FOLATE METABOLISM IN THE FEMALE WEANLING RAT

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A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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SUMMARY

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The metabolism of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid was studied in female weanling rats. Intact folates and folate catabolites were excreted in the urine. Folate poly-glutamates were found in the tissues. Rats treated with the oestrogen diethylstilbestrol and 17α-ethynyloestradiol exhibited marked changes in the metabolic handling of folic acid and folate catabolism was greatly increased compared to controls. Allopurinol treatment gave greater label retention in the gut, with a substantial increase in catabolism compared to normals. A dose response relationship was illustrated between allopurinol dose and folate catabolism. After lead acetate dosing there was little radioactivity in the urine and tissues over 72h and more radioactivity was retained in the faeces compared to normals. Excretion of intact folates was depressed, especially 5MeTHF and 10CHOTHF. A tenfold increase in both lead and folic acid dosage resulted in an even further decrease of radioactivity in the tissues and urine over 72h. Excretion in the faeces was further elevated. Ferrous sulphate administration resulted in increased catabolism. The retention of radioactivity in the liver, kidney and gut was greatly reduced.

A new method of folate analysis; Sephadex LH-20 was introduced.

In vitro superoxide anion formation was illustrated using an allopurinol/xanthine oxidase system.

Histological studies were employed to qualitatively and quantitatively illustrate the oxidative status in livers and brains of allopurinol and ferrous sulphate dosed rats.

Increased dose related formazan deposition was observed when livers of pretreated animals were incubated with nitroblue tetrazolium. Formazan deposition was reduced in pretreated animals also treated with the anti-oxidants vitamin E, mannitol or 4-hydroxy-methyl-4,6-ditertiary-butylphenol.

A possible route of folate catabolism is scission by a non-enzymic oxidation involving active oxygen species.

KEY WORDS

Folate metabolism, folate catabolism, superoxide anion, oxidation.

TO MY PARENTS

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ABBREVIATIONS:

DHF	-	Dihydrofolic acid
THF	-	Tetrahydrofolic acid
DHFR	-	Dihydrofolate Reductase
DHPR	-	Dihydropteridine Reductase
pAB-L-GLU	-	pAmino-benzoyl-L-glutamate
pAcAB-L-GLU	-	pAcetamidobenzoyl-L-glutamate
pAcAB	-	pAcetamidobenzoic acid

CHAPTER 1

INTRODUCTION

1.1 FOLIC ACID

Folic acid (figure 1a) is the parent molecule of a large number of compounds known as the folate coenzymes. These coenzymes are of key importance to all cells and are required as vitamins by mammals (reviewed by Blakley, 1969; Hoffbrand 1976; Turner, 1977; Rowe 1978; 1983 and Blakley and Benkovic 1984), because of their inability to synthesise de novo the basic molecular structure from simple precursors. Folates are derived from the reduction of and, addition to, folic acid. (Blakley 1969 and Cossins 1984).

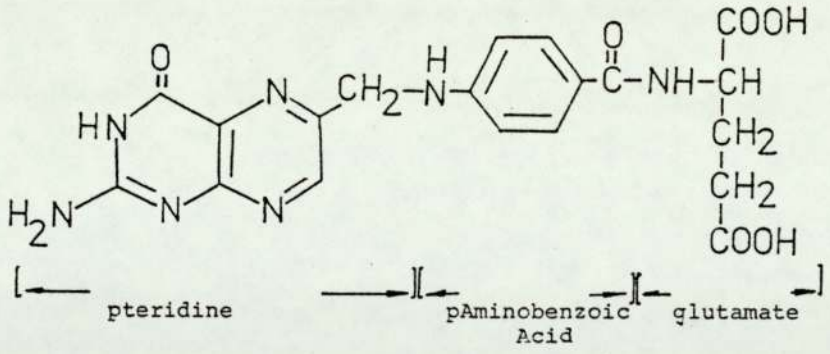


Figure 1a. Folic acid

1.2 FOLATE COENZYMES AND ONE-CARBON UNIT TRANSFER

The active coenzymes differ in three respects.The

pyrazine ring of the pterin moiety is reduced to either the 7,8-dihydro or 5,6,7,8-tetrahydro-level (figure 1b)

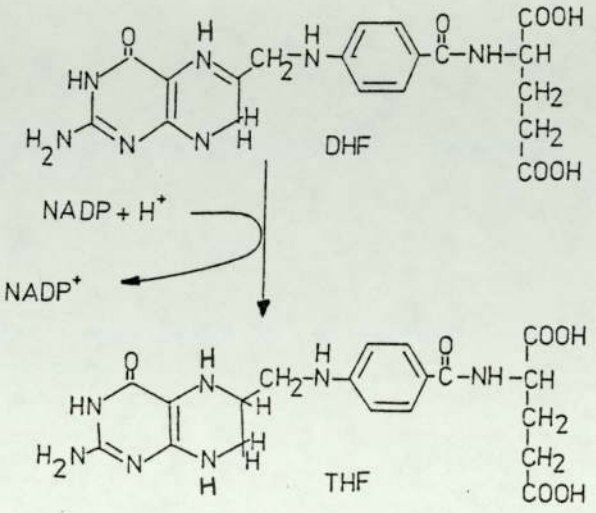


Figure 1b. Reduction of folic acid by DHFR

(Harper, 1975; Coleman and Herbert, 1980). A substiuent group may be present at N5 or N10 as in 5-methyltetrahydrofolate (figure 1c) or 10-formyltetrahydrofolate

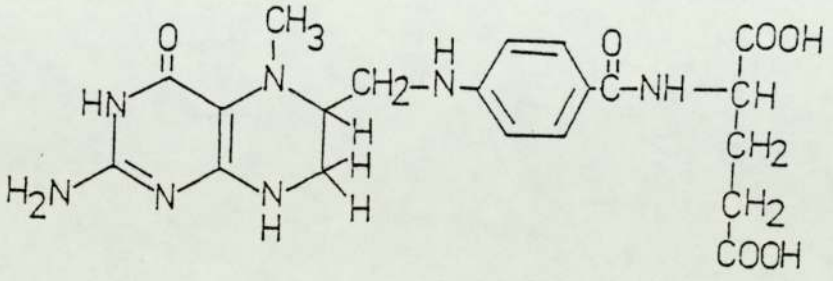


Figure 1c. 5-Methyltetrahydrofolic acid

(figure 1d), the major plasma and urinary folates (Ratanasthive, et al., 1974). Finally, addition of glutamate residues to the γ-carboxyl of the terminal glutamate forming folate polyglutamate (figure 1e);

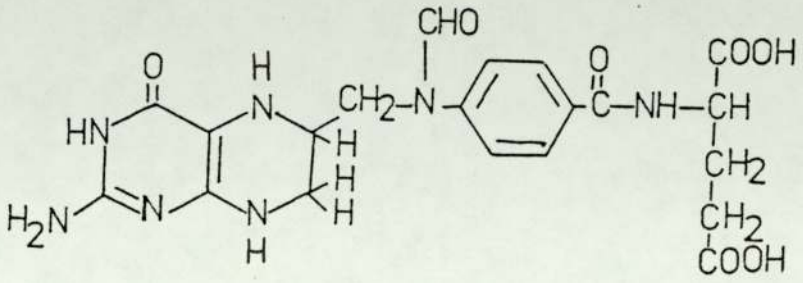


Figure 1d. 10-Formyltetrahydrofolic acid

these represent the major form of intracellular folate (Butterworth, et al., 1963; Corrocher, et al., 1972; Barford, et al., 1977 and Kesavan and Noronha, 1983). In vivo enzymes can utilise both the monoglutamate and polyglutamate forms, the latter being better substrates (Hoffbrand, 1976; Chanarin 1969 and 1979). Folate coenzymes are essential to a number of one-carbon unit transfer reactions in many areas of metabolism. A simplified scheme showing the major folate requiring reactions and interconversion routes in the mammal is illustrated in Scheme 1.

One way they fulfil this role is by participation in

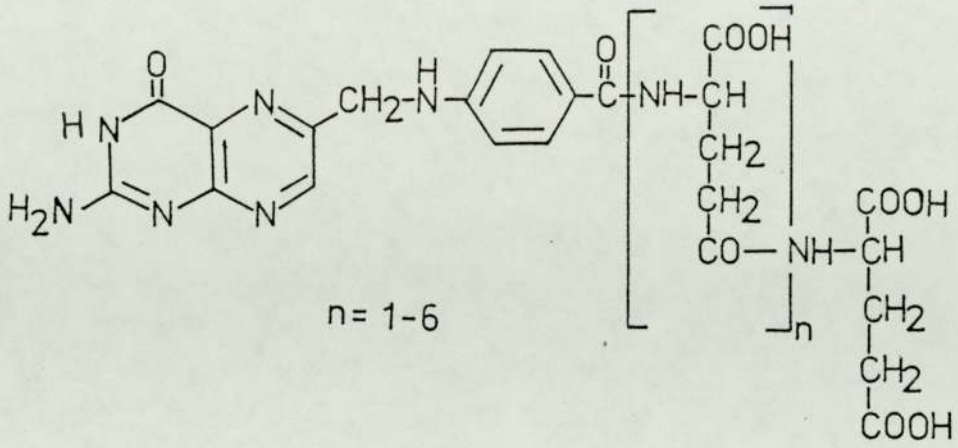


Figure 1e. Folate polyglutamate

three reactions in DNA synthesis; one in pyrimidine nucleotide biosynthesis employing 5,10-CH_2THF (figure 1f) in the transfer of a methylene (-CH_2-) group to

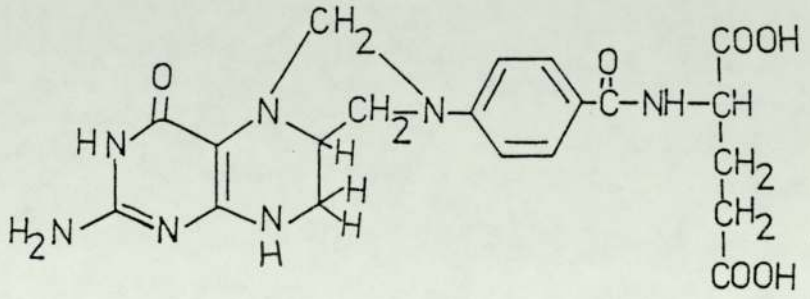


Figure 1f. 5,10-Methylenetetrahydrofolic acid

uridylate in the synthesis of thymidine and tetrahydrofolate (Hoffbrand, 1976; McGuire, et al., 1979), in Scheme 2.

Secondly, two reactions in de novo purine synthesis. Here, folate coenzymes donate carbon atoms 2 and 8 into the purine ring where 10-CHOTHF and 5-10CH = THF (figure

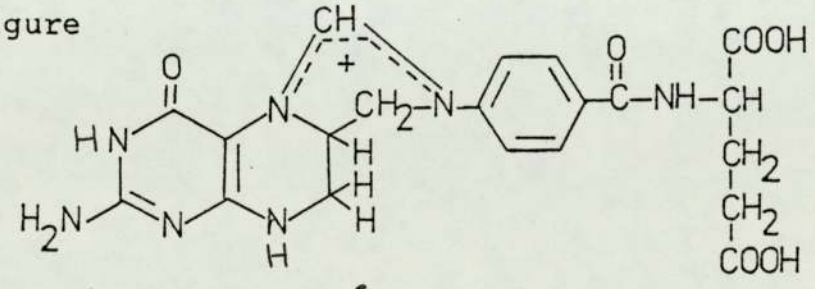
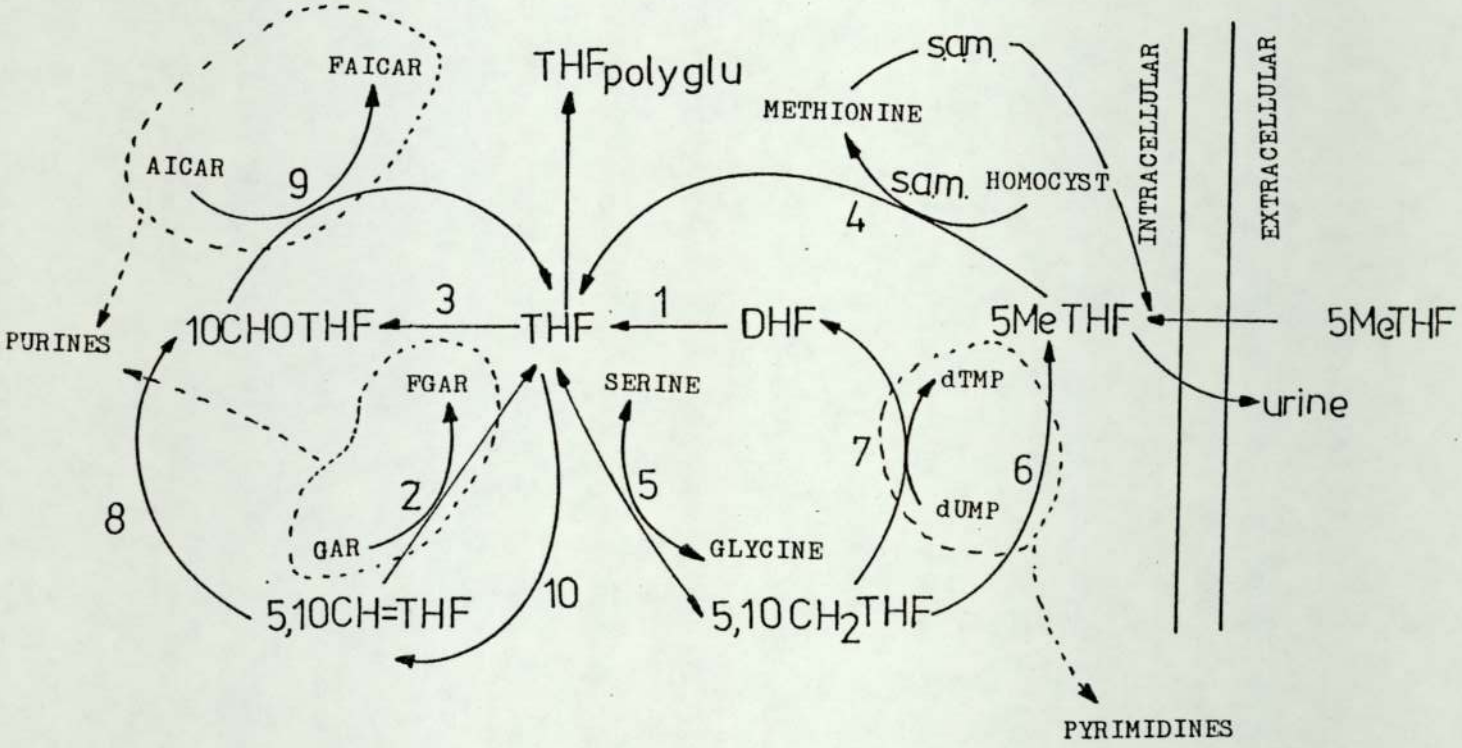


Figure 1g. 5,10-Methenyltetrahydrofolic acid

1g) appear to be the donors (scheme 1).



Scheme 1. Folate coenzyme interconversion and function.

ABBREVIATIONS (for scheme 1)

DHF = Dihydrofolate

THF = Tetrahydrofolate

THF polyglu = THF polyglutamate

5-MeTHF = 5-Methyltetrahydrofolate

10-CHOTHF = 10-Formyltetrahydrofolate

5,10,CH_2THF = 5,10-Methylenetetrahydrofolate

5,10CH = THF = 5,10-Methenyltetrahydrofolate

AlCAR = 5-Amino-4-Imidazole carboxamide ribonucleotide

FAICAR = 5-Formamido-4-imidazole carboxamide ribonucleotide

GAR = Glycinamide ribonucleotide

FGAR = Formyl glycinamide ribonucleotide

dUMP = Deoxyuridine monophosphate

dTMP = Deoxythymidine monophosphate

SAM = S.Adenosyl-methionine

ENZYMES IN SCHEME 1

1 Dihydrofolate reductase

2 CHO-transferase

3 10CHOTHF synthetase

4 Methionine synthetase

5 Serine transhydroxymethylase

6 Methylene tetrahydrofolate reductase

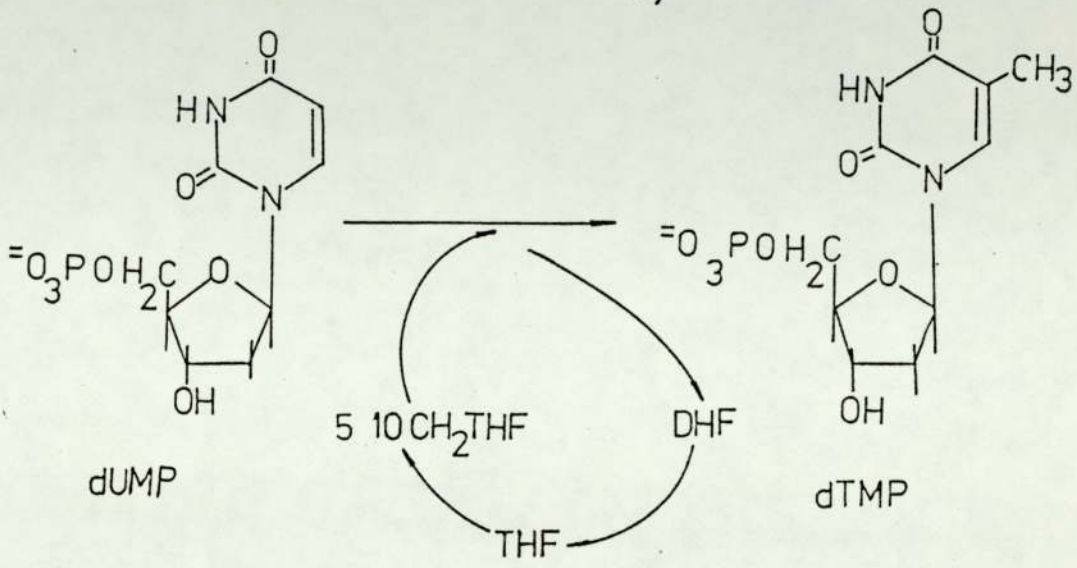
7 Thymidylate synthetase

8 Cyclohydrolase

9 Formyl THFA formyltransferase

10 Methenyltetrahydrofolate dehydrogenase

The exact mechanism is not clear (reviewed by Hartman and Buchanan, 1959; Devland Harvey, 1978a and b;



Scheme 2. The formation of thymidine

Mackenzie and Baugh, 1980 and Caperelli, et al., 1980). Folate coenzymes are concerned with the formation of methionine from homocysteine (Mangum and Scrimgeour, 1962; Taylor, et al., 1974). Serine and glycine interconversion also utilises folate coenzymes, whereby one α-carbon of serine is transferred to THF, forming 5,10CH_2-THF and glycine (Schirch, et al., 1977). About 71% of serine is catabolised via this mechanism (Kretchmar and Price, 1969).

Finally, folate coenzymes play a role in histidine catabolism, this is a major source of C-1 units for addition to THF. 5-Formino-THF (figure 1h) is formed by

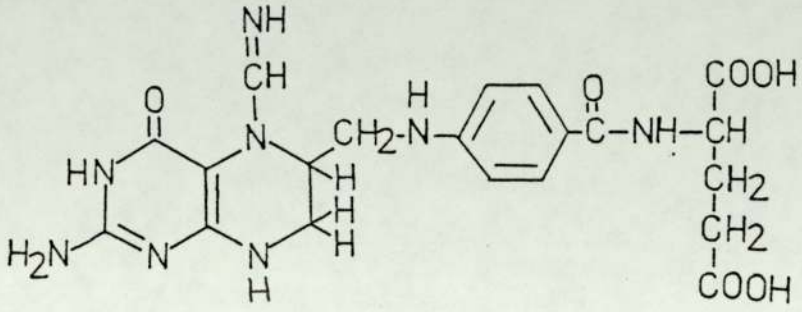


Figure 1h. 5-Forminotetrahydrofolic acid

the action of the enzyme formino-THF-forminotransferase (EC 2.1.2.5). 5-Formino-THF is unstable and is hydrolysed to 5,10-CH = THF. (Tabor and Rabinowitz, 1956). This is the normal route of histidine catabolism (Krebs, et al., 1976) and can be used to diagnose folate deficiency (Harper, 1975).

1.3 FOLATE OXIDATION

The oxidation of labile species such as the folate coenzymes may have neurological and haematological sequelae (reviewed by Castle, 1978; Green; 1977 and Chanarin, 1979) and has led to several investigations

of these compounds (Rowe , 1983; Coleman and Herbert, 1980; Pheasant et al., 1981; Cossins 1984 ; Guest, 1984 Al-Haddad 1984).

Due to the reactivity and such minute quantities (10^{-6}-10^{-8}M), these compounds are difficult to work with; their extreme susceptibility to light, temperature, pH, oxygen and endogenous conjugase results in partial or complete degradation (Chippel and Scrimgeour 1970; Maruyama, et al., 1978; Reed and Archer, 1980 and Cossins, 1984).

However, under anaerobic conditions, folic acid is stable to alkali, mild conditions of pH, and temperature(Blakley 1969 and Cossins 1984). 5-MeTHF oxidises to 5-methyl, 5,6-DHF (Rowe, 1978) and a pyrazino-s-triazine derivative (Jongejan, et al., 1979). 10CHOTHF is oxidised by oxygen to 10-formylfolate. Folic acid is oxidised aerobically to p-aminobenzoyl-L-glutamate (figure 1i) and pterin-6-carboxylic acid (Stokstad, et al., 1948) THF oxidises rapidly in air to form p-aminobenzoyl-L-glutamate, a number of pterins, folic acid, and DHF (Blakley, 1957; Zakrzewski 1966 a, b and Morar, 1985). DHF oxidises to yield folic acid, formaldehyde, p-aminobenzoyl-L-glutamate and dihydroxanthopterin. (Hillcoat, et al.,

1967: Morar, 1985 and Sahota 1985, personal communication).

FOLATE CATABOLISM

In 1949, following earlier chemical work (Bloom, et al., 1944 and Stokstad, et al., 1947) which had suggested that folate can be cleaved between C9 and N10., Lowry, et al., 1949, suggested that such cleavage proceeded as follows:

Folate ⟶ pterin-6-carboxyaldehyde ⟶ 6 carboxypterin ⟶ pterin.

And conversion of pterin (figure 1j) to isoxanthopterin

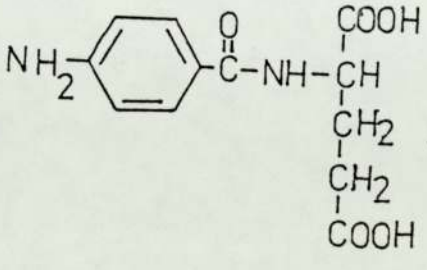


Figure 1i. pAminobenzoyl-L-glutamate

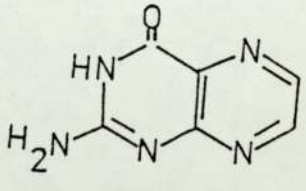


Figure 1j. Pterin

(figure 1k) in vivo by xanthine oxidase. Matsuda, (1950) detected folate in silkworm pupae by microbiological assay, and studies of Aruga and Yoshitake (1954) suggested that pteridines found in silkworm Larva arise by catabolism of folates. Blair, (1958), detected the presence of isoxanthopterin in urine. On the basis of these observations and the earlier suggestions (Jukes, et al., 1947) folate scission in vivo was proposed.

Initially, folate analysis was carried out by employing microbiological assay (Blakley 1969 and Cossins 1984). This assay could be affected by many factors and has been questioned (Rodriguez, 1978). This was followed by the introduction of radiolabelling of the folate

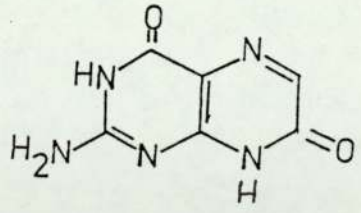


Figure 1k. Isoxanthopterin

•=^{3}H
×=^{14}C

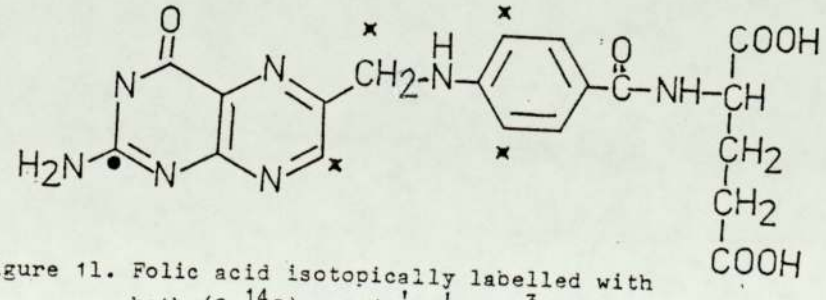


Figure 11. Folic acid isotopically labelled with both (2-^{14}C) and (3',5',7,9-^{3}H).

molecule (figure 1L), initially with tritium [^{3}H] and later with carbon-14 [^{14}C], therefore the fate of the pterin and p-aminobenzoyl-L-glutamate moieties could be followed in the body. Coupling this with sequential chromatography techniques such as thin layer chromatography (T.L.C) (Blair and Dransfield 1971); paper chromatography, (Connor, et al., 1979); Ion-exchange chromatography, (Barford, et al., 1978) and gel permeation chromatography, folates can be analysed. Although problems such as inadequate separation still occur, improvements are being made very rapidly. Recently Sephadex LH-20 chromatographic separation with organic solvents has been introduced. (Surdhar and Barford, 1984, unpublished observation),

These techniques coupled with the administration of radiolabelled folates to man, the rat, the guinea pig and the Syrian golden hamster has led to the identification of a series of catabolites in the urine (Reviewed by Krumdieck, et al., 1978; Murphy, et al., 1976 and 1978; Connor, et al., 1979; Pheasant, et al., 1981; Choolun, 1980; Kennelly, 1980; Saleh, et al., 1980 and 1982; Guest, 1984 and Al-Haddad, 1984. (Table1.1.1 illustrates the various catabolites detected).

These studies all suggest that folate breakdown *in vivo* could arise via cleavage of the C9-N10 bond. The cleavage yields p-aminobenzoyl-L-glutamate and a pterin fragment. Connor *et al.*, 1979 have shown the presence of acetylated derivatives of p-aminobenzoyl-L-glutamate

TABLE 1.1.1

CATABOLITES FOUND IN THE URINE FOLLOWING THE ADMINISTRATION OF FOLATE

Catabolite	Species	Identified by
Xanthopterin	Man	Koschara (1939)
Isoxanthopterin	Man	Blair (1958)
		Fukushima and Shiota(1972)
	Mouse	Knipe and McCormack (1977)
Pterin	Man	Floystrup, *et al.*, 1949
		Krumdieck, *et al.*, 1978
	Rat	Saleh, *et al.*, 1981
	Hamster	Al-Haddad (1984)
Acetylated diazotizable amines	Rat	Dinnings, *et al.*, 1957

p-Aminobenzoyl	Man	Johns, et al., 1961
- L-glutamate	Rat	Dinnings, et al., 1957
pAcABGlu	Man	Saleh, et al., 1980
	Rat	Murphy, et al., 1979
	Hamster	Al-Haddad (1984)
pAcAB	Man	Saleh, et al., 1980
	Rat	Connor, et al., 1979
	Guineapig	Choolun, et al., 1980
	Hamster	Al-Haddad (1984)
H_2O	Rat	Connor, et al., 1979
	Hamster	Al-Haddad (1984)
Urea	Rat	Connor, et al., 1979
	Hamster	Al-Haddad (1984)

and p-aminobenzoic acid in the urine of rats dosed with folic acid. Similar results have been obtained in the Syrian golden hamster (Al-Haddad, 1984). Krumdieck and co-workers in the late 1970's have demonstrated the presence of pterin in the urine of man as a result of catabolism of folic acid.

In adult rats Pheasant, et al., 1981 have shown that p-acetamidobenzoic acid (figure 1 m) is the major

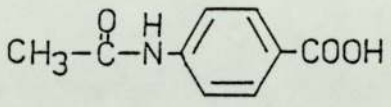


Figure 1m. pAcetamidobenzoic acid

catabolite in early urine samples but levels decrease with time whereas p-acetamidobenzoyl-L-glutamate (figure 1 n) levels

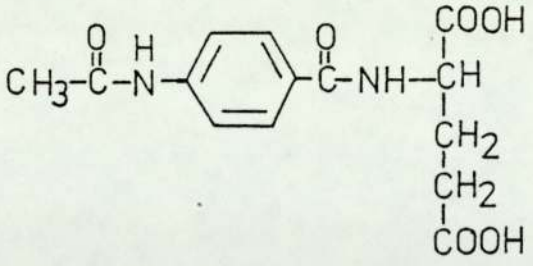
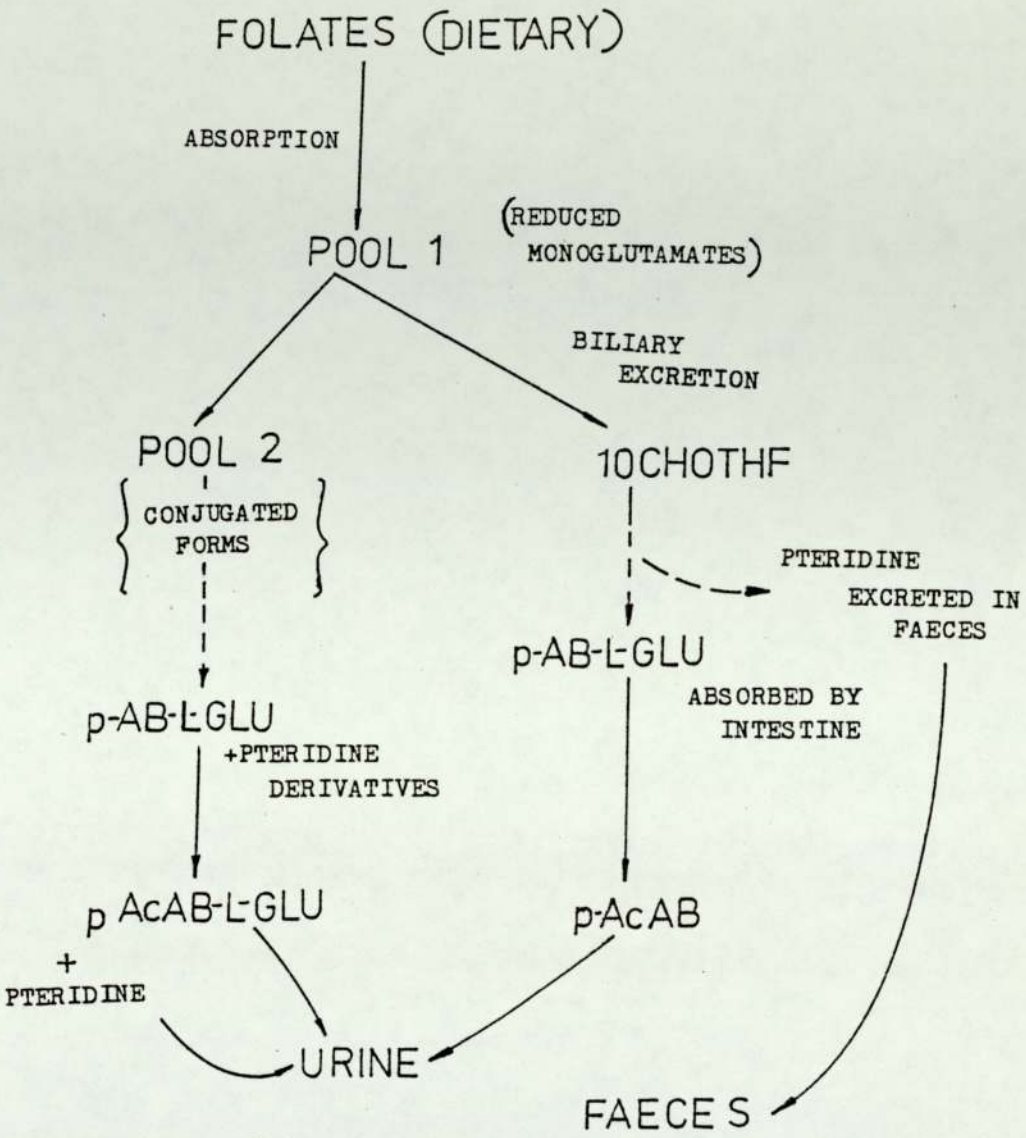


Figure 1n. pAcetamidobenzoyl-L-glutamic acid

increase with time. This has been explained as a pathway where catabolites formed within the body are derived from one of two distinct pools of folate coenzymes each having differenct biological half lives (Pheasant, et al., 1981): (Scheme 3 illustrates the



Scheme 3. Folate pools and their major catabolites.

two pools).

POOL 1 - Folates within this pool are derived from recent absorption, secreted in the bile and catabolised. These have a half life of approximately 1 day in the rat (Saleh et al., 1981) and 31.5 hours in man (Krumdieck, et al., 1978). The major catabolite of this pool is pAcAB.

POOL 2 - Folates within this pool constitute folate polyglutamates. With a half life of approximately 11 days in the rat (Saleh, et al., 1981) and 100 days in man (Krumdieck, et al., 1978). The major catabolite of this pool is pAcABGlu.

Folates found in the bile are largely derived from free (unconjugated) tissue and plasma folate (Hillman, et al., 1977) hence pAcAB is detected in the early urine samples.

1.5 FACTORS WHICH MAY ALTER FOLATE METABOLISM

The catabolism of folates in vivo is a normal phenomenon, the rate of this breakdown may vary with various factors (reviewed by Careye et al., 1968; Blakley, 1969; Hoffbrand and Peters, 1977; and Scott, 1984). Folate metabolism is affected by

dietary lack, malabsorption as a result of disease; such as tropical sprue and coeliac disease (reviewed by Anderson, et al., 1960; Klipstein, 1963; Freedmen, et al., 1973 and Halsted, et al., 1978); the lack of enzyme(s) responsible for folate coenzyme interconversion which leads to mental retardation particularly in children (Erbe, 1979); the lack of vitamin B_{12}, resulting in megaloblastic changes (Rosenberg et al., 1974) and non-specific central nervous system changes, (Herbert, 1972 and 1977); and chemotherapeutic agents which can significantly effect the folate status (reviewed by Maxwell, et al., 1972; Reynolds, et al., 1976 and 1980; Figuera, et al., 1980; Sharvon, et al., 1980; Trimble, et al., 1980 and Rowe, 1983).

1.6 METHOTREXATE AND THE ANTIFOLATES

The central role of folates in metabolism has made them targets for the antifolates. The most successful used in cancer chemotherapy is methotrexate (MTX) 4-amino-10-methyl folic acid (figure 1o). MTX is a

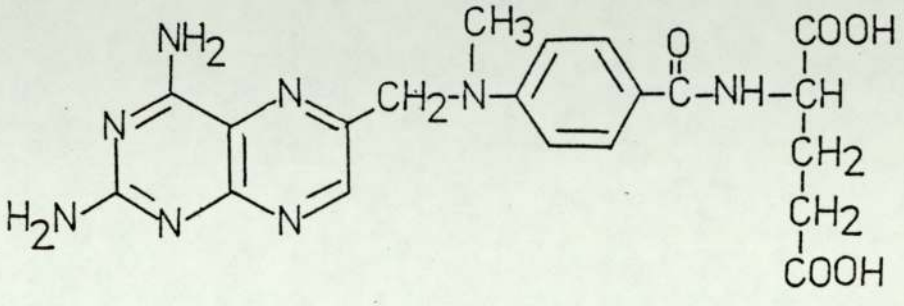


Figure 1O. Methotrexate

powerful inhibitor of DHFR (Bertino, et al., 1964; Hillcoat, et al., 1967; Waxman, et al., 1970; Johns and Bertino, 1973 and Chabner, 1975).

MTX causes a depletion of the cellular pool of folate coenzymes and hence a lack of purines, pyrimidines and ultimately cell death (Borsa and Whitmore, 1969; Craine, et al., 1972; Magnum, et al., 1979 and Saleh, et al., 1981 and 1982). MTX increases folate

catabolism (Barford, et al., 1980) and itself undergoes catabolism by the loss of glutamic acid residue yielding a pteroate residue (Valerino, et al., 1972 and Connor, 1979). The exact mechanism of breakdown is not clear but it could be as suggested by Pheasant, et al., 1981 or metabolism by the gut microflora (Valerino, 1972). Although an enzyme has not been reported in the mammal both the bacterial enzyme known as carboxypeptidase G1 (Albrect, et al., 1978) and the related protozoan (Crithidia) enzyme (Iwai, et al., 1979) will cleave folic acid and other folate derivatives. The biliary excretion of folate is extensive (Lavoie and Cooper, 1974; Hillman et al., 1977; Pheasant et al., 1981 and Guest 1984), hence catabolism by the gut bacteria could occur during enterophepatic circulation. Recent studies employing antibiotics (Pheasant, et al., 1983) suggest gut microflora contribute very little to folate catabolism.

1.7 RATIONALE FOR THE PRESENT STUDY

Recent studies (Saleh, 1981; Pheasant, et al., 1981; Guest, 1984; Al-Haddad, 1984 and Blair, et al., 1984) have extensively studied folate metabolism in health and malignancy. These studies suggest folate catabolism to be via an oxidative cleavage of the C9-N10 bond, possibly via superoxide as there are many biological sources in vivo (Hill, 1981).

This research was directed at the elucidation of a mechanism of folate catabolism in the female weanling rat using certain xenobiotics (diethylstilboestrol, (D.E.S)

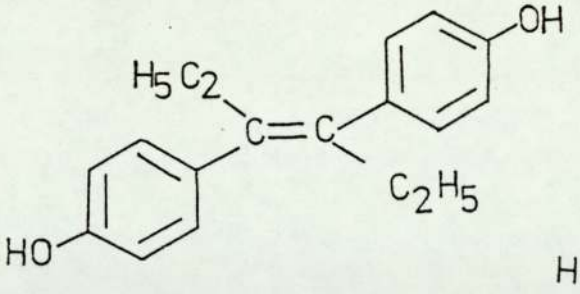


Figure 1p. Diethylstilboestrol

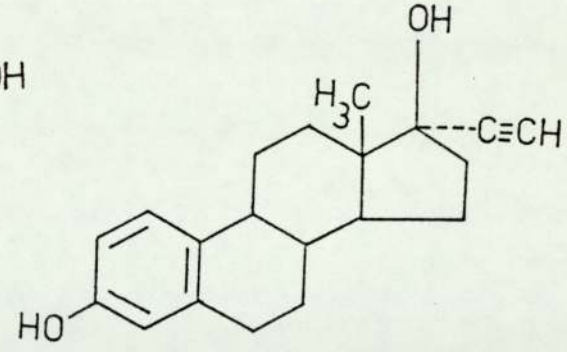


Figure 1q. 17∝-ethynyloestradiol

figure 1p, 17∝-ethynyloestradiol (figure 1q); lead acetate; ferrous sulphate and allopurinol) known to

interfere with folate catabolism at present in an unknown way. These xenobiotics either induce increased cellular proliferation (D.E.S, 17 ∝-ethynyloestradiol) or generate superoxide *in vivo* (allopurinol and ferrous sulphate) Gutteridge, 1984; and Surdhar, *et al.*, 1986 (submitted for publication). The oxidative status in the female weanling rat was investigated using nitro-blue

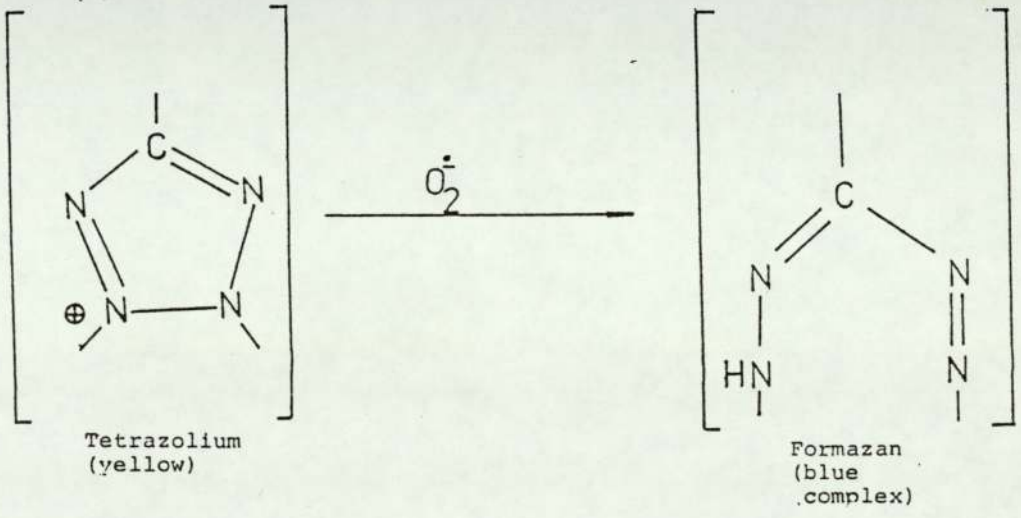


Figure 1r. The conversion of tetrazolium into formazan

tetrazolium (NBT) as proposed by Baehner and Nathan, 1968; Nishikimi, *et al.*, 1972; Auclair, *et al.*, 1978 and Picker and Fridovich, 1984. (Figure 1.r) and compared with the qualitative and quantitative aspects of folate catabolism.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

The following materials were obtained commercially as described; folic acid and pterin from Koch-Light Laboratories Limited [Colnbrook, Bucks, UK]; p-amino-benzoyl-L-glutamic acid, dithiothreitol, diethyl-stilboestrol, 17α-ethynyloestradiol, allopurinol, oxipurinol, vitamin E, xanthine, mannitol and xanthine oxidase from Sigma Chemical Co Limited, (London, UK); p-aminobenzoic acid and p-acetamidobenzoic acid from the Aldrich Chemical Co Limited (Wembley, Middlesex, UK); 5-methyl-tetrahydrofolic acid from Eprova Research Laboratories, (Basle, Switzerland); [2-^{14}C]-folic acid (specific activity 58.2 μCi/mMol) (96% radiochemically pure) and [3',5',7,9-^{3}H]-folic acid (specific activity 500 μCi/mMol) (95% radiochemically pure and Iron-59 (^{59}Fe) (specific activity 3-20 μCi/mg Iron), as iron sulphate (100% radio-chemically pure) from the radiochemical Centre (Amersham, Bucks, UK). Other standard pteridines were obtained from the following organisations; 5-formyl-THF from Lederle Laboratories Division Cynamid of Great Britain Limited (London); 6-methyl pterin from Roche Products Limited (Welwyn Garden City, UK). Pterin-6-carboxylic acid (Zakrewski, et al., 1970); p-acetamido-benzoyl-L-glutamate (Baker, et al., 1964) and 10-formyl-folate (Blakley, 1969) were prepared by Dr R N Mazhir in this

Laboratory.

4-hydroxy-methyl 2.6. ditertiary butyl-phenol was a gift from Dr Al-Malaika, University of Aston in Birmingham. Other chemicals are laboratory reagents and of Analar grade.

CHROMATOGRAPHY

2.2.1 ION-EXCHANGE CHROMATOGRAPHY

Diethylaminoethyl cellulose DE 52, (Whatman Limited, Maidstone, Kent UK) 80-100g was washed with distilled water and equilibrated in 0.05M sodium phosphate buffer, pH 7.0, containing dithiothreitol (D.T.T., 5 mg % w/v) until the washings were of constant ionic strength and pH 7.0. After decanting off the fines and degassing under reduced pressure the prepared DE 52 was packed into glass columns (University of Aston, Glass Blowing Department)., 2 cm X 50 cm plugged with glass wool. Samples (5-30 mls) and appropriate standards were diluted to the conducting value of the starting buffer (0.05M sodium phosphate buffer) with distilled water before loading onto columns. Standard linear gradients (0-1.2M NaCl in starting buffer) were eluted automatically using an LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon,

Surrey, UK). The eluent was passed through a UV monitor., λ= 254 nm. (LKB Uvicord II, LKB Instruments). 5 ml fractions were collected using an LKB ultra rac fraction collector (LKB Instruments). The gradient, eluted over eight hours, was determined by measuring the conductivity of every tenth fraction with a Mullard conductivity cell. Aliquots (0.5 ml) of each fraction were counted as described below. All fractions were stored at -20°C in the presence of sodium ascorbate until analysis was complete. A summary of the elution pattern of relavent folate derivatives used is given in table 2.1.1.

ii **SEPHADEX G-15 GEL FILTRATION**

Sephadex G-15 (Pharmacia, Uppsala, Sweden) was placed in excess 0.05M sodium phosphate buffer, pH 7.0, containing D.T.T. (5mg % w/v) and left to swell overnight at room temperature. After degassing under reduced pressure the slurry was poured into perspex columns; 2 x 60 cm (Wright Scientific Limited, Surrey, UK) and allowed to pack under pressure. After loading samples and standards (5-25ml), elution was achieved using 0.05M sodium phosphate buffer, pH 7.0. The eluent was monitored and collected as above. A summary of the elution pattern of relevant folate derivatives used is given in Table 2.1.1.

Folate Compound	DE 52 NaCl molarity(M)	G-15 Fraction No.	LH-20 Fraction No.
Folic acid	0.96	37	56
10CHO-folic acid	0.53	21	39
10CHOTHF	0.45	18	-
5CHOTHF	0.59	28	37
5MeTHF	0.67	37	35
Folate X	0.84	36	-
Pterin	0.30	35	95
$6CH_3$pterin	-	44	102
pterin-6-CHO	0.70	43	84
pterin-6-COOH	0.60	30	78
pNH_2.Benzoic acid	0.40	35	77
pAc.AB	0.43	36	78
pNH_2.B.Glu	0.40	18	48
pAcABGlu	0.43	19	48
pNH_2hippuric acid	-	33	69
$3H_2O$	0.0	21	81
Urea	0.0	21	-
Metabolite Y	0.84	34	-
Folate polyglutamate(B)	0.60	11	64
Metabolite A1	-	-	38-44
Metabolite A2	-	-	48-51
Metabolite C	-	-	76
Metabolite C1	-	-	72
Metabolite D	-	-	105
Metabolite E	-	-	98
Metabolite F	-	-	102

TABLE 2.1.1 The elution properties of folates and metabolites on DE 52 ion exchange, sephadex G-15 gel filtration and sephadex LH-20 chromatography.

iii SEPHADEX LH-20

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was placed in excess 20% w/v methanol containing 0.05M - sodium phosphate buffer pH 7.0 with 5 mg % w/v D.T.T. and left to swell overnight at room temperature. After degassing under reduced pressure the slurry was poured into perspex columns (2 x 60 cm; Wright Scientific Limited, Surrey, UK) and allowed to pack under pressure. After loading sample and standards (5-25 ml) elution was achieved using 20% w/v methanol containing 500 µM - sodium phosphate buffer pH 7.0 and D.T.T. (5 mg % w/v). The eluent was monitored and collected as above. A summary of the elution pattern of relevant folate derivatives used is given in table 2.1.1

iv PAPER CHROMATOGRAPHY

Decending paper chromatography was performed in glass tanks equilibrated with two different solvent systems (table 2.2.1) using Whatman 3MM chromatography paper and developed in the dark for 6 hours. Samples and standards (10CHO-folate, pABGlu, pABA and their acetyl derivatives) were applied as spots using glass micro-pipettes. Where multiple applications were required samples were dried in situ with a stream of cold

air. Standards were observed as dark absorbing or fluorescing spots by viewing under UV light at 254 nm or 355 nm. The chromatograms were cut into 1 cm^2 strips and counted. Table 2.2.1 shows the chromatographic behaviour of standard compounds.

2.2.3. ANIMALS

All experiments were conducted on female weanling Wistar grade IV* animals. Both prior to and during experimentation, animals were kept at 21°C in a sealed room having a fixed 12 hours dark and 12 hours light cycle and allowed free acess to food (Pilsburys Breeding diet, Pilsbury's Limited UK) and water. Rats were dosed orally by stomach intubation using specially prepared steel dosing needles or by intraperitoneal injection. Administered folates were dissolved in 0.05 M sodium phosphate buffer, pH 7.0 containing sodium ascorbate (2% w/v). The volumes administered did not exceed 0.35 ml. Metabolism studies were carried out by housing single rats in cages designed for the separate collection of faeces and urine (Jencons metabowls; Jencons (Scientific) Limited; Hemel Hempstead, Herts, UK). Urine samples were collected into flasks containing 10 ml 0.05M sodium phosphate buffer pH 7.0, containing sodium ascorbate (2% w/v). To prevent light degradation the

Compound	Rf values	
	Solvent A	Solvent B
p-aminobenzoic acid	0.39	0.21
p-acetamidobenzoic acid	0.54	0.35
p-aminobenzoyl-L-Glutamate	0.22	0.05
p-acetamidobenzoyl-L-glutamate	0.29	0.09
p-aminohippurate	0.40	0.26
10-formylfolic acid	0.18	0.02

Solvent A = propanol/aq. NH_3 sp.gr. 0.88/water
(200 : 1 : 99 by vol.)

Solvent B = Butanol/ethanol/aq. NH_3 sp.gr. 0.88/water
(10 : 10 : 1 : 4 by vol.)

TABLE 2.2.1 The chromatographic behaviour of p-aminobenzoic acid derivatives and 10-formylfolic acid on Whatman 3MM paper

flasks were wrapped in aluminium foil. Rats were killed by cervical dislocation followed by surgical opening of the thorax and tissues removed for direct determination of radioactivity or for quantitative examination of retained radioactivity.

* Grade IV - refers to the quality of the animals, these animals are the next best to those raised directly from the womb (microorganism free), (Banting and Kingman Limited UK).

2.2.4 MEASUREMENT OF RADIOACTIVITY

β-counting: Prepared samples were counted in a Beckman LS 7500 liquid scintillation counter (Beckman Instruments Inc; Scientific Instruments Division, Insine California 92173). Aqueous samples, eg column eluants and urine samples were made upto 1 ml with water and 10 mls of Beckman ready-solve "E.P" scintillation cocktail added. 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. Tissue radioactivity was measured after solubilisation

(see page 54).

γ-counting: Prepared samples were counted directly in a LKB 1282 Compugamma counter (LKB Limited, London

UK). Samples were counted for 60 seconds or 10,000 counts. Appropriate corrections were made for background and radioactive decay for [^{59}Fe].

2.2.5 SOLUBILISATION

This was carried out by the method of Wunderly and Mooney (1981). Tissue samples, homogenised or minced (liver, kidney and the gastrointestinal tract) (100 mg) were placed in a glass scintillation vial and 1.0 ml of Beckmans tissue solubiliser (BTS - 450, P/N 580691). The samples were incubated in a 40°C water bath until thoroughly digested, 0.5 ml of 30% H_2O_2 was added as a decolourising agent and allowed to stand for several hours, (this step may be deleted if little colouration is evident, as this induces chemiluminescence). Samples were counted in scintillation cocktail containing glacial acetic acid (7 ml/litre) (to degrade the chemiluminescence which might be generated by tissue solubilisers).

Freeze-dried, homogenised faeces (20 mg) were put into glass scintillation vials with 0.1 ml distilled water and allowed to stand for 30 minutes, 1 ml of BTS - 450 solubiliser was added. Following incubation at 40°C for 2 hrs, 0.5 ml of isopropanol and 0.5 ml of 30% H_2O_2 were added. After 10 minutes the samples were

again incubated at 40°C for 2 hrs and diluted with 5 ml distilled water. Samples were counted as above. Prior to counting the samples were dark adapted overnight.

2.2.6 OXIDATIVE ACTIVITY ANALYSIS

After the administration of appropriate agents, animals and tissues were removed as described in section 6. Liver sections or homogenates (for histology) were covered in 0.5 ml of 0.1% w/v nitroblue tetrazolium (NBT) in Hanks balanced salt solution (Table 2.3.1) incubations were carried out for 20 minutes at 37°C in a standard water bath. Then either a qualitative or quantitative procedure was observed as described in section 6. A similar procedure was observed for brain samples.

HANKS BALANCED SALT SOLUTION (MODIFIED)

NaCl	80 g/l
KCl	4 g/l
Glucose	10 g/l
KH_2PO_4	600 g/l
Na_2HPO_4	475 mg/l

Table 2.3.1 Hanks balanced salt solution.

2.3.1 IN VITRO ILLUSTRATION OF SUPEROXIDE ANION PRODUCTION IN THE XANTHINE AND/OR ALLOPURINOL/XANTHINE OXIDASE SYSTEM

This assay was carried out according to the method of McCord and Fridovich (1969). All assays were carried out in potassium phosphate buffer 0.05M pH 7, containing 0.1 mM disodium EDTA, at 25°C. The reduction of cytochrome C (10^{-6}M) at 550 nM was observed over initial 30s of reaction.

(Cytochrome C ϵ_{550} = 22.7 mol^{-1} cm^{-2} pH 7 Lehninger 1978).

Systems containing either xanthine (0.1 mM), allopurinol (0.1 mM), oxipurinol or a mixture (as described in table 2.3) were used. Superoxide dismutase (S.O.D) 200 mg/ml was used for inhibition studies.

In in vitro the xanthine oxidase system with xanthine and allopurinol, superoxide anions are produced (cytochrome C was reduced as shown in table 2.4.1). Oxipurinol exhibited no reaction with the enzyme. Allopurinol inhibits xanthine oxidase and it also acts as a substrate resulting in the production of oxipurinol (chapter 5). Oxipurinol only inhibits

[Substrate] μm	μM/min at 550 nm
Xanthine 35	0.051 ± 0.002
allopurinol 35	0.020 ± 0.003
oxipurinol 35	0 -
oxipurinol 70	0 -
Xanthine 35 + oxipurinol 10	0.0033 ± 0.001
Xanthine 35 + allopurinol 10	0.038 ± 0.002
Xanthine 35 + allopurinol 35	0.025 ± 0.003
allopurinol 35 + oxipurinol 10	0.014 ± 0.002
Xanthine 35 + 200 μg/ml S.O.D	0.002 ± 0.001
allopurinol 0.35 + 200 μg/ml S.O.D	0.001 ± 0.001

(n = 5 ± S.E.M. where appropriate)

TABLE 2.4.1 The illustration of Superoxide anion production in the Xanthine/Xanthine oxidase and Allopurinol/Xanthine oxidase systems

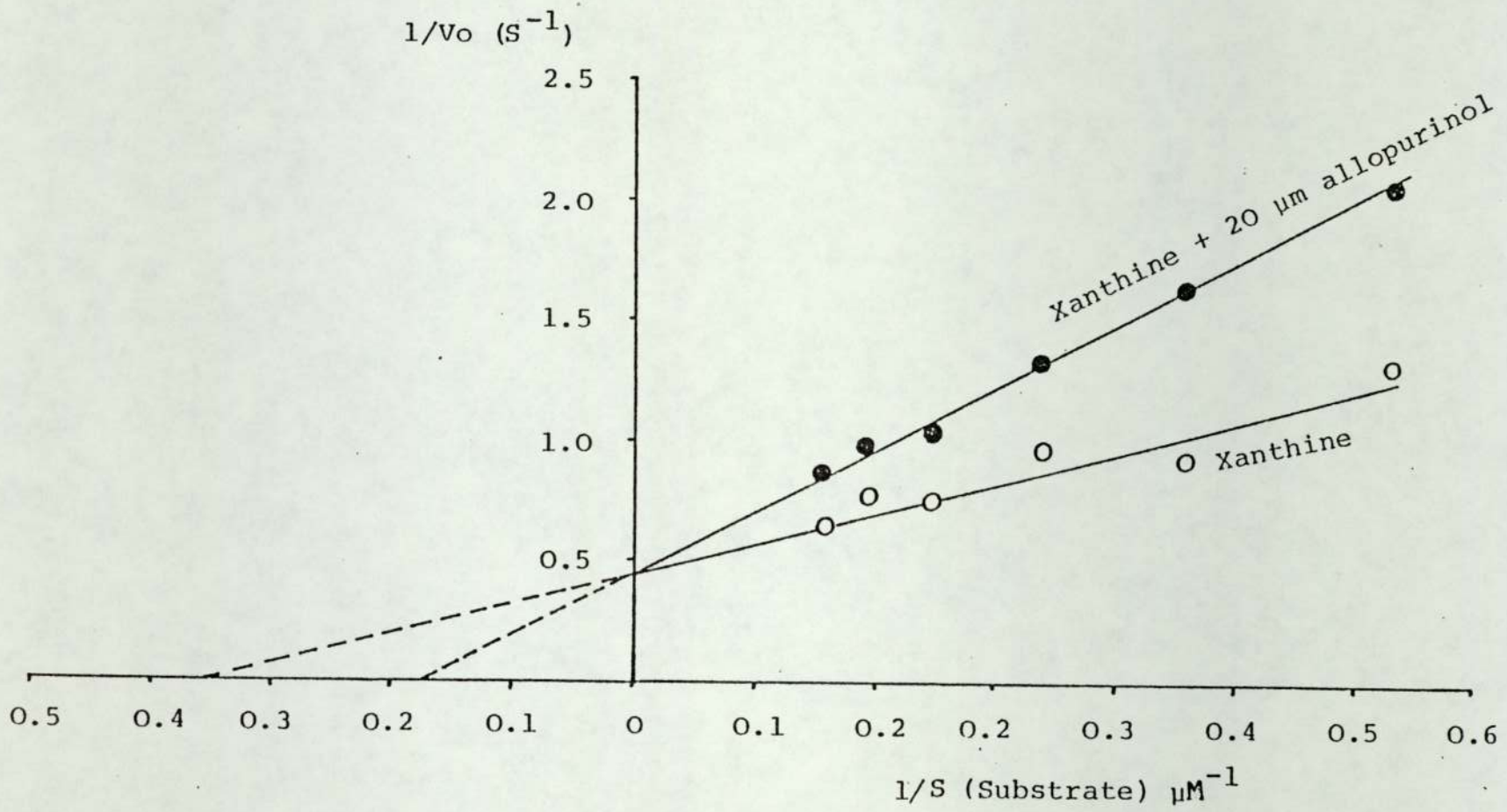


Figure 2.3.1

Lineweaver - Burk plot: Xanthine oxidase activity.

xanthine oxidase.

K_m Xanthine/xanthine oxidase = 2.56 μm

K_m Allopurinol/xanthine oxidase = 4.50 μm

K_i Xanthine/allopurinol = 5.26 μm

2.4.1 STATISTICAL ANALYSIS

Throughout the text in all experiments, all tests of statistical significance were performed using Students "t" test and Wilcoxon's sum of ranks test unless otherwise stated in the text.

CHAPTER 3

FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

EXPERIMENTAL

This chapter describes the metabolic fate of folic acid in the female weanling rat. The normal pattern of folate metabolism and catabolism was determined in healthy female weanling rats dosed orally with a mixture of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid and compared to that found in similarly dosed rats with diethylstilboestrol (D.E.S) and 17α-ethynyloestradiol compounds which are claimed to cause folate deficiency (Lakshmaiah and Bamji; 1979 and Eggar, et al., 1983).

3.1.1 FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

The normal metabolism and incorporation of an oral dose of radiolabelled folic acid into the reduced folate coenzyme pool has been described (see chapter 1 for references). The mechanisms of transfer and breakdown of folates remain controversial and need to be fully elucidated. The catabolic phenomenon (Murphy, et al., 1978., Pheasant, et al., 1981; Guest 1984 and Al-Haddad 1984) is investigated here with a description of the metabolic fate of oral [3',5',7,9-^{3}H] and [2-^{14}C] folic acid in the normal female weanling rat upto 72 hours after dosing.

MATERIALS AND METHODS

Fifteen female Wistar grade IV weanling rats (50-100 g body wt) were dosed orally with a mixture of [3',5',7,9-^{3}H] (2.0 µCi) and [2-^{14}C] (1.0 µCi) folic acid (100 µg/kg body wt) and 0.3 ml corn oil. Urine and faeces were collected from 3 groups of 5 animals; 0-24h after dosing for the first group, 24-48h for the second group and 48-72h for the third as described in chapter 2. At the end of each time period the rats were killed by cervical dislocation, the collection flasks were changed after collecting from each group. Liver, kidneys and the gut samples were removed for the determination of retained radioactivity. The major portion of the livers were quickly removed, washed in ice cold buffer and extracted as follows:

(a) Chopped into small pieces, dropped into 4 vols of boiling 0.05M sodium phosphate buffer pH 7.0 containing sodium ascorbate (2% w/v) and maintained at 100°C for 5 minutes, cooled, homogenised and centrifuged. The supernatant was examined by Sephadex G-15 gel filtration.

All samples were stored at -20°C in the presence of the antioxidant sodium ascorbate (2% w/v) until analysed by DE-52 ion exchange, Sephadex G-15 gel

filtration or liquid scintillation counting.

RESULTS

Table 3.1.1 illustrates the recovery of radioactivity in the urine, faeces and the distribution of retained radioactivity in the tissues. The majority of excreted radioactivity appeared in the 0-24h urine and faeces, later excretion levels being far lower. For each time period, quantitative analysis of the urine and faeces revealed an imbalance in the excretion of the two isotopes, as more [^{3}H] than [^{14}C] was excreted in the urine and more [^{14}C] than [^{3}H] was found in the faeces. The differences in the recovery of [^{3}H] and [^{14}C] in the urine are not statistically significant over 0-72h. The differences in the recovery of [^{14}C] and [^{3}H] in the faeces were statistically significant ($P<0.05$) over 0-72h.

However similar levels of ^{3}H and ^{14}C were found in the kidneys and gastrointestinal tract samples. The slight elevation of ^{14}C levels seen in the livers was not statistically significant.

URINARY METABOLITES

Table 3.1.2 summarise the metabolites found in the

Tissue Sample	0-24 h ^{3}H	0-24 h ^{14}C	24-48 h ^{3}H	24-48 h ^{14}C	48-72 h ^{3}H	48-72 h ^{14}C	0-72 h ^{3}H	0-72 h ^{14}C
Urine	39.51 (1.63)	36.77 (1.78)	5.75 (0.90)	4.30 (0.52)	2.50 (0.31)	2.21 (0.38)	47.76	43.28
Faeces	7.21 (0.80)	22.7 (1.20)	0.98 (0.66)	4.10 (0.38)	0.35 (1.60)	2.40 (0.25)	8.54	29.20
Liver	16.85 (0.53)	18.96 (0.20)	16.98 (0.32)	20.21 (0.32)	21.22 (1.60)	20.40 (0.42)	21.22	20.40
Kidney	2.00 (0.40)	2.65 (0.35)	2.22 (0.60)	2.55 (0.42)	2.00 (1.80)	2.01 (1.50)	2.00	2.01
Gut	5.60 (0.30)	5.40 (0.20)	6.21 (0.50)	6.00 (0.42)	2.30 (1.50)	3.21 (0.20)	2.30	3.21
TOTAL							81.82	98.10

TABLE 3.1.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 24 h, 48 h and 72 h after the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid (100 µg/Kg body wt.). Data are expressed as a percentage of the dose administered (± S.E.M.) n = 5.

Metabolites	0-24 h		24-48 h		48-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	3.13	3.01	0.18	0.12	0.08	0.065
5MeTHF	17.82	16.50	1.36	1.29	0.48	0.39
10CHO-Folate	15.25	16.31	2.38	3.01	0.41	0.40
Metabolite X	ND	ND	ND	ND	ND	ND
Metabolite Y	1.0	1.0	0.1	0.09	TRACE	
pAcABGlu	5.31	-	0.51	-	0.12	-
pAcAB	3.21	-	0.52	-	0.25	-
^{14}C only labelled Catabolite	-	3.80	-	0.96	-	0.09
Urea	-	0.6	-	0.1		0.1
$^{3}H_2O$	0.3	-	0.01	-	ND	-

TABLE 3.1.2 Metabolites present in the urine of healthy female weanling rats (50-100 g body wt.) 24 h, 48 h and 72 h after the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid (100 µg/Kg body wt.). Results are expressed as a percentage of the dose administered. n = 5 ND = not detectable

urine samples over 72h. The first 24h samples contain predominately intact folates with low levels of the single labelled scission products. However over the later 48h the levels of intact folates fall sharply.

DE-52 ion exchange chromatography of urine samples is illustrated in figures 3.1.1., 3.1.2 and 3.1.3. The 0-24h urine samples contained a mixture of labelled metabolites, four of which retained both the ^{3}H and ^{14}C labels, had almost identical isotope ratios and three were identified as (iv) 10-formylfolate (10CHOfolate), (v) 5 methyltetrahydrofolate (5MeTHF) and (vii) folic acid. Peak (vi) had the ^{3}H : ^{14}C ratio of an intact folate and was designated metabolite Y. The ratio of ^{3}H : ^{14}C in the folate derivatives was higher than in the folic acid administered.

The other radioactive peaks (i), (ii) and (iii) had different ^{3}H : ^{14}C ratios and appeared to be scission products. Sephadex gel filtration of peak (iii) resolved the radioactivity into 3 components (figure 3.1.4) each with very different ^{3}H : ^{14}C ratios. The first component, peak (a) eluted at Fraction 19 had a high ^{3}H : ^{14}C ratio and was a mixture of 10 formylfolate, present due to the overlap of peak (iv)

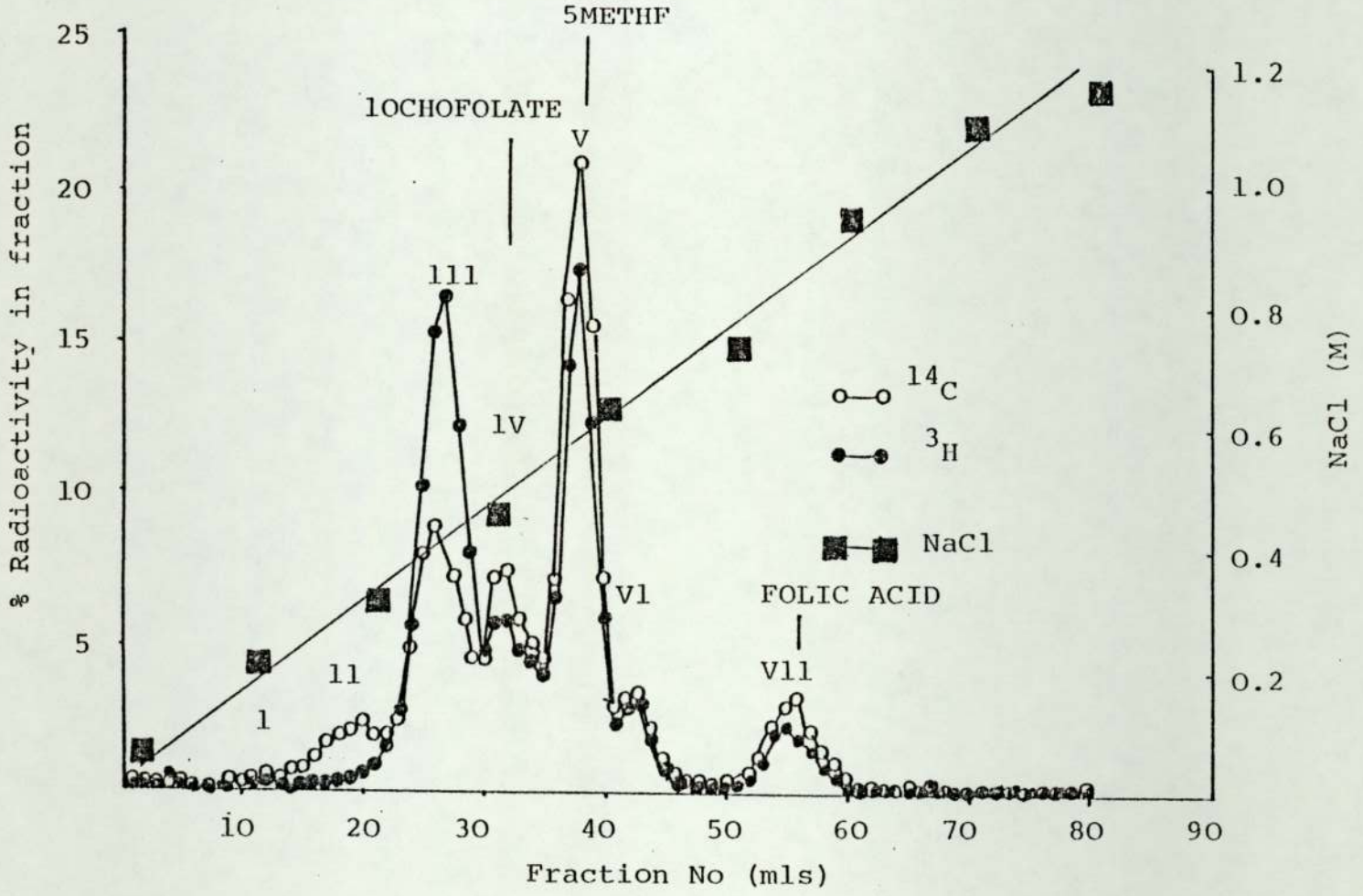


Figure 3.1.1.

DE-52 Chromatography of female weanling rat urine samples collected 0-24h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid.

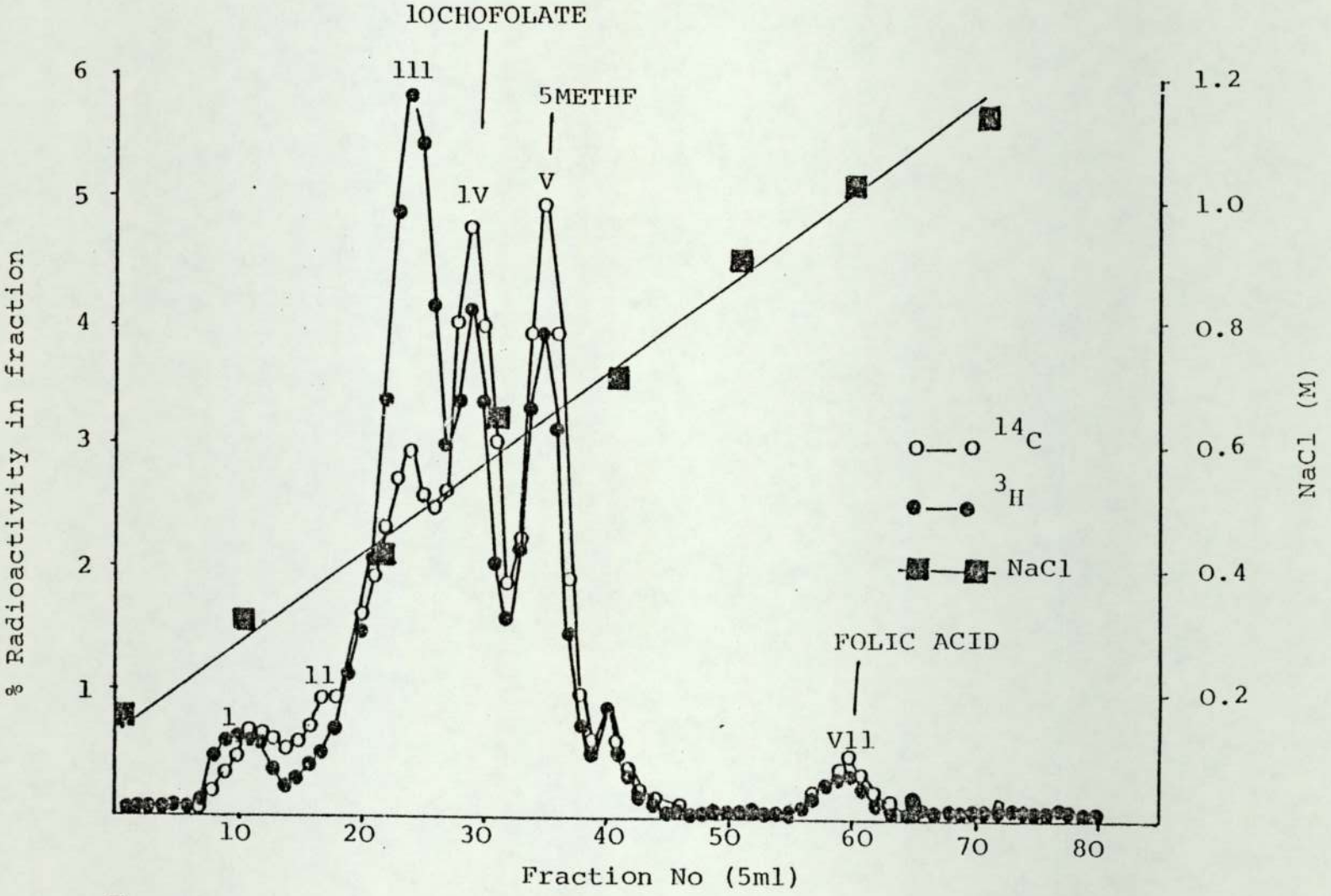


Figure 3.1.2 DE52

Chromatography of female weanling rats urine samples collected 24-48 hours after administration of a mixture of (2-C^{14}) and (3',5',7,9-H^3) folic acid.

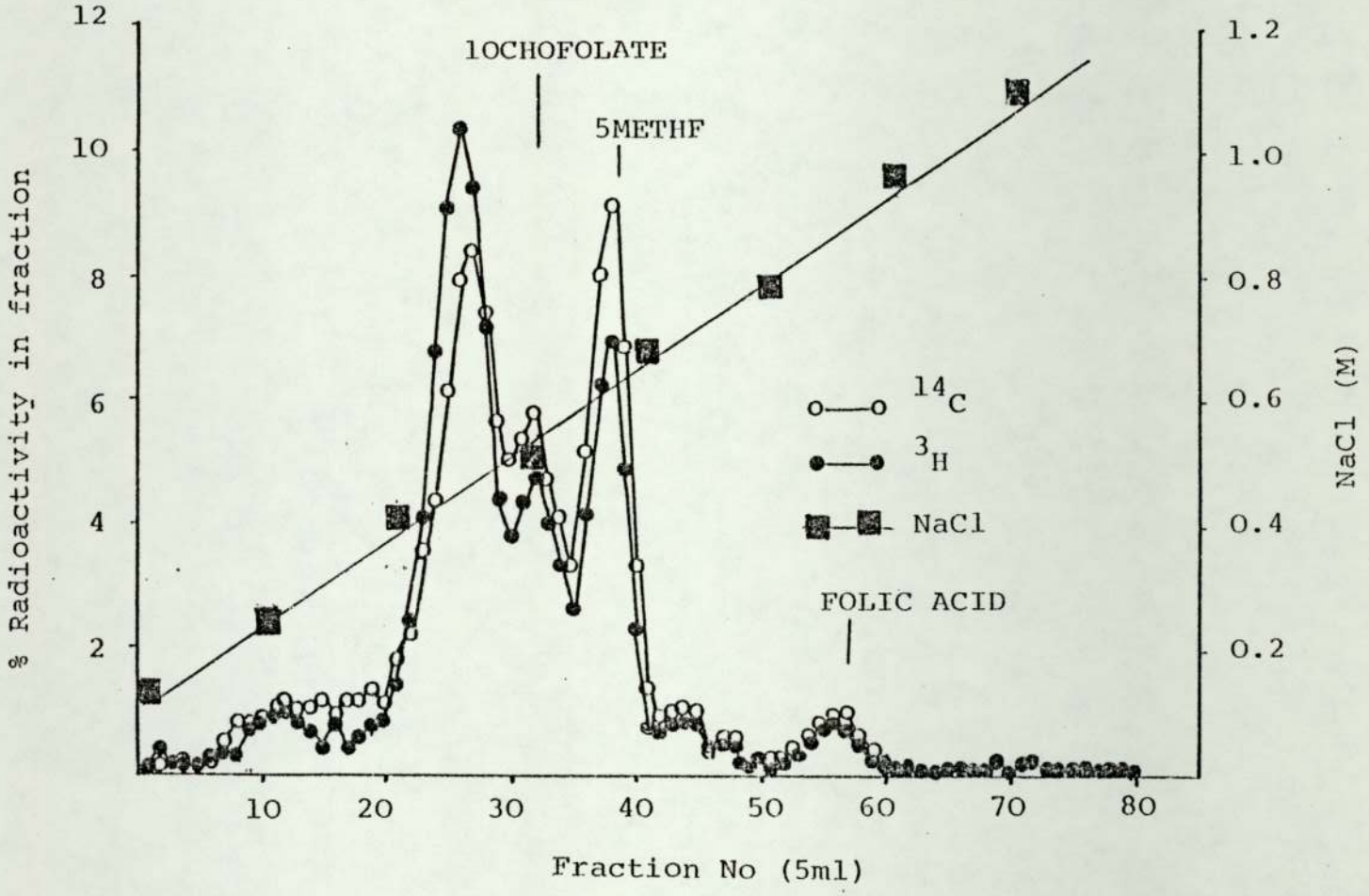


Figure 3.1.3.

DE-52 Chromatography of female weanling rat urine samples collected 48-72h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid.

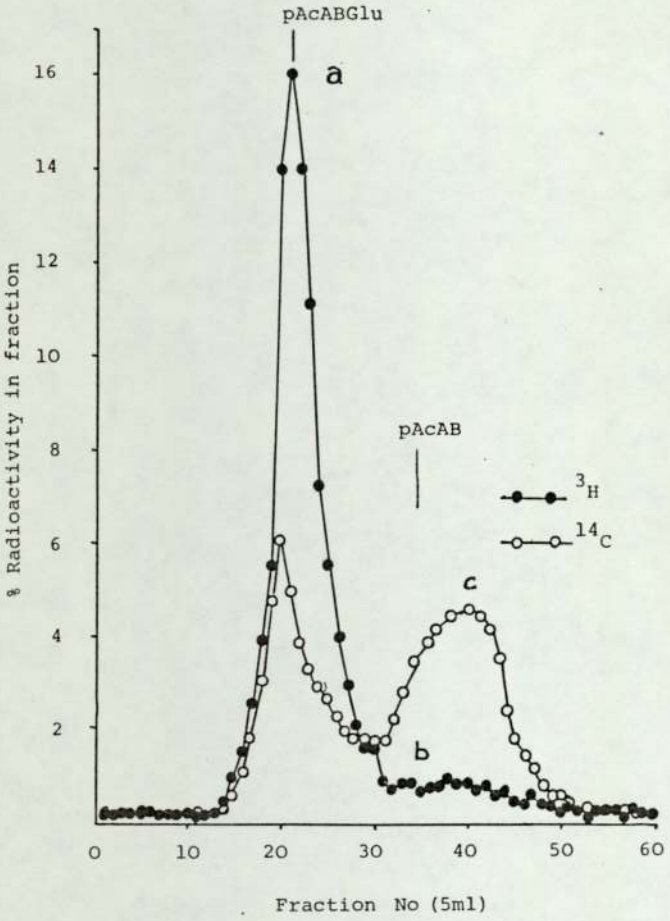


Figure 3.1.4.

Sephadex G-15 chromatography of peak iii from DE52 chromatography of urine (0-24h) from female weanling rats after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100 µg/kg body wt).

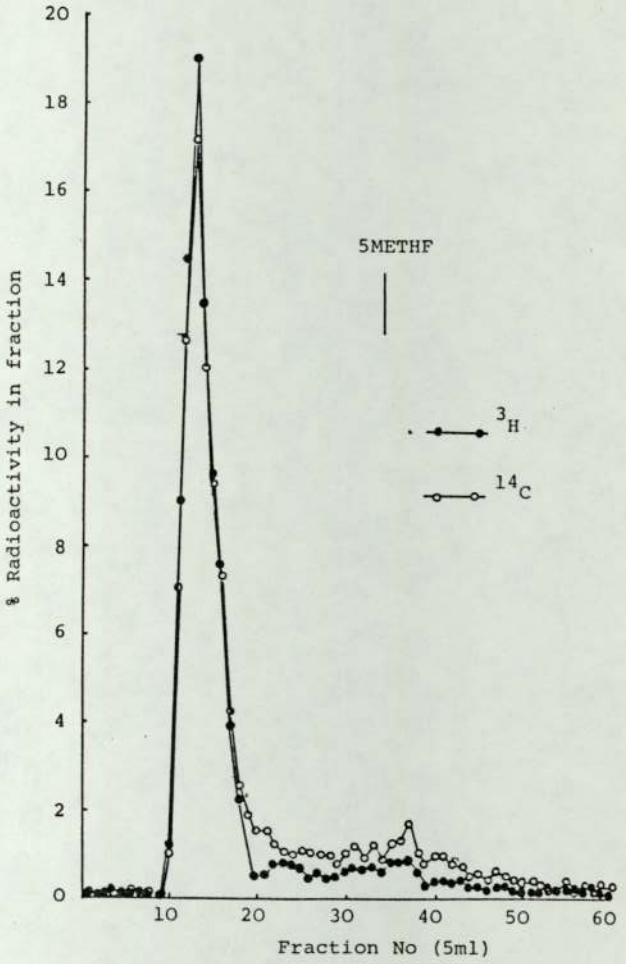


Figure 3.1.5.

Sephadex C-15 chromatography of the liver extract from female weanling rats 24 hours after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100 µg/kg body wt).

with peak (iii) on the DE-52 column and another metabolite labelled only with ^{3}H, (pAcABGlu). The second peak eluted at Fraction 36, appeared to contain only ^{3}H and co-chromatographed with pAcAB (peak b). The third component (peak c) had a much reduced ^{3}H : ^{14}C ratio and eluted at fraction no 41. Earlier efforts to identify this compound failed but it was characterised (Connor, 1979; Pheasant, et al., 1981 and Al-Haddad, 1984) as a reduced pteridine which has retained the ^{3}H label at C7 and is known as pterin A. The metabolite eluting as peak (i) from DE-52 at the void volume contained tritiated water, as the ^{3}H label could be removed by evaporation or freeze-drying, and a ^{14}C species peak (ii) previously identified as urea (Connor, et al., 1979; Saleh 1981 and Al-Haddad 1984). DE-52 ion exchange chromatography of the later urine samples showed a progressive simplification in the pattern of metabolites (figures 3.1.2 and 3.1.3). The relative amounts of folic acid (vii), 5MeTHF, 10CHOfolate (iv) and metabolite Y decreased while the scission product peaks (i), (ii) and (iii) progressively increased. Sephadex G-15 of this peak (iii) showed that in the 24-48h sample, pAcABGlu was the major ^{3}H-containing catabolite along with pAcAB.

LIVER EXTRACTS

Sephadex G-15 gel filtration of hot liver extracts is illustrated in figure 3.1.5. At each time period the major radioactive component eluted close to the void volume in the position of the high molecular weight folate polyglutamate. No folate monoglutamates were detected over 0-72h.

3.2.1 THE EFFECT OF DIETHYLSTILBOESTROL ON FOLIC ACID METABOLISM IN THE FEMALE WEANING RAT

In 1944 even before the chemical structure of folic acid had been established, Hertz and Sebrell (1944) reported that a dietary deficiency of the "L.casei" factor in chicks impaired the normal growth response of the oviduct to diethylstilboestrol stimulation. Subsequently it was shown that folate is essential for placental maintenance and that in its absence fetal resorption occurs, (Nelson and Evans 1949); 20% of women using oral steroid hormones for fertility regulation may display biochemical evidence of impaired folic acid metabolism (Doctor and Trunnel (1955)., Shojania, et al., 1968 and 1982).

Snyder, et al., 1969 and Streiff (1970) have suggested that megaloblastic anaemia is caused by oral contraceptive agents. Alteration in serum folate levels, impaired absorption, altered tissue uptake, increased excretion, diminished conversion to the coenzyme forms, binding to folate binders, induction of increased microsomal enzyme metabolism have all been suggested but experimental evidence is either absent or limited, (Anderson, et al., 1976; Krumdieck, et al., 1975; and Guest, 1984). Folate scission could be affected (Guest et al., 1983) hence experiments employing D.E.S. and 17α-ethynyloestradiol were undertaken to investigate these apparently conflicting trends.

MATERIALS AND METHODS

15 female weanling Wistar grade IV rats (50-100g body wt) each received daily oral doses of diethylstilboestrol (D.E.S) (416 mg/kg body wt) in Mazola corn oil (0.3 ml) for 3 days prior to receiving a single oral dose of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100 µg/kg body wt).

Urine was collected for periods of 0-24h, 24-48h as described in section 3.1. 5 animals were killed 24h, 48h and 72h after dosing with the folic acid. Animals

and samples were handled as described previously (section 3.1).

RESULTS

Table 3.2.1 shows the recovery of radioactivity in the urine, faeces and the distribution of retained radioactivity in the tissues. Most of the urinary radioactivity was found in the 0-24h samples with 51.50% ^{3}H and 54.12% ^{14}C but later samples contained lower levels; 6.09% ^{3}H and 7.37% ^{14}C over 24-48h with 3.37% ^{3}H and 4.02% ^{14}C over 48-72h respectively. Although more ^{14}C than ^{3}H was present in each urine sample there is no significant difference in isotope balance but urinary excretion is statistically higher $P<0.001$ over whole 0-72h period compared to normals.

There was also significantly more ^{14}C and ^{3}H in the faeces ($P<0.001$), more ^{3}H is excreted in the faeces of the D.E.S pretreated animals when compared to normals. After 24h there is no significant difference in the ^{3}H : ^{14}C in the faeces. Similar levels of ^{3}H and ^{14}C were found in the kidney and the G.I tract . Less radioactivity is retained in the livers over 72h ($P<0.05$) but the kidneys retained more radioactivity over the first 24h ($P<0.05$) with later levels of retention being far lower and

statistically non-significant. The gastrointestinal tract retained less radioactivity over the first 48h ($P<0.001$) but there was no statistical difference after 48-72h.

URINARY METABOLITES

Table 3.2.2 summarises the metabolites detected in the urine of the D.E.S treated rats upto 72h after the administration of radiolabelled folic acid. More folic acid (5.30% ^{3}H and 5.33% ^{14}C) was excreted after 0-24h and over 72h than in the normals. Slightly more 5MeTHF is excreted but significantly more 10-formylfolate was excreted in the urine, especially in the last 48 hours. After 48-72h 10-formylfolate is the major intact folate metabolite. After 48-72h a new metabolite appeared as a shoulder on the left hand side of the 5MeTHF peak, this had the ^{3}H : ^{14}C ratio of an intact folate and was designated metabolite X; (3.11% ^{3}H and 3.80% ^{14}C). pAcABGlu was the main scission product excreted in the urine, 6.15% ^{3}H was excreted as pAcABGlu over 0-24h and a total of 7.70% ^{3}H was excreted as pAcABGlu over 72h. pAcAB was not detected over 0-24h but 1.04% ^{3}H was present as pAcAB over the last 48h. 2.21% ^{14}C was excreted as a ^{14}C only labelled scission products over 72h. 0.1% ^{14}C was detected as urea over 72h and 0.2% ^{3}H as $^{3}H_2O$.

Tissue Sample	0-24 h ^{3}H	0-24 h ^{14}C	24-48 h ^{3}H	24-48 h ^{14}C	48-72 h ^{3}H	48-72 h ^{14}C	0-72 h ^{3}H	0-72 h ^{14}C
Urine	51.50 (11.41)	54.12 (9.76)	6.09 (3.05)	7.37 (3.95)	3.37 (0.81)	4.02 (1.26)	60.96	61.49
Faeces	10.59 (0.61)	23.02 (4.93)	2.68 (0.88)	3.78 (0.45)	2.06 (0.49)	2.68 (0.26)	15.33	29.48
Liver	17.70 (1.33)	22.73 (2.66)	11.66 (2.43)	13.31 (3.11)	9.04 (0.27)	10.47 (0.21)	9.04	10.47
Kidney	4.41 (2.19)	5.38 (2.52)	1.78 (0.34)	1.34 (0.33)	1.46 (0.11)	1.28 (0.14)	1.46	1.28
Gut	4.80 (0.34)	4.48 (0.61)	1.92 (0.4)	1.23 (0.09)	2.65 (0.34)	2.02 (0.23)	2.65	2.02
TOTAL							89.44	104.74

TABLE 3.2.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 24 h, 48 h and 72 h after being dosed with diethylstilboestrol (416mg/Kg body wt.) in Mazola corn oil (0.3 ml) daily for 3 days prior to the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) Folic acid (100μg/Kg body wt.)

Results are expressed as a percentage of the dose administered. n = 5

Metabolite	0-24 h		24-48 h		48-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	5.30	5.55	0.40	0.56	0.20	0.42
5MeTHF	20.26	23.14	1.98	2.49	0.88	1.10
10CHO-Folate	17.56	15.40	3.14	2.13	3.11	3.80
Metabolite X	ND	ND	ND	ND	0.31	0.25
Metabolite Y	ND	ND	0.51	0.47	ND	ND
pAcABGlu	6.15	-	1.29	-	0.26	-
pAcAB	ND	-	0.57	-	0.47	-
^{14}C labelled only Catabolite	-	0.90	-	0.95	-	0.36
Urea	-	0.1	-	ND	-	ND
$^{3}H_2O$	0.1	-	0.1	-	ND	-

TABLE 3.2.2 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with diethylstilboestrol (416 mg/Kg body wt.) in Mazola corn oil (0.3 ml) daily for 3 days prior to the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid (100 µg/Kg body wt.).

Results are expressed as a percentage of the dose administered. n = 5

LIVER EXTRACTS

Sephadex G-15 filtration of hot liver extracts revealed chromatograms similar to that illustrated in figure 3.1.5, at each time period the major radioactive component eluted close to the void volume in the position of the high molecular weight folate polyglutamate. No folate monoglutamates were detected in any sample.

3.3.1 THE EFFECT OF 17α-ETHYNYLOESTRADIOL ON FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

The reasons for the investigation of synthetic oestrogens and folic acid have been outlined in section 3.2. This section investigates the effects of one of the consituents of the oral contraceptive pill, the synthetic oestrogen, 17α-ethynyloestradiol, on the metabolism of folic acid.

MATERIALS AND METHODS

Fifteen female Wistar grade IV weanling rats (50-100g body wt) each received daily oral doses of 17α-ethynyl-oestradiol (20 mg/kg body wt) in 0.3 ml Mazola corn oil for 5 days prior to receiving a single oral dose of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid

(100 µg/kg body wt). Urine was collected for periods of 0-24h, 24-48h and 48-72h as described in section 3.1. 5 animals were killed 24h, 48h and 72h after dosing with folic acid. Animals and samples were handled as described previously (section 3.1).

RESULTS

Table 3.3.1 illustrates the recovery of radioactivity from the urine, faeces, and the distribution of retained radioactivity in the tissues. Most of the urinary radioactivity was found in the 0-24h samples (34.11% ^{3}H and 32.11% ^{14}C) but later samples contained lower levels, 2.77% ^{3}H and 2.80% ^{14}C over 24-48h which increased after 48-72h to 4.17% ^{3}H and 3.97% ^{14}C. The former two illustrate a decreased level of excretion $P<0.05$) while the later is an elevation in urinary excretion over 48-72h $P<0.05$) when compared to the normals. Although more ^{3}H than ^{14}C was present in each urine sample except over 24-48h, this excess is not statistically significant. There was also an imbalance of the two isotopes in the faeces over 0-24h, 24-48h and 48-72h, this was of statistical significance ($P<0.001$). Similar levels of ^{3}H and ^{14}C were found in the kidney and the G.I tract, without statistical significance. The level of radioactivity retained in the livers over 0-72h and the

slight elevation of ^{3}H levels seen in the livers were not statistically significant.

URINARY METABOLITES

DE-52 ion exchange chromatography of urine samples was similar to those illustrated in figure 3.1.1., 3.1.2 and 3.1.3. Table 3.3.1 summarises the metabolites identified following ion exchange chromatography and gel filtration as described in detail in section 2. Folic acid was detected in each sample upto 72h in the urine of the 17α-ethynyloestradiol treated animals. Less folic acid was excreted over 0-24h, 2.01% ^{3}H and 1.92% ^{14}C, with later levels being lower. 5MeTHF was the major intact folate detected after 0-24h (14.50% ^{3}H and 16.49% ^{14}C) followed by 10CHOfolate 4.59% ^{3}H 4.08% ^{14}C (this represents a very marked decrease).

A new metabolite X previously identified as 5,10-CH_2 THF (section 1) was detected in the 24-48h and 48-72h urine samples (analysis here was incomplete due to very low levels of radioactivity). Over 0-72h the total amount of ^{3}H-only labelled catabolites was 12.2% ^{3}H of the dose. This represents an increase due to the high levels of pAcAB excreted 0-24h after the administration of folic acid and the high levels of pAcABGlu excreted 24-48h after the administration of the folic acid. While 3.93% ^{14}C of the actual dose

Tissue Sample	0-24 h ^{3}H	0-24 h ^{14}C	24-48 h ^{3}H	24-48 h ^{14}C	48-72 h ^{3}H	48-72 h ^{14}C	0-72 h ^{3}H	0-72 h ^{14}C
Urine	34.11 (4.85)	32.11 (5.18)	2.77 (0.43)	2.80 (0.46)	4.17 (0.54)	3.97 (0.47	41.05	38.88
Faeces	4.19 (0.67)	14.68 (2.2)	0.91 (0.47)	2.73 (0.65)	0.345 (0.048)	1.78 (0.21)	5.45	19.19
Liver	19.52 (3.67)	15.10 (5.78)	13.94 (1.58)	15.67 (1.60)	18.28 (1.65)	15.41 (2.26)	18.28	15.41
Kidney	1.74 (0.19)	1.36 (0.24)	1.52 (0.24)	1.45 (0.21)	1.22 (0.16)	1.20 (1.25)	1.22	1.20
Gut	4.31 (0.20)	2.72 (0.22)	2.62 (0.74)	2.06 (0.25)	3.98 (1.25)	1.60 (0.18)	3.98	1.60
TOTAL							69.98	76.28

TABLE 3.3.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 24 h, 48 h, and 72 h after being dosed with 17α ethynyloestradiol (20 mg/Kg body wt.) in Mazola corn oil (0.3 ml) daily for 3 days prior to the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid. (100 µg/Kg body wt.). Results are expressed as a percentage of the dose administered. n = 5

Metabolite	0-24 h		24-48 h		48-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	2.01	1.92	0.11	0.15	0.18	0.17
5MeTHF	14.50	16.49	0.71	0.87	1.15	1.15
10CHO-Folate	4.59	4.08	0.13	0.25	0.61	0.27
Metabolite X	ND	ND	0.13	0.12	0.06	0.05
Metabolite Y	ND	ND	0.10	0.10	TRACE	
pAcABGlu	5.09	-	1.41	-	0.18	-
pAcAB	5.04	-	0.30	-	0.18	-
^{14}C labelled only Fragment	-	3.01	-	0.87	-	0.05
Urea	-	0.1	-	0.1	-	ND
$^{3}H_2O$	0.31	-	0.12	-	0.1	-

TABLE 3.3.2 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with 17α-ethynyloestradiol (20 mg/Kg body wt.) in Mazola corn oil (0.3 ml) daily for 3 days prior to the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid (100 μg/Kg body wt.). Results are expressed as a percentage of the dose administered. n = 5

was excreted as ^{14}C only labelled catabolites over 0-72h. 0.2% ^{14}C was detected as urea over 0-48h. More $^{3}H_2O$ was also found in the 17α-ethynyloestradiol treated animals. (0.53% ^{3}H over 0-72h).

LIVER EXTRACTS

Sephadex G-15 gel filtration of hot liver extracts were similar to the one illustrated in section 3.1. At each time period the major radioactive component eluted close to the void volume in the position of the high molecular weight folate polyglutamates. No folate monoglutamates were detected in any sample.

SUMMARY

Folic acid was administered to normal D.E.S and 17 ethynyloestradiol pretreated rats. Qualitatively the handling of folic acid was similar to normal rats but the following significant differences were observed.

NORMAL ANIMALS

(i) The majority of excreted radioactivity appeared in 0-24h urine and faeces.

(ii) 24-72h excretion levels were far lower.

(iii) Radioactivity was found in the livers, kidney and the gastrointestinal tract.

(iv) Similar levels of ^{3}H and ^{14}C were detected in all tissues.

(v) Four intact folates were detected in the urine (5MeTHF, 10CHOfolate, folic acid and metabolite Y).

(vi) The ratio of ^{3}H : ^{14}C in the folate derivatives was higher than in the folic acid administered.

(vii) Folate catabolites were detected (pAcAB, pAcABGlu, pterin A, urea and $^{3}H_2O$) over 72h.

(viii) Folate polyglutamates were detected in the liver over 72h. No folate monoglutamates were detected in any sample.

D.E.S. PRETREATED ANIMALS

(i) Higher recovery of radioactivity in urine and faeces.

(ii) Higher retention of radioactivity in the kidneys.

(iii) greater reduction in the liver and the kidneys with each time period.

(iv) Intact folate excretion was higher.

(v) A new metabolite X was detected after 48h.

(vi) No pAcAB was detected after 0-24h.

(vii) Scission product excretion was increased.

17α-ETHYNYLOESTRADIOL PRETREATED ANIMALS

(i) Lower recovery of radioactivity in the urine and faeces after 0-24h.

(ii) Lower retention of radioactivity in tissues in general.

(iii) Intact folate excretion was lower especially 10CHO-folate.

(iv) A new metabolite X was detected over 24-72h.

(v) pAcAB excretion was increased after 24h. Scission product excretion was enhanced.

CHAPTER 4

THE EFFECT OF METAL IONS ON FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

4.1.1 FOLATE METABOLISM IN NORMAL WEANLING RATS

Section 3.1 describes the fate and metabolic handling of folic acid in rats dosed with Mazola corn oil and folic acid, in this section rats were given 0.3 ml distilled water followed by the usual folic acid (100 µg/Kg body wt.) dose. Qualitatively and quantitatively the handling of folic acid in all samples were similar to section 3.1 without statistically significant differences. Hence experimental data, results and summary will not be repeated. All references to controls will therefore be referred to data in section 3.

Only H^3-folic acid was used, due to the non-availability of ^{14}C-folic acid from the suppliers.

4.2.1 THE EFFECT OF FERROUS SULPHATE ON FOLATE METABOLISM IN THE FEMALE WEANLING RAT

Transition metal ions are known to catalyse the propagation reactions involving partially reduced forms of dioxygen or "oxy-radicals". Gutteridge, et al., 1982 and 1983; Aust, et al., 1985. Triggs and Willmore (1984) have suggested that iron salts ($Fe^{2+}(aq)$) added to tissue suspensions result in the formation of free radical oxidants

including superoxide radicals (Aisen, 1977), singlet oxygen (Svingen, et al., 1978) peroxides and hydroxy radicals (Fong, et al., 1976). These "oxy-radicals are capable of oxidising biomolecules such as DNA, proteins and tetrahydrofolates in vitro, (Blair and Pearson (1974)). Since many vitamin supplements administered orally to pregnant women contain a combination of ferrous sulphate and folic acid the effect of ferrous sulphate administration could provide a possible mechanism of folate scission. Hence the role of iron in folate metabolism was investigated.

MATERIALS AND METHODS

Fifteen female (Wistar grade IV) weanling rats (50-100 g body wt) each received a single oral dose of ferrous sulphate 10 mg/100g body wt in 0.3 ml distilled water prior to receiving a single oral dose of [3',5',7,9-^{3}H]-folic acid (100 µg/kg body wt). Urine was collected from 3 groups of 5 animals; 0-24h after dosing for the first 5 animals, 24-48h for the second and 48-72h for the last 5 as described in section 2.1. The animals and samples were handled as described previously section 3.1.

RESULTS

All statistical differences are compared with the data in section 3.1 unless otherwise stated.

Table 4.2.2 illustrates the recovery of radioactivity from the urine, faeces and the radioactivity retained in the tissues following the administration of [3',5',7,9-^{3}H]-folic acid to a group of healthy rats dosed with ferrous sulphate. Excretion of radioactivity into the urine took place mainly over 0-24h, while 9.18% ^{3}H and 4.74% ^{3}H were recovered in the 24-48h and 48-72h urine samples respectively. The difference in these levels when compared with those of controls was significantly different. Faecal radioactivity was detected up to 72h, the highest level of excretion was over 0-24h, 7.54% ^{3}H andly non-this was statistically non-significant.
Later levels of faecal ^{3}H excretion were lower.
Hepatic radioactivity was also detected upto 72h, 7.19% ^{3}H over 0-24h, 5.83% ^{3}H over 24-48h and 4.34% ^{3}H over 48-72h respectively. These levels were significantly lower ($P<0.001$) when compared to normals at 0-24 and 48-72h). The level of radioactivity retained in the kidneys and the gut were statistically significantly lower over 72h in the treated animals ($P<0.05$).

Metabolite	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H
Folic acid	3.02	0.16	0.07
5MeTHF	18.36	1.36	0.06
10CHOFolate	15.21	2.01	0.21
Metabolite X	ND	ND	ND
Metabolite Y	0.11	0.03	ND
pAcABG	3.16	0.31	0.09
pAcAB	5.10	0.72	0.09
$^{3}H_2O$	0.29	0.02	0.01

TABLE 4.2.1 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with Ferrous sulphate (10 mg/100 g body wt.) prior to the administration of(3', 5', 7, 9 - ^{3}H) Folic acid (100 µg/Kg body wt.). Results are expressed as a percentage of the dose administered.

n = 5, ND = Not Detected

URINARY METABOLITES

The result of DE-52 ion exchange chromatography of urine samples from female weanling rats pre-dosed with ferrous sulphate, were similar to the controls in figures 3.1.1., 3.1.2. and 3.1.3. Sephadex G-15 gel filtration of the peaks containing the intact folates and the scission products, were also similar to those in section 3.1. The quantitative data derived from the analysis is listed in table 4.2.1. The bulk of the urinary radioactivity was found to be 5MeTHF and 10-CHO-folate, 18.36% ^{3}H was found to be 5MeTHF after 24h, and 15.25% ^{3}H was detected as 10-CHO folate. Later levels of excretion of these metabolites were much lower. Folic acid was present as 3.02% ^{3}H over 0-24h, 0.16% ^{3}H over 24-48h and 0.07% ^{3}H over 48-72h respectively. Metabolite X was not detected over 72h but 0.11% ^{3}H and 0.03% ^{3}H of metabolite Y was detected over 0-24h and 24-48h respectively. Metabolite Y was not detected 43-72h after dosing. 9.47% of the ^{3}H dose appeared as the labelled scission products with 3.56% ^{3}H as pAcABGlu and 5.91% ^{3}H as pAcAB. The early urine samples mainly contain intact folates while later samples were composed of scission products. 0.32% ^{3}H was detected as $^{3}H_2O$ over 72h. Folate scission was increased over 0-72h.

Tissue Sample	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H	0-72 h H^{3}
Urine	33.12 (4.88)	9.18 (1.63)	4.74 (1.00)	47.04
Faeces	7.54 (1.43)	2.20 (0.59)	0.37 (0.06)	10.11
Liver	7.19 (1.00)	5.83 (0.46)	4.34 (0.54)	4.34
Kidney	1.34 (0.12)	0.96 (0.05)	0.85 (0.08)	0.85
Gut	2.61 (0.30)	1.42 (0.041)	0.97 (0.033)	0.97
TOTAL				63.31

TABLE 4.2.2 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 24 h, 48 h and 72 h after being dosed with Ferrous sulphate (10 mg/100 g body wt.) prior to the administration of (3', 5', 7, 9 - ^{3}H) Folic acid (100 µg/Kg body wt.). (Results are expressed as a percentage of the dose administered, n = 5)

LIVER EXTRACTS

Sephadex G-15 gel filtration of the hot liver extracts at 24h, 48h and 72h after dosing was similar to figure 3.1.5., only polyglutamates were detected.

4.3.1 THE UPTAKE AND DISTRIBUTION OF ^{59}Fe IN THE FEMALE WEANLING RAT.

The aim of this section was to illustrate the uptake and retention of ferrous sulphate in the tissues. Section 4.2 illustrates the increase in folate scission as a result of ferrous sulphate administration. (discussed p 91 -2). Partridge (1986) has shown Fe^{2+} uptake and retention in adult male rats.

MATERIALS AND METHODS

Fifteen female weanling (Wistar grade IV rats 50-100 g body wt) were given a single oral dose of ^{59}Fe in a ferrous sulphate carrier (10 mg/100g body wt), urine was collected from 3 groups of 5 animals and treated as in section 2.1. The animals and tissue samples (plus brains) were handled as described previously in section 2.1.

RESULTS

Table 4.3.1. illustrates the distribution and uptake of ^{59}Fe in the urines, faeces and the tissues in the female weanling rat. The majority of radioactivity is excreted in the faeces. 33.28% ^{59}Fe at 0-24h, 9.19% ^{59}Fe over 24-48h and 1.52% ^{59}Fe over 48-72h respectively. The hepatic radioactivity levels were 1.13% ^{59}Fe over 0-24h. These reached a maximum at 24-48h, 7.47% ^{59}Fe. This increase was statistically significant ($P<0.05$). The levels of radio-activity in gut followed a similar pattern to the urines while the kidneys exhibited a optimum retention over the 24-48h time period. Total recovery of % ^{59}Fe was 36.38% over 0-24h, 18.41% over 24-48h and 9.71% ^{59}Fe respectively (a progressive decrease).

4.4.1 THE EFFECT OF LEAD ACETATE ON FOLATE METABOLISM IN THE FEMALE WEANLING RATS

Folates, like many other dietary constituents may be potential chelating agents. Coleman, etal., (1979) and Partridge (1986) have suggested that the transport of lead across the gastrointestinal tract by forming a lead-folate complex. The daily uptake of lead through the gut ranges from 200 - 500 mg but only 10% of this is retained and the rest being excreted (Morar,

Tissue Sample	% ^{59}Fe Recovered as % Of. Actual Dose		
	0-24 h	24-48 h	48-72 h
Livers	1.13 (0.33)	7.47 (2.09)	7.40 (1.76)
Brains	0.035 (0.003)	0.026 (0.005)	0.025 (0.003)
Gut	1.52 (0.26)	0.90 (0.1)	0.58 (0.09)
Kidney	0.048 (0.006)	0.075 (0.006)	0.045 (0.09)
Urine	0.37 (0.13)	0.75 (0.54)	0.14 (0.05)
Faeces	33.28 (4.30)	9.19 (3.59)	1.52 (0.46)
Total Recovery of Dose	36.38	18.41	9.71

(Results are expressed as a percentage of the dose (± S.E.M. where appropriate), n = 5)

TABLE 4.3.1 The distribution of ^{59}Fe in female weanling rats dosed with 10 µCi of ^{59}Fe in 10 mg/100 g body wt. of ferrous sulphate.

1984). This still represents an elevation of lead levels and it has been shown to inhibit the enzyme dihydropteridine reductase (DHPR) in the weanling rat (Eggar, 1985).

DHPR and DHFR can act in conjunction to salvage THF from either DHF or quinonoid DHF which are easily oxidised (Chippel and Scrimgeour, 1970). A decrease in the activity of DHPR would result in an increased loss of folate. Thus it was decided to investigate the possible effects lead might have on the general metabolism of folic acid, its coenzymes and folate scission.

MATERIALS AND METHODS

Fifteen female (Wistar grade IV) weanling rats (50-100 g body wt) each received a single oral dose of lead acetate 3.1 µg/100g body wt in 0.3 mls distilled water prior to receiving a single oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (100 µg/kg body wt). Urine was collected from 3 groups of 5 animals; 0-24h after dosing for the first 5, 24-48h for the second 5 and 48-72h for the last 5 as described in section 2.1. Animals and samples were handled as previously described in section 3.1.

RESULTS

Table 4.4.1. illustrates the recovery of radioactivity from the urine, faeces and the radioactivity retained in the tissues following the oral administration of a mixture [3',5',7,9-^{3}H] and [2-^{14}C] folic acid to a group of healthy rats dosed with lead acetate. The excretion of radioactivity into the urine took place mainly over the first 24 h, thus 17.46% ^{3}H, 16.43% ^{14}C was detected over 0-24 h while only 3.98% ^{3}H, 3.63% ^{14}C and 1.12% ^{3}H, 0.97% ^{14}C were recovered in the 24-48h and 48-72h urines respectively. This is lower than the controls ($P<0.001$) . Faecal radioactivity was detected upto 72h after the radiolabelled dose. Although the 0-24h sample contained the highest level; 8.53% ^{3}H and 13.97% ^{14}C this is not statistically significant. Hepatic radioactivity retention was depressed over 0-72h and this is statistically significant ($P<0.05$). The radioactivity retained in the kidneys over 72h was halved ($P<0.05$). The levels of radioactivity retained in the gut were significantly($P<0.001$) depressed. The total recovery of [^{3}H] and [^{14}C] was greatly depressed; 55.78% and 55.38% ^{14}C over 0-72h.

Tissue Sample	0-24 h		24-48 h		48-72 h		0-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C		
Urine	17.46 (1.79)	16.43 (1.68)	3.98 (0.73)	3.63 (1.68)	1.12 (0.12)	0.97 (0.67)	22.56	21.03
Faeces	8.53 (0.64)	13.97 (1.47)	4.15 (0.72)	2.36 (0.28)	2.68 (0.29)	2.06 (0.65)	15.36	18.39
Livers	14.49 (1.20)	13.93 (1.19)	10.83 (1.67)	10.87 (0.90)	14.47 (1.71)	13.21 (1.98)	14.47	13.21
Kidney	1.60 (0.20)	1.36 (0.21)	1.75 (0.20)	1.46 (0.16)	1.57 (0.17)	1.28 (0.16)	1.57	1.28
Gut	3.13 (0.16)	2.76 (0.15)	2.0 (0.19)	1.58 (0.12)	1.82 (0.36)	1.47 (0.31)	1.82	1.47
TOTAL							55.78	55.38

TABLE 4.4.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.), 24 h, 48 h and 72 h after being dosed with lead acetate (3.1 µg/100 g body wt.) prior to the administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2 - ^{14}C) folic acid (100 µg/Kg body wt.). Results are expressed as a percentage of the dose administered.

n = 5 ($\pm$ S.E.M.)

URINARY METABOLITES

Table 4.4.2 summarises the metabolites detected in the urine of the lead acetate treated rats upto 72h after the administration of the radiolabelled folic acid. The pattern of metabolites found in the urine from normal rats also occurs in the urine of the lead acetate dosed rats, prominent differeces are evident, less folic acid was present in urine of treated animals after the first 24 h although more folic acid was detected in the urine of treated animals after 48h and 72h. In contrast, 5MeTHF levels fall drastically over the whole 72h, a similar observation is made with the 10-CHOfolate levels over 72h. A new metabolite X previously identified as 5,10CH_2THF was detected after 48-72h, 0.03% ^{3}H and 0.03% ^{14}C respectively. Over 0-72h the total amount of ^{3}H only labelled catabolites was 10.02% ^{3}H of the dose after lead acetate treatment. This slight increase was due to high levels of pAcABGlu excreted 0-24h after administration of folic acid. No ^{14}C only labelled catabolites were detected in the pretreated animals.
Only trace amounts of urea and $^{3}H_2O$ was detected.
(Analysis here were incomplete due to low levels of radioactivity).

Metabolites	0-24 h		24-48 h		48-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	2.50	2.70	0.41	0.40	0.03	0.02
5MeTHF	6.0	6.40	0.97	0.98	0.28	0.11
10CHO-Folate	1.50	1.61	0.25	0.20	0.08	0.09
Metabolite X	ND	ND	ND	ND	0.03	0.03
Metabolite Y	1.20	1.21	0.89	0.83	0.02	0.02
pAcABGlu	7.0	-	0.84	-	0.10	-
pAcAB	1.84	-	0.15	-	0.09	-
^{14}C only labelled Catabolite	ND	ND	-	0.60	ND	ND
Urea	TRACE		-	0.1	0.3	
$^{3}H_2O$	TRACE			TRACE	TRACE	

Results are expressed as a percentage of the dose administered
ND - Not detected

TABLE 4.4.2 Metabolites present in the urines of healthy female weanling rats (10-100 g body wt.) pretreated with a single oral dose of lead acetate (3.1 µg/100 g body wt.) prior to the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) Folic acid (100 µg/Kg body wt.).
n = 5

4.5.1 THE METABOLISM OF AN INCREASED FOLIC ACID DOSE IN THE FEMALE WEANLING RAT

The normal metabolism and incorporation of an increased (tenfold) oral dose (100 µg/100g body wt) of radiolabelled folic acid into the reduced folate coenzyme pool is described in normal female weanling rats upto 72h after dosing. A new chromatographic technique (Sephadex LH-20) was introduced.

MATERIALS AND METHODS

Fifteen female (Wistar grade IV) weanling rats (50-100g body wt) were given a single oral dose of [3',5',7,9-^{3}H]-folic acid (100 µg/100 g body wt). Animals and samples were handled as described in section 2.1. The major portion of the livers were quickly removed, washed in ice cold buffer and extracted in one of the following ways:

(a) Chopped into small pieces and dropped into 4 volumes of boiling 0.05M sodium phosphate buffer pH 7.0, containing sodium ascorbate (2% w/v) and maintained at 100°C for 5 minutes, cooled, homogenised and centrifuged. The supernatant was examined by Sephadex LH-20 organic solvent chromatography.

(b) A small portion of the livers were chopped into small pieces and dropped into 4 volumes I_2/KI in 1N HCl, homogenised and left for 1 hour at 20°C. The iodine was then removed with sodium thiosulphate, followed by centrifugation, the supernatant was recovered followed by Sephadex LH-20 chromatography.

(c) As in (b) except the supernant collected was heated in a water bath at 100°C for 1 hour in the presence of I_2/KI in 1N HCl.

All samples were stored at -20°C in the presence of the antioxidant sodium ascorbate (2% w/v) until analysed by Sephadex G-15, Sephadex LH-20 organic solvent chromatography and liquid scintillation counting.

RESULTS

Table 4.5.1. illustrates the recovery of radioactivity from the urine, faeces and the distribution of retained radioactivity in the tissues. The majority of excreted radioactivity appeared in the 0-24h urine [43.85% ^3H] and faeces [8.05% ^3H], later excretion levels being far lower. These are significantly higher, $P<0.001$ for the urine at 0-24h and $P<0.05$ for the faeces at 0-24h when compared to normals. Hepatic radioactivity retention in all three groups are similar.

Tissue Sample	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H	0-72 h
Urine	43.85 (3.33)	8.47 (2.29)	1.45 (0.37)	53.77
Faeces	8.05 (0.52)	2.04 (0.19)	1.06 (0.05)	11.15
Liver	16.12 (0.82)	17.64 (0.87)	18.3 (0.95)	18.30
Kidney	2.32 (0.17)	1.94 (0.15)	1.87 (0.035)	1.87
Gut	5.70 (0.76)	5.87 (0.52)	6.33 (0.94)	6.33
TOTAL				91.42

TABLE 4.5.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50/100 g body wt.) 0-24 h, 24-48 h, and 48-72 h after being dosed with a single oral dose of (3', 5', 7, 9 - ^{3}H) folic acid (100 µg/100 g body wt.).

Results are expressed as a percentage of the dose administered. (n = 5 $\pm$ S.E.M.)

Similar observations can be made over 72h for all the other tissues without any statistical difference compared to normals. The increased dosage of folate, yields greater total recovery of the ^{3}H dose [91.42% ^{3}H].

URINARY METABOLITES

The results of the DE - 52 ion exchange chromatography were similar to those obtained with urine samples from the low-dosed normals described in section 3.1.

The 0-24h urine samples contained a mixture of ^{3}H labelled metabolites, four of which were also detected in section 3.1. Three were identified as 10-formyl-folate (10CHO-folate), 5MeTHF and folic acid, increased folic acid dosage results in more folic acid in the urine over 0-72h (6.11% ^{3}H) similar observations are also made with 5MeTHF and 10CHO-folate over 72h. 1.30% ^{3}H of metabolite X was detected after 0-24h but this was absent over the next 48h, 1.60% ^{3}H of metabolite Y was also detected after 0-24h and only trace amounts were detected after 48-72h. 5.86% of the [^{3}H] dose appeared as pAcABGlu after 72h, while only 3.06 of ^{3}H was detected as pAcAB over 72h. 0.33% ^{3}H was detected as $^{3}H_2O$ during the first 48h while only traces were present during 48-72h.

Metabolite	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H
Folic acid	5.31	0.50	0.3
5MeTHF	19.01	3.0	0.6
10CHO-Folate	18.21	2.6	0.5
Metabolite X	1.30	ND	ND
Metabolite Y	1.60	ND	Trace
pAcABGlu	5.10	0.6	0.16
pAcAB	2.30	0.5	0.26
$^{3}H_2O$	0.30	0.03	Trace

TABLE 4.5.2 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with a single oral dose of (3', 5', 7, 9 - ^{3}H) folic acid (100 µg/100 g body wt.). Results are expressed as a percentage of the dose administered. n = 5.

A general increase in excretion of intact ^{3}H metabolites is evident.

LIVER EXTRACTS

Sephadex G-15 gel filtration and Sephadex LH-20 chromatography of the hot liver extracts (method a) are illustrated in figures 4.5.1 and 4.5.2. The major radioactive component in the G-15 chromatogram elutes close to the void volume in the position of the high molecular weight folate polyglutamates. The Sephadex LH-20 chromatography (figure 4.5.2) of the polyglutamate peak (b) from the G-15 elutes at fraction 64 (b), but peaks A1 and A2 elute earlier (identification incomplete due to unavailability of known comparable standards).

However, upon the application of method (b) and Sephadex LH-20 chromatography (figure 4.5.3), two new peaks were observed (C and D). These did not match existing standards. Peak D suggests the presence of oxidation products, further oxidation (method C) and Sephadex LH-20 chromatography (figure 4.5.4) results in 3 new peaks C1, E and F. Peak F eluted in fraction no 102 and was identified as $6CH_3$-pterin.

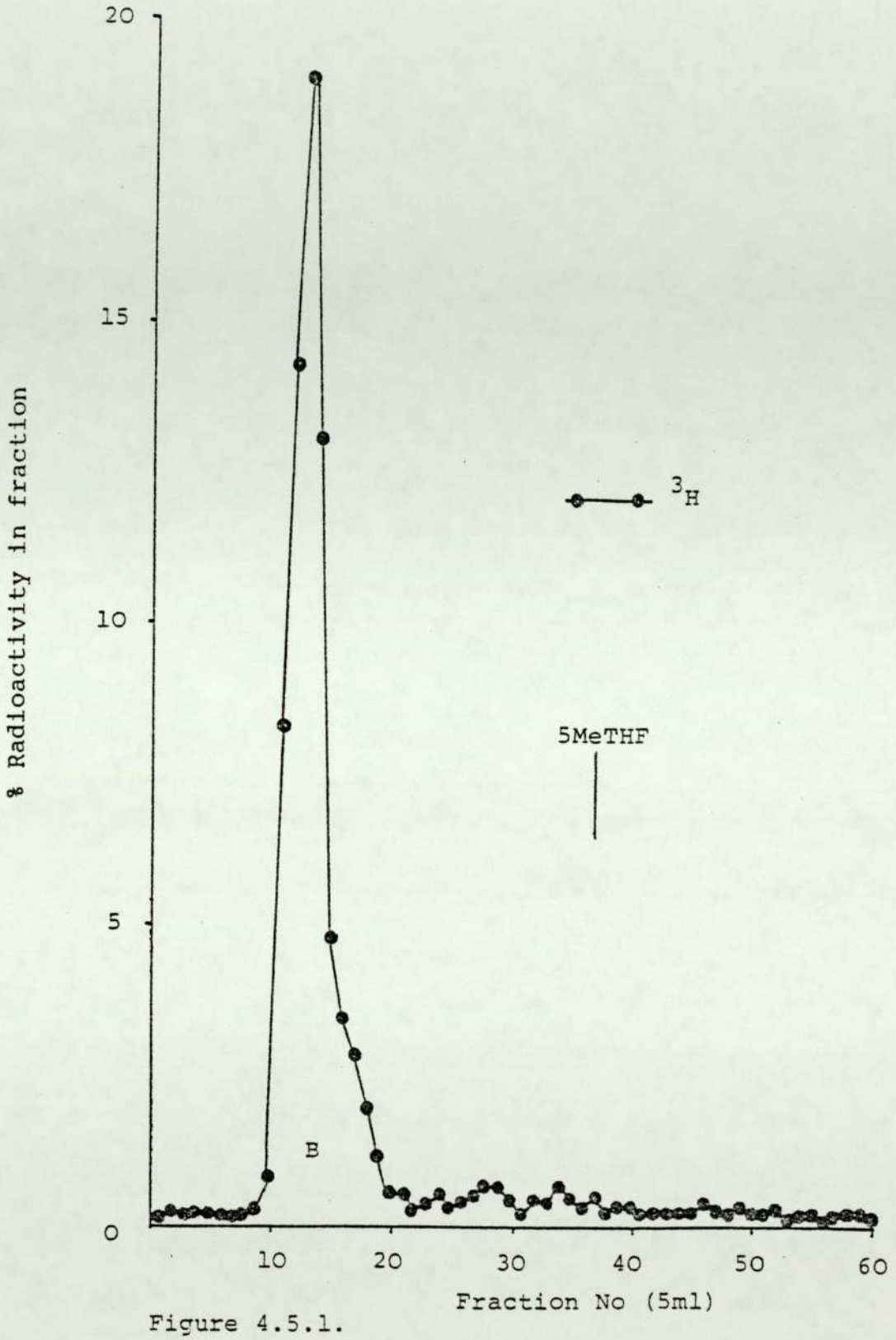


Figure 4.5.1.

Sephadex G15 chromatography of hot liver extracts (method a) from livers from pool 1 (0-24h) of female weanling rats (50-100g body wt) dosed with (3',5',7,9-^{3}H) folic acid.

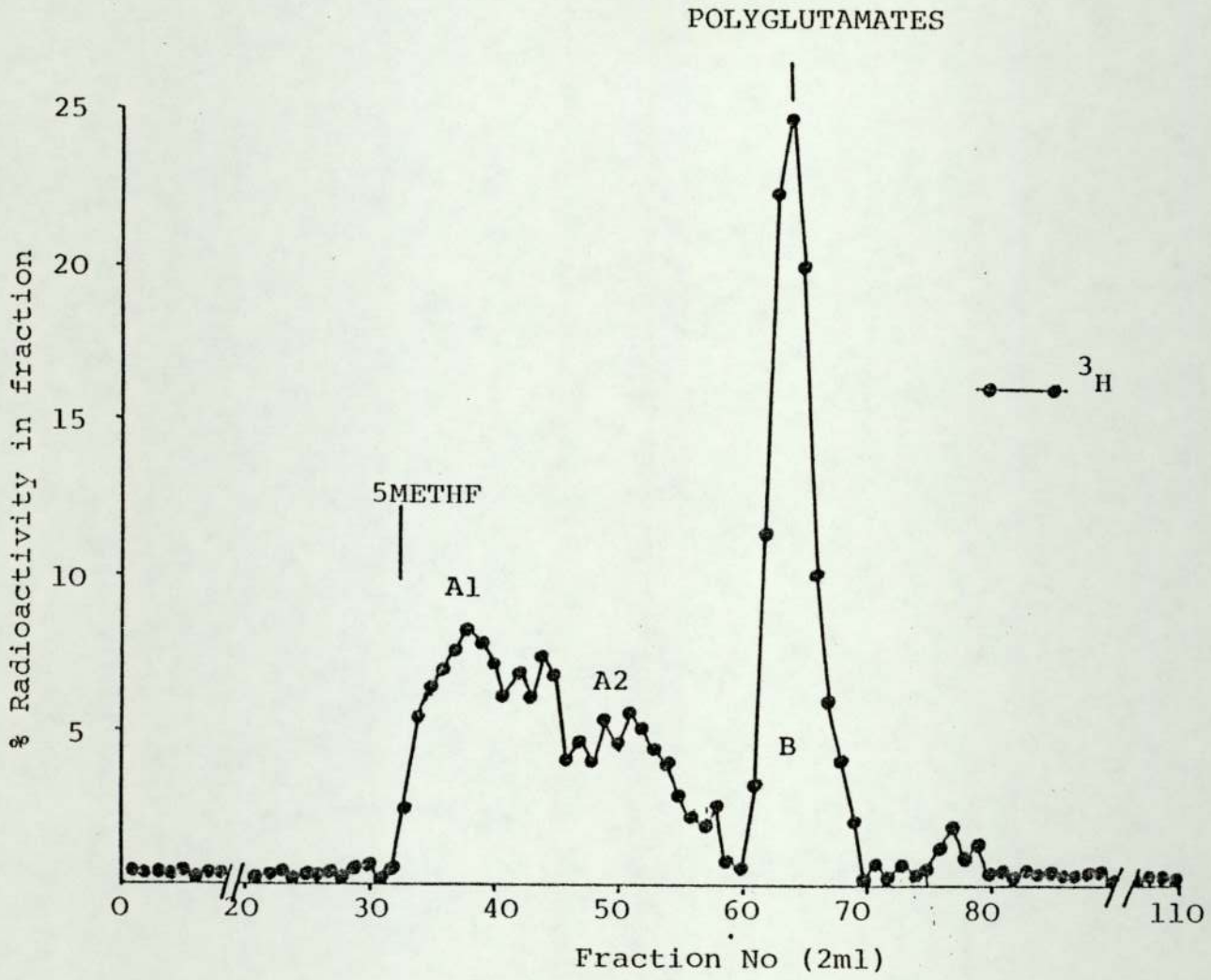


Figure 4.5.2.

Sephadex LH-20 chromatography of hot liver extracts (method a) from livers from pool 1 (0-24h) of female weanling rats (50-100g body wt) dosed with 100 µg/100g (3',5',7,9-^{3}H) folic acid.

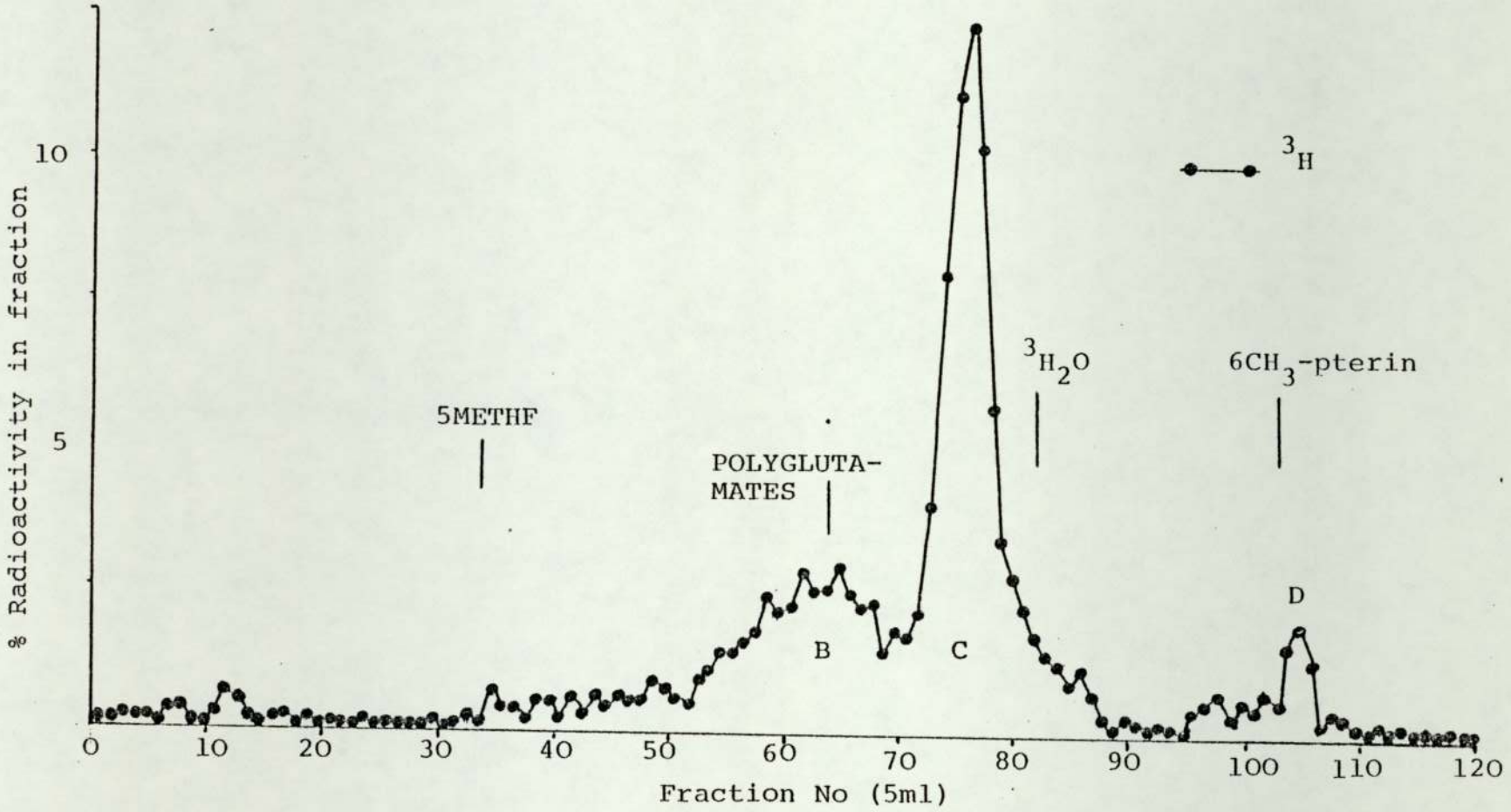


Figure 4.5.3.

Sephadex LH-20 chromatography of hot liver extracts (method b) from livers from pool 1(0-24h) of female weanling rats (50-100g body wt) dosed with (100 µg/100g body wt.) (3',5',7,9-^{3}H) folic acid.

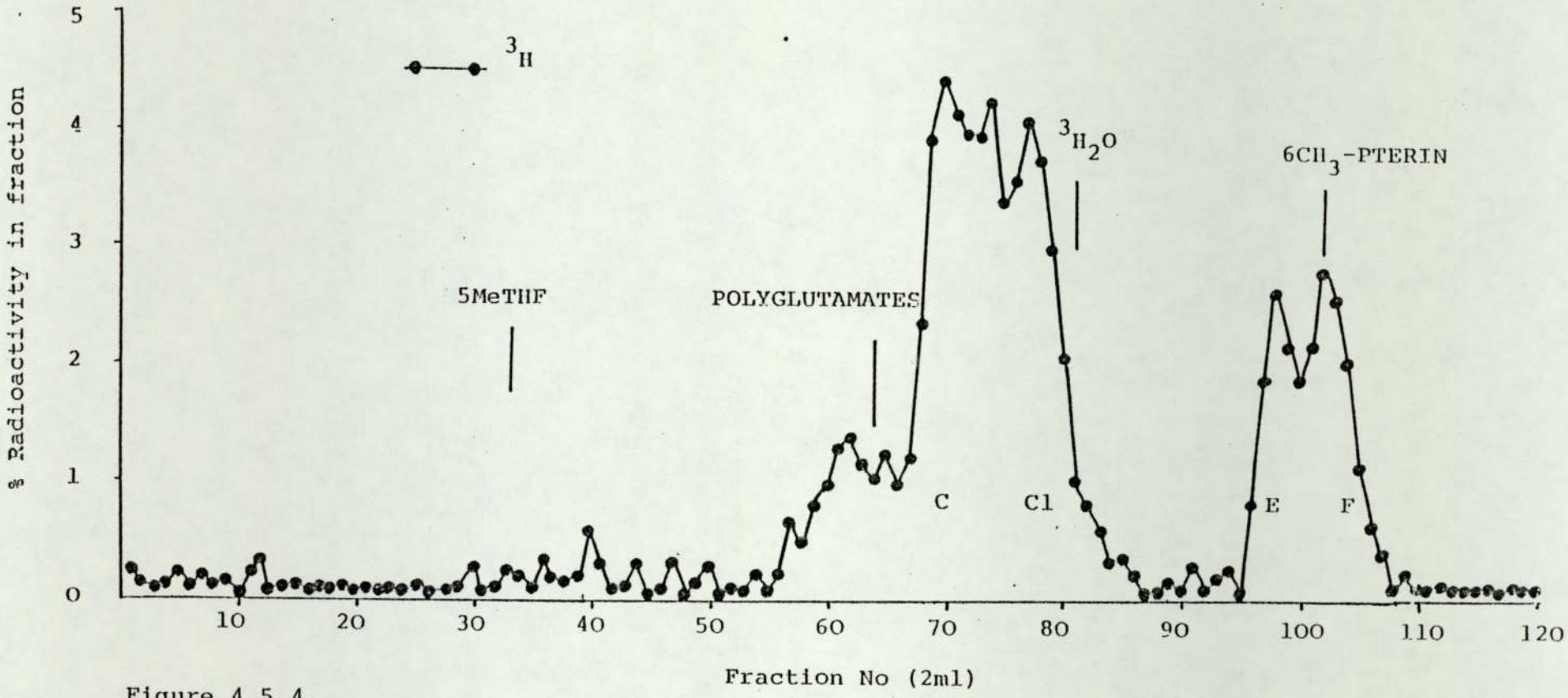


Figure 4.5.4.

Sephadex LH-20 chromatography of hot liver extracts (method C) from livers from poll 1 (0-24h) of female weanling rats (50-100g body wt.) dosed with (100 µg/100g body wt.) (3',5',7,9-^3H) folic acid.

Due to the unavilability of known folate polyglutamate and other standards, the analysis here is incomplete, but no known folate monoglutamates were detected.

4.6.1 THE EFFECT OF INCREASED LEAD ACETATE DOSE IN FEMALE WEANLING RATS DOSED WITH INCREASED FOLATE

Lead acetate administration results in decreased folate retention in the female weanling rat (section 4.4). Partridge,1986 (personal communication) has suggested that this could be due to an increased folate lead complex being formed. An increased dosage (tenfold) of both lead acetate and folic acid might lead to increased excretion or retention in the tissues. Lead appears to interfere with folate metabolism in an as yet unknown way. Lead is not known to induce any oxidative activity like ferrous salts (Halliwell and Gutteridge, 1984). A knowledge of the effects of increased dosage on folate catabolism would be of great value.

MATERIALS AND METHODS

Fifteen female Wistar grade IV weanling rats (50-100 g body

wt) were each given a single oral dose of [3',5',7,9-^{3}H]-folic acid (100 µg/100 g body wt) prior to the administration of a single oral dose of lead acetate (31 µg/100 body wt). Urine, and faeces were collected for 24h periods upto 72h as described earlier (section 3.1). Animals and samples were handled as previously described (section 3.1).

RESULTS

All statistical differences are compared with the data in section 4.5 unless otherwise stated.

The recovery of radioactivity from the urine and faeces and the distribution of the retained radioactivity in the tissues is summarised in table 4.6.1. The majority of excreted radioactivity appeared in the 0-24h urine and faeces, 6.47% ^{3}H and 10.66% ^{3}H respectively. The fall in % ^{3}H levels in the urine is highly significant ($P<0.001$) while the increase in the faecal excretion is also significant ($P<0.05$). The later levels of excretion in the higher dosed animals being far lower in the urines; faecal % ^{3}H levels at 48h and 72h were significantly higher ($P<0.05$). Similar to the urine samples, the hepatic radioactivity retention at the higher dose was depressed, 3.77 % ^{3}H over 0-24h, 3.48 % ^{3}H over 24-48h and 4.68 % over 48-

Tissue Sample	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H	0-72 h
Urine	6.47 (1.69)	2.29 (0.044)	0.77 (0.12)	9.53
Faeces	10.66 (0.74)	4.85 (0.57)	3.05 (0.45)	18.56
Liver	3.77 (0.42)	3.48 (1.02)	4.68 (0.33)	11.93
Kidney	0.31 (0.035)	0.26 (0.05)	0.19 (0.05)	0.76
Gut	3.65 (1.47)	4.47 (1.08)	3.23 (0.48)	11.35
TOTAL				52.13

TABLE 4.6.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 0-24 h, 24-48 h and 48-72 h after a single oral dose of (3', 5', 7, 9 - ^{3}H) folic acid (100 µg/100 g body wt.), pretreated with a single oral dose of lead acetate (31 µg/100 g body wt.). The results are expressed as a percentage of the dose administered n = 5 ($\pm$ S.E.M.)

72h respectively ($P<0.001$). The retention of radioactivity in the kidney over 72h was significantly lower ($P<0.05$). Only 52.13 % ^{3}H was recovered over 0-72h.

URINARY METABOLITES

DE-52 ion exchange chromatography of urine samples were similar to those illustrated in section 4.4, in the 0-24h urine samples 4 intact folate metabolites were detected; folic acid (0.50 % ^{3}H), 5MeTHF (2.30 % ^{3}H), 10CHO-folate (1.40 % ^{3}H) and metabolite Y (0.40 % ^{3}H). A significant fall in the level of all metabolites was observed. Metabolite X was not detected except in trace at the 48-72h time period, metabolite Y was detected over 0-72h. 1.68 % ^{3}H was present as pAcABGlu over 0-72h while 1.35 % ^{3}H was present as pAcAB in these samples. Only trace amounts of $^{3}H_2O$ was detected in the treated animals. Due to very low levels of radioactivity in the urines the analysis here was incomplete.

LIVER EXTRACTS

Sephadex G-15 filtration of hot liver extracts revealed chromatograms similar to that illustrated in figure 3.1.4.

Metabolite	0-24 h ^{3}H	24-48 h ^{3}H	48-72 h ^{3}H
Folic acid	0.50	0.02	0.006
5MeTHF	2.30	1.20	0.21
10CHO-Folate	1.40	0.42	0.08
Metabolite X	ND	ND	*TRACE
Metabolite Y	0.40	0.30	0.08
pAcABGlu	1.30	0.18	0.20
pAcAB	1.10	0.15	0.10
$^{3}H_2O$	* TRACE	* TRACE	* TRACE

*Analysis here is incomplete due to low level of radioactivity in the urines

TABLE 4.6.2 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with a single oral dose of (3', 5', 7' 9, - ^{3}H) folic acid (100 µg/100 g body wt.), pretreated with a single oral dose of lead acetate (31 µg/100 g body wt.). Results are expressed as a percentage of the dose administered. n = 5.

SUMMARY

Qualitatively the handling of folic acid in all rats in section 4 was similar to normal rats but the following differences in the following sub-sections were observed.

(a) **FERROUS SULPHATE ADMINISTRATION** (10 mg/100g body wt)

(ai) Lower recovery of radioactivity in the urines at 24h.

(aii) Decreased retention in all tissue samples.

(aiii) Increased production of pAcAB over 72h.

(aiv) Overall increase in scission was observed.

(b) **LEAD ACETATE ADMINISTRATION** (3.1 µg/100g body wt)

(bi) Lower recovery of radioactivity in urine and faeces over 0-72h.

(bii) Decreased retention of radioactivity in the tissues.

(biii) Decreased excretion of intact folates especially 5MeTHF and 10CHO-folate.

(bv) pAcABGlu is the major ^{3}H catabolite over 0-48h.

(bvi) $^{3}H_2O$, urea, ^{14}C-only labelled catabolites detected in trace amounts.

(c) **INCREASED FOLIC ACID DOSE** (100 µg/100g body wt)

(ci) Increased radioactivity excretion in the urine especially after 24h.

(cii) Increased excretion of all intact folates.

(ciii) A general increase in ^{3}H-labelled catabolites.

(civ) Metabolite X was detected after 0-24h.

(cv) $^{3}H_2O$ detected over 0-72h.

(cvi) A new technique for folate analysis was introduced (section 2.1). New oxidation and/or non-oxidised folate polyglutamate or metabolites. A1, A2, C, D, E and F were detected.

(d) **INCREASED FOLIC ACID (100 µg/100 g BODY WT) AND INCREASED LEAD ACETATE ADMINISTRATION (31 µg/100g BODY WT)**

(di) A general decreased recovery of radioactivity in the

urine and all tissues over 72h.

(dii) Increased recovery of radioactivity in the faeces over 72h.

(diii) Decreased excretion of all metabolites, particularly intact folates.

(dv) Metabolite Y was detected in all urine samples.

(dvi) Folate catabolism is depressed and $^{3}H_{2}O$ was detected only as traces.

CHAPTER 5

THE EFFECT OF ALLOPURINOL ON FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

5.1.1 THE EFFECT OF ALLOPURINOL ON FOLIC ACID METABOLISM IN THE FEMALE WEANLING RAT

The administration of certain drugs has been associated with the developed of folate deficiency. (Hoffbrand (1977); Reynolds (1980); Saleh (1980); Guest, et al., (1983) and Al-Haddad (1984)). One such chemotherapeutic agent, allopurinol (used to treat gout), inhibits the enzyme xanthine oxidase and also acts as a substrate leading to the production of oxipurinol and superoxide (Figure 5.1.1), Pryor (1976).

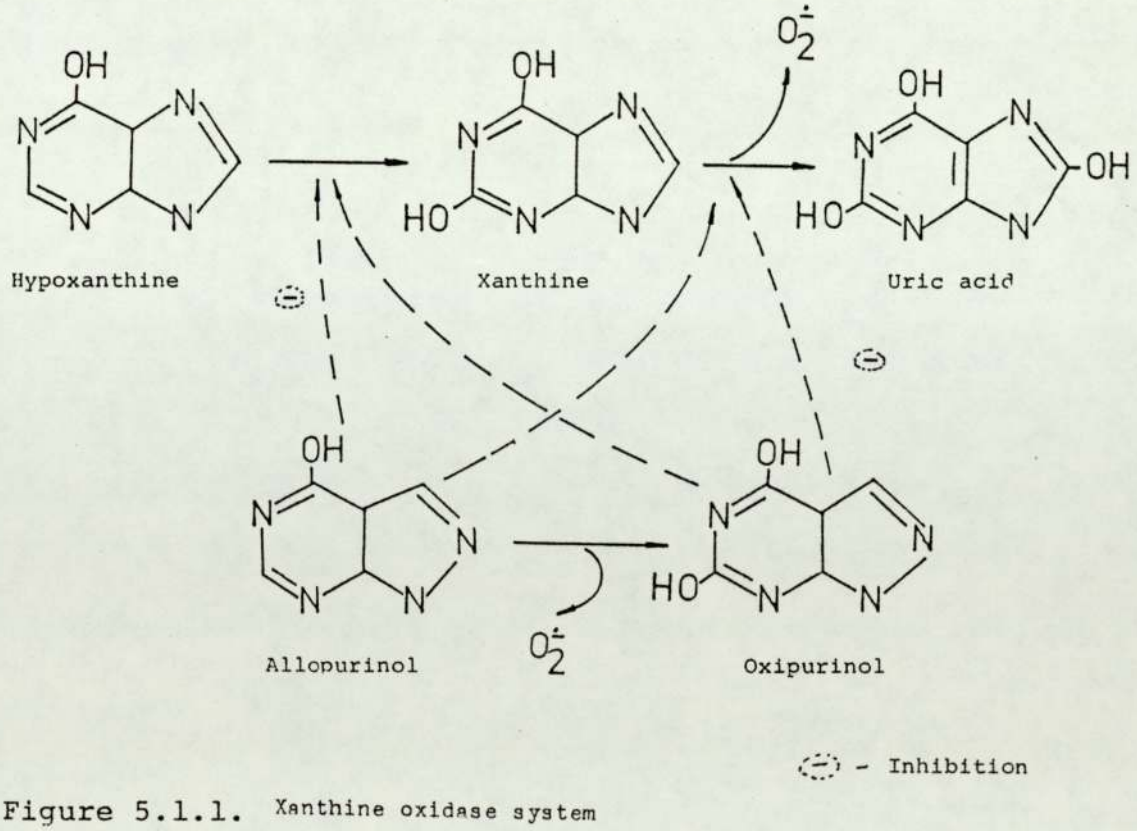


Figure 5.1.1. Xanthine oxidase system

EXPERIMENTAL

Solid tumour cells are known to exhibit anoxic conditions and have less xanthine oxidase activity. This could be a characteristic of proliferating cells which exhibit decreased scission, Saleh (1980), and Blair, et al., 1984. Administration of allopurinol to female weanling rat might provide valuable information regarding the mechanism of folate catabolism. This might also support the work carried out in section 4 which suggests oxidative activity due to Fe^{2+} administration resulting in increased catabolism. Hence there is a need to study allopurinol administration.

MATERIALS AND METHODS

Fifteen female Wistar grade IV weanling rats (50-100 g body wt) were dosed orally with allopurinol (20 mg/kg body wt) in 0.3 ml Mazola corn oil for 5 days prior to the administration of a single oral dose of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid (100 µg/kg body wt). Urine and faeces were collected for 24h periods upto 72h as described earlier (section 3.1). Groups of 5 animals were killed 24, 48 and 72h after the folic acid dose. Animals, tissues and samples were handled as previously described (section 3.1).

RESULTS

(All data is compared with normals). Table 5.1.1. illustrates the recovery of radioactivity in the urine and faeces and the tissue distribution of retained radioactivity from the group of rats, treated with allopurinol, after the administration of the isotopically labelled folic acid. The majority of excreted radioactivity appeared in the 0-24h urine and faeces, later excretion levels being far lower. The radioactivity excreted in the urine was significantly lower in the pretreated samples; 33.0 % ^{3}H and 30.85 % ^{14}C after 24h ($P<0.001$). For each time period quantitative analysis of the urine reveals an imbalance in the excretion of the two isotopes as more ^{3}H than ^{14}C was excreted, but this is not statistically significant over the entire 72h. More ^{14}C than ^{3}H was found in the faeces over 72h, this was only statistically significant over 0-24h ($P<0.05$). Similar levels of ^{3}H and ^{14}C were found in the liver, kidneys and the gut. The slight elevation of ^{3}H levels seen in the tissues were not statistically significant. Over the first 24h high levels of radioactivity are retained in the gut of the treated animals; 11.06 % ^{3}H and 10.75 % ^{14}C, this is statistically significant ($P<0.001$). The radioactivity retained in kidneys over the first 24h is high 4.11 % ^{3}H and 3.60 % ^{14}C ($P<0.05$).

Tissue Sample	0-24 h		24-48 h		48-72 h		0-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C		
Urine	33.0 (3.52)	30.85 (3.20)	5.54 (1.83)	5.40 (1.84)	2.25 (0.42)	2.10 (0.38)	40.79	38.35
Faeces	7.52 (0.51)	19.14 (0.56)	3.98 (0.15)	4.79 (0.15)	1.07 (0.36)	1.52 (0.36)	12.57	25.45
Liver	23.42 (1.50)	19.72 (2.71)	17.52 (1.37)	19.72 (2.11)	21.11 (2.49)	21.21 (2.24)	21.11	21.21
Kidney	4.11 (1.09)	3.60 (0.60)	1.80 (0.25)	1.97 (0.31)	2.39 (0.23)	2.48 (0.33)	2.39	2.48
Gut	11.06 (0.96)	10.75 (0.98)	6.06 (0.73)	5.80 (0.65)	3.67 (0.45)	3.65 (0.55)	3.67	3.65
TOTAL							80.53	91.14

TABLE 5.1.1 Radioactivity in the urine, faeces and tissues of healthy female weanling rats (50-100 g body wt.) 0-24 h, 24-48 h, and 48-72 h after being dosed with allopurinol (20 mg/Kg body wt) in Mazola corn oil (0.3 ml) daily for 5 days prior to the administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2 - ^{14}C) folic acid (100 µg/Kg body wt). Results are expressed as a percentage of the dose administered. (n = 5; $\pm$S.E.M.)

URINARY METABOLITES

DE-52 ion exchange chromatography of urine samples after 0-24h and 24-48h were similar to those illustrated in figures 3.1.1 and 3.1.2, the differences were noted on the 48-72h chromatogram. The 0-24h urine samples contained a mixture of labelled metabolites, folic acid excretion in pretreated animals is depressed to 1.80% ^{3}H and 2.00% ^{14}C. 5MeTHF was present in all samples, the highest being excreted after 24h (10.61% ^{3}H and 11.91% ^{14}C). The levels of 5MeTHF after 24-48h and 48-72h were unaltered, however, the 10CHO-folate levels were vastly depressed, over 0-24h only 6.10% ^{3}H and 8.31% ^{14}C were detected while later excretion values were much lower; 0.60% ^{3}H and 0.71% ^{14}C over 24-48h and 0.15% ^{3}H and 0.20% ^{14}C over 48-72h. Metabolite X was detected over 72h, 1.0% ^{3}H and 1.0% ^{14}C over 0-24h 0.30% ^{3}H, 0.23% ^{14}C over 24-48h and 0.11% ^{3}H, 0.09% ^{14}C over 48-72h. Metabolite Y was not detected after the first 24h but traces were evident over the next 48h. pAcAB was the main scission product excreted in the urine 8.54% ^{3}H was excreted as pAcAB after 0-24h. Over 0-72h a total of 11.44% ^{3}H was excreted as pAcAB. pAcABGlu was present in lower levels, 8.40% ^{3}H over 0-24h and over 0-72h a total of 9.68% ^{3}H was excreted as pAcABGlu. 3.46% ^{14}C was excreted as ^{14}C

Metabolite	0-24 h		24-48 h		48-72 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	1.80	2.00	0.16	0.17	0.03	0.02
5MeTHF	10.61	11.91	1.87	2.01	0.20	0.30
10CHO-Folate	6.10	8.31	0.60	0.71	0.15	0.20
Metabolite X	1.0	0.90	0.30	0.23	0.11	0.09
Metabolite Y	ND	ND	TRACE		TRACE	
pAcABGlu	8.40	-	0.98	-	0.30	-
pAcAB	8.54	-	2.10	-	0.80	-
^{14}C only labelled catabolite	-	1.70	-	1.00	-	0.76
Urea	-	0.1	-	0.1	-	ND
$^{3}H_2O$	1.0	-	0.3	-	0.2	-

TABLE 5.1.2 Metabolites detected in the urine of healthy female weanling rats (50-100 g body wt.) dosed with allopurinol (20 mg/Kg body wt.) in Mazola corn oil (0.3 ml) daily for 5 days prior to the administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid (100 µg/Kg body wt.). Results are expressed as a percentage of the dose administered. n = 5

only labelled scission products over 72h and 0.2% ^{14}C was detected as urea over 72h. $^{3}H_2O$ comprised 1.5% ^{3}H of the dose over 72h. Overall scission had increased to 32.0%.

LIVER EXTRACTS

Similar results were obtained using Sephadex G-15 gel filtration as in section 3.1.

5.2.1 THE EFFECT OF AN INCREASING ALLOPURINOL DOSE REGIME ON FOLATE CATABOLISM IN THE FEMALE WEANLING RAT

Allopurinol increases folate catabolism in the hamster (Al-Haddad, 1984) and in the female weanling rat (section 5.1). The aim of this section is to try to establish a relationship between an increasing allopurinol dose regime and a fixed folic acid dose (100 µg/kg body wt), the possible effects on folate catabolism and percentage retained label.

MATERIALS AND METHODS

Four groups of 15 animals were dosed with allopurinol, 5,8,10 and 15 mg/100g body weight respectively (as

described in section 5.1). Animals, tissue and urine samples were collected and treated as described in section 3.1.

RESULTS

Table 5.2.1 illustrates the recovery of radioactivity of radioactivity in the livers at 0-24 h, 24-48 h and 48-72 h. All results are compared with the normals. The extent of folate breakdown in terms of percentage pAcABGlu and pAcAB is also illustrated. The distribution of retained radioactivity in the kidneys, gut and the faeces will not be discussed as no significant changes from section 5.1 were observed. As the concentration of the allopurinol dose increased, the percentage ^{3}H of the dose retained in the livers descreased from 23.14% ^{3}H to 20.40% ^{3}H and leveled off at 20.30% ^{3}H (0-24h).
After 24-48h the percentage ^{3}H retained in the livers falls to 15.85% ^{3}H, an increase in the allopurinol dose did not exhibit any further fall in the ^{3}H levels. The overall fall is statistically significant ($P<0.05$). After 48-72h there is a slight elevation in the retained percentage ^{3}H but this is not statistically significant even when the dose is increased.

Allopurinol (mg/100 g body wt.)	% ^{3}H of the dose retained in the livers			Extent of Breakdown at 24-72 h		Total Scission
	0-24 h	24-48 h	48-72 h	% pAcABG	% pAcAB	
2	23.14 (±1.50)	17.56 (±1.37)	21.13 (±1.20)	9.97	22.03	32.0
5	21.83 (±1.0)	16.55 (±1.10)	19.20 (±1.31)	22.41	13.59	36.0
8	20.40 (±1.30)	16.37 (±1.0)	20.20 (±1.10)	27.89	10.41	38.30
10	20.30 (±1.25)	15.89 (±0.50)	16.86 (±0.80)	28.89	10.11	39.0
15	20.30 (±1.30)	15.85 (±1.20)	16.87 (±2.0)	27.85	10.36	38.21
Controls	16.85 (±0.53)	16.98 (±1.0)	21.22 (±1.60)	10.65	6.79	17.44

* Results are expressed as a percentage of the dose ± S.E.M. where appropriate

* n = 5

$$\text{Extent of breakdown} = \frac{\text{\% dose excreted as pAcABGlu 24-48h x 100\%}}{\text{\%Radioactivity retained at 24h.}}$$

TABLE 5.2.1 Percentage breakdown of folic acid in rats pretreated with allopurinol

URINARY METABOLITES

DE-52 ion exchange chromatography of urine samples after 0-24h and 24-48h were similar to those illustrated in figures 3.1.1 and 3.12, again all the differences were noted on the 48-72h chromatogram as in figure 5.1.1. The pattern of the metabolites detected was similar to that described in section 5.1.

However, as the dose increased the levels of percentage pAcAB falls rapidly from 22.03% ^{3}H (at the 2 mg dose) to 10.41% ^{3}H (8 mg dose) and then levels off. The percentage pAcABG detected increases from 9.97% ^{3}H (at 2 mg dose) to 28.89% ^{3}H (10 mg dose) this corresponds to an increase in dose.

LIVER EXTRACTS

Similar results were obtained using Sephadex G-15 gel filtration at all dose levels as shown in section 3.1.

SUMMARY

Folic acid was administered to allpurinol dosed rats (2 mg, 5,8,10 and 15 mg/100 g body wt). Qualitatively the handling of folic acid was similar in all dose regimes to normal rats but the following differences

were observed.

(i) Lower recovery of radioactivity in urine and faeces.

(ii) Higher retention of radioactivity in kidney and the gastrointestinal tract after 24 hours.

(iii) Intact folate excretion was lower especially 10CHO-folate.

(iv) Increased allopurinol dosage resulted in decreased radioactivity retention in the livers over 72h.

(v) A substantial increase in all scission products with pAcAB dominating at low allopurinol doses, while pAcABGlu dominates at all higher allopurinol dose levels.

(vi) Increase in the allopurinol dose levels leads to an increase in total folate scission.

CHAPTER 6

QUALITATIVE AND QUANTITATIVE OXIDATIVE ACTIVITY ANALYSIS

6.0.1 IN VIVO OXIDATIVE STUDIES IN THE FEMALE WEANLING RAT

Experimental data presented in sections 4 and 5 illustrate the increase in folate catabolism in rats pretreated with ferrous sulphate and allopurinol. While in section 2 *in vitro* studies illustrate the production of superoxide anion by the xanthine oxidase system. Ferrous sulphate administration is known to induce increased oxidative activity due to the production of "oxy-radicals", Aust *et al.*, 1985; Gutteridge (1984) and Anderson and Means (1985). Evidence for increased oxidative activity in weanling rats dosed with allopurinol or ferrous sulphate is limited. Hence there is a need to investigate increased oxidative activity in weanling rats dosed with ferrous sulphate and allopurinol.

Oxidative activity can be investigated by two main methods:

(i) Chemiluminescence assay. Arneson (1970); Allen *et al.*, (1972); Van Dyke *et al.*, (1977); Nelson *et al.*, 1977 and Boveris *et al.*, 1983).

(ii) Nitroblue tetrazolium reduction (Repine *et al.*, (1979)

and Lewis (1984) personal communication).

The former technique is expensive and not quantitative and therefore not suitable for our investigations hence the nitroblue tetrazolium (NBT) reduction was employed. This technique can be qualitative and quantitative. Oxidative activity is followed by a histological technique (qualitative) whereby increased oxidation results in the NBT being reduced from yellow to blue formazan (Figure 1.r). Secondly this can be followed quantitatively by modifying the method of Repine, et al., (1979) whereby homogenised tissue samples are used. The aims and objectives of this section were:

To qualitatively illustrate oxidative activity (if any) as a result of "oxy-radicals" in ferrous sulphate and allopurinol dosed rats.

To quantitatively investigate the oxidative activity (if any) in the presence of ferrous ions.

Thirdly to obtain dose response relationships in the allopurinol and ferrous sulphate pretreated rats.

Finally to confirm the presence of "oxy-radicals" by using known scavengers of the suspected oxy-radicals.

EXPERIMENTAL

This experimental approach was chosen as it may help to elucidate the mechanism of folate catabolism as initially proposed by Blair (1958), Pheasant, et al., 1981, Guest (1984) and Al-Haddad (1984).

6.1.1 HISTOLOGICAL ILLUSTRATION OF OXIDATIVE ACTIVITY IN ALLOPURINOL AND FERROUS SULPHATE PRETREATED FEMALE WEANLING RATS

This section aims to illustrate qualitatively the in vivo oxidative activity due to superoxide anions in the female weanling rat. The livers of these animals were chosen because it is the major site of metabolism and detoxification as shown by earlier studies (sections 3,4 and 5).

MATERIALS AND METHODS

Thirty rats were divided into six groups of five. The first group of five were allowed access to normal Pilsbury rat breeding diet (Pilsbury Limited, UK) and water, the second group were treated as the first but were then dosed orally (0.3 ml) with Mazola corn oil for 5 days. The third group of animals were dosed orally with allopurinol (20 mg/kg body wt) for 5 days and allowed free access to food and water as with the second group. The fourth group of animals were

treated as with the third group, but these animals were also given a single oral dose (each day for 5 days) of vitamin E (α-tocopherol acetate) 5 mg/100g body wt. The fifth group of rats were given a single oral dose of ferrous sulphate 10 mg/100g body wt and allowed free access to food and water. The last group of animals were treated as the fifth group but were also dosed with vitamin E as in group four.

After each dose regime the animals were killed by cervical dislocation, the livers were removed and washed in ice-cold isotonic saline. They were then cut into small pieces and mounted on a microtome (M.S.E. Limited, London, UK) via an epoxy resin and liquid nitrogen. The frozen pieces were sectioned (6-8 μm thick) and mounted onto glass microscope slides and incubated with NBT using a modified method of Repine, et al., (1979) (as described earlier 2). The prepared slides were photographed using an Olympus 35 mm SLR camera mounted on Zeiss microscope (Karl Zeiss Jena Co. E. Germany). The film used was a standard Kodak 35 mm - 400 ASA.

RESULTS

Figure 6.1.1 illustrates the histological results obtained from both the control (groups 1 and 2).

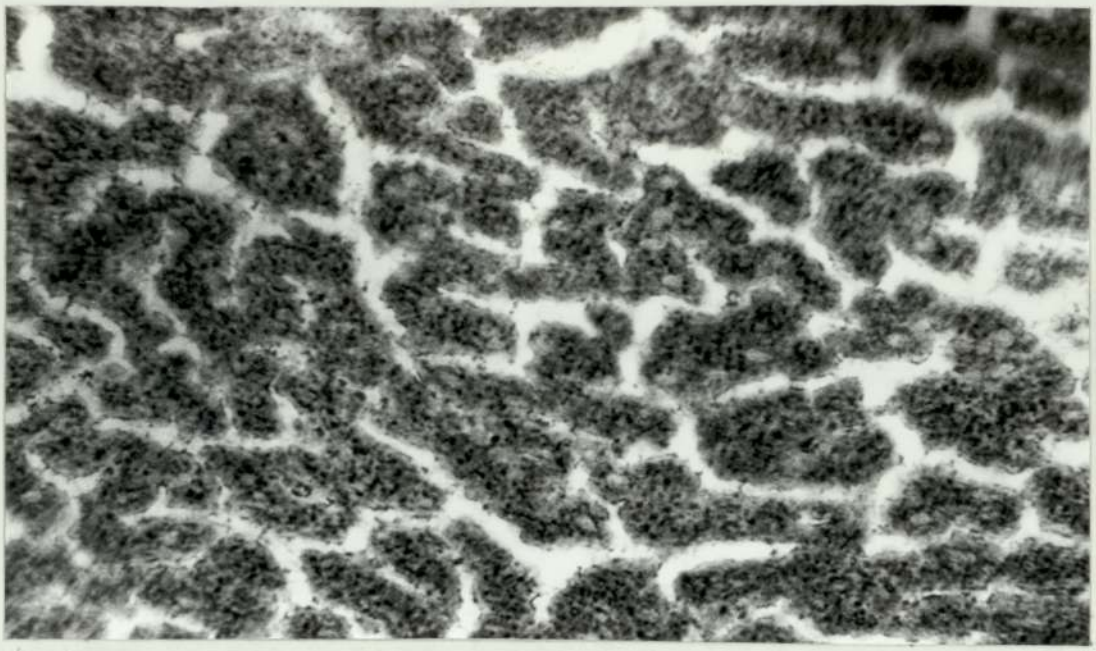


Figure 6.1.1. Livers of control rats.
(x 160 magnification)

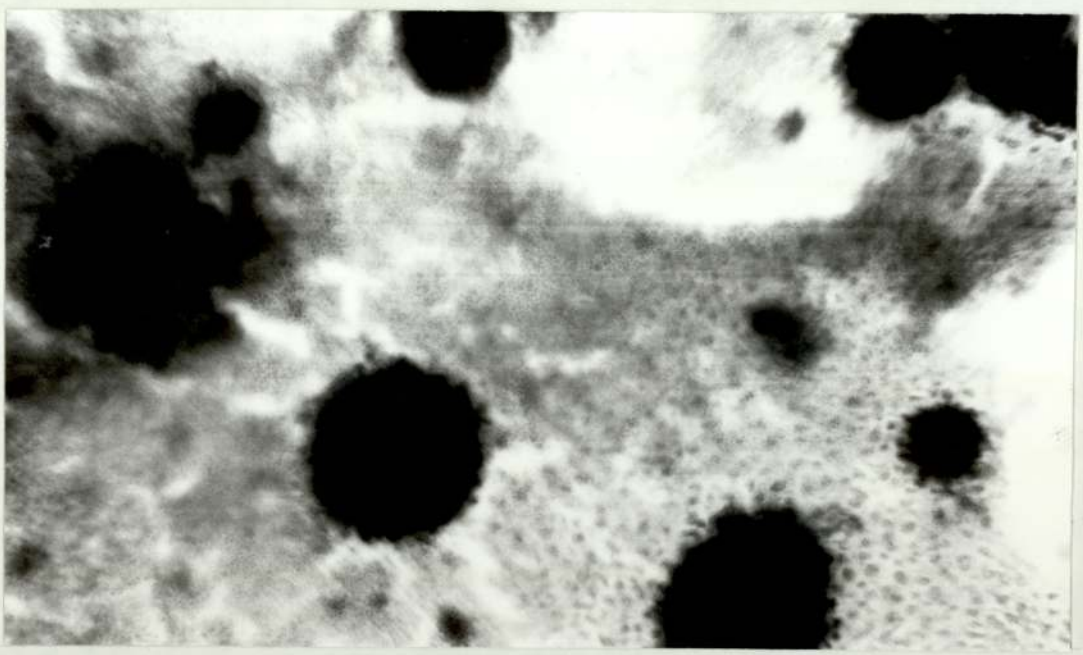


Figure 6.1.2. Livers of allopurinol pretreated rats.
(x 160 magnification)

Small dark blue crystalline granules were present in these liver samples. Oxidative activity was evident in all liver parechyma cells. Activity was not specifically localised.

However, upon examination of liver samples from allopurinol pretreated animals (group 3), the intensity and size of the granules were larger and localised. This is clearly illustrated in figures 6.1.2 and 6.1.3. Figure 6.1.4. illustrates the results obtained in rats pretreated with allopurinol and then dosed with vitamin E. In these samples the oxidative activity was greatly reduced but formazan granule deposition was localised. The liver samples of ferrous sulphate dosed rats (figure 6.1.5) exhibited even greater activity and deposition of formazan granule cluster. Formazan deposition was localised into large clusters, the whole cells were stained with formazan deposition. Administration of vitamin E to ferrous sulphate pretreated rats resulted in the decreased size of the granule clusters (figure 6.1.6). The intensity of colouration was not reduced to the normal levels as shown in figure 6.1.1. These effects in figures 6.1.4. and 6.1.6 could be due to more than one oxidative species.

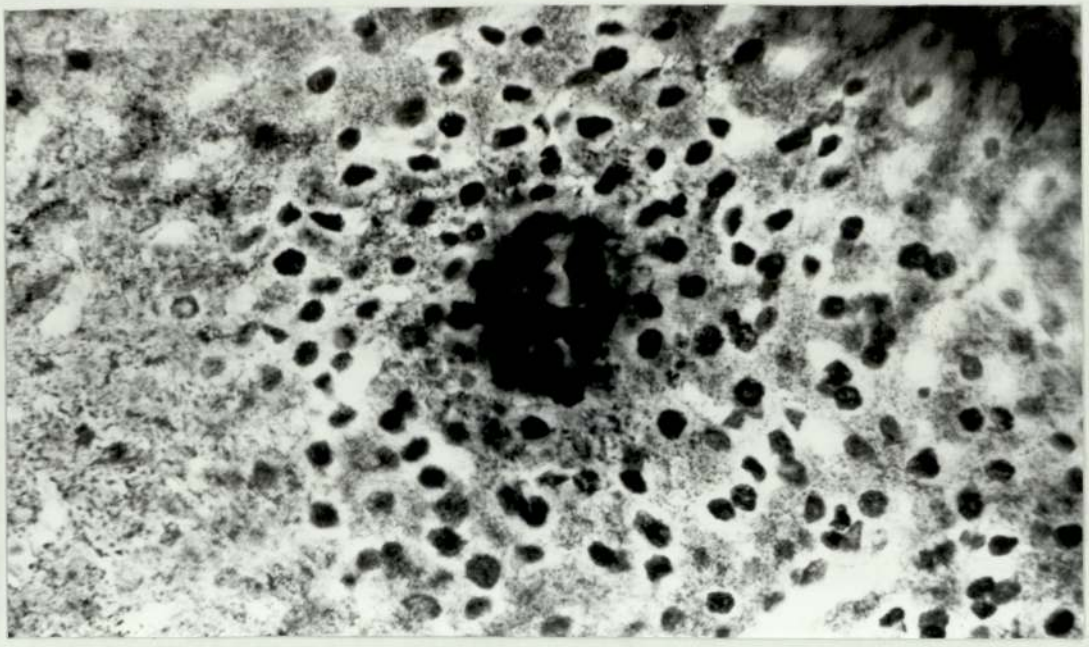


Figure 6.1.3. Livers of allopurinol pretreated rats. (x 400 magnification)

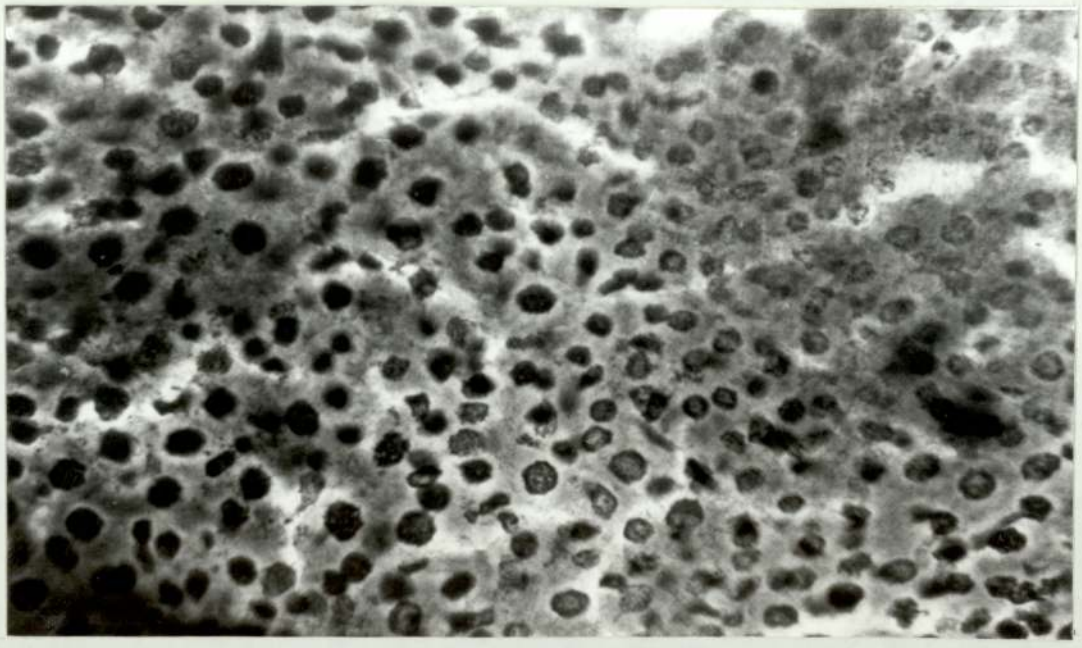


Figure 6.1.4. Livers of allopurinol and vitamins E treated rats. (x 160 magnification)

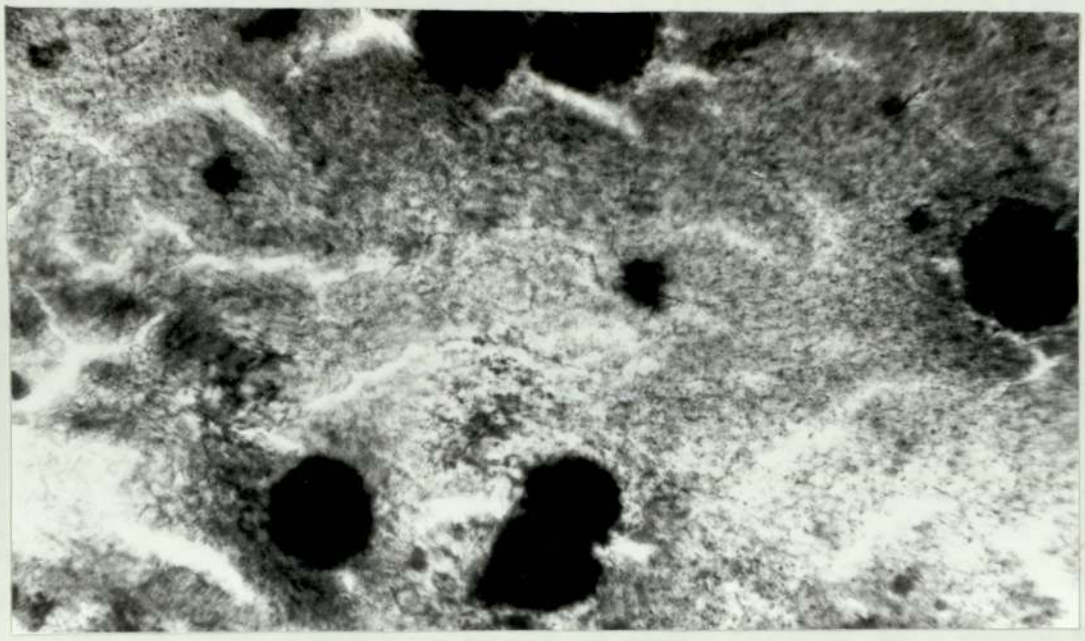


Figure 6.1.5. Livers of ferrous sulphate pretreated rats. (x 160 magnification)

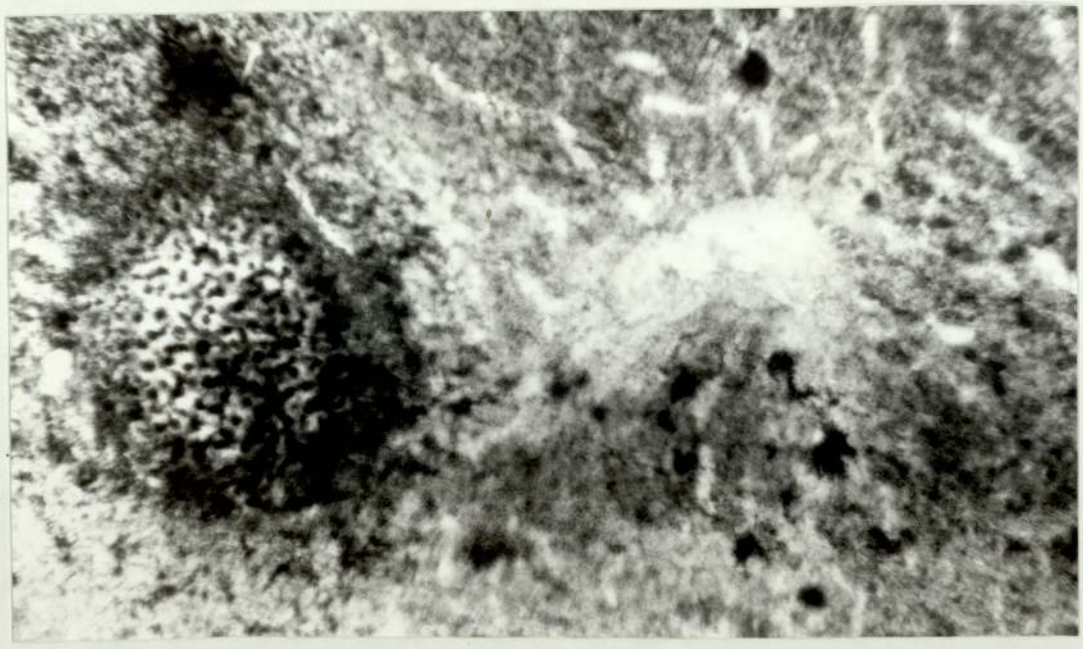


Figure 6.1.6. Livers of ferrous sulphate dosed rats pretreated with vitamin E. (x 160 magnification)

6.2.1 QUANTITATIVE ESTIMATION OF OXIDATIVE ACTIVITY IN THE FEMALE WEANLING RAT

The aims and objectives of this section were to quantitatively investigate oxidative activity in ferrous sulphate and allopurinol pretreated rats. The presence of "oxy-radicals" was investigated by using known radical scavengers. The possibility of dose-reponse relationships in allopurinol and ferrous sulphate pretreated rats was also investigated.

MATERIALS AND METHODS

(a) Seven groups of 5 rats were used, the first group of 5 were treated as the first group in section 6.1, but the livers and brains were removed. The next five groups of 5 animals (in each) were dosed orally with the following doses of allopurinol, 2,5,8,10 and 15 mg/100g body wt respectively. A further group of five animals were dosed with 2 mg/100g body wt allopurinol followed by vitamin E, all for 5 days as above.

(b) Eight groups of 5 animals were used, the first five groups were given a single oral dose of ferrous sulphate at the following doses; 5,10,15,18 and 20 mg/100 body wt. The final 3 groups were dosed orally with 10 mg/100g body wt ferrous sulphate followed by

vitamin E (5 mg/100g body wt), and intraperitoneally with mannitol (10 mg/100 g body wt), and 4 hydroxy-methyl 4.6 ditertiarybutyl phenol (10 mg/100g body wt) respectively.

At the end of each period rats from each group were killed by cervical dislocation and the livers and brains removed for analysis.

The removed tissues were washed with Hanks balanced media and homogenised, centrifuged (4000 g) and incubated at 37°C with Hanks balanced media containing 0.1% w/v NBT for 20 minutes. After incubation the samples were centrifuged (4000g) for 20 minutes and the supernatant discarded. The pellet was resuspended in boiling absolute pyridine, the colour extracted and analysed at 560-580 nm using Pye-Unicam spectrometer (Pye-Unicam Limited, UK). Repine, et al., (1979).

RESULTS

Table 6.2.1 illustrate the effect of increased ferrous sulphate concentration on oxidative activity measured as formazan (m mol/g tissue) in brains and livers of female weanling rats. Upto a concentration of 18 mg/100g body wt, oxidative activity in the livers is higher than the brains but

Ferrous Sulphate (mg/100g body wt)	Oxidative Activity as [Formazan] m mol/g tissue	
	Livers	Brains
5	0.37 ($\pm$ 0.065)	0.26 ($\pm$ 0.047)
10	0.46 ($\pm$ 0.05)	0.25 ($\pm$ 0.065)
15	0.87 ($\pm$ 0.044)	0.66 ($\pm$ 0.06)
18	1.17 ($\pm$ 0.22)	1.08 ($\pm$ 0.014)
20	1.43 ($\pm$ 0.12)	1.65 ($\pm$ 0.32)
CONTROLS	0.31 ($\pm$ 0.034)	0.32 ($\pm$ 0.03

(Results are expressed as a mean of 5, $\pm$ S.E.M.)

TABLE 6.2.1 To illustrate the effect of increased Ferrous sulphate dose on oxidative activity as [Formazan] m mol/g tissue

at a concentration of 20 mg/100g body wt ferrous sulphate appears to induce increased oxidative activity in the brain (1.65 (± 0.32) formazan mmol/g tissue) while the activity in the livers is lower (1.43 (± 0.12) formazan mmol/g tissue). Hence a dose response relationship is evident in both the brain and liver samples. The increased response over 5-20 mg/100g ferrous sulphate is statistically significant ($P<0.05$).

Table 6.2.2 illustrate the effect of increased allopurinol concentration on oxidative activity measured as formazan (mmol/g tissue) in the brains and livers of female weanling rats. The livers exhibit a higher level of oxidative activity (0.62 (± 0.039) mmol/g tissue of formazan) than the brains 0.35 (± 0.015) mmol/g tissue of formazan. This is statistically significant over the whole dose regime ($P<0.001$). The highest levels of oxidative activity is detected in the livers (2.85 (± 0.11) mmol/g tissue of formazan at a 10 mg/100g body wt allopurinol dose). From table 6.2.2 it is also evident that a higher dose of allopurinol is required to induce an optimum level of oxidative activity in the brain of the female weanling rat. Table 6.2.2 illustrates the dose-response relationship obtained in the livers and

Allopurinol dose (mg/100g body wt)	Oxidative Activity as Formazan m mol/g tissue		* % Total Folate Scission
	Livers	Brains	
2	0.62 (± 0.039)	0.35 (± 0.015)	32.0
5	0.66 (± 0.085)	1.0 (± 0.11)	36.0
8	1.752 (± 0.075)	1.11 (± 0.13)	38.30
10	2.85 (± 0.11)	1.16 (± 0.06)	39.0
15	2.82 (± 0.215)	1.31 (± 0.095)	38.21
CONTROLS	0.31 (± 0.034)	0.32 (± 0.03)	17.44

* [Extent of polyglutamate breakdown = $\frac{\text{\% dose excreted as pAcABGlu 24-48h x 100\%}}{\text{\%Radioactivity retained at 24h.}}$]

Results are expressed as a percentage of the dose (n = 5, ± S.E.M. where appropriate)

TABLE 6.2.2 To illustrate the effect of increased allopurinol dose on oxidative activity and folate scission.

brains of allopurinol dosed female wearling rats. A biphasic effect is evident. Oxidative activity in the livers levels off at the 10 mg/100g body wt dose level. This could be due to saturation levels of allopurinol.

When allopurinol pretreated rats table 6.2.3 were also dosed with vitamin E oxidative activity in the brains and livers was depressed (brain $P<0.05$; livers; $P<0.001$) this confirms the presence of "oxy-radical"(s).

In comparison, when ferrous sulphate pretreated rats (table 6.2.3) were given vitamin E a fall in oxidative activity was evident, this is not statistically significant for the brains, however in the livers this is highly significant ($P<0.001$). Known scavengers of OH ions administered to ferrous sulphate pretreated rats, (eg, mannitol) depressed the oxidative activity in both the brains and the livers. This was only significant ($P<0.001$) for the livers.

4, hydroxy-methyl 4,6 ditertiarybutyl phenol administration to ferrous sulphate dosed rats resulted in decreased oxidative activity only in the liver samples and this was statistically significant ($P<0.001$). This is a positive test for OH radicals).

Experimental Regime	Oxidative Activity as Formazan m mol/g tissue	
	Brain	Liver
a) Normal	0.32 (± 0.315)	0.31 (± 0.034)
Allopurinol	0.35 (± 0.015)	0.62 (± 0.039)
Ferrous sulphate	0.25 (± 0.065)	0.46 (± 0.05)
b) Calculations based on radical scavenger experiments		
Allopurinol + vitamin E	0.22 (± 0.025)	0.39 (± 0.08)
Ferrous sulphate + vitamin E	0.21 (± 0.027)	0.17 (± 0.012)
Ferrous sulphate + mannitol	0.23 (± 0.02)	0.29 (± 0.012)
Ferrous sulphate + 4 hydroxy-methyl 4,6 ditertiarybutyl phenol	0.25 (± 0.015)	0.30 (± 0.02)

Results are expressed as a mean (n = 5; ± S.E.M.)

TABLE 6.2.3 Quantitative estimation of Formazan m mol/g tissue in normal, allopurinol and Ferrous sulphate pretreated rats.

SUMMARY

Oxidative activity measured as [formazan] mmol/g tissue detected was investigated in allopurinol and ferrous sulphate pretreated animals.

The following qualitative and quantitative observations were made:

(i) Qualitative studies histologically illustrated increased oxidative activity in the livers of allopurinol and ferrous sulphate dosed animals.

(ii) Oxidative activity was depressed, upon vitamin E administration.

(iii) Quantitatively, increased allopurinol dosage resulted in increased oxidative activity, a dose response was obtained.

(iv) The livers of allopurinol pretreated rats exhibited more oxidative activity than the brains.

(v) Similar observations as (iii) and (iv) were made for ferrous sulphate pretreated animals.

(vi) Radical scavenger experiments suggest the presence of $O_2^{\cdot-}$, $OH^{\cdot}$ and other "oxy species.

(vii) Overall increased oxidative activity as a result of allopurinol and ferrous sulphate administration.

CHAPTER 7

DISCUSSION

DISCUSSION

These studies highlight the complexity of folate metabolism in the female weanling rat. Folic acid is metabolised to a variety of reduced folate monoglutamate derivatives and to high molecular weight polyglutamate forms in the tissues. Several catabolites are also found in the urine. Similar observations have already been made in the adult rat, (Barford, et al., 1978; Connor, 1979; Pheasant, et al., 1981; Saleh, 1982 and Guest, 1984), the guinea pig (Choolun, 1980) and the Syrian golden hamster (Al-Haddad, 1984).

The scission products are produced by cleavage of the folate molecule (figure 7.1.1) giving ^{3}H-labelled

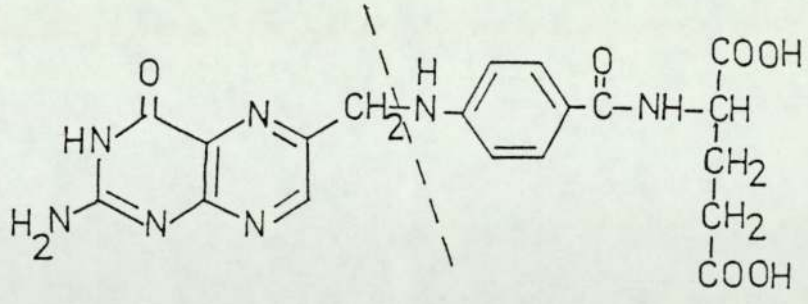


Figure 7.1.1 C9-N10 scission of folic acid

pABGlu derivatives and a dual labelled pterin which undergoes further metabolism losing the ^{3}H label before being excreted as ^{14}C - only labelled fragment. The pteridine ring is also cleaved as indicated by the detection of a ^{14}C labelled urea in the urine samples. These observations indicate the

existence of a catabolic process in the female weanling rat. These studies also support the presence of two metabolically distinct folate pools (Krumdieck, et al., 1978). Pheasant et al., 1981 suggested a scheme to explain these observations whereby folate derivatives in vivo are divided into two pools:

One with a half life of about 1 day in the rat (Saleh, 1981) and 31.5 h in man (Krumdieck, et al., 1978) and another with an estimated half life of 11 days in the rat (Saleh, 1981) and 100 days in man Krumdieck, et al., (1978). (Details of the consituents of these pools are discussed in chapter 1).

Analysis of urinary folate metabolites in the female weanling rats dosed with D.E.S and 17α-ethynyloestradiol revealed quantitative differences when compared with the normals. The marked increase in the urinary folate in the D.E.S pretreated animals was similar to the results obtained in women using oral contraceptive agents (Shojania, et al., 1975; Prasad, et al., 1975 and Shojania 1982).

Tollomelli, et al., (1972) have suggested that there is decreased excretion of intact folates in rats pretreated with β-estradiol. A similar effect was found

in the present work on the 17α-ethynyloestradiol pretreated rats where there is a marked decrease in 10 formylfolate; from 16.40% ^{3}H and 17.70% ^{14}C in the normals at 24h to 4.59% ^{3}H and 4.08% ^{14}C in pretreated rats. Similar observations have been made by Laffi, et al., 1972. In contrast, the 10-formylfolate levels in the D.E.S treated animals are similar to the normals (17.56% ^{3}H and 15.40% ^{14}C over 0-24h).

Eggar et al., 1983 have suggested that there is a major shift towards 10-formylfolate in adult female rats but work carried out in the chick by Burns and Jackson (1982) have supported the findings in the female weanling rats, where 17β-oestradiol administration to chicks caused a statistically significant ($P < 0.001$) decrease in 10-formylfolate synthetase activity. The activity of dihydrofolate reductase (DHFR) was also found to be elevated, this explains the reduction in urinary excretion of unchanged folic acid. Anderson, et al., 1976 and Burns and Jackson (1982) have suggested that oral contraceptive agents can diminish conversion of folic acid to the coenzyme forms. Experimental evidence up to now is limited. The kidneys of the D.E.S pretreated rats exhibit an increase in radioactivity retention, this is in agreement with results obtained by Lakshmaiah and Bamji (1979).

They also show as presented here that faecal excretion of folates is lower in ethynyloestradiol pretreated rats. It could be that the equilibrium between circulating folates and retained folates is a slow process (Barford, et al., 1977). The results presented suggest that the half life of the rapid turnover rate of the labile pool (circulatory folates) appear to be reduced whereas that of the slow turnover rate, stable pool (retained folates) is raised by estrogen treatment.

Estrogen treatment appears to impair this further by a mechanism not clear at present. This impairment may lead to increased urinary excretion as in D.E.S pretreated rats. (Dacosta and Rothenberg 1974). The induction of microsomal enzymes by contraceptive agents (Labadarios, et al., 1978 and Maxwell, et al., 1972) might accelerate folate metabolism. This has been shown in mice, Briatico, et al., (1976).

The folate polyglutamate body burden may be used to assess the extent of polyglutamate breakdown using the formula:-

Extent of breakdown

$$= \frac{\text{\% dose excreted as pAcABGlu 24-48h x 100\%}}{\text{\%Radioactivity retained at 24h.}}$$

Group	% Scission as pAcAB (at 24-28 h)	% Scission as pAcABGlu (at 24-28 h)	% Scission as pAcAB (at 48-72 h)	% Scission as pAcABGlu (at 48-72 h)	Extent of Total Scission %
Normal Female	9.66	4.66	1.44	1.68	17.44
diethylstilboestrol treated	6.44	14.56	8.06	4.44	33.50
17 ethynyloestradiol treated	3.08	14.44	2.58	2.58	22.68

(Calculations are based on radioactivity retained in the livers at 24 h and 48 h)

TABLE 7.1.1 Extent of scission in terms of polyglutamate breakdown following the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2-^{14}C) folic acid to normal rats and those treated with diethylstilboestrol and those treated with 17α ethynyloestradiol

DISCUSSION

Administration of estrogens induces increased folate scission, 17.44% in the normals, 33.50% and 22.68% in the D.E.S and 17α-ethynyloestradiol pretreated rats respectively. (Table 7.1.1). The precise mechanism by which cleavage of the folate molecule occurs is not known. Increased scission *in vivo* could produce folate deficiency, expressed as the clinical condition of megaloblastic anaemia, this is substantiated by a high histidine dose resulting in increased formimino glutamate being excreted in the urine, Shojania, *et al.*, (1982). Increased folate catabolism is one of the main conditions that results in folate deficiency. It was therefore decided to investigate potential agents which may provide a valuable insight into a possible mechanism for folate scission. *In vivo* evidence provided in section 2 suggests that increased superoxide anion production occurs in the reactions promoted by the enzyme xanthine oxidase *In vivo* both xanthine and allopurinol appear to produce; superoxide anion, hydrogen peroxide and hydroxyl ions (Massey and Verger, 1963; McCord and Fridovich, 1968; Yamazaki 1969; Massey, *et al.*, (1970); Pryor, 1976; Bannister and Hall, 1979; Blair, *et al.*, 1984 and Al-Haddad, (1984). The administration of allopurinol (20 mg/kg body wt) resulted in a decrease in 5-MeTHF levels, 13.70% ^{3}H and 17.83% ^{14}C in the normals while 10.61% ^{3}H and

11.91% ^{14}C was detected in the allopurinol pretreated animals. The percentage folic acid recovered was similar to the normals, but as shown in the Syrian golden hamster (Al-Haddad, 1984) there was a marked increase in the recovery of pAcABGlu and pAcAB in the allopurinol pretreated animals. This was evident over 72 h and is reflected in the total extent of scission (Table 7.1.2).

Total scission in the normal weanling rats was 17.44%, while in the allopurinol pretreated animals the total scission increased to 32.0%, 36.0%, 38.30%, 39.0% and then falls to 38.21% for a 20-150 mg/kg body wt dose regime respectively.

Allopurinol increased the oxidative status of the liver over the dose range of 20-100 mg/kg (figure 7.1.2). Correspondingly the hepatic retention of radioactivity was greater in the allopurinol pretreated rats than undosed controls over the first 24h. At the lowest dose of 20 mg/kg body wt the amount of radioactivity retained was (37%) greater than in the controls. At higher doses of allopurinol the amount of ^{3}H declined. At the two highest doses of 100 mg and 150 mg allopurinol per kg the ^{3}H detected in liver was still 20% greater than in the controls.

Group/Treatment	% Scission as (at 24-48 h)		% Scission as (at 48-72 h)		Extent of Total Scission %
	pAcAB	pAcABGlu	pAcAB	pAcABGlu	
Normals	9.66	4.66	1.44	1.68	17.44
Allopurinol	17.94	8.36	3.42	2.28	32.0
Ferrous sulphate	16.82	7.24	2.60	2.60	29.26
Lead acetate	1.75	9.73	1.39	1.55	14.42

(Calculations are based on radioactivity retained in the livers at 24 h and 48 h)

TABLE 7.1.2 To illustrate total scission in normal, allopurinol, ferrous sulphate and lead acetate pretreated rats

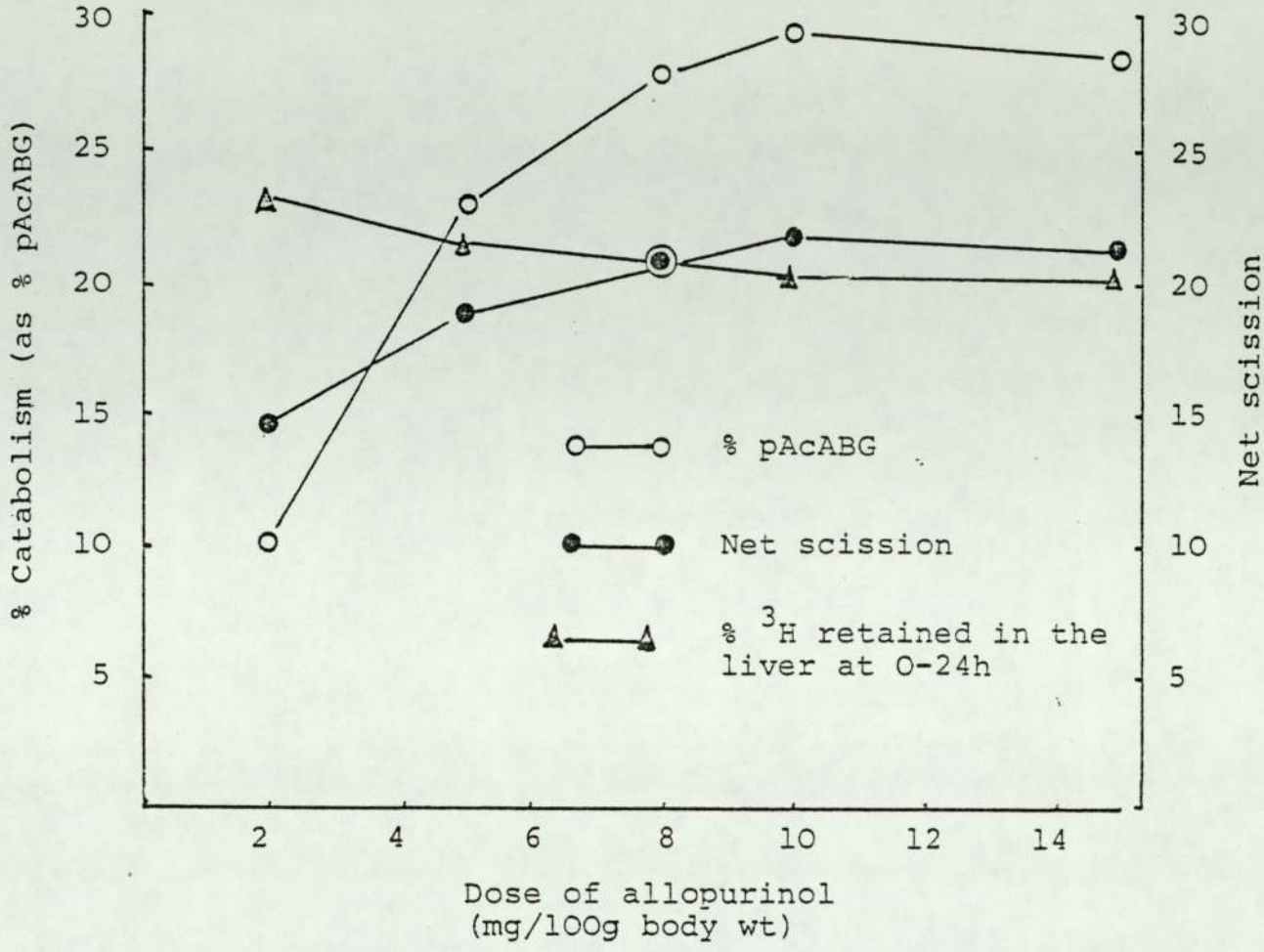


Figure 7.1.2.

Folate breakdown and scission in relation to % ^{3}H retained in the livers of allopurinol dosed rats.

DISCUSSION

The hepatic retention and the increase in scission appears to be related to an increase in oxidative activity in the livers animals pretreated with allopurinol.

The results of the histological examination described in section 6 shows the effect of allopurinol (20 mg/kg body wt) administration on oxidative function in liver. Treatment with NBT results in formazan deposition. These large aggregates were absent in the untreated rats, or rats that had been dosed with both allopurinol and vitamin E where formazan granules were small, infrequent and diffuse. Quantitatively formazan deposition was increased in livers after allopurinol administration but not in brains.

Triggs and Willmore (1984), and Aust et al., 1985, have demonstrated the involvement of oxy-radicals in the induction of in vivo lipid peroxidation in the adult rat treated with ferrous salts. It was decided to investigate the effect of ferrous sulphate on folic acid metabolism. (Section 4). Qualitatively the handling of folic acid was similar to normal female weanling rats but the levels of radioactivity recovered from the urine of dosed animals was lower; 33.12% ^{3}H compared to 39.51% ^{3}H in the normals, ($P<0.001$) over 0-24h. More radioactivity was

recovered in the urines over the 48 hours, suggesting delayed transport of folates. The greatest fall in retained radioactivity was observed in the livers ($P < 0.001$ over 72h). This suggests an interference in the long term folate pool (retained folates). A significantly lower ($P < 0.05$) degree of retention was observed in the kidneys and the gut. The most striking feature in this experiment was the high extent of folate catabolism 32.0% in the treated rat compared to only 17.44% in the controls. Histological studies show that ferrous sulphate treatment increased formazan deposition both qualitatively and quantitatively (section 4 and 6). Since ferrous sulphate was administered orally it was necessary to study the uptake and distribution of iron in vivo (section 4). Most of the administered iron is not absorbed but is excreted with the faeces but the liver which is the major drug metabolizing organ took up 7% of the iron (24-72h). Up to 24h it appears likely that the bulk of absorbed iron was circulating in the blood. There are four possible mechanisms whereby the metabolism of folate can be altered by ferrous sulphate, these are:

i. Folate/Fe^{2+} complex in the gut which is not absorbable. This could explain the lower levels of folate in the urine and tissues.

ii. The oxidation of Fe^{2+} to Fe^{3+} which could be a potential agent for reduced folate oxidation.

iii. The oxidation of Fe^{2+} to Fe^{3+} leading to the production of $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ radicals all contributing to increased folate scission. (Hill, 1981 and Gutteridge et al., (1983).

iv. A combination of i, ii and iii. This would be more favourable and can be supported by the studies presented here.

The complexing of metal ions with folates would make them less amenable for transport due to molecular size, Coleman (1979).

Waprir, et al., (1975) and Nechay and Saunder (1978) have demonstrated that low levels of lead inhibit sodium - potassium dependent ATPase resulting in a decreased hydrolysis of ATP and reduced production of hydrogen ions, consequently the folate zwitterions formed would be reduced hence decreasing intestinal transport of folate species.

It was decided to investigate the metabolism of folates in lead pretreated rats because lead does

(section 4) not produce oxy-species (Blair, 1985 personal communication). Radioactivity detected in the urine was markedly down over 0-72h; 17.46% ^{3}H and 16.43% ^{14}C was present in the urine of treated animals compared to 39.51% ^{3}H and 36.77% ^{14}C in the normals ($P < 0.001$).

There was an increase in the radioactivity detected in the faeces upto 72h after dosing. The difference in the ^{3}H: ^{14}C ratio over 24-48h and 48-72h was not statistically significant, suggesting impaired metabolism. Lead also appeared to reduce the levels of radioactivity in the G.I tract, kidneys and livers (section 4). There were distinct differences in the levels of metabolites detected in the urine. Less intact folic acid appeared in the urine after 24h with levels rising in the last 48h compared to the normals. The excretion of intact folates was lowered over 72h and a new metabolite X previously identified as 5,10CH_2THF was detected after 48h (0.03% ^{3}H and 0.03% ^{14}C). This fall in the levels of metabolites could be due to an increased lead/folate complex formations as has been demonstrated in lead acetate pretreated female weanling rats (Partridge,1986).

Reciprocal experiments using radiolabelled lead and folic acid have yielded similar data (Table 7.1.3),

Tissue/Sample	Control	+ Folate
Urine	0.98 (±1.05)	0.14 (±0.06)
Liver	1.46 (±1.15)	0.47 (±0.36)
Kidney	2.24 (±1.89)	0.67 (±0.54)
Faeces	94.05 (±14.41)	86.33 (±16.8

* Results are expressed as a percentage of the dose ± S.E.M.

* n = 5

TABLE 7.1.3 The effect of folic acid (100 µg/Kg body wt.) on lead acetate (3.1 µg/Kg body wt.) uptake. (studies used ^{203}Pb as a carrier)

(Data supplied by Partridge, 1986)

Partridge (1986). Folate appeared to reduce lead uptake in the urine, liver, kidneys and faeces.

However, the extent of total folate scission was lower than the in normals (14.42% in lead treated animals while it was 17.44% in the normals) although lead acetate pretreated animals did exhibit increased pAcABGlu excretion over 24-48h. Coleman, et al.,(1979) have shown that folic acid and 5MeTHF transport into the serosal compartments was unaffected in the presence of lead.

Saleh (1981) has shown that increasing the folate dose in adult rats results in the increased excretion of intact folate in the urine ($P < 0.001$). Increasing the dose tenfold resulted in the detection of metabolite X and metabolite Y. The levels of radioactivity retained in the tissues at both dose levels (section 4) were similar and no significant differences were observed except in the faeces after 0-72h where excretion was elevated ($P < 0.05$).

However, the levels of radioactivity recovered in rats dosed with a higher dose of lead and folate (both tenfold) resulted in a significant fall in all tissue

levels except the faeces. The levels of radioactivity retained by the faeces over 72h were significantly higher ($p<0.05$) when compared to the normals.

Folate scission in these animals was elevated to 28.39%. Although at the low dose of lead acetate total scission falls to 14.42% (Table 7.1.4).

Dihydrofolate (DHFR) reductase and dihydropteridine reductase (DHPR) act in conjunction to salvage THF from DHF or quinoid-DHF which are easily oxidised (Chippel and Scrimgeour, 1970). (Figure 7.1.3).

A decrease in DHPR and/or DHFR activity would result in increased loss of folate. (Bertino, et al, 1964 and Craine, et al, 1972). Lead is an inhibitor of DHPR in vitro (Purdy, et al, 1981) and in vivo (McIntosh, et al, 1985) This could explain the increased folate scission in the lead dosed animals. These observations need further study. Partridge (1986) has compared iron and lead distribution in adult male rats (Table 7.1.5) and the data are similar to the weanlings.

Quantitative and qualitative investigations presented here have shown that scission is increased in the rats pretreated with various compounds. This increase was

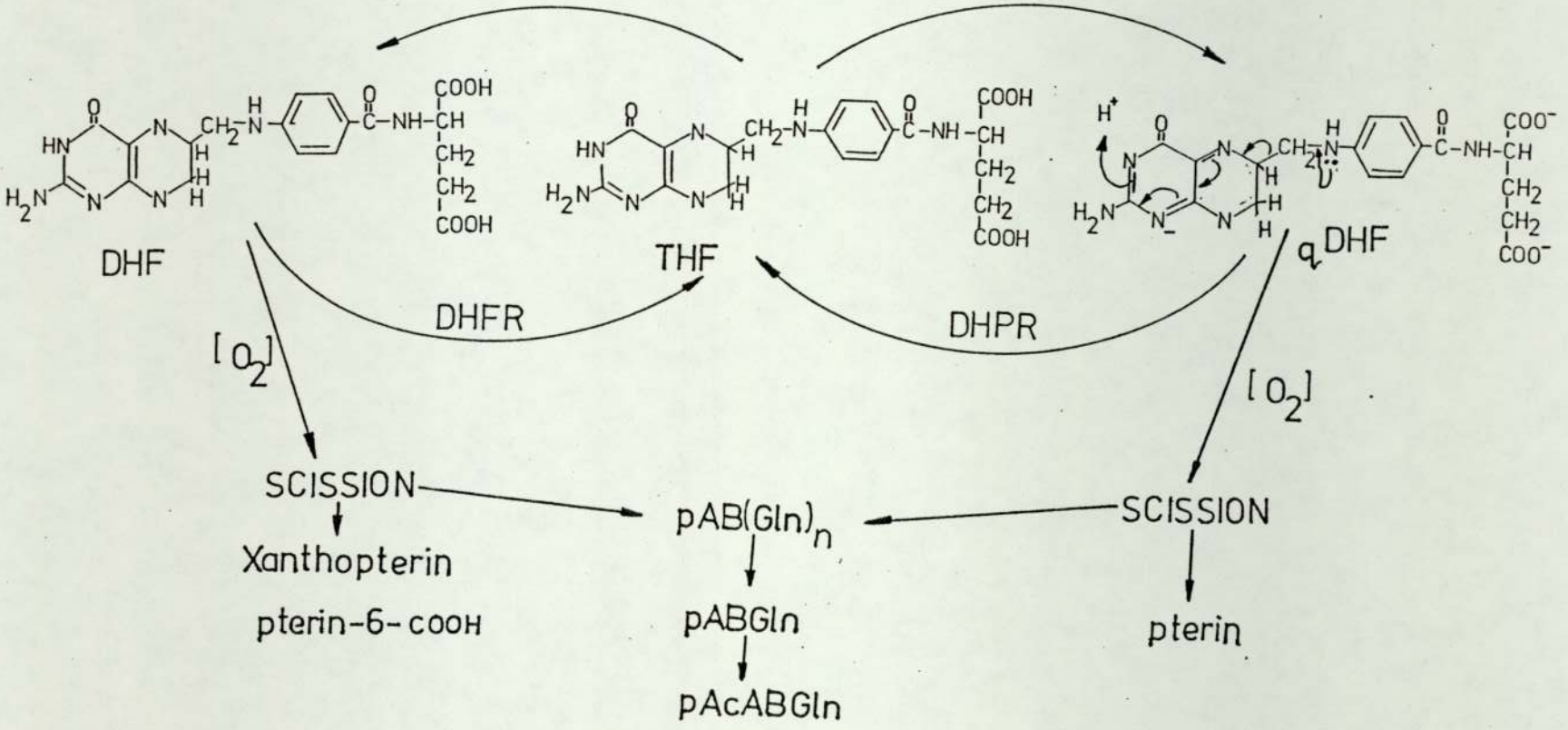


figure 7.1.3. DHPR and DHFR in A possible route of folate catabolism.

Group	% Scission as pAcAB (at 24-48 h)	% Scission as pAcABGlu (at 24-48 h)	% Scission as pAcAB (at 48-72 h)	% Scission as pAcABGlu (at 48-72 h)	Extent of Total Scission %
Normal Female Weanling rats	5.27	6.32	2.50	1.53	15.62
Lead acetate treated	6.77	8.09	4.50	9.03	28.39

(calculations are based on radioactivity retained in the livers at 24 h and at 48 h)

TABLE 7.1.4 Extent of scission in terms of polyglutamate breakdown following the oral administration of (3', 5', 7, 9 - ^{3}H) folic acid (100 µg/100 g body wt.) to normal and lead acetate treated (31 µg/100 g body wt.) female weanling rats.

more evident in allopurinol and ferrous sulphate pretreated weanling rats. It appears to be related to an increase in oxidative activity in the liver. The oxidation of xanthine or hypoxanthine and allopurinol (section 2) by xanthine oxidase results in the production of superoxide, hydrogen peroxide and hydroxyl radical (Massey et al., 1970); Elim et al., 1966; Pryor 1976, Bannister and Hall 1979 and Blair et al., 1984). Although in vivo many enzyme can contribute to the generation of "oxy-radicals" (Table 7.1.6). The hydroxyl radical is also produced by Fe^{2+} by the iron catalysed Haber - Weiss reaction (Czapski and Ilan, 1978). Ferrous salts added to tissue suspensions in vitro produce free radical oxidant (figure 7.1.4) including superoxide anions, singlet oxygen, various peroxides and hydroxyl radicals (O'Brien 1969, Fong et al., 1976, Aisen 1977, Svingen et al., 1978). Although not directly proven it appears probable that the elevated superoxide anion levels in liver were also accompanied by increases in the levels of other oxygen species. If this was so then the conditions for the chemical scission of the C9-N10 bond were also present. An alternative possibility is that scission occured via the cyt-p450 mixed function oxidase system. This appears unlikely for a number of reasons. When rats were treated with phenytoin and phenobarbitone which induce mixed

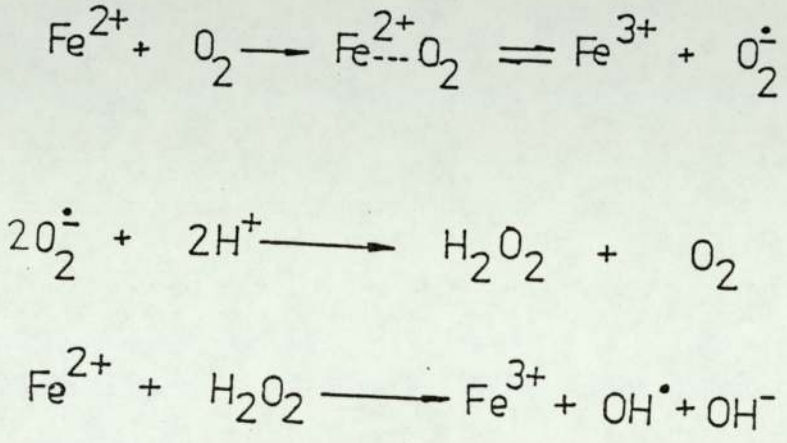


Figure 7.1.4. The production of superoxide anion formation by Fe^{2+} salts. (Taken from Gutteridge, 1982).

Tissue Sample	Percentage of whole dose recovered	
	10^{-6}m Fe (n = 9)	10^{-6}m Pb (n = 6)
Liver	± 2.06 ± 0.99	± 6.04 ± 3.09
Kidney	± 0.43 ± 0.36	± 5.00 ± 2.49
Gut (Absorptive)	± 11.70 ± 6.92	± 20.07 ± 3.66
Gut (Non Absorptive)	± 24.31 ± 17.52	± 27.08 ± 18.13
Stomach	± 0.84 ± 0.72	± 1.34 ± 0.53
Total Recovered	39.34	59.53

(± S.D)

TABLE 7.1.5 A comparison of 10^{-6}m Fe and 10^{-6}m Pb distribution in the adult rat 4 hours after dosing.

(Data supplied by Partridge 1986)

function oxidases (Conney, 1967, Eling et al., 1970, Latham et al., 1973, Levine, 1973 and Guest et al., 1983) they have no effect on folate catabolism.

In contrast Scott (1984) found that ethanol administration to rats increased folate catabolism. Ethanol administration results in a 86% elevation in cytosolic superoxide anion levels, Boveris, et al., (1983). One possible reason for this difference is that lipid peroxidation may be induced by superoxide-dependent hydroxyl radicals in the presence of iron and hydrogen peroxide or by decomposition of lipid peroxides to activate intermediate radicals which initiate destructive chain reactions by abstracting hydrogen ions. Lipid peroxidation results in a decrease in mixed function oxidase activity possibly due to inactivation of NADPH cytochrome C reductase or a decrease in Cyt. P450 levels (Wills 1971, Brogan, et al., 1983 and Gutteridge 1984). Also allopurinol has been reported to inhibit microsomal enzymes (Vesal et al., 1979, Fried et al., 1973, and Rowlins and Smith, 1973). If catabolism is the result of oxidative breakdown, changes in the redox potential in the tissues could alter the rate of folate degradation. Pheasant, et al., 1981 and Saleh 1982 have shown that folate catabolism (expressed as the % of the retained ^{3}H radioactivity excreted as pAcABGlu

as presented in this report) increases with the hepatic cytosolic NAD/NADH ratio. In comparason D.E.S and 17α-ethynyloestradiol would increase anabolic activity and enzyme activity, this could lead to increased metabolism of purines and pyrimidines, hence increased folate metabolism. This is further supported by the absence of some of the enzyme illustrated in table 7.1.6 in solid tumours where anoxia is also prevalent. Folate catabolism in this situation is depressed.

Hamsters pretreated with growth hormone also exhibited increased folate catabolism (Al-Haddad, 1984). On the basis of these observations and the findings presented, it appears likely that folate scission is due to simple chemical oxidation as suggested by Blair, et al., 1984.

Enzyme system:	Identified by:	Oxidative species
Alcohol dehydrogenase	Pryor (1976)	O_2^-, OH^-
Xanthine oxidase	Massey and Verger 1963 McCord and Fridovich (1968a); Fong, *et al.*,1976	O_2^-, OH H_2O_2
NADPH oxidase	Bannister, *et al.*, 1982	O_2^-, OH
NADPH cytochrome P450 reductase	Aziz, *et al.*, 1975 Morehouse, *et al.*, 1984	O_2^-, H_2O_2
Dihydro-orotic acid dehydrogenase	Handler, *et al.*, 1984 Forman and Kennedy (1975)	O_2^-, H_2O_2
Aldehyde oxidase	Fried, *et al.*, 1973	O_2^-, OH^-

Table 7.1.6. To illustrate enzyme systems known to produce "oxy-species".

FURTHER WORK

The results presented here highlight a number of areas where further experimentation might yield information about the handling of folate coenzymes by the rat. These are:

(i) The administration of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid to allopurinol pretreated rats dosed with broadspectrum antibiotics and vitamin E.

(ii) Histological and oxidative studies on rats pretreated with oestrogens and alcohol (to support experiments in section 3.1.)

(iii) DHPR and/or DHFR studies in iron and allopurinol pretreated animals (to elucidate the role of these enzymes in folate catabolism).

(iv) Further analysis of metabolites and catabolites using Sephadex LH-20 chromatography. And to use LH-20 to seperate the overlapping peaks in ion exchange chromatography.

(v) Finally, further experimental investigations employing metal ions (Cu^{2+}, Ca^{2+}) and the administration of sodium nitrite (Pearson, 1974). To demonstrate the oxidative effects of these metal ions and compare with iron and lead.

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