Teratogenic Properties of L-2,4-Diaminobutyric Acid and Polyamine Levels in the Murine Neural Tube Defect Model.

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Potchefstroom

2007

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ACKNOWLEDGEMENTS

My sincere appreciation to the following people who contributed positively towards the completion of this study:

- My supervisor, Prof. HC Potgieter, for his leadership and support throughout the project;
- Prof. LJ Mienie for his assistance in the execution of the metabolic experiments and interpretation of the GC-MS data;
- Mr. E Erasmus for his assistance with the ESI-MS-MS analyses and interpretation of the acquired data;
- Prof. HF Kotze, Dr. FH van der Westhuizen and Dr. Roan Louw for their contributions;
- The Biochemistry Department and Metabolic Screening Laboratory personnel for their assistance;
- Mr. Cor Bester, Mrs. Antoinette Fick and other support personnel at the experimental animal facility for their valuable assistance with this project;
- The National Research Foundation (NRF), Medical Research Council (MRC) and the Department of Biochemistry for financial support;
- My family (Dad, Mom, Karabo, Keitumetse) for their unwavering support;
- My husband and my daughters for always understanding;
- My friends and colleagues (Boitumelo, Anni, Ntombi, Andy, Ouma, Justice, Kgomotso) for their support.

I also, herewith, want to declare my deepest gratitude towards my Creator for the talent and opportunities He awarded me throughout the duration of this project and for my whole life.

SUMMARY

L-2,4-Diaminobutyric acid (DABA) is a non-protein amino acid and constituent of numerous plants (i.e. *Lathyrus* species) and microorganisms. This relatively abundant amino acid is a known neurotoxin, neurolathyrogen and urea cycle inhibitor and commonly occurs in the food chain of vulnerable populations in the rural regions of Africa (i.e. Ethiopia, South Africa) and India. In spite of the fact that DABA is a well-studied toxic agent, it has not yet been reported to be associated with birth defects in man or domestic animals. DABA is a structural homologue of L-ornithine (Orn) and it was subsequently proposed that this potential Orn anti-metabolite may be teratogenic by acting as an inhibitor of ornithine decarboxylase (ODC) and subsequently by modulating polyamine concentrations in experimentally exposed pregnant female mice. The objectives of this investigation were to investigate the purported teratogenic properties of DABA in a murine NTD model, to establish the effect of DABA on hepatic and whole embryo ornithine ODC and to broadly investigate its effect on the general metabolism of pregnant female mice and their embryos.

Pregnant Hanover.NMRI female mice were dosed (*per os*) with DABA (total dose: 450 - 1350 mg/kg body mass) on gestation days 7, 8, and 9. Controls animals received a saline solution. The females were kept in metabolic cages - 24-hour urine samples were collected (gestation day 10) and stored (-70 ^{0}C). Following decapitation of the pregnant females on (gestation day 18), their livers and embryos were harvested and the livers immediately frozen in at -70 ^{0}C. The harvested embryos were stereomicroscopically examined to assess the occurrence, incidence and nature of congenital defects and then stored at -70 ^{0}C. DABA proved to be highly toxic to mouse foetuses at all administered dose levels and foetal mortality was extremely high. A relatively high proportion of foetuses displayed growth retardation and congenital defects. Neural tube defects (i.e. exencephali) occurred in 5 -17% of DABA-exposed foetuses. These results corroborate the notion that DABA is an embryotoxic and teratogenic agent.

Urinary organic acids, acylcarnitines and amino acids were quantified using GC-MS, ES-MS-MS and the Phenomenex EZ:faast® GC-MS procedures respectively. The detrimental effects of DABA on the metabolism of female Hanover.RPMI mice were obvious from the complexity of and profound changes in the urinary metabolic profiles of DABA-treated females, relative to those obtained for control animals. DABA had clearly left an enormous, but non-specific footprint on the general biochemistry of DABA-treated females and a variety of crucial metabolic processes (glycolysis, tricarboxylic acid cycle, urea cycle, etc.) were adversely affected.

Enzyme assays were developed and optimised to assess the catalytic activity of ODC in hepatic and whole embryo cytosols and to quantify the total polyamine concentration in tissue (liver, embryo) and urine samples. The catalytic activity of ODC in maternal livers and whole embryos, harvested from DABA-treated pregnant females, were significantly inhibited (-53%; $p<0.0001$). Total polyamine concentrations in the liver and whole embryo cytosols and maternal urine samples were similarly depressed (-70%; $p<0.05$). These results unambiguously demonstrated that DABA can act as an Orn anti-metabolite, inhibit the ODC-catalyzed production of Put from Orn and depress the biosynthesis of polyamines (Put, Spd, Spm). The relatively low levels of polyamines during the critical stages of neural tube closure may have adversely affected polyamine-directed regulation of the cell cycle, subsequently causing the dysmorphogenesis (i.e. NTD) observed in some of the embryos of DABA-treated pregnant female mice.

OPSOMMING

L-2,4-Diaminobottersuur (DABA) is `n nie-proteïen aminosuur en komponent van `n groot verskeidenheid van plante (bv. *Lathyrus* species) en mikroorganismes. DABA is 'n bekende neurotoksien, neurolatirogeen en ureumsiklusinhibitor en kom algemeen voor in die voedselketting van blootgestelde bevolkings in the plattelandse streke van Afrika (bv. Ethiopië, Suid-Afrika) en Indië. Ten spyte van die feit dat DABA 'n goed-bestudeerde toksiese verbinding is, is dit tot dusver nog glad nie verbind met enige geboortedefekte by mense of plaasdiere nie. DABA is 'n strukturele analoog van L-ornitien (Orn) en daar is gepostuleer dat hierdie potensiële Orn-antimetaboliet as 'n teratogeen mag optree wat ornitiendekarboksilase (ODC) kan inhibeer en gevolglik daartoe in staat is om die konsentrasies van poliamiene in DABA-behandelde dragtige muise te kan moduleer. Die oogmerke van hierdie ondersoek was om die beweerde teratogeniese eienskappe van DABA in 'n muismodel vir neuraalbuisdefekte (NTD) te bestudeer, om die effek van DABA op lewer- en embrio-ODC te bepaal en om 'n breë ondersoek na die effek van DABA op die algemene metabolisme van dragtige wyfiemuise te loods.

Dragtige Hanover.NMRI wyfies is op gestasiedae 7, 8 en 9 *per os* met DABA gedoseer (totale dosis: 450 – 1350 mg/kg liggaamsmassa). Kontrolediere is met 'n fisiologiese soutoplossing gedoseer. Die dragtige wyfiemuise is in metaboliese hokke aangehou - 24-uur urienmonsters is versamel (gestasiedag 10) en dadelik ingevries (-70 ^{0}C). Na dekapitering van die dragtige wyfies (gestasiedag 18) is hulle lewers en embrios ge-oes en dadelik by -70 ^{0}C ingevries en gestoor. Die geoesde embrios is stereomikroskopies ondersoek en die voorkoms, insidensie en aard van kongenitale afwykings geassesseer waarna die embrios dadelik ingevries en gestoor (-70 ^{0}C) is. DABA was hoogs-toksies vir ontwikkelende embrios teen al die dosisvlakke wat toegedien is en fetale mortaliteit was buitengewoon hoog. 'n Relatief hoë proporsie van die embrios het tekens van groeivertraging en kongenitale afwykings gehad. Neuraalbuisdefekte (NTD) het voorgekom in 5 – 17% van die embryos wat blootgestel was aan DABA. Hierdie resultate bevestig die vermoede dat DABA 'n embriotoksiese en teratogeniese verbinding is.

Urinêre organiese sure, asielkarnitiene en aminosure is respektiewelik d.m.v. gaschromatografie-massaspektrometrie (GC-MS), elektrosprei-tandemmassaspektrometrie (ES-MS-MS) en die Phenomenex EZ:faast® GC-MS metodiek gekwantifiseer. Die nadelige invloed van DABA op die metabolisme van dragtige Hanover.NMRI muiswyfies was duidelik sigbaar in die kompleksiteit van en

omvattende veranderings in die metaboliese profiele van DABA-behandelde wyfies, vergeleke met die van kontrolewyfies. DABA het 'n baie groot, maar onspesifieke effek op die algemene biochemie van DABA-behandelde dragtige wyfies uitgeoefen. 'n Verskeidenheid van belangrike metaboliese prosesse (glikolise, trikarboksielsuursiklus, ureumsiklus, ens.) is ernstig benadeel.

Ensiemanalises is ontwikkel en geoptimiseer om die katalitiese aktiwiteit van ODC in hepatiese en heel-embrio sitosolle te kan meet en om die totale poliamienkonsentrasie in weefsel (lewer, embrio) en urienmonsters te kwantifiseer. Die spesifieke aktiwiteit van ODC in die lewers van DABA-behandelde dragtige muise en hulle embrios is betekenisvol geïnhibeer (-53%; $p<0.0001$). Totale poliamienkonsentrasie in die weefsel (lewer, embrio) en urienmonsters van DABA-behandelde dragtige wyfies is ook sterk onderdruk (-70%; p,0.05). Hierdie resultate het ondubbelsinnig bewys dat DABA as `n Orn antimetaboliet kan optree, die ODC-gekataliseerde produksie van Put vanaf Orn kan rem en sodoende die biosintese van poliamiene (Put, Spd, Spm) nadelig kan beïnvloed. Die relatief lae konsentrasies van poliamiene gedurende die kritiese stadium van neuraalbuissluiting het waarskynlik `n nadelige effek gehad op die rol wat poliamiene in die selsiklus in die selsiklus speel, met die gevolglike ontstaan dismorfogeneses (bv. NTD) in sommige van die ontwikkelende embrios van DABA-behandelde dragtige muiswyfies.

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LIST OF ABBREVIATIONS

AMDIS	automated mass spectral deconvolution and Identification system
ATP	adenosine-5´-triphosphate
ADP	adenosine-5´-diphosphate
BSTFA	bis(trimethylsilyl)trifluoroacetamide
°C	degrees centigrade
CO_2	carbon dioxide
CoA	coenzyme A
CID	collision induced dissociation
Cyt *c*	cytochrome *C*
DCA	dicarboxylic acids
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DHP	dorsolateral hinge point
dpm	disintegration per minute
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
e^-	electron
EDTA	ethylenediaminetetraacetic acid
EZ:faast®	Easy-Fast amino acid sample testing kit
FAD	flavin adenine dinucleotide (oxidised)
$FADH_2$	flavin adenine dinucleotide (reduced)
5-Formyl-THF	5-formyl-tetrahydrofolate
FPGS	folylpoly-γ-glutamate synthetase
g	gram
g'	gravitatational constant
GABA	γ-amino butyric acid
GABA-T	γ-amino butyric acid transaminase
GC	gas (liquid) chromatography
GC-MS	gas chromatography-mass spectrometry
GCS	glycine cleavage system.
GHD	γ-hydroxybutyric acid
HCl	hydrochloric acid
HoCys	homocysteine
h	hour
HP	Hewlett Packard
IS	internal standard
kg	kilogram
LC-MS-MS	liquid chromatography tandem mass spectrometry
LDH	lactate dehydrogenase
µM	micromolar
mg	milligram
min	minutes
MHP	median hinge point
ml	millilitre
mM	millimolar
MPT	mitochondrial permeability translation
MS	mass spectrometer
MSD	mass selective detector
MTHFR	methylene tetrahydrofolate reductase
n	sample size
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised)

NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NTD	neural tube defect(s)
NMDA	*N*-methyl-D-aspartate
PDH	pyruvate dehydrogenase
PGA	2-propyl-glutaric acid
p.c.	post coitus
PSA	2-propylsuccinic acid
RNA	ribonucleic acid
ROS	reactive oxygen species
SAM	*S*-adenosylmethionine
SAH	S-adenosylhomocysteine
SD	serine dehydratase
SHMT	serine hydroxymethyltransferase.
cSHMT	cytosolic serine hydroxymethyltransferase.
mSHMT	mitochondrial serine hydroxymethyltransferase.
SSADH	succinic semi-aldehyde dehydrogenase
TCA	tricarboxylic acid
TCA cycle	tricarboxylic acid cycle, citric acid cycle, Krebs cycle.
THF	tetrahydrofolate, H_4-folate.
TMCS	trimethylchlorosilane
PLP	pyridoxal-5′-phosphate.
s	seconds
SD	standard deviation
t	time
UCS	unit citrate synthase, units citrate synthase
UV	ultraviolet
VPA	valproic acid, valproate, dipropylacetic acid, 2-propylvaleric acid.

CHAPTER 1
LITERATURE REVIEW

1.1 RATIONALE AND MOTIVATION OF THE STUDY

Birth defects or congenital defects are abnormalities in the structure, function and/or or metabolism of neonates. These defects may result in mild to severe physical and/or mental disabilities and even high incidences of mortality in affected children. Most birth defects in humans originate during the first three months of pregnancy, when the organs of the embryo are still developing. However, numerous other birth defects may occur either earlier or later in the pregnancy (Morris-Kay & Sokolova, 1996; Berkow *et al.*, 1997; O`Rahilly & Muller, 2001; Moore & Persaud, 2003a, 2003b; Brauer, 2003; Kaplan, 2004; Dolk, 2005; Bailey & Barry, 2005).

A wide variety of birth defects or congenital defects can occur (i.e. heart defects, cleft lip and/or cleft palate, neural tube defects, gastro-intestinal defects, foetal alcohol syndrome, congenital hypothyroidism, Down syndrome, phenylketonuria). Nearly eight thousand different birth defects, ranging from those with a relatively mild to others with a very serious and often fatal outcome, have already been described (Moore & Persaud, 2003a; Schriver *et al.*, 2004). Although many birth defects can be treated by medical intervention, they are still the leading cause of death in the first year of post-natal life. According to the *March of Dimes*, an organization involved in world wide birth defect awareness and prevention campaigns, approximately 150 000 babies with birth defects are born in the United States annually (March of Dimes, 1997). The American College of Obstetricians and Gynaecologists (ACOG) calculated that the mean incidence of congenital defects in the American population is approximately 3% (ACOG, 1993). These figures are known to be significantly higher in other areas of the world, especially in the rural populations of Africa, China and Mexico (Ncayiyana, 1986; Moore, 1997; Hendricks, 1999; McDonnell, 1999).

Neural tube defects (i.e. spina bifida, microcepahly, anencephaly, encephalocoele) are a variety of multi-factorially derived, congenital defects that frequently occur in humans (Lemire, 1988; Leech, 1991; Sulik, 1993; O`Rahily & Muller, 2001). The various forms of NTD are characterized by incomplete development of the central nervous system and related surrounding structures, apparently due to defects in neurulation. Neurulation is

the process through which an embryo develops a neural tube, the rudimentary structure from which the central nervous system develops. The neural tube of the human embryo closes during the first three weeks of pregnancy and eventually develops into the central nervous system (brain, spinal cord, etc.) of the baby (Moore & Persaud, 2003b; Brauer, 2003).

NTD prevalence varies internationally and between different socioeconomic and ethnic groups (Vanaerts, 1995; Wasserman, 1998; Kloeblen & Batisch, 1999). The global incidence of neural tube defects (NTD) fluctuates between 0.6 and 6.12 cases per 1000 live births and the highest reported incidences were recorded in Ireland, Mexico, China and South Africa. Rural communities in China and the Republic of South Africa appear to be particularly prone to pregnancies complicated by NTD (Ncayiyana, 1986; Moore, 1997; Delport *et al.*, 1995; Venter, 1995; Moore *et al.*, 1997; Hendricks, 1999; McDonnell, 1999; Li *et al.*, 2006).

Although NTD are among the most common congenital malformations, relatively very little is known about the underlying causal factors and mechanisms in humans and experimental animals. The aetiology or causes of birth defects (i.e. neural tube defects) may be environmental (i.e. maternal hyperthermia, nutrient deficient diet, teratogens, tobacco usage, radiation, occupational), genetic (i.e. Down syndrome, phenylketonuria) or idiopathic in nature. Birth defects are often caused by a combination of genetic, environmental and/or idiopathic factors (Warkany, 1971; Smithels, 1976; Op't Hoff, 1985; Lemire, Persaud, 1990; 1988; Leech, 1991; Bruni JE *et al.*, 1994; Corcoran, 1998; Lewis DP, 1998; Nash & Persaud, 1988; Gos & Szpecht-Potocka, 2002; Yerby MS, 2003; Finnell RH *et al.*, 2003). One of the first dietary factors associated with NTD was folic acid. Dietary or metabolically induced folic acid deficiencies, occurring during the process of neural tube closure, may be important factors impacting on the development and relatively high incidence of NTD in selected populations The British Medical Council and the Budapest trials conclusively proved that a woman's risk for a recurring NTD-affected pregnancy is reduced substantially (>70%) by periconceptionally supplemented folic acid (Medical Research Council Vitamin Study Group, 1991; Czeizel, 1992; Eskes, 2000; Czeizel *et al.*, 2004).

Folate intermediates (i.e. 5-methyltetrahydrofolate, 5,10-methylenetetrahydrofolate) play a crucial role in numerous metabolic pathways, i.e. one-carbon metabolism (Girgis, 1997; Rosenblatt, 1995; Bailey, 1999; van der Put, 1999; Herbig, 2002). Numerous

studies have linked disturbances in one-carbon metabolism (i.e. remethylation of homocysteine, folate cycle) as probable causes of several folate responsive congenital defects (i.e. NTD, orofacial defects) (Coelho & Klein, 1990; Pulikkunnel & Thomas, 2005; Dunlevy *et al.*, 2006a, 2006b). One-carbon derivatives of THF are involved in the interconversion of serine and glycine (Xue *et al.*, 1999; Cetin, 2001), the metabolism of histidine and formate (Stover *et al.*, 1993; Pasternak *et al.*, 1994; Fowler, 2001), the biosynthesis of purines and thymidine (Scott *et al.*, 1994; Refsum, 2001; Tai, *et al.*, 2004) and the remethylation of homocysteine to methionine (Dunlevy *et al.*, 2006a). S-adenosylmethionine (SAM) is synthesized by the condensation of ATP and methionine through the action of methionine adenosyltransferase (MAT) (Chiang, 1996; Loenen, 2006). SAM is a very important biomethylating reagent in living organisms and basically acts as a methyl (one-carbon) donor in the biosynthesis of carnitine, polyamines, glutathione, neurotransmitters and numerous other biomolecules (Heby, 1981; Selhub, 1992; Bailey, 1999; Avila, 2002; Herbig, 2002; Mattson, 2003). SAM is also responsible for the methylation of DNA and consequently plays an important role in the regulation of gene expression (Haaf, 2006; Schob & Grossniklaus, 2006;Serman *et al.*, 2006).

In general, the health of the mother plays an important role in determining the well being of the developing embryo or foetus. Some congenital defects are closely associated with the presence of prenatal infectious disease in the mother (i.e. rubella, toxoplasmosis, genital herpes) (Moore & Persaud, 2003b; O`Rahilly & Muller, 2001) or the dietary presence of toxins, produced by fungi (Finnell, 2003; Marasas *et al.*, 2004; Cabrera *et al.*, 2004; Padmanabban, 2006). Dietary mycotoxins (i.e. fumonisin B1, moniliformin, aflatoxins, ochratoxins, etc.) are increasingly suspected of being involved in the development of birth defects, i.e. neural tube defects. Inhabitants of the Transkei region in the Eastern Cape Province of South Africa display the highest reported incidence and prevalence rates for oesophageal cancer in the world today. This phenomenon appears to be linked to the presence of relatively high levels of the mycotoxin, fumonisin B_1, a fungal contaminant in maize, consumed in this region (Sydenham *et al.*, 1990; Marasas *et al.*, 2004; Marasas, 1995; Makaula *et al.*, 1995). Fumonisin B_1, a disrupter of sphingolipid metabolism, occurs in maize crops all over the world and is now known to be present in relatively high concentrations in crops produced in regions where the local population display high incidences of NTD (Transkei region in the Eastern Cape, China, Mexico) (Somdyala *et al.*, 2003; Marasas *et al.*, 2004; Gelineau-van Waes, 2005). A number of disease conditions of the mother, i.e. non-ketotic hyperglycaemia,

phenylketonuria, hyperhomocysteinemia and diabetes may facilitate or cause the abnormal development of an embryo or foetus. This may, in part, be due to the toxicity of high levels of a variety of metabolic intermediates in the blood of the mother and/or the amniotic fluid of the embryo/foetus (Reece *et al.*, 1985; Levy & Ghavami, 1996; Rouse *et al.*, 2000; Nissenkorn *et al.*, 2001; Finnell *et al.*, 2003; Chen, 2005).

The anti-epileptic drug, sodium valproate (Epilim®, Depakine®; VPA), a branched, short-chain fatty acid is a known cause of neural tube defects in babies, born to epileptic mothers (Naruse *et al.*, 1988; Wegner, 1992; Elmazar *at al.*, 1995; Finnell, 1999; Alonso-Aperte, 1999; Frey & Hauser, 2003; Padmanabban & Shafiullah, 2003; Padmanabban *et al.*, 2006). A close relationship between the teratogenic properties of VPA and serious disturbances in a number of metabolic pathways (one carbon metabolism, serine-glycine conversion, etc.) was recently demonstrated in a murine model (Brits, 2005). The toxic, non-protein amino acid, 3-hydroxynorvaline, induced neural tube defects in chicken and mouse embryos and this phenomenon was strongly associated with anomalies observed within one-carbon metabolism of pregnant mothers and their embryos (Louw, 2004; Louw *et al.*, 2005).

Several other xenobiotic chemicals have been identified as probable aetiological factors involved in the epidemiology of NTD, i.e. anti-epileptic agents (valproic acid, diazepam), barbiturates, hypoglycin-A, concanavalin-A, ethanol, retinoic acid, retinol (vitamin A), methotrexate, nitric oxide, short-chain carboxylic acids, arsenic and many more (Persaud, 1968, 1969; Persaud & Henderson, 1970; Persaud & Kaplan, 1970; Leonard & Lauwerys, 1980; Coakley, 1986; Nash & Persaud, 1988; Gilani & Persaud, 1992; Elmazar, 1995; Vorster, 1995; Morriss-Kay & Sokolova, 1996; Inagaki, 1996; Lewis, 1998; Emmanouil-Nikoloussi *et al.*, 2000; Yerby, 2003; Finnell, 2003). However, in spite of the fact that the aetiology of a large number of congenital defects are becoming clearer, the causal factor(s) and biochemical mechanisms, involved in more than 70% of all cases of congenital defects, have still not been identified (Hwang & Jaakkola, 2003; Kaplan, 2004; Anderson, 2005). It is therefore evident that the role of phytotoxins, mycotoxins and all other possible xenobiotic agents in the induction of congenital disorders should be rigorously investigated.

Communities in the rural areas of Southern Africa, Ethiopia and India are known to consume the edible parts of a large number of indigenous plants (i.e. legumes) to supplement their protein intake. Rural inhabitants in most of the third world countries and

also in developed countries use herbal remedies in alternative medicine to treat a wide variety of illnesses. Most of the plants used for these purposes contain a variety of different types of chemical compounds that display chronic or acute toxicity towards animals and man, while some are definitive teratogens (Rosenthal, 1982; Rozan, *et al.*, 1999, 2000, 2001; Jurgens, 2003; Gallo *et al.*, 2003). Since ancient times, it was known that the consumption of certain wild legumes may cause acute or chronic diseases in animals and man. Neurolathyrism is a neurodegenerative condition characterized by progressive paralysis of the lower limbs, often reaching epidemic proportions in some communities in Ethiopia and India (Spencer & Schaumburg, 1983; Haimannot *et al.*, 1990; Gettahun *et al.*, 1999). Neurolathyrism is especially rife in rural populations where wild legumes of the genus *Lathyrus* are often consumed as part of the staple diet in these communities. A number of bioactive agents (beta-(N)-oxalylamino-L-alanine, BOAA; L-3-oxalylamino-2-aminopropionic acid, ODAP; β-aminopropionitrile, BAPN) potentially responsible for some of the pathology have been isolated, identified and investigated in experimental animals (Spencer & Schaumburg, 1983; Spencer *et al.*, 1987; Pai *et al.*, 1993; Ravindranath, 2002). Although DABA proved to be a potent neurolathyrogen in experimental animals, it is not yet certain if it does play a role in human Lathyrism (Rosenthal, 1982).

The well-known neurotoxin, L-2,4-diaminobutyric acid (DABA), is a relatively abundant constituent of most of the plants mentioned above and many more. Apart from often being produced by plants in copious quantities, DABA also acts as a building block in numerous peptide antibiotics, mureins and glycopeptides in the cell walls of a wide variety of microorganisms (Perkins, 1971; Fiedler & Kandler, 1973; Sasaki *et al.*, 1998; Vertesy *et al.*, 2000; Martin *et al.*, 2003). In spite of its apparent natural abundance and the fact that is known to be neurotoxic, hepatotoxic and/or lathyrogenic to experimental animals (O`Neal *et al.*, 1968; Rosenthal, 1982), it is not yet clear whether DABA is a teratogen capable of inducing congenital defects in humans or experimental animals (Fowden & Bryant, 1958; Ressler *et al.*, 1961; Bell, 1962; Bell & Tirimanna, 1965; O`Neal *et al.*, 1968; Sutton & Simmonds, 1974; Rosenthal, 1982; Foster *et al.*, 1987; Rowe *et al.*, 1993; Rasmussen *et al.*, 1993; Bell *et al.*, 1996; Rozan *et al.*, 2001). The main objective of this investigation, therefore, was to investigate the teratogenic potential of DABA in a murine model and the effect the toxin may have on selected metabolic pathways (urea cycle, polyamine synthesis, etc.).

1.2 BRIEF SYNOPSIS OF MURINE EMBRYOLOGY

Embryonic development of the mouse begins with fertilization of the egg by the sperm. The period of early development commences with the formation of the zygote, followed by cleavage divisions, and then blastulation. The common feature of the early developmental stages in mammals, is the formation of a cavitated (blastocoel-bearing) embryo. Cleavage divisions that occur in the oviduct are holoblastic and synchronous. During the first cleavage, the ovum is split into two equal blastomeres by a meridional cleavage, extending from the animal to the vegetal pole. The two blastomeres adhere to each other very closely, being held together by an albuminous envelope (zona pellucida) (Burki K, 1986; Roberts, 1990; Van Allen, 1996; Gilbert & Raunio, 1997; Moore & Persaud, 2003b).

The second cleavage is irregular, first splitting one of the blastomeres in a longitudinal plane, at right angles to the first cleavage, a process which is repeated with the next blastomere in line, until the four-blastomere stage is completed. Cleavage proceeds irregularly, until a small sphere of blastomeres, the size of a pin head, has been formed. At the 16-celled stage, the embryo resembles a mulberry (morula) (Huettner, 1965; Burki K, 1986; Roberts, 1990; Van Allen, 1996; Gilbert & Raunio, 1997; Moore & Persaud, 2003b).

Gastrulation in the mouse begins at about 6.5 days post coitum (p.c.) when there are about 1000 cells in the primitive ectoderm. Cells delaminate from the epithelial layer of individual mesoderm cells, between the primitive ectoderm and visceral endoderm. Gastrulation consists of a complex series of morphogenetic (i.e., form generating) actions in which the three primary germ layers are established and during which cells are brought together in new combinations, allowing cell-cell interactions to occur (Burki K, 1986; Moore & Persaud, 2003b; Hogan *et. al*, 1986; Roberts, 1990). The accumulating cells form a wedge-like structure, with rostrally orientated apex points and a caudal base. This wedge-like structure forms the initial primitive streak. It elongates and becomes cord-like over the next few days of development. The primitive streak is the site at which cells leave the epiblast and migrate into the interior of the blastoderm during formation of the three primary germ layers, the ectoderm, the mesoderm, and the endoderm (Fig. 1.2). Movement of cells through the streak is termed ingression (Burki K, 1986; Moore & Persaud, 2003b; Hogan *et. al*, 1986; Roberts, 1990).

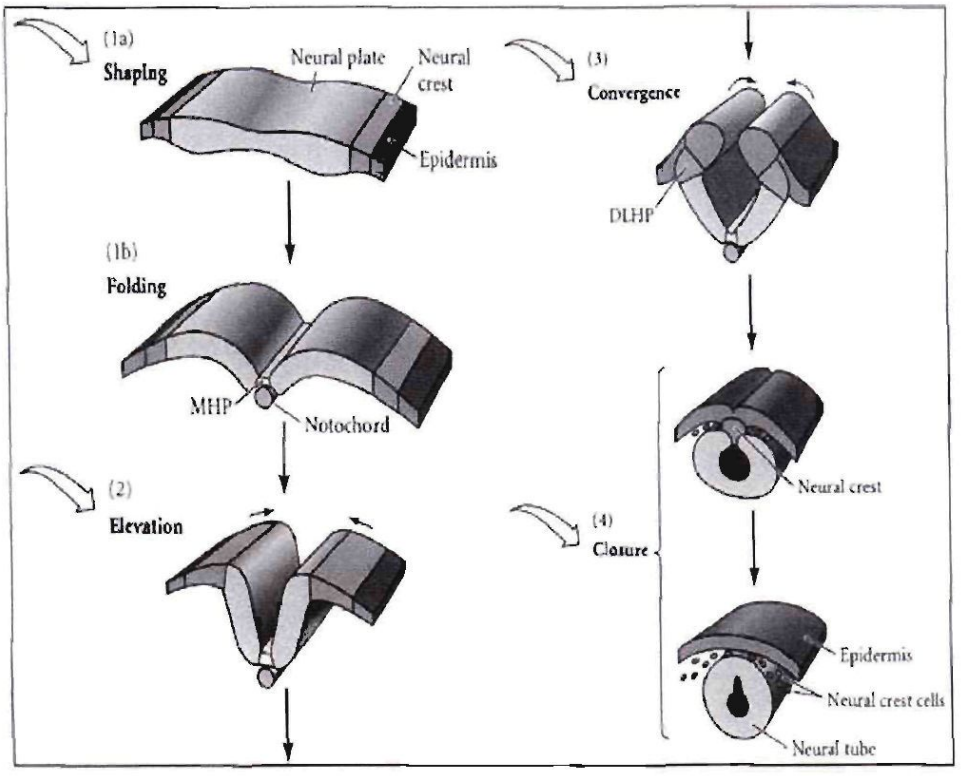

Figure 1.1: *Formation and closure of the neural tube (Smith & Schoenwolf, 1997).*

The first cells to ingress through the primitive streak form the endoderm and extra-embryonic mesoderm. As ingression occurs, the primitive streak begins to shorten or regress. During regression a specialized portion of the primitive streak, Hensen's node (rostral end of the streak), moves caudally, leaving cells behind which then develop into the notochord. The notochord plays an important role in the induction of neural plate formation. Upon completion of gastrulation, all prospective endodermal and mesodermal cells have ingressed and the primitive streak has largely disappeared (Burki K, 1986; Roberts, 1990; Moore & Persaud, 2003b; Gilbert and Raunio, 1997).

The first major neural plate structure which is formed is the neural tube. The ectoderm above the notochord thickens (i.e. neural plate) and two ridges on opposite sides of each other begin to rise up, forming a channel down the middle of the plate. These two ridges move closer together, eventually fusing and disconnecting from the ectoderm to form a tube-like structure, the neural tube. From the neural tube the complete central nervous system, the brain and spinal cord of the embryo and eventually the fetus develops. The process of gastrulation results in the gradual establishment of a sequence of about 65

paired blocks of mesodermal cells, the somites, along the anterior-posterior body axis. These somite blocks eventually differentiate into vertebrae, ribs muscles and the dermis of the skin. Subsequently each somite block becomes subdivided into three regions: the sclerotome, the dermatome and myotome. The next major structure to form is the primitive gut, followed by the heart, limb buds, tail and head until the whole mouse has completely developed (Burki K, 1986; Roberts, 1990; Moore & Persaud, 2003; Gilbert and Raunio, 1997; Swarthmore, 1998). The last stages of the closure of the neural tube are illustrated in Fig. 1.1 above.

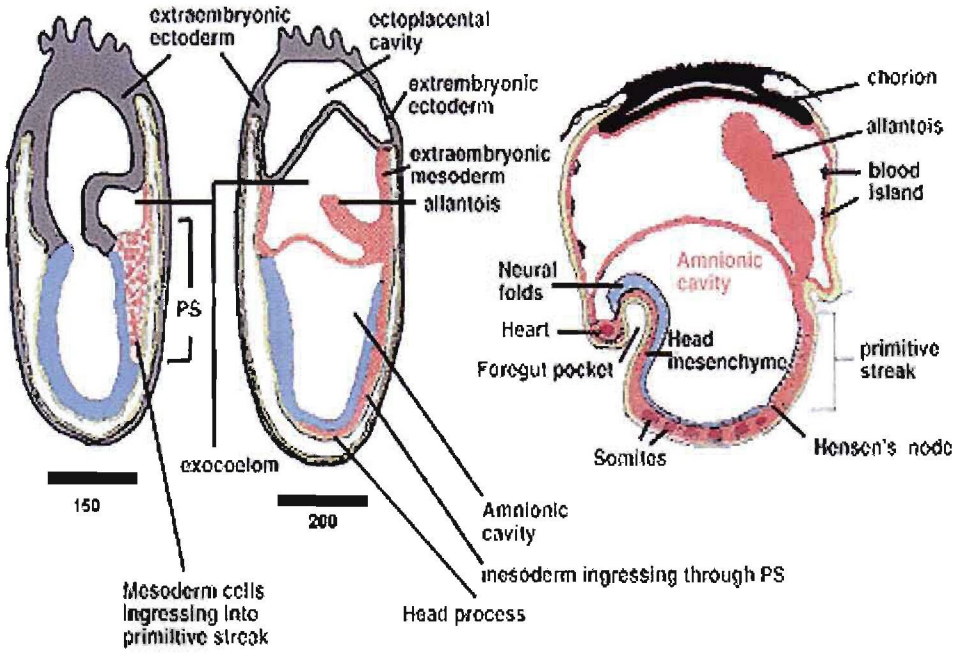

Figure 1.2: *Early post implantation development of the mouse embryo (Moore & Persaud, 2003).*

1.3 TERATOGENS AND THE AETIOLOGY OF CONGENITAL DEFECTS

Prenatally, developing organisms are continuously exposed to a vast array of chemical and physical factors (i.e. industrial and food-borne chemicals, chronic medication, alcohol, recreational drugs, chemicals inhaled by tobacco smoking, low level non-ionic radiation, excessive heat, etc.) impinging on their embryonic and foetal development (Wilson & Fraser, 1977; Paul, 1993; Briggs *et al.*, 1998; Koren, 1994; Niesink *et al*, 1998; O`Rahilly & Muller, 2001).

Table 1.1: Somite regions in the developing embryo (Gilbert & Raunio, 1997).

Somite Block Region	Tissue
Sclerotome	Vertebrae and ribs in the thoracic regions. The anterior sclerotomes of one pair of somites and the posterior sclerotomes of the preceding pair co-operate to form one vertebra.
Dermatome	Connective tissue in the dermis of the skin dermal component of the hair follicles.
Myotome	Muscles in the body wall, the myotomes of the four to five somites, adjacent to the fore and hind limb buds, give rise to muscles in the limbs.

Teratology is essentially the study of environmentally induced congenital anomalies. A teratogen is an agent which, by acting on the developing embryo or foetus, can cause structural anomalies or defects. To date, only a relatively small number of known chemicals, or regularly used drugs have been proven to be teratogens in man. Malformations induced by chemicals and drugs are potentially preventable. The following basic principles of teratology were outlined (Wilson & Fraser, 1977; Finnell, 1999):

- Susceptibility to teratogenesis depends on the genotype of the conceptus and the manner in which it interacts with the environmental agents.
- Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure.
- Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate abnormal embryogenesis (pathogenesis).
- The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent).
- Manifestations of deviant development increase in degree as dosage increases from the no effect to the totally lethal level.
- The final manifestations of abnormal development are malformation, growth retardation, functional disorder and death.

At first, studies in experimental teratogenesis appeared to indicate that particular agents produce characteristic types of malformations. Such associations of patterns of defects

with causative factors were sometimes referred to as "agent specificity", implying that each agent had the capability to elicit specific defects. As more and more causative factors were described, however, it became apparent (Schardein, 1985; Rubin *et al.*, 1986; Teratological Society Affairs Committee, 1994; Koren, 1994) that the profiles of the patterns of defects overlapped, i.e. different agents often produced a range of defects in different degrees and proportions, or sometimes even the same defects in similar incidences. It was subsequently concluded that, since each causative factor did not necessarily produce a distinctive array of defects, there must be some commonality in the developmental pathways between the process of induction and the final expression of abnormality (Wilson & Fraser, 1977; Finnell, 1999).

Evidence assembled from a vast experimental literature base support the fact that numerous teratogenic agents can induce abnormal development in a large number of different species. The vast array of teratogenic risks to which both humans and animals are frequently exposed to, necessitate exhaustive screening tests to ensure the safety of food and drugs. A thorough knowledge of the mechanisms, involved in teratogenesis may eventually help to prevent congenital diseases (Wilson and Fraser, 1977; Dansky *et al.*, 1992; Juchau, 1993; 1997; Finnell, 1999; Azarbayjani, 2001).

1.3.1 TERATOGENS AND NEURAL TUBE DEFECTS

Neural tube defects (NTD) are among the most distressing congenital defects known, mainly because of the life long implications that these effects pose for the affected child and his/her family (Cate, 2002; Coakley, 2002; Padua, 2002). The term NTD refers to a variety of malformations of the embryonic brain, the spinal cord and surrounding protective structures (i.e. scull, spinal column) (Sarawak, 1996; Dias, 1998; Drolet, 2000). NTD originate during the first three weeks of gestation, when the neural tube is formed, following the zipper-like closure of the neural groove on the neural plate structure. NTD can be classified as either open or closed. Open NTD occur when the neural tissue (i.e. spinal cord , brain) remain completely exposed to the external environment of the amnionic fluid in the amnionic sac of the uterus, or else is only covered by a membrane. NTD are termed closed when the defect is covered by normal skin (Botto *et al.*, 1999). Two main groups of NTD can be distinguished: cranial neural-tube defects and spinal neural-tube defects (Persaud, 1981; Vintzileos *et al.*, 1983; Nicolaides & Campbell, 1989; Stover *et al.*, 1997).

1.3.1.1 Cranial neural-tube defects.

(a) Anencephaly.

Anencephaly is one of the most lethal NTD and is caused by a malformation of the bony parts of the cranial vault, leading to the partial or complete absence of the brain. Only a small amount of exposed, undifferentiated vascular and dysplastic brain tissue usually remain attached to the base of the skull (Locksmith & Duff, 1998; Peter & Fieggen, 1999; Guille *et al.*, 2006). Infants with this severe form of NTD are either stillborn or die shortly after birth (Persaud, 1981; Vintzileos *et al.*, 1983; Nicolaides & Campbell, 1989; Stover *et al.*, 1997).

(b) Encephalocoele.

Exencephaly results when the malformation of the cranium causes the formation of an opening, through which the brain and/or the meninges can herniate, to form a sac-like structure at the back of the head. Two forms of encephalocoele can be distinguished: meningocoele or meningomyelocoele form (Persaud, 1981; Vintzileos *et al.*, 1983; Nicolaides & Campbell, 1989; Stover *et al.*, 1997). When the membranes of the spinal cord, the meninges and/or the brain tissue or spinal cord tend to bulge through the opening in the skull or vertebrae, the defect can result in a meningocoele or a myelomeningoecele. In the case of a typical meningocele, no brain or spinal cord elements are contained in the cerebrospinal fluid-filled sac. Myelomeningocoele contains brain tissue, as well as spinal cord and nerve route components in the sac-like structure. These conditions may be associated with caudal displacement of the medulla oblongata and cerebellum and hydrocephalus (Arnold-Chiari malformation) (Shuman, 1995; Peter & Fieggen, 1999; Guille *et al.*, 2006).

(c) Craniorachischisis.

Craniorachischisis is characterised by anencephaly, accompanied by severe defects in the closure of the vertebrae in the spinal column, resulting in the extreme exposure of neural tissue. This type of NTD is relatively rare, however, its incidence appears to be significantly higher in northern China than anywhere else globally (Botto *et al.*, 1999; Stover *et al.*, 1997). The morbidity of this form of NTD is grave (Peter & Fieggen, 1999; Drolet 2000; Guille *et al.*, 2006).

1.3.1.2 Spinal neural tube defects.

Spinal NTD entail the most common form of NTD (>80%) and refers to a range of congenital defects of the vertebral column, caused by failure of the bony structures,

dorsal to the spinal cord to fuse (Botto *et al.*, 1999; Stover *et al.*, 1997; Drolet, 2000). Two of the most frequent forms of spinal NTD are spina bifida occulta and spina bifida.

(a) Spina bifida occulta:

Spina bifida occulta is a very mild form of spina bifida but its real prevalence is unclear. The spinal cord is usually uncompromised and the only clinical sign of the presence of the defect is often a tuft of hairs, or just a dimple in the skin at the site of the defect. Neurological deficit is rare and only subtle neurological abnormalities, such as enuresis or incontinence may be present (Persaud, 1981; Vintzileos *et al.*, 1983; Nicolaides & Campbell, 1989).

(b) Spina bifida

The term spina bifida refers to a variety of vertebral and neural tube defects and this form of NTD results following the failure of a posterior vertebral arch to fuse. The defect most commonly occurs in the lumbo-sacral region of the baby. The main defect of spina bifida is an abnormal opening in the bony vertebral column (spinal column) through which the spinal cord passes. An abnormal opening, somewhere along the vertebral column, leaves the spinal cord unprotected and vulnerable to mechanical and/or chemical injury as well as infection (Botto *et al.*, 1999; Stover *et al.*, 1997; Northrup & Volcik, 2000).

Spina bifida may be associated with other abnormalities such as talipes and hip dislocation. The spinal defect is clinically obvious and may result in various degrees of limb weakness, sensory loss below the level of the defect, joint dislocation and contractures. Urinary disorders are common in spina bifida patients and may extend to partial or complete loss of bladder and anal sphincter control, causing urinary and faecal incontinence (Persaud, 1981; Vintzileos *et al.*, 1983; Nicolaides & Campbell, 1989).

1.3.2 ONE-CARBON METABOLISM AND NEURAL TUBE DEFECTS

The aetiologies of NTD are complex and include genetic, dietary, and environmental factors (Section 1.1). One particular environmental factor which has been epidemiologically linked to the incidence of NTD in several populations is the level of dietary folate (Smithells, 1985; Medical Research Council Vitamin Study Group, 1991; Czeizel & Dudas, 1992; Steegers-Theunissen, 1993; Eskes, 2000; Shoob, 2000; Christensen & Rosenblatt, 1995). Periconceptional folate administration reduces the

occurrence as well as the recurrence risk of NTD (Smithells, 1985; Czeizel & Dudas, 1992). However, the precise mechanisms, involved in the prevention of NTD by folic acid supplementation, are not yet completely understood. Supplementation may very well overcome a transient or chronic folate deficiency or even a metabolic disturbance in the folate or one carbon metabolism (Van der Put and Blom, 2000; Blom *et al.*, 2006).

Pregnant women are more prone to develop a folate deficiency and subsequently pregnancy anaemia, due to increased folate demands by the developing foetus and maternal tissues. Other factors that may provoke a folate deficiency include a poor diet, physiological haemodilution occurring during pregnancy, increased plasma clearance, and hormonal influences (Steegers-Theunissen, 1995; Sifakis & Pharmakides, 2000; Mcmullin *et al.*, 2001). Low dietary levels of methionine and vitamin B_{12} or a combination of these factors and low dietary folate may lead to an increased occurrence of NTD (Coelho & Klein, 1990; Czeizel & Dudas, 1992; Goh *et al.*, 2006; Haider & Bhutta, 2006).

The importance of vitamins, i.e. folic acid, vitamin B_{12} and vitamin B_6, as essential dietary factors in the pathology of NTD, are well known. In the early 1930`s it was demonstrated that pregnancy anemia can be corrected with brewer's yeast (Wills, 1991). Folate (vitamin B_9) was eventually identified as one of the specific bioactive substances in brewer's yeast, responsible for alleviating the anaemic condition. Folic acid, vitamin B_{12} and other water soluble B-group vitamins (i.e. vitamin B_6) were subsequently found to be abundant in marmite, a savoury smear produced from yeast extract and demonstrated to cure transient megaloblastic anaemia (Forshaw *et al.*, 1964; Vinke, 1964; Wills, 1991). The term folic acid is derived from the Latin word for leaf, *folium*. Major natural dietary sources of folate are fresh green leafy vegetables (spinach, turnip greens, asparagus, legumes, peas, etc.), wheat, dry beans, fruit (citrus, fruit juices, etc.), whole wheat bread and animal tissues (liver, kidney, etc.) (Zittoun, 1993; Eskes, 1997). Folic acid was eventually extracted from spinach leaves in 1941 and its total synthesis achieved in 1946 (Whiteley, 1971; Hoffbrand, 1975 Rowe, 1983; Steegers-Teunissen, 1995).

Dietary folates are absorbed from the gut and converted from the various polyglutamate forms to the respective monoglutamates, followed by a complete reduction to 5,6,7,8-tetrahydropteroylmonoglutamate (tetrahydrofolate;THF), the parent compound of all biologically active folates. THF is the primary one-carbon carrier in all known biological systems, responsible for the transfer of one-carbon groups in various oxidation states (methyl-, methylene-, methenyl-, formyl-, formimino-) to a variety of one-carbon

acceptors (Fowler, 2001; Lucock, 2000). The predominant folate intermediate in serum and in tissue is 5-methyl-THF (Horne, 1993; Donnelly, 2001). Folate and folate intermediates actively participate in a number of important metabolic routes, namely purine biosynthesis, thymidine biosynthesis, methionine biosynthesis, the remethylation of homocysteine, interconversion of serine and glycine and the metabolism of histidine and formate (Horne, 1993; Lucock, 2000; Donnelly, 2001; Fowler, 2001; Refsum, 2001; Daly *et al.*, 2005). Since folate is involved in the formation of methionine, it is also indirectly involved in numerous methylation reactions with S-adenosylmethionine (SAM). SAM is probably the most important biomethylating agent in living systems, participating in DNA methylation and therefore playing an important role in carnitine biosynthesis, polyamine biosynthesis, the synthesis of cysteine and glutathione through the transsulfuration route and numerous other methylation reactions, as well as gene regulation (Heby, 1981; Selhub, 1992; Bailey, 1999; Avila, 2002; Herbig, 2002; Mattson, 2003) (Figure 1.3).

Requirements of folate can be met by a variety of folate derivatives, as long as the essential structure of pteroylglutamic acid remains intact. Human cells need a critical concentration of folates to allow normal activity of folate-dependant enzymes. The demand of folate is greatly increased during infancy, adolescence and pregnancy (Horne, 1993; Steegers-Theunissen, 1995; Donnelly, 2001). Folate mainly acts as a cofactor/co-substrate for a number of enzymes involved in *de novo* purine and pyrimidine (i.e. thymidine) synthesis, essential building blocks for DNA. A compromise in folate metabolism could therefore result in defective DNA synthesis, resulting from deficiencies in the availability of sufficient quantities of deoxyribonucleotide bases. Problems in the synthesis of intact DNA may negatively impact upon the development of the embryo, due to impairment in the transcription of genes activated during the process of neurulation. The correct level of DNA methylation is also essential for the regulation of gene activity and since folate intermediates play an important role in the methylation process, defects in folate metabolism may negatively affect the expression of specific genes (Friso & Choi, 2002, 2005; Fang & Xiao, 2003).

The highly negatively charged polyglutamate forms of folate are poorly transported across cell membranes. Specific folate binding proteins and receptors are therefore involved in the transfer of folate against concentration gradients from the mother to the

foetus, which the foetus is able to accumulate folate (Jansen *et al.*, 1997; Sierra & Goldman, 1999; Matherly & Goldman, 2003).

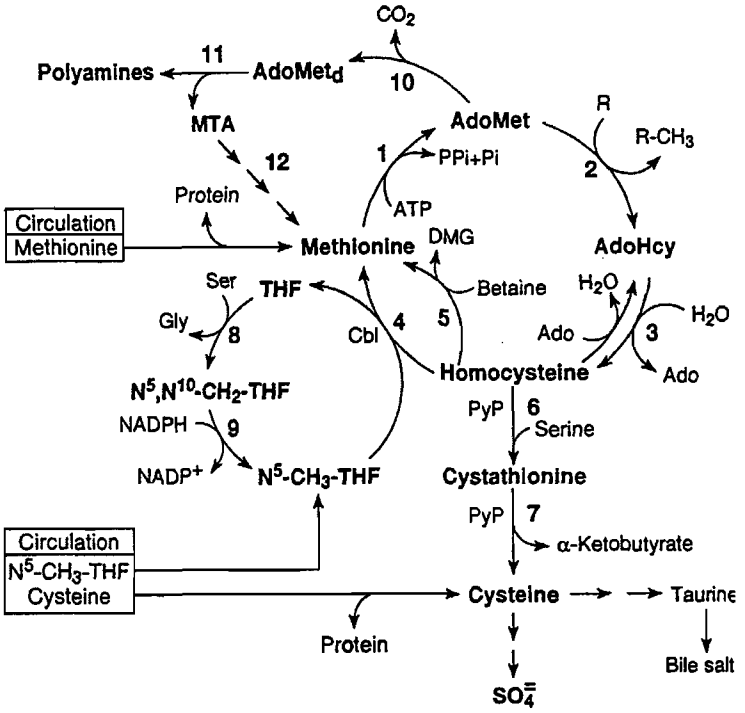

Figure 1.3: *The Folate cycle: Interaction with the methylation cycle and transsulfuration pathway (Scott & Weir, 1998).*

The placenta seems able to supply the foetus with adequate folate (Carrocher *et al.*, 1991; Henderson *et al.*, 1995). The enzymic interconversion of pteroylmonoglutamates and pteroylpolyglutamates in the mucosa, during the uptake of folates from the gut, enables cells to maintain intracellular folate levels at relatively high concentrations. Specialized enzymes, i.e. folylpolyglutamate synthetase and folylpolyglutamate hydrolase (glutamate hydrolase) are responsible for the maintenance of the delicate balance between free and bound folates (Jansen *et al.*, 1997; Sierra & Goldman, 1999; Matherly & Goldman, 2003). Folate is effectively trapped inside the cell by polyglutamation and this process contributes to the metabolic control of intracellular and extracellular folate dependent biochemical reactions. The chain length of the glutamate also plays a huge role on the affinity of the folate-dependant enzyme for the substrate. Polyglutamate folates with the correct number of glutamate residues have much lower K_m values for some of the folate-dependant reactions than the monoglutamate forms - this allows folate metabolism to progress at the normal concentrations of folates in the cell (Rosenblatt, 1995; Atkinson, 1997). Defects in the

structure and function of any of the enzymes and/or binding proteins, involved in the absorption, high-affinity binding and biochemical interconversion of folates may also precipitate folate deficiency syndromes (Carrocher *et al.*, 1991; Lucock, 2000; Donnelly, 2001; Fowler, 2001; Matherly & Goldman, 2003).

The metabolic intermediate, homocysteine, lies at an important metabolic branch point (see Fig. 1.3). It can be either catabolised to cystathionine *via* the transsulfuration pathway or remethylated to methionine. The latter pathway is folate dependent and homocysteine is known to be a sensitive marker of the folate and vitamin B_{12} status in humans (Steegers-Theunissen *et al.*, 1994; Ubbink *et al.*, 1994, 1995, 1999). Maternal hyperhomocysteinaemia is a known risk factor for neural tube defects and associated with a nearly 3-fold increase in the relative risk for NTD-affected pregnancies (Harmon, 1996; Daly *et al.*, 2005; Gellekink *et al.*, 2005). Women with NTD affected offspring have been demonstrated to display increased levels of plasma and amnionic fluid homocysteine (Steegers-Theunissen, 1994; Mills, 1995). Mild hyperhomocysteinaemia may be caused by deficiencies in folate, vitamin B_6, vitamin B_{12}, or by a metabolic abnormality in one or more of three enzymes involved in homocysteine metabolism, i.e. 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine-β-synthase (CBS) or methionine synthase (MS).

Methylenetetrahydrofolate reductase (MTHFR) defects are known risk factors for neural tube defects in caucasians. A 677C→T mutation in the MTHFR gene, leading to the formation of a heat-sensitive mutant form of this enzyme was identified in caucasians (Kang *et al.*, 1991). The presence of the 677C→T mutation, a relatively common mutation in the MTHFR gene, was found to be associated with an increased incidence of spina bifida offspring in the Dutch population (Van der Put *et al.*,1996a). The thermolabile MTHFR gene is associated with a 7.2 fold increase in the risk for NTD (Ou *et al.*, 1996). These observations demonstrate that a single nucleotide substitution in the coding region of MTHFR can reduce the activity of the enzyme and, as a result, impair homocysteine and folate metabolism and increase the risk of the NTD affected pregnancies (van der Put, 1996b). A recent South African investigation did not find any homozygotes for the 677C→T mutation in the MTHFR gene in mothers with NTD affected offspring, or the controls employed in the study (Ubbink *et al.*, 1998). Heterozygote carriers for this mutation were detected in both the black and caucasian sample groups, confirming that the 677C→T mutation occurs in the South

African population. The prevalence of the 677C→T mutation in the black population appeared to be very rare, compared to that in the caucasian population. The latter population displayed incidence characteristics, similar to that of the Dutch population.

Homocysteine can also be remethylated to methionine by betaine-homocysteine methyltransferase (BHMT), using betaine as a methyl-group donor. During this reaction, betaine (trimethylglycine) is converted to dimethylglycine and homocysteine methylated to methionine. The betaine employed during this reaction is usually derived from the catabolism of choline (Ueland *et al.*, 2005). A study of choline metabolism in mouse embryos indicated that BHMT was not yet genetically expressed in the 10 day-old embryos (Fisher *et al.*, 2002). Closure of the neural tube is almost complete at 10 days *p.c.* and it therefore seems unlikely that BHMT will play any role in providing one-carbon units during closure of the neural tube in the developing mouse embryo (Fisher *et al.*, 2002; Collinsova *et al.*, 2006; Pajares & Perez-Sala, 2006; Zeisel, 2006).

As indicated before, homocysteine is currently regarded as a risk factor for NTD (Harmon, 1996; Daly *et al.*, 2005; Gellekink *et al.*, 2005). Folate supplementation decreases homocysteine levels by boosting the remethylation of homocysteine to methionine (Ubbink *et al.*, 1994, 1995, 1999). During transsulfuration, homocysteine is irreversibly converted to cystathionine by the vitamin B_6-dependent enzyme cystathionine β-synthase (CBS). Recent studies indicated the presence of a 31 base pair (bp) variable number tandem repeat (VNTR) in the CBS gene could be associated with decreased CBS activity, accompanied by an increase in total homocysteine (tHcy) levels following methionine loading in humans cardiovascular patients (Kraus *et al.*, 1998; Lievers *et al.*, 2001; Afman *et al.*, 2003). Five different alleles with 16, 17, 18, 19, and 21 times the 31 bp repeat, constituting 10 different genotypes were observed. The 18/18 VNTR genotype appeared to be the most common allele and subjects with this CBS mutation displayed significantly higher tHcy levels than the levels observed in subjects displaying either the 17/18 or the 18/19 VNTR genotypes. Vitamin B_6 levels did not appear to influence this association. A further increase in homocysteine levels of the 18-18 allele carriers, relative to the 17-18 subjects, homozygous for the MTHFR 677C→T polymorphism was observed. The results clearly indicated that the number of 31 bp repeat elements in the CBS gene influences the levels of tHcy.

Recent research reports indicated that some other candidate enzymes may also be implicated in the aetiology of NTD, i.e. glutamate formiminotransferase-cyclodeaminase

(GFTC), dihydrofolate reductase (DHFR), ribonucleotide reductase (RR), HMG-CoA-reductase (HMGCOAR), serine hydroxymethyltransferase (SHMT) and the glycine cleavage complex (GCC) (Zittoun J, 1995; Chen, 2003; Richter *et al.,* 2002; Matsuda, *et al.* , 1994, Copp *et al.* , 2003). Brink (2005) observed that the teratogenic antiepileptic, sodium valproate (VPA), modulated the specific activity of the glycine cleavage system (GCS), the mitochondrial serine hydroxymethyltransferase (mSHMT), as well as the cytosolic serine hydroxymethyltransferase (cSHMT), in the livers of pregnant Hanover.NMRI female mice and whole foetuses. The same enzymes were also significantly modulated in the livers and whole embryos of pregnant Hanover.NMRI mice (*per os* dosage) and chicken embryos (*in ovo* dosage), following exposure to the toxic non-protein amino acid, hydroxynorvaline (Potgieter, 1999; Louw, 2004; Louw *et al.*, 2005).

1.3.3 POLYAMINE METABOLISM AND NEURAL TUBE DEFECTS

1.3.3.1 Introduction

Polyamines are aliphatic amine compounds with a multitude of functions (Nilsen *et al.*, 1997; Bryson & Greenall, 2000; Oliver *et al.*, 2000; Usherwood, 2000; Thomas & Thomas, 2001; Seiler & Raul, 2005; Takao *et al.*, 2006). These compounds are present in and essential for the well being of cells of all known living systems (Feistner, 1994; Ruiz-Herrera, 1994; Pandy *et al.*, 2000; Walters, 2003). Putrescine, spermidine and spermine are the most abundant polyamines (Fig. 1.4).

Cellular polyamine levels are regulated by cellular uptake mechanisms, salvaging polyamines from the diet, *de novo* synthesis from amino acid precursors, production by symbiotic intestinal micro-organisms, as well as by catabolic degradation and efflux (Fig. 1.5) (Seiler *et al.*, 1996; Morgan, 1999; Coffino, 2001; Seiler, 2004).

Studies employing inhibitors of polyamine biosynthesis demonstrated that alterations in cellular polyamine levels can modulate the growth of normal and cancer cells (Bachrach, 2004; Moinard *et al.*, 2005; Seiler & Raul, 2005). Transgenic mice, overexpressing polyamine biosynthetic enzymes, have been employed to study the role of polyamines in the process of carcinogenesis (Stabellini *et al.*, 2003; Bachrach, 2004). Numerous signal transduction pathways intersect with polyamine biosynthetic pathways and the regulation of intracellular polyamine levels (Morgan, 1999; Urdiales *et al.*, 2001; Moinard *et al.*, 2005).

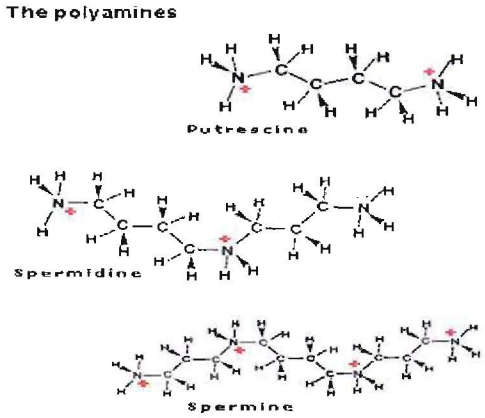

Figure 1.4: *Structures of putrescine spermidine and spermine.*

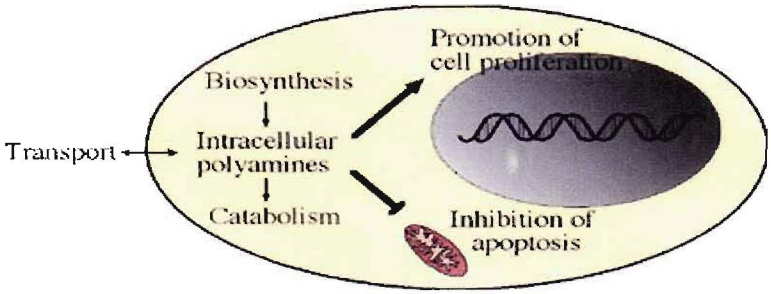

Figure 1.5: *Functions of the polyamines (Department of Physiology, Lund University, 2001; URL: www.biol.lu.se/zoofysiol/Cellprolif/).*

Direct binding of polyamines to DNA and their ability to modulate DNA-protein interactions appear to be important in the molecular mechanisms of polyamine action in cell proliferation (Heby, 1995; Thomas & Thomas, 2001). Polyamines are essential facilitators of cell growth and several studies have demonstrated the ability of these compounds to protect cells from apoptotic cell death. At the same time it must also be recognized that polyamines also play an important role in facilitating cell death (Thomas & Thomas, 2001; Seiler & Raul, 2005; Takao *et al.*, 2006). The precise mechanisms involved in these diverse cellular responses are not yet clear. Cell death responses may in part be attributed to the production of hydrogen peroxide during the catabolism of the polyamines (Parchment, 1993). Additionally, the ability of polyamines to alter DNA-protein and protein-protein interactions may be disruptive for cellular functions. This may be possible when abnormally high levels of polyamines have accumulated, because of defects in catabolic or efflux pathways (Thomas & Thomas, 2001; Seiler, 2004). The polyamine biosynthetic pathway may be exploited as a potential molecular target for

therapeutic intervention in several types of cancers. Inhibitors of polyamine biosynthesis, i.e. polyamine analogues and oligonucleotide:polyamine analogue combinations, make promising drug candidates for chemoprevention and/or the treatment of cancer (Thomas & Thomas, 2001; Huang *et al.*, 2005).

1.3.3.2 Polyamines and the cell cycle

Cell proliferation involves two major processes: cell growth and division. Cellular growth leads to a doubling of all the structural elements and functional capacities of the cell. As soon as the cell has grown to its full size it will go into mitosis. Duplication of cellular DNA is a key event in cell growth and an absolute genetic prerequisite for successful cell division. During the cell-division cycle, chromosomal DNA is precisely duplicated, followed by segregation and the formation of two daughter cells. Accuracy of these two processes is maintained by two tightly interlinked cycles, i.e. the replication licensing cycle and the cohesion cycle. The replication licensing cycle ensures the precise replication of the cellular DNA, while the cohesion cycle ensures the correct segregation of the original and replicated copies into the mother and daughter cells respectively (Heby, 1981; Blow & Tanaka, 2005).

The cell cycle can be divided into four successive phases, namely the G1, S, G2, and M phases. The G1 phase (Gap 1) constitutes the period which transpires between the initiation of mitosis (M) and DNA synthesis (S). The G2 phase (Gap 2) refers to the period between DNA synthesis (S) and mitosis (M). The intermitotic phase (G1, S, and G2) is characterized by continuous cell growth (Heby, 1981; Blow & Tanaka, 2005). Figure 1.6 below depicts the different phases of the cell cycle.

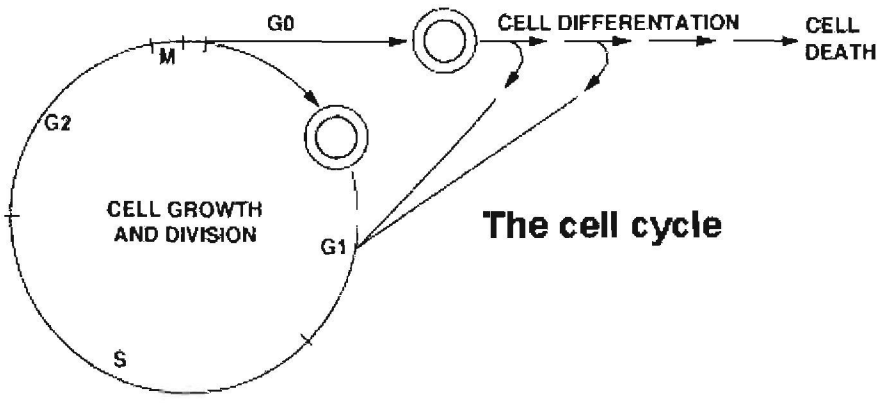

Figure 1.6: *The cell cycle (Department of Physiology, Lund University, 2001;URL:www.biol.lu.se/zoofysiol/Cellprolif/).*

The G1 phase of the cell cycle contains a succession of events, some of which are essential to the initiation of DNA replication. The synthesis of polyamines is strongly activated during the G1 period and has been implicated to actively take part in the cell's preparation for DNA synthesis. A drastic increase in the activity of the enzyme, ornithine decarboxylase (ODC), appears to be one of the very first changes taking place when a cell in the G0, or quiescent phase, enters into the G1 state after being stimulated to proliferate. ODC activity first peaks in early G1 phase and again in late G1 phase to early S phase. A similar pattern is observed for S-adenosylmethionine decarboxylase (SAMDC) activity in some experimental systems (Heby *et al.*, 1980; Kai *et al.*, 2003).

Recent observations imply that early G1 phase polyamine synthesis stimulation may be unrelated to cell proliferation. Some biochemical stimuli, not responsible for cell proliferation, can also induce a rapid increase in ODC activity, corresponding in time to an early G1 phase stimulation. It has also been suggested that the early G1 increase in ODC activity, particularly in conditions associated with a general increase in protein synthesis, may be a consequence of the high rate of turn over of ODC. ODC is one of a number of mammalian proteins, possessing the PEST sequences, i.e., amino acid sequences in the primary structure of enzymes and other proteins enriched in proline, glutamic acid, serine, and threonine, which are found in most rapidly turning over enzymes. PEST sequences range from 12 to 60 amino acid residues in length and are frequently flanked by positively charged amino acid side chains (i.e. lysyl, arginyl residues). All PEST proteins are important regulatory molecules (i.e. oncogene products, key enzymes, signal pathways) and their high level of turnover may be indicative of a metabolic requirement for rapid changes in their intracellular concentrations. Numerous permutations and combinations of transcriptional control, translational control and rapid protein (enzyme) degradation play a role in these processes. (Goyns, 1982; Rechsteiner, 1988; Oredsson, 2003; Rechsteiner & Rogers, 1996; Sehkar & Freeman, 1998).

The intracellular half-lives of PEST containing regulatory proteins are typically less than two hours. ODC displays an extremely short half-life (i.e. 39 min in rat liver), implying that it is not only degraded quickly but also synthesized more rapidly than other enzymes. Large variations in ODC concentration can therefore occur in relatively short periods of time. The activity of ODC is negatively regulated by intracellular polyamines. The polyamines mediate a feedback inhibition on the very first enzyme in their synthetic

pathway, a phenomenon which is, in part, due to translational regulation (Van Daalen Wetters *et al.*, 1989; Kopitz *et al.*, 1990).

Late G1 stimulation of polyamine synthesis is involved in cell's preparation for DNA synthesis. In comparison to other cellular processes DNA replication tends to be the more sensitive one for polyamine limitations. DNA synthesis and cell division are more adversely affected by polyamine limitations in the sense that rate of polyamine synthesis reaches peak levels just prior to these cell cycle events. Some cell types are blocked in G1 phase whereas others are blocked in G2 phase, the restriction points in these phases of cell cycle. Sufficiently high intracellular levels of the polyamines will favour continued progression of the cell cycle (Heby, 1981; Scorcioni *et al.*, 2001).

1.3.3.3 Polyamines and programmed cell death: apoptosis

Programmed cell death (apoptosis), and cell growth are narrowly interlinked. Pathways regulating these processes overlap to some extent: a signal inducing cell proliferation in one cell type or during a specific physiological state can lead to apoptotic death in a different cell type or physiological state. In addition to modulating cell growth, polyamines are also involved in the regulation of programmed cell death. These polycationic compounds can either promote or modulate apoptosis, or they can protect cells against apoptosis. Under certain conditions polyamines may even be toxic to cells. Abnormally high polyamine concentrations are detrimental to cell growth and may even invoke programmed cell death. The oxidation of spermidine and spermine by serum amine oxidase or by the intracellular FAD-dependent polyamine oxidase produces hydrogen peroxide (H_2O_2) and aminoaldehydes that are strong inducers of apoptosis. Polyamines can also induce apoptosis in the absence of oxidation, however, it is unclear which mechanism plays the major role in cells (Parchment, 1991, 1993; Kai *et al.*, 2003; Oredsson, 2003; Moinard *et al.*, 2005; Loikkanen, *et al.*, 2005; Janne *et al.*, 2006).

Induction of cellular apoptosis by a variety of apoptopic stimuli (i.e. reactive oxygen species, oncogene products) invariably leads to a modulation of cellular polyamine levels. Characteristically, a marked increase in the activity levels of ODC can be observed in a variety of cells, prior to the onset of apoptosis. Accumulation of intracellular putrescine is frequently associated with the initiation of apoptosis. However, a decrease in spermidine and/or spermine levels appears to be a more common feature in apoptosis (Erez *et al.*, 2002; Takao *et al.*, 2006). An important step in polyamine-

induced apoptosis is the oxidation of spermidine and spermine, either by the copper containing enzyme, serum amine oxidase (SAO), or by the intracellular FAD-dependent polyamine oxidase (PAO). Hydrogen peroxide (H_2O_2) is produced in these reactions and the resultant aminoaldehydes appear to be strong inducers of apoptosis. The co-addition of catalase and polyamine analogues strongly inhibited apoptosis. The addition of specific inhibitors of the FAD-dependent polyamine oxidase (i.e. MDL72527) also leads to an inhibition of apoptosis (Kahl *et al.*, 2004; Agnostinelli *et al.*, 2006).

Results from these studies and alternative investigations executed on a spermidine:spermine acetyl transferase (SSAT) overproducing transgenic mice indicated that in some cell types, polyamines and/or their analogues do not necessarily need to be oxidized in order to initiate the induction of apoptosis. Excessive accumulation or depletion of polyamines may disrupt many cellular functions, including DNA-protein interactions, protein-protein interactions and mitochondrial integrity, leading to apoptosis. Cell types that undergo apoptosis in the presence of ODC over-expression, appear to be highly sensitive to apoptotic stimuli. ODC over-expression is also frequently observed in a variety of cells, following their transformation to a malignant phenotype (Heby *et al.*, 1980; Thomas and Thomas, 2001; Kai *et al.*, 2003; Filomeni & Ciriolo, 2006; Meyer *et al.*, 2006).

Recent studies also reveal an alternative pathway where the accumulation of putrescine also leads to the induction of apoptosis. Hypusine, a compound formed when the butylamine moiety from spermidine is transferred to a lysine residue in the eukaryotic initiation factor, eIF-5A, appears to play an important part in the normal functions attributed to spermidine. In the presence of excess levels of putrescine, hypusine formation is inhibited with a concomitant decrease in the concentration of modified eIF-5A. The lack of hypusine, along with other functional abnormalities caused by accumulation of putrescine, led to apoptosis in a hepatoma cell line selected for α-difluoromethylornithine (DFMO) resistance. The generality of this phenomenon in other cell types remains to be determined. In spite of these examples of ODC- and polyamine-induced apoptosis, the major role of polyamines in most cell types is to stimulate cell proliferation (Park *et al.*, 1997; Thomas and Thomas, 2001).

It is highly likely that extremely low levels of polyamines may promote apoptosis. In the first instance, a depletion of polyamine levels can lead to either cell cycle arrest or apoptosis by affecting the p53/p21/p27 cell cycle regulatory pathway. Secondly,

polyamines are important in the regulation of ion transport and the stabilization of important cellular components, such as cell membranes and chromatin structures. Depletion of polyamine levels may therefore induce destabilization of important cell structures, with a resultant loss in cell integrity and finally the induction of programmed cell death, or apoptosis. On the other hand, the depletion of polyamines, even after cell-cycle arrest, may not be sufficient to induce apoptosis alone, but may sensitize cells to apoptosis induced by other factors. The altered susceptibility of the cells, following polyamine depletion appears to be inducer-specific, implicating that polyamines may be involved in the regulation of some of the apoptotic pathways, but not necessarily all of them. The protective effect may be specific to the pathway inducing apoptosis and mediated by activating the transcription of genes, required for cell proliferation and survival (Park *et al.*, 1997; Kramer *et al.*, 1998; Ray *et al.*, 1999; Thomas and Thomas, 2001; Thomas and Thomas, 2001).

α-Difluoromethylornithine (DFMO) is an ornithine analogue and irreversible inhibitor of ODC and inhibits apoptosis in nearly all cell systems investigated. DMFO-like inhibitors (DFMO, α-methylornithine, etc.) up-regulate the expression of S-adenosylmethionine decarboxylase (SAMDC), another important key enzyme in the polyamine biosynthetic pathway. Although putrescine and spermidine levels are decreased by DFMO, spermine levels are often increased. Inhibitors of SAMDC (i.e. 5'-deoxy-5'-[N-methyl-N-[2-(amino-oxy)ethyl])amino-adenosine, 5'-deoxy-5'-[N-methyl-N-[3-hydrazinopropylamino)]amino-adenosine), as well as spermidine and spermine analogues (i.e. S-adenosyl-1,8-diamino-3-thio-octane, S-methyl-5'-methylthioadenosine) generally tend to induce or increase apoptosis. Modulation of ODC activity mainly affects putrescine levels, whereas SAMDC provides decarboxylated S-adenosylmethionine for spermidine and spermine synthesis. This may suggest that putrescine and the higher polyamines may have different roles to play in the processes involved in apoptotic cell death (Madhubala *et al.*, 1988; Holm *et al.*, 1989; Park *et al.*, 1997; Thomas and Thomas, 2001).

Although the mechanistic role(s) of polyamines in apoptosis are still unclear, it is obvious that excessive levels as well as extremely low levels of polyamines may affect their specific cellular interactions and influence their important physiological functions. Many researchers agree that the real physiological significance of polyamine synthesis may be focused on the control of critical steps in the cell cycle and in cellular survival. In conclusion, the early induction of ODC, which is often observed, immediately prior to or

during apoptosis, may be related to the progression of the cell into a new phase in the cell cycle. A specific cell cycle checkpoint is reached from where apoptosis is triggered, upon receipt of death-inducing signals. Under conditions where apoptosis is inhibited and/or where cells possess specific genetic lesions, constitutive ODC expression can lead to cell transformation and deregulated cell growth. After reaching the cell cycle checkpoint polyamine levels may actually decrease during the apoptotic process (Cooper, 2006).

1.3.3.4 Metabolism of polyamines in mammals

The importance of the polyamines in cell function is reflected in a strict regulatory control of their metabolism and resultant intracellular levels. A careful balance must be struck between biosynthesis, degradation, and uptake to guarantee optimal cellular polyamine levels at all times. Some of the regulatory mechanisms involved in maintaining a balance in the intracellular and systemic polyamine pools are truly unique. Most living organisms are capable of synthesizing polyamines from precursor amino acids like arginine, ornithine and methionine. Putrescine (Put), the precursor of spermidine (Spd) and spermine (Spm), is produced, following the direct decarboxylation of L-ornithine (Orn). This reaction is catalyzed by ornithine decarboxylase (ODC) and is the rate limiting step in biosynthesis of polyamines in non-proliferating cells. However, in rapidly dividing cells (i.e. embryonic cells, gut mucosa cells, tumour cells) ODC expression and hence intracellular ODC activity are significantly increased and ODC activity is therefore not considered to be the rate limiting parameter. ODC requires pyridoxal-5'- phosphate (vitamin B_6) for its catalytic activity and is characterized by an extremely short half-life (~20 minutes) (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

Spd and Spm are synthesized from Put by the action of spermidine synthase (SpdS) and spermine synthase (SpmS) respectively. The biosynthesis of the two higher polyamines is accomplished through the addition of aminopropyl moieties to Put or Spd respectively. The aminopropyl groups are derived from L-methionine which is converted to S-adenosyl-L-methionine (SAM) by L-methionine-S-adenosyltransferase (MAT). Decarboxylation of SAM is catalyzed by S-adenosylmethionine decarboxylase (SAMDC) to form methylthioadenosine, while the aminopropyl moiety is transferred to Put by the enzyme spermidine synthase to form Spd. Condensation of Spd with another SAM-derived aminopropyl group (catalysed by SpmS) lead to the formation of Spm. The intracellular concentration of decarboxylated SAM is usually very low in mammalian cells

and the enzyme SAMDC is activated by putrescine (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

Ornithine, the main precursor of the polyamines is primarily produced from glutamate in mitochondria, following the acetylation of the amino group and the phosphorylation and reduction of the acetylated derivative to N-acetylglutamic-γ-semialdehyde. Subsequent transamination produces α-N-acetylornithine, which is deacetylated to L-ornithine and regenerated N-acetylglutamate. In liver mitochondria, L-ornithine can enter the urea cycle and react with the carbamoyl phosphate to form L-citrulline. The production of carbamoyl phosphate in mammalian cells can be catalysed by either one of two allosterically regulated carbamoyl phosphate synthetases (CPS: Forms I and II). CPS I is localized in the mitochondria, demonstrates a preference for ammonia as substrate and is employed in the biosynthesis of L-citruline, a precursor for the production of L-arginine. L-Arginine is eventually converted to L-ornithine, a lower homologue of L-lysine and polyamine precursor, while urea is produced. In the cytosol L-citrulline is first converted to L-arginosuccinate by the enzyme L-argininosuccinate synthetase and then to L-arginine. L-arginine is subsequently degraded by arginase to form urea and L-ornithine. Cytosolic L-ornithine can be either decarboxylated to putrescine by ODC, or it can be converted to proline, *via* glutamic-γ-semialdehyde. Unmetabolised cytosolic L-ornithine may also return to the mitochondrial compartment (Teti *et al.*, 2002; Seiler 2004).

Polyamine biosynthesis (Fig. 1.7) is mainly accomplished by four enzymes, two of which are highly regulated, i.e. ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC). The two remaining enzymes, spermidine synthase (SpdS) and spermine synthase (SpmS) are constitutively expressed. The biological half-lifes of the two regulatory enzymes, ODC and SAMDC (5-60 min), are among the shortest known for mammalian enzymes. The unique properties of these enzymes enable cells to rapidly modulate cellular levels of the polyamines. Polyamines can also be synthesized by the retro-conversion of spermidine to putrescine and of spermine to spermidine, following the initial N'-acetylation of spermidine and spermine to N'-acetylspermidine and N'-acetylspermine respectively. N'-Acetylspermine can be converted to N^1,N^{12}-diacetylspermine. The acetylation reactions are catalysed by cytosolic spermidine/spermine N'-acetyltransferase, with acetyl CoA as co-substrate. Polyamine oxidase (PAO), on the other hand, releases aminopropyl moieties from N'-acetylspermidine, N'-acetylspermine to yield putrescine, spermidine and N'-

acetylspermidine respectively. Nuclear acetyltransferase converts putrescine to N-acetyl putrescine and spermidine to N^8-acetyl spermidine, which may subsequently be retro-converted to putrescine and spermidine, respectively, following hydrolysis by acetylhydrolase (Teti *et al.*, 2002; Seiler, 2004).

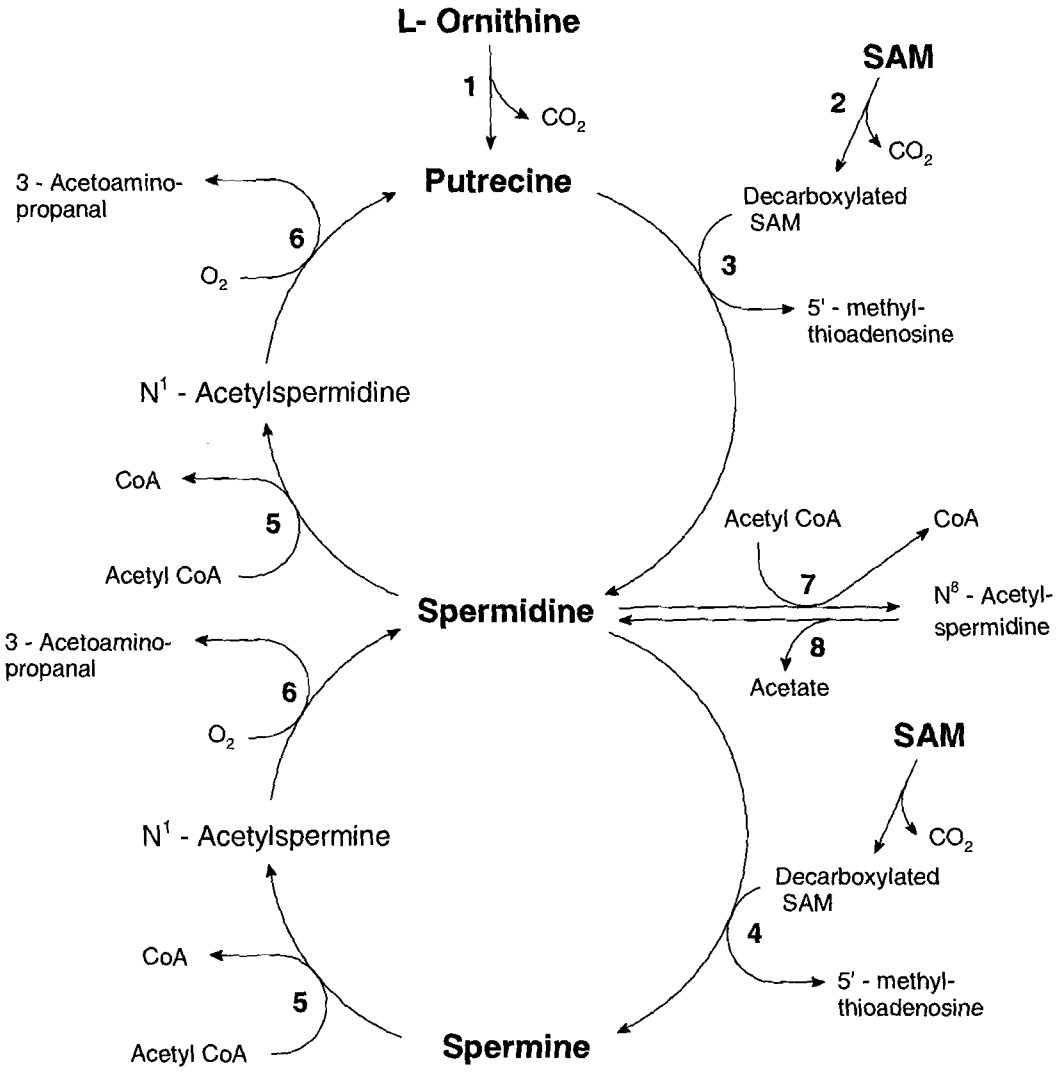

Figure 1.7: *Biosynthesis of polyamines. (1) Ornithine decarboxylase (ODC), (2) S-adenosyl-L-methionine decarboxylase (SAM-DC), (3) Spermine synthase, (4) Spermidine synthase, (5) Spermine/spermidine N^1-acetyltransferase, (6) Polyamine oxidase (PAO), (7) Spermidine N^8-acetyltransferase, (8) N^8-Acetylspermidine acetylhydrolase. SAM: S-Adenosyl-methionine. (Modified from Seiler, 1987).*

Terminal degradation, consisting of an oxidative deamination of primary amino groups of polyamines and their N-acetyl derivatives may also be catalyzed by copper-containing amine oxidases, such as diamine oxidase (DAO) and spermine oxidase (SpmO). The resulting aldehyde derivatives are subsequently oxidized by aldehyde dehydrogenase to the corresponding organic acids. Putrescine is converted to γ-aminobutyric acid (GABA), spermidine to isoputreanine, putreanine and spermic acid-2, while N'-acetylspermidine is metabolised to N'-acetylisoputreanine. Spermine was broken down to spermic acid-1 and spermic acid-2, while N'-acetylspermine was converted to N'-acetylspermic acid. The oxidative deamination of GABA produces succinic acid which is shunted into the citric acid cycle, finally producing carbon dioxide and water. Polyamine degradation in mammals also involves the production of 1,3-diaminopropane (DAP), N'-acetyl-1,3-DAP, β-alanine, 2-hydroxyputrescine and the alcohol derivatives of the n-α-amino acids (Teti et al., 2002; Seiler, 2004).

1.3.3.5 Polyamines in embryonic development

The metabolism of polyamines during the embryonic development of polychaetes, sea urchins, amphibians, chicks, mice and rats has been investigated by numerous researchers (Emmanuelsson & Heby, 1983; Joseph & Baby, 1991; Taibi *et al.*, 1995; Robinson & Mayne, 2000). Inhibitors of polyamine synthesis (i.e. α-difluoromethylornithine, α-methylornithine) have made it possible to analyze the importance of polyamines in embryonic development more precisely. In the chicken embryo, polyamine biosynthesis is activated in the late cleavage phase and reaches maximum levels during gastrulation. α-Difluoromethylornithine (DFMO) imposed inhibition during the early up-regulation of ODC activity largely prevents a rise in the concentrations of all three of the polyamines (Put, Spd, Spm). The consequences of polyamine limitation during chick embryo morphogenesis appears to be severe – embryonic development does not progress past the primitive streak stage (gastrulation) and neurulation, as well as somite formation, are suppressed (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

The effects of polyamine synthesis inhibitors on the pre-implantation development of the mouse have serious consequences in embryonic development. Treatment of mouse embryos cultured *in vitro* from the 2-cell stage, with α-methylornithine (AMO) does not affect cleavage prior to the formation of the blastocyst. Methylglyoxal bis(guanylhydrazone) (MGBG) treatment, on the other hand, can induce an irreversible

metabolic quiescence (diminished DNA synthesis and cell proliferation) in mouse embryos during the 8-16-cell stage. At a suitable dose MGBG causes the complete inhibition of cavitation during blastocoel formation. A good correlation can be observed between MGBG dose, the blastocyst number and the mean cell number of blastocysts. MGBG treatment also slows down the mitotic rate very early during cleavage and the cells appear to be arrested in the interphase of the cell cycle. Although the rates of DNA synthesis and cell proliferation appear to decrease at an early stage following MGBG exposure, the rate of RNA synthesis decreases only after an extended treatment period (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Wallace & Frazer, 2004; Seiler & Raul, 2005).

During the post implantation development of the mouse embryo DMFO-induced suppression of ODC up-regulation in the implant (decidual tissue plus embryo) can lead to an arrest of embryonic development (Teti *et al.*, 2002; Seiler, 2004). Cleavage of the fertilized ovum and the formation of the blastocyst appears to be unaffected by a limitation in polyamine biosynthesis. Decidual tissue formation proceeds normally after implantation, but embryonic development fails to progress beyond the primitive streak stage. Inhibition of mouse embryo development is associated with a suppression of embryo growth and mass gain, because of a fall in the synthesis of DNA, RNA, and protein. Developmentally arrested embryos and surrounding decidual tissue is eventually resorbed or lost from the uterus and no sign of pregnancy persists. Sustainable increases in the biosynthesis of putrescine and /or spermidine and spermine therefore appears to be essential during early embryonic development, in order to ensure continued embryonic development in all animals. Polyamines play a fundamental role during gastrulation, the pivotal developmental process delineating the inception of embryonic autonomy, but are equally essential role players in other developmental processes (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

1.3.3.6 Polyamines and ornithine decarboxylase

As pointed out earlier in this section (Section 1.3.3.4, Fig. 1.7), ornithine decarboxylase (ODC) catalyzes the decarboxylation of L-ornithine (Orn) to putrescine (Put). Putrescine is the basic precursor of the higher polyamines, spermidine (Spd) and spermine (Spm). ODC requires pyridoxal-5'- phosphate (vitamin B_6) for its catalytic activity and as a PEST sequence protein, has an extremely short half-life (~20 minutes). In normal cells the ODC catalyzed decarboxylation of Orn is the rate limiting step in the biosynthesis of the

polyamines. In rapidly dividing cells, such as embryonic cells, gut mucosa cells and tumour cells, the expression of ODC and its subsequent intracellular activity are equally rapidly and significantly elevated. However, in the latter case scenario the catalytic activity of ODC does not appear to be the rate limiting step in polyamine biosynthesis. ODC is effectively regulated on the transcriptional level (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

1.3.3.6.1 Molecular structure of ornithine decarboxylase (ODC)

Native mammalian ODC is a homodimer with 2-fold symmetry and the monomers have two clearly defined structural domains. Residues 46-283 give rise to an α/β-barrel domain, while the remaining carboxyl terminal residues pack into a β-sheet domain, consisting of two separate sheet regions. The two active sites in the homodimer are formed at the interface between the α/β-barrel domain of one monomer and the β-sheet domain of the second subunit. The single polypeptide monomer of human ODC consists of 461 amino acids, has a molecular mass of approximately 51 kDa and displays a relatively high level of homology with that of the mouse. In comparison, the hamster ODC polypeptide is constructed from 455 amino acids. However, the homology between the three species (man, mouse, hamster) is still very high. Several amino acids in the primary structure of the ODC subunit are essential, or of great importance for the maintenance of the catalytic activity of the enzyme. Proper alignment of the two subunits in the formation of the catalytically active dimeric ODC requires the interaction of the side chains of lysine 69, lysine 169 and histidine 197 at the interface of the two subunits and part of the other subunit containing cysteine 360. At the same time lysine 69 is also crucial for the binding of the co-enzyme, 5′-pyridoxal phosphate (vitamin B_6). PLP is bound through a Schiff base, formed between lysine 69 and the aldehyde function within the PLP. This structural requirement is essential for the proper positioning of the substrate (L-ornithine) during decarboxylation (Coleman *et al.*, 1994; Pegg *et al.*, 1994; Kern *et al.*, 1999; Almrud *et al.*, 1999).

All known ODC enzymes require pyridoxal 5′-phosphate (PLP) as a cofactor. The mammalian enzyme binds the cofactor relatively weakly and it can be removed by a rather simple procedure (i.e. dialysis). The Michaelis-Menten constant (K_m value) for the co-substrate, PLP is 0.2 - 0.3 µM. The catalytic mechanism of ODC is typical to all PLP-dependent decarboxylases. In the absence of substrate the holoenzyme contains an internal aldimine functional group where the PLP is bound to lysine 69, in the active of

the enzyme by a Schiff-base linkage. Ornithine first binds to the cofactor, following a transaldimination reaction to form an external aldimine. Intra-molecular rearrangement and subsequent decarboxylation are followed by the release of CO_2 and the formation of a quinonoid intermediate. The quinonoid moiety is subsequently protonated to the external aldimine, consisting of the putrescine residue and PLP, bound in a Schiff-base structure (Seely et al., 1992). When the natural amine in the Schiff-base is replaced by an amine analogue bearing an α-substituent in the R-configuration (i.e. vinyl, acetylene, halomethyl) the enzyme is inhibited by a suicide substrate-like mechanism. Acetylenic amines are very effective suicide substrate inhibitors of decarboxylases (Barret et al., 1985).

1.3.3.6.2 Degradation and inhibition of ODC

Polyamines are involved in the regulation of ODC levels by means of a negative feedback mechanism. Antizyme, a polyamine inducible protein, plays a key role in the feedback regulation of ODC (Fig. 1.8).

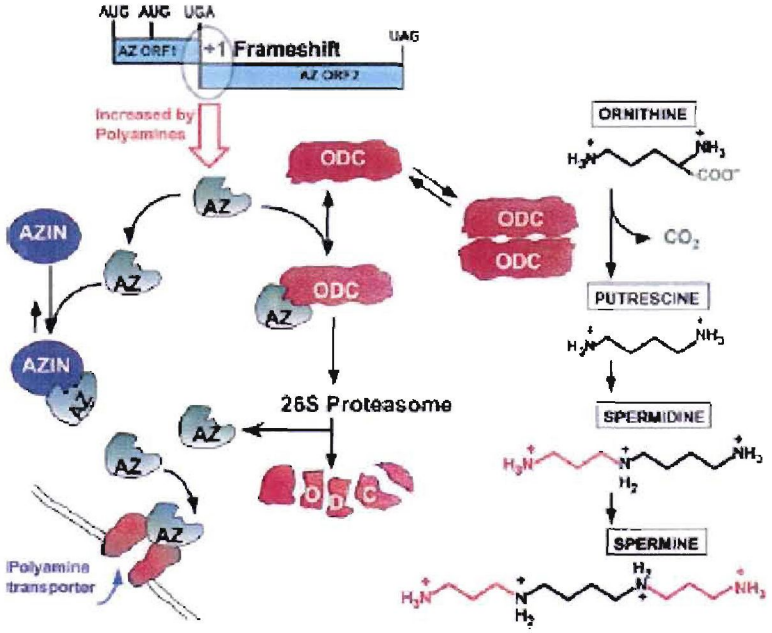

Figure 1.8: *Regulation of ODC: ODC catalyzes production of putrescine (Put); Put is converted to higher polyamines. Following an a+1 frameshift in the transition of the mRNA, fusing ORF 1 and ORF 2, a process which is stimulated by polyamines, antizyme 1 is synthesized. Antizyme (AZ) binds to ODC and initiates the degradation of the 26S proteasome or to antizyme inhibitor (AZIN) (Pegg, 2006).*

Antizyme stoichiometrically binds to ODC to form an inactive complex, the 26S proteasome. The proteasome promotes an ATP-dependent, ubiquitin-independent degradation of ODC, functioning by means of a recycling procedure (Fig. 1.8). The up-regulation of ODC expression can be induced by a variety of stimuli, such as hormones, growth factors and tumour promoters. Over-expression of ODC is usually strongly associated with an oncogenic transformation of cells.

Irreversible inhibition of ODC by DFMO leads to the formation of covalent adducts with the cysteine 360 (Cys-360) and lysine 69 (Lys-69) residues, indicating that these amino acid residues are located at, or very close to, the active site. Irreversible suicide inhibition of ODC by DFMO is depicted in Fig. 1.9. During catalysis the Cys-360 residue can function as a proton donor. Other potential proton donor groups occur in the Lys-69 and more unlikely the histidine 197 (His-197) side chains. Conservation of the intact structure of the Cys-360 side chain is absolutely essential for the specificity of the catalytic reaction. Stuctural modification of the catalytic site can result in the diminished catalytic activity of ODC, while the major decarboxylation-dependent transamination reaction culminates in the formation of pyridoxamine 5'-phosphate (PMP) and γ-aminobutyraldehyde, rather than PLP and putrescine. The latter result is possible if the C4'-carbon in PLP is protonated, instead of the Cα-carbon in Orn. Glycine 387 (Gly-387) is essential for the formation of dimeric structure of ODC, however, the reason for this structural requirement is not yet clear.

The catalytic activity of ODC is significantly decreased when its acidic side chains, i.e. aspartate 88 (Asp-88), glutamate 94 (Glu-94), aspartate 233 and most significantly the glutamate 274 (Glu-274) are modified. The Glu-274 residue interacts with N1-nitrogen of the PLP co-enzyme, creating an acidic cluster in conjunction with Asp-88, Asp-233 and three hydrogen bonded water molecules. A network of hydrogen bonds is formed which most probably influences the electron-withdrawing properties of the cofactor residue. Two other residues that also exert an effect on the catalytic activity of ODC are the lysine 115 (Lys-115) and lysine 169 (Lys-169) residues. Lys-169 is involved in the formation of a salt-bridge, which stabilises the ODC dimer, however, the role of the Lys-115 residue is not yet clearly comprehended.

Following the ODC catalyzed decarboxylation of Orn, the resulting product, a putrescine molecule, is released from the active site and the PLP molecule re-establishes the formation of a Schiff base with Lys-69.

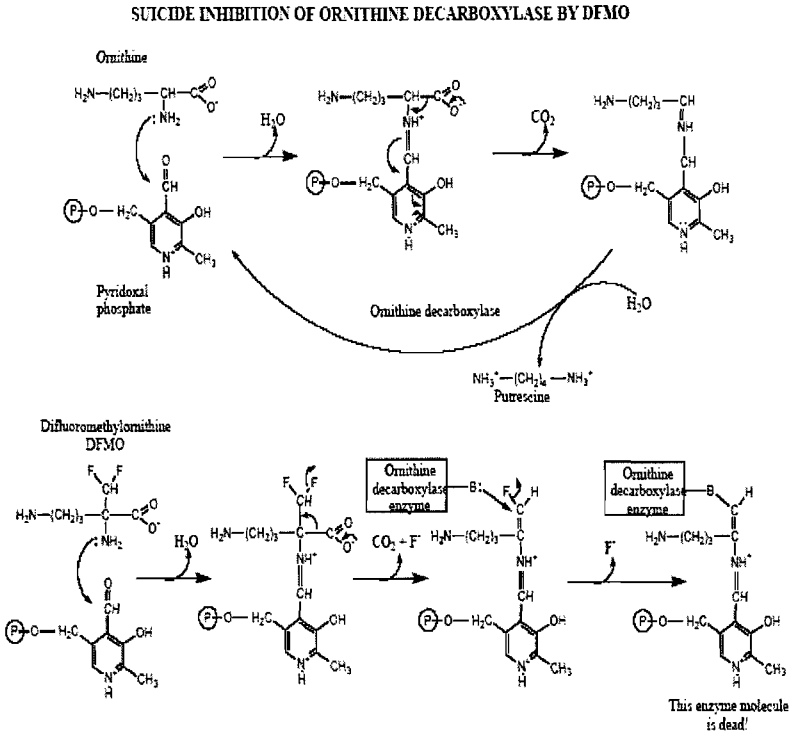

Figure 1.9: *Mechanism of the suicide inhibition of ODC by DFMO (Kern et al., 1999).*

PLP-dependent enzymes effect reaction specificity by positioning specific residues or molecules (i.e. Cys-360 in ODC), interacting with each group surrounding the C_α-carbon of the substrate in a geometry which favours cleavage of one specific bond. If this is not the case any of three bonds around the C_α-carbon may be cleaved, enabling a broad range of non-specific reactions including transamination, racemization, retro-aldo cleavage and deamination in addition to decarboxylation. In decarboxylase enzymes transaldimination leads to the orientation of the α-carboxylate group perpendicular to the pyridine ring of the PLP moiety, leading to cleavage of the bond between the C_α-carbon and the carboxylate group (Kern et al., 1999).

The biosynthesis of ODC can be induced by a variety of stimuli, and its suppression by specific inhibitors (i.e. DFMO) or diminished activity due to a mutation, will lead to an inhibition of cellular growth and transformation. ODC has therefore been identified as a promising potential target for the use of highly specific chemotherapeutic strategies in the battle against cancer and parasitic diseases (Pegg *et al.*, 1994; Kern *et al.*, 1999).

1.3.4 DIAMINOBUTYRIC ACID (DABA)

L-α,γ-diaminobutyric or L-2,4-diaminobutyric acid (DABA) acid (Fig. 1.10) is one of well over six hundred non-protein amino acids, discovered over the last fifty or more years. It is synthesised by a number of plants and microorganisms (*L. latifolius, L. cicera,* etc.) (Fowden & Bryant, 1958; Ressler *et al.*, 1961; Bell, 1962; O`Neal *et al.*, 1968; Sutton & Simmonds, 1974; Rosenthal, 1982; Barret et. al. 1985; Foster *et al,* 1987; Rowe *et al.*, 1993; Rasmussen *et al.*, 1993; Bell *et al.*, 1996; Rozan *et al.*, 2001). Not only is DABA synthesized in plants, but it is also produced by a large variety of micro-organisms. It functions as an important building block in the primary structure of numerous peptide antibiotics, antifungal peptides (i.e. polypeptin, comirin, polymixin, colistin, circulin) and cell wall peptidoglycans (Nigam et al, 1966; Sasaki, 1998; Bisacchi, 1987; Harrison, 1991; Martin, 2003).

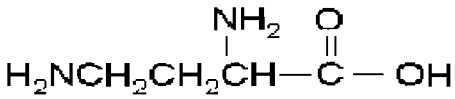

Figure 1.10: *Chemical structure of DABA.*

DABA is currently recognized as a unique anti-tumour agent, but regular employment of this compound as a systemic anti-cancer drug has been hampered by its toxicity (Ronquist *et al.*, 1980, 1984; Panasci *et al.*, 1988; Blind *et al.*, 2003; Bergenheim *et al.*, 2006). *In vitro* studies have demonstrated that, compared to normal glial cells, glioma cells display a higher transport capacity for amino acids, since they utilize the sodium dependent amino acid transport system A. Glioma cells are therefore prone to exhibit uncontrollable uptake of DABA. DABA is vigorously transported into the cells through the sodium dependent amino acid transport system A, however, the net exodus of L-DABA from the cell, through the L-amino acid transport system, appears to be negligible, resulting in extreme turgor and eventually cellular lysis (Antoni *et al.*, 1997).

When lethal doses of DABA have been administered (*per os*) to experimental animals (i.e. male weanling Sherman rats), acute toxic symptoms first become apparent after a delay of 12 - 20 hours. DABA causes a weakness in the hind legs of the animals, within 48 hours. Upper extremity tremors subsequently occur, immediately prior to the onset of convulsions and subsequent death. DABA appears to be selectively concentrated in the liver of the affected animals, effectively impairing the function of the liver. Impairment of liver function leads to the pathological elevation of ammonia levels in the blood and

brain, culminating in convulsive seizures and eventually death. The basic molecular mechanism behind DABA toxicity appears to be the result of the dose-dependent competitive inhibition of ornithine carbamoyltransferase (OCT), a key enzyme in the urea cycle (Rosenthal, 1982). The neurotoxic activity of DABA appears to be a function of the toxin's ability to block the inhibitory action of γ-aminobutyric acid in the brain of affected animals. DABA acts as a highly potent and selective inhibitor of GABA uptake, precipitating the accumulation of GABA in the brain. This mechanism probably forms the basis of the anti-epileptic properties known to be displayed by DABA (Amabeoku *et al.*, 1999). (O`Neal *et al.*, 1968; Levi *et al.*, 1976; Taberner & Roberts, 1978; Fugler-Domenico *et al.* 1990; Sitte *et al.*, 2002).

Non-protein amino acids sometimes display a high level of structural and stereochemical similarities to some of the natural protein constituents and in rare cases may even be incorporated into proteins (Barret *et. al.*, 1985). Incorporation of amino acid analogues (one or more) into structural proteins or enzymes may compromise the secondary, tertiary and even the quaternary structure of the protein. Changes in the physical and chemical properties of the protein will compromise the normal biochemical and physiological functions of the protein, i.e. diminished catalytic activity of an enzyme. Structurally, DABA is a lower homologue and anti-metabolite of the essential amino acid, L-lysine, and the urea cycle intermediate, L-ornithine. It proved to be hepatotoxic to experimental rats and mice, capable of inducing chronic secondary hyperammonemia. Investigators could illustrate that the secondary hyperammonemia was the result of DABA inhibiting carbamoyltransferase, one of the regulating enzymes in the urea cycle (O`Neal *et al.*, 1968; Rao *et al.*, 1969). However, despite DABA`s striking structural and stereochemical resemblance to Orn (natural substrate of ODC), no scientific report refers to its potential to act as an inhibitor of ODC. No empirical investigation of this potential property of DABA seems to have been undertaken yet.

Information on the metabolism of DABA appears to be very limited but the available experimental evidence indicates that it may be synthesized from ß-cyanoalanine (Fig. 1.11). ß-Cyanoalanine is a potent neurolathyrogen when chronically ingested by humans and experimental animals and appears to be the only free, non-protein amino acid occurring in eukaryotic organisms, which contains a cyano functional group. It is produced as a reaction intermediate during the chemical conversion of asparagine to α,γ-diaminobutyric acid (DABA). Dehydration of asparagine leads to the production of ß-

cyanoalanine, which was subsequently reduced to DABA. It has also been proposed that in certain eukaryotic systems DABA can be synthesized as an intermediate during the biosynthesis of the proline analogue, azetidine-2-carboxylic, acid from homoserine (Rosenthal, 1982).

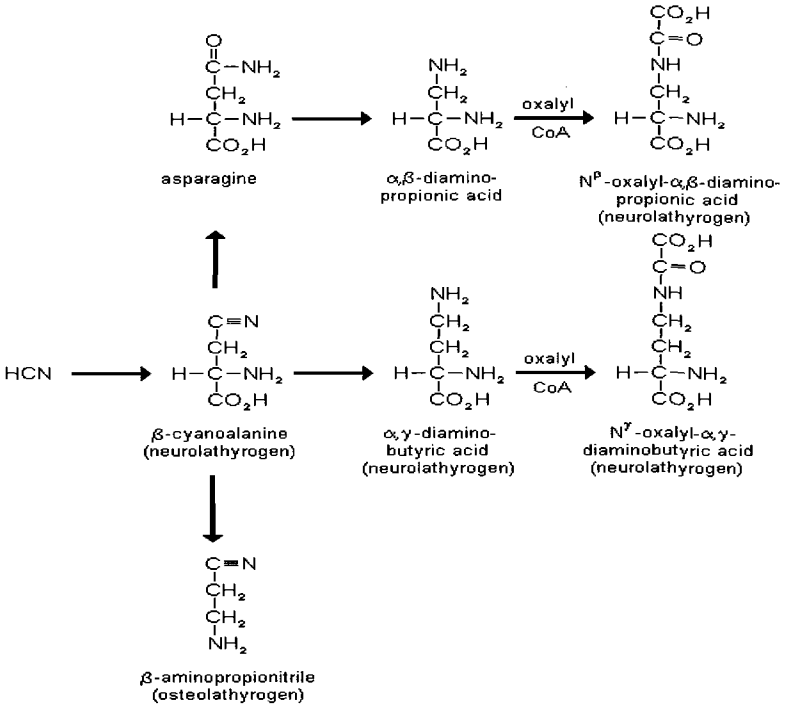

Figure 1.11: *Cyanoalanine is a reaction intermediate in the chemical conversion of asparagine to α,γ-Diaminobutyric acid (Rosenthal, 1982).*

DABA acid can be catabolized by certain *Xanthomonas* species, a process which leads to the formation of aspartic β-semialdehyde, aspartic acid and oxaloacetic acid (Fig 1.12). Aspartic β-semialdehyde is synthesized from DABA, following a pyruvate-dependent γ-transamination. The *Xanthomonas* γ-transaminase exhibits a high degree of substrate specificity. Analogues of DABA acid and pyruvate are not decarboxylated by the enzyme and therefore appear to be inert in the system. The γ-transaminase can be inhibited by hydroxylamine, a carbonyl-binding agent. Aspartic acid is produced after the NAD^+-dependent dehydrogenation of aspartic β-semialdehyde. The dehydrogenase is a thiol-containing enzyme and displays a high level of specificity for aspartic β-semialdehyde as substrate, but NAD^+-analogues, such as 3-acetylpyridine adenine-dinucleotide and deamino-NAD appear to be only weakly bound by the enzyme.

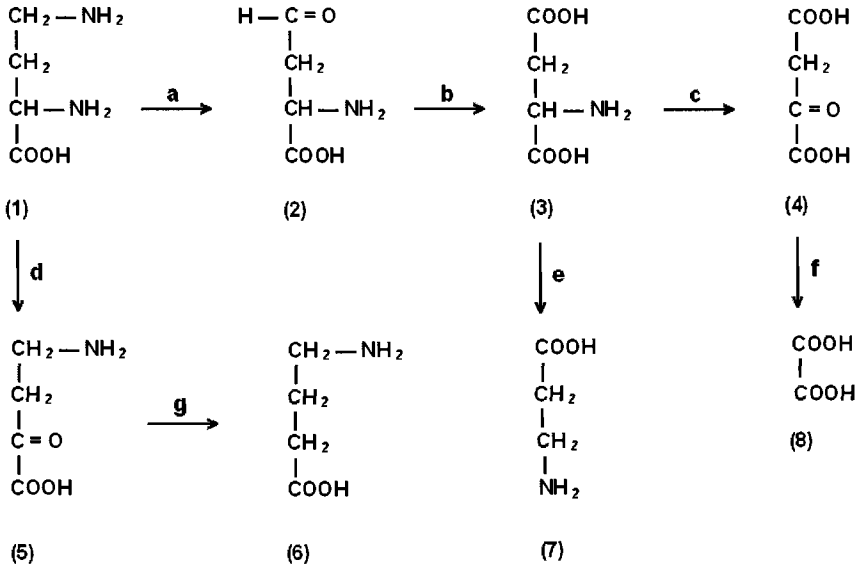

Fig. 1.12: *Proposed metabolism of DABA by Xanthomonas species and Holtman rats:(1) L-2,4-Diaminobutyric acid (DABA); (2) Aspartic β-semialdehyde; (3) Aspartic acid; (4) oxaloactetate; (5) 2-keto-4-aminobutyric acid; (6) γ-Aminobutyric acid (GABA); (7) β-Alanine; (8) Oxalic acid. Enzymes and/or enzyme reactions purportedly involved in the metabolism of DABA: a = pyruvate dependent γ-transaminase; b = NAD^+-dependent dehydrogenation of aspartic β-semialdehyde; c = β-transamination of aspartic acid; d = β-transamination of DABA; e = aspartate 1-decarboxylase (Rosenthal, 1982Mushahwar & Koeppe, 1963).*

When 2-^{14}C-labelled L-DABA was peritoneally injected into male Holtman rats 19 – 44% of the ^{14}C was exhaled as $^{14}CO_2$, while 13 – 23% was excreted in the urine within 24 hours (Mushahwar & Koeppe, 1963). Approximately 10% of the unchanged 2-^{14}C-DABA occurred in the urine. In contrast, only 3 – 9% of the 2-^{14}C-D-isomer was completely oxidised to $^{14}CO_2$, while 28 – 61% was excreted in the urine. More than 30% of the D-isomer appeared intact in the urine. Both the isomers were metabolised in the liver, but some breakdown seemed to occur in the kidneys of the rats. Around 5% of both the L- and D-isomers appeared to have been converted to β-1-^{14}C-alanine, which was excreted via the urine. Radiolabel was also present in varying proportions of tissue glutamate, aspartate and alanine. Evidence indicated that not all the 2-^{14}C-DABA was catabolized *via* β-1-^{14}C-alanine, but that a significant portion was at some time converted into 2-^{14}C-aspartate, or a closely related substance (Fig. 1.12).

1.4 AIMS AND OBJECTIVES OF THIS INVESTIGATION

Food borne xenobiotic compounds (i.e. mycotoxins, non-protein amino acids, organic acids) may be involved in the aetiology of NTD. Complex mixtures of a large variety of natural ingredients are present in the food chain on which the daily diet of man and animals is based. It is a known fact that the neurotoxic, xenobiotic compound, DABA, is contained in a variety of forms (free, bound) in plant and microbial organisms (see Section 1.1), but it is not yet clear if this compound is teratogenic in nature. The main focus of this study is therefore to:

1. investigate the potential of L-2, 4-diaminobutyric acid (DABA) to induce NTD in the Hanover.NMRI mouse embryos, during the window period for neural tube development (days 7-9);

2. investigate the effect of DABA on the catalytic acitivity of ODC in pregnant female mice and their embryos and the concomitant influence of modulated ODC activity on polyamine levels in the urine of pregnant females.

3. investigate the effect of DABA on selected metabolic processes in pregnant female mice in pregnant female mice;

    (a) effect of DABA on the urinary organic acid profiles of pregnant females;

    (b) effect of DABA on the urinary amino acid profiles of pregnant females;

    (c) effect of DABA on the urinary acylcarnitine profiles of pregnant females.

CHAPTER 2

INDUCTION OF NEURAL TUBE DEFECTS IN A MURINE MODEL WITH L-2,4-DIAMINOBUTYRIC ACID

2.1 Rationale for using the murine model in this study

As stated previously, neural tube defects (NTD) are a family of multifactorially derived, folate-responsive congenital defects occurring in humans with a relatively high frequency (see Sections 1.1, 1.3.1, 1.3.2) (Lemire, 1988; Leech, 1991, Sulik, 1993; O`Rahily & Muller, 2001; Cate, 2002; Coakley, 2002; Padua, 2002; Vanaerts, 1995; Wasserman, 1998; Kloeblen & Batisch, 1999). Some important probable causes of these defects are infectious diseases (i.e. rubella, toxoplasmosis, genital herpes), cofactor deficiencies (i.e. folate, vitamin B_{12}, vitamin B_6), genetic defects in key enzymes involved in the metabolism of the mother and the developing embryo or foetus (methyltetrahydrofolate reductase, cystathionine β-synthase, methionine synthase, etc.) or teratogenic agents (sodium valproate, diazepam, non-protein amino acids, fumonisin B1, etc.) Medical conditions, affecting the health and basic metabolic profile of the mother (i.e. epilepsy, diabetes, obesity, inborn errors of metabolism), may also play a crucial role in the induction of congenital defects (Imanuel, 1986; Naruse *et al.*, 1988; Wegner, 1992; Elmazar *at al.*, 1995; Finnell, 1997; Alonso-Aperte, 1999; Nissenkorn *et al.*, 2001; O`Rahilly & Muller, 2001; Frey & Hauser, 2003; Moore & Persaud, 2003b; Finnell, 2003; Padmanabban & Shafiullah, 2003; Marasas, 2004; Cabrera *et al.*, 2004; Chen, 2005; Padmanabban, 2006; Padmanabban *et al.*, 2006).

A prophylactic approach in the treatment of disorders may help to reduce the disease load in affected populations (i.e. genetic counseling, specific dietary regimes, folate supplementation) (Greene & Copp, 2005; Czeizel & Dudas, 1992). In order for such a prophylactic approach to be effective it is essential to understand the nature of the molecular processes involved in the initiation, progression and outcome of diseases, including those effecting teratogenesis. However, understanding the pathological processes involved in the pathogenesis of congenital defects (i.e. neural tube defects), require an insight into the basic molecular mechanisms, invoked during embryogenesis and teratogenesis (Wilson and Fraser, 1977; Dansky *et al.*, 1992; Juchau, 1993; 1997; Finnell, 1999; Azarbayjani, 2001). Ethically, humans may never be used in research to discover the underlying aetiology of congenital defects (i.e. neural tube defects, etc.), the

possible outcomes of teratogen exposure or the molecular mechanisms of the processes leading up to the observed defects in human neonates. Scientists are therefore resigned to the use of appropriate animal models to address the problems posed by identifying causal factors and unraveling the molecular mechanisms involved in the induction and development of NTD in human embryos (Wilson & Fraser, 1977; Paul, 1993; Koren, 1994; Frey, 1997; Briggs *et al.*, 1998; Niesink *et al*, 1998; O`Rahilly & Muller, 2001; Bliton, 2003; Burdan *et al.*, 2006).

Experimental animal models have been successfully employed to evaluate the teratogenic potential and action mechanisms of numerous physical and chemical agents on embryo and foetal development. A variety of different types of animal models have been developed to study neural tube defects. These include the rhesus monkey, rat, rabbit, mouse and chicken models. For the sake of biological relevance, however, it is desirable to employ experimental animals demonstrating close similarities to man in respect to placentation, reproduction pattern, neurulation and metabolic enzyme systems. Chicken and mouse embryos proved to be the most suitable models because of interspecies similarities in the process of neurulation in human embryos (Naruse, 1988; Copp & Bernfield, 1994; George & McLone, 1995; George & Fuh, 2003; Green & Copp, 2005; George & McLone, 1995; George & McLone, 1995; Corcoran, 1998; Juriloff & Harris, 2000; Basu & Wireman, 2000; Basu & Wireman, 2000; Gos & Szpecht-Potocka, 2002; George & Fuh, 2003; Bhogal & Combes, 2006).

Aetiological investigations, employing experimental teratogenesis, are often complicated by genetic variability in the animal species or strain used, inconsistencies in the foetal response to the teratogenic insult(s) and the relatively high incidence rates resulting from spontaneous malformations (Copp, 1994; Opitz, 1996; Molloy, 2001; Bhogal & Combes, 2006). Because of these potential species related differences, two or more animal species need to be used in the teratological screening of a suspected teratogenic agent (i.e. drugs, xenobiotics, radiation), since experimental results obtained with one species may not always be readily extrapolated to another species. Due to these complications, relatively large samples of experimental animals are usually required in aetiological investigations before significant and relevant conclusions can be drawn from the results. Excessive or non-physiological dose levels of suspected potential teratogens, often used in experiments, may forcefully precipitate biological defects not often associated with the agent under investigation and may even induce premature abortion or resorption of

foetuses. Experimental doses of drugs should therefore be closely related to more realistic doses encountered in the environment, food chain, or employed in clinical practice (Sisodia, 1972; Rogers, 1987; Finnell, 1999; Burdan *et al.*, 2006).

More than sixty different mouse mutants and strains have been developed to study the aetiology and molecular mechanisms in the formation and prevention of NTD. NTD mutations at specific loci proved to be widely heterogeneous in function. Most of the mutations demonstrate variable low penetrance, while some demonstrated highly complex inheritance patterns (i.e. SELH/Bc, Abl/Arg, Mena/Profilin1). The most common NTD observed in the mouse models are exencephaly, the human equivalent of anencephaly and spina bifida, or both. This dysmorphogenic phenomenon reflects failure of neural fold elevation in well defined and mechanistically distinct elevation zones of the neural plate. In a number of these mouse models the risk of the occurrence of dysmorphogenesis in the foetuses can be reduced by maternal nutrient supplementation, including folic acid (i.e. Pax3, Cart1, Cd mutants), inositol (ct) or methionine (Axd) (Copp, 1994; Copp & Bernfield, 1994; Jurloff & Harris, 2000; Frey & Hauser, 2003; Greene & Copp, 2005).

Deficiencies in *de novo* methylation in certain embryos (Dnmt3b-null) may increase the risk for NTD. This may indicate a potential link between methylation and the observed higher prevalence of female embryos with cranial NTD in several models. Results obtained with some unique NTD mutants (i.e. Gadd45a, Terc, Trp53) suggest that genes controlling basic mitotic functions may also play a role in neural fold elevation. Some of the mutated genes occurring in a number of mouse NTD models, function as regulators of the muscle protein, actin (i.e. Abl/Arg, Macs, Mena/Profilin1, Mlp, Shrm, Vcl). This finding supports the notion that actin may play a key role in neural fold elevation and that this gene may be a feasible candidate for exploring another human gene which may be involved in NTD (Copp, 1994; Copp & Bernfield, 1994; Jurloff & Harris, 2000; Frey & Hauser, 2003; Greene & Copp, 2005).

NTD in mice may be caused by genetic mutations (knockouts) or as a result of exposure to teratogenic agents. Some mouse NTD models have been used to investigate the primary prevention of NTD by supplementation with exogenous agents, such as folic acid. NTD models like crooked tail, Cart1, Cited2 and splotch, as well as NTD induced by teratogens (i.e. sodium valproate, fumonisin B1), respond positively to maternal folate supplementation. These folate-responsive models can be employed to investigate

possible mechanisms underlying the prophylactic qualities of folic acid in the prevention of NTD in humans. Some models, i.e. curly tail, axial defects and the Ephrin-A5 knockout do not respond to folic acid, a phenomenon similar to that observed in a subset of folate insensitive human NTD. Alternative preventive agents, such as methionine and/or inositol, however, proved to exert a protective effect against NTD inducing teratogens. Data from mouse models also suggests that *in utero* therapy may be employed successfully to alleviate the gross impact of NTD on a relatively large proportion of affected foetuses (Jurloff & Harris, 2000; Van Straaten & Copp, 2001; Frey & Hauser, 2003; Greene & Copp, 2005).

Previous studies in this laboratory investigated the teratogenicity of the toxic, non-protein amino acid 3-hydroxynorvaline (Louw, 2004; Louw *et al.*, 2005). This compound proved to be a potent teratogen in the Hanover.NMRI mouse line, while the C57Black mouse line and certain rat models proved to be developmentally resistant to its effects. Embryotoxic effects included retarded growth and mortality, but no NTD in the C57Black mouse line, while several types of dose-response related NTD were observed in the Hanover.NMRI line. The following experiments were executed to establish if the neurotoxic, non-protein amino acid, 2,4-diaminobutyric acid (DABA) can induce congenital defects in developing mouse embryos. For this purpose the more fertile and developmentally more susceptible Hanover.NMRI (Han.NMRI) mouse line was employed (Wegner, 1992; Elmazar, 1995; Kotwani *et al.*, 1995; Brits, 2005).

2.2 MATERIALS AND METHODS

2.2.1 Chemicals employed in the induction of neural tube defects in Hanover.NMRI mouse embryos:

- L-2,4-Diaminobutyric acid (Sigma-Aldrich)
- Physiological saline solution (NaCl;154mM)
- Distilled water prepared by reverse osmosis, sterilised by filtration through a 0.2 µm filter

2.2.2 Experimental animals:

Hanover-NMRI (Han.NMRI) were bred and kept at the North-West University Animal Testing Facility on the Potchefstroom Campus. Only pathogen free and healthy animals were employed in all of the experiments. Approval for this investigation and all other

subsequent experiments was obtained from the Ethics Committee of the North-West University, Potchefstroom (Approval number: 01D02). Han.NMRI mice were bred and kept under well controlled conditions at an ambient temperature of 21 ± 1 °C, relative humidity of 55 ± 5% and a 12 hour light:dark cycle (06h00 - 18h00). Total volume of air in the room was replaced 15 – 20 times per hour and light intensity maintained at 350 – 400 lux, approximately 100 cm above the floor level. A standard, nutritionally balanced laboratory diet, obtained from Rainbow Farms (Pty) Ltd), was fed to the mice. All experimental animals had free access to food and water at all times.

2.2.3 Determination of the optimal dose levels of DABA for non-pregnant females

2.2.3.1 Experimental procedure:

To reliably determine the teratogenicity of DABA towards developing mouse embryos it is of the utmost importance to select dose levels that will not induce any acute toxicity in the pregnant females and/or their embryos. Generally speaking, only mild toxic effects should be observed at the highest selected dose level of the toxin, i.e. slight weight loss and preferably no mortality. At worst a maximum of a 10% mortality rate for the mothers may be tolerated (Kotwani et al., 1995). At the lower dose levels of the toxin, no observable effects should be observed in the mothers. No information concerning the potential LD_{50} of DABA for any animal model could be gleaned from the literature. It was therefore decided to execute a dose-response study on non-pregnant females to estimate the optimal dosage levels to be employed in the teratogenic investigation.

Four groups of five females per group were selected and the body masses of individual animals recorded. They were subsequently placed in separate metabolic cages and kept there for the duration of the study under the same conditions previously described (Section 2.2.2). All animals were allowed to consume a balanced food ration and drink water *ad lib*. A 24 hour base line urine sample was collected from each mouse during day 5 and the body masses of the mice recorded. Body mass values were used to calculate the doses to be applied. On day 6, the females were dosed with 250 µl of DABA, dissolved in sterile saline at four different concentration levels: final dosage levels were 450, 900, 1350 and 1800 mg/kg body mass respectively. Controls received 250 µl of a sterile saline solution. The DABA and sterile saline solutions were administered to the experimental animals with a 1 ml syringe, fitted with a curved, blunt needle. A short piece of silicone rubber tubing was slipped over the tip of the blunt needle to prevent any

trauma to the oesophagi of experimental animals during the gavage procedure. The silicone rubber protected needle was gently pushed down the oesophagus of a mouse until it reached its stomach, before the DABA or saline solutions were released. This method guaranteed the administration of relatively accurate doses of the toxin, while the females were exposed to the minimum levels of stress possible under the circumstances. Following the administration of the DABA and saline solutions, the dosed females were frequently observed for the occurrence of any abnormal behaviour, including seizures and death.

Phenol was added into the urine collectors in the metabolic cages, prior to the beginning of all experiments, in order to preserve the collected urine samples. Phenol prevents the accumulation of microbially derived organic products that may confound the metabolic information derived from the profiles. Twenty four hour urine samples were collected on days 10 and 12 respectively and immediately stored at -74 ^{0}C. The urine samples were left frozen at -74 ^{0}C until metabolic analyses could be executed on them (Chapter 3).

2.2.3.2 Results and discussion: dose response investigation

Table 2.1: *Dose response effect of DABA on pregnant Hanover.NMRI females.*

Status of Females in Groups	**Dose of DABA (mg/kg body mass)**				
	0	**450**	**900**	**1350**	**1800**
Number (%) of live females at start of experiment	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
Number (%) of live females at end of experiment	5 (100)	5 (100)	5 (100)	4 (80)	0 (0)
Number (%) of dead females at end of experiment	0 (0)	0 (0)	0 (0)	1 (20)	5 (100)

Mice receiving DABA at doses of 1800 mg/kg body mass were acutely affected and all the animals in this group died within 24 hours (100% mortality) of dosing (Table 2.1). Some of the animals experienced epileptic-like seizures, prior to death. Ten percent of the females receiving DABA at a dose of 1350 mg/kg body mass died within 48 hours. Mice treated with a dose of 450 and 900 mg/kg body mass DABA displayed no immediately visible behavioural signs of toxicity. None of the control animals were

affected by the saline solutions and a 100% of them survived without any ill effects being observed. From the results it could be concluded that the maximum dose of 1800 mg/kg body mass was far too lethal to use in a teratogenic study. The teratogenic investigation was subsequently executed at the 450, 900 and 1350 mg/kg body mass doses respectively.

2.3.3 Induction of neural tube defects in mouse embryos with DABA

2.3.3.1 Experimental procedure:

Virgin females (age: 40 – 60 days; mean body mass: 55.5 ± 2.5 g) were mated overnight (16h00 to 09h00) with experienced males. The presence of post-coital vaginal plugs served as indicators of successful copulation and all the females displaying vaginal plugs were subsequently regarded as potentially pregnant. The ensuing 24 hours were designated as day one of gestation. It was decided to treat the pregnant females with L-2,4-diaminobutyric acid (DABA), or saline alone on days 7, 8 and 9 *post coitus* (*p.c.*). This period in the embryonic development of the mouse is generally regarded as the stage during which closure of the neural tubes is executed (Burki , 1986).

A total of 30 pregnant female mice were used for this experiment (mean body mass: 51 ± 2.75 g). The animals were kept in metabolic cages for the duration of the experiment and had free access to food and water for the duration of the experiment. Baseline urine samples were collected for the 24 h prior to administration of the DMBA and control solutions. The urine samples were frozen in at -70 ^{0}C until the metabolic profiles could be assessed (Chapter 3). Fifteen pregnant females were divided into three groups of 5 animals/group and the groups designated to receive doses of 450, 900, or 1350 mg of DABA/kg body mass/day. DABA was dissolved in saline and the solution cold sterilised through a 0.22 μm filter. Each individual female received three separate 250 μl doses of DABA on gestation days 7, 8 and 9. The accumulated volume of administered doses was 750 μl and totals of 450, 900 or 1350 mg of DABA/kg body mass were administered respectively. Pregnant controls received 3 doses of 250 μl sterile saline solution on the same days. Urine samples were again collected during the 24 hours of the 10th day of gestation and frozen in at -70 ^{0}C until analyses could be executed (Chapter 3). All DABA and sterile saline solutions were administered to the mice with a 1 ml syringe, as described in Section 2.2.3.1. Toxin and control saline solutions were administered at approximately the same time of day (09:00 ± 30 min) on the three consecutive days.

Following the administration of the DMBA and saline doses, all the animals were regularly observed for any visible signs of toxicity, abortion or premature delivery. Females displaying any indications of abortion or premature delivery were immediately sacrificed and examined by necropsy. On gestation day 18 all surviving females were sacrificed by decapitation and their uteri removed. Embryos were subsequently harvested, weighed, sexed and examined for any congenital defects by stereomicroscopy. The livers of all the females were also removed, frozen in at -70 ^{0}C and used for enzyme analyses later on (Chapter 4).

For the purpose of this study all embryos, including those in a single litter, were considered to be independent of each other and exposed to exactly the same doses of either DABA and saline (experimental animals), or saline alone (controls). This approach was used in the statistical analysis of mouse embryo data obtained from experiments designed to determine the embryotoxicity of DABA and its effect(s) on the growth and embryonic development of the embryos.

2.3.3.2 Statistical analysis of data

Relatively small experimental samples were employed in the study. The statistical analysis of experimental data was executed using the Student`s t-test as well as an analysis of variance (ANOVA) (Ellis and Steyn, 2003). Results are expressed as the mean ± standard error of the mean (SEM).

2.3.3.3 Results and discussion of the teratogenic investigation

Table 2.2: *Effect of DABA on the pregnant females: gestation day 18.*

Status of Pregnant Females	Total Dose of DABA (mg/kg body mass)			
	0*	450	900	1350
Number (%) of surviving females	15 (100)	5 (100)	4 (80)•	3 (60)•
Number (%) of mortalities	0 (0)	0 (0)	1 (20)•	2 (40)•
Number (%) of pregnant females	8 (53.3)	2 (40)	3 (60)	4 (80)
Number (%) of mothers that resorbed their foetuses	1 (6.7)	0 (0)	1 (20)	0 (0)

*Control group; • Significantly different from controls @ 95% confidence level ($p<0.05$).

Table 2.3: Con centration dependent effect of DABA on foetuses: gestation day 18.

Status of foetuses	Total dose of DMBA Administered (mg/kg body mass)			
	0*	450	900	1350
Number (%) of foetuses	60 (100)	21 (100)••	20 (100)••	24 (100)••
Number (%) of dead foetuses	0 (0)	12 (33)•	8 (40)•	8 (57)•
Number (%) of foetuses with congenital defects	0 (0)	0 (0)	5 (25)•	12 (50)•
Number (%) of foetuses with encephalocoele (NTD)	0 (0)	0 (0)	1 (5)•	4 (17)•

*Control group; •Significantly different from controls @ 95% confidence level ($p<0.05$); ••Not significantly different from other dosage levels ($p>0.05$).

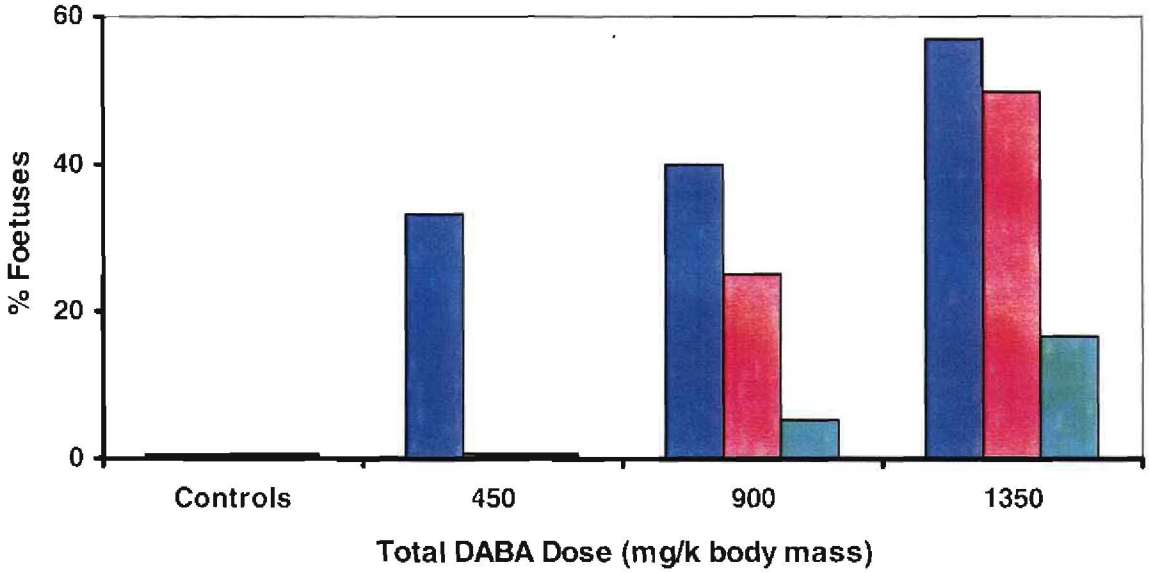

Figure 2.1: *Effect of DABA on 18 day old foetuses:* ( ) % dead foetuses; ( ) % congenital defects; ( ) % neural tube defects.

Table 2.4: *Descriptive statistics of mouse embryos ($n^{\#} = 125$)*

Groups	n#	Total DABA Dose (mg/kg body mass)	Body Mass ± SEM* (g)
1	60	0	1.28 ± 0.42
2	20	450	1.57 ± 0.15^{a}
3	21	900	0.88 ± 0.26^{b}
4	24	1350	0.77 ± 0.31^{c}

n = number of foetuses in group; * SD = standard error of the mean; a = not significantly different from controls @ 95% confidence level; b & c = significantly different from controls ($p < 0.05$).

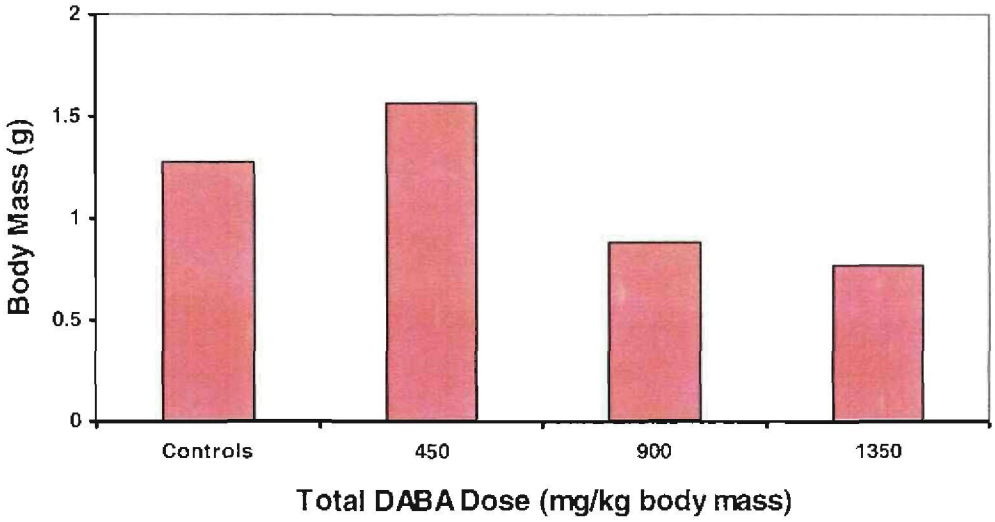

Figure 2.2: *Dose-response effect of DABA on embryo growth.*

The toxic and teratogenic effects of DABA on mouse embryos are summarised in Tables 2.2 and 2.3, as well as in Figures 2.1 and 2.1 above. From the outset it was clear that the pregnancy rates (40 – 80%) and mean litter size (4.0 ± 1) of the Hanover.NMRI females were abnormally low and erratic. The use of vaginal plugs as pregnancy indicators did not appear to be very reliable. Body mass increments, as an indicator of pregnancy proved to be equally unreliable. Although sensitive hormonal indicators of pregnancy, such as the chorionic gonadothrophin (CG) may have been more reliable, the unavailability of any pregnancy kits based on antibodies for mouse chorionic gonadothrophin (mCG), made it impossible to employ this technology. Louw (2005) also previously found that the mCG antigen in confirmed pregnant mouse urine samples reacted poorly with anti-human chorionic gonadothrophin (anti-hCG). Efforts to use pregnancy assay kits, aimed at measuring the level of hCG in mouse urine samples were subsequently abandoned.

Special care was taken not to use dose levels of DABA that were too toxic for the pregnant females, however, 20 – 40% of the animals died at the two highest dose levels (900 & 1350 mg/kg body mass respectively; statistically significant at 95% confidence level). This phenomenon was clearly precipitated by the toxic effects of DABA, but the result may also indicate that these individuals have experienced more stress during the execution of the experiment compared to the majority of females or the non-pregnant females used in the teratogenic (Table 2.2) and dose-response studies (Table 2.1) respectively. Resorption of foetuses occurred in two of the 30 pregnant females (6.7%) -

one in the control group (6.7% of group) and another one (20% of group) in the 900 mg/kg body mass DABA dose level group. In the case of the control group the observed effect may be related to stress alone, while both stress and the toxic effects of DABA could have played a role in the resorption of the foetuses at the 900 mg/kg body mass dose level.

DABA proved to be highly toxic to the mouse foetuses at all the administered dose levels (450 – 1350 mg/kg body mass). Foetal mortality was extremely high (33 – 57%) and an apparent non-linear, but directly proportional relationship between dosage level and foetal mortality was observed (Fig. 2.1). The effect of the three DABA dose levels differed significantly ($p<0.05$) from the control group. Differences between the various DABA dose levels also appeared to be statistically significant ($p<0.05$). A very high percentage of the foetuses (25 – 50%) displayed evidence of congenital defects, but only 5 -17% of the animals had neural tube defects (i.e. exencephali). The teratogenic dose-response relationship was statistically significant ($p<0.05$), non-linear and directly proportional to the administered dose of DABA.

The mean body mass of the embryos previously proved to be a fairly reliable indicator of the growth and development of chicken and mouse embryos (Louw, 2005, Louw *et al.*, 2006). The mean body mass of the control embryos at 18 days of gestation (1.28 ± 0.42 g; Table 2.2) was slightly higher than that previously reported for full term mouse embryos (1.1 ± 0.25 g; -16.4%), but was clearly indicative of normal growth and development (Transgenic animal web, 1999). However, in contrast to this observation, the mean embryo body mass of the group of embryos exposed to the lowest dose of DABA (450 mg/kg body mass) appeared to be higher than that recorded for the controls (1.57 ± 0.15 g), but this result was not statistically significant ($p>0.05$). Overall the dose-response effect of DABA on embryo growth appeared to be statistically significant ($p<0.05$) and directly proportional, although clearly non-linear. The apparent non-significant ($p>0.05$) higher body mass of embryos exposed to DABA at the lowest dose level could be a statistical artefact due to the relatively small sample sizes employed in the study.

From these results one can conclude that DABA possesses teratogenic properties and that it can cause congenital defects (i.e. NTD) when pregnant Hanover.RPMI mice are exposed to it during critical stages in embryo development.

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CHAPTER 3

EFFECT OF DABA ON METABOLIC PROCESSES IN PREGNANT MICE.

3.1 INTRODUCTION.

As previously described in Sections 1.1 and 1.4, L-2,4-diaminobutyric acid (DABA) is a well-known, toxic (i.e. neurotoxic, hepatotoxic), non-protein amino acid, produced by a large variety of plants and micro-organisms. It is an important building block in the primary structure of numerous peptide antibiotics, antifungal peptides and cell wall peptidoglycans (Nigam et al, 1966; Sasaki, 1998; Bisacchi, 1987; Harrison, 1991; Martin, 2003). DABA possesses unique anti-tumour (tumorolytic) properties but is efficacy as an anti-cancer drug is hampered by its systemic toxicity (Ronquist *et al.*, 1980, 1984; Panasci *et al.*, 1988; Antoni *et al.*, 1997; Blind *et al.*, 2003; Bergenheim *et al.*, 2006).

DABA's hepatotoxic mechanism appears to relate to a dose-dependent, competitive secondary hyperammonemia, caused by the inhibition of ornithine carbamoyltransferase (OCT), a key enzyme in the urea cycle. (Rosenthal, 1982). Its neurotoxic characteristics on the other hand seem to result from a metabolic blockation of the inhibitory action of γ-aminobutyric acid in the brain of affected animals. DABA causes an accumulation of GABA in the brain due to a selective inhibition of the uptake of GABA. This mechanism is probably responsible for DABA's anti-epileptic action (O`Neal *et al.*, 1968; Rao *et al.*, 1969; Amabeoku *et al.*, 1999).

Metabolic data about DABA appears to be very scarce but available experimental evidence indicates that it can synthesized from ß-cyanoalanine in some plants and microorganisms (Fig. 1.11). ß-Cyanoalanine is a metabolic intermediate in the dehydration of asparagine to ß-cyanoalanine, which is subsequently reduced to DABA. DABA is a purported intermediate in the biosynthesis of azetidine-2-carboxylic acid from homoserine (Rosenthal, 1982). Certain *Xanthomonas* species can catabolise DABA to aspartic β-semialdehyde, aspartic acid and oxaloacetic acid (Fig 1.12 and 3.1). Aspartic acid is produced, following the NAD^+-dependent dehydrogenation of aspartic β-semialdehyde. The dehydrogenase is a thiol-containing enzyme and displays a high level of substrate specificity for aspartic β-semialdehyde. NAD^+-Analogues, such as 3-acetylpyridine adenine- dinucleotide and deamino-NAD are only weakly bound by the enzyme.

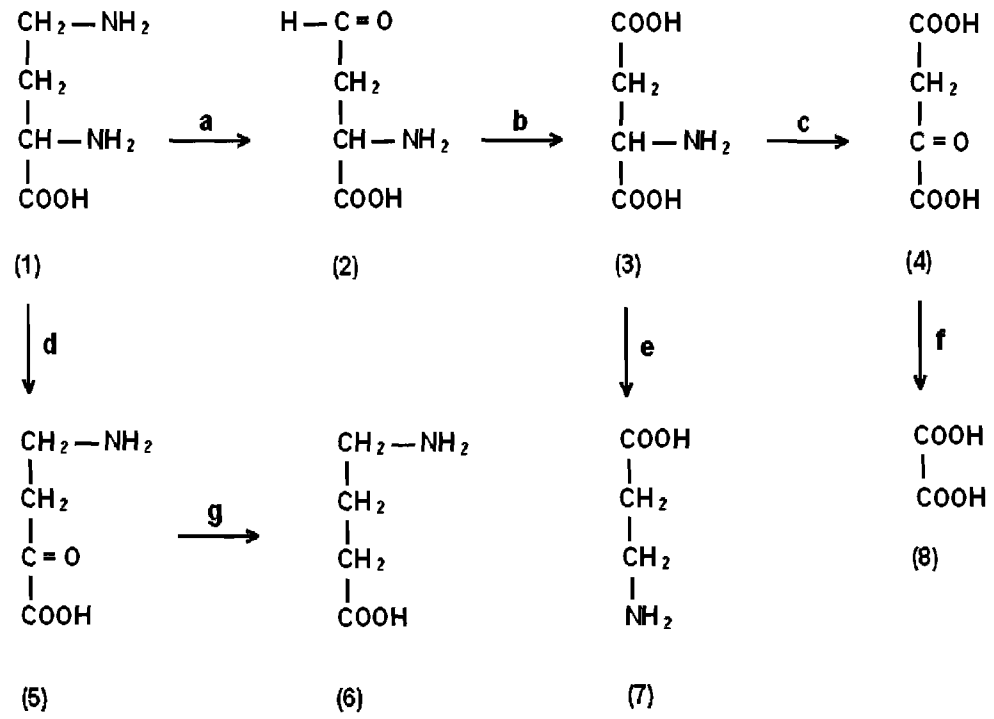

Fig. 3.1: *Purported metabolic fate of DABA (Xanthomonas species, Holtman rats):(1) L-2,4-Diaminobutyric acid (DABA); (2) Aspartic β-semialdehyde; (3) Aspartic acid; (4) oxaloactetate; (5) 2-keto-4-aminobutyric acid; (6) γ-Aminobutyric acid (GABA); (7) β-Alanine; (8) Oxalic acid. Enzymes and/or enzyme reactions purportedly involved in the metabolism of DABA: a = pyruvate dependent γ-transaminase; b = NAD^+-dependent dehydrogenation of aspartic β-semialdehyde; c = β-transamination of aspartic acid; d = β-transamination of DABA; e = aspartate 1-decarboxylase (Mushahwar & Koeppe, 1963; Rosenthal, 1982).*

Studies with ^{14}C-labelled L- and D-DABA isomers in rats indicate that the L-isomer of DABA is preferentially oxidised to $^{14}CO_2$ and water, while intermediates such as aspartic β-semialdehyde (2), aspartic acid (3), 2-keto-4-aminobutyric acid, oxaloacetate (5), γ-aminobutyric acid (GABA) (6), β-alanine (7) and oxalic acid (8) are produced and/or utilized (Fig. 1.12 and 3.2). Approximately 10% of the unchanged ^{14}C-labelled DABA is excreted in the urine. DABA is mainly metabolised in the liver, but some breakdown seem to occur in the kidneys of the rats. Around 5% of both the L- and D-isomers are converted to β-1-^{14}C-alanine, which is excreted *via* the urine. Radiolabel can also be found in varying proportions of tissue glutamate, aspartate and alanine (Mushahwar & Koeppe, 1963).

The effect of DABA on the general metabolism of pregnant female mice was subsequently studied. The basic approach of the investigation was to determine the effects of DABA on the basic metabolic profiles of urinary organic acids, amino acids and acylcarnitine conjugates.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals employed in experiments:

- Phenol, ethylacetate, diethylether, butanol, hydrochloric acid (HCl), acetonitrile, acetyl chloride and formic acid were obtained from Merck Chemical Co. (Darmstadt, Germany)
- Trimethylchlorosilane (TMCS) Bis(trimethylsilyl)trifluoroacetamide (BSTFA), sodium sulphate (Na_2SO_4) and 3-phenyl butyric acid were all obtained from Sigma-Aldrich Co. (St. Louis, USA).
- Phenomenex EZ:faast® (rapid amino acid analysis kit) was obtained from Separations (Johannesburg, South Africa).
- Sodium sulphate was purchased from BDH Laboratory Supplies.

3.2.2 Collection of urine samples:

Urine samples were collected while animals were kept in metabolic cages, during the execution of two experiments described in Sections 2.2.3.1 (dose-response study on non-pregnant females) and 2.3.3.1 (dose-response study on pregnant females). Non-pregnant and pregnant females were placed in separate metabolic cages and kept there for the duration of the study under conditions previously described in Section 2.2.2. All animals were allowed to consume a balanced food ration and drink water *ad lib*. Phenol was added into the urine collectors in the metabolic cages, prior to the beginning of all experiments, in order to preserve the collected urine specimens. It prevents the accumulation of microbially derived organic products that may confound metabolic information to be derived from the samples. Twenty four hour urine samples were collected on days 5 (dose-response study on non-pregnant females) or on days 10 and 12 (dose-response study on pregnant females). The samples were filtered to remove any food, faecal or any other foreign materials that may be present, placed in separate identifiable tubes, immediately frozen in at -30 ^{0}C and then stored until metabolic analyses could be executed.

A vast amount of analytical work is usually required to execute all the analyses and the accumulated cost of the analyses can be daunting. A strategic decision was therefore taken to initially focus only on urine samples collected from five (n = 5) pregnant females, dosed at 450 mg DABA/kg body mass and five pregnant controls (n = 5).

3.3 Analysis of organic acids in mouse urine samples

3.3.1 Basic principle underlying the organic acid analysis.

Urinary organic acid analysis by gas chromatography (GC) and gas chromatography mass spectrometry (GC-MS) is a well-established, standardized procedure. These analyses are applied to investigate the nature and quantities of excreted organic acids, produced in the course of general metabolic activity in animals or humans. Important metabolic information can be gleaned from the resulting metabolic profiles. The extraction of organic acids by a liquid-liquid extraction procedure is still the most commonly used method, although it is less effective than column extraction techniques (Charmers & Lawson, 1982). By using a combination of more than one solvent (i.e. diethyl ether, ethyl acetate) organic acids can be extracted much more efficiently (Erasmus, 1987). Urinary organic acids are usually present in urine as a mixture of dissociated salts (>99%) and partially dissociated free acids. Prior to extraction, all the organic acids must be quantitatively displaced from their salts by acidification (HCl) of the urine samples. Because of associated volatility problems the acids then need to be derivatized to obtain more volatile derivatives (i.e. trimethylsilyl ethers, etc.) that can be successfully fractionated and quantified by means of GC and GC-MS. 3-Phenyl butyric acid is routinely employed as an internal standard for the quantification of organic acids.

Following derivitization, injection and chromatographic fractionation (GC) of the highly complex mixture of organic acid trimethylsilyl ether derivatives the compounds enter into the ionization source of a mass selective detector (MSD), where they are fragmented by means of electron impact (70 eV). Resulting fragmentation patterns are analysed and compared to an on board compound library, to identify the individual components in the gas chromatograms. Ion abundance values of the molecular ions of individual components, relative to the abundance of the molecular ion of a non-physiological fatty acid derivative (internal standard: i.e. phenylbutyric acid) are employed in the calculation of the concentrations of the individual components.

The concentration of individual organic compounds in the urine samples of individual animal or human subjects may vary considerably in the course of any 24 h day and between different days. Because of wide temporal fluctuations the quantities of the acids, or any other bio-analyte, for that matter, a natural biomarker (reference compound) must be employed, relative to which all other organic compounds in the urine sample can be quantified. In healthy, functioning kidneys, creatinine is freely filtered through the glomeruli and is not reabsorbed by the tubules. This compound

is normally rapidly and quantitatively cleared from the blood, via the kidneys at a predetermined rate (creatinine clearance), which remains relatively constant for individual animal or human subjects (i.e. 90 - 120 mL/min for humans). Creatinine clearance is related to the glomerular filtration rate (GFR) and creatinine can therefore be used as a reliable biomarker, or reference compound, against which the concentration of urinary organic compounds can be normalised (Cockcroft & Gault, 1976; Ware, 1981; Perrone *et al.*, 1992).

By calculating the concentrations of urinary organic compounds with reference to the creatinine content of a urine sample, the intra- and interbatch data and inter-individual data can be made comparable. The principle is based on the assumption that the individual animal or human subject must have healthy kidneys and can maintain a healthy (normal) clearance rate for creatinine over a 24 h period. In the case of unhealthy kidneys, the clearance rate of creatinine may deviate from the norm and the relative intra- and interbatch, as well as interindividual data (i.e. urinary concentration of organic compounds) may then not be comparable to that of healthy subjects. The concentration of creatinine in urine samples is usually measured by means of the Jaffé method, with a uniquely designed assay kit in an auto-analyser (Jaffé, 1886; Rosano *et al.*, 1990).

3.3.2 Methodology of organic acid analysis:

Organic acid analysis can be subdivided into four basic subroutines or procedures:

(a) Liquid-liquid or solvent extraction of the organic acids from an acidified urine sample;

(b) Derivatization of the organic acids (i.e. trimethylsilyl ethers) in the extract;

(c) Analysis of the derivatized organic acids by GC-MS;

(d) Processing of MS fragmentation and abundance data to identify and quantify the organic acids in the organic acid profiles, employing automated mass spectral deconvolution and identification system software (AMDIS).

(a) Solvent extraction of organic acids from mouse urine samples.

Stored urine samples were thawed at room temperature, thoroughly mixed and 1 ml of each sample transferred to clean, dry Kimax tubes. Hundred microlitres of the internal standard (IS: 3-phenyl-butyric acid, M_r 164.21) was added to each urine sample. The IS-spiked sample was subsequently acidified (pH < 2) following the addition of approximately 6 drops of 5 N HCl. Six millilitres of ethylacetate was added to the IS-spiked urine sample, the aqueous and organic phases were thoroughly mixed for 30 minutes and then centrifuged (2000 × *g* x 3 min). The phases were

separated and organic phase carefully transferred to a clean, dry Kimax tube. The remaining aqueous phase was re-extracted with 3 ml dry diethylether (10 min) and the solvent mixture centrifuged at 2000 × *g* for 3 minutes. The diethylether organic phase was carefully removed and combined with the ethylacetate organic phase. Anhydrous sodium sulphate (Na_2SO_4) was added to the combined organic phases and the mixture thoroughly shaken up to absorb residual water. The sodium sulphate was subsequently removed by centrifugation (2000 × *g*, 3 min) and the dried organic phase decanted into a clean, dry Kimax tube, to conclude the extraction procedure.

(b) Derivatization of organic acids in the mouse urine samples.

The organic phase was dried at approximately 70 °C under a constant stream of nitrogen. The derivatizing reagents, BSTFA (40 µl) and TMCS (8 µl) were added to the dry sample residue in the Kimax tube and the reaction mixture incubated in the tightly sealed tube (70 °C, 45 min). Volatile trimethylsilyl (TMS) derivatives of the extracted organic acids were produced during incubation with the BSTFA/TMCS reagent.

(c) Organic acid analysis of mouse urine samples.

One microlitre of the derivatized sample was injected into a Hewlett Packard (HP) 6890 series gas chromatograph, fitted with a HP 5973 mass selective detector (MSD) and a split/splitless injector. A fused silica SE30 capillary column (25m × 0.32 mm) was used to separate the derivatized organic compounds at a carrier gas (helium) flow rate of 1 ml/min. Initial column temperature was 70 °C and it was kept at this temperature for 2 minutes. Column temperature was gradually increased (5 °C/min) to 280 °C and then kept constant at this temperature for 3 minutes, before re-equilibration at the initial temperature of 70 °C, prior to the next run. The TMS derivatives of the organic acids were detected and analysed (fragmentation and quantification) by a quadropole mass selective detector (HP 5973 MSD), following electron impact at 70 eV in the ion source. Unique fragmentation patterns (mass spectra) of each constituent organic acid TMS derivative were generated, making it possible to positively identify each of the eluting compounds.

(d) Processing of fragmentation and abundance data: identification and quantification of organic acids.

Organic acids represented by numerous peaks in the gas chromatogram, were identified, following automated mass spectral deconvolution of the information generated in the mass spectrometer (AMDIS). A sophisticated library containing mass spectral data of approximately 1150 organic acids, previously compiled and regularly employed in the metabolic laboratory (North-west University, Potchefstroom

Campus), aided in the identification of the organic acids in the organic acid profile. To quantify the identified organic acids, the following equation was used:

$$[Organic\ Acid] = (Area\ of\ Peak_X / Area\ of\ peak\ IS) \times [IS]$$

Where:

- [Organic Acid] = concentration of organic acid (μmole/L)
- Area of $Peak_X$ = Area of Organic Acid peak X
- IS = Internal Standard
- [IS] = concentration of internal standard

The concentrations of identified organic acids, relative to the creatinine content of individual urine samples were calculated, employing the formula below:

$$[Organic\ Acid] = \frac{[Area\ of\ Peak\ X / Area\ of\ I.S.\ Peak] \times [I.S.]}{mmol / L\ Creatinine}$$

Where:

- [Organic Acid] = concentration of organic acid per mmol creatinine
- $[Organic\ Acid_X]$ = concentration of organic acid X (μmole/L)
- $[Creatinine_{24h}]$ = Creatinine content of 24 h urine sample (mol/L)

3.4 Quantification of acylcarnitine conjugates in mouse urine samples with electrospray tandem mass spectrometry (ESI-MS-MS).

3.4.1 Basic principle of the analysis.

Acylcarnitines in blood reflect the primary accumulation of mitochondrial acyl-CoA metabolites in disorders of fatty acid and amino acid catabolism. Acylcarnitine profiles enable clinicians and researchers to diagnose nearly all possible metabolic defects in these pathways. Classical GC-MS methods fail to detect acylcarnitines, but these metabolites are readily detectable and can be quantified with tandem mass spectrometry (MS/MS), following a "soft" ionization technique, i.e. electrospray (ESI) or fast atom bombardment (FAB) (Koeberl *et al.*, 2003).

Butylated amino acids and acylcarnitines display characteristic fragmentation profiles with commonly shared ions, following collision induced decomposition (CID). These ions can be employed in the detection of specific groups of metabolites, I.e. butylated acylcarnitines can be identified by the presence of a positive precursor ion with a *m/z* of 85. Butylated neutral and acidic amino acids display the loss of a neutral fragment of 102 Da, due to the elimination of a butylformate group under CID conditions. Basic amino acids, on the other hand, always loose an ammonia group as well a butylformate group, a total neutral loss of 119 Da (Millington *et al.*, 1990).

In the course of typical analyses, patient or animal derived samples are first derivatized by butylation, generating butyl esters of all carboxylic acid groups in the molecules of interest (i.e. amino acids, fatty acids). The derivatized samples are then introduced into the ionization source of an electrospray tandem mass spectrometer (ESI-MS-MS) through a fused silica capillary tube, connected to an HPLC injector (flow rate 200 μl/min). A high voltage (3.5 kV) is applied to the tip of the capillary, once the probe is present in the source of the mass spectrometer. Derivatized samples, emerging from the capillary are dispersed into an aerosol of highly charged droplets under the influence of the strong electric field. The electrospray process is aided by an inert, nebulizing gas (nitrogen), flowing down the probe around the outside of the capillary. The nitrogen gas helps to disperse the solvent-solute spray mixture, emerging from the capillary tip, into the MS/MS. Highly charged droplets in the spray diminish in size as a result of rapid evaporation, aided by the flow of warm nitrogen gas. Charged, solvent-free sample ions eventually enter into the quadropole mass spectrometer (MS). The first MS scan for previously specified mother ions, according to preset mass values. Selected mother ion(s) subsequently pass into the collision chamber where it is kinetically bombarded and fragmented by collision induced dissociation (CID). Either nitrogen or argon may be used as collision gases.

Fragmentation of the butylated, ionized compounds in the collision chamber leads to the formation of breakdown products, known as daughter ions. The daughter ions are physically derived from the original ionized compounds (mother ions), and their specific mass to charge ratios (m/z) quantified by the second MS detector. The molecular masses of the various daughter ions will vary according to the different mother ions bombarded in the collision chamber. The butylated acyl carnitines derivatives were analysed by selective ion monitoring (SIM) positive mother ions producing a neutral entity with a m/z ratio of 85 atomic mass units (amu).

3.4.2 Sample preparation.

Urine samples previously assayed for the presence and quantities of organic acids, were used for the analysis of amino acids and carnitine/acylcarnitines.

3.4.3. Method

3.4.3.1 Reagents

Deuterated carnitine and acylcarnitines (Table 3.1) were purchased from Cambridge Isotope Laboratories, Inc or were synthesised and supplied by Dr. Herman J. ten Brink, Academic Hospital, VU, The Netherlands.

3.4.3.2 Carnitine and acylcarnitine analysis of mouse urines samples

Aliquots (100 µl) of homogeneous urine samples were transferred into an Eppendorf vial and clarified by centrifugation (30 minutes @ 16 000 × *g*, room temperature). Ten microlitre aliquots of each of the clear urine samples were transferred to another clean, dry Eppendorf tube and thoroughly mixed with a stable isotope labelled acylcarnitine mixture (400 µl) (Table 3.1).

Table 3.1: *Stable isotope standards for acylcarnitine ananlysis.*

Carnitines	Concentration (µmol/L)
Carnitine- (methyl-d_3)	1.52
Acetyl carnitine- (d_3)	0.51
Propionyl carnitine- (d_3)	0.13
Isovaleryl carnitine- (d_9)	0.11
Octanoyl carnitine- (d_3)	0.10
Palmitoyl carnitine- (d_3)	0.04

The mixtures of samples and internal standards were re-centrifuged for 30 minutes at 16000 × *g*. An aliquot (100 µl) of the clear supernatant mixture of sample and internal standards spiked sample supernatant evaporated to dryness under a stream of nitrogen at 55°C for 45 minutes. Butanolic HCl (3 N) was prepared with the addition of 12.5 ml acetyl chloride to 50 ml ice cold butanol and the reaction mixture left on ice for 20 minutes. Two hundred microlitres of the 3N butanolic-HCl preparation was added to the dry residue and the reaction mixture incubated at 70°C for 15 minutes. Solvent was removed under a constant stream of dry nitrogen gas at a temperature of 55°C and the resulting dry residue subsequently re-dissolved in 100 µl acetonitrile:water (80:20) mixture, containing 1% formic acid. An aliquot (10 µl) of the derivatized sample solution was injected into a VG Quatro Micromass HRGC 8000ESI-MS-MS system (Rashed *et al.,* 1994). The second MS for carnitine analysis scanned for mother ions losing a neutral fragment with m/z 85.40. To quantify the identified carnitine conjugates, the equation below was used:

$$[Acyl\ Carnitine] = \frac{[Area\ of\ Peak\ X\ /\ Area\ of\ I.S.\ Peak] \times [I.S.]}{mmol\ /\ L\ Creatinine}$$

Where:

- *[Acyl Carnitine]* = Concentration of specific acylcarnitine (mmol/mol Creatinine)

- X = Specific butylated acyl carnitine derivative
- IS = Internal Standard

Table 3.2: *Conditions employed in the ESI-MS-MS analysis of acylcarnitines.*

		Source (ES^+)		
Capillary:		3.50 kVolts		
HV Lens:		0.50 kVolts		
Cone:		34 Volts		
Skimmer Offset:		5 Volts		
Skimmer:		1.5 Volts		
RF Lens:		0.2 Volts		
Source Temperature:		85 °C		
		MS1		**MS2**
Ion Energy:		1.0 Volts		1.0 Volts
Ion Energy Ramp:		0.0 Volts		0.0 Volts
LM Resolution:		14.0		13.5
HM Resolution:		14.0		13.5
Lenses:	(# 5)	100 Volts	(# 7)	250 Volts
	(# 6)	5 Volts	(# 8)	40 Volts
			(# 9)	5 Volts
Multiplier:		650 Volts		

Nitrogen was used as drying and nebulizing gas. Flow rates for drying and nebulising were set at 350 and 20 $L.h^{-1}$, respectively.

3.5 Analysis of amino acids in mouse urine samples: The Phenomenex EZ:faast® GC- MS assay.

3.5.1 Basic principle of the analysis.

The EZ:faast assay kit was employed in the extraction and derivitization of amino acids in mouse urine samples. Amino acids can be rapidly and reliably quantified with the Phenomenex EZ:faast amino acid analysis kit. With the exception of L-arginine, all the other amino acids can be detected and quantified. The procedure consists of a patented solid phase extraction step and derivatization procedure, followed by a liquid/liquid extraction step. Following extraction and derivitization of the amino acids present in the urine samples, the derivatized samples (2 μl) were analysed by GC-MS. The amino acids were positively identified and subsequently quantified, using automated mass spectral deconvolution of the data generated in the mass spectrometer (AMDIS).

3.5.2 Reagents, materials, buffers and solutions.

- Phenomenex EZ: faast® amino acid analysis kit (Phenomenex, Inc.).
- All solvents, buffers and reagents were supplied with the Phenomenex EZ: faast® amino acid analysis kit (Phenomenex, Inc., United Kingdom).

3.5.3 Method

3.5.3.1 Derivatization of amino acids for GC-MS analysis in mouse urine samples

Hundred microlitre (100 µl) aliquots of urine were used in the analysis of amino acids with the EZ:faast assay kit. Amino acids were first extracted onto a solid phase support, desorbed and subsequently derivatized using the designated patented reagents in the Phenomenex EZ:faast® amino acid analysis kit. The method was used exactly as prescribed in the protocols provided by the manufacturer.

3.5.3.2 GC-MS analysis of amino acids in mouse urine samples.

A Hewlett Packard 5890 GC, equipped with a split/splitless auto injector (HP 7673) was employed in the analysis. A ZB-AAA GC column (provided in the Phenomenex EZ:faast® amino acid analysis kit, Phenomenex, Inc.) was used. The flow rate of the carrier gas (helium) was 1 ml/min. Derivatized samples (2 µl) were injected into the GC column at an initial column temperature of 110 °C. The column temperature was gradually increased to 320 °C at a rate of 30°C/min. A quadrupole mass spectrometer (MSD: HP 5989A) was used for the GC-MS analyses. Electron impact ionisation spectra were generated at a source temperature of 240 °C and the electron beam energy set at 70 eV. To quantify the identified amino acids, the equation below was used:

$$[Amino\ Acid] = \frac{[Area\ of\ Peak\ X\ /\ Area\ of\ I.S.\ Peak] \times [I.S.]}{mmol\ /\ L\ Creatinine}$$

Where:

- [Organic Acid] = Concentration of specific organic acid X
- X = Specific organic acid
- IS = Internal Standard

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3.6 Results and discussion: The effect of DABA on the organic acid profile of female Hannover.NMRI mice

A gas chromatogram, displaying a typical organic acid profile observed in the urines of control mice appears in Fig. 3.2. The effect of DABA on the organic acid profile of female mice is depicted in Fig. 3.3.

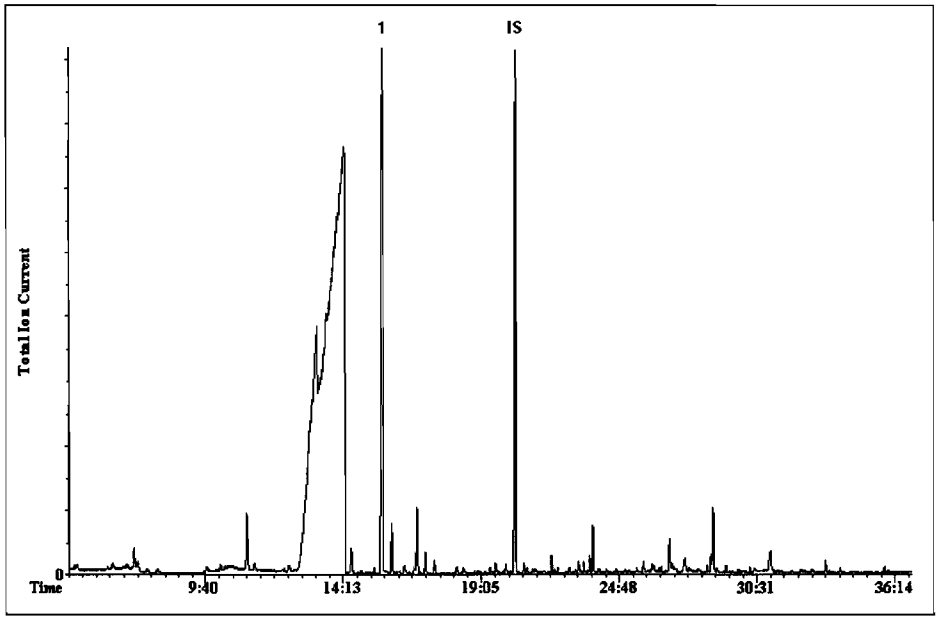

Figure 3.2: *Urinary organic acid profile of a control mouse:(1) Thymol; (IS) 3-phenylbutyric acid (internal standard).*

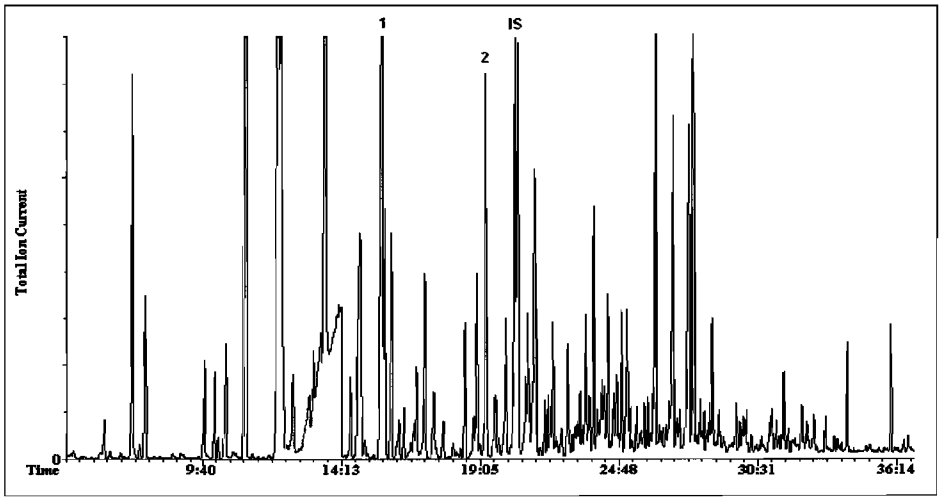

Figure 3.3: *Urinary organic acid profile of a pregnant female mouse treated with DABA. (1) Thymol; (IS) 3-phenylbutyric acid (internal standard).*

Table 3.3: *DABA induced changes in selected mouse urinary citric acid cycle and glycolysis pathway intermediates.*

Metabolites	Controls[a]	DABA Treated mice[a]	% Change[a]
1. Lactate	196.3(12.4)	928.8(67.5)	373.2(24.9)[b]
2. Pyruvate	1.01(3.9)	0.1(0.6)	- 90.1(10.3)[c]
3. Oxalate	31.6(11.7)	504.7(34.6)	1497.1(112.5)[b]
4. cis-Aconitate	180.9(14.6)	1068.2(10.3)	490.5(34.8)[b]
5. Malate	21.9(3.8)	96.4(7.9)	340.2(23.4)[b]
6. Citrate	209.8(11.3)	1649.2(234.6)	686.1(78.5)[b]
7. Succinate	184.6(12.8)	615.8(87.2)	233.6(33.5)[b]
8. Fumarate	13.5(3.3)	113.8(19.3)	743.0(59.1)[b]

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p< 0.05$); c = not statistically significant ($p>0.05$); n.d. = not detected; ∞: infinitely high value due to metabolite not being detected in controls; All values expressed as Mean (± SEM).

Table 3.4: *DABA induced changes[d] in selected mouse urinary urea cycle metabolites.*

Metabolites	Controls[a]	DABA Treated mice[a]	% Change[a]
1. Urea	105.7(9.4)	131.9(13.4)	24.8(3 .3)[c]
2. Citrulline	208. 3(11.9)	121.8(14.4)	- 41.5(8.2)[b]
3. Ornithine	738.4(91.3)	366.8(23.1)	- 50.3(6.2)[b]

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p< 0.05$); c = not statistically significant ($p>0.05$); n.d. = not detected; ∞: infinitely high value due to metabolite not being detected in controls; All values expressed as Mean (± SEM).

Table 3.5: *DABA induced changes[d] in the excretion of selected mouse urinary hydroxyacids.*

Metabolites	Controls[a]	DABA Treated mice[a]	% Change[a]
2-Methyl –3-hydroxybutyrate	16.4(2.3)	49.3(5.2)	200.6(9.3)[b]
3-Hydroxybutyrate	90.8(6.6)	311.4(12.7)	243.0(17.8)[b]
2-Hydroxybutyrate	14.0(2.2)	40.2(3.8)	187.1(13.2)[b]
2-Hydroxyvalerate	55.5(6.2)	15.1(2.2)	- 72.8(5.3)[b]

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p< 0.05$); c = not statistically significant ($p>0.05$); n.d. = not detected; (: infinitely high value due to metabolite not being detected in controls; All values expressed as Mean (± SEM).

Table 3.6: *DABA induced changes in amino acids and some selected metabolites urinary amino acid profiles of pregnant female mice.*

Amino Acids/Metabolites	Controls[a]	DABA Treated mice[a]	% Change[a]
1. Alanine	129.0(11.5)	132.0(9.7)	2.3(1.6)[c]
2. Sarcosine	70.8(8.9)	269.7(11.0)	280.9(13.9)[b]
3. Glycine	127.3(7.7)	144.5(9.1)	13.5(8.4)[c]
4. Valine	85.7(9.9)	160.9(12.8)	87.7(10.5)[b]
5. Isoleucine	89.1(8.9)	141.0(11.8)	58.3(7.0)[b]
6. Leucine	126.0(11.4)	133.3(5.7)	5.8(2.8)[c]
7. Allo-Isoleucine	51.6(4.6)	63.4(7.2)	22.9(9.4)[c]
8. Threonine	120.9(11.1)	81.1(8.9)	-32.9(5.3)[b]
9. Proline	82.0(6.7)	78.1(9.3)	-4.8(2.2)[c]
10. Asparagine	78.8(8.3)	77.0(10.1)	-2.3(1.8)[c]
11. Pipecolic acid	241.0(78.3)	252.3(23.1)	4.7(3.3)[c]
12. Aspartic-acid	43.1(6.2)	31.8(4.1)	-26.2(5.3)[c]
13. Methionine	93.8(9.3)	71.5(5.9)	-23.8(5.5)[c]
14. Glutamic acid	70.4(6.5)	68.4(7.2)	-2.8(2.5)[c]
15. Phenylalanine	105.5(9.8)	141.8(12.4)	34.4(6.3)[b]
16 α-Aminoadipic acid	60.0(8.4)	31.2(6.3)	-48.0(9.4)[b]
17. Cysteine	79.0(8.2)	165.1(12.5)	109.0(11.7)[b]
18. Cadaverine	61.7(7.4)	363.0(56.4)	488.8(33.6)[b]
19. Glutamine	83.6(9.5)	n.d.	-100.0(8.6)[b]
20. Ornithine	738.5(112.4)	366.8(89.5)	-50.3(8.3)[b]
21. Creatinine	67.3(6.4)	51.6(10.1)	-23.4(4.3)[c]
22. Lysine	184.3(13.3)	98.7(10.3)	-46.5(9.6)[b]
23. Histidine	94.7(10.7)	39.2(6.3)	-58.7(6.2)[b]
24. Tyrosine	91.2(11.3)	51.9(11.2)	-43.1(8.4)[b]
25. Phenylalanylproline	n.d.	33.0(11.1)	∞ [b]
26. Cystathionine	60.8(7.3)	n.d.	-100.0(8.2)[b]
27. Citrulline	208.3(98.3)	121.8(11.2)	-41.5(6.0)[b]
28. DABA	n.d.	302.3(5.7)	∞ [b]
29. ß-alanine	112.6(10.7)	332.0(35.7)	194.8(28.5)[b]
30. Internal-Standard	200.0	200.0	0

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p < 0.05$); c = not statistically significant ($p > 0.05$); n.d. = not detected;∞: infinitely high value due to metabolite not being detected in controls; All values expressed as Mean (± SEM).

Table 3.7: *DABA induced changes in selected dicarboxlic fatty acids observed in the urine of pregnant female mice.*

Metabolites	Controls[a]	DABA Treated mice[a]	% Change[a]
1. Adipate	11.6(2.9)	18.0(3.1)	55.2(6.6)[b]
2. Suberate	6.1(1.4)	20.4(3.7)	234.4(98.5)[b]
3. Ethylmalonate	65.0(7.4)	74.9(9.2)	15.2(3.6)[c]

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p< 0.05$); c = not statistically significant ($p>0.05$); n.d. = not detected; ∞: infinitely high value due to metabolite not being detected in controls; All values expressed as Mean (± SEM).

Table 3.8: *DABA induced changes in selected acylcarnitine and free carnitine levels observed in the urine of pregnant female mice.*

Metabolites	Controls[a]	DABA Treated Mice[a]	% Change[a]
1. Acylcarnitines	59.5 (7.3)	64.6(8.4)	-8.6(1.9)[c]
2. Free Carnitine	29.2(3.8)	36.8(8.7)	26.0(3.7)[c]
3. Acylcarnitines/Free carnitine	3.8(1.9)	1.8(0.7)	-52.6 (13.5)[c]
4. Acetyl carnitine	23.7(9.9)	41.5(15.9)	75.6(19.9)[b]
5. Butyryl carnitine	9.3(0.9)	3.7(0.4)	-60.2(18.6)[c]
6. C14:2-carnitine	0.9(0.1)	0.69(0.08)	-33.3(4.8)[c]
7. C14:1-carnitine	1.8(0.2)	0.5(0.07)	-72.2(17.5)[c]
8. Propionyl carnitine	2.9(0.7)	0.9(0.1)	-69.0(18.9)[c]
9. Hexanoyl carnitine	2.1(0.95)	1.1(0.08)	-47.6(17.6)[c]
10. Octenoyl carnitine	1.3(0.8)	0.4(0.06)	-69.2(13.8)[c]
11. Octanoyl carnitine	2.7(0.75)	5.8(1.1)	114.8(33.9)[b]

% Control= {(Experimental value – Control value)/Control value x 100}; a = Units: mM/mmol creatinine; b = Statistically significant ($p< 0.05$); c = not statistically significant ($p>0.05$); n.d. = not detected; ∞: infinitely high value due to metabolite not being detected in controls; @: Ratio; All values expressed as Mean (± SEM).

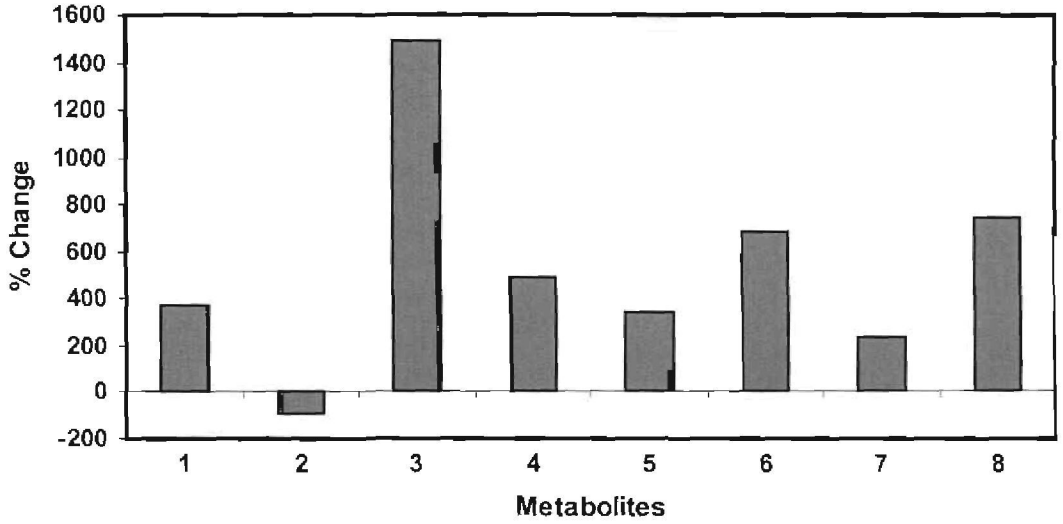

Figure 3.4: *DABA induced effects on selected citric acid cycle and glycolysis intermediates observed in the urine of pregnant female mice (Table 3.3).*

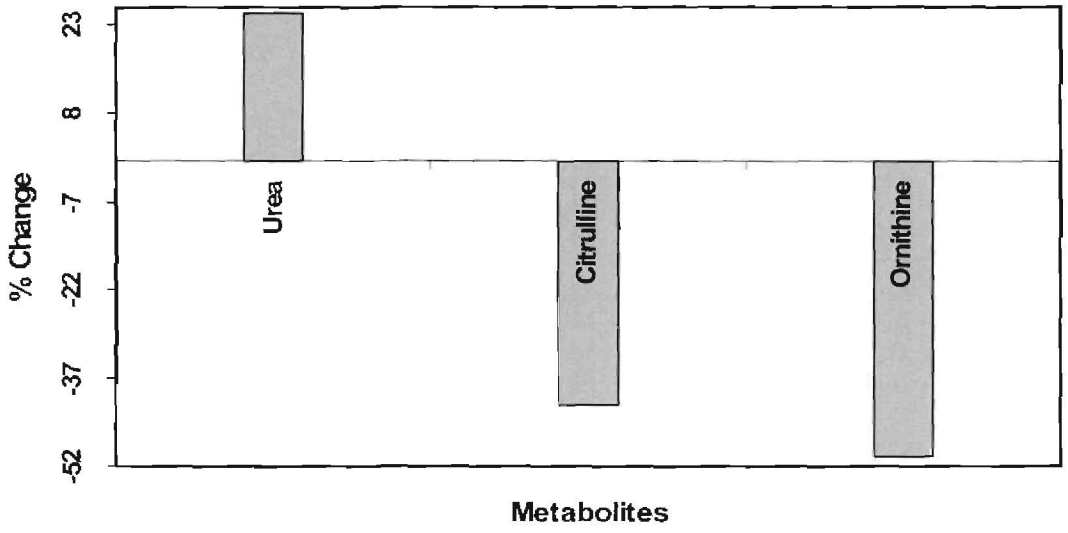

Figure 3.5*: DABA induced changes in selected urea cycle metabolites observed in the urine of pregnant female mice (Table 3.4).*

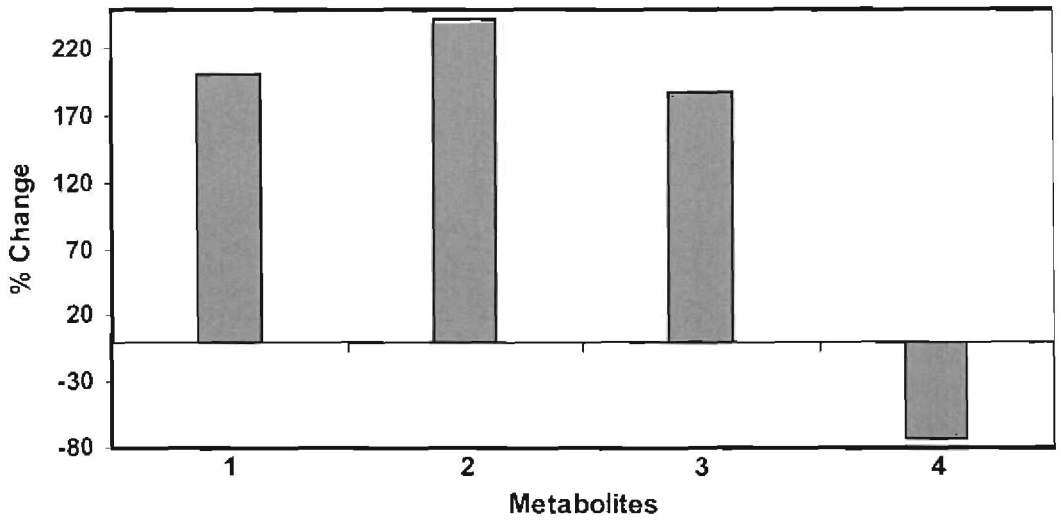

***Figure 3.6**: DABA induced changes in selected hydroxyacids observed in the urine of pregnant female mice (Table 3.5).*

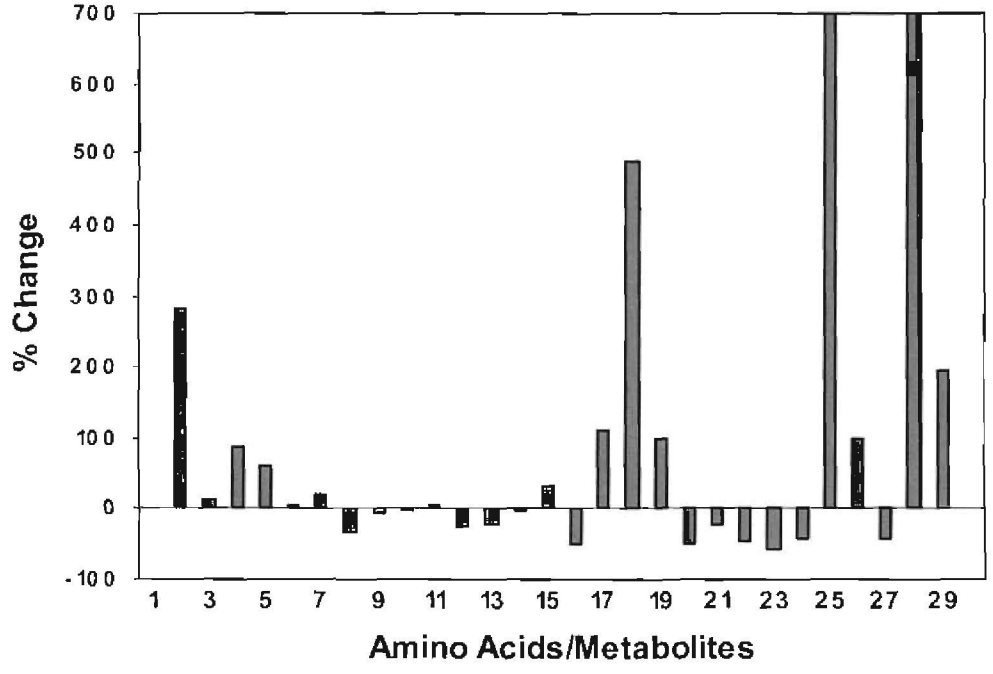

***Figure 3.7**: DABA induced changes in the urinary amino acid profile of pregnant female mice (Table 3.6).*

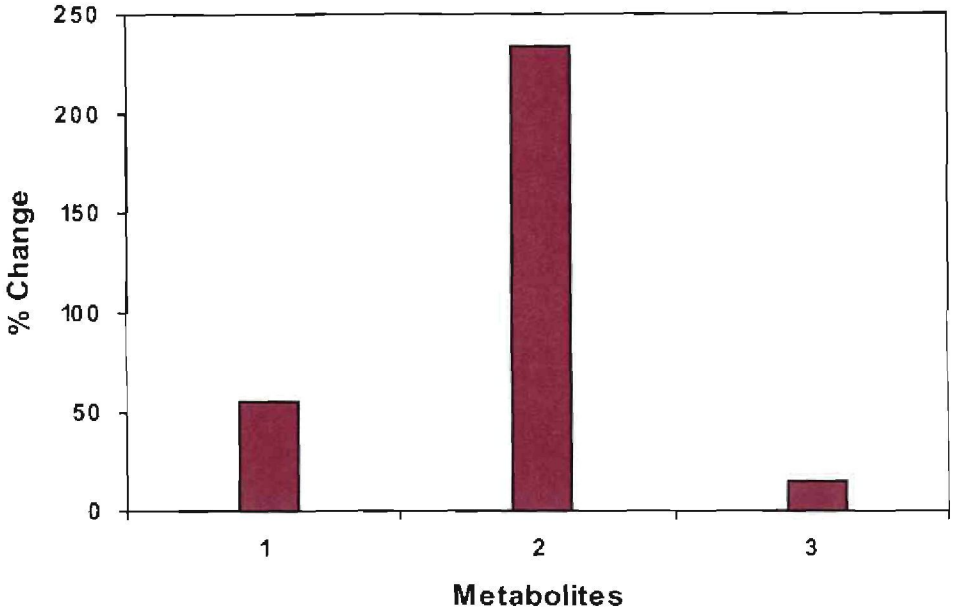

Figure 3.8: *DABA induced effects on selected dicarboxylic acids in the urine of pregnant female mice (Table 3.7).*

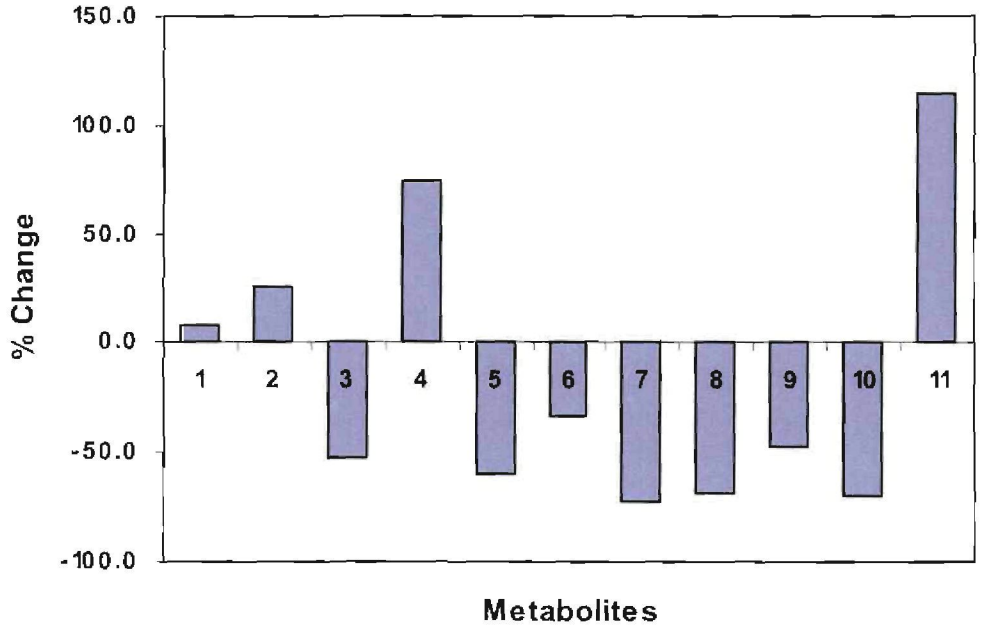

Figure 3.9: *DABA induced changes in the concentrations of free carnitine, acylcarnitines and the acylcarnitine/free carnitine ratio in the urine of pregnant female mice (Table 3.8).*

The effect of DABA on the metabolism of female Hanover.RPMI mice can be clearly observed in the abundance of metabolites present in GC chromatograms of the DABA treated females (Fig. 3.3), relative to that obtained for the control animals (Fig. 3.2). The vast number of metabolites and the relatively high concentrations of some of these metabolites in the urine samples indicate that DABA had left an enormous, though non-specific metabolic footprint on the general biochemistry of the DABA treated females and that a large number of different metabolic pathways were affected. Since it is virtually impossible to highlight and discuss all the observed changes in the scope of this thesis, only a select few of the most important changes will be summarised and briefly discussed.

The effect of DABA on selected glycolysis and citric acid cycle metabolites is represented in Table 3.3 and Fig. 3.4. Lactate appears to have accumulated (+373.2%; $p<0.05$) at the cost of pyruvate (-90.1%; $p>0.05$). Although the observed change in lactate appears to be significant, the low level of pyruvate detected - in both DABA treated and control animals - rendered the measured change in pyruvate statistically insignificant. However, in spite of the low pyruvate concentration all the selected citric acid cycle metabolites (cis-aconitate, malate, citrate, succinate, fumarate) appear to have increased significantly ($p<0.05$). Oxalate concentration has drastically increased (+1497.1%) to more than 14 times the levels observed in control animals. This hyperoxaluric condition is most probably caused by an inhibition of lactic dehydrogenase (LDH) and pyruvate carboxylase by oxalate (Hagler & Herman, 1973).

DABA is a known inhibitor of ornithine transcarbamylase (OTC), responsible for the conversion of ornithine to citrulline (Sections 1.1 and 1.3.4). It is therefore logical to anticipate changes in urea cycle metabolites, following exposure of experimental animals to systemic DABA. Table 3.4 and Fig. 3.5 briefly summarises the effect of DABA treatment on selected urea cycle metabolites, present in the urine of pregnant female mice. Only a relative small and statistically non-significant increase in urea (+24.8%; $p>0.05$) was observed as a result of DABA treatment. Both citrulline (-41.5%) and ornithine (-50.3%) were significantly decreased ($p<0.05$). Inhibition of arginase by DABA or derived metabolites may explain the lower citrulline level, while the decrease in ornithine may be attributed to the inhibition of ornithine decarboxylase by the toxic amino acid.

DABA induced changes in selected hydroxyacids are illustrated in Table 3.5 and Fig. 3.6. 2-Methyl-3-hydroxybutyrate, (200.6%), 3-hydroxybutyric (β-hydroxybutyric

acid; 243%) and 2-hydroxy butyric (187.1%) are all significantly elevated ($p<0.05$) in the urine of pregnant, DABA treated female mice. This observation may be indicative of increased ketosis due to an inhibition of carbohydrate metabolism by DABA or DABA metabolites. In contrast to the observed increase in the hydroxyacids, mentioned above, 2-hydroxyvaleric (-72.8 %; $p<0.05$) levels decreased as a result of DABA ingestion. Excess levels of all of these metabolites may indicate the presence of toxin induced metabolic ketosis or the presence of mitochondrial disorders.

The effect of DABA on amino acids and other nitrogenous metabolites in the urine of pregnant female mice, dosed with DABA, is summarised and depicted in Table 3.6 and Fig. 3.7 respectively. The Phenomenex EZ:faast® methodology demonstrated one unfortunate drawback - it failed to detect and quantify arginine, an essential amino acid and urea cycle intermediate. Statistically significant changes were observed in 62.1% of the selected amino acids and nitrogenous metabolites, while relatively small or no changes were observed in the rest (37.9%). Relatively large and statistically significant changes (±50% or more) were observed in sarcosine (+280.9%), valine (+87.7), isoleucine (+58.3%), cysteine (+109%), cadaverine (+488.8%), glutamine (-100%), ornithine (+-50.3%), histidine (-58.7%), phenylalanylproline (+∞%), cystathionine (-100%) and β-alanine (+194.8%). Although the changes are below 50% the effect on lysine (-46.5%) seems to be mirrored by a nearly equal change in α-aminoadipic acid (-48%), one of the catabolic products derived from lysine (Papes *et al.*, 2001). ß-Alanine is one of the main catabolic products of DABA metabolism (Section 1.3.4; Mushahwar & Koeppe, 1999). It is therefore possible that a large proportion of the excess ß-alanine may have been produced from DABA.

The effect of DABA ingestion on some important long-chain dicarboxylic acids is illustrated in Table 3.8 and Fig. 3.7. Adipate (+55.2%) and suberate (-234.4%) levels appear to be significantly elevated ($p<0.05$), but ethylmalonate (15.2%) was only slightly and non-significantly increased in DABA treated animals. Adipate and suberate are products of incomplete oxidation in the ω-oxidation pathway of long-chain fatty acids. All three intermediates may be elevated in cases of severe carnitine deficiency. The influence of DABA on the conjugation of acyl groups with carnitine is illustrated in Table 3.8. and Fig. 3.9.

Most of the selected carnitine conjugates, observed in the urines of DABA treated animals, were decreased. Our results seem to demonstrate a tendency of free

carnitine levels to increase (26%) as a result of DABA intoxication, while acyl carnitine levels (-8.6) and the acylcarnitine/free carnitine ratio decreased (-23%). Free carnitine levels increased by 26% in the urines of pregnant DABA treated mice, while the acylcarnitine levels decreased by 8.6%. None of these results were statistically significant ($p>0.05$). Significantly increased concentrations of acetylcarnitine (75.6%) and octanoylcarnitine (115%) were observed ($p<0.05$). Butyrylcarnitine (-60.2%), C14:2-carnitine (-33.3%), C14:1-carnitine (-72.2%) propionylcarnitine (-69%), hexanoylcarnitine (-47.6%) and octenoylcarnitne (-69.2%) were all non-significantly lowered in DABA-treated pregnant females than in control animals.

CHAPTER 4

THE EFFECT OF DABA ON ORNITHINE DECARBOXYLASE AND POLYAMINES METABOLISM IN PREGNANT MICE

4.1 INTRODUCTION

Ornithine decarboxylase (ODC) is recognised as an important biomarker for the risk of neoplasia (i.e. colorectal cancer) and ODC catalyzed putrescine biosynthesis is currently regarded as a reliable indicator for a number of different pathological and physiological processes (Harinder *et al.*, 1992; Sharma *et al.*, 1997). Studies with inhibitors of polyamine biosynthesis indicated that alterations in the intracellular concentration of polyamine levels can modulate the growth of normal and cancer cells and affect the embryonic and foetal development of most organisms (Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005; Bachrach, 2004; Moinard *et al.*, 2005; Seiler & Raul, 2005). Intracellular polyamine concentrations are regulated by cellular uptake mechanisms, the salvaging of polyamines from the diet, the *de novo* synthesis from amino acid precursors, intestinal production by symbiotic micro-organisms, as well as catabolic degradation and efflux (Fig. 1.5) The exact molecular mechanisms of polyamines in cellular processes (i.e. cell division, apoptotic cell death) are still unclear. Excessively high or low intracellular polyamine levels may alter their specific intracellular interactions and subsequently their physiological functions. The physiological significance of polyamine synthesis may be centralized in the ability of these compounds to control certain critical steps in the cell cycle and subsequently cellular survival (Seiler *et al.*, 1996; Morgan, 1999; Coffino, 2001; Seiler, 2004).

Polyamine biosynthesis (Fig. 1.7) is the combined result accomplished by four enzymes - ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), spermidine synthase (SpdS) and spermine synthase (SpmS). The first two of these enzymes are highly regulated, while the latter two are constitutively expressed. The biological half-lives of the two regulatory enzymes, ODC and SAMDC (5 - 60 min), are among the shortest known for mammalian enzymes. The unique properties of these enzymes enable cells to rapidly modulate cellular levels of the polyamines. Polyamine biosynthesis in the chicken embryo has been shown to be activated in the late cleavage phase and reaches maximum levels during gastrulation. α-Difluoromethylornithine (DFMO) imposed inhibition during the early up-regulation of ODC activity largely

prevents a rise in the concentrations of Put, Spd and Spm. Polyamine limitation during chick embryo morphogenesis has a severe outcome – embryonic development does not progress beyond the primitive streak stage (gastrulation). As a result, neurulation and somite formation are suppressed (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

Polyamine synthesis inhibitors affect the pre-implantation development of the mouse embryo, imposing serious consequences for embryonic development. The *in vitro* treatment of mouse embryos with methylglyoxal (bis)guanylhydrazone (MGBG) can cause diminished DNA synthesis and cell proliferation in mouse embryos during the 8-16-cell stage. At the right dose MGBG can cause complete inhibition of cavitation during blastocoel formation. MGBG treatment also slows down the mitotic rate very early during cleavage and the cells appear to be arrested in the interphase of the cell cycle. Although the rates of DNA synthesis and cell proliferation appear to decrease at an early stage following MGBG exposure, the rate of RNA synthesis decreases only after an extended treatment period. Inhibition of mouse embryo development is associated with a suppression of embryo growth and mass gain, because of a fall in the synthesis of DNA, RNA, and protein. Developmentally arrested embryos and surrounding decidual tissue is eventually resorbed or lost from the uterus and no sign of pregnancy persists. Polyamines play a fundamental role during gastrulation, the pivotal developmental process delineating the inception of embryonic autonomy, but are equally essential role players in other developmental processes. Sustainable increases in the biosynthesis of putrescine, spermidine and spermine therefore appear to be essential for embryonic development in all animals (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Wallace & Frazer, 2004; Seiler & Raul, 2005).

In normal, healthy cells ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine (Orn) to form putrescine (Put). This is the major rate-limiting step in the biosynthesis of naturally occurring polyamines (Section 1.3.3.4, Fig. 1.7). Putrescine is the basic precursor of the higher polyamines, spermidine (Spd) and spermine (Spm). ODC requires pyridoxal-5'- phosphate (vitamin B_6) for its catalytic activity and as a PEST sequence protein, has an extremely short half-life (~20 minutes). In rapidly dividing cells, i.e. embryonic, gut mucosa and tumour cells, the expression of ODC and its subsequent intracellular activity are equally rapidly and significantly elevated. However, in the latter case scenario the catalytic activity of ODC does not appear to be the rate limiting step in

polyamine biosynthesis. ODC is effectively regulated on the transcriptional level (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Seiler & Raul, 2005).

The effect of DABA on the metabolism of female Hanover.RPMI mice is obvious from the abundance of metabolites present in GC chromatograms of DABA-treated females (Fig. 3.3), compared to control animals (Fig. 3.2). The vast number of metabolites and the relatively high concentrations of some of these metabolites in the urine of DABA-treated pregnant female mice clearly demonstrate that DABA had left an enormous, though non-specific metabolic footprint on the general biochemistry of DABA-treated females. Significant effects on important metabolic pathways (glycolysis, tricarboxylic acid cycle, urea cycle, etc.) were observed (Chapter 3, Section 3.6). Citrulline and ornithine levels were significantly lower in DABA-treated pregnant females mice. Cadaverine (diaminopentane), a homologue of putrescine and decarboxylation product of L-lysine appeared to be nearly five times higher in the DABA-treated females than in the control animals. Since DABA is an ornithine homologue it may be able to act as a potential antagonist of ornithine metabolism and subsequently also of polyamine biosynthesis. As an ornithine analogue DABA may therefore be regarded as a potential inhibitor of ODC activity on its natural substrate (Orn) and subsequently on polyamine metabolism.

In the course of this investigation we have been able to demonstrate that DABA can induce congenital defects (i.e. neural tube defects) in developing mouse embryos and that it can modulate numerous biochemical processes in the pregnant mothers. The focus of this part of the investigation was to determine the effect of DABA on ODC activity and polyamine metabolism. We wanted to investigate the notion that systemic exposure to DABA may be able to affect polyamine biosynthesis by modulating the level of polyamines in tissue (i.e. liver, embryo) and urine samples collected from DABA-treated pregnant females.

4.2 Standardization and optimisation of a spectrophotometric assay of the assessment of ODC activity.

4.2.1 Principle of the assay:

ODC can be detected and its catalytic activity quantified with a variety of methods, i.e. manometry (Umbreit *et al.*, 1951), radiometric analyses (Clark, 1976; Maderdrut & Oppenheim, 1978; Djurhuus, 1981; Harinder *et al.*, 1992; Tabib, 1998), GC analyses (Lambert & Moss, 1973), HPLC analyses (Haraguchi *et al.*, 1980; Beeman &

Rossomando, 1989) and coupled enzyme reactions with colorimetric detection (Scoccianti *et al.*, 1990; Badolo *et al.*, 1998). The measurement of CO_2-release as a function of ODC activity by means of a manometric assay, was one of the first procedures to be developed (Umbreit *et al.*, 1951), followed by a radiometric $^{14}CO_2$-release assay with 1-^{14}C-ornithine as substrate (Clark, 1976). Carbon dioxide release methodologies are notoriously unreliable and investigators reported variations in ODC activity as high as 10 to 20-fold. Following a large number of unsuccessful trials with fluorometric HPLC (dansylchloride as fluorochrome) and radiometric assays (1-^{14}C-ornithine as substrate), we decided to employ one of the most recently published spectrophotometric procedures, purported to demonstrate high levels of specificity, sensitivity and reliability (Badolo *et al.*, 1999). The selected ODC assay employs a coupled enzyme reaction, executed in 3 steps {(a) → (c): Fig. 4.1}.

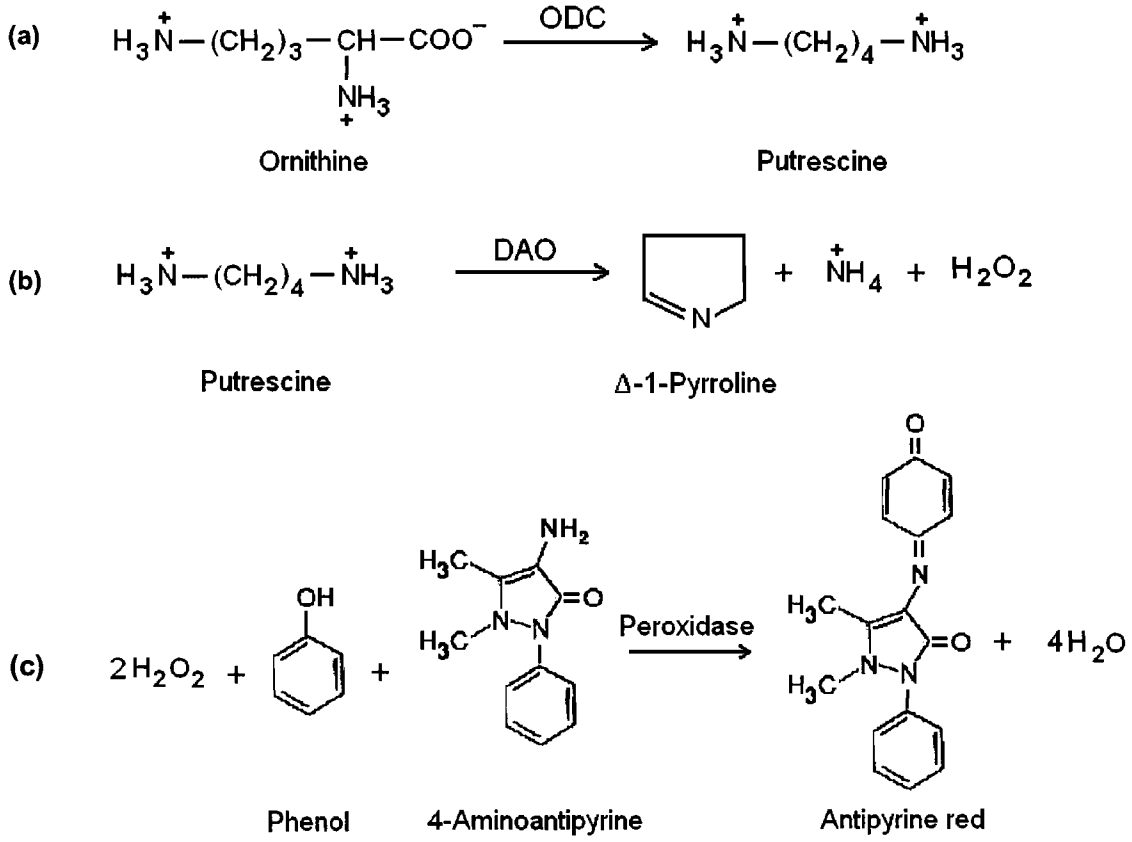

***Figure 4.1**: Schematic representation of the reactions involved in the quantitative measurement of ODC activity in the livers of non-pregnant and pregnant females and whole embryos. Females were dosed with DABA or saline: (a) Orn is decarboxylated by ODC; (b) Put is oxidised by DAO: Δ-1-pyrroline, ammonia and hydrogen peroxide are produced; (c) Peroxidase releases oxygen from hydrogen peroxide; liberated oxygen, phenol and 4-amino-antipyrine react and antipyrine red (λ_{max} = 485 nm) is produced.*

Putrescine (Put), produced from L-ornithine (Orn) by the catalytic activity of ODC {step one; (a)} is oxidised by diamine oxidase (DAO; porcine kidney), yielding Δ-1-pyrroline (condensed from the putrescine aminoaldehyde), ammonia and H_2O_2 {step two; (b)}. Peroxidase (horse radish) releases oxygen from hydrogen peroxide and in a complex chemical reaction between the liberated oxygen, phenol and 4-amino-antipyrine, a strongly coloured product, antipyrine red (λ_{max} = 485 nm) is produced {step three; (c)}.

4.2.2 The assay of ODC activity in maternal livers and whole embryos

(a) Chemicals employed in experiments:

- Diamine oxidase (DAO) purchased from Separations (Johannesburg, South Africa);
- Horse raddish peroxidase, (HRP) obtained from Roche Diagnostics (California, USA);
- Phenol purchased from Merck Chemicals (Darmstadt, Germany);
- 4-Aminoantipyrine obtained from Sigma-Aldrich Co. (St. Louis, USA);
- Sodium monobasic phosphate and tris–(hydroxymethyl)aminoamethane were bought from BDH Chemicals (Mid Rand, South Africa);
- Putrescine and Ornithine hydrochloride were obtained from Sigma Aldrich (St. Louis, USA);
- Pyridoxal phosphate was purchased from Sigma-Aldrich Co. (St. Louis, USA).
- Bio-Rad® protein analysis reagent.

(b) Preparation of hepatic and foetal cytosols:

Livers and foetuses, collected and stored (-70 ^{0}C) during the experiments designed to explore the potential teratogenic nature of DABA were used in this part of the investigation (Chapter 2). Some of the experiments executed in the course of the ODC studies required the use of freshly harvested livers from non-pregnant females.

Frozen livers were thawed on ice (0 - 4 ^{0}C), weighed and suspended in nine volumes of ODC buffer (20 mM phosphate buffer, 0.2 M EDTA, 0.1 mM PLP; pH 9.0). Freshly harvested mouse livers were removed, weighed and immediately placed in ice-cold (0 - 4 ^{0}C) ODC buffer, prior to homogenization (Fig. 4.2). The livers were finely minced with a scalpel and homogenized (Potter Elvehjem homogenizer). Homogenates were cooled on ice during the preparation and then centrifuged at 600 × *g'* for 10 minutes (4°C). The 600 x *g'* pellet, containing nuclei and cell debris, was discarded and the 600 x *g'* supernatant re-centrifuged at 9500 × *g'* for 25 minutes at 4 °C. The 9500 x *g'* supernatant fraction

was immediately placed on ice or stored frozen at -70^{0}C until the catalytic activity of the cytosolic ODC could be assayed.

Aliquots (100 µl) of the cytosols were removed and appropriately diluted for quantification with the Bio-Rad® microplate protein assay. The Bio-Rad® protein assay, based on the method of Bradford (1976) is a simple and accurate procedure for determining the concentration of solubilized protein. The principle behind the assay is based on involves the adsorption of an acidic dye (Coomassie® Brilliant Blue G-250) to protein in solution. Subsequent measurement of optical density (O.D.) at 595 nm (spectrophotometer, microplate reader) renders O.D. values directly proportional to the concentration of protein in solution. Comparison of values obtained for the protein solution (i.e. hepatic cytosol) to a standard curve (i.e. bovine serum albumin; BSA) provides a relative measurement of protein concentration. The Coomassie blue dye binds to primarily to basic and aromatic amino acid residues, especially arginine. The extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range (Spector, 1978). Beer's law can therefore be applied for the accurate quantification of protein. Interferences may be caused as a result of interactions between the chemicals and the protein or between the chemicals and the dye.

Five dilutions of the BSA protein standard (1.2 - 10 µg/ml) were prepared. Standard and cytosol sample solutions were pipetted into clean, dry test tubes. Bio-Rad® dye reagent concentrate (200 µl) was added to each tube and vortexed. The reaction mixtures contained samples and standards respectively were incubated at room temperature for at least 15 minutes. Protein solutions were assayed in duplicate. Absorbance was measured in a Biotek microplate reader at 595 nm. The data was plotted and the graph (Fig. 4.5) used to calculate the protein concentration of the hepatic and whole embryo cytosols.

(c) Executing the ODC assay:

ODC activity was measured as a function of the concentration of Put produced as a result of the decarboxylation of Orn. The concentration of Put was measured as a function of the amount of H_2O_2 released following the *in situ* oxidation of putrescine by DAO.

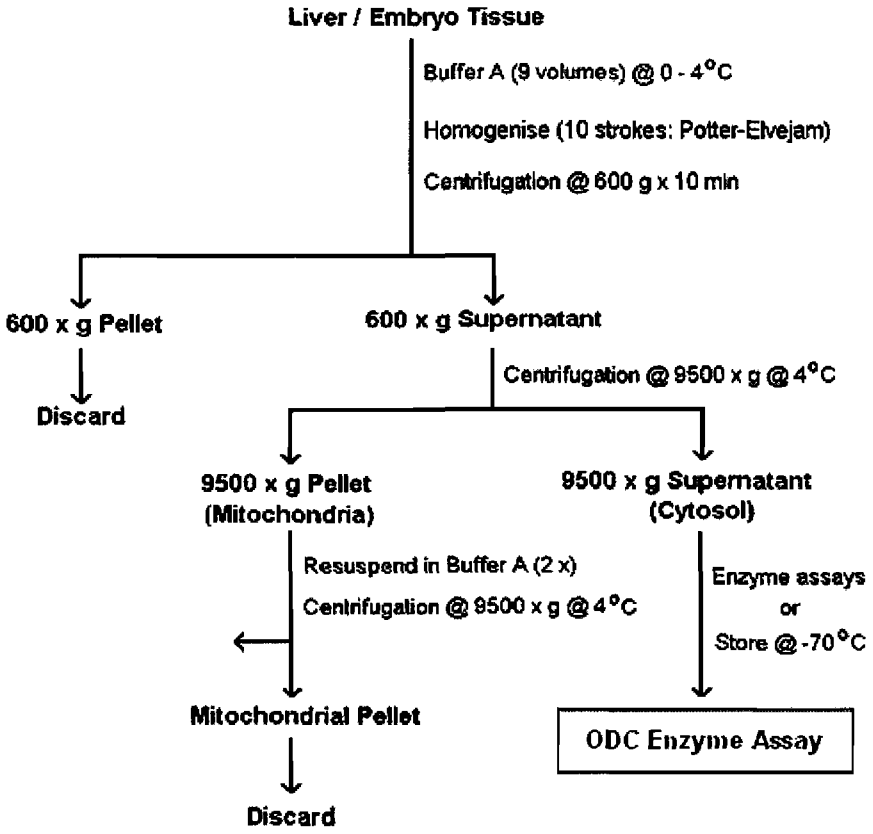

Figure 4.2: *Flow diagram for the preparation of cytosols from maternal liver and whole embryo tissue, prior to execution of the coupled enzyme assay for ODC.*

Assays were performed in clean, sterile 8 x 12 matrix microplates on a Biotek Microplate reader (485 nm). Each well contained 230µl of the reaction mixture (ODC containing cytosol, 50 µg phenol, 40µg aminoantipyrine, 2.2 mg DAO, 5 µl HRP suspension, L-ornithine (Orn). Orn concentrations varied from 1 - 50 nM. DABA, Put, Spd and Spm were employed as alternative substrates (1 nM - 50 nM). Enzyme reactions were initiated with the addition of the substrate and allowed to run for 40 minutes at 37 ^{0}C. The specific activity of the ODC enzyme was calculated, using the equation below:

Where

$$\text{ODC activity} = \frac{(A_2 - A_1)}{\varepsilon_\lambda} \times \frac{CF}{TP}$$

- A_2 = the absorbance reading of the sample
- A_1= the absorbance of the blank
- ε_λ = the molar absorption coefficient
- TP = the total amount of protein (mg) in the reaction mixture
- CF = Correction factor
- Enzyme units: nmol/mg protein/min

4.3. Standardization of the coupled enzyme assay for ODC activity

ODC analyses were performed as previously described by Badolo *et al.* (1999). The method was first standardized for optimum performance in our laboratory.

4.3.1 Verification of ODC activity in assay samples

It was necessary to verify that the coupled enzyme assay (Fig. 4.1) actually measured the catalytic activity of ODC and that the observed 485 nm absorbance was not an artefact of the release/presence of H_2O_2 by some other unknown process. At first a cytosol was prepared from the liver of a non-pregnant female (Fig. 4.2) and then subjected to a coupled enzyme assay using the following protocol: The freshly prepared cytosol sample was volumetrically divided into three separate samples: one part sample was stored on ice (0 - 4 ^{0}C), the second part was first boiled in hot water (~96 ^{0}C) for 15 minutes to denature the enzyme and then put back on ice with the non-boiled sample to cool down to 0 - 4 ^{0}C. The third part sample was immediately frozen and stored at -70 ^{0}C for two weeks. Both of the first two samples were thouroughly vortexed and analysed for ODC. The third sample was thawed, re-vortexed and analyzed two weeks later. The necessary buffer blanks were included in all of the assays. All assays were executed in duplicate. The outcome of this experiment is illustrated in Fig. 4.3 and the mean values of the results clearly corroborated that ODC activity can be reliably assessed with the coupled enzyme assay. The sample, stored for two weeks at -70 ^{0}C, appeared to have lost approximately 15.4% of the initial ODC activity, while the denatured sample lost 100% activity.

4.3.2 Optimizing the protein concentration for the ODC assay

The optimal protein content for individual assays was determined by incubating a linear range of increasing volumes of hepatic cytosol, produced from a non-pregnant female, at the same time diminishing the amount of buffer added to the reaction mixtures. Alternatively a linear range of various dilutions of the cytosol, containing a linear range of the protein concentration was first prepared and the diluted cytosols used for the assay. The final protein content in the reaction mixture varied from 0.25 -12.0 mg protein/microplate well. All assays were executed in duplicate and protein content of undiluted and diluted samples was measured using the microplate Bio-Rad assay. A linear increase in the absorbance units (AU_{485nm}), as a function of increased ODC activity in the cytosolic fraction was observed. This result is depicted in Fig. 4.3.3.

4.3.3 Enzyme-kinetic properties of ODC

The kinetic properties of ODC (K_m, V_{max}) were ascertained, using the coupled enzyme assay (see Fig. 4.1). Freshly prepared hepatic cytosol, from a non-pregnant female was assayed for ODC activity, with Orn as substrate at seven (7) different concentrations (0 - 6 mM). The effect of substrate concentration on reaction velocity was measured and graphically analysed. The enzyme kinetic results are presented in Fig. 4.4 (Michaelis-Menton plot) and Fig. 4.5 (Lineweaver-Burke plot). The K_m value for of ODC for Orn as substrate was calculated to be 1.02 mM and the V_{max} under these conditions was 0.58 nmol Put produced per min.

4.3.4 Precision and accuracy of the coupled enzyme ODC assay

The reliability of an analytical method is a function of its precision and accuracy. To determine the precision of the coupled enzyme assay for ODC activity, eight (8) assays were simultaneously executed on the same sample of cytosols, previously prepared from the liver of a pregnant female and a whole embryo (gestation day 18) and stored at -70 ^{0}C for up to one month. Assay conditions were similar to that described in Section 4.2.2(c). The accuracy of an enzyme reaction is difficult to measure and variation can be very high. It was therefore decided to use only precision data as an indicator of assay reliability. The results of the precision experiment are depicted in Table 4.1. Relatively low % coefficients of variation (%CV) of between 9.4% and 10.3% were obtained for assays on maternal liver and whole embryo cytosols respectively, relating to a precision of between 89.7% and 90.6% for the coupled enzyme assay for ODC in these matrices.

4.3.5 Linearity of the coupled enzyme ODC assay for the measurement of putrescine concentrations in reaction mixtures.

As mentioned before in Section 4.3.4 above, Orn is the natural substrate for ODC, while Put is the product of decarboxylation. Effective conversion of Put, produced by ODC, into detectable levels of H_2O_2 is critical for the quantification of ODC activity. DAO in the assay is specific for diamines like Put and necessary for the quantitative oxidation of Put to Δ-1-pyrroline and H_2O_2 {step (b)}. The linearity of steps 2 and 3 of the coupled enzyme assay for hepatic cytosolic ODC is therefore important for the accurate measurement of Put concentration and subsequently for the assessment of ODC activity. In these assays no Orn was added to reaction mixtures. Put was employed as the only "substrate" for the coupled enzyme reaction in concentrations varying between

0.5 - 10 nM. The coupled enzyme assay was performed as described before (Section 4.2.2(c)) and each assay was duplicated. The outcome of this experiment is depicted in Fig. 4.9. The coupled enzyme assay proved to be linear for the measurement of Put (R = 0.999).

4.3.6 *In vitro and in vivo* effects of DABA on ODC activity in maternal liver and whole embryo cytosols.

Results obtained in the course of this investigation indicated that the systemic exposure of pregnant female mice to DABA (450 - 1800 mg DABA/kg body mass; day 7 to 9) can lead to a disruption of normal embryonic development in some Hannover.RPMI embryos. Congenital defects, i.e. neural tube defects (NTD) occurred (Chapter 2, Section 2.3.3.1) as a result of the *in utero* exposure of the developing embryos to DABA and DABA metabolites, present in the blood and other biological fluids of the mothers. Metabolic profiling of the urines of DABA-treated mothers indicated that DABA caused serious disturbances in some metabolic pathways (Chapter 3). Since DABA is a structural homologue of Orn, the notion that it may also be an inhibitor of ODC was investigated.

The *in vitro* effect of DABA on the catalytic activity of ODC in hepatic cytosol of a non-pregnant female was first determined. Fresh hepatic cytosol samples were prepared (Fig. 4.2) and the cytosols then subjected to a coupled enzyme assay to measure ODC activity in the absence or presence of three different concentrations of DABA (1, 10 and 100 mM). DABA was added to the reaction mixture on ice (0 - 4 ^{0}C), immediately before the reaction mixture was initiated by the addition of Orn and incubated at 37 ^{0}C for 40 minutes. Buffer was added to control samples to determine the uninhibited activity level of hepatic ODC. The result of the *in vitro* inhibition experiment is illustrated in Fig. 4.10. Significant inhibition of the hepatic ODC (69.0-90.5%) was observed at all the concentrations of DABA employed in the experiment.

The *in vivo* effect of DABA (total dose: 1350 mg/kg body mass) on the activity of ODC in the mice was assessed by measuring the degree of suppression of ODC activity in cytosols prepared from the livers (controls = 8; DABA-treated = 10) and whole embryos (controls = 21; DABA exposed = 30), harvested from pregnant Han.NMRI mice on gestation day 18 (Section 2.3.3.1). On day 18 of gestation the mothers were sacrificed and embryos harvested. Cytosolic samples were prepared as described in Fig. 4.2 and Section 4.2.2(b). The protein content of the cytosolic fractions was determined with the

Bradford microplate assay (Bradford, 1976). The outcome of this experiment is depicted in Fig. 4.11. Both hepatic and whole embryo ODC appeared to be significantly inhibited (69 - 90.5 %; $p<0.0001$) due to the systemic effects of DABA.

4.4 Results:

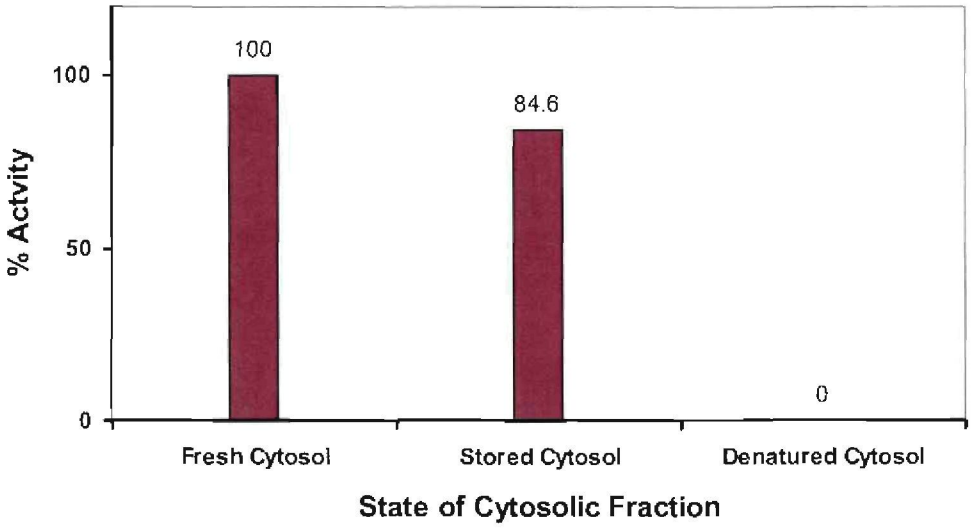

Figure 4.3: *ODC activity in the hepatic cytosol of a non-pregnant female:* ***Fresh cytosol****: stored on ice (0 - 4 ^{0}C) for 15 - 20 min after preparation, prior to assay;* ***Stored Cytosol****: freshly prepared cytosol frozen and stored at -70 ^{0}C for 14 days, prior to assay;* ***Denatured Cytosol****: freshly prepared cytosol boiled (96 ^{0}C) for 15 min and cooled down(0 - 4 ^{0}C) prior to assay (Section 4.3.1).*

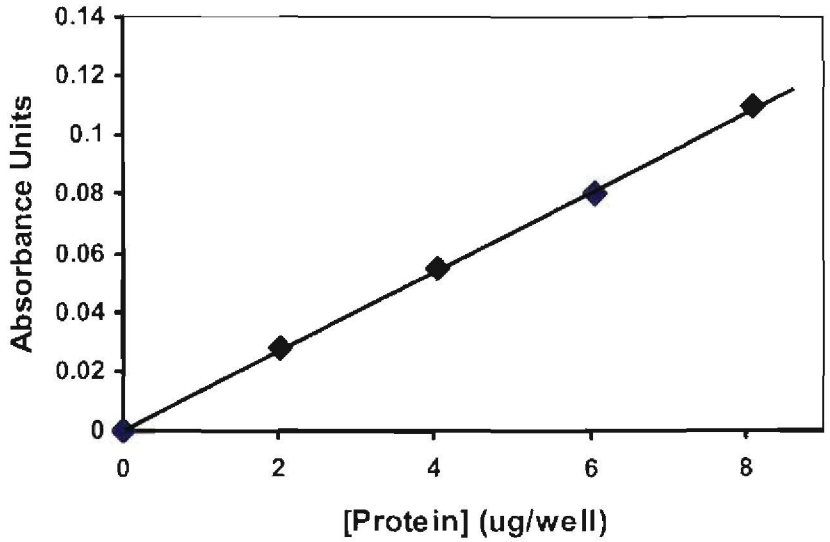

Figure 4.4: *Absorbance at 485 nm due to ODC activity in the hepatic cytosol of a non-pregnant female as a function of increasing protein concentration (Section 4.3.2).*

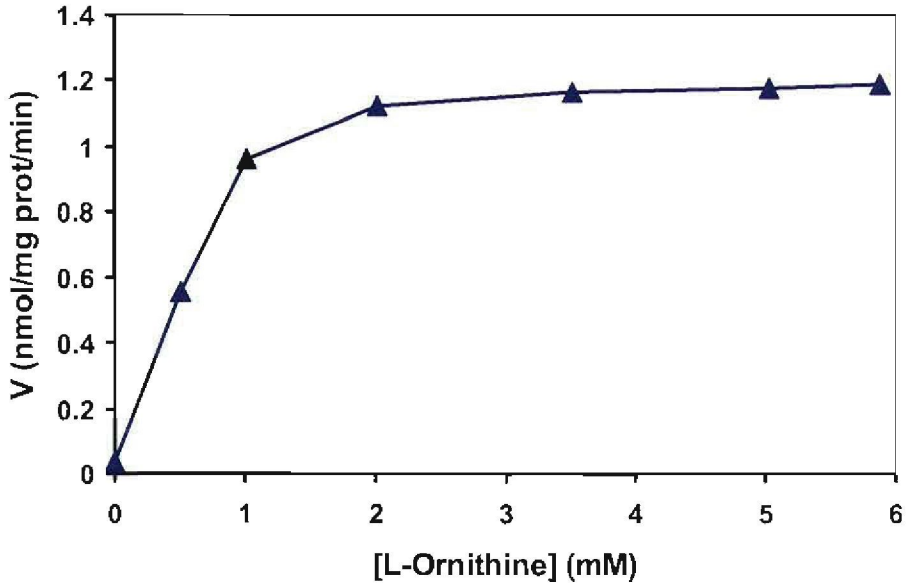

Figure 4.5: *Michaelis-Menten plot of ODC activity in the hepatic cytosol of a non-pregnant female: L-ornithine as substrate. Specific activity of ODC expressed as nmol putrescine produced per mg cytosolic protein per minute (Section 4.3.3).*

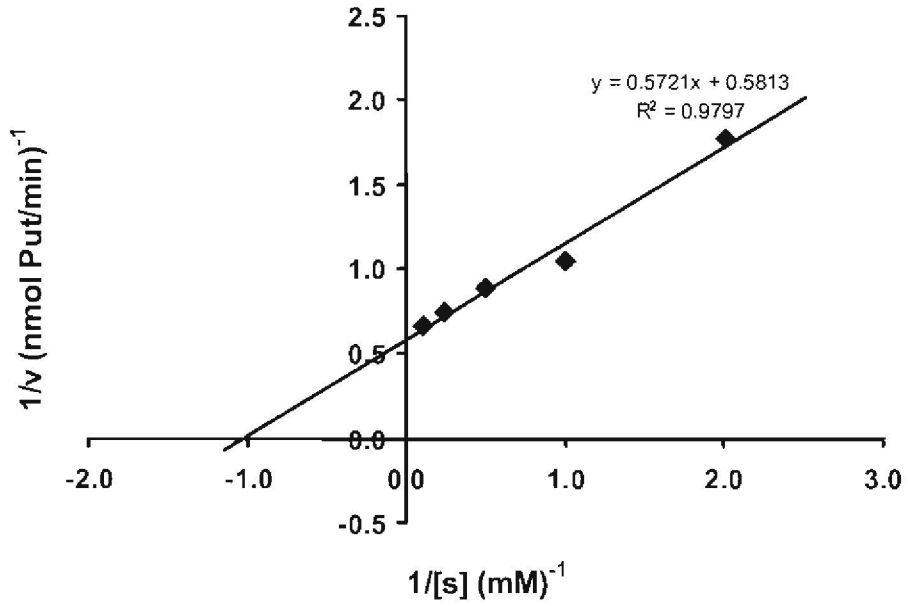

Figure 4.6: *Lineweaver-Burke plot of ODC activity in hepatic cytosol of non-pregnant female mouse, with L-ornithine as substrate. Derived from Michelis-Menton plot (Fig. 4.4); Km =1.02 mM (Section 4.3.3).*

Table 4.1: *Precision of the ODC assay, executed on cytosolic fractions from a pregnant female liver and whole embryo at gestation day 18 (Section 4.3.4).*

Assay Nr.	Specific Activity of ODC (nmol/μg protein/min)	
	Hepatic Tissue	Whole Embryo
1	91.0(7.0)	99.7(10.5)
2	110.4(9.4)	115.8(8.1)
3	98.6(6.2)	97.6(9.6)
4	106.2(8.4)	119.3(11.6)
5	101.0(10.2)	106.3(11.1)
6	86.9(7.8)	111.9(9.6)
7	94.6(5.8)	92.1(8.5)
8	118.4(8.2)	97.0(9.1)
Mean:	**100.9(7.9)**	**105.0(9.8)**
SD:	**10.4**	**9.9**
%CV:	**10.3**	**9.4**

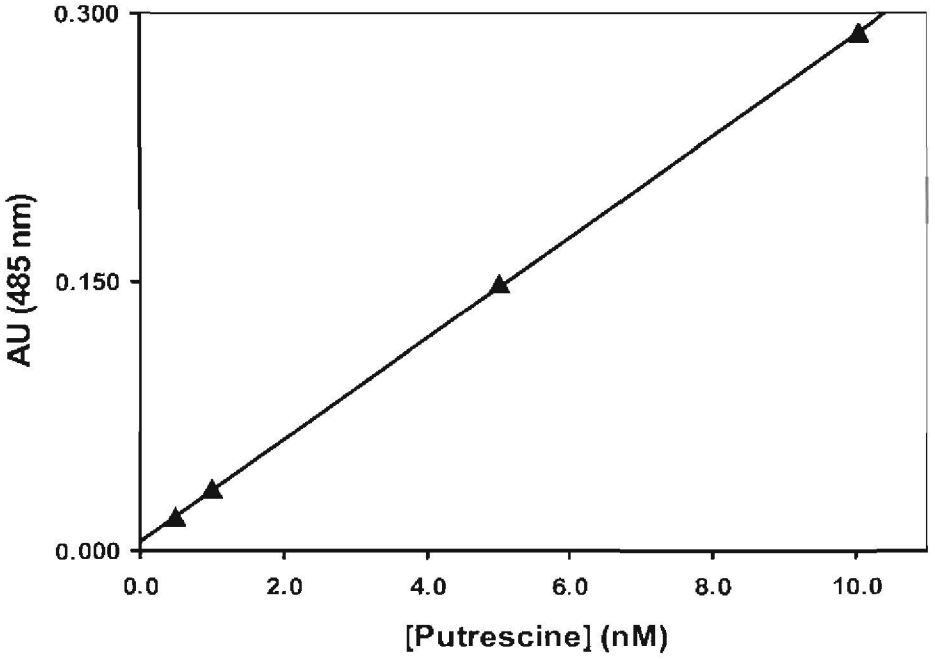

Figure 4.7: *Linearity of steps 2 and 3 (DAO oxidation of Put) of the coupled enzyme assay for hepatic cytosolic ODC: Assessment of Put concentration. Orn was not added to reaction mixtures; Put was employed as an apparent substrate for the reaction (Section 4.3.5).*

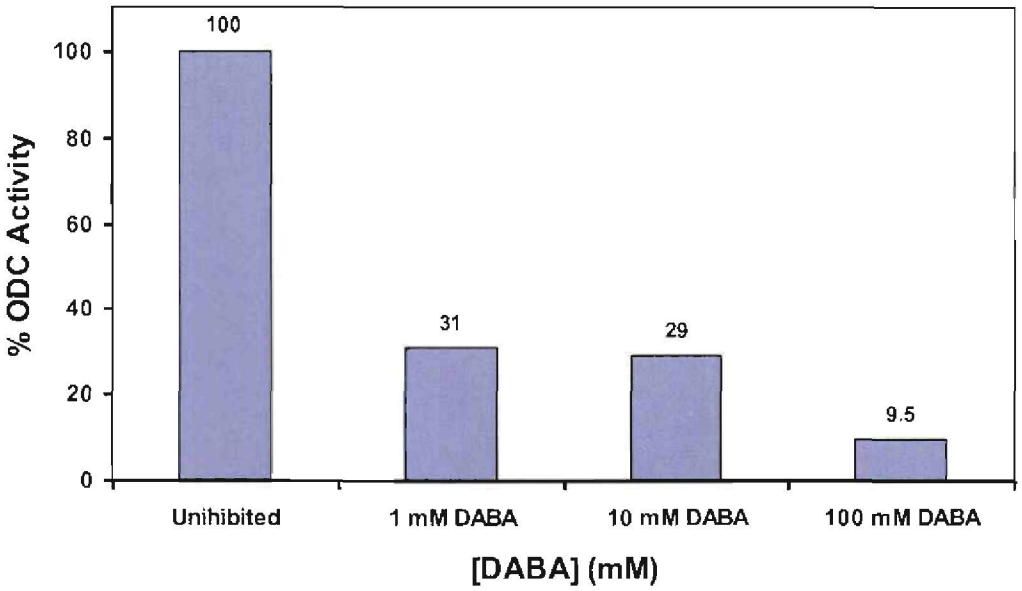

Figure 4.8: *Effect of DABA on hepatic ODC activity in a cytosol, prepared from the liver of a non-pregnant female mouse. DABA (1, 10,100 mM) was added to the reaction mixture on ice (0 - 4 0C), immediately before the reaction was initiated by the addition of Orn (Section 4.3.6) and incubated at 37 ^{0}C for 40 min (Section 4.3.6).*

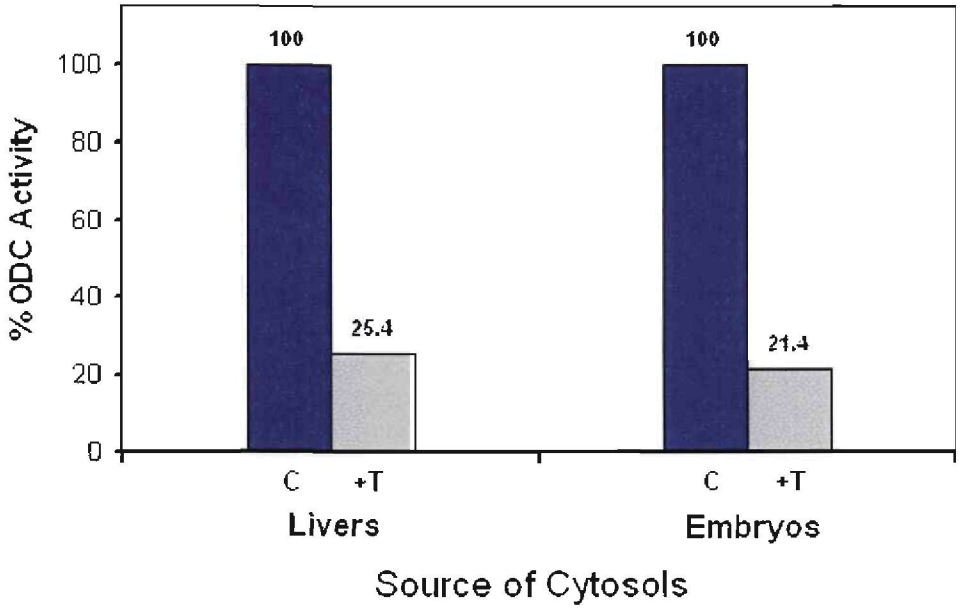

Figure 4.9: *The effect of DABA (1350 mg/kg body mass) on ODC activity in the livers and whole embryos of pregnant females at gestation day18. DABA (1, 10,100 mM) was added to the reaction mixture on ice (0 - 4 0C), immediately before the reaction was initiated by the addition of Orn (Section 4.3.6) and incubated at 37 ^{0}C for 40 min: C = control livers (n = 8), control embryos (n = 10); +T = DABA exposed livers (n = 20), DABA exposed embryos (n = 30) (Section 4.3.6).*

4.4.1 Brief summary of results:

The coupled enzyme assay for ODC appeared to perform well under the conditions employed in the study and ODC activity could be reliably assessed with this assay. Cytosolic samples appear to lose some of the initial ODC activity (15.4%) after two weeks of storage in a freezer at -70 ^{0}C (Section 4.3.1; Fig. 4.3).

ODC activity appeared to be directly proportional to cytosolic protein concentration in the region of 0.25 - 12 mg protein per microplate well. Enzyme activity was completely lost, following denaturation in boiling water (96 ^{0}C) for 15 min (Section 4.3.2; Fig. 4.3). This experiment proved that the proportional increase in absorbance (AU_{585nm}) was a function of ODC activity.

Enzyme kinetic results are presented in Fig. 4.4 (Michaelis-Menton plot) and Fig. 4.5 (Lineweaver-Burke plot). The K_m value for of ODC for Orn as substrate was calculated to be 1.02 mM and the V_{max} under these conditions was 0.58 nmol Put produced per min (Section 4.3.3; Fig. 4.5 & Fig 4.6)

Relatively low % coefficients of variation (%CV) of between 9.4% and 10.3% were obtained for assays on maternal liver and whole embryo cytosols respectively, relating to a precision of between 89.7% and 90.6% for the coupled enzyme assay for ODC in these matrices (Section 4.3.4; Table 4.1). The coupled enzyme assay proved to be linear for the measurement of Put (R = 0.999) (Section 4.3.5; Fig. 4.7).

Significant *in vitro* inhibition of the ODC (69.0-90.5%) was observed at all the concentrations of DABA employed. Both hepatic and whole embryo ODC appeared to be significantly inhibited (69 - 90.5 %; $p<0.0001$) due to the systemic effects of DABA and/or DABA metabolites (Section 4.3.6; Fig. 4.8 & 4.9)

4.5 Quantification of total polyamines in maternal hepatic tissue, whole embryos and urine of pregnant female mice.

Inhibition of ODC activity will affect polyamine concentration in cells, tissues and organs. An imbalance in the otherwise intricately controlled polyamine metabolism can pose severe consequences for organisms (Heby, 1981; Khuhawar *et al.*, 2001; Seiler, 2004; Wallace & Frazer, 2004; Seiler & Raul, 2005). Embryonic development can also be severely affected by the presence of either too high or too low concentrations of selected polyamines during critical stages in the cell cycle and other important processes.

We successfully illustrated that DABA, an inhibitor of ODC (Section 4.3.6), can cause major metabolic disturbances (Chapter 3) and induce congenital defects in a relatively large proportion of DABA-exposed embryos (Chapter 2). It was deemed important to ascertain if polyamine concentrations in DABA-treated pregnant females and subsequently their embryos, were concomitantly affected. Systemic changes in polyamine concentrations may be reflected in the levels of polyamines detected in the urine of DABA-treated pregnant females (Matsumoto *et al.*, 1981; Bachrach, 2004). The purpose of the last part of this investigation was therefore to measure changes in the total concentration of polyamines in the livers, whole embryos and urine samples of pregnant females as a function of systemic DABA exposure.

The four main polyamines, Put, Spd, Spm, Cad and their N-acetylated conjugates can be analysed by a plethora of methodologies (Matsumoto *et al.*, 1981; Otsuji *et al.*, 1985; Jiang, 1990; Inoue *et al.*, 2005; Seiler & Raul, 2005). Most of the methods endeavour to separate and quantify the individual members of the polyamine family, prior to ascertaining the concentrations of individual members. A previous study in our lab revealed that potential inhibitors of one-carbon and subsequently polyamine metabolism can severely depress Put levels, rendering it nearly impossible to measure this analyte precisely and accurately (Louw, 2005). We therefore decided to determine the effect of DABA on total polyamine concentration in the urines of pregnant females dosed with DABA during the investigation of the teratogenic potential of this toxic amino acid.

4.5.1 Principle of the total polyamine assay:

Putrescine is the precursor of all polyamines. Because most of it is so rapidly converted to higher polyamines (i.e. Spd, Spm) and their N-acetylated conjugates., Put is usually present in very low in concentrations in tissues and biological fluids (Seiler, 2004; Seiler & Raul, 2005). To determine the total polyamine content of a tissue or body fluid the method of choice must be capable of quantatively converting all polyamines and their N-acetylated conjugates to free Put and polyamines.

In principle N-acetylated polyamines, present in the samples were first hydrolysed by hydrochloric acid to render the unconjugated (free) polyamines. The free polyamines were then differentially oxidized by two amine oxidases, i.e. maize polyamine oxidase (MPAO) and diamine oxidase (DAO). MPAO is specific for the higher polyamines Spm and Spd, converting these higher polyamines to Put and H_2O_2., DAO targets Put and

from that reaction Δ-1-pyrroline and H_2O_2 are produced (Fig. 4.1). Phenol, 4-amino-antipyrine and the released H_2O_2 react in the presence of horse radish peroxidase (HRP) to form the coloured antipyrine red solution exhibiting a λ_{max} at 485 nm (Fig. 4.1).

Matsumoto *et al.* (1981) used a soybean seedling amine oxidase (SSAO) in their assay of total polyamines in the urine of cancer patients. This enzyme nor any equivalent replacement enzyme was commercially available and had to be freshly isolated from a rich source, such as maize seedlings. Maize seeds (*Zea Mays*) were wrapped in wet brown paper dolls, the folded paper dolls packed tightly into a plastic bag and then incubated in the dark for 10 days at room temperature. The seedlings were collected and processed as described in the flow diagram below (Fig. 4.10).

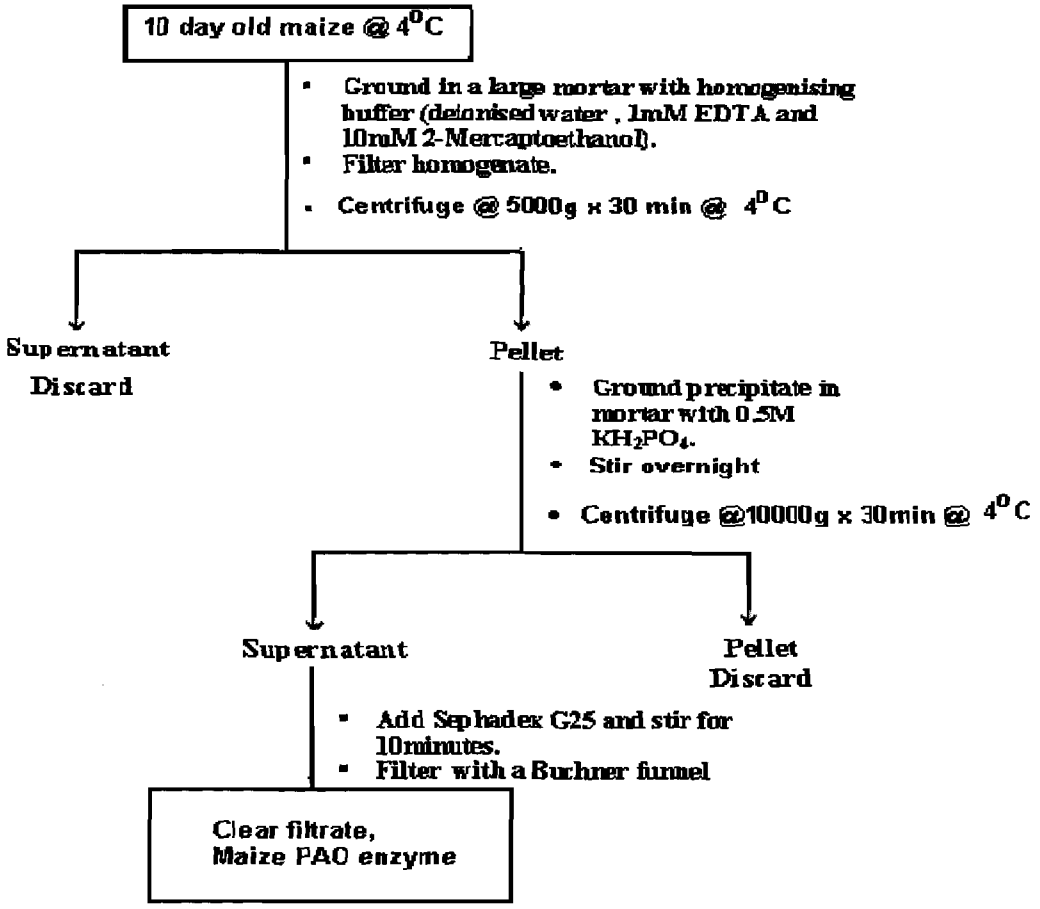

Figure 4.10 *Isolation of the maize polyamine oxidase (MPAO) from Zea mays seedlings (Matsumoto et al.,1981).*

4.5.2 Reagents

- Maize seeds (*Zea mays*)
- Paper dolls and plastic bags
- 1mM solution of EDTA (Merck, Darmstadt, Germany)
- 10mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, USA)
- 1 M KH_2PO_4 solution
- 12 M Hydrochloric acid (Sigma–Aldrich, St. Louis, USA)
- deionised H_2O
- Sephadex G25
- All the reagents and materials cited in section 4.2.2(a).

4.5.3 Method of analysis

Several methods were blended into one to create a relatively simple procedure for the analysis of total urinary polyamines (Matsumoto *et al.*, 1980; Otsuji *et al.*, 1985; Badolo, 1999). Urine samples were collected from pregnant females while they were kept in metabolic cages, during the investigation of the teratogenic potential of DABA (Chapter 2, Section 2.3.3.1). Maternal livers and whole embryos were also harvested, during that investigation after decapitation of the pregnant females on gestation day 18. All samples were immediately frozen and stored at -70 ^{0}C, until the analyses could be executed. Aliquots of urine samples were removed to measure the concentration of creatinine in the samples with an automated Jaffe-analysis (Jaffe, 1886). Samples of hepatic and whole embryo cytosols were removed to measure the protein concentration with a microplate version of the Bradford assay (Section 4.2.2(b); Bradford, 1976).

Conjugated polyamines, present in urine, maternal liver and whole embryo cytosols were hydrolysed with 6M HCl at 80 ^{0}C for 24 h to produce the free polyamines. Aliquots of urine and/or maternal liver and whole embryo samples were transferred to clean, dry Kimax tubes. An equal volume of 12 M HCl was added to the hydrolysates sample, the tubes were tightly sealed and incubated at 80 ^{0}C for 24 h. The pH of the hydrolysates was adapted to 9.0 by the addition of a small volume of 1 M potassium phosphate. Aliquots of the hydrolysates were transferred to 90-well microtitre plates. An aliquot of a solution of the enriched MPAO was added to each well and the rest of the reagents used in the ODC assay followed (Section 4.2.2(c); Fig. 4.1). In this part of the method all of the higher polyamines, Spd, Spm were oxidized by MPAO to form Put and H_2O_2. Subsequent oxidation of Put by DAO lead to the formation of Δ-1-pyrroline and more H_2O_2 was produced (Fig. 4.1). Subsequent co-oxidation of phenol and 4-amino-

antipyrine by reactive oxygen, released through the enzyme action of HRP on the H_2O_2, led to the formation of a brick-red coloured complex known as antipyrine red (Fig. 4.1). The absorbance intensity of the reaction mixture in the microtitre plate wells was measured in a Biotek microplate reader at 595 nm and the total polyamine concentration calculated using the principles of the Beer-Lambert law.

4.5.4 Results:

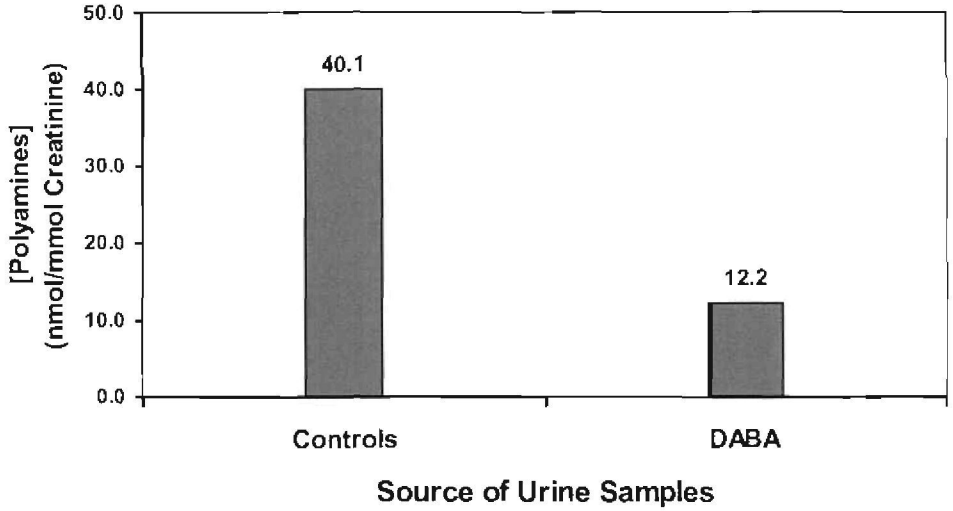

Figure 4.11 *The effect of DABA on total polyamine levels in the urine of pregnant female mice. Controls: pregnant females received saline only; DABA: pregnant females were dosed with DABA (1350 mg/kg body mass) on days 7, 8,9 of the gestation (Section 4.5.3) .*

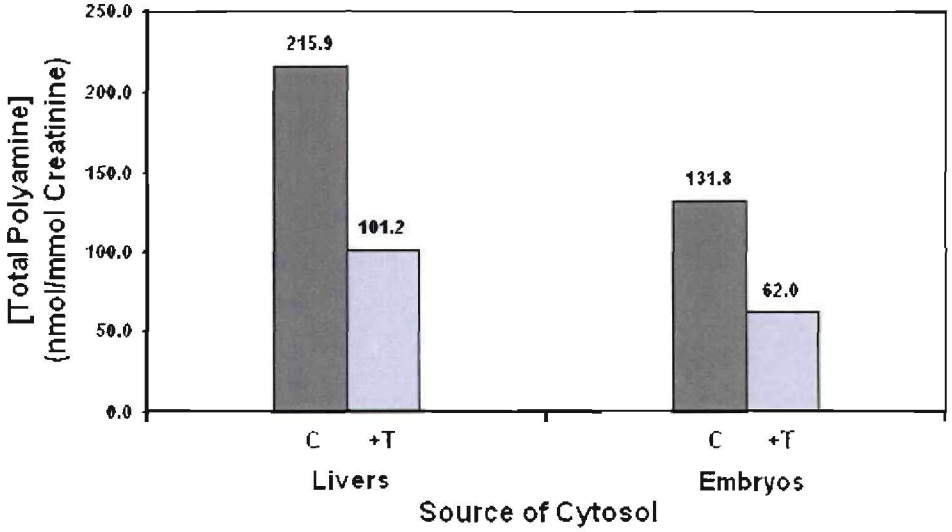

Figure 4.12 *The effect of DABA on total polyamine levels in cytosols prepared from livers and whole embryos, harvested from pregnant female mice on gestation day 18. C = controls, females received saline only; +T = females received DABA @ 1350 mg/kg body mass dosed on days 7, 8, 9 of the gestation (Section 4.5.3).*

4.5.5 Brief discussion:

The effect of DABA on the total polyamine concentration in the urine samples of pregnant female mice dosed with either saline or DABA (1350 mg/kg body mass) is depicted in Fig. 4.11. A statistically significant ($p<0.05$) difference (-69.6%) was observed between the mean concentration of polyamines in the urine of DABA-treated females (12.2 nmol/mmol creatinine; n = 4) and control animals (40.1 nmol/mmol creatinine; n = 4).

DABA also exerted a significant effect (-53.1%; $p<0.0001$) on the mean polyamine content of cytosols, prepared from livers of DABA-treated (101.2 nmol/mg protein; n = 10) and control (215.9 nmol/mg protein; n = 8) females (Fig. 4.12). A similarly significant change (-53.0%; $p<0.001$) was observed between cytosols derived from whole embryos of DABA-exposed (62.0 nmol/mg protein; n = 10) and control (131.8 nmol/mg protein; n = 9) embryos.

Chapter 5

Discussion of Study Outcomes

Congenital defects (i.e. neural tube defects) display a multi-factorial aetiology, most probably the result of a complex interaction between genetic and environmental factors (i.e. inborn errors of metabolism, dietary deficiencies, teratogenic xenobiotics) (Gos & Szpecht-Potocka, 2002; Yerby MS, 2003; Finnell RH *et al.*, 2003). L-2,4-Diaminobutyric acid (DABA) is a non-protein amino acid and common metabolite and constituent of numerous plants and microorganisms (Perkins, 1971; Fiedler & Kandler, 1973; Sasaki *et al.*, 1998; Vertesy *et al.*, 2000; Martin *et al.*, 2003). This relatively abundant amino acid is a known toxin (i.e. neurotoxin, neurolathyrogen, urea cycle inhibitor) and commonly occurs in the food chain of vulnerable populations in Ethiopia and India (O`Neal *et al.*, 1968; Rosenthal, 1982; Gettahun *et al.*, 1999). In spite of the awareness that DABA is a toxic agent, it has not yet been reported to be associated with birth defects in man or domestic animals. None of the reports studied referred to DABA as a potential L-ornithine (Orn) anti-metabolite with the potential to inhibit ornithine decarboxylase (ODC), nor is it known if DABA can modulate polyamine levels in experimentally exposed animals. The objectives of this investigation, therefore, were:

(a) to investigate the potential teratogenic properties of 2,4-diaminobutyric acid (DABA), employing a murine NTD model (i.e. Hanover.NMRI mouse embryos);

(b) to establish the effect of DABA on hepatic and whole embryo ornithine decarboxylase (ODC) - a key enzyme of the urea cycle;

(c) To investigate the potential anti-metabolic properties of DABA, focusing on selected metabolic processes, i.e. glycolysis, the citric acid cycle, the urea cycle, polyamine metabolism.

A dose-response experiment was executed on non-pregnant females to approximate optimal dosage levels for the teratogenic experiments, followed by a study of the teratogenic potential of DABA (Sections 2.2.3 & 2.3.3) on pregnant Hanover.NMRI females. DABA proved to be acutely toxic at doses of 1800 mg/kg body mass - all the non-pregnant females in this group died within 24 hours (100% mortality) (Tables 2.1, 2.2 & 2.3; Figures 2.1 & 2.1). Epileptic-like seizures occurred in some of the females prior to coma and death. Ten percent of the females receiving DABA at a dose of 1350 mg/kg body mass died within 48 hours. No visible signs of toxicity were

observed in the groups receiving a total dose of 450 and 900 mg/kg body mass. None of the control animals, dosed with saline alone were affected.

Experimental outcomes in general were unfavourably influenced by the relatively small number of confirmed, impregnated females in the course of the experiments. Pregnancy rates (40 – 80%) and mean litter size (4.0 ± 1) among the Hanover.NMRI females were abnormally low and erratic. Vaginal plugs and body mass increments proved to be unreliable pregnancy indicators. No commercial pregnancy kits for mouse chorionic gonadothrophin (mCG) were available and it was not feasible to monitor blood or urinary mCG as an indicator of pregnancy in experimental mice. Louw (2004) previously reported poor cross reactivity of mCG antigen with anti-human chorionic gonadothrophin (anti-hCG) and efforts to use human pregnancy assay kits to detect mCG in the urine of female mice were abandoned.

Although care was taken not to use extreme toxic dose levels of DABA in the experiments designed to investigate the teratogenic potential of DABA, 20% – 40% of the pregnant females died at the highest selected dosages (900 & 1350 mg/kg body mass respectively; $p<0.0001$). Foetal resorption occurred in two cases (6.7%) of the 30 pregnant females - one in the control group (6.7% of group) and one (20% of group) in the 900 mg/kg body mass DABA dose level group. The affected pregnant females may have experienced higher levels of stress than the majority of experimental animals during the execution of the experiment (Tables 2.1 & 2.2). DABA proved to be highly toxic to mouse foetuses at all administered dose levels (450 – 1350 mg/kg body mass) and foetal mortality was extremely high (33 – 57%). A directly proportional, but non-linear relationship was observed between the DABA dosage level and foetal mortality (Fig. 2.1; $p<0.05$). The observed toxic effects were statistically significant ($p<0.05$). Differences between the various DABA dosage levels also appeared to be statistically significant ($p<0.05$). A relatively high proportion of foetuses (25 – 50%) displayed congenital defects - neural tube defects (i.e. exencephali) occurred in only 5 -17% of the experimental group. The teratogenic dose-response relationship was statistically significant ($p<0.05$), directly proportional to the administered dose of DABA, but non-linear in trend (Fig. 2.1 & 2.2).

Mean body mass can be regarded as a fairly reliable indicator of the growth and development of chicken and mouse embryos (Louw, 2004, Louw *et al.*, 2005). At 18 days of gestation the control Hanover.MMRI embryos proved to be slightly heavier (1.28 ± 0.42 g; Table 2.2) than previously reported (Louw, 2004, 2005; Brits, 2005) for full term Hanover.NMRI mouse embryos (1.1 ± 0.25 g; -16.4%). In contrast to this observation, the mean embryo body mass of the embryos exposed to the lowest

dose of DABA (450 mg/kg body mass) appeared to be higher (1.57 ± 0.15 g; +30%) than that recorded for the control embryos in our experiment. However, this result was not statistically significant ($p>0.05$). Overall the dose-response effect of DABA on embryo growth ($p<0.05$) appeared to be directly proportional with a non-linear tendency (Fig. 2.2). The apparently higher body mass of embryos exposed to DABA at the lowest dose level ($p>0.05$) may be regarded as a statistical artefact resulting from the relatively small sample sizes employed in the study.

The results of this study (Tables 2.1 – 2.4; Fig. 2.1 & 2.2) clearly proved that the administration of DABA to pregnant female mice, during the second week of pregnancy (gestation days 7 - 9) may cause congenital defects (i.e. NTD) and growth retardation in developing embryos. This is one of the most critical periods in embryonic development, when differentiation of the embryonic neural plate and closure of the embryonic neural tube occur (Morris-Kay & Sokolova, 1996; Moore & Persaud, 2003a). Our finding strengthens previously reported evidence that DABA is toxic to animals and potentially also to man (O`Neal *et al.*, 1968; Rosenthal, 1982).

Analysis of 24-hour urine samples, collected from pregnant females during gestation day 10 in metabolic cages demonstrated that DABA can cause gross perturbations in the metabolism of pregnant female Hanover.RPMI mice (Fig. 3.2 & Fig. 3.3; Tables 3.2 – 3.8; Fig. 3.4 – 3.9). The abundance and intensity of urinary metabolite peaks observed in the metabolic profiles (i.e. GC chromatograms, etc.) of DABA-treated females, compared to the metabolic profiles of saline-treated controls (Fig. 3.2 *versus* Fig. 3.3; Tables 3.2 – 3.8; Fig. 3.4 – 3.9) clearly underscore these results. DABA evidently exerted an enormous impact on the general biochemistry of the DABA-treated females - a variety of crucial metabolic processes appeared to have been affected.

DABA effected changes in a number of glycolytic and citric acid cycle metabolites (Table 3.3 & Fig. 3.4). Lactate accumulated (+373.2%; $p<0.05$) at the cost of pyruvate (-90.1%; $p>0.05$), probably causing some degree of lactic acidosis. Although the observed change in lactate appears to be impressive, the change in the pyruvate concentration was statistically insignificant ($p>0.05$). Most of the citric acid cycle metabolites, cis-aconitate (+491%), malate (+340%), citrate (+686%), succinate (+234%) and fumarate (+743%) were significantly increased ($p<0.05$). Oxalate concentration also significantly increased (+1497.1%; $p<0.05$) to more than 14 times the levels observed in control animals. It is not clear whether the excess oxalate was produced from oxaloacetate through the dismutase-like activity of lactate dehydrogenase (LDH) or if some unknown metabolic effect was precipitated by

DABA and/or its metabolites (Sharma & Schwille, 1992). Excess levels of oxalate may have caused a shift in the pyruvate:lactate ratio, because of its inhibitory action on lactate dehydrogenase (LDH) and/or pyruvate carboxylase (Emerson & Wilkinson, 1965; Hagler & Herman, 1973).

DABA is an inhibitor of ornithine transcarbamylase (OTC) (O`Neal *et al.*, 1968; Rosenthal, 1982). This key enzyme in the urea cycle is responsible for the conversion of ornithine to citrulline (Sections 1.1 & 1.3.4). DABA is a structural homologue and potential anti-metabolite of ornithine and we hypothesised that it may also be a potential inhibitor of ODC. Systemic exposure of pregnant females to DABA caused changes in some of the urea cycle metabolites (Table 3.4 & Fig. 3.5), present in urine samples of the DABA-treated pregnant females. A relatively small but statistically non-significant increase in urea (+24.8%; $p>0.05$) was observed, while both citrulline (-41.5%) and ornithine (-50.3%) were significantly decreased ($p<0.05$). The inhibition of arginase by DABA or DABA-derived metabolites may explain the lower citrulline level, while the decrease in ornithine may be attributed to the inhibition of ornithine decarboxylase (ODC). However, these inhibitory actions should have caused a lowering of urea production and not an increase in urea (Ash, 2004; Meerarani & Shanmugasundaram, 1987). Systemically administered DABA most probably exerted its effect on the urea cycle by simultaneously inhibiting two of the key enzymes in the urea cycle, i.e. OTC and ODC (O`Neal *et al.*, 1968; Rosenthal, 1982).

DABA induced changes in some of the hydroxyacids in the urine of pregnant, DABA-treated pregnant female mice (Table 3.5 & Fig. 3.6). Significant changes ($p<0.05$) were observed in 2-methyl-3-hydroxybutyrate (+200.6%), 3-hydroxybutyrate (β-hydroxybutyrate; +243%) and 2-hydroxy-butyrate (+187.1%). In contrast 2-hydroxyvaleric (-72.8 %) levels significantly decreased ($p<0.05$), following DABA ingestion. Excess levels of all of these metabolites may be indicative of a DABA-induced metabolic ketosis or mitochondrial malfunction with concomitant interference in the generation of energy (ATP). Prolonged or chronic ketosis can cause modulations in the citric acid cycle, limiting the production of reactive oxygen species (ROS). These processes are often associated with a boost of energy production in the brain and a concomitant increase in the synthesis of GABA (Yudkoff *et al.*, 2004; Bough KJ & Roh JM, 2007).

A large number of DABA-induced changes were observed in the concentrations of urinary amino acids and certain nitrogenous metabolites, collected from DABA-treated pregnant female mice (Table 3.6 and Fig. 3.7). Statistically significant

changes occurred in 62.1% of the amino acids and selected nitrogenous metabolites. Relatively small or no changes were observed in 37.9% of the amino acids and some targeted nitrogenous metabolites. The method used to quantify amino acids in the urine samples (Phenomenex EZ:faast®), failed to detect and quantify arginine, one of the essential amino acids and a critically important urea cycle intermediate. The effect of DABA on arginine levels in the assayed samples is therefore unknown. Relatively large and statistically significant changes (>50%) were observed in sarcosine (+280.9%), valine (+87.7), isoleucine (+58.3%), cysteine (+109%), cadaverine (+488.8%), glutamine (-100%), ornithine (-50.3%), histidine (-58.7%), phenylalanylproline (+∞%), cystathionine (-100%) and β-alanine (+194.8%). The change in lysine concentration (-46.5%) appears to be mirrored by a similar change in α-aminoadipic acid (-48%), a catabolic product derived from lysine (Papes *et al.*, 2001). Lysine is an important precursor in the biosynthesis of L-carnitine and decreased levels of lysine may be indicative of potential effects on carnitine metabolism (Vaz & Wanders, 2002). ß-Alanine is a catabolite of pyrimidine metabolism (Traut & Jones, 1996) and precursor in the synthesis of co-enzyme A (CoA) (Tahiliani & Beinlich, 1991), but also one of the main catabolic products of DABA metabolism (Mushahwar & Koeppe, 1999). A fraction of the increased levels of DABA may therefore have been derived from DABA (Section 1.3.4, Fig. 3.1).

DABA ingestion also effected changes in some of the short and long-chain dicarboxylic acids and their carnitine conjugates (Table 3.7, Fig. 3.8). Adipate (+55.2%) and suberate (+234.4%) concentrations appear to be significantly elevated ($p<0.05$). Ethylmalonate (15.2%) was only slightly and non-significantly ($p>0.05$) increased in DABA-treated animals. Adipate and suberate are both products of incomplete oxidation in the ω-oxidation pathway of long-chain fatty acids. All three intermediates may be elevated in cases of metabolically induced carnitine deficiency (Michalak & Qureshi, 1995). Most of the selected carnitine conjugates, quantified in the urine samples of DABA-treated pregnant female mice, were decreased (Table 3.8). Our results demonstrated a tendency of free carnitine levels to increase (+26%) following DABA intoxication, while acyl carnitine levels (-8.6%) and the acylcarnitine/free carnitine ratio decreased (-23%). None of these results were statistically significant ($p>0.05$). Significantly increased concentrations of acetylcarnitine (75.6%) and octanoylcarnitine (115%) were, however, observed ($p<0.05$). Butyrylcarnitine (-60.2%), C14:2-carnitine (-33.3%),C14:1-carnitine(-72.2%) propionylcarnitine (-69%), hexanoylcarnitine (-47.6%) and octenoylcarnitne (-69.2%),

on the other hand, were all non-significantly ($p>0.05$) suppressed in DABA-treated pregnant females.

Carnitine plays an essential role in the transfer of long-chain fatty acids across the inner mitochondrial membrane, in the detoxification of acyl moieties, and in the maintenance of free coenzyme A levels. Besides its role as a carrier of activated acyl groups, L-carnitine functions as a buffer for acetyl groups which may be present in excess in different tissues during ketosis and hypoxic muscular activity. L-carnitine also protects membrane structures, stabilizes the CoA-SH/acetyl-CoA ratio and reduces the danger of lactic acidosis. The protective effects of L-carnitine system can be compromised by a variety of xenobiotics, i.e. 3-hydroxynorvaline (Louw, 2004, 2005) and sodium valproate (Brits, 2005). These alterations are often responsible for most of the observed intracellular toxic effects (Thurston *et* al., 1985; Zeyner & Harmeyer, 1999; Arrigoni-Martelli & Caso, 2001; Michalak & Qureshi, 1995).

We have successfully demonstrated that DABA can induce congenital defects (i.e. neural tube defects) in developing mouse embryos and modulate numerous biochemical processes in the pregnant mothers. The notion that systemic exposure to DABA may affect polyamine biosynthesis through the modulation of the activity of maternal and whole embryo ODC was investigated. ODC activity in maternal livers and whole embryos, as well as the concentration of polyamines in tissue (i.e. liver, embryo) and urine samples were quantified. A coupled enzyme assay (Fig. 4.1) was developed and employed in the analysis of ODC activity in the livers and whole embryos, harvested from pregnant female mice, treated with either DABA or saline. ODC activity was measured as a function of the concentration of Put produced, following the decarboxylation of Orn. The concentration of Put was measured as a function of the amount of H_2O_2 released following the *in situ* oxidation of putrescine by DAO (Badolo *et al.*, 1999).

The coupled enzyme assay was rigorously investigated and modified to obtain optimal precision, reliability and linearity and the stability of the ODC scrutinised. Cytosolic samples lost a fraction of the initial ODC activity (15.4%) after two weeks of storage in a freezer at -70 ^{0}C (Section 4.3.1; Fig. 4.3), indicating that the ODC activity in tissue samples should be assayed soon after tissue collection. Enzyme activity was completely lost, following denaturation in boiling water (96 ^{0}C) for 15 min (Section 4.3.2; Fig. 4.3). This result proved that the observed changes in the absorbance of reaction mixtures at 485 nm were due to heat labile enzymatic activity, attributed to the oxidation of Put, produced by the decarboxylation of Orn. Relatively low %CV's were obtained for assays on maternal liver (10.3%) and whole embryo

(9.4%) cytosols respectively. The precision of the coupled enzyme assay for ODC in these matrices was between 89.7% and 90.6% (Section 4.3.4; Table 4.1). The coupled enzyme assay for ODC proved to be linear for the measurement of increasing enzyme concentration (0.996; Fig. 4.4) and the concentration of Put (R = 0.999; Section 4.3.5; Fig. 4.7). The K_m value for of ODC for Orn as substrate was calculated to be 1.02 mM and the V_{max} was 0.58 nmol Put produced per min under the conditions used for the assays (Section 4.3.3; Fig. 4.5 & Fig 4.6). A significant *in vitro* inhibition of the ODC activity (69.0-90.5%) was observed at all the concentrations of DABA employed in the experiment. Both hepatic and whole embryo ODC activity were significantly inhibited (69 - 90.5 %; $p<0.0001$), due to the systemic effects of DABA and/or DABA metabolites (Section 4.3.6; Fig. 4.8 & 4.9) on pregnant mice and their embryos.

We have successfully illustrated that DABA, an inhibitor of ODC (Section 4.3.6), can cause major metabolic disturbances (Chapter 3) and induce congenital defects in a relatively large proportion of DABA-exposed embryos (Chapter 2). It was deemed important to ascertain if polyamine concentrations in DABA-treated pregnant females and their embryos were concomitantly affected. Systemic changes in polyamine concentrations may be reflected in the levels of polyamines detected in the urine of DABA-treated pregnant females (Matsumoto *et al.*, 1981; Bachrach, 2004). Changes in the total concentration of polyamines in the livers, whole embryos and urine samples of pregnant females, as a function of systemic DABA exposure, were subsequently quantified.

A coupled enzyme assay for ODC was developed. In principle N-acetylated polyamine conjugates, present in the samples were first hydrolysed by hydrochloric acid to render the free, unconjugated polyamines. Free polyamines were then differentially oxidized by successive steps employing a amine oxidases, i.e. maize polyamine oxidase (MPAO; Fig. 4.10) and diamine oxidase (DAO). MPAO is specific for the higher polyamines Spm and Spd, converting these higher polyamines to Put and H_2O_2. DAO targets Put and from that reaction Δ-1-pyrroline and H_2O_2 are produced (Fig. 4.1). Phenol, 4-amino-antipyrine and the released H_2O_2 react in the presence of horse radish peroxidase (HRP) to form the coloured antipyrine red solution exhibiting a λ_{max} at 485 nm (Fig. 4.1).

A statistically significant difference (-69.6%; $p<0.05$) was observed between the mean concentration of polyamines in the urine of DABA-treated (1350 mg/kg body mass) pregnant females (12.2 nmol/mmol creatinine; n = 4) and control animals (40.1 nmol/mmol creatinine; n = 4) (Fig. 4.11). DABA also exerted a significant ($p<0.001$)

effect (-53.1%) on the mean polyamine content of cytosols, prepared from livers of DABA-treated (101.2 nmol/mg protein; n = 10) and control (215.9 nmol/mg protein; n = 8) females. Similar changes (-53.0%; $p<0.001$) were observed between cytosols, derived from whole embryos of DABA-exposed (62.0 nmol/mg protein; n = 10) and control (131.8 nmol/mg protein; n = 9) embryos (Fig. 4.12).

This investigation successfully demonstrated that DABA is a teratogen with the potential to induce congenital defects (i.e. neural tube defects) in developing mouse embryos. DABA and/or DABA metabolites can apparently modulate a variety of biochemical processes when systemically administered to pregnant female mice. Inhibition of two of the enzymes in the urea cycle, i.e. OTC (O`Neal *et al.*, 1968; Rosenthal, 1982) and ODC (this investigation) may be partly responsible for some of the observed metabolic disturbances. Inhibition of ODC led to an inhibition in the production of polyamines in tissues (i.e. liver, whole embryo) and their excretion in the urine of DABA-treated animals.

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