real kinetic change during the intoxification.

2. Further study of the role of oxidative stress and bioenergetic disorders in DNB-induced neurotoxicity

Because of the coupling of oxidative stress and bioenergetic disorders, redox disruption and bioenergetic disorders may need to be considered as of equal importance in terms of the mechanism underlying DNB-induced brain stem lesions. It is likely that oxidative stress may also cause damage by a more subtle manner in which no direct free radical attack can be observed, such as gross tissue lipid peroxidation and GSH depletion. Further investigation in this direction will not only improve our understanding of DNB neurotoxicity, but also provide more information on the interaction between redox balance and bioenergetic function. Technically brain slices may be used to test the activation of PPP due to intoxication by DNB, and the NMR technique may be used in non-invasive studies in an animal model to investigate the kinetic alteration, if any, of energy metabolism.

3. CNS antioxidant systems and brain susceptibility towards neurotoxins

Heterogeneity of GSH distribution and GSH turnover across brain regions has been demonstrated in this study. Further study is required for understanding whether and how GSH shares the antioxidation responsibility in the brain with other endogenous antioxidants, such as ascorbic acid and α-tocopherol, and whether there are reciprocal or parallel changes in these systems.

4. Metabolism and the importance of brain GSH

(1) Brain GSH and CNS excitability

In addition to its involvement in redox processes, GSH has been found to modulate CNS excitability. It is as yet unclear how GSH works, though a number of reports have reported on the action of GSH on NMDA receptors.

(2) Regulation of GSH synthesis

Growth factors have been reported to increase the intracellular GSH level of *in vitro* cultures (Pan and Perez-Polo, 1993). It was also shown that there is a parallel

increase of intracellular cysteine in EGF-treated endothelial cells. These suggest that growth factors may increase GSH synthesis by up-regulating the uptake of cysteine by the cells. It needs to be made clear whether this regulation is also true in cell cultures from CNS. Further study will provide useful information for understanding the phenomenon that a low level of brain cysteine parallels a low level of brain GSH in developmental rats (Chapter 7).

(3) Identification of an unknown substance in the CNS

An effort needs to be made to identify an interesting substance whose content in cerebellum is 50 times higher than in neocortex. This specially high concentration is possibly in relation to some specific metabolic process or function of the cerebellum. Since this substance can be altered by the treatment of BSO, it may be related to GSH metabolism and/or susceptible to the disruption of redox balance.

5. Common factors and differences among the brain lesions produced by chlorosugars, acute thiamine deficiency and neurotoxic nitrocompounds

The suggestion of regional GSH as a determining factor for regional susceptibility should be tested with some other neurotoxic agents producing pathologically analogous lesions. The rat model of brain GSH depletion induced by BSO can be used to determine whether depletion of brain GSH sensitises the brain to lesions caused by chlorosugars and thiamine deficiency.

6. GSH involvement in microvessel function and cerebral haemorrhage

Sustained brain GSH depletion caused mild haemorrhage in vestibular nuclei, which is the only morphological change observed after GSH depletion. The nature of the vascular damage will be further examined with this model. This may provide clues for the understanding of vascular damage under some pathological conditions, including those caused by gliotoxic nitrocompounds.

Most recently, a paper reported an action of GSH and GSSG in mediating cation channel activation in calf vascular endothelial cells during oxidant stress (Koliwad et al., 1996). Brain GSH is mainly distributed within the non-neuronal cells, especially in astrocytes. The next logical question to answer would be how much GSH exists in the brain microvessels, in comparison with the GSH in brain tissue. Astrocytes are believed

to play an essential role in maintaining cerebral homeostasis and the microenvironment. They have also been reported in murine CNS to be the primary source of tissue factor (TF), which is the primary cellular initiator of the coagulation protease cascades. It is worthwhile looking at the effects of oxidative stress (induced by DNB or other compounds) or GSH depletion on the expression of TF by astrocytes, in order to learn more about the haemorrhage under these conditions.

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APPENDIX
LIST OF PUBLICATIONS, PRESENTATIONS AND PRIZE BASED ON THIS PROJECT

Publications:

1. Hu H.L. and Ray D.E. Metabolism of *m*-dinitrobenzene in brain studied *in vitro*. *Hum. Exp. Toxicol.* 1995; 14: 363.

2. Hu H.L., Bennett N., Lister T., Nolan C.C., Holton J.L., and Ray D.E. Increased susceptibility of brain towards *m*-dinitrobenzene by glutathione depletion. *Hum. Exp. Toxicol* 1996; 15: 145.

3. Hu H.L., Bennett N., Holton J.L., Nolan C.C., Lister T., and Ray D.E. Regional heterogeneity in turnover and novel functions of glutathione in rat brain. *Hum. Exp. Toxicol* 1996; 15: 658.

4. Hu H.L., Bennett N., Lamb J.H., Ghersi-Egea J.F., Schlosshauer B., and Ray D.E. Metabolism of *m*-dinitrobenzene by brain: an *in vitro* study. *Neurotoxicology* 1997; 18: 363-370.

5. Hu H.L., Bennett N., Holton J.L., Lister T., Nolan C.C., Cavanagh J.B., and Ray D.E. Glutathione depletion Increases brain susceptibility to *m*-dinitrobenzene neurotoxicity. *Neurotoxicology*, 1997, in press.

6. Hu H.L., Bennett N., Lister T., and Ray D.E. Heterogeneity in regional distribution of glutathione, ascorbic acid and endogenous lipid peroxidation in brain. *Brain Research* 1997; finalising.

7. Hu H.L., Bennett N., Holton J.L., Nolan C.C., Lister T., Ray D.E. Brain glutathione depletion is related to seizures in rats. *Brain Research* 1997; Submitted.

Presentations:

1. **Oral presentation** in BTS Edinburgh meeting in September 1994.
2. **Oral presentation** in BTS Oxford meeting in September 1995.
3. **Invited Speaker,** in The Fourth Conference of the Life Science Society for Chinese Bioscientists in UK, Cambridge, 24-25 August 1995.
4. **Oral presentation,** Wednesday Seminar, MRC Toxicology Unit, 18 October 1995.
5. **Oral presentation,** Tuesday Seminar, CMHT Postgraduate Society, MRC Toxicology Unit, 27 February 1996.
6. **Poster presentation,** 13th Brain Research Association Meeting, Newcastle, 25-27 March 1996
7. **Poster presentation,** BTS meeting in York, 1-3 April 1996.
8. **Oral presentation,** 2nd Conference of Chinese Medical Society in UK, London , 14-15 September, 1996.
9. **Poster presentation,** BTS meeting in Warwick, 24-26 April, 1997.

Prize:

Won the prize for the best oral presentation in British Toxicology Society Meeting in Oxford, 1995. Winning topic: Increased susceptibility of brain towards *m*-dinitrobenzene by glutathione depletion.