STUDIES OF FOLATE CATABOLISM IN THE RAT

BY

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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 adult male and adult female rats. Both sexes were found to metabolise folic acid to various compounds. Intact folates and catabolites were excreted in the urine and folate polyglutamates were found in the tissues. The extent of tissue uptake and catabolism were found to vary between the male and female rat. Female rats treated with diethylstilboestrol and mazola corn oil showed marked changes in the metabolic handling of folic acid. Less radioactivity was excreted in the urine and catabolism was greatly reduced. There was also a major shift towards 10CHO-folate in the urinary metabolites.

Male rats treated with phenytoin showed malabsorption of the folic acid. The absorption of ^{3}H-folic acid across the gut wall was affected by phenytoin. The extent of catabolism was also reduced.

Almost total recovery of the dose was achieved from the male rats treated with antibiotics demonstrating the importance of the intestinal bacteria in the handling of folic acid by the rat. Tissue uptake of folates was also increased in these rats.

In vitro oxidation of THFA to pterin and p-Aminobenzoyl-L-glutamic acid was found to occur very rapidly. Nitrous acid, formed during the Bratton and Marshall Assay for diazotisable amines, reacted with THFA in vitro to produce quinonoid DHF which quickly broke down suggesting a previously unrecognised site of loss for ingested folates.

Folate catabolism is proposed to be a primarily chemical process as the induction of microsomal oxidising enzymes did not increase scission. The possibility that superoxide anion produced by various endogenous enzymes during cell metabolic processes might be involved in the in vivo oxidation of THFA is discussed. Intestinal bacteria and the production of nitrous acid within the gut from nitrite are of importance in folate catabolism by the rat.

Key Words

Folate chemical oxidation, intestinal bacteria, nitrite, oestrogen.

To my family - for their encouragement

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Chapter 1

INTRODUCTION

The term "folic acid" was introduced in 1940 to designate a nutritional factor found in spinach leaves which was essential for the growth of Streptococcus faecalis (Mitchell et al., 1941). It has since been found that folic acid is the parent compound of a large family of closely related chemical coenzymes that play a fundamental role in cell growth and division.

Folic acid (I) consists of a pteridine ring linked by a methylene bridge to p-aminobenzoic acid, which is joined by amide linkage to glutamic acid. Folic acid is not normally found in foods or biological tissues and is without known biochemical activity. Reduction of the pteridine ring and substitution of one-carbon adducts onto the N^5 and/or N^{10} positions confers biological activity. Further modification may occur by the linkage of additional glutamate residues to the terminal glutamic acid group forming folate polyglutamates (II).

Although folate polyglutamates form the majority of dietary and tissue folates (Connor and Blair, 1980) human cells cannot directly absorb these macromolecules from the blood. Plasma and cerebrospinal fluid folates are in the monoglutamate form with 5 methyltetrahydrofolate (5 MeTHF) (III) and 10 formyltetrahydrofolate (10CHO-THF) (IV) as the major folates in plasma. 10CHO-THF is usually maintained at a constant level under normal conditions whereas 5 MeTHF acts as a circulatory storage form (Ratanasthein, et al., 1974). It was initially thought that folate polyglutamates acted as a relatively stable storage form and that the monoglutamates, which are rapidly inter-converted in vivo, functioned as the coenzymes (Blair, 1976). However more recent kinetic evidence has proved that although most folate dependent enzymes will

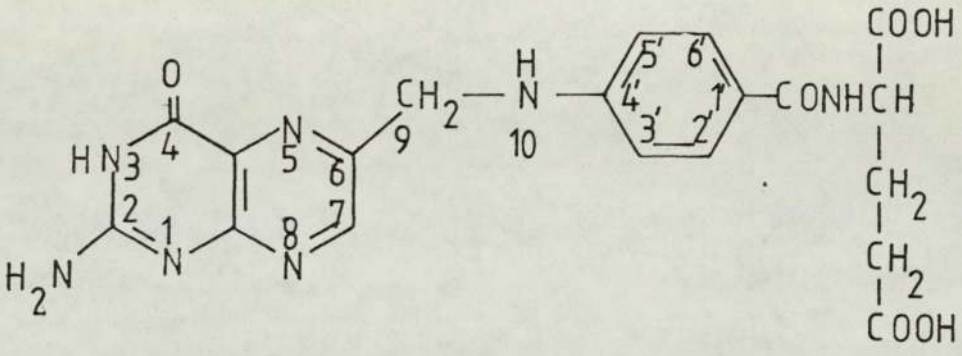


Figure 1. Folic Acid.

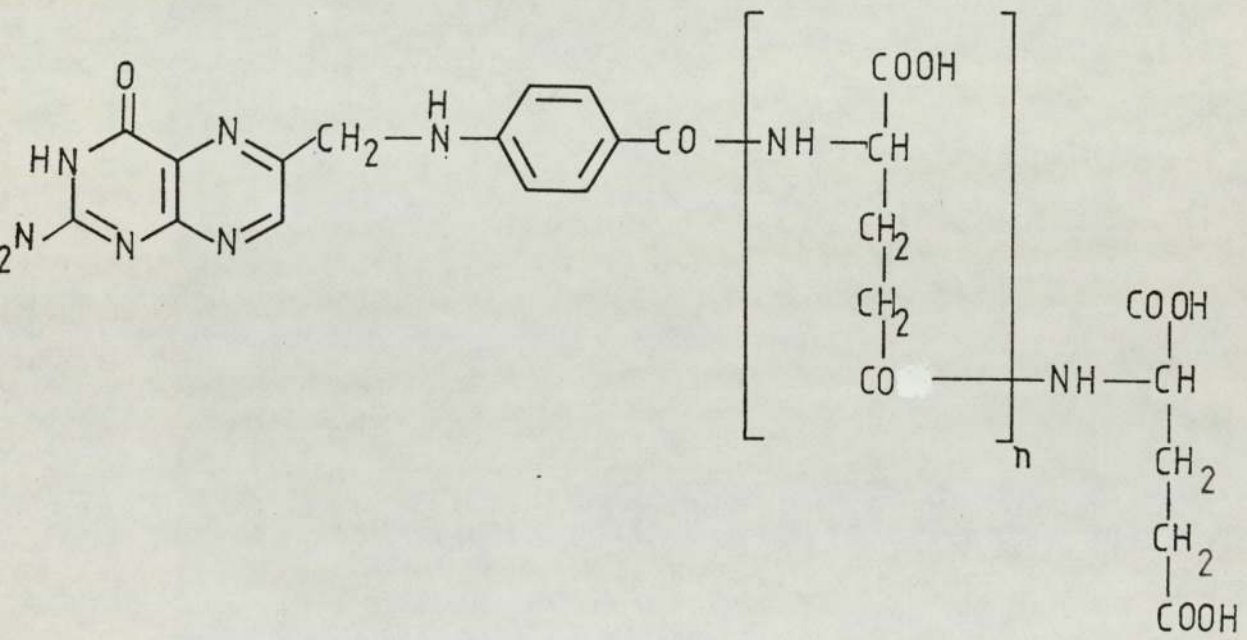


Figure II: Folate Polyglutamates

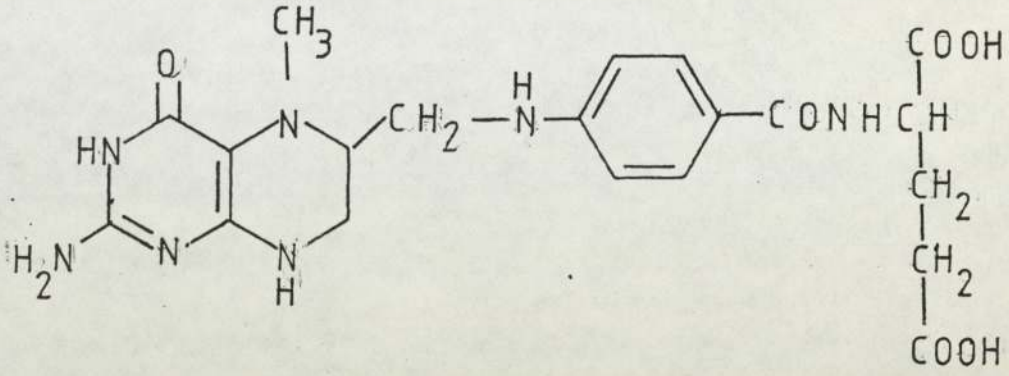


Figure III: 5Methyltetrahydrofolic Acid

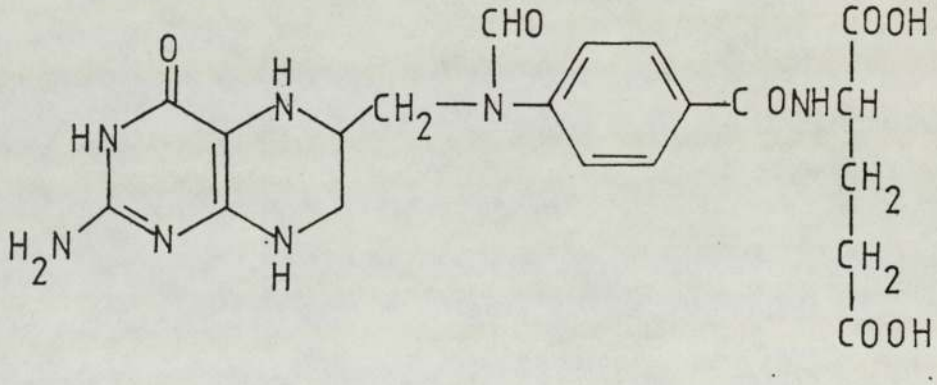


Figure IV: 10-Formyltetrahydrofolic Acid

utilise both mono- and polyglutamate forms of folate derivatives the latter form better substrates than the corresponding monoglutamate derivatives (Hoffbrand, 1976; Rowe, 1983; Chanarin, 1979).

The enzymes responsible for folate polyglutamate synthesis in vivo are called folate polyglutamate synthetases or ligases. They are unevenly distributed among the intracellular compartments with the highest activity occurring in the cytosol (Gawthorne, 1980), the main site of folate linked enzyme activity. The optimal substrates for the rat enzyme appear to be L-glutamic acid and 10CHO-THF (Spronk, 1973; d'Urso-Scott and Makulu, 1973), although 5MeTHF is probably the main form of folate entering the cell.

1.1. Interconversion of Folate Coenzymes

Folic acid entering the body must be reduced to a biologically active form before it can function as a coenzyme. In vivo reduction is catalysed by the enzyme dihydrofolate reductase (DHFR) [5,6,7,8:THFA NAD(P) oxidoreductase (EC 1.5.1.3.)] which is widely distributed in most mammalian tissues. It is located in the cytoplasm and high levels have been detected in liver, kidney, embryonic tissues (Roberts and Hall, 1965) and in tumours (Blakley, 1969). The pyrazine ring of the pterin moiety is first reduced to 7,8 - dihydrofolate (DHF) (V) and then to 5,6,7,8 - tetrahydrofolate (THFA VI). DHFR is strongly inhibited by methotrexate (VII) (Bertino et al., 1964) and other folate analogues causing cell death through acute folate deficiency. This property has been exploited in cancer chemotherapy.

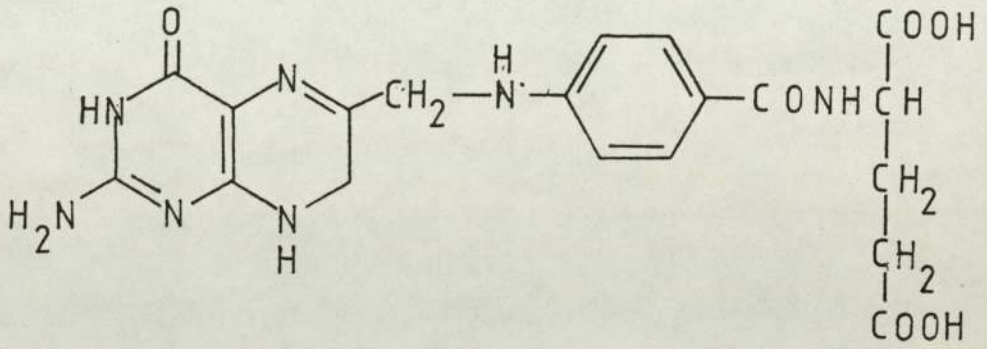


Figure V: 7,8-Dihydrofolic Acid

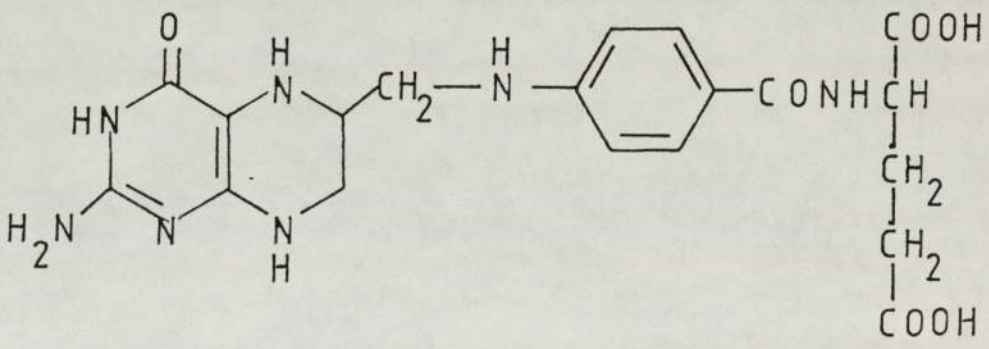


Figure VI: 5,6,7,8-Tetrahydrofolic Acid

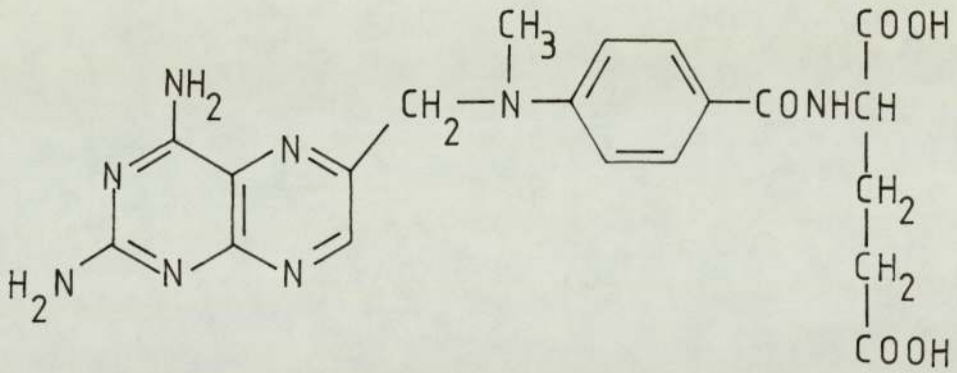


Figure VII. Methotrexate

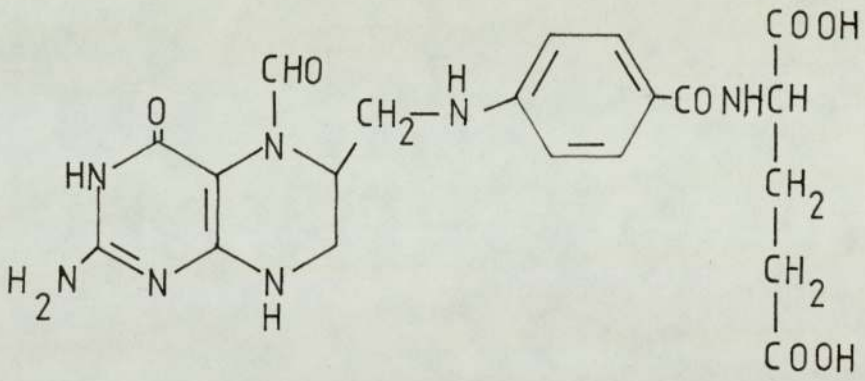
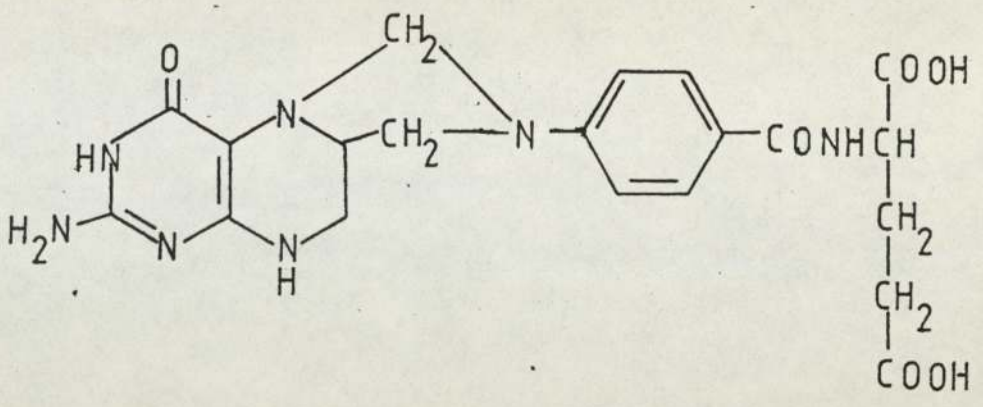


Figure VIII. 5-Formyltetrahydrofolic Acid



FigureIX : 5,10-Methylenetetrahydrofolic Acid

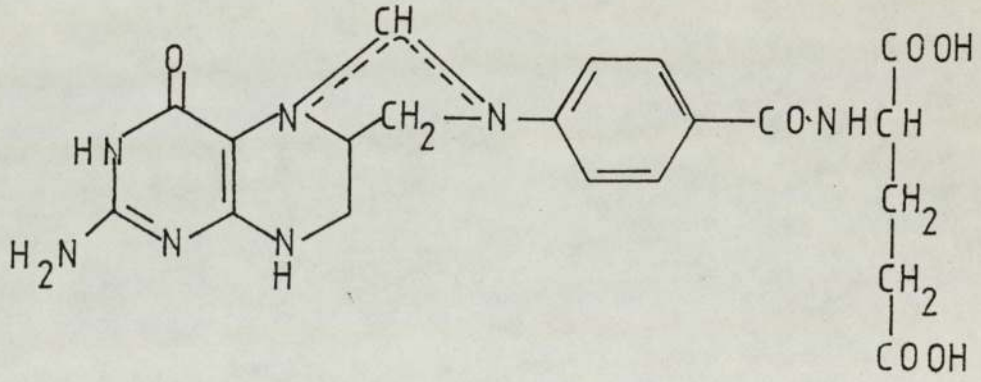


Figure X: 5,10 = methenyltetrahydrofolic Acid

1. DHFR
2. Formyl transferase
3. Methylene tetrahydrofolate dehydrogenase
4. Methylene tetrahydrofolate reductase
5. Serine transhydroxymethylase
6. Methionine synthetase
7. Thymidylate synthetase
8. Formimino tetrahydrofolate formimino transferase
9. Formimino tetrahydrofolate cyclo deaminase
10. Cyclohydrolase
11. Formyl THF formyl transferase.

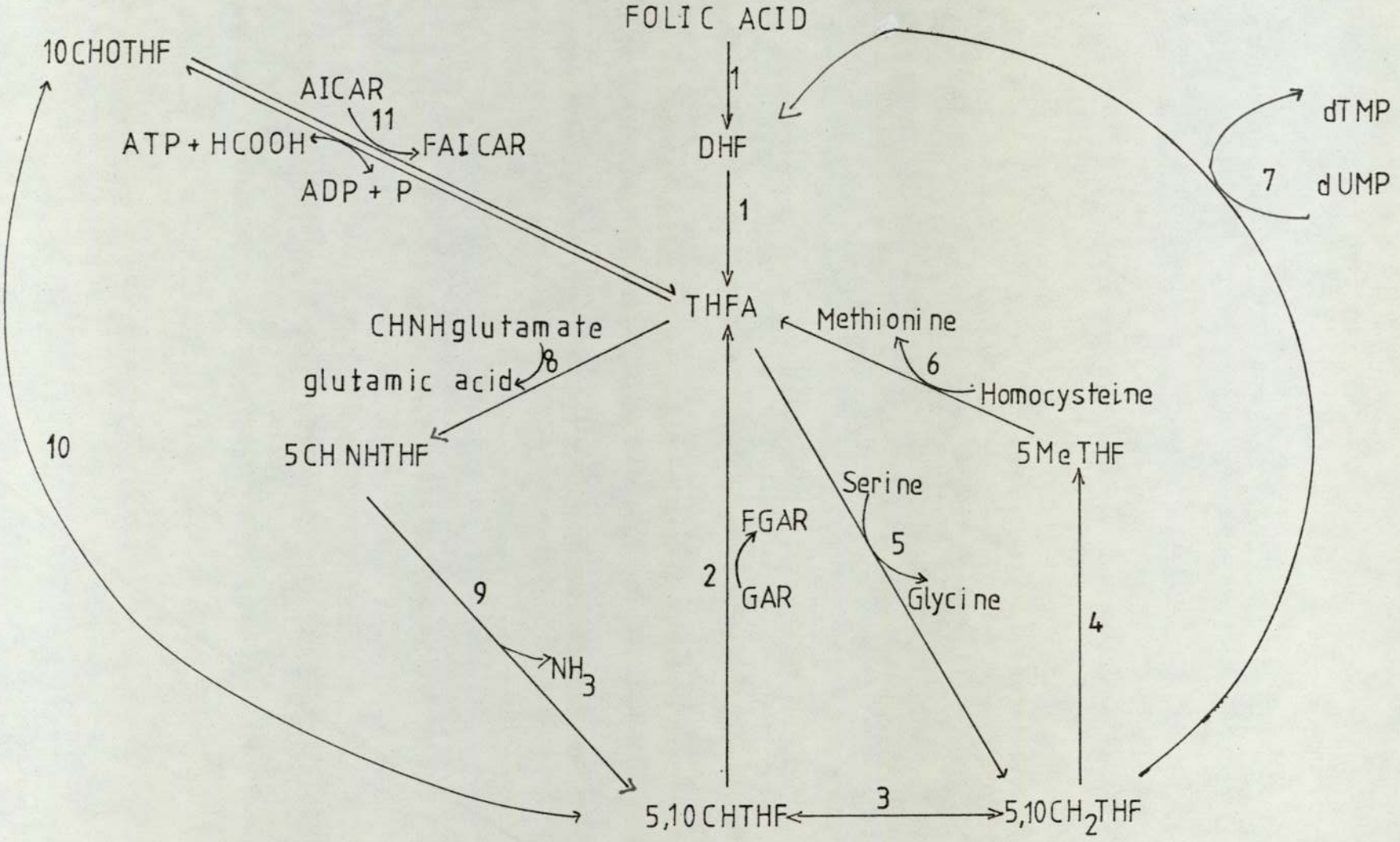


Figure 1.1: Interconversion pathways of folate coenzymes.

Following reduction to THFA a number of carbon containing groups including formyl (CHO-), methenyl (-CH=), methylene ($-CH_2-$), formimino (CHNH) and methyl (CH_3) may be substituted onto the molecule at position N5 and/or N10 to yield for example 5 formyltetrahydrofolate, (5CHO-THF) (VIII) and 5,10 methylene tetrahydrofolate (5,10CH_2-THF) (IX) and 5,10 methenyl tetrahydrofolate (5,10CH = THF) (X). The folate pathways are a complex series of interlocking enzyme reactions which generate a number of relatively unstable coenzymes that are critical for several important biosynthetic reactions. Figure 1.1. is a simplified scheme showing the major folate requiring reactions and coenzyme interconversions.

1.2. Role of Folate Coenzymes In One-Carbon Unit Transfers

The primary function of folate coenzymes is to transport one-carbon units. They fulfill this role by participating in three reactions in DNA synthesis: one in pyrimidine nucleotide biosynthesis and two in de novo purine synthesis. They are involved in methionine synthesis, serine-glycine interconversion, histidine breakdown and the generation and utilisation of formate.

The methylation of uridylate to thymidylate is a rate-limiting step in cellular DNA synthesis (Hoffbrand, 1976). The enzyme thymidylate synthetase (EC 2.1.1.6.) transfers a methylene group ($-CH_2-$) from 5,10 CH_2-THF to uridylate and the methylene ($-CH_2-$) group is simultaneously reduced to methyl (CH_3) forming thymidylate and DHF (Figure 1.2.).

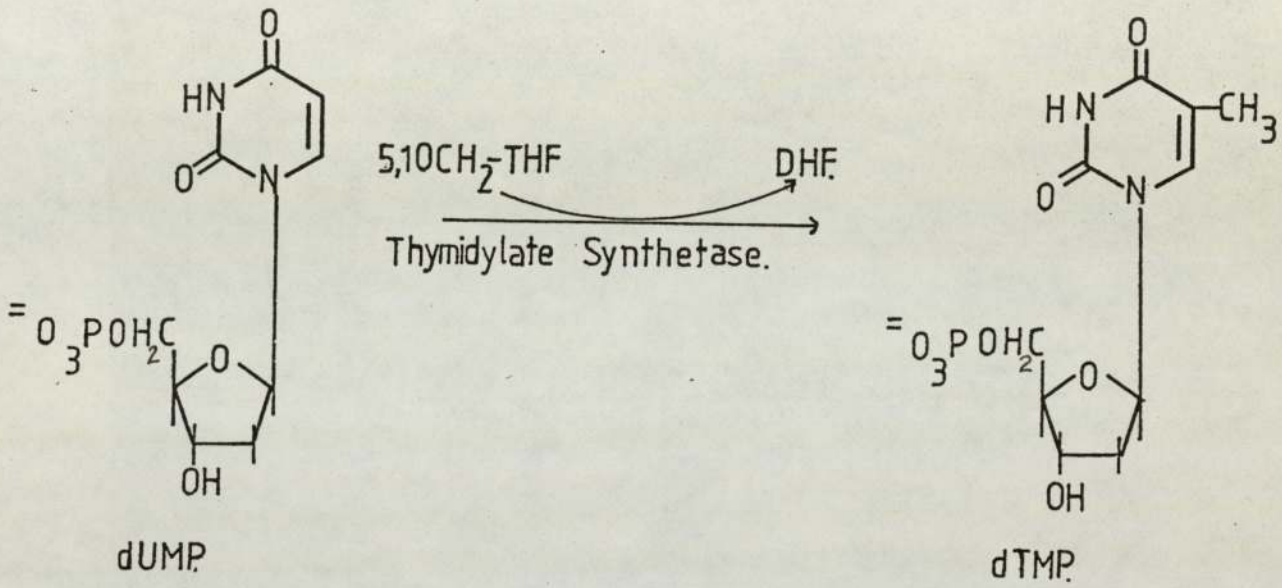


Figure 1.2: The methylation of deoxyuridine catalysed by thymidylate synthetase is accompanied by formation of dihydrofolate from 5, $10CH_2$-THF.

In this process 5,10CH_2-THF is not only demethylated but is converted to DHF. Therefore DHFR is required for the reconversion to THFA which may in turn be converted to 5,10CH_2-THF or participate in further coenzymic reactions. Thus, thymidylate synthetase and DHFR function in sequence to allow the cyclic nature of these reactions.

The introduction of carbon atoms 2 and 8 into the purine ring was thought to be a two step reaction involving the formylation of glycinamide ribonucleotide by 5,10CH = THF followed by the formylation of aminoimidazolecarboxamide ribotide (AICAR) by 10CHO-THF to form inosinic acid (Hartman and Buchanan, 1959). However, more recent evidence from E. Coli (Dev and Harvey, 1978a) suggests that 10CHO-THF is the required coenzyme in each reaction. It is now thought that in the earlier studies the 5,10CH-THF used was hydrolysed <u>in vitro</u> to 10CHO-THF either chemically or by the enzyme cyclohydrolase,

5,10CH = THF + H_2O $\Rightarrow$ 10CHO-THF + H+

It has been reported that methylenetetrahydrofolate dehydrogenase and cyclohydrolase activities are present on a single protein together with 10-formyltetrahydrofolate synthetase (Dev and Harvey, 1978b). Thus, during the conversion of 5,10CH_2-THF to 10CHO-THF, 5,10CH = THF would only have a transient existence as an intermediary and would thus be unlikely to play a major donor role <u>in vivo</u> in a formylation reaction.

The reversible reaction by which one β-carbon of serine is transferred to THFA with the formation of 5,10CH_2-THF and glycine is the principal route of serine catabolism in the mammal (Figure 1.3.). Kretchmar and Price (1969) have estimated that approximately 71 percent of serine

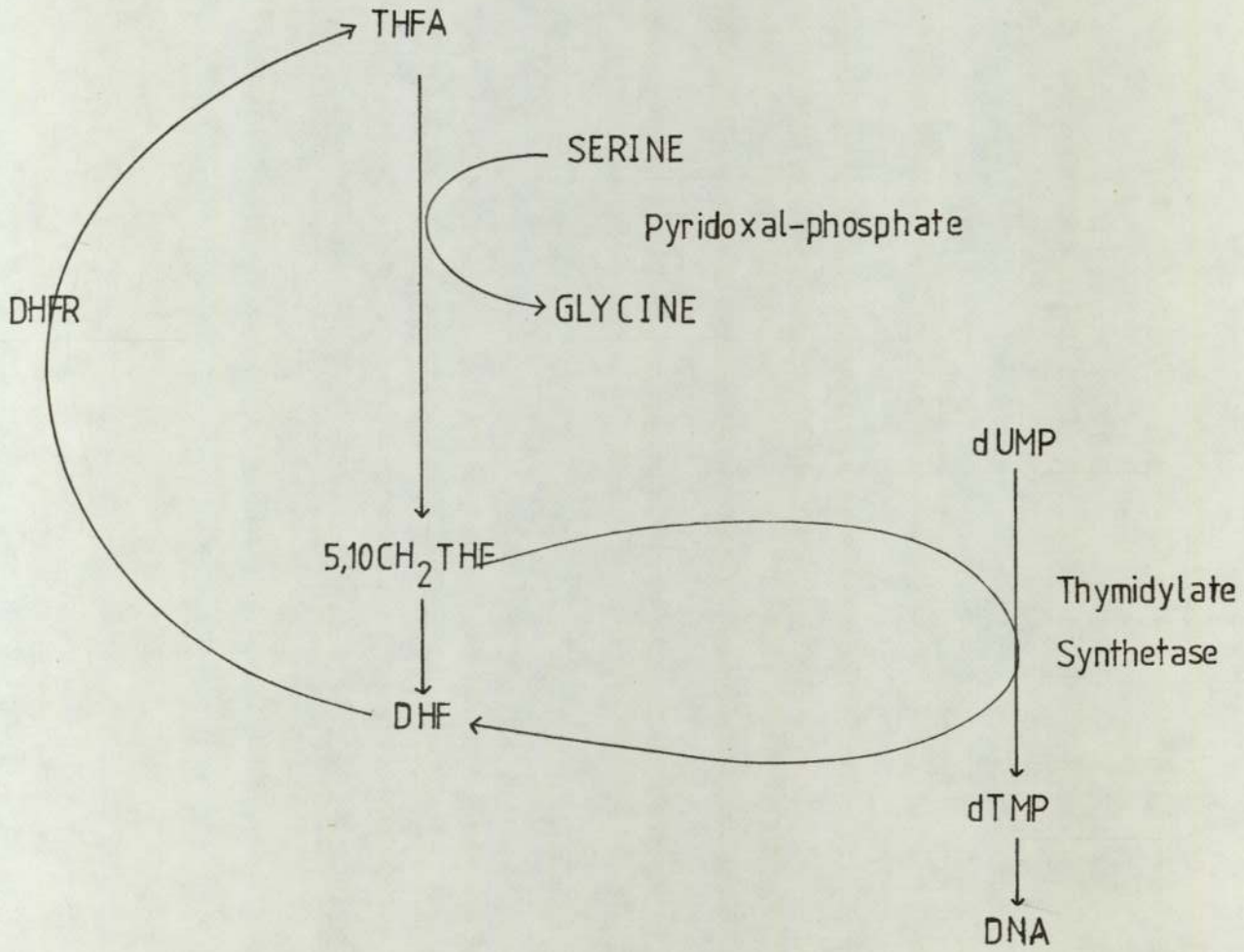


Figure 1.3: Conversion of serine to glycine by serine transhydroxymethylase showing relationship to thymidylate synthetase reaction.

breakdown is by way of the folate coenzyme pathway, although alternate degradation pathways are available and become of importance in cases of folate deficiency (Rowe, 1983). The conversion is catalysed by serine transhydroxymethylase (EC 2.1.2.1.), an enzyme which has been detected in many species with especially high levels occuring in vertebrate livers. The bulk of enzyme activity is cytosolic but an enzyme with almost identical physical and kinetic properties has been found in rabbit liver mitochondria (Rowe, 1983). The reaction requires pyridoxal 5-phosphate (Vitamin B6).

Another major source of one-carbon units for addition to THFAis the breakdown of formiminoglutamic acid (FIGLU) during the catabolism of histidine (Figure 1.4.). In this reaction the formimino group is transferred to THFAto yield 5 formiminotetrahydrofolate (5CHNH-THF) (XI) and glutamic acid by the enzyme formiminotetrahydrofolate formiminotransferase (EC 2.1.2.5.).

Figure 1.4.

THFA + CHNHglutamic acid $\longrightarrow$ 5CHNH-THF + glutamic acid.

5CHNH-THF is unstable and is rapidly converted to 5,10CH = THF by formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4.).

5CHNH-THF $\longrightarrow$ 5,10CH = THFA + NH_3

It was thought that these enzymes existed in mammalian tissues as an extremely tight complex (Rowe, 1978) but there is recent evidence that pig liver contains a single multifunctional enzyme which catalyses the

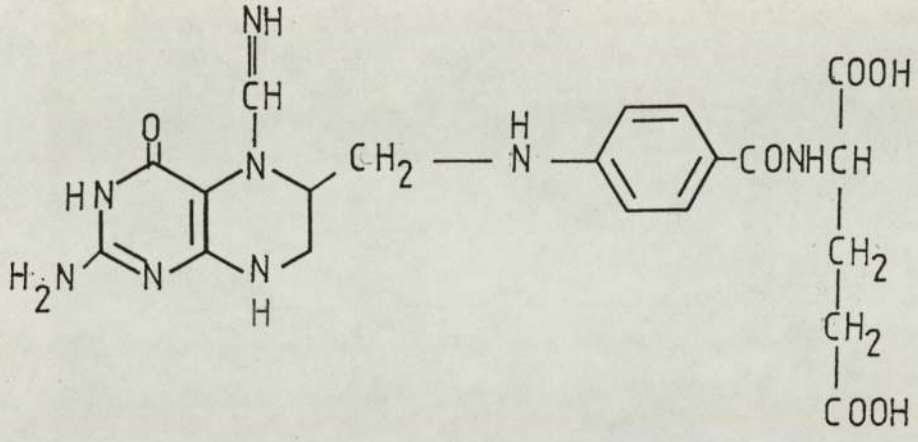


Figure XI: Formiminotetrahydrofolic Acid

two reactions (Beaudet & Mackenzie, 1976; Drury & Mackenzie, 1977 and Mackenzie & Baugh, 1980).

The formation of the important coenzyme 5MeTHF by the enzymatic reduction of $5,10CH_2$-THF is also the first step in the biosynthesis of methyl groups. The reaction is catalysed by the enzyme methylene tetrahydrofolate reductase (EC 1.1.1.68) in the presence of FAD and NADPH and is virtually irreversible (Figure 1.5.).

Figure 1.5.

$$5,10CH_2\text{-THF} + NADPH + H^+ \xrightarrow{FAD} 5CH_3THF + NADP^+$$

THFA may be regenerated by the transfer of the methyl group to homocysteine thus forming methionine. This is catalysed by 5 MeTHF - homocysteine methyl transferase (EC 2.1.1.13) (Methionine synthetase) and requires cobalamin (Vit. B12) $FADH_2$ and S-adenosylmethionine (SAM).

$$5MeTHF + \text{homocysteine} \xrightarrow[SAM + FADH_2]{B12} THFA + \text{methionine}$$

The haematological damage found in pernicious anaemia may be corrected by therapeutic doses of folic acid. In these cases serum 5MeTHF is elevated (Herbert et al., 1962; Thenen et al., 1973) and this block in coenzyme interconversion reduces the available pool of coenzymes, as THFA cannot be regenerated by methionine synthetase owing to B12 deficiency and $5,10CH_2$-THF cannot be formed from 5MeTHF as this reverse reaction does not occur in mammalian cells. This metabolic trapping of folate coenzymes as 5MeTHF was put forward as the "methyl trap

hypothesis" (Herbert & Zalusky, 1962 and Noronha and Silverman, 1962) and has been confirmed by more recent observations (Krebs et al., 1976). However, Rowe (1983) has claimed that the hypothesis does not explain why megaloblastic anaemia is not found with congenital methylmalonicaciduria despite the defect in cobalamin metabolism and methionine synthesis.

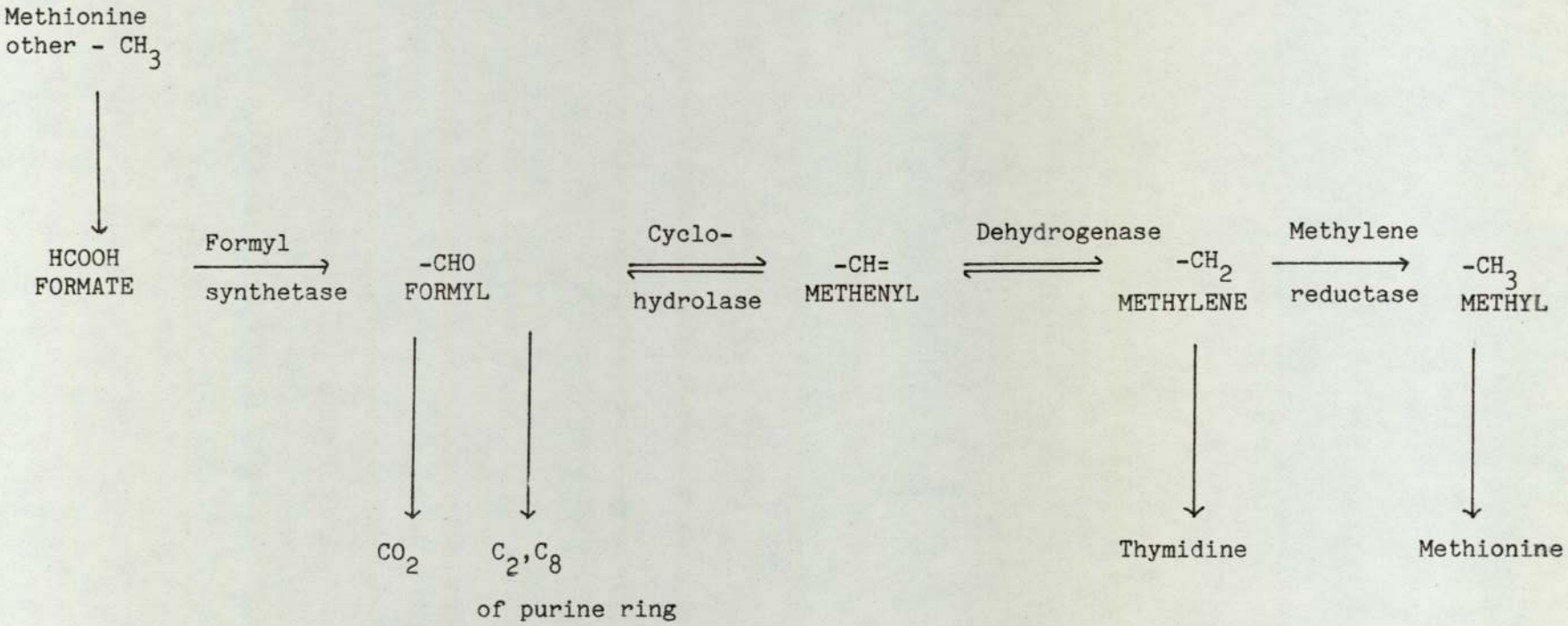
In vivo studies of B12/folate inter-relationships are hampered by the great difficulty that lies in producing nutritional deficiency of either vitamin, especially B12, in experimental animals. However, a series of unrelated observations have led to the discovery that nitrous oxide (N_2O), usually an inert compound, is cleaved by transition metal complexes such as vitamin B12, releasing free nitrogen and electrons which oxidise Cob(I)alamin to Cob(III)alamin thereby inactivating methionine synthetase (Deacon et al., 1978, 1980a and 1982). This inactivation parallels the situation found in B12 deficiency in man. Observations using the animal model have indicated that B12 deficiency may have another effect on folate metabolism (Perry et al., 1979; Deacon et al., 1980b, 1980c; Lumb et al., 1980; McGing et al., 1978; Scott et al., 1979).

Chanarin et al., (1980) and Deacon et al., (1982) have put forward an alternative hypothesis. Rats treated with N_2O and thereby deficient in B12, show a deficiency in formate supply. They claim that formate (HCOOH) arises from the oxidation of methyl groups, particularly the methyl group derived from methionine. Thus, inhibition of methionine synthetase by N_2O decreases the availability of formate. Normally

formate groups are reduced to formyl groups by the enzyme 10CHO-THF synthetase which in turn are reduced to methenyl groups (-CH =) by 5,10 CH = THF cyclohydrolase (see Figure 1.6.). Rats treated with N_2O show an increased formyl synthetase activity but decreased cyclohydrolase activity - the net effect being a conservation of formyl coenzymes and a reduction in methenyl, methylene and methyl coenzymes and therefore a general interference with folate metabolism.

However, these results may also be interpreted in terms of the methyl trap hypothesis. A series of experiments investigating the catabolism of histidine to glucose using isolated hepatocytes or perfused rat livers showed that in the presence of insufficient methionine, histidine breakdown leads to the production of excess formiminoglutamate. As described earlier, this indicates a lack of THF coenzyme. The addition of methionine to the medium promoted both the degradation of formiminoglutamate and the production of glucose from histidine. The addition of methionine raises the concentration of S-adenosyl methionine (SAM) which is known to inhibit methylene tetrahydrofolate reductase (Kutzbach and Stokstad, 1967) (Figure 1.7.) causing an increase in CH_2-THF and the coenzymes in equilibrium with it, including 10CHO-THF. The increased concentration of 10CHO-THF accelerates the formyl tetrahydrofolate dehydrogenase reaction.

Thus, when the concentration of methionine is high SAM indirectly increases the release of 1-C units as CO_2 by raising the CHO-THF dehydrogenase reaction, but when methionine levels are low 1-C units are preserved and utilised for the production of methionine, purines and pyrimidines.



(from Deacon et al., 1982)

Figure 1.6: Utilisation of formate and its ultimate conversion to methionine.

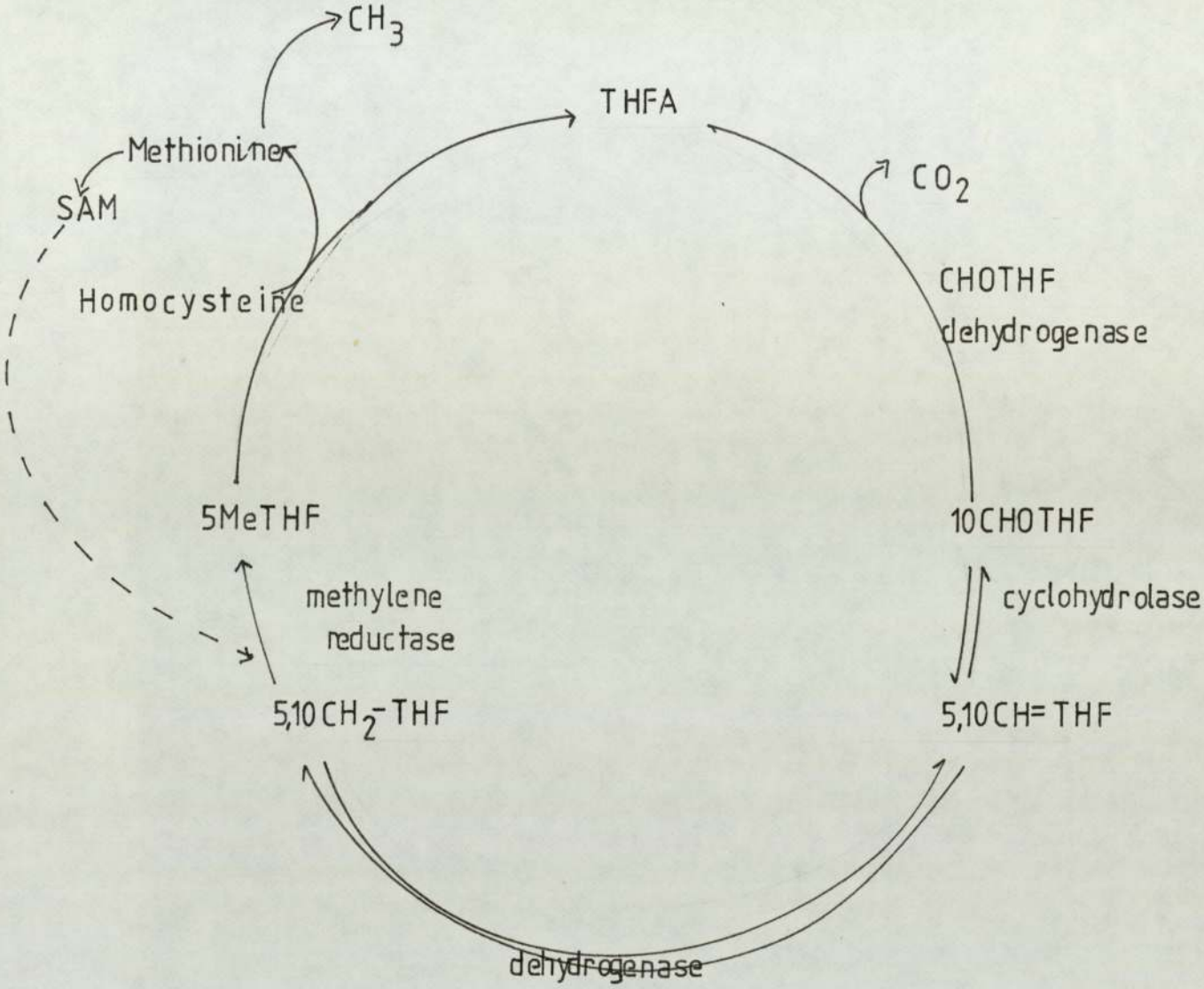


Figure 1.7: The Methyl trap hypothesis.

1.3. Studies on Folate Catabolism

The reduced forms of folates are of biochemical importance because of their high reactivity. However, this very property renders these chemicals very difficult to work with as they are photolabile, susceptible to aerobic oxidation and are only present in biological tissues in trace amounts. Early studies used microbiological assays based on the growth promoting properties of folates and the differing requirements of various bacteria (see Table 1.1). The introduction of radioisotope labelling of the folate molecule, initially with tritium (^{3}H) and later with ^{14}Carbon (^{14}C) improved the sensitivity and specificity of results as it became possible to follow the fate of an administered dose of a folate derivative without interference from folates already present within the body. More recently improvements in chromatographic techniques have led to the introduction of DEAE - cellulose ion exchange chromatography, Sephadex gel filtration and high performance liquid chromatography (HPLC) systems to folate research. Although problems, such as inadequate separations, still occur improvements are being made very rapidly.

Bearing in mind the difficulties encountered by the early workers in folate biochemistry and the vast improvements in experimental techniques that have occurred, I shall restrict the following review of folate catabolism to work conducted from 1975 onwards.

Experimental administration of labelled folates to man, the rat and the guinea pig has led to the identification of a series of catabolites in the urine (Krumdieck *et al.*, 1978; Murphy *et al.*, 1976, 1978; Connor

Table 1.1.

Response of Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae to folate derivatives. (Taken from Blakley, 1969).

Compound	Growth activity for		
	L. casei	P. cerevisiae	S. faecalis
Folic Acid	+	-	+
DHF	+	-	+
THF	+	+	+
5CHO-THF	+	+	+
10CHO-folate	+	-	+
10CHO-DHF	+	-	+
10CHO-THF	+	+	+
5MeTHF	+	-	-
Folyglutamate	+	-	+
Folyldiglutamate	+	-	-
THF diglutamate	+	+	-

et al., 1979; Pheasant et al., 1979, 1981; Choolun et al., 1980; Kennelly, 1980; Saleh et al., 1980, 1982) (see Table 1.2).

In 1958 Blair proposed that folate breakdown in vivo could arise from either chemical oxidation or enzymic activity giving cleavage of the C9 NIO bond forming p-ABGlu and a pterin fragment. No evidence to support this suggestion was found until the late 1970's. Murphy et al., (1978) have more recently put forward the same suggestion.

Acetylated derivatives of p-ABGlu (XII) and p-aminobenzoic acid (p-ABA) (XIII) appear in the urine of the rat after the administration of folic acid, 10CHO-folic acid (Connor, 1979) or 5MeTHF (Kennelly, 1980). p-ACBA is the major catabolite in early urine samples but levels quickly decrease whereas p-ACBGlu excretion levels begin at a low level but increase with time to eventually become the major urinary catabolite (Connor, 1979). Pheasant et al., (1981) have explained these results in a hypothesis in which catabolite formation within the body is derived from one of two distinct pools of folate coenzymes each having different biological half lives.

The first pool consists of folate derived from recent absorption and is called the short term pool. Folates within this pool have a half life of approximately 1 day in the rat (Saleh, 1981) and 31.5h in man (Krumdieck et al., 1978). Enzymic degradation or chemical oxidation of these folates generates p-ACBA which is excreted in the early urine samples.

Table 1.2.

Catabolic products detected in the urine following the administration of folates.

CATABOLITE	SPECIES	IDENTIFIED BY:-
Isoxanthopterin	MAN	Blair, 1958; Fukushima and Shiota, 1972.
	MAN	Krumdieck, et al., 1978.
Pterin	MAN	Krumdieck, et al., 1978.
p-aminobenzoyl-L-glutamate	MAN	Johns, et al., 1961.
	RATS	Dinning, et al., 1957.
p-acetamidobenzoyl-L-glutamate	MAN	Saleh, et al., 1980.
	RATS	Murphy, et al., 1976. Connor, et al., 1979. Kennelly, 1980.
	GUINEA-PIG	Choolun, et al., 1980.
p-acetamidobenzoate	MAN	Saleh, et al., 1980.
	RATS	Connor, et al., 1979.
	GUINEA-PIG	Choolun, et al., 1980.

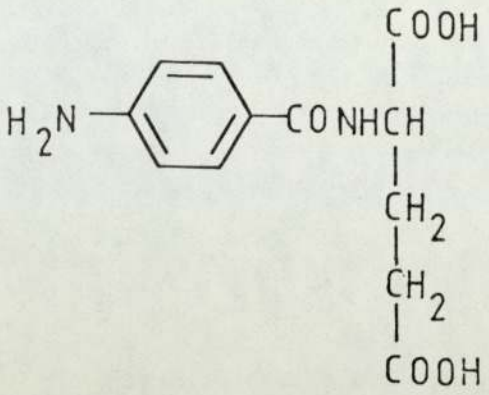


Figure XII. p-Aminobenzoyl-L-glutamic acid.

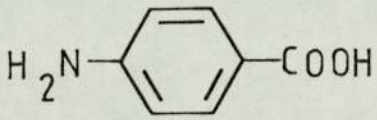


Figure XIII. p-Aminobenzoic Acid.

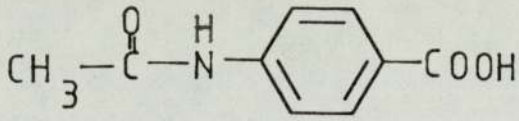


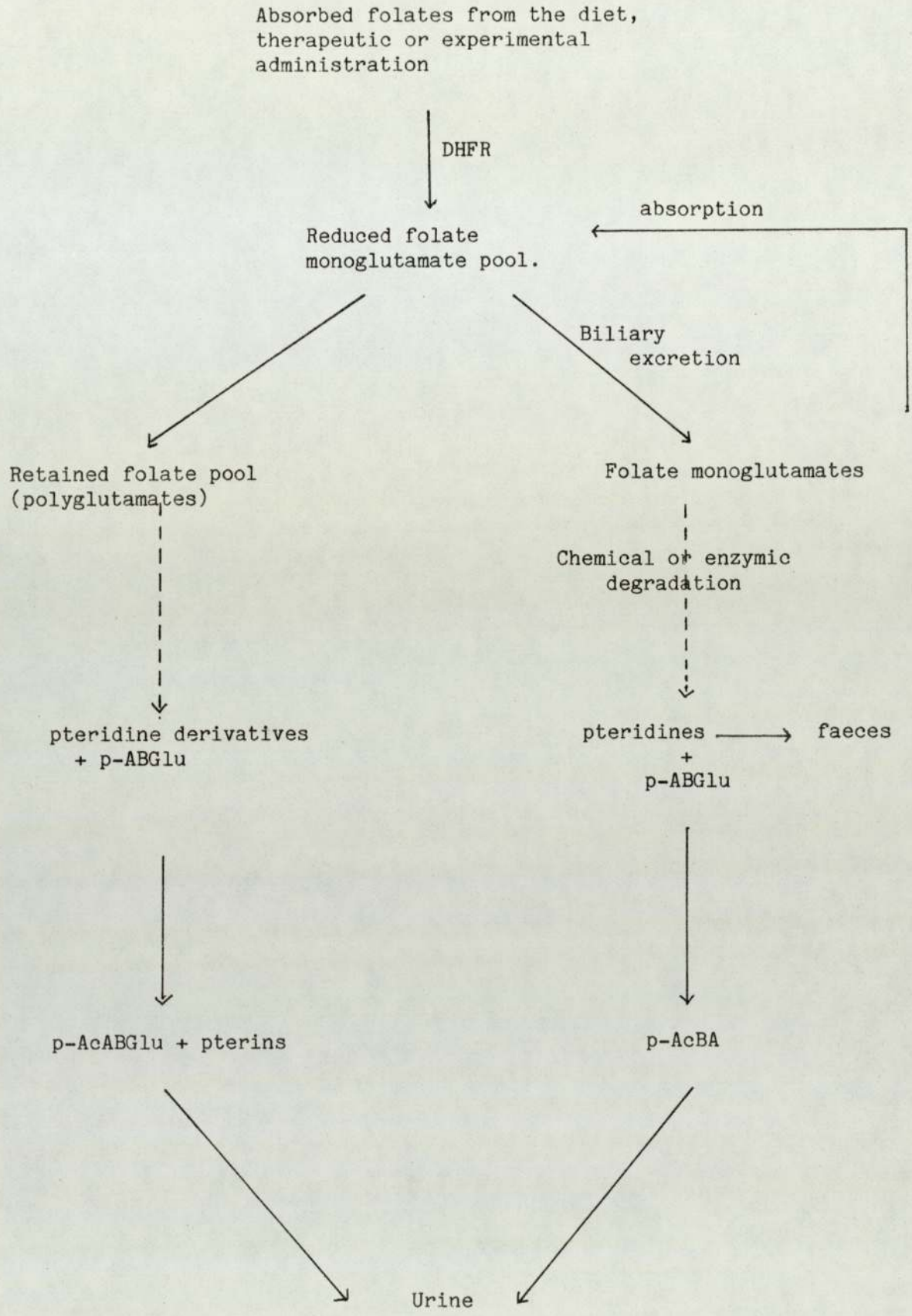
Figure XIV p-Acetamidobenzoic Acid.

The second pool constitutes a long term pool of folate coenzymes that exist in the tissues as folate polyglutamates for a relatively long period of time. Half lives have been estimated as 11 days in the rat (Saleh, 1981) and 100 days in man (Krumdieck et al., 1978). The principal catabolite of this pool is p-ABGlu which is excreted acetylated in later urine samples.

There is an enterohepatic circulation of folates within the body. Folates secreted in the bile are derived from free tissue (monoglutamate) and plasma folates (Hillman et al., 1977). Within the intestine these compounds undergo C9 N10 scission to give p-ABGlu and a pterin. Due to the low lipid solubility of the pterins formed they are largely excreted in the faeces whereas the p-ABGlu is well absorbed. Acetylation and loss of the glutamate moiety occurs during reabsorption and these catabolites appear in the urine as p-acetamidobenzoic acid (p-AcBA) (XIV). This scheme is summarised in Figure 1.8.

1.4. Factors which may alter folate metabolism

The complex interactions between the folate coenzymes may be modified either directly or indirectly by a number of factors. As already described nitrous oxide interferes with the equilibrium between coenzymes by oxidation of the cobalamin required by methionine synthetase (Deacon et al., 1982). Methotrexate, Trimethoprim and Triamterene inhibit the enzyme dihydrofolate reductase to produce a form of folate deficiency which has been exploited in cancer chemotherapy. Disease states such as pernicious anaemia, coeliac disease and tropical sprue may also cause megaloblastic anaemia



(Taken from Pheasant et al., 1981)

Figure 1.8: Routes of folate catabolism in vivo.

(Rosenberg et al., 1974), the first through inadequate provision of Vitamin B12 and the last two through insufficient absorption of dietary folate due to intestinal tract abnormalities. In pregnancy (Chanarin , 1979; Cooper et al., 1970) and malignant disease (Blakley, 1969; Poirier, 1973) folate deficiency may arise because of an increased demand which is not fulfilled from normal dietary sources. The various causes of folate deficiency have been extensively reviewed (Stokstad, 1968; Blakley, 1969; Herbert, 1972; Hoffbrand et al., 1977). The minimum adult requirement for folate is 50 μg/day and the recommended dietary allowance is 300 μg/day (Herbert, 1977). Prolonged folate deficiency produces haematological abnormalities leading to megaloblastic anaemia and nonspecific central nervous system changes including insomnia, forgetfulness and irritability which may be reversed by the therapeutic administration of folic acid (Herbert, 1972).

It has been observed that epileptic patients receiving chronic anticonvulsant therapy have a higher incidence of megaloblastic anaemia than normal and there is increasing concern that the mental processes of these patients are being adversely affected (Reynolds, 1976, 1980; Trimble and Reynolds, 1976; Figueroa et al., 1980; Shorvon et al., 1980 and Trimble et al., 1980). Folic acid has potent central nervous system excitatory properties (Shorvon et al., 1980) and the treatment of anaemic epileptic patients often causes an increase in their fit-frequency. These effects are usually found in those patients taking phenytoin (XV) either alone or in combination with phenobarbitone (XVI) (Reynolds, 1976) but the mechanism(s) by which these compounds alter

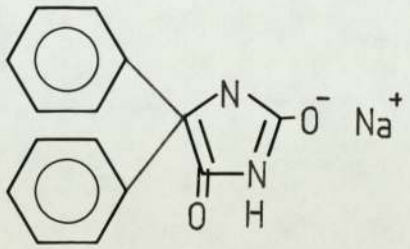


Figure XV. Phenytoin

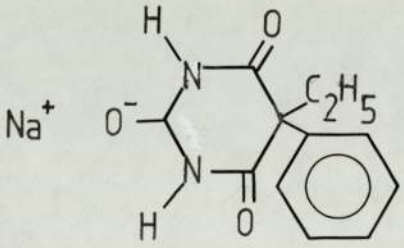


Figure XVI. Phenobarbitone

folate metabolism is (are) unknown. Current theories however include an action which decreases the absorption of dietary folate (Hoffbrand and Necheles, 1968; Rosenberg et al., 1968; Gerson et al., 1970; Benn et al., 1971); the induction of enzyme pathways requiring folate coenzymes (Richens, 1976; Maxwell et al., 1972 Labadarios et al., 1978) and lastly a direct effect which increases folate catabolism in vivo (Kelly et al., 1979).

Alcohol also alters folate homeostasis (Steinberg et al., 1980; Wilkinson and Shane, 1982). The effects of chronic alcoholism are thought to be due to inadequate dietary folate as megaloblastic anaemia is rarely found in the well nourished alcoholic (Wilkinson and Shane, 1982). However, acute alcohol administration has been shown to cause a rapid depression of serum folate levels (Eichner & Hillman, 1976; Lane et al., 1976). Although the mechanism of action is not known it has been suggested that ethanol may block the release of folate stores (Lane et al., 1976), interfere with the folate enterohepatic cycle (Hillman et al., 1977) or stimulate the tissue uptake of serum folates (Horne et al., 1979 and Wilkinson and Shane, 1982).

In human subjects oral contraceptives and oestrogens have been shown to affect folate metabolism (Krumdieck et al., 1975; Lakshmaiah and Bamji, 1979; Burns and Jackson, 1982 and Shojania, 1982). More than 20% of women using oral steroid hormones for fertility regulation may show biochemical evidence of impaired folate metabolism which may readily be corrected by oral folate administration even though serum folate levels are not depressed (Krumdieck et al., 1975), suggesting a localised interference with folate metabolism in oestrogen target tissues. Women

taking oral contraceptives excrete significantly more formiminoglutamic acid in the urine after a dose of histidine, suggesting that they are folate deficient (Shojania, 1982). However, the overall effects are very mild and are not likely to develop into megaloblastic anaemia unless there are other associated contributory factors.

Because of the importance of the folate coenzymes in cancer chemotherapy the elucidation of the precise mechanisms by which agents like alcohol, steroid hormones and anticonvulsants affect folate catabolism would be of great value.

1.5. Rationale for the present study

Folate metabolism in man, the rat (Connor, 1979; Kennelly, 1980; Saleh, 1981) and the guinea pig (Choolun, 1982) has been extensively studied in health and malignant disease. The folate coenzymes are of critical importance to DNA synthesis and therefore cell division and growth. Methotrexate and other antifolate agents have been successfully employed in the treatment of malignant disease but a more extensive understanding of the mechanism of folate catabolism in the normal situation would be of great help in the development of new ways in which to combat malignant cell growth. It was decided to study a group of compounds known to interfere with folate metabolism in as yet unknown ways so that any alterations in coenzyme levels could be carefully monitored.

Chapter 2

Materials and Methods

2.1. Chemicals and Reagents.

The following materials were obtained commercially as described: folic acid, xanthine, pterin from Koch-Light Laboratories Limited (Colnbrook, Bucks, U.K.); p-aminobenzoyl-L-glutamic acid, THFA, Xanthine Oxidase, Phenytoin, Diethylstilboestrol, Bacitracin, Neomycin, Tetracycline, and dithiothreitol from the Sigma Chemical Co. Limited (London, U.K.); p-acetamidobenzoic acid and p-aminobenzoic acid from the Aldrich Chemical Co. Limited (Wembley, Middlesex, U.K.); 5MeTHF from Eprova Research Laboratories (Basle, Switzerland). [2-^{14}C] - folic acid (specific activity 58 mCi/mM) and [3',5',7,9-^{3}H] - folic acid (specific activity 500 mCi/mM) were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.).

Other chemicals are laboratory reagents and of Analar grade.

The following folate derivatives were prepared by Dr. A. Saleh: 10-formylfolic acid (using the method of Blakely (1959) and p-acetamidobenzoyl-L-glutamate (p-AcBGlu) (by the method of Baker et al., (1964).

2.2. In vivo methods

2.2.1. Chromatography

(i) Ion Exchange Chromatography

Diethylaminoethyl cellulose (DE52, Whatman Limited, Maidstone, Kent, UK) (80g) was washed with distilled water and equilibrated in

(0.05 M)
sodium phosphate buffer, pH 7.0, containing dithiothreitol (DTT) (5 mg % w/v) until the washings were of constant ionic strength and at pH 7.0. Ionic strength measurements were made using a Mullard conductivity cell. After decanting off the fines and degassing under reduced pressure the prepared DE52 was packed into glass columns (University of Aston Glass Blowing Department) (2 cm x 50 cm) plugged with glass wool. Samples (5-50ml) and appropriate standards were diluted to the conductivity of the starting buffer (0.05 M-sodium phosphate buffer) with distilled water before loading onto columns. Standard linear gradients (0 - 1.2 M-NaCl in starting buffer) were eluted automatically using an LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon, Surrey, UK). The eluant was passed through a U.V. monitor (LKB Uvicord II, LKB Instruments). 5 ml fractions were collected using an LKB Ultrarac 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 (0.5ml) of each fraction were counted as described below. All fractions were stored at -15°C in the presence of sodium ascorbate until analysis was complete. A summary of the elution pattern of relevant folate derivatives is given in Table 2.1.

(ii) Gel Filtration

Sephadex G15 (Pharmacia, Uppsala, Sweden) was placed in excess 0.05 - sodium phosphate buffer, pH 7.0, containing DTT (5 mg % w/v) and left to swell for 4 hours at room temperature. After degassing under reduced pressure the slurry was poured into Perspex columns (2 cm x 60

TABLE 2.1.

The elution properties of folates, p-aminobenzoic acid derivatives and unknown metabolites on DE52 ion exchange chromatography and Sephadex G15 gel filtration.

Compound	DE52 (molarity NaCl)	Sephadex G15 (fraction number)
Folic Acid	0.96	37
IOCHO-FA	0.53	21
IOCHO-THF	0.45	18
5CHO-THF	0.59	28
5MeTHF	0.67	37
5,IOCH$_2$-THF	0.64	25
Metabolite (X)	0.70	36
Pterin	0.30	35
Xanthopterin	0.57	57
p-aminobenzoic acid	0.40	35
p-acetamidobenzoic-acid	0.43	36
p-aminobenzoyl-L-glutamate	0.40	18
p-acetamidobenzoyl-L-glutamate	0.43	19
p-aminohippuric acid*	-	33
p-acetamidohippuric acid*	0.35	27
^3H_2O	0.0	21
Urea	0.0	21
New Peaks	0.84 - 0.90	34
^{3}H-only labelled peak	0.40 - 0.43	25

Elution conditions are described in the text.
* Elution positions taken from Connor (1979).

cm) (Wright Scientific Limited, Surrey, UK) and allowed to pack under pressure. Samples and standards were eluted from the base of the column upwards using 0.05 M-sodium phosphate buffer, pH 7.0. The eluant was monitored and collected as above. A summary of the elution pattern of relevant folate derivates is given in Table 2.1.

(iii) Paper Chromatography

Descending paper chromatography was performed in glass tanks equilibrated with a solvent composed of aq Ammonia (Sp.gr. 0.88)/ water / Propan-2-ol (1:99:200) using Whatman 3 MM chromatography paper and developed overnight in the dark. Samples and standards (10CHO-folate p-ABGlu, p-ABA and their acetyl derivates) were applied as spots using glass micropipettes. 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 U.V. light at 254nm or 355nm. The chromatograms were cut into strips and counted.

2.2.2. Animals

Experiments were conducted on WAB/Not rats supplied by Dr. M. Pimm (Department of Cancer Studies, University of Nottingham).

Rats were dosed orally by stomach intubation using specially prepared steel dosing needles. Where possible the rats were dosed without the use of anaesthetic but occasionally a vicious rat necessitated the use of diethyl ether.

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 0.35ml. The animals were then transferred to metabolism cages (Jencons Metabowls; Jencons (Scientific) Limited, Hemel Hempstead, Herts, UK) designed to allow the separate collection of urine and faeces. During experiments the rats were at 21°C in a sealed room having a fixed 12 hour dark and 12 hour light cycle and allowed free access to food (Breeding Diet, Pilsbury's Limited) (Table 2.2.) and water.

Urine samples were collected into flasks containing 10 ml 0.05 M-sodium phosphate buffer, pH 7.0, containing sodium ascorbate (2% w/v). To prevent light degradation the flasks were wrapped in aluminium foil.

Rats were killed by cervical dislocation, with the occasional use of diethyl ether where necessary. The thorax was then opened and tissues removed for freeze-drying for direct determination of radioactivity or for extraction for qualitative examination of retained radioactivity.

2.2.3. Measurement of Radioactivity

Initially, prepared samples were counted in a Nuclear Enterprises Liquid Scintillation Counter Type 8310 (Nuclear Enterprises Limited, Edinburgh) using a scintillation cocktail composed of toluene (one litre) and Fisons Emulsifier mix No.1 (Fisons, Loughborough, Leics) (500ml) in which were dissolved 2,5-diphenyloxazole (PPO) (5g) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (0.1g). Later experimental samples were counted on a Beckman LS7500 liquid scintillation counter

Table 2.2.

Pilsbury's Modified Rat and Mouse Breeding Diet.

Crude Oil	3.26%
Crude Protein	21.23%
Crude Fibre	3.48%
Digestible Oil	3.24%
Digestible Protein	17.60%
Digestible Fibre	2.10%
Digestible Carbohydrate	46.80%

Fats

Saturated Fatty Acids	0.73%
Linoleic Acid	0.99%
Other unsaturated fatty acids	1.54%

(Beckman Instruments Inc; Scientific Instruments Division, Irvine, California 92713) using Fisosolve liquid scintillation cocktail.

All aqueous samples eg. urine samples and column eluants were made up to 1 ml with distilled water, and 10 ml of the relevant scintillation cocktail. 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.

Freeze-dried tissue and faecal samples were ground to a fine powder and their radioactive content determined by combustion in a Beckman Biological Material Oxidiser. ^{14}Carbon was trapped as carbon dioxide in 15 ml of Fisons Fisosorb II. ^{3}H was collected as tritiated water in a dry ice/methanol cold trap and counted in 10ml of Fisons tritium absorber "H".

2.3. In Vitro Methods

2.3.1. The Bratton and Marshall Assay for Diazotizable Amines

Materials

Trichloroacetic acid (15% solution) (w|v)
Sodium nitrite (0.1% solution) (w|v)
N(-1-napthyl)ethylenediamine dihydrochloride (0.1% solution)(w|v)
Hydrochloric acid
Ammonium sulphamate (0.5% solution) (w|v)
Phosphate buffer

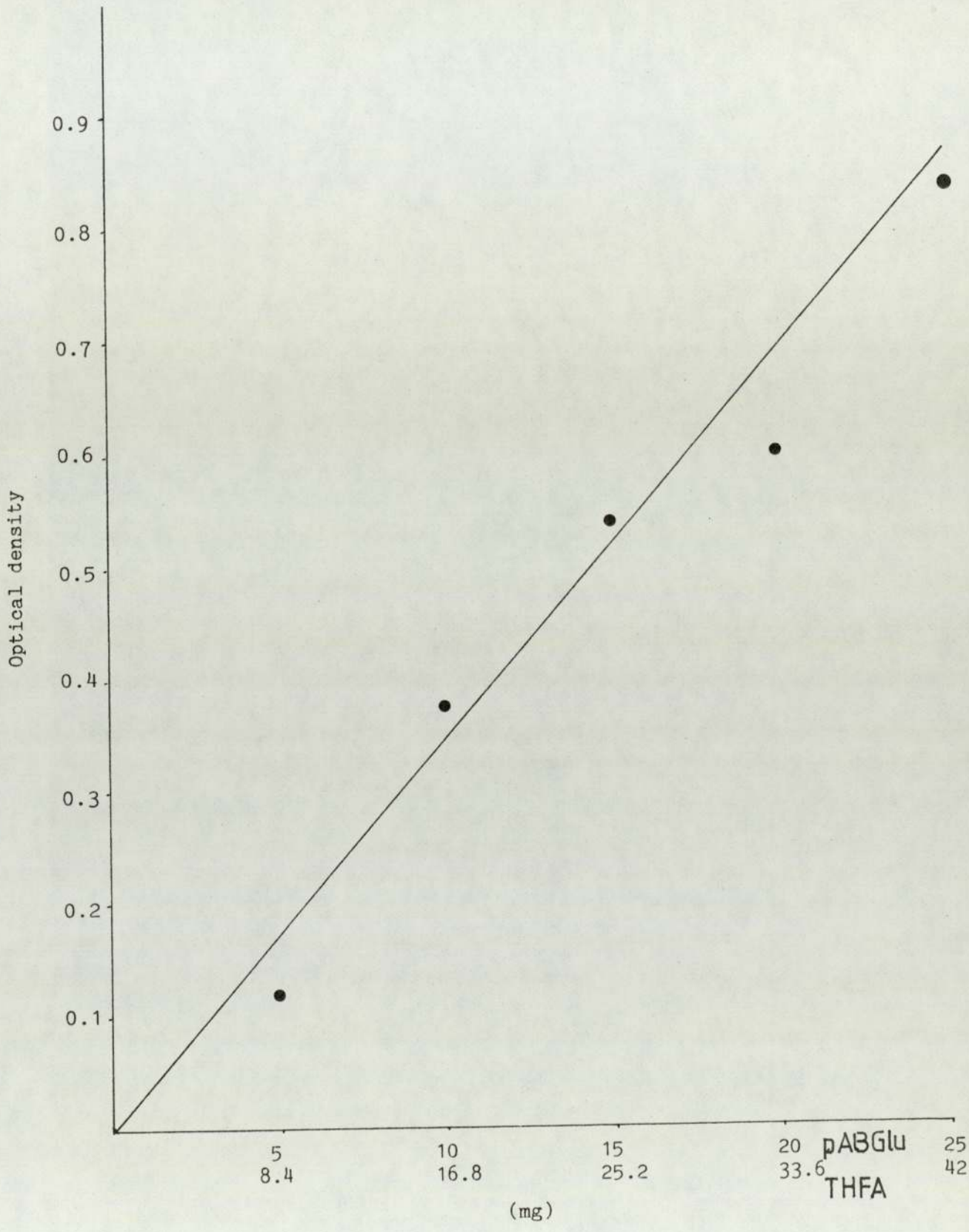


Figure 2.1: Calibration curve for p-ABGlu.

Tetrahydrofolic acid

p-Aminobenzoyl-L-glutamate

Xanthine

Xanthine oxidase.

Method

All reactions were conducted in an ice bath and protected from light. The tubes were shaken after each addition.

0.5 ml Hydrochloric acid was added to a 2.0 ml aliquot of the test solution. This mixture was then made up to 10.0 ml with phosphate buffer. 1.0 ml sodium nitrite was added and after 3 mins 1.0 ml of ammonium sulphamate was added. 2 mins later 1.0 ml of the dye solution was added. The reaction mixture developed a purple colour which was read 10 mins later on a colorimeter at 545nm.

A calibration curve was constructed using p-ABGlu (Fig. .2.1.). Initially the p-ABGlu was dissolved in phosphate buffer containing sodium ascorbate (5% w/v) but the ascorbate was found to interfere with the assay and was therefore omitted.

Triplicate incubations of THFA alone (1mg/ml); THFA plus Xanthine oxidase (20μl of a 1.0 unit/ml solution) and THFA plus Xanthine oxidase plus Xanthine (3mM) were carried out in an ice bath and protected from the light. Aliquots were removed and assayed at the start of the incubation (time 0) and at set time intervals up to 40 minutes.

2.3.2. Thin Layer Chromatography

Materials

Solvent systems:-

A. 5% acetic acid (w/v) containing 5% sodium ascorbate (w/v).

B. 10% acetic acid (v/v) containing 1% sodium ascorbate. (w/v).

C. 10% acetic acid (v/v) containing 10% sodium ascorbate. (w/v).

Incubations:-

Tetrahydrofolic acid 1mg/ml solution in 0.01 Molar phosphate buffer; Xanthine (3mM); Xanthine oxidase (20 μl); sodium ascorbate; phosphate buffer containing sodium ascorbate (1% w/v).

Methods

All incubations were protected from the light and conducted at 37°C. THFA was incubated either alone or with Xanthine oxidase and/or Xanthine as shown in Table 2.3.

Table 2.3.

Incubation used in in vitro experiments to investigate the oxidation of THFA in the presence of superoxide anion.

	THFA (ml)	Xanthine Oxidase (μl)	Xanthine	Buffer (ml)	Total (ml)
1.	7	0	0	1	8
2.	7	20	0	1	8
3.	7	20	1	0	8

0.5 ml aliquots were removed at 0, 3, 7 and 10 minutes from the start of the incubation and immediately mixed with 2.0 ml phosphate buffer containing sufficient sodium ascorbate to give a final concentration of 10% and then plunged into a methanol/dry ice bath. Solutions were stored frozen and protected from light.

T.l.c. (thin layer chromatography) plates were spotted with approximately equal amounts from each of the reaction mixtures and run in a variety of solvents. When dry the results were read in dark room using an ultra-violet light at 365nm.

CHAPTER 3

FOLIC ACID METABOLISM IN THE ADULT MALE RAT

This chapter describes the metabolism of folic acid by the male rat. The normal pattern of folate metabolism was determined in healthy male rats dosed orally with a mixture of [2- ^{14}C] and [3',5',7,9- ^{3}H] folic acid and compared to that found in another group of rats pretreated with the anticonvulsant drug phenytoin which can cause folate deficiency. The influence of the gastrointestinal microflora upon folate metabolism was investigated in rats pretreated with antibiotics.

3.1. Folic Acid Metabolism in the Adult Male Rat

Considerable incorporation into the reduced folate coenzyme pool of an oral dose of radiolabelled folic acid has been described (see Chapter 1 for references). The breakdown of folates within the body has likewise been shown to be a normal phenomenon but the precise mechanism has yet to be fully elucidated. This section describes the metabolic fate of oral [2-^{14}C] and [3',5',7,9- ^{3}H] folic acid in the normal male rat up to 72 hours after dosing.

Materials and Methods

15 male WAB/Not rats (180-210g body weight) were dosed orally with a mixture of [2- ^{14}C] and [3',5',7,9- ^{3}H] folic acid (100μg/kg body weight). 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 and the next 5 rats were placed into the metabowls. Liver, kidney and gut samples were removed for the

determination of retained radioactivity. The major portion of the removed livers was quickly washed in ice cold buffer, chopped into small pieces and dropped into 4 volumes of boiling 0.05M phosphate buffer, pH 7, containing sodium ascorbate (2% w/v) and maintained at 100°C for 5 minutes. This inactivated the enzyme folate polyglutamate conjugase which would otherwise have rapidly broken down the hepatic polyglutamates. Homogenisation and centrifugation of this hot liver extract followed by Sephadex gel filtration of the supernatant allowed an estimation of the hepatic polyglutamate burden. 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 chromatography, Sephadex G15 gel filtration or liquid scintillation counting.

Results

Table 3.1.1. shows the recovery of radioactivity excreted in the urine and 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 were statistically significant (at 24h $p = < 0.05$, at 48h $p = < 0.001$ and at 72h $p = < 0.05$). However similar levels of ^{3}H and ^{14}C were found in the kidneys and gastrointestinal tract samples. The slight elevation of ^{3}H levels seen in the livers was not statistically significant.

Table 3.1.1.

Radioactivity in the urine, faeces and tissues of healthy male rats (180 - 210g body weight) 24h, 48h and 72h after the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2 - ^{14}C) folic acid (100μg/kg body weight). Results are expressed as a percentage of the dose administered ($\pm$ SE) n=5.

	24h		48h		72h		0-72h	
Sample	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine	23.4 (2.3)	31.8 (3.2)	1.6 (0.3)	8.1 (2.3)	0.3 (0.07)	1.0 (0.3)	25.3	40.9
Faeces	15.3 (4.3)	6.7 (2.1)	1.3 (0.4)	0.8 (0.2)	0.1 (0.04)	0.6 (0.2)	16.7	8.1
Liver	15.7 (1.4)	18.6 (1.6)	19.1 (1.8)	21.6 (2.3)	17.9 (1.8)	20.6 (2.2)	17.9	20.6
Kidney	2.5 (0.8)	3.4 (0.6)	1.6 (0.17)	2.0 (0.5)	1.9 (0.2)	2.4 (0.4)	1.9	2.4
Gut	3.2 (0.3)	3.9 (0.4)	2.3 (0.3)	2.5 (0.2)	1.5 (0.2)	2.4 (0.3)	1.5	2.4
Total							63.3	74.4

Urinary Metabolites

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, 4 of which retained both the ^{3}H and ^{14}C labels, had almost identical isotopic ratios and were identified as (IV) 10-formylfolate, (V) 5,10 CH_2-THF, (VI) 5 MeTHF and (VIII) folic acid. The ratio of ^{3}H : ^{14}C in the folate derivatives was higher than in the folic acid administered. The other radioactive peaks 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 eluted at fraction number 19 had a high ^{3}H : ^{14}C ratio and was mixture of 10CHO-folate, present due to overlap of peak (IV) with peak (III) on the DE 52 column, and another metabolite labelled only with ^{3}H, p-AcBGlu. The second peak eluted at fractions 36-37, appeared to contain only ^{3}H and co-chromatographed with p-AcAB. The third component had a much reduced ^{3}H : ^{14}C ratio and eluted at fraction number 41. Previous efforts to identify this compound have failed but it has been characterised (Connor, 1979; Pheasant et al., 1981) as a reduced pteridine which has retained the ^{3}H label at C^{7} 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 previously identified as urea (Connor et al., 1977).

The small peak (VII) eluting from DE 52 as a shoulder of the 5 MeTHF containing peak (VI) had a ^{3}H : ^{14}C ratio similar to the intact folates

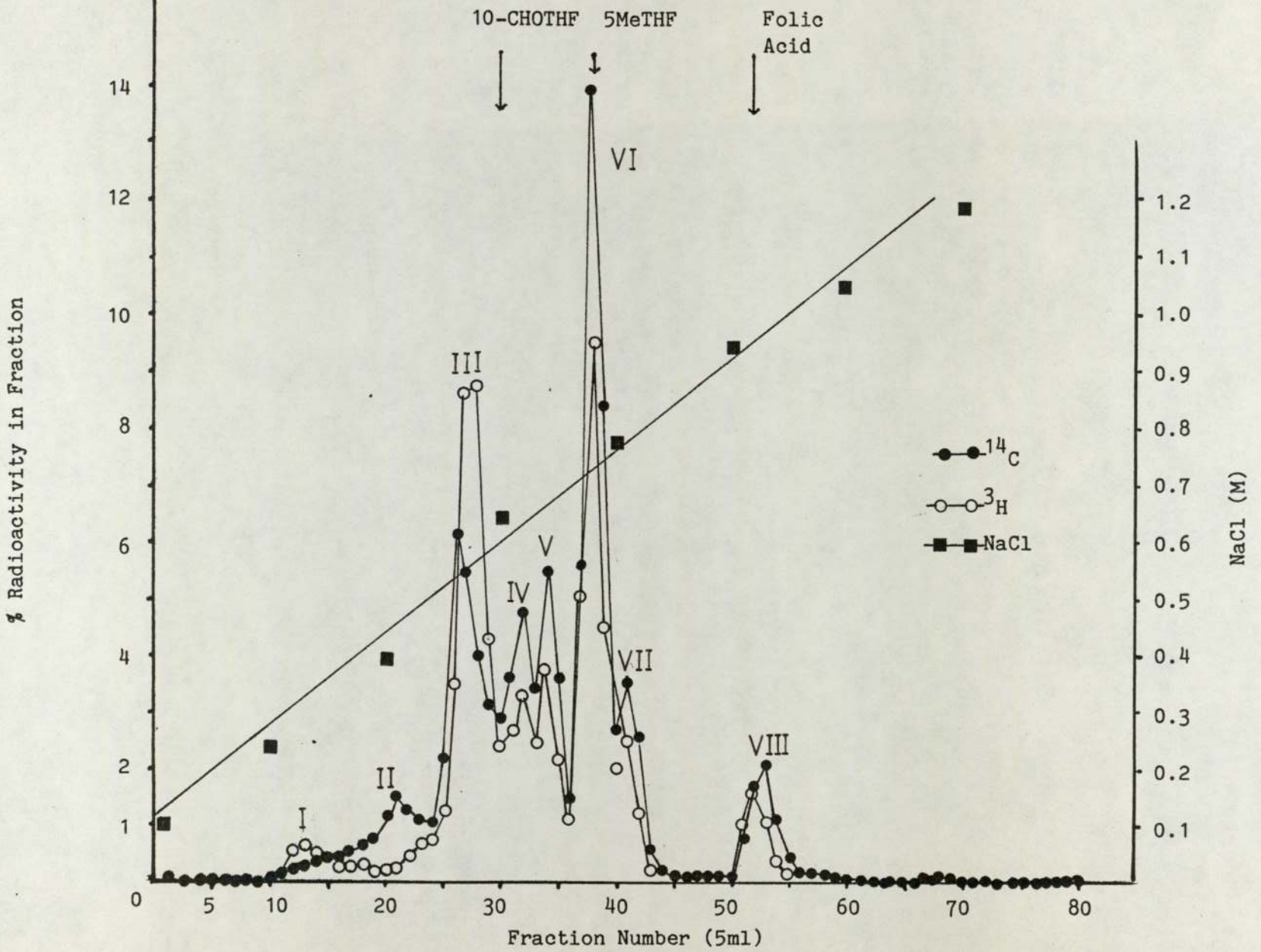


Figure 3.1.1. DE52 chromatography of adult male rat urine samples collected 0-24h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) Folic acid.

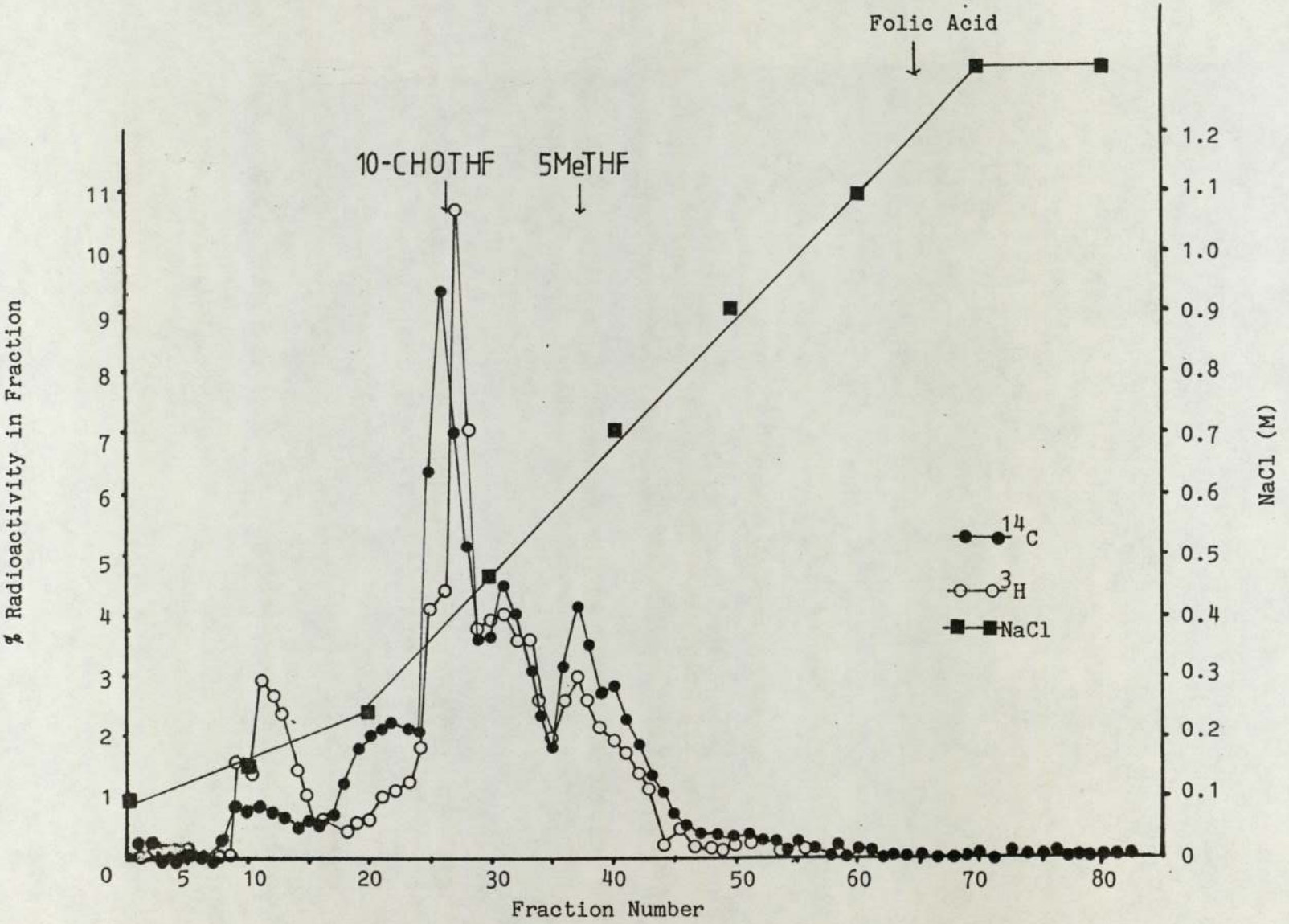


Figure 3.1.2. DE52 chromatography of adult male rat urine samples collected 24-48h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) Folic acid.

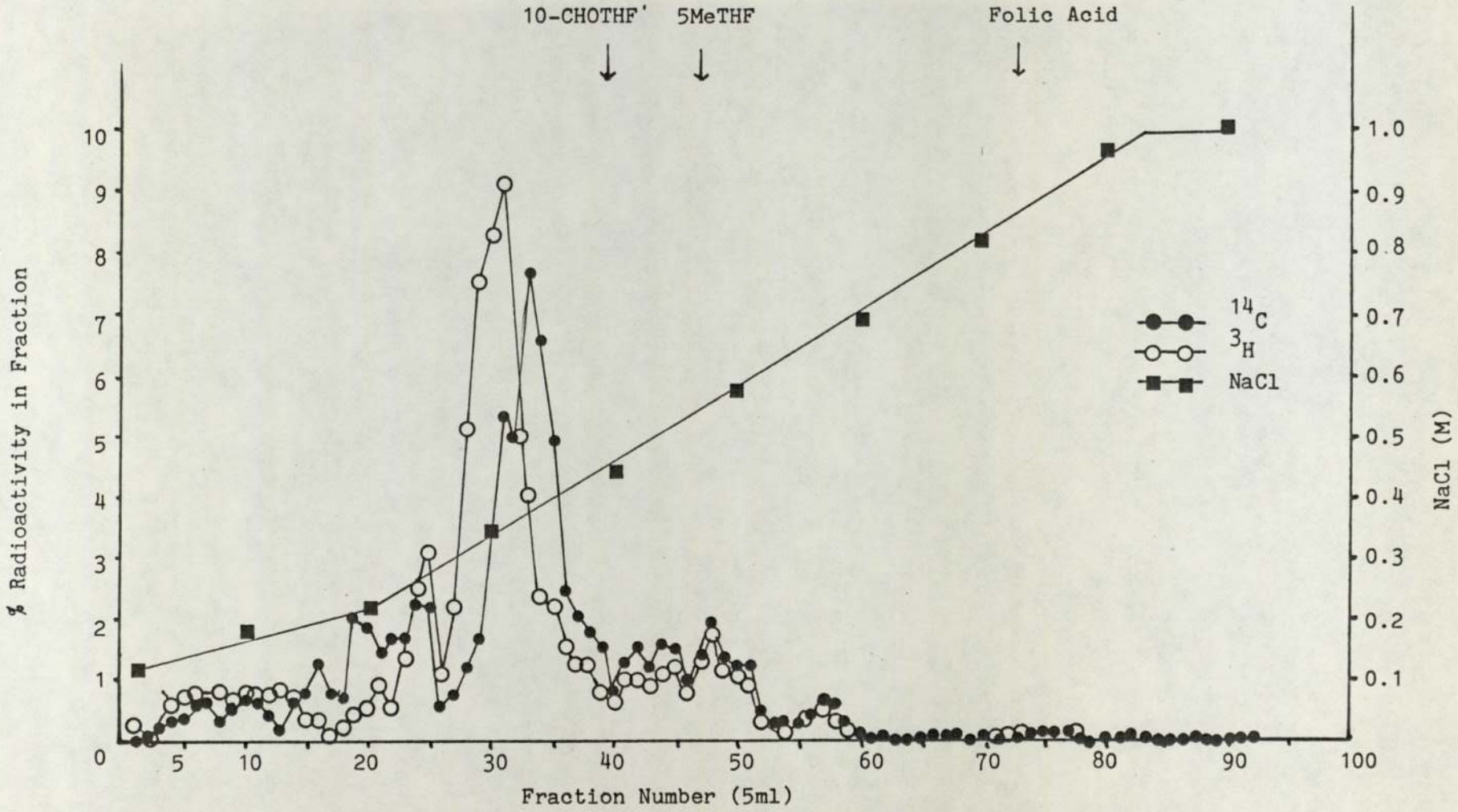


Figure 3.1.3: DE52 chromatography of adult male rat urine samples collected 48-72h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid.

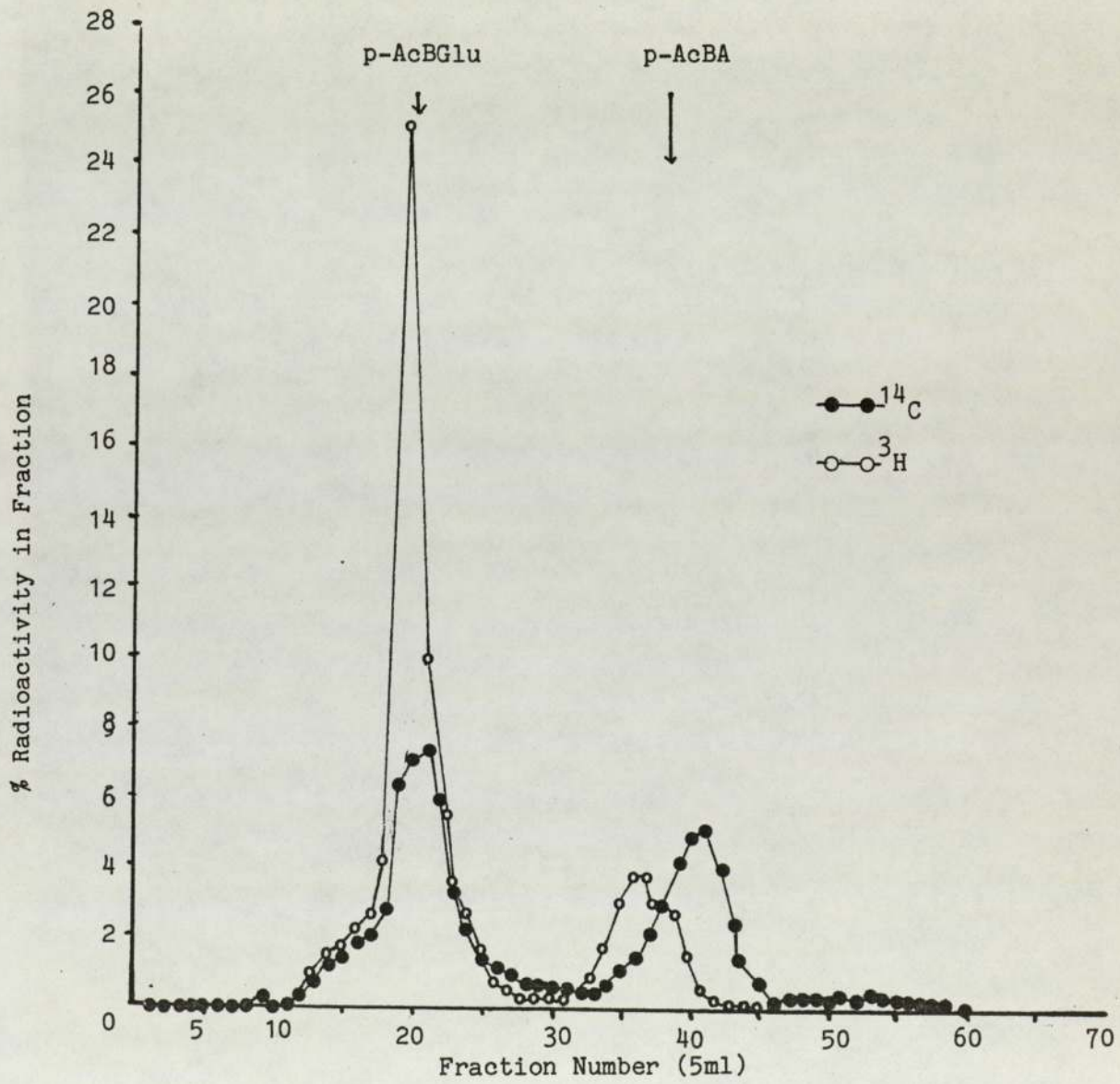


Figure 3.1.4. Sephadex G15 chromatography of peak (III) from DE52 chromatography of urine (0-24h) from adult male rats.

and was designated Metabolite X.

DE 52 chromatography of the later urine samples showed a progressive simplification in the pattern of metabolites (Figures 3.1.2. and 3.1.3.). Folic acid (VIII) and 5,10 CH_2 THF (V) disappeared, the relative amounts of 5 MeTHF (VI), 10CHO-folate (IV) and Metabolite X (VII) decreased while the scission product peak (III) progressively dominated the picture. Sephadex G15 gel filtration of this peak showed that in the 24-48h sample (Figure 3.1.5.) p-AcBGlu was the major ^{3}H containing metabolite as levels of p-AcBA fall both in comparison to that seen in the 0-24h urine sample (Figure 3.1.4.) and in relation to pAcBGlu.

Table 3.1.2. summarises the metabolites found in the urine samples over 0-72h. Early samples contain predominantly intact folates with low levels of the single labelled scission products. However, with time the levels of intact folates fall sharply with scission products, especially pAcBGlu dominating later samples.

Liver Extracts

Sephadex G15 gel filtration of hot liver extracts are illustrated in Figures 3.1.6., 3.1.7. and 3.1.8. 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.

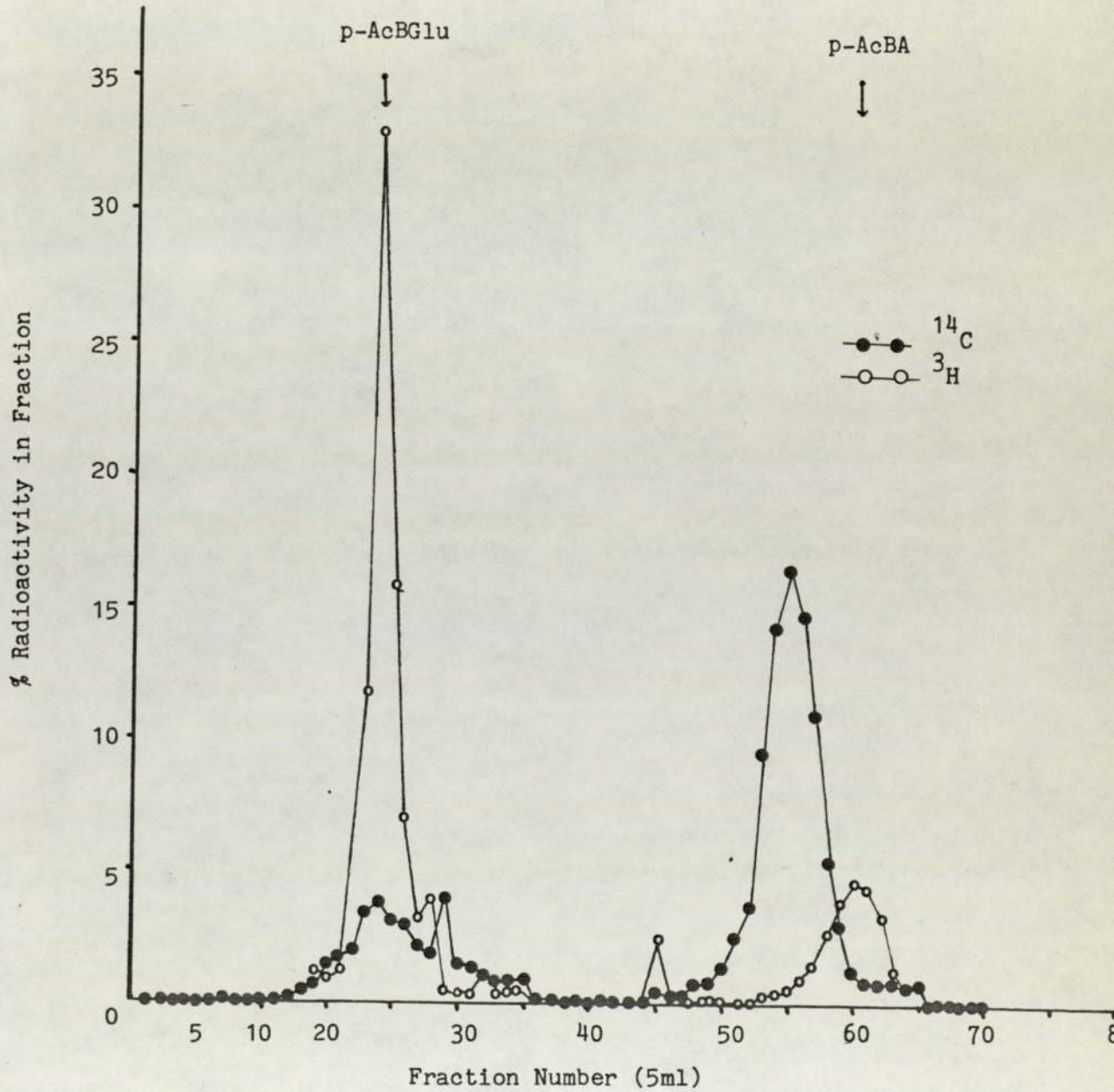


Figure 3.1.5. Sephadex G15 chromatography of peak III from DE52 chromatography of urine (24-48h) from adult male rats.

Table 3.1.2.

Metabolites present in the urine of healthy male rats (180 - 210g body weight) 24h, 48h and 72h after the oral administration of a mixture of (3', 5', 7, 9 - ^{3}H) and (2 - ^{14}C) folic acid (100µg/kg body weight). Results are expressed as a percentage of the dose administered. n=5. ND = not detectable.

	0-24h		24-48h		48-72h		0-72h	
Metabolites	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	1.5	1.8	ND	ND	ND	ND	1.5	1.8
5MeTHF	7.4	8.7	0.2	1.1	0.1	0.1	7.7	9.9
10CHO-Folate	6.3	8.2	0.4	1.9	ND	ND	6.7	10.1
5,10CH_2THF	3.3	3.8	ND	ND	ND	ND	3.3	3.8
Metabolite X	2.2	2.3	0.2	0.8	ND	ND	2.4	3.1
p-AcBGlu	-	5.3	-	1.4			-	7.5
p-AcBA	-	2.3	-	0.5		0.8*	-	2.8
^{14}C only labelled catabolites	2.9	0.2	0.5	-	0.2*		3.6	0.2
Urea	0.9	-	0.1	-			1.0	0
$^{3}H_2O$	-	0.9	-	1.3			0	2.2

* Incomplete analysis is due to due low level of radioactivity present.

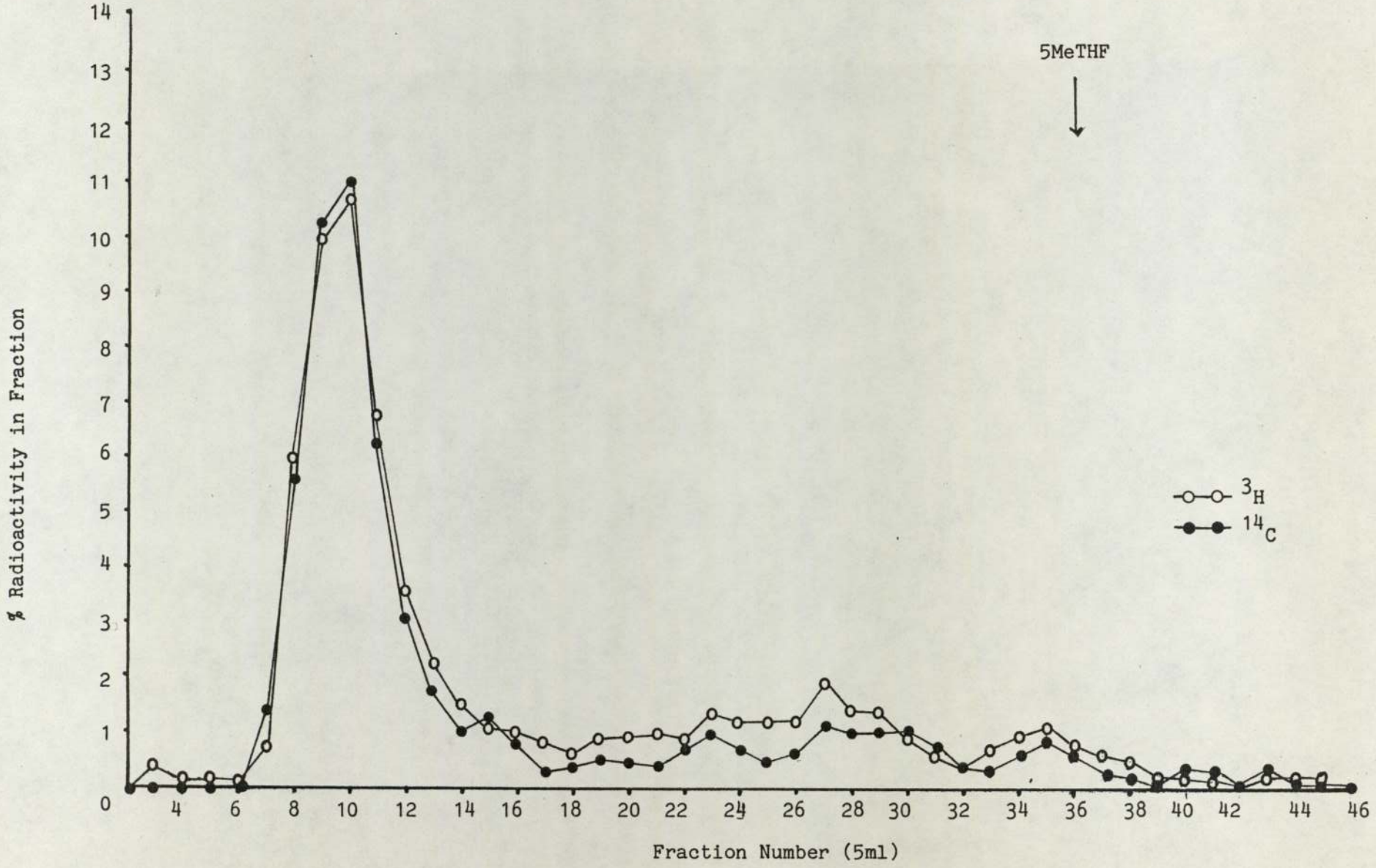


Figure 3.1.6. Sephadex G15 chromatography of the liver extract from adult male rats 24h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100μg/kg b.wt).

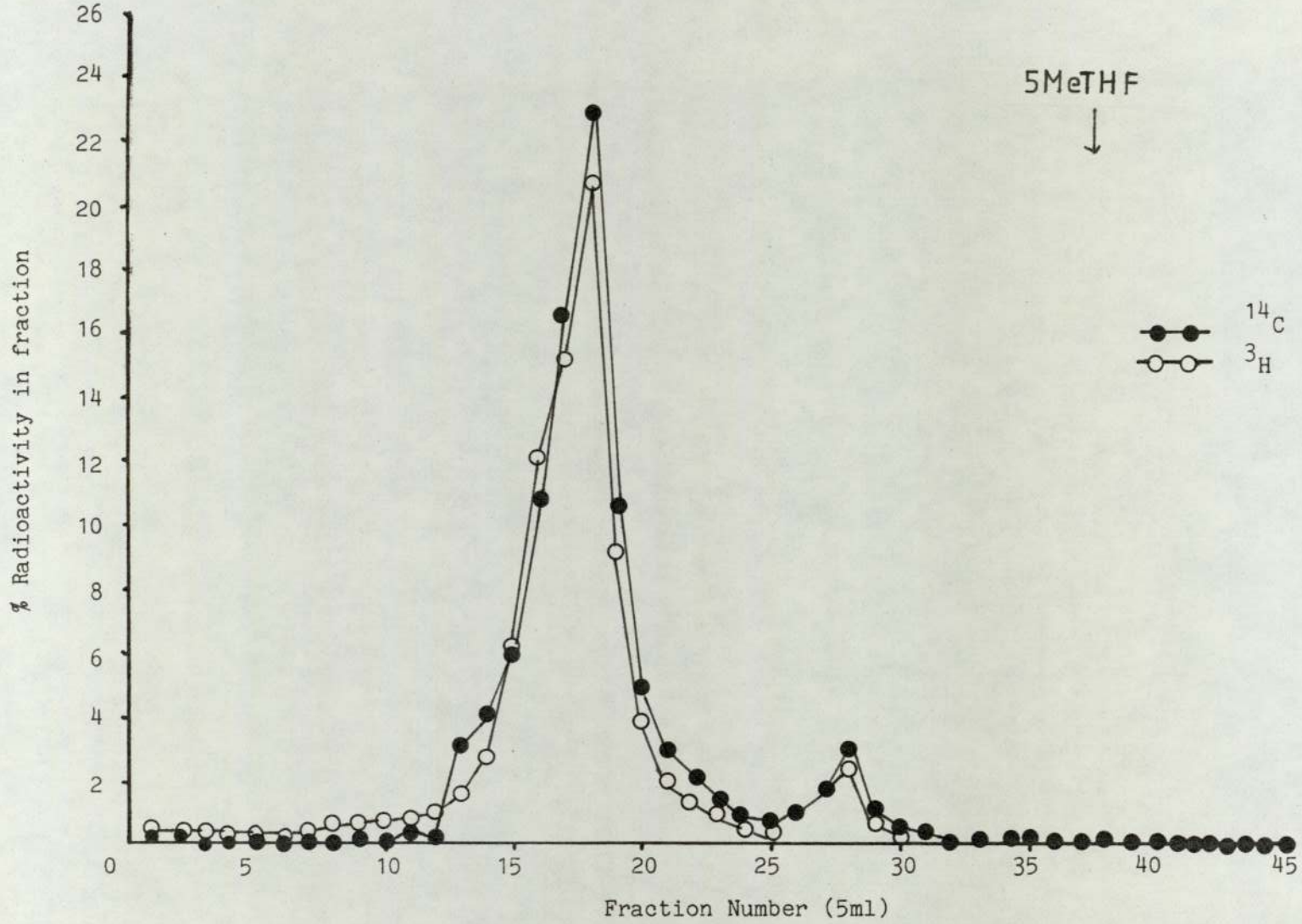


Figure 3.1.7: Sephadex G15 chromatography of the liver extract from adult male rats 48h after the administration of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid.

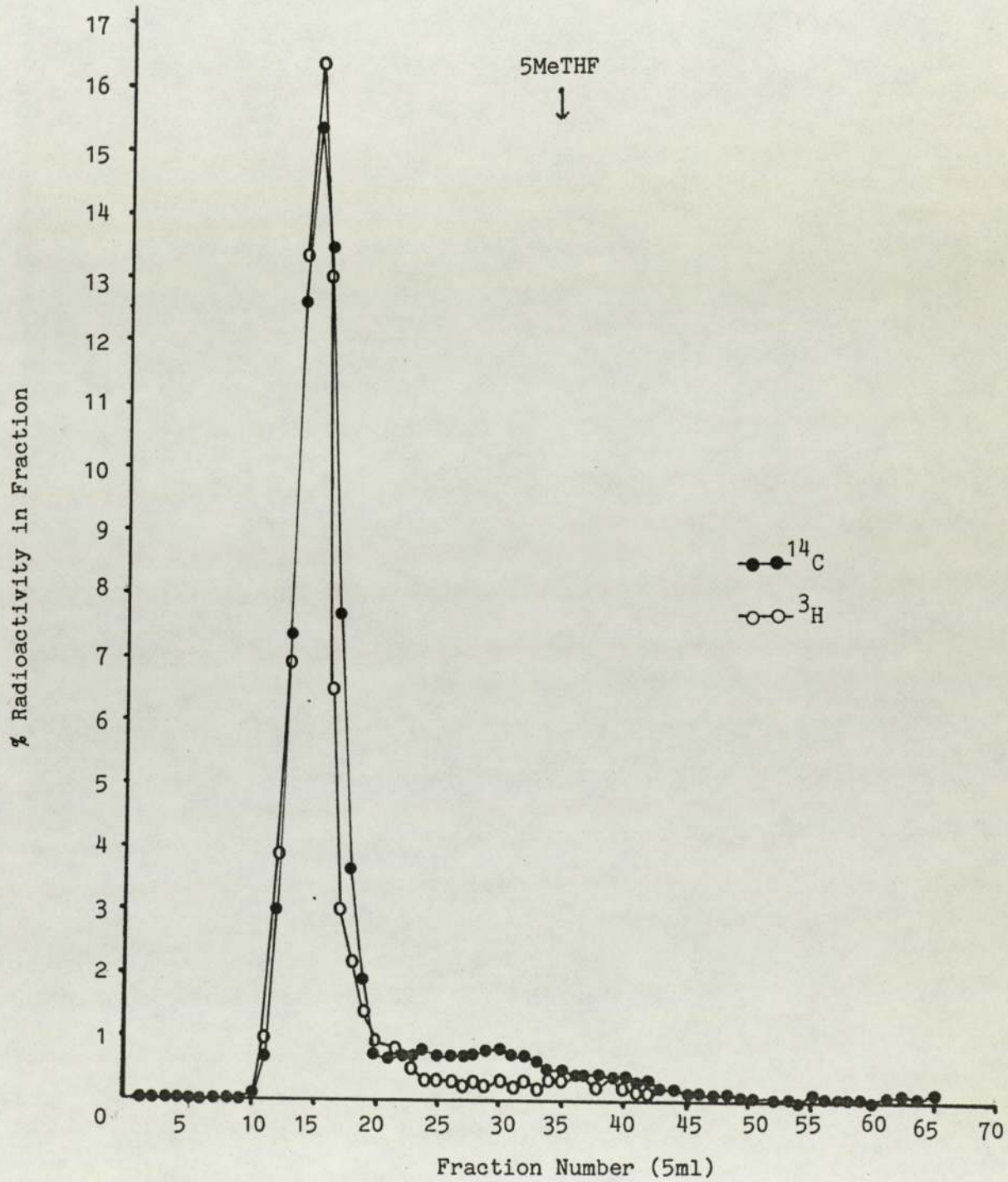


Figure 3.1.8. Sephadex G15 chromatography of the liver extract from adult male rats 72h after the administration of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid.

3.2. The Effect of Phenytoin on Folic Acid Metabolism in The Male Rat

Chronic administration of anticonvulsant drugs to epileptic patients has been associated with the development of folate deficiency (Reynolds, 1980). This side effect is most commonly observed in those patients prescribed phenytoin, either alone or in combination with phenobarbitone. Deficiency may develop after any duration of therapy and may be reversed by the concurrent administration of folic acid. The mechanism by which phenytoin causes folate deficiency is not known but suggestions have included an effect on the absorption of folates from the gut (Benn et al., 1971; Hoffbrand and Necheles, 1968; Hepner et al., 1970) by various mechanisms; enzyme induction of pathways requiring folate leading to increased utilization (Maxwell et al., 1972; Labadarios et al., 1978); and an increased rate of breakdown of folates within the body (Kelly et al., 1979; Krumdieck et al., 1978). It was therefore decided to investigate the effect of phenytoin on folate metabolism in the rat in an attempt to finally elucidate the way in which folate deficiency is produced.

Materials and Methods

15 male WAB/Not rats (180-210g body weight) were pretreated with phenytoin (diphenylhydantoin sodium) (100 mg/Kg body weight/day in 0.05M phosphate buffer) administered as an intraperitoneal injection each day for 10 days prior to a single oral dose of [2- ^{14}C] and [3',5',7,9- ^{3}H] folic acid (100μg/kg body weight). Daily phenytoin treatment was then continued until the end of the experiment. Urine

and faeces were collected for periods of 0-24h, 24-48h and 48-72h as described previously (Section 3.1.). 5 animals were killed 24h, 48h and 72h after the administration of folic acid. Animals and tissues were handled as described previously (Section 3.1.).

Results

Table 3.2.1. shows the recovery of radioactivity in the urine and faeces and the distibution of retained radioactivity in rats treated with phenytoin prior to oral [2- ^{14}C] and [3',5',7,9- ^{3}H] folic acid. Total overall recovery figures are close to those found in the normal rat (71.8% and 63.3% ^{14}C and 78.6% and 74.4% ^{3}H). Therefore a direct comparison of the results from each group may be made without correction.

Overall excretion of ^{14}C in the urine was similar to that found in the normal rats (27.3% in the treated group and 25.3% in the controls). However the excretion of ^{14}C into the urine differs when compared on a 24h basis with less appearing in the initial 24h urine (19.4% as compared to 23.4% in the controls) but more appearing in the 24-48h urine (4.7% as compared to 1.6%) and the 48-72h urine (3.2% as compared to 0.3%). Less ^{3}H is present in all urine samples from the rats receiving phenytoin, except that collected 48-72h after the folic acid was administered. This is reflected in a lower overall recovery (30.6% as compared to 40.9%). The large excess of ^{3}H over ^{14}C found in the urine of normal rats does not occur when the rats have been treated with phenytoin and there is no statistical significance between ^{3}H and ^{14}C recoveries in this group.

Table 3.2.1.

Radioactivity in the urine, faeces and tissues of healthy male rats (180 - 210g body weight) treated with phenytoin (100 mg/kg body weight i.p./day in 0.05M phosphate buffer for 10 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 weight). Results are expressed as a percentage of the dose administered (± S.E.). n=5.

	24h		48h		72h		0-72h	
Sample	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine	19.4 (2.7)	20.5 (3.0)	4.7 (1.3)	5.4 (1.6)	3.2 (1.3)	4.7 (1.8)	27.3	30.6
Faeces	22.4 (2.5)	13.6 (1.6)	5.5 (0.8)	4.6 (0.6)	4.8 (1.0)	4.1 (1.0)	32.7	22.3
Liver	19.9 (2.9)	20.3 (2.6)	11.8 (1.4)	11.9 (1.9)	19.3 (3.8)	22.9 (4.0)	19.3	22.9
Kidney	2.0 (0.3)	1.7 (0.3)	1.6 (0.3)	1.1 (0.2)	1.0 (0.3)	1.3 (0.2)	1.0	1.3
Gut	1.6 (0.1)	1.9 (0.1)	2.1 (0.3)	2.1 (0.2)	1.5 (0.3)	1.5 (0.2)	1.5	1.5
Total							71.8	78.6

More radioactivity is excreted in the faeces by the rats treated with phenytoin. Overall recoveries are greater for ^{14}C (32.7% as compared to 16.7% in the control group) and ^{3}H (22.3% as compared to 8.1% in the control group). This trend is also found in the 24h collections of faeces with considerable radioactivity present in the 48-72h sample (4.8% ^{14}C and 4.1% ^{3}H) whereas only low levels could be detected at this point in faeces from the normal rats (0.1% ^{14}C and 0.6% ^{3}H). The excess of ^{14}C over ^{3}H seen in the faeces from the normal rats only occurs in the 0-24h samples ($p = 0.05$) from the phenytoin treated rats.

The tissue retention of radioactivity following phenytoin treatment is similar to that found in normal rats. Most radioactivity was found in the liver (19.3% ^{14}C and 22.9% ^{3}H at 72h) with much lower levels occuring in the kidneys (1.0% ^{14}C and 1.3% ^{3}H at 72h) and gut (1.5% ^{14}C and 1.5% ^{3}H at 72h).

Urinary Metabolites

Table 3.2.2. summarises the metabolites identified following ion exchange chromatography and gel filtration as described in detail in Section 3.1.. It may be seen that although broadly speaking the pattern of metabolites found in the urine from normal rats also occurs in the urine of the phenytoin treated rats, there are certain differences.

Folic acid was only detectable in the 0-24h urine sample from normal rats but it was found in each sample up to 72h in the urine of the phenytoin treated group. The levels found in the 0-24h urine samples

Table 3.2.2.

Metabolites present in the urine of healthy male rats (180 - 210g body weight) treated with phenytoin (100 mg/kg body weight i.p./day in 0.05m phosphate buffer for 10 days) prior to the oral administration of a mixture of (3', 5', 7, 9 -^{3}) and (2-^{14}C) folic acid (100μg/kg body wt.) Results are expressed as a percentage of the dose administered. n=5. ND = not dectable.

	0-24h		24-48h		48-72h		0-72h	
Metabolites	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic acid	4.9	3.2	0.5	0.4	0.2	0.3	5.6	3.9
5MeTHF	5.5	3.6	0.7	0.6	0.2	0.2	6.4	4.4
10CHO-folate	4.4	4.7	0.7	0.7	0.3	0.3	5.4	5.7
5,10CH$_2$THF	ND	ND	ND	ND	ND	ND	ND	ND
Metabolite X	ND	ND	ND	ND	ND	ND	ND	ND
New Peaks	ND	ND	1.6	1.6	1.0	1.3	2.6	2.9
p-AcABGlu	-	2.7	-	1.3	-	0.6	-	4.6
p-AcBA	-	0.6	-	0.6	-	0.5	-	1.7
^{14}C only catabolites	1.8	-	0.5	-	0.9	-	3.2	-
Urea	2.0	-	0.7	-	0.4	-	3.1	-
$^{3}H_2O$	0	0.8	-	0.4	0	1.1	-	2.3

were also different with more folic acid present in the treated group (4.9% ^{14}C and 3.2% ^{3}H) than in the control group (1.5% ^{14}C and 1.8% ^{3}H). In contrast less 5 MeTHF and 10CHO-folate were present in the urine of the phenytoin treated rats and 5,10CH_2-THF and Metabolite X were undetectable. However, two previously unseen dual labelled peaks occur in the 24-48h and 48-72h urines eluting from DE 52 cellulose between 0.6 and 0.75M NaCl (Figures 3.2.1. and 3.2.2.). Sephadex G15 gel filtration of these peaks is illustrated in Figure 3.2.3..

Table 3.2.2. shows that over 72h the total amount of ^{3}H - only labelled catabolites is 8.6% of the dose after phenytoin treatment as compared to 12.5% in the normal rats. This reduction is due to the low levels of p-AcBGlu and p-AcBA excreted 0-24h after the administration of the folic acid. Later levels of excretion are similar to those found in the normal rat. Equal amounts of ^{14}C-only labelled catabolites are present in both the treated rat urine and the normal rat urine but higher levels of ^{14}C-urea are excreted following phenytoin treatment.

3.3. The Effect of Antibiotics on Folic Acid Metabolism in The Male Rat

Intestinal bacteria are known to play an important role in the metabolism of some compounds, an example being the metabolism of salicylazosulfapyridine in the rat (Peppercorn and Goldman, 1972). The intestinal microflora cleave the azo bond yielding 5-aminosalicylate and sulfapyridine both of which, unlike the parent compound, may then be absorbed and further metabolised. Germ-free rats fed this same drug

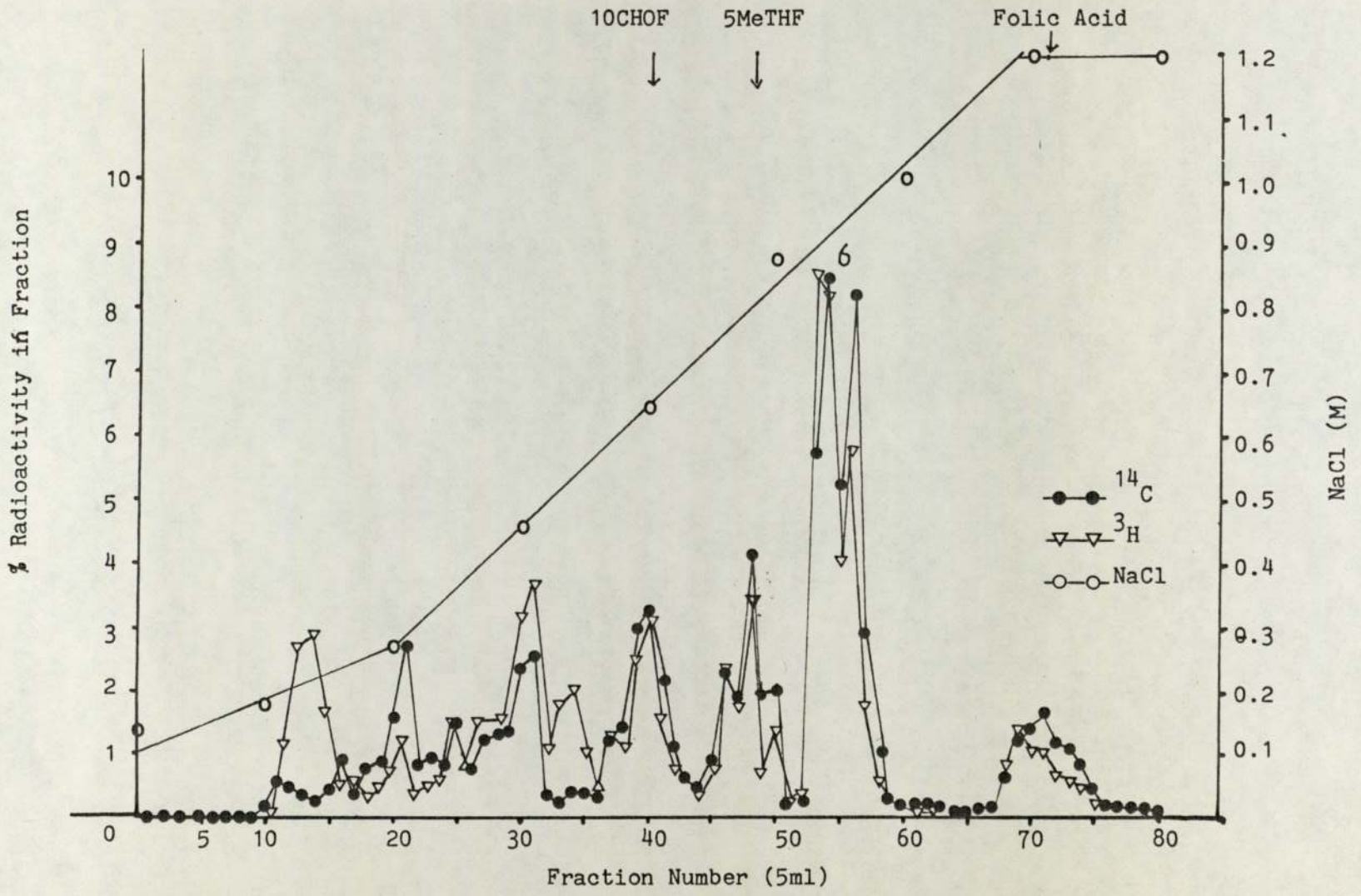
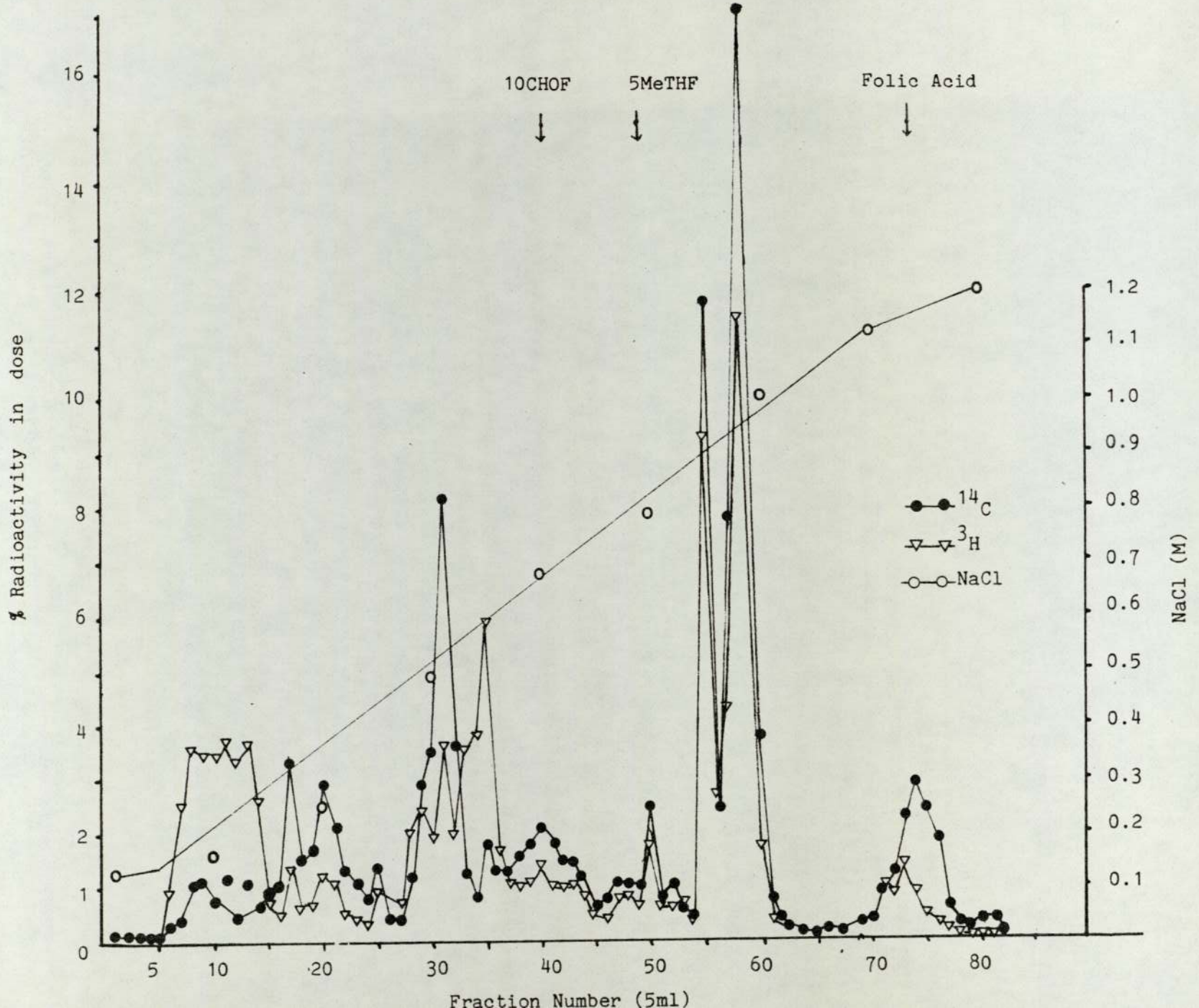


Figure 3.2.1: DE52 chromatography of urine samples collected 24-48h after the administration of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid to adult male rats treated with phenytoin.

Figure 3.2.2: DE 52 chromatography of urine samples collected 48-72h after the administration of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid to adult male rats treated with phenytoin.


10CHOF
5MeTHF
Folic Acid
% Radioactivity in dose
NaCl (M)
Fraction Number (5ml)
^{14}C
^{3}H
NaCl
0
1
2
4
6
8
10
12
14
16
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
1.2
5
10
20
30
40
50
60
70
80

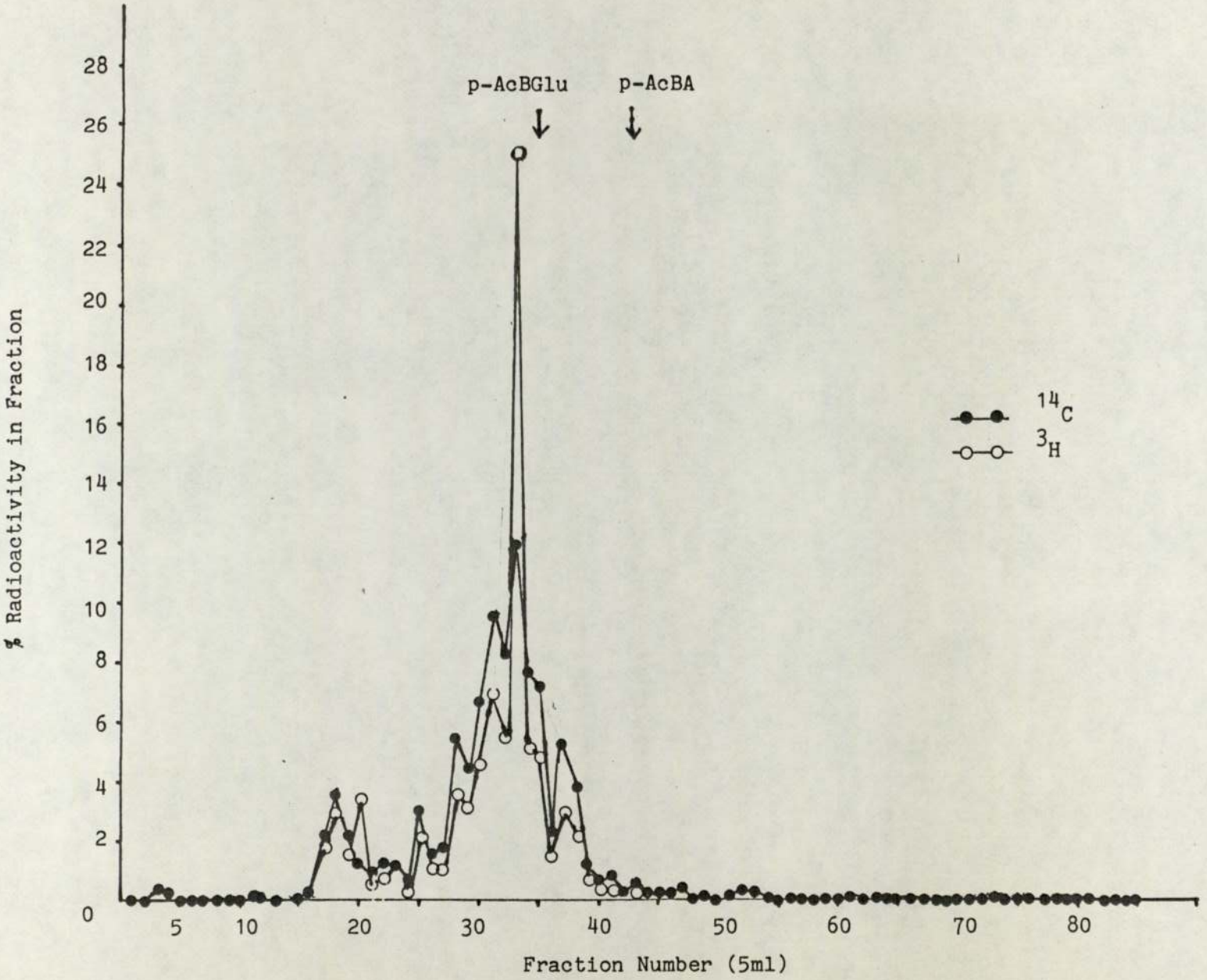


Figure 3.2.3: Sephadex G15 chromatography of peak 6 from DE52 chromatography of urine (48-72h) from adult male rats treated with phenytoin.

excrete none of the usual metabolites and virtually all of the dose is recovered unchanged in the faeces (Peppercorn and Goldman, 1972).

Folate coenzymes are extremely labile and there are a number of ways that intestinal bacteria could influence their metabolism in the rat. The bacteria could break down the folic acid directly following oral administration or they could initially reduce the folic acid prior to breaking it down. Many of the coenzyme forms are likely candidates for interaction with intestinal bacteria, including folic acid, dihydrofolate, tetrahydrofolate, 10CHO-folate or 5MeTHF. As tetrahydrofolate is very readily oxidised it is highly likely to undergo breakdown (Blair and Pearson, 1974). Recent experiments in the guinea pig, using antibiotics in the drinking water, showed an alteration in the pattern of urinary scission products (Choolun, 1982). It was therefore decided to investigate the influence of intestinal bacteria on folic acid metabolism in the rat using a regime of antibiotics known to minimise the microfloral population in this species (Gingell et al., 1971).

Materials and Methods

15 Male WAB/Not rats (190-210g body weight) received twice daily oral doses of a mixture of neomycin sulphate (20mg), tetracycline hydrochloride (10mg) and bacitracin (10mg) for two days (Gingell et al, 1971) prior to the administration of folic acid. This was given as a single oral dose of a mixture of [2 - ^{14}C] and [3',5',7,9 - ^{3}H] folic acid (100μg/kg body weight). Antibiotic administration was continued until the end of the experiment. Urine and faeces were collected for

24h periods up to 72h as described earlier (Section 3.1.). Groups of 5 animals were killed 24h, 48h and 72h after the folic acid dose. Animals, tissues and samples were handled as previously described (Section 3.1.).

Results

Table 3.3.1. shows the recovery of radioactivity in the urine and faeces and the tissue distribution of retained radioactivity from the group of rats treated with antibiotics before the administration of the isotopically labelled folic acid. The total overall recovery of both ^{3}H and ^{14}C is very high (91.5% ^{14}C and 88.0% ^{3}H). The 0-72h recovery figures show that this increase is due to more radioactivity being retained by the liver and a greater excretion of radioactivity into the faeces. These differences will be highlighted later.

There is no difference in the amount of ^{14}C excreted into the urine over 72h by either group but the antibiotic treated group excrete significantly more ^{14}C into the 48-72h urine ($p = 0.05$). However, there is much less ^{3}H in the urine of the antibiotic treated group in the first and second samples and over 0-72h. There is no urinary ^{3}H excess in contrast to that previously observed in the control urine.

The 0-24h ^{14}C content of the faeces is unchanged by antibiotic treatment but later samples contain considerably more and the 0-72h ^{14}C recovery is almost double that found in the control group of rats. The faeces also contain higher levels of ^{3}H (25.0% as compared to 8.1%)

Table 3.3.1.

Radioactivity in the urine, faeces and tissues of healthy male rats (190 - 210g body weight) treated with oral antibiotics (neomycin 20mg; tetracyline 10mg and bacitracin 10mg) twice daily for two 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 weight). Results are expressed as a percentage of the dose administered ± SE. n=5.

Sample	24h		48h		72h		0-72h	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine	20.0 (7.1)	21.1 (6.9)	2.4 (1.0)	2.6 (1.0)	1.9 (0.3)	2.1 (0.3)	24.3	25.8
Faeces	15.6 (7.1)	12.2 (5.8)	9.6 (2.6)	7.4 (2.2)	7.3 (1.2)	5.4 (2.0)	31.5	25.0
Liver	26.9 (3.7)	30.8 (3.9)	26.2 (4.5)	30.8 (5.0)	28.8 (1.7)	30.1 (2.7)	28.8	30.1
Kidney	5.3 (0.4)	5.2 (0.4)	4.0 (0.1)	4.1 (0.2)	3.2 (0.2)	3.3 (0.2)	3.2	3.3
Gut	8.5 (1.5)	9.8 (1.6)	5.5 (0.4)	6.0 (0.5)	3.6 (0.3)	3.8 (0.2)	3.6	3.8
Total							91.47	88.0

thereby reducing the faecal ^{14}C excess found in the control group.

Faecal excretion of radioactivity by the control group was mainly in the initial 24h and had fallen to only low levels by the 48-72h sample (0.1% ^{14}C and 0.6% ^{3}H). The faeces from the antibiotic treated group however contain considerable levels of radioactivity throughout the experiment with even the last sample containing 7.3% ^{14}C and 5.4% ^{3}H.

Livers taken from the antibiotic treated rats contained more radioactivity than normal livers at each time period. This trend was also repeated in the gut and kidney tissues.

Urinary Metabolites

Table 3.3.2. lists the metabolites identified following DE 52 ion exchange chromatography of the urine of rats pretreated with antibiotics prior to receiving oral radiolabelled folic acid. As was found in the urine of the control rats (Table 3.1.2.) there are a number of dual labelled metabolites and several that contain only a single isotope. Most of the differences are quantitative with one important exception, that is a new ^{3}H-only labelled compound present mainly in the 0-24h urine.

Higher levels of folic acid were excreted into the urine following antibiotic pretreatment. These increases were found in each time period, with this compound being detected right through to the 48-72h urine (excretion of labelled folic acid was only detected up to 24h in

Table 3.3.2.

Metabolites detected in the urine of healthy male rats (190 - 210g body weight) treated with oral antibiotics (neomycin 20mg; tetracycline 10mg and bacitracin 10mg) twice daily for 2 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 weight). Results are expressed as a percentage of the dose administered. n = 5. ND = not detectable.

	0.24h		24-48h		48-72h		0.72h	
Metabolites	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic acid	3.0	3.3	0.3	0.2	0.1	0.1	3.4	3.6
5MeTHF	4.5	4.9	0.7	0.6	0.2	0.3	5.4	5.8
10CHO-FOLATE	6.5	5.5	ND	ND	ND	ND	6.5	5.5
5,10CH_2THF	1.9	2.1	0.1	0.1	ND	ND	2.0	2.2
Metabolite X	1.1	1.3	ND	ND	ND	ND	1.1	1.3
p-AcBGlu	-	ND	-	0.8	-	1.1	-	1.9
p-AcBA	-	1.8	-	0.3	-	0.3	-	2.4
^{3}H-Only Pk	-	3.7	-	0.1	-	0.1	-	3.9
^{14}C - Labelled catabolites	3.0	-	1.3	0.4	0.5	-	4.8	0.4
Urea	1.8	-	0.1	-	0.5	-	2.4	-
$^{3}H_{20}$	-	0.3	-	0.1	-	0.2	-	0.6

the normal group). The overall recovery of folic acid was found to be approximately double that of the control group.

In contrast the overall urinary excretion of 5 MeTHF was decreased by antibiotic pretreatment. This was due to a lower level in the 0-24h urine (4.5% ^{14}C and 4.9% ^{3}H as compared to 7.4% ^{14}C and 8.7% ^{3}H) as later levels were slightly greater than those found in the normal rat urine. The urinary excretion of 10CHO-folate, $5,10CH_2$-THF and Metabolite X were unchanged by antibiotic pretreatment. G15 gel filtration of peak (III) from the 0-24h urine (Figure 3.3.1.) shows that unlike the control rat urine this sample contained no p-AcBGlu. This compound elutes from Sephadex G15 at fractions 18-19 (see Figure 3.1.4.) and p-ABA elutes at fraction 35-36. The 0-24h urine from the antibiotic treated rats contained p-AcABA and a ^{14}C-containing compound, possibly pterin A together with a ^{3}H-only labelled species, eluting at fraction 36, constituting 3.7% of the dose. This species was also detected in the later samples but at far lower levels. p-AcBGlu was detected in the 24-48h and 48-72h urines together with p-AcBA and the pteridine.

The overall excretion of ^{14}C-only containing compounds (urea and pteridines) was higher in the urine of the antibiotic pretreated rats with 4.6% of the dose from the normal rat urine and 7.2% from the antibiotic group.

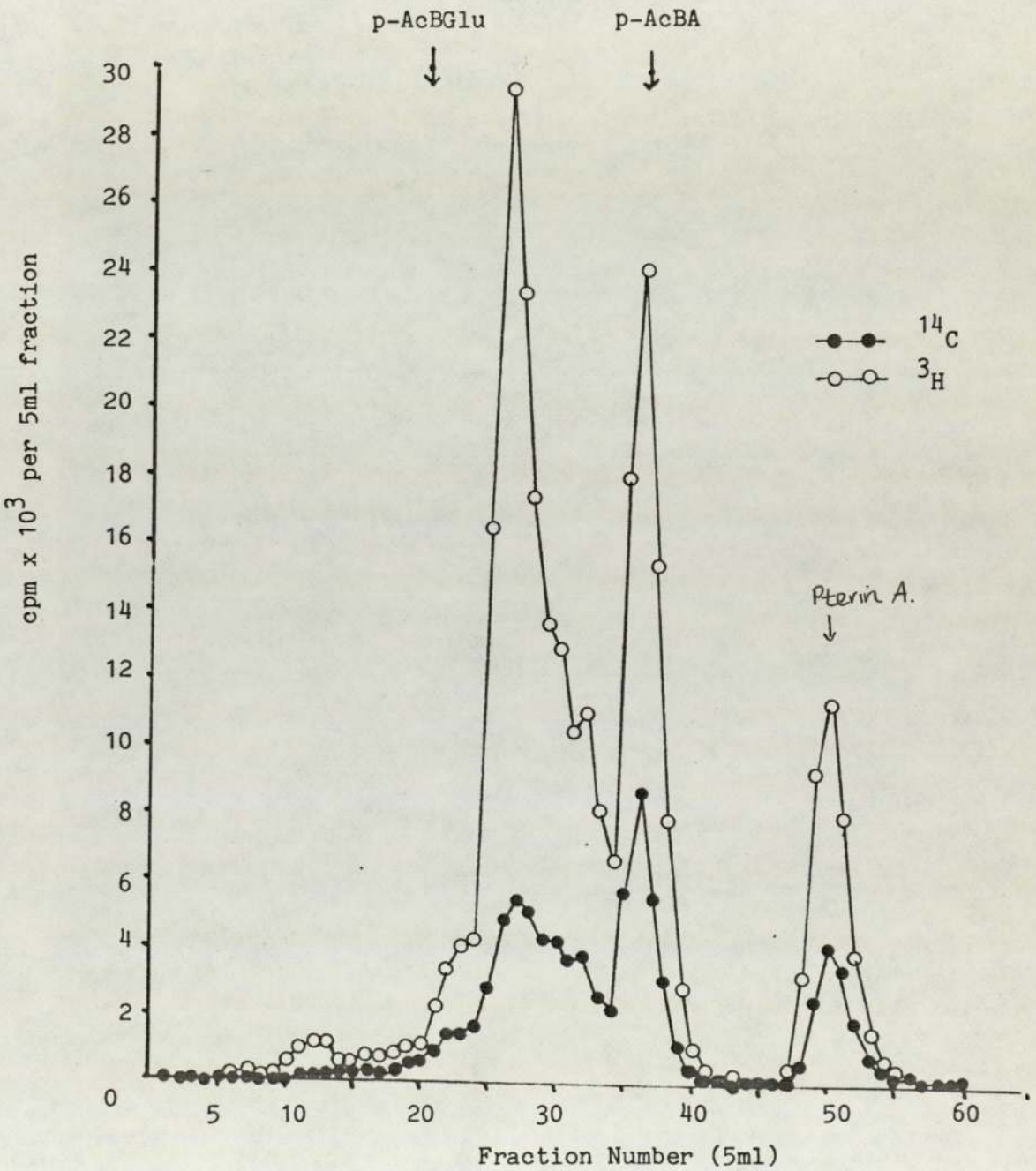


Figure 3.3.1: Sephadex G15 chromatography of peak III from DE52 chromatography of urine (0-24h) from adult male rats treated with antibiotics.

Liver Extracts

Sephadex G15 gel filtration of hot liver extracts showed that in each case the major radiolabelled component was folate polyglutamate.

Summary

Following the administration of radioactive folic acid to normal adult male rats, male rats pretreated with phenytoin and male rats pretreated with antibiotics, radioactivity was found in the urine, faeces, kidneys, liver and gut.

The majority of the urinary excretion of radioactivity occurred over the first 24 hours in each group. However, phenytoin pretreatment was found to decrease ^{3}H and ^{14}C levels over this period of time and antibiotics lowered ^{3}H excretion throughout the experiment but especially in the first 24 hours. Overall excretion of radioactivity into the urine appeared to be delayed by phenytoin as initial ^{3}H and ^{14}C levels were low but later samples contained more radioactivity than did that of the controls.

The urinary excess of ^{3}H which was found in the normal rats was not seen in either of the two treated groups.

Both of the treated groups of rats excreted more ^{14}C and ^{3}H in the faeces than the control group. The control rat faeces contained more ^{14}C and ^{3}H at 24, 48 and 72h after dosing. This ^{14}C excess was repeated in the faeces collected from the antibiotic pretreated rats but the

difference between the two isotopes was smaller. However the phenytoin treated group only excreted more ^{14}C than ^{3}H over the first 24h, after this the isotope levels were similar.

Chromatographic analysis revealed the presence of folic acid, 5 MeTHF, 10CHO-folate, 5,10CH_2-THF, Metabolite X, p-AcBGlu, p-AcBA, pteridine fragments and $^{3}H_2O$ in the urine of the normal rats. The scission products, present at low levels in the early samples were the major components of the 48-72h urine.

Phenytoin raised and prolonged urinary folic acid excretion but depressed 5 MeTHF and 10CHO-folate levels. No 5,10CH_2-THF or Metabolite X were detectable in the urine of the phenytoin treated group but two new peaks were found. Lower levels of scission products were present.

Similarly antibiotic treatment raised and prolonged folic acid excretion but lowered 5 MeTHF levels. However, no p-AcBGlu was detectable in the urine until after 24h while a new ^{3}H-only labelled catabolite was present in all the samples together with increased amounts of ^{14}C-only labelled fragments. Antibiotic treatment also raised the tissue retention of radioactivity.

Therefore both phenytoin and antibiotic treatments altered folate metabolism in the rat but in separate ways which will be analysed in the final discussion.

Chapter 4

Folic Acid Metabolism in The Adult Female Rat

This chapter describes a study of the metabolism of oral radiolabelled folic acid in the adult female rat in order to evaluate the influence of hormones upon folate handling. Female rats sleep longer than their male counterparts after an equal dose of hexobarbital (Kato et al., 1970) and male rats treated with oestrogens show a decreased rate of metabolic activity. It was decided to see if these metabolic differences are extended to folate metabolism.

The mortality rates for breast cancer are greater for women than men (Lilienfeld, 1976) suggesting an hormonal involvement. Epidemiological studies have also revealed that the incidence of breast cancer varies widely from one country to another with Japan having the lowest death rate (Segi et al., 1962; Willett et al., 1984). Although data from the human is limited, experiments using animal models have linked the intake of dietary fat to an increased incidence of mammary tumours. Therefore a group of adult female rats were treated with diethylstilboestrol (DES) and mazola corn oil (lipid) before the administration of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid to see if this produced any alterations in the handling of folates.

4.1 Folic Acid Metabolism in the Female Rat

Materials and Methods

15 female WAB/Not rats (150 ± 10g body weight) were dosed orally with a mixture of [2 - ^{14}C] and [3',5',7,9 - ^{3}H] folic acid (100μg/kg body weight) containing 5μCi ^{3}H and 2μCi ^{14}C). 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 Chapter 2. At the end of each time period the rats were killed by cervical dislocation, the collection flasks were changed and the next 5 rats were placed in the metabowls. All samples were stored at -20°C in the presence of sodium ascorbate (2% w/v) until required. Analytical techniques used included DE 52 ion exchange chromatography, G15 gel filtration, paper chromatography and liquid scintillation counting.

Results

Table 4.1.1. shows the recovery of radioactivity excreted in the urine and faeces and the radioactivity retained by the liver following the administration of [2 - ^{14}C] and [3',5',7,9 - ^{3}H] folic acid to a group of 15 healthy female rats. The levels of radioactivity retained by the gut and kidney were not determined as all the necessary information on folate catabolism could be obtained without these figures.

Excretion of radioactivity into the urine took place mainly over the first 24h as 36.5% ^{14}C and 43.0% ^{3}H were detected 0-24h but only 3.2% ^{14}C and 3.5% ^{3}H and 1.8% ^{14}C and 2.0% ^{3}H were recovered in the 24-48h and 48-72h urines respectively. In each sample more ^{3}H than ^{14}C was present in the urine and over 0-24h this ^{3}H excess was statistically significant ($p = 0.01$).

Faecal radioactivity was detected up to 72h after the radiolabelled dose but the 0-24h sample contained the highest levels (13.3% ^{14}C and 8.8% ^{3}H). In all the samples there was more ^{14}C than ^{3}H but none of

Table 4.1.1.

Radioactivity detected in the urine, faeces and liver of healthy female rats (150 ± 10g body weight) 24h, 48h and 72h after the oral administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100µg/Kg body weight). Results are expressed as a percentage of the dose administered ± SE. n = 5.

	0-24h		24-48h		48-72h		0-72h	
Sample	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
URINE	36.5 (2.1)	43.0 (2.4)	3.2 (0.7)	3.5 (0.8)	1.8 (0.2)	2.0 (0.3)	41.5	48.5
FAECES	13.3 (2.7)	8.8 (2.2)	2.2 (0.4)	1.7 (0.3)	0.7 (0.2)	0.6 (0.1)	16.2	11.1
LIVER	9.8 (1.9)	9.6 (0.5)	10.7 (0.9)	12.0 (1.3)	6.2 (0.4)	9.2 (0.6)	6.2	9.2

these differences were statistically significant. Hepatic radioactivity was also detected up to 72h with more ^{3}H than ^{14}C present in all but the 24h livers. The levels were maximal at 48h and lowest 72h after dosing.

Urinary Metabolites

DE 52 ion exchange chromatography of urine samples from female rats are illustrated in Figures 4.1.1., 4.1.2. and 4.1.3. Sephadex G15 gel filtration of the peaks containing the scission products are shown in Figures 4.1.4., 4.1.5. and 4.1.6. The quantitative data derived from this analysis is listed in Table 4.1.2. The bulk of the urinary radioactivity was found to be 5 MeTHF and 10CHO-folate. Folic acid was present at less than 1% of the dose over the first 24h and was undetectable after 48h. No $5,10CH_2$-THF and only trace amounts of Metabolite X were found. The remainder of the radioactivity was present as $^{3}H_2O$ (1.4% over 72h), urea (1.7% over 72h) and single labelled scission products. 4.4% of the ^{14}C dose appeared in pteridine derivatives. 12.4% of the ^{3}H dose appeared as ^{3}H-only labelled scission products with 8.8% as p-ABGlu and 3.6% as p-AcBA. As was found in the urinary metabolites from the male rats the early urines mainly contain intact folates while the later samples are mostly composed of single labelled scission products.

Liver Extracts

Sephadex G15 gel filtration of the hot liver extracts at 24, 48 and 72h after dosing are illustrated in Figures 4.1.7., 4.1.8. and 4.1.9. From

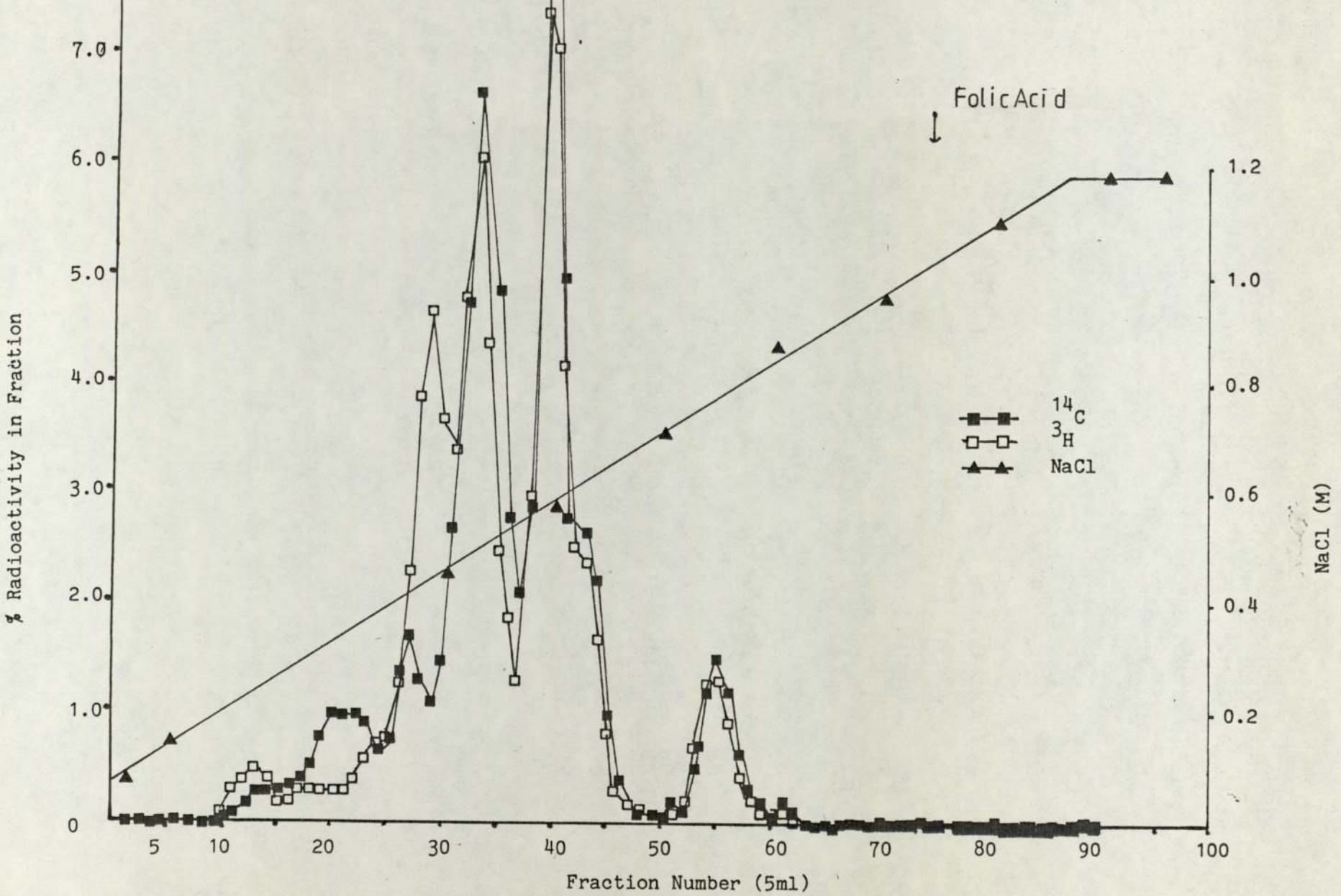


Figure 4.1.1. DE 52 chromatography of urine samples collected from adult female rats 0-24h after the administration of (2-^{14}C) and (3',5',7,9-^{3}H) Folic acid.

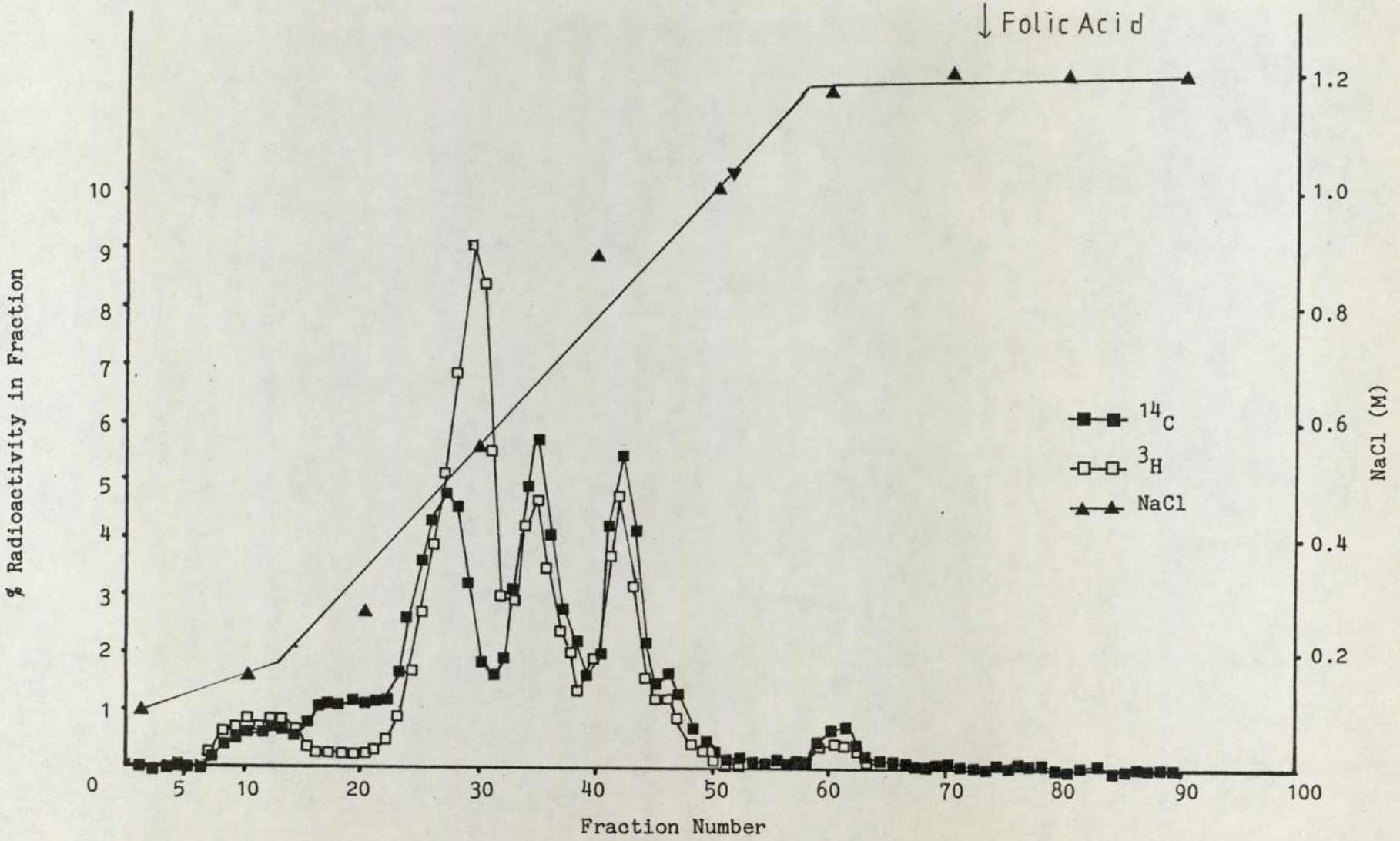


Figure 4.1.2: DE52 chromatography of urine samples collected from adult female rats 24-48h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) Folic acid.

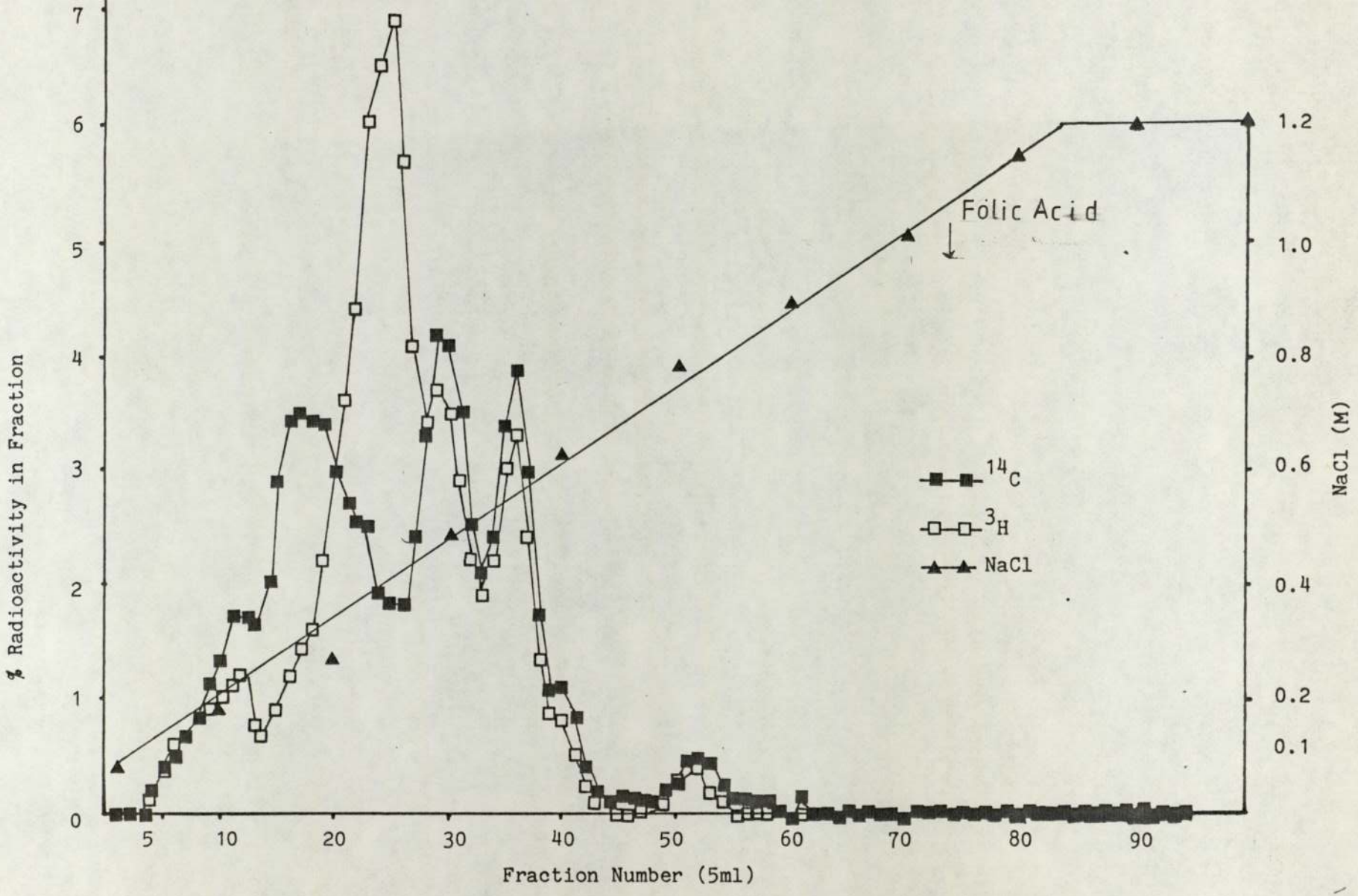


Figure 4.1.3: DE52 chromatography of urine samples collected from adult female rats 48-72h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) Folic acid.

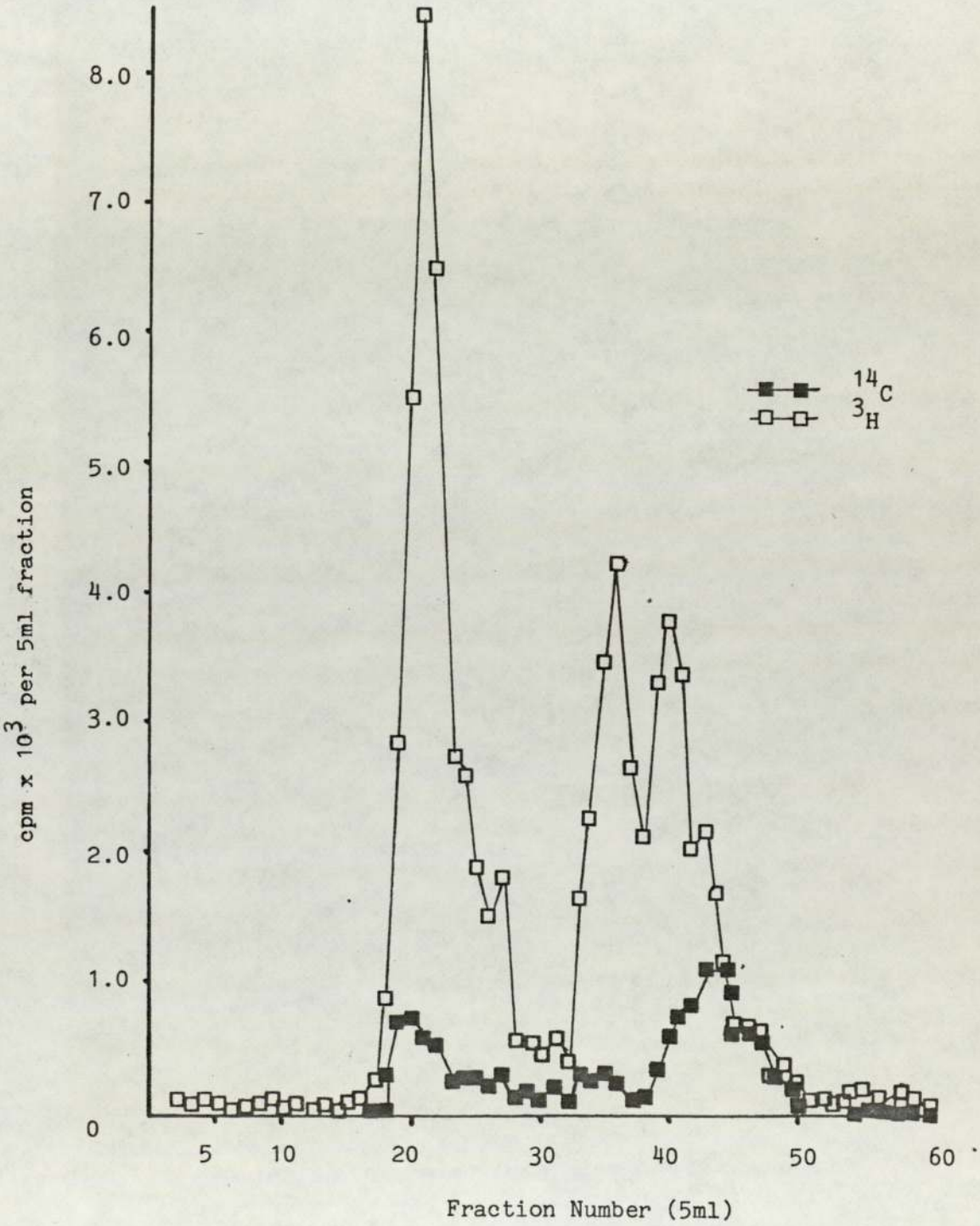


Figure 4.1.4: G15 chromatography of peak 2 from DE52 chromatography of urine (0-24h) from adult female rats.

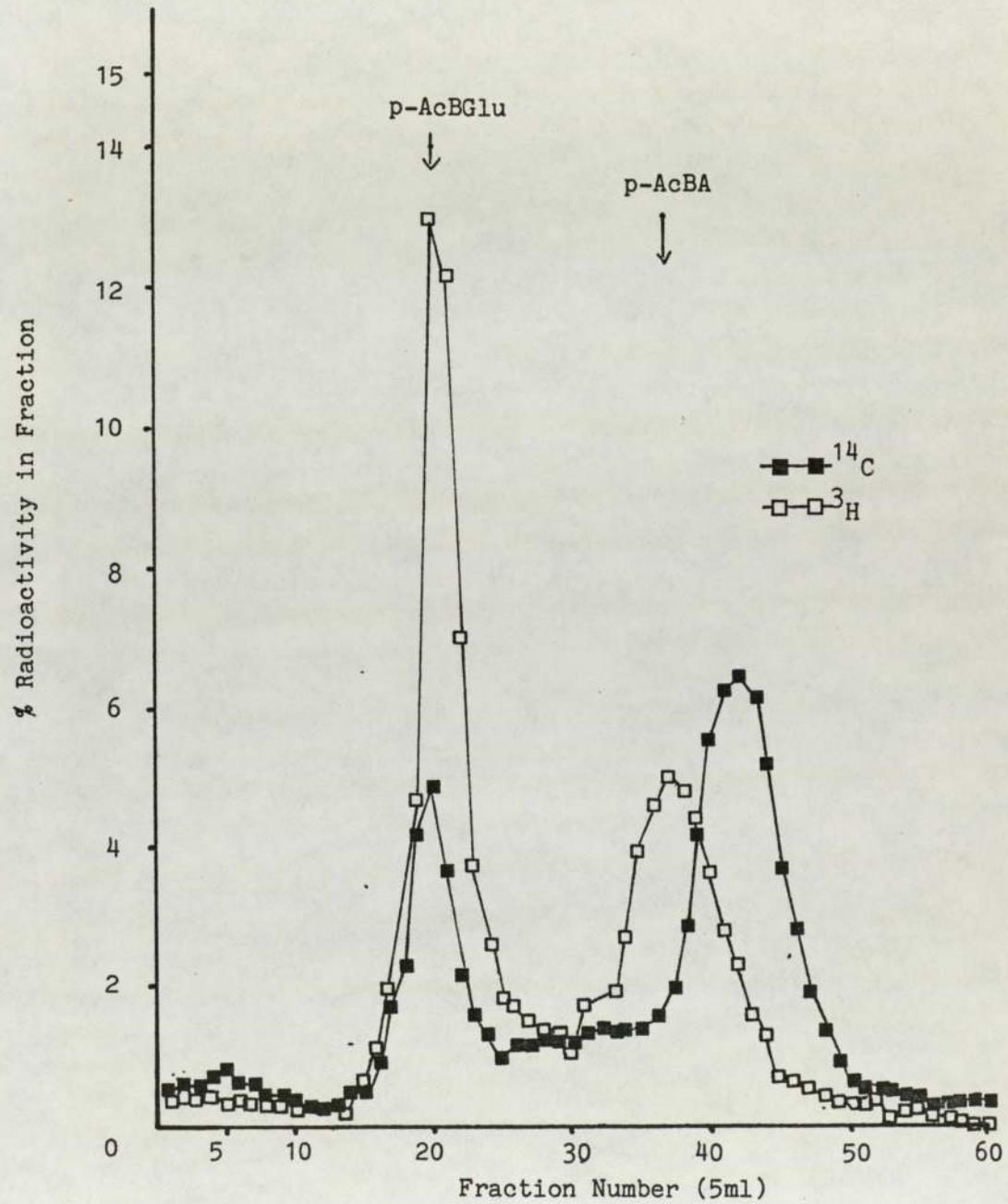


Figure 4.1.5: Sephadex G15 chromatography of peak 3 of DE52 chromatography (24-48h) of adult female rat urine.

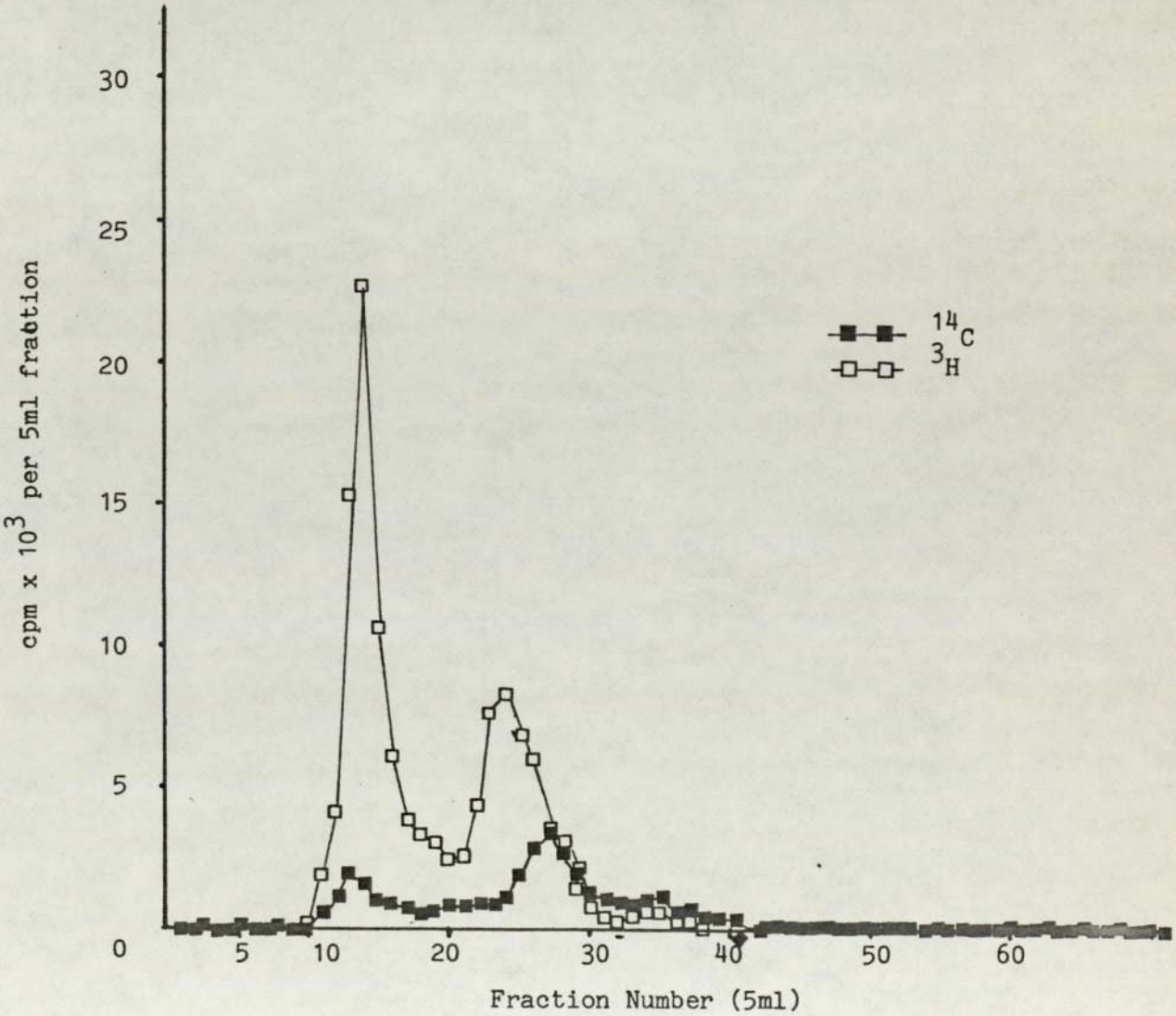


Figure 4.1.6: Sephadex G15 chromatography of peak 3 of DE52 chromatography of urine (48-72h) from adult female rats.

Table 4.1.2.

Metabolites present in the urine of healthy female rats (150 ± 10g body weight) 24h, 48h and 72h after the oral administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight). Results are expressed as a percentage of the dose administered. n = 5.

Metabolite	0-24h		24-48h		48-72h		0-72h	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	0.1	0.07	ND	ND	ND	ND	0.1	0.07
5MeTHF	21.1	15.3	0.8	0.8	0.1	0.1	22.0	16.2
10CHO-folate	15.5	18.0	0.3	0.6	0.3	0.2	16.1	18.8
5,10CH_2THF	ND	ND	ND	ND	ND	ND	ND	ND
Metabolite X	T	T	T	T	T	T	T	T
p-AcBA	-	2.5	-	0.4	-	0.7	-	3.6
p-AcBGlu	-	7.2	-	0.6	-	1.0	-	8.8
Pteridine Derivatives	3.4	-	0.4	-	0.6	-	4.4	-
^{14}C Urea	1.2	-	0.4	-	0.1	-	1.7	-
$^{3}H_2O$	-	1.1	-	0.2	-	0.1	-	1.4

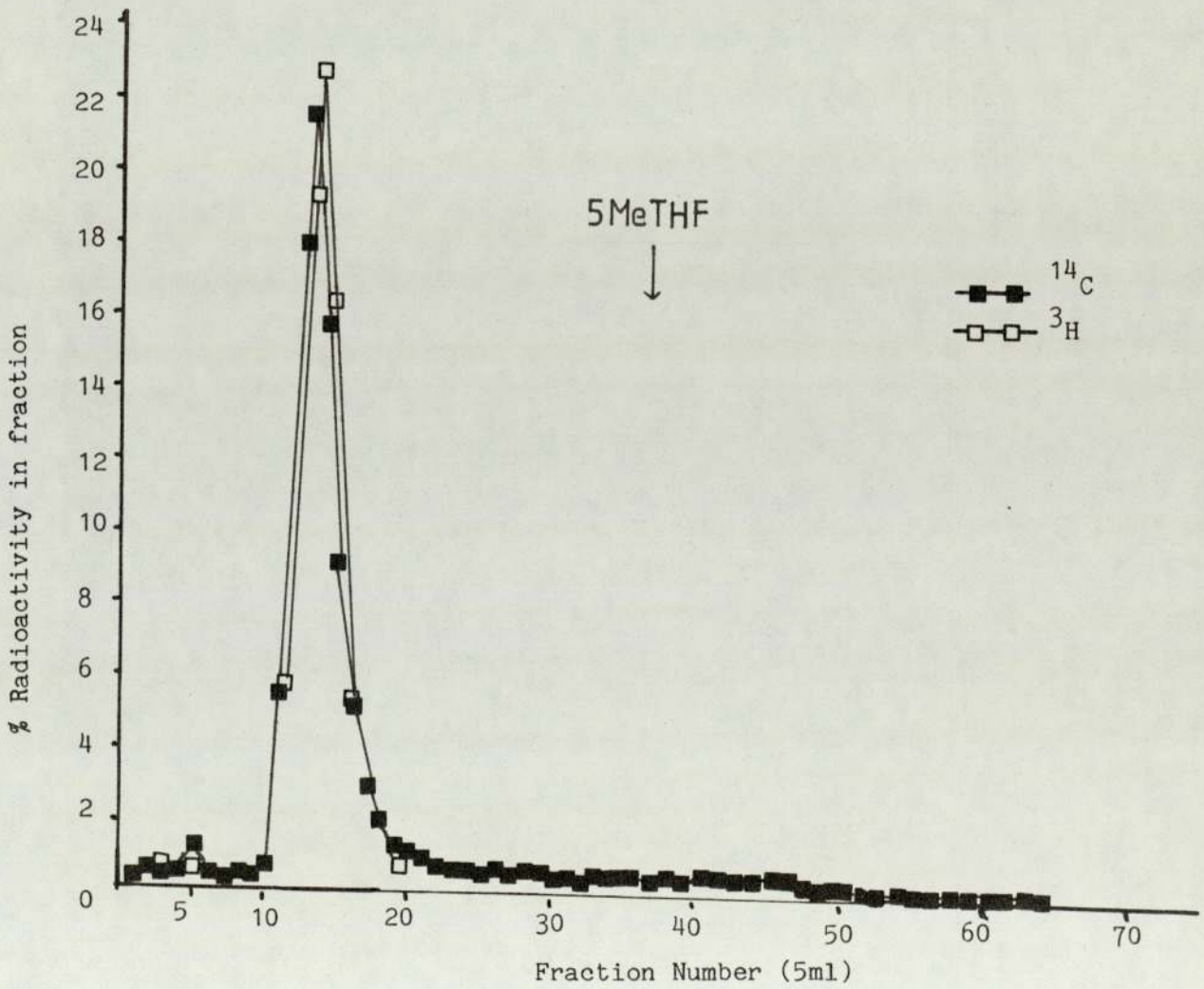


Figure 4.1.7: Sephadex G15 chromatography of liver extracts (24h) from adult female rats dosed with (2-^{14}C) and (3',5',7,9-^{3}H).

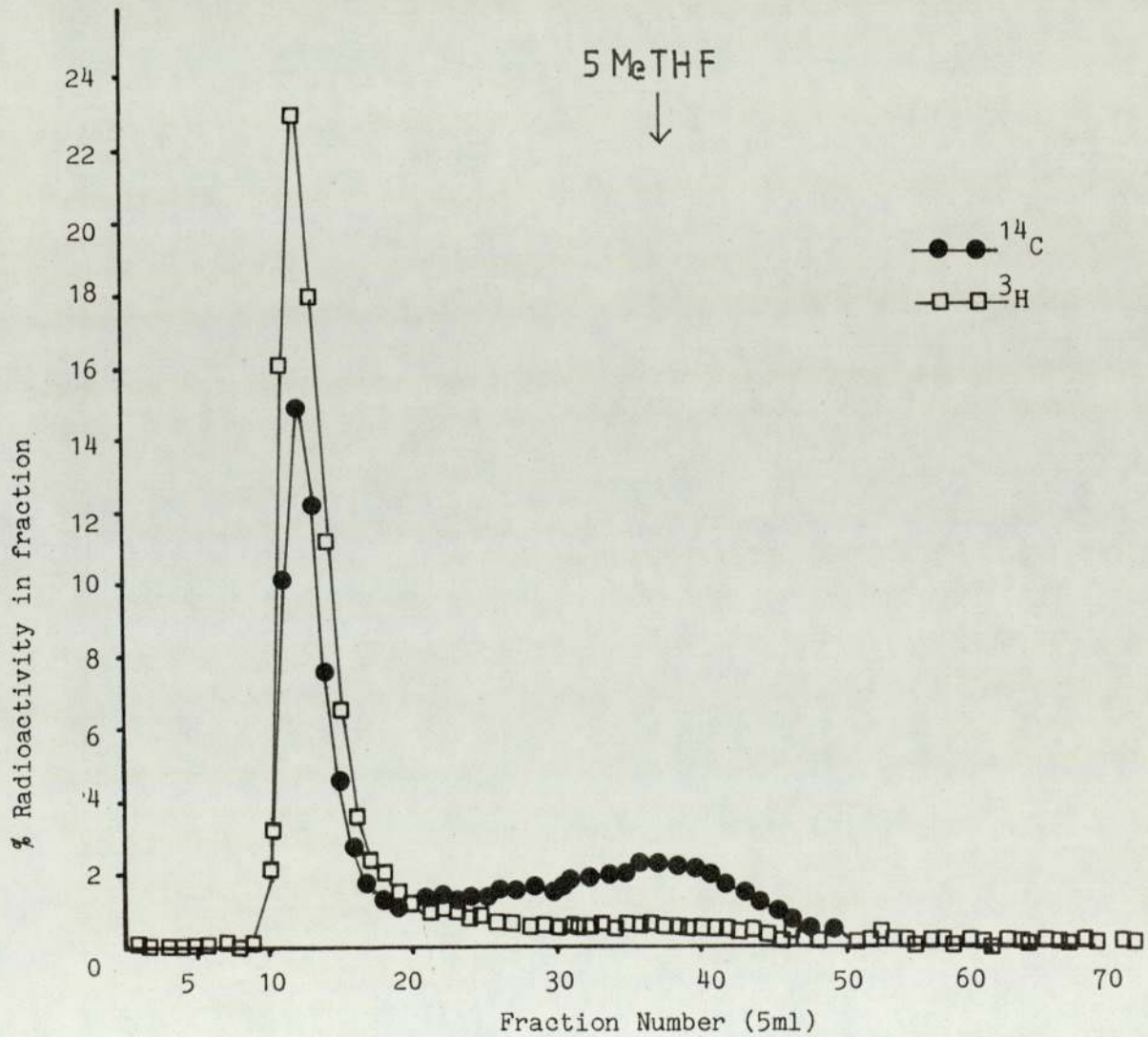


Figure 4.1.8: Sephadex G15 chromatography of liver extracts (48h) from adult female rats dosed with (2-^{14}C) and (3',5',7,9-^{3}H).

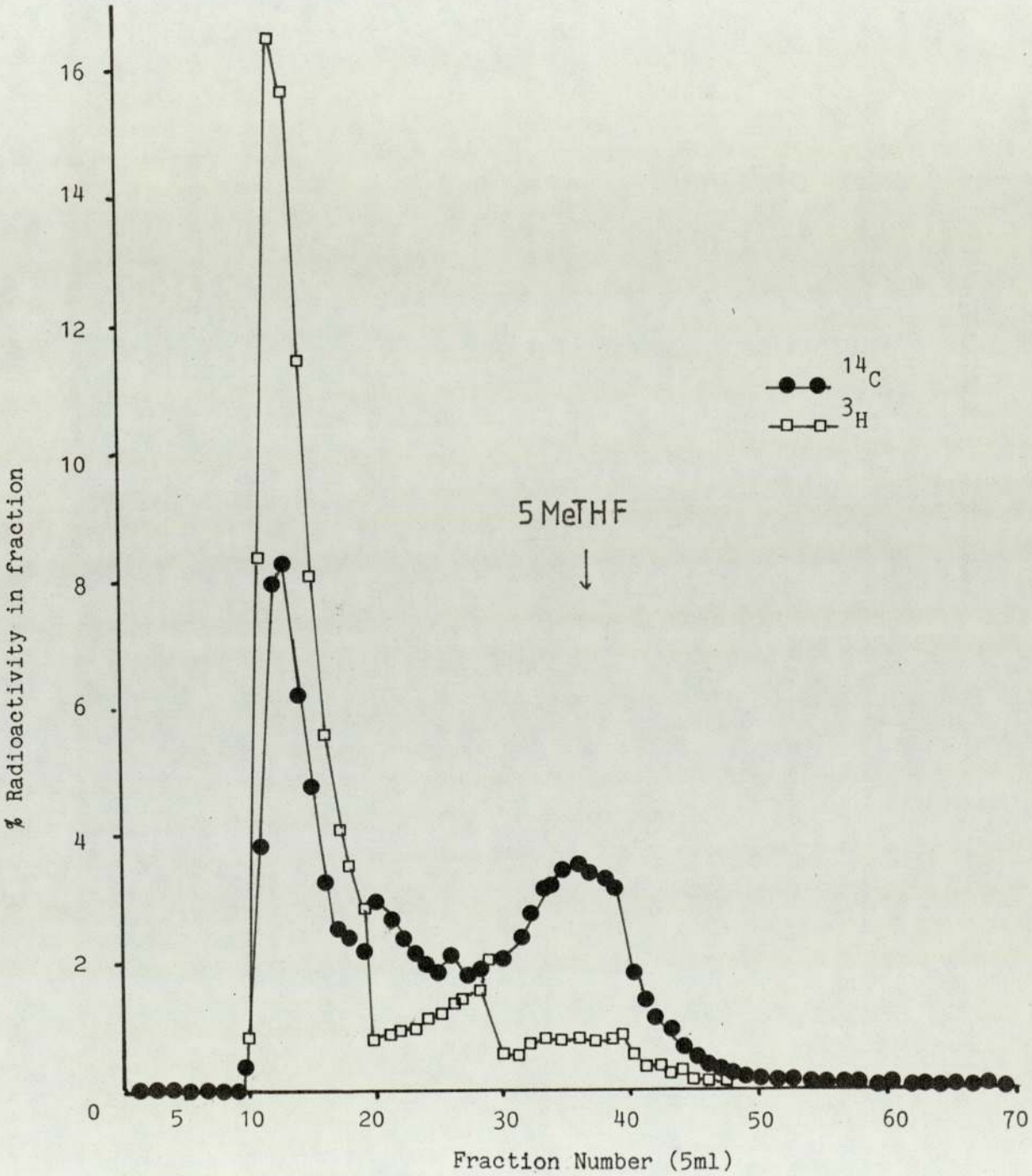


Figure 4.1.9: Sephadex G15 chromatography of liver extracts (72h) from adult female rats dosed with (2-^{14}C) and (3',5',7,9-^{3}H).

these it can be seen that the major radioactive component was folate polyglutamate.

Paper Chromatography

Figures 4.1.10., 4.1.11. and 4.1.12. show the results of the paper chromatography carried out on the 0-24h, 24-48h and 48-72h urines from normal female rats. A number of radioactive peaks are distinguishable. In the 0-24h urine (Figure 4.1.10.) a large dual labelled peak corresponding to the 10CHO-folate standard and two smaller ^{3}H-only labelled peaks coincidental to the p-AcBGlu and p-AcBA standards are visible. These 3 peaks are not as clear in the 24-48h urine (Figure 4.1.11.) but the specific activity of this urine was much lower than the 0-24h urine. The 48-72h urine (Figure 4.1.12.) shows a peak coincidental to the 10CHO-folate standard which is just distinguishable from the background but no more. Because of this the fractions from the DE 52 ion exchange chromatography that contained the relevant radiolabelled metabolites were also run as paper chromatograms in an identical way. Figures 4.1.13., 4.1.14. and 4.1.15. illustrate the results obtained. It may be seen that although a peak corresponding to the 10CHO-folate standard is visible in the 0-24h urine (Figure 4.1.13.) and the 48-72h urine (Figure 4.1.15.) the results are still by no means conclusive.

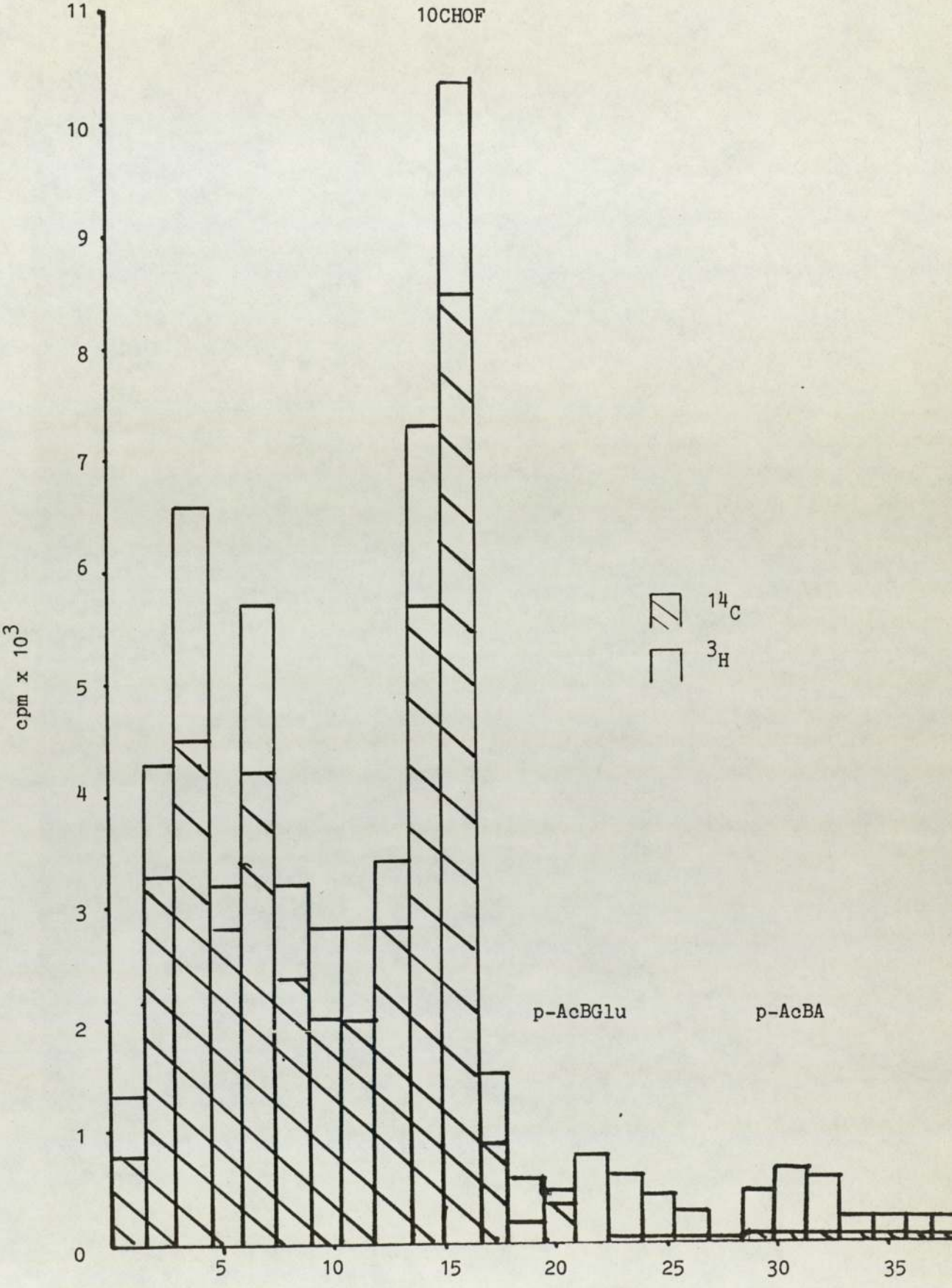


Figure 4.1.10: Paper chromatography of urine samples (0-24h) collected from adult female rats.

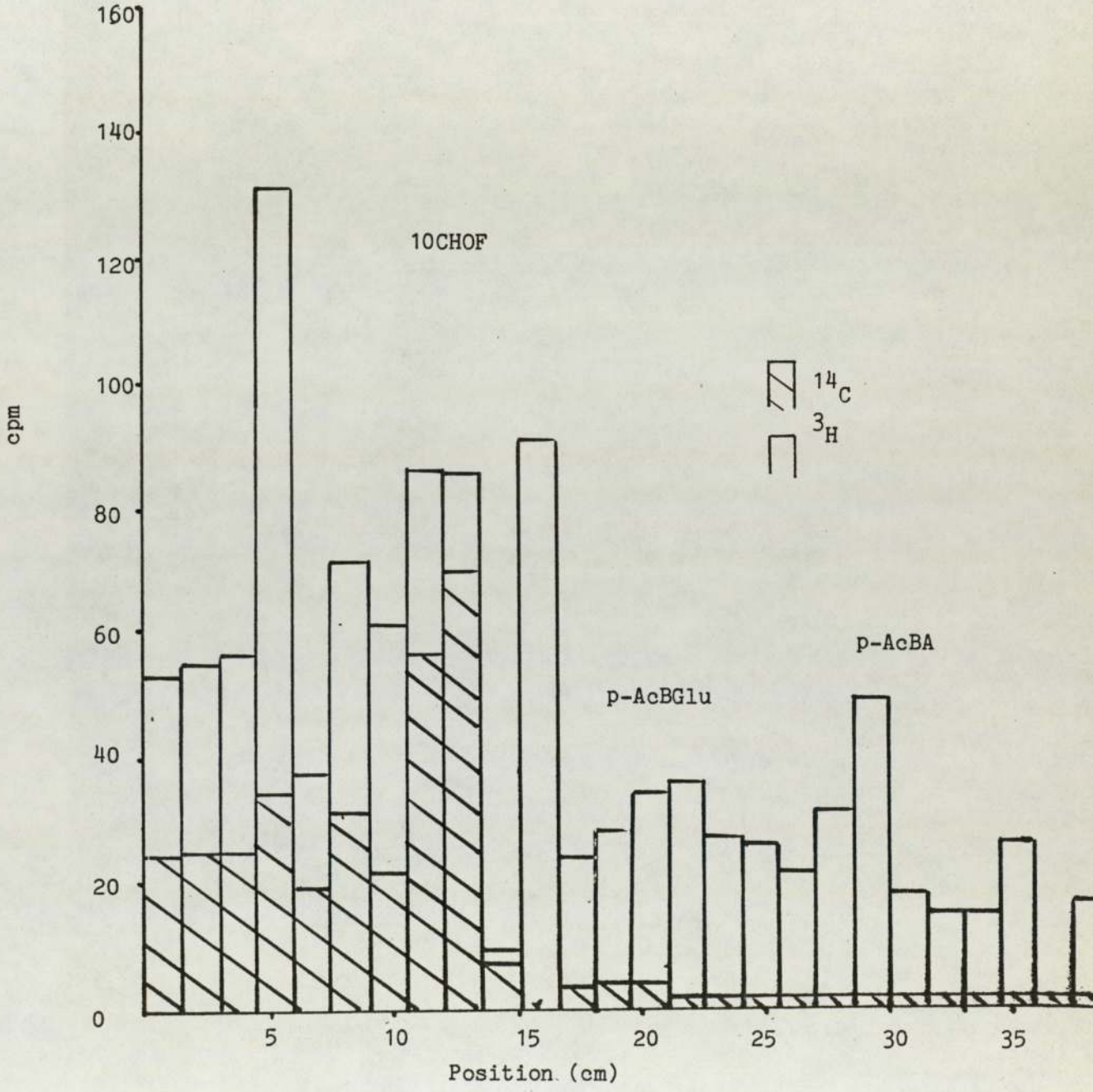


Figure 4.1.11: Paper chromatography of urine samples (24-48h) collected from adult female rats.

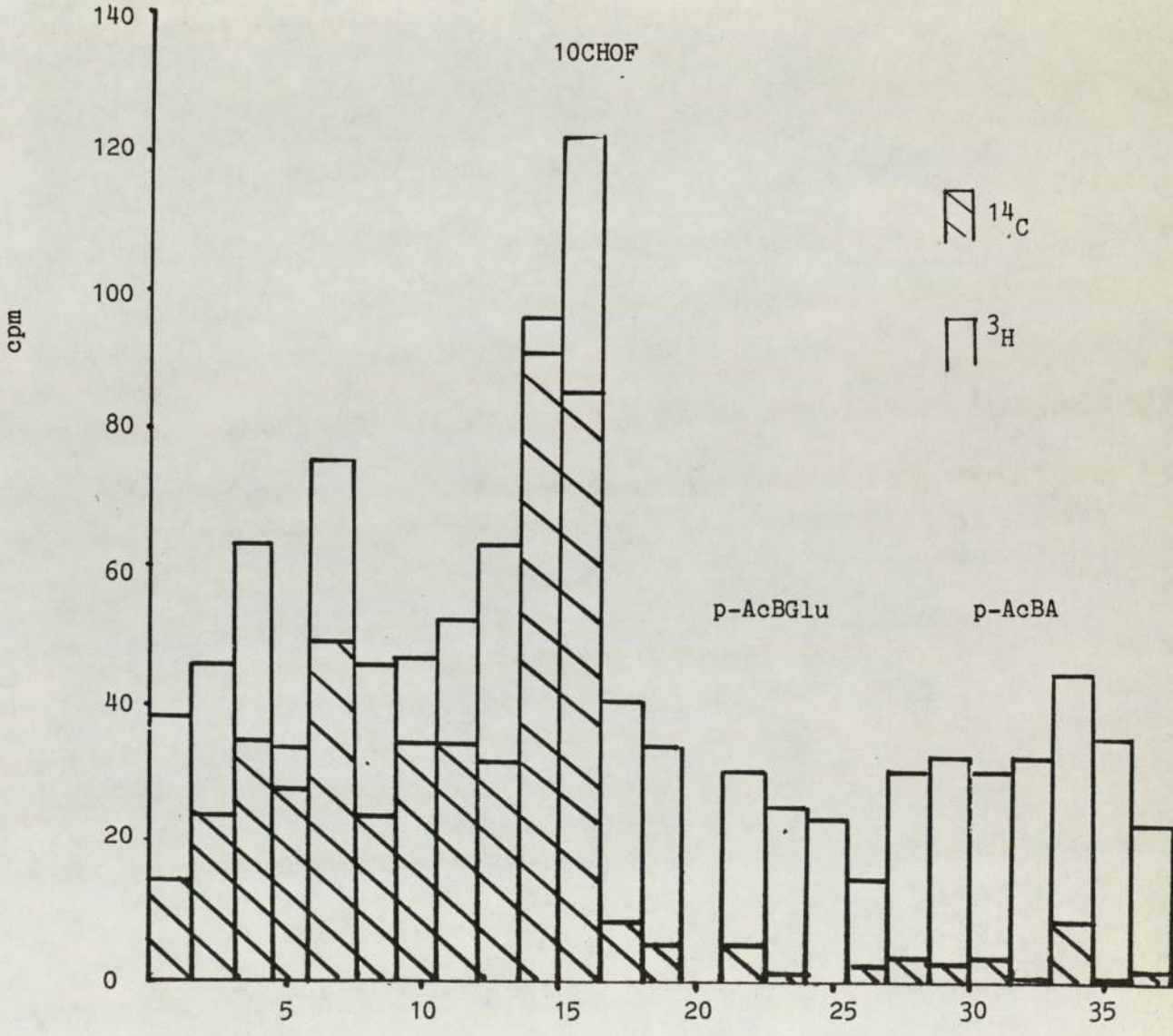


Figure 4.1.12: Paper chromatography of urine samples (48-72h) collected from adult female rats.

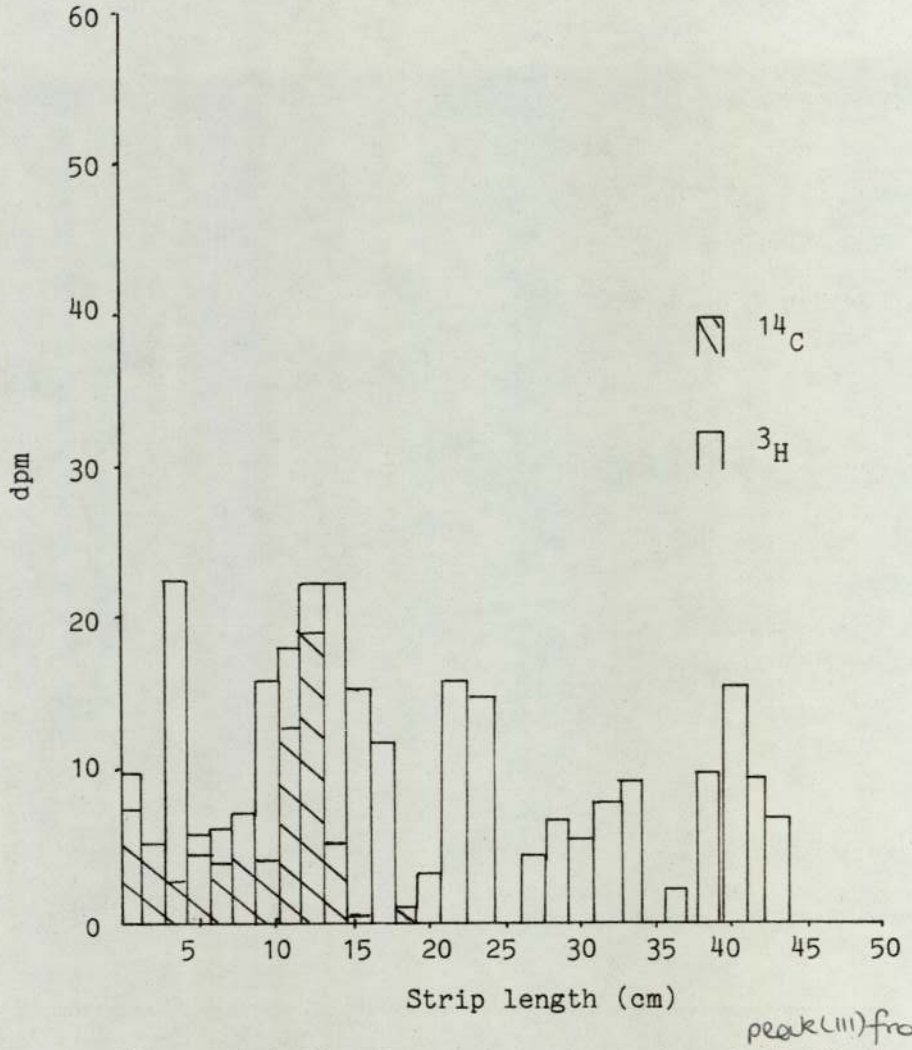


Figure 4.1.13: Paper chromatography of peak (III) from DE 52 chromatography of urine (0 - 24h) collected from adult female rats.

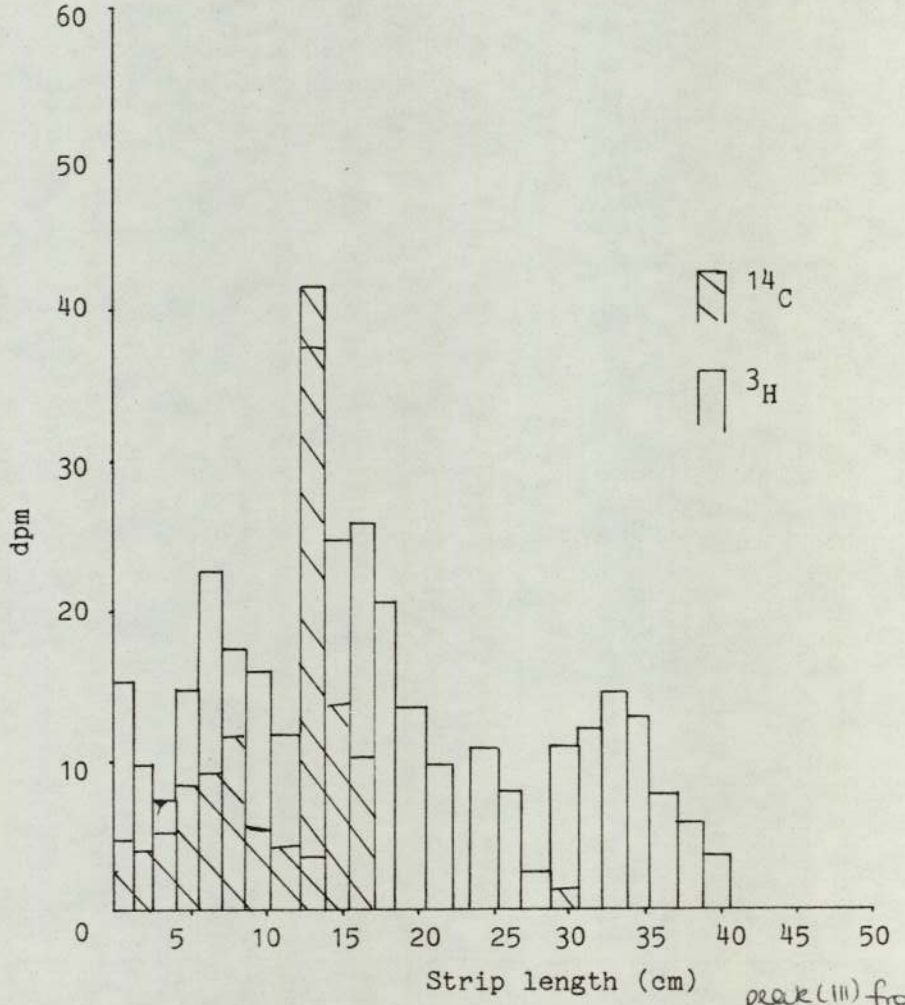


Figure 4.1.14: Paper chromatography of peak (III) from DE 52 chromatography of urine (24 - 48h) collected from adult female rats.

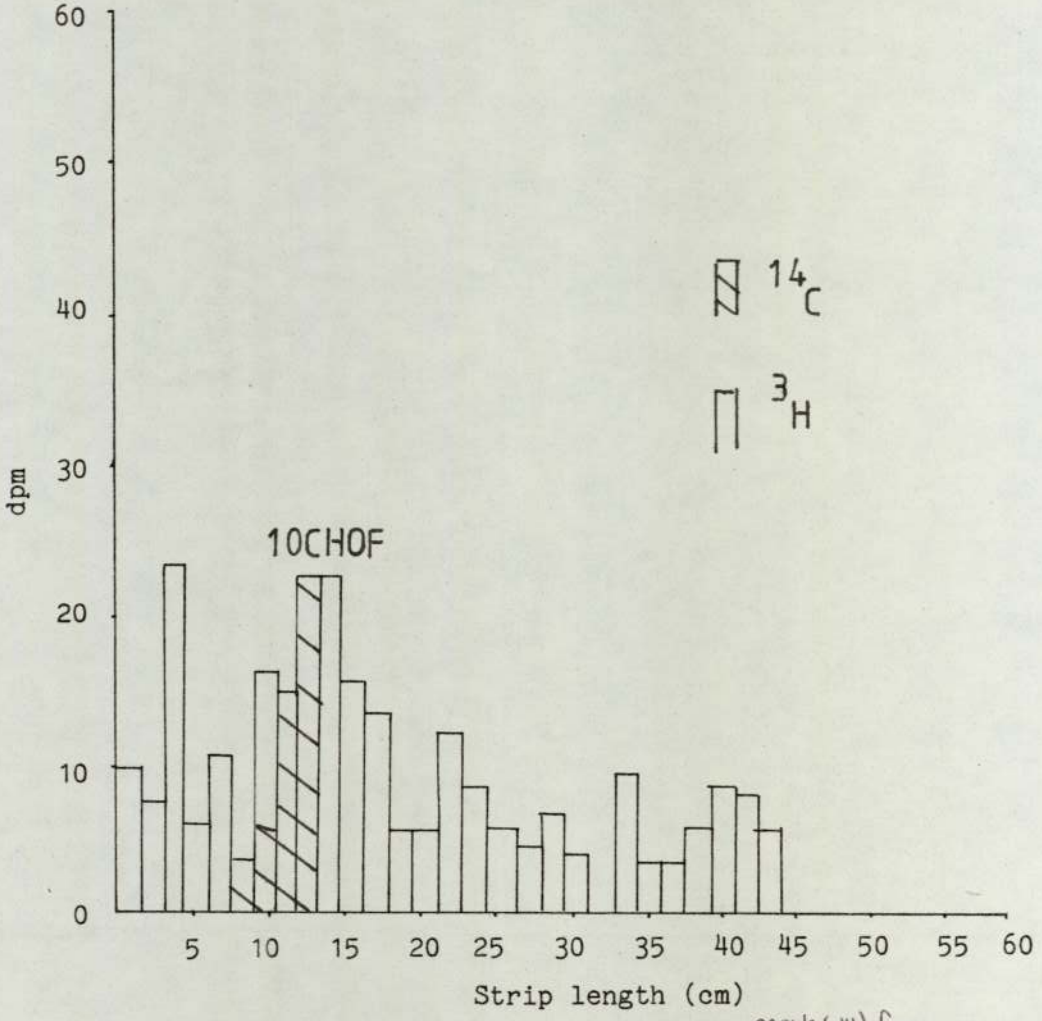


Figure 4.1.15: Paper chromatography of peak (III) from DE 52 chromatography of urine (48 - 72h) collected from adult female rats.

4.2. The Effect of Diethylstilboestrol Plus Lipid on Folate Metabolism in the Female Rat

Materials and Methods

15 female WAB/Not rats (150 ± 10g body weight) each received daily oral doses of diethylstilboestrol propionate (350 mg/kg body weight) in mazola corn oil (0.3ml) for 3 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 weight).

Urine was collected for periods of 0-24h, 24-48h and 48-72h as described in Section 4.1. 5 animals were killed 24h, 48h and 72h after dosing with the folic acid. Animals and samples were handled as described previously (Section 4.1.).

Results

Table 4.2.1. shows the recovery of radioactivity from the urine of rats treated with diethylstilboestrol plus lipid prior to the administration of radiolabelled folic acid. Most of the urinary radioactivity was found in the 0-24h sample with 30.0% ^{14}C and 35.8% ^{3}H but later samples contained lower levels (3.3% ^{14}C and 4.0% ^{3}H over 24-48h and 2.3% ^{14}C and 2.7% ^{3}H over 48-72h). More ^{3}H than ^{14}C was present in each urine sample and over 0-24h this ^{3}H excess was statistically significant.

Table 4.2.1.

Radioactivity in the urine of healthy female rats (150 ± 10g body weight) dosed with diethylstilboestrol (350mg/Kg body weight) in mazola corn oil (0.3ml) 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 weight). Results are expressed as a percentage of the dose administered ± SE. n = 5.

Time (h)	0-24	24-48	48-72	0-72
Isotope				
^{14}C	30.0 (2.6)	3.3 (0.5)	2.3 (0.2)	35.6
^{3}H	35.8 (3.0)	4.0 (0.7)	2.7 (0.3)	42.5

Urinary Metabolites

Table 4.2.2. summarises the metabolites detected in the urine of the DES plus lipid treated rats up to 72h after the administration of radiolabelled folic acid. Folic acid was present at very low levels in the 0-24h urine but none was detectable after 24h. 5 MeTHF is present in all the samples but the major metabolite was 10CHO-folate especially over 0-24h where it comprised 23.8% ^{14}C and 28.25% ^{3}H. Later excretion values are much lower (1.39% ^{14}C, 2.2% ^{3}H over 24-48h and 1.1% ^{14}C, 2.2% ^{3}H over 48-72h) but 10CHO-folate is still the major metabolite. No $5,10CH_2$-THF and only trace amounts of Metabolite X were detected in the urine. p-AcBGlu was the main scission product excreted in the urine. 1.0% ^{3}H was excreted as p-AcBGlu over 0-24h and over 0-72h a total of 1.5% ^{3}H was excreted as p-AcBGlu. p-AcBA was present in lower levels (0.5% ^{3}H over 0-72h). 3.1% ^{14}C was excreted as ^{14}C-only labelled scission products over 72h, 1.0% as pteridine fragments and 2.1% as urea. $^{3}H_2O$ comprised 0.79% of the dose.

Paper Chromatography

Figures 4.2.1., 4.2.2. and 4.2.3. show the results of paper chromatography of urine samples from female rats treated with DES plus lipid prior to an oral dose of radiolabelled folic acid. All three urines contain a large dual labelled peak corresponding to the 10CHO-folate marker. There are no peaks which could be ascribed to either p-AcBGlu or p-AcBA. Figures 4.2.4., 4.2.5. and 4.2.6. show the results of the paper chromatography of the pooled fractions from DE 52 ion exchange chromatography of the urine from the DES plus lipid treated

Table 4.2.2.

Metabolites detected in the urine of healthy female rats (150 ± 10g body weight) dosed with diethylstilboestrol (350mg/Kg body weight) in mazola corn oil (0.3ml) 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 weight). Results are expressed as a percentage of the dose administered. n = 5.

Metabolite	0-24h		24-48h		48-72h		0-72h	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	0.4	0.4	ND	ND	ND	ND	0.4	0.4
5MeTHF	3.54	3.94	0.3	0.3	0.13	0.12	3.97	4.36
10CHO-folate	23.8	28.25	1.39	2.2	1.1	2.2	26.3	32.6
5,10CH_2-THF	ND	ND	0.5	0.6	-	-	0.5	0.6
Metabolite X	ND	ND	ND	ND	ND	ND	ND	ND
p-AcBA	-	0.3	-	0.1	-	0.1	-	0.5
p-AcBGlu	-	1.0	-	0.3	-	0.2	-	1.5
$^{3}H_2O$	-	0.65	-	0.1	-	0.04	-	0.79
Pteridine Fragments	0.53	-	0.37	-	0.1	-	1.0	-
^{14}C Urea	1.63	-	0.4	-	0.08	-	2.1	-

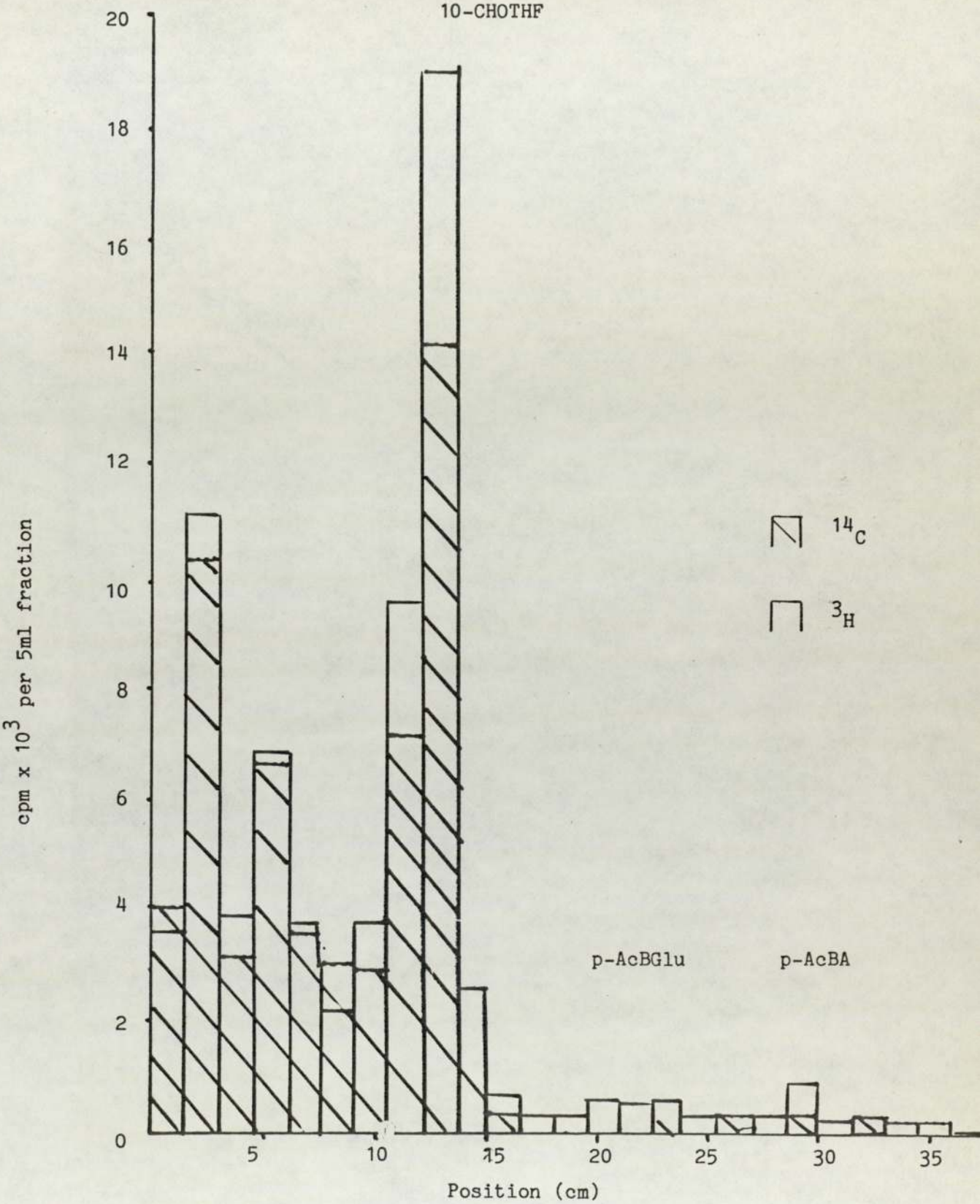


Figure 4.2.1: Paper chromatography of urine samples (0-24h) from adult female rats treated with DES.

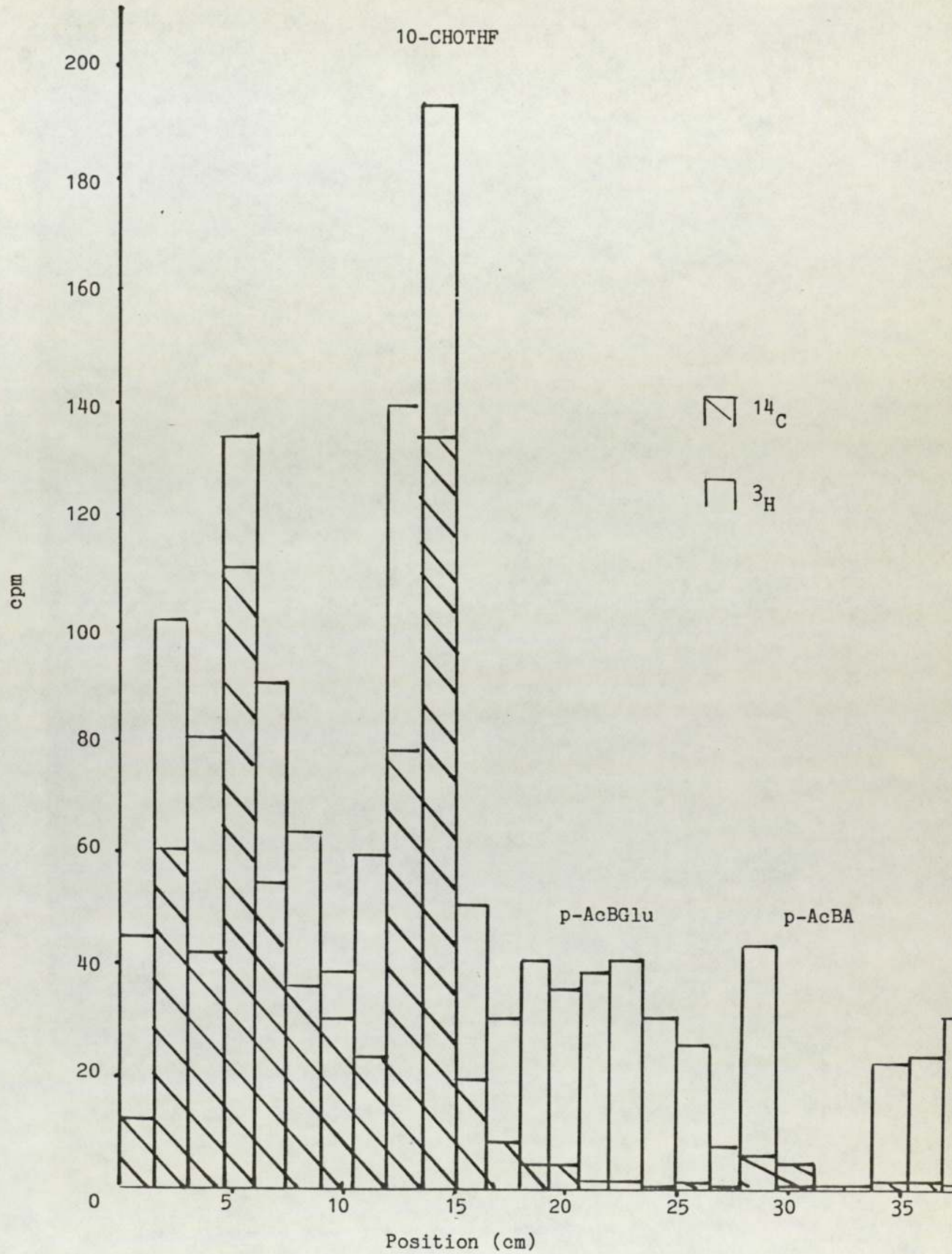


Figure 4.2.2: Paper chromatography of urine samples (24-48h) from adult female rats treated with DES.

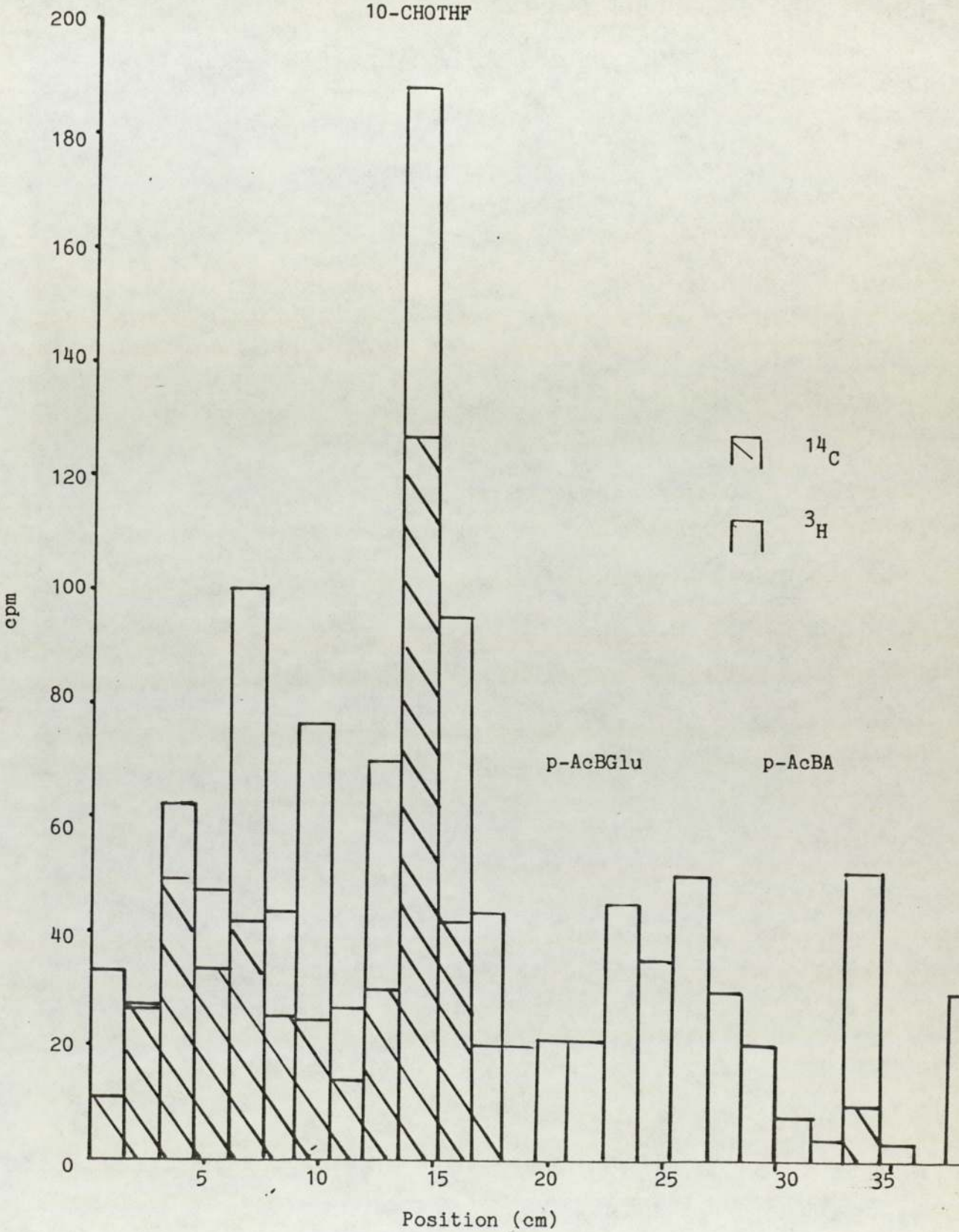


Figure 4.2.3: Paper chromatography of urine samples (48-72h) from adult female rats treated with DES.

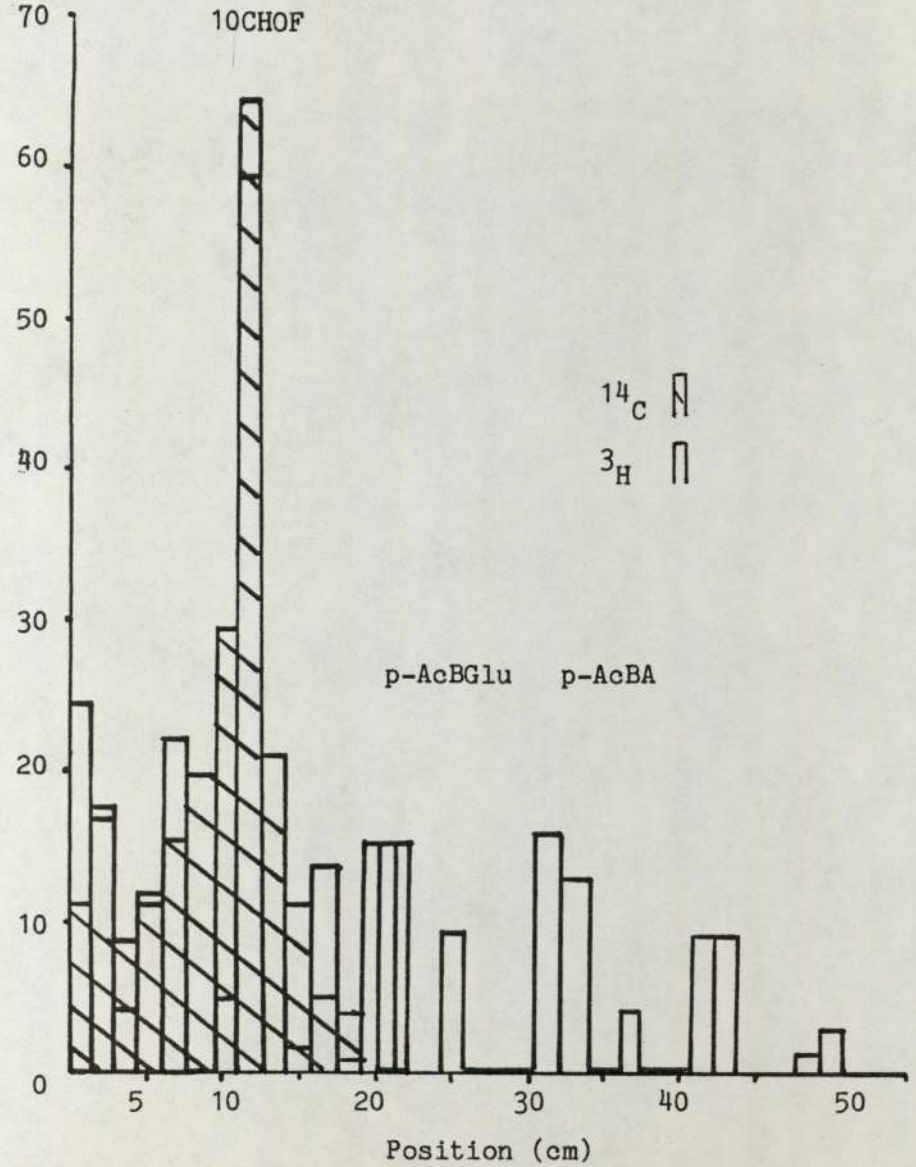


Figure 4.2.4: Paper chromatography of peak 2 of DE52 Chromatography of urine (0-24h) from adult female rats treated with DES.

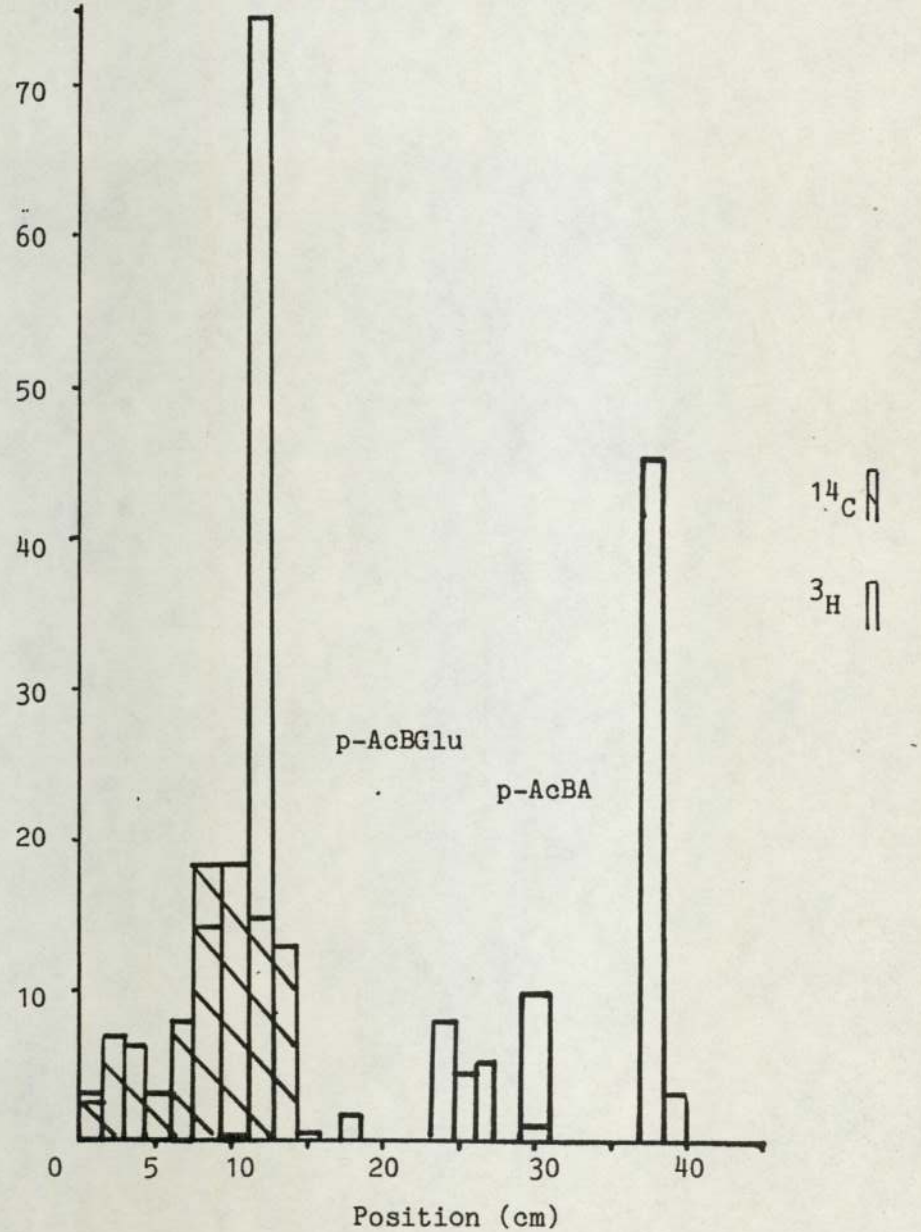


Figure 4.2.5: Paper chromatography of peak 2 of DE52 chromatography of urine (24-48h) from adult female rats treated with DES.

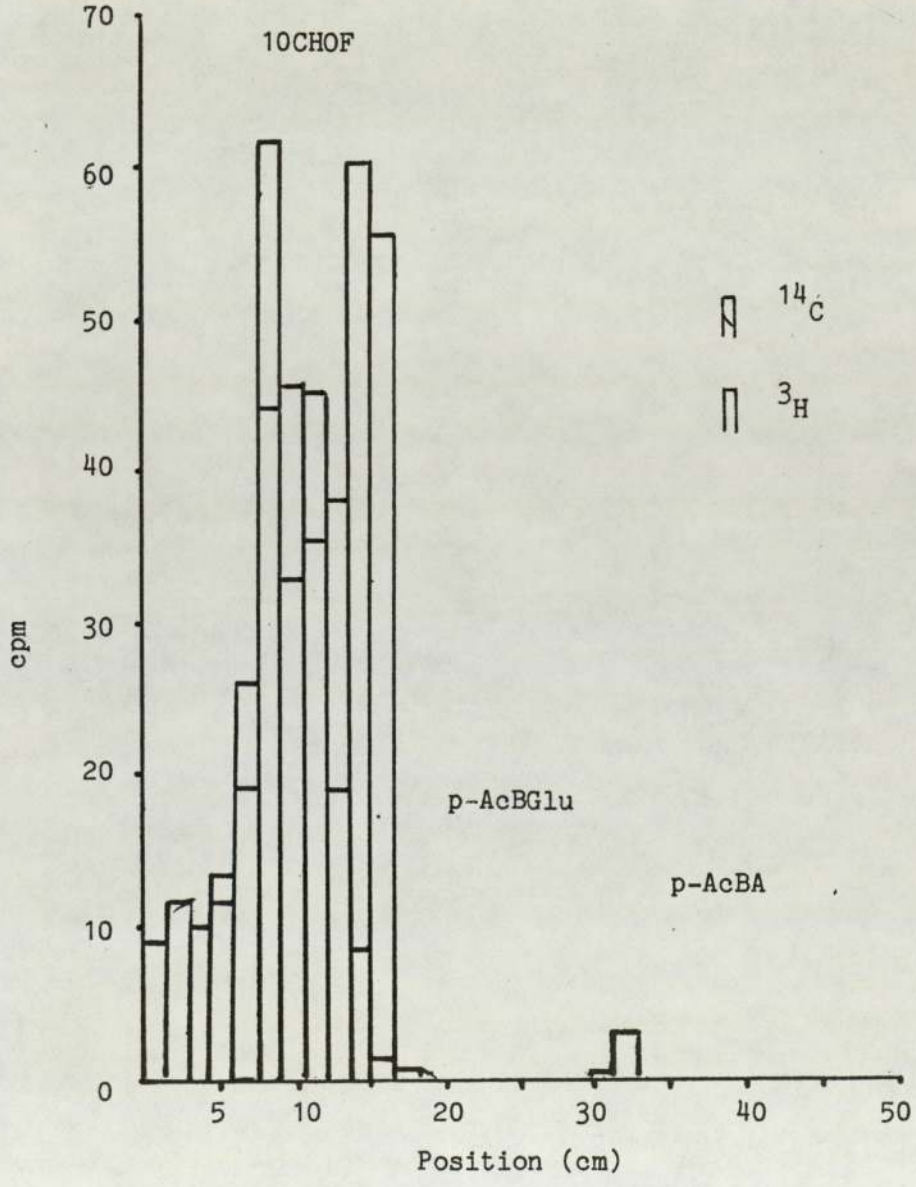


Figure 4.2.6: Paper chromatography of peak 2 of DE52 chromatography of urine (48-72h) from adult female rats treated with DES.

female rats. In each a major dual labelled peak corresponding to the 10CHO-folate standard was clearly visible. There is no evidence of ^{3}H-only labelled peaks corresponding to p-AcBA or p-AcBGlu in the 48-72h urine (Figure 4.2.6.) but the 0-24h and 24-48h urines (Figures 4.2.4. and 4.2.5.) do contain small ^{3}H-only peaks corresponding to the p-AcBA and p-AcBGlu standards.

Summary

Qualitative differences in the handling of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid were found between normal adult female rats and adult females treated with diethylstilboestrol (DES) plus lipid. The treated group excreted less radioactivity into the urine over 0-72h after dosing. Analysis showed that the urine from the treated group contained slightly more unchanged folic acid, less 5MeTHF but a lot more 10CHO-folate than the untreated group. Scission was also depressed by the administration of DES plus lipid when compared with the normal females.

Chapter 5

In Vitro Studies of Folate Catabolism

5.1. Previous studies of folate metabolism have shown that breakdown occurs in man, the rat and the guinea-pig (Krumdieck et al., 1978; Murphy et al., 1978; Connor et al., 1979; Pheasant et al., 1979, 1981; Choolun et al., 1980; Saleh et al., 1980, 1982) giving rise to a series of catabolites.

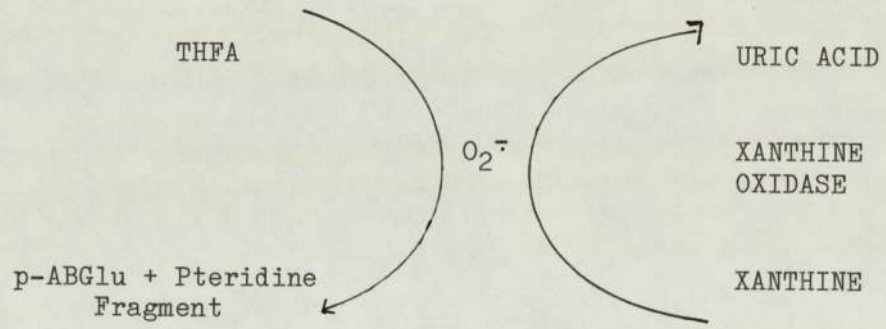
Jukes et al. (1947), Blair (1958) and more recently Murphy et al., (1978) have suggested that in vivo folate catabolism may rise from scission of the molecule via cleavage of the C9N10 bond, forming p-ABGlu and a pteridine fragment. This cleavage could be effected by either chemical or enzymic means. Some studies carried out in this department, reported in this thesis (Chapter 3) and elsewhere (Guest et al., 1983), have shown that phenobarbitone and phenytoin do not increase folate catabolism although they are known to induce hepatic enzyme activity (Eling et al., 1970). It was therefore decided to investigate the chemical oxidation of folates in vitro. Tetrahydropterins react with molecular oxygen to produce free radicals (Pearson, 1974; Pryor, 1976; Jakoby, 1980). The endogenous metabolism of a cell produces a small yield of these highly reactive molecules as many enzymes including the cytochrome oxidase system, alcohol dehydrogenase and the electron transport chain all generate free radicals (Pryor, 1976). To counteract this, aerobic organisms have evolved a unique set of defensive enzymes that scavenge free radicals. Superoxide dismutases remove the superoxide radical producing hydrogen peroxide (Figure 5.1.1.) which is in turn converted to less reactive compounds by catalase and peroxidase (Hassan and Friedovich, 1980). Could free radicals be involved in the break-down of THFA in vivo? To test this hypothesis a series of experiments were conducted to examine

Figure 5.1.1.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} H_2O_2 + O_2$$

Superoxide dismutase scavenges the free radicals.

Figure 5.1.2.



Hypothetical involvement of superoxide anion in the scission of THFA.

the rate of scission of THFA alone and in the presence of an enzyme system known to produce free radicals. (Figure 5.1.2.). (Pryor, 1976). The in vivo conversion of xanthine to uric acid is catalysed by the enzyme xanthine dehydrogenase. This reaction does not generate free radicals as the electrons are transferred to hydrogen unlike the oxidase which in vitro transfers electrons to oxygen thus producing superoxide free radicals. The experiments were carried out in vitro using two methods. The first was the Bratton and Marshall colorimetric assay for diazotizable amines (1939). This gave a quantitative estimate of the rate of scission by measuring the rate of appearance of p-ABGlu. The second method, thin layer chromatography, (tlc), was purely qualitative and allowed the identification of the other scission products produced together with p-ABGlu. Both of these methods are described in detail in Chapter 2.

5.2. Results

5.2.1. The Bratton and Marshall Assay For Diazotizable Amines

Using a standard solution of p-ABGlu a calibrat ion curve was constructed (Figure 5.2.1.). By assuming that 1 mole of THFA breaks down to produce 1 mole of p-ABGlu the percentage scission of THFA was calculated and the equivalent amount of THFA broken down is shown on the x axis.

The standard p-ABGlu solution was initially prepared using a phosphate buffer containing sodium ascorbate (5% w/v) as it was thought it would be useful in later experiments as an antioxidant. The ascorbate was

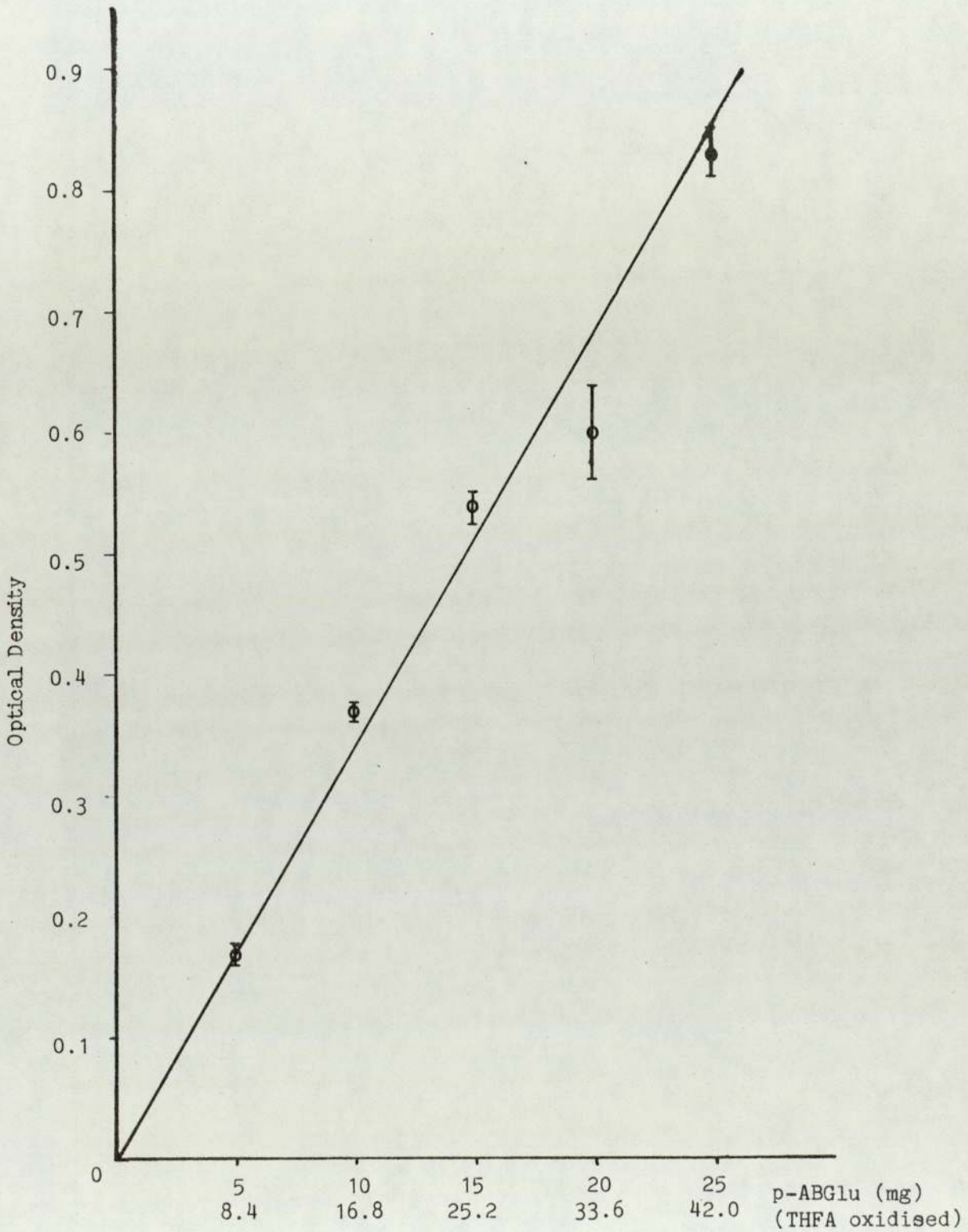


Figure 5.2.1: Calibration for p-ABGlu showing the equivalent amount of THFA oxidised.

found to block the development of colour and was therefore omitted from later solutions. Table 5.2.1.1. shows the results of these incubations. As the maximum purity of the THFA was 80% (Sigma, 1983) almost complete oxidation had occurred in all the solutions, including those read at time zero. The incubations were therefore repeated using a more diluted buffer solution (0.01M phosphate buffer, pH7) to reduce any acceleration of oxidation of the THFA by metal ions present in the buffer (Pearson, 1974). The results of these experiments are shown in table 5.2.1.2. The mean oxidative scission was slightly lower in these incubations but there was no measurable change with time. When the impurity of the THFA was taken into account the corrected results of table 5.2.1.2 became; THFA alone 73.1%; THFA plus xanthine oxidase 66.7%; THFA plus xanthine oxidase plus xanthine 63.9%.

5.2.2. Thin Layer Chromatography

Table 5.2.2.1. shows the results of the thin layer chromatograms run. Aliquots were taken from the incubations at time zero and 3,7 and 10 minutes after this but no changes were found with time. Therefore table 5.2.2.1. shows the overall data with no reference to time. A series of standards were also run in each solvent system including a solution of THFA that had been left at room temperature overnight to allow total oxidation to occur.

THFA is known to cleave to form p-ABGlu and pterin (Pearson, 1974). This series of experiments confirmed these observations as both of these scission products were tentatively identified in each of the

Table 5.2.1.1.

Oxidative scission of THFA with xanthine and xanthine Oxidase using 0.05M phosphate buffer, pH7.

TIME (mins)	p-ABGlu (mg) produced	THFA (mg) oxidised	% Scission
a) THFA alone			
0	10.5	17.6	70.6
10	9.5	16.0	63.8
20	12.0	20.2	80.6
40	9.5	16.0	63.8
			Mean 69.7 ± 3.4
b) THFA + XANTHINE OXIDASE			
0	10.25	17.2	68.9
10	10.0	16.8	67.2
20	9.5	16.0	63.8
40	9.3	15.5	62.0
			Mean 65.5 ± 1.6
c) THFA + XANTHINE + XANTHINE OXIDASE			
0	11.0	18.5	73.9
10	12.0	20.2	80.6
20	6.0	10.1	40.3
40	8.5	14.3	57.1
			Mean 63.0 ± 9.0

Table 5.2.1.2.

Oxidative scission of THFA with xanthine and xanthine oxidase using 0.01 M phosphate buffer, pH7.

TIME (mins)	p-ABGlu produced (mg)	THFA oxidised (mg)	% Oxidative Scission
a) THFA alone			
0	8.0	13.4	67.2
15	7.8	13.0	65.1
30	8.0	13.4	67.2
45	6.0	10.1	50.5
90	6.0	10.1	50.5
135	6.0	10.1	50.5
			Mean=58.5 ± 3.9
b) THFA + XANTHINE OXIDASE			
0	7.0	11.8	58.8
15	7.0	11.8	58.8
30	7.0	11.8	58.8
45	6.0	10.1	50.5
90	6.0	10.1	50.5
135	6.5	10.9	54.6
			Mean=53.3 ± 1.8
c) THFA + XANTHINE + XANTHINE OXIDASE			
0	8.0	13.4	67.2
15	6.0	10.1	50.5
30	6.0	10.1	50.5
45	6.0	10.1	50.5
90	5.0	8.4	42.0
135	5.5	9.2	46.2
			Mean=51.2 ± 3.8

Table 5.2.2.1.

Thin layer chromatography of solutions containing a) THFA b) THFA plus Xanthine Oxidase c) THFA plus Xanthine Oxidase plus Xanthine.

Rfs in solvent systems

Standard/ incubation	5% Acetic Acid 5% Sod Ascorbate	10% Acetic Acid 1% Sod Asorbate	10% Acetic Acid 10% Sod Ascorbate
Oxidised THFA	0.19	0.65	0.36
p-ABGlu	0.03	0.63	0.66
pterin	0.19	0.63; 0.84	0; 0.88
xanthine	0.09; 0.38	0.66; 0.84	0.9
Incubation a)	0.03; 0.19	0.65	0.36
Incubation b)	0.03; 0.19	0.84	0.9
Incubation c)	0.03; 0.19; 0.5	0.84	0.9

incubations. However no changes with time were observed as the reactions appeared to be complete at time zero.

SUMMARY

THFA is readily oxidised (Pearson, 1974) and it was hoped to show that the rate of this oxidation was increased in the presence of superoxide anion generated from xanthine and xanthine oxidase (Pryor, 1976). If this increased rate could be demonstrated then it would suggest that any process within the cell generating superoxide anion could increase folate scission.

The thin layer chromatography showed p-ABGlu and pterin to be the two major scission products as demonstrated earlier (Pearson, 1974). Kinetic analysis of the scission reaction was impossible using the measurement of the rate of appearance of the p-ABGlu formed as the nitrous acid produced during the colorimetric assay apparently also oxidises ascorbic acid. The mechanisms of these oxidations will be discussed in the final chapter.

Chapter 6

Discussion

These studies in the administration of radiolabelled folic acid to groups of adult male (Chapter 3) and adult female rats (Chapter 4) have shown that radioactivity is found in the tissues, urine and faeces. Further analysis of the urine samples revealed a similar pattern of metabolites in male and female rats. In both sexes there was a urinary excess of ^{3}H over ^{14}C and an excess of ^{14}C over ^{3}H in the faeces (Table 6.1.). Folic acid, 5MeTHF, IOCHO-folate, 5,$IOCH_2$-THF, Metabolite X, p-AcBGlu, p-AcBA, $^{3}H_2O$, ^{14}C-urea and ^{14}C-only labelled fragments (possibly pteridine derivatives) were found in male and female rat urine. Similar metabolites have previously been detected in the urine of male rats (Murphy et al., 1976; Barford et al., 1978; Pheasant et al., 1981; Saleh et al., 1981) humans (Pheasant et al., 1981; Saleh et al., 1982) and the guinea-pig (Choolun et al., 1980). The scission products are produced by cleavage of the C9N10 bond of the folate molecule (Figure 6.1.) giving ^{3}H-labelled p-ABGlu derivatives and a dual labelled pterin which undergoes further metabolism losing the ^{3}H label before being excreted as a ^{14}C-only labelled fragment. p-ABGlu has been shown to be converted to p-AcABA in vivo (Murphy et al., 1976; Connor et al., 1979; Pheasant et al., 1981). Pheasant et al., (1981) suggested a scheme to explain these experimental observations in which folate derivatives in vivo are divided into two pools: one with a half life of about 1 day in the rat (Saleh, 1981) and 31.5h 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). Folate monoglutamates are the sole components of the pool participating in the enterohepatic circulation with p-AcBA being the major tritiated catabolite formed in the gut whereas monoglutamates and polyglutamates within the body form a second pool of which p-AcBGlu is the main tritiated catabolite.

Table 6.1.

Radioactivity recovered in the urine and faeces of adult male (180 - 200g body weight) and adult female rats (140 - 160g body weight) orally dosed with a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100μg/Kg body weight). Results are expressed as a percentage of the dose administered. ($\pm$ S.D.).
(Statistical significance. 1. Comparison of ^{14}C : ^{3}H within a sample *p = 0.05, **p = 0.01 2. Comparison of ^{14}C or ^{3}H between similar samples of each group + p = 0.05, ++ p = 0.01).

SAMPLE	MALES		FEMALES	
	^{14}C	^{3}H	^{14}C	^{3}H
URINE				
0 - 24h	23.4 (2.3)*++	31.8 (3.2)*+	36.5 (2.1)*++	43.0 (2.4)*+
24 - 48h	1.6 (0.3)*	8.1 (2.3)*+	3.2 (0.7)	3.5 (0.8)+
48 - 72h	0.3 (0.1)*+	1.0 (0.3)*	1.8 (0.2)+	2.0 (0.3)
0 - 72h	25.3	40.9	41.5	48.5
FAECES				
0 - 24h	15.3 (4.3)**	6.7 (2.1)**	13.3 (2.7)	8.8 (2.2)
24 - 48h	1.3 (0.4)	0.8 (0.2)+	2.2 (0.4)	1.7 (0.3)+
48 - 72h	0.1 (0.1)	0.6 (0.2)	0.7 (0.2)	0.6 (0.1)

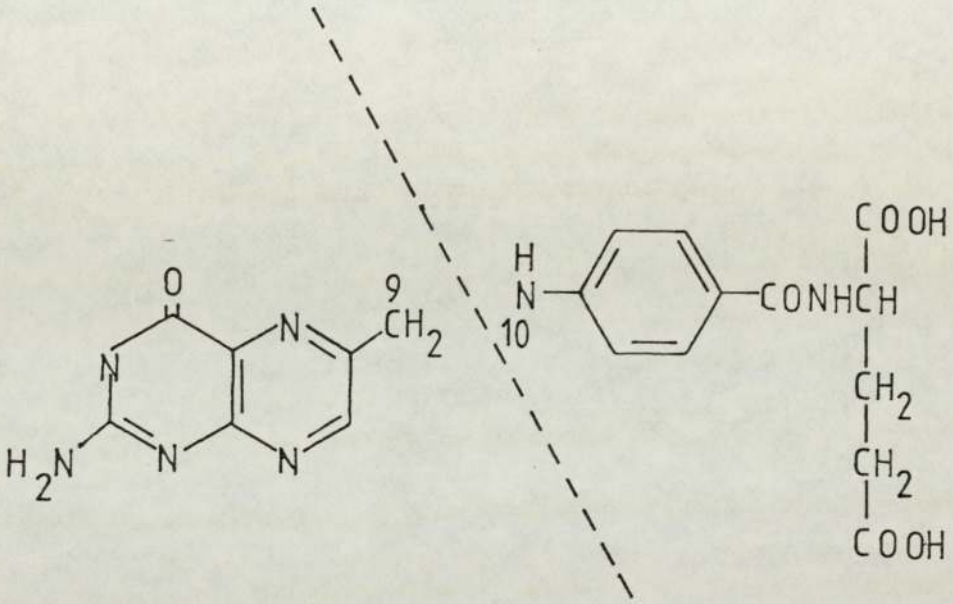
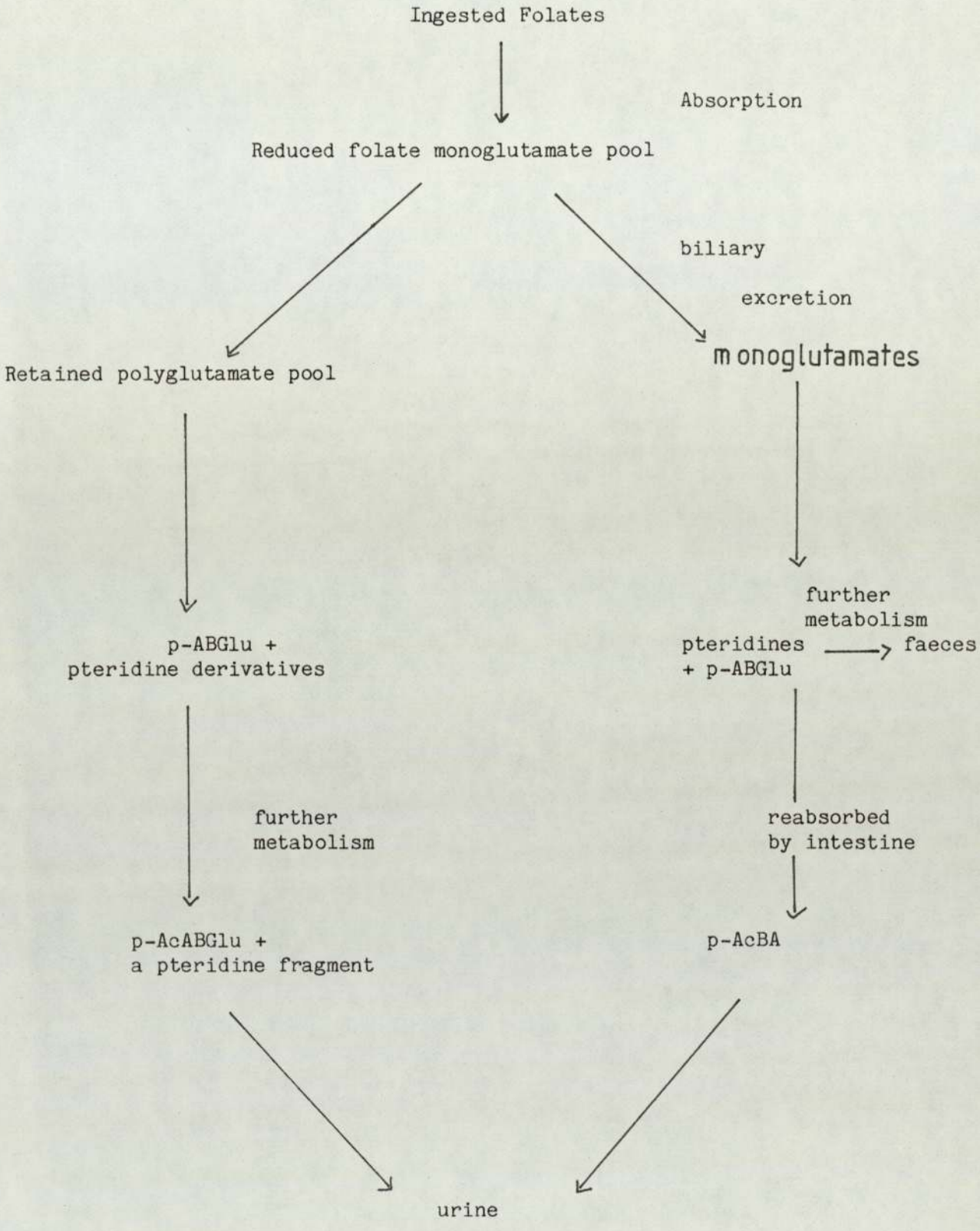


Figure 6.1. C9N10 Scission of the folate molecule.

Figure 6.2. shows the formation, interaction and breakdown of these two pools. Analysis of urinary folate metabolites revealed some quantitative differences between the male and female rats (Table 6.3.). The only metabolite not present in both of the urines was 5,$IOCH_2$-THF. This was absent from the female rat urine and Metabolite X was also reduced to trace amounts. The amount of unchanged folic acid excreted was lower in the female rat urine than in the male rat urine (1.5% ^{14}C and 1.8% ^{3}H in the male; 0.1% ^{14}C and 0.07% ^{3}H in the female over 72h). 5MeTHF and IOCHO-folate were the two main metabolites found in the urines of both sexes but the females excreted almost twice as much as the males (5MeTHF: 7.7% ^{14}C, 9.9% ^{3}H in the male; 22.0% ^{14}C, 16.2% ^{3}H in the female. IOCHO-folate: 6.7% ^{14}C, 10.1% ^{3}H in the male and 16.1% ^{14}C, 18.8% ^{3}H in the female urine). If the ratio of 5MeTHF/IOCHO-folate is taken at 24 and 72 hours it becomes apparent that the proportion of these two metabolites differs in the urines from the two sexes (table 6.2.). Thus not only is more 5MeTHF excreted by the females but the level of this metabolite exceeds that of IOCHO-folate. 5,$IOCH_2$-THF was not found in the female rat urine and as figure 6.3. shows 5MeTHF, along with DHF and THFA may be formed from this folate intermediate. Table 6.3. summarises the urinary catabolites detected in the urine from the adult male and adult female rats



(from Pheasant et al., 1981)

Figure 6.2: Possible routes of folate catabolism in vivo

Table 6.2.

5MeTHF and 10CHO-folate content of urine collected from adult male and female rats dosed orally with a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight). Figures are expressed as a percentage of the dose administered.

Sex	Sample	5MeTHF	10CHO-folate	Ratio $\frac{\text{5MeTHF}}{\text{10CHO-folate}}$
Male	24h urine	7.4	6.3	1.17
	0 - 72h urine	7.7	6.7	1.15
Female	24h urine	21.0	15.5	1.36
	0 - 72h urine	22.0	16.1	1.37

Figure 6.3.

The routes by which THFA, DHF and 5MeTHF may be derived from $5,10CH_2$-THF.

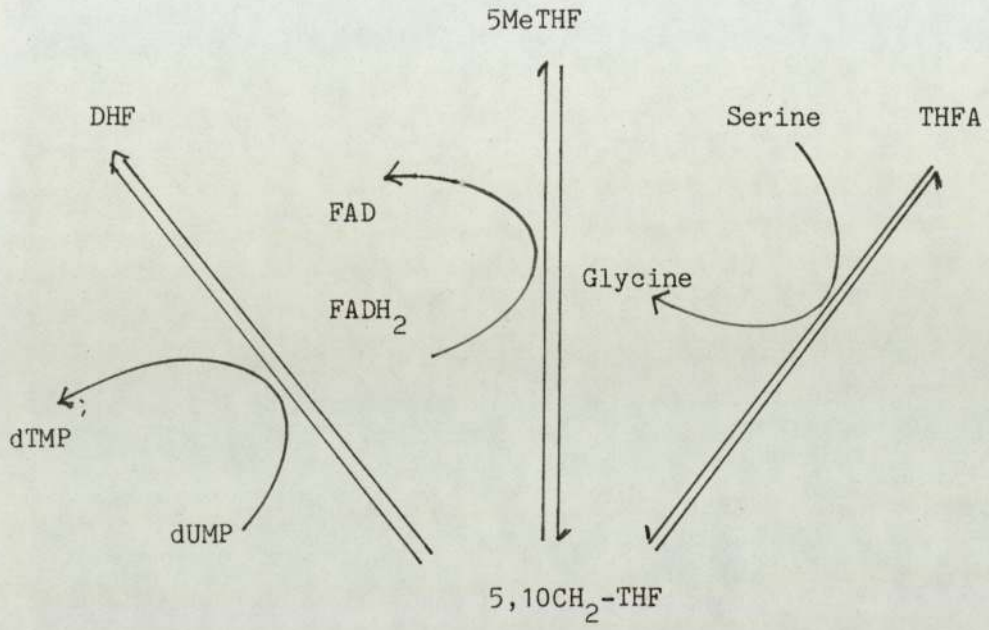


Table 6.3.

Metabolites present in the urine of adult male and adult female rats 0 - 72h after the oral administration of a mixture of [3',5',7,9^{3}H] and [2-^{14}C] folic acid (100μ g/Kg body weight). Results are expressed as a percentage of the dose administered. n=15, ND = Not detectable, T = trace.

METABOLITES	MALES 0-72h		FEMALES 0-72h	
	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	1.5	1.8	0.1	0.07
5MeTHF	7.7	9.9	22.0	16.2
IOCHO-Folate	6.7	10.1	16.1	18.8
5,IOCH$_2$-THF	3.3	3.8	ND	ND
METABOLITE X	2.4	3.1	T	T
p-AcBA	-	2.8	-	3.6
p-AcBGlu	-	7.5	-	8.8
^{14}C-only labelled fragments	3.6	-	5.4	-
^{14}C Urea	1.0	-	1.7	-
$^{3}H_2O$	-	2.2	-	1.4

0-72h after the dose of radiolabelled folic acid. More p-AcBGlu, p-AcBA, ^{14}C-urea and ^{14}C-only labelled pteridine fragments were excreted over 0-72h by the female rats. $^{3}H_2O$, probably derived from the C9 position during cleavage of the C9N10 bond, is present in greater amounts in the urine from the male rats. Table 6.4. shows the radioactivity recovered in the faeces from each group of rats. The excess ^{14}C found in the male rat faeces is not as pronounced in the female faeces as there is more ^{3}H present in the latter. The presence of ^{14}C-urea in the urine indicates that degradation of the pterin ring system is occuring in vivo but the mechanism by which this occurs is not known. No attempt to quantify the loss of radioactivity in the expired air was made in these experiments but Connor et al., (1977) and Choolun (1982) have recovered $^{14}CO_2$ in the breath of the rat and the guinea-pig respectively.

Table 6.5. shows the hepatic radioactivity of the male and female rat. Male rat liver contains significantly more ^{3}H and ^{14}C than female rat liver. The same trend of slightly raised levels at 48h followed by a fall in the levels of each isotope at 72h may be observed in both male and female rat livers. G15 gel filtration analysis showed that in each case the major radiolabelled hepatic component was folate polyglutamate. Therefore these experiments have shown that the female rat converts less of the total available folate to the polyglutamate form than the male rat and that the female has higher levels of 5MeTHF and IOCHO-folate, the latter being the preferred substrate for polyglutamate formation. The polyglutamate body burden may be calculated in a number of ways (Table 6.6.). If the assumption is made that all of the administered radioactivity, excepting that lost in the

Table 6.4.

Faecal radioactivity recovered from adult male and female rats dosed with (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight). Figures are expressed as a percentage of the dose administered ($\pm$ SD) (Statistical significance 1. p = 0.01; 2. p = 0.05).

Sample	Faecal radioactivity recovered from:-			
	Male Rats		Female Rats	
	^{14}C	^{3}H	^{14}C	^{3}H
0 - 24h	15.3 (4.3)[1.]	6.7 (2.1)[1.]	13.3 (2.7)[2.]	8.8 (2.2)[2.]
0 - 72h	16.7	8.1	16.2	11.1

Table 6.5.

Radioactive content of adult male and female rat liver 24h, 48h and 72h after the administration of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight). Figures are expressed as a percentage of the dose administered. ($\pm$ SD). (Statistical significance * p = 0.05, ** p = 0.01 for differences between ^{14}C or ^{3}H content of male and female rat livers).

LIVER	24h		48h		72h	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Male Rat	15.7 (1.4)	18.6 (1.6)	19.1 (1.8)	21.6 (2.3)	17.9 (1.8)	20.6 (2.2)
Female Rat	9.8** (1.9)	9.6** (0.5)	10.7** (0.9)	12.0** (1.3)	6.2** (0.4)	9.2** (0.6)

urine and faeces, is converted to folate polyglutamate the resulting body burden is shown in Table 6 section (a). As this gives an exaggerated estimate a second calculation may be performed where the radioactivity retained by the gut, kidney and liver 24h after dosing is taken to represent the body burden of polyglutamate (Table 6.6.(b)). These tissues have been shown to take up the majority of radioactivity following the administration of folic acid (Pheasant et al., 1981) and Bates et al., (1980) demonstrated that conversion of the administered radiolabelled folates to polyglutamates was maximal 24h after dosing. A third method is to solely use the liver burden as this is the main source of folate polyglutamate in the body (Table 6.6.(c)).

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

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as pAcBGlu 24-48h}}{\%\ \text{Radioactivity retained at 24h}} \times 100\%$$

The distribution of the tritium label throughout the folate molecule is such that C9N10 cleavage leaves 42.5% of the label in the glutamyl-containing fragment (figure 6.1.). Appropriate correction must therefore be made for this. The resulting figures are also shown in table 6.6. The male rat has the greater extent of polyglutamate breakdown and a larger polyglutamate body burden than the female regardless of which calculation is made.

Krumdieck et al., (1975) reported that in the rat uterus the enzyme which shortens the polyglutamate chain length (conjugase) undergoes

Table 6.6.

The extent of polyglutamate breakdown following the oral administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid to adult male and adult female rats where:-

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as pAcBGlu 24 - 48h}}{\%\ \text{Radioactivity retained at 24h}} \times 100\%$$

Group	% ^{3}H dose retained in the body after 24h Polyglutamate body burden	% ^{3}H dose excreted as p-AcBGlu 24 - 48h	Extent of breakdown (%)
a) Calculations based on total radioactivity retained in the body at 24h i.e. Dose administered - (Total excreted in urine and faeces over 0 - 24h)			
Males	61.5	3.3	5.4
Females	48.2	1.4	2.6
b) Calculations based on radioactivity found in the liver, kidney and gut at 24h.			
Males	25.9	3.3	12.7
Females	13.13*	1.4	11.0
c) Calculations based on radioactivity in the liver at 24h.			
Males	18.6	3.3	17.7
Females	9.6	1.4	13.8

* Figures available for liver and kidney only.

rhythmic variation during the reproductive cycle reaching a peak at proestrus. At this time the polyglutamate forms of the folate coenzymes are minimal, monoglutamate forms are maximal and oestrogen secretion is at its peak. They further state that during oestrus the circulating folate is retained by the uterus and is then rapidly converted to polyglutamates thereby increasing the total folate content of the uterus. The importance of folate co-factors in reproductive metabolism was recognised even before the structure of folate had been established, as a dietary deficiency of the "L casei factor" impaired the normal growth response of chick uterus to oestrogen stimulation (Hertz and Sebrell, 1944). Since that time numerous reports have shown a close interrelationship between folates and reproductive endocrinology. Oral contraceptive agents which have been reported to induce megaloblastic anaemia (Streiff, 1970, Snyder and Necheles, 1969, Whitehead et al., 1973), alter serum folate levels, (Lakshmaiah and Bamji, 1979; Shojania, 1982) increase the excretion of FIGLU after a dose of histidine (Shojania, 1982) and castration may depress folate metabolism in the female rat (Tolomelli et al., 1972). In these experiments the female rats excreted more radioactivity into the urine and faeces than the males. This observation confirms the earlier reports that oestrogens increase the excretion of folates. Doctor and Trunnell (1955), [illegible], Streiff and Greene (1970), Fleming (1972) Shojania et al., (1975) and Lakshmaiah and Bamji (1979) all reported an increase in urinary folate excretion following oral contraceptive or oestrogen administration and during pregnancy. However Tolomelli et al (1972) found that castration increased the urinary excretion of folates by female rats, that the administration of

oestrogen to normal females reduced the urinary folate levels and that oestrogens restored the excretion rates of castrated rats to levels close to that of normal females. In the experiments reported here the female rat urine contained more 5MeTHF and IOCHO-folate than the urine from the male rats, but 5,$IOCH_2$-THF levels had fallen to trace amounts. Figure 6.4. shows the interconversion and regulation by glycine of 5,$IOCH_2$-THF, IOCHO-THF and THFA biosynthesis. Glycine is important in the regulation because when there are high glycine levels the production of N-Methyl-glycine is enhanced which increases the conversion of THFA to 5,$IOCH_2$-THF and then the biosynthesis of IOCHO-THF[A] and 5MeTHF. The later step may proceed because the removal of SAM (S-adenosyl methionine) due to N-methylglycine formation reduces the inhibition of 5,$IOCH_2$-THF reductase (Kutzbach and Stokstad, 1967). Oestrogens increase the activity of serine transhydroxy methyl transferase (Tolomelli et al., 1972; Burns and Jackson, 1982) which will enhance the biosynthesis of 5,$IOCH_2$-THF and glycine thereby leading to increased levels of both 5MeTHF and IOCHO-THF as seen in Table 6.3. In the Chick oestrogens increase the uptake of ^{3}H-folic acid into isolated epithelial cells (Eliam et al., 1982) and raise the activity of dihydrofolate reductase (DHFR) (Burns and Jackson, 1982). Remembering the species difference, if the same were true of the female rat this could explain the reduction in urinary excretion of unchanged folic acid, due to the increased activity of DHFR, and the greater total excretion of radioactivity, due to an increased absorption of the dose across the intestine. An increase in DHFR activity would also protect against loss of THFA through oxidation to DHF and might explain why the level of scission products is only slightly greater in the

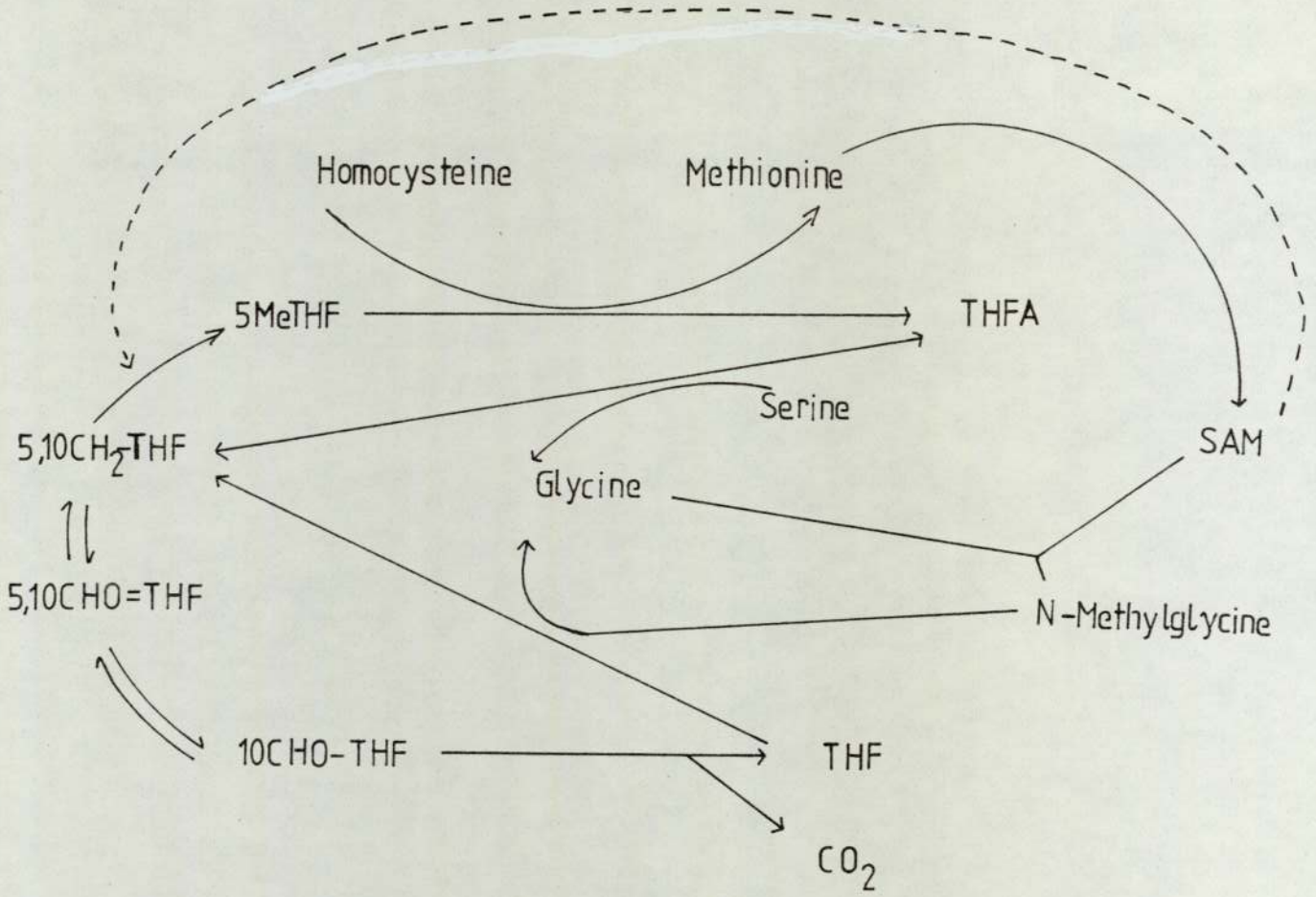


Figure 6.4. The role of glycine levels in regulating the production of $5,10CH_2$-THF, 10CHO-THF and THFA.

female rat urine despite the low conversion of monoglutamates to polyglutamates.

Having established a pattern for the distribution, metabolism and excretion of folates in the adult male rat it was then possible to determine that this pattern was quantitatively different in the adult female rat. One of several possible reasons for this may be the hormonal differences between males and females as oestrogens have been reported to alter the activity of some enzymes in the folate metabolic pathways (Krumdieck et al., 1975; Tolomelli et al., 1972). Folates and oestrogens have both been connected with malignant disease. Antifolates, such as Methotrexate, are used in the treatment of certain cancers and anti-oestrogens, such as Tamoxifen, have been employed to combat breast cancer (Allegra et al., 1980; Coezy et al., 1982). Breast cancer itself has been linked with the intake of dietary fat (for review see Willett and Mac Mahon, 1984) as most undeveloped countries have rates of breast cancer far lower than those of the United States and Northern Europe where the national per capita consumption of fat is much greater. However recent increases in the consumption of fat in Japan has not led to an increased incidence of breast cancer whereas the descendants of Japanese imigrants to USA have breast cancer rates that are close to those of the general American population. The results from animal experiments are clearer as the incidence of mammary tumours increases when there is a higher intake of dietary fat. This correlation is only appar ent when part of the dietary fat is polyunsaturated (Willett et al., 1984).
(Kutzbach and Stokstad, 1967).

Adult female rats were treated with diethylstilboestrol (DES) and mazola corn oil in an experiment designed to investigate the effect of both oestrogens and raised dietary fat intake upon folate metabolism (Chapter 4). Table 6.7.a. shows the composition of the normal diet fed to laboratory rats. In Table 6.7.b. corn oil is shown to contain a large proportion of polyunsaturated fats.

As the major differences between folate metabolism in the adult male rat and adult female rat had been in the urinary folates these aspects were concentrated upon in this experiment. Table 6.8. a) shows the excretion of radioactivity into the urine by untreated female rats and females treated with DES plus lipid. The normal females excreted significantly more ^{3}H and ^{14}C in the urine over 0-24h than the females treated with DES plus lipid (36.5% ^{14}C and 43.0% ^{3}H by the normal females as compared to 30.0% ^{14}C and 35.8% ^{3}H by the treated females). However the 24-48h urine samples contain similar levels of each isotope and the 48-72h urine samples from the treated rats contain slightly higher levels of radioactivity. The overall decrease in the urinary radioactivity of the treated group seen over 0-72h is therefore due to the changes over the initial 24h.

Section (b) of Table 6.8. lists the metabolites detected in the urine samples over 0-72h. The females treated with DES plus lipid excreted slightly more unchanged folic acid and 5,IOCH_2-THF than the normal females. There is considerably less 5MeTHF (3.97% ^{14}C and 4.36% ^{3}H as compared to 22.0% ^{14}C and 16.2% ^{3}H) in the urine from the treated rats but the opposite is true of IOCHO-folate which is present in much greater amounts (26.3% ^{14}C and 32.6% ^{3}H as compared to 16.1% ^{14}C and

Table 6.7.a)

Pilsbury's Modified Rat and Mouse Breeding Diet.

Crude Oil	3.26%
Crude Protein	21.23%
Crude Fibre	3.48%
Digestible Oil	3.24%
Digestible Protein	17.60%
Digestible Fibre	2.10%
Digestible Carbohydrate	46.80%
Fats	
Saturated Fatty Acids	0.73%
Linoleic Acid	0.99%
Other unsaturated fatty acids	1.54%

Table 6.7.b)

Composition Corn Oil.

Fat g/100g	Saturated Fatty Acids	Monounsaturated Fatty Acids	Polyunsaturated Fatty Acids
99.9	16.0	29.0	49.0

18.8% ^{3}H). 5,$IOCH_2$-THF is the coenzyme required for the production of dTMP an essential step in DNA synthesis and IOCHO-THF, from which the urinary IOCHO-folate is derived, is necessary for purine biosynthesis. Both of these processes are required for cell growth and both of these cofactors are found in greater quantities in the urine following oestrogen with lipid treatment. The urine from the DES plus corn oil treated female rats contained less scission products than the urine from the normal females (Table 6.8.b.). Therefore the administration of DES plus corn oil not only decreased the overall excretion of radioactivity into the urine but also reduced the breakdown of available folate. The excretion of radioactivity into the faeces 0-24h basically represents the unabsorbed portion of the dose. (Later faecal excretion includes radioactivity derived from the enterohepatic circulation of folates). As there are no major differences between the faecal radioactivity recovered 0-24h from the male or female rats (Table 6.9.) there appears to be no evidence of malabsorption in either sex. The urinary excretions do vary as the female rats excrete more ^{3}H and ^{14}C than the males. This corresponds to a lower tissue retention in the females (Table 6.9.) for if absorption is similar but excretion is greater less must be retained. No values for faecal or hepatic radioactivity are available for the females treated with DES plus corn oil but as their urinary excretion lies between that of the males and untreated females it may be assumed that the tissue retention of radioactivity would also be greater than in the untreated female but less than that of the male. Therefore the DES plus corn oil treatment has possibly increased tissue uptake, reduced scission and decreased urinary excretion of folates when compared to untreated females. When

SAMPLE	FEMALES		FEMALES + DES + LIPID	
	^{14}C	^{3}H	^{14}C	^{3}H
a) Urinary Excretion				
0 - 24h	36.5* (2.1)	43.0* (2.4)	30.0 (2.6)	35.8 (3.0)
24 - 48h	3.2 (0.7)	3.5 (0.8)	3.3 (0.5)	4.0 (0.7)
48 - 72h	1.8 (0.2)	2.0 (0.3)	2.3 (0.2)	2.7 (0.3)
0 - 72h	41.5	48.5	35.6	42.5
b) Urinary Metabolites (0 - 72h)				
Folic Acid	0.1	0.07	0.4	0.4
5MeTHF	22.0	16.2	3.97	4.36
10CHO-Folate	16.1	18.8	26.3	32.6
5,10CH_2-THF	ND	ND	0.5	0.6
Metabolite X	T	T	ND	ND
p-AcBA	-	3.6	-	0.5
p-AcBGlu	-	8.8	-	1.5
^{14}C-labelled compounds	7.1	-	3.1	-
$^{3}H_2O$	-	1.4	-	0.79

Table 6.9.

Radioactivity excreted in the urine and faeces and retained by the liver 0 - 72h following the oral administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight) to adult male, adult female and adult female rats treated with DES plus corn oil. ($\pm$ SD where appropriate).

Sample	Males		Females		Females Plus DES Plus Corn Oil	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Total Urinary Excretion						
0 - 72h	25.3	40.9	41.5	48.5	35.6	42.5
Total Faecal Excretion						
0 - 24h	15.3 (4.3)	6.7 (2.1)	13.3 (2.7)	8.8 (2.2)	-	-
Hepatic Radioactivity						
72h	17.9 (1.8)	20.6 (2.2)	6.2 (0.4)	9.2 (0.6)	-	-

radiolabelled folic acid was administered to male WAB/Not rats bearing Mc/103B sarcoma it was found that compared to normal rats there was a lower recovery of radioactivity in the urine and faeces, a higher tissue retention and scission was depressed (Saleh et al., 1981). Wistar rats bearing Walker 256 tumours also showed decreased scission (Barford and Blair, 1978). Therefore the administration of an oestrogenic compound together with a raised fat intake has produced changes in the metabolism of folic acid similar to those seen in tumour-bearing rats.

Eggar et al., (1983) reported that the administration of DES plus corn oil to female rats significantly raised the activity of hepatic dihydropteridine reductase (DHPR) and as already discussed oestrogens increased the activity of DHFR in the chick (Burns and Jackson, 1982). These enzymes act in conjunction to salvage THFA from either DHF or quinonoid DHF which are easily oxidised (Chippel and Scrimgeour, 1970) as shown in figure 6.5. An increase in the activity of DHFR and/or DHPR would result in a decreased loss of folate. The antifolate methotrexate which inhibits both DHFR (Bertino et al., 1964) and DHPR, (Craine et al., 1972) increases scission in man and the rat (Saleh et al., 1981). The reduced excretion of scission products found in the DES plus corn oil treated rats may therefore be due to an increase in the efficiency of this salvage pathway through the stimulation of DHPR and DHFR.

Therefore the administration of DES plus corn oil to adult female rats produced a number of changes in the metabolism of folic acid similar to those found in the tumour bearing rat. The reduced scission may be due

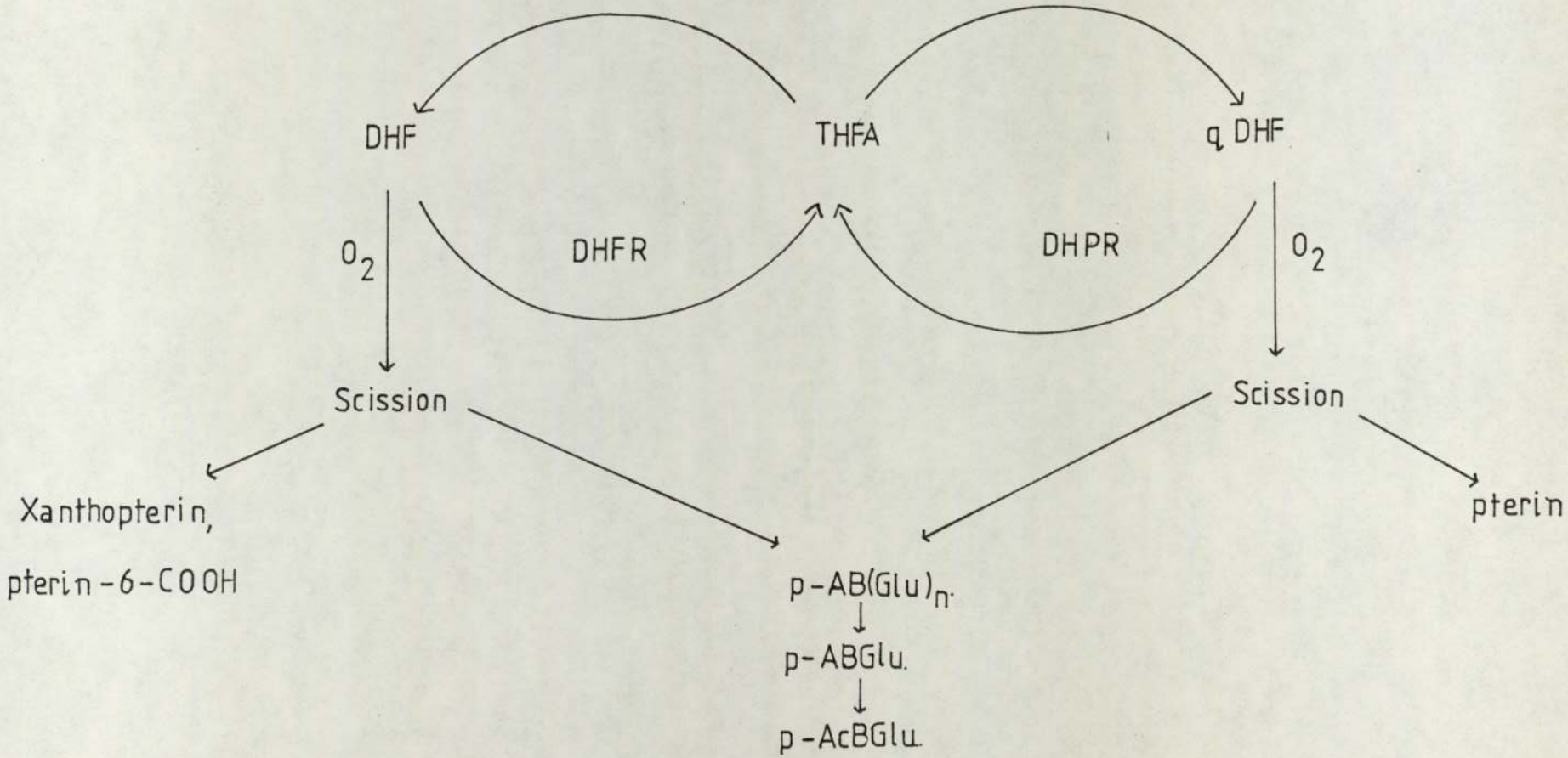


Figure 6.5. Involvement of DHFR and DHPR in folate biochemistry.

to increased activity of DHPR and DHFR an effect which has already been observed *in vivo* (Eggar *et al.*, 1983; Burns and Jackson, 1982). The suspected correlation between a high fat intake and the incidence of breast cancer in humans is partly supported by these experimental findings as the changes induced by the treatment were reduced scission and excretion of folates, increased tissue retention of radioactivity and a change in the equilibruim of the coenzymes with more 5,10CH_2-THF and 10CHO-THF being produced all of which are necessary for growth. The precise mechanism by which C9 N10 cleavage of the folate molecule occurs is not known. Scission could proceed by means of either enzymic catalysis or by chemical oxidation of these labile molecules. Increased scission *in vivo* would theoretically produce folate deficiency, expressed as the clinical condition of megaloblastic anaemia. Many conditions are known to produce this condition by various means only one of which may be an altered rate of catabolism. It was therefore decided to investigate the effects of an agent known to cause megaloblastic anaemia on the pattern of folate metabolism in the rat.

Drug induced megaloblastic anaemia is a well reported complication of anticonvulsant therapy in epilepsy (Hawkins & Maynell, 1958; Klipstein, 1964 and Reynolds, 1975, 1980). Where as less than 1% of drug treated epileptics actually develop megaloblastic anaemia, macrocytosis occurs in between 8-53% and low serum folate levels are found in 27-91% of nonanaemic drug treated epileptic patients (Reynolds 1976). These changes are usually associated with patients taking phenytoin, either alone or in combination with phenobarbitone (Reynolds, 1976). The mechanisms by which these changes are brought about are unclear and may

include malabsorption of folate (Hoffbrand and Necheles, 1968; Rosenberg et al 1968; Gerson et al., 1970; 1972; Benn et al., 1971), which has been disputed, (Baugh & Krumdieck, 1969; Bernstein et al., 1970; Doe et al., 1971; Houlihan et al., 1972; Perry & Chanarin, 1972; & Fehling et al., 1973;), an induction of the enzymes involved in folate metabolism (Richens and Walters, 1971) or by an increased demand for folate coenzymes because of the induction of folate-requiring pathways (Maxwell, et al., 1972; Labadarios et al., 1978).

Phenytoin induces microsomal enzyme activity in man (Conney, 1967; Latham et al., 1973 and Levine, 1973) and the rat (Eling et al., 1970) and is widely used in the treatment of epilepsy (Laurence, 1973). Guinea-pigs fed a folate deficient diet showed a marked decrease in the microsomal metabolising activity of their intestinal mucosa (Clement et al., 1981). Female rats have already been shown to produce a different pattern of folate metabolism (Chapter 4). Contraceptive steroids also induce microsomal enzymes in rats (Briatico et al., 1974) and although folate cofactors have not conclusively been shown to participate in steroid hydroxylations the studies of Hagerman (1964) and Lehoux et al., (1972) suggest such a possibility exists.

Phenytoin (100 mg/Kg body weight i.p.) was administered to healthy male rats (180-200g body weight) for 10 days prior to a single oral dose of radiolabelled folic acid (Chapter 3) Table 6.10 shows the radioactivity recovered in the urine and faeces and the distribution of retained radioactivity in normal rats and those treated with phenytoin. The urine excreted by the rats treated with phenytoin does not show the excess of ^{3}H over ^{14}C found in the normal rat urine. There is

Table 6.10.

Radioactivity recovered in the urine, faeces and tissues of normal male rats and male rats treated with phenytoin (100 mg/Kg body weight, i.p. for 10 days) following the administration of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100 μg/Kg body weight). Figures are expressed as a percentage of the dose administered ($\pm$ SD)
(Statistical significance p = 0.05* p = 0.01** for comparison of ^{3}H or ^{14}C of similar samples between groups).

SAMPLE	NORMAL GROUP		PHENYTOIN TREATED GROUP	
	^{14}C	^{3}H	^{14}C	^{3}H
Urine				
0 - 24h	23.4 (2.3)	31.8 (3.2)*	19.4 (2.7)	20.5 (3.0)*
24 - 48h	1.6 (0.3)**	8.1 (2.3)	4.7 (1.3)**	5.4 (1.6)
48 - 72h	0.3 (0.1)**	1.0 (0.3)	3.2 (1.3)**	4.7 (1.8)
0 - 72h	25.3	40.8	27.3	30.6
Faeces				
0 - 24h	15.3 (4.3)**	6.7 (2.1)*	22.4 (2.5)**	13.6 (1.6)*
24 - 48h	1.3 (0.4)**	0.8 (0.2)*	5.5 (0.8)**	4.6 (0.6)*
48 - 72h	0.14(0.1)**	0.6 (0.2)**	4.8 (1.0)**	4.1 (1.0)**
0 - 72h	16.7	7.1	32.7	22.3
Liver				
24h	15.7 (1.4)	18.6 (1.6)	19.9 (2.9)	20.3 (2.6)
48h	19.1 (1.8)**	21.6 (2.3)**	11.8 (1.4)**	11.9 (1.9)**
72h	17.9 (1.8)	20.6 (2.2)	19.3 (3.8)	22.9 (4.0)
Kidney				
24h	2.5 (0.8)	3.4 (0.6)	2.0 (0.3)	1.7 (0.3)
48h	1.6 (0.2)*	2.0 (0.5)*	1.6 (0.3)*	1.1 (0.2)*
72h	1.9 (0.2)	2.4 (0.4)	1.0 (0.3)	1.3 (0.2)
Gut				
24h	3.2 (0.3)	3.9 (0.4)	1.6 (0.1)	1.9 (0.1)
48h	2.3 (0.3)	2.5 (0.2)	2.1 (0.3)	2.1 (0.2)
72h	1.5 (0.2)	2.4 (0.3)	1.5 (0.3)	1.5 (0.2)

significantly less ^{3}H in the phenytoin treated rat urine over 0-24h than in the normal rat urine but over 24-48h and 48-72h the phenytoin treated rats excreted significantly more ^{14}C. There is also significantly more ^{3}H in the 48-72h urine from the phenytoin treated rats. Therefore the overall excretion of ^{14}C into the urine is quantitatively unaffected by phenytoin treatment (25.3% ^{14}C excreted over 0-72h by normal rats, 27.3% ^{14}C excreted over 0.72h by phenytoin treated rats) but early samples contain less radioactivity and later samples contain significantly more radioactivity than normal rat urine. Less ^{3}H is excreted 0 - 24h, 24 - 48h and 0 - 72h after phenytoin treatment.

The changes in urinary excretion of radioactivity after phenytoin are reflected by opposite changes in the faecal excretion of radioactivity. There is significantly more ^{3}H and ^{14}C present in the faeces of the phenytoin treated group in all samples. Therefore phenytoin appears to have decreased and delayed the absorption of folic acid.

The tissue levels are very similar to normal following phenytoin treatment with the exception of the 48h liver radioactivity where there is significantly less ^{3}H and ^{14}C, and 24h kidney and gut radioactivity where the levels are lower but not significantly so.

Table 6.11. lists the metabolites detected in male rat urine and phenytoin treated rat urine 0-24h, 24-48h and 48-72h after a dose of radiolabelled folic acid. The major differences are the greatly increased excretion of unchanged folic acid together with slightly less

Table 6.11.

Metabolites detected in the urine (24, 48 and 72h) of normal male rats and males treated with phenytoin (100 mg/Kg i.p. for 10 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 weight). Figures are epxressed as a percentage of the dose administered. ND = Not detectable.

Sample	Normal Males						Males Treated with Phenttoin					
Metabolite	0 - 24h		24 - 48h		48 - 72h		0 - 24h		24 - 48h		48 - 72h	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	1.5	1.8	ND	ND	ND	ND	4.9	3.2	0.5	0.4	0.2	0.3
5MeTHF	7.4	8.7	0.2	1.1	0.1	0.1	5.5	3.6	0.7	0.6	0.2	0.2
10CHO-Folate	6.3	8.2	0.4	1.9	ND	ND	4.4	4.7	0.7	0.7	0.3	0.3
5,10CH_2-THF	3.3	3.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Metabolite X	2.2	2.3	0.2	0.8	ND	ND	ND	ND	ND	ND	ND	ND
New peaks	ND	ND	ND	ND	ND	ND	ND	ND	1.6	1.6	1.0	1.3
Total Intact Folates	20.7	24.8	0.8	3.8	0.1	0.1	14.8	11.5	3.5	3.3	1.7	2.1
p-AcBGlu	-	5.3	-	1.4			-	2.7	-	1.3	-	0.6
p-AcBA	-	2.3	-	0.5		0.8	-	0.6	-	0.6	-	0.6
Pteridines derivatives	2.9	-	0.5	-	0.2		1.8	-	0.5	-	0.9	-
Urea	0.9	-	0.1	-			2.0	-	0.7	-	0.4	-
$^{3}H_2O$	-	0.9	-	1.3			-	0.8	-	0.4	-	1.1
Total Scission Products	3.8	8.5	0.6	3.2	0.2	0.8	3.8	4.1	1.2	2.3	1.3	2.3

5MeTHF, IOCHO-folate, no detectable 5,$IOCH_2$THF or Metabolite X and less p-AcBGlu. There are also the two new dual labelled peaks which were not present in the normal rat urine and constituted 2.6% ^{14}C and 2.9% ^{3}H dose. These peaks were found in both the 24-48h and 48-72h urines. Their delayed appearance suggesting that they may originate from folate polyglutamates, especially as the major polyglutamate derived scission product is present in reduced amounts (p-AcBGlu 7.5% in the normal rat urine, 4.6% in the phenytoin treated rat urine)(see Table 3.22.).

These experiments were conducted to investigate the effect of phenytoin upon folate catabolism in the rat. Reports that phenytoin-treated epileptics developed megaloblastic anaemia due to folate deficiency suggested that perhaps this anticonvulsant was increasing folate breakdown in vivo. Indeed Kelly et al., (1979) reported that phenytoin but not phenobarbitone increased folate catabolism in the mouse. Table 6.11. shows the urinary metabolites present in normal rat urine and in urine from rats treated with phenytoin. The sum of the ^{3}H only labelled scission products from the normal males is 12.7% dose as compared to 7.4% dose from the phenytoin treated group. The extent of breakdown of folate polyglutamates is shown in table 6.12. The phenytoin treated group again show a lower extent of breakdown but the polyglutamate body burden of each group is very similar. This experiment has therefore not only failed to confirm that phenytoin increases folate catabolism but it has produced evidence to show that catabolism is actually reduced in the rats treated with phenytoin. In a long term study the excretion of folates by one human subject treated with phenytoin Krumdieck et al., (1978) found an increase in the urinary excretion of intact folates but no evidence to suggest that

Table 6.12.

The extent of polyglutamate breakdown following the administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight) to normal male rats and male rats treated with phenytoin (100 mg/Kg body weight i.p. for 10 days) where:-

$$\text{Extent of breakdown} = \frac{\%\text{ Dose excreted as p-AcBGlu 24 - 48h}}{\%\text{ Radioactivity retained at 24h}} \times 100\%$$

Group	% ^{3}H dose retained in the body after 24h Polyglutamate body burden	% ^{3}H dose excreted as p-AcBGlu 24 - 48h	Extent of Breakdown (%)
a) Calculations based on total radioactivity retained in the body at 24h i.e. Dose administered - (Total excreted in urine and faeces over 0 - 24h)			
Males	61.5	3.3	5.4
Phenytoin Treated	65.9	3.1	4.7
b) Calculations based on radioactivity found in the liver, kidney and gut at 24h.			
Males	25.9	3.3	12.7
Phenytoin Treated	24.9	3.1	12.5
c) Calculations based on radioactivity in the liver at 24h.			
Males	18.6	3.3	17.7
Phenytoin Treated	20.3	3.1	15.3

phenytoin increases the breakdown of folates *in vivo*. The group that found an increased

catabolism following phenytoin treatment in mice (Kelly *et al.*, 1979) used a method where all the urinary folate derivatives were hydrolysed to p-ABA (Reed *et al.*, 1977). They reported an increase in total urinary radioactivity but assumed this was solely due to the excretion of more scission products because urine from control mice contained no intact folates at this time. Similarly the male rats excreted only 0.1% ^{14}C and 0.1% ^{3}H as intact folates over 48-72h but the group treated with phenytoin excreted considerably more intact folates over the same time period, 1.7% ^{14}C and 2.1% ^{3}H (Table 6.11.) Krumdieck *et al.*, (1978) reported that phenytoin raised the excretion of intact folates in a long term study using a human subject. Therefore it is highly likely that the increase in total radioactivity which was observed by Kelly *et al.*, (1979) was in fact due to the presence of intact folates not increased scission products.

Phenobarbitone, another compound known to induce microsomal enzyme activity (Conney, 1967; Latham *et al.*, 1973), has also been shown to have no effect on folate catabolism in the rat (Guest *et al.*, 1983; Saleh, 1981) or the mouse (Kelly *et al.*, 1979). Therefore, it is unlikely that folate catabolism *in vivo* is mediated via enzymic activity as two agents known to induce enzymes do not increase scission. The excess of ^{3}H over ^{14}C recovered in the urine of normal rats and humans is derived from three sources (Figure 6.6.). Part is due to the presence of ^{3}H-only labelled scission products, part to ^{3}H in association with pteridine fragments and part is due to the so called secondary isotope effect. This was initially discovered when experiments using

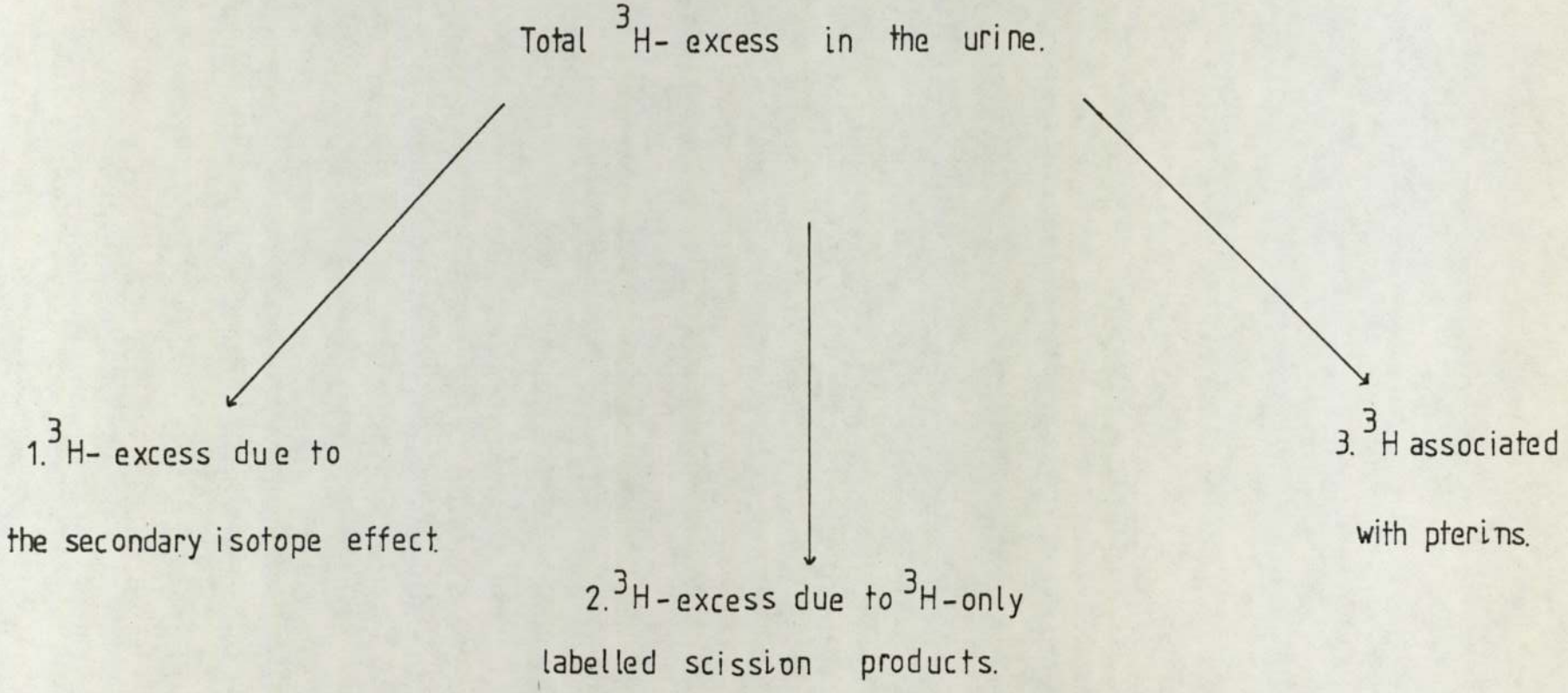


Figure 6.6. Composition of the urinary ^{3}H-excess.

isotopically labelled folates revealed that some isotopomers are biologically distinguishable from unlabelled molecules and from other isotopomers both in vitro and in vivo. There is a partial separation of ^{3}H- and ^{14}Cfolic acid on ion exchange chromatography (Figure 6.7.), the ^{3}H- containing species eluting slightly earlier than the ^{14}C- or unlabelled folic acid. Connor et al., (1980) reported that ^{3}H-labelled folic acid shows differences in biological handling from unlabelled folic acid due to the ^{3}H- substitution at the C-9 position despite the fact that the minimum perturbation that can be made in a molecule is the substitution of one iosotope for another. Folates excreted in the urine by man & the rat exhibit a higher ^{3}H: ^{14}C ratio than the administered folic acid (Table 6.13.) (Pheasant, 1981; Saleh, 1981).

Absorption experiments conducted in in vitro using everted gut sac preparations showed that [3',5',7,9-^{3}H]-folic acid was absorbed faster than [2-^{14}C]-folic acid and unlabelled folic acid (Connor et al., 1980). Folic acid absorption is believed to depend on the formation of an 'acid microclimate' at the surface of the gut, the electrically neutral form of folic acid formed within this region being readily absorbed (Blair & Matty, 1974). Such an effect may be due to changes in the electron density of the pterin ring caused by the reduced hyperconjugative effect of ^{3}H compared to ^{1}H (Forsyth et al., 1982). This would reduce the pka of N^{1} and hence increase the proportion of the uncharged species which being more lipid soluble is thought to be the form of folic acid that is absorbed. The changes in electron densities within the folate molecule may also account for the differing

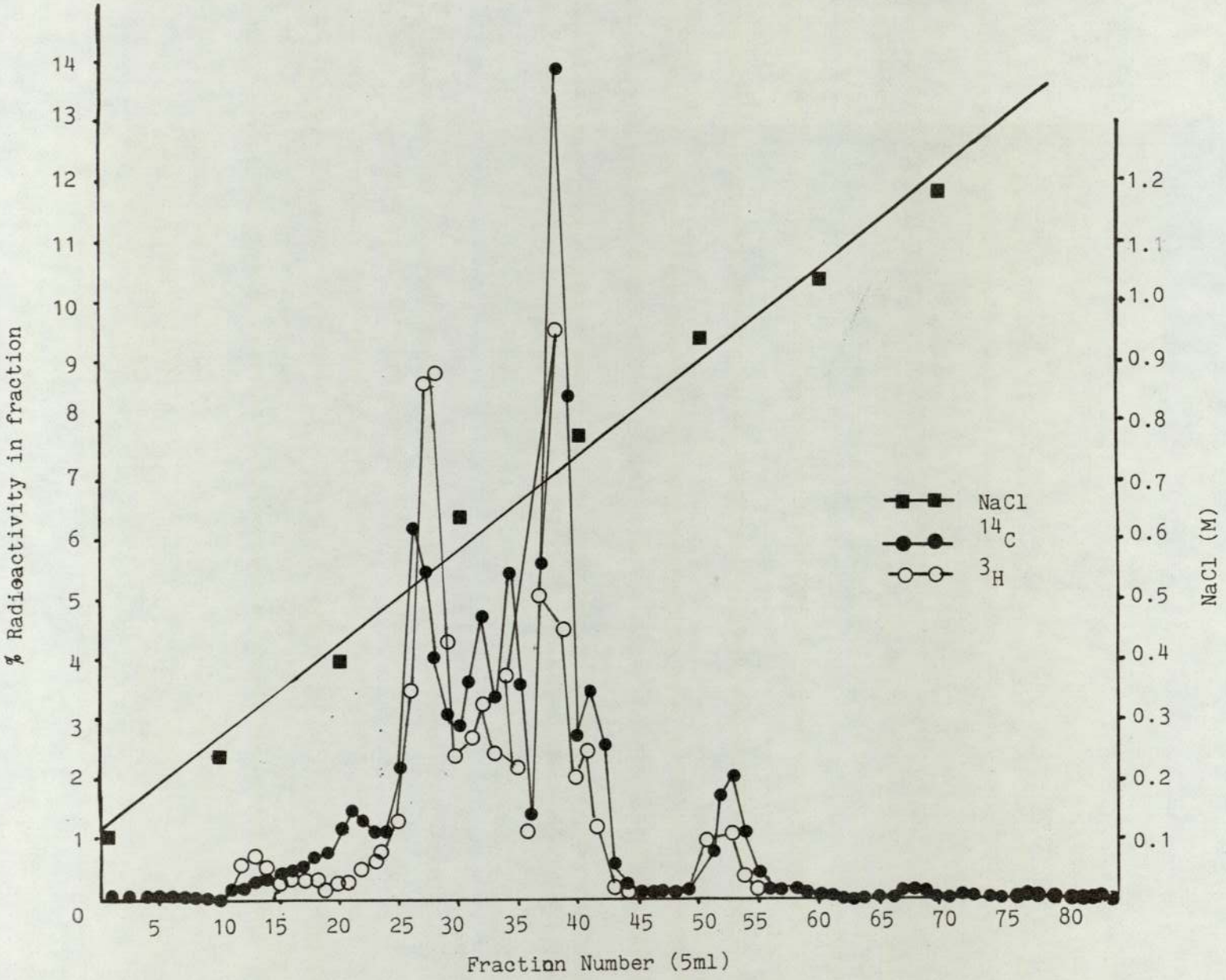


Figure 6.7: DE 52 ion exchange chromatography of normal urine (0 - 24h) from adult male rats dosed orally with (3',5',7,9-^{3}H) and (2-^{14}C)-folic acid (100μg/Kg body weight).

Table 6.13.

Folates recovered in the urine (0-24h) following the oral administration of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid to normal rats (100μmg/kg body weight).

Compound in urine	% Dose	
	% ^{14}C	% ^{3}H
Folic acid	1.5	1.8
5MeTHF	7.7	9.9
IOCHO-folate	6.7	10.1

biological activities of folic acid isotopomers observed in the binding to various enzymes (Whitburn et al., 1983).

The contribution of the secondary isotope effect to the total urinary ^{3}H excess may be calculated over 0-24h as 3.7% ^{3}H enrichment in the normal male rat. This is due to the preferential absorption of ^{3}H through the gut wall (Connor et al., 1980). However, the % ^{3}H enrichment found in the urinary folates excreted by the phenytoin treated rats is only 0.5%. This shows that there is little preferential absorption of ^{3}H and indicates that phenytoin is interfering with the absorption of folates across the intestine. The earlier observation that phenytoin reduces and retards the urinary excretion of radioactivity but increases faecal excretion also suggests that absorption is affected. These findings agree with Gerson et al., (1972) who found that phenytoin caused an inhibition of ^{3}H-folic acid absorption and Benn et al., (1971) who ascribed phenytoin-induced folate malabsorption to intraluminal alkalinisation as they found that oral bicarbonate could produce a similar reduction. However Perry and Chanarin (1972) and Doe et al., (1971) failed to support these observations and Gerson et al., (1972) claimed that the concentration of phenytoin found to reduce the absorption of ^{3}H-folic acid was too low to alter intraluminal pH. Lucas et al., (1978) and Blair et al., (1981) found that phenytoin altered neither intraluminal pH nor folate absorption in vitro.

Phenytoin is very alkaline in solution (pH 10-11) and the intestinal absorption of folates is highly pH dependent (Smith et al., 1970; Binder and Whiting, 1975; Russell et al., 1979; Rose et al., 1978).

Smith et al., (1970) found the absorption of ^{3}H-folic acid was maximal at a luminal pH of 6.0 and decreased by half at pH 5.0 or pH 7.0. Mackenzie and Russell (1976) found increasing intraluminal pH decreased folate absorption in humans (for review see Selhub et al., 1983). As stated earlier the absorption of folic acid is believed to depend on the formation of an "acid microclimate" at the surface of the gut, the electrically neutral form of folic acid formed within this region being absorbed (Blair and Matty, 1974). Gerson et al., (1970) and Hepner et al., (1970) found evidence to suggest that phenytoin inhibits intestinal ATP-ase activity. As ATP is essential for the production and maintenance of the intestinal surface acid microclimate (Said and Strum, 1983) which is very pH sensitive (Said, 1981), phenytoin may be producing conditions unfavourable to the absorption of folic acid from the proximal jejunum. The unabsorbed folic acid would then have to travel further down the gastrointestinal tract to regions where the surface acid microclimate does not extend and absorption is correspondingly less efficient. This would produce the observed effects of an increased amount of the dose excreted in the faeces and a prolonged period of absorption.

The most striking qualitative effect of phenytoin on folate metabolism was the excretion of the two new dual labelled compounds in the urine over 24-72h. As it was not possible to identify these compounds it is hard to assess their effect on the internal equilibrium of folate cofactors. It is not known if they possess biological activity but if they do not their production would be conducive to the development of megaloblastic anaemia. Tissue levels of folates were slightly

depressed by phenytoin treatment, especially the 48h liver levels, but the uptake of folate into the tissues did not seem to be greatly affected.

In establishing that phenytoin does not increase folate catabolism in the rat through enzyme induction it was discovered that it delays the absorption of folates across the intestinal wall by producing an unfavourable environment for absorption. The retained folates were therefore retained in the presence of the intestinal bacteria which are known to catabolise [2^{14}C] pteridines to $^{14}CO_2$ (Jenkins and Spector, 1976; Tsusue et al., 1979; Fukushima and Nixon, 1980; Bacher et al., 1980) are thought to be the main cause of degradation of unconjugated pteridines (Pheasant and Pearce, 1981). However, as the contribution of the intestinal bacteria to folate and pteridine catabolism in vivo is not fully known it was decided to investigate the role of these bacteria upon folate catabolism in the rat (Chapter 3).

The influence of intestinal bacteria upon folate metabolism in the rat was determined by treating a group of male rats with a mixture of antibiotics which are poorly absorbed from the gut (neomycin sulphate, tetracycline hydrochloride and bacitracin). The antibiotics were administered twice daily for two days before the radiolabelled folic acid and continued afterwards until the end of the experiment. This dosing regime sufficiently depresses both anaerobic and aerobic intestinal organisms to determine their influence in drug metabolism (Gingell et al., 1971).

Table 6.14. summarises the recovery of radioactivity from the urine and faeces and the distribution of tissue radioactivity in normal rats and rats treated with antibiotics. The ^{14}C recovered in the urine is very similar in each group but the urine from the antibiotic treated rats does not show the excess of ^{3}H over ^{14}C that occurs in the normal rat urine (25.3% ^{14}C, 40.9% ^{3}H in the normal rat urine compared to 24.4% and 25.8% in the antibiotic treated rat urine). The urine from the antibiotic treated rats over 72h contains less ^{3}H than the normal rat urine (25.8% ^{3}H as compared to 40.9% ^{3}H). The faecal radioactivity is increased by antibiotic treatment probably due to the removal of the intestinal micro-organisms known to degrade [2-^{14}C] pteridines to $^{14}CO_2$ (Fukushima and Nixon, 1980). The increase from 16.1% ^{14}C over 0-72h in the faeces from the normal rats to 31.5% ^{14}C shows that the loss through bacterial degradation is considerable. The higher ^{3}H content of the faeces from the antibiotic treated group suggests that the bacteria may also degrade ^{3}H containing moietics.

The results obtained from the group of rats treated with antibiotics indicate that the intestinal bacteria play a considerable role in the metabolism of radiolabelled folic acid in this species. When the total recovery of radioactivity from the urine, faeces and tissues is compared in groups of rats treated in various ways with that from the rats treated with the antibiotics it is clear that less of the dose is lost in the experiments using antibiotic treated rats. This is unlikely to be due to experimental error as earlier experiments conducted in the rat (Connor, 1979; Saleh, 1981) and guinea-pig (Choolun, 1982; Choolun et al., 1980) also show low recoveries in animals in which the intestinal bacteria are intact (Table 6.15.).

Table 6.14.

Radioactivity recovered in the urine, faeces and tissues of normal male rats and rats treated with antibiotics (neomycin sulphate 100 mg/Kg body weight; tetracycline hydrochloride 100 mg/Kg body weight and bacitracin 100 mg/Kg body weight twice daily for 2 days before and after) an oral dose of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid (100 g/Kg body weight). Figures are for 0 - 72h after dosing. Results are expressed as a percentage of the dose.

SAMPLE	NORMAL RATS		ANTIBIOTIC TREATED RATS	
	^{14}C	^{3}H	^{14}C	^{3}H
Urine	25.3	40.9	24.4	25.8
Faeces	16.7	8.1	31.5	25.0
Liver	17.9	20.6	28.8	30.1
Gut	1.9	2.4	3.2	3.3
Kidney	1.5	2.4	3.6	3.8

Table 6.15.

Total radioactivity recovered from the urine, faeces, gut, kidneys and liver in various experiments following the oral administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid to rats and guinea-pigs.

"Folic Acid Metabolism In . . . . "	Total Recovery of Radioactivity	
	^{14}C	^{3}H
Adult male rats	63.3	74.4
Adult male rats treated with phenytoin	71.8	78.6
Adult female rats*	63.9	68.8
Adult male rats treated with antibiotics	91.5	88.0
Saleh, 1981		
Adult male rats	75.2	67.7
Adult male rats bearing tumours	51.4	48.3
Adult male rats treated with Methotrexate	53.9	55.4
Adult male rats treated with Phenobarbitone	71.4	75.2
Choolun, et al., 1980*		
Adult male guinea pigs	38.5	33.4

* No figures available for gut and kidney radioactivity.

The absence of the urinary excess of ^{3}H over ^{14}C could be due to a decreased formation and/or excretion of ^{3}H-only labelled scission products, an increased excretion of ^{14}C-only labelled fragments or an interference with the absorption of ^{3}H-folic acid (see the section of the discussion concerned with phenytoin). The percentage ^{3}H enrichment of urinary folates due to the secondary isotope effect is 3.8% in normal male rat urine and 2.9% in the urine from the antibiotic treated rats. This slight decrease is unlikely to totally remove the urinary ^{3}H excess. Table 6.16. shows that the extent of polyglutamate breakdown is greatly reduced by antibiotic treatment and that the polyglutamate body burden is increased when compared to normal rats.

The sum of ^{3}H-only labelled scission products in the urine of the antibiotic treated rats is lower than in the normal rat urine (12.5% ^{3}H in normal rat urine as compared to 8.8% ^{3}H following antibiotic treatment). However the opposite is true of ^{14}C-only labelled scission products (4.6% ^{14}C in the normal rat urine as compared to 7.2% ^{14}C after antibiotic treatment). Therefore there is no urinary excess of ^{3}H after antibiotic treatment as the decrease in ^{3}H-only catabolites together with the increase in the ^{14}C-only catabolites cancels the usual excess out.

Table 6.17. summarises the metabolites present in the urine from each group of rats. Antibiotic treatment increased the excretion of unchanged folic acid but decreased that of all the reduced folate coenzymes. This suggests that the intestinal bacteria aid in the reduction of folic acid and therefore the incorporation of these folates into the reduced folate pool. The antibiotics were

Table 6.16.

Extent of polyglutamate breakdown following the oral administration of a mixture of (3',5',7,9-^{3}H) and (2-^{14}C) folic acid (100μg/Kg body weight) to normal male rats and male rats treated with antibiotics.

Group	% dose retained in the body after 24h Polyglutamate body burden	% dose excreted as p-AcBGlu, 24 - 48h	Extent of Breakdown (%)
a) Calculations based on total radioactivity retained in the body at 24h i.e. dose administered - (Total excreted in the urine and faeces over 0 - 24h).			
Normal males	61.5	3.3	5.4
Antibiotic treated	66.7	1.9	2.8
b) Calculations based on radioactivity found in the liver, kidney and gut at 24h.			
Normal males	25.9	3.3	12.7
Antibiotic treated	45.8	1.9	4.2
c) Calculations based on radioactivity in the liver at 24h.			
Normal males	18.6	3.3	17.7
Antibiotic treated	30.8	1.9	6.2

Table 6.17.

Metabolites detected 0 - 72h after the administration of a mixture of (2-^{14}C) and (3',5',7,9-^{3}H) folic acid to normal male rats and rats treated with a mixture of antibiotics (neomycin sulphate 100mg/Kg body weight; tetracycline hydrochoride 100mg/Kg body weight and bacitracin 100mg/Kg body weight) twice daily two days prior to and after receiving the radiolabelled dose. Figures are expressed as a percentage of the dose.

Metabolite	Normal Rats		Antibiotic Treated Rats	
	^{14}C	^{3}H	^{14}C	^{3}H
Folic Acid	1.5	1.8	3.4	3.6
5MeTHF	7.7	9.9	5.4	5.8
10CHO-Folate	6.7	10.1	6.5	5.5
5,10CH$_2$-THF	3.3	3.8	2.0	2.2
Metabolite X	2.4	3.1	1.1	1.3
p-AcBGlu	-	7.5	-	1.9
p-AcBA	-	2.8	-	2.4
New ^{3}H-only species			-	3.9
Pteridine derivatives	3.6	0.2	4.8	0.4
Urea	1.0	-	2.4	-
$^{3}H_2O$	-	2.2	-	0.6

administered over 4 days prior to the dose of folic acid. During this time as the bacterial population was progressively lowered by the antibiotics the microbial reduction of folic acid within the gut would be decreased. Therefore the levels of circulating reduced folates within the body would also have fallen causing a slight deficiency. Therefore when the bolus dose of folic acid (100μg/Kg body weight) was administered the absorbed, reduced folates were taken up by the tissues of the antibiotic treated rats more readily than by normal rat tissues. Table 6.14. shows that the levels of radioactivity in the gut and kidney are elevated slightly in the treated group and that the liver radioactivity is significantly higher.

The new ^{3}H-only labelled catabolite was detected in each urine sample up to 72h after the administration of the radiolabelled folic acid, but the levels were highest over 0-24h. For this reason it is unlikely to be derived from folate polyglutamates. It may be a scission product which is normally degraded by the intestinal bacteria or converted by them to p-ABA. The position at which it eluted from Sephadex G15 suggests that it might be p-acetamido hippuric acid. However it was not possible to verify this.

The reduction in folate catabolism following antibiotic treatment to minimise the metabolic interference of the intestinal bacteria highlighted the complexity of investigations carried out in vivo. The experiments reported here have shown that the oxidative cleavage of folates in vivo in the adult rat is not carried out enzymically, as phenytoin not only failed to increase catabolism but actually reduced

the excretion of scission products. This strengthened the view that chemical oxidation might be responsible for catabolism as the folates are all labile compounds known to oxidise readily. The experiments were conducted *in vitro* to allow the chemical oxidation of THFA to be studied in isolation from influences such as the intestinal bacteria (Chapter 5). Two methods were used. The first was quantitative as it followed the production of p-ABGlu a known scission product of THFA by means of the Bratton and Marshall colorimetric assay for diazotisable amines. The second, TLC was purely qualitative and this showed that the THFA was breaking down to pterin and p-ABGlu. The inclusion of the Xanthine Oxidase System, which generated superoxide anion from the convers ion of Xanthine to Uric Acid (Bannister and Hall, 1979), was designed to evaluate the effect of free radicals upon the oxidation rate of THFA. However, the speed of the oxidation reaction defeated this as complete breakdown occurred before measurements could be taken. Experiments recently conducted have confirmed that folate catabolism *in vitro* is accelerated by superoxide anion generated by Xanthine plus Xanthine Oxidase and that this oxidation is significantly reduced by superoxide dismutase (Blair *et al.*, 1984). In the hamster and rat folate catabolism is significantly increased after the administration of compounds such as allopurinol and dihydrorotate whose *in vivo* metabolism may increase *in vivo* superoxide anion formation (Blair *et al.*, 1984).

In vivo the superoxide anion would not be generated by the conversion of Xanthine to Uric Acid by Xanthine Oxidase (the experimental system used *in vitro*) but by the many enzymes known to produce O_2^- during their normal function e.g. the cytochrome oxidase system, alcohol

dehydrogenase, and the electron transport system (Pryor, 1976).

Thus, O_2^- may promote the oxidation of THFA to p-ABGlu and pteridine fragments which may then undergo either further catabolism or direct excretion. In a similar reaction the folate coenzyme 10CHO-THFA may be oxidised to the inactive 10CHO-folate (Figure 6.8.). As 10CHO-folate inhibits the enzyme DHFR the recirculation of DHFA or FA would also be reduced.

As superoxide anions are produced within the cell during normal metabolic processes the effect that they will have upon folate catabolism is likely to be extensive. The enzymes superoxide dismutase, catalase and peroxidase all act to scavenge the free radicals (Pryor, 1976; Bannister and Hill, 1979) in vivo thereby minimising their destructive potential. However, to what extent they protect against folate catabolism is yet to be assessed.

In the colorimetric assay it was noted that the presence of sodium ascorbate stopped the reaction proceeding. The details of the Bratton and Marshall Assay (1939) are described in Chapter 2 but the initial stage involve the acidification of the test solutions prior to the addition of sodium nitrite thus producing nitrous acid (Figure 6.9.).

Figure 6.9.

$$HNO_2 \xrightarrow{H+} H_2O^+ \text{———} N = O$$

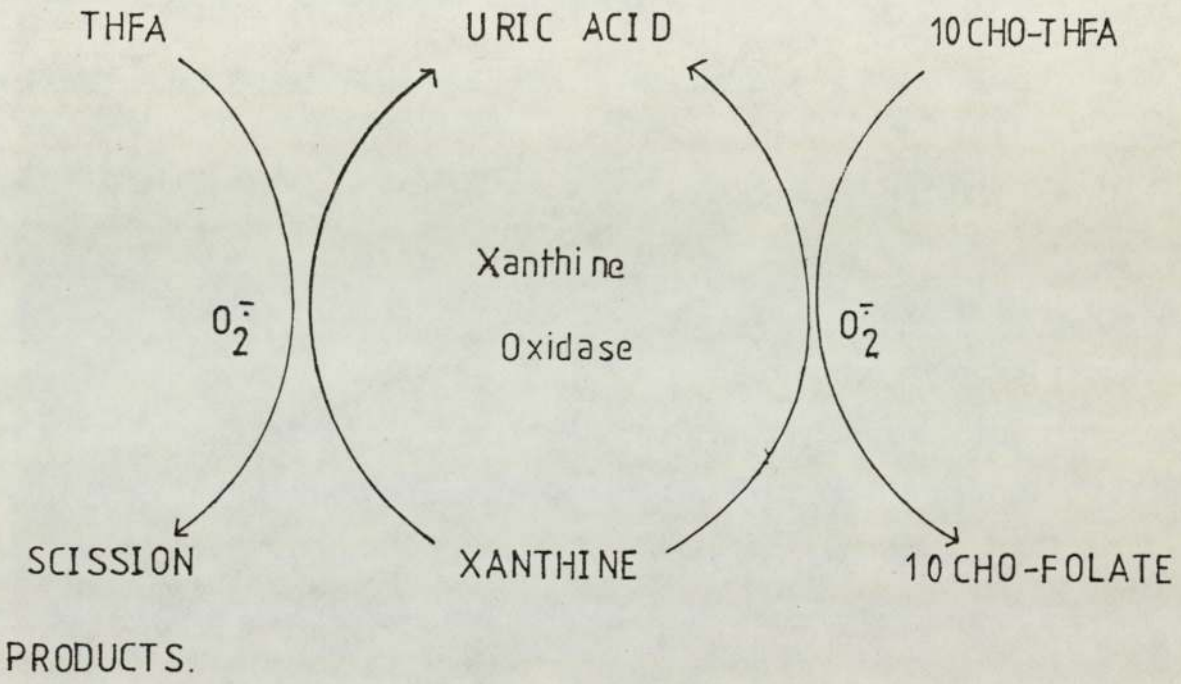


Figure 6.8. Role of superoxide anion in the oxidation of folates.

This acidification is an essential step in the assay. Dahn et al., (1960) and Mirvish (1975) have reported that ascorbate is oxidised by nitrous acid to dehydroascorbate with the concomitant production of water and nitrous oxide (Figure 6.10.).

Figure 6.10.

$$AH_2 + H_2O^+ - N = O \longrightarrow AH + H_2O + H^+ + N = O$$

Therefore the addition of ascorbate to the reaction mixture was stopping the assay by removing the nitrous acid. Dahn et al., (1960) showed that at slightly acid pH the rate of the acid catalysed reaction of ascorbate with nitrous acid is independent of reductone concentration and is very close to the rate found for the diazotization of amines.

THFA reacts with the nitrous acid in a similar manner to finally produce quinonoid dihydrofolate (q DHF) (Figure 6.11.).

Figure 6.11.

$$FH_4 + H_2O^+ - N = O \longrightarrow FH_3 + H_2O + H^+ + N = O$$

$$FH_3 + H_2O^+ - N = O \longrightarrow qFH_2 + H_2O + H^+ + N = O$$

The quinonoid DHF is unstable and will breakdown to p-ABGlu and pteridine fragments (Pearson, 1974). This therefore explains why virtually total oxidation of the THFA was found in all the reactions

carried out as the THFA was reacting with the nitrous acid produced during the Bratton and Marshall assay procedure.

Unfortunately it is unlikely that this acid catalysed breakdown of THFA is restricted to the test tube. For nitrous acid to be generated nitrite needs to be present in an acidic aqueous environment, such as the stomach. The intestine contains nitrate-reducing bacteria capable of converting nitrate to nitrite. There is considerable concern that the nitrite generated *in situ* in the human gut may be the precursor of various N-nitroso compounds many of which may be carcinogenic (Fine *et al.*, 1977; Mirvish *et al.*, 1972). However these bacteria may also be reducing nitrates present in foods (Tannenbaum *et al.*, 1978) and human saliva (Tannenbaum *et al.*, 1974; White, 1975) which contains about 10mg nitrate/L. The nitrous acid produced is therefore capable of reacting with THFA present in food and breaking it down and thereby consitutes a site of loss for dietary folates which has not previously been recognised.

Further Work.

The results presented in this thesis highlight a number of areas where further experimentation might yield information about the handling of folate coenzymes by the rat. The administration of [3',5',7,9-^{3}H] and [2-^{14}C] folic acid to immature females, females treated with either a natural oestrogen or an antioestrogen, for example Tamoxifen or a progesterone would help to clarify the influence of hormones upon folate metabolism. An extension of the experiment where mazola corn oil was administered to female rats would be to use another fat with a higher proportion of saturated fatty acids such as olive oil.

Nitrous acid formed from nitrite was found to oxidise THFA *in vitro* it would therefore be of interest to see whether nitrate reducing bacteria present in the intestine were significantly involved in folate catabolism especially as broad spectrum antibiotics have been shown to decrease scission and increase the total recovery of administered folates.

The suggestion that superoxide anion might be involved in the *in vivo* oxidation of folates presents many experimental possibilities including the induction or inhibition of superoxide dismutases *in vivo* or the application of more sensitive methods to evaluate their role *in vitro*. For example, the oxidation of adrenaline by superoxide anion is accompanied by a change in colour (Valerino and McCormack, 1971). Competition for the O_2^- *in vitro* by THFA could therefore be followed colorimetrically.

Although the induction of the microsomal oxidising enzymes by phenytoin decreased rather than increased folate catabolism this aspect could be examined further by looking at the oxidation of THFA by isolated P450 with or without prior treatment with phenytoin, phenobarbitone or SKF 525A an enzyme inhibitor.

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DIHYDROPTERIDINE REDUCTASE LEVELS IN HUMAN NORMAL AND NEOPLASTIC TISSUES.

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Introduction

The known role of dihydropteridine reductase is to reduce quinonoid dihydrobiopterin generated in certain biological oxidation reactions. Pollock & Kaufman (1) have suggested that dihydropteridine reductase may be involved in maintaining cellular tetrahydrofolate pools by reducing any quinonoid dihydrofolate produced either enzymically or by chemical oxidation. Saleh et al (2) looked at the effect of methotrexate on folate metabolism in normal animals and animals with tumours. They showed that folate catabolism was decreased in tumour-bearing animals, and increased in tumour-bearing animals dosed with methotrexate, and suggest that these findings could be achieved by inhibition of dihydropteridine reductase as well as inhibition of dihydrofolate reductase. Dhondt (3) has shown a positive correlation between dihydropteridine reductase and oestrogen receptors in human breast tumours. If dihydropteridine reductase is partially responsible for maintaining cellular reduced folate pools, then increased levels of dihydropteridine reductase could play an important role in the malignant process by increasing the levels of reduced folates available for synthesis of purines and pyrimidines. We have assayed normal and neoplastic human tissue, from the breast and from the large intestine, for dihydropteridine reductase activity. We also administered a synthetic oestrogen to rats, and report the results here.

Results

1) Assay of dihydropteridine reductase in human tissues. Tissue samples were obtained immediately after surgical removal of the tumour. For controls apparently normal tissue adjacent to the tumour was used. High speed supernatants of tissue 25% homogenates were prepared. Dihydropteridine reductase was assayed by the method of Cotton & Jennings (4). Protein was measured by the method of Lowry. The reaction was followed by monitoring the decrease in absorption at 340nm in a double beam recording spectrophotometer. In tissue samples prepared in this way there was considerable oxidation of NADH in the absence of pterin. Dihydropteridine reductase activity was taken as the difference between the rate in the presence of pterin and that in the absence of pterin. Control cuvettes contained no protein. The assay cuvettes contained 10^{-4}M NADH, 10^{-3}M H_2O_2, 8µg horseradish peroxidase, 2.5 x 10^{-4}M sodium azide, $\pm$ 10^{-4}M 6,7- dimethyltetrahydropterin in 0.05M tris buffer pH 7.6 and 0.02ml of the tissue fraction. In all cases reductase activity with NADPH was less than 4% of that with NADH. There is a significant increase in dihydropteridine reductase activity in neoplastic breast tissue as compared to apparently normal tissue from the same breast ($p < 0.002$ by Wilcoxson's signed ranks test), (See tables 1 and 2) but there is no significant increase in dihydropteridine reductase activity in neoplastic tissue from human large intestine as compared to apparently normal adjacent tissue. In all tissues there was no increase in non-specific oxidation of NADH in neoplastic tissue as compared to apparently normal tissue.

2. Effect of diethylstilboestrol on folate metabolism and dihydropteridine reductase activity in the rat. Female rats were pretreated with diethylstilboestrol (416mg/Kg) for 3 days. 24h after the last dose of diethylstilboestrol these animals, together with untreated animals, were given a single dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (100 µg/Kg body wt). Urine samples collected for the following 72h were analysed on DEAE cellulose and Sephadex G.15. A second group of animals were dosed with diethylstilboestrol then killed and liver and uteri were removed, supernatants prepared and assayed for dihydropteridine reductase as described above. The results are shown in tables 3 and 4. Pretreatment of normal female rats with diethylstilboestrol reduced folate catabolism and increased the levels of 10-formyltetrahydrofolate. Oestrogen treatment also raised dihydropteridine reductase activity in liver but not in the uterus.

Discussion

Dihydropteridine reductase activity is significantly raised in neoplastic breast tissue but not in neoplastic tissue from the large intestine. There is no rise in non-specific oxidation of NADH in neoplastic tissue from either source. There is apparently a specific rise in dihydropteridine reductase in neoplastic breast tissue. The decreased folate scission seen in rats treated with an oestrogen can be explained by raised levels of dihydropteridine reductase in the liver, since this enzyme most probably helps to maintain cellular tetrahydrofolate levels (2) and reverses the oxidative steps that could lead to scission of the folate molecule (5). Increased dihydropteridine reductase activity could contribute to the neoplastic process by increasing the availability of reduced folates for the biosynthesis of

Table 1. Dihydropteridine reductase activity in apparently normal and neoplastic tissue from human breast.

Patient	(Specific activity (η moles NADH used/mg protein/min)). apparently normal tissue	neoplastic tissue.
1. B.S.	24.4	25.2
2. B.L.	16.3	99.0
3. B.T.	13.3	19.8
4. J.H.	8.8	28.9
5. R.M.	15.7	27.2
6. H.E.	11.5	20.9
7. M.B.	26.7	151.2
8. D.W.	8.5	5.3
9. G.L.	16.3	127.0.
10. A.G.	17.6	39.3
11. D.P.	13.1	21.0
12. E.Y.	50.3	341.0
13. G.B.	0.8	16.6

Table 2. Dihydropteridine reductase activity in apparently normal and neoplastic tissue from human large intestine.

Patient	Source of tumour	(Specific activity (η moles NADH used/mg protein/ min)). Apparently normal tissue	tumour tissue
1. B.L.	Rectum	8.8	22.8
2. E.M.	Colon	24.4	7.1
3. C.J.	Colon	10.2	39.5
4. H.S.	Colon	16.7	9.6
5. J.C.	Caecum	45.6	30.0
6. A.H.	Colon	28.2	18.7
7. A.T.	Rectum	33.1	34.4

Table 3. Distribution of metabolites of radiolabelled folic acid in the urine of normal female rats, and normal female rats dosed with diethylstilboestrol. Number of animals in brackets.

Compound	Percentage of dose recovered in urine in 72h			
	Normal female Rats (5)		Female Rats +diethystil-boestrol.(5)	
	^{14}C	^{3}H	^{14}C	^{3}H
Folic acid	0.10	0.07	0.4	0.4
5-methyltetra-hydrofolate	22.0	16.1	4.6	5.0
10-formyltetra-hydrofolate	16.1	18.8	30.7	35.5
Total intact folates	38.2	35.0.	35.6	41.0
p-amino benzoate	-	3.6	-	0.5
p-aminobenzoyl-l-glutamate	-	8.8	-	1.5
pterin fragments	4.4	-	1.0	-

Table 4. Dihydropteridine reductase activity in normal rat livers, and livers from rats receiving diethylstilboestrol. Number of animals in brackets.

	Specific activity, mean ± S.D, (ηmoles NADH used/mg protein/min)
Normal rats (3)	108.2 ± 37.4
DES treated rats (3)	254 ± 68.5

purines and pyrimidines, so increasing the rate of cellular proliferation. The specific increase in dihydropteridine reductase in breast tumours, and its increase in oestrogen treated rats indicates that this enzyme may be responsive to oestrogens. This could explain the effectiveness of oestrogen antagonists in the treatment of oestrogen sensitive breast tumours.

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POSSIBLE MECHANISMS OF FOLATE CATABOLISM

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Introduction

The catabolism of folate to biologically inactive scission products is now well established and has been shown to occur in the rat, the guinea-pig and man (1-5). However the mechanism of this breakdown is, as yet, unresolved. Two pools of folate exist (short and long term) which break down in the lumen of the gut and in the tissues to give p-acetamidobenzoate (pAcBA) and p-acetamidobenzoyl-L-glutamate (pAcBG) respectively as the major urinary catabolites. Our present model of folate breakdown has been set out in detail elsewhere (1).

Several investigators have suggested that the catabolic route of folate is via cleavage of the C9-N10 bond. There are a number of possible ways this could be brought about.

(1) Breakdown in the gut may be carried out by the intestinal microflora.

(2) Enzyme catalysed cleavage of the C9-N10 bond may occur in the gut and other tissues.

(3) Chemical oxidative scission of the C9-N10 bond of a labile reduced folate could also explain the observed catabolites.

We have investigated these possibilities by a number of approaches. The extent of folate catabolism in different groups of animals was estimated by monitoring the urinary excretion of the two major tritiated catabolites (pAcBA and pAcBG) following an oral dose of [2-^{14}C] + [3',5',7,9-^{3}H] folic acid over a period of 2 days. (For details of methods see references (1) and (2).

Effect of antibiotics

A group of 15 rats was pretreated with a mixture of antibiotics (neomycin sulphate, 100 mg ; tetracycline HCl, 50 mg ; bacitracin,50 mg) twice daily for 2 days before administration of radiolabelled folic acid and treatment was continued throughout the experiment, Antibiotic treated rats excreted less pAcBA than normal animals (2.1% ^{3}H of the dose compared to 2.8%) thus suggesting that the intestinal microflora are at least partly responsible for the breakdown of the short term folate pool.

Effect of enzyme inducers

In an attempt to determine whether the microsomal oxidative enzymes are involved in folate catabolism, the effects of phenobarbitone and phenytoin were studied. Groups of rats were pretreaed with phenobarbitone (20 mg/day, i.p) for 3 days or with phenytoin (100 mg/kg body weight/day, i.p.) for 10 days prior to administration of radiolabelled folic acid. Phenobarbitone treatment caused a transient increase in the excretion of ^{3}H-catabolites, both pAcBA and pAcBG excretion being raised in the first 24h but falling back to normal values on the second day (Table 1). In view of the long acting enzyme inducing effects of phenobarbitone, this suggests that the induction of the microsomal enzymes has little or no effect on folate catabolism.

Phenytoin treatment caused a fall in the excretion of ^{3}H-catabolites (pAcBA + pAcBG) (Table 1) again offering no support for microsomal enzymic mechanism of folate catabolism.

TABLE 1 Excretion of ^{3}H-catabolites following the administration of (2-^{14}C) + (3',5',7,9-^{3}H) folic acid (100 ug/kg body weight).

	Excretion of ^{3}H-catabolites (% dose)	
Normal rats :	0 - 24h	24-48h
pAcBA	2.75	0.56
pAcBG	4.0	1.2
Phenobarbitone-treated rats :		
pAcBA	4.4	0.8
pAcBG	5.2	1.1
Phenytoin-treated rats :		
*pAcBA)) pAcBA)	3.1	<1.9

* The two catabolities could not be separately quantified.

Effect of the redox state of the cell

Since evidence of a folate cleaving enzyme is lacking at present, spontaneous chemical scission of the folate molecule seems the simplest and most probable mechanism of tissue folate breakdown bearing in mind the extreme lability of the reduced folate coenzymes. The folate derivative which undergoes scission has not yet been identified but tetrahydrofolate and dihydrofolate polyglutamates are the most likely candidates due to their inherent chemical instability. If catabolism is the result of oxidative breakdown, changes in the redox potential of the tissues could alter the rate of folate degradation. A comparison of the species studies shows that tissue folate catabolism increases with the hepatic cytosolic NAD/NADH ratio (Table 2) thus lending support to the hypothesis of breakdown by chemical oxidation.

TABLE 2 The extent of tissue folate breakdown in different species

Species	NAD/NADH RATIO	extent of folate breakdown*
Man	1871	2.0
Rat	725	0.9
Guinea-pig	293	0.2

* expressed as % of retained ^{3}H-radioactivity excreted as pAcBG

Effect of a tumour and of methotrexate

The presence of a tumour in both man (3) and the rat (2) reduces folate catabolism to approximately half its normal level possibly by stabilisation of the labile reduced

folates by the more reducing conditions known to exist in tumour cells (see reference 2).

Methotrexate treatment of normal rats leads to a 4 fold increase in folate breakdown (2). The catabolites excreted suggests that both dihydrofolate and tetrahydrofolate polyglutamates are breaking down probably due to the inhibition by methotrexate of dihydrofolate reductase (DHFR) and dihydropteridine reductase (DHPR) respectively This supports the hypothesis that DHPR acts in the maintenance of the reduced folates (6). DHPR activity has been found to be increased in human breast tumour tissue compared to normal breast tissue (7)

Thus the evidence at present supports chemical oxidation of dihydrofolate and tetrahydrofolate derivatives as the mechanism of catabolism of tissue folates. This oxidation is usually kept to a low level by DHFR and DHPR. The gut micoflora may also increase breakdown of folate monoglutamates following biliary excretion.

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EFFECTS OF PHENOBARBITONE AND PHENYTOIN ON FOLATE CATABOLISM IN THE RAT

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Abstract—The effects of phenobarbitone and phenytoin on the catabolism of oral [2-^{14}C] and [3′,5′,7,9-^{3}H] folic acid were investigated. Normal rats were found to excrete an excess of ^{3}H-labelled compounds into the urine and ^{14}C-labelled compounds into the faeces. Phenytoin abolished this urinary ^{3}H imbalance and also delayed and prolonged the overall excretion of radioactive material. Phenobarbitone appeared to increase the amounts of urinary scission products in the first 24 hr but over the 0–72 hr period both anticonvulsants decreased folate polyglutamate catabolism.

As the anticonvulsants used in these experiments decreased folate catabolism in the rat it is unlikely that the megaloblastic anaemia caused by chronic anticonvulsant therapy is due to induction of the enzymes responsible for folate breakdown *in vivo*.

Folate metabolism in man and the rat has been extensively studied and considerable catabolism has been shown to occur [1–3]. This catabolism is subject to alteration by several factors including malignancy [4]. Pheasant *et al.* [3] proposed a scheme to describe the physiological distribution of oral folic acid. Reduced folates are absorbed to form part of a folate monoglutamate pool. They are then either excreted in the bile to undergo enterohepatic circulation or are taken into the tissues where they are converted to polyglutamate derivatives. These latter compounds are eventually degraded to *p*-aminobenzoyl-L-glutamate (*p*-ABG) and various pteridines via scission of the C9-N10 bond [5] and excreted into the urine after acetylation to *p*-acetamidobenzoyl-L-glutamate (*p*-AcBG). Folates excreted into the bile may also undergo C9–N10 scission. The resulting pteridines are poorly reabsorbed from the gastro-intestinal tract and are excreted mainly in the faeces, although some may be further metabolised by the gut microflora [6]. The glutamate moiety is removed forming *p*-aminobenzoic acid (*p*-ABA) which is acetylated during reabsorption and then excreted in the urine as *p*-acetamidobenzoic acid (*p*-AcBA).

The actual mechanisms of scission are not known. Oxidative cleavage of the C9–N10 bond may be achieved by a chemical process or via catalysis by the microsomal mixed function oxidases.

Phenobarbitone and phenytoin are known to induce these enzymes in the rat [7], and are also widely used as anticonvulsant drugs to treat epilepsy [8]. Reynolds [9] noted that the drug-induced megaloblastic anaemia occurring in epileptics is usually associated with phenytoin therapy, either alone or in combination with phenobarbitone. In all cases this anaemia responds well to folic acid therapy. It was therefore decided to further investigate the effects of phenytoin and phenobarbitone on folate catabolism in the rat.

MATERIALS AND METHODS

Three groups of healthy male WAB/Not rats (180–280 g body weight) supplied by Dr M. Pymm (University of Nottingham) were used.

(A) Fifteen rats were dosed orally with a mixture of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight).

(B) Fourteen rats were dosed intraperitoneally with phenobarbitone sodium (80 mg/kg body weight) at 24 hr-intervals for 3 days. On the 4th day they received oral radiolabelled folic acid as per group A.

(C) Fifteen rats were dosed intraperitoneally with phenytoin (100 mg/kg body weight) for 10 days prior to oral dosing with radiolabelled folic acid as per group A. Treatment with phenytoin was then continued until the end of the experiment.

After the radioactive dose the animals were housed in metabolism cages (Jencons Metabowls, Jencons (Scientific) Ltd.) designed to allow the separate collection of urine and faeces. Samples were collected for periods of 0–24 hr, 24–48 hr for group B, and for a further 24 hr for groups A and C. Urine was collected into 0.05 M sodium phosphate buffer, pH 7.0, containing (2% w/v) sodium ascorbate and protected from light. At the end of each collection period 5 rats from each group were killed by cervical dislocation and the gut, liver and kidney removed to determine the distribution of retained radioactivity. All samples were stored at −15°C in the presence of sodium ascorbate until analysed.

Measurement of radioactivity. The radioactivity retained in the tissues and faeces was determined after freeze drying using a Beckman biological material oxidiser (10). Urine and column effluents were counted using a Beckman LS7500 liquid scintillation counter.

Column chromatography. DE52-cellulose chromatography and Sephadex G15 gel filtration

were performed as described by Barford *et al.* [11]

Extraction of retained folates. Livers were removed, quickly chilled and washed in ice-cold buffer. Retained folates were extracted into 4 volumes of boiling 0.05 M-sodium phosphate buffer, pH 7.0, containing sodium ascorbate (2% w/v). After boiling for 10 min the extract was cooled, homogenised, centrifuged to remove preciptated protein and the supernatant retained.

Chemicals. (2-^{14}C) Folic acid (specific activity 58.2 mCi/mmol) (96% radiochemically pure) and (3′,5′,7,9-^{3}H) folic acid (specific activity 500 mCi/mmol) (95% radiochemically pure) were obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). The distribution of the tritium label was found to be 42.5% at the 3′ and 5′ positions on the benzene rine, 32% at position 9 and 25.5% at the 7 position by tritium nuclear magnetic resonance spectroscopy (Radiochemical Centre batch analysis sheet H/2392) (Fig. 1). Compounds for calibration purposes were

*Denotes position of ^{3}H, T denotes ^{14}C

..... shows the site of C9 − N10 scission

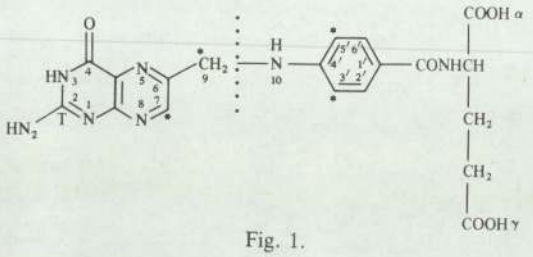

Fig. 1.

obtained as follows: folic acid was purchased from Koch Light (Colnbrook, Bucks); *p*-aminobenzoic acid, *p*-aminobenzoyl-L-glutamic acid, phenytoin sodium and phenobarbitone sodium from Sigma Chemical Company Limited (Kingston-upon-Thames) and *p*-acetamidobenzoic acid from Aldrich Chemical Company (Middlesex). 10-Formylfolate was prepared by the method of Blakley [12] and *p*-acetamidobenzoyl-L-glutamate by the method of Baker *et al.* [13]

RESULTS

The excretion of radioactivity into the urine and faeces of each group of rats is shown in Table 1. The normal animals excreted large amounts of radioactivity into the urine and faeces over the first 24 hr after which excretion rates were far lower. The excretion of the two isotopes was unbalanced in each sample with an excess of ^{3}H appearing in the urine and of ^{14}C in the faeces. However, if excretion is summed over 0–24 hr then the total isotope excretion figures are very close at 38.7% ^{14}C and 38.5% ^{3}H. The rats pretreated with phenobarbitone showed qualitatively the same pattern seen in normal rats. However, the 0–24 hr urinary excretion figures were greater than normal ($p = 0.01$), whereas the 0–24 hr faecal radioactivity was lower than normal ($p = 0.05$). Phenytoin pretreatment abolished the urinary ^{3}H excess. Significantly less ^{3}H appeared in both the 0–24 hr period ($p = 0.05$) and over the total 0–72 hr period ($p = 0.05$). The 48–72 hr urinary ^{3}H radioactivity, however, was significantly higher than normal. Greater amounts of ^{14}C also appeared in later urine samples. This suggests a delayed and prolonged excretion of radioactive material into the urine. Faecal radioactivity was greater in each time period for both isotopes ($p = 0.01$) and the imbalance between ^{14}C and ^{3}H levels was not as great as that found in normal rats.

Table 2 shows the tissue distribution of radioactivity together with overall recovery figures. No major differences are apparent except that the radioactive content of the liver appears lower in the animals pretreated with phenytoin ($p = 0.05$) and slightly higher in the phenobarbitone pretreated rats. This latter elevation is not statistically significant. Sequential chromatography of urine samples on DE52-cellulose and Sephadex G 15 columns allowed the identification of a number of metabolites listed in Table 3 (described in greater detail elsewhere [3]). Both groups of rats treated with anticonvulsants excreted more unchanged folic acid than normal rats. The two major urinary metabolites in all three groups were 5-methyltetrahydrofolate (5-MeTHF) and 10-formyl-tetrahydrofolate (10-CHOTHF). The

Table 1. Radioactivity recovered in the urine and faeces after the administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Samples	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine						
0–24 hr	23.4 (2.3)	31.8 (3.2)	30.3 (2.6)	37.5 (1.6)	19.4 (2.7)	20.5 (3.0)
24–48 hr	1.6 (0.3)	8.1 (2.3)	1.9 (0.1)	2.9 (0.2)	4.7 (1.3)	5.4 (1.6)
48–72 hr	0.3 (0.1)	1.0 (0.3)	n.d.	n.d.	3.2 (1.3)	4.7 (1.8)
Faeces						
0–24 hr	15.3 (4.3)	6.7 (2.1)	6.9 (2.3)	4.6 (1.5)	22.4 (2.5)	13.6 (1.6)
24–48 hr	1.3 (0.4)	0.8 (0.2)	1.8 (0.9)	1.7 (0.9)	5.5 (0.78)	4.6 (0.6)
48–72 hr	0.14 (0.04)	0.6 (0.2)	n.d.	n.d.	4.8 (0.95)	4.1 (1.0)
Total 0–48 hr	41.6	47.4	40.9	46.7	52.0	44.1
Total 0–72 hr	42.0	49.0	n.d.	n.d.	60.0	52.9

Results are expressed as a percentage of the dose (± S.E.M.) n.d. = Not determined.

Table 2. Recovery of radioactivity from urine and faeces up to 48 hr and in the tissues at 48 hr after administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid to rats (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Sample	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Urine	25.0	39.9	32.2	40.4	24.1	25.9
Faeces	16.6	7.5	8.7	6.3	27.9	18.2
Liver	19.1 (1.8)	21.6 (2.3)	25.6 (1.5)	23.6 (1.4)	11.8 (1.4)	11.9 (1.9)
Kidney	1.6 (0.2)	2.0 (0.5)	2.1 (0.1)	2.1 (0.1)	1.6 (0.3)	1.1 (0.2)
Small intestine + colon	2.3 (0.3)	2.5 (0.2)	2.8 (0.1)	2.8 (0.1)	2.1 (0.3)	2.1 (0.2)
Total	64.6	73.5	71.4	75.2	67.5	58.2

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

phenobarbitone pretreated rats excreted the highest levels of these and the phenytoin treated the lowest. 5,10-Methylenetetrahydrofolate (5,10-CH_2THF) was present in similar amounts in the urine of normal and phenobarbitone treated rats but was undetectable in the urine of the phenytoin treated animals. However, two unidentified species were present in the 24–72 hr urine of these rats. The peaks eluted separately from DE52-cellulose at a molarity of 0.8–0.85 M and from Sephadex G15 at fraction number 33 as a single peak. Increased amounts of C9–N10 scission products (^{14}C-only and ^{3}H-only labelled compounds; ^{3}H at C9 is lost in scission process [4] and ^{3}H at C7 is lost in subsequent hydroxylation) were found in the phenobarbitone pretreated rats whereas the opposite occurred in the phenytoin treated animals. Sephadex G15 gel filtration of liver homogenates from each group showed the major radioactive species present to be folate polyglutamate.

DISCUSSION

Theories put forward to explain anticonvulsant-induced anaemia include an impairment of absorption of folates from the gastrointestinal tract [14,15]; the induction of enzymes which require folate coenzymes [16] and the induction of enzymes responsible for the breakdown of the folate molecule via C9–N10 scission [17].

In this paper we have compared the metabolism of an oral dose of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid in normal rats and in rats pretreated with two compounds known to induce hepatic mixed function oxidases. If degradation of the folate molecule through oxidative cleavage of the C9–N10 bond is catalysed by these microsomal enzymes then we would expect to see a greater proportion of the urinary metabolites present as scission products (Fig. 1). However, the phenytoin and phenobarbitone treated rats excreted more unchanged folic acid than the normal group and similar amounts of 5-MeTHF and 10-CHOTHF.

The phenobarbitone treated group does appear to excrete more ^{3}H-only labelled fragments, 11.5% of the dose as opposed to the normal value of 7.6% but his increase was restricted to the first 24 hr and returned to normal on the second day. In contrast the phenytoin treated group excrete less (5.0%). These figures may only reflect changes in the short term turnover of folates within the rat. A better estimate of the extent of tissue folate catabolism may be obtained using the formula

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as } p\text{-AcBG 24–48 hr}}{\%\ \text{Radioactivity retained at 24 hr}} \times 100\%.$$

The distribution of the ^{3}H label throughout the folate molecule is such that C9–N10 cleavage leaves 42.5% of the label in the glutamyl containing fragment. Appropriate correction must therefore be made for this. This allows an estimation to be made of the breakdown product of tissue folate polyglutamates as a percentage of the total body burden of polyglutamate. There are a number of ways of assessing the

Table 3. Metabolites present in the urine 0–48 hr following the administration of (2-^{14}C) and (3′,5′,7,9-^{3}H) folic acid (100 μg/kg body weight)

	Normal rats		Phenobarbitone treated		Phenytoin treated	
Metabolites	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Folic acid	1.5	1.8	3.4	3.8	5.4	3.7
5MeTHF	7.6	9.8	10.5	11.1	6.2	4.2
10CHOTHF	6.7	10.1	8.0	8.8	5.1	5.4
5,10-CH_2THF	3.3	3.8	2.5	2.6	0	0
Novel Folates	0	0	0	0	1.6	1.6
^{14}C-only fragments	4.3	0	6.7	0	3.2	0
^{3}H-only fragments	0	9.52	0	11.5	0	5.0
$^{3}H_2O$	0	2.2	0	0.6	0	0.8

The results are expressed as the percentage of the dose given.

Table 4. Excretion of the catabolites of folate polyglutamates

Group	% ^{3}H of the dose retained in the body after 24 hr	% ^{3}H dose excreted as *p*-AcBG at 24–48 hr	Extent of breakdown (%)
(a) Calculations based on total radioactivity retained in the body at 24 hr i.e. Dose administered − (Total excreted in urine and faeces over 0–24 hr)			
Normal	61.5	3.3	5.4
Phenobarbitone	57.9	2.58	4.5
Phenytoin	65.9	1.7	2.6
(b) Calculations based on radioactivity found in the liver, kidney and gut at 24 hr			
Normal	25.9	3.3	12.7
Phenobarbitone	27.0	2.58	9.6
Phenytoin	24.0	1.7	7.1
(c) Calculations based on radioactivity in the liver at 24 hr			
Normal	18.6	3.3	17.7
Phenobarbitone	21.4	2.58	12.1
Phenytoin	20.3	1.7	8.4

The results are given as the percentage of tissue radioactivity excreted as pAcBG where

$$\text{Extent of breakdown} = \frac{\%\ \text{Dose excreted as } p\text{-AcBG 24–48 hr}}{\%\ \text{Radioactivity retained at 24 hr}} \times 100\%.$$

polyglutamate burden, as shown in Table 4. If the assumption is made that there is no loss of labelled folate polyglutamate from the body over 24 hr except through the urine and faeces and that the remainder of the dose is totally converted to polyglutamates then the extent of breakdown is shown in Table 4(a). As this assumption exaggerates the total body folate the catabolism may also be calculated using the radioactivity retained in the gut, liver and kidney at 24 hr (Table 4b) or the liver only at 24 hr (Table 4c).

Table 4 shows that no matter how the calculation is done the rats pretreated with the two anticonvulsants show a lower breakdown of polyglutamates than the normal animals. Phenytoin produces a greater decrease in breakdown than phenobarbitone and this trend is found in all three types of calculation.

Krumdieck *et al.* [18] and Kelly *et al.* [17] also investigated the effects of anticonvulsant therapy on folate metabolism. In a long term study [18] it was found that in man phenytoin accelerated the urinary loss of newly absorbed folate but did not increase the rate of catabolism. Although these results agree with those presented here, Kelly *et al.* [17] found that in the mouse phenytoin increased scission but phenobarbitone had no effect. This apparent discrepancy may be an example of species variation in the handling of folates or may be due to differing experimental procedures.

In conclusion, the induction of the hepatic microsomal enzyme system does not lead to an increase in folate catabolism. Both phenobarbitone and phenytoin did influence folate handling by the rat but as their actions were different the changes seem to be the result of some intrinsic property of the drugs themselves as opposed to an effect on hepatic mixed function oxidases. The apparent delay in absorption and increased loss of folates in the urine of phenytoin treated animals may offer a partial explanation for the folate deficiency sometimes associated with phenytoin therapy.

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