OXIDATIVE CATABOLISM OF TETRAHYDROPTERINS

Margaret Jean Stankiewicz

Doctor of Philosophy

The University of Aston in Birmingham
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SUMMARY:

Mixed labelled folic acid was administered to rats. Exposure to N_2O was used to give an insight into the major route of scission within the monoglutamate pool, results suggest that THF formed during transport from the gut lumen to the plasma is the major route of scission within the gut.

Peroxides in corn oil and arising as a result of lipid peroxidation and autoxidation increase catabolism of the monoglutamate pool and decrease incorporation of administered folates into the polyglutamate pool. It is suggested that peroxides may oxidise B_{12} resulting in inhibition of methionine synthetase, this results in diminished polyglutamation and increased urinary excretion of 5 CH_3THF.

Fats undergo peroxidation within tissues, the resulting peroxides increase catabolism of the polyglutamate pool. It is suggested that the NBT assay may reflect polyglutamate breakdown.

Antioxidants such as vitamin E (and DES) decrease catabolism of the monoglutamate pool.

Administration of DES resulted in changes similar to those observed during malignancy, it is suggested that these changes may precede the onset of tumour development.

Vitamin E elevates brain DHPR activity. Since lowered DHPR levels and disturbed THB metabolism have been observed in aging and Down's syndrome it is proposed that vitamin E therapy may prove beneficial in situations where oxidative stress is increased.

Brain DHPR activity was increased on administration of peroxides suggesting that in situations of oxidative stress (which may result in increased catabolism of THB) the salvage pathway may be stimulated and loss of THB minimised.

N_2O exposure had no effect on THB metabolism suggesting that the stimulatory role of 5 CH_3THF is due to its role as a methyl donor.

KEY WORDS:- Tetrahydrofolate, Peroxides, Antioxidants, Tetrahydrobiopterin, Catabolism.

TO MY PARENTS

Acknowledgements

I would like to thank Professor J. A. Blair (Department of Pharmaceutical Sciences, Biology Division) for his valued supervision throughout my research and my colleagues within the research group for their help. I thank Aston University for financial support.

ABBREVIATIONS

pABGlu	=	p-aminobenzoyl-l-glutamic acid.
pAcABA	=	p-acetamidobenzoic acid.
pAcABGlu	=	p-acetamidobenzoyl-l-glutamic acid.
BHT	=	butylated hydroxytoluene.
5.10 CHTHF	=	5.10 methenyl tetrahydrofolate.
5,10 CH_2THF	=	5,10 methylene tetrahydrofolate.
5 CH_3THF	=	5-methyl tetrahydrofolate.
10 CHOTHF	=	10-formyl tetrahydrofolate.
5 CHNHTHF	=	5-formimino tetrahydrofolate.
cAMP	=	cyclic adenosine 3'.5'-monophosphate.
CNS	=	central nervous system.
CSF	=	cerebrospinal fluid.
DES	=	diethylstilboestrol.
DHB	=	7,8 dihydrobiopterin.
qDHB	=	quinonoid dihydrobiopterin.
DHF	=	7,8 dihydrofolate.
qDHF	=	quinonoid dihydrofolate.
DHFR	=	dihydrofolate reductase.
DHPR	=	dihydropteridine reductase.
$DMPH_4$	=	6,7-dimethyl 5,6,7,8 tetrahydropterin.
DNA	=	deoxyribonucleic acid.
L-Dopa	=	l-3,4-dihydroxyphenylalanine.
DTT	=	dithiothreitol.
EC	=	Enzyme Commission.
EDTA	=	ethylenediaminetetraacetic acid.
FAD(H_2)	=	flavin adenine dinucleotide (reduced form).
GSH	=	glutathione (reduced form).
GSH(Px)	=	glutathione (peroxidase).
GTP	=	guanosine triphosphate.
HPLC	=	high performance liquid chromatography.
LDL	=	low density lipoprotein.
MTX	=	methotrexate.
n	=	sample size.
NAD(H)	=	nicotinamide adenine dinucleotide (reduced form).
NADP(H)	=	nicotinamide adenine dinucleotide phosphate (reduced form).
NBT	=	nitro blue tetrazolium.
NH_2TP	=	7,8 dihydroneopterin triphosphate.
N_2O	=	nitrous oxide.
OCA	=	oral contraceptive agents.
PUFA	=	polyunsaturated fatty acids.
SAM	=	S-adenosyl methionine.
SD	=	standard deviation.
SDAT	=	senile dementia of the Alzheimer type.
SOD	=	superoxide dismutase.
TCA	=	trichloroacetic acid.
THB	=	tetrahydrobiopterin.
THF	=	tetrahydrofolate.
Tris	=	tris-(hydroxymethyl)-aminomethane.
dTMP	=	deoxythymidine monophosphate.
dUMP	=	deoxyuridine monophosphate.
uv	=	ultraviolet.
VLDL	=	very low density lipoprotein.

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INTRODUCTION

CHAPTER ONE.

1.1 INTRODUCTION

1.1.1 PTERIDINES

Pterins are compounds having in common the 2-amino-4-oxo-pteridine structure (1), these are of two types; conjugated - having a p-aminobenzoyl-l-glutamic acid (pABGlu) linkage at position 6 such as folic acid and are termed folates, or they may remain unconjugated - having no linkage or a small substituent at position 6. The basic pteridine structure (1) and the structures of folic acid (2) (a conjugated pterin) and biopterin (3)(an unconjugated pterin) are shown in figure 1.1. The ability to conjugate pABGlu to pterins has been lost by vertebrates and folate is a vitamin for these.

1.2 FOLATES

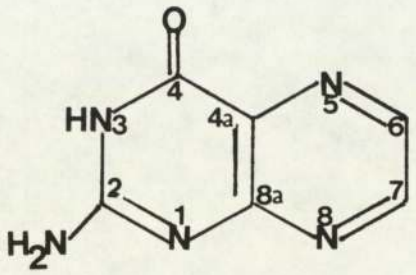
1.2.1 NUTRITIONAL REQUIREMENTS

Pteroylglutamic acid (folic acid) (2) is the parent compound of a group of molecules known collectively as folates. Folic acid may undergo several types of modifications; these include the state of reduction of the pteridine group (such as in dihydrofolate (DHF) (4) and tetrahydrofolate (THF) (5) the structures of which are shown in figure 1.2), substitution at N5 and N10 (such as in 5-methyl THF (6) and 10-formyl THF (7) the structures of which are shown in figure 1.3) and the addition of glutamic acid residues to form polyglutamates. Folic acid does not occur naturally, folates are present in the diet as reduced folate polyglutamates (McGuire *et al.*, 1979), rich dietary sources of folates are liver, kidney and dark green vegetables.

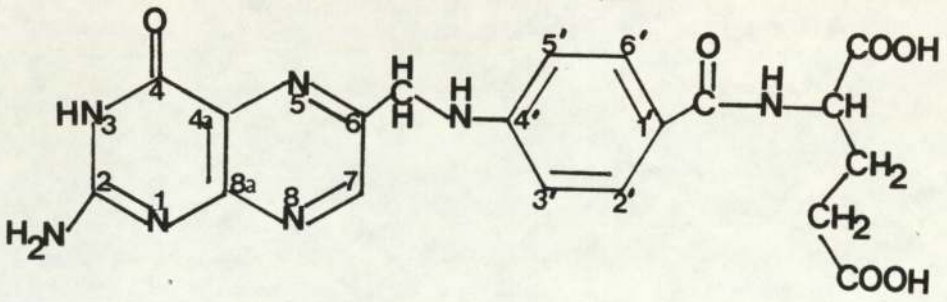
The minimum daily requirement is 50µg in the adult (Herbert, 1964), there are no official guidelines in Britain for daily requirements (Gilli, 1987) but in the U.S.the recommended daily intake is 400µg in the adult which is doubled during pregnancy [Recommended Dietary Allowances, Nutritional Academy of Sciences, Washington. D.C. (1980)].

The daily intake of folate falls short of the recommended daily allowance at 200µg (Lindenbaum, 1980). Folates are susceptible to oxidation and photodecomposition (Blakley, 1969) so methods of food preparation and the

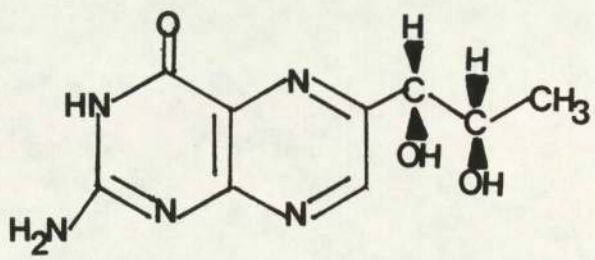
FIGURE 1.1



(1) 2-amino-4-oxo-pteridine

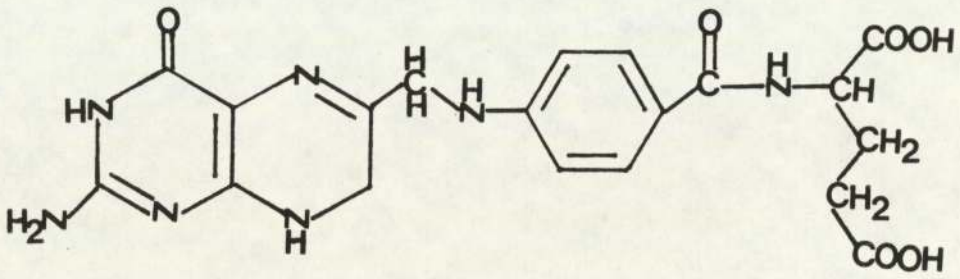


(2) Folic acid (pteroyl glutamic acid)
4[pteridine-6-yl-(methyl, benzoyl glutamic acid)]

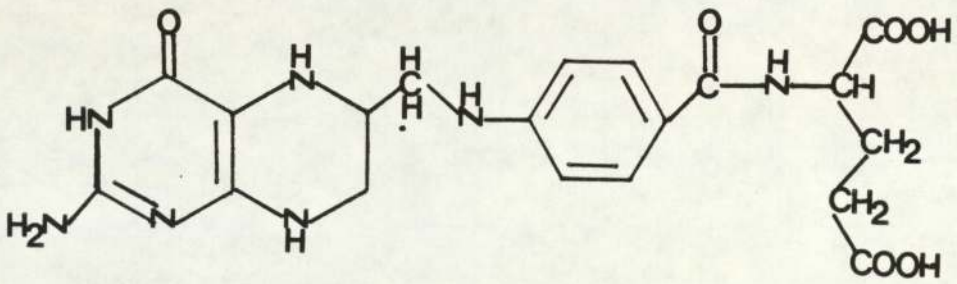


(3) Biopterin [6'(2,3-dihydroxypropyl) pterin]

FIGURE 1.2

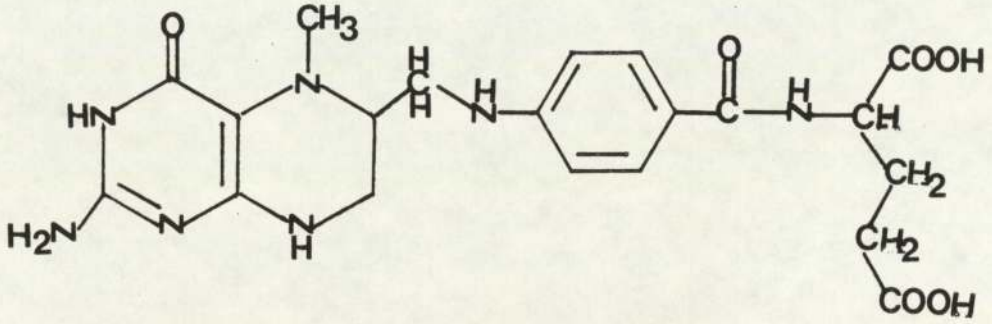


(4) 7,8, Dihydrofolic acid (DHF)

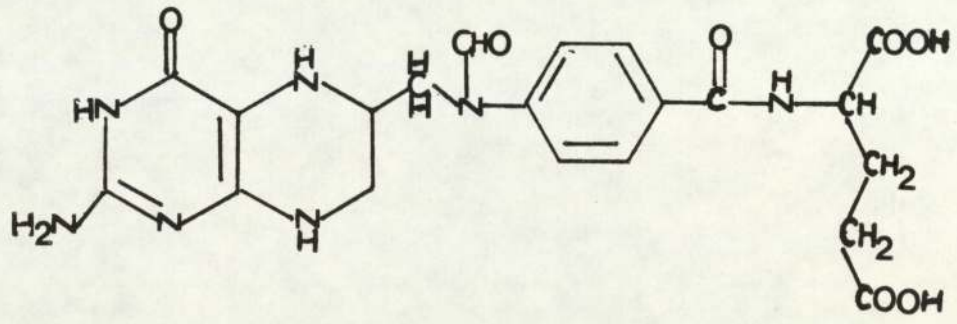


(5) 5,6,7,8 Tetrahydrofolic acid (THF)

FIGURE 1.3



(6) 5-methyl-THF



(7) 10-formyl-THF

presence of ascorbate in the body will affect the availability of dietary folates (Stokes *et al.*, 1975, Ratansthien *et al.*, 1977).

1.2.2 ABSORPTION AND TRANSPORT

Dietary folates must first be deconjugated to monoglutamates prior to transport from the intestinal lumen to the portal circulation. Two types of conjugase enzyme appear to exist, an intracellular lysosomal enzyme with a pH optimum of 4.5 and a brush border enzyme with a pH optimum of 7.5 (Rosenberg and Godwin, 1971 and Lindenbaum,1980). The reduced monoglutamates may be absorbed by a pH sensitive carrier mediated active transport process (Weir *et al.*, 1973, Strum and Said, 1983) or a pH sensitive passive diffusion process associated with the acid microclimate of the rat jejunal glycocalyx (Smith *et al.*, 1970, Blair and Matty, 1974, Russell *et al.*, 1979, Said, 1981) which is enhanced by glucose (Momtasi and Herbert, 1973).

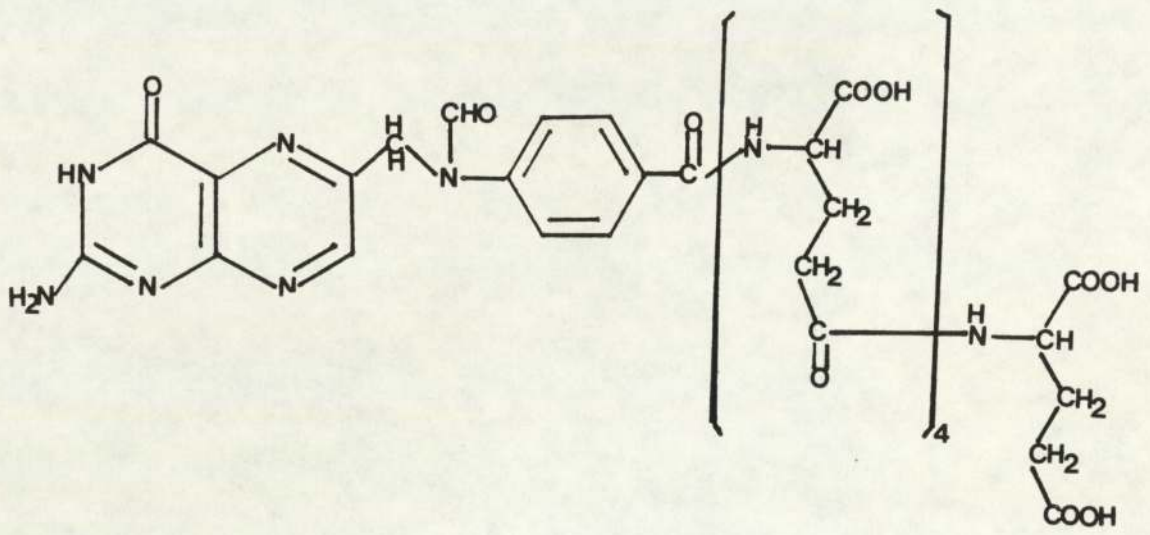
Reduced folates are largely metabolised to $5CH_3THF$ during transport across the gut and may then be transported either in the free form or loosely bound to serum proteins (Rowe, 1983). Systems of hepatic uptake and bile excretion of folates are energy dependent and carrier mediated (Strum *et al.*, 1979).

A specific active transport system for $5CH_3THF$ has been demonstrated in the choroid plexus, folates concentrate in the spinal fluid and brain levels are maintained even when serum levels fall (Herbert, 1964, Reynolds, 1976).

1.2.3 POLYGLUTAMATES

Polyglutamation is essential for cellular retention. Polyglutamates are predominantly present in the liver where the activity of folylpolyglutamyl synthetase is highest (McGuire *et al.*, 1979). The optimal substrates for synthesis of polyglutamates are L-glutamic acid and 10-formyl THF (Spronk, 1973, d'Urso-Scott and Makulu, 1973) or THF (McGuire *et al.*, 1979), $5CH_3THF$ is a poor substrate for polyglutamation (Lavoie *et al.*, 1974). Position 10 of the folate molecule appears to be specified for membrane transport inwards and polyglutamation (Samuels *et al.*, 1985). Polyglutamates are predominantly present in the pentaglutamate form, the major form isolated from the liver has been identified as 10-formyl-pteroyl-pentaglutamate (8)(Connor and Blair, 1980), the structure of which is shown in figure 1.4.

FIGURE 1.4



(8) 10-formyl-pteroyl-pentaglutamate

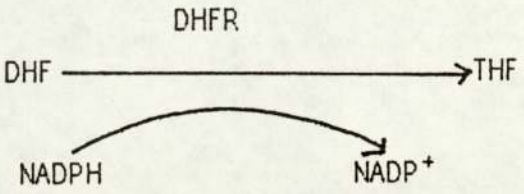
Polyglutamates were once believed to be storage forms but this is no longer thought to be correct since levels do not decrease at an increased rate in states of deficiency (Scott *et al.*, 1983). Polyglutamates are the major coenzyme forms (Hoffbrand, 1976) and are believed to serve a regulatory role (Kisliuk and Gaumont, 1974, Krumdieck *et al.*, 1975).

1.2.4 FUNCTIONS

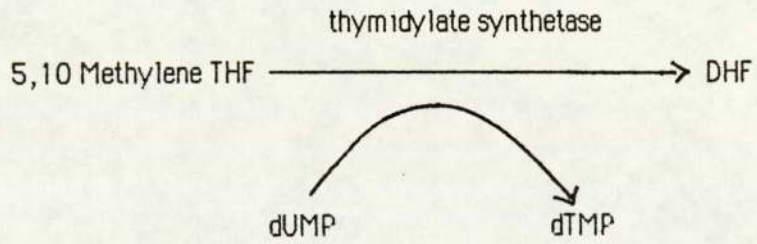
Folates are involved in several important biosynthetic reactions, their primary function is the transport of one-carbon units. Folates are required for; purine and pyrimidine biosynthesis, methionine synthesis, serine-glycine interconversion, histidine breakdown and the generation and utilisation of formate (Rowe, 1983). The interconversion of folate coenzymes and the reactions in which they are involved is summarised in figure 1.5.

1.2.4.1 DIHYDROFOLATE REDUCTASE (DHFR)

DHFR catalyses the reduction of folic acid and dihydrofolate (DHF) to tetrahydrofolate (THF).



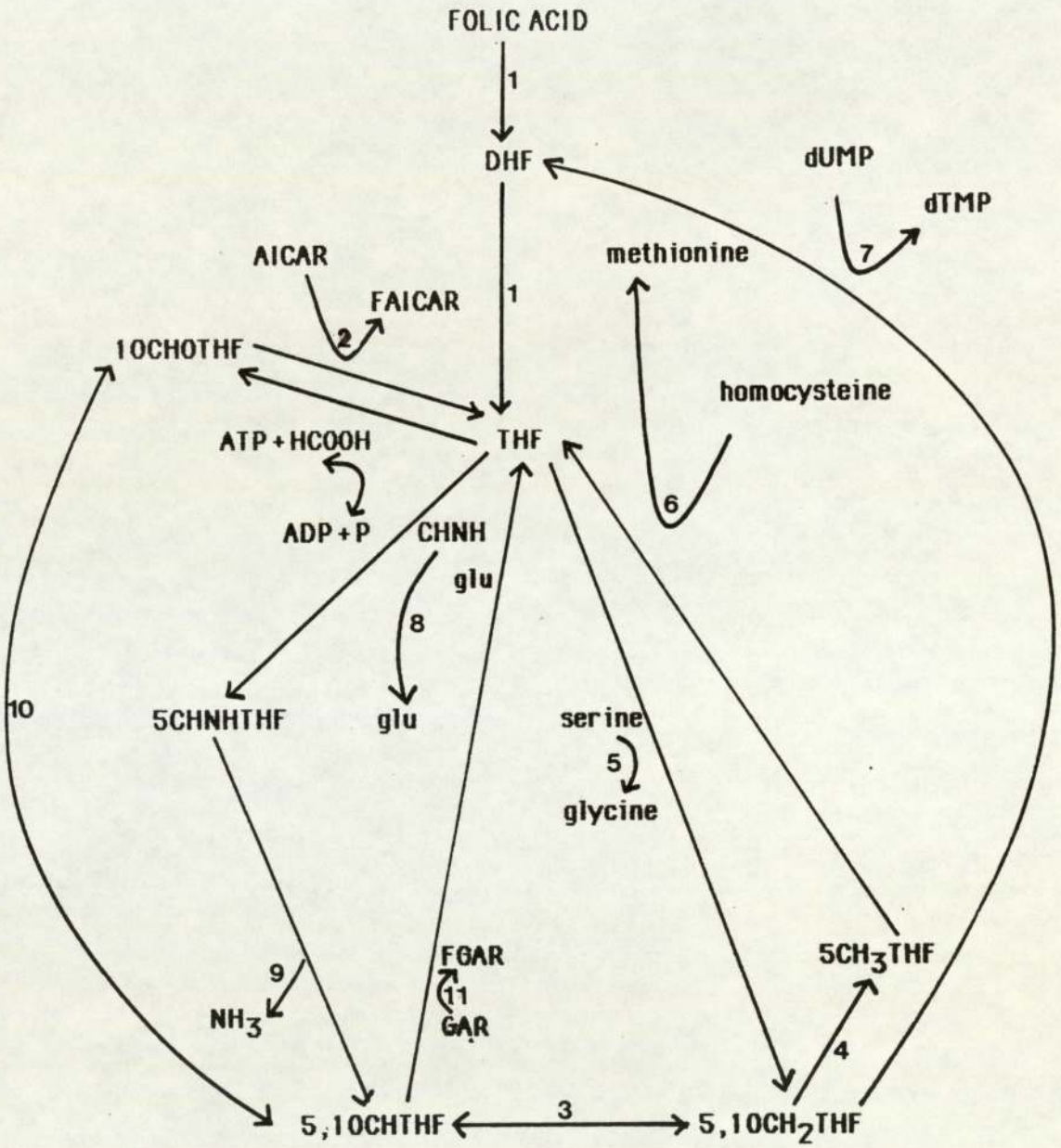
DHFR and thymidylate synthetase are important in regulating DNA synthesis. Thymidylate synthetase is important in methylation of uridylate to thymidylate which is the rate limiting step in DNA synthesis and results in the generation of DHF which is reduced back to THF by DHFR.



DHFR also has a role in the salvage of THB. Quinonoid 7,8 DHB (qDHB) readily isomerises to 7,8 DHB which is not a substrate for DHPR but is salvaged back to

FIGURE 1.5

Interconversions of folate coenzymes and the reactions catalysed by these.



AICAR =5-aminoimidazole-4-carboxamide ribonucleotide

FAICAR=5-formiminoimidazole-4-carboxamide ribonucleotide

GAR =B-glycinamide ribonucleotide

FGAR =N-formyl-glycinamide ribonucleotide

glu = glutamic acid

FIGURE 1.5 (legends contd.,)

Enzymes involved in folate interconversions

1 = DHFR

2 = formyl-THF-dehydrogenase

3 = methylene-THF-dehydrogenase

4 = methylene-THF-reductase

5 = serine-transhydroxymethylase

6 = methionine synthetase

7 = thymidylate synthetase

8 = formimino-THF-formiminotransferase

9 = formimino-THF-cyclodeaminase

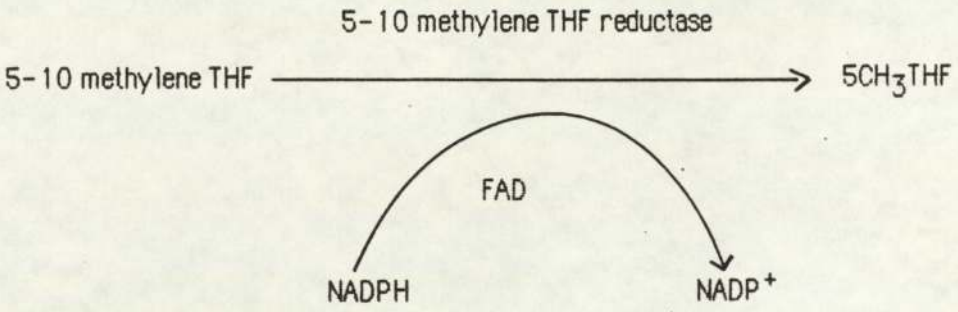
10 = cyclohydrolase

11 = formyl transferase

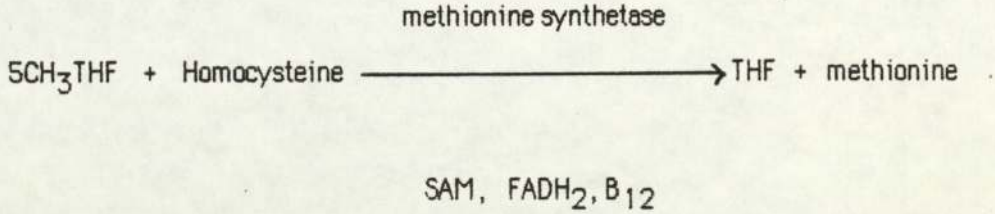
THB by DHFR (Craine *et al.*, 1972, Webber and Whiteley, 1985).

1.2.4.2 5-10 METHYLENE THF REDUCTASE

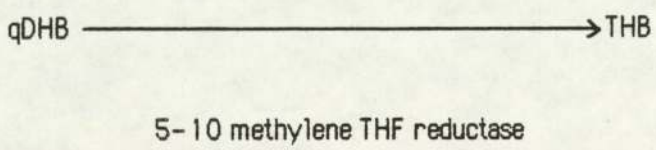
The reduction of 5-10 methylene THF to $5CH_3THF$ is the first committed step in the biosynthesis of methyl groups and is catalysed by the enzyme 5-10 methylene THF reductase.



The reaction is virtually irreversible and is subject to feedback inhibition by S-adenosyl methionine (SAM) and DHF (Matthews and Haywood, 1979, Kutzbach and Stokstad, 1967, 1971). SAM is required for the synthesis of methionine by the enzyme methionine synthetase.



5-10 methylene THF reductase also has a role in salvage of THB from qDHB (Nichol *et al.*, 1983, Smith *et al.*, 1986(a), Hyland *et al.*, 1986).



1.2.5 DEFICIENCY

Folate deficiency in the Western population is associated with old age, poverty and alcoholism (Weir *et al.*, 1985). It is particularly common during pregnancy when the incidence of deficiency in the West approaches 50% and 33% worldwide (Chanarin, 1979, Rowe, 1983).

Adequate folate intake during pregnancy is important for normal brain development and prevention of congenital defects. The fetus accumulates folate stores during the later stages of pregnancy (Herbert and Tisman, 1973, Morgan and Winick, 1978, Rowe, 1983). It has also been suggested that folate catabolism may be increased during pregnancy and this may contribute to folate deficiency states (Scott *et al.*, 1986)

Deficiency may arise due to inadequate folate intake, defects in absorption or metabolism, inhibition of deconjugation, displacement from serum and increased excretion- these may be the result of prolonged drug use (Rowe, 1983, Lambie and Johnson, 1985). Early signs of folate deficiency are low serum folate levels (<3ng/ml) and low red blood cell folate levels (<140ng/ml) which is accompanied by declining liver stores, failure of nucleic acid synthesis, impaired serine/glycine metabolism with decreasing methionine levels and a decline in protein synthesis resulting in megaloblastic anaemia (Chanarin, 1979). Neurological and psychological disorders may also occur in severe cases (Reynolds, 1976), although the deficiency in dementia and depression is probably the result of a secondary nutritional effect (Sneath *et al.*, 1973, Reynolds, 1976).

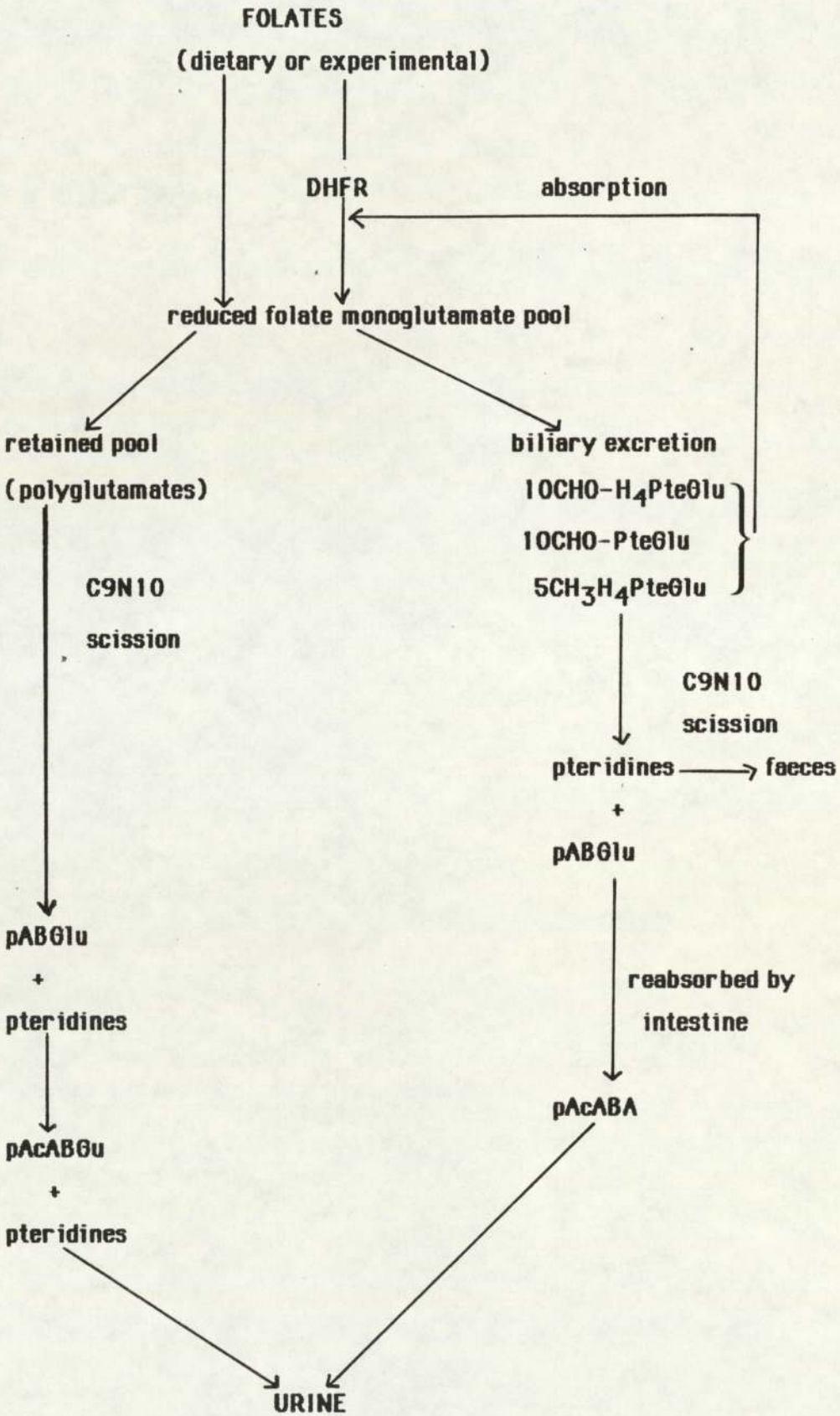
1.2.6 CATABOLISM

It has long been suggested that folate catabolism occurs by way of scission of the C9N10 bond (Jukes *et al.*, 1947, Blair, 1958). It was only with the introduction of labelled folates that this was confirmed (Murphy *et al.*, 1976, Murphy *et al.*, 1978, Connor *et al.*, 1979 and Pheasant *et al.*, 1981) with the identification of p-acetamidobenzoate (pAcABA) as an early urinary catabolite (Connor *et al.*, 1979) and pAcABGlu as a later urinary catabolite (Murphy *et al.*, 1976, Murphy *et al.*, 1978, Pheasant *et al.*, 1981).

Pheasant *et al.*, (1981) have proposed a route for *in vivo* folate catabolism. Catabolites may arise from one of two distinct pools (figure 1.6). The short term monoglutamate pool has a half life of one day in the rat (Saleh, 1981) and arises

FIGURE 1.6

Proposed routes of folate catabolism *in viva*



from biliary excretion, the principle urinary catabolite is pAcABA. The long term polyglutamate pool has a half life of eleven days in the rat (Saleh, 1981) and arises from retained polyglutamates, the principle urinary catabolites are pAcABGlu and pteridines. P-AcABGlu is the predominant species of later urine samples. The structures of these acetylated derivatives are shown in figure 1.7.

The mechanism of folate catabolism is believed to be an oxidative process which may be chemical or enzymic (Blair *et al.*, 1984, Al-Haddad *et al.*, 1986, Surdhar, 1987). The induction of microsomal enzymes does not result in increased catabolism suggesting that a chemical oxidative process is a strong possibility, (Guest *et al.*, 1983, Pheasant *et al.*, 1983, Guest, 1984).

THF is readily oxidised via qDHF which is a substrate for DHPR (Pollock and Kaufman, 1978). Administration of methotrexate (MTX) results in inhibition of both DHFR and DHPR, (Craine *et al.*, 1972) and increased scission with the excretion of pterin derivatives which are consistent with the breakdown of both THF and DHF pools (Saleh *et al.*,1981). Both DHFR and DHPR may have a role in salvaging DHF species from oxidative scission. The possible involvement of DHFR and DHPR in protecting folates from scission is shown in figure 1.8.

1.2.7 METHODS OF STUDYING FOLATE CATABOLISM

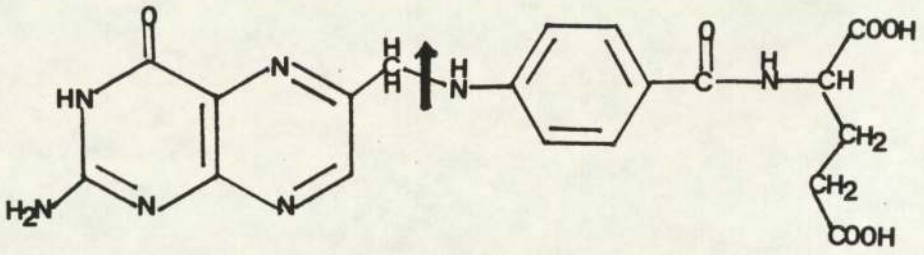
Studies of folate catabolism *in vivo* utilise commercially available radioactive folic acid. Although folic acid is not naturally occurring it enters the folate pool after reduction by DHFR. The use of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (figure 1.9) enables both portions of the molecule to be detected after scission. Urinary species are determined by sequential ion-exchange chromatography and gel filtration (Barford and Blair, 1978).

1.3 BIOPTERIN

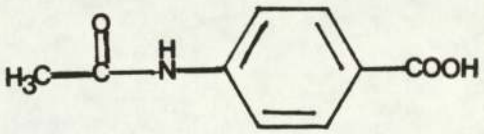
Biopterin is the predominant unconjugated pterin in man. It is present in the reduced form as dihydrobiopterin (DHB)(9) and tetrahydrobiopterin (THB)(10) the structures of which are shown in figure 1.10. Biopterin is found in the brain, pineal, liver, bone marrow, spleen, pituitary, adrenals, plasma and erythrocytes and is excreted in the urine (Fukushima and Nixon, 1980, Nichol *et al.*, 1985).

FIGURE 1.7

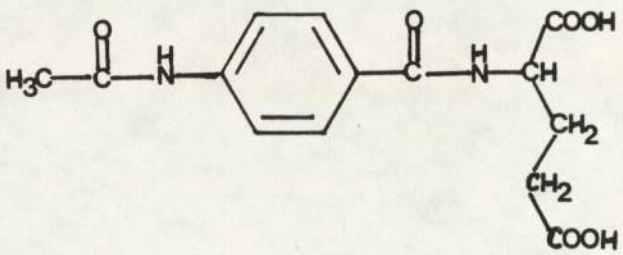
Site of folate scission and structures of resulting acetylated derivatives



Site of scission of folate molecule



(9) p-acetamidobenzoic acid (pAcABA)



(10) p-acetamidobenzoyl-l-glutamic acid (pAcABGlu)

FIGURE 1.8

Possible routes of folate breakdown (showing the involvement of DHPR and DHFR).

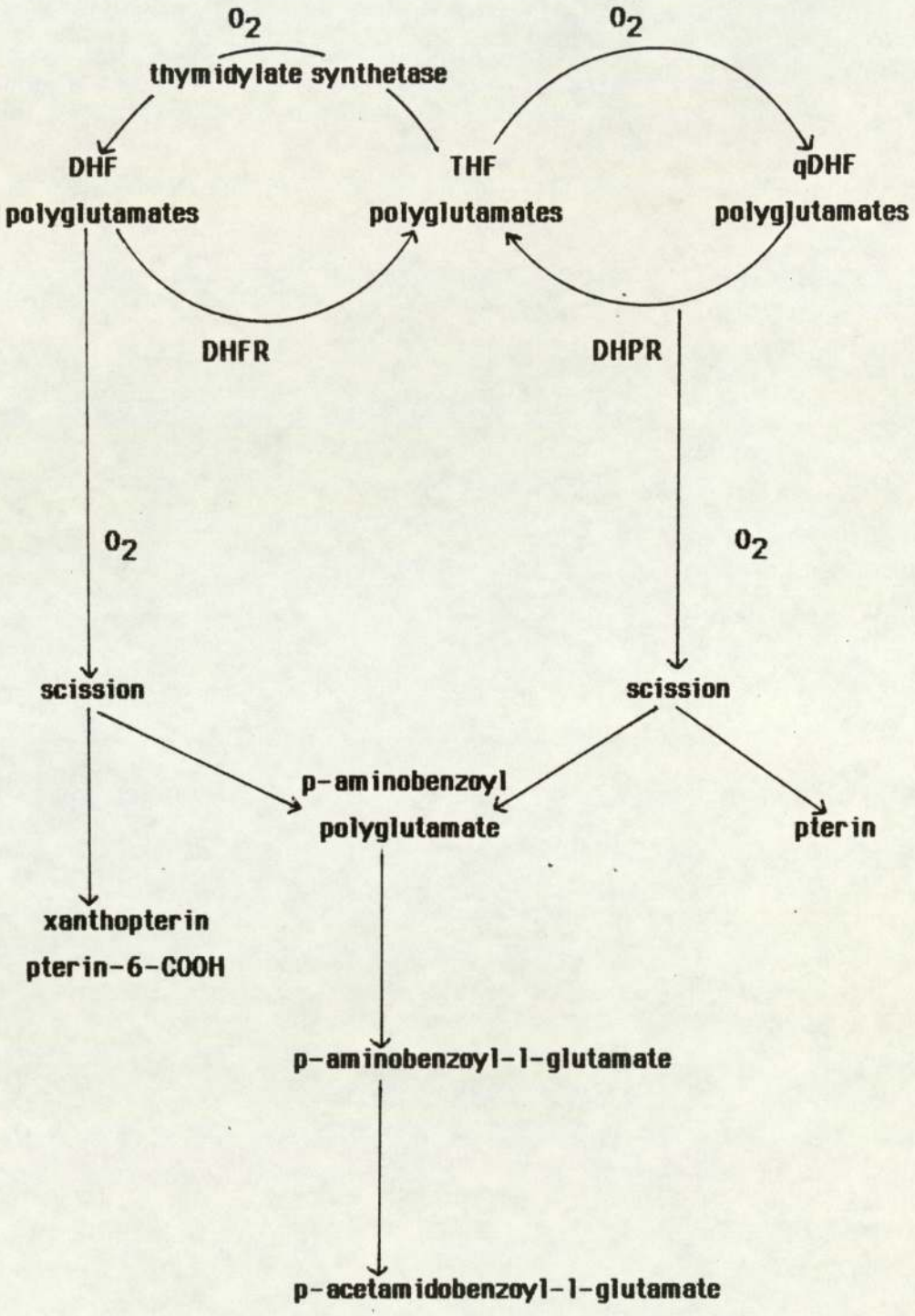
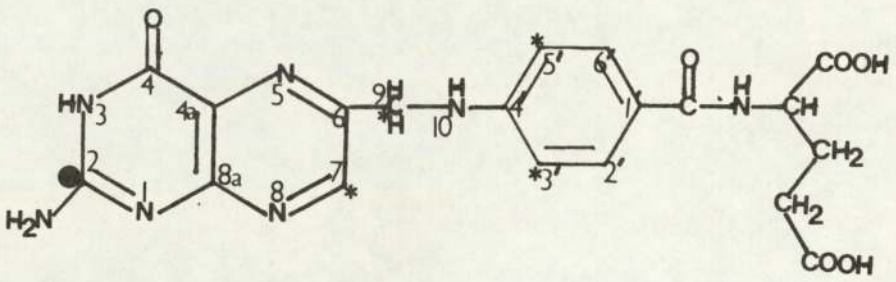


FIGURE 1.9

Sites of labelling of folate molecule

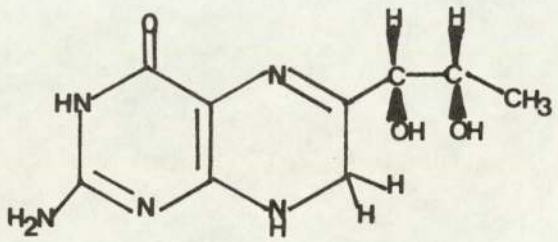


● ^{14}C=[2-^{14}C] folic acid (88% isotopic abundance)

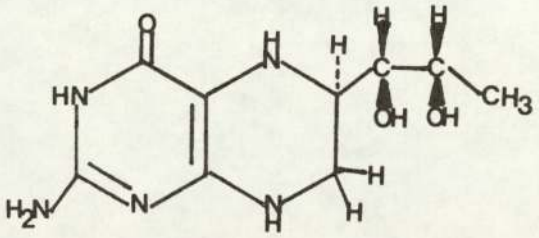
* ^{3}H=[3',5',7,9-^{3}H] folic acid

(distribution of label as determined by tritium nuclear magnetic resonance spectroscopy = 42.5% at positions 3',5',
= 25.5% at position 7,
= 32% at position 9)

FIGURE 1.10



(11) 7,8 dihydrobiopterin (DHB)



(12) l-erythro-5,6,7,8 tetrahydrobiopterin (THB)

1.3.1 FUNCTIONS

THB is an essential cofactor for the amino acid hydroxylases and is therefore necessary for the biosynthesis of catecholamine neurotransmitters and serotonin. THB is also involved in the cleavage of alkyl acyl glycerol ethers (Nichol *et al.*, 1985).

Its cofactor function is related to its ability to reduce molecular oxygen by formation of a 4a-hydroxyperoxy-tetrahydropterin or similar species involving Fe at the active site (Hasegawa *et al.*, 1978).

Intracellular levels of THB are maintained by *de novo* synthesis from GTP and salvage by DHPR - both pathways are necessary for normal neurological function (Nichol *et al.*, 1985).

1.3.2 *DE NOVO* SYNTHESIS OF THB

The first step in the synthesis of THB from GTP is the formation of 7,8 dihydroneopterin-triphosphate (NH_2TP). The reaction is catalysed by the enzyme GTP cyclohydrolase and involves imidazole ring opening, loss of C8 as formate, an Amadori type rearrangement to place a keto function at 2'C and formation of a N5-C6 double bond, (Burg and Brown, 1968).

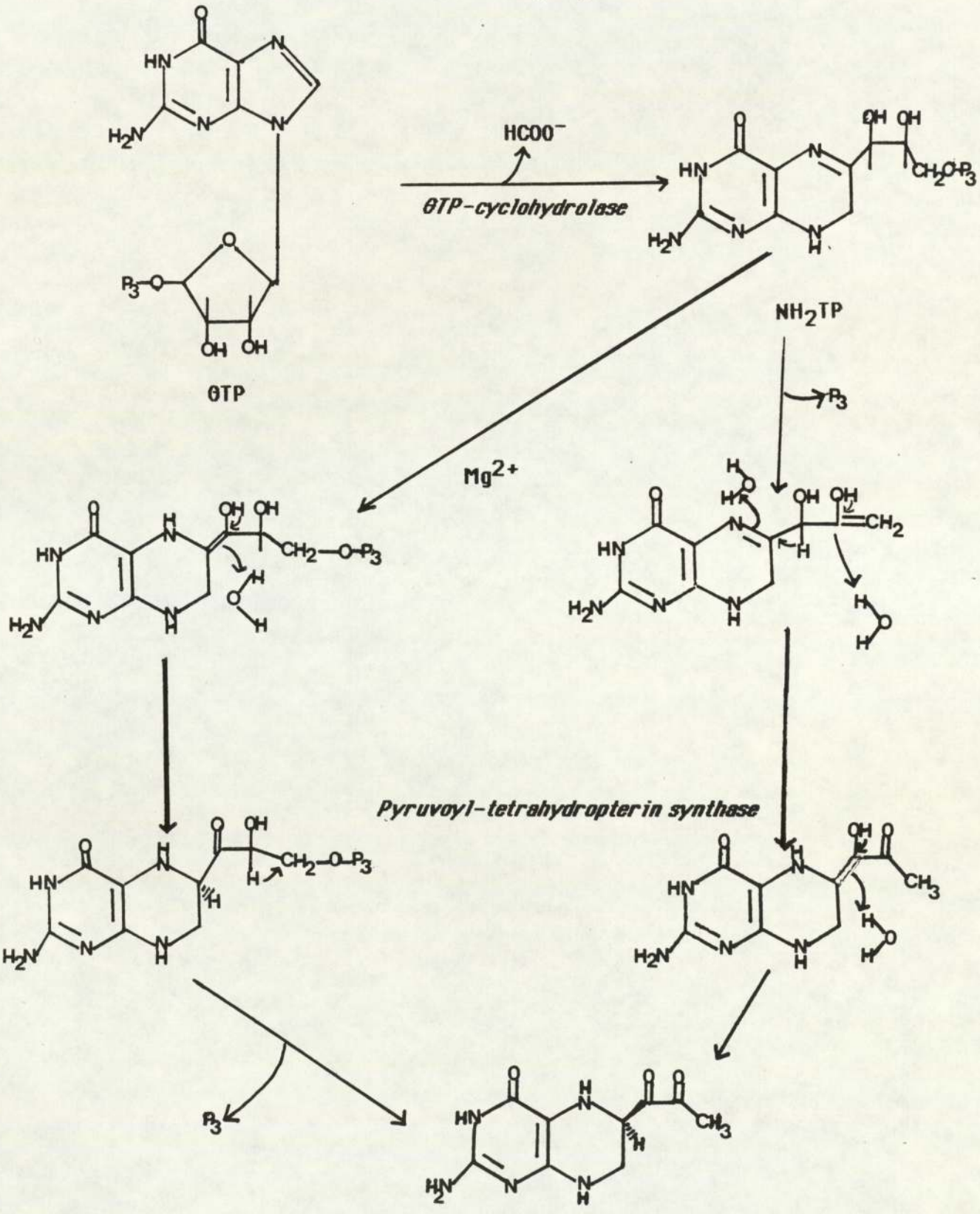
Further intermediates in the biosynthetic pathway are tetrahydropterins. DHFR has no role in *de novo* biosynthesis, (Nichol *et al.*,1983, Switchenko and Brown, 1985, Brown *et al.*, 1986, Curtius *et al.*, 1986, Milstien and Kaufman, 1986, Smith *et al.*, 1986(b)). 6-Pyruvoyl-tetrahydropterin synthase catalyses the formation of 6-pyruvoyl-tetrahydropterin, the reaction requires Mg^{2+} and involves the elimination of triphosphate and ketonisation to form a tetrahydropterin having a diketo side chain at position 6, (Brown *et al.*, 1986, Curtius *et al.*, 1986, Milstien and Kaufman, 1986, Smith *et al.*, 1986(b)). Figure 1.11 shows the formation of 6-pyruvoyl-tetrahydropterin from GTP.

The final stage in the biosynthetic pathway is the two stage reduction catalysed by sepiapterin reductase and requiring NADPH, (Brown *et al.*, 1986, Curtius *et al.*, 1986, Milstien and Kaufman, 1986, Smith *et al.*, 1986(b)). A second reductase has also been identified, this catalyses the formation of 6-lactoyl-tetrahydropterin from 6- pyruvoyl-tetrahydropterin which may be reduced to THB by sepiapterin reductase - this is believed to be a minor pathway

FIGURE 1.11

De novo synthesis of THB

Synthesis of 6-pyruvoyl-tetrahydropterin from GTP



in THB synthesis (Brown *et al.*, 1986, Curtius *et al.*, 1986, Milstien and Kaufman, 1986, Smith *et al.*, 1986(b)). Figure 1.12 shows the formation of THB from 6-pyruvoyl-tetrahydropterin.

1.3.3 SALVAGE PATHWAY FOR THB

THB is oxidised to quinonoid dihydrobiopterin (qDHB) during amino acid hydroxylation, levels of THB are maintained by DHPR which reduces qDHB back to THB (Craine *et al.*, 1972).

Failure to convert qDHB back to THB results in non-enzymic tautomerization to 7,8 dihydrobiopterin, which is not a substrate for DHPR. As levels build up in the cell 7,8 DHB passes out into the serum and is ultimately lost in the urine. This loss of biopterin in the urine may be decreased by DHFR. DHFR has a role in salvaging 7,8 DHB back to THB, (Craine *et al.*, 1972). The salvage of THB is shown in figure 1.13.

1.3.4 DEFECTS IN THB METABOLISM

Several genetic defects in THB metabolism have been identified. These include deficiency of GTP cyclohydrolase, defect in the biosynthetic pathway beyond NH_2TP and deficiency of DHPR. All result in impaired neurological function due to depletion of brain precursors of catecholamine and serotonin neurotransmitters. Therapy for these 'Malignant Hyperphenylalaninaemias' includes a low phenylalanine diet and neurotransmitter precursors, (Armarego *et al.*, 1984, Nichol *et al.*, 1985). It has been shown that in DHPR deficients some turnover of THB occurs in the liver possibly via DHFR and 5-10 methylene THF reductase, (Smith *et al.*, 1986(a), Hyland *et al.*, 1986).

1.4 RATIONALE FOR STUDY

The prevalence and causes of folate deficiency have been discussed. Factors which affect the extent of catabolism will affect folate availability and may result in deficiency. The fate of orally administered folic acid was monitored after the administration of substances known to affect the oxidising species within the body in an attempt to elucidate their effects on folate catabolism and the mechanism by which this occurs. Oxidising species were monitored using a modified assay employing nitro blue tetrazolium (NBT) (Surdhar, 1987).

FIGURE 1.12

De novo synthesis of THB

Synthesis of THB from 6-pyruvoyl-tetrahydropterin

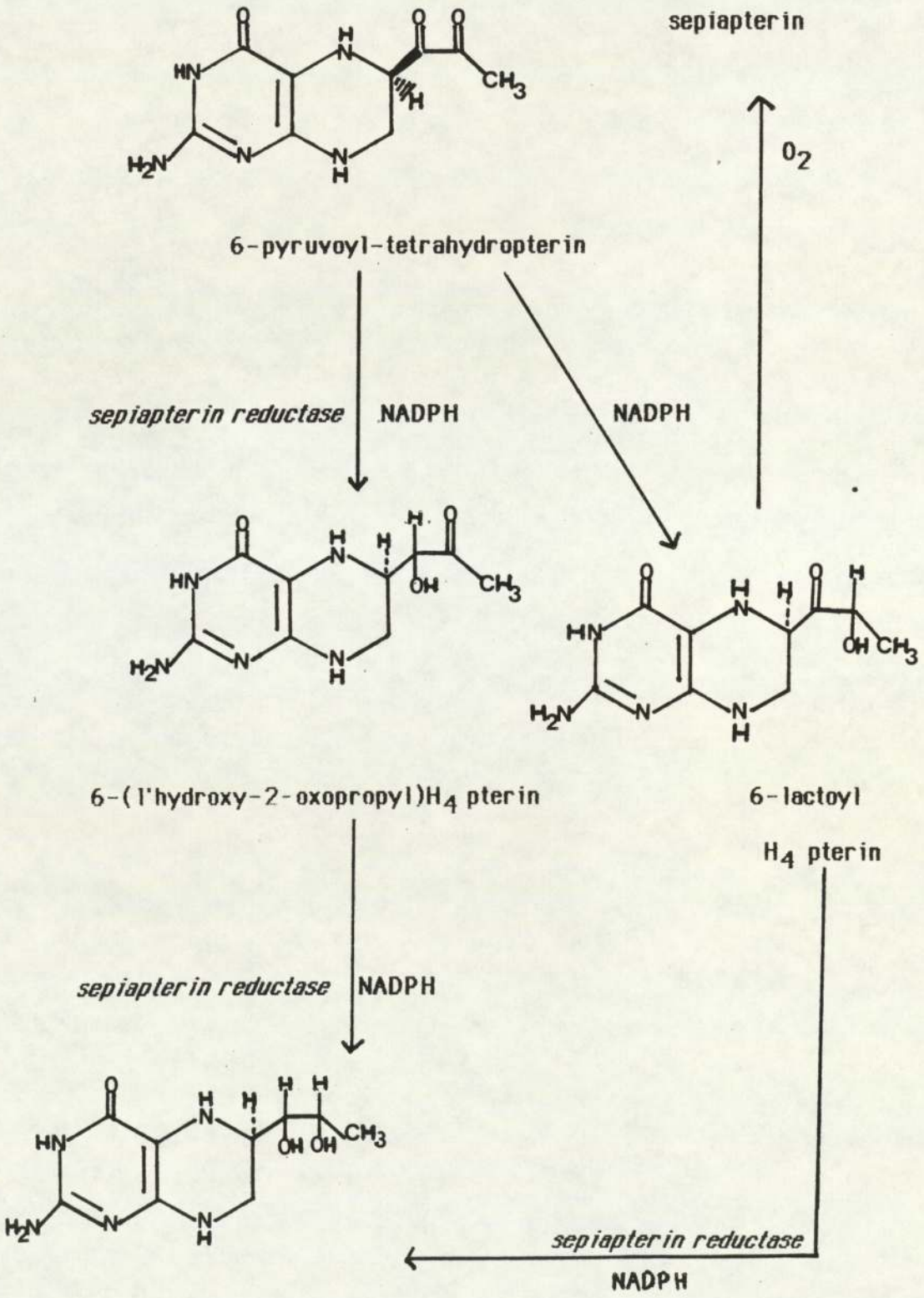
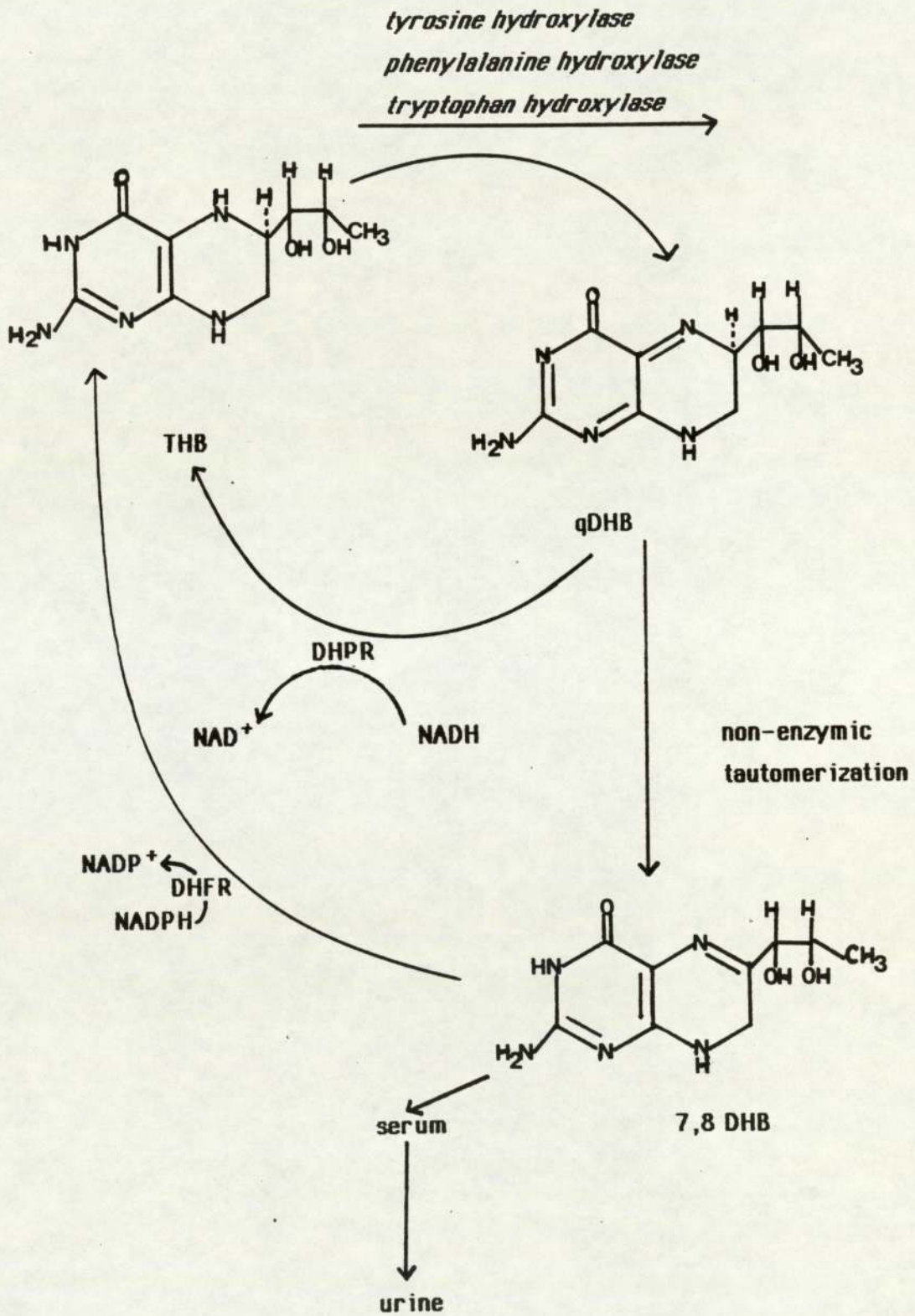


FIGURE 1.13

Salvage of THB



THB is a readily oxidisable species (Blair and Pearson, 1974). The effects of the antioxidant dl-α-tocopherol acetate (Vitamin E) on total tissue biopterin, DHPR and sepiapterin reductase were observed to see if this may have a protective role in THB metabolism.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO.

2.1 MATERIALS AND METHODS

2.1.1 MATERIALS (CHEMICAL)

The following materials were obtained commercially as described: folic acid, Koch-Light Laboratories Limited (Bucks); 5-methyltetrahydrofolate, Eprova Research Institute (Basle, Switzerland); p-amino-benzoyl-L-glutamate, p-amino-benzoic acid, diethylstilboestrol, NADH, NADPH, $DMPH_4$, BHT, pterin, horseradish peroxidase, dithiothreitol, Hanks Balanced Salts Solution, Nitroblue tetrazolium, ferricytochrome C, FAD, Menadione, EDTA, heparin and dl-α - tocopherol acetate, Sigma Chemical Company (Poole, Dorset.); p-acetamido-benzoic acid, Aldrich Chemical Company (Dorset). 'Optiphase Safe', Fisosolve Tissue Solubilizer, chloroform, methanol (HPLC grade) and hydrogen peroxide, Fisons Scientific Apparatus (Loughborough); dimethylsulphoxide, glacial acetic acid, formaldehyde and Folin and Ciocalteus Reagent, British Drug House (BDH) Ltd., (Dorset); .L-sepiapterin, L-erythrobiopterin were obtained from Dr B Schircks (Switzerland). All other reagents were of AnalaR Grade.

2.1.2 MATERIALS (RADIOCHEMICAL)

[2-^{14}C] Folic acid (potassium salt) specific activity 55mCi/mmol and 95% radiochemical purity, [3',5',7,9-^{3}H] folic acid (potassium salt) specific activity 500mCi/mmol and 96% radiochemical purity and 5-[$^{14}CH_3$] methyl-tetrahydrofolic acid (barium salt) specific activity 60mCi/mmol and 95% radiochemical purity were obtained from Amersham International p.l.c. (Amersham, Bucks.,)

The purity of the folic acid was checked by ion-exchange chromatography prior to use and was found to be 87.3% for ^{3}H and 86.7% for ^{14}C (figure 2.1). It was observed that ^{3}H species elute earlier than ^{14}C species, as has been observed previously by others, (Connor, 1979, Said, 1980) and is believed to be a secondary isotope effect due to labelling at C9, (Connor *et al.*,1980).

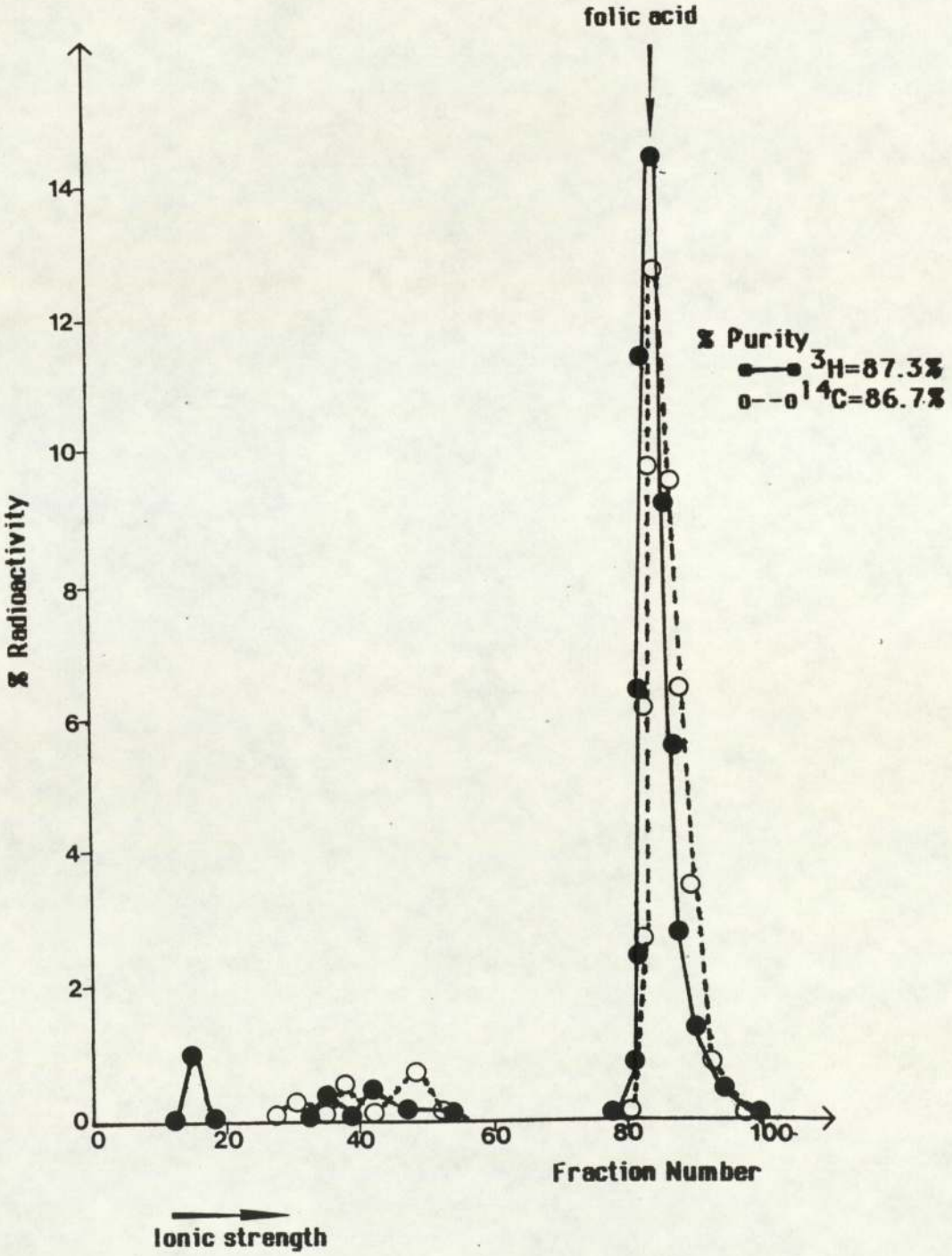
2.2 METHODS

2.2.1 ANIMALS

Healthy female Grade (iv) Wistar rats (Bantin and Kingman Ltd., Hull.) 250±20g body weight were used.

FIGURE 2.1

DE52 chromatogram. Radiochemical purity of o--o [2-^{14}C] folic acid and •--• [3',5',7,9-^{3}H] folic acid.
(samples were eluted in a gradient of 0-1.2M NaCl in 0.05M sodium phosphate, pH 7. Gradient was eluted over 8h.)



Rats were dosed orally by stomach intubation using specially prepared steel dosing needles. Diethylether was used as a restraint.

Folates were administered in 0.05M sodium phosphate buffer, pH 7, containing sodium ascorbate 2%(w/v). The animals were then transferred to individual metabolism cages (Jencons Metabowls, Jencons (Scientific) Limited, Hemel Hempstead, Herts.,) designed to allow the separate collection of urine and faeces. During experiments the animals were housed at 21°C in a sealed room having a fixed 12 hour dark and 12 hour light cycle and allowed free access to food (Rat and Mouse breeding diet (code 422) Pilsbury's Ltd.,) and water.

Urine was collected into $5cm^3$ 0.05M sodium phosphate buffer, pH 7, containing sodium ascorbate 2%(w/v), flask contents were protected from light degradation with aluminium foil.

Rats were killed by cervical dislocation and tissues rapidly removed. Radioactivity of urines was determined after which urines were made up to a constant volume with 0.05M sodium phosphate buffer, pH 7, containing sodium ascorbate 2%(w/v) and pooled. All samples were stored at -20°C.

2.2.2. DETERMINATION OF RADIOACTIVITY

Samples were counted in a total volume of $1cm^3$ with $10cm^3$ of scintillation cocktail (Fisons 'Optiphase Safe') in a Beckman LS 7500 Liquid Scintillation Counter (Beckman Instruments Inc., Scientific Instruments Division, Irvine, California). Samples were counted for 10 minutes or 10,000 counts. Appropriate corrections were made for background, quenching and overlap of ^{14}C into the ^{3}H channel.

URINES

Urinary radioactivity was determined by counting $0.1cm^3$ aliquots of each sample as described above.

TISSUES

0.1g tissue samples were solubilized in $1cm^3$ tissue solubilizer (Fisons, 'Fisosolve'), hydrogen peroxide was added dropwise to discolour, $10cm^3$ of scintillation cocktail containing $7cm^3$/litre glacial acetic acid was added and samples counted after 24 hours.

FAECES

Faecal samples were freeze dried and ground to a homogenous powder. 0.02g samples were solubilized in $1cm^3$ tissue solubilizer (Fisons 'Fisosolve'), $0.5cm^3$ of isopropanol was added to enhance solubility. Hydrogen peroxide was added dropwise to discolour, $10cm^3$ of scintillation cocktail containing $7cm^3$/litre glacial acetic acid was added and samples were counted after 24 hours as a gel by the addition of $10cm^3$ water.

2.2.3 CHROMATOGRAPHY

ION-EXCHANGE CHROMATOGRAPHY

Urinary folates were separated by ion-exchange chromatography as described by Barford *et al.*, (1977).

Diethylaminoethyl cellulose (DE52, Whatman Ltd., Maidstone, Kent) equilibrated in 0.05M sodium phosphate buffer, pH 7, containing dithiothreitol (DTT) 5mg%(w/v) was packed into glass columns (University of Aston, Glass Blowing Department) (2cmx40cm) plugged with glass wool. Samples and appropriate standards were loaded onto the column and eluted with a linear gradient of 0-1.2M NaCl in 0.05M sodium phosphate buffer, pH 7 containing DTT 5mg% (w/v). The gradient was eluted automatically over 8 hours using an LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon, Surrey.) The eluant was passed through a UV monitor (LKB Uvicord II, LKB Instruments, Croydon, Surrey.) $5cm^3$ fractions were collected using an LKB Ultrarac fraction collector (LKB Instruments, Croydon, Surrey.) Aliquots ($0.5cm^3$) of each fraction were counted as described. A typical elution profile of pooled urines is shown in figure 2.2.

GEL FILTRATION

Peaks obtained from ion-exchange chromatography were further resolved by gel filtration as described by Barford *et al.*, (1977).

Sephadex G15 (Pharmacia Ltd.,) in 0.05M sodium phosphate buffer, pH 7, containing DTT 5mg% (w/v) was packed in Perspex columns (2cmx60cm) (Wright Scientific Ltd., Surrey.) Samples and standards were eluted from the base of the column upwards with 0.05M sodium phosphate buffer, pH 7, containing DTT 5mg% (w/v). The eluant was monitored and collected as for ion-exchange chromatography. Aliquots ($1cm^3$) of each fraction were counted as described.

A typical elution profile showing resolution of urinary scission products (peak 3) on G15 is shown in figure 2.3.

FIGURE 2.2

DE52 chromatogram. Urines 0-24h after administration of •--•[3',5',7,9-^{3}H] folic acid and o--o[2-^{14}C] folic acid. (samples were eluted in a gradient of 0-1.2M NaCl in 0.05M sodium phosphate, pH 7. Gradient was eluted over 8h.)

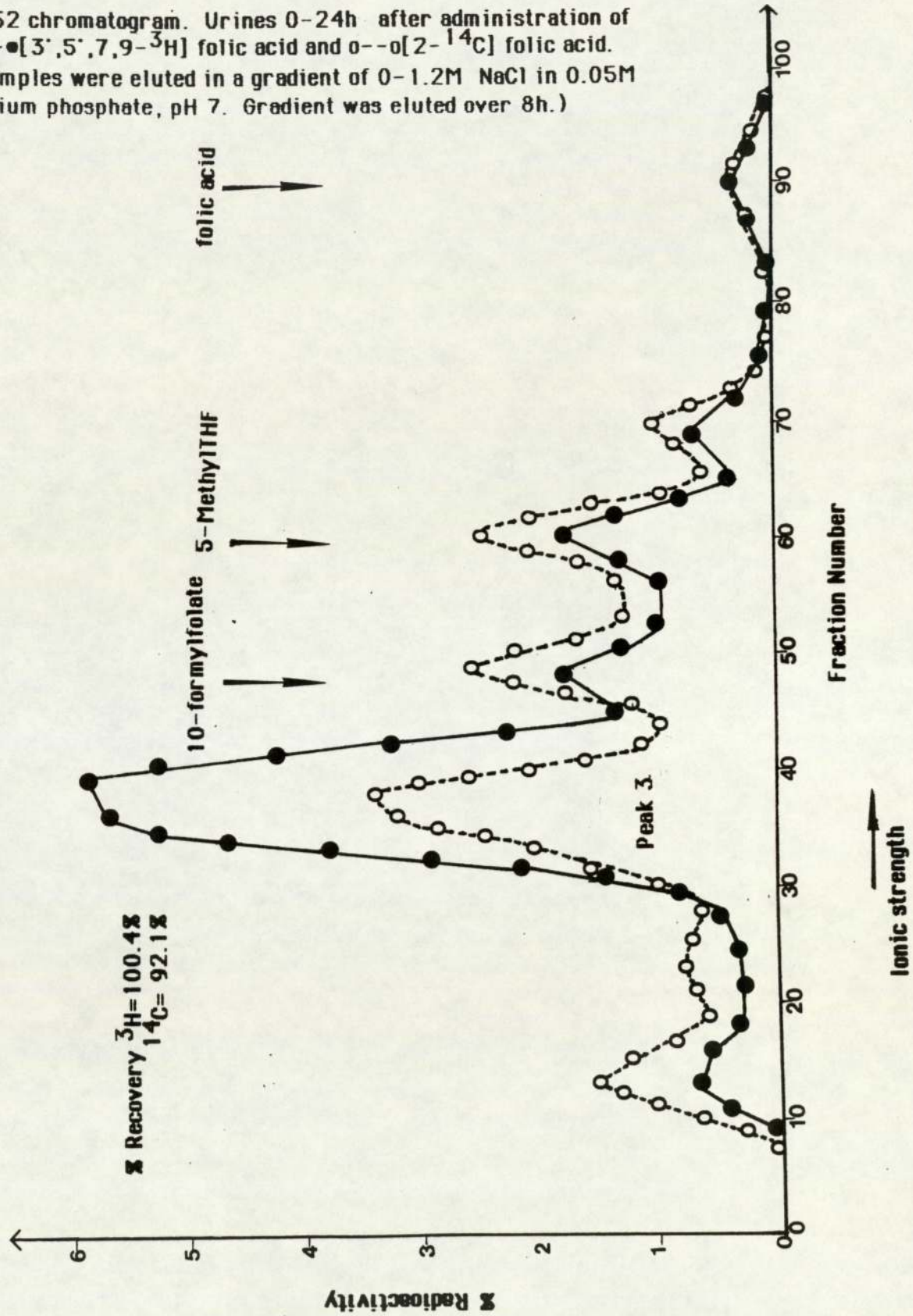
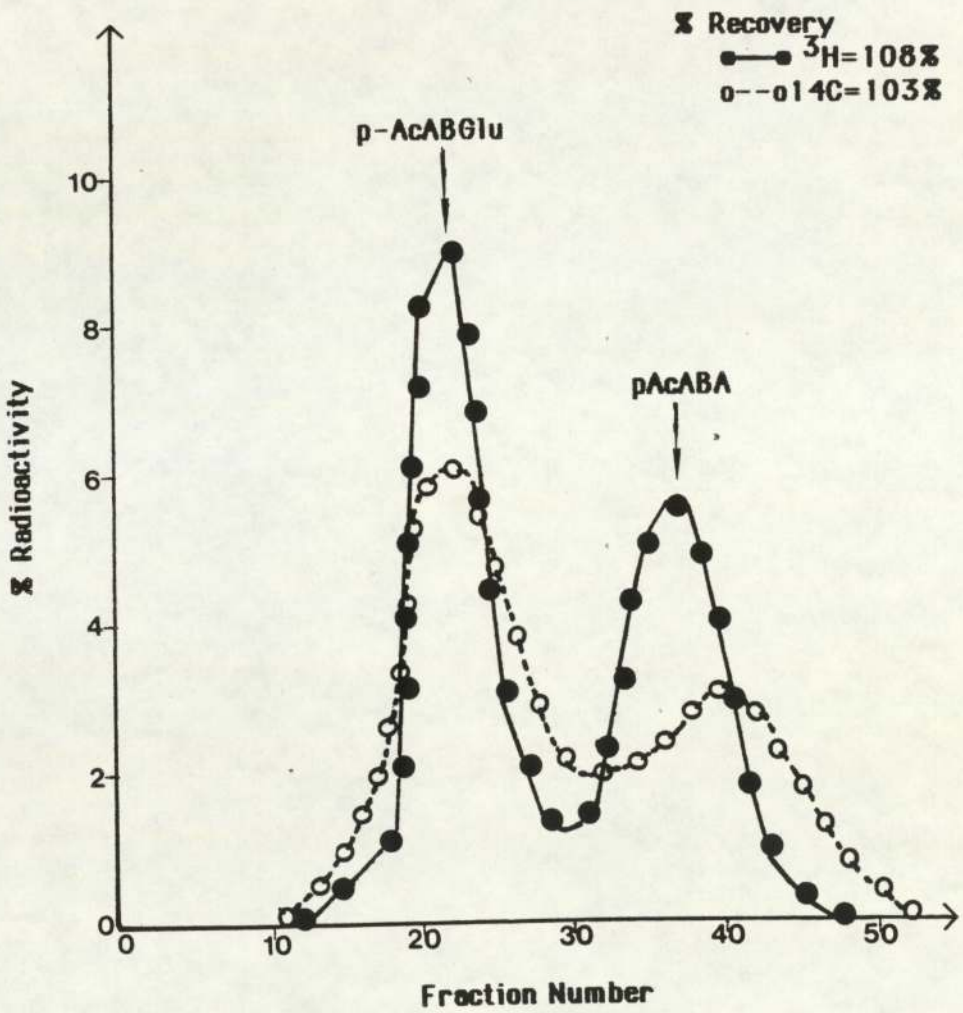


FIGURE 2.3
Sephadex G15 chromatogram of Scission peak



Liver folates were analysed by gel filtration as described by Barford *et al.* (1977).

Livers were removed rapidly, washed in ice cold buffer and portions dropped into 4 volumes of boiling 0.05M sodium phosphate buffer, pH 7, containing sodium ascorbate 2% (w/v) and maintained at 100°C for 5 minutes. This inactivates the conjugase enzymes which would rapidly break down the polyglutamates. The extract was homogenised and centrifuged prior to loading onto Sephadex G15. A typical elution profile of liver extracts is shown in figure 2.4.

2.2.4 ENZYMES

2.2.4.1 SAMPLE PREPARATION

Tissues were homogenised in assay buffer 30% (w/v) using a Potter-Elvehjem homogeniser. The homogenate was centrifuged for 45 minutes at 4°C, 100,000xg in a MSE Superspeed 50 ultracentrifuge using a MSE 10x10 cm^3 angle rotor (Measuring and Scientific Equipment Ltd., London.) Supernatants were stored at -20°C.

2.2.4.2 DIHYDROPTERIDINE REDUCTASE (EC 1.6.99.7) IN TISSUES

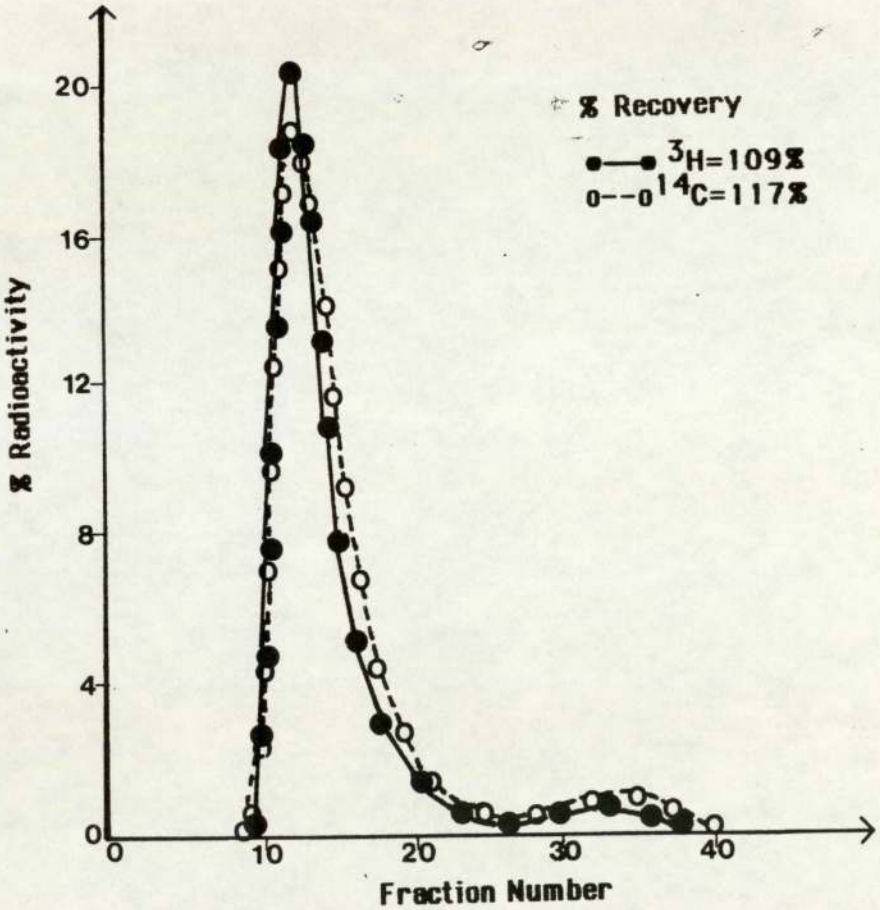
Dihydropteridine reductase (DHPR) was determined in brains and livers using the peroxidase method as described by Craine *et al.*, (1972).

The quinonoid dihydropterin was generated from the tetrahydropterin with peroxidase and hydrogen peroxide. The reaction was followed by measuring the decrease in absorbance at 340nm due to oxidation of NADH.

The assay cuvettes (1cm path length) contained the following in a final volume of 1cm^3; $2.5x10^{-4}$M sodium azide (which inhibits catalase activity), 8µg horseradish peroxidase, $1x10^{-4}$M NADH, $1x10^{-3}$M H_2O_2, $1x10^{-4}$M 6,7 Dimethyl 5,6,7,8 tetrahydropterin ($DMPH_4$) in 0.05M Tris-maleate buffer, pH 6.8 and 0.02cm^3 of tissue supernatant. All reagents other than $DMPH_4$ were preincubated for 90s at 37°C, the reaction was initiated by addition of $DMPH_4$ and the decrease in absorbance at 340nm was measured after 30s against an enzyme free mixture using a Unicam SP1700 UV Spectrophotometer and recorded using a Unicam AR55 Linear Recorder. Proteins were determined by the biuret method (Gornall *et al*, 1949). Results were expressed as nmoles NADH oxidised /min/mg protein using a molar extinction coefficient of $6.22x10^3M^{-1}$ cm^{-1} for NADH at 340nm.

FIGURE 2.4

Sephadex G15 chromatogram of liver extract 24h after dosing with •—•[3',5',7,9-^{3}H] folic acid and o--o[2-^{14}C] folic acid.



2.2.4.3. DIHYDROPTERIDINE REDUCTASE (EC 1.6.99.7) IN WHOLE BLOOD

Blood was collected from rats by cardiac puncture into heparinised syringes (500 IU/ml isotonic saline.) Blood was hemolysed by diluting 25 fold with distilled water.

Dihydropteridine reductase was determined by the method described by Narisawa *et al*, (1981).

The quinonoid dihydropterin was generated from the tetrahydropterin with ferricytochrome C. The reaction was followed by measuring the increase in absorbance at 550nm due to reduction of ferricytochrome C.

The assay cuvettes (1cm path length) contained the following in a final volume of $1cm^3$; $5x10^{-5}M$ ferricytochrome C, $1x10^{-4}M$ NADH, $1x10^{-5}M$ $DMPH_4$ in 0.05M Tris-maleate buffer, pH 6.8 and $0.1cm^3$ hemolysed blood. All reagents other than $DMPH_4$ were preincubated for 90s at 37°C, the reaction was initiated by addition of $DMPH_4$ and the increase in absorbance at 550nm was measured after 30s against an enzyme free mixture using a Unicam SP1700 UV Spectrophotometer and recorded using a Unicam AR55 Linear Recorder. Proteins were determined by the method of Lowry *et al*, (1951). Results were expressed as nmol cytochrome C reduced/min/mg protein using a molar extinction coefficient of $21x10^3M^{-1}cm^{-1}$ for cytochrome C at 550nm.

2.2.4.4 DIHYDROFOLATE REDUCTASE (EC 1.5.1.3)

Dihydrofolate reductase (DHFR) levels were determined in the liver. The method was based on that described by Osborn and Huennekens, (1958).

Dihydrofolic acid (DHF) was prepared by dithionite reduction of folic acid as described by Blakley (1960), this was stored frozen as a suspension in $1x10^{-3}M$ HCl containing $5x10^{-2}M$ mercaptoethanol for a period not exceeding 5 weeks.

The assay cuvettes (1cm path length) contained the following in a final volume of $1cm^3$; $4x10^{-5}M$ DHF, $8x10^{-5}M$ NADPH, $7.2x10^{-3}M$ mercaptoethanol in 0.05M potassium phosphate buffer, pH 7.4 and $0.02cm^3$ of tissue supernatant. All reagents other than NADPH were preincubated for 120s at 37°C, the reaction was initiated by addition of NADPH and the decrease in absorbance at 340nm was measured after 180s against an enzyme free mixture using a Unicam SP1700 UV spectrophotometer. Proteins were determined by the biuret method (Gornall

et al,1949). Results were expressed as µmoles DHF reduced/min/mg protein using a molar extinction coefficient of $5.8 \times 10^{3} M^{-1} cm^{-1}$ for DHF at 340nm.

2.2.4.5 5,10-METHYLENE TETRAHYDROFOLATE REDUCTASE (EC 1.1.99.15)

The method for 5,10 methylenetetrahydrofolate reductase was based on that described by Kutzbach and Stokstad, (1971).

5,10 Methylenetetrahydrofolate reductase was assayed in the back direction, [5-$^{14}CH_3$] methyl tetrahydrofolic acid was oxidised in the presence of FAD and the artificial electron acceptor menadione to yield 5,10 methylenetetrahydrofolate which spontaneously dissociates to tetrahydrofolate and ^{14}C formaldehyde which was measured by liquid scintillation counting after extraction in toluene.

The reaction mixture contained $5 \times 10^{-4} M$ [5-$^{14}CH_3$] methyltetrahydrofolic acid, $5 \times 10^{-3} M$ FAD, $2.5 \times 10^{-3} M$ menadione, $1 \times 10^{-3} M$ EDTA in 0.02M potassium phosphate buffer, pH 7 and $0.1 cm^3$ tissue supernatant. The mixture was incubated for 60min at 37°C and the reaction terminated by the addition of $0.4 cm^3$ 1M sodium acetate pH 4.5 containing 0.25M formaldehyde. Blank tubes contained no tissue supernatant. Tubes were stoppered, heated for 5min at 100°C, cooled in ice after which $5 cm^3$ water saturated toluene was added. ^{14}C formaldehyde was extracted in the upper toluene layer by centrifugation following vigorous shaking. $2 cm^3$ of the upper toluene layer was counted in $10 cm^3$ of Fisons 'Optiphase Safe' using a Beckman LS 700 Liquid Scintillation Counter (Beckman Instruments Inc., Scientific Instruments Division, Irvine, California). Samples were counted for 10 minutes or 10,000 counts. Appropriate corrections were made for background and quenching. Protein was determined by the biuret method (Gornall *et al*, 1949). Results were expressed as nmoles 5-methyltetrahydrofolate oxidised/h/mg protein.

2.2.4.6 SEPIAPTERIN REDUCTASE (EC 1.1.1.153)

Sepiapterin reductase was determined by the method of Katoh, (1971).

The assay cuvettes (1cm path length) contained the following in a final volume of $1 cm^3$; $5 \times 10^{-5} M$ sepiapterin, $1 \times 10^{-4} M$ NADPH in 0.1M Tris-HCl buffer, pH 7.4 and $0.05 cm^3$ tissue supernatant. The decrease in absorbance at 420nm was measured at 37°C against an enzyme free mixture using a Pye Unicam PU 8800 spectrophotometer. Proteins were determined by the biuret method (Gornall *et al*., 1949). Results were expressed as nmoles sepiapterin reduced /min/mg protein using a molar extinction coefficient of $10.2 \times 10^{3} M^{-1} cm^{-1}$ for sepiapterin at 420nm.

2.2.5 PROTEIN ESTIMATION

2.2.5.1 BIURET METHOD

The method was that of Gornall *et al*, (1949). 2cm^3 of biuret reagent (copper sulphate pentahydrate 0.075%(w/v), sodium potassium tartrate 0.3%(w/v) and sodium hydroxide 2.5%(w/v)) was added to 0.5cm^3 of sample. Samples were mixed and left at room temperature for 30min. Absorbance at 550nm was determined against a distilled water blank using a Shimadzu UV 240/UV visible recording spectrophotometer (Japan). Bovine serum albumin was used as standard.

2.2.5.2 FOLIN AND CIOCALTEUS METHOD

The method was that of Lowry *et al*, (1951). Solutions of sodium carbonate 2%(w/v) in 0.1M sodium hydroxide and copper sulphate pentahydrate 0.5%(w/v) in 1%(w/v) sodium potassium tartrate were mixed 50:1. 2cm^3 of the above reagent was mixed with 0.4cm^3 of sample and incubated at room temperature for 10min. 0.2cm^3 of Folin and Ciocalteus phenol reagent 50%(v/v) was added and mixed. The absorbance at 750nm was read after 30min against a distilled water blank using a Shimadzu UV 240/UV visible recording spectrophotometer (Japan). Bovine serum albumin was used as standard.

2.2.6 DETERMINATION OF OXIDISING SPECIES

Oxidising species in tissues were determined using nitroblue tetrazolium (NBT). The method was based on that of Baehner and Nathan, (1968).

The assay was based on the reduction of NBT to blue formazan. 0.5g tissue samples were washed and homogenised in 2cm^3 of Hanks Balanced Salt Solution (Hanks BSS) using a Potter-Evelhjem homogeniser. Homogenates were incubated at 37°C for 20min with 8×10^{-4}M NBT and 0.8%(v/v) dimethylsulphoxide. The reaction was stopped by centrifugation and the blue deposition due to formation of formazan was extracted for 10min with 1cm^3 of pyridine in a boiling water bath. The amount of formazan formed was determined from the absorbance at 578nm against a Hanks blank using a Unicam SP1700 UV spectrophotometer. Results were expressed as µmoles formazan/g wet weight using a molar extinction coefficient of $16.9 \times 10^3 M^{-1} cm^{-1}$ for formazan at 578nm.

2.2.7 MEASUREMENT OF PEROXIDES IN OILS

Peroxides were determined in oils by the method developed by Lea as described by Egan *et al.*, (1981).

The method was based on the reaction of potassium iodide in acid solution with the bound oxygen followed by titration of the liberated iodine with sodium thiosulphate.

To 1g of oil was added 1g of powdered iodide and $20cm^3$ of solvent mixture (2 volumes glacial acetic acid and 1 volume chloroform). The tube was placed into a boiling water bath and the mixture allowed to boil within 30s for not more than 30s. The contents were mixed with $20cm^3$ potassium iodide 5%(w/v) and $50cm^3$ water. The solution was titrated against standardised 0.002M sodium thiosulphate using starch 1%(w/v) as indicator. Results were expressed as mEquivalents of peroxide oxygen/kg oil (mEq/kg).

2.2.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Total tissue pterins and biopterins were determined by HPLC after acid/iodine oxidation using the method of Fukushima and Nixon, (1980).

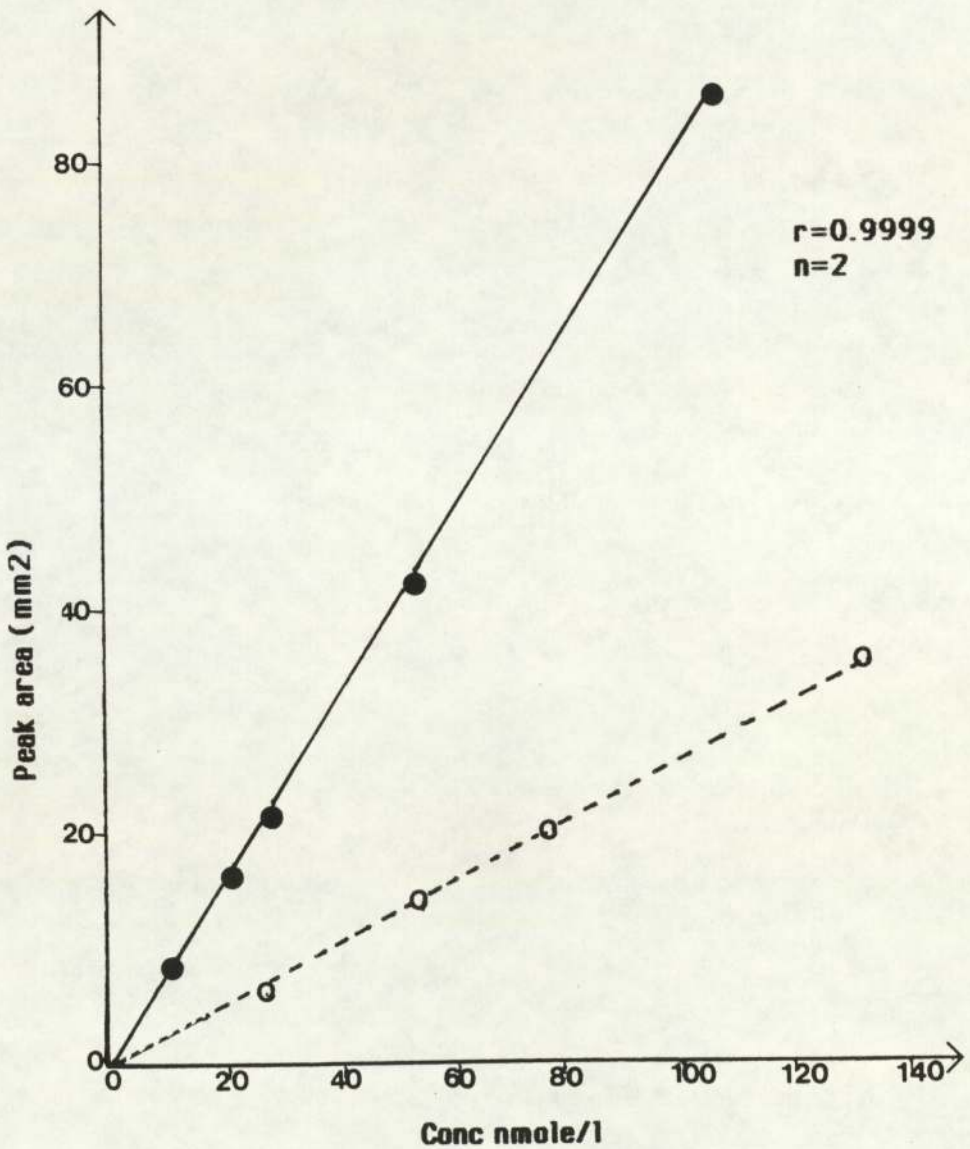
BRAINS 1g of tissue was homogenised with $1cm^3$ trichloroacetic acid (TCA) 20%(w/v) and $4cm^3$ of 0.1M HCl and centrifuged for 5min at 4°C, 100,000xg in a MSE Superspeed 50 Ultracentrifuge using a MSE $10x10cm^3$ angle rotor (Measuring and Scientific Equipment Ltd., London). $2cm^3$ of supernatant was incubated in the dark at room temperature for 1h with $0.5cm^3$ of iodine 3%(w/v). The reaction was stopped by the addition of ascorbic acid to discolour. The solution was analysed by HPLC.

LIVERS Livers were analysed essentially as above except 1g of tissue was homogenised with $1cm^3$ TCA 20%(w/v) and $9cm^3$ of 0.1M HCl.

The system consisted of a dual-piston pump (Laboratory Data Control, Milton Roy Constametric III pump, LDC, Staffs.,) auto sampler (Waters Intelligent Sample Processor, WISP710B, Waters Associate Inc., USA.) a spectrofluoromonitor SFM/23 (Kontron LDC fluoromonitor III model 1311, USA) with an excitation wavelength of 350nm and emission at 410nm and a W+W chart recorder (Model 302). The column used was a Spherisorb ODS reverse column (25cmx0.46cm) with a particle size of 5µm and a pre-column with similar packing. The solvent used was 5% aqueous methanol.

FIGURE 2.5

Biopterin ●——● and Pterin o---o standard curve



The system was calibrated before use with a series of biopterin and pterin standards of known concentrations. The concentration was determined by measuring the absorbance at pH 13 at 362nm for biopterin (molar extinction coefficient of $8.3x10^3M^{-1}cm^{-1}$) and 358nm for pterin (molar extinction coefficient of $6.6x10^3M^{-1}cm^{-1}$) using a Shimadzu UV 240/UV visible spectrophotometer (Japan). A typical standard curve is shown in figure 2.5. The injection volume was 20µl. Results were expressed as nmoles/g wet weight.

2.2.9 STATISTICAL ANALYSIS

Samples were analysed using the Students t-test on an Epson HX20 computer unless otherwise stated.

CHAPTER THREE

THE EFFECT OF LIPID PEROXIDES ON FOLATE CATABOLISM IN THE RAT

CHAPTER THREE.

3 THE EFFECT OF LIPID PEROXIDES ON FOLATE CATABOLISM IN THE RAT

3.1 INTRODUCTION

Epidemiological evidence has suggested that dietary fat may be linked with cancer promotion, (Willett and MacMahon, 1984, Gregorio *et al.*, 1985, Hopkins and Carroll, 1985, Lubin *et al*, 1985, Hill, 1987). This has been supported by animal studies. Fats - particularly polyunsaturated fats-have a powerful promoting effect on the development of chemically induced tumours, tumours are observed after a shorter latent period if the level of dietary fat is increased (Shamberger, 1980, Carrington and Hosick, 1985, Hopkins and Carroll, 1985, Oyaizu *et al.*, 1985, Hill, 1987).

Several mechanisms for tumour promotion by fats have been proposed. Cancer is a multistage, multifunctional process and more than one mechanism may be operating (Klurfeld and Kritchevsky, 1986). Mechanisms which have been proposed include:-increased bile acid concentrations (these are thought to be tumour promoters), (Hopkins and Carroll, 1985, Hill, 1987), altered hormone levels and hormone responsiveness (Gregorio *et al.*, 1985, Welsch *et al.*, 1985), increased prostaglandin synthesis (Kollmorgen et al., 1983) and altered membrane properties and functions as a result of incorporation of dietary fats into membranes (Booyens *et al.*, 1985, Solaini *et al.*, 1985, Wade *et al.*, 1985). The tumour promoting effect of fats is not a calorific effect (King and McCay, 1986, Klurfeld and Kritchevsky, 1986) but is due to fatty acid composition, a threshold level of linoleic acid (which is the predominant fatty acid in corn oils) is required (Cohen *et al.*, 1986).

Fats undergo lipid peroxidation and autoxidation resulting in the production of free radicals (Shamberger, 1980, Coultate, 1984), which may have a role in cancer promotion and may alter the extent of folate catabolism. Folate deficiency may arise during malignant disorders, this may be a result of increased demand or increased catabolism of folates. Inhibitors of DHFR have found use in chemotherapy. The effects of corn oil treatment and the peroxide content of corn oils on folate catabolism were investigated.

3.2 MATERIALS AND METHODS

Materials were obtained as described in Chapter 2.

Female Wistar rats (250g) were divided into three groups of 15 rats each. The control group were dosed orally with 2µCi of [2-^{14}C] and 5µCi of [3',5', 7,9-^{3}H] folic acid (83µg/kg body wt) in 0.05M sodium phosphate buffer, pH 7, containing 2% (w/v) sodium ascorbate. A second group of rats were dosed orally with 0.3cm^3 of refined corn oil (containing 0.01% (w/v) butylated hydroxytoluene (BHT) (2,6 di-tertiary butyl-4-methyl phenol)) for 3 days prior to being dosed with the same dose of radiolabelled folic acid. The third group of rats were dosed orally with 0.5cm^3 of pure Mazola corn oil (containing 0.02% (w/v) α-tocopherol) twice daily for 3 days prior to being dosed with the same dose of radiolabelled folic acid. 5 rats from each group were transferred to individual Metabowls and urines and faeces were collected as described in Chapter2. 24h after dosing with radiolabelled folic acid animals were killed by cervical dislocation and brains and livers were removed for analysis. The collection procedure was repeated for the next 2 groups of 5 animals such that for each treatment group 0-24h, 24-48h and 48-72h samples were collected. Throughout the experiment animals were allowed free access to food and water and were kept on grids to prevent coprophagy. Urines and faeces were analysed for radioactivity. Brains and livers were analysed for oxidising species, DHFR, DHPR and retention of radioactivity as described in Chapter 2. The peroxide value of the corn oils was determined as described in Chapter 2.

3.3 RESULTS

3.3.1 EXCRETION OF RADIOACTIVITY IN URINES.

The excretion of radioactivity in urines after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 3.1. The bulk of the radioactivity was excreted in the early urine samples (0-24h), with progressively less radioacivity being excreted over 24-48h and 48-72h. In each sample there is an excess of ^{3}H compared to ^{14}C excreted in the urines (31.03% ^{3}H and 21.31% ^{14}C in the 0-24h sample of the control group ($p<0.02$), 32.06% ^{3}H and 21.48% ^{14}C in the 0-24h sample of the refined corn oil dosed group ($p<0.05$) and 25.58% ^{3}H and 16.1% ^{14}C in the 0-24h sample of the Mazola corn oil dosed group).

TABLE 3.1

The effect of corn oils on the excretion of radioactivity in the urines of rats after the oral administration of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt).

EXCRETION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	31.03[2] ±5.89	21.31 ±4.64	32.06[1] ±7.34	21.48 ±4.6	25.58 ±8.12	16.1 ±6.01
24-48h	2.91[1] ±1.07	1.52 ±0.74	5.44 ±3.61	3.11 ±1.37	3.26 ±1.4	1.88 ±0.88
48-72h	2.24 ±0.65	1.65 ±1.04	3.56 ±2.36 (*)	1.66 ±1.03	2.77 ±1.5	1.42 ±0.67

(Results are expressed as mean±SD where n=5 except * where n=4.)

^{3}H over ^{14}C excess. [1] = $p<0.05$

[2] = $p<0.02$

3.3.2 EXCRETION OF RADIOACTIVITY IN THE FAECES.

The excretion of radioactivity in faeces after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 3.2. For each sample collected it is observed that there is an excess of ^{14}C compared to ^{3}H excreted, this excess is significant for all 0-24h samples, (3.2% ^{3}H and 16.96% ^{14}C in the control group ($p<0.05$), 6.98% ^{3}H and 24.08% ^{14}C in the refined corn oil dosed group ($p<0.001$) and 4.21% ^{3}H and 17.87% ^{14}C in the Mazola corn oil dosed group ($p<0.001$)). The ^{14}C excess continues to be significant for all samples after dosing with refined corn oil. More radioacivity is excreted after corn oil dosing, this increase is significant after dosing with refined corn oil (3.2% ^{3}H in the controls compared with 6.98% ^{3}H after dosing with refined corn oil ($p<0.001$) (0-24h), 3.31% ^{14}C in the controls compared with 11.61% ^{14}C after dosing with refined corn oil ($p<0.05$) (24-48h) and 1.86%^{14}C in the controls compared with 5.78%^{14}C after dosing with refined corn oil ($p<0.02$) (48-72h). More radioactivity is excreted after dosing with refined corn oil when compared to dosing with Mazola corn oil (6.98% ^{3}H and 24.08% ^{14}C after dosing with refined corn oil compared with 4.21% ^{3}H and 17.87% ^{14}C after dosing with Mazola corn oil ($p<0.01$ for ^{3}H and $p<0.05$ for ^{14}C (0-24h)).

3.3.3 RETENTION OF RADIOACTIVITY IN THE LIVER

The retention of radioactivity in the liver after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 3.3. There is a consistent excess of ^{3}H retained compared to ^{14}C. Retention of radioactivity was decreased after corn oil treatment, 18.52% ^{3}H and 14.32% ^{14}C in the controls compared to 12.63% ^{3}H ($p<0.05$) and 9.61% ^{14}C ($p<0.01$) after dosing with refined corn oil and 13.13% ^{3}H and 12.24% ^{14}C after dosing with Mazola corn oil (for liver retentions 24h after dosing with radiolabelled folic acid).

Analysis of liver extracts by Sephadex G15 chromatography resulted in elution of a high molecular weight peak compatible with the elution of a polyglutamate species, there was no difference in the pattern of elution between the three groups.

3.3.4 RETENTION OF RADIOACTIVITY IN THE BRAIN.

The retention of radioactivity in the brain after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 3.4. There is a significant excess of ^{3}H retained compared to ^{14}C retained in all samples

TABLE 3.2

The effect of corn oils on the excretion of radioactivity in the faeces of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

EXCRETION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	3.2 ±0.58	16.96[1] ±10.87	6.98[#c] ± 0.94	24.08[S3] ±1.82	4.21[#] ±1.55	17.87[S3] ±4.61
24-48h	2.27 ±1.35	3.31 ±2.1	3.57 ±2.06	11.61[a1] ±6.84	2.21 ±1.48	6.86 ±4.51
48-72h	1.46 ±0.84	1.86 ±1.97	2.48 ±0.77 (*)	5.78[#b2] ±1.73	1.77 ±0.64	2.0[#] ±0.58

(Results are expressed as mean±SD where n=5 except * where n=4.)

^{14}C over ^{3}H excess. 1=p<0.05
2=p<0.02
3=p<0.001

Comparing corn oil treated with control. a=p<0.05
b=p<0.02
c=p<0.001

Comparing refined corn oil with Mazola corn oil. S=p<0.05
#=p<0.01

TABLE 3.3

The effect of corn oils on the retention of radioactivity in the liver of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

RETENTION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
24h	18.52[1] ±3.72	14.32 ±1.55	12.63[a] ±2.79	9.61[c] ±2.28	13.15 ±4.61	12.24 ±3.49
48h	21.46[1] ±6.1	13.61 ±3.76	16.08[2] ±2.21	12.2 ±1.67	12.22[b] ±3.38	11.45 ±3.59
72h	18.81[4] ±2.93	11.44 ±1.87	15.78[3*] ±1.48	11.97 ±1.29	9.56[d*] ±1.49	10.52 ±2.08

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1]=p<0.05
[2]=p<0.02
[3]=p<0.01
[4]=p<0.002

Comparing corn oil treated with control. [a]=p<0.05
[b]=p<0.02
[c]=p<0.01
[d]=p<0.001

Comparing refined corn oil with Mazola corn oil. [*]=p<0.001

TABLE 3.4

The effect of corn oils on the retention of radioactivity in the brain of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

RETENTION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
24h	0.11[5] ±0.02	0.05 ±0.01	0.08[1] ±0.03	0.04 ±0.01	0.11[5] ±0.01	0.04 ±0.01
48h	0.11[5] ±0.02	0.05 ±0.01	0.08[5b] ±0.01	0.04 ±0.01	0.09[4] ±0.02	0.04 ±0.01
72h	0.18[2] ±0.06	0.09 ±0.03	0.10[3a] ±0.03	0.04[c] ±0.01	0.11[5a] ±0.01	0.04[c] ±0.01

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1]=p<0.05
[2]=p<0.02
[3]=p<0.01
[4]=p<0.002
[5]=p<0.001

Comparing corn oil treated with control. [a]=p<0.05
[b]=p<0.02
[c]=p<0.01

(0.11% ^{3}H compared to 0.05% ^{14}C in the control group ($p<0.001$), 0.08% ^{3}H compared to 0.04% ^{14}C after dosing with refined corn oil ($p<0.05$) and 0.11% ^{3}H compared to 0.04% ^{14}C after dosing with Mazola con oil ($p<0.001$) (for samples 24h after dosing with radiolabelled folic acid)). Less ^{3}H is retained after dosing with refined corn oil when compared to controls, this is significant at 48h ($p<0.02$) and at 72h ($p<0.05$).

3.3.5 METABOLITES IDENTIFIED IN 0-24h URINES.

The following species were identified in 0-24h urines:- folic acid, two unidentified dual labelled species (A and B), $5CH_3THF$, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 3.5. Intact folates and products of catabolism were detected in all groups.

Less folic acid was excreted after dosing with corn oil (1.83% ^{3}H and 1.71% ^{14}C in controls, 1.03% ^{3}H and 0.83% ^{14}C after refined corn oil and 0.21% ^{3}H and 0.17% ^{14}C after Mazola corn oil). Metabolite A was not detected in the corn oil dosed groups. Excretion of $5CH_3THF$ was increased after dosing with corn oil, 13.88% ^{3}H and 10.53% ^{14}C after refined corn oil and 11.17% ^{3}H and 8.1% ^{14}C after Mazola corn oil compared to 8.72% ^{3}H and 7.24% ^{14}C in the controls. Metabolite B was not detected after dosing with refined corn oil and was decreased after dosing with Mazola corn oil (1.88% ^{3}H and 1.15% ^{14}C compared to 2.38% ^{3}H and 1.83% ^{14}C in the control group). The excretion of pAcABGlu and pAcABA was increased after dosing with refined corn oil (8.6% and 4.27% compared with 5.01% and 3.9% in the controls) and was decreased after dosing with Mazola corn oil (3.53% pAcABGlu and 3.53% pAcABA). The total ^{3}H only catabolites were increased after dosing with refined corn oil (12.87%) but decreased after dosing with Mazola corn oil (7.06%) compared to controls (8.91%). The excretion of urinary pterins was increased after corn oil dosage, (3.99% after refined corn oil and 2.38% after Mazola corn oil compared with 1.35% in the controls).

3.3.6 METABOLITES IDENTIFIED IN 24-48h URINES.

The following species were identified in 24-48h urines:- folic acid, metabolites A and B, $5CH_3THF$, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is given in table 3.6.

Folic acid was detected in control urines but was not present in urines after

TABLE 3.5

The effect of corn oil administration on metabolites detected in 0-24h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE					
	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
FOLIC ACID	1.83	1.71	1.03	0.83	0.21	0.17
METABOLITE A	0.44	0.4	ND		ND	
5CH$_3$THF	8.72	7.24	13.88	10.53	11.17	8.1
METABOLITE B	2.38	1.83	ND		1.88	1.15
10-CHO-FOLATE	5.0	5.23	6.73	7.6	4.01	3.51
pAcABGlu	5.01		8.6		3.53	
pAcABA	3.9		4.27		3.53	
total ^{3}H only metabolites	8.91		12.87		7.06	
PTERINS		1.35		3.99		2.28
^{14}C UREA		0.95		0.66		0.26
$^{3}H_2O$	0.58		0.75		0.75	

ND=not detected.

(Each value was determined by analysis of pooled urine from 5 rats.)

TABLE 3.6

The effect of corn oil administration on metabolites detected in 24-48h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE					
	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
FOLIC ACID	0.12	0.17	ND		ND	
METABOLITE A	0.25	0.28	0.86	0.86	ND	
5CH$_3$THF	0.11	0.1	0.45	0.23	0.72	0.51
METABOLITE B	ND		ND		0.09	0.07
10-CHO-FOLATE	0.37	0.18	0.3	0.32	0.61	0.5
pAcABGlu	0.18		1.71		0.92	
pAcABA	0.13		1.29		0.79	
total ^{3}H only metabolites	0.31		3.0		1.71	
PTERINS		0.11		0.57		0.37
^{14}C UREA		0.14		0.33		0.11
$^{3}H_2O$	0.23		0.77		0.23	

ND=not detected.

(Each value was determined by analysis of pooled urine from 5 rats.)

corn oil dosage. Excretion of metabolite A was increased after dosing with refined corn oil (0.86% ^{3}H and 0.86% ^{14}C compared to 0.25% ^{3}H and 0.28% ^{14}C in controls). Excretion of $5CH_3THF$ continued to be increased after dosing with corn oil, (0.45% ^{3}H and 0.23% ^{14}C after refined corn oil and 0.72% ^{3}H and 0.51% ^{14}C after Mazola corn oil compared to 0.11% ^{3}H and 0.1% ^{14}C in controls). Trace amounts of metabolite B were present in urines after dosing with Mazola corn oil. Increased excretion of pAcABGlu and pAcABA was observed after corn oil dosage, (1.71% and 1.29% after refined corn oil and 0.92% and 0.79% after Mazola corn oil compared to 0.18% and 0.13% in controls). The total ^{3}H only catabolites were increased after corn oil dosage, refined corn oil (3%), Mazola corn oil (1.71%) compared to controls (0.31%). The excretion of urinary pterins was increased after dosing with refined corn oil (0.57%) and after dosing with Mazola corn oil (0.37%) compared to controls (0.11%). More extensive breakdown of the folate molecule is suggested after dosing with refined corn oil since excretion of both ^{14}C urea and $^{3}H_2O$ is increased (0.33% and 0.77% compared to 0.14% and 0.23% in controls).

3.3.7 METABOLITES IDENTIFIED IN 48-72h URINES.

The following species were identified in 48-72h urines:- $5CH_3THF$, metabolite B, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 3.7.

The excretion of $5CH_3THF$ continued to be increased after dosing with refined corn oil, (0.64% ^{3}H and 0.63% ^{14}C compared with 0.24% ^{3}H and 0.21% ^{14}C in controls). Trace amounts of metabolite B continued to be excreted after dosing with Mazola corn oil. Excretion of 10-formyl folate was increased after corn oil dosage, (0.59% ^{3}H and 0.55% ^{14}C after refined corn oil, 0.53% ^{3}H and 0.42% ^{14}C after Mazola corn oil compared to 0.2% ^{3}H and 0.19% ^{14}C in controls). Increased excretion of pAcABGlu and pAcABA was observed after corn oil dosage, (0.63% and 0.84% after dosing with refined corn oil, 0.68% and 0.69% after dosing with Mazola corn oil compared to 0.22% and 0.06% in the control group). The total ^{3}H only catabolites were increased after both refined corn oil (1.47%) and Mazola corn oil (1.37%) compared to controls (0.28%). Excretion of ^{14}C urea (0.22%) and $^{3}H_2O$ (1.05%) were increased after dosing with refined corn oil compared to controls (0.18% and 0.23%) suggesting that more extensive breakdown of the folate molecule occurs after dosing with refined corn oil.

TABLE 3.7

The effect of corn oil administration on metabolites detected in 48-72h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE					
	CONTROL		REFINED CORN OIL		MAZOLA CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
5CH$_3$THF	0.24	0.21	0.64	0.63	0.27	0.2
METABOLITE B	ND		ND		0.15	0.15
10-CHO-FOLATE	0.2	0.19	0.59	0.55	0.53	0.42
pAcABGlu	0.22		0.63		0.68	
pAcABA	0.06		0.84		0.69	
total ^{3}H only metabolites	0.28		1.47		1.37	
PTERINS		0.32		0.25		0.43
^{14}C UREA		0.18		0.22		0.11
^{3}H$_2$O	0.23		1.05		0.39	

ND=not detected.

(Each value was determined by analysis of pooled urine from 5 rats.)

3.3.8 TOTAL EXCRETION OF SCISSION PRODUCTS.

The total pAcABGlu and pAcABA (^{3}H only catabolites) and 'pterins' excreted over the 72h collection period are shown in table 3.8. The excretion of these catabolites increases after dosing with corn oils, more catabolites were excreted after dosing with refined corn oil than with Mazola corn oil, (^{3}H only catabolites 9.5% (controls), 10.14% (Mazola corn oil) and 17.34% (refined corn oil) and 'pterins' 1.78% (controls), 3.08% (Mazola corn oil) and 4.81% (refined corn oil)).

3.3.9 OXIDISING SPECIES.

The oxidising species detected after corn oil and folic acid administration are shown in table 3.9. Oxidising species in the brain and liver increase after corn oil administration, this increase is greater after dosing with Mazola corn oil. There is a significant increase in liver oxidising species at 24h ($p<0.001$ for both refined and Mazola corn oils) and at 48h ($p<0.02$ for refined corn oil dosed rats and $p<0.01$ for Mazola corn oil dosed rats). Oxidising species are increased in the brains, this is significant only for the Mazola corn oil dosed group ($p<0.01$ at 24h, $p<0.05$ at 48h and $p<0.001$ at 72h when compared to controls).

3.3.10 EXTENT OF LIVER POLYGLUTAMATE BREAKDOWN.

The extent of liver polyglutamate breakdown may be calculated if it is assumed that any pAcABGlu excreted in urines after 24h arises from the liver polyglutamate fraction, this may be calculated where the percentage scission occurring over 24-48h:-

$$= \frac{\text{pAcABGlu excreted over 24-48h}}{\%\text{ of } ^{3}H \text{ in the liver at 24h}} \times 100$$

(it is necessary to correct for the ^{3}H retained in the liver since after scission 42.5% of the label remains in the glutamyl fraction).

The extent of liver polyglutamate breakdown is shown in table 3.10. The percentage scission over 24-48h is increased after corn oil dosage, control (2.29%), Mazola corn oil (16.46%) and refined corn oil (31.86%). The percentage scission over 48-72h continues to increase after corn oil dosage, control (2.41%), Mazola corn oil (13.09%) and refined corn oil (9.22%). If the net scission occurring is calculated it is observed that although corn oil increases

TABLE 3.8

The effect of corn oil administration on the excretion of urinary scission products over 0-72h after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt).

	% OF DOSE		
	CONTROL	**REFINED CORN OIL**	**MAZOLA CORN OIL**
total pAcABGlu and pAcABA 0-72h	9.5	17.34	10.14
total pterins 0-72h	1.78	4.81	3.08

(Results were determined from analysis of pooled urine samples from 5 rats for each 24h group.)

TABLE 3.9

Oxidising species detected by NBT assay after corn oil and radiolabelled folate administration

	CONTROL	REFINED CORN OIL	MAZOLA CORN OIL
		(µmoles formazan/g wet wt/20 min)	
24h LIVER	0.81±0.07	1.85±.33[d]	2.22±0.38[d]
48h LIVER	0.85±0.19	1.48±0.43[b]	2.18±0.82[c]
72h LIVER	1.15±0.2	1.07±0.3	1.84±0.7
24h BRAIN	1.27±0.39	0.9±0.63[1]	2.16±0.38[c1]
48h BRAIN	1.05±0.26	1.18±0.22	1.65±0.48[a]
72h BRAIN	0.68±0.13	0.66±0.19[2]	1.53±0.25[d2]

(Results are expressed as mean±SD where n=5.)

Comparing corn oil treated with control. [a]=$p<0.05$
[b]=$p<0.02$
[c]=$p<0.01$
[d]=$p<0.001$

Comparing refined corn oil with Mazola corn oil. [1]=$p<0.01$
[2]=$p<0.001$

scission of the folate molecule the extent to which this occurs depends on the type of corn oil administered, control (4.7%), Mazola corn oil (29.55%) and refined corn oil (41.08%).

3.3.11 PEROXIDES.

The peroxide content of the corn oils is shown in table 3.11. A ten fold increase in the peroxide value is observed in the refined corn oil (1142mEq/kg oil) compared to Mazola corn oil (112mEq/kg oil).

3.3.12 TISSUE DHPR.

The effects of corn oil and folic acid administration on tissue DHPR are shown in table 3.12. Dosage with refined corn oil results in a significant increase in brain DHPR compared to controls ($p<0.05$ at 24h, 48h and 72h). Dosage with Mazola corn oil results in a significant decrease in liver DHPR when compared to controls ($p<0.02$ at 24h, $p<0.05$ at 48h and $p<0.002$ at 72h). This decrease is also significant if compared to the refined corn oil dosed group ($p<0.001$ at 24h and $p<0.05$ at 48h).

3.3.13 LIVER DHFR.

The effects of corn oil and folic acid administration on liver DHFR are shown in table 3.13. There appears to be a decrease in DHFR activity with time after dosing with folic acid. There is significantly less DHFR activity in the refined corn oil group at 72h ($p<0.05$) and in the Mazola corn oil group at 48h ($p<0.002$) when compared to controls. The activity in the Mazola corn oil treated group is significantly less than the refined corn oil group at 24h and 48h ($p<0.01$).

3.4 DISCUSSION.

The effects of corn oil dosage on the patterns of retention and excretion of radiolabelled folic acid were observed. It was observed that there is a consistent excess of ^{3}H over ^{14}C excretion in the urines and an excess of ^{14}C over ^{3}H excretion in the faeces, in agreement with previously observed results, (Connor, 1979, Saleh, 1981, Al-Haddad, 1984, Guest, 1984, Surdhar, 1987). This imbalance in excretion is due to catabolism of the folate molecule by scission at C9N10 resulting in excretion of ^{3}H only catabolites in the urine and excretion of ^{14}C only catabolites in the faeces (Pheasant *et al.*, 1981). Part of the ^{3}H

TABLE 3.10

Extent of liver polyglutamate breakdown* after corn oil treatment

	CONTROL	REFINED CORN OIL	MAZOLA CORN OIL
% SCISSION 24-48h	2.29	31.86	16.46
% SCISSION 48-72h	2.41	9.22	13.09
NET SCISSION 24-72h	4.7	41.08	29.55

$$*\%\ \text{scission}_{24-48h} = \frac{\text{pAcABGlu excreted 24-48h} \times 100}{\%\ ^{3}H \text{ in liver at 24h}}$$

(^{3}H in the liver being corrected for since after scission 42.5% of ^{3}H remains in the glutamyl fraction.)

TABLE 3.11

Peroxide value of corn oils

	PEROXIDE VALUE (Peroxide oxygen (mEq/kg oil)
REFINED CORN OIL	1142
MAZOLA CORN OIL	112

TABLE 3.12

The effect of corn oil and radiolabelled folic acid administration (83µg/kg body wt) on tissue DHPR

	DHPR+		
	CONTROL	**REFINED CORN OIL**	**MAZOLA CORN OIL**
24h BRAIN	191±55	261±16[1]	218±51
48h BRAIN	180±45	234±21[1]	218±39
72h BRAIN	239±32	290±30[1]	274±85
24h LIVER	317±83	366±59[b]	199±37[2b]
48h LIVER	266±59	340±116[a]	186±31[1a]
72h LIVER	352±50	285±75	220±38[3]

(Results are expressed as mean±SD where n=5.)

+ DHPR activity=nmole NADH oxidised/min/mg protein.

Comparing controls with corn oil treated. [1]=p<0.05
[2]=p<0.02
[3]=p<0.002

Comparing refined corn oil with Mazola corn oil. [a]=p<0.05
[b]=p<0.001

TABLE 3.13

The effect of corn oil and radiolabelled folic acid administration (83µg/kg body wt) on liver DHFR

	DHFR++		
	CONTROL	**REFINED CORN OIL**	**MAZOLA CORN OIL**
24h LIVER	7.43±3.92	5.95±0.78[a]	4.37±0.48[a]
48h LIVER	4.92±0.67	5.2 ±1.09[a]	3.21±0.45[2a]
72h LIVER	5.4 ±1.65	3.52±0.34[1]	3.73±1.29

(Results are expressed as mean±SD where n=5.)

++DHFR activity=µmole DHF reduced/min/mg protein.

Comparing controls with corn oil treated. [1]=$p<0.05$
[2]=$p<0.002$

Comparing refined corn oil with Mazola corn oil. [a]=$p<0.01$

imbalance which is observed is due to a secondary isotope effect as a result of ^{3}H substitution at the C-9 position (Connor *et al.*, 1980). This secondary isotope effect may also account for the consistent ^{3}H over ^{14}C excess observed in tissues.

There was no significant difference in the total urinary excretion of radioactivity between controls and after corn oil treatment. Further urinary analysis did reveal differences in the pattern of excretion of metabolites which suggest that corn oil administration results in increased folate catabolism.

The administration of corn oils results in increased folate catabolism - the excretion of pterins was increased over 0-24h and 24-48h and the excretion of pAcABGlu and pAcABA over 24-48h and 48-72h was increased after both types of corn oils. More extensive catabolism occurs after dosage with refined corn oil, the excretion of pAcABGlu, pAcABA, ^{14}C urea and $^{3}H_{2}O$ were all increased as was the excretion of ^{14}C in the faeces. If the total excretion of pterins and ^{3}H only catabolites over the 72h collection period is considered it is observed that corn oils result in increased catabolism but more catabolites were excreted after dosage with refined corn oil.

The percentage scission may be determined by calculating the extent of liver polyglutamate breakdown. Scission was increased after corn oil treatment, net scission was greater after administration of refined corn oil than after the administration of Mazola corn oil.

Liver retentions were decreased after corn oil dosage - this could be indicative of impaired polyglutamation although analysis of liver extracts revealed no differences in chromatographic separation between corn oil dosed and control groups. Reduced uptake may be occurring after corn oil dosage and indeed levels of $5CH_{3}THF$ (the predominant circulatory form of folates) were increased in the urines of corn oil dosed animals, however this observation was not consistent since lowest retentions were observed in 72h livers of rats dosed with Mazola corn oil whilst excretion of $5CH_{3}THF$ in 48-72h urines was not different from controls. The most likely cause of decreased liver retentions is increased catabolism of the folate molecule - levels of catabolites were increased in the urines of rats dosed with corn oil. A similar pattern of decreased retention of radiolabel may be observed in the brain-the differences are less marked due to the low overall retentions in the brain.

Oxidising species in the brain and the liver were detected using a NBT assay. Oxidising species were increased in tissues after dosage with corn oils -this is expected since polyunsaturated fats undergo autoxidation resulting in the production of free radicals (Shamberger, 1980, Coultate, 1984). It was observed that more oxidising species were present after dosage with Mazola corn oil, this would be expected since the dose of this was higher than the dose of refined corn oil ($1cm^3$/day compared to $0.3cm^3$/day) hence more fats would be available for autoxidation after dosage with Mazola corn oil, however more catabolites were excreted after dosage with the refined corn oil. The NBT assay is a relatively non-specific assay since it detects various oxidising species, it may therefore be concluded that the assay may at best be used as an indicator of increased catabolism.

The peroxide content of the refined corn oil was found to be 10 fold higher than the Mazola corn oil. The dosage of Mazola corn oil was 3.3 times that of the refined corn oil therefore the difference in the administered peroxides was 3.3 fold. Refined corn oil resulted in increased folate catabolism, however the difference in the extent of catabolism was not as great as the difference in peroxide content. It may be concluded that the free radicals generated by lipid peroxidation and autoxidation and the peroxide content of the corn oils may both contribute to the extent of folate catabolism which occurs. Both the quantity of unsaturated fats and the quality of these in the diet may affect the bioavailability of folates. Lipid peroxidation has been proposed as a possible mechanism in cancer promotion and it has been observed that cancers may result in folate deficiency - this could be the result of increased oxidising species.

Brain DHPR activity was increased after dosage with refined corn oil. This could be a result of the protective role of the added antioxidant BHT -since the active site is believed to contain easily oxidisable thiol groups (Armarego *et al.*, 1984) however this is unlikely since BHT is a poor antioxidant in the body (London *et al.*, 1985) and later work with higher doses (Chapter 7) revealed no effect on brain DHPR. It is proposed that in the presence of increased oxidising species there may be some form of compensatory mechanism operating which results in elevated DHPR activity - it has been observed that short term dosage with an agent which depletes glutathione levels results in unaltered DHPR activity whilst long term dosage of the drug results in elevated levels of DHPR (Heales, 1987).

In conclusion further evidence is presented to suggest that folate catabolism is an oxidative process. Oxidising species arising as a result of dietary intake of fats will affect folate status and deficiency could arise as a secondary dietary effect.

Corn oils are frequently used as a vehicle for dosage of other substances and it is suggested that the peroxide content of these should be monitored before use to ensure that corn oils with a low peroxide value are used as a vehicle.

CHAPTER FOUR

THE EFFECT OF DIETHYLSTILBOESTROL ON FOLATE CATABOLISM IN THE RAT

CHAPTER FOUR.

4 THE EFFECT OF DIETHYLSTILBOESTROL ON FOLATE CATABOLISM IN THE RAT

4.1 INTRODUCTION

It has been suggested that prolonged use of oral contraceptive agents (OCA) may result in folate deficiency (Krumdieck *et al.*, 1975, Lakshmaiah and Bamji, 1979, Lakshmaiah and Bamji, 1981, Shojania, 1982). The precise mechanism by which folate deficiency may arise as a consequence of oral contraceptive use still remains unclear although impaired absorption, altered tissue uptake, increased excretion, decreased conversion to coenzyme forms and increased binding to folate binders have all been proposed (Lakshmaiah and Bamji, 1979, Shojania, 1982).

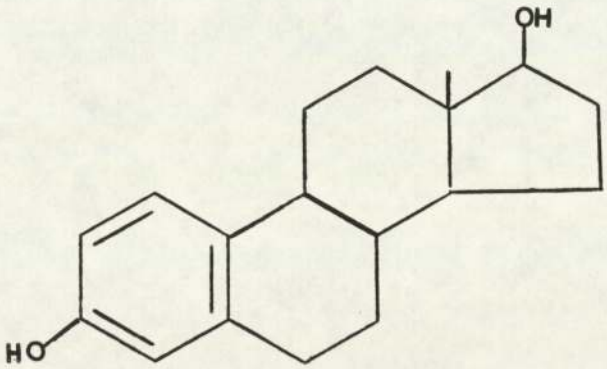
Diethylstilboestrol (DES) is a synthetic non-steroidal oestrogen with similar activity to naturally occurring oestrogens (Dodds *et al.*, 1938, Noller and Fisher, 1974). Despite their structural and immunological differences both 17-β oestradiol and DES bind to the same receptor sites with similar affinities. The increased potency of DES is believed to be due to its increased nuclear retention (Campbell *et al.*, 1980). DES was previously used in the prevention of threatened abortions but is now recognised to be carcinogenic (Noller and Fisher, 1974). Carcinogenicity does not correlate with oestrogenic effect; DES is considered to be more carcinogenic than 17-β oestradiol (Sumi *et al.*, 1984). Oxidative metabolism is believed to play an important role in the carcinogenicity of DES. DES undergoes peroxidase mediated oxidation in its target tissues to a quinone which may undergo adduct formation with DNA and initiate tumours (Liehr *et al.*, 1983, Metzler, 1984, Sumi *et al.*, 1984, Ross *et al.*, 1985). The structures of 17-β oestradiol, DES and DES quinone are shown in figure 4.1.

OCA and DES have been implicated in breast cancer (Greenberg *et al.*, 1984, Greenman *et al.*, 1984, Lipnick *et al.*, 1986), although this is disputed (Lipsett, 1979, Ory *et al.*, 1983). A long latent period of 14-25 years appears to exist between exposure to DES and the onset of breast cancer and may account for the conflicting views (Lipsett, 1979).

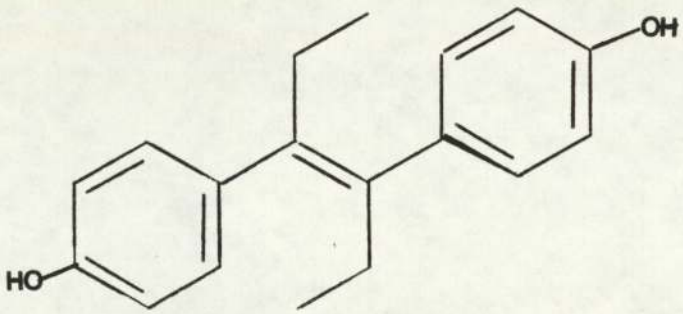
The mechanism of DES induced cancer and the role of folates remains unclear. Oestrogens induce increased synthesis of DHFR whilst the antioestrogen Tamoxifen decreases DHFR synthesis (Levine *et al.*, 1985). A correlation between DHPR

FIGURE 4.1

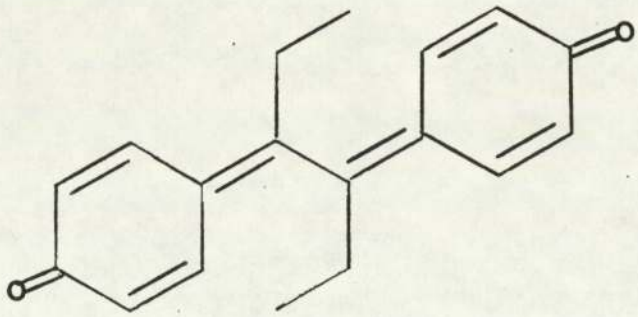
Structure of the naturally occurring oestrogen 17-β oestradiol, the synthetic non-steroidal oestrogen DES and its quinone.



17-β oestradiol



DES



DES quinone

activity and hormonal dependence has been observed (Dhondt *et al.*, 1981) and increased DHPR activity has also been observed in neoplastic tissue from breast cancer patients (Eggar *et al.*, 1983). These results suggest that altered pteridine metabolism may occur in hormonally induced cancers.

It was therefore decided to study the effects of DES on folate catabolism in the female rat. Due to the difficulties encountered with long term dosage regimes a high dose of DES was administered over a short period. The activities of DHPR and DHFR were monitored since both have been observed to be elevated in cancer. The effects on 5-10 methylene THF reductase was also monitored since this is a key enzyme in the biosynthesis of methyl groups and an increased demand for methyl groups has been reported to result in folate deficiency in rats with tumours (Poirier, 1973).

4.2 MATERIALS AND METHODS

Materials were obtained as described in Chapter 2.

Female Wistar rats (250g) were divided into two groups of 15 rats each. The control group were dosed orally with 0.3cm^3 of refined corn oil (containing 0.01% (w/v) butylated hydroxytoluene (BHT)) for 3 days, the test group were dosed orally with 415mg/kg body wt diethylstilboestrol (DES) as a suspension in 0.3cm^3 of refined corn oil for 3 days. On the fourth day all rats were dosed orally with 2µCi of [2-^{14}C] and 5µCi of [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) in 0.05M sodium phosphate buffer, pH 7, containing 2% (w/v) sodium ascorbate. 5 rats from each group were transferred to individual Metabowls and urines and faeces were collected as described in Chapter 2. 24h after dosing with radiolabelled folic acid animals were killed by cervical dislocation and brains and livers were removed for analysis. The collection procedure was repeated for the next 2 groups of 5 animals such that for each treatment group 0-24h, 24-48h and 48-72h samples were collected. Throughout the experiment animals were allowed free access to food and water and were kept on grids to prevent coprophagy. Urines and faeces were analysed for radioactivity whilst brains and livers were analysed for oxidising species, DHFR, DHPR , 5-10 methylene THF reductase and retention of radioactivity as described in Chapter 2.

4.3 RESULTS

4.3.1 EXCRETION OF RADIOACTIVITY IN URINES

The excretion of radioactivity in urines after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 4.1. The bulk of the radioactivity was excreted in the early urine samples (0-24h), with progressively less radioactivity being excreted over 24-48h and 48-72h. In each sample there is an excess of ^{3}H compared to ^{14}C excreted in the urines (32.06% ^{3}H and 21.48% ^{14}C in the 0-24h sample of the corn oil dosed group ($p<0.05$) and 21.05% ^{3}H and 15.24% ^{14}C in the 0-24h sample of the DES and corn oil dosed group). Although less radioactivity was excreted after dosing with DES and corn oil this was not statistically significant.

4.3.2 EXCRETION OF RADIOACTIVITY IN THE FAECES

The excretion of radioactivity in the faeces after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7, 9-^{3}H] folic acid (83µg/kg body wt) is shown in table 4.2. For each sample collected it is observed that there is an excess of ^{14}C compared to ^{3}H excreted (6.98% ^{3}H and 24.08% ^{14}C after dosing with refined corn oil ($p<0.001$) and 3.34% ^{3}H and 11.28% ^{14}C after dosing with DES and corn oil ($p<0.01$) -for 0-24h samples), this excess of ^{14}C over ^{3}H persists in all samples after dosing with corn oil but is only significant for 0-24h and 24-48h samples after dosing with DES and corn oil. Less radioactivity was excreted in the faeces after dosing with DES and corn oil, this is significant for 0-24h samples (3.34% ^{3}H compared to 6.98% ^{3}H after dosing with refined corn oil ($p<0.01$) and 11.28% ^{14}C compared to 24.08% ^{14}C after dosing with corn oil ($p<0.001$)).

4.3.3 RETENTION OF RADIOACTIVITY IN THE LIVER

The retention of radioactivity in the liver after an oral dose of 2µCi [2-^{14}C] and 5µCi [3', 5', 7, 9-^{3}H] folic acid (83µg/kg body wt) is shown in table 4.3. There is a consistent excess of ^{3}H retained compared to ^{14}C. Retention of radioactivity was increased after dosing with DES and corn oil when compared to dosing with corn oil alone, (29.4% ^{3}H and 20.85% ^{14}C compared to 12.63% ^{3}H and 9.61% after dosing with corn oil ($p<0.002$) -for liver retentions 24h after dosing with radiolabelled folic acid), (32.14% ^{3}H compared to 16.08% ^{3}H after dosing with corn oil ($p<0.002$) and 23.06% ^{14}C compared to 12.2% ^{14}C after dosing with corn oil ($p<0.001$) -for liver retentions 48h after dosing with

TABLE 4.1

The effect of DES on the excretion of radioactivity in the urines of rats after the oral administration of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	EXCRETION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	32.06[1] ±7.34	21.48 ±4.6	21.05 ±9.46	15.24 ±6.78
24-48h	5.44 ±3.61	3.11 ±1.37	4.86 ±3.64	3.28 ±2.13
48-72h	3.56* ±2.36	1.66* ±1.03	4.37 ±3.81	1.82 ±0.97

(Results are expressed as mean±SD where n=5 except * where n=4.)

^{3}H over ^{14}C excess. [1] = $p<0.05$

TABLE 4.2

The effect of DES on the excretion of radioactivity in the faeces of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

EXCRETION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	6.98 ±0.94	24.08[4] ±1.82	3.34[a] ±1.6	11.28[3b] ±4.97
24-48h	3.57 ±2.06	11.61[1] ±6.84	3.56 ±2.3	10.02[1] ±4.97
48-72h	2.48* ±0.77	5.78*[2] ±1.73	2.18 ±1.14	3.62 ±1.63

(Results are expressed as mean±SD where n=5 except * where n=4.)

^{14}C over ^{3}H excess. [1]=p<0.05
[2]=p<0.02
[3]=p<0.01
[4]=p<0.001

Comparing DES and corn oil treated with corn oil treated. [a]=p<0.01
[b]=p<0.001

TABLE 4.3

The effect of DES on the retention of radioactivity in the liver of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	RETENTION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
24h	12.63 ±2.79	9.61 ±2.28	29.41[a] ±7.0	20.85[a] ±4.98
48h	16.08[2] ±2.21	12.2 ±1.67	32.14[1a] ±7.16	23.06[b] ±4.39
72h	15.78[3] ±1.48	11.97 ±1.29	25.49[3b] ±3.77	17.69[a] ±2.52

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1]=p<0.05
[2]=p<0.02
[3]=p<0.01

Comparing DES and corn oil treated with corn oil treated. [a]=p<0.002
[b]=p<0.001

radiolabelled folic acid) and (25.49% ^{3}H compared to 15.78% ^{3}H after dosing with corn oil ($p<0.001$) and 17.69% ^{14}C compared to 11.97% ^{14}C after dosing with corn oil ($p<0.002$) for liver retentions 72h after dosing with radiolabelled folic acid).

Analysis of liver extracts by Sephadex G15 chromatography resulted in the elution of a high molecular weight peak compatible with the elution of a polyglutamate species. There was no difference in the pattern of elution between the two groups.

4.3.4 RETENTION OF RADIOACTIVITY IN THE BRAIN

The retention of radioactivity in the brain after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 4.4. There is a consistent excess of ^{3}H compared to ^{14}C retained in all samples, this is significant for all samples after dosing with corn oil but is only significant at 24h after dosing with DES and corn oil (0.08% ^{3}H and 0.04% ^{14}C ($p<0.05$) for corn oil dosed and 0.06% ^{3}H and 0.04% ^{14}C ($p<0.01$) for DES and corn oil dosed). Less ^{3}H was retained after dosing DES and corn oil when compared with dosing with corn oil alone, however this difference was not statistically significant.

4.3.5 METABOLITES IDENTIFIED IN 0-24h URINES

The following species were identified in 0-24h urines:-folic acid, $5CH_3THF$, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 4.5. Intact folates and products of catabolism were detected in both groups.

There was little difference in the excretion of folic acid after dosing with DES and corn oil (1.13% ^{3}H and 0.88% ^{14}C compared with 1.03% ^{3}H and 0.83% ^{14}C after dosing with corn oil). Less $5CH_3THF$ was excreted after dosing with DES and corn oil (10.51% ^{3}H and 8.11% ^{14}C compared to 13.88% ^{3}H and 10.53% ^{14}C after dosing with corn oil). Less 10-formyl folate was excreted on dosing with DES and corn oil (2.75% ^{3}H and 2.78% ^{14}C compared with 6.73% ^{3}H and 7.6% ^{14}C after dosing with corn oil). The excretion of pAcABGlu and pAcABA was decreased after dosing with DES and corn oil (4.58% and 0.67% compared with 8.6% and 4.27% after dosing with corn oil). The total ^{3}H only catabolites were decreased after dosing with DES and corn oil (5.25%) compared to dosing with corn oil alone

TABLE 4.4

The effect of DES on the retention of radioactivity in the brain of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	RETENTION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
24h	0.08[1] ±0.03	0.04 ±0.01	0.06[2] ±0.01	0.04 ±0.00
48h	0.08[3] ±0.01	0.04 ±0.01	0.06 ±0.02	0.04 ±0.01
72h	0.10[2] ±0.03	0.04 ±0.01	0.07 ±0.02	0.05 ±0.01

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1]=$p<0.05$
[2]=$p<0.01$
[3]=$p<0.001$

TABLE 4.5

The effect of DES administration on metabolites detected in 0-24h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µ g/kg body wt)

	% OF DOSE			
	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
FOLIC ACID	1.03	0.83	1.13	0.88
5CH$_3$THF	13.88	10.53	10.51	8.11
10-CHO-FOLATE	6.73	7.6	2.75	2.78
pAcABGlu	8.6		4.58	
pAcABA	4.27		0.67	
total ^{3}H only metabolites	12.87		5.25	
PTERINS		3.99		1.8
^{14}C UREA		0.66		0.46
$^{3}H_2O$	0.75		0.35	

(Each value was determined by analysis of pooled urine from 5 rats.)

(12.87%). The excretion of urinary pterins was decreased after dosing with DES and corn oil (1.8%) compared to dosing with corn oil (3.99%). Less ^{14}C urea and $^{3}H_2O$ was excreted after dosing with DES and corn oil (^{14}C urea 0.46% and $^{3}H_2O$ 0.35% compared with ^{14}C urea 0.66% and $^{3}H_2O$ 0.75% after dosing with corn oil).

4.3.6 METABOLITES IDENTIFIED IN 24-48h URINES

The following species were identified in 24-48h urines:- Metabolite A, $5CH_3THF$, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 4.6.

The excretion of Metabolite A was increased after dosing with DES and corn oil (1.58% ^{3}H and 1.36% ^{14}C compared with 0.86% ^{3}H and 0.86% ^{14}C after dosing with corn oil). More $5CH_3THF$ was excreted after dosing with DES and corn oil (0.55% ^{3}H and 0.36% ^{14}C compared with 0.45% ^{3}H and 0.23% ^{14}C after dosing with corn oil). More 10-formyl folate was excreted after dosing with DES and corn oil (0.56% and 0.93% ^{14}C compared with 0.3% ^{3}H and 0.32% ^{13}C after dosing with corn oil). The excretion of pAcABGlu and pAcABA was decreased after dosing with DES and corn oil (1.2% and 0.38% compared with 1.71% and 1.29% after dosing with corn oil). The total ^{3}H only catabolites excreted were decreased after dosing with DES and corn oil (1.58%) compared to dosing with corn oil alone (3%). The excretion of urinary pterins was decreased after dosing with DES and corn oil (0.4%) compared to dosing with corn oil (0.57%). Less ^{14}C urea and $^{3}H_2O$ was excreted after dosing with DES and corn oil (^{14}C urea 0.21% and $^{3}H_2O$ 0.39% compared with ^{14}C urea 0.33% and $^{3}H_2O$ 0.77% after dosing with corn oil).

4.3.7 METABOLITES IDENTIFIED IN 48-72h URINES

The following species were identified in 48-72h urines:-Metabolite A, $5CH_3THF$, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 4.7.

Metabolite A was detected in urines after dosing with DES and corn oil (0.89% ^{3}H and 0.45% ^{14}C) but was not detected after dosing with corn oil. Less $5CH_3THF$ was excreted after dosing with DES and corn oil (0.41% ^{3}H and 0.13% ^{14}C

TABLE 4.6

The effect of DES administration on metabolites detected in 24-48h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE			
	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
METABOLITE A	0.86	0.86	1.59	1.36
5CH$_3$THF	0.45	0.23	0.55	0.36
10-CHO-FOLATE	0.3	0.32	0.56	0.93
pAcABGlu	1.71		1.2	
pAcABA	1.29		0.38	
total ^{3}H only metabolites	3.0		1.58	
PTERINS		0.57		0.4
^{14}C UREA		0.33		0.21
^{3}H$_2$O	0.77		0.39	

(Each value was determined by analysis of pooled urine from 5 rats.)

TABLE 4.7

The effect of DES administration on metabolites detected in 48-72h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE			
	CORN OIL		DES AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
METABOLITE A	ND		0.89	0.45
5CH$_3$THF	0.64	0.63	0.41	0.13
10-CHO-FOLATE	0.59	0.55	0.97	0.48
pAcABGlu	0.63		1.73	
pAcABA	0.84		0.36	
total ^{3}H only metabolites	1.47		2.09	
PTERINS		0.25		0.56
^{14}C UREA		0.22		0.15
$^{3}H_2O$	1.05		0.49	

ND=not detected.

(Each value was determined by analysis of pooled urine from 5 rats.)

compared with 0.64% ^{3}H and 0.63% ^{14}C after dosing with corn oil). Excretion of 10-formyl folate was increased after dosing with DES and corn oil (0.97% ^{3}H and 0.48% ^{14}C compared with 0.59% ^{3}H and 0.55% ^{14}C after dosing with corn oil). Increased excretion of pAcABGlu was observed after dosing with DES and corn oil (1.73%) compared to dosing with corn oil (0.63%) whilst less pAcABA was excreted after dosing with DES and corn oil (0.36%) compared to dosing with corn oil (0.84%). The total excretion of ^{3}H only catabolites was increased after dosing with DES and corn oil (2.09%) compared with dosing with corn oil (1.47%). Excretion of urinary pterins was increased after dosage with DES and corn oil (0.56%) compared with dosage with corn oil (0.25%). Excretion of ^{14}C urea and $^{3}H_2O$ was decreased after dosing with DES and corn oil (^{14}C urea 0.15% and $^{3}H_2O$ 0.49% compared with ^{14}C urea 0.22% and $^{3}H_2O$ 1.05% after dosing with corn oil).

4.3.8 TOTAL EXCRETION OF SCISSION PRODUCTS

The total pAcABGlu and pAcABA (^{3}H only catabolites) and 'pterins' excreted over the 72h collection period are shown in table 4.8. The excretion of these catabolites was decreased after dosing with DES and corn oil (^{3}H only catabolites 8.92% and 'pterins' 2.76% compared with ^{3}H only catabolites 17.34% and 'pterins' 4.81% after dosing with corn oil).

4.3.9 OXIDISING SPECIES

The oxidising species detected after corn oil and DES and corn oil administration prior to the administration of folic acid are shown in table 4.9. After dosing with DES and corn oil less oxidising species were detected in the livers at 24h ($p<0.001$) and 48h whilst at 72h oxidising species were increased ($p<0.05$), this trend was similar in the brains, less oxidising species were detected after dosage with DES and corn oil ($p<0.05$ at 24h and 48h) whilst at 72h oxidising species were increased when compared to dosing with corn oil.

4.3.10 EXTENT OF LIVER POLYGLUTAMATE BREAKDOWN

The extent of liver polyglutamate breakdown may be calculated if it is assumed that any pAcABGlu excreted in the urines after 24h arises from the liver polyglutamate fraction, this may be calculated where the percentage scission occurring over 24-48h:-

TABLE 4.8

The effect of DES administration on the excretion of urinary scission products over 0-72h after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE	
	CORN OIL	**DES AND CORN OIL**
total pAcABGlu and pAcABA 0-72h	17.34	8.92
total pterins 0-72h	4.81	2.76

(Results were determined from analysis of pooled urine samples from 5 rats for each 24h group.)

TABLE 4.9

Oxidising species detected by NBT assay after corn oil and DES and corn oil administration prior to the administration of radiolabelled folate

	CORN OIL	DES AND CORN OIL
	(µmoles formazan/g wet wt/20 min)	
24h LIVER	1.85±0.33	0.45±0.27[b] (*)
48h LIVER	1.48±0.43	1.31±0.16
72h LIVER	1.07±0.30	1.61±0.30[a]
24h BRAIN	0.90±0.63	0.12±0.19[a]
48h BRAIN	1.18±0.22	0.74±0.36[a]
72h BRAIN	0.66±0.19	0.80±0.41

(Results are expressed as mean±SD where n=5 except (*) where n=3.)

Comparing corn oil treated with DES and corn oil treated. [a]=$p<0.05$

[b]=$p<0.001$

$$= \frac{\text{pAcABGlu excreted over 24-48h}}{\%\ \text{of}\ ^{3}H\ \text{in the liver at 24h}} \times 100$$

(it is necessary to correct for the ^{3}H retained in the liver since after scission 42.5% of the label remains in the glutamyl fraction)

The extent of liver polyglutamate breakdown is shown in table 4.10. The percentage scission over 24-48h was decreased after dosing with DES and corn oil (9.6%) compared with dosing with corn oil (31.86%). The percentage scission over 48-72h was increased after dosing with DES and corn oil (12.67%) compared with dosing with corn oil (9.22%). If the net scission over 24-72h is calculated it is observed that scission was decreased after dosing with DES and corn oil (22.27%) compared with dosing with corn oil alone (41.08%).

4.3.11 TISSUE DHPR

The effects of corn oil and DES and corn oil administration prior to the administration of radiolabelled folic acid on tissue DHPR activity are shown in table 4.11. No effect on DHPR activity in either the brain or the liver was observed after DES and corn oil administration when compared to corn oil administration.

4.3.12 LIVER DHFR

The effects of corn oil and DES and corn oil administration prior to the administration of radiolabelled folic acid on liver DHFR activity are shown in table 4.12.. Increased activity was observed after dosing with DES and corn oil when compared to dosing with corn oil alone, this was significant at 72h (7.37µmoles DHF reduced/min/mg protein compared to 3.53µmoles DHF reduced/min/mg protein ($p<0.001$)).

4.3.13 TISSUE 5-10 METHYLENE THF REDUCTASE

The effects of corn oil and DES and corn oil administration prior to the administration of radiolabelled folic acid on tissue 5-10 methylene THF reductase are shown in table 4.13. Increased activity was observed in the brain at 48h ($p<0.01$) and at 72h ($p<0.01$) after dosing with DES and corn oil. Increased activity was also observed in the liver after dosing with DES and corn oil when compared to dosing with corn oil alone, this was only significant at 48h ($p<0.02$)

TABLE 4.10

Extent of liver polyglutamate breakdown* after DES treatment.

	CORN OIL	DES AND CORN OIL
% SCISSION 24-48h	31.86	9.6
% SCISSION 48-72h	9.22	12.67
NET SCISSION 24-72h	41.08	22.27

* **% scission** $= \frac{\text{pAcABGlu excreted 24-48h}}{\text{24-48h}} \times \frac{100}{\text{\% } ^3\text{H in liver at 24h}}$

(^{3}H in the liver being corrected for since after scission 42.5% of ^{3}H remains in the glutamyl fraction.)

TABLE 4.11

The effect of corn oil and (DES and corn oil) administration prior to the administration of radiolabelled folic acid (83µg/kg body wt) on tissue DHPR

	DHPR+	
	CORN OIL	**DES AND CORN OIL**
24h BRAIN	261±16	278±42
48h BRAIN	234±21	274±44
72h BRAIN	290±30	288±67
24h LIVER	366±59	354±67
48h LIVER	340±116	339±78
72h LIVER	285±75	313±42

(Results are expressed as mean±SD where n=5.)

+DHPR activity = nmole NADH oxidised/min/mg protein.

TABLE 4.12

The effect of corn oil and (DES and corn oil) administration prior to the administration of radiolabelled folic acid (83µg/kg body wt) on liver DHFR

	DHFR[++]	
	CORN OIL	**DES AND CORN OIL**
24h LIVER	5.95±0.78	6.27±1.05
48h LIVER	5.20±1.09	5.91±1.20
72h LIVER	3.53±0.34	7.37±0.64[a]

(Results are expressed as mean±SD where n=5.)

[++] DHFR activity = µmole DHF reduced/min/mg protein.

Comparing corn oil treated with DES and corn oil treated. [a]=$p<0.001$

TABLE 4.13

The effect of corn oil and (DES and corn oil) administration prior to the administration of radiolabelled folic acid (83µg/kg body wt) on tissue 5-10 methylene THF reductase.

5-10 METHYLENE THF REDUCTASE

(nmole $5CH_3$THF oxidised/h/mg protein)

	CORN OIL	DES AND CORN OIL
24h BRAIN	0.44±0.55	0.32±0.21 (*)
48h BRAIN	0.14±0.10	0.38±0.07[b] (*)
72h BRAIN	0.11±0.05	0.23±0.04[b]
24h LIVER	0.31±0.09	0.47±0.12 (*)
48h LIVER	0.35±0.17	0.64±0.14[a]
72h LIVER	0.74±0.32	1.0 ±0.35

(Results are expressed as mean±SD where n=5 except (*) where n=4.)

Comparing corn oil treated with DES and corn oil treated. [a]=$p<0.02$

[b]=$p<0.01$

(0.64nmole $5CH_3THF$ oxidised/h/mg protein compared to 0.35 nmole $5CH_3THF$ oxidised/h/mg protein).

4.4 DISCUSSION

The effects of DES administration on the patterns of retention and excretion of radiolabelled folic acid were observed. It was again observed that there was an excess of ^3H over ^{14}C excreted in the urines and an excess of ^{14}C over ^3H excreted in the faeces, this imbalance of excretion of radiolabel was discussed in Chapter 3.

Less radioactivity was excreted in the faeces after DES administration, this suggests that less breakdown of the folate molecule or increased retention is occurring - this is in agreement with the work of Lakshamaiah and Bamji, 1981 who observed lowered faecal excretion of ^3H folate in rats treated with OCA.

Although there was no significant difference in the urinary excretion of radioactivity further urinary analysis did reveal differences in the pattern of excretion of metabolites which suggests that DES administration results in decreased catabolism (which is in agreement with the observations of Eggar *et al.*, 1983). Less intact folates and products of scission were detected in 0-24h urines - less folate is entering the reduced pool, more is being retained and less is available for scission. In 24-48h samples less pAcABA, pAcABGlu, pterins, ^{14}C urea and ^3H_2O were excreted after DES administration suggesting that less scission of the folate molecule is occurring after DES treatment. However in 48-72h urines more pAcABGlu and pterins were detected after DES administration suggesting that more polyglutamate breakdown is occurring after DES treatment. This increase could be a consequence of increased metabolism of DES by the liver resulting in the generation of free radicals (Ross *et al.*,1985), which may be available for scission of the folate molecule. Over 72h less scission products were excreted after DES administration suggesting DES results in decreased folate catabolism.

Less oxidising species were detected in the liver at 24h after DES treatment - this correlates with less catabolites being excreted in the urines whilst at 72h more oxidising species were detected after DES treatment - this also correlates with the increased excretion of catabolites over this period after DES treatment. These results suggest that the NBT assay may be used as a good indicator of the extent of folate catabolism which is occurring and confirms that scission is indeed an oxidative process.

Increased liver retention of folate was observed after DES treatment, in agreement with the work of Lakshamaih and Bamji, 1979 and 1981, and Tolomelli *et al.*, 1972. Less radioactivity was retained in the brain after DES treatment although this was not significant, in agreement with Lakshamaiah and Bamji, 1981.

The extent of liver polyglutamate breakdown was calculated. It was observed that the extent of scission was decreased over 24-48h but increased over 48-72h after DES treatment, net scission over 72h was virtually halved after DES treatment compared to treatment with corn oil alone.

It was observed that DES administration had no effect on brain or liver DHPR activity whilst liver DHFR was increased. The increase in DHFR activity was significant at 72h and is supported by the observations of Lakshamaih and Bamji, 1981, Burns and Jackson, 1982 and Levine *et al.*, 1985.

Brain and liver 5-10 methylene THF reductase was increased after DES treatment. This results in increased production of $5CH_3THF$ which would result in a change in methylated forms, this has been observed (Poirier, 1973, Lakshamaiah and Bamji, 1979). This increase in 5-10 methylene THF reductase activity could be due to increased DHFR since DHF inhibits the enzyme (Matthews and Haywood, 1979) and increased DHFR activity would ensure lifting of this inhibition.

In conclusion DES administration results in increased liver retention, decreased excretion and folate catabolism and increased DHFR and 5-10 methylene THF reductase activities. Saleh *et al.*, 1980 and1981, have observed decreased urinary excretion and scission of folates and increased folate retention in cancer patients and rats bearing tumours, the reverse is observed with methotrexate administration suggesting that the observed effects may be due to increased DHFR activity. Similar results were observed with DES administration suggesting that the effects may be due to DHFR and precede malignancy.

CHAPTER FIVE

THE EFFECT OF VITAMIN E ON FOLATE CATABOLISM IN THE RAT

CHAPTER FIVE.

5 THE EFFECT OF VITAMIN E ON FOLATE CATABOLISM IN THE RAT

5.1 INTRODUCTION

Vitamin E (tocopherol) is an important antioxidant within the body. It has a role in protecting biomembranes from damage arising from the autoxidation of free fatty acids by forming stable complexes with these (Pieri *et al.*, 1986, Skrypin *et al.*, 1987). α-Tocopherol (figure 5.1) is the most biologically active of the tocopherols and is the major and possibly only chain breaking antioxidant interfering with the propagation of a free radical chain within the blood. Its activity is due to its ability to donate hydrodgen from its hydroxyl group at position 6 (Burton *et al.*, 1983, Burton and Ingold, 1986). Vitamin E is a more potent antioxidant than superoxide dismutase and glutathione peroxidase. Deficiency of vitamin E results in increased lipoperoxide levels (Yoshioka *et al.*, 1987).

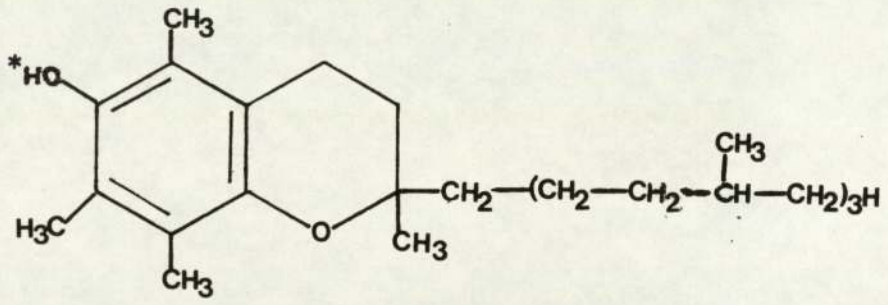
After the administration of vitamin E the levels of natural antioxidants increase and it has been suggested that some interchange may be occurring via redox reactions (Gallo-Torres, 1980). It has been shown that the antioxidant activity of vitamin E is enhanced by ascorbate - partially oxidised tocopherol in the bilayer is regenerated by ascorbate present in the aqueous phase (Burton and Ingold, 1986).

Vitamin E is fat soluble and is transported via the lipoproteins VLDL and LDL (Gallo-Torres, 1980). Absence of β-lipoproteins results in a severe vitamin E deficiency (Vuilleumier *et al.*, 1983, Harding *et al.*, 1985). Deficiency of vitamin E usually arises as a result of malabsorption syndromes (Sokol *et al.*, 1985, Satya-Murti *et al.*, 1986) although deficiencies as a result of increased utilisation or degradation (Yokota *et al.*, 1987) or absence of apoprotein β-100 (Harding *et al.*, 1985) have also been reported.

Deficiency results in increased peroxidation of membranes rich in polyunsaturated fatty acids, which results in impaired vision and pigment retinopathy (Satya-Murti *et al.*, 1986) and neurological abnormalities (Sokol *et al.*, 1985, Gordon, 1987). The neurological aspects of vitamin E will be considered in Chapter 7. In the premature infant deficiency may result in hemolytic anaemia which may increase folate requirements (Oski and Barness, 1967, Ritchie *et al.*, 1968).

FIGURE 5.1

α-tocopherol



CHAIN BREAKING PROPERTIES OF VITAMIN E

Vitamin E donates a hydrogen atom * to a free radical such as ROO

$$AH + ROO^{\circ} \longrightarrow ROOH + A^{\circ}$$

A is resonance stabilized and too unreactive to continue the chain but may react with a second free radical.

$$ROO^{\circ} + A^{\circ} \longrightarrow ROOA$$

Low serum levels of vitamin E have been noted to predispose patients to lung and breast cancers (London *et al.*, 1985, Menkes *et al.*, 1986). Decreased neoplasia has been observed in vitamin E treated rats and it has been suggested that vitamin E may have a role in mammary gland cells to prevent free radical oxidation in the nucleus (London *et al.*, 1985). High levels of antioxidants have been reported in tumours, these are believed to enable growth since products of lipid peroxidation can inhibit DNA synthesis and cell division (Burton *et al*, 1983, Burton and Ingold, 1986).

Folate catabolism is believed to be an oxidative process (Blair *et al.*, 1984). It is decreased during malignancy whilst folates accumulate in tumours (Saleh *et al.*,1980, Saleh *et al.*,1981). It would therefore be expected that the administration of vitamin E - a potent antioxidant *in vivo* would result in decreased folate catabolism, particularly since it has been shown that peroxides and lipids (which undergo peroxidation) result in increased folate catabolism (Chapter 3).

The effects of administering vitamin E (as dl-α-tocopherol acetate) were observed to see if this does indeed decrease the extent of folate catabolism.

5.2 MATERIALS AND METHODS

Materials were obtained as described in Chapter 2.

Female Wistar rats (250g) were divided into two groups of 15 rats each. Rats were dosed twice daily at 0900h and 2100h throughout the experiment. Control rats were dosed with 0.5cm^3 of Mazola corn oil (containing 0.02% (w/v) vitamin E), test rats were dosed with 7.5mg dl-α-tocopherol acetate (vitamin E) in 0.5cm^3 Mazola corn oil. On the fourth day rats were additionally dosed at 1000h with 2μCi of [2-^{14}C] and 5μCi [3',5',7,9-^{3}H] folic acid (83μg/kg body wt) in 0.05M sodium phosphate buffer, pH 7, containing 2%(w/v) sodium ascorbate. 5 rats from each group were transferred to individual Metabowls and urines and faeces were collected as described in Chapter 2. 24h after dosing with radiolabelled folic acid animals were killed by cervical dislocation and brains and livers were removed for analysis. The collection procedure was repeated for the next 2 groups of 5 animals such that for each treatment group 0-24h, 24-48h and 48-72h samples were collected. Throughout the experiment animals were allowed free access to food and water and were kept on grids to prevent coprophagy. Urines and faeces were analysed for radioactivity whilst brains and livers were analysed for oxidising species, DHFR, DHPR and retention of radioactivity as described in Chapter 2.

5.3 RESULTS

5.3.1 EXCRETION OF RADIOACTIVITY IN URINES.

The excretion of radioactivity in urines after an oral dose of 2μCi [2-^{14}C] and 5μCi [3',5',7,9-^{3}H] folic acid (83μg/kg body wt) is shown in table 5.1. The bulk of the radioactivity was excreted in the early (0-24h) urine samples, although less appeared to be excreted after dosing with vitamin E this difference was not statistically significant (23.74% ^{3}H and 14.09% ^{14}C in the corn oil dosed group compared with 15.7% ^{3}H and 11.16% ^{14}C after dosing with vitamin E and corn oil for 0-24h samples). In each sample there is an excess of ^{3}H compared to ^{14}C excreted in the urines. This excess was less significant after dosing with vitamin E, (3.37% ^{3}H and 1.69% ^{14}C in the 48-72h sample of the corn oil dosed group ($p<0.001$) and 3.3% ^{3}H and 1.41% ^{14}C in the 48-72h sample of the vitamin E and corn oil dosed group ($p<0.05$).

5.3.2 EXCRETION OF RADIOACTIVITY IN THE FAECES.

The excretion of radioactivity in the faeces after an oral dose of 2μCi [2-^{14}C] and 5μCi [3',5',7,9-^{3}H] folic acid (83μg/kg body wt) is shown in table 5.2. The bulk of the radioactivity was excreted over 0-24h. For each sample collected it is observed that there is an excess of ^{14}C compared to ^{3}H excreted, this excess appears to be more pronounced after dosing with vitamin E (3.03% ^{3}H and 14.86% ^{14}C after dosing with corn oil and 3.37% ^{3}H and 13.43% ^{14}C after dosing with vitamin E and corn oil ($p<0.05$) - for 0-24h samples). Less radioactivity was excreted after dosing with vitamin E, this was significant for the ^{3}H species only at 48-72h (2.5% ^{3}H after dosing with corn oil compared to 1% ^{3}H after dosing with vitamin E and corn oil ($p<0.002$)).

5.3.3 RETENTION OF RADIOACTIVITY IN THE LIVER

The retention of radioactivity in the liver after an oral dose of 2μCi [2-^{14}C] and 5μCi [3',5',7,9-^{3}H] folic acid (83μg/kg body wt) is shown in table 5.3. There is a consistent excess of ^{3}H retained compared to ^{14}C. Retention of radioactivity was decreased after dosing with vitamin E and corn oil compared to dosing with corn oil alone (13.47% ^{3}H compared to 17.23% ^{3}H after dosing with corn oil ($p<0.05$) and 12.3% ^{14}C compared to 15.75% ^{14}C after dosing with corn oil alone - for liver retentions 48h after dosing with radiolabelled folic acid) and (12.3% 3H

TABLE 5.1

The effect of vitamin E administration on the excretion of radioactivity in the urines of rats after the oral administation of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	EXCRETION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	23.74 ±12.17	14.09 ± 7.59	15.7 ± 5.35	11.16 ± 3.59
24-48h	4.21[2] ± 1.11	2.13 ± 0.62	2.66 ± 1.39	1.54 ± 0.65
48-72h	3.37[3] ± 0.59	1.69 ± 0.29	3.3[1] ± 1.59	1.41 ± 0.63

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1] = $p<0.05$
[2] = $p<0.01$
[3] = $p<0.001$

TABLE 5.2

The effect of vitamin E administration on the excretion of radioactivity in the faeces of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

EXCRETION OF RADIOACTIVITY (% OF DOSE)

SAMPLE	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
0-24h	3.03 ± 3.69	14.86 ±12.7	3.37 ± 2.93	13.43[1] ± 8.82
24-48h	2.27 ± 1.14	4.46 ± 2.33	1.95 ± 1.28	3.05 ± 1.81
48-72h	2.5 ± 0.73	4.29 ± 1.79	1.0[a] ± 0.11	3.55[2] ± 1.93

(Results are expressed as mean±SD where n=5.)

^{14}C over ^{3}H excess. [1]=p<0.05

[2]=p<0.02

Comparing Vitamin E and corn oil treated with corn oil treated.

[a]=p<0.002

TABLE 5.3

The effect of vitamin E administration on the retention of radioactivity in the liver of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	RETENTION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
24h	15.82 ±3.71	14.41 ±2.73	14.33 ±2.46	13.13 ±3.13
48h	17.23 ±1.84	15.75 ±2.82	13.47[a] ±2.27	12.31 ±2.74
72h	16.38[1] ±1.04	14.55 ±1.32	12.3[b] ±1.94	11.46[a] ±2.36

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1]=$p<0.05$

Comparing vitamin E and corn oil treated with corn oil treated. [a]=$p<0.05$

[b]=$p<0.01$

compared to 16.38% ^{3}H after dosing with corn oil alone ($p<0.01$) and 11.46% ^{14}C compared to 14.55% ^{14}C after dosing with corn oil alone ($p<0.05$) - for liver retentions 72h after dosing with radiolabelled folic acid. It was observed that retention of radioactivity decreased with time after dosing with vitamin E and corn oil whilst there was an increase in retention at 48h after dosing with corn oil alone.

Analysis of liver extracts by Sephadex G15 chromatography resulted in the elution of a high molecular weight peak compatible with the elution of a polyglutamate species. There was no difference in the pattern of elution between the two groups.

5.3.4 RETENTION OF RADIOACTIVITY IN THE BRAIN

The retention of radioactivity in the brain after an oral dose of 2µCi [2-^{14}C] and 5µCi [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) is shown in table 5.4. There is a significant excess of ^{3}H compared to ^{14}C retained in all samples. Less ^{3}H was retained after dosing with vitamin E and corn oil when compared to dosing with corn oil alone, this difference was significant at 24h (0.1% ^{3}H compared to 0.16% ^{3}H after dosing with corn oil alone ($p<0.02$)).

5.3.5 METABOLITES IDENTIFIED IN 0-24h URINES

The following species were identified in 0-24h urines:- folic acid, $5CH_3THF$, Metabolite B, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 5.5. Intact folates and products of catabolism were detected in both groups.

Less folic acid was excreted after dosing with vitamin E and corn oil (0.08% ^{3}H and 0.11% ^{14}C compared with 0.25% ^{3}H and 0.22% ^{14}C after dosing with corn oil alone). Less $5CH_3THF$ was excreted after dosing with vitamin E and corn oil (6.52% ^{3}H and 5.3% ^{14}C compared with 8.94% ^{3}H and 6.34% ^{14}C after dosing with corn oil alone). Metabolite B was not detected after dosing with vitamin E and corn oil whilst the excretion of 10-formyl folate was similar in both groups (3.62% ^{3}H and 3.56% ^{14}C after dosing with vitamin E and corn oil compared with 3.89% ^{3}H and 4.14% ^{14}C after dosing with corn oil alone). The excretion of pAcABGlu and pAcABA was decreased after dosing with vitamin E and corn oil (2.54% and 2.42% compared with 4.6% and 4.42% after dosing with corn oil alone). The total ^{3}H only catabolites were decreased after dosing with vitamin E

TABLE 5.4

The effect of vitamin E administration on the retention of radioactivity in the brain of rats after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	RETENTION OF RADIOACTIVITY (% OF DOSE)			
SAMPLE	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
24h	0.16[2] ±0.04	0.08 ±0.03	0.10[1a] ±0.02	0.07 ±0.01
48h	0.12[2] ±0.02	0.07 ±0.02	0.11[4] ±0.01	0.07 ±0.01
72h	0.14[2] ±0.04	0.07 ±0.02	0.11[3] ±0.01	0.08 ±0.01

(Results are expressed as mean±SD where n=5.)

^{3}H over ^{14}C excess. [1] = $p<0.02$
[2] = $p<0.01$
[3] = $p<0.002$
[4] = $p<0.001$

Comparing vitamin E and corn oil treated with corn oil treated. [a] = $p<0.02$

TABLE 5.5

The effect of vitamin E administration on metabolites detected in 0-24h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

% OF DOSE

	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
FOLIC ACID	0.25	0.22	0.08	0.11
5CH$_3$THF	8.94	6.34	6.52	5.3
METABOLITE B	0.99	0.75	ND	
10-CHO-FOLATE	3.89	4.14	3.62	3.56
pAcABOlu	4.6		2.54	
pAcABA	4.42		2.42	
total ^{3}H only metabolites	9.02		4.96	
PTERINS		1.51		0.58
^{14}C UREA		0.56		0.27
$^{3}H_2O$	0.95		0.29	

(Each value was determined by analysis of pooled urine from 5 rats.)

ND= not detected.

and corn oil (4.96%) compared to dosing with corn oil alone (9.02%). The excretion of urinary pterins was decreased after dosing with vitamin E and corn oil (0.58%) compared to dosing with corn oil alone (1.51%). Less ^{14}C urea and $^{3}H_2O$ was excreted after dosing with vitamin E and corn oil (^{14}C urea 0.27% and $^{3}H_2O$ 0.29% compared with ^{14}C urea 0.56% and $^{3}H_2O$ 0.95% after dosing with corn oil alone.)

5.3.6 METABOLITES IDENTIFIED IN 24-48h URINES

The following species were identified in 24-48h urines:- Metabolite A, $5CH_3THF$, Metabolite B, 10-formyl folate, pAcABGlu, pAcABA, 'pterins'. ^{14}C urea and $^{3}H_2O$. The distribution of these metabolites is shown in table 5.6.

Metabolites A and B were detected after the administration of vitamin E and corn oil but not after the administration of corn oil alone. Less $5CH_3THF$ was excreted after dosing with vitamin E and corn oil (0.32% ^{3}H and 0.22% ^{14}C compared with 0.57% ^{3}H and 0.44% ^{14}C after dosing with corn oil). Less 10-formyl folate was excreted after dosing with vitamin E and corn oil (0.6% ^{3}H and 0.55% ^{14}C compared with 0.77% ^{3}H and 0.77% ^{14}C after dosing with corn oil). The excretion of pAcABGlu and pAcABA was slightly decreased after dosing with vitamin E and corn oil (0.59% and 0.84% compared with 0.63% and 0.96% after dosing with corn oil). The total excretion of ^{3}H only catabolites was slightly decreased after dosing with vitamin E and corn oil (1.43%) compared to dosing with corn oil alone (1.59%). The excretion of urinary pterins was decreased after dosing with vitamin E and corn oil (0.53%) compared to dosing with corn oil alone (1.09%). Less ^{14}C urea and $^{3}H_2O$ was excreted after dosing with vitamin E and corn oil (^{14}C urea 0.09% and $^{3}H_2O$ 0.33% compared with ^{14}C urea 0.18% and $^{3}H_2O$ 1.1% after dosing with corn oil).

5.3.7 METABOLITES IDENTIFIED IN 48-72h URINES

The following species were identified in 48-72h urines:- Metabolite A, $5CH_3THF$, Metabolite B, 10-formyl folate, pAcABGlu, pAcABA, 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is shown in table 5.7.

Small amounts of Metabolites A and B were detected after the administration of

TABLE 5.6

The effect of vitamin E administration on metabolites detected in 24-48h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE			
	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
METABOLITE A	ND		0.13	0.11
5CH$_3$THF	0.57	0.44	0.32	0.22
METABOLITE B	ND		0.18	0.12
10-CHO-FOLATE	0.77	0.77	0.6	0.55
pAcABGlu	0.63		0.59	
pAcABA	0.96		0.84	
total ^{3}H only metabolites	1.59		1.43	
PTERINS		1.09		0.53
^{14}C UREA		0.18		0.09
$^{3}H_2O$	1.10		0.33	

(Each value was determined by analysis of pooled urine from 5 rats.)

ND= not detected.

TABLE 5.7

The effect of vitamin E administration on metabolites detected in 48-72h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE			
	CORN OIL		VITAMIN E AND CORN OIL	
	^{3}H	^{14}C	^{3}H	^{14}C
METABOLITE A	ND		0.05	0.04
5CH$_3$THF	0.33	0.25	0.22	0.16
METABOLITE B	ND		0.10	0.13
10-CHO-FOLATE	0.75	0.68	0.52	0.60
pAcABGlu	0.73		0.57	
pAcABA	0.82		0.61	
total ^{3}H only metabolites	1.55		1.18	
PTERINS		2.39		0.44
^{14}C UREA		0.08		0.18
$^{3}H_2O$	0.48		1.19	

(Each value was determined by analysis of pooled urine from 5 rats.)

ND= not detected.

vitamin E and corn oil but not after administration of corn oil alone. Less $5CH_3THF$ was excreted after dosing with vitamin E and corn oil (0.22% ^{3}H and 0.16% ^{14}C compared with 0.33% ^{3}H and 0.25% ^{14}C after dosing with corn oil). Less 10-formyl folate was excreted after dosing with vitamin E and corn oil (0.52% ^{3}H and 0.6% ^{14}C compared with 0.75% ^{3}H and 0.68% ^{14}C after dosing with corn oil). The excretion of pAcABGlu and pAcABA was decreased after dosing with vitamin E and corn oil (0.57% and 0.61% compared with 0.73% and 0.82% after dosing with corn oil). The total ^{3}H only catabolites were decreased after dosing with vitamin E and corn oil (1.18%) compared to dosing with corn oil alone (1.55%). The excretion of urinary pterins was decreased after dosing with vitamin E and corn oil (0.44%) compared to dosing with corn oil (2.39%). Increased excretion of ^{14}C urea and $^{3}H_2O$ was observed after dosing with vitamin E and corn oil (^{14}C urea 0.18% and $^{3}H_2O$ 1.19% compared with ^{14}C urea 0.08% and $^{3}H_2O$ 0.48% after dosing with corn oil).

5.3.8 TOTAL EXCRETION OF SCISSION PRODUCTS

The total pAcABGlu and pAcABA (^{3}H only catabolites) and 'pterins' excreted over the 72h collection period are shown in table 5.8. The excretion of these catabolites decreases after dosing with vitamin E and corn oil (^{3}H only catabolites 7.57% and 'pterins' 1.55% compared with ^{3}H only catabolites 12.16% and 'pterins' 4.99% after dosing with corn oil).

5.3.9 OXIDISING SPECIES

The oxidising species detected after corn oil and (vitamin E and corn oil) administration prior to the administration of folic acid are shown in table 5.9. Increased oxidising species were detected in the livers and the brains after administration of vitamin E and corn oil, a greater increase in oxidising species was observed in the livers than in the brains.

5.3.10 EXTENT OF LIVER POLYGLUTAMATE BREAKDOWN

The extent of liver polyglutamate breakdown may be calculated if it is assumed that any pAcABGlu excreted in the urines after 24h arises from the liver polyglutamate fraction, this may be calculated where the percentage scission occurring over 24-48h:-

TABLE 5.8

The effect of vitamin E administration on the excretion of urinary scission products over 0-72h after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE	
	CORN OIL	**VITAMIN E AND CORN OIL**
total pAcABGlu and pAcABA 0-72h	12.16	7.57
total pterins 0-72h	4.99	1.55

(Results were determined from analysis of pooled urine samples from 5 rats for each 24h group.)

TABLE 5.9

Oxidising species detected by NBT assay after corn oil and vitamin E and corn oil administration prior to the administration of radiolabelled folate

	CORN OIL	VITAMIN E AND CORN OIL
	(µmoles formazan/g wet wt/20 min)	
24h LIVER	1.23±0.37	2.10±0.35[1]
48h LIVER	1.18±0.65	1.39±0.41
72h LIVER	1.26±0.28	2.29±0.23[2]
24h BRAIN	1.01±0.40	1.08±0.36
48h BRAIN	0.72±0.09	1.25±0.26[1]
72h BRAIN	1.01±0.24	1.07±0.14

(Results are expressed as mean±SD where n=5.)

Comparing vitamin E and corn oil treated with corn oil treated. [1]=$p<0.01$
[2]=$p<0.001$

$$= \frac{\text{pAcABGlu excreted over 24-48h}}{\text{\% of } ^3\text{H in the liver at 24h}} \times 100$$

(it is necessary to correct for the ^3H retained in the liver since after scission 42.5% of the label remains in the glutamyl fraction)

The extent of liver polyglutamate breakdown is shown in table 5.10. The percentage scission over 24-48h was increased after dosing with vitamin E and corn oil (9.69%) compared with dosing with corn oil (9.37%). The percentage scission over 48-72h was decreased after dosing with vitamin E and corn oil (9.95%) compared with dosing with corn oil (9.97%). Net scission over 24-72h was increased after dosing with vitamin E and corn oil (19.64%) compared with dosing with corn oil alone (19.34%).

5.3.11 TISSUE DHPR

The effects of corn oil and (vitamin E and corn oil) administration prior to the administration of radiolabelled folic acid on tissue DHPR activity are shown in table 5.11. Vitamin E administration resulted in a significant increase in brain DHPR activity ($p<0.02$ at 24h, $p<0.002$ at 48h and $p<0.01$ at 72h), liver DHPR activity was also elevated, this was significant at 72h (335 nmoles NADH oxidised/min/mg protein compared with 247 nmoles NADH oxidised/min/mg protein when dosed with corn oil alone ($p<0.01$))

5.3.12 LIVER DHFR

The effects of corn oil and (vitamin E and corn oil) administration prior to the administration of radiolabelled folic acid on liver DHFR activity is shown in table 5.12. Increased activity was observed after dosing with vitamin E and corn oil when compared to dosing with corn oil alone (5.86µmoles DHF reduced/min/mg protein compared with 3.95µmoles DHF reduced/min/mg protein after dosing with corn oil at 24h ($p<0.02$) and 6.8µmoles DHF reduced/min/mg protein compared with 3.92µmoles DHF reduced/min/mg protein after dosing with corn oil at 48h ($P<0.01$)).

5.4 DISCUSSION

The effects of vitamin E administration on the patterns of excretion and

TABLE 5.10

Extent of liver polyglutamate breakdown* after vitamin E treatment

	CORN OIL	VITAMIN E AND CORN OIL
% SCISSION 24-48h	9.37	9.69
% SCISSION 48-72h	9.97	9.95
NET SCISSION 24-72h	19.34	19.64

$$*\%\ \text{scission}_{24-48h} = \frac{\text{pAcABGlu excreted 24-48h}}{\%\ ^{3}H \text{ in liver at 24h}} \times 100$$

(^{3}H in the liver being corrected for since after scission 42.5% of ^{3}H remains in the glutamyl fraction.)

TABLE 5.11

The effect of corn oil and (vitamin E and corn oil) administration prior to the administration of radiolabelled folic acid (83µg/kg body wt) on tissue DHPR

	DHPR[+]	
	CORN OIL	**VITAMIN E AND CORN OIL**
24h BRAIN	199±17	283±54[1]
48h BRAIN	178±38	285±33[3]
72h BRAIN	207±22	298±46[2]
24h LIVER	263±20	290±82
48h LIVER	222±20	287±112
72h LIVER	247±36	335±38[2]

(Results are expressed as mean± SD where n=5.)

[+]DHPR activity = nmole NADH oxidised/min/mg protein.

Comparing vitamin E and corn oil treated with corn oil treated. [1]=p<0.02
[2]=p<0.01
[3]=p<0.002

TABLE 5.12

The effect of corn oil and (vitamin E and corn oil) administration prior to the administration of radiolabelled folic acid (83µg/kg body wt) on liver DHFR

	DHFR^{++}	
	CORN OIL	**VITAMIN E AND CORN OIL**
24h LIVER	3.95±1.09	5.86±0.84[1]
48h LIVER	3.92±0.54	6.8 ±1.42[2]
72h LIVER	4.9 ±1.15	6.23±1.13

(Results are expressed as mean ± SD where n=5.)

$^{++}$DHFR activity = µmole DHF reduced/min/mg protein.

Comparing vitamin E and corn oil treated with corn oil treated. [1]=$p<0.02$
[2]=$p<0.01$

retention of radiolabelled folic acid were observed. An imbalance of excretion of ^{14}C in the faeces and ^{3}H in the urines was observed - this was discussed in Chapter 3.

Less radioactivity was excreted in the urines after the administration of vitamin E, however this difference was not statistically significant. Further urinary analysis did reveal that less of the catabolites pAcABGlu, pAcABA and pterins were excreted suggesting that catabolism is decreased after vitamin E treatment.

Less radioactivity was retained in the livers at 48h and 72h and the extent of liver polyglutamate scission was increased after vitamin E administration. The liver is the site of temporary storage of vitamin E (Gallo-Torres, 1980). Although increased vitamin E levels would be expected to result in decreased scission of the folate molecule (since this is an oxidative process) it has been observed that linoleic acid (the predominant fatty acid in corn oils) decreases the bioavailability of tocopherol (Gallo-Torres, 1980), possibly increased scission may not have been observed if a vehicle other than corn oil had been used. Wispe *et al.*, 1986, have observed that a single injection of α-tocopherol in the newborn rabbit resulted in increased liver but not lung peroxides, this is believed to be the result of proton donation by tocopherol converting reactive lipid species to more stable lipid peroxides. It was observed that oxidising species (as detected by the NBT assay) were increased in the liver after vitamin E administration, this is in agreement with the observations of Wispe *et al.*, 1986, and explains the increased scission of the liver polyglutamate fraction. It is suggested that the NBT assay may be used as an indicator of tissue polyglutamate breakdown.

DHPR activity was increased in both the brain and the liver after vitamin E administration. These results suggest that vitamin E (and possibly other antioxidants) result in increased DHPR levels as a result of protection of thiol groups within the active site from oxidation. Liver DHFR activity was also increased after vitamin E administration suggesting that this may have a similar protective role for DHFR. Urinary catabolites were decreased whilst the activities of both DHPR and DHFR were elevated after vitamin E treatment, this further suggests that these may have a role in protecting oxidisable species from catabolism.

In conclusion vitamin E results in decreased folate catabolism - possibly as a result of elevated DHPR and DHFR levels whilst the extent of liver polyglutamate breakdown was increased as a result of formation of lipid peroxides. It is suggested

that increased intake of dietary antioxidants (such as vitamin E) may prove beneficial in conserving folate stores.

CHAPTER SIX

THE EFFECTS OF NITROUS OXIDE ON FOLATE CATABOLISM AND TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

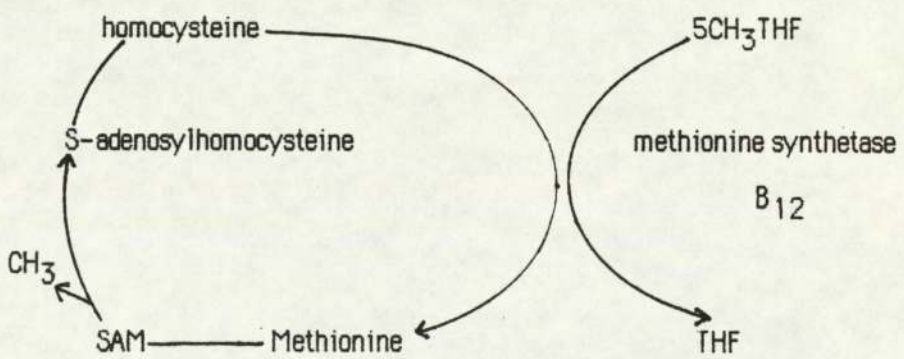
CHAPTER SIX.

6 THE EFFECTS OF NITROUS OXIDE ON FOLATE CATABOLISM AND TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

6.1 INTRODUCTION

Exposure to the anaesthetic gas nitrous oxide (N_2O) results in oxidation of the cobalt 1 centre of vitamin B_{12} (cob(I)alamin) (Banks *et al.*, 1968). This results in selective inactivation of the enzyme methionine synthetase (EC 2,1,1,13) for which cob(I)alamin is a cofactor (Deacon *et al.*, 1978). Inhibition of the enzyme is maximal after 6h and persists as long as exposure to N_2O is continued (Deacon *et al.*, 1980a, Deacon *et al.*, 1982).

Methionine synthetase is required in the regeneration of THF from $5CH_3THF$ and is a key regulator in THF dependent processes (Black and Tephly, 1983). Inactivation of methionine synthetase results in depletion of serum methionine, S-adenosylmethionine (SAM) is the main source of methyl groups within the body and provides the active formate required for the synthesis of 10 formyl THF which is a precursor of 5-10 methylene THF (the essential carbon donor in the conversion of deoxyuridine to deoxythymidine). Thus N_2O interferes with DNA synthesis which is the cause of megaloblastic anaemia and leucopenia which have been observed in patients with prolonged exposure to N_2O (Nunn, 1984). N_2O is implicated in the fetotoxic aspects of its occupational exposure (Chanarin, 1982). Methionine depletion has been implicated in the neurological deficit associated with chronic N_2O abuse (Dinn *et al*, 1978, Layzer, 1978).



Exposure to N_2O results in decreased tissue levels of THF (McGing *et al.*, 1978, Perry *et al*, 1979, Wilson and Horne, 1986), accumulation of $5CH_3THF$ in the plasma (Lumb *et al*, 1981a), decreased nucleic acid synthesis (Amess *et al.*,1978), reduced synthesis of folate polyglutamates (McGing *et al.*,1978, Perry *et al*, 1979, Deacon *et al*, 1980b, Lumb *et al.*,1981b) and increased excretion of folate in the urine (McGing *et al.*,1978, Lumb *et al.*,1982). Inhibition of THB biosynthesis *in vitro* has also been reported (Hamon *et al*, 1986).

It is not established whether the increased excretion of folates is due to increased catabolism of the folate molecule or solely due to increased excretion of $5CH_3THF$ as a result of impaired tissue retention (Lumb *et al.*,1981b). Since N_2O exposure results in impaired polyglutamation, reduced levels of THF and accumulation of $5CH_3THF$ the contribution of this monoglutamate species to folate catabolism was observed.

Rats were exposed to N_2O/O_2 for 8h (to inactivate methionine synthetase) prior to receiving an oral dose of radiolabelled folic acid, the effects of this on folate catabolism were observed after a further 24h exposure. Total tissue biopterins and DHPR activity were measured to determine whether there is an *in vivo* effect of N_2O on THB metabolism.

6.2 MATERIALS AND METHODS

Materials were obtained as described in Chapter 2.

Female Wistar rats (250g) were placed individually in sealed Metabowls and exposed to N_2O/O_2 (80%/20%(v/v)). After 8h exposure rats were dosed orally with 2µCi of [2-^{14}C] and 5µCi of [3',5',7,9-^{3}H] folic acid (83µg/kg body wt) in 0.05M sodium phosphate buffer, pH7, containing 2%(w/v) sodium ascorbate, animals were returned to metabolism cages and exposure to N_2O/O_2 was continued for a further 24h. Control rats breathed air. Urines and faeces were collected as described in Chapter 2. Throughout the experiment animals had free access to food and water. 24h after dosing rats were killed by cervical dislocation and brains, livers and kidneys removed for analysis. Urines, faeces and kidneys were analysed for radioactivity whilst brains and livers were analysed for total biopterins, oxidising species, DHFR, DHPR and retention of radioactivity as described in Chapter 2.

6.3 RESULTS

6.3.1 DISTRIBUTION OF RADIOACTIVITY

The distribution of retained and excreted radioactivity 24h after the oral administration of 2μCi of [2-^{14}C] and 5μCi of [3',5',7,9-^{3}H] folic acid is given in table 6.1. It is observed that more radioactivity is excreted in urines after N_2O/O_2 exposure (46.18% ^{3}H compared to 20.79% ^{3}H in the control group ($p<0.01$) and 24.02% ^{14}C compared to 10.65% ^{14}C in the control group ($p<0.05$)). There is an excess of ^{3}H excreted in the urines of both groups ($p<0.01$ for controls and $p<0.02$ for the N_2O/O_2 treated group). The excretion of radioactivity in the faeces is similar (11.34% ^{3}H and 25.36% ^{14}C in the controls and 7.87% ^{3}H and 16.66% ^{14}C after N_2O/O_2 exposure). Less radioactivity is retained in the livers after N_2O/O_2 exposure (0.93% ^{3}H and 0.75% ^{14}C compared to 4.62% ^{3}H and 5.57% ^{14}C in the controls ($p<0.01$)). Less radioactivity is retained in the kidneys but this is only significant for ^{3}H ($p<0.05$), (0.89% ^{3}H and 0.73% ^{14}C compared to 1.39% ^{3}H and 1.27% ^{14}C in the controls). There is no difference in the retention of radioactivity in the brain (0.18% ^{3}H and 0.04% ^{14}C compared to 0.18% ^{3}H and 0.03% ^{14}C in the controls), the imbalance of ^{3}H retention is significant in both groups ($p<0.001$).

6.3.2 IDENTIFICATION OF URINARY METABOLITES

The following species were identified in urines:-folic acid, an unidentified dual labelled species, 5 CH_3THF, 10 formyl folate, p-acetamidobenzoate (pAcABA), p-acetamidobenzoyl-1-glutamate (pAcABGlu), 'pterins', ^{14}C urea and $^{3}H_2O$. The distribution of these is given in table 6.2. Intact folates and products of catabolism were excreted by both groups. Excretion of folic acid was similar in both groups (0.45% ^{3}H and 0.44% ^{14}C in controls and 0.52% ^{3}H and 0.4% ^{14}C after N_2O/O_2 exposure). The unidentified dual labelled species was not detected in urines of rats exposed to N_2O/O_2. 5 CH_3THF is increased in urines after N_2O/O_2 exposure (24.1% ^{3}H and 17.52% ^{14}C compared to 2.45% ^{3}H and 2.02% ^{14}C in the control group). The products of catabolism are increased after N_2O/O_2 exposure:-pAcABGlu 5.94% compared to 4.78% and pAcABA 6.99% compared to 5.3%in the control group. The excretion of urea and H_2O is increased after N_2O/O_2

TABLE 6.1

The effect of N_2O/O_2 exposure on the distribution of radioactivity recovered 24h after the oral administration of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt).

	DISTRIBUTION OF RADIOACTIVITY (% OF DOSE)			
	AIR BREATHING		N_2O/O_2	
	^{3}H	^{14}C	^{3}H	^{14}C
URINES	20.79±4.55[1]	10.65± 4.96	46.18±12.2[2a]	24.02± 9.75[b]
FAECES	11.34±9.37	25.36±21.44	7.87± 3.87	16.66± 8.68
LIVER	4.62± 1.9	5.57± 2.95	0.93± 0.2[a]	0.75± 0.19[a]
KIDNEY	1.39± 0.43	1.27± 0.57	0.89± 0.21[b]	0.73± 0.31
BRAIN	0.18± 0.07[3]	0.03± 0.01	0.18± 0.05[3]	0.04±0.01

(Results are expressed as mean±SD where n=5.)

Comparing air breathing versus N_2O/O_2. [a]=$p<0.01$

[b]=$p<0.05$

^{3}H over ^{14}C excess [1]=$p<0.01$

[2]=$p<0.02$

[3]=$p<0.001$

TABLE 6.2

The effect of N_2O/O_2 exposure on metabolites detected in 0-24h urines of rats given an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (83µg/kg body wt)

	% OF DOSE			
	AIR BREATHING		N_2O/O_2	
	^{3}H	^{14}C	^{3}H	^{14}C
FOLIC ACID	0.45	0.44	0.52	0.40
METABOLITE A	0.84	0.61	ND	
5 CH_3THF	2.45	2.02	24.1	17.52
10 CHOFOLATE	4.05	3.88	3.82	2.75
pAcABGlu	4.78		5.94	
pAcABA	5.3		6.99	
total ^{3}H only catabolites	10.08		12.93	
PTERINS		1.27		0.62
^{14}C UREA		1.68		1.84
$^{3}H_2O$	1.32		5.5	

ND=not detected.

(Each value was determined by analysis of pooled urine from 5 rats.)

exposure (^{14}C urea 1.84% and $^{3}H_2O$ 5.5% compared to ^{14}C urea 1.68% and $^{3}H_2O$ 1.32% in controls) suggesting that more extensive breakdown of the folate molecule occurs after N_2O/O_2 exposure.

6.3.3 ANALYSIS OF LIVER EXTRACTS

Liver extracts were chromatographed on Sephadex G15. The elution profiles are shown in figure 6.1. The pattern of elution differs but in both groups a high molecular weight peak eluting close to the void volume and compatible with the elution of a polyglutamate species was identified. Due to the absence of the appropriate standards the species could not be further identified.

6.3.4 TOTAL TISSUE BIOPTERINS AND PTERINS

The effect of radiolabelled folic acid administration (83µg/kg body wt) and N_2O/O_2 exposure on total brain and liver biopterin and pterin is shown in table 6.3. Total tissue biopterins were unaffected by N_2O/O_2 exposure but liver pterins (which may be used as a measure of THF content, but is not derived solely from THF, Heales, 1987) is decreased after exposure (420ng/g wet wt compared to 552ng/g wet wt in controls ($p<0.05$)), brain pterin was unaffected.

6.3.5 DHPR AND DHFR

The effect of folic acid administration (83µg/kg body wt) and N_2O/O_2 exposure on brain and liver DHPR and liver DHFR is shown in table 6.4, both enzymes were unaffected by N_2O in the tissues examined.

6.3.6 OXIDISING SPECIES

Oxidising species detected by NBT assay after exposure to N_2O/O_2 are shown in table 6.5. Less oxidising species were detected in the livers after exposure to N_2O/O_2 (1.03µmoles formazan/g wet wt/20 min compared to 1.42µmoles/g wet wt/20 min).

6.4 DISCUSSION

Following the administration of mixed label folic acid to rats after 8h exposure

FIGURE 6.1

Sephadex G-15 chromatography of radioactivity from rat liver

(a) Air breathing

(b) N_2O/O_2

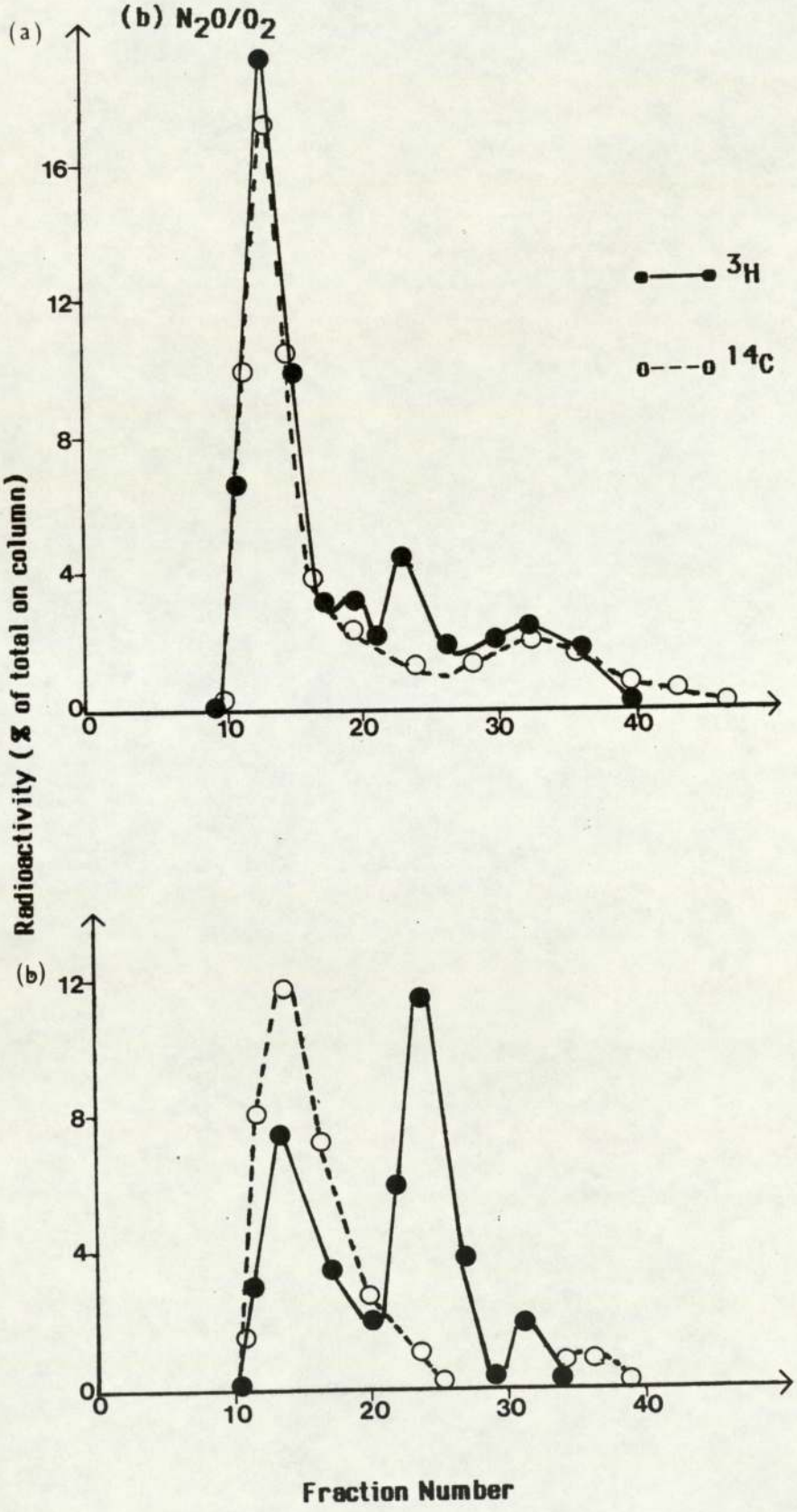


TABLE 6.3

The effect of oral administration of 83µg/kg body weight radiolabelled folic acid and N_2O/O_2 exposure on total tissue bioptern and pterin

	BRAIN		LIVER	
	BIOPTERIN	PTERIN	BIOPTERIN	PTERIN
	(ng/g wet wt)			
CONTROL	49±2	37±4	503±56	552±95
N_2O/O_2	52±9	34±6	500±77	420±43[a]

(Results are expressed as mean±SD where n=5.)

Comparing air breathing versus N_2O/O_2. [a]=$p<0.05$

TABLE 6.4

The effect of oral administration of 83µg/kg body weight radiolabelled folic acid and N_2O/O_2 exposure on tissue DHPR and DHFR.

	DHPR[+]		DHFR[++]
	BRAIN	**LIVER**	**LIVER**
CONTROL	135±45	291±66	1.61±0.36
N_2O/O_2	156±51	323±82	1.17±0.56

(Results are expressed as mean±SD where n=5.)

[+] DHPR activity=nmole NADH oxidised/min/mg protein.

[++] DHFR activity=µmole DHF reduced/min/mg protein.

TABLE 6.5

The effct of oral administration of 83µg/kg body weight radiolabelled folic acid and N_2O/O_2 exposure on oxidising species detected by NBT assay.

	OXIDISING SPECIES	
	AIR BREATHING	**N_2O/O_2**
	(µmoles formazan/g wet wt/20 min)	
BRAINS	2.90±0.73 (n=4)	2.18±0.35 (n=5)
LIVERS	1.42±0.16 (n=5)	1.03±0.25[1] (n=4)

(Results are expressed as mean±SD.)

Comparing air breathing versus exposure to N_2O/O_2. [1]=$p<0.05$

to N_2O/O_2 and continued exposure for a further 24h, increased urinary excretion of radioactivity was observed, the bulk of which was identified as $5CH_3THF$ in agreement with Lumb *et al.*, 1982. Faecal excretion was unaffected suggesting that N_2O does not affect intestinal absorption.

Folate catabolism occurs in both the polyglutamate and monoglutamate pools (Pheasant *et al.*, 1981), the monoglutamate pool (as $5CH_3THF$) is the major circulatory form. Exposure to N_2O results in inhibition of methionine synthetase, accumulation of $5CH_3THF$ and a reduction of polyglutamates and THF (McGing *et al.*, 1978, Perry *et al.*, 1979, Deacon *et al.*, 1980b, Lumb *et al.*, 1981b, Wilson and Horne, 1986). The catabolites pAcABA, pAcABGlu, urea and H_2O were all increased after N_2O exposure - these were not detected by other workers presumably due to the use of [2-^{14}C] labelled folic acid (Lumb *et al.*,1982), the use of mixed label folic acid ensured that both portions of the folate molecule could be followed after scission. There is no evidence to suggest that N_2O functions as an oxidising species for tetrahydropterins (Heales, 1987), indeed levels of oxidising species detected by NBT assay were lower after N_2O/O_2. It may be concluded that the increased catabolites arise as a result of oxidation of $5CH_3THF$, however since the excretion of this in the urines was greatly increased more catabolites were expected if this were the sole monoglutamate species to undergo catabolism. It is suggested that part of the folate deficiency which arises after N_2O treatment may be due to increased catabolism.

Exposure to N_2O/O_2 has been reported to result in decreased polyglutamation (McGing *et al.*, 1978, Perry *et al.*,1979, Deacon *et al.*, 1980b, Lumb *et al.*,1981b), this is confirmed here by observing decreased tissue retention of radioactivity and identifying labelled polyglutamate species in liver extracts.

Acid/iodine oxidation and HPLC analysis of pterin levels may be used as an indicator of tissue THF (and not 5 CH_3THF) content (Heales, 1987). N_2O/O_2 exposure results in decreased levels of THF in tissues (McGing *et al.*, 1978, Perry *et al.*, 1979, Wilson and Horne, 1986, Heales, 1987). It is observed that if folate is administered during N_2O/O_2 exposure liver retention of radioactivity is decreased as is liver THF content whilst brain retention and THF content is

unaffected. It has been reported that the brain will conserve folate more tenaciously than other tissues (Lumb *et al.*, 1981a). Clearly polyglutamation must be occurring since only polyglutamate species are retained in cells. Polyglutamation is not abolished during N_2O exposure (Perry *et al.*, 1979) but is diminished due to a lack of supply of folates at the formate level (Chanarin *et al.*, 1980, Deacon *et al.*, 1980b, Perry *et al.*, 1980) which are the preferred substrates for polyglutamation. Demethylation is a prerequisite to polyglutamation (McGing *et al.*, 1978, Kennelley *et al.*, 1982). Since the brain takes up folates as 5 CH_3THF and folates are retained demethylation and polyglutamation must be occurring. It has been shown by McGing and Scott, (1980) that some demethylation occurs during N_2O exposure. However demethylation may not be a prerequisite for polyglutamation as Lumb *et al.*, 1985, have shown that 5CH_3THF may be used as a substrate for methyl polyglutamates. It may be concluded that if folate is supplied during N_2O/O_2 exposure the brain has the capacity to maintain levels at the expense of other tissues.

It has been reported that 5CH_3THF stimulates THB synthesis *in vitro* (Leeming *et al.*, 1982, Hamon *et al.*, 1986) and that N_2O abolishes THB synthesis *in vitro* Hamon *et al.*, 1986. N_2O exposure *in vivo* results in accumulation of folates as 5CH_3THF, however no increase in total brain or liver biopterin or DHPR activity was observed suggesting that either the effect is not reproducible *in vivo* or that the stimulatory effect of 5CH_3THF is as a result of its role as a methyl donor. The requirement for this may be met from betaine methyl transferase (EC 2, 1, 1, 5) which is induced on inhibition of methionine synthetase (Chanarin *et al.*, 1985).

CHAPTER SEVEN

THE EFFECT OF ANTIOXIDANTS ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

CHAPTER SEVEN.

7 THE EFFECT OF ANTIOXIDANTS ON TETRAHYDROBIOPTERIN METABOLISM IN THE RAT

7.1 INTRODUCTION

Deficiency of vitamin E results in neurological disorders which include axonopathy (Yatassery *et al.*, 1986) and spinocerebellar degeneration (Harding *et al.*, 1985), the administration of vitamin E at an early stage prevents irreversible neurological damage (Sokol *et al.*, 1985, Gordon, 1987).

Vitamin E may have a role in the sympathetic nervous system. In chronic vitamin E deficiency the urinary excretion of noradrenaline and adrenaline is increased (Nakashima and Esashi, 1987). It has been proposed that vitamin E may have a role in the protection of catecholamine storage vesicles and may be an essential cofactor for one of the enzymes involved in the biosynthesis of noradrenaline (Behrens *et al.*, 1986). Tetrahydrobiopterin is an essential cofactor for the biosynthesis of noradrenaline (Leeming *et al.*,1981).

Lowered serum vitamin E levels have been observed in patients suffering from Alzheimer's disease (Burns and Holland, 1986) although no evidence of vitamin E deficiency was observed in the temporal cortex of Alzheimer patients (Muller *et al.*,1986). Tetrahydrobiopterin metabolism is disturbed in Alzheimer's disease (Anderson *et al.*, 1986. Cowburn, 1988).

Lipofuscin deposition which is a consistent manifestation of aging and dementia is observed in vitamin E deficiency (Towfighi, 1981, Sarter and Van der Linde, 1987). Tetrahydrobiopterin metabolism is impaired in the aged and demented brain (Anderson *et al.*, 1987. Anderson, 1987).

The involvement of tetrahydrobiopterin (THB) and vitamin E in catecholamine biosynthesis, impaired tetrahydrobiopterin metabolism in Alzheimer's disease, the aging brain and dementia coupled with deficiencies of vitamin E in the above disorders, the ease of oxidation of terahydrobiopterin (Blair and Pearson, 1974, Heales, 1987) and the antioxidant properties of vitamin E suggest that vitamin E may have a protective role in tetrahydrobiopterin metabolism.

The effects of vitamin E and the synthetic antioxidant BHT (2,6 di-tertiary

butyl-4-methyl phenol) (figure 7.1) on THB metabolism were observed.

7.2 MATERIALS AND METHODS

Materials were obtained as described in Chapter 2.

DOSE RESPONSE

Female Wistar rats (250g) were dosed orally at 0900h and 1600h for 4 days with antioxidants (butylated hydroxytoluene (BHT) or dl-α-tocopherol acetate (vitamin E)) at doses ranging from 0-30mg in 0.5cm^3 Mazola corn oil. Rats were killed by cervical dislocation 18h after the final dose. Brains, livers and blood were analysed for DHPR activity, brains and livers were analysed for sepiapterin reductase activity and livers were analysed for DHFR activity as described in Chapter 2. Brain biopterin and pterins were determined for the maximum dose of vitamin E administered (30mg) as described in Chapter 2. Throughout the experiment animals were allowed free access to food and water and were kept on grids to prevent coprophagy.

TIME RESPONSE FOR VITAMIN E.

Female Wistar rats (250g) were dosed orally at 0900h with 15mg dl-α-tocopherol acetate in 0.5cm^3 Mazola corn oil. Rats were killed by cervical dislocation at various times after dosage. Biochemical parameters (other than biopterin and pterin levels) were determined as above. Throughout the experiment animals were allowed free access to food and water and were kept on grids to prevent coprophagy.

7.3 RESULTS

7.3.1 THE EFFECT OF BHT ON DHPR ACTIVITY

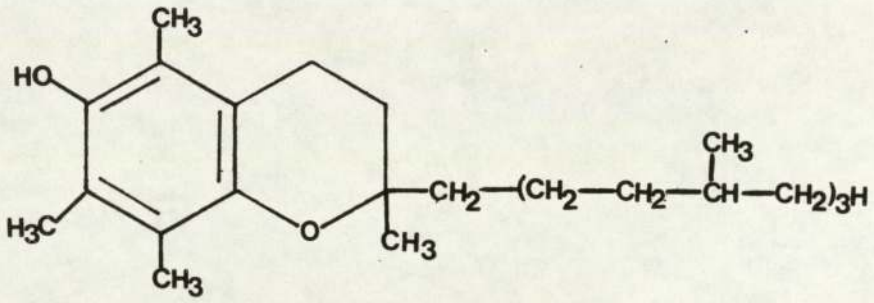
The effect of BHT at doses ranging from 0-15mg on tissue DHPR activity is shown in table 7.1. BHT was observed to have no effect on DHPR activity in the brain, liver or blood at the doses administered.

7.3.2 THE EFFECT OF BHT ON SEPIAPTERIN REDUCTASE ACTIVITY

The effect of BHT at doses ranging from 0-15mg on brain and liver sepiapterin

FIGURE 7.1

α-tocopherol



BHT (2,6 di-tertiary butyl-4-methyl phenol)

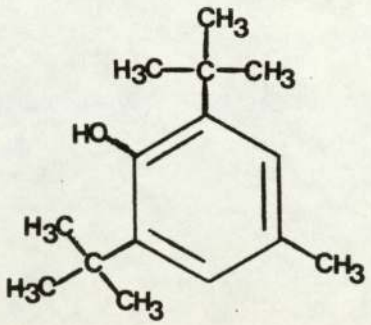


TABLE 7.1

The effect of BHT on DHPR activity.

DOSE (mg)	DHPR BRAIN[+]	DHPR LIVER[+]	DHPR BLOOD[++]
0	183±20	392±74	2.3±0.8
3.75	181±10	411±24	2.5±0.8
7.5	210±24	329±34	2.9±1.3
15.0	161±9	342±35	2.6±0.5

(Results are expressed as mean±SD where n=5 except at 15mg where n=4.)

[+] DHPR activity =nmole NADH oxidised/min/mg protein.

[++] DHPR activity=nmole cytochrome C reduced/min/mg protein.

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of BHT.
Rats were killed 18h after the final dose.

reductase activity is shown in table 7.2. BHT was observed to have no effect on sepiapterin reductase activity in the brain or liver.

7.3.3 THE EFFECT OF BHT ON LIVER DHFR ACTIVITY

The effect of BHT at doses ranging from 0-15mg on liver DHFR activity is shown in table 7.3. Activity of DHFR decreased in a dose dependent manner, this was significant at 7.5mg BHT/dose (2.34µmoles DHF reduced/min/mg protein) and at 15mg BHT/dose (2.29 µmoles DHF reduced/min/mg protein) when compared to 2.93µmoles DHF reduced/min/mg protein when no BHT was administered.

7.3.4 THE EFFECT OF VITAMIN E ON DHPR ACTIVITY

The effect of vitamin E at doses ranging from 0-30mg on tissue DHPR activity is shown in table 7.4. Although a significant decrease was observed in the liver at 3.75mg/dose and in the blood at 15mg/dose these results appear to be inconsistent with other values. Brain activity increased in a dose dependent manner reaching a maximal activity of 266nmoles NADH oxidised/min/mg protein at 15mg/dose ($r=0.484$, $p<0.001$, $n=54$), no further increase in activity was observed at 30mg/dose ($r=0.403$, $p<0.01$, $n=64$). The individual activities are plotted versus administered dose in figure 7.2.

7.3.5 THE EFFECT OF VITAMIN E ON SEPIAPTERIN REDUCTASE ACTIVITY

The effect of vitamin E at doses ranging from 0-30mg on brain and liver sepiapterin reductase activity is shown in table 7.5. Brain activity decreases in a dose dependent manner, this decrease is significant at 15mg/dose (0.35nmoles sepiapterin reduced/min/mg protein) and at 30mg/dose (0.33nmoles sepiapterin reduced/min/mg protein) when compared to 0.41nmoles sepiapterin reduced/min/mg protein when no vitamin E was administered. Vitamin E had no effect on liver activity.

7.3.6 THE EFFECT OF VITAMIN E ON LIVER DHFR ACTIVITY

The effect of vitamin E at doses ranging from 0-30mg on liver DHFR activity is shown in table 7.6. Vitamin E was observed to have no effect on liver activity.

TABLE 7.2

The effect of BHT on sepiapterin reductase activity.

DOSE (mg)	SEPIAPTERIN REDUCTASE *nmole sepiapterin reduced/min/mg protein*	
	BRAIN	**LIVER**
0	0.42±0.14	0.48±0.23
3.75	0.31±0.04	0.63±0.05
7.5	0.33±0.03	0.67±0.16
15.0	0.36±0.03	0.74±0.25

(Results are expressed as mean±SD where n=5 except at 15mg where n=4.)

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of BHT.
Rats were killed 18h after the final dose.

TABLE 7.3

The effect of BHT on liver DHFR activity.

DOSE (mg)	DHFR *µmoles DHF reduced/min/mg protein*
0	2.93±0.28
3.75	2.52±0.3
7.5	2.34±0.44[1]
15.0	2.29±0.24[2]

(Results are expressed as mean±SD where n=5 except at 15mg where n=4.)

Comparing to dosing with 0mg BHT [1]=$p<0.05$
[2]=$p<0.01$

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of BHT.
Rats were killed 18h after the final dose.

TABLE 7.4

The effect of vitamin E on DHPR activity.

	DHPR		
DOSE (mg)	**BRAIN[+]**	**LIVER[+]**	**BLOOD[++]**
0	216±32 (24)	263±53 (15)	2.18±0.49 (14)
3.75	220±25 (10)	196±31[2] (5)	1.85±0.48 (5)
7.5	250±45[1] (10)	264±88 (5)	1.79±0.33 (5)
15.0	266±46[2] (10)	222±43 (5)	1.48±0.14[3] (5)
30.0	256±39[2] (10)	246±65 (10)	2.26±0.48 (10)

(Results are expressed as mean±SD where the number in brackets refers to the number of animals.)

[+] DHPR activity =nmole NADH oxidised/min/mg protein.

[++] DHPR activity=nmole cytochrome C reduced/min/mg protein.

Comparing to dosing with 0mg vitamin E [1]=$p<0.05$

[2]=$p<0.01$

[3]=$p<0.001$

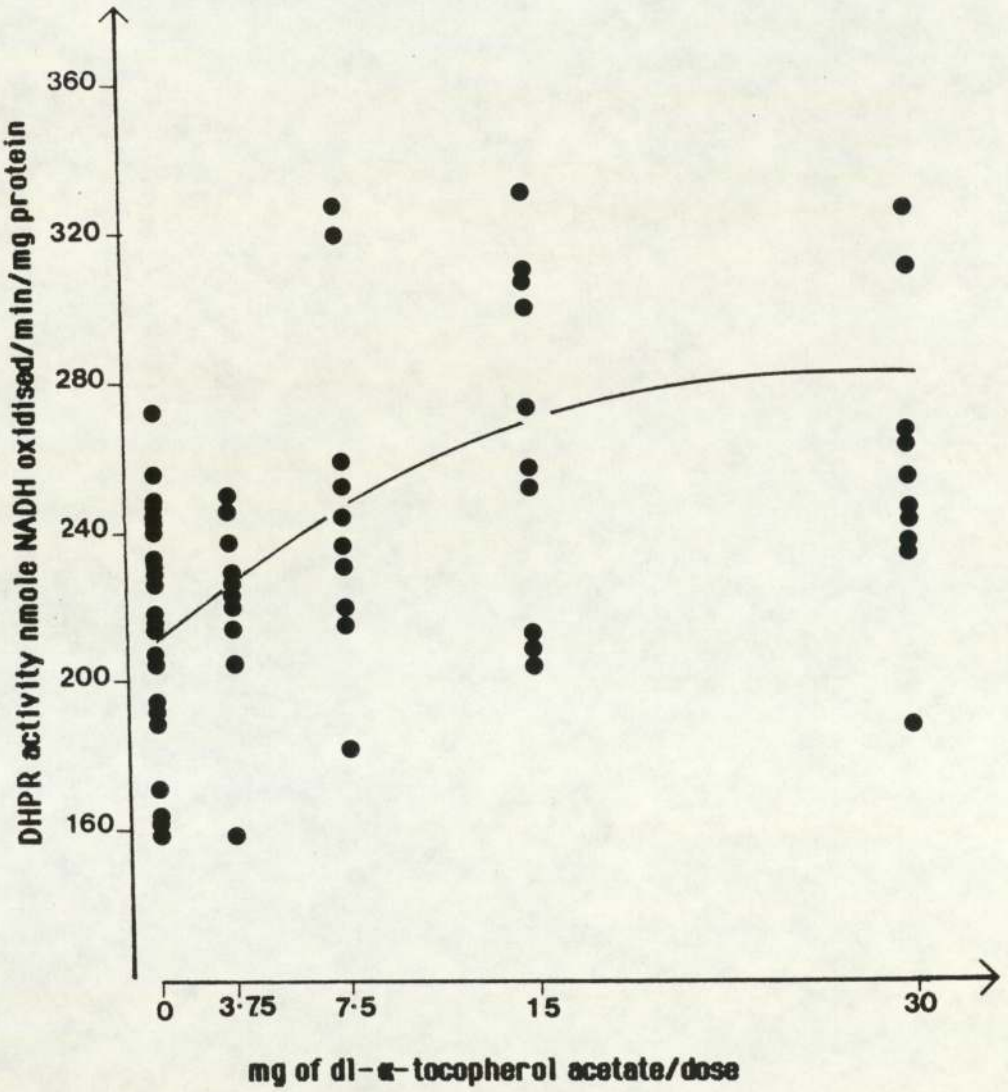
Female Wistar rats (250g).

Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of vitamin E.

Rats were killed 18h after the final dose.

FIGURE 7.2

Effect of administration of dl-α-tocopherol acetate at different doses on brain DHPR activity.



r=0.403

n=64

p<0.01

Female Wistar rats (250g)
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of vitamin E.
Rats were killed 18h after the final dose.

TABLE 7.5

The effect of vitamin E on sepiapterin reductase activity.

DOSE (mg)	SEPIAPTERIN REDUCTASE *nmole sepiapterin reduced/min/mg protein*	
	BRAIN	**LIVER**
0	0.41±0.09 (14)	0.48±0.21 (14)
3.75	0.40±0.04 (5)	0.51±0.11 (5)
7.5	0.38±0.02 (5)	0.33±0.05 (5)
15.0	0.35±0.03[1] (5)	0.41±0.10 (5)
30.0	0.33±0.06[1] (10)	0.44±0.23 (10)

(Results are expressed as mean±SD where the number in brackets refers to the number of observations.)

Comparing to dosing with 0mg vitamin E [1]=$p<0.02$

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of vitamin E.
Rats were killed 18h after the final dose.

TABLE 7.6

The effect of vitamin E on liver DHFR activity.

DOSE (mg)	DHFR *µmoles DHF reduced/min/mg protein*
0	1.58±0.52 (14)
3.75	1.88±0.71 (10)
7.5	1.70±0.47 (10)
15.0	1.61±0.56 (9)
30.0	1.66±0.29 (5)

(Results are expressed as mean±SD where the number in brackets refers to the number of observations.)

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of vitamin E.
Rats were killed 18h after the final dose.

7.3.7 THE EFFECT OF VITAMIN E ON TOTAL BRAIN BIOPTERINS AND PTERINS

The effect of vitamin E (at 30mg/dose) on total brain biopterins and pterins is shown in table 7.7. Vitamin E was observed to have no effect on biopterin or pterin levels in the brain.

7.3.8 DHPR ACTIVITY AT VARIOUS TIMES AFTER DOSING WITH VITAMIN E

Tissue DHPR activity analysed at various times after a single oral dose of vitamin E is shown in table 7.8. Brain DHPR activity increased linearly with time after dosage reaching a peak activity of 286 nmoles NADH oxidised/min/mg protein at 12h ($p<0.002$ compared to activity at 0.5h). No effect was observed on liver or blood activity.

7.3.9 SEPIAPTERIN REDUCTASE ACTIVITY AT VARIOUS TIMES AFTER DOSING WITH VITAMIN E

Tissue sepiapterin reductase activity analysed at various times after a single oral dose of vitamin E is shown in table 7.9. No effect on brain or liver acivity was observed over the 24h period.

7.3.10 LIVER DHFR ACTIVITY AT VARIOUS TIMES AFTER DOSING WITH VITAMIN E

Liver DHFR activity analysed at various times after a single oral dose of vitamin E is shown in table 7.10. No effect on liver activity was observed over the 24h period.

7.4 DISCUSSION

The effects of the antioxidants BHT and α-tocopherol (vitamin E) on tetrahydrobiopterin metabolism were observed.

BHT is widely used in industry although it is 100 times less potent than vitamin E in biological systems (Burton *et al.*, 1983). BHT was observed to have no effect on tissue DHPR or sepiapterin reductase activity although a significant decrease in liver DHFR was observed at doses of 7.5mg and 15mg, this may be indicative of toxicity at higher levels (Shlian and Goldstone, 1986). BHT enhances tumour formation as a result of induction of mixed function oxidases which could affect

TABLE 7.7

The effect of vitamin E on total brain biopterins and pterins.

	ng/g wet wt	
DOSE (mg)	**BIOPTERIN**	**PTERIN**
0	63.70±10.67 (10)	50.71±12.36 (10)
30	64.74±14.39 (9)	48.09±11.6 (9)

(Results are expressed as mean±SD where the number in brackets refers to the number of observations.)

Female Wistar rats (250g).
Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil ±30mg vitamin E.
Rats were killed 18h after the final dose.

TABLE 7.8

DHPR activity at various times after dosing with vitamin E.

	DHPR		
HOURS after dosing	**BRAIN**[+]	**LIVER**[+]	**BLOOD**[++]
0.5	232±24	408±65	2.21±0.45
4	258±36	349±77	2.64±0.48
8	259±32	423±45	2.06±0.38
12	286±11[1]	411±56	1.98±0.67
24	268±31	434±53	2.19±0.66

(Results are expressed as mean±SD where n = 5.)

[+] DHPR activity =nmole NADH oxidised/min/mg protein.

[++] DHPR activity=nmole cytochrome C reduced/min/mg protein.

Comparing to activity 0.5h after dosing [1]=$p<0.002$

Female Wistar rats (250g).

Animals were dosed at 0900h with 0.5cm^3 Mazola corn oil containing 15mg dl-α-tocopherol acetate (vitamin E).

TABLE 7.9

Sepiapterin reductase activity at various times after dosing with vitamin E

HOURS after dosing	SEPIAPTERIN REDUCTASE *nmole sepiapterin reduced/min/mg protein*	
	BRAIN	**LIVER**
0.5	0.16±0.07	0.61±0.08
4	0.17±0.05	0.50±0.15
8	0.19±0.01	0.49±0.16
12	0.18±0.04	0.55±0.13
24	0.16±0.03	0.64±0.01

(Results are expressed as mean±SD where n=5.)

Female Wistar rats (250g).
Animals were dosed at 0900h with 0.5cm^3 Mazola corn oil containing 15mg dl-α-tocopherol acetate (vitamin E).

TABLE 7.10

Liver DHFR activity at various times after dosing with vitamin E.

HOURS after dosing	DHFR *µmoles DHF reduced/min/mg protein*
0.5	1.62±0.54
4	1.83±0.44
8	2.21±0.49
12	2.18±0.5
24	2.19±0.62

(Results are expressed as mean±SD where n=5.)

Female Wistar rats (250g).
Animals were dosed at 0900h with 0.5cm^3 Mazola corn oil containing 15mg dl-α-tocopherol acetate (vitamin E).

pathways in metabolism of carcinogens (Witschi, 1985), this may account for the decrease in DHFR at higher doses of BHT since it has been observed that increased oxidising conditions result in lower activities (Chapter 3).

Brain DHPR increased with dose of vitamin E with maximal activity being observed at 15mg/dose (equivalent to 30mg daily) and 12h after dosing. Increased DHPR activity in the presence of antioxidants may be the result of protection of thiol groups from oxidation, increased DHPR acivity has been observed on dosing with L-cysteine (Heales, 1987). Increased oxidising conditions result in decreased DHPR activity, starvation results in increased oxidising species (Appendix 1) and decreased DHPR levels (Cutler, 1986, Heales, 1987). Declining DHPR levels have been observed in aging and Down's syndrome, THB metabolism is disturbed in aging, Down's and Alzheimer's disease (Anderson *et al.*, 1986, Anderson *et al.*, 1987, Cowburn,1988). Vitamin E therapy has been proposed to be beneficial in Parkinson's disease since levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase are decreased whilst the metabolism of L-Dopa (which is used in therapy) may generate free radicals (Cadet, 1986).

Although the administration of vitamin E had no effect on total brain biopterins in this study the ease of oxidation of tetrahydropterins and the protective role of glutathione (Heales, 1987) suggests that vitamin E may also have a protective role in THB metabolism, particularly in situations where oxidative stress is increased.

Vitamin E affects the sympathetic nervous system. Increased excretion of noradrenaline metabolites is observed with age and deficiency of vitamin E (Nakashima and Esashi, 1986, Nakashima and Esashi, 1987), this may be the result of increased oxidising conditions resulting in catecholamine breakdown. Noradrenaline levels are decreased on starvation (Schweiger *et al.*, 1985) possibly as a result of increased oxidising species (Appendix 1). Dietary vitamin E results in elevated tissue noradrenaline levels (Behrens *et al*, 1986). Noradrenaline inhibits brain sepiapterin reductase *in vitro* (Katoh *et al*, 1982) this may account for the observed decrease in brain sepiapterin reductase activity on vitamin E treatment.

In conclusion vitamin E administration results in increased reducing conditions with resultant increased brain DHPR levels, increased salvage of qDHB to THB may occur resulting in increased production of noradrenaline which inhibits brain sepiapterin reductase.

CHAPTER EIGHT

GENERAL DISCUSSION

CHAPTER EIGHT.

8 GENERAL DISCUSSION

8.1 FOLATES

Man is unable to biosynthesise the folate skeleton and folates are therefore an essential dietary requirement. Folates are involved in several important biosynthetic reactions, these include:- purine and pyrimidine biosynthesis, methionine biosynthesis, serine-glycine interconversions, histidine breakdown and the generation and utilisation of formate (Rowe, 1983). Deficiency results in a wide spectrum of biochemical disturbances and ultimately causes megaloblastic anaemia (Chanarin, 1979) and in more severe cases neurological disturbances (Reynolds, 1976). Folate deficiency is associated with old age, poverty, alcoholism and pregnancy and may be the result of inadequate intake, impaired absorption or metabolism or increased catabolism.

Folate catabolism is an oxidative process and occurs by scission of the C9N10 bond (Blair *et al.*, 1984, Al-Haddad *et al.*, 1986). It is well established that during catabolism the folate molecule is fragmented and that scission products such as p-acetamidobenzoate (pAcABA) and p-acetamidobenzoyl-l-glutamate (pAcABGlu) appear in the urine (Murphy *et al.*, 1976 and 1978, Connor *et al.*, 1979, Pheasant *et al.*, 1981). The polyglutamate pool contributes little to these catabolites particularly in early urine samples (Pheasant *et al.*, 1981). Several molecules could be available for scission - these include dihydrofolate (DHF), tetrahydrofolate (THF) and 5-methyl tetrahydrofolate ($5CH_3THF$), whether each contributes equally or if one particular species predominates is still unclear.

Folate catabolism may be studied *in vivo* by following the fate of orally administered mixed labelled folic acid, labelled in the pterin ring with ^{14}C (at position 2) and ^{3}H (at position 7), labelled with ^{3}H at the site of scission (at position 9) and labelled with ^{3}H in the benzoic acid portion (at positions 3' and 5'). This enables both portions of the folate molecule to be followed after scission (Barford and Blair, 1978). It has been established that folic acid is metabolised in a similar manner in man and the rat and that folate catabolism is a normal phenomenon in both (Saleh, 1981). Using the rat as a model for man the effects of various agents were observed on folate catabolism. Situations which affect folate catabolism will affect folate status and may have clinical consequences.

Studies were performed to identify species which make a major contribution to catabolism. Exposure to N_2O was used to give an insight into the major route of scission within the monoglutamate pool (first 24h).

8.1.1 EFFECT OF NITROUS OXIDE

Exposure to nitrous oxide results in oxidation of the cobalt 1 centre of vitamin B_{12} resulting in inactivation of methionine synthetase for which this is a cofactor (Banks *et al.*, 1968, Deacon *et al.*, 1978). This alters the distribution of folates - the polyglutamate pool is decreased as are all monoglutamate species other than $5CH_3THF$ which is increased (McGing *et al.*, 1978, Perry *et al.*, 1979, Lumb *et al.*, 1981a, Lumb *et al.*, 1982).

N_2O does not oxidise the tetrahydropterins (Heales, 1987) and there is no evidence to suggest that it functions as an oxidising species for these. Indeed it was observed that less oxidising species were detected by NBT assay after N_2O exposure (Table 6.5).

Following the administration of mixed label folic acid to rats after 8h exposure to N_2O and 24h continued exposure it was observed that faecal excretion was unaffected suggesting that N_2O does not affect intestinal absorption. Urinary excretion was increased to 200%, the bulk of this was identified as $5CH_3THF$ (which was increased to1000%) in agreement with Lumb *et al.*, 1982. Products of catabolism pAcABA and pAcABGlu were also increased but much less so (a 30% increase) (Table 8.1).

This slight increase most probably arises as a result of oxidation of $5CH_3THF$ as the excretion of this in the urines was greatly increased (Table 8.1). These results suggest that $5CH_3THF$ is not the major route of scission. DHF is an unlikely candidate for scission since this is oxidised to pterin -6-carboxylic acid which is unmetabolised in the rat and the absence of this in the urines suggests that oxidative breakdown of DHF does not occur under normal circumstances (Pheasant and Pearce, 1981). On the basis of these results it is suggested that THF (which is formed during transport from the gut lumen to the plasma and very susceptible to oxidation and whose formation at this stage and site is not affected by N_2O) is the major route of scission within the gut.

TABLE 8.1

Fate of mixed labelled folic acid during N_2O/O_2 exposure

	% OF DOSE			
	AIR BREATHING		N_2O/O_2	
	^3H	^{14}C	^3H	^{14}C
*faecal excretion	11.34±9.37	25.36±21.44	7.87±3.87	16.66±8.68
*urinary excretion	20.79±4.55	10.65±4.96	46.18±12.2[1]	24.02±9.75[2]
**urinary $5CH_3THF$	2.45	2.02	24.1	17.52
**urinary catabolites (pAcABA+ pAcABGlu)	10.08		12.93	

Female Wistar rats (250g) exposed to N_2O/O_2 (80%/20% (v/v)) for 8h prior to being dosed with 83µg/kg body wt mixed labelled folic acid.
Exposure was continued for a further 24h.

* mean±SD where n=5.
** analysis of pooled urine from 5 rats.

Comparing air breathing versus N_2O/O_2 [1]=$p<0.01$
[2]=$p<0.05$

Acid/iodine oxidation and HPLC analysis of pterin levels may be used as an indicator of tissue THF(and not $5CH_3THF$) content although some pterin may arise from 7,8 dihydropterin (Heales, 1987). It was observed that N_2O exposure resulted in decreased liver retentions and THF levels in agreement with McGing *et al.*, 1978, Perry *et al.*, 1979, Wilson and Horne, 1986, although brain retentions and THF levels were unaffected (Table 8.2). Clearly therefore if folate is supplied during N_2O exposure the brain may conserve levels at the expense of other tissues, since only polyglutamate species may be retained in tissues this further confirms that N_2O exposure does not abolish polyglutamation.

TABLE 8.2

Effect of N_2O/O_2 exposure on retention of radioactivity in tissues and pterin levels derived by acid/iodine oxidation.

	AIR BREATHING			N_2O/O_2		
	% of DOSE		PTERIN	% of DOSE		PTERIN
	^3H	^{14}C	ng/g wet wt	^3H	^{14}C	ng/g wet wt
LIVER	4.62 ±1.9	5.57 ±2.95	552 ±95	0.93[2] ±0.2	0.75[2] ±0.19	420[1] ±43
BRAIN	0.18 ±0.07	0.03 ±0.01	37 ±4	0.18 ±0.05	0.04 ±0.01	34 ±6

Female Wistar rats (250g) exposed to N_2O/O_2 (80%/20% (v/v)) for 8h prior to being dosed with 83µg/kg body wt mixed labelled folic acid.
Exposure was continued for a further 24h.
Results are expressed as mean±SD where n=5.

Comparing air breathing versus N_2O/O_2 [1]=$p<0.05$
[2]=$p<0.01$

8.1.2 EFFECTS OF LIPID PEROXIDES, VITAMIN E AND DIETHYLSTILBOESTROL.

The effects of agents which will affect the oxidative state *in vivo* were observed.

Fats undergo lipid peroxidation and autoxidation, resulting in the generation of free radicals (Shamberger, 1980, Coultate, 1984). Previous studies on folate catabolism have employed corn oil as a vehicle for the administration of other substances (Guest,1984, Surdhar, 1987). Corn oils are high in polyunsaturated fatty acids (PUFA). The fatty acid composition of corn oils is shown in table 8.3. The effects of corn oil administration on folate catabolism were studied since these may contain peroxides which could increase catabolism and therefore affect folate availability. Autoxidation of administered fats may also increase tissue peroxide content.

TABLE 8.3

Fatty acid composition of corn oil.

FATTY ACID			**mole %**
saturated fats:-			
16:0	(palmitic)		14
18:0	(stearic)		2
unsaturated fats:-			
18:1	(oleic)	(9)	34
18:2	(linoleic)	(9, 12)	48
18:3	(α-linolenic)	(9, 12, 15)	1
20:1			1

Vitamin E interferes with free radical chain propagation in the blood and is an important antioxidant *in vivo* (Burton *et al.*, 1983, Burton and Ingold, 1986). Vitamin E deficiency results in increased levels of lipoperoxides (Yoshioka *et al.*, 1987) and since these have been shown to result in increased folate catabolism

(Chapter 3) a protective role for vitamin E is suggested.

It has been suggested that prolonged use of oral contraceptive agents (OCA) results in folate deficiency although the mechanism by which this may arise remains unclear (Krumdieck *et al.*, 1975, Lakshamaiah and Bamji, 1979 and 1981, Shojania, 1982). The synthetic non-steroidal oestrogen diethylstilboestrol (DES) which is now recognised to be carcinogenic (Noller and Fisher, 1974) undergoes oxidative metabolism (Liehr *et al.*, 1983, Metzler, 1984, Sumi *et al.*, 1984, Ross *et al.*, 1985) and as such may function as an antioxidant *in vivo*.

MONOGLUTAMATE POOL.

Studies with N_2O suggest that THF may be the species which undergoes catabolism during the first 24h after folate administration. The effects of the above agents during this period will be considered to reflect catabolism of the circulating monoglutamate pool. Liver retentions at 24h will be considered to reflect the extent of polyglutamation (and therefore the extent of catabolism of the monoglutamate fraction) which occurs during this period.

Administration of Mazola corn oil had little effect on the excretion of catabolites arising from the monoglutamate pool, however dosing with refined corn oil resulted in a 44% increase in urinary catabolites when compared to control values. Vitamin E and DES administration resulted in decreased excretion of catabolites (44% and 59% respectively when compared to controls (Table 8.4)). The peroxide content of the corn oils was determined and found to be 1142mEq/kg oil for the refined corn oil and 112mEq/kg oil for the Mazola corn oil (a tenfold difference), the difference in administered dose was infact threefold ($0.3cm^3$ refined corn oil compared with $1cm^3$ Mazola corn oil) and therefore the difference in administered peroxides was threefold however the difference in extent of catabolism was not as great as this (the difference was infact less than twofold). The peroxide content of the administered oil contributes to the extent of catabolism which occurs but any fats which are administered may themselves undergo peroxidation and result in the production of peroxides. Decreased catabolism as a result of administration of vitamin E and DES may be attributed to their antioxidant properties, vitamin E is an important antioxidant in the body and has an important role in scavenging free radicals. DES is a diphenol and will therefore be oxidised itself in preference to folates.

TABLE 8.4

Effect of corn oils, vitamin E and diethylstilboestrol on catabolism of the folate monoglutamate pool.

	% of DOSE					
	control	**refined corn oil**	**Mazola corn oil (A)**	**Mazola corn oil (B)**	**VitaminE +Mazola (B)**	**DES+ refined**
^{3}H catabolites*						
0-24h	8.91	12.87	7.06	9.02	4.96	5.25
^{3}H retained in liver at 24h	18.52 ±3.72	12.63[1] ±2.79	13.15 ±4.61	15.82 ±3.71	14.33 ±2.46	29.41[2] ±7.0
^{14}C retained in liver at 24h	14.32 ±1.55	9.61[3] ±2.28	12.24 ±3.49	14.41 ±2.73	13.13 ±3.13	20.85[1] ±4.98
peroxide oxygen mEq/kg oil		1142	112			

Results are expressed as mean ±SD where n=5 except *which are means of pooled urines from 5 rats.

Comparing to controls [1]=$p<0.05$ ($p<0.002$ when compared to refined corn oil) [2]=$p<0.02$ [3]=$p<0.01$.

Controls = dosed 83µg/kg body wt mixed labelled folic acid (containing 6mg sodium ascorbate).
Refined corn oil = dosed 3 days 0.3cm^3 refined corn oil (0.01% BHT (w/v)) prior to above dose of folic acid.
Mazola (A) = dosed 1cm^3 Mazola corn oil (contains 0.02% (w/v) vitamin E) for 3 days prior to same dose of folic acid.
Mazola (B) = dosed as above except continued dosing throughout experiment.
Vitamin E + Mazola (B)= 7.5mg dl-α-tocopherol acetate in 0.5cm^3 Mazola corn oil twice daily for 3 days prior to above dose of folic acid and throughout the experiment.
DES+refined corn oil= 415mg /kg body wt DES in 0.3cm^3 refined corn oil for 3 days prior to above dose of folic acid.

Liver retentions were considered to reflect the extent of polyglutamation which occurs (Table 8.4). Mazola corn oil at either dose or vitamin E had no significant effect on liver retentions at 24h. Administration of refined corn oil resulted in decreased liver retentions at 24h. Since the excretion of catabolites was increased during this period this suggests that increased catabolism of the monoglutamate pool has resulted in decreased availability of folates for incorporation into the polyglutamate fraction. DES administration resulted in increased retentions of both ^{3}H and ^{14}C species in the liver ($p<0.02$ compared to controls).

These results suggest that peroxides result in increased catabolism of the monoglutamate pool and decreased incorporation of folates in the polyglutamate pool. Antioxidants (such as vitamin E) decrease catabolism of the monoglutamate pool. DES administration resulted in increased incorporation of folates into the polyglutamate pool.

It is proposed that increased peroxide content as a result of corn oil administration may be oxidising vitamin B_{12} and resulting in inhibition of methionine synthetase since increased urinary excretion of 5 CH_3THF and decreased tissue retentions were observed in situations where peroxide administration was the highest (Table 8.5).

POLYGLUTAMATE POOL

Previous workers have shown that excretion of catabolites continues at a diminished rate at 48h and 72h after folate administration (Pheasant *et al.*, 1981). A similar pattern of excretion was observed (Table 8.6). Since at 24h most of the dose is in the polyglutamate pool and polyglutamation is complete at 48h these catabolites are believed to arise from breakdown of the polyglutamate pool or from breakdown of monoglutamates which have been derived from the polyglutamate pool.

Excretion of catabolites decreases with time after administration of mixed labelled folic acid reflecting a slower breakdown of the tissue polyglutamate fraction. The extent of catabolism over 24-48h and 48-72h was increased in all groups when compared to controls (Table 8.6) and may be attributed to dosing with corn oils. Dosing with corn oils will increase the tissue lipid content. These may undergo autoxidation and peroxidation resulting in the generation of peroxides which will be available for scission of the folate molecule. Thus corn oil administration affects both the monoglutamate and polyglutamate pool.

TABLE 8.5

Urinary excretion of 5 CH_3THF and liver retentions 24h after mixed labelled folic acid administration.

	% of DOSE					
	control	refined corn oil	Mazola corn oil (A)	Mazola corn oil (B)	VitaminE +Mazola (B)	DES+ refined
5 CH_3THF* excreted 0-24h ^3H	8.72	13.88	11.17	8.94	6.52	10.51
^3H retained in livers at 24h	18.52 ±3.72	12.63[1] ±2.79	13.15 ±4.61	15.82 ±3.71	14.33 ±2.46	29.41[2] ±7.0

Results are expressed as mean ±SD where n=5 except *which are means of pooled urines from 5 rats.

Comparing to controls [1]=$p<0.05$ [2]=$p<0.02$

Controls = dosed 83µg/kg body wt mixed labelled folic acid (containing 6mg sodium ascorbate).
Refined corn oil = dosed 3 days 0.3cm^3 refined corn oil (0.01% BHT (w/v)) prior to above dose of folic acid.
Mazola (A) = dosed 1cm^3 Mazola corn oil (contains 0.02% (w/v) vitamin E) for 3 days prior to same dose of folic acid.
Mazola (B) = dosed as above except continued dosing throughout experiment.
Vitamin E + Mazola (B)= 7.5mg dl-α-tocopherol acetate in 0.5cm^3 Mazola corn oil twice daily for 3 days prior to above dose of folic acid and throughout the experiment.
DES+refined corn oil= 415mg /kg body wt DES in 0.3cm^3 refined corn oil for 3 days prior to above dose of folic acid.

TABLE 8.6

Effect of corn oils, vitamin E and DES on catabolism of the liver polyglutamate fraction.

	% of DOSE					
	control	**refined corn oil**	**Mazola corn oil (A)**	**Mazola corn oil (B)**	**VitaminE +Mazola (B)**	**DES+ refined**
^{3}H catabolites* 24-48h	0.31	3.0	1.71	1.59	1.43	1.58
^{3}H catabolites* 48-72h	0.28	1.47	1.37	1.55	1.18	2.09
^{3}H retained in liver 48h	21.46 ±6.1	16.08 ±2.21	12.22[1] ±3.38	17.23 ±1.84	13.47[2] ±2.27	32.14[2] ±7.16
^{14}C retained in liver 48h	13.61 ±3.76	12.2 ±1.67	11.45 ±3.59	15.75 ±2.82	12.31 ±2.74	23.06[3] ±4.39
^{3}H retained in liver 72h	18.81 ±2.93	15.78 ±1.48	9.56[4] ±1.49	16.38 ±1.04	12.3[3] ±1.94	25.49[1] ±3.77
^{14}C retained in liver 72h	11.44 ±1.87	11.97 ±1.29	10.52 ±2.08	14.55[2] ±1.32	11.46 ±2.36	17.69[3] ±2.52
% scission 24-48h	2.29	31.86	16.46	9.37	9.69	9.6
% scission 48-72h	2.41	9.22	13.09	9.97	9.95	12.67

Results are expressed as mean ±SD where n=5 except *which are means of pooled urines from 5 rats.

Comparing to controls [1]=p<0.02 [2]=p<0.05 [3]=p<0.01 [4]=p<0.001

Controls = dosed 83µg/kg body wt mixed labelled folic acid (containing 6mg sodium ascorbate).
Refined corn oil = dosed 3 days 0.3cm^3 refined corn oil (0.01% BHT (w/v)) prior to above dose of folic acid.
Mazola (A) = dosed 1cm^3 Mazola corn oil (contains 0.02% (w/v) vitamin E) for 3 days prior to same dose of folic acid.
Mazola (B) = dosed as above except continued dosing throughout experiment.
Vitamin E + Mazola (B)= 7.5mg dl-α-tocopherol acetate in 0.5cm^3 Mazola corn oil twice daily for 3 days prior to above dose of folic acid and throughout the experiment.
DES+refined corn oil= 415mg /kg body wt DES in 0.3cm^3 refined corn oil for 3 days prior to above dose of folic acid.

The extent of catabolism may also be determined by calculating the extent of polyglutamate breakdown, this may be calculated where:-

$$\% \text{ scission } 24\text{-}48h = \frac{\text{pAcABGlu excreted } 24\text{-}48h}{\%\,^{3}H \text{ in liver at } 24h} \times 100$$

(^{3}H in the liver is corrected for since after scission 42.5% of the ^{3}H dose remains in the glutamyl fraction).

The extent of scission of the polyglutamate fraction was increased for all treatment groups when compared to controls (Table 8.6). Since all these were dosed with agents in corn oil it may be concluded that corn oils result in increased scission of the polyglutamate fraction. This must be as a result of production of peroxides during autoxidation and peroxidation of fats. From these results it may be concluded that corn oil administration may result in increased catabolism of both folate pools.

Both the quantity and quality of fats will affect folate status, thus highlighting the importance of different methods of food preparation. The peroxide content of fats increases as these are subjected to high temperatures - particularly when these are used for deep frying (Lundberg, 1962). Not only will foods prepared in such a manner be low in folates but a high intake of such fats may have a detrimental effect on folates within the body.

Oxidising species in the liver were determined using the NBT assay (Table 8.7). Increased oxidising species were observed on treatment except at 24h after DES+ refined corn oil administration when these were decreased (as was the excretion of catabolites). These results suggest that the NBT assay may be used qualitatively to reflect changes in catabolism of the polyglutamate pool. Oxidising species did not correlate with the extent of scission and the assay may not be used quantitatively. This is perhaps not surprising since the assay itself not specific for any particular species.

It has been suggested that DHFR and DHPR may have a role in protecting oxidisable species from undergoing catabolism and the possible involvement of these in folate catabolism was considered (Table 8.7). The results did not appear to be consistent with this theory. If these are involved in protecting species from oxidation other factors must also be involved.

TABLE 8.7

Possible contributions of liver oxidising species ,DHPR and DHFR in oxidative scission of the liver polyglutamate fraction.

	% of DOSE					
	control	**refined corn oil**	**Mazola corn oil (A)**	**Mazola corn oil (B)**	**VitaminE +Mazola (B)**	**DES+ refined**
oxidising species at 24h	0.81±0.07	1.85±0.33[5]	2.22±0.38[5]	1.23±.037[1]	2.10±0.35[5]	0.45±0.27 (n=3)
oxidising species at 48h	0.85±0.19	1.48±0.43[2]	2.18±0.82[3]	1.18±0.65	1.39±0.41[1]	1.31±0.16[3]
oxidising species at 72h	1.15±0.2	1.07±0.3	1.84±0.7	1.26±0.28	2.29±0.23[5]	1.61±0.3[1]
% scission 24-48h	2.29	31.86	16.46	9.37	9.97	9.6
% scission 48-72h	2.41	9.22	13.09	9.69	9.95	12.67
DHFR						
24h	7.43±3.92	5.95±0.78	4.37±0.48	3.95±1.09	5.86±0.84	6.27±1.05
48h	4.92±0.67	5.2±1.09	3.21±0.45[4]	3.92±0.54[1]	6.8±1.42[1]	5.91±1.2
72h	5.4±1.65	3.52±0.34[1]	3.73±1.29	4.9±1.15	6.23±1.13	7.37±0.64[1]
DHPR						
24h	317±83	366±59	199±37[2]	263±20	290±82	354±67
48h	266±59	340±116	186±31[1]	222±20	287±112	339±78
72h	352±50	285±75	220±38[4]	247±36[3]	335±38	313±42

Results are expressed as mean ±SD where n=5.

Oxidising species = µmoles formazan/g wet wt/20 min.
DHPR activity = nmoles NADH oxidised/min/mg protein.
DHFR activity = µmole DHF reduced/min/mg protein.

Comparing to controls [1]=p<0.05 [2]=p<0.02 [3]=p<0.01 [4]=p<0.002 [5]=p<0.001

TABLE 8.7 (contd)

Controls = dosed 83µg/kg body wt mixed labelled folic acid (containing 6mg sodium ascorbate).
Refined corn oil = dosed 3 days 0.3cm^3 refined corn oil (0.01% BHT (w/v)) prior to above dose of folic acid.
Mazola (A) = dosed 1cm^3 Mazola corn oil (contains 0.02% (w/v) vitamin E) for 3 days prior to same dose of folic acid.
Mazola (B) = dosed as above except continued dosing throughout experiment.
Vitamin E + Mazola (B) = 7.5mg dl-α-tocopherol acetate in 0.5cm^3 Mazola corn oil twice daily for 3 days prior to above dose of folic acid and throughout the experiment.
DES+refined corn oil = 415mg /kg body wt DES in 0.3cm^3 refined corn oil for 3 days prior to above dose of folic acid.

8.1.3 INCREASED ENZYME ACTIVITIES AFTER DES ADMINISTRATION

Both DHFR and 5-10 methylene THF reductase activity was elevated after DES treatment (Table 8.8). 5-10 methylene THF reductase is a key enzyme in the formation of methyl groups and it has been observed that tumours result in an increased demand for methyl groups (Poirier, 1973, Lakshamaiah and Bamji, 1979). This rise in 5-10 methylene THF reductase may serve to accommodate this increased demand and clearly precedes tumour formation. Alternatively increased DHFR activity may contribute to the elevated levels of 5-10 methylene THF reductase which is inhibited by DHF (Matthews and Haywood, 1979).

DHFR activity is increased on administration of oestrogens (Lakshamaiah and Bamji, 1981, Burns and Jackson, 1982, Levine *et al.*, 1985) and during malignancy. Saleh *et al.*, 1981, observed that treating rats with the DHFR inhibitor MTX resulted in increased catabolism whilst catabolism was decreased in tumour bearing rats. Since administration of DES resulted in elevated DHFR levels and decreased catabolism this further suggests a role for DHFR in protecting oxidisable species from undergoing catabolism. Increased tissue retentions, elevated DHFR levels and decreased catabolism are observed during malignant disorders and since these were observed on administration of a carcinogen (DES) these may precede tumour development.

TABLE 8.8

Elevated liver DHFR and 5-10 methylene THF reductase levels after DES administration

	DHFR[+]		5-10 methylene THF reductase[++]	
	CORN OIL	**DES+CORN OIL**	**CORN OIL**	**DES+CORN OIL**
24h	5.95±0.78	6.27±1.05	0.3±0.09	0.47±0.12 (n=4)
48h	5.2±1.09	5.91±1.2	0.35±0.17	0.64±0.14[1]
72h	3.52±0.34	7.37±0.64[2]	0.74±0.32	1.0±0.35

Female Wistar rats (250g) dosed with 0.3cm^3 **refined** corn oil ±415mg/kg body wt DES for 3 days prior to being dosed with 83µg/kg body wt mixed labelled folic acid.

Each value represents mean±SD where n=5 unless otherwise stated.

[+] DHFR activity= µmoles DHF reduced/min/mg protein.

[++] 5-10 methylene THF reductase= nmole $5CH_3$THF oxidised/h/mg protein.

Comparing corn oil treated versus DES and corn oil [1]=$p<0.02$ [2]=$p<0.001$

8.2 TETRAHYDROBIOPTERIN

Tetrahydrobiopterin is an essential cofactor for the amino acid hydroxylases and is therefore necessary for the biosynthesis of catecholamine neurotransmitters and serotonin. Intracellular levels of THB are maintained by *de novo* synthesis from GTP and salvage by DHPR, (Nichol *et al.*, 1985). Defects in metabolism result in impaired neurological function due to depletion of brain precursors of catecholamine and serotonin neurotransmitters.

THB is susceptible to oxidation (Blair and Pearson, 1974), therefore the effects of agents which may effect the state of the oxidising environment *in vivo* were observed on THB metabolism. The rat was used as an experimental model for man.

8.2.1 NITROUS OXIDE

Nitrous oxide is commonly employed as an anaesthetic gas, neurological disorders are associated with its abuse and this is believed to arise as a result of methionine depletion (Dinn *et al.*, 1978, Layzer, 1978). It has been reported that $5CH_3THF$ stimulates THB biosynthesis (Leeming *et al.*, 1982, Hamon *et al.*, 1986) and that N_2O abolishes THB biosynthesis *in vitro* (Hamon *et al.*, 1986). Exposure to N_2O *in vivo* results in accumulation of $5CH_3THF$ (Lumb *et al.*, 1981a), therefore the effects of N_2O exposure on THB biosynthesis were observed *in vivo*.

N_2O exposure was observed to have no effect on total brain or liver biopterins or DHPR activity (Table 8.9), this suggests that either the effect is not reproducible *in vivo* or the stimulatory effect of $5CH_3THF$ is as a result of its role as a methyl donor and this requirement is met *in vivo* by betaine methyl transferase (EC 2, 1, 1, 5) which is induced on exposure to N_2O (Chanarin *et al.*, 1985).

TABLE 8.9

Effect of N_2O/O_2 exposure on total tissue biopterins and DHPR activity.

	TOTAL BIOPTERNS[+]		DHPR ACTIVTY[++]	
	control	N_2O/O_2	control	N_2O/O_2
BRAIN	49±2	52±9	135±45	156±51
LIVER	503±56	500±77	291±66	323±82

Female Wistar rats (250g) exposed to N_2O/O_2 (80%/20% (v/v)) for 8h prior to being dosed with 83µg/kg body wt mixed labelled folic acid. Exposure was continued for a further 24h.

(Results are expressed as mean±SD where n=5).

[+] total biopterins = ng/g wet wt.

[++] DHPR activity = nmoles NADH oxidised/min/mg protein.

8.2.2 VITAMIN E

Vitamin E deficiency may result in neurological disorders whilst the administration of vitamin E at an early stage may prevent irreversible neurological damage (Sokol *et al.*, 1985, Gordon, 1987). A role for vitamin E has been implicated in the sympathetic nervous system since in chronic vitamin E deficiency increased excretion of adrenaline and noradrenaline is observed (Nakashima and Esashi, 1987). THB is essential for the biosynthesis of noradrenaline (Leeming *et al.*,1981) , is easily oxidised and lowered vitamin E levels have been observed in patients suffering from Alzheimer's disease (Burns and Holland, 1986). THB metabolism is disturbed in the aged and demented brain (Anderson *et al.*, 1986 and 1987). It is suggested that vitamin E may have a protective role in THB metabolism.

Brain DHPR activity was elevated in a dose dependant manner after vitamin E administration, brain sepiapterin reductase (which is involved in *de novo* synthesis of THB) was decreased (Table 8.10). The increase in DHPR activity was observed to be maximal 12h after vitamin E administration (Table 8.11). Increased DHPR activity in the presence of antioxidants may be the result of protection of thiol groups from oxidation, increased DHPR activity has been observed on dosing with L-cysteine (Heales, 1987). Since lowered DHPR levels and disturbed THB metabolism have been observed in aging and Down's syndrome (Anderson *et al.*, 1986 and 1987) it is proposed that vitamin E therapy may prove beneficial. Although no effect was observed on total biopterins the ease of oxidation of tetrahydropterins and the protective role of glutathione (Heales, 1987) suggests that vitamin E may also have a protective role in THB metabolism.

Brain sepiapterin reductase was observed to be decreased after vitamin E administration (Table 8.10) this may be the result of inhibition by noradrenaline (Katoh *et al.*, 1982) which is increased on vitamin E administration (Behrens *et al.*, 1986).

TABLE 8.10

Effect of vitamin E administration on brain DHPR and sepiapterin reductase.

DOSE (mg) (half daily dose/rat)	Brain DHPR activity[+]	Brain sepiapterin reductase[++]
0	216±32 (24)	0.41±0.09 (14)
3.75	220±25 (10)	0.40±0.04 (5)
7.5	250±45[1] (10)	0.38±0.02 (5)
15	266±46[3] (10)	0.35±0.03[2] (5)
30	256±39[3] (10)	0.33±0.06[2] (10)

Female Wistar rats (250g). Animals were dosed at 0900h and 1600h for 4 days with 0.5cm^3 Mazola corn oil containing varying amounts of vitamin E. Rats were killed 18h after the final dose.

(Results are expressed as mean±SD where the number in brackets refers to the number of observations).

[+] DHPR activity = nmoles NADH oxidised/min/mg protein.

[++] Sepiapterin reductase activity = nmoles sepiapterin reduced min/mg protein.

Comparing to dosing with 0mg vitamin E [1]=$p<0.05$

[2]=$p<0.02$

[3]=$p<0.01$

TABLE 8.11

Brain DHPR activity at various times after dosing with vitamin E.

Hours after dosing	DHPR (nmoles NADH oxidised/min/mg protein)
0.5	232±24
4	258±36
8	259±32
12	286±11[1]
24	268±31

Female Wistar rats (250g). Animals were dosed at 0900h with 0.5cm^3 Mazola corn oil containing 15mg dl-α-tocopherol acetate (vitamin E).

(Results are expressed as mean±SD where n=5)

Comparing to activity 0.5h after dosing [1]=$p<0.002$.

In conclusion vitamin E administration results in increased reducing conditions with resultant increased brain DHPR levels, increased salvage of qDHB to THB may occur resulting in increased production of noradrenaline which inhibits brain sepiapterin reductase and THB levels are maintained.

8.2.3 STARVATION

Starvation was observed to result in increased brain and liver oxidising species as detected by the NBT assay (Table 8.12). Starvation results in depletion of liver reduced glutathione levels (Tateishi *et al.*, 1974, Isaacs and Binkley, 1977) with

resultant increased levels of oxidising species which may be detected by the NBT assay. Starvation may have neurological consequences - decreased noradrenaline turnover is observed on starvation (Young and Landsberg, 1977) this could be the result of depressed brain tyrosine levels (Schweiger *et al.*, 1985) since tyrosine hydroxylase is the rate limiting enzyme in catecholamine synthesis. Edwards *et al.*, 1987, have observed decreased THB biosynthesis and DHPR activity in the rat brain on starvation, these could arise as a result of the increased oxidising species which occur on starvation. It has been observed that situations where oxidising conditions are increased - aging, Down's and Alzheimer's disease may result in disturbed THB metabolism (Anderson, 1987).

TABLE 8.12

Effect of 18h starvation on oxidising species detected by NBT assay.

	FED	STARVED
	(µmoles formazan/g wet wt/ 20 min)	
LIVER	1.58±0.23	2.06±0.39[1]
BRAIN	0.71±0.35	1.36±0.23[2]

Female Wistar rats (250g) starved for 18h on grids (1600h to 1000h).
Control rats were allowed free access to food. All animals were allowed free access to water.

(Results are expressed as mean±SD where n=5).

Comparing fed with starved [1]=$p<0.05$
[2]=$p<0.01$

It is proposed that therapy of vitamin E or other antioxidants may prove beneficial in such situations since this results in elevated DHPR levels, indeed it has been proposed by Cadet, 1986 to be beneficial in Parkinsonism since conventional therapy of L-Dopa generates free radicals.

8.2.4 STIMULATION OF DHPR ACTIVITY WITH PEROXIDES

Brain DHPR activity was increased on administration of refined corn oil with a high peroxide value (Table 8.13), this suggests that in the presence of increased oxidising species a compensatory mechanism operates resulting in increased DHPR levels. Heales, 1987, observed a similar increase in liver DHPR on chronic dosage with L-buthionine sulfoximine which depletes liver glutathione levels, this suggests that in situations of increased oxidative stress (which would presumably result in increased catabolism of THB) the salvage pathway is stimulated and loss of THB may be minimised.

TABLE 8.13

Increased brain DHPR activity after dosing with corn oil with a high peroxide value.

	DHPR (nmoles NADH oxidised /min /mg protein)	
	control	**corn oil dosed**
BRAIN	191±55	261±16[1]

Female Wistar rats (250g) dosed orally with 0.3cm^3 refined corn oil (peroxide value 1142 mEq/kg oil) for 3 days prior to being dosed with 83µg/kg body wt mixed labelled folic acid. Brains were analysed for 24h after dosing with folic acid.

Results are expressed as mean±SD where n=5.

Comparing to controls [1]=$p<0.05$

8.3 GENERAL CONCLUSIONS

1 The small increase in excretion of catabolites observed after N_2O exposure may be the result of oxidation of $5CH_3THF$, since this pool was greatly increased a greater increase in catabolites was expected. THF is proposed to be the monoglutamate

species which is susceptible to oxidative scission.

2 Vitamin E administration results in decreased catabolism of the folate monoglutamate pool and may therefore be beneficial in conserving folate stores if the intake of dietary fats is minimal (since with increased fat intake reactive lipid species may be converted to lipid peroxides which may contribute to folate catabolism).

3 Both the free radicals generated by lipid peroxidation and autoxidation and the peroxide content of corn oils may result in increased folate catabolism, therefore the quantity and quality of fats may affect folate status.

4 DES administration results in increased tissue retentions and decreased folate catabolism since these observations are similar to those observed in malignancy this suggests that these changes may precede malignancy.

5 DES administration results in increased 5-10 methylene THF reductase suggesting that the increased demand for methyl groups which is observed during malignancy precedes tumour formation.

6 Peroxides may result in oxidation of vitamin B_{12} and inhibition of methionine synthetase.

7 The NBT assay reflects changes in oxidative states within tissues and may be used to reflect changes in polyglutamate breakdown although it may not be used quantitatively.

8 The brain has the capacity to maintain folate levels at the expense of other tissues.

9 N_2O exposure has no effect on THB metabolism - suggesting that $5CH_3THF$ has no effect on THB biosynthesis.

10 Vitamin E administration elevates brain DHPR, this may be the result of protection of thiol groups from oxidation.

11 Vitamin E administration results in inhibition of brain sepiapterin reductase, (increased salvage capacity as a result of increased brain DHPR activity may result in increased catecholamine synthesis resulting in increased noradrenaline levels

which may inhibit sepiapterin reductase and maintain THB levels).

12 Oxidising species are increased on starvation, these could account for the disturbances in THB metabolism which have been observed on starvation.

13 In situations where there is an increase in oxidising species (such as high peroxide levels) stimulation of DHPR activity may occur, the resulting increased salvage of qDHB may help to maintain THB levels.

8.4 FURTHER WORK

1 Long term exposure to N_2O to see whether the observed effects persist beyond 24h.

2 Measurement of methionine synthetase levels after the administration of peroxides to observe whether peroxide administration does indeed result in inhibition of methionine synthetase as a result of oxidation of vitamin B_{12}. This would account for the observed increased excretion of $5CH_3THF$ in situations where high doses of fats (and therefore peroxides) were administered.

3 Repeat vitamin E experiment but administering vitamin E in a small volume of corn oil with a low peroxide content.

4 Repeat DES experiment but administering DES in a vehicle having a low peroxide content.

5 Further long term studies may be performed to see whether the NBT assay does indeed correlate with the extent of polyglutamate breakdown.

6 Further studies on the effects of agents which affect catabolism to see whether DHPR and DHFR may have a role in protecting species from oxidation.

7 Prolonged exposure to N_2O to observe whether there is an effect on THB metabolism *in vivo*.

8 N_2O exposure which results in increased levels of 5 CH_3THF had no effect on THB metabolism suggesting that the stimulatory role of $5CH_3THF$ on THB biosynthesis

may be as a result of its function as a methyl donor, the effects of methionine supplementation may be observed *in vitro*.

9 Evaluation of the possible use of vitamin E supplementation in situations of increased oxidative stress (such as starvation) on THB metabolism.

APPENDIX ONE

THE EFFECT OF ASCORBATE AND STARVATION ON OXIDISING SPECIES DETECTED BY NBT ASSAY

APPENDIX ONE.

A1 THE EFFECT OF ASCORBATE AND STARVATION ON OXIDISING SPECIES DETECTED BY NITROBLUE TETRAZOLIUM ASSAY.

A1.1 INTRODUCTION.

In the presence of oxidising species nitroblue tetrazolium (NBT) is reduced to insoluble blue formazan (Baehner and Nathan, 1968), this may be used as a basis for a qualitative assay for the detection of oxidising species in tissues (Surdhar, 1987). Since folate catabolism is an oxidative process (Blair *et al.*, 1984, Al-Haddad *et al.*, 1986) the assay was employed to observe whether changes in oxidising species correlated with changes in folate catabolism. Increased levels of ascorbate and starvation affect the levels of tissue oxidising species, the effects of these treatments were monitored by NBT assay to observe whether these changes may be detected by the assay.

A1.2 MATERIALS AND METHODS.

Materials were obtained as described in Chapter 2.

ASCORBATE

5 female Wistar rats (250g) were dosed orally with 0.3cm^3 2% (w/v) sodium ascorbate (equivalent to a dose of 6mg/rat), rats were killed 24h after dosing, livers were analysed for oxidising species as described in Chapter 2 (brains were not analysed). Animals were allowed free access to food and water and were kept on grids to prevent coprophagy.

STARVATION.

5 female Wistar rats (250g) were deprived food but not water for a period of 18h from 1600h to 1000h. Animals were kept on grids to prevent coprophagy. Rats were killed by cervical dislocation and brains and livers were removed and assayed for oxidising species employing the NBT assay as described in Chapter 2.

A1.3 RESULTS AND DISCUSSION.

Dosing with ascorbate results in a significant decrease in liver oxidising species detected by NBT assay (0.64µmoles formazan/g wet wt/20 min compared to 1.58µmoles formazan/g wet wt/20 min in the control group) (Table A1). These

TABLE A1

Effect of the oral administration of ascorbate on oxidising species in the liver as detected by NBT assay.

	OXIDISING SPECIES (µmoles formazan/g wet wt/20 min)
CONTROL	1.58±0.23
ASCORBATE DOSED	0.64±0.19[1]

(Results are expressed as mean±SD where n=5.)

Comparing ascorbate dosed with controls. [1]=$p<0.001$

Female Wistar rats (250g) dosed with 0.3cm^3 2% (w/v) sodium ascorbate (equivalent to 6mg). Rats were killed 24h after dosing. All animals were allowed free access to food and water.

results are due to the antioxidant properties of ascorbate.

Ascorbic acid is a powerful reducing agent, although at low concentations ascorbate may have oxidant properties as a result of its reaction with Fe^{2+} and O_2 to produce active oxygen species. At higher tissue concentrations ascorbate may raise tissue sulfhydryl levels and have a role in regenerating vitamin E (Lewin, 1976, Cadenas, 1985, Burton and Ingold, 1986). This cooperativity between antioxidants is reflected in the observed reduction of oxidising species after the administration of ascorbate.

18h starvation results in a significant increase in oxidising species in both the brain and the liver (Table A2). This increase in oxidising species arises as a result of the depletion of reduced glutathione levels which occurs on starvation (Tateishi *et al.*, 1974, Isaacs and Binkley, 1977, Brigelius, 1983). Cyclic AMP levels increase on starvation, this results in decreased reduced glutathione and catalase levels and a corresponding increase in hepatic peroxides which are directed into the glutathione peroxidase system resulting in increased levels of oxidised glutathione (Isaacs and Binkley,1977).

In conclusion the altered levels of tissue oxidising species arising as a result of dosing with ascorbate or starvation may be detected by NBT assay, this suggests that the assay may be used qualitatively to reflect changes in tissue oxidising species.

TABLE A2

Oxidising species detected by NBT assay after 18h starvation.

SAMPLE	FED	STARVED
	(µmoles formazan/g wet wt/20min)	
LIVER	1.58±0.23	2.06±0.39[1]
BRAIN	0.71±0.35	1.36±0.23[2]

(Results are expressed as mean±SD where n=5.)

Comparing fed with starved. [1]=$p<0.05$
[2]=$p<0.01$

Female Wistar rats (250g) starved for 18h on grids (1600h to 1000h).
Control rats were allowed free access to food and water.

APPENDIX TWO

EFFECT OF FOOD INTAKE ON LIVER FOLATE RETENTION

APPENDIX TWO.

A2 EFFECT OF FOOD INTAKE ON LIVER FOLATE RETENTIONS

It was observed that retention of radiolabelled folate in the livers of animals depended on the time of dosing. Rats dosed at 10.00h were observed to retain considerably more label than rats dosed at 18.00h. Rats are nocturnal and will be feeding at 18.00h, these results suggest that food intake affects liver folate retention.

	^{3}H	^{14}C
RATS DOSED AT 10.00	18.52±3.72	14.32±1.55
RATS DOSED AT 18.00	4.62±1.9	5.57±2.95

(Results are expressed as mean±SD where n=5)

Female Wistar rats (250g) dosed [2-^{14}C] and [3', 5', 7,9-^{3}H] folic acid (83µg/kg body wt)

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