THE METABOLISM OF 5 METHYLTETRAHYDROFOLIC ACID AND RELATED COMPOUNDS IN THE RAT

BY

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D E C L A R A T I O N

This thesis describes work done at the Chemistry Department, University of Aston from October 1976 to December 1979. The work was done independently and has not been submitted for any other Degree.

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A C K N O W L E D G E M E N T S

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SUMMARY

The metabolism of 5Methyltetrahydrofolic acid (5MeTHF) and its oxidation products were investigated in the rat using (^{14}C methyl) and mixed label (2^{14}C)(3',5',7,9,^{3}H) species.

The 5(^{14}C)MeTHF preparation is a racemic mixture of the C_6 epimers, however, they are absorbed from the gut without discrimination. The methyl group is removed from one active epimer forming various metabolites having the same chromatographic behaviour as those produced from (^{14}Cmethyl) methionine. No evidence was observed for the oxidation of 5MeTHF methyl to -CH_2- or the formation of 5(^{14}C)MeTHF polyglutamate types.

Mixed label (2^{14}C)(3',5',7,9,^{3}H)5MeTHF is metabolised to various compounds including the formyl folates and polyglutamates.

The compound is cleaved to produce urinary ^{3}H labelled scission products, which demonstrates that scission reactions previously observed with folic acid are a function of normal folate metabolism.

5MeTHF was oxidised to produce its triazine oxidation product and 5Methyl 5,6, dihydrofolate (5Me 5,6,DHF). The triazine is biologically inactive, not being metabolised to any other compounds by the rat. 5Me 5,6,DHF is metabolised in a similar manner to 5MeTHF after I.P. dosage, however, only scission metabolites are observed in urine after oral dosing. In vitro acidification was observed to result in several products from 5 Me 5,6,DHF, including p.aminobenzoyl-glutamate. These oxidation products are unlikely to contribute to available food folate.

In rats bearing the Walker 256 carcinosarcoma the production of non-folate compounds from 5(^{14}C)MeTHF is increased, possibly due to the increased mass of the tumour, or because of this tumour's specific demand for methionine. There is less scission of mixed label 5MeTHF and diminished total excretion of radioactivity. The scission products differ from the normal.

The normal metabolism of 5 MeTHF is discussed with reference to the 'methyl trap hypothesis', the role of vitamin C, and in comparison with prior studies using folic acid. The 5MeTHF metabolism of the tumour bearing rat is contrasted with the normal.

Key terms: folate metabolism and catabolism
folate oxidation
methyl metabolism
tumour bearing rats

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1.1 Folate Metabolism

The folates are a ubiquitous group of pterin based coenzymes involved in the intermediate metabolism of one-carbon moeities (reviewed by Blakley 1969, Rowe 1978). The naturally occuring folate coenzymes are derivatives of tetrahydrofolic acid (fig. 1.1) with one-carbon substituents on or between the N^5 and N^{10} atoms which are interconverted and donated during the course of folate-catalysed reactions. Mammals, including man, are unable to synthesise these compounds de novo, and thus they are required as vitamins, (Rodriguez 1978). In therapeutic supplements folate is usually supplied as folic acid, an oxidised synthetic derivative of the naturally occuring folates (Fig 1.2).

One carbon units enter the folate pool as the C_2 atom of serine, which is ultimatelyderived from carbohydrate and quantitatively the most important source (Blakley 1969 p289, Newman et al 1974). The C_3 atom of histidine and formate are also sources (Blakley 1969 p. 291). These one carbon units are consumed in reactions producing the C_2 and C_8 atoms of the purine ring, the methyl groups of deoxythymidylate and methionine, and CO_2 with recycling of THF (fig. 1.3).

In fig 1.3 the coenzyme donating the C_8 atom of purines is given as 5, 10,methenyl THF . and shown in more detail in fig 1.4.

However, Dev and Harvey (1978a) have shown that this reaction in E.coli requires 10CHO THF rather than 5,10CH=THF

Fig.1.1. 5,6,7,8,Tetrahydrofolic acid (THF)

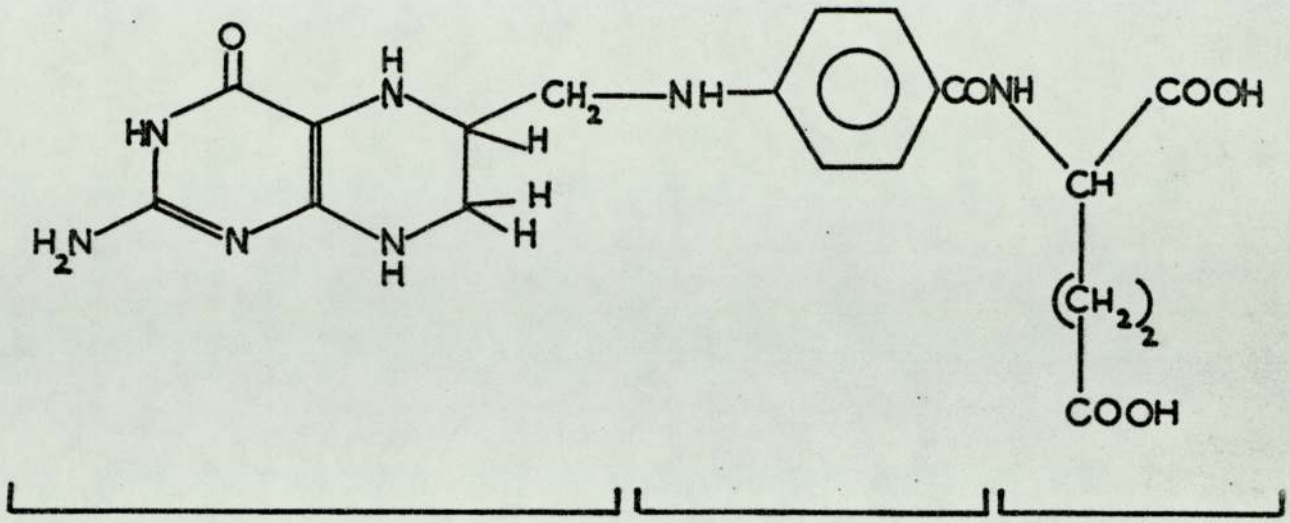


Fig.1.2 Folic acid (FA)

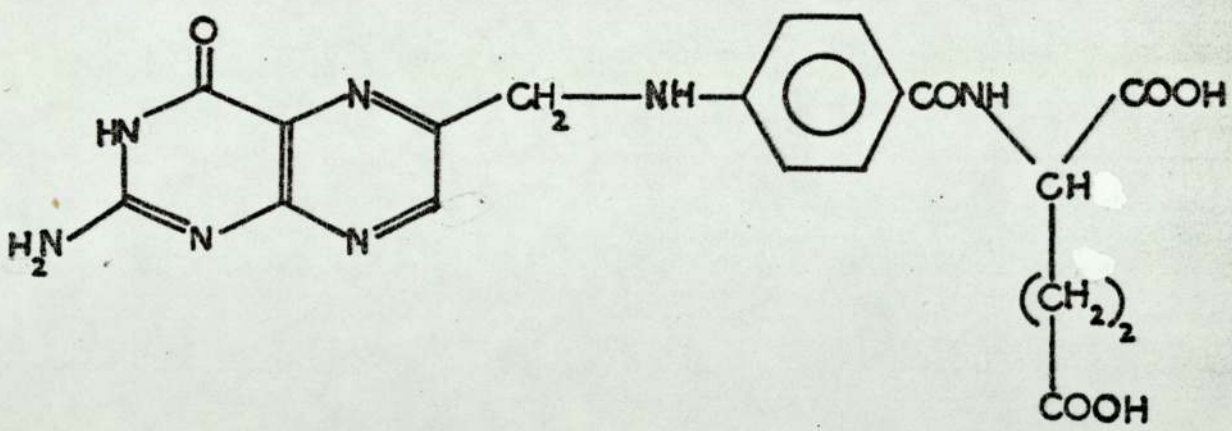


Fig.1.3 Metabolic Reactions of Folates (Key to enzymes overleaf -)

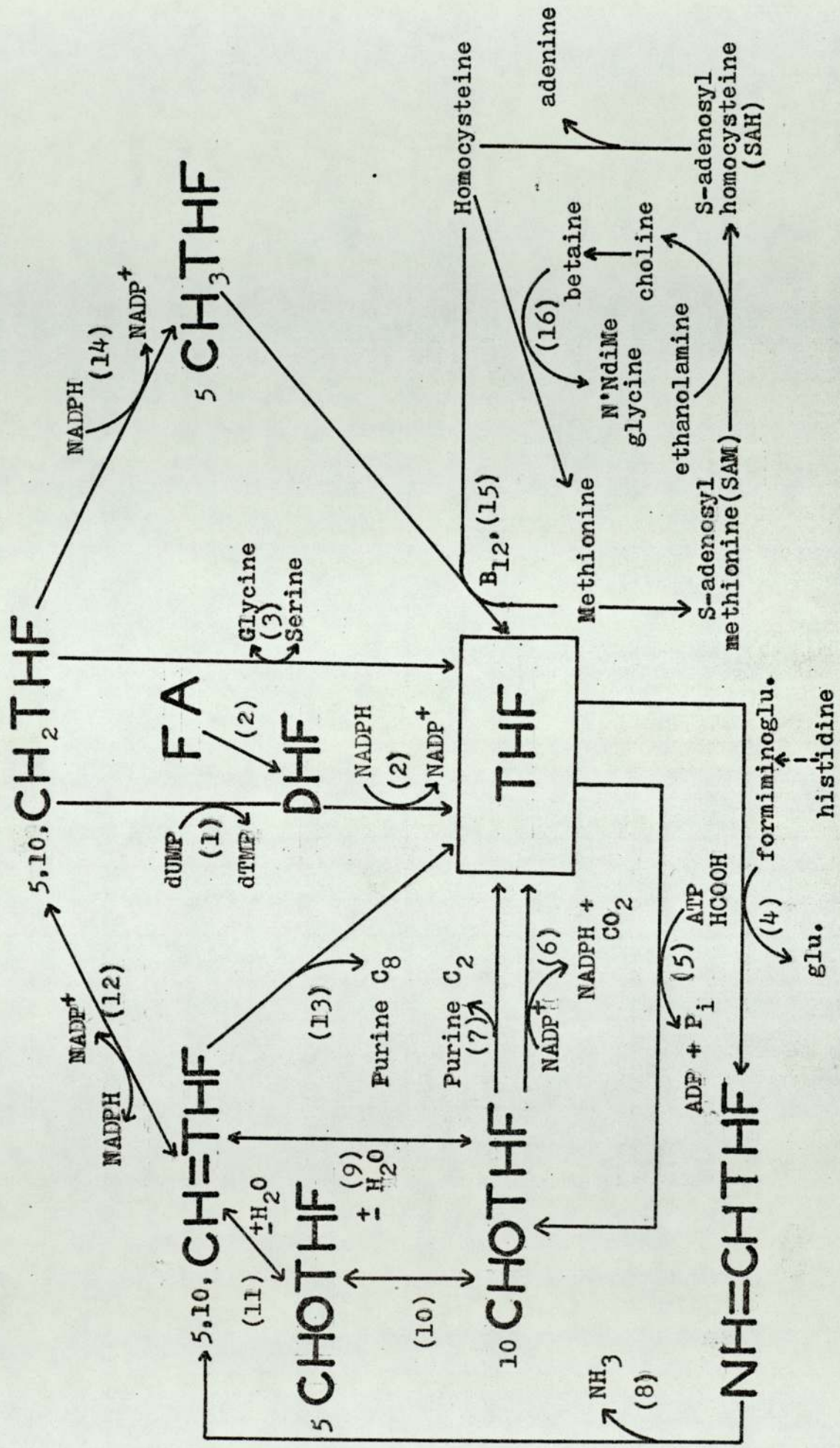


Fig.1.3. Key : Enzymes of Folate and One-Carbon Reactions

Enzyme (1) Thymidylate Synthetase - 5,10,Methylene THF : dUMP C Methyltransferase EC 2.1.1.6

(2) Dihydrofolate Reductase - 5,6,7,8,THF : NAD(P) Oxidoreductase EC 1.5.1.3

(3) Serine Transhydroxymethylase - 5,10,Methylene THF : Glycine Hydroxymethyltransferase EC 2.1.2.1

(4) Formiminotransferase - N-formimino-L-glutamate : THF 5-formiminotransferase EC 2.1.2.5

(5) 10 formyl THF Synthetase - Formate : THF ligase (ADP) EC 6.3.4.3

(6) 10 Formyl THF Dehydrogenase - 10 Formyl THF $NADP^+$ Oxidoreductase EC 1.5.1.6

(7) 10 formyl THF AICAR Transformylase - 10 formyl THF : 5-amino 1 ribosyl 4 imidazolecarboxamide 5' phosphate Transforylase

(8) 5 formimino THF cyclodeaminase - 5 formimino THF : Ammonia lyase (cyclising) EC 4.3.1.4

(9) 5,10,Metheneyl THF cyclohydrase - 5,10,CH=THF 5 Hydrolase (decyclising) EC 3.5.4.9

(10) 5 formyl THF : 10 formyl THF mutase

(11) 5 formyl THF cyclohydrase - 5 formyl THF cyclo-ligase (ADP forming) EC 6.3.3.2

(12) Methylene THF dehydrogenase - 5,10,Methylene THF NADP oxidoreductase EC 1.5.1.5

(13) Glycinamide riboside transformylase EC 2.1.2.21

(14) 5,10,Methylene THF reductase - 5MeTHF:(acceptor) oxidoreductase EC 1.1.99.16

(15) Methionine synthetase - 5MeTHF : L-homocysteine S-methyltransferase EC 2.1.1.13

(16) Betaine-homocysteine methyltransferase - Betaine : L-homocysteine S-methyltransferase EC 2.1.1.5

Note: the substrates for folate catalysed reactions are formally given as the monoglutamates, however, it is likely that they, in vitro, are mainly polyglutamates (see text)

Fig.1.4. Formation of Purine C_8

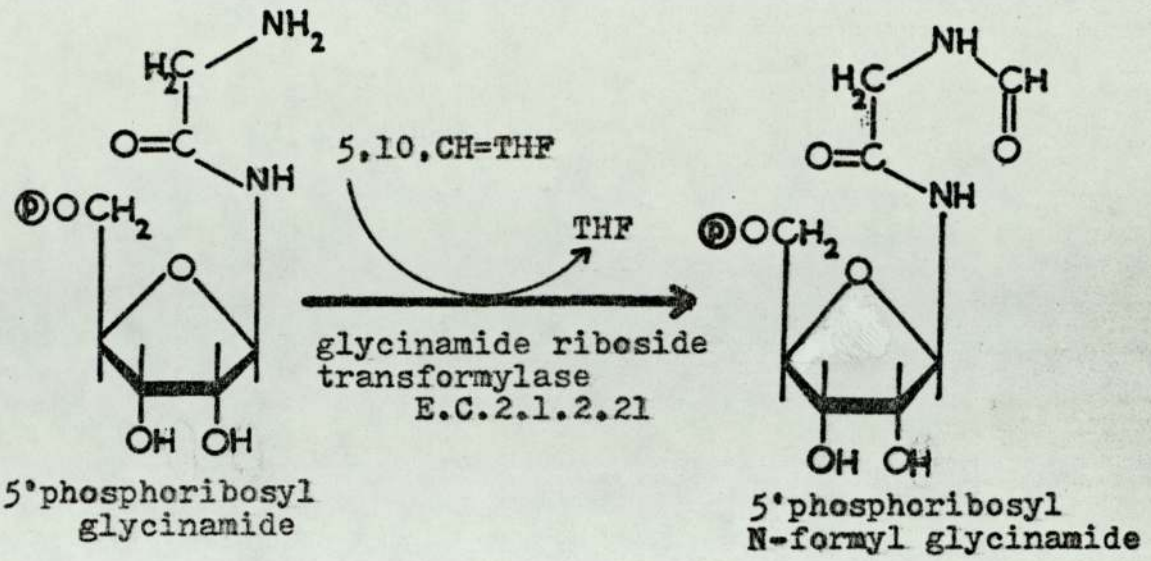
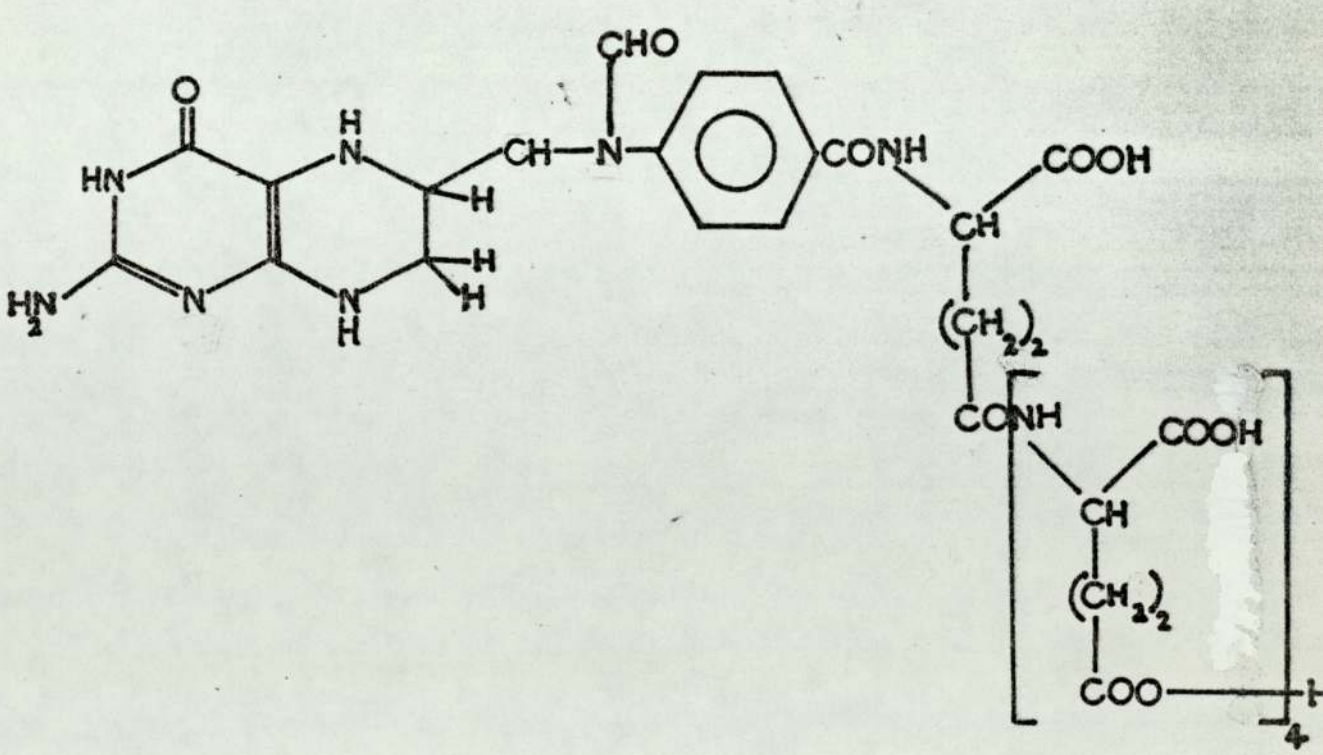


Fig.1.5. 10formylTHF tetraglutamate (10formyl tetrahydropteroyl pentaglutamate)



by the use of enzyme preparations which are free of cyclohydrase (enzyme 9 fig. 1.3) which catalyses the hydrolysis of 5,10CH = THF to 10CHOTHF, and under pH conditions which precludes their non-enzymic interconversion. They suggest that previous experiments which reported 5,10CH = THF as the required coenzyme, without these precautions result in 5,10CH = THF being rapidly converted to 10CHOTHF, the active donor. They also report (Dev and Harvey 1978b) that methylene THF dehydrogenase (enzyme 12 fig. 1.3) and cyclohydrase activities are present on a single protein, such that 5,10CH=THF would have only a transient existence during the conversion of $5,10CH_2THF$ to 10CHOTHF, and this would be unlikely to have a donor role <u>in vivo</u> for transformylations. Since these two enzyme activities are also on a single protein together with 10 CHOTHF synthetase (enzyme 5 fig 1.3) in pig (Tan et al 1977, Tan and Mackenzie 1979) and sheep liver (Paubert 1976) which suggests 10CHOTHF may also be the exclusive formyl donor in mammalian purine biosynthesis.

The flow of one-carbon units through the folate pool is branched at the formation of 5 MeTHF, the reduction of $5,10CH_2THF$ having an equilibrium position ($Keq = 10^7$) which precludes substantial formation of $5,10,CH_2THF$ from 5MeTHF under physiological redox conditions (Kutzbach and Stokstad 1971). As a result 5MeTHF, unlike the bridged and formyl forms, cannot be directly converted to other folate types without loss of its one-carbon substituent, and does not directly provide any one carbon species for

thymidylate and purine synthesis. The methyl group of 5 MeTHF is donated to homocysteine catalysed by the cobalamin (vitamin B_{12}) - requiring methionine synthetase (enzyme 15 fig 3) in mammals (Dickerman et al 1964). The adenyl derivative of methionine, S-adenosyl methionine (SAM). is the methyl donor in numerous biosynthetic reactions (Cantoni 1975, see chapter 3). Some labile methyl groups are provided in the diet as methionine and choline which may furnish SAM (fig. 1.3), with the rest of the body methyl group requirements being synthesised de novo from the folate pool. Mudd and Pool (1975) calculate that human volunteers on a nitrogen equilibrium diet synthesised approximately 24% of their methyl requirement de novo which rose to 67% when the labile methyl content of their diet was reduced by 62%. Regulation of methyl biosynthesis with dietary methyl is achieved at two points; control of methionine synthetase production and regulation of methylene THF reductase activity. The synthesis of methionine synthetase is repressed by methionine (Kutzbach et al 1967 Finkelstein et al 1971) and stimulated by homocysteine (Kamely et al 1973). Methylene THF reductase activity is allosterically inhibited by SAM (Kutzbach and Stokstad 1971) and stimulated by S-adenosyl homocysteine (SAH) (Osifo 1978). Thus the rate of de novo methyl synthesis will be modulated by the levels of the methyl donor SAM and its precursor methionine, versus that of the methyl acceptor homocysteine and its precusor SAH.

The level of one carbon units in the folate pool is regulated by the high activity of 10CHOTHF reductase (Krebs et al 1976, Scrutton and Beis 1979). Should an increase occur in the formyl or bridged folate forms which are in equilibrium with 10CHOTHF, due to a decreased rate of purine synthesis or by inhibition of methylene THF reductase by increased SAM, the concomitant rise of 10CHOTHF results in the loss of C_1 units as CO_2. This mechanism may explain the constancy of 10CHOTHF levels compared to 5MeTHF observed in human serum by Ratanasthein et al (1974).

Extraction of folates from cellular material under conditions which preclude significant enzymatic degradation produces folate species with supernumerary glutamate residues (Pfiffner et al 1946 Connor et al 1977, Connor and Blair 1980) termed folylpolyglutamates. Numerous other reports have been given of extraction of tissue folates which have high molecular weight and either chromatograph with synthetic polyglutamates with γlinked glutamate peptide chains, or support the growth of folate - requiring bacteria after treatment by glutamate γcarboxypeptidase (conjugase) e.g. Bird et al(1965), Shane et al(1977), Baugh and Krumdiek (1971) Perry and Chanarin (1977). Fig 1.5 gives a typical polyglutamate structure.

In vitro studies have shown synthetic polyglutamate folates to be superior to monoglutamates as coenzymes in a number of folate requiring reactions viz: thymidylate synthetase (Kisluik et al 1974), methionine synthetase

(Coward et al 1975), dihydrofolate reductase (Coward et al 1974) and AICAR formyl transferase (Baggot and Krumdiek 1979). Also the polyglutamates of dihydrofolate and 10 formylfolate are more effective inhibitors of thymidylate synthetase compared with the monoglutamate (Kisluik et al 1974, Friedkin 1973) this, together with their cosubstrate activity varies with chain length, from which it is possible to speculate that the ratio of intracellular polyglutamyl types may exert a controlling function on folate metabolism. Hilton et al (1979) showed that cultured cells showed a higher ratio of polyglu:monoglu during log growth phase than confluent cells, however, the ratios of various polyglutamates was not investigated. The importance of polyglutamates to folate metabolism is illustrated by a mutant strain of baby hamster kidney cells (BHK) described by MacBurney and Whitmore (1974) which were found to be auxotrophic for glycine, thymine and adenine. Taylor and Hanna (1977) showed that these cells were unable to synthesise polyglutamate which resulted in the observed deficiencies. It was determined that the deficiency was due to a lack of appropriate folyl polyglutamate coenzymes by that fact that these metabolic defects persist even when the cells are provided with excess folate in the medium. In liver there is an observed turnover of stored polyglutamates to extracellular folate (Shane et al 1977), thus there would be a net loss of tissue folate should the rate of polyglutamate synthesis decrease.

Work on the polyglutamates is hampered by their complexity. Bauch and Krumdiek (1971) estimated that with varying states of oxidation, one-carbon substituents and up to seven glutamate residues, the possible number of folates is 150. These present a considerable challenge to analysis. In earlier studies (reviewed by Blakley 1969 p.34) tissues were allowed to autolyse, endogenous conjugase liberating folates as monoglutamate, chromatography and microbiological assay used to estimate the various folate types. Besides the problems associated with microbiological assay methods (see section 1.4), under autolysis there is likely to be interconversion of folate types. Similarily Norohana and Aboobaker (1963) showed that freezing and thawing of tissue samples results in a greater proportion of formyl folates and a decrease in 5MeTHF. The method of Bird et al (1965) prevents autolysis by enzyme inactivation in boiling ascorbate, with use of conjugase to facilitate microbiological assay of the chromatographed polyglutamate peaks. However, conjugase has only recently been extracted, purified to homogeniety and characterised (Rao and Norohana 1977), previous use of impure preparations suggests the possibility of folate interconversions during analysis (Connor and Blair 1980). Thus there are discrepancies between the reported tissue folates depending on the methods of extraction and analysis viz: Norohana and Silverman (1962), Bird et al (1965) report that 5MeTHF polyglutamates are

the major liver folates, Shin et al (1972) extracted a mixture of formyl and methyl polyglutamates with up to six glutamate residues, while Connor et al(1977), Connor and Blair(1980) showed only polyglutamates of 10 formyl folic acid. The polyglutamyl folates do not elute from gel filtration or ion-exchange in a pattern consistent with either their one-carbon substituents or polyglutamate chain length (Baugh and Krumdiek 1971). Attempts have been made to simplyfy the species derived from tissue by cleaving the C^9 - N^{10} bond of polyglutamates producing a series of p aminobenzoyl glutamyl - (γ glutamyl)$_n$ peptides which may be chromatographed with relative ease. Recently, however, it has been demonstrated that reductive (Lewis and Rowe 1979) and oxidative (Maruyama et al 1978; Lewis and Rowe 1979) 'cleavage' techniques, although able to produce amino benzoyl glutamates from synthetic folic acid polyglutamates, cannot do so consistently from reduced folate types.

Connor (1979) was unable to demonstrate the presence of 5MeTHF polyglutamates, under extraction conditions where they would be expected to be stable, in rat liver. The high molecular weight folate was shown to be mainly 10CHOFA glu$_4$ by chemical analysis (presumably the oxidation product of 10CHO THF glu$_4$) with some 10CHOFA glu$_3$ which might be an artifact of extraction (Connor et al 1977, Connor and Blair 1980). From this result Connor (1979) suggests that the polyglutamate tissue pool consists of formyl and bridgedfolates providing the coenzymes for purine and

thymidylate synthesis with methionine synthesis the exclusive domain of monoglutamate 5MeTHF. In support of this hypothesis it is observed that the BHK mutant strain reported by MacBurney and Whitmore (1974) while auxotrophic for glycine purines and thymine, the products of bridged and formyl types, shows no requirement for methionine, product of 5MeTHF. As the requirements are associated with polyglutamate deficiency (Taylor and Hanna 1977) this would indicate only 5MeTHF coenzyme activity is independent of polyglutamate synthesis. Similarly, rat intestine lacks methionine synthetase (Finkelstein et al 1971, 1978) yet apparently forms polyglutamates (Connor 1979) and synthesises 5MeTHF (Blakley 1969 p. 370). Should 5MeTHF polyglutamates be formed, they would be unable to be demethylated in the gut, effectively trapping folate and stopping one carbon transfer reactions.

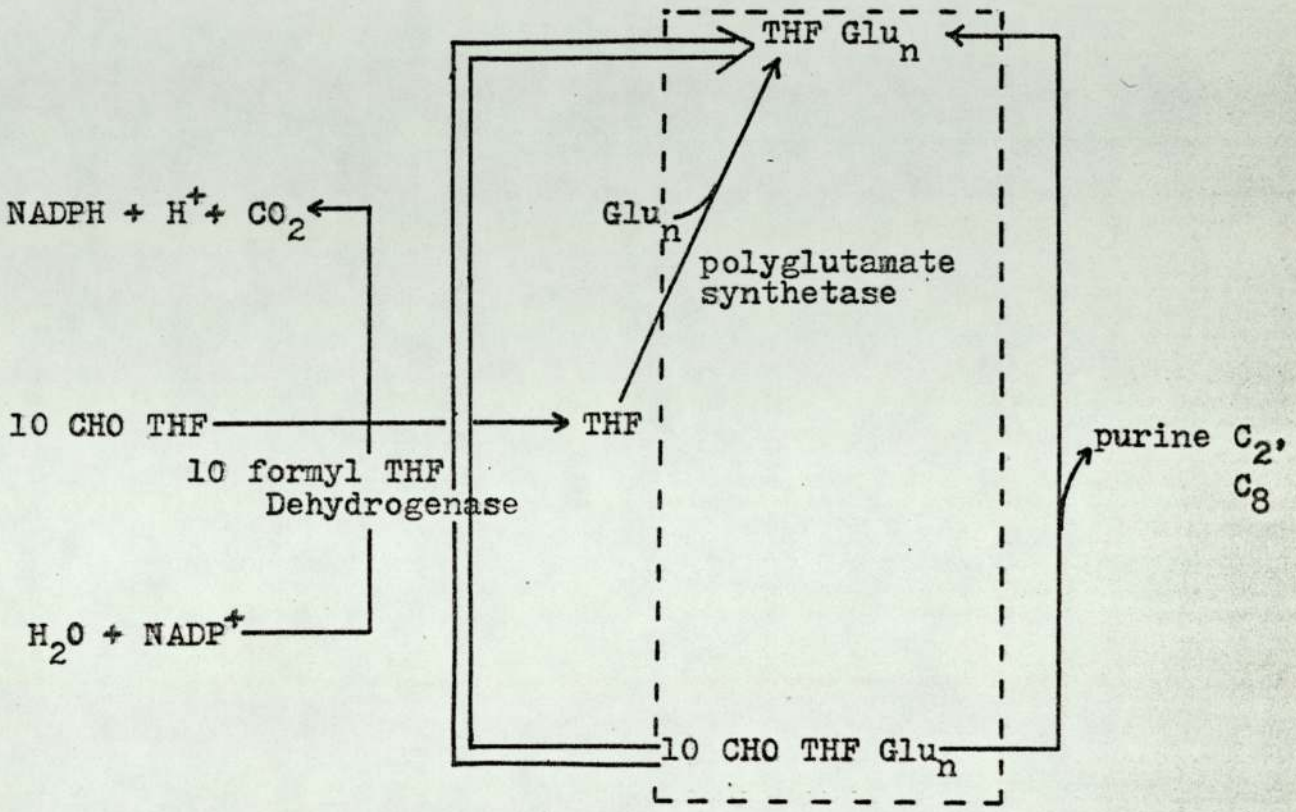
The apparent primacy of 10CHOTHF polyglutamate as tissue folates invites speculation about the control mechanism of folate metabolism which distributes folate between the mono - and polyglutamate pool (Connor 1979). The work of Krebs et al (1976) and Scrutton and Beis (1979) proposed a role for 10 formyl THF dehydrogenase in controlling the level of one carbon units in the folate pool, releasing excess units as CO_2 (fig 1.3). If as is found with other enzymes, the polyglutamyl folate is the preferred substrate (see above) 10CHOTHF polyglutamate would be the primary substrate of the pathway, competing with monoglutamyl

10CHOTHF. When the demand for 10CHOTHF polyglutamate is increased during phases of nucleotide synthesis, a greater proportion of monoglutamyl 10CHOTHF will be utilised by the dehydrogenase, generating THF. The increase in THF concentration would result in enhanced polygutamate synthesis, as in vitro studies (Sakami et al 1973, Spronk 1973 McGuire et al 1979) have indicated that this folate is the optimal substrate for polyglutamate synthetase (fig 1.6). This mechanism would enable nucleotide synthesis to modulate folate conjugation and provide a rationale for the observation of Hilton et al (1979) that the polyglutamate to monoglutamate ratio is increased in cultured cells during their logarithmic growth phase.

The oxidation of 10CHOTHF glu_4 may have physiological consequences as its oxidation product 10CHOFA glu_4 is found to be a powerful inhibitor of dihydrofolate reductase (Friedkin et al 1975). Thus on occasions when the oxidation of 10CHOTHF types is increased, as observed in Vitamin C deficiency (Stokes et al 1975) increased dihydrofolate reductase inhibition may interfere with cell division contributing to anaemia. Conversely cell division is likely to be enhanced by thymidylate synthetase activity when 10CHOTHF oxidation is diminished, as may occur in tumour cells which are observed to have a higher redox potential then normal (Schwartz et al 1974, Schwartz and Johnson 1976).

Rigorous investigation of these postulates awaits a technique for the routine examination of cellular folates without the ambiguities of present analytic methods.

Fig.1.6. Relationship between the Mono- and Polyglutamate Folate Pools



1.2 Folates in Physiology

The folate coenzymes are involved at three points in the synthesis of nucleic acids; purine and thymidylate synthesis and the de novo production of modifying methyl groups (Nau 1976). This, and possibly their role in amino acid metabolism makes them essential to cell division. Lack of folate in man results in a disease primarily characterised by deranged red blood cell formation, megaloblastic anaemia. The circulating red cells become macrocytic and their bone marrow precursors megaloblastic. These cells, together with transformed lymphocytes and dividing epithelial cells show chromosome anomalies. As a result of decreased erythropoesis anaemia occurs. The decreased proliferation of the epithelial surfaces may, in acute cases, result in the ulceration of the mouth and upper intestinal mucosa, and the impairment of gonadial cell division cause male and female sterility(Hoffbrand 1977).

Deficiency of vitamin B_{12} also gives rise to megaloblastic anaemia, histologically and haematologically indistinguishable from that produce by folate deficiency (Blakely 1969 p439). Their interrelationship is shown by the fact that B_{12} deficient anaemia may be alleviated by pharmacological doses of folic acid and folate deficient megalobastosis by vitamin B_{12}. However, neurological disturbances and degeneration associated with B_{12} deficiency are not treated by folate therapy and may even be exacerbated by it (Rowe 1978). To explain the relationship of B_{12} and folate

deficient anaemias, Herbert and Zalusky (1962) and Norohana and Silverman (1962) proposed the methyl tetrahydrofolate trap' hypothesis. Noting that B_{12} is required as a cofactor for methionine synthetase (fig 1.3) they suggest that during B_{12} deficiency the rate of methionine synthesis thus 5MeTHF demethylation, falls. This results in available folate being trapped as 5MeTHF at the expense of the purine and thymidylate forming types, this functional folate deficiency gives rise to the observed megaloblastosis.

That the anaemia is not immediately fatal is possibly due to residual methionine synthetase activity, or as a result of the diet compensating for the block by provision of folates other than 5MeTHF which would enable vital folate functions to continue at a low level, yet not prevent the development of anaemia.

Evidence for the 'methyl trap' hypothesis has been provided by Thenen et al (1973) who reported that the serum of pernicious (B_{12} deficient) anaemia patients showed elevated 5MeTHF. Blakely in his review (1969 p. 456) considered the evidence to date for increased serum 5MeTHF to be equivocal. However, Rowe (1978) suggested that the majority of serum folate being normally 5MeTHF (Ratanasthein et al 1977), the proportional increase may not be readily detected. This is especially true should microbiological methods be used which may show high variability (Rothenberg et al 1972).

More directly Lavoie et al (1974) showed a decreased incorporation of ^{14}C activity from 5MeTHF into proteins in cobalamin deficient human cells and Sakamoto et al (1975) observed a decreased synthesis of thymidylate in cultured

B_{12} deficient lymphocytes. The hypothesis has been extended by the observation that 5(^{14}C)MeTHF is not incorporated into polyglutamates with its methyl group intact (Lavoie et al 1974, Blair et al 1976).

This could result either from demethylation of the 5(^{14}C) methyl THF proceeding at a much faster rate compared to its rate of conjugation, so that (^{14}C methyl) labelled polyglutamates are not readily observed by this technique, or that 5MeTHF is a poor direct substrate for polyglutamate synthetase. In vitro studies of bacterial and rat liver polyglutamate sythetase (d'Urso - Scott and Malaku 1973 Spronk 1973) show that the enzyme is unable to utilise 5MeTHF. A later report (McGuire et al 1979) observed some in vitro 5MeTHF conjugation but at a rate some 20% of that observed with THF. Thus accumulation of 5MeTHF during B_{12} deficiency would result in decreased polyglutamate synthesis and reduced tissue folate eg. in liver (Thenen et al1973). Perry and Chanarin (1977) report that the folates of red blood cells from pernicious anaemia patients show a different spectrum of polyglutamyl chain lengths from normal which could have consequences for folate enzyme regulation. However, the detailed analysis of chain lengths should be treated with caution, as their analysis was based on cochromatography with synthetic 5MeTHF polyglutamates which may be inappropriate markers for tissue folates (see section 1.1). In a later report (Perry et al 1979a) they observe that B_{12} deficient rats show a marked decrease in incorporation of folate into polyglutamate when the source is 5MeTHF, compared to formyl and methylene

THF.

The methyletrahydrofolate trap thus explains the haematological effects of B_{12} deficiency as a consequence of disturbed folate metabolism, yet Rowe (1978) suggests the hypothesis is limited in that with congenital methylmalonicaciduria there is defective cobalamin metabolism and methionine synthetase activity (Mahoney et al 1971, Dillon et al 1974) but those affected do not automatically show the expected megaloblastic anaemia.

Folate deficiency may arise due to a variety of causes. Blakely (1969 p.411) described nutritional folate deficiency as possibly the commonest example of vitamin deficiency in man. However, human folate requirements are not as yet well characterised (Rodriguez 1978); although estimates have been made of the minimum amount of folate, as folic acid, required to forestall the appearance of deficiency symptoms. Hansen and Weinfield (1962) gave a value of 100µg daily, Zalusky and Herbert (1961) of the order of 50µg and Banerjee et al (1975) 75µg. However, it has to be noted that these values are obtained from populations of ill defined nutritional status. Folate deficiency is also observed in malabsorbtion syndromes (Rodriguez 1978), during the clinical use of folate antagonists (Blakely 1969 p 484, Waxman et al 1970) and anticonvulsants (Chien et al 1974). Folate deficiency is commonly associated with conditions characterised by increased cell proliferation v12; Haemolytic anaemia, myosclerosis, inflammatory disease and malignant neoplasia (Hoffbrand 1977), and especially

pregnancy.

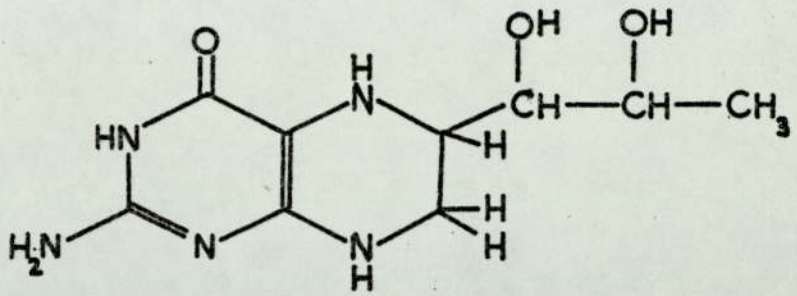
It has been estimated that the folate requirements of a pregnant woman is in the order of 400 µg/day, some 4 to 8 times that of the non pregnant adult woman(Lowenstein et al 1966) The association of folate deficient anaemia with pregnancy has been studied since the 1930's and the observations of Wills (1931). Chanarin et al (1958) reported that the majority of women in pregnancy and all mothers of multiple births show folate deficiency. Cooper et al (1970) reported that studies to date had showed that 50% of pregnant women had low serum folate and studies in Britain, Canada and South Africa showed 25% of women with megaloblastic changes in the bone marrow during pregnancy. Reports differ as to the effects of maternal folate deficiency on the foetus, for it is apparently able to avail itself of folate at the expense of the mother even when the maternal stores are depleted (Rowe 1978). Gatenby and Lillie (1960), Hibbard and Hibbard (1968) report an increase in prematurity and congenital defects in the children of folate deficient mothers, while Pritchard et al (1970), Varadi et al (1966), Giles (1966) and Cooper et al (1970) could not show any increase in perinatal mortality or morbidity with maternal folate deficiency. This question will be difficult to resolve now that folic acid supplements are given routinely during pregnancy. Folic acid supplements have been shown to result in a significant decrease in premature births to Black South African mothers while no significant difference was observed among white women with a more

substantial diet (Braumslag et al 1970). The study indicates that one cannot study folate deprivation amongst human populations in isolation from other dietary factors.

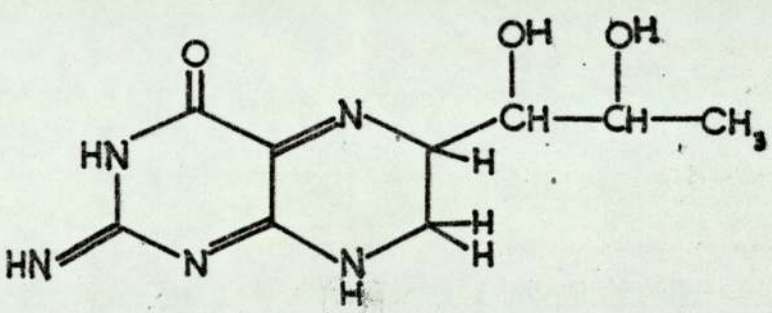
Folates have an important role in mental development evidenced by the congenital folate metabolic diseases. In his review Rowe (1978) cites several folate enzyme deficiencies viz congenital malabsorbtion; dihydrofolate reductase formiminotransferase/cyclodeaminase, methylene THF reductase and methionine synthetase deficiencies. Although slowing of physical growth was an occasional feature, mental deficiency was almost invariably present to some degree. From animal studies folate deficiency in early life may be associated with abnormal mental development; Arakawa (1970) reported that induced folate deficiency in the neonate rat causes a delay in the maturation of brain electrical activity. Possibly deranged folate activity affects brain development by decreasing nucleic acid synthesis during a phase of nerve proliferation. However, folate and one-carbon metabolism also have a role in mature brain activity where growth is minimal.

Both folate deficiency and excess have been observed to cause neuropsychiatric disturbances in adults. Folate deficiency in man is associated with neuropathy (Manzoor and Runcie 1976) and mild depression and fatigue (Botez et al 1978) Pharmacological doses of FA (15mg/day) are observed to cause gastrointestinal disorders, sleep difficulties, vivid dreams and irritability which can interfere with normal working life (Hunter et al 1970). Further work

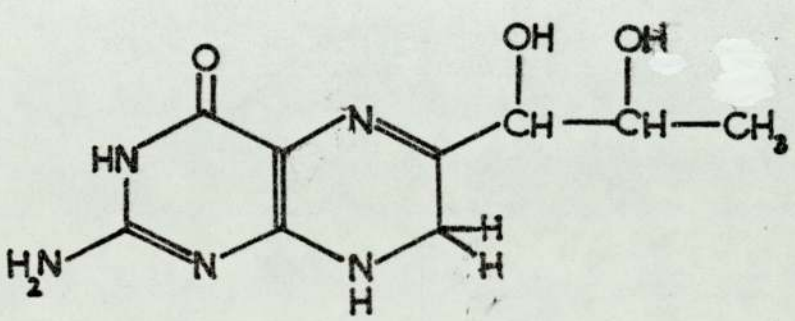
Fig.1.7. Tetra- and Dihydrobiopterins



Tetrahydrobiopterin (BH_4)

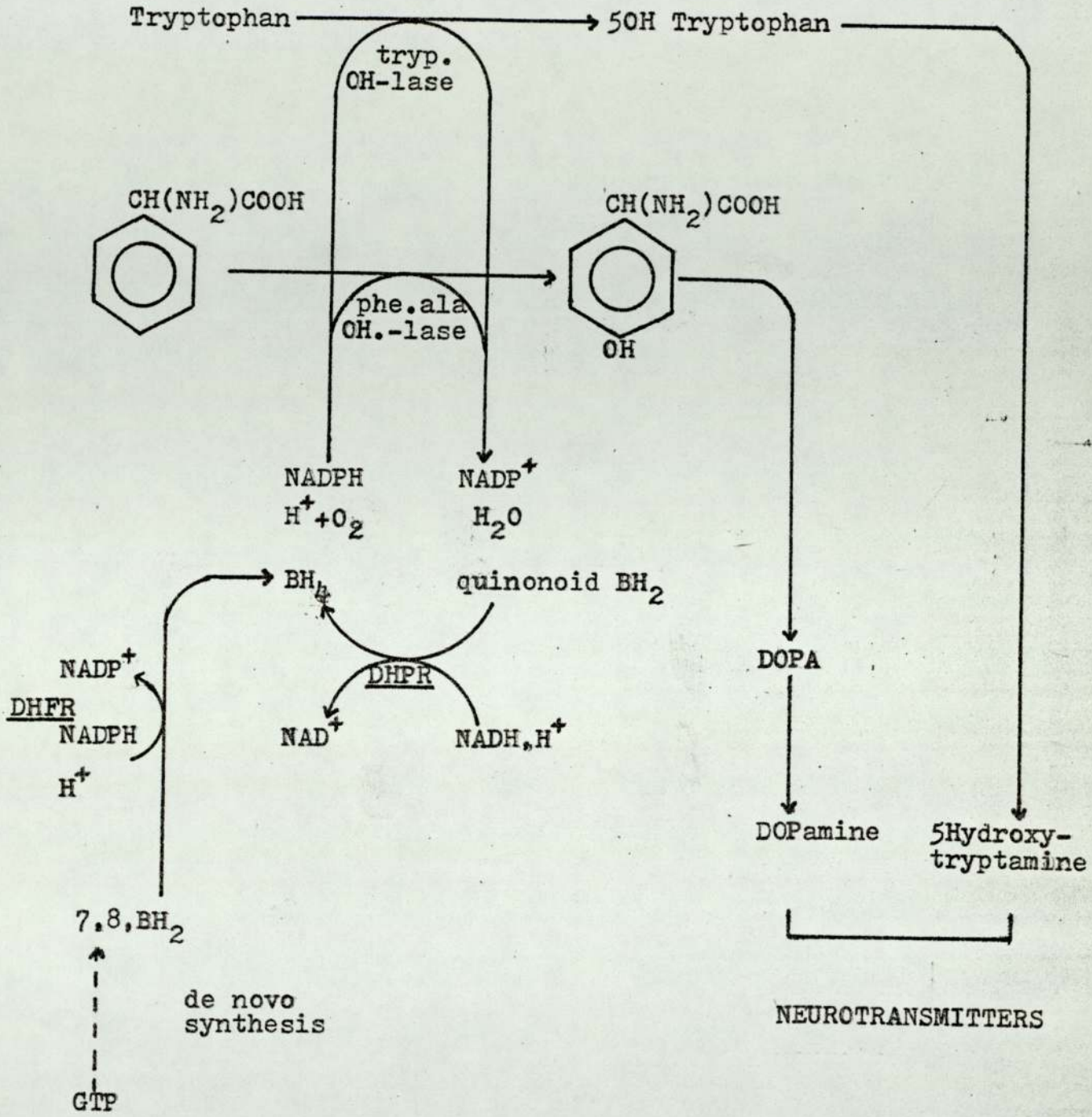


Quininoid Dihydrobiopterin (qBH_2)



7,8,Dihydrobiopterin ($7,8,BH_2$)

Fig.1.8. Role of Biopterin in Neurotransmitter Synthesis



DHFR - Dihydrofolate Reductase
DHPR - Dihydropteridine Reductase

on humans (Hunter et al 1971) showed that 30 mg FA by mouth resulted in a significant fall in cerebrospinal fluid levels of hydroxyvanillic acid, a dopamine breakdown product indicating a decreased turnover of the neurotransmitter. In rats Botez et al (1979) report a decrease in brain levels of the neurotransmitter 5 hydroxtryptamine (5HT) in animals maintained on a folate excess or folate deficient diet. They postulate that in folate depletion an associated thiamine def ciency (Thomson et al 1971) results in the decreased 5HT production. Spector et al (1978) report that dihydrofolate reductase (DHFR) is required for the reduction of 7,8. dihydrobiopterin in the de novo synthesis of tetrahydropterin (fig 1.7) the pterin redox cofactor in tryptophan (Lovenberg et al 1967), phenylalanine and tyrosine hydroxylation (Costa and Meck 1974) in the synthesis of 5HT and dopamine (fig 1.8). Botez at al (1979) proposed that when high doses of FA are administered the preferential reduction of FA to DHF and THF decreases the rate of tetrahydrobiopterin (BH_4) by DHFR, consequently decreasing subsequent 5HT production as BH_4 concentration is thought to be rate-limiting in hydroxylating systems (Costa and Meck 1974). Another possible interaction of FA with biopterin metabolism is via dihydropteridine reductase (DHPR) the enzyme which recycles the quinoid dihydrobiopterin product of BH_4 requiring hydroxylations (Lund 1972) fig 1.8. Folic acid is observed to be an in vitro inhibitor of this enzyme (Cheema et al 1973), thus the high transient body concentrations of FA after a pharmacological dose would be expected to result in DHPR inhibition. Thus it is possible to envisage folates interacting both with the de novo synthesis and salvage

pathways for BH_4. These postulates are still to be tested by experiment, however, the biopterin hypothesis suggests an interesting association between the two areas of mammalian pterin metabolism.

Folates and one carbon metabolism are involved in the two commonest chronic mental disorders, epilepsy and schizophrenia. Epileptics treated with anticonvulsant drugs often develop folate deficiency and megaloblastic anaemia (Klipstein 1964, Waxman et al 1970). It has not been established whether anti-convulsant drug action is primarily due to antifolate activity, however, Ch'ien et al (1975) note that seizures and EEG abnormalities may be induced in some drug treated epileptics by high (75mg) intravenous folic acid doses.

Schizophrenia is of interest in that 40% of chronic schizophrenics when given methionine doses of 20g/day (normal adult requirement = 10mg/day) suffer acute psychotic attacks (Smythies 1975). Opinions as to the mechanism of this effect differ, Osmond and Smythies (1952) proposed the transmethylation hypothesis where abnormal methylation of neurotransmitters occurs in schizophrenia to produce natural analogues of N- and O- methylated psychomimetic substances such as dimethyl tryptamine (DMT), bufotenine 5 methoxy DMT, and mescaline (Rosengarten and Friedhoff 1976). However, Smythies (1976) reported that there is little evidence that schizophrenics produce greater amounts of methyl modified neurotransmitters than normals, and suggested the methionine effect may be due to action on 5HT metabolism,

by competing with tryptophan uptake and or by psychotoxic effects of homocysteine. They note the schizophrenia-like symptoms associated with methylene THF reductase deficiency (Freedman et al 1975) which gives rise to homocysteinuria, and that administration of serine a source of one carbon units for homocysteine methylation abolishes some of the methionine effect symptoms in schizophrenics (Beaton et al 1975). However, conversely simple homocysteine accumulation is not observed to cause schizophrenia-like effects in subjects with cyst thionine synthetase deficiency (Laster et al 1965). In contrast to the transmethylation hypothesis, Levi and Waxman (1975) proposed that the biochemical lesion of schizophrenia is under methylation of neurotransmitters. Reynolds (1968a) noted schizophrenic episodes in epileptics undergoing anticonvulsant drug treatment, which lowers folate, thus presumably transmethylation. Levi and Waxman also note the methylene THF reductase deficient schizophreniform syndrome (Freedman et al 1975) where there would be diminished de novo synthesis of methyl groups. They propose that schizophrenics are deficient in activating methionine for transmethylation. Leukocytes from schizophrenics produce less $^{14}CO_2$ from (^{14}C methyl) methionine than normal, suggesting a general abnormality of transmethylation or oxidation of methionine methyl (Latife et al 1978). Measurement of one carbon pathway enzymes (Carl et al 1978) shows blood cells of schizophrenics to have diminished serine hydroxymethyltransferase (enzyme 3 fig 1.3) and methionine adenosyl transferase (SAM synthetase). Deficiency of the former would result in less de novo methyl group formation, the

latter less SAM production. However, Levi and Waxman (1975) do not account for the effect of methionine load, which simplistically, should allieviate schizophrenia psychosis caused by under-methylation rather than potentiate it. However, schizophrenia is a complex disease state which is diagnosed by behavioural and psychiatric criteria rather than physiology and may have several biochemical mechanisms involved in its aetiology (Smithies 1975, 1976). Folate and one carbon metabolism are demonstrably altered in at least some affected patients, and thus further research in this field would be valuable.

An adequate supply of folate, B_{12} and one carbon donors plays an important role in the maintenance of an effective immune response (Newberne 1977). Although immune deficiency is observed with protein calorie (Chandra 1975) and other vitamin deficiencies (Axelrod 1971), that of folate deficiency may be especially important due to its widespread incidence. Folate deficient women have a decreased cell-mediated immune response as measured by dinitrochlorobenzene skin tests and depressed phytohaemaglutinin (PHA) stimulation of peripheral lymphocytes (Gross et al 1975). In animal experiments damage occurs to the rat immune reponse following subclinical deprivation of folate, B_{12} and methyl donors (choline and methionine) in the mother (Newberne 1977). The offspring, in adult life, show significantly greater mortality when challenged with Salmonella typhimurum infection, low serum protein and white cell counts, and a reduced leukocyte response to PHA

stimulation. In the same series of experiments pre- and post-natal B_{12} depletion also supresses the rat immune response , however, excess B_{12} improves the immune response above normal, and the cytotoxic ability of challenged lymphocytes from post-weaning folate depleted animals was correlated with their serum folate levels. In the later experiments of Williams et al (1979) rats were marginally deprived of methionine and choline during various growth and devopmental stages, and it was found that the severest immunological impairment was associated with deprivation during gestation and lactation, the period of maximal lymphoid proliferation. If these studies are extrapolated to humans, they indicate that folate deficiency should be avoided during the early stages of life, as even subclinical deficiency of folate, B_{12} and one carbon donors may lead to long term immune system impairment.

The requirement of folate for cell division is exploited therapeutically to destroy invasive or malignant cells. The sulphonamides, structural analogues of p.aminobenzoic acid, have antibiotic activity by competitively inhibiting bacterial synthesis of dihydrofolate (Brown 1962). 2-diamino pyrimidines are antimalarials which function by inhibiting Plasmodium dihydrofolate reductase (Blakely 1969 p.466). Methotrexate (MTX), fig. 1.9 is widely used in the treatment of neoplastic disease and can give complete remission of Burkitts lymphoma and choriocarcinoma with substantial remission of acute lymophoblastic leukaemia (Condit 1971).

Fig.1.9. Methotrexate (MTX)

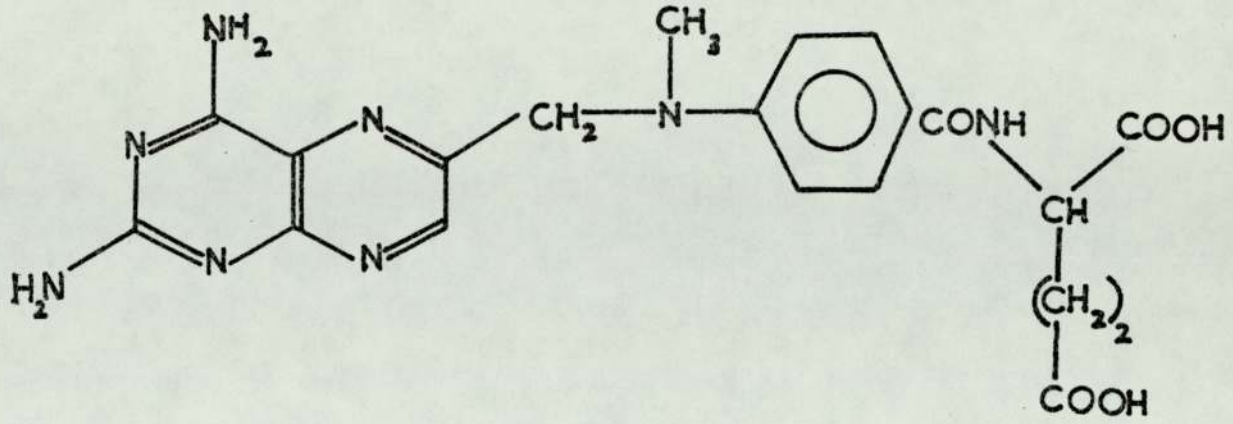
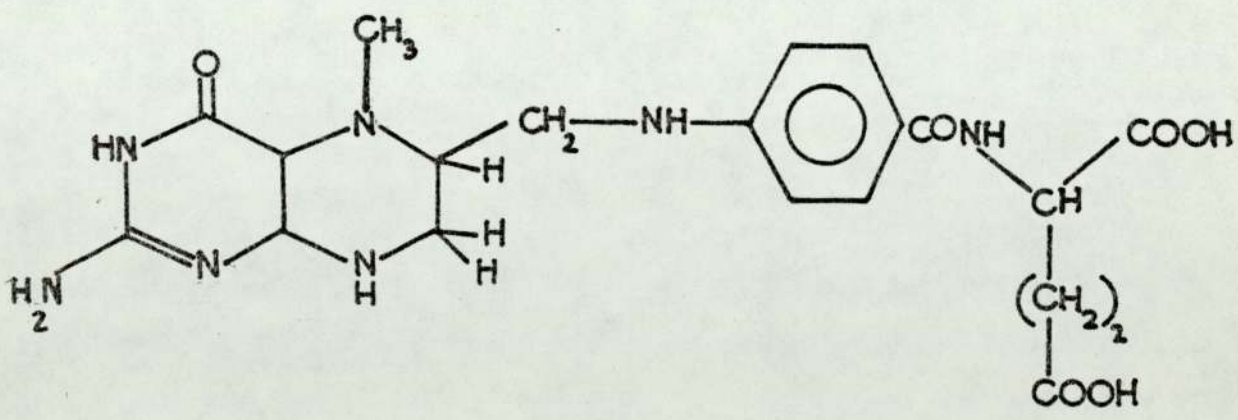


Fig.1.10.5Methyltetrahydrofolic acid (5MeTHF)



Methotrexate is a phase specific cancer chemotherapeutic halting the synthesis of nucleic acids in 'S' phase of the cell cycle by DHFR inhibition. The recycling of folate coenzymes ceases with the result that the cell undergoes 'thymineless death' (Cohen 1971). Methotrexate cytoxicity is proliferation dependent, the kill rate obtained with cell cultures in log growth phase is 6.7 times that obtained with resting cell cultures (Hrynuik et al 1969). In mouse fibroblasts 10^{-6}M MTX is observed to give a 95% kill of cells passing through the cell cycle after 24 hours, whereas contact inhibited non-dividing cells are immune (Johnson et al 1978a). In theraputic use the differential kill between normal and malignant cells is enhanced by folate rescue treatment. Tumours are observed to have a smaller fraction of cells in Go or 'resting' with respect to the mitotic cycle (Valenote and Van Putten 1975) which are thus not subject to S phase inhibition by MTX. Methotrexate is administered at high dose to destroy all mitotically active cells. Provision of 5CHOTHF or occasionally 5MeTHF after the period of a cell cycle will act as an antidote for those cells in G_0, especially those which can repopulate the gut epithelium and the haemopoetic system, as they reenter the mitotic cycle.

Thus folates are seen to be involved in several major areas of normal and diseased human physiology.

1.3 5 Methyltetrahydrofolic acid

5 MeTHF(fig. 1.10) since its discovery as prefolic A by Donaldson and Keresztesy(1959) has been recognised as quantitatively the most important folate of serum (Herbert 1961, Ratanasthein et al 1974), cerebrospinal fluid (Levitt et al 1971) of autolysed or stored mammalian tissues (Bird et al 1965, Shin et al 1972), various foodstuffs (Rodriguez 1978) and the conjugase treated folate content of a typical British diet (Perry 1971). Since 5MeTHF would be the major exogenous folate form available to humans, this would suggest it as the most appropriate folate for investigations of folate metabolism. However, 5MeTHF and its radiotracers have only relatively recently become commercially available for widespread research use. Previously most studies have used folic acid or occasionally 5CHOTHF the most chemically stable and readily available forms. Folic acid is not a naturally occuring folate and it is only by the fortuitous ability of dihydrofolate reductase (DHFR) to catalyse its reduction to DHF which enables the compound to enter the folate pool. This unusual reaction however, is slow, proceeding at 2-19% of the rate observed with DHF (Blakley 1969 p.146) and is the rate limiting step for the incoporation of folic acid into the pool. Interestingly the orientation of folic acid on the active site of DHFR is inverted with respect to DHF yet the enzyme is able to utilise both as substrates(Gready 1979). So the use of folic acid tracers may not give valid observations of the normal activities of of the folate coenzymes. A similar reservation applies to

5CHOTHF which although reported to be present in foodstuffs (Butterworth and Santini 1963, Perry 1971) it has not been confirmed to have any one carbon donor role in mammals (Rowe 1978).

5MeTHF has only one characterised metabolic reaction, that of donation of its methyl group to homocysteine forming methionine (Sakami and Uskins 1961). This is the major methionine synthesis route, although there is a betaine: homocysteine methyltransferase (fig. 1.3) this is confined to the liver in rats (Finkelstein et al 1971, 1978, Cantoni 1977, Balinska and Grzelakowska-Sztabert 1978). The deficiency of cobalamin (vitamin B_{12}) in pernicious megaloblastic anaemia is thought to result in the decreased rate of 5MeTHF demethylation, with the result that folate accumulates as 5MeTHF which is unable to participate in polyglutamate or purine and thymidylate synthesis (the methyltetrahydrofolate trap see section 1.2). Underpinning this hypothesis is the assumption that the reduction of $5,10CH_2THF$ is irreversible <u>in vivo</u> as calculated by Kutzbach and Stokstad (1971). However several reports were published suggesting 5MeTHF might have a direct methylating role in neurotransmitter metabolism (Laduron 1972, Hsu and Mandel 1973, Banerjee and Snyder 1974). It was later shown (Meller et al 1975) that the 'methylating' effect was due to methylene THF reductase reacting in the reverse direction under the oxidising conditions of the assay employed which included FAD or menadione. The $5,10,CH_2$ THF formed undergoes non-enzymic dissociation producing

formaldehyde which condenses with e.g. tryptamine and dopamine to produce compounds which mimic the pharmacological effects of methylated biogenic amines (Taylor and Hanna 1975, Pearson and Turner 1975, Fuller 1976, Rosengarten and Friedhof 1976)

Biochemical evidence for the in vivo formaldehyde route was strengthened by Burton and Sallach (1975) who showed that the distribution of the putative 5MeTHF requiring methyl-transferase activity was the same as that of CH_2THF reductase. The physiological significance of this reaction is unclear, the isoquinoline and β carbolines produced are not known as natural products of brain metabolism, and the oxidation of $5CH_3$THF to 5, $10,CH_2$THF is unlikely (Keq = 10^7 in favour of reduction, Kutzbach and Stokstad 1971) at the redox potential observed in both normal and transformed cells (Williamson et al 1967, Schwartz et al 1974, Schwartz and Johnson 1976). Although Stebbins et al (1976) postulated that the reaction may occur under special conditions viz: the metabolism of typtamine by blood platelets, the pathway has not yet been demonstrated in vivo or in cell free extracts without added oxidising agents. Were this pathway quantitatively important it would provide a route to bypass the 'methyltetrahydrofolate trap' releasing 5 MeTHF one carbon units by means other than methionine synthesis.

A second area of controversy involving 5MeTHF metabolism has been in the apparent methionine auxotrophy of tumour cells. A methionine deficient diet was observed to inhibit

the growth of an implanted Walker 256 carcinosarcoma in the rat (Sugimura et al 1959). Halpern et al (1974) showed that replacement of methionine by homocysteine in cell culture did not affect normal cells, however, it caused cell death with human monocyte leukaemia cells (J111), mouse lymphoid carcinoma (L1210) and Walker 256. They proposed that there is a functional methionine synthetase deficiency in tumour cells (Ashe et al 1975) and that a more effective methotrexate regimen would be the simultaneous dosage of 5MeTHF with MTX to protect normal cells with adequate methionine synthetase, while the tumour cells would be unguarded against MTX inhibition (Halpern et al 1975).

However, these experiments were critised in that not all tumour cells show the methionine requirement, HEp2 and Hela can survive methionine replacement (Magnum et al 1969) as can mouse lymphoma TLX5 (Tisdale 1979) and human mammary carcinoma A1A6 showing that methionine auxotrophy is a poor guide to malignancy (Kries and Goodenow 1978). Hoffman and Erbe (1976) showed that although Walker 256 and the SV40 transformed human skin fibroblasts SV80 and W18VA2 show a requirement for methionine, both FA and 5MeTHF are equally effective in stimulating the folate depleted cells. They are able to take up 5MeTHF normally and use it for methionine synthesis at rates similar to or greater than normal cells; after 26 hours 59-83% of 5(^{14}C)MeTHF activity is found as ^{14}C methionine. Also if otherwise limiting amounts

of methionine are provided in the medium the cells will thrive on homocysteine. Peytremann et al (1975) found the level of methionine synthetase in human blood cell lines to be chronic lymphocytic leukaemia lympocytes > normal lymphocytes > normal granulocytes > chronic myelocytic leukaemia granulocytes, and suggested the level of the enzyme may be related to the cell's potential for division.

Goff and Blakley (1978) have criticised the 5MeTHF protection experiments of Halpern et al (1975) on the grounds of the MTX concentrations used. They showed that under conditions more resembling the clinical usage of MXT that both 5MeTHF and 5CHOTHF have similar efficacy in protecting rescuing normal and tumour cell lines, as they have been observed to do so in the whole mouse (Blair and Searle 1970). Thus methionine auxotrophy is observed as a characteristic of only some tumour cells, although when the tumour cells revert to normal methionine independence they also show loss of other tumour cell characteristics (Hoffman et al 1979), which implies the methionine requirement is a function of transformation in those cells. The mechanism does not involve methionine synthetase, but presumably some as yet unknown function of methionine or change in the folate pool.

5 MeTHF is oxidised by air to 5Methyl 5,6, dihydrofolate (Donaldson and Keresztesy 1962, Scrimgeour and Vitrols 1966) the rate of oxidation is increased by the presence of Cu^{2+} and the polarity of the solvent (Blair et al 1975). Further

oxidation of this compound by H_2O_2 gives rise to a species originally designed as 4a hydroxy- 5methyltetrahydrofolate (4aOH5MeTHF) by Gapski et al (1971) later reformulated as a pyrazino-s-triazine by Jongejan et al (1979). As the folate content of foodstuffs is likely to undergo oxidation in storage (Blair 1976) these 5 MeTHF oxidation products may be significant in the diet, and their metabolism in mammals bears investigation to asses their physiological importance.

1.4. Research Objectives and Methods

There are two major methods commonly used in folate metabolism studies, microbiological assay and radiotracers. The low concentration of folates in physiological fluids does not lend them to spectral analysis without lengthy purification and concentration proceedures which may cause degradration. The folates are involatile which renders them unsuitable for mass spectrometry or gas chromatography. Microbiological assay, although a sensitive technique, may be criticised on the grounds of high variability (Rothenberg et al 1972). In one study (Butterfield and Calloway 1972) a coefficient of variation of 25% was obtained for total folate determination. The technique relies on an assumed 'all or nothing' response by each test organism to a particular folate monoglutamate which will give a differential estimate of the sample under investigation when organisms with different folate requirements are used viz: Lactobacillus casei responds to FA and the reduced folates, Streptococcus faecalis to all L casei folates except 5MeTHF, and Pediococcus cervisiae. to the formyl THF's and THF (Baugh and Krumdiek 1971) They are also assumed to have no response to polyglutamates higher than the triglutamate.

Pollock and Kaufman (1978) showed that on the contrary, different folates elicit varying growth responses from test organisms assumed to be equally sensitive to them. Also L. Casei does show some response to synthetic polyglutamates; folic acid and FA glu and FA glu_2 showing an equal response

with FAglu_3 (65.6% FA growth), FA glu_4 (19.9%), FA glu_5 (3.5%) and FA glu_6 (2.4%) also giving a response (Tamura et al 1972). Thus determination of individual folate monoglutamates and studies attempting to assay the proportion of monoglutamate to polyglutamates in tissue which rely on the microbiological activity before and after conjugase treatment are likely to be suspect.

In this project radiochemical methods are used to investigate folate metabolism. Both (3', 5', 7, 9, ^3H) and ($2^{14}C$) labelled folic acid are commercially available, but their use has been criticised on the grounds of possible 3H exchange reactions (Blair 1976) for the former, and difficulties in distinguishing intact ($2^{14}C$) folates from their pterin scission products (Murphy et al 1976). In this study a mixture of the two folic acid tracers was used to produce mixed label 5MeTHF from the rat, which in use avoids most of the problems associated with the single labels. 5(^{14}C) MeTHF is available commercially and may be used to follow the metabolism of the methyl group, although caution has to be observed as this compound is provided as a mixture of the natural and unnatural C_6 epimers

After administration to the rat, radiolabelled species from urine and tissue were separated by sequential ion exchange and gel filtration chrommatography which enables substantially greater amounts of fluid to be analysed than would be possible with alternative chromatography methods e.g. thin layer (Beavon 1973). Experiments were undertaken with suitable radiolabels to a) determine the role of 5MeTHF as a one carbon donor in comparison with methionine, b) by use of dual

labelled 5MeTHF to trace the normal disposition, metabolism and excretion of a naturally occuring folate. c)the synthesis of dual and methyl labelled oxidation products of 5MeTHF to determine their bioavailability and metabolism in the rat d) comparison studies on rats with an implanted Walker 256 tumour to observe changes in 5MeTHF metabolism which might be exploited diagnostically or therapeutically.

2. METHODS AND MATERIALS

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2.1 Animals

The experimental animals used were 200g male Wistar albino rats maintained on rat breeding diet (table 2.1). Experiments concerned with tumour metabolism used male Wistars obtained from the Chester-Beatty Institute, London, which had been implanted under the skin of the right flank with 10^6 cells of Walker 256 carcinosarcoma in suspension. These animals were kept for seven days after implant prior to dosing with radiotracer, by which time the tumour mass was palpable.

2.2 Chemicals

The reagents used routinely during experiments were of 'Anala R' grade or equivalent obtained from various commercial sources. 5Methyltetrahydrofolic acid (Magnesium salt) was obtained from Eprova Research Laboratories (Basle, Switzerland). Radiolabelled, non-radioactive folates and related compounds were either bought, extracted or synthesised; 5(^{14}C)MeTHF (Ca salt 58 μCi/μM),(^{14}C methyl) methionine (58μCi/μM), (2^{14}C) folic acid (55μCi/μM), (3',5',7,9,^{3}H) folic acid(45Ci/mM) were obtained from the Radiochemical Centre, (Amersham, Bucks). The following compounds were prepared:

Mixed label (2^{14}C)(3',5',7,9,^{3}H)5MeTHF

2^{14}C(55 μCi/μM) and 3',5', 7,9,^{3}H folic acid (45Ci/mM), were each dissolved in 50mM Na phosphate buffer pH7 containing 2% Na ascorbate and mixed to give a solution containing 10μCi ^{14}C, 25μCi ^{3}H, and 80.5μg folic acid per cm^3. Six rats were orally dosed with 0.2cm^3 (2μCi ^{14}C 5μCi ^{3}H) of the solution daily for three days with daily collection of urine as described below (Section 2.3). The pooled urines were chromatographed by DE52 ion-exchange as in Section 2.5. The 5MeTHF fraction was pooled,

Table 2(1) Composition of Rat Diet
(Rat and Mouse Breeding Diet, Pilsbury's Ltd.)

Crude oil	%	3.26
Crude protein	%	21.23
Crude fibre	%	3.48
Digestible Crude Oil	%	2.48
Digestible Crude Protein	%	17.60
Digestible Crude Fibre	%	2.10
Digestible Carbohydrates	%	46.80
Gross Energy Cals/Kg		4073.00
Metabolisable Energy Cals/kg		3666.00
Saturated Fatty Acid	%	0.73
Linoleic Acid	%	0.99
Other Unsaturated Acids	%	1.54
Calcium	%	1.30
Phosphorus	%	1.00
Sodium Chloride	%	0.635
Magnesium	%	0.24
Potassium	%	0.80
Sulphur	%	0.23
Iron mg/Kg		171.60
Copper mg/Kg		14.50
Manganese mg/Kg		86.60
Cobalt µg/Kg		104.70
Zinc mg/Kg		39.60
Iodine µg/Kg		600.00
Arginine	%	1.31
Lysine	%	1.14
Methionine	%	0.36
Cystine	%	0.33
Tryptophan	%	0.23
Glycine	%	1.57
Histidine	%	0.51
Threonine	%	0.71
Isoleucine	%	0.84
Leucine	%	1.49
Phenylalanine	%	0.89
Valine	%	1.07
Tyrosine	%	0.69
Aspartic Acid	%	1.64
Glutamic Acid	%	3.96
Proline	%	1.53
Serine	%	1.00
Vitamin 'A' i.u./Kg		1,1587.00
Carotene mg/Kg		0.79
Vitamin B1 (Thiamine) mg/Kg		9.10
Vitamin B2 (Riboflavine) mg/Kg		9.90
Vitamin B6 (Pyridoxin) mg/Kg		10.10
Vitamin B12 µg/Kg		17.00
Vitamin 'E' mg/Kg		77.00
Vitamin 'K' mg/Kg		3.00
Folic Acid mg/Kg		0.70
Nicotinic Acid mg/Kg		78.00
Pantothenic Acid mg/Kg		27.80
Choline Chloride mg/Kg		2.22
Biotin mg/Kg		0.12
Vitamin D3 i.u./Kg		859.00
Vitamin 'C' mg/Kg		-

freeze dried and desalted by passage through a G15 column equilibrated with 0.2% Na Ascorbate. After freeze drying the compound was stored as the solid prior to use. An aliquot of the stored compound was chromatographed with authentic unlabelled 5MeTHF to ensure its identity.

5 Methyl 5,6 Dihydrofolate (see fig 5.2, Chapter 5)

This compound was prepared by a modification of the method of Gapski et al (1971). 50mg 5MeTHF (for unlabelled compound) or 25μCi ^{14}C, 62.5μCi ^{3}H (for mixed label preparations) was dissolved in 20cm^3 0.5M phosphate buffer pH5.6 previously gassed with nitrogen. 100 mg potassium ferricyanide was added, and the mixture left to react for 30 minutes under nitrogen. The reaction mixture was diluted with N_2 gassed water to a conductivity of less than 10^{-1} mhos cm^{-1} and chromatographed on DE52 (section 2.5). The reaction product was detected by its UV absorbance or radioactivity as it eluted from the column, collected and freeze dried. The unlabelled compound was observed to give the UV spectrum of 5Me,5,6,DHF as published by Gapski et al (1971). 5MeDHF was held as the solid at -20°C until required for dosing, when it was made up in nitrogen gassed water.

Pyrazino-s-triazine oxidation product of 5MeTHF "4aOH 5MeTHF" (see fig 5.2 Chapter 5)

This compound is described as 4aOH 5MeTHF by Gapski et al (1971) but this structure was revised by Jongejan et al (1979) as a pyrazino-s-triazine. The compound

was prepared as in the proceedure given by Gapski et al (1971). 50mg of 5MeTHF or 10 μCi 5(^{14}C)MeTHF was dissolved in 10 cm^3 of 0.1M sodium acetate. 0.5cm^3 of 30% v/v H_2O_2 was added to 10cm^3 of 0.1M sodium acetate, followed by the 5MeTHF solution. The pH of the solution was adjusted to 6 with 1M acetic acid, and the reaction proceeded for 1 hour at room temperature. The compound was then purified by DE52 ion exchange (section 2.5) and desalted by passage through a G15 column equilibrated with water. The UV spectrum of the unlabelled compound agreed with that given by Gaspki et al (1971) for 4aOH5MeTHF, and the ^{14}C labelled species was chromatographically inseparable from it on G15 and DE52 columns.

The radiolabelled species was stored at -20^{o}C, the non-labelled compound was recrystallised, after addition of excess barium nitrate, from methanol as the barium salt.

2.3 Experimental

The radiotracer solutions were made up in 50mM Na phosphate buffer pH7 containing 2% sodium ascorbate, with the exception of 5Me 5, 6, DHF solutions, and the animals were lightly anaesthetised with ether prior to oral or intraperitoneal (IP) dosage. The animals were usually dosed with 0.2 cm^3 of solution with a maximum volume of 0.4 cm^3 for oral dosing. The oral dose was via a modified steel horse serum needle, made curved with a bulbous tip to enable it to be introduced down the animal's oesophagus without damage. IP dosage was via a short hypodermic needle, inserted at a shallow angle through the spread skin, into the abdomen to one side of the mid-line to avoid the bladder.

The animals were placed in wire-floored glass 'metabowl' metabolism cages (Jencons Ltd. Herts), which enabled collection of urine and faeces in separate flasks. Carbon dioxide was collected by passing a stream of air through the apparatus and trapping the expired CO_2 in 100 cm^3 of 2M NaOH. The flasks for urine collection were covered with silver foil and contained 5cm^3 of 50mM Na phosphate buffer pH7 plus 100 mg of sodium ascorbate to minimise photolysis and oxidation of urinary folates. Typically urines were collected from 0-6hr, 6-24hr, 24-48hr, and 48-72 hr., with faeces and CO_2 collected daily. During the period of the experiment the animals had access to food and water ad libitum. Throughout the experiment

the metabowls were housed in a constant temperature ventilated, windowless animal room which was illuminated from above on a 12 hour light/12 hour dark cycle. This was to avoid the differences in folate excretion between animals under differing light conditions (Beavon 1973). Furthermore the time of dosing was kept within the period 9.30 to 10.30 a.m. to avoid any interexperimental differences due to the effects of circadian rhythm.

After three days the animals were killed by stunning and cervical dislocation. Various organs were excised, typically liver, kidney, muscle (from hind leg), brain, spleen, small intestine and tumour.

Some tissue, usually liver and tumour was extracted by the method of Barford et al (1977), a modification of the proceedure of Bird et al (1965). The excised tissue was diced into 2mm cubes and dropped immediately into boiling 50mM phosphate buffer pH7 containing 2% ascorbate. After cooling, the extract was homogenised and spun down, the supernatant being retained for chromatography.

2.4 Estimation of Radioactivity

The individual rat urines were collected and made up to a known volume (usually 25cm^3) with phosphate buffer. Aliquots (0.5cm^3) in duplicate, were diluted with an equal volume of water and 10cm^3 of scintillant (1 litre of toluene containing 5g PPO 0.1g POPOP plus 500 cm^3 of Fisons emulsifier mix no 1) added. Tissue extracts were similarly prepared. The scintillation vials were counted in an NE 8310 scintillation counter (Nuclear Enterprises, Edinburgh) with the windows set for similtaneous estimation of ^{14}C and ^{3}H activity. Corrections for quenching and 14C overlap were made by the external standard method. These settings gave an efficiency of counting of 74% for ^{14}C and 31% ^{3}H. NaOH CO_2 traps were counted by taking 200µl aliquots, diluting to 1cm^3 and estimated in the same manner as urines. The individual rat urines were pooled, after sampling, for chromatographic analysis. For counting of strips from paper chromatographs, the paper was added to 10cm^3 of scintillation cocktail from which the emulsifier had been omitted.

Faeces and tissue were freeze dried and ground up for radiactivity estimation. Samples, = 100 mg, of the tissue or faece were weighed out into quartz boats and burned catalytically in the stream of oxygen of a biological materials oxidiser (Beckman Ltd). The resultant gases passed through two traps; the first in a dry ice/acetone bath to freeze water vapour, the second containing 15cm^3

of alkaline absorber (Absorber P; Fisons , Loughborogh , Leics.) to trap CO_2 . The first trap was washed out with 10cm^3 of Fison's Absorber H, which contains dimethyl sulphoxide to take up water, into a scintillation vial. These two scintillant solutions were counted in the NE 8300 set wide open to give a 91% efficiency for ^{14}C and 35% for ^{3}H.

2.5 Chromatography

Ion exchange : Urines were fractionated (25 to 150cm^3) on diethylaminoethyl cellulose (DE52 ; Whatman Ltd., Maidstone, Kent) ion exchange gel. The gel was equilibrated with several changes of 50mM phosphate buffer pH7 until the filtrate had the same conductivity as the equilibrating buffer. The gel was allowed to stand and the suspended fines decanted off, degassed, and typically, packed in a 50 x 2 cm glass column plugged with glass wool. Non radioactive markers e.g. 5MeTHF and 10CHOFA were added to the urine and its conductivity adjusted to that of 50mM Na phosphate with water. The solution was loaded onto the column by peristaltic pump and the effluent collected for counting to ensure that the capacity of the column was not exceeded. The adsorbed substances were eluted by a linear salt gradient (0 to 1.2M NaCl in 50mM Na phosphate pH7) provided by a mixer system (Ultragrad gradient maker, LBK instruments, Croydon). The eluant was passed through a scintillation detector flow cell (Nuclear Enterprises) to follow radioactivity,and an ultraviolet monitor, (UVcord II LBK instruments) set to 254nm. to detect the

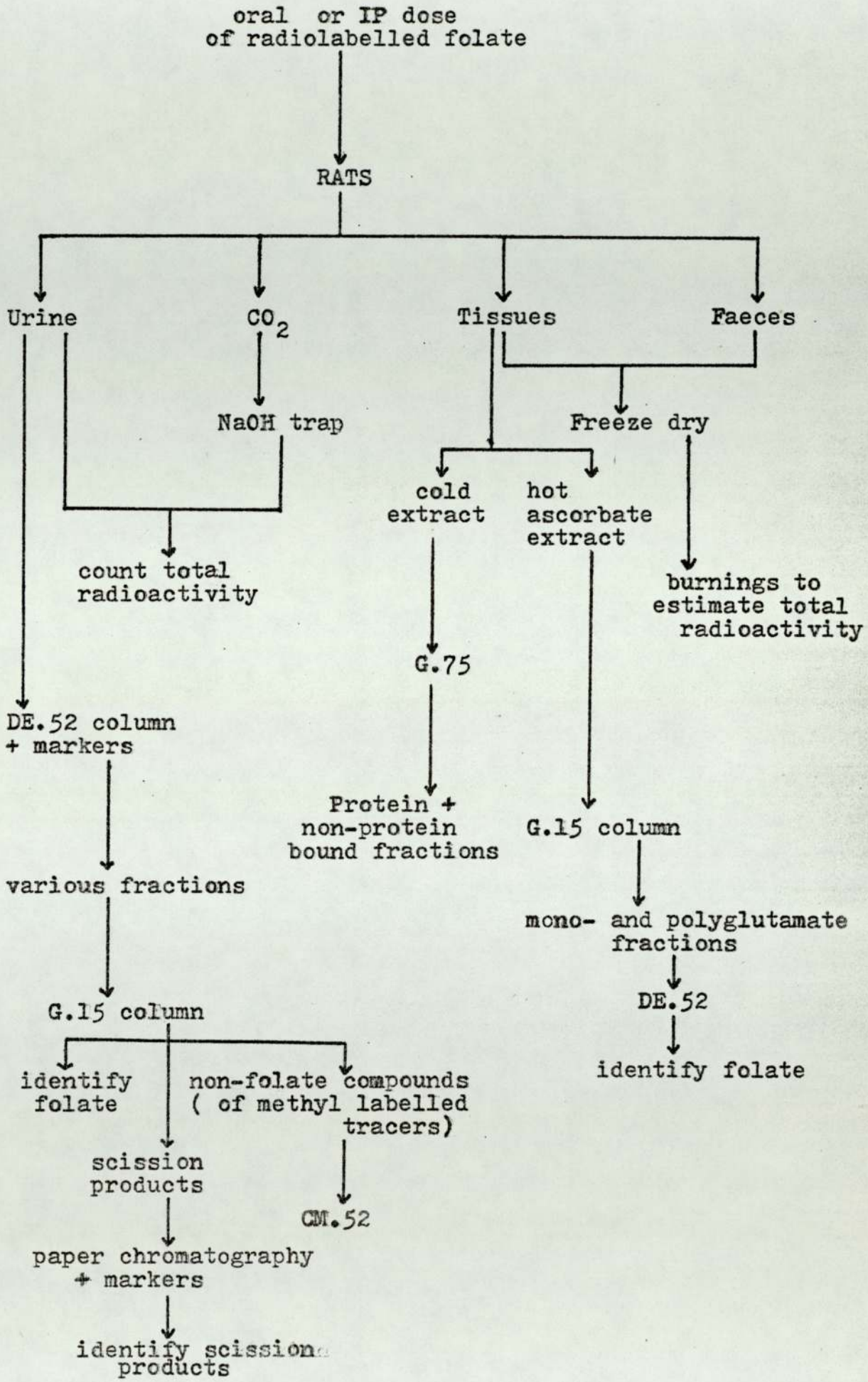
folate markers. The eluant was automatically collected in 5cm^3 aliquots, usually 100, by an ULTRARAC fraction collector (LBK instruments). The gradient was set to run for eight hours overnight. Occasionally gel of the opposite charge was used, carboxymethyl cellulose (CM 52: Whatman Ltd., Maidstone, Kent) for fractionation of positively charged urinary metabolites derived from 5(^{14}C)MeTHF, the non-folate fraction (NFF). The gel was prepared and elution carried out using the same buffer systems and apparatus as DE52.

0.5cm^3 of each aliquot were diluted with an equal volume of water and counted in the same manner as urine samples. The salt gradient was determined by pooling every 10th and 11th sample and determing its electrical conductivity compared to the initial and final buffers.

Gel Filtration: Routinely G15 and occasionally G75 Sephadex (Pharmacia, Uppsala, Sweden) were used. Chromatography on G15 was used to further fractionate the peaks from DE52 columns and for primary fractionation of hot ascorbate tissue extracts, separating early eluting high molecular weight polyglutamate from later eluting monoglutamate. G75 columns were used to resolve cold tissue extracts into protein bound (eluting at the void volume) and non-protein bound (later eluting) fractions. (A flow diagram of the course of a typical folate metabolic experiment is given in figure 2.1)

The Sephadex gel was swollen in 50mM phosphate buffer pH7 for three hours, degassed, and packed under pressure

Fig.2.1. Protocol of Folate Metabolism Study



in purpose designed Perspex columns (Wright Scientific Ltd., Surrey) 1.5cm x 60cm. The sample, 25cm^3 or less, was loaded and eluted with degassed phosphate buffer. The eluant was monitored and collected by the same system as for DE52 columns, or by a UVcord II and REDIRAC automatic fraction collector (LBK instruments). Usually 60 5 cm^3 fractions were collected, of which 1cm^3 of each was taken undiluted for scintillation counting.

Paper chromatography: Paper chromatograms were used to resolve ^3H scission products from mixed label folate experiments. The freeze dried sample was dissolved in a minimum volume of methanol/H_2O (95:5 by volume) and standards (p.aminobenzoyl glutamate, p.aminobenzoic acid and their acetyl derivates) added. The resulting solution was spotted onto 3MM paper (Whatman Ltd, Maidstone, Kent.) as were the individual standards as markers. The paper was developed overnight in a tank pre equilibrated with the appropriate solvent, either propanol/0.880* ammonia/water (200:1:99) or butanol/ethanol/0.880 ammonia/water (10:10:1:4). The paper was dried and the position of the markers noted. The paper was then cut into 1cm wide strips for scintillation counting.

Detection of Chromatography markers: Unlabelled standard folates were located in the chromatography column eluants by their UV absorbance trace from the UVcord II. Their identity was confirmed by determining their UV spectrum in an SP 1700 spectrophotometer (Unicam Instruments, Cambridge.

* Specific gravity

Creatine and creatinine markers were detected via the Jaffe reaction for creatinine as described by Selgison (1961). To $3cm^3$ of the column effluent $1cm^3$ of picrate reagent (16g/l picric acid heated to $80^{\circ}C$, cooled and 690 cm^3 of the saturated supernatant made up to 1l with water) and $4cm^3$ of ether were added with shaking for 30 seconds. $3cm^3$ of the clear lower phase were drawn off and 0.5 cm^3 of 0.75M NaOH added. After 20 minutes the absorbance of the solution was read at 520nm. For determination of creatine $0.5cm^3$ of picrate solution was added to $3cm^3$ of column effluent and boiled for $1\frac{1}{4}$ hours. A further 0.5 cm^3 of picrate was added to the cooled solution its volume adjusted to $4cm^3$ and then treated in the same manner as creatinine solutions.

The positions of standard compounds on paper chromatographs were marked by their fluoresence or absorbance under ultraviolet light.

3. THE ROLE OF 5METHYLTETRAHYDROFOLIC ACID AS A ONE CARBON DONOR

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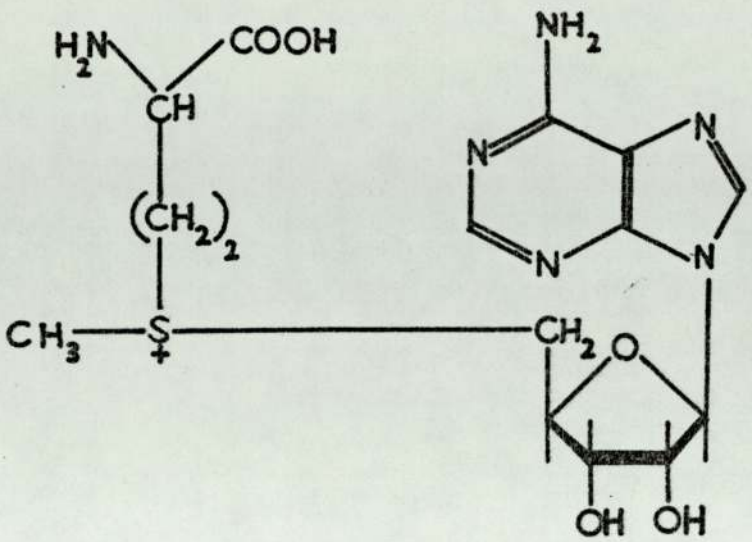
3.5.	Discussion	91

3.1 Introduction: S-Adenosyl methionine (SAM) fig 3.1 is the major methylating agent in biological systems, the widespread occurence of methylation reactions in metabolic pathways results in SAM being second only to ATP in the variety of reactions in which it is a cofactor (Salvatore et al 1977). SAM provides methyl groups for the synthesis of choline, steroids, creatine, carnitine, fatty acids, biogenic amines and nucleic acids (Cantoni 1975) fig 3.2. The methylation of catechol amine neurotrasmitter substances, noradrenalin and dopamine plays an important role in nerve transmission (Axelrod 1957) and disturbances of methylation reactions in the brain has been implicated in the aetiology of mental disease (see chapter 1(2)). The specific methylation of nucleic acids is suggested to have a function in determining the tertiary structure of tRNA's (Nau 1976) and as a protection mechanism for bacterial DNA where a 'restriction endonuclease' catalyses the lysis of inappropriately methylated foreign DNA (Meselson et al 1972). In higher animals DNA methylation had been proposed as a mechanism involved in cell differentiation (Scarano et al 1977).

There are only two other characterised methylating systems in mammals, both of which synthesise methionine from homocysteine. The betaine: L homocysteine methyltransferase (EC 2.1.1.5) system is a salvage pathway for preformed methyl groups, however, it is restricted to the liver in the rat (chapter 1(3)). The other, 5-methyltetrahydropteroyl L-glutamate; L homocysteine 5-methyltransferase (EC 2.1.1.13 'methionine synthetase') donates denovo synthesised methyl

Fig.3.1. S-Adenosyl Methionine and S-Adenosyl Homocysteine

S-adenosyl Methionine (SAM)



S-adenosyl Homocysteine (SAH)

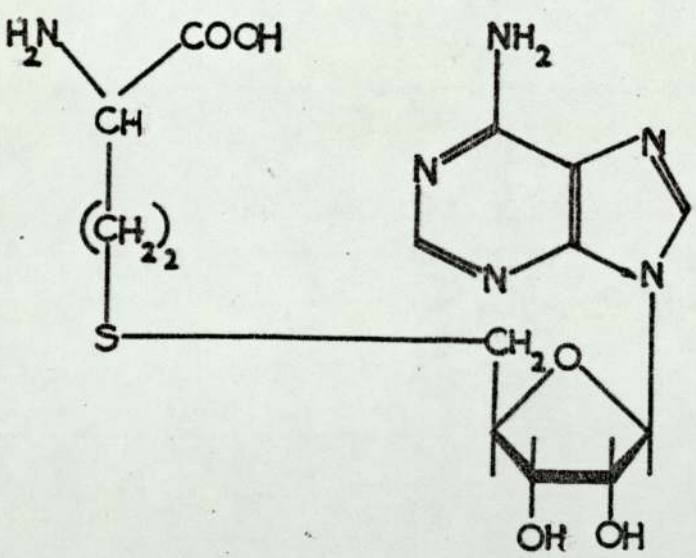
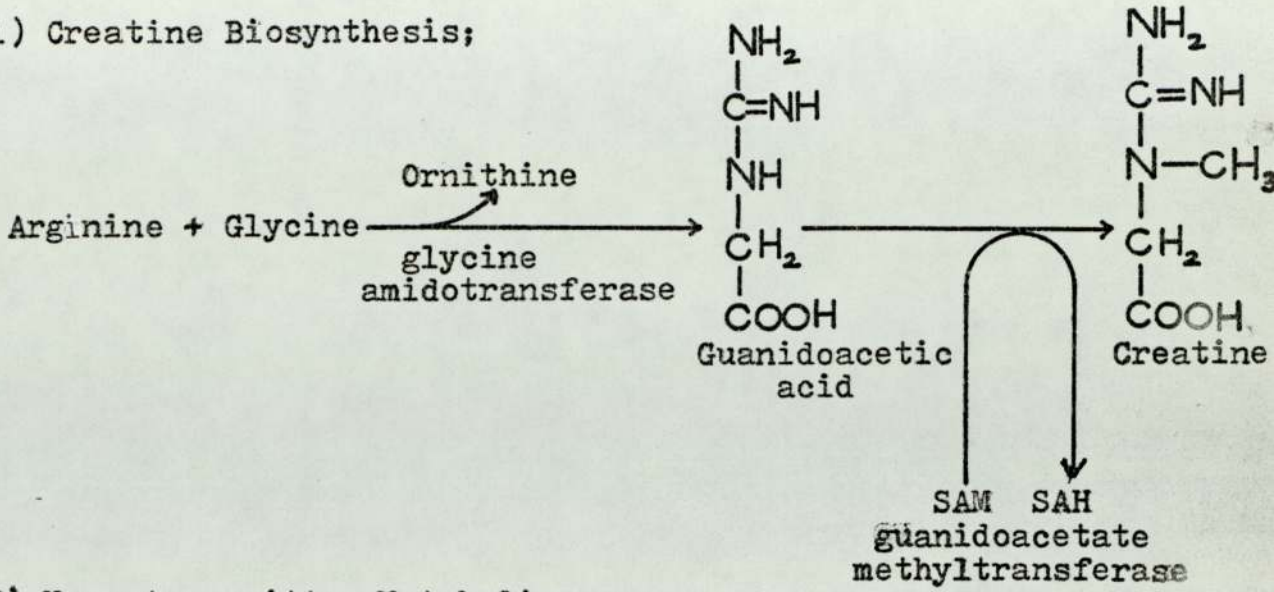
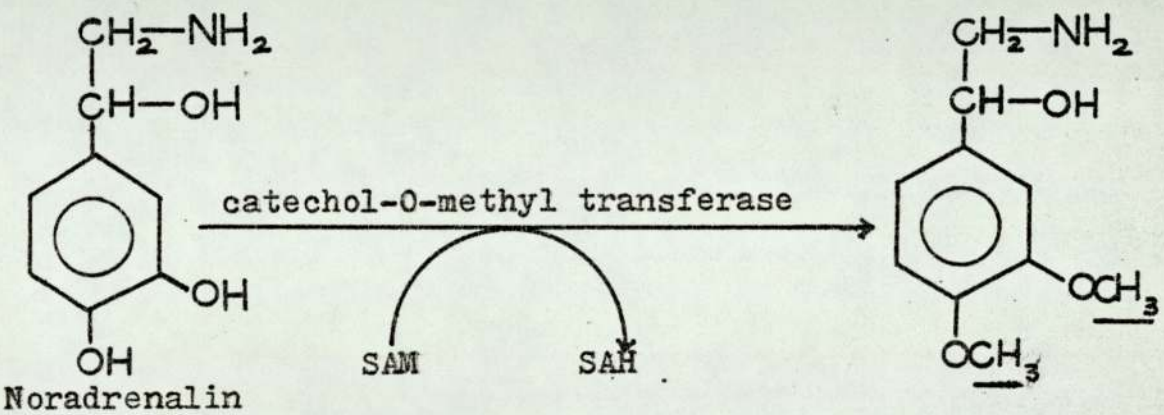


Fig.3.2. Methyl Reactions of SAM

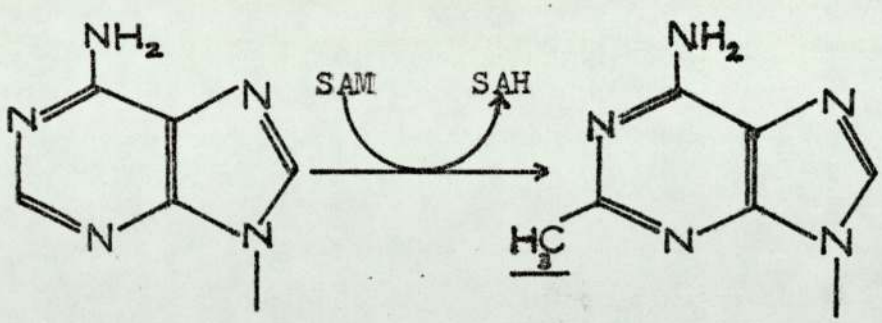
1) Creatine Biosynthesis;

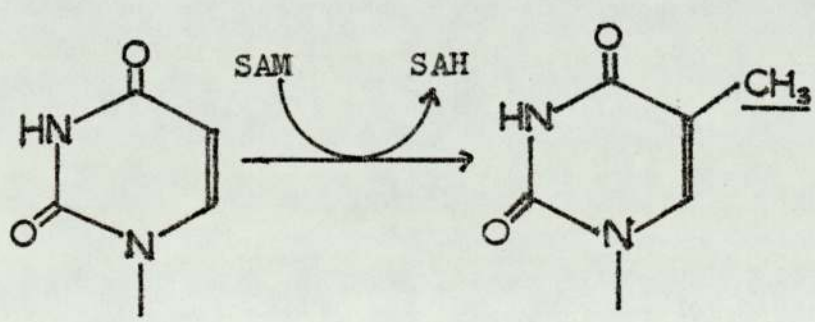


2) Neurotransmitter Metabolism eg:



3) Nucleic Acid Methylation



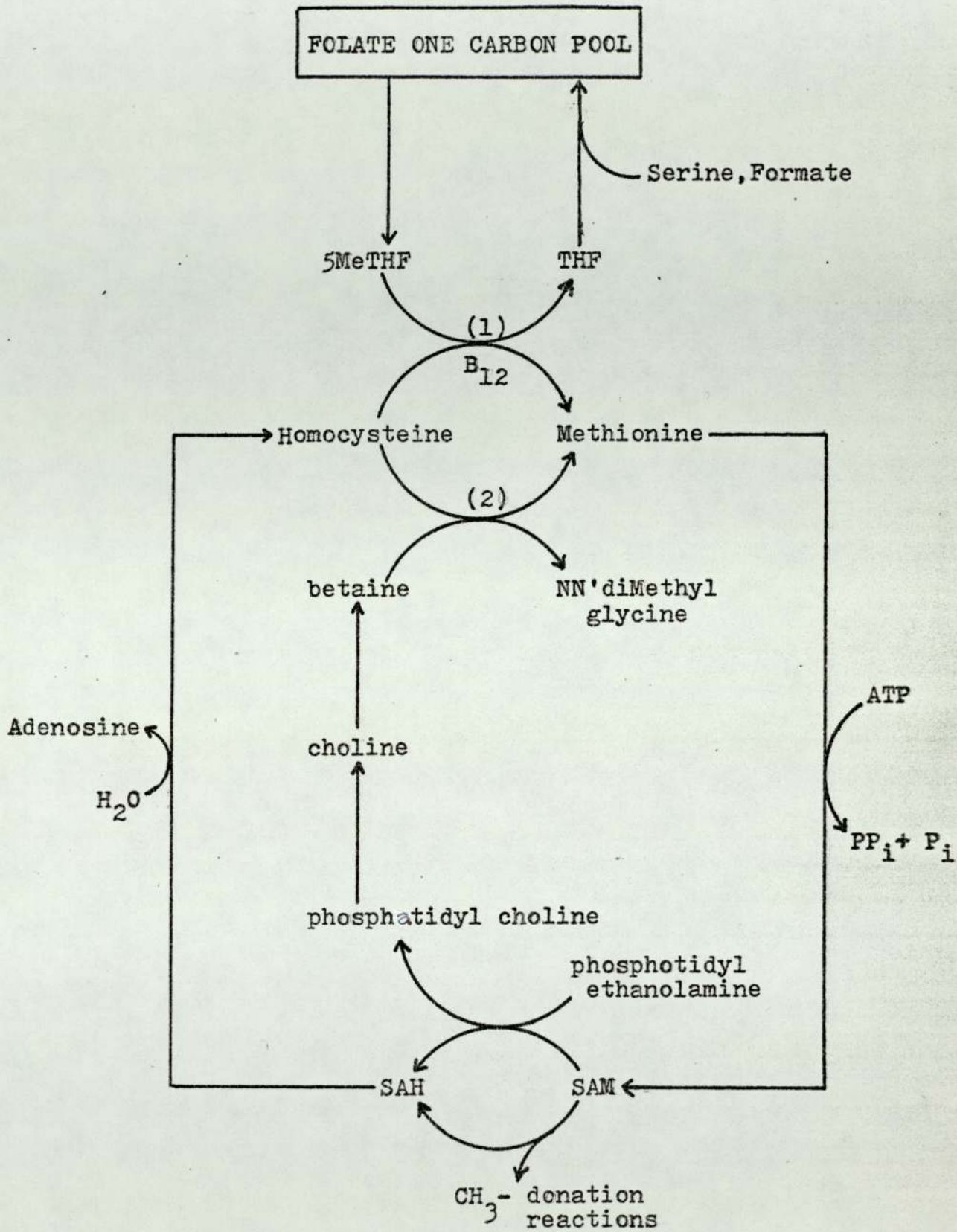


groups furnished from the folate one carbon pool (fig 3.3) The work of Mudd and Poole (1975) (Chapter 1.1) indicates that the entry of folate one carbon into the labile methyl pool is regulated by the body requirements for transmethylation, denovo synthesis contributing from 24 to 67% of the body methyl turnover depending on the labile methyl (methionine and choline) present in the food intake.

In animal systems cobalamin, vitamin B_{12}, is required as a cofactor in the methionine synthetase reaction (Amstein and Neuberger 1953). In E.coli a non-cobalamin requiring pathway using 5MeTHF triglutamate as the methyl donor is reported (Salem and Foster 1972) in addition to the cobalamin requiring route. Non-cobalamin requiring pathways have been observed in Aerobacter aerogenes (Morningstar et al 1965), Salmonella typhimurium (Cauthen et al 1966), Sacchromyces cerevisiae (Botsford and Park 1967) and the higher plants (Guest et al 1964), however, such pathways have not been reported in animals.

Deficiency of cobalamin in man, which may occasionally arise due to nutritional deficiency, or more usually, by lack of the cobalamin transport protein the 'intrinsic factor' in the gut may result in pernicious anaemia which is haematologically indistinguishable from megaloblastic anaemia caused by folate deficiency (Blakley 1969 P.439). Vitamin B_{12} deficiency however, has neurological symptoms not observed in cases of folate deficiency which may be exacerbated by doses of folate inappropriately administered to correct the megaloblastic anaemia (Rowe 1978).

Fig.3.3. Synthesis and Metabolism of S-adenosyl Methionine



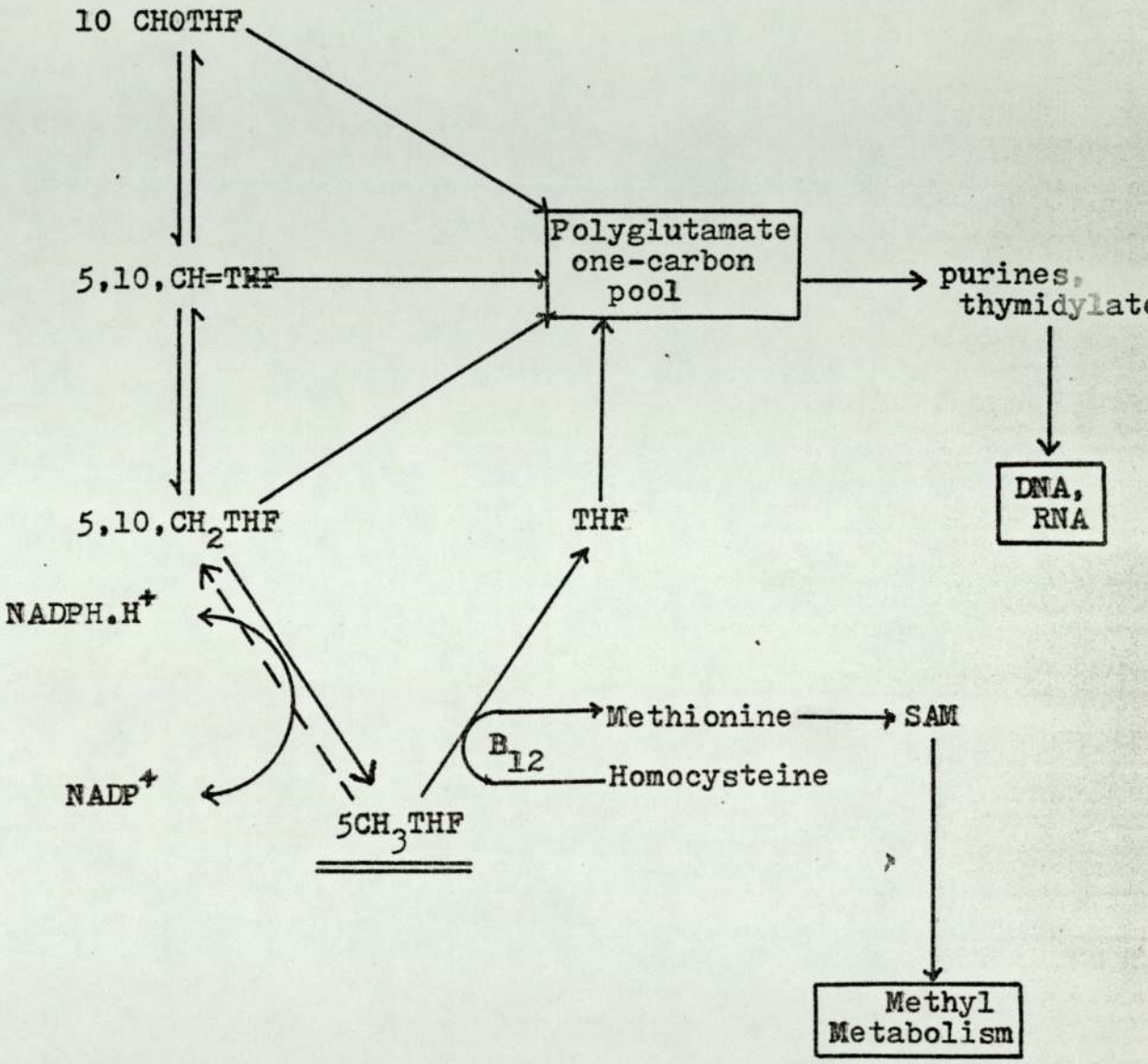
(1) Methionine Synthetase - 5MeTHF:Homocysteine methyltransferase EC 2.1.1.13

(2) Betaine:Homocysteine methyltransferase EC 2.1.1.5

The 'methyl tetrahydrofolate trap' proposed by Norohana and Silverman (1962) and Herbert and Zalusky (1962) with its later modifications (see chapter 1) explains the haematological effects of B_{12} deficiency as the trapping of folate as 5-Methyltetrahydrofolate due to decreased methionine synthetase activity, with consequential falls in the formyl and bridged monoglutamates and polyglutamates. Underlying the theory is the assumed irreversability of methylene THF reduction under normal redox conditions, as shown by Kutzbach and Stokstad (1971) in vitro. However, Stebbins et al (1976) noting the work in which in vitro tissue preparations, under oxidising conditions, could generate formaldehyde suggested the reaction may have an in vivo role in specific instances viz: the metabolism of tryptamine in blood platelets. This reaction if widespread would invalidate the 'methyltetrahydrofolate trap' by enabling one carbon units to be released from 5MeTHF other than by methionine synthesis, (fig 3.4) enabling other purine and thymidylate forming species to be produced.

Experiments were undertaken on rats using 5(^{14}C)MeTHF to observe the distribution, metabolism and excretion of the methyl group and its fate compared to the methyl group of (^{14}C methyl) methionine. If, as the 'methyl trap' hypothesis postulates, methionine synthesis is the sole route of the 5MeTHF one carbon, the methyl groups of both methionine and 5MeTHF ought to follow the same qualitative pathway. Also animals were dosed with a mixture of 5(^{14}C)MeTHF and (^{3}H)FA to simultaneously label the labile methyl and folate pools,

Fig.3,4. Possible entry of 5MeTHF methyl into folate one-carbon pool



in order to observe any entry of ^{14}C from 5MeTHF into other folates

3.2 Dosing of Rats with 5(^{14}C)MeTHF

Male Wistar rats (200g) were dosed either orally or intraperitoneally with 2μCi of 5(^{14}C)MeTHF in 50mM Na phosphate buffer pH7 containing 2% Na Ascorbate (80μg/Kg oral, 60μg/Kg I.P.). The collection of urine, faeces, CO_2, and tissue was as described in Chapter 2. The distribution of radioactivity and chromatographic separation of urinary activity is given in tables 3(1) to 3(4).

Results and Discussion

The radiotracer 5(^{14}C)MeTHF administered to the animals had been synthesised after the method of Keresztesy and Donaldson (1961) which produces an equimolar mixture of the epimers about C_6 , only one of which is active for methionine synthetase (Blakley 1969). The faecal activity collected 24 hrs. after oral administration was low, 2.3% table 3(3), indicating absorbtion of 5MeTHF is in excess of 97%, however, the value may be nearer 100% as the first day faecal ^{14}C activity after I.P. dosage (table 3(1)) is not significantly different (P 0.05 'Students' t test). This indicates 5MeTHF absorbtion from the gut proceeds without discrimination between the C_6 epimers.

Chromatography of the pooled urines separated the ^{14}C activity of the first day urines into two peaks on both G15 and DE52 columns (see fig.3.5, table 3(2) I.P. dose, table 3(4) oral dose). The first, minor, fraction eluted from DE52 ion exchange columns at salt values of less than 0.1M NaCl and was termed the non folate fraction (NFF), as

Table 3(1)

Distribution of ^{14}C activity of Rats IP Dosed with 60 µg/Kg 5(^{14}C)MeTHF

% dose ^{14}C; vlaues mean of 4 rats ± S.E.M.

	Day 1	Day 2	Day 3	Total
Urine	38.7 ±2.6	2.4 ± 0.5	1.1 ±0.15	42.2
Faece	1.4 ±0.4	1.4 ± 0.4	0.5 ±0.1	3.2
Liver			0.9	0.9
Kidney			1.1	1.1
Spleen			0.5	0.5
	40.1	3.8	4.1	47.9

Table 3(2)

Chromatographic separation of urinary ^{14}C activity from Rats IP dosed 60 µg/Kg with 5(^{14}C)MeTHF

G15 Column:

	Fraction	Identity	%dose ^{14}C	% urine ^{14}C
Day 1	I	NFF	2.4	6.2
	II	5MeTHF	35.0	90.5
Day 2	I	NFF	0.6	24.4
	II	5MeTHF	1.6	65.1
Day 3	I	NFF	0.2	18.9
	II	5MeTHF	0.6	50.3
Total			40.4	

DE52 Column:

	Fraction	Identity	%dose ^{14}C	%Urine ^{14}C
Day 1	I	NFF	2.6	7.0
	II	TRIAZ*	32.7	88.5
Day 2	I	NFF	0.5	20.0
	II	TRIAZ	1.9	79.9
Day 3	I	NFF	0.4	32.1
	II	TRIAZ	0.6	46.4
Total			38.7	

*TRIAZ: triazine oxidation product of 5 MeTHF, shown to be an analylical artifact.

Table 3(3)

Distribution of ^{14}C activity of Rats orally dosed 80 µg/Kg with 5(^{14}C)MeTHF

% dose ^{14}C activity values mean of 4 Rats ± SEM

	Day 1	Day2	Day 3	Total
Urine	50.9 ±4.6	1.9 ±0.2	1.2 ±0.2	54.0
Faeces	2.3 ±1.0	0.1 ±0.03	0.02 ±0.01	2.4
CO_2	2.0	0.4	0.4	2.8
Liver			0.1	0.1
Muscle			13.2*	13.2
Kidney			0.1	0.1
Spleen			0.01	0.01
Total	55.2	2.4	15.0	72.6

*Calculated assuming muscle = 40% body weight.

Table 3(4)

Chromatographic fractionation of ^{14}C urinary activity from rats orally dosed 80 µg/Kg with 5(^{14}C)MeTHF

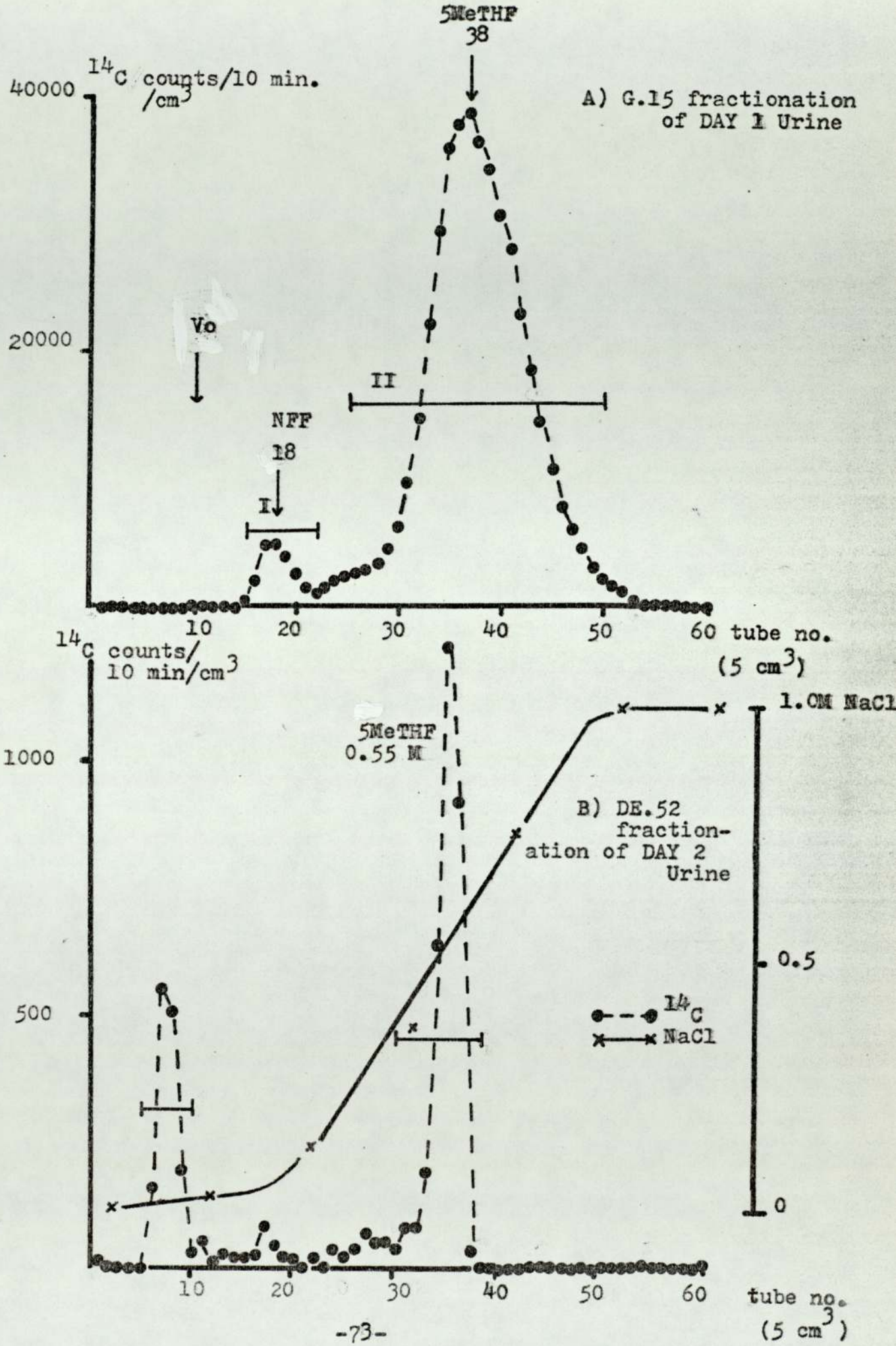
a) G15 Column:

	Fraction	Identity	%dose ^{14}C	%Urine ^{14}C
Day 1	I	NFF	2.7	5.0
	II	5MeTHF	48.0	88.9
Day 2	I	NFF	0.58	30.4
	II	A	0.36	18.8
	III	5MeTHF	0.65	34.1
Day 3	I	NFF	0.32	24.7
	II	A	0.26	19.6
	III	5MeTHF	0.59	45.1
			53.5	

b) DE52 Column

	Fraction	Identity	%dose ^{14}C	%Urine ^{14}C
Day 1	I	NFF	3.2	5.9
	II	5MeTHF	47.8	88.5
Day 2	I	NFF	0.52	27.0
	II	5MeTHF	1.07	55.8
Day 3	I	NFF	0.52	44.7
	II	5MeTHF	0.42	32.3
			53.6	

Fig.3.5. Typical Chromatograms of ^{14}C urine activity from rats dosed orally with 80µg/Kg 5(14C)MeTHF



all known folates are observed to elute from DE52 in excess of 0.2M. The elution of NFF indicates it did not contain the major methylated purines and pyrimidines found in human urine (Chheda 1975) fig 3.6, however, both methionine and creatine elute at the same position as NFF from DE52 and G15 columns (fig 3.7a). When treated with acid (pH1) which catalyses the dehydration of creatine to creatinine (Edgar and Striver 1925) the day 1 NFF from the orally dosed animals showed 38% of ^{14}C eluting with added creatinine with 56% remaining at the methionine elution position fig 3.7b. On days 2 and 3 of the oral dose experiment G15 shows a third non-folate peak eluting after the NFF and creatinine marker at tube 25 or 26 (Table 3(4)a), designated'A'.

The major urinary fraction observed after oral dosing eluted from both G15 (tube 36-39) and DE52 (0.5M-0.6M NaCl) with added unlabelled 5 MeTHF table 3(4). In the earlier IP dosage experiment, table 3(2), showed the major day 1 peak eluting with 5MeTHF from G15. However, when chromatographed on DE52 the major ^{14}C urinary fraction was observed to elute at 0.25M NaCl, well below the elution position of 5MeTHF. Rechromatography of the ^{14}C peak on G15 and DE52 showed it to be inseparable from the peroxide oxidation product of 5MeTHF described as 4aOH5MeTHF by Gapski et al (1971), later reformulated as a pyrazino-s-triazine by Jongejan et al (1979), when used as a marker. Thus the difference in chromatographic behaviour of the urine radioactivity with time is ascribed to the oxidation of 5MeTHF to its corresponding triazine, possibly resulting from

Fig.3.6. Elution of Methylated Bases from G15 columns

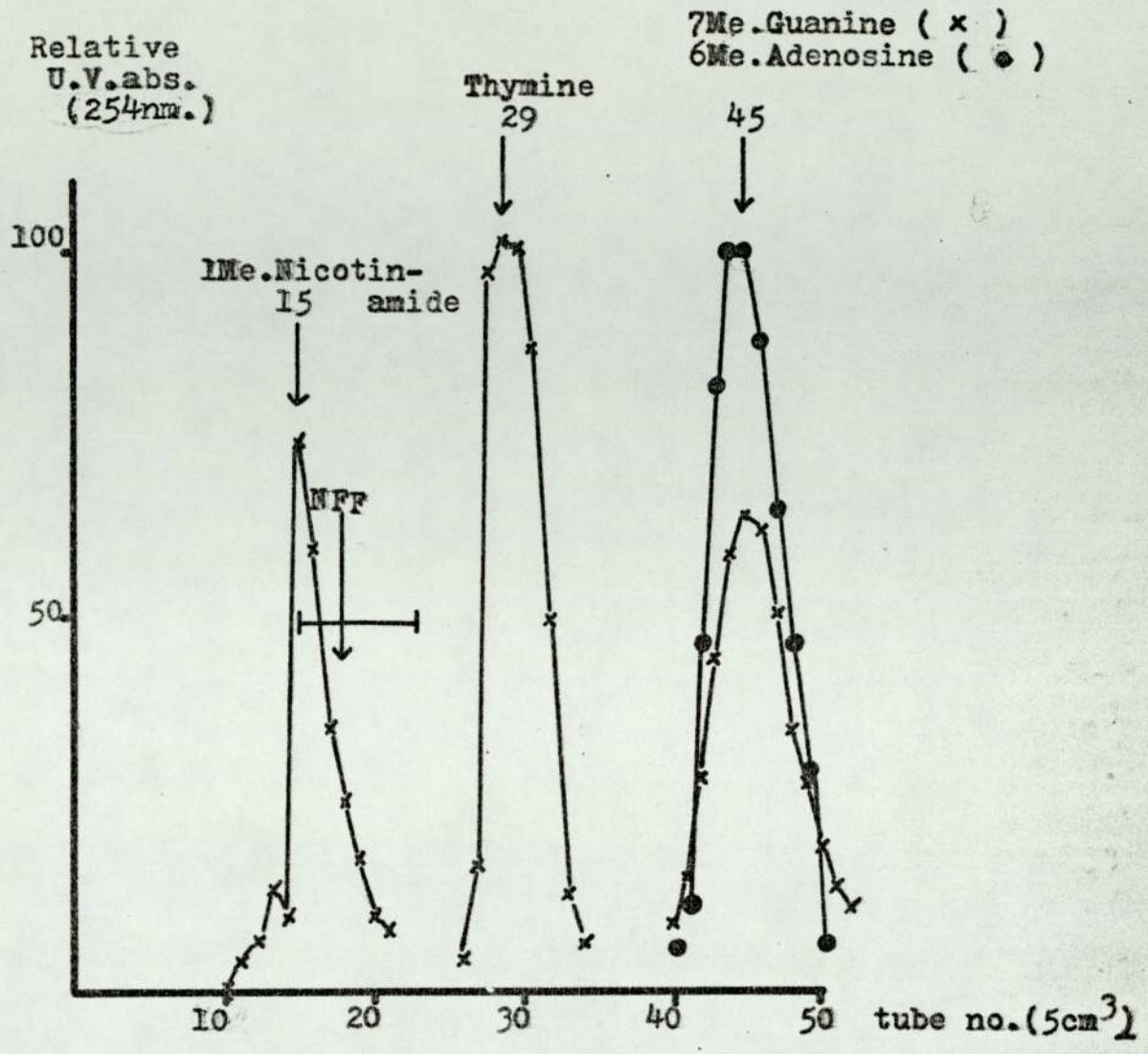
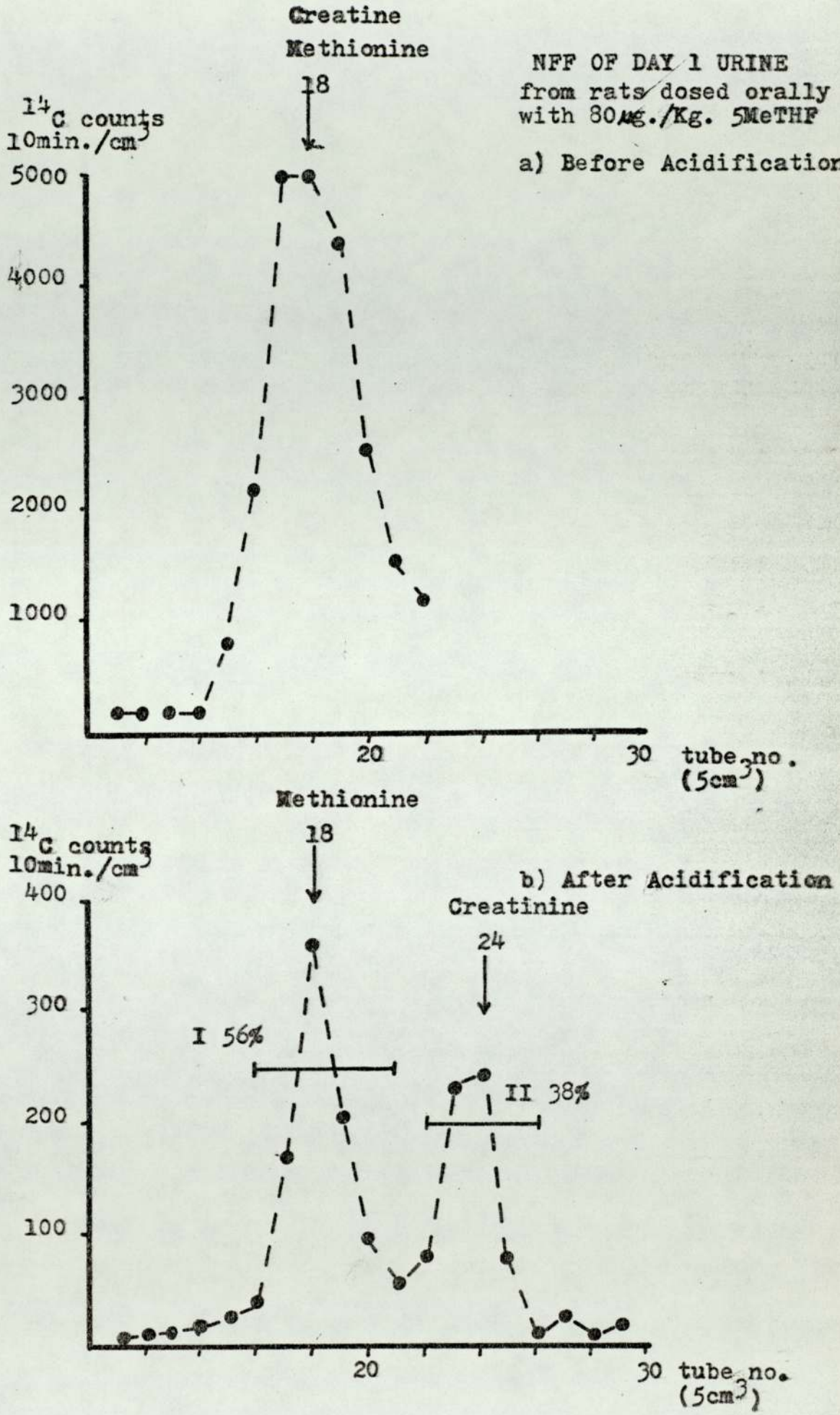


Fig. 3.7 - Elution of Non-folate fraction (NFF) from G15 columns



the depletion of ascorbate in the sample while handling in air. This effect also may explain the discrepancy of the 5MeTHF proportion of the day 2 urine of the orally dosed rats between G15 and DE52 columns (0.65% compared to 1.07% dose ^{14}C table 3(4)), as the triazine elutes at tube 18-22 and may coelute with the NFF which elutes at tube 16-18 (fig 3.7). Following this observation the level of antioxidant was maintained by additions of supplementary sodium ascorbate whenever the urines were sampled for chromatography. This method followed during the oral dose experiment, showed only the presence of 5MeTHF in addition to the non-folate compounds, with the possible exception of the second day urine, suggesting that when observed (Barford and Blair 1976) that the triazine is an artifact and not a normal metabolite of the folate pool.

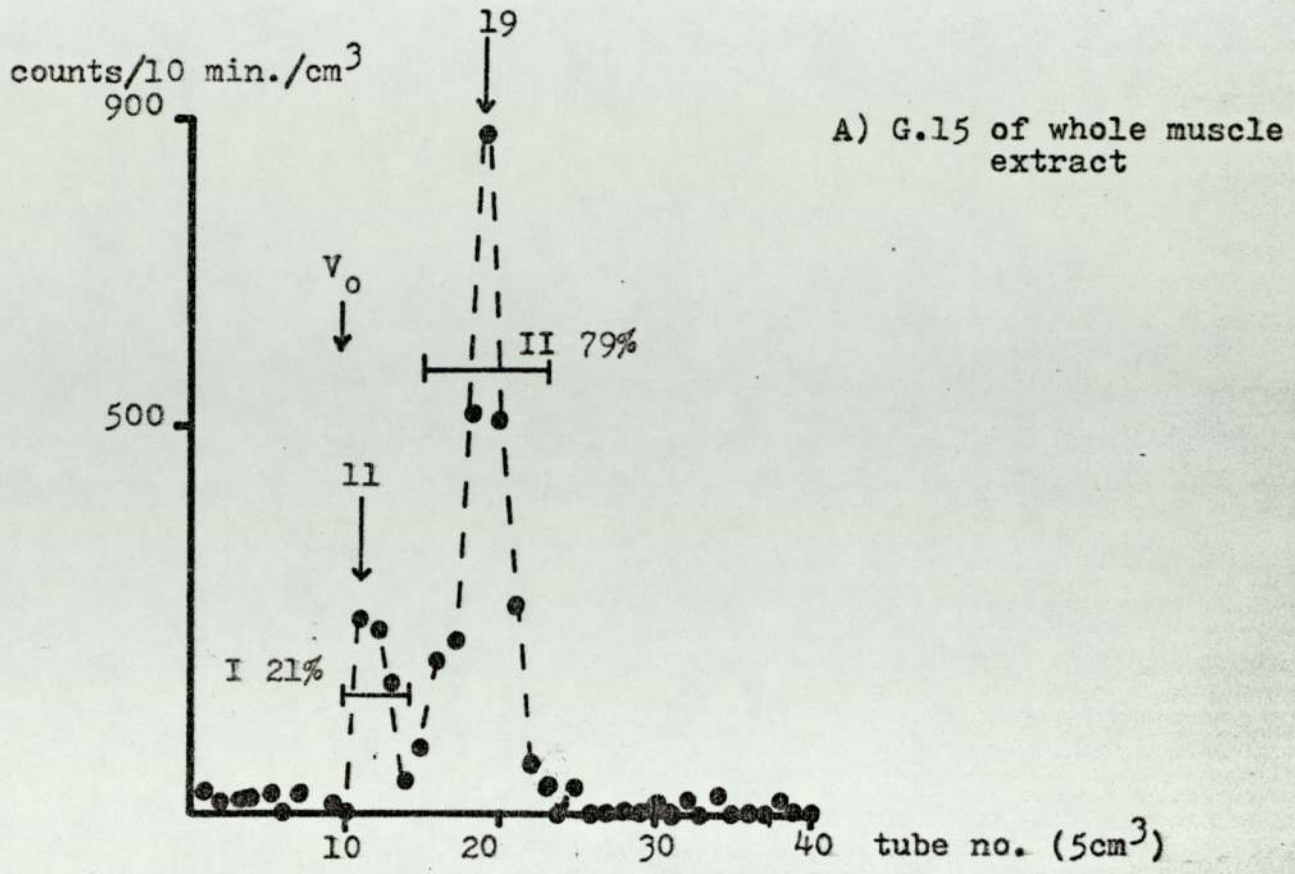
Retention of 5MeTHF ^{14}C activity in the liver and kidney was low, of the order of 1% (tables 3(1), 3(3))., while hot ascorbate extracts of the day 3 liver had no ^{14}C labelled polyglutamates, the retained activity eluting from DE52 at less than 0.1M NaCl, while polyglutamates elute in excess of 0.5M. This would be expected as 5MeTHF is observed to be a poor direct substrate for polyglutamate synthesis(Lavoie et al 1974, Spronk et al 1973, Sakami et al 1973, Blair et al 1976, McGuire et al 1979).

Alternatively, this experiment may be inadequate to detect 5(^{14}C)MeTHF polyglutamates, should the rate of synthesis be slow compared to the rate of demethylation of the monoglutamate, or if once formed the demethylation of 5MeTHF glu_x proceeds at such a rate to preclude its detection

after 3 days. The greatest ^{14}C retention was in the muscle (Table 3(3)), 21% of muscle ^{14}C as high molecular weight compounds and 79% eluting at the position of urinary NFF on G15 (fig 3.8a). The high molecular weight compounds are unlikely to consist of negatively charged species such as nucleic acids, for the muscle extract radioactivity shows little retention on the DE52 ion exchange gel, thus more likely they are proteins. Acidification of the low molecular weight peak (fig 3.8b) resulted in 93% of the activity eluting with creatinine. Thus the majority of retained ^{14}C from 5(^{14}C)MeTHF is as creatine, the synthesis of creatine being the singlemost consistent demand for methyl groups (Mudd and Poole 1975).

The coincidence that approximately 50% of the oral 5(^{14}C)MeTHF activity is recovered in the day 1 urine as 5MeTHF (table 3(4)) and that half of the dose is biologically inactive suggests that the urinary 5MeTHF is as a result of preferential excretion of the inactive diastereoisomer. To test this possibility the 5MeTHF fraction of the first day urine was desalted, collected, freeze dried, and readministered to rats. Its fate was compared to that of stock 5(^{14}C)MeTHF orally dosed at a similar level. Table 3(5) shows that both 5(^{14}C)MeTHF samples are metabolised to urinary non folate compounds in comparable animals, with a similar ^{14}C retention in tissue, thus indicating they have similar bioavailability in the rat. However, when the 5MeTHF fraction from the second day urine of the 80 µg/Kg oral dose experiment was subsequently readministered to rats, the high recovery as 5MeTHF (85% dose) in the first

Fig.3.8. G.15 fractionation of 50mM phosphate extract of rat muscle after 80µg/Kg oral dose 5(^{14}C)MeTHF



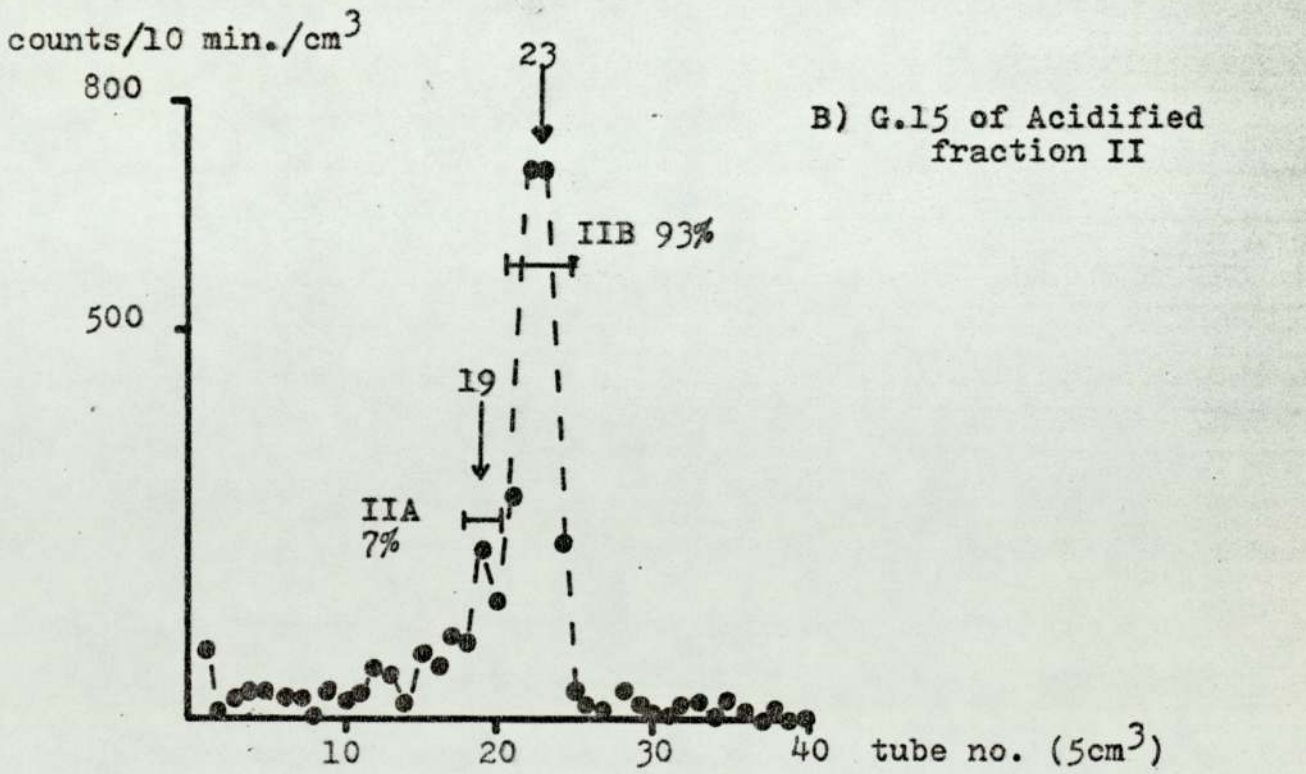


Table 3(5)

Distribution and excretion of stock 5(^{14}C)MeTHF and readministered urinary 5(^{14}C)MeTHF radioactivity

	Urine Extract	Stock Compound
Number of animals	3	7
Dose µg/Kg	1.25	2.0
% dose activity in		
day 1 Urine	12.1	10.8
2	4.0	4.2
3	3.9	2.6
day 1 CO_2	-	2.7
2	-	2.3
3	-	2.4
day 3 Tissue		
Spleen	1.6	0.1
Muscle	4.6	6.8
Kidney	2.2	1.6
Liver	3.0	4.5
Fractionation of 1st day urine; % activity as NFF	17.6	22.1
5MeTHF	71.3	68.3

day urine with no observed non-folate compounds indicated the 5(^{14}C)MeTHF had no detectable biological activity. From these experiments the mixture of diastereoisomers in the first day urine following 80 µg/Kg of oral 5MeTHF is due to renal overloading, with subsequent excretion of mainly the inactive diasteroisomers over the next two days as the active form loses its ^{14}C label in methionine synthesis and there is no excretory mechanism specific to the unnatural epimer.

3.3 L(^{14}C Methyl) Methionine

In order to compare the metabolism of methionine methyl with that of 5MeTHF, rats were dosed orally (22 µg/Kg) or IP (11 µg/Kg) with L(^{14}C methyl)methionine. These doses were the molar equivalent of 80 and 40 µg/Kg doses of 5MeTHF, however, the diet of the rats (Table 2(1) Chapter 2) provided 3200 µg methionine /g while only providing 0.7 µg/g of folate as folic acid. Thus at these levels, 2-5 µg per rat, the (^{14}C methyl) methionine is serving to label dietary methionine, while 16 µg 5(^{14}C)MeTHF tracer per animal would be a load of folate in addition to the diet.

Table 3(6) gives the recovery of ^{14}C activity from (^{14}C methyl) methionine dosed rats, fig 3.10 typical chromatographs and table 3(7) the G15 separation of urinary ^{14}C.

The total urinary radioactivity is lower compared to 5(^{14}C)MeTHF experiments, presumably due to the lower effective dose with labelled methionine. The retained tissue radioactivity shows that after a dose of (^{14}C methyl) methionine the muscle shows a greater ^{14}C retention than liver.

Table 3(6)

Distribution and excretion of ^{14}C activity of (^{14}C methyl) methionine dosed rats 11 µg/Kg IP, 22 µg/Kg oral

Values as % dose activity

		Day 1	Day 2	Day 3	
Urines	IP	3.5	1.6	0.9	6.0
	Oral	4.1	1.2	0.8	6.1
Faeces	IP	2.3	0.3	ND	2.6
	Oral	-	-	ND	-
CO_2	IP	3.5	0.6	ND	4.1
	Oral	3.0	0.9	0.6	5.8
Liver	IP			0.8	0.8
	Oral			ND	-
Muscle	IP			ND	-
	Oral			7.9	7.9

ND: not determined

Table 3(7)

G15 chromatography of ^{14}C activity of urine from (^{14}C methyl) methionine dosed rats

	Fraction	Tube No.	IP dose %Urine	IP dose %dose	Oral dose %urine	Oral dose %dose
Day 1	I	16-19	46.9	1.64	79.2	3.24
	II	25-26	43.0	1.51	17.2	0.73
	Total		89.9	3.15	96.4	3.97
Day 2	I	16-19	52.6	0.84	67.0	0.80
	II	25-26	40.9	0.65	28.3	0.34
	Total		93.5	1.49.	95.3	1.14
	Total			4.64		5.11

Fig.3.9. G.15 Chromatograph of 50mM phosphate extract of muscle from rats dosed 22μg/Kg ^{14}C(methyl) methionine

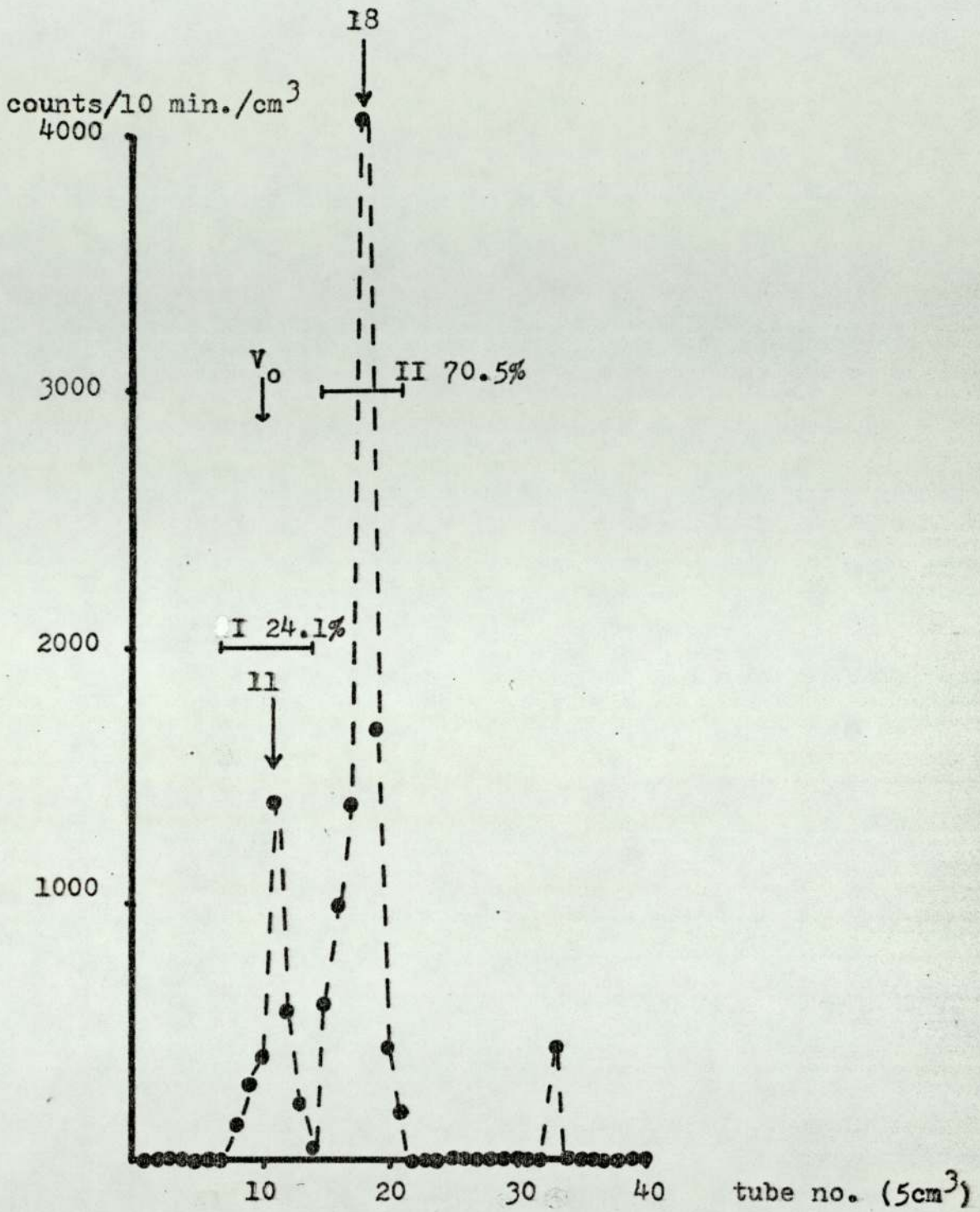


Fig.3.10. Typical Chromatograms of ^{14}C Urine activity from rats dosed I.P. 11µg/Kg (^{14}C methyl) methionine

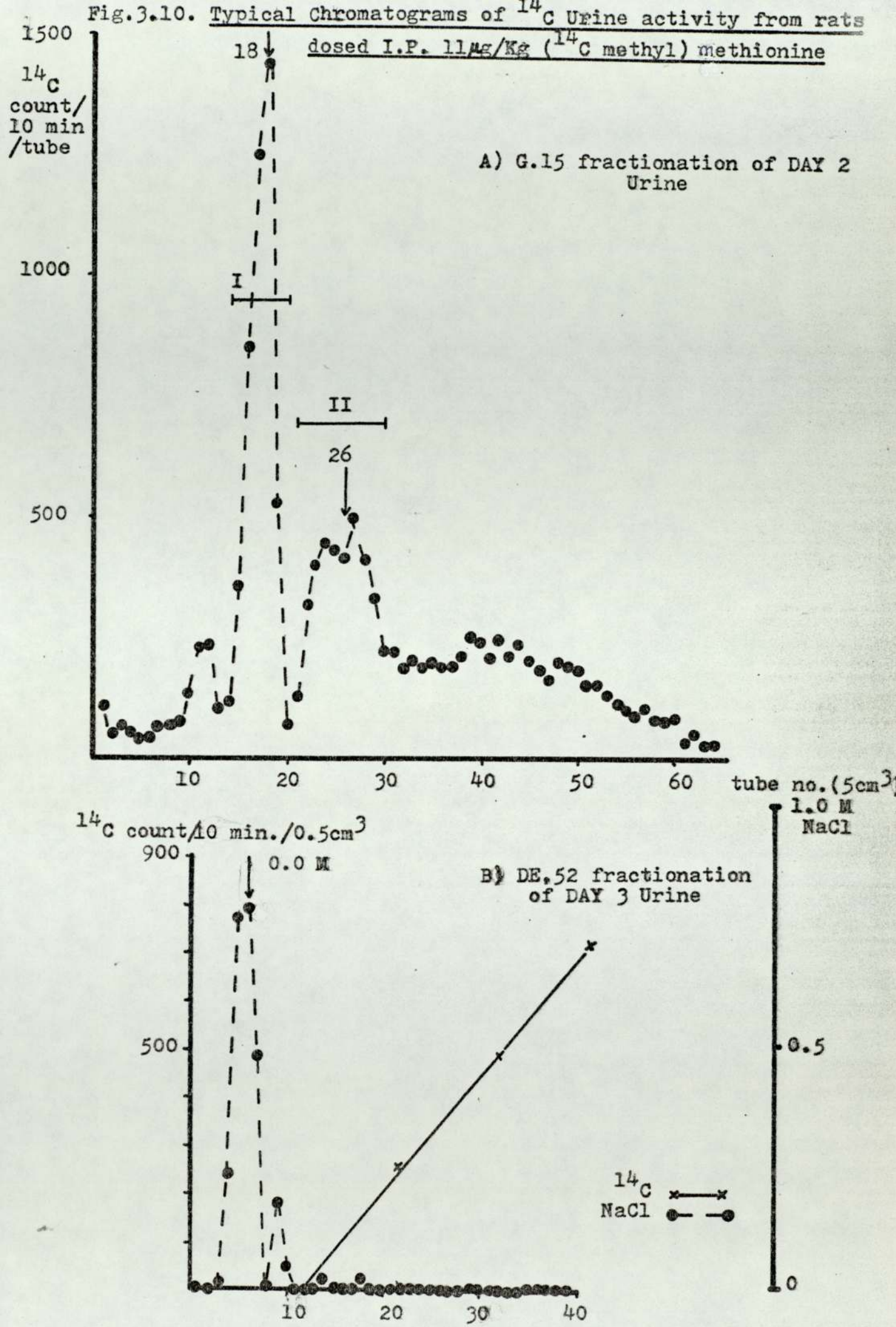
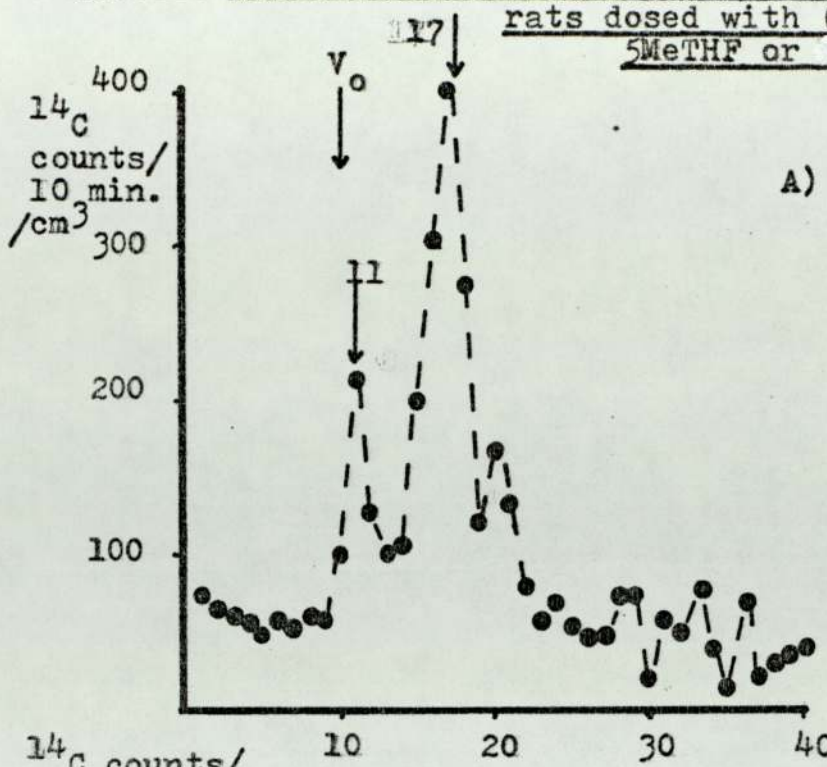
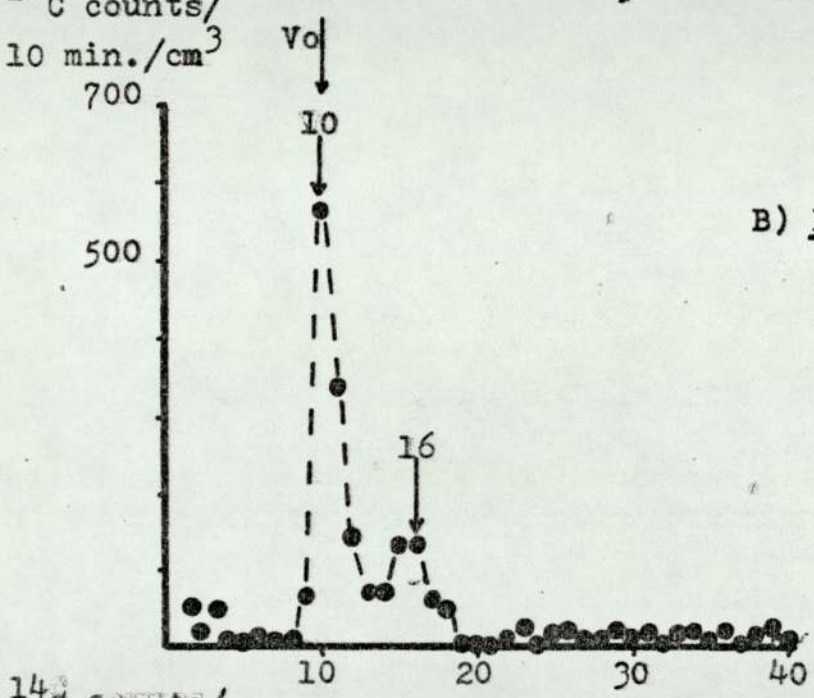


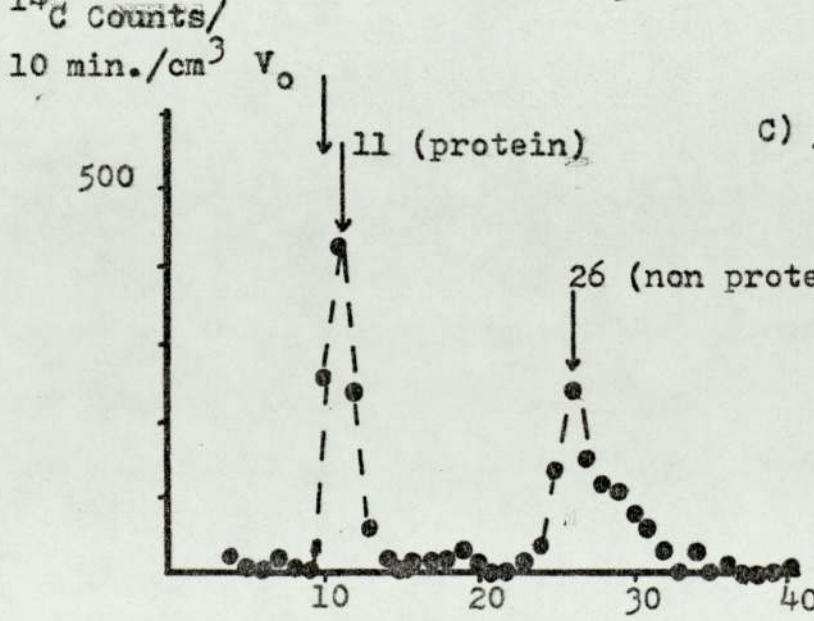
Fig.3.11. Chromatographs of hot Ascorbate extracts of liver from rats dosed with (14C methyl) labelled 5MeTHF or ethionine



A) 60µg/Kg I.P. 5(^{14}C)MeTHF
G.15 fractionation



B) 11µg/Kg I.P. (^{14}C methyl) methionine
G.15 fractionation



C) 11µg/Kg I.P. (^{14}C methyl) methionine
G.75 fractionation

Chromatography of the urinary activity gives a single ^{14}C peak on DE52 eluting at the beginning of the NaCl gradient, indicating it consists of neutral or positively charged species (fig 3.10B). G15 chromatographs give two fractions; I eluting at tube 18 the position of methionine and creatine markers and II at tube 25-26 the position at which the minor peak 'A' eluted from rats orally dosed with 5(^{14}C)MeTHF (fig 3.10A). In both oral and IP dosed animals fraction I was the major urinary ^{14}C peak (table 3(7)), the oral route differing from the IP in the total excretion of fraction II over 0-48 hours; 2.2% dose in urine of IP dosed rats, 1.1% in orally dosed animals.

Hot ascorbate extracts of the third day liver gave a single peak at low salt concentrations (0-0.1 NaCl) on DE52, with a peak at the void volume and one at tube 16-18 on G15 (fig 3.11B). Similar G15 chromatographs are obtained from the liver extract of 5(^{14}C)MeTHF dosed rats (fig 3.11A). Although ^{14}C methyl activity shows a proportionately greater incorporation into high molecular weight forms G.75 fractionation of the ^{14}C methyl)methionine liver extract, which separates protein fron non-protein constituents, shows the high molecular weight ^{14}C liver activity is protein bound (fig 3.11C). An extract of retained muscle activity (fig 3.9) gives a high molecular weight fraction and a fraction coeluting with creatine similar to that observed with 5(^{14}C)MeTHF dosed animals (fig 3.8a)

Thus the ^{14}C activity after a dose of ^{14}C methyl methionine is observed to produce urinary compounds which have the same chromatographic behaviour on DE52 and G15

as the non folate products (NFF and 'A') of 5(^{14}C)MeTHF. Tissue extracts of 5(^{14}C)MeTHF and (^{14}C methyl) methionine dosed animals are also similar, although the ^{14}C methyl activity of methionine dosed animals shows greater incorporation into proteins (fig 3.11). This may be as a result of the (^{14}C)methionine being used directly for protein synthesis while the methyl group of 5MeTHF has to be transmethylated to homocysteine resulting in a slower labelling of protein.

3.4. 5(^{14}C)MeTHF plus (3',5';7,9,^{3}H) folic acid

In order to better observe any incorporation of ^{14}C methyl activity from 5(^{14}C)MeTHF into the folate pool, 6 rats were each dosed orally with 8 µg/Kg (3', 5',7,9,^{3}H) FA, 80µg/Kg 5MeTHF Table 3(8) gives the result of sequential DE52 and G15 fractionation of the urines, fig 3.12 the DE52 chromatographs of the 0-6 and 6-24 hr. urine. The ^{14}C activity is observed to coincide with the ^{3}H from folic acid in the 5MeTHF urinary factions, however, 10CHOFA in the urine shows no significant labelling with ^{14}C, the ^{14}C/^{3}H ratio of the fraction being similar to that observed for unmetabolised folic acid (Table 3(8)).

G15 chromatography of the hot ascorbate extract of day 3 liver shows a high molecular weight ^{3}H peak with minimal ^{14}C labelling (fig 3.13a) further chromatography on DE52 gave a peak eluting at 0.5M the position of polyglutamate species, and on earlier eluting ^{3}H peak which may be a breakdown product (fig 3.13B).

While folic acid is readily metabolised into formyl folate and polyglutamyl types, no significant entry of methyl

with 80μg/Kg 5(^{14}C)MeTHF plus (3;5;7.9.^{3}H)FA (8μg/Kg)

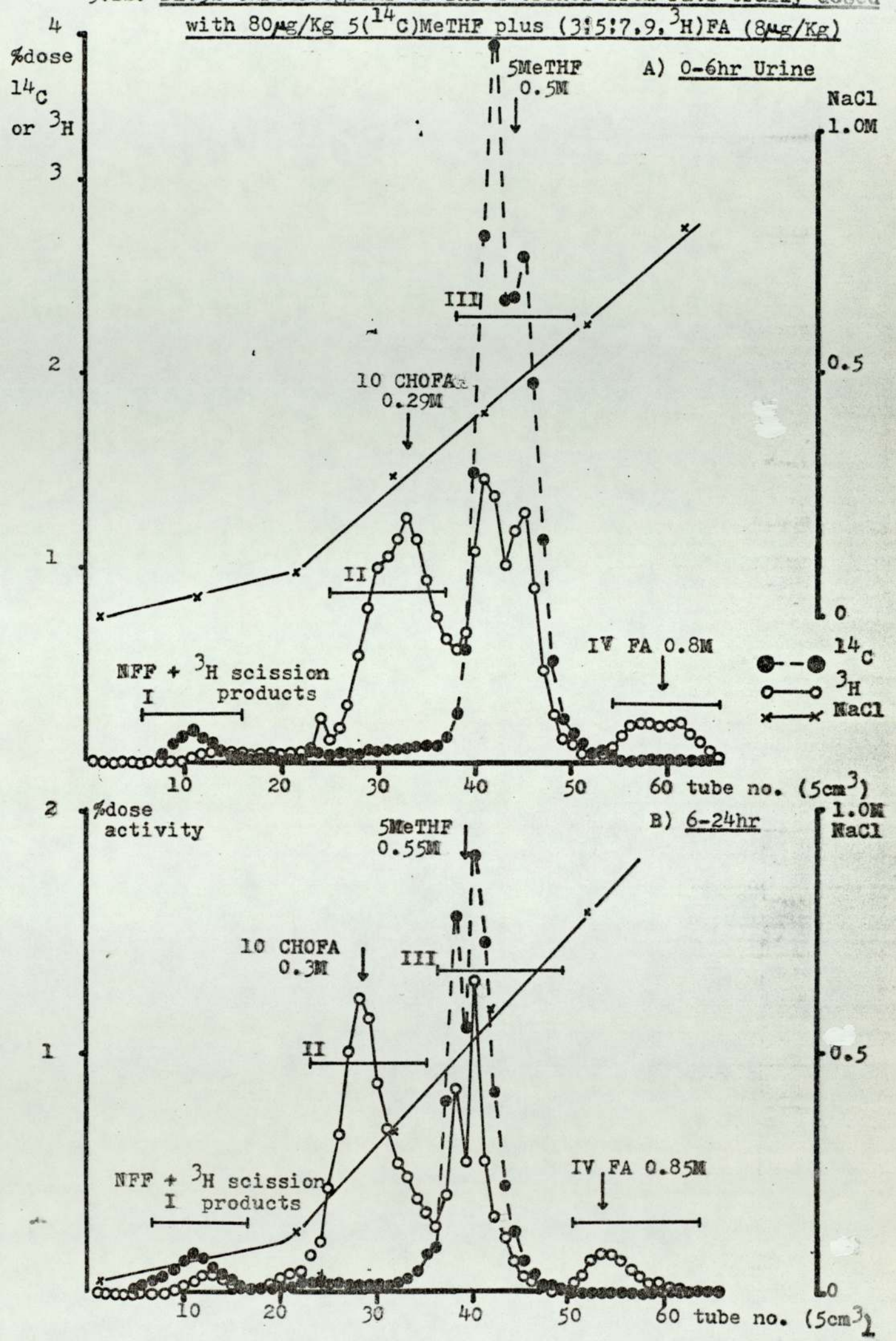


Table 3(8)

Recovery and chromatography of urinary radioactivity from rats orally dosed with 80 μg/Kg 5(^{14}C) MeTHF plus 8 μg/Kg (3', 5', 7,9,^{3}H)FA

0-6 hr Urine 25.8± 1.4% ^{14}C dose, 29.8± 1.6% ^{3}H dose (mean of six rats±SEM)

Fraction	% Urine Activity ^{14}C	% Urine Activity ^{3}H	% Dose Activity ^{14}C	% Dose Activity ^{3}H	Ratio ^{14}C/^{3}H
NFF	3.3	-	1.01	-	-
^{3}H scission products	-	1.1	-	0.41	-
10CHOFA	4.4	34.4	1.4	12.33	0.11
5MeTHF	74.1	32.4	22.7	11.6	1.96
FA	6.6	5.8	0.2	2.06	0.08

6-24 hr Urine 11.9 ± 1.6% ^{14}C dose 15.7 ±1.0% ^{3}H dose

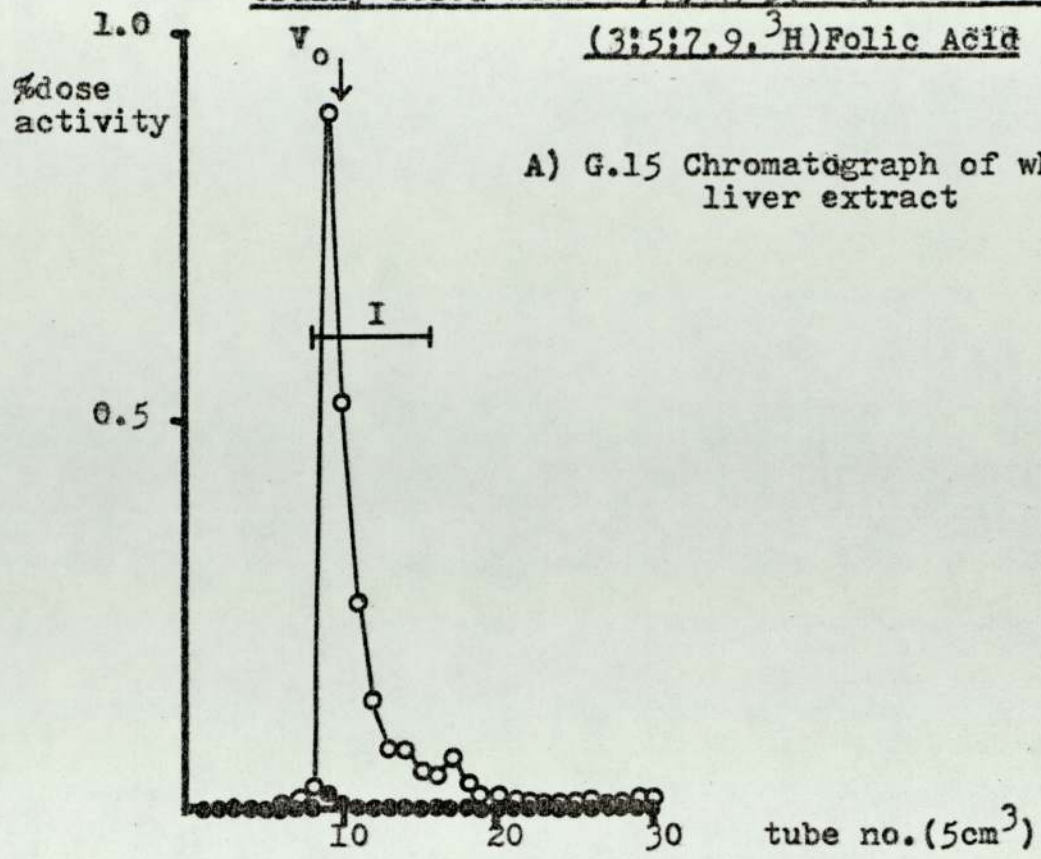
Fraction	% Urine Activity ^{14}C	% Urine Activity ^{3}H	% dose activity ^{14}C	% dose activity ^{3}H	Ratio ^{14}C/^{3}H
NFF	9.8	-	1.04	-	-
^{3}H scission products	-	2.9	-	0.43	-
10CHOFA	6.2	53.2	0.66	7.92	0.08
5MeTHF	83.9	32.8	8.89	4.89	1.81
FA	0.4	7.5	0.04	1.12	0.04

Day 2 Urine 2.6± 0.5% ^{14}C 3.5± 0.3% ^{3}H

Fraction	% Urine Activity ^{14}C	% Urine Activity ^{3}H	% Dose Activity ^{14}C	% Dose Activity ^{3}H	Ratio ^{14}C/^{3}H
NFF	33.6	-	0.84	-	-
^{3}H scisson products	-	41.1	-	1.48	-
10CHOFA	6.4	50.8	0.16	1.83	0.09
5MeTHF	53.6	22.0	1.34	0.79	1.70
FA	0	4.4	0	0.16	

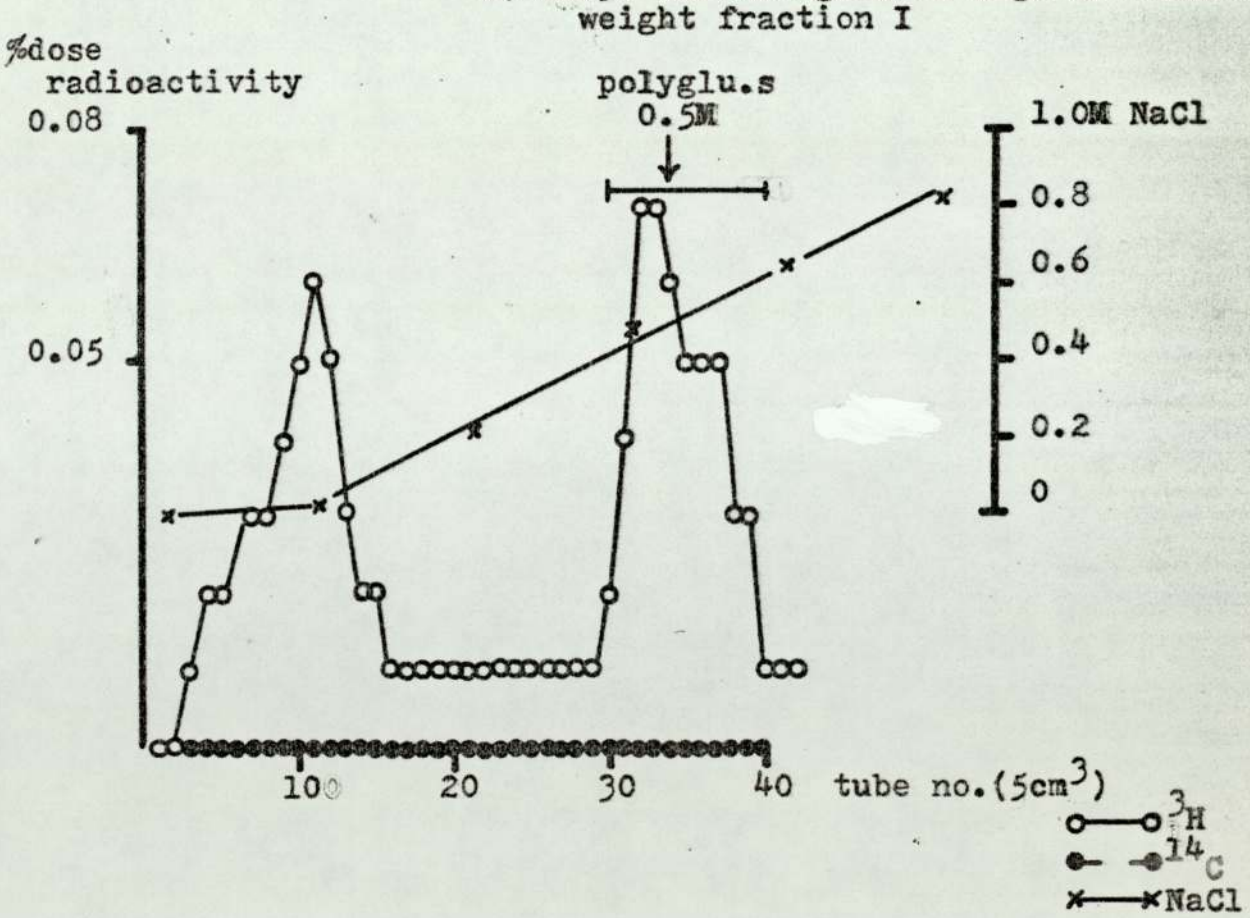
Day 3 Urine 0.9% ^{14}C 1.9% ^{3}H.

Fig.3.13. Chromatographs of hot ascorbate liver extract from rats orally dosed with 80μg/Kg 5(^{14}C)MeTHF + 8μg/Kg (3;5;7,9,^{3}H)Folic Acid



A) G.15 Chromatograph of whole liver extract

B) DE.52 chromatogram of high molecular weight fraction I



group activity into such species in liver and urine could be demonstrated.

3.5 Discussion

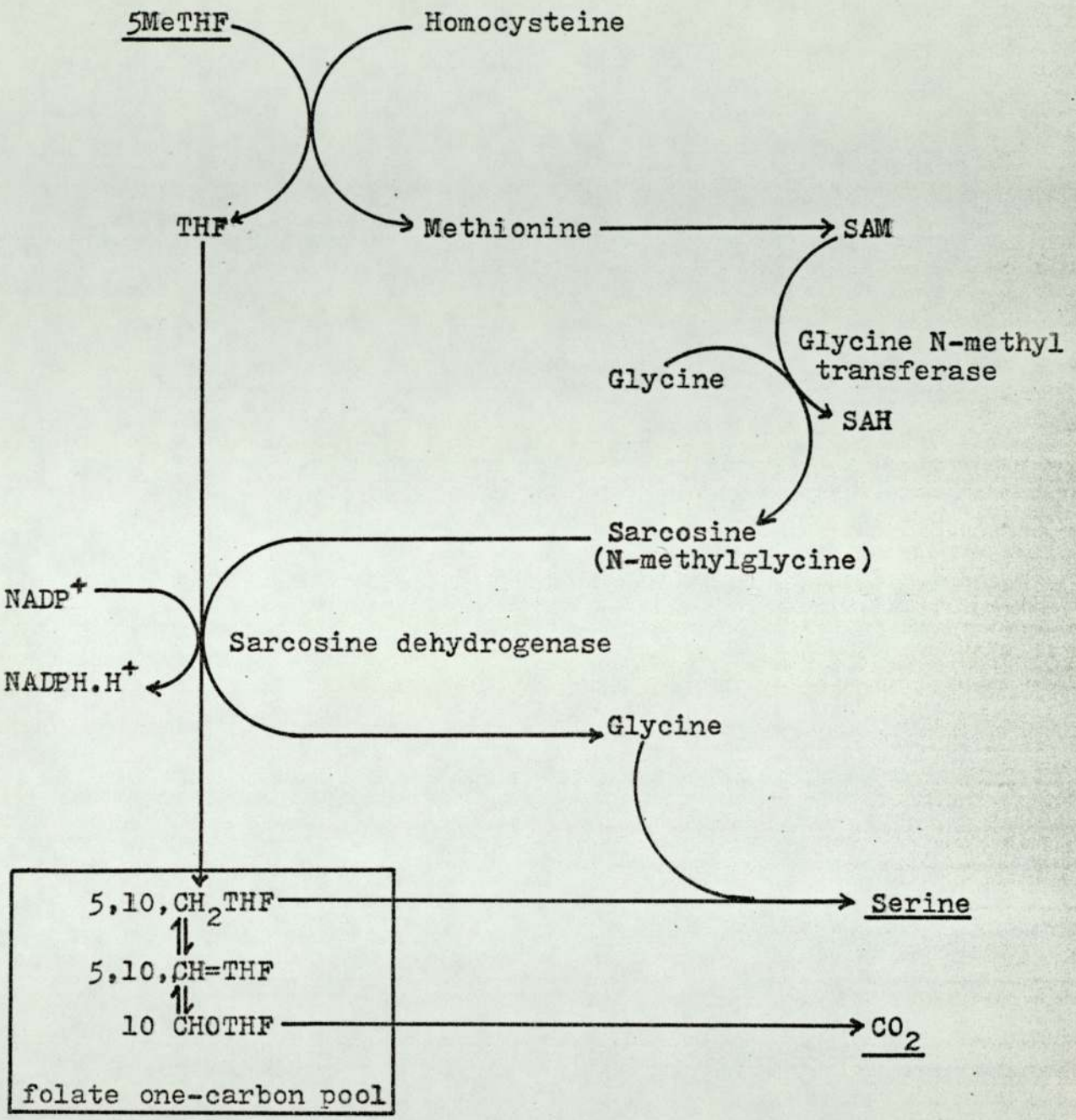
Comparison of the two oral and IP dosages of 5(^{14}C)MeTHF indicates that the compound is completely absorbed from the rat small intestine, in spite of the radiotracer being a mixture of C_6 epimers. Weir and Scott (1973) report that in human subjects absorbtion favours the biologically active isomer, in contrast to these results with the rat. However, their method involved the collection of urinary 5MeTHF activity following a 'flushing dose' of folic acid, and comparing results from subjects who had prior oral doses with one or other of the different 5MeTHF isomers. This method of 'flushing' retained labelled folate has not however, been found to be effective, at least in the rat (Blair and Dransfield 1971; Barford et al 1977), and the more direct method of comparing faecal radioactivity after oral and IP doses would be expected to give a better estimate of absorbtion. Such a non-discrimination between C_6 epimers would be expected if the absorbtion of 5MeTHF proceeds by the passage of the electrically neutral form across the gut wall, the neutralisation taking place within an acid microclimate generated at the gut surface (Blair and Matty 1974, Coleman et al 1979) where the pKa of both epimers is the same, rather than stereospecific carrier protein system.

On entry into the body active 5MeTHF is demethylated to produce a variety of non folate metabolities, the

majority of retained activity is present in the muscle as creatine. Creatine and methionine are excreted in the urine, and the methyl group is oxidised to CO_2 which is excreted in the breath. The non-folate metabolites of 5(^{14}C)MeTHF are observed to chromatograph in qualitatively the same manner as those of (^{14}C methyl) methionine. Furthermore simultaneous dosage of 5(^{14}C)MeTHF + (^{3}H)FA showed no entry of methyl activity into polyglutamate or formyl folates. Thus the one carbon metabolism of 5MeTHF is observed to proceed via the methyl transfer route, rather than by oxidation to methylene THF. However, the techniques employed may be inappropriate to show production of ^{14}CHO folate forms from 5MeTHF. Methyl carbon is able to re-enter the folate pool via mitochondrial sarcosine dehydrogenase, which judging from its products (serine, formaldehyde and formate), involves 5,10,CH_2THF formation (Lewis et al 1978). Sarcosine is synthesised by an SAM requiring reaction from glycine(Blumenstein and Williams 1960) catalysed by glycine N-methyltransferase (fig. 3. 14). The high concentration of this enzyme in rabbit liver, 0.9 -3% total extracted protein, suggests the reaction may be important in regulating intracellular SAM levels (Heady and Kerr 1973).

Although this pathway is considered an important source of folate one carbon (Lewis et al 1978) the production of urinary or tissue formyl folates could not be demonstrated from (^{14}C methyl) labelled methionine or 5MeTHF. It is possible that formation of 5,10,CH_2THF from methyl groups takes place at a subcellular level and may not be observed in these experiments, however, it is likely that production of folate one-carbon from

Fig.3.14. Entry of Methyl carbon into the folate pool



methyl groups is small compared to transmethylation reactions, and that normally such activity is insufficient to circumvent the 'methyl tetrahydrofolate trap' in B_{12} deficient anaemia.

4. THE ENTRY OF 5'METHYLTETRAHYDROFOLIC ACID INTO THE FOLATE POOL

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4.1 Introduction

The majority of investigations of folate metabolism have used either folic acid or 5CHOTHF which are not normal constituants of the folate pool (Chapter 1 (3)). Experiments were undertaken using mixed label (2^{14}C)(3',5',7,9,^{3}H)5MeTHF in order to investigate the contribution of a fully reduced naturally occuring folate to thebody folate pool. Prior studies on 5MeTHF metabolism in humans and cell culture systems have used (3',5', 7,9, ^{3}H) 5MeTHF tracer (Weir et al 1973) or the tritiated form mixed with 5(^{14}C)MeTHF (Nixon and Bertino 1972, Nixon et al 1973) to observe the entry of the tetrahydrofolate moiety of 5MeTHF into folate metabolic pathways. Earlier experiments involving (2^{14}C) 5MeTHF were carried out in this laboratory by Dransfield (1972). There are problems associated with the use of a single (2^{14}C) or (^{3}H) label, outlined in Chapter 1 and the 5(^{14}C)(^{3}H) system, while useful in comparing the fate of the methyl group with that of the tetrahydrofolate portion of 5MeTHF(Nixon et al 1973), will have the same effective labelling of the folate pool as the (^{3}H)-only tracer, as the ^{14}C label is lost during methionine synthesis.

Recent work in this laboratory has used mixed label (2^{14}C)(3',5',7,9,^{3}H) folic acid (Barford et al 1977, Connor and Blair 1980, Pheasant et al 1980) and the use of mixed label 5MeTHF would indicate whether the general conclusions obtained from these studies are valid for a naturally occuring folate.

Mixed label 5MeTHF was extracted from the urine of rats dosed with (2^{14}C)(3',5',7,9,^{3}H) mixed label folic acid as

described in Chapter 2 diluted with non radioactive 5MeTHF and orally dosed (in 50mM Na phosphate buffer pH7 containing 2% sodium ascorbate) to 5 animals at 8 μg/Kg and 4 rats at 80 μg/Kg with a single rat dosed at 209 μg/Kg The experiments followed the protocol outlined in Chapter 2.

4.2 Results

Tables 4(1) and 4(2) give the retained and excreted radioactivity, Tables 4(3) and 4(4) fig 4.1 and 4.3 the chromatographic fractionation of urinary radioactivity after 8 and 80 μg/Kg oral dose of mixed label 5MeTHF (0·04μCa ^{14}C, 0·1μCa ^{3}H

The recovery of radioactivity in the first day and subsequent day faeces (Table 4(1)) total 30.2% ^{14}C 21.5% ^{3}H) is much higher at 80 μg/Kg than observed with 5(^{14}C) MeTHF (2.4% over three days table 3(3)). Experiments with the (^{14}C methyl) labelled species have indicated that 5MeTHF is absorbed 100% (Chapter 3), thus the increased faecal activity may be due to increased biliary excretion. The faecal radioactivity of both dose experiments shows an excess of ^{14}C over ^{3}H, which is statistically significant on days 1 and 2 at 80 μg/Kg, and day 1 at 8 μg/Kg with the isotope imbalance reversed in urine (Table 4(1) 4(2)). As the ^{14}C label is associated with the pterin portion of the folate, the faecal imbalance may indicate a preferential excretion of pterin-derived products in the bile. The imbalance of isotope recovery in urine and faeces is not totally due to preferential biliary pterin excretion, for notably in the chromatographs of the 8 μg/Kg dose urines (fig 4.1 A,B) the dual labelled peaks (0-6 hr I, II, III and 6-24 hr II, III, IV) show an excess of ^{3}H over ^{14}C

Table 4(1)

Oral dose 80 μg/Kg($2^{14}C$)(3',5', 7,9,^{3}H) mixed label 5MeTHF retained and excreted radioactivity (%dose activity)

Mean of 4 rats		0-6hr	6-24hr	Day2	Day 3	Total
Urine	^{14}C	17.0[1]	23.7[1]	2.9[2]	3.0[1]	46.6
	^{3}H	18.6	25.7	4.5	3.8	52.6
			Day 1			
Faeces	^{14}C		17.0[3]	10.7[4]	2.5[1]	30.2
	^{3}H		10.6	6.8	4.1	21.5
Liver	^{14}C				2.5	2.5
	^{3}H				2.4.	2.4
Kidney	^{14}C				0.1	0.1
	^{3}H				0.3	0.3
Brain, Spleen, Muscle, gut.	^{14}C				NS	0
	^{3}H				NS	0
Total	^{14}C		57.7	13.6	8.1	79.4
	^{3}H		54.9	11.3	10.6	76.8

't' test recovery of ^{3}H vs ^{14}C

1 not significant $p > 0.05$
2 significant $p < 0.001$
3 " $p < 0.0001$
4 " $p < 0.05$

NS Radioactivity not significantly higher than background

Table 4(2)

Oral dose 8 µg/Kg ($2^{14}C$)(3',5',7,9,^{3}H) mixed label 5 MeTHF
retained and excreted radioactivity (% dose activity)

Mean of 5 rats			0-6hr	6-24hr	Day 2	Day 3	Total
Urine	^{14}C	^{14}C	39.1[1]	28.5[2]	3.2	3.4[4]	74.2
	^{3}H	^{3}H	41.0	40.4	4.7	5.6	91.7
				Day 1			
Faeces	^{14}C			24.5[4]	8.5[3]	-	33.0
	^{3}H			18.1	6.4	-	24.5
Liver	^{14}C					6.8	6.8
	^{3}H					ND	-
Kidney	^{14}C					0.8	0.8
	^{3}H					0.4	0.4
Gut	^{14}C					1.1	1.1
	^{3}H					0.8	0.8
Total	^{14}C			92.1	11.7	12.1	115.9
	^{3}H			99.5	11.1	6.8	117.3

't' test recovery ^{3}H vs ^{14}C 1 not significant $p > 0.05$
2 significant $p < 0.01$
3 " $p < 0.001$
4 " $p < 0.05$

ND: not determined

Table 4(3)

Chromatographic fractionation of urinary activity from rats dosed 8 µg/Kg mixed label ($2^{14}C$)(3',5',7,9,^{3}H)5MeTHF

0-6hr Urine: 40.6% ^{14}C, 42.4% ^{3}H dose

	%Urine activity		%dose activity		Ratio dose
	^{14}C	^{3}H	^{14}C	^{3}H	$^{3}H/^{14}C$
I 10CHOFA	27.0	26.3	11.0	11.2	1.02
II 5MeTHF	39.4	48.2	16.0	20.4	1.28
III 'Compound D'	26.2	28.2	10.6	12.0	1.13
Total	92.6	102.7	37.6	43.6	

6-24hr Urine: 26.3% ^{14}C, 40.3% ^{3}H dose

	%urine Activity		%dose activity		Ratio dose
	^{14}C	^{3}H	^{14}C	^{3}H	$^{3}H/^{14}C$
I Scission Products	24	40	5.3	16.2	3.06
II 10CHOFA	12	9	3.1	3.7	1.19
III 5MeTHF	32	31	8.4	12.5	1.49
IV 'Compound D'	20	19	5.3	7.7	1.45
Total	88	99	22.1	40.1	

Table 4(4)

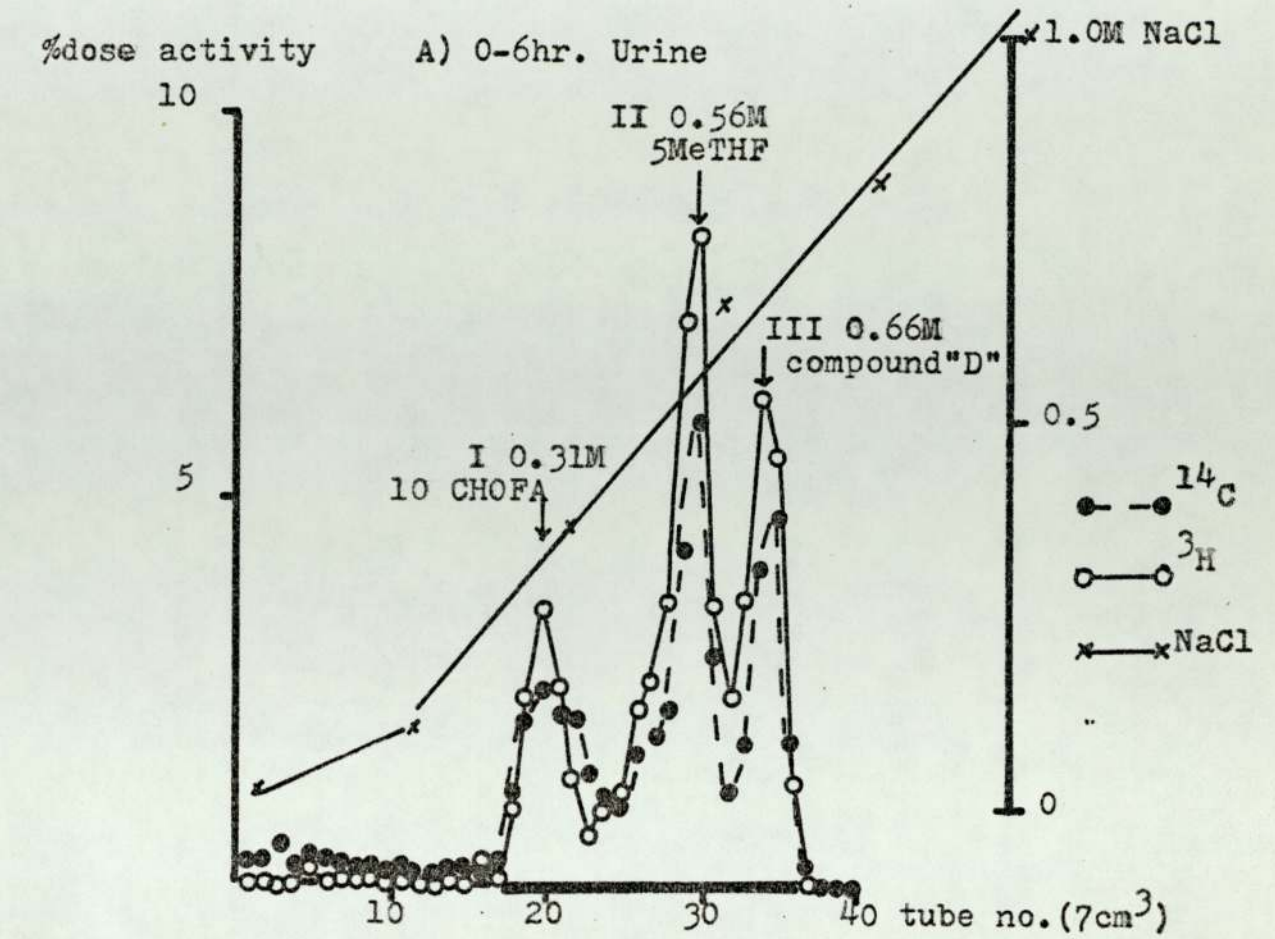
Chromatographic fractionation of 6-24 hr urine from rats dosed 80 μg/Kg ($2^{14}C$)(3',5',7,9,^{3}H)5MeTHF

	%Urine Activity		%Dose Activity		Ratio dose
	^{14}C	^{3}H			$^{3}H/^{14}C$
I Scission products	21.8	22.2	5.2	5.7	1.04
II 10CHOFA	18.7	17.2	4.4	4.4	1.0
III 5MeTHF	28.3	26.9	6.7	6.9	1.03
IV 'Compound D'	20.4	16.4	4.8	4.2	0.89
Total	89.3	82.7	21.1	21.2	

Table 4(5) Tritiated 6-24hr urinary metabolites of mixed Label ($2^{14}C$)(3',5',7,9,^{3}H)5MeTHF dosed rats

	Compound	%dose ^{3}H	%urine ^{3}H	Fraction pAcetABA + pAcetABglu	%dose folate cleaved
80 μg/Kg	pAcetABglu	2.7	10.3	0.47	6.4
	pAcetABA	3.0	11.7	0.53	7.1
		5.7	22.0		13.5
8 μg/Kg	pAcetABglu	10.7	26.4	0.66	25.2
	pAcetABA	5.5	13.6	0.34	12.9
		16.2	40		38.1

Fig.4.1. DE.52 Chromatograms of Urine from rats dosed 8µg/Kg ($2^{14}C$)(3;5;7,9,^{3}H) mixed label 5MeTHF



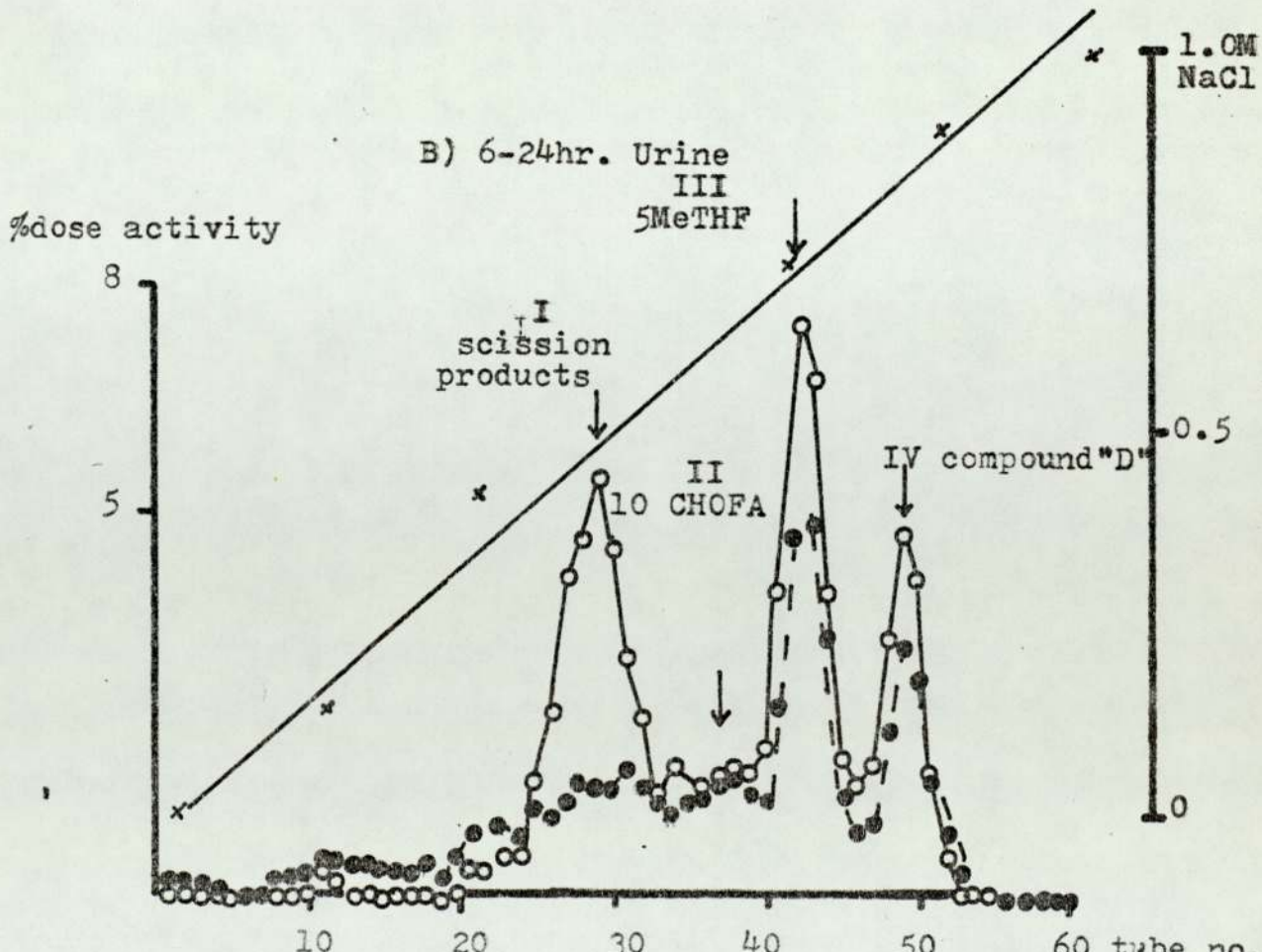


Fig.4.2. Portion of DE.52 Chromatogram of Urine from rats dosed with mixed label (2 ^{14}C)(3;5;7.9, ^{3}H) Folic Acid

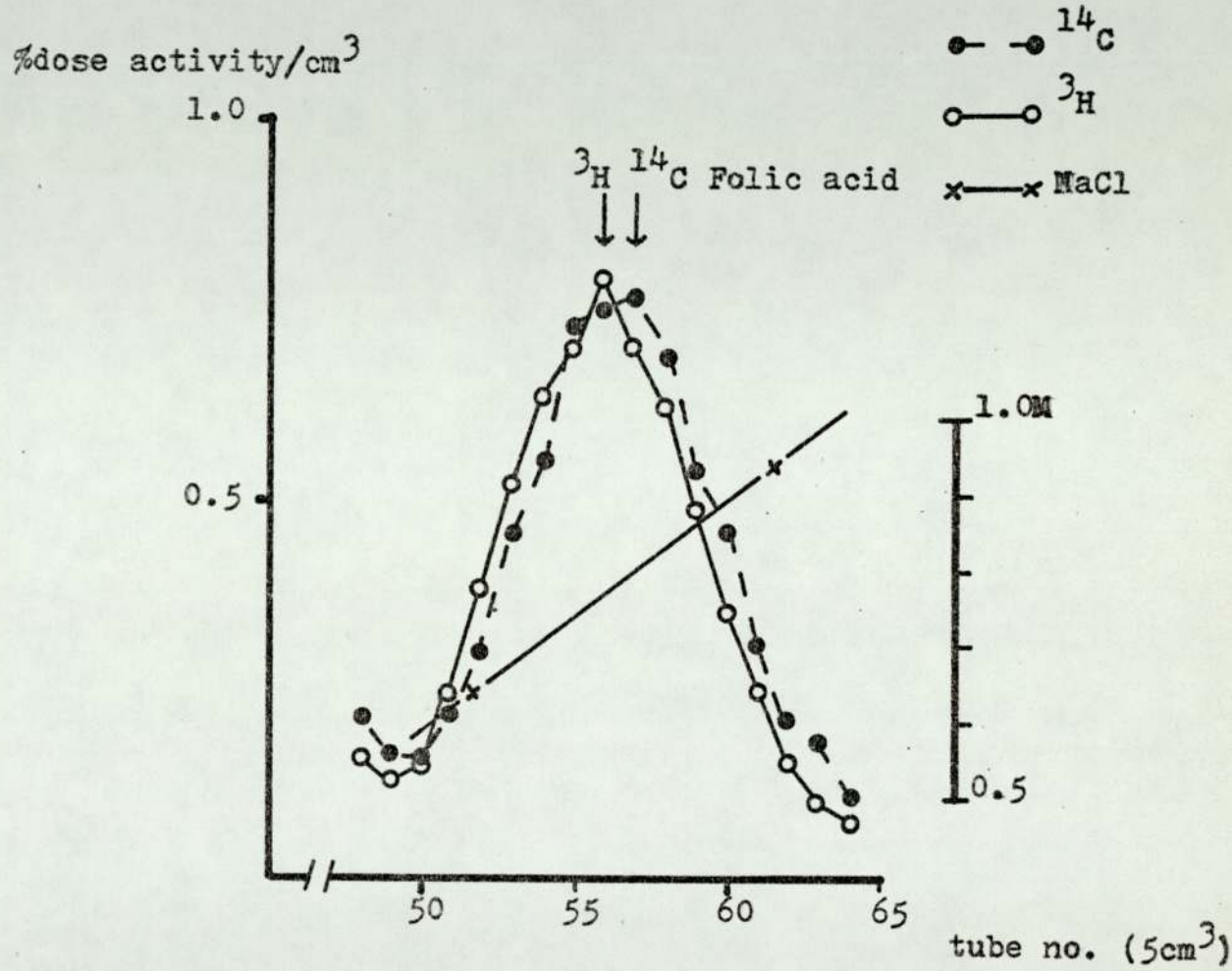
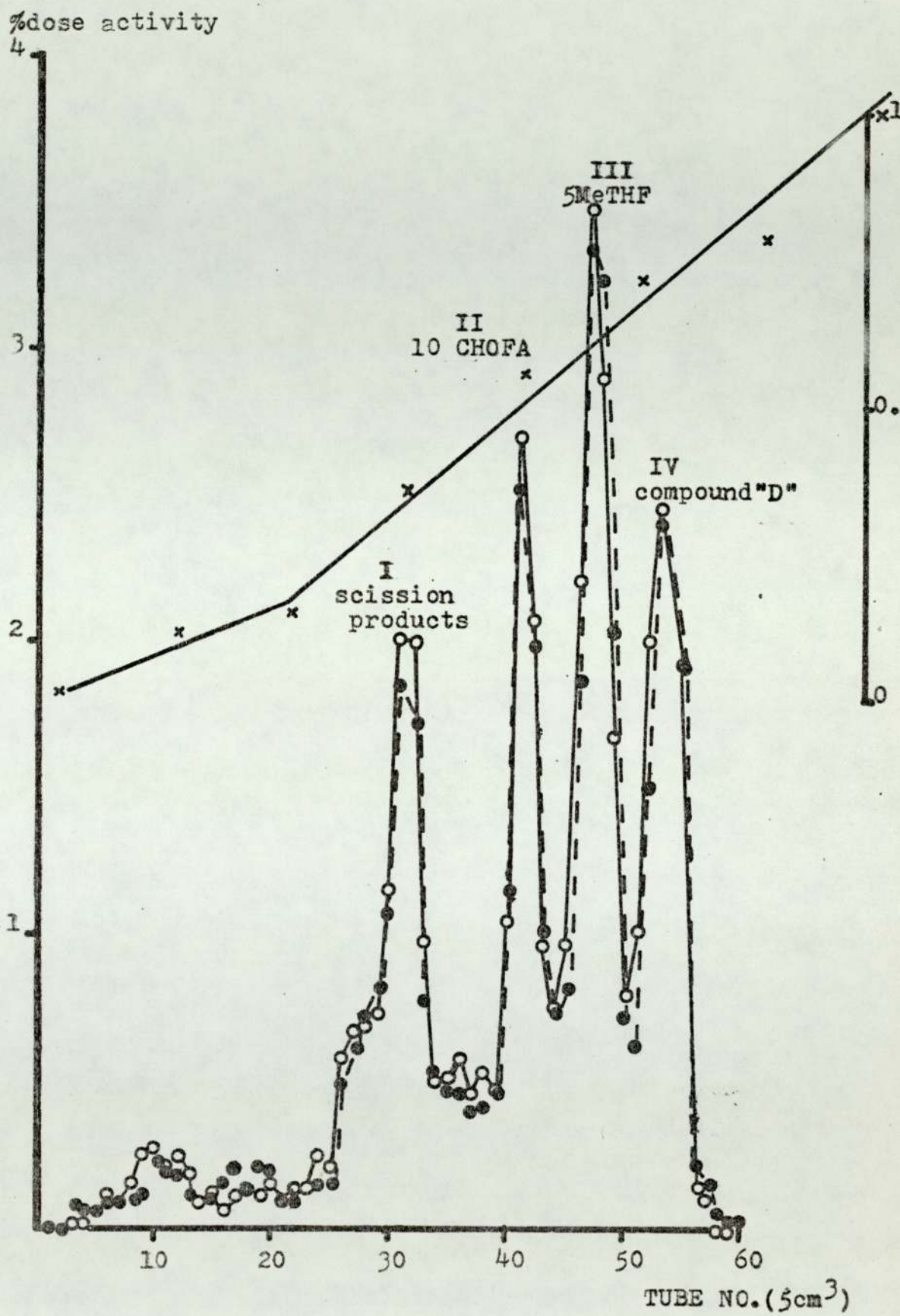


Fig.4.3. DE.52 Chromatogram of 6-24hr Urine from rats orally dosed with 80μg/Kg ($2^{14}C$)(3;5;7,9,^{3}H) mixed label 5MeTHF

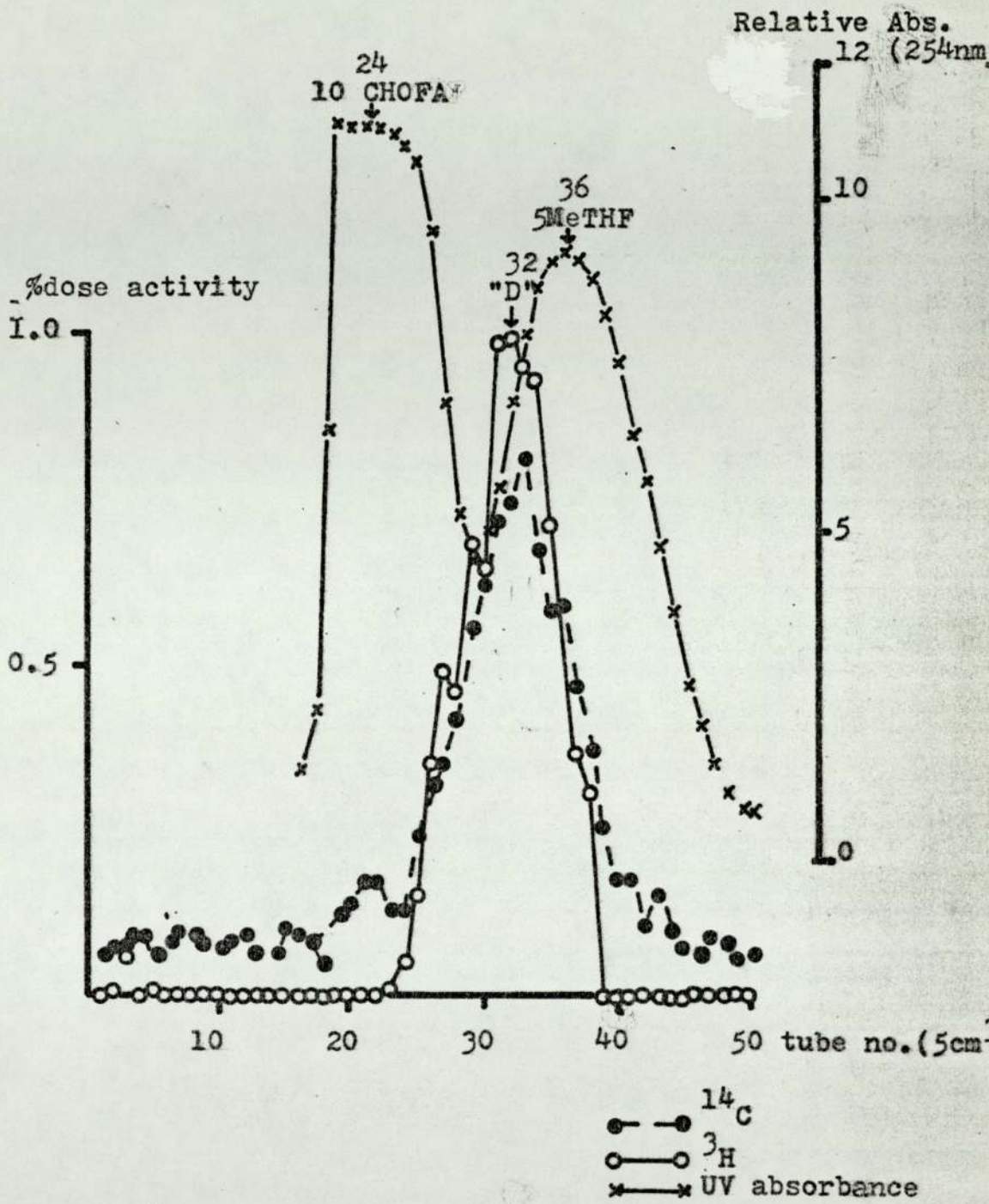


which are not further resolved by subsequent G15 chromatography into soley ^{3}H and ^{14}C labelled fragments. However, in the 0-24 hours urine after the 8 µg/Kg, there is an excess of 15.8% of dose ^{3}H over ^{14}C. 4.9% of ^{3}H is accounted for by the excess of ^{3}H in the dual labelled peaks, while 10.9% ^{3}H dose is as a result of the excretion of solely ^{3}H labelled species, thus 69% of the urinary imbalance at 8 µg/Kg can be ascribed to folate scission. Since cannulated bile after IP injection of folic acid contains only intact folates (Hillman et al 1977. Pheasant and Blair 1979, Steinberg et al 1979, Pheasant et al 1980) the cleavage of folates occurs within the gut lumen, the isotopic imbalances would result from the pterin derived fragment being less well absorbed than the ^{3}H labelled fragment. The complete absorbtion of 5 MeTHF from the intestine implies that the scission substrate is another folate, possibly the more labile 10CHOTHF or THF.

The isotope effect, which produces dual labelled peaks with a ^{3}H excess at 8 µg/Kg, is dose dependent as at 80 µg/Kg the chromatographic peaks show equal isotope recovery (Table 4(4) fig 4.3). The two labels of the parent folic acid tracer (2^{14}C) and (3',5',7,9, ^{3}H)FA show separation on DE52 column (Fig 4.2) so there is a demonstrable chemical difference between the labels which may result in the biological system discriminating between them.

DE52 chromatography of the 0-6hr and 6-24 hr urines give threee dual labelled peaks at both doses which subsequently remain intact on G15 chromatography (8 µg/Kg 0-6 hr I, II, III; 6-24 hr II III, IV and 80 µg/Kg 6-24 hr

Fig.4.4. G.15 chromatogram of compound "D" (DE.52 fraction III of 0-6hr. Urine from rats dosed 8µg/Kg mixed label 5MeTHF)



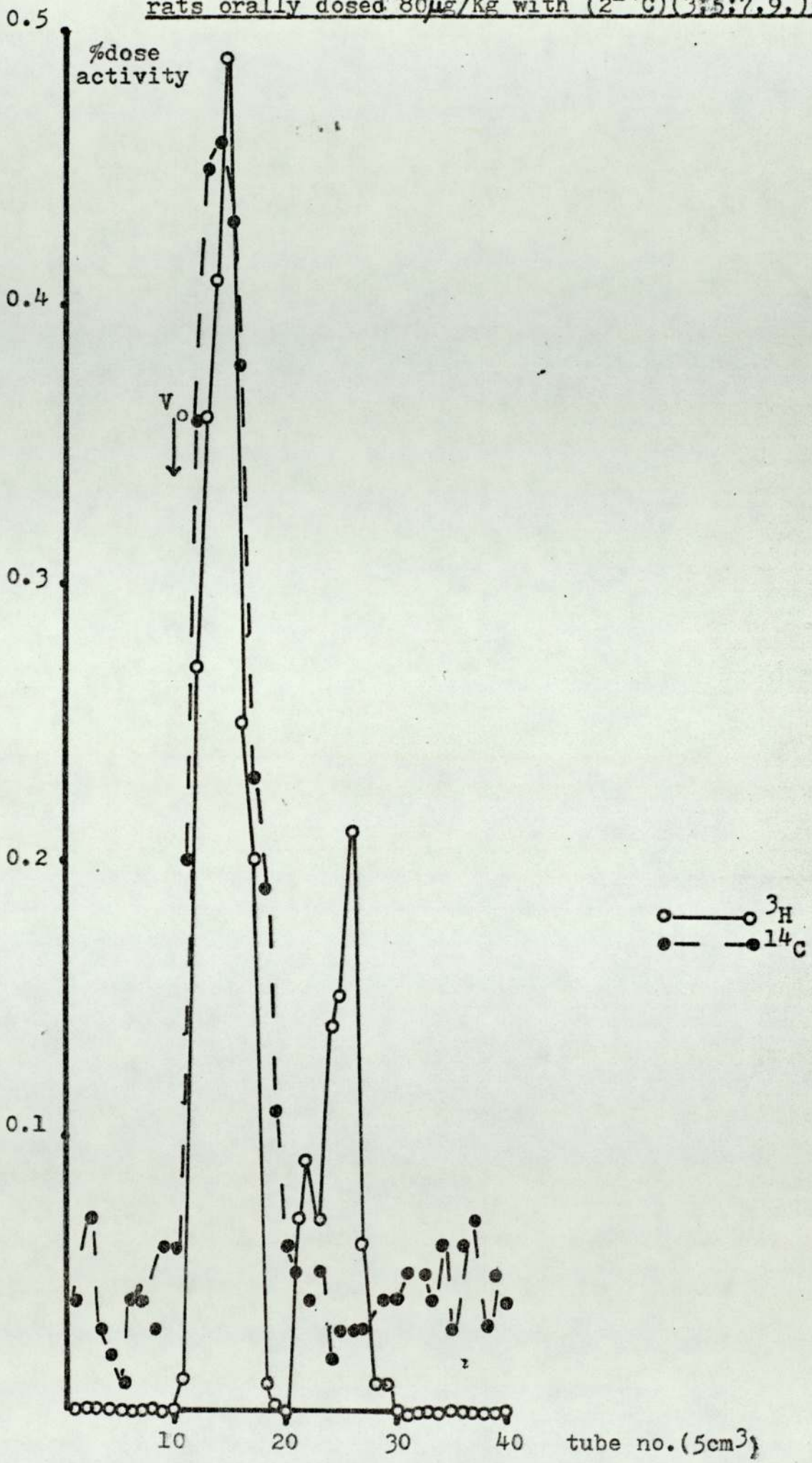
II, III, IV fig 4.1 and 4.3). The first two of these peaks chromatograph with 10CHOFA and 5MeTHF on G15 (Tables 4(3) and 4(4)), however, the third peak (compound D) eluting after 5MeTHF on DE52 does not chromatograph at a position compatible with the formyl folates, folic acid, or 5MeTHF and its known oxidation products (fig 4.4). A similar product has also been observed after dosing rats bearing the Novikoff hepatoma with folic acid (Pheasant and Blair 1979). The 6-24 hr urines show a peak eluting before 10CHOFA (fig 4.1 B peak I and fig 4.3 peak I) These peaks when chromatographed on G15 result in the ^{3}H peak activity splitting into two, one fraction eluting at tube 19 with added p.acetamidobenzoylglutamate (pacetABglu) and the other at tube 33 with pacetamidotobenzoic acid (pacetABA) marker (table 4(5)). After freeze drying the pooled ^{3}H peaks were dissolved in methanol/water and spotted onto paper with p cetABA and pacetABglu standards. When developed with BuOH/EtOH/0.880 NH_3/water (10:10:1:4 by volume) the two metabolites cochromatograph with the pAcetABglu and pAcetABA markers clear of the p'ABA and pABglu standards.

After three days the majority of retained radioactivity is in liver, kidney and gut (tables 4(1) and 4(2)). Proportionally greater retention occurs at 8 µg/Kg, at the higher dose 80 µg/Kg renal overloading would result in proportionally greater excretion (Chapter 3). Hot ascorbate extract of the third day livers shows the majority of retained activity corresponds to high molecular weight compounds eluting at the void volume (fig 4.5) on G15. Rechromatography of the dual labelled peak on DE52 shows elution at approximately 0.7M NaCl, a position compatible with polyglutamates.

5.3 Discussion

The pattern of metabolites found after an oral dose

Fig.4.5. G.15 Chromatogram of hot ascorbate extract of liver from rats orally dosed 80μg/Kg with (2^{14}C)(3;5;7,9,)5MeTHF



of mixed label (2^{14}C)(3',5',7,9,^{3}H)5MeTHF is broadly in agreement to that observed after dosing with similarly labelled folic acid (Barford et al 1977, Connor et al 1979; Pheasant et al 1980). The experiments show that 5MeTHF, a naturally occuring reduced folate, undergoes the same scisson pathway as folic acid (Connor et al 1979). Scisson is not an artefact of loading, for the degree of scisson as measured by the ^{14}C/^{3}H imbalance in the urine and faeces, or more directly by the excretion of ^{3}H only labelled metabolites in the urine (tables 4(1), 4(2) and 4(5)) show cleavage to be greater at the lower dose. Thus C^{9}-N^{10} scisson is observed as a normal folate catabolic pathway.

Retained folates are distributed between two kinetic pools, the first with a short half life is presumed to consist of free monoglutamates and the second long term tissue polyglutamate folates (Blair 1976). Krumdiek et al (1978) have calculated the $t_{\frac{1}{2}}$ for the pools in man; 31.5 hours for the short life and 100 days for the long life kinetic pools.

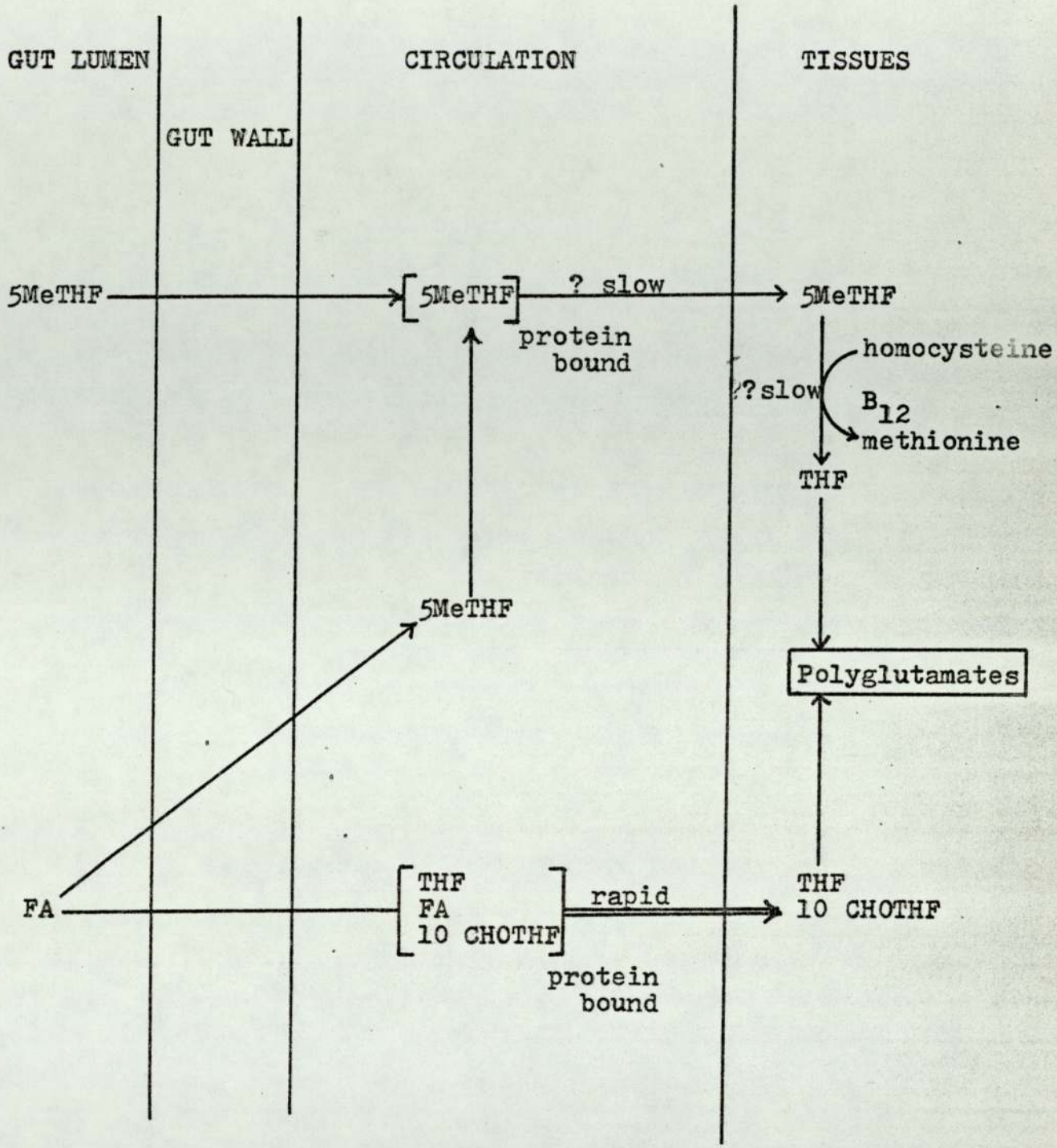
Pheasant et al (1980) report that after 100 µg/Kg oral dose of folic acid there is excretion of 28% ^{14}C, 37% ^{3}H dose in 0-48 hours urine with 11.4% ^{14}C and ^{3}H retained in liver. In contrast with a similar dose (80 µg/Kg) of mixed label 5MeTHF there is an excretion of 43.6% ^{14}C and 48.8% ^{3}H in urine over the same period with 2.5% ^{14}C, 2.4% ^{3}H retained in the liver over three days (If $t_{\frac{1}{2}}$ for polyglutamates = 100 days the retention values for day 3 will not be significantly different from day 2, differing by less than 1%). These results indicate that 5MeTHF is taken up in a greater

proportion than FA into the short life (monoglutamate) pool and a lesser proportion than FA into the long life polyglutamate pool. A similar discrepancy between the liver uptake of FA and 5MeTHF (^{3}H) was observed by Steinberg et al (1979), and between the liver uptake of 5MeTHF and THF in the South African fruit bat (Perry et al 1979).

This effect may be a result of 5MeTHF being a poor substitute for polyglutamate synthesis (Spronk et al 1973, McGuire et al 1979). However, demethylation of 5MeTHF during methionine synthesis would furnish THF the prefered substrate for polyglutamate production (McGuire et al 1979). Thus the conclusion is that 5MeTHF demethylation is a slow process compared to polyglutamate synthesis from THF. However, Nixon et al (1973) observed that L1210 cells where able to remove 85% of 5(^{14}C)MeTHF label to other forms after 5 minutes while 87% of (^{3}H)5MeTHF label was still recovered as 5MeTHF after 60 minutes. In contrast in rat liver polyglutamate synthesis from a pulse of folic acid is only 30% complete after 2 hours (Bates, Blair and Connor unpublished results). Should such a rapid demethylation of 5MeTHF occur in the rat, there ought to be similar incorporation of ring labelled 5MeTHF and FA via THF in the rat. To explain the slower 5MeTHF incorporation into polyglutamate it is possible to postulate that methionine synthesis in the normal rat takes place at a much slower rate than in L1210 murine lymphoma. This may be a result of decreased enzyme activity in the rat or because 5MeTHF is held in storage prior to demethylation. Waxman (1976) reports that substantial protein binding of folates occurs in the plasma. If 5MeTHF is taken into

protein binding and released at a slower rate than folic acid, this would effectively slow its rate of demethylation hence polyglutamate synthesis from a 5MeTHF pulse. If this is the case it could be envisaged that 5MeTHF could act as a short term protein bound folate store in the circulation with polyglutamates acting as long term store of tissue folate fig 4.6. This phenomenum may be explained by further investigations into plasma folate binding proteins and methionine synthetase in the normal animal.

Fig.4.6. Incorporation of 5MeTHF and FA into polyglutamate



5. THE OXIDATION PRODUCTS OF 5MeTHF

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5.1 Introduction

As the folate content of the diet in developed nations may only be adequate (Blakley 1969 p.44) and when in underdeveloped nations the majority of the population suffers from nutritional folate deficiency (Blakley p.411), the bioavailability of the folate types in food bears investigation. Tetrahydrofolates are readily oxidised *in vitro* (Blair and Pearson 1975) and thus the folate content of foodstuffs would be expected to contain a proportion of oxidised folate forms may be important in determining the ability of various diets to meet folate vitamin requirements.

Differential assay of food folates indicated that the major folate form estimated by microbiological techniques is 5MeTHF (Cossins and Shah 1972, Rodriguez 1978). Perry (1971) estimated that a Western mixed diet contained 60% of total folate as 5MeTHF with 38% as formyl folates after conjugase treatment. The major formyl folate form was found to be 5CHOTHF, however, Perry (1971) suggests this may arise from 10 CHOTHF rearrangement during extraction. Other studies reviewed by Rodriguez (1978), report that 10CHOTHF types are the major folate forms after 5MeTHF. However, with the variable response of microbiological assay organisms and the use of impure conjugase preparations (chapter 1.4) the precise values for food folate as 5MeTHF and 10CHOTHF needs to be treated with caution, although it is likely both are normal food constituents.

10CHOTHF readily oxidises in air to 10CHOFA (fig 5.1, Robinson 1971 Blair 1976) this oxidation may be prevented by the prescence of antioxidants notably ascorbate. Stokes et al (1975) investigating a scorbutic subject reported the prescence of 10CHOFA in the urine when the compound is not normally present in serum. They

Table 5(1)

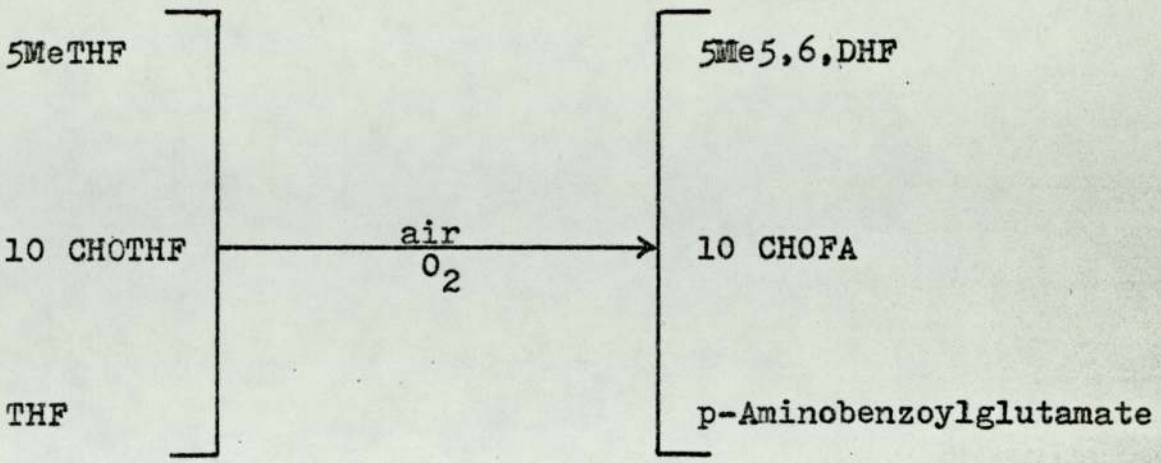
Lactobacillus casei folate content of foodstuffs prior to and after acidification

	Before acid µg/100g	After acid µg/100g
Watercress	2.7	0.8
Spring cabbage	2.6	0.8
White bread	0.09	0.08
Lambs Liver	2.6	2.6
Lettuce	0.4	0.3
Egg yolk	1.2	1.2

Difference between the two values i 5Me 5,6, DHF content (p.117).

After Hanson and Blair (1978) unpublished observations

Fig.5.1. Oxidation of Food Folate



propose that ascorbate in humans has a role in protecting serum folates from oxidation, and that in scorbutic subjects lack of ascorbate results in oxidation of 10CHOTHF, which results in depletion of the body folate pool, leading to the megaloblastic anaemia observed in some cases of scurvy (Andrews 1977).

5MeTHF is known to be oxidised by air or H_2O_2 to give 5Methyl 5,6 dihydrofolate (Donaldson and Keresztesy 1962, Gupta and Huennekens 1967, Scrimgeour and Vitols 1966), an oxidation which is reversed by thiols or ascorbate. In vitro studies have shown that the rate of oxidation is increased by the polarity of the solvent system and the presence of Cu^{2+} ions (Blair et al 1975). Thus samples of 5 Me 5,6DHF when assayed in the presence of ascorbate will have the microbiological activity of 5MeTHF. However, if assayed without ascorbate; or if acidified to pH3 prior to ascorbate addition, 5Me5,6DHF samples show no microbiological activity for the common assay organisms (Ratanasthein et al 1977). Thus if duplicate samples of a foodstuff or tissue are taken, one being acidified prior to both having added ascorbate, the difference in L.casei activity between them will measure the 5Me5,6,DHF content, as the other microbiological activities are unaffected by the acidification. (Ratanasthein et al 1977). By this technique it is observed (Blair and Hansen unpublished data (1978) Table 5(1)) that 5Me 5,6 DHF may comprise the major portion of free folate in stored food. Similarly Ford et al (1978) showed that the

depletion of folate in stored milk was mainly the result of 5MeTHF oxidation to 5Me5,6,DHF when then produces biologically inactive forms, possible due to the loss of protecting ascorbate by aerial oxidation.

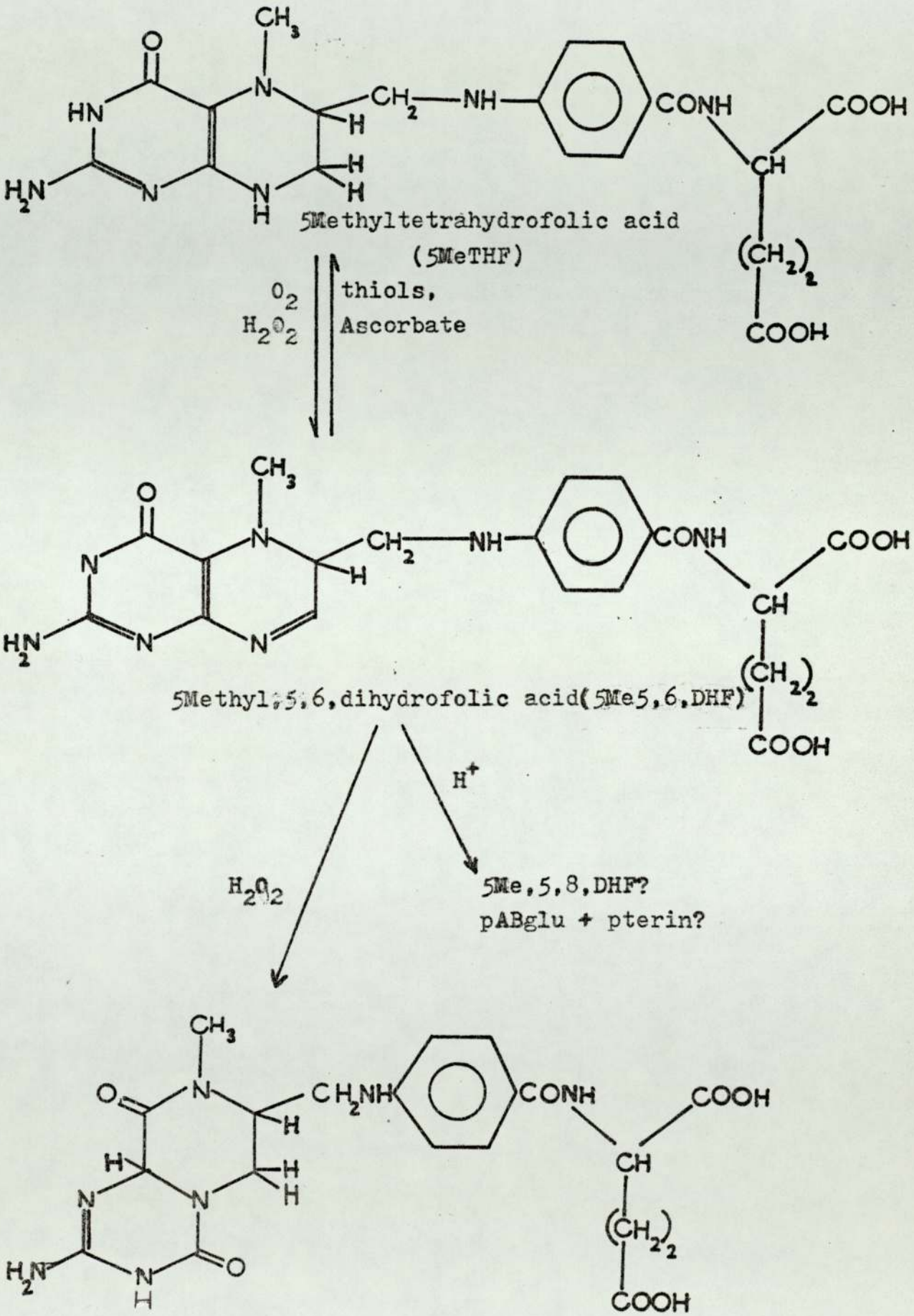
5Me 5,6, DHF gives a small rise in serum folate when administered orally to normal humans (Ratanasthein et al 1977). However, when dosed with sodium bicarbonate, 5Me 5,6, DHF resulted in a significantly greater rise in serum folate. This implies gastric H^+ renders 5Me 5,6, DHF inactive, however, should any portion survive the gastric enviroment it can be assimilated into the folate pool.

The product of acidified 5Me 5,6,DHF was at first formulated as 5Me 5,8, DHF (Blair et al 1975) by analogy with the acid rearrangement of 5,6 dihydropterines, later workers (Deits et al 1976) suggested there is a C^9-N^{10} scission in acid to give non-folate products.

5Me 5,6, DHF may also be further oxidised to another compound by H_2O_2 (Gupta and Huennekens 1967) originally described as 4aOH5MeTHF by Gapski et al (1971). However, Jongejan et al (1979) working with folate analogues report that oxidation is followed by an intramolecular rearrangement to form a pyrazino-s-triazine structure (fig 5.2). As mentioned in Chapter 3 this compound was found in stored 5MeTHF samples when the level of ascorbate was not maintained. The triazine "4aOH5MeTHF" is not active for the common folate microbiological assay organisms (Gapski et al 1971, Ratanasthein et al 1977) and will not be demonstrated by microbiological methods.

Thus the 5MeTHF of stored folate could give rise to varied

Fig.5.2 Oxidation of 5MeTHF



compounds; 5Me 5,6, DHF, the triazine, or acid rearrangement products. The oxidation of 5MeTHF is not yet completely characterised, as stored 5Me 5,6, DHF during these experiments was observed to give products whose UV spectra are not compatible with the triazine, p-aminobenozyl glutamate nor 5Me 5,6. DHF. These diverse products may have varying biological activity.

Experiments were undertaken to observe the effect of the absence of antioxidant on the metabolism of 5MeTHF and to investigate the metabolism and disposition of the well-characterised 5MeTHF oxidation products in the rat. Animals were orally dosed with 5(^{14}C)MeTHF dissolved in water and compared with the results from animals given 5MeTHF in 2% Na ascorbate in 50mM Na phosphate buffer pH7. The metabolism of 5Me 5,6, DHF was investigated by IP dosage of (3',5', 7,9,^{3}H)5(^{14}C)Me 5,6, DHF mixed label and the effect of gastric acid observed by oral dosage of mixed (2^{14}C) and (3',5',7,9,^{3}H)5Me 5,6, DHF. In vitro samples of 5Me 5,6, DHF labels were acidified and chromatographed to determine the reaction products. Finally rats were orally dosed with (^{14}C methyl) pyrazino-s-triazine to observe whether this compound which is microbiologically inactive, undergoes metabolism by the mammal.

5.2. Metabolism of 5MeTHF in the absence of ascorbate

5(^{14}C)MeTHF (barium salt) was dissolved in distilled water immediately prior (5 Minutes) to oral dosing at 80 µg/Kg of six male Wistar rats. The collection of urine, faeces and CO_2 was as described in methods (Chapter 2).

Table 5(2)

Recovery of ^{14}C activity from rats orally dosed 80 µg/Kg with 5(^{14}C)VeNVT in the presence or absence of ascorbate.

values % dose ^{14}C ± S.E.M.

	With ascorbate (4 rats)	without ascorbate (6 rats)	Pvalue 't' test
Urines			
Day 1	50.9 ± 4.0	33.1 ± 2.9	p < 0.002*
Day 2	2.0 ± 0.2	4.7 ± 0.4	p < 0.001*
Day 3	1.2 ± 0.2	2.2 ± 0.2	p < 0.01*
Total	54.1	40.0	
Faeces			
Day 1	2.4 ± 1.0	13.0 ± 2.4	p < 0.005*
Day 2	0.1 ± 0.03	12.8 ± 3.8	p < 0.025*
Day 3	0.02 ± 0.01	-	
Total	2.5	25.8	
CO_2			
Day 1	2.0	2.3	
Day 2	0.4	0.3	
Day 3	0.4	-	
Total	2.8	2.6	

* significant difference

Table 5(3)

DE52 Fractionation of urinary activity of rats dosed orally 80 µg/Kg 5(^{14}C)MeTHF with or without ascorbate

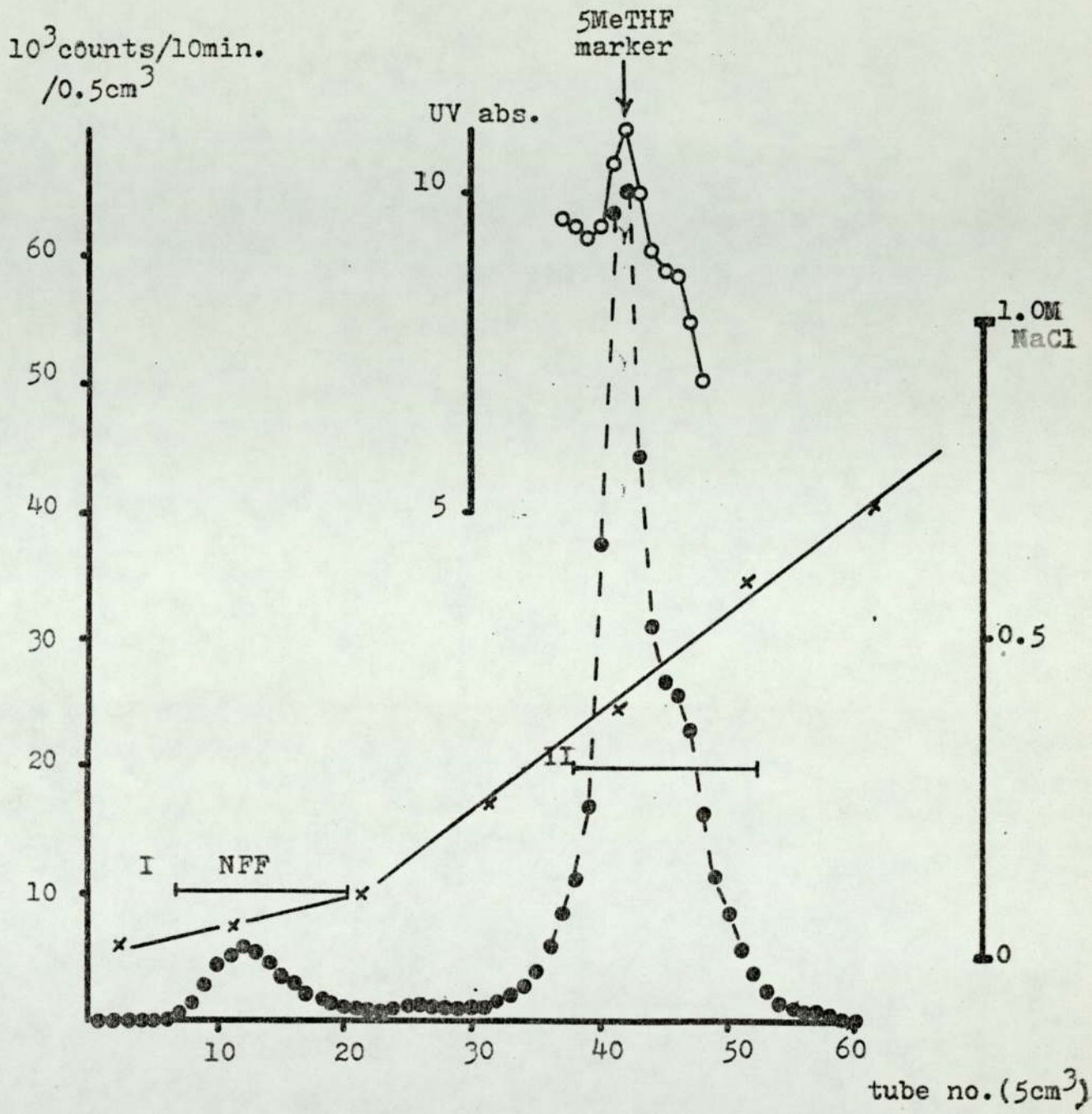
	With ascorbate		Without ascorbate	
	% urine activity	% dose activity	% urine activity	% dose activity
Day 1				
HFF	5.9	3.2	9.5	2.7
5MeTHF	88.5	47.8	81.7	23.6
HFF/5MeTHF	0.07		0.12	
Day 2				
HFT	27.0	0.5	16.6	0.8
5MeTHF	55.8	1.1	78.9	3.7
HFF/5MeTHF	0.48		0.21	
Day 3				
HFF	44.7	0.6	26.3	0.6
5MeTHF	32.3	0.4	66.9	1.5
HFF/5MeTHF	1.38		0.39	

Tables 5(2) and 5(3) give the recovery of radioactivity and fractionation of urinary ^{14}C compared to that observed when rats are dosed 80 µg/Kg 5(^{14}C)MeTHF dissolved in 2% ascorbate in pH7 50mM Na phosphate buffer.

From table 5(2) it is observed on the first day the non-ascorbate group show significantly less activity in the urine, with significantly more ^{14}C in the first day faeces. Thus the absence of ascorbate decreases the efficiency of absorbtion of 5MeTHF. The higher second day ^{14}C faecal activity of the (-)ascorbate group may be explained by a delayed gut passage of unabsorbed dose material or more likely by an increase in biliary excretion of ^{14}C methyl derived products.

DE52 fractionation of the urines from both groups of rats resolves the ^{14}C activity into two peaks; I, non folate compounds as described in chapter 3 and II, a peak which cochromatographs with added 5MeTHF on DE52 and also on subsiquent G15 elution Table 5(3). Notably no triazine was observed on the urinary chromatographs fig 5.3 , indicating that it is not produced under these conditions, or that if formed it is not absorbed and excreted in the urine at levels sufficient to be detected. With the exception of day 1, the urinary activity of the non ascorbate rat group shows a lower NFF/5MeTHF ratio Table 5(3) indicating a slower rate of 5MeTHF demethylation. In the case of the first day urine, from Chapter 3 it is observed that the 5MeTHF present in the first day urine following oral dosing of 80 µg/Kg 5MeTHF is probably due to renal overloading. However, when dosed in

Fig.5.3. DE.52 Chromatogram of 0-24hr urine from rats orally dosed with 80μg/Kg 5(^{14}C)MeTHF without ascorbate



the absence of ascorbate (table 5(2)) the ^{14}C activity of 5(^{14}C)MeTHF is less efficiently absorbed, which would decrease renal overloading and tend to diminish the excretion of 5MeTHF in the urine, and increase the ratio of NFF/5MeTHF.

The slower rate of demethylation of 5MeTHF in the non-ascorbate group of rats indicates the ^{14}C label is less biologically active. As only 5MeTHF is found in addition to NFF in the urines it is possible to speculate that a portion of the ^{14}C label is absorbed as 5Me 5,6, DHF which is not an in vitro substrate for methionine synthetase (Deits et al 1976), and thus has to be reduced to 5MeTHF before demethylation. If this reduction is slow compared to methionine sythetase activity, the apparent rate of 5MeTHF demethylation would decrease. If 5Me 5,6,DHF is excreted intact in the urine, it will be chromatographed as 5MeTHF by reduction in the collecting flask.

These results indicate biological disposition of 5(^{14}C)MeTHF is significantly altered when ascorbate antioxidant is ommitted.

5.3. Metabolism of 5Me 5,6, DHF

Mixed label (2^{14}C)(3',5',7,9,^{3}H)5Me 5,6,DHF was prepared from similarly labelled 5MeTHF by ferricyanide oxidation, and purified as described in Chapter 2, made up in N_2 aerated water and orally dosed to six rats at 0.06μg/Kg. The retention and excretion of the radioactivity is given in Table 5(4). There is apparently no detectable retention of ^{14}C or ^{3}H in the examined tissue, although this may be due to the low total (3×10^{4}c/10min) radioactivity dosed. The urinary activity shows a significant excess of ^{3}H over ^{14}C

Table 5(4)

Oral dosing of rats with 0.06 µg/Kg (2^{14}C)(3',5', 7,9, ^{3}H) 5Me 5,6, DHF; retained and excreted radioactivity

Values % dose

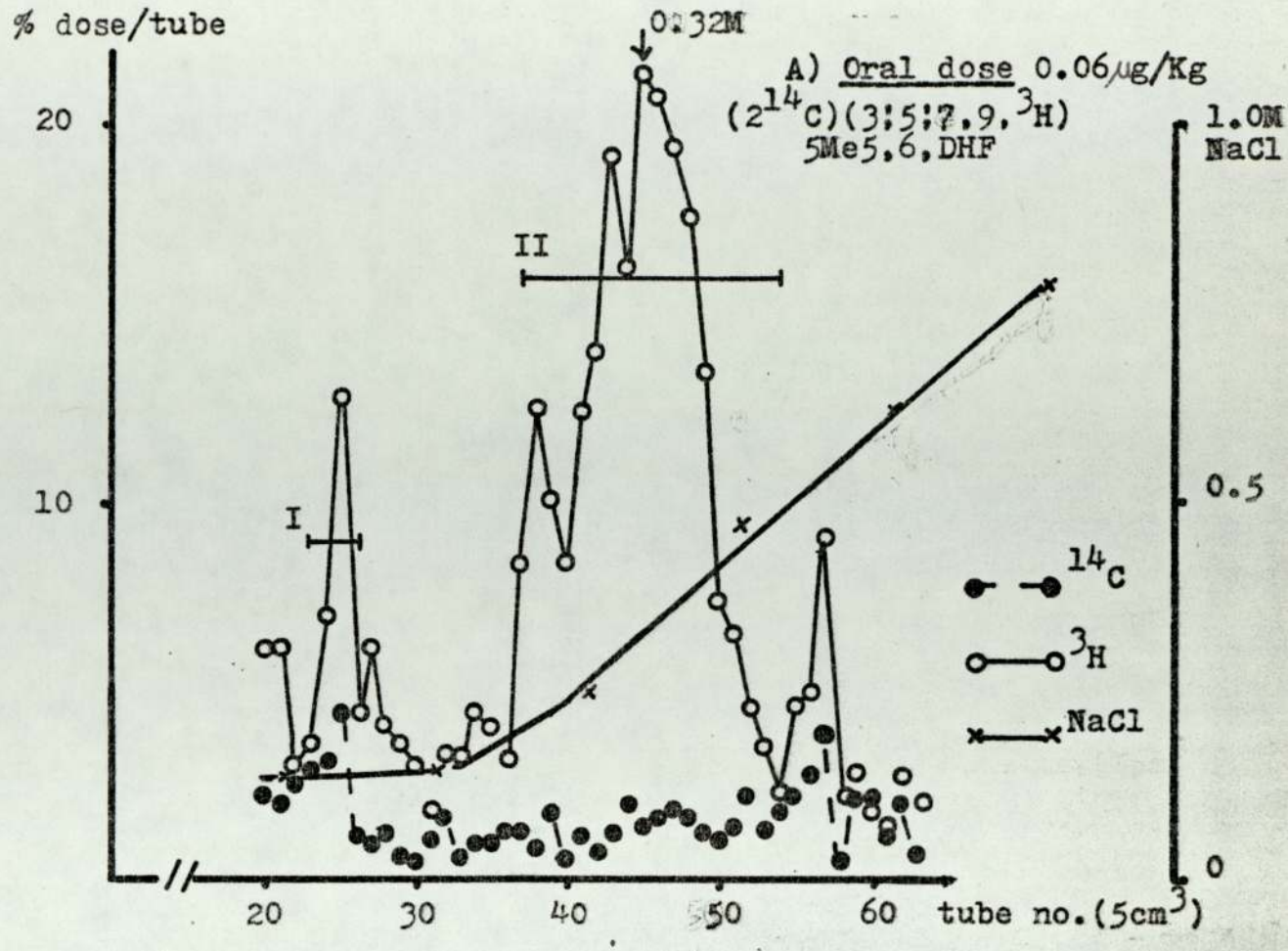
6 rats 0.06 µg per Kg		0-6hr	6-24hr	Day 2	Day 3	Total
Urine	^{14}C	22.9[1]	25.4[1]	NS	NS	48.3
	^{3}H	39.1	46.2	NS	NS	85.3
			Day 1			
Faeces	^{14}C		45.1[2]	10.0	9.3[3]	64.4
	^{3}H		28.1	8.1	4.3	40.5
Kidney, Spleen, Gut, Brain, Muscle, Liver	^{14}C				NS	-
	^{3}H				NS	-
Total	^{14}C					112.7
	^{3}H					125.7

NS: not significant compared to background

1 t test $p < 0.001$ ^{3}H recovery vs ^{14}C
2 $p < 0.025$
3 $p < 0.01$

and vise versa in the faeces. This would be consistent with scission of the folate with preferential excretion of the ^{3}H labelled fragment(s) in the urine with biliary excretion or diminished absorbtion of the ^{14}C species in the gut. This is confirmed by the observation of p-acetamidobenzoyl-glutamate in the 0-6hr and 6-24 hr urines and p-acetamido-benzoate in the 6-24hr urine (fig 5.6) identified by paper chromatography (chapter 2(4)). These compounds are observed as products of 5MeTHF catabolism; however, after oral dosing of 5Me 5,6,DHF ^{3}H scission products are the only well resolved peaks on DE52 chromatographs of the first day urines (fig 5.4a, fig 5.5a). These results suggest that 5Me 5,6, DHF is poorly assimilated into the folate pool when given orally at this dose. To investigate whether this is a result of acid degradation in the stomach as proposed by Ratanasthein et al (1977), six rats were IP dosed 0.4 µg/Kg with mixed label (3',5',7,9,^{3}H)5(^{14}C)Me 5,6,DHF. DE52 chromatography of the first day urines is in marked contrast to those of orally dosed animals (figs 5.4b, 5.5b) the ^{3}H label showing incorporation into the folate pool and excreted as 10CHOFA and 5MeTHF. These intact folates make up the majority of first day urine ^{3}H activity (Table 5.5). The 5^{14}C label has been transfered to non-folate compounds in the 6-24 hr urine fig 5.5b peak I indicating reduction of 5Me 5,6, DHF to 5MeTHF has taken place within the animal. Some triazine is observed in the 0-6 hr urine 5.4b peak I, however, this may be as a result of the compound being an impurity of the dose solution, for in the experiment where 5MeTHF is dosed without ascorbate (section 5.2) which is likely

Fig.5.4. Chromatographs of 0-6hr urines from rats dosed orally or I.P. with 5Me5,6,DHF



B) Oral dose 0.4µg/Kg 5(^{14}C)Me(3;5;7,9,^{3}H)DHF

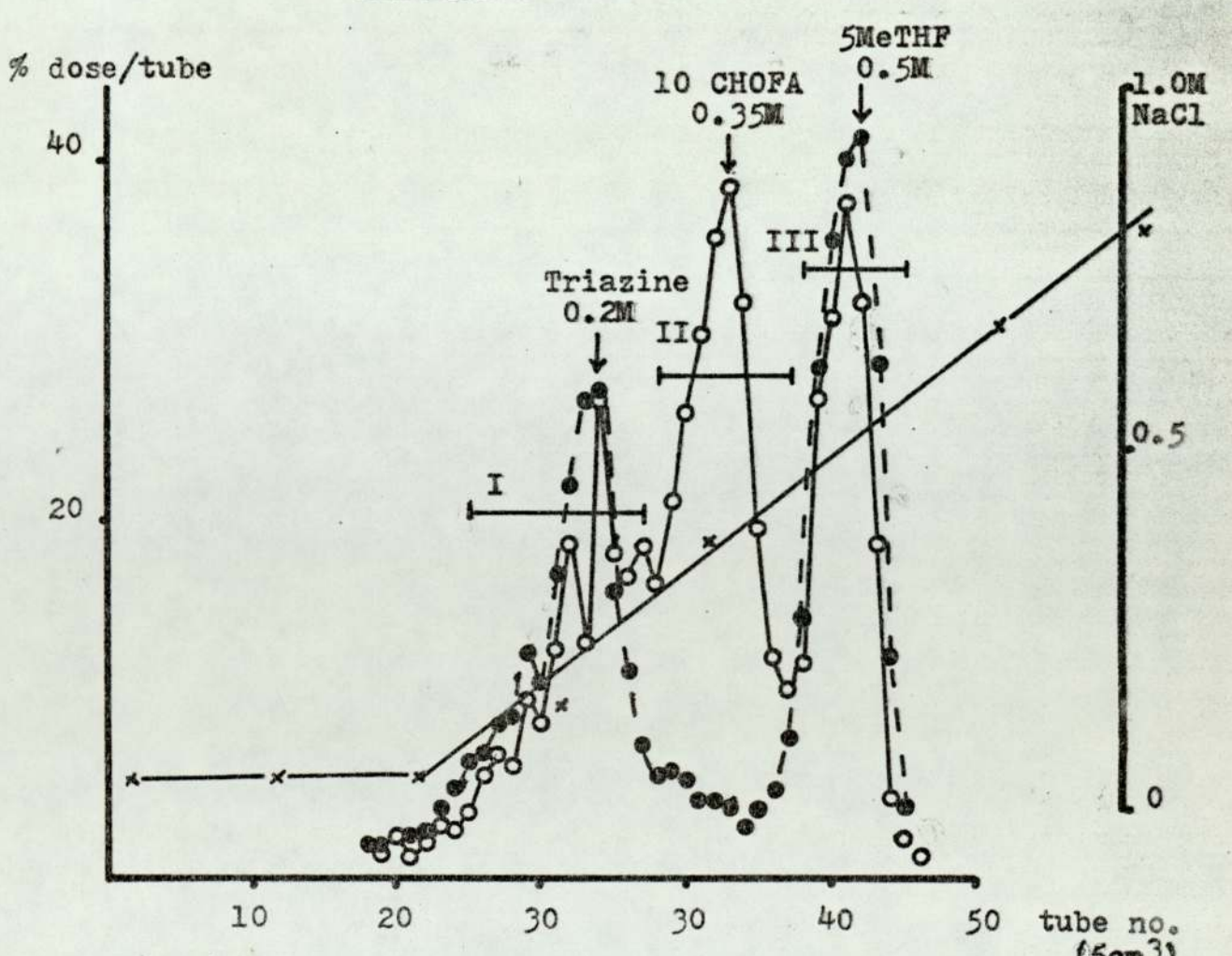


Fig.5.5. DE.52 chromatograms of 6-24hr urine from rats dosed orally or I.P. with 5Me5,6,DHF

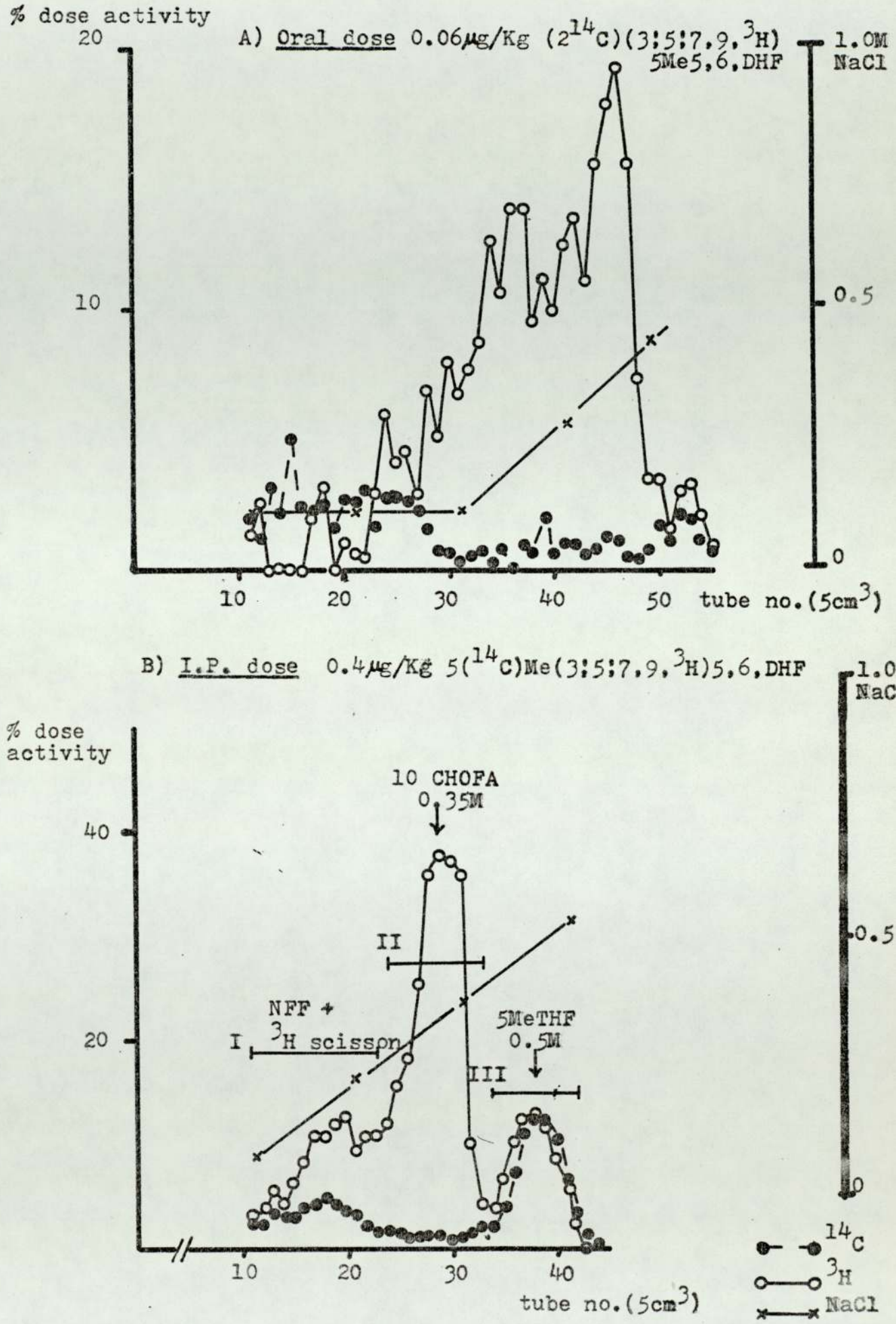
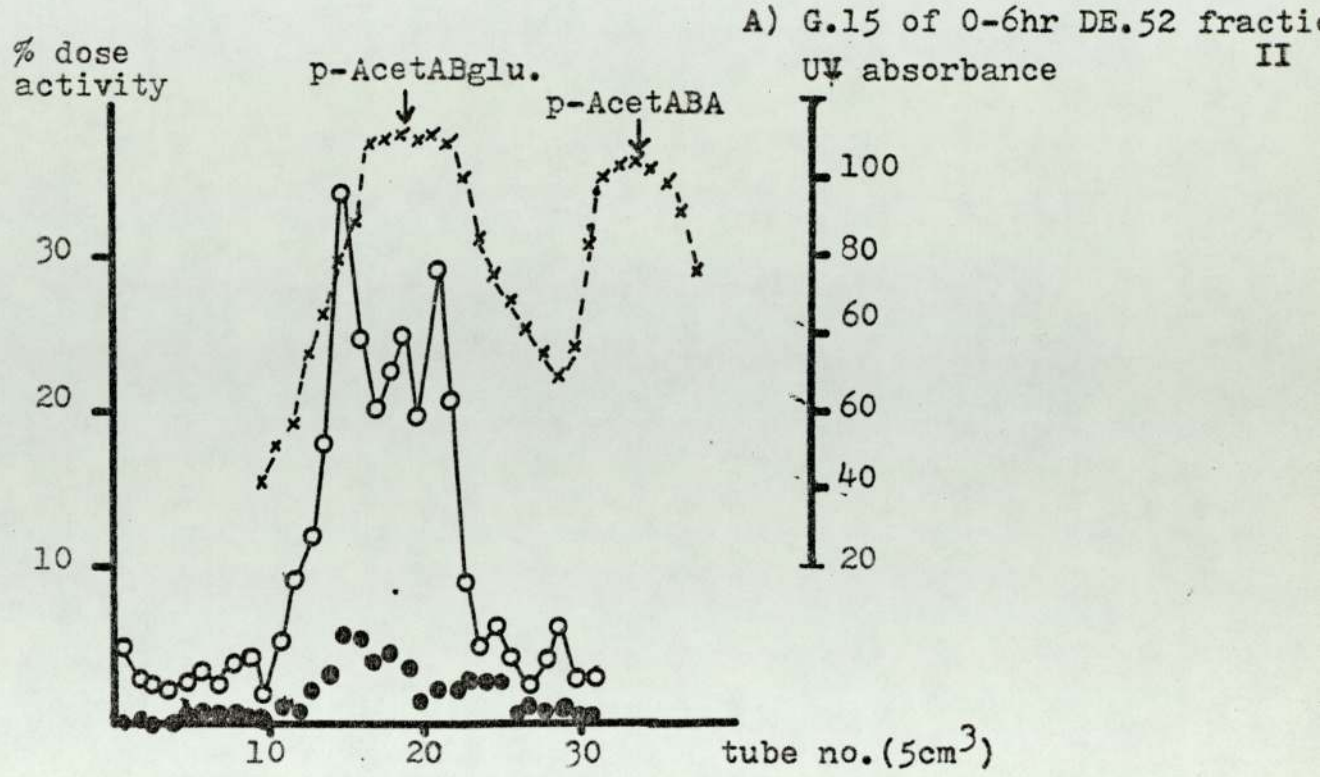


Fig.5.6. G.15 Chromatography of ^{3}H fractions from day 1 urines of rats orally dosed with 0.06µg/Kg ($2^{14}C$)(3;5;7,9,^{3}H) 5Me5,6.DHF



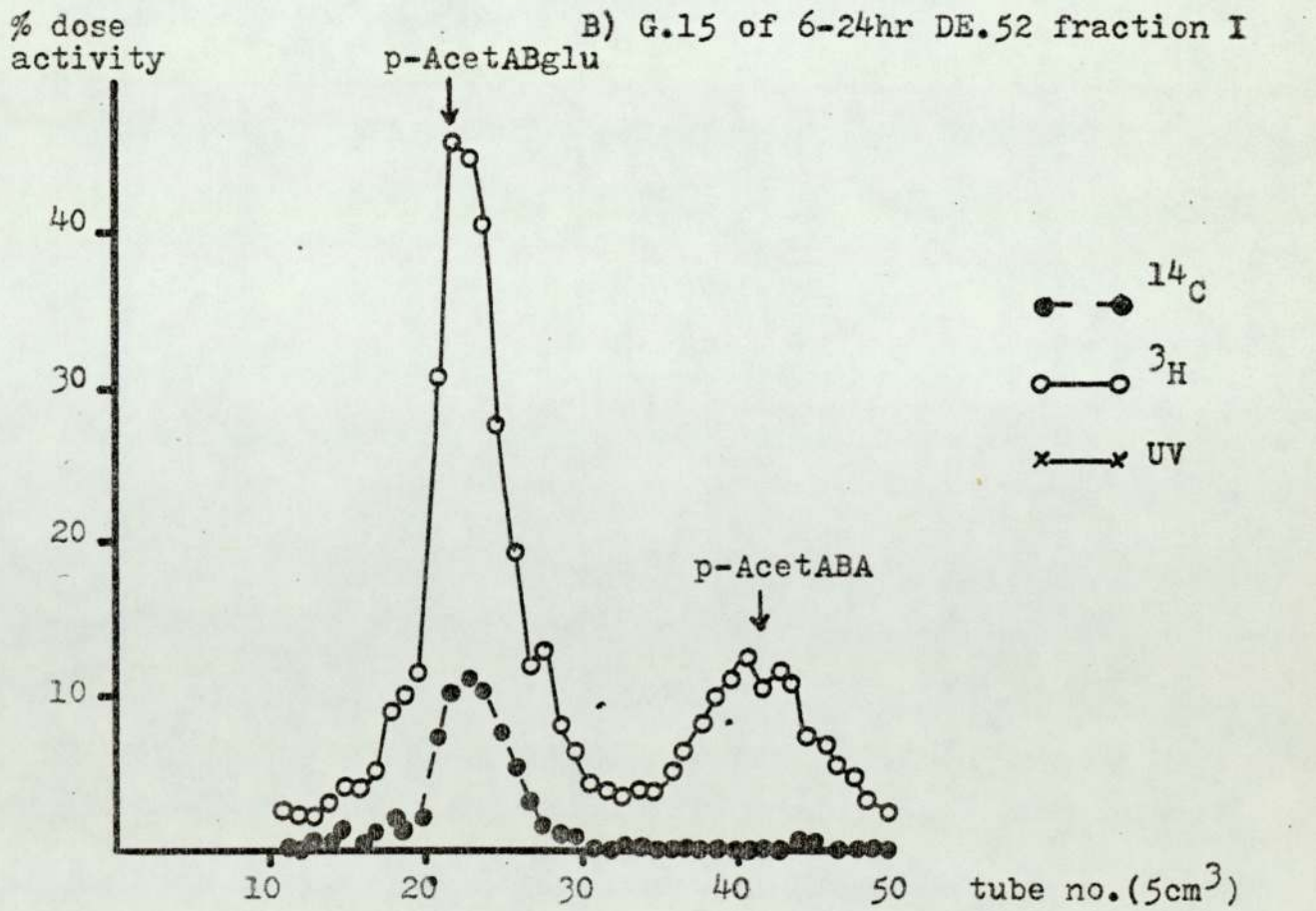


Table 5(5)
DE52 Fractionation of first day urine of rats dosed IP with 0.4 µg/Kg (3',5',7,9,^{3}H) 5(^{14}C)Me 5,6,DHF

0-6 hr urine: 68.2% dose ^{14}C 59.3% dose ^{3}H

Fraction	% urine		%dose	
	^{14}C	^{3}H	^{14}C	^{3}H
I triazine	43.3	30.6	29.5	18.1
II 10CHOFA	11.1	41.0	7.6	24.3
III 5MeTHF	44.4	27.8	30.3	16.5
Total	98.8	99.4	67.4	58.9

6-24 hr urine: 14.3% dose ^{14}C 44.8% dose ^{3}H

	% urine		% dose	
	^{14}C	^{3}H	^{14}C	^{3}H
I NFF	34.7	-	5.0	-
I ^{3}H scission	-	27.2	-	12.2
II 10CHOFA	9.8	54.5	1.4	24.5
III 5MeTHF	54.8	18.2	7.8	8.1
Total	99.3	99.9	14.2	44.8

to result in 5Me 5,6, DHF formation there is no observed urinary excretion of triazine.

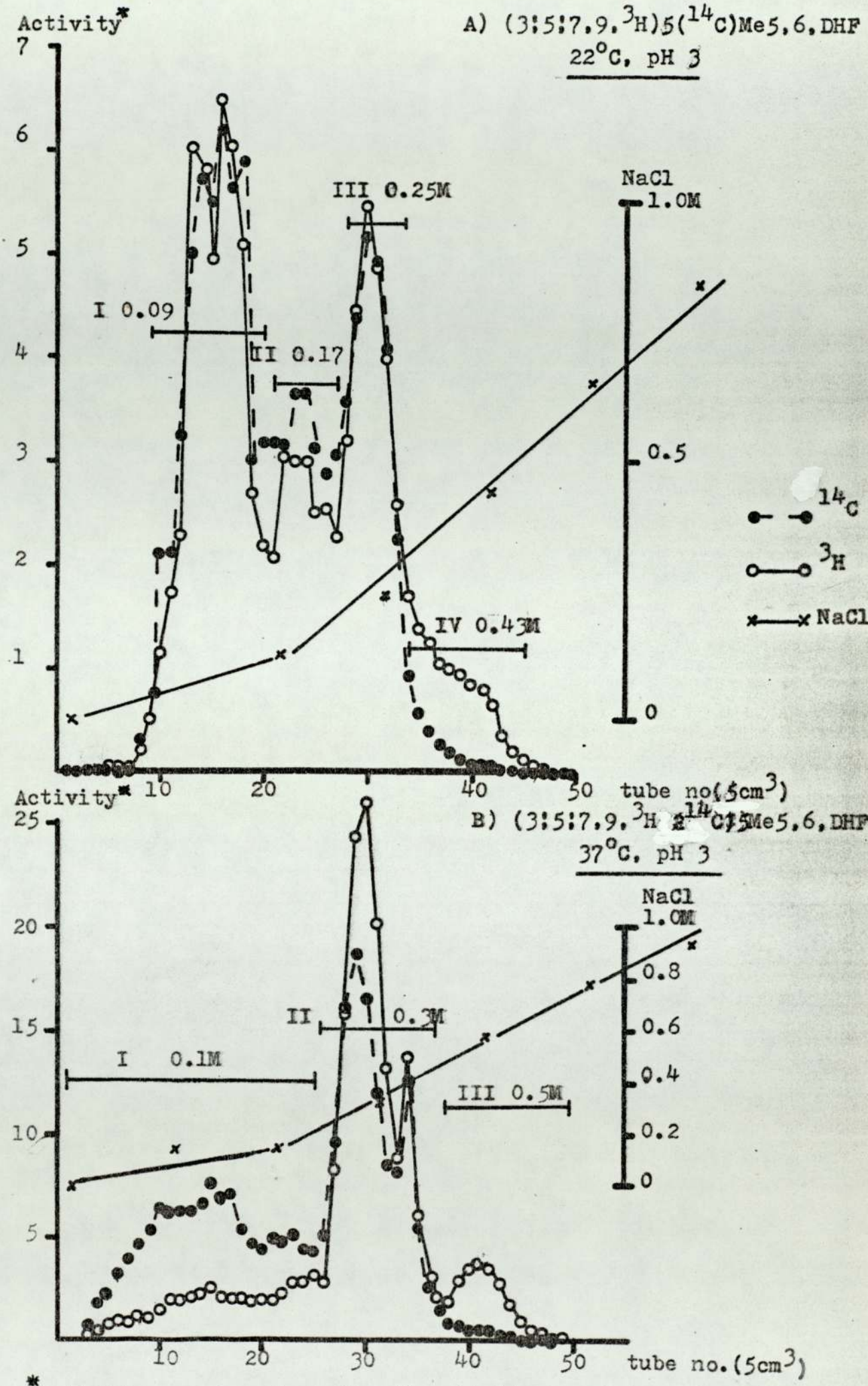
To conclude, an IP dose of 5Me5,6, DHF may be assimilated into the rat folate pool, most probably via reduction to 5MeTHF. However, passage across the gut results in the compound being excreted also 100% as scission products in the urine and faeces.

5.4 Acidification of 5Me 5,6, DHF

In order to determine the effect of gastric acid on 5Me 5,6,DHF the labelled compound was acidified in vitro and chromatographed on DE52 to determine the reaction products. Two samples of mixed label 5Me5,6,DHF were prepared ($2^{14}C$)(3',5', 7,9, ^{3}H) and (3',5',7,9,^{3}H)($5^{14}C$ methyl) forms. The freeze dried solid was dissolved in 25 cm^3 of N_2 gassed water and the pH adjusted to 3 with HCl. After 30 minutes under nitrogen the reaction mixture was neutralised with NaOH, diluted with N_2 gassed water to a conductivity of less than 0.1 mhos and applied to a DE52 column. The incubation of the ($5^{14}C$ methyl) sample (6 µg) was carried out at room temperature (22°C), that of the ($2^{14}C$) labelled sample (0.25 µg) at 37°C.

Fig 5.7 gives the chromatograph traces and table 5(6) the fractionation of the acidified 5Me 5,6, DHF on DE 52. At 22°C 5Me 5,6,DHF elutes as four peaks; the late eluting peak IV (fig 5.7a) is labelled with tritium only, and rechromatographs on G15 with p-aminobenzoyl glutamate, peaks I and II which have approximately equal recovery of ^{14}C and ^{3}H (Table 5(6)a) and peak III which shows an excess of ^{14}C over ^{3}H. Peak I may be a rearrangement or

Fig.5.7. DE.52 Chromatographs of acidified 5Me5,6,DHF



*Activity expressed as % radioactivity dosed to rats in metabolism experiments

Table 5(6)

DE52 Column separation of acidified 5Me 5,6, DHF

a) (3',5',7,9,^{3}H) 5(^{14}C) Me 5,6,DHF pH3 22°C

Fraction	%^{14}C	%^{3}H	Ratio $^{14}C/^{3}H$
I	48.93	45.29	1.08
II	22.89	18.81	1.22
III	25.45	26.4	0.96
IV (pABglu)	2.6	8.71	0.30

b) (2^{14}C)(3',5',7,9,^{3}H)5Me 5,6, DHF pH3 37°C

Fraction	%^{14}C	%^{3}H	Ratio $^{14}C/^{3}H$
I	47.63	19.34	2.46
II	49.38	67.92	0.73
III (pABglu)	2.6	12.16	0.21

possibly an oxidation product of 5Me5,6, DHF as yet uncharacterised; rechromatography on G15 shows that it does not elute with the triazine. Peak III may contain unreacted 5Me 5,6, DHF, as after addition of ascorbate a dual-labelled portion of this fraction cochromatographs with 5MeTHF, although the majority of the fraction activity had produced other compounds after storage. Fraction II may be the other fragment produced by cleavage of 5Me 5,6, DHF, the addition of the activities of peaks II and IV gives a $^{14}C/^{3}H$ ratio of 1.08. The structure of II remains indeterminate, from the published labelling of the parent ^{3}H folic acid (25.5% C_7, 32% C_9 and 42.5% 3',5', Radiochemical Centre, Amersham.) a 5(^{14}C)(7,9,^{3}H) pterin moeity would have a $^{14}C/^{3}H$ ratio of 1.74 where II had a value of 1.22. However the mixed label 5MeTHF used to produce 5Me 5,6,DHF was extracted from rat urine which may alter the relative abundance of ^{3}H at the various positions. Using the value of 42.5% for ^{3}H at 3', 5', positions, the apparent cleavage of 5Me 5,6, DHF is 20.5%.

At 37°C with the (2^{14}C)(3',5',7,9,^{3}H) mixed label the apparent cleavage to pABglu is increased, with 12.2% of ^{3}H as pABglu (fraction III fig 5.7b) 28.6% of 5Me 5,6,DHF Fraction II elutes at a similar position to fraction III of the 5(^{14}C methyl) label although the peak shows a ^{3}H excess. Peak I may be a pterin which has lost some ^{3}H at C_9, its $^{14}C/^{3}H$ ratio 2.46 is between that of a (2^{14}C)(7,9,^{3}H) pterin (1.74) and that of a (2^{14}C) (C_7 ^{3}H) species (3.92). The excess ^{3}H may have been lost as $^{3}H_2O$ during loading of the DE52 column.

Thus as observed by Deits et al (1976), mild acid conditions can cause cleavage of 5Me 5,6,DHF, however, the reaction is not complete after 30 minutes at 37°C (30% cleavage at pH3) and produces other products. However from these results it is possible to predict that at least some cleavage will occur in the stomach prior to absorbtion.

5.5 Metabolism of the triazine oxidation product of 5MeTHF

The triazine was synthesised by H_2O_2 oxidation of 5MeTHF after Gapski et al (1971) both ^{14}C methyl and unlabelled types being produced as described in Chapter 2.

The ^{14}C methyl compound was diluted with unlabelled triazine and orally dosed at 41.5 µg/Kg (4 animals) and 415 µg/Kg to a single rat. The retention and excretion of the ^{14}C activity is given in table 5(7). There is little retention of ^{14}C in the examined tissues, at the low dose 96% of ^{14}C is lost via urine and faeces. The high faecal activity found of the first day indicates the triazine is less well absorbed compared to 5MeTHF (25% compared to 2%). The continued high faecal activity of days 2 and 3 suggest a biliary excretion route.

Chromatography of the low dose group urines (fig 5.8c) showed a single ^{14}C peak coeluting with added triazine marker. A boiling ascorbate extract of a liver from the low dose group of animals showed no incorporation of ^{14}C into polyglutamate forms.

To investigate whether the faecal activity contains triazine metabolites formed by the rat or its gut microflora, a sample of dried first day faeces was extracted with 1% ammonia solution containing 1% (v/v) mercaptoethanol.

Table 5(7)

Retained and excreted radioactivity in rats orally dosed with (^{14}C methyl) triazine

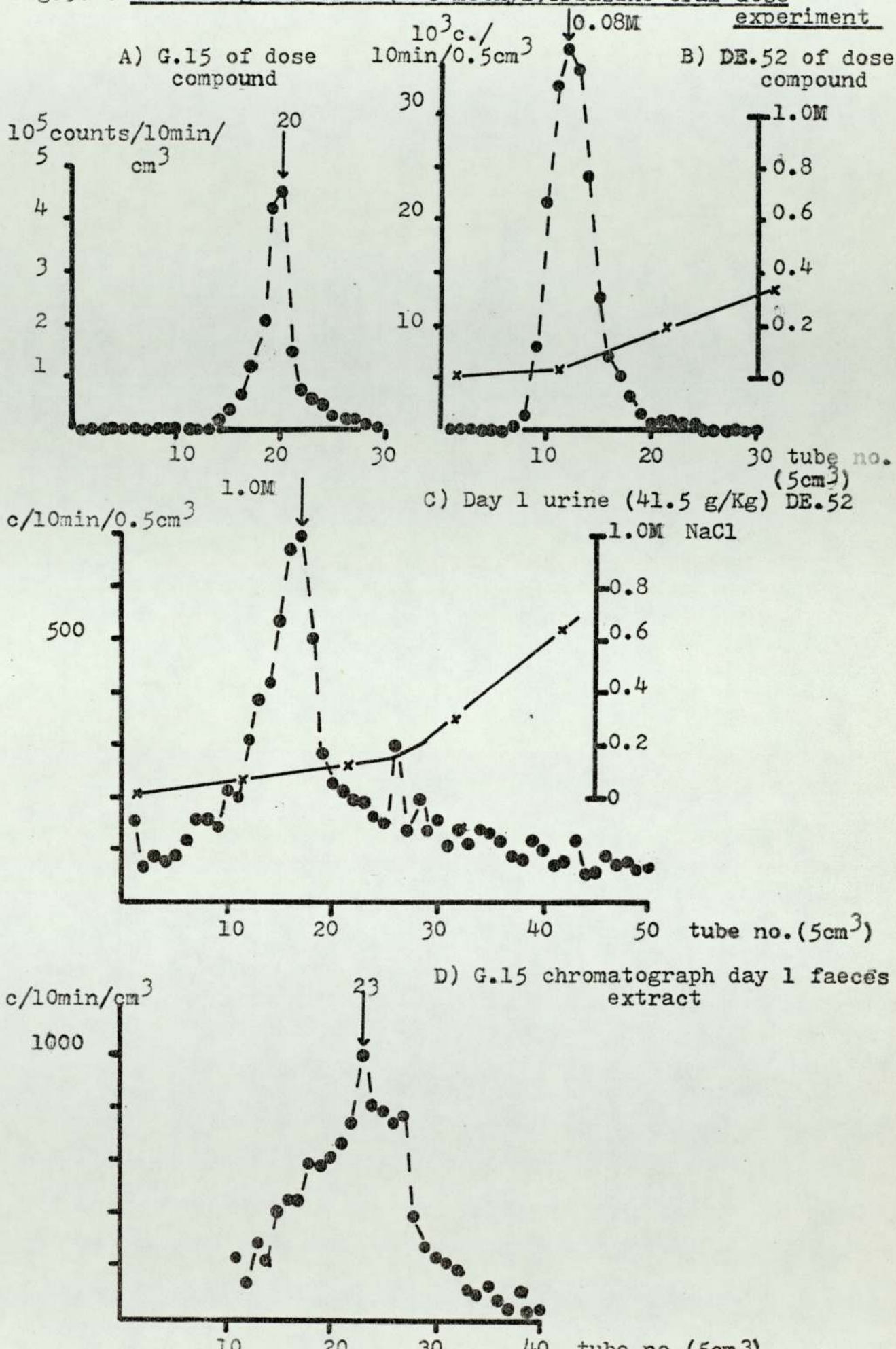
Values as % dose activity, the high dose values are for a single rat.

Low dose 41.5 µg/kg	urine	faeces	CO_2	Liver	Kidney	Spleen	Muscle	Total
number of rats	4	4	2	3	4	4	4	
Day 1	37.0	24.7	0					61.7
2	3.9	17.5	0					21.4
3	3.7	9.0	0	0.5	0.5	0.1	1.0*	14.8
Total	44.6	51.2	0	0.5	0.5	0.1	1.0	97.9

High dose 415 µg/Kg	urine	faces	CO_2	liver	kidney	spleen	muscle	Total
Day 1	21.1	17.5	-					38.6
2	3.6	10.7	-					14.3
3	1.2	11.3	-	0.4	-	-	2.2*	15.1
Total	25.9	39.5		0.4			2.2	68.0

*Calculated assuming muscle = 40% body weight

Fig.5.8. Chromatograms from (^{14}C methyl)Triazine oral dose experiment



Counting indicated this proceedure extracted 88% of faecal ^{14}C. The neutralised extract was chromatographed on G15 (fig 5.8d) and DE52 with triazine standard, and its radioactivity was observed as a single peak inseparable from the marker.

The triazine is excreted unchanged in the urine and faeces, over three days 96% of ^{14}C activity is recovered at the low dose, 65% with the high dose and biliary excretion is a major route. No incorporation of the triazine into high molecular weight was observed. The evidence suggests the triazine makes no contribution to the folate pool or one carbon metabolism.

5.6 Discussion

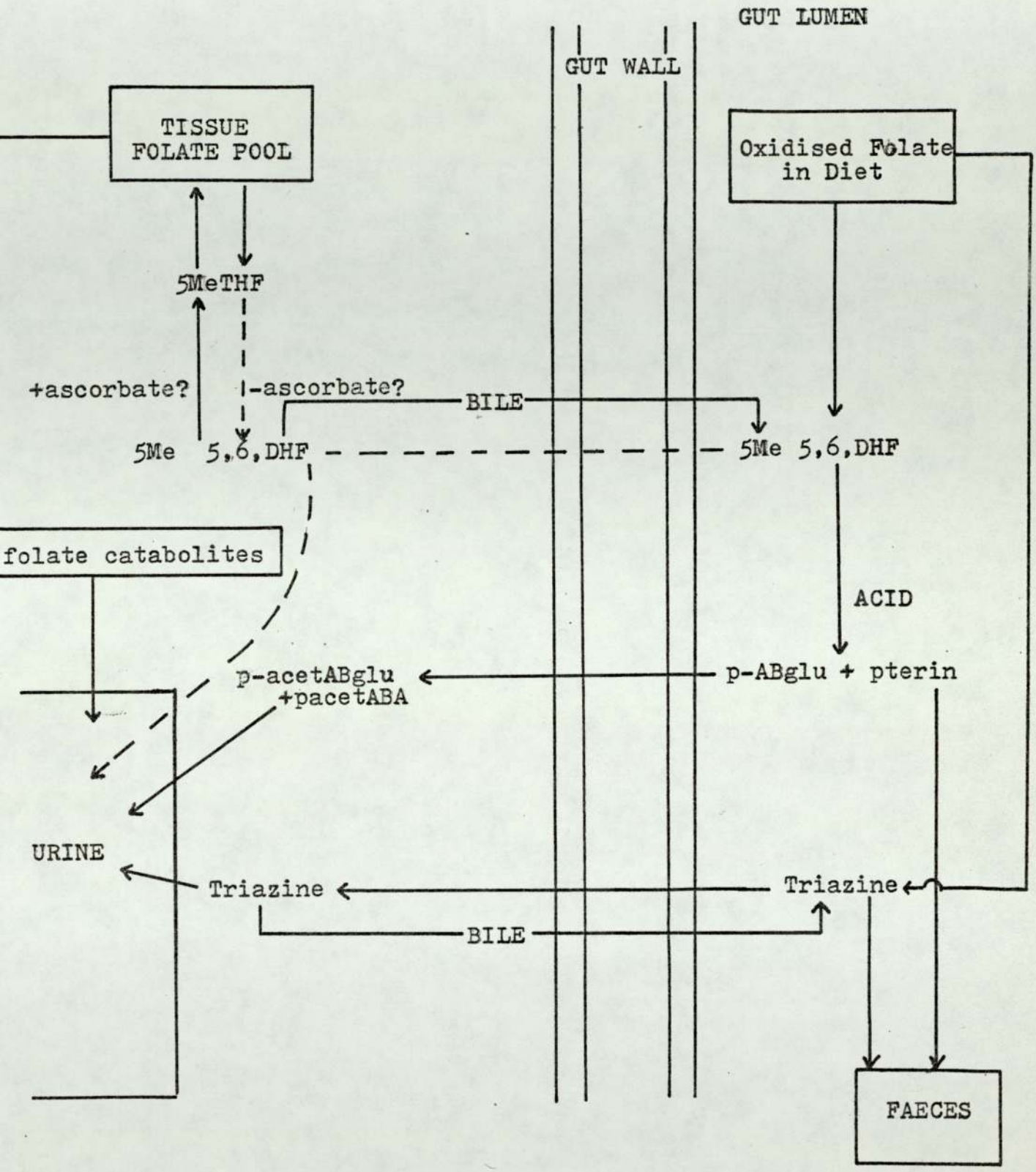
These experiments have investigated the effect of the absence of antioxidants on the metabolism of oral 5MeTHF, and the metabolism of its two well characterised oxidation products, 5Me 5,6, DHF and the triazine. An oxidised sample of 5MeTHF may contain both these compounds in various proportions.

The triazine is less well absorbed than 5MeTHF and some will be lost in the faeces; that which is absorbed will not be further metabolised in the rat and will be excreted unchanged via bile and urine. 5Me 5,6,DHF will undergo at least partial scission in the acid conditions of the stomach. The preferential excretion of ^{14}C in the faeces of rats orally dosed with mixed label $(2^{14}C)(3',5',7,9,^{3}H)$ 5Me 5,6,DHF indicates the pterin portion of $C^{9}N^{10}$ scission is less well absorbed compared to the pAEglu fragement. From the IP dosage experiment absorbed 5Me 5,6,DHF may be assimilated into the folate pool by reduction to 5MeTHF.

However, should the 5Me5,6,DHF persist or be generated in the body, as may occur in scorbutic humans by analogy with the observed oxidation of 10CHOTHF to 10CHOFA (Stokes et al 1975), the compound is likely to enter the enterohepatic circulation with other folates (Hillman et al 1977; Pheasant and Blair 1979, Pheasant et al 1980). Entry into the acidic conditions of the small intestine and more particularly the acid microclimate generated at the surface of the gut mucosa (Blair and Matty 1974) would facilitate further scission of 5Me 5,6,DHF (Fig 5.9). Acidic scission of 5Me 5,6,DHF is likely to be the major route of folate depletion during Vitamin C deficiency, as 5MeTHF is the major circulating folate monoglutamate. Oxidation of 5MeTHF to 5Me5,6,DHF will result in scission to pterines with irreparable loss of folates, however, 10CHOFA is stable and may re-enter the folate pool by dihydrofolate reductase activity (Pheasant et al 1980) albeit at a slower rate than folic acid.

The microbiological assay of foodstuffs may overestimate the L.casei folate content as in the presence of ascorbate it will give the same response for 5Me5,6,DHF, as 5MeTHF, however, these experiments have shown oral 5Me5,6,DHF has diminished activity compared to 5MeTHF in the rat. Similarly, 5MeTHF shows a marked decrease in biological activity if allowed to remain in solution without ascorbate five minutes prior to dosing.

Fig.5.9. The Disposition of 5MeTHF Oxidation Products



6. THE METABOLISM OF 5METHYLTETRAHYDROFOLIC ACID IN TUMOUR BEARING RATS

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6.1 Introduction

The clinical use of methotrexate illustrates the rationale of cancer chemotherapy, the exploitation of a physiological difference between normal and malignant cells to facilitate selective therapeutic cytotoxicity. MTX is a phase specific agent destroying cells entering the S phase of the mitotic cycle by dihydrofolic reductase inhibition. As such it is thus most effective against rapidly dividing cells which have a greater cell fraction passing through S phase at any one time period; in culture log phase cells have a MTX kill rate 6.7 times that of confluent cells (Hryniuk et al 1967). Differential cell kill with phase specific agents is heightened in that normal cells have a greater proportion of cells in G_o, or 'resting' with respect to mitosis, which are immune to phase specific inhibition, than tumour cells (Valenote and Van Putten 1975). These cells in G_o may be rescued from MTX inhibition as they re-enter the cell cycle by the provision of exogenous folate antidote, usually 5CHOTHF.

The success of MTX as an antifolate cancer chemotherapeutic and the observed importance of folates to cell division (Chapter 1) has prompted further research into the role of folate coenzymes in tumour cells to observe any metabolic variation from normal which may have diagnostic or therapeutic applications.

The increased requirement for purines and thymidylate during cell division results in observable changes in the folate-requiring enzymes with the state of mitotic activity within the cell or tissue. Thymidylate synthetase (TS), an

essential and possibly rate-limiting enzyme for DNA synthesis (Friedkin 1973) is observed to increase 2.4 times when cells in culture change from resting to logarithmic cell growth stage (Rosengarten et al 1971) Similarly in normal adult rat liver cells Maley and Maley (1960) were unable to demonstrate thymidylate synthetase activity until the liver had undergone partial hepatectomy. Jackson and Niehammer (1979) were able to show a 2,000 to 12,000 increase of TS in various hepatoma cell lines compared to normal liver. In cell culture dihydrofolate reductase increases thirty fold when resting cells begun logarithmic growth (Johnson et al 1978b). A tumour load may affect the metabolism of the host, rats with implanted tumours having increased levels of liver methylene THF reductase (Albrecht 1979).

The enzymic changes caused by the continuous mitosis of a malignant tumour would be expected to result in a displacement in the equilibria between the various coenzymes, if it is large enough. Halpern et al (1977) and Stea et al (1978) report novel pterin cleavage products from tumour cell culture and cancer patients given large doses of folic acid. Following oral FA Barford and Blair (1978) and Pheasant and Blair (1979) note a change in the ratios of urinary folates of rats bearing implanted Walker 256 carcinosarcoma and Novikoff hepatoma. Similar studies with 5MeTHF would indicate whether such effects are also associated with a naturally occurring folate.

More specifically, there have been reports concerning the tumour metabolism of 5MeTHF. The experiments of Halpern's group (Chapter 1) showed some tumour cell lines in culture

Table 6 (1)

Excreted and retained radioactivity after oral 80 μg/Kg dose of 5(^{14}C)MeTHF in normal and W-256 implanted male rats

% dose ^{14}C activity	Normal rats(4)	W256 implanted(6)	't' test
Day 1 urine	54.0	47.9	NS
2	1.9	4.2	$p < 0.01$
3	1.3	2.5	NS
Day 1 CO_2	2.0	4.3	
2	0.4	2.1	
3	0.4	2.5	
Day 1 faeces	2.3	2.6	NS
2	0.1	0.1	NS
3	0.02	0.04	NS
Day 3 Tissues			
Liver	0.1	0.6	
Kidney	0.1	-	
Spleen	0.01	0.04	
Muscle	13.2	2.5	
Tumour	-	0.6	
Total	75.9%	69.9%	

Value in ( ), number of rats in experiment
NS: not significant.

Table 6(2)

Fractionation of urine from normal and tumour bearing rats orally dosed 80 μg/Kg 5(^{14}C)MeTHF

	Normals			W256 implanted		
	%urine	%dose	NFF/5MeTHF	%urine	%dose	NFF/5MeTHF
Day 1						
NFF	5.9	3.2		10.8	5.2	
5MeTHF	88.5	47.8		71.7	34.3	
Total	94.4	51.0	0.07	82.5	39.5	0.15
Day 2						
NFF	27.0	0.52		54.3	2.3	
5MeTHF	55.8	1.07		36.3	1.5	
Total	82.8	1.6	0.49	90.6	3.8	1.77
Day 3						
NFF	44.7	0.59		-	-	
5MeTHF	32.3	0.42		-	-	
Total	77.0	1.0	1.4	-	-	

to have a requirement for preformed methionine not observed with most normal cells. These results are of especial interest as the experimental implant used in this project the Walker 256 carcinosarcoma displays this *in vitro* methionine requirement. Thorndyke and Beck (1977) report a greater *in vitro* rate of production of formaldehyde from 5MeTHF methyl groups in malignant lymphocytes compared to normals. This reaction is a result of methylene THF reductase catalysed oxidation of 5MeTHF to 5,10CH_2THF with subsequent chemical hydrolysis to produce formaldehyde. However, the occurence of this reaction *in vivo* has not been demonstrated. The technique may be merely assaying the level of methylene THF reductase which is observed to be increased in the livers of tumours implanted rats (Albrecht 1979), rather than the increased rate of 5MeTHF methyl oxidation *per se*.

Rats were obtained from the Chester Beatty Institute (London) implanted with 2 x 10^6 cells of Walker 256 carcinsarcoma. After seven days the tumour mass may weigh 5% of the animals body weight and the animals were dosed with radiotracer. The use of the Walker 256 in a model system may be criticised in that it is a rapidly growing tumour in comparison with the majority of human clinical cancers. However, conversely the use of such a tumour may be advantageous as its growth rate is likely to highlight quantitative differences between normal and tumour cells.

Experiments were performed with Walker 256 (W256) implanted animals using 5(^{14}C)MeTHF and 5(^{14}C)MeTHF/(3',5',7,^{3}H) folic acid mixture to observe the course of methyl metabolism in the tumour bearing rat, and using (2^{14}C)(3',5',7,9,^{3}H)5MeTHF

to observe the behaviour of the folate pool under such conditions.

6.2 Metabolism of 5(^{14}C)MeTHF in W256 implanted Rats

The implanted rats were orally dosed with 80μg/Kg 5(^{14}C)MeTHF in 50mM phosphate buffer pH7 containing 2% sodium ascorbate, with the experimental proceedure followed as with normal rats (chapter 3).

Table 6(1) gives the distribution and excretion of ^{14}C activity over three days in normal and Walker 256 implanted rats. The W-256 implated rats show an increased production of $^{14}CO_2$, increased ^{14}C retention in liver and a decrease in retention of methyl ^{14}C label in muscle.

Chromatography of the urines from the two groups shows that on DE52 (table 6(2)) there is a proportionally and absolutely greater excretion of non folate compounds (NFF table 6(2)) by tumour rats on days 1 and 2. In normal rat urine NFF and 5MeTHF are excreted in the ratios of 1:14.9 and 1:2.1 on days 1 and 2 while for W256 rats the values are 1:6.6 and 1:0.66. The excretion of 5MeTHF in the first day urine at this dose is substantially due to renal overloading (chapter 3), so the decrease of 5MeTHF excretion in the W256 rats urine may be due to more rapid clearance of 5MeTHF from the blood, possibly as a result of increased folate demands caused by the presence of the tumour mass. However, on day 2, although there is a greater total excretion of 5MeTHF is Walker 256 implanted rat urine than normals, there is also an absolute and proportional increase of NFF, thus indicating there is a more rapid demethylation of 5MeTHF to produce non-folate compounds in the tumour bearing rat.

The difference observed in tissue ^{14}C retention

between the groups can be explained by two possible mechanisms. That suggested by Thorndyke and Beck (1977); that in tumour cells there is an increase oxidation of 5MeTHF to 5,10, CH_2THF which would provide folate coenzymes suitable for purine and thymidylate synthesis. This would explain the decrease in muscle ^{14}C retention, which is mainly products of methionine methyl (chapter 3). Substantial 5MeTHF one carbon oxidation would divert methyl groups from methionine synthesis, thus decreasing creatine and muscle protein labelling. Krebs et al (1976) proposed that 10 formyl THF dehydrogenase regulates the level of one-carbon units in the folate pool, catalysing their release as CO_2. Should there be substantial production of 5, 10, CH_2THF from 5MeTHF, the concomittant rise in 10 CHOTHF would be expected to result in increased $^{14}CO_2$ production, as observed.

However, this analysis is at variance with the observations of a two fold greater output of NFF in the 0-48hr urine of W-256 implanted rats, for one carbon oxidation of 5MeTHF should decrease the number of methyl groups available for transmethylation. The decrease in muscle labelling may be due to the observed preformed methionine requirements of the rapidly growing W-256 cell mass(5.0% of the body weight after ten days) diverting methyl groups from other tissues. The increased output of CO_2 may be a result of the increased turnover of methyl groups to result in increased CO_2 derived from the methyl group of e.g. methionine (Chapter 3). Jackson and Niehammer (1979) report that hepatomas show diminished 10 CHO THF dehydrogenase activity compared to normal liver cells, thus the production of CO_2 from

10CHOTHF is likely to be less in these cells. Walker 256 implanted rats show a greater production of formyl folates (Barford and Blair 1978), thus it is likely that this tumour cell line is also 10CHOTHF dehydrogenase deficient. This could make the scheme 5(^{14}C)MeTHF ⟶ 5,10$^{14}CH_2$THF ⟶ 10^{14}CHOTHF ⟶ $^{14}CO_2$ + THF less likely. Chromatography of a hot ascorbate extract from the liver of a Walker 256 rat indicates that the increased ^{14}C retention is not as polyglutamates, as may possibly occur when there is substantial formation of formyl and bridged folate species from 5MeTHF. The liver retention of ^{14}C methyl activity may be due to increased hepatic methionine synthesis to cope with the requirements of the tumour.

6.3 Metabolism of 5(^{14}C)MeTHF plus (^{3}H)folic acid by W 256 implanted rats

In order to clarify the metabolism of 5MeTHF methyl group in tumour bearing animals, W256 rats and controls were dosed with 80 µg/Kg 5(^{14}C)MeTHF together with 0.24 µg/Kg of (3',5',7,9,^{3}H) folic acid mixed in 2% ascorbate in 50mM phosphate pH7 (2 µCi ^{14}C, 5 µCi ^{3}H per rat). The use of folic acid was to label the folate pool such that entry of the methyl group of 5MeTHF into other folate species might be observed by association of ^{14}C and ^{3}H.

Table 6(3) gives the recovery of ^{14}C and ^{3}H activity in urine. There is a similar recovery from both groups in all cases except for the 6-24 hr urine where the tumour bearing group shows a significant increase in ^{3}H excretion. Chromatography of the urines by DE52 (fig 6.1) and G15 columns (table 6(4)) shows no evidence of the incorporation

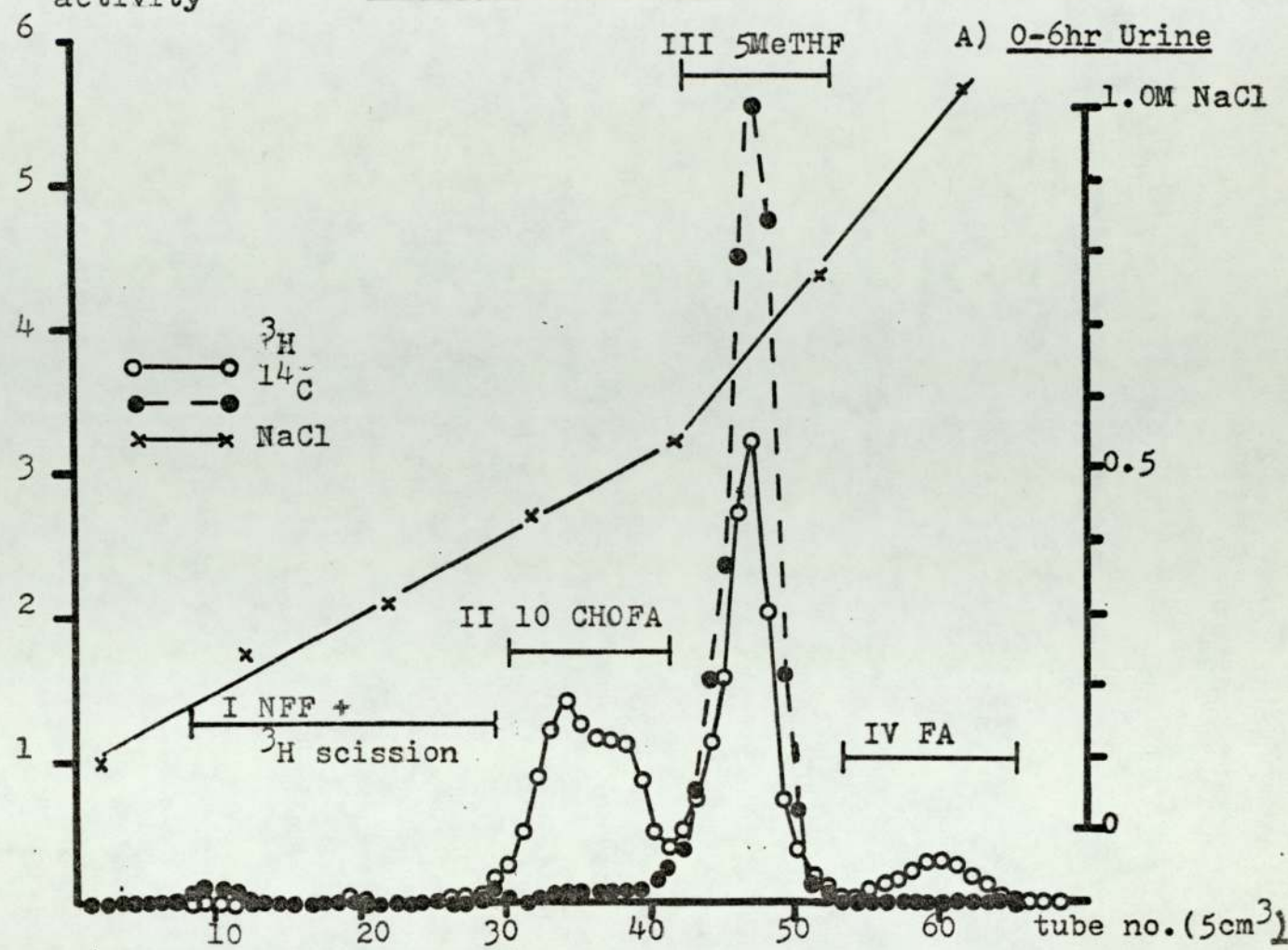
Table 6(3)

Urinary radioactivity following an oral dose 80 μg/Kg 5(^{14}C)MeTHF plus 0·2 μg/Kg (3',5',7,9,^{3}H) folic acid

Urine	Normal rats (6)		W-256 implanted (6)	
	^{14}C	^{3}H	^{14}C	^{3}H
0-6hr	25.8	29.8	25.1	30.6
6-24hr	11.0	15.7*	15.8	27.2*
Day 2	2.6	3.5	2.2	3.4
Day 3	0.9	1.4	1.4	2.1
Total	40.1	50.4	44.6	63.3

values as % dose radioactivity
figures in parenthesis = no of rats in experiment
* t test $p < 0.01$ significant; comparing W256 with normals

Fig.6.1. DE.52 Chromatographs of day 1 urines from Walker-256 implanted rats orally dosed with 80µg/Kg 5(^{14}C)MeTHF plus (3;5;7,9,^{3}H) folic acid(0.24µg/Kg)



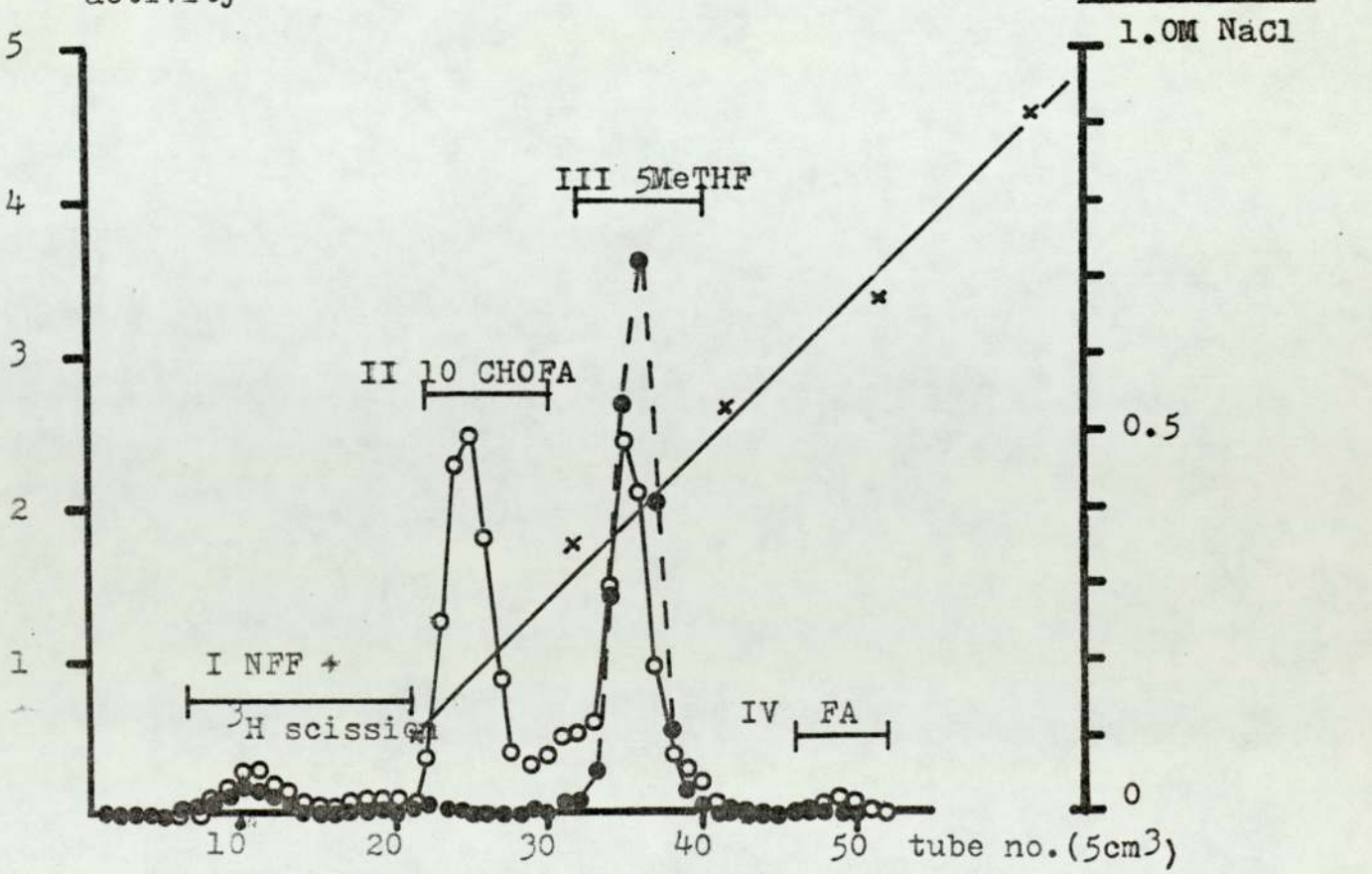


Table 6(4)

Chromatographic fractionation of urines from W256 implanted rats orally dosed 80 μg/Kg 5(^{14}C)MeTHF plus(^{3}H)FA

0-6hr Urine

		%Urine activity		%dose activity		Ratio
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C/^{3}H
I	NFT	4.6	-	1.22	-	
	^{3}H scission products	-	5.0	-	1.59	
II	10CHOFA	4.5	37.0	1.10	11.65	0.09
III	5MeTHF	92.0	42.0	22.37	13.27	1.69
IV	FA	1.0	7.0	0.22	2.19	0.10

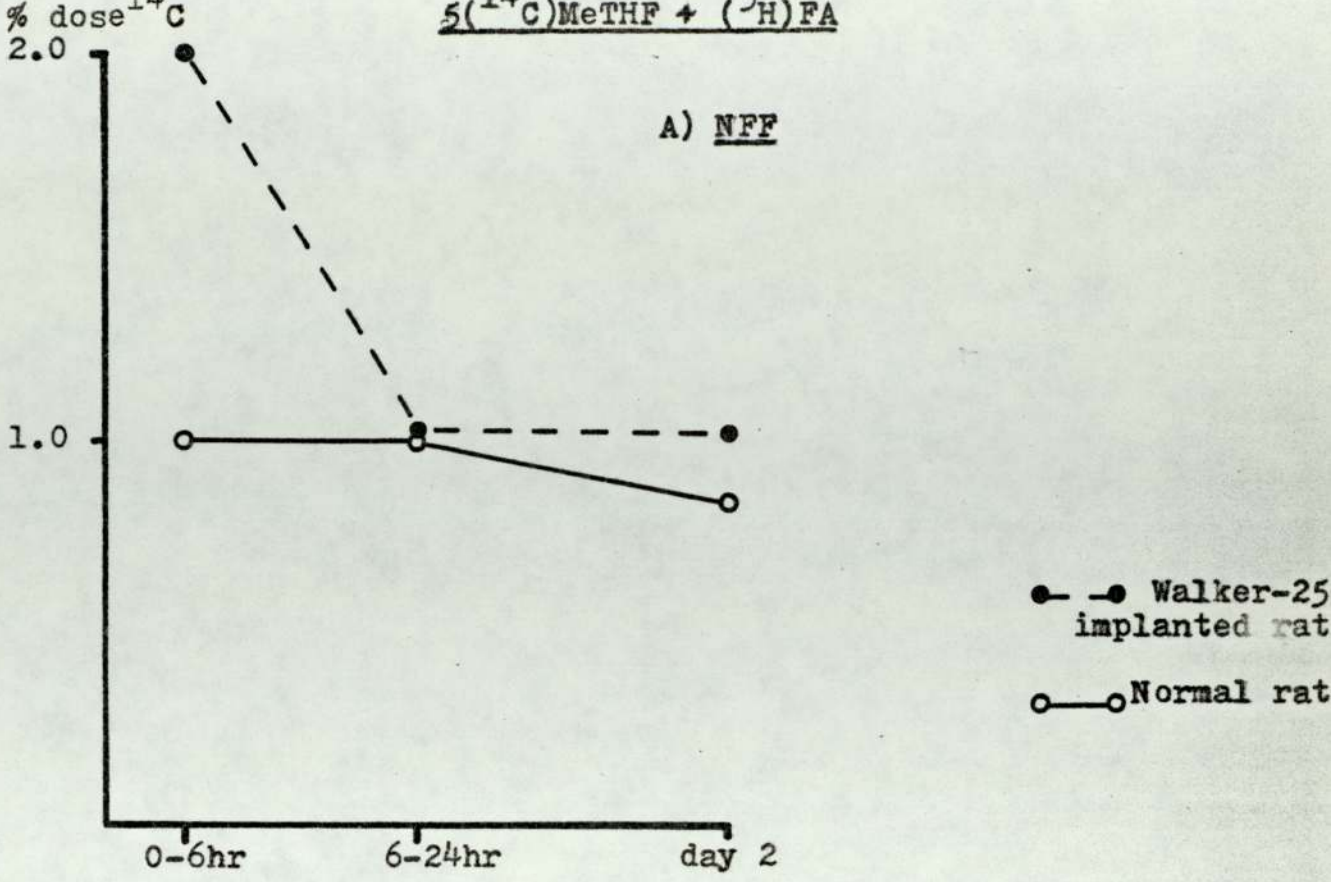
6.-24hr Urine

		%urine activity		%dose activity		Ratio
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C/^{3}H
I	NFT	7.6	-	1.17	-	
	^{3}H scission products	-	8.5	-	2.0	
II	10CHOFA	1.7	37.1	0.26	9.83	0.03
III	5MeTHF	71.8	34.1	10.98	9.06	1.21
IV	FA	0.4	1.5	0.06	0.41	0.14

Day 2 Urine

		% urine activity		%dose activity		Ratio
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C/^{3}H
I	NFT	53.2	-	1.17	-	
	^{3}H scission products	-	20	-	0.68	
II	10CHOFA	11.8	54.4	0.26	1.85	0.14
III	5MeTHF	32.3	15	0.71	0.51	1.39
IV	FA	0	0	0	0	-

Fig.6.2. Excretion of Urinary ^{14}C Metabolites from rats dosed with 5(^{14}C)MeTHF + (^{3}H)FA



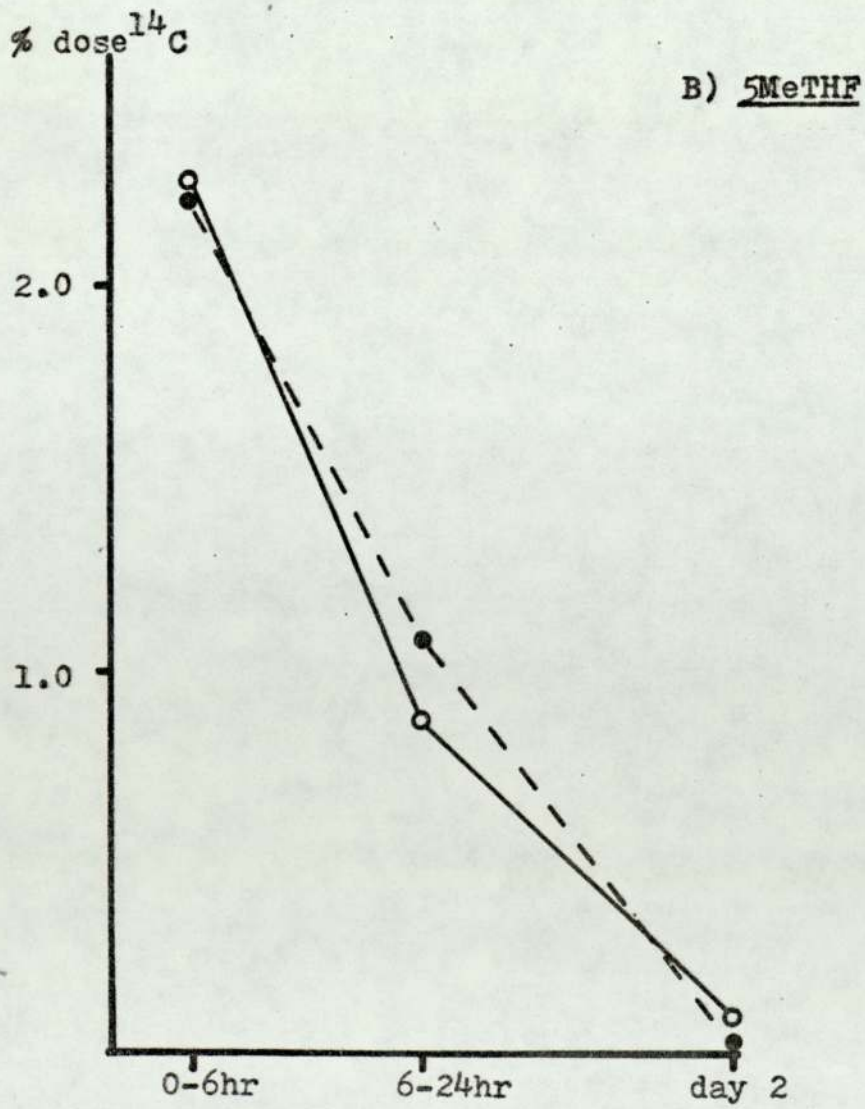
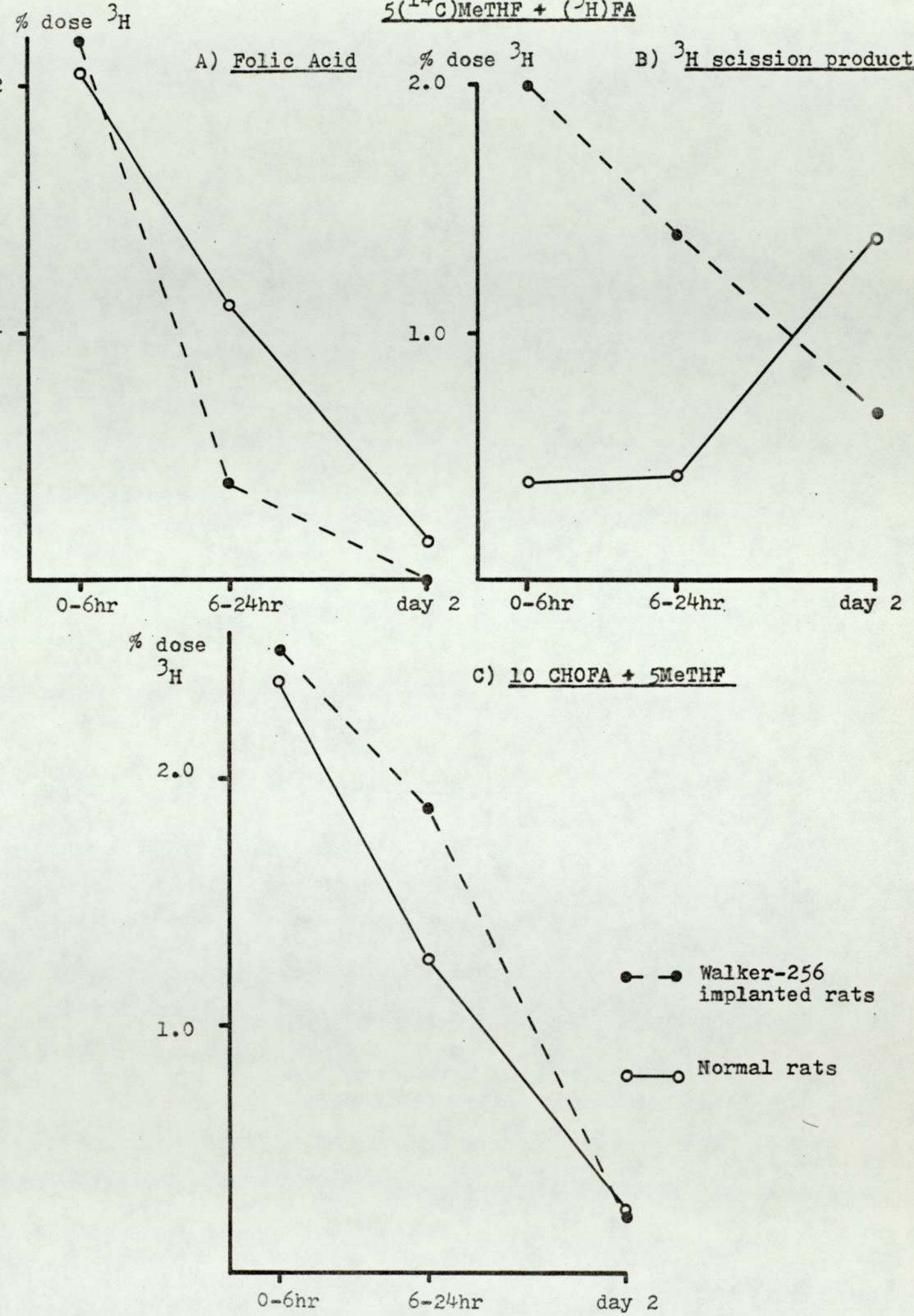


Fig.6.3. Excretion of ^{3}H metabolites from rats orally dosed with 5(^{14}C)MeTHF + (^{3}H)FA



of ^{14}C from 5(^{14}C)MeTHF into urinary 10CHOFA in both normals (see table 3(8) chapter 3) and W-256 implanted animals (table 6(4)), the $^{14}C/^{3}H$ ratio for 10CHOFA in both groups having a mean value of 0.07, the same as that of unmetabolised urinary folic acid. Similarly, chromatography of hot ascorbate extracts of liver show only inclusion of ^{3}H into polyglutamates.

These results may be criticised on the ground that any 10^{14}CHOTHF or 5,10 CH_2THF produced from 5 MeTHF would be metabolised too quickly to appear in the urine. However, Nixon et al (1973) reported that 85% of 5 MeTHF methyl group is lost within five minutes, yet it is found in the whole rat (chapter 3) that at 80μg/Kg active 5MeTHF can be demonstrated in the 0-24 hr urine. Apparently the extracellular pool of 5MeTHF is large enough to enable a pulse of ^{14}C methyl active diastereoisomer to persist in the circulation long enough to be excreted. Possibly the same may occur with 10^{14}CHOTHF if produced in significant amounts. A more satisfactory resolution of this question would be facilitated by examination of intracellular folates following 5(^{14}C)MeTHF dosage, and determining the fate of the administered ^{14}C. In this respect it is of note that Nixon et al (1973) did not report any significant association of 5(^{14}C) Me with other folates in L1210 murine lymophoma, after 5 minutes.

As in the previous experiment the NFF:5MeTHF ratio is greater for W-256 animals (1st day urine 1:11.1, day 2 1:0.61) than normals (1st day urine 1:15.4 day 2 1:1.6) and there is a greater total excretion of NFF in the W-256 implanted rats (fig 6.2a). The W256 animals, as might be expected due to their load of a rapidly growing tumour mass, show a more

complete incorporation of (^{3}H) folic acid into the reduced folate pool as shown by their increased output of (^{3}H) 10CHOFA and (^{3}H)5MeTHF (fig 6.3 (c)) and decreased excretion of unmetabolised folic acid (fig 6.3(a)). The excretion of ^{3}H scission products differs; that of the W256 declines over the period examined, while in normals they are increasing (fig 6.3(b)) this presumably a consequence of demand for folate decreasing the net rate of scission.

6.4 Metabolism of mixed label ($2^{14}C$)(3',5',7,9,^{3}H)5MeTHF by Walker 256 implanted rats

($2^{14}C$)(3',5',7,9,^{3}H) mixed label 5MeTHF was prepared as in Chapter 2 and orally dosed 6 μg/Kg in 2% ascorbate 50mM phosphate pH7 buffer, Table 6(5) gives the urinary and faecal recovery of radioactivity compared to normals dosed 8 μg/Kg (Chapter 4).

The excretion of both ^{14}C and ^{3}H is significantly decreased in the W-256 implanted rat group (table 6(5)a). Similarly the faecal radioactivity is also decreased (table 6(5)b). This suggests the tumour is promoting greater tissue retention of folate thus less is being excreted in the urine or entering the enterohepatic circulation to be recovered in the faeces (chapter 4). Examination of retained radioactivity shows that W256 show a higher retention of tissue ^{14}C and ^{3}H although its significance cannot be statistically tested (table 6(6)), the tumour shows a similar retention to that of liver.

Chromatography of the first day urines (fig 6.4) reveals essentially the same metabolities in the urines of W256 rats (table 6(7)) as observed in those of normal animals(chapter 4

Table 6(5)

Excreted. Radioactivity from rats orally dosed with (2-^{14}C)(3',5',7,9,-^{3}H)5MeTHF;Normal and W256 implanted

a) Urinary radioactivity % dose activity ( ) = no of rats
dose 8 μg/Kg normals, 6 μg/Kg W-256

	Normals (5)		W-256(6)		't'test(comparing same labels)
	^{14}C	^{3}H	^{14}C	^{3}H	
0-6hr	39.1	41.0	16.1	18.4	$p < 0.001$
6-24hr	28.5	40.4	7.3	8.8	"
Day 2	3.2	4.7	2.8	2.4	"
Day 3	3.4	6.3	2.1	1.2	"
Total	74.2	92.4	28.3	30.8	

b) Faecal radioactivity % dose activity

	Normals		W.256	
	^{14}C	^{3}H	^{14}C	^{3}H
Day 1	22.0	18.1	3.2	3.1
Day 2	5.1	3.9	1.5	0.9
Day 3	-	-	0.6	0.4
Total	27.1	22.0	5.3	3.4

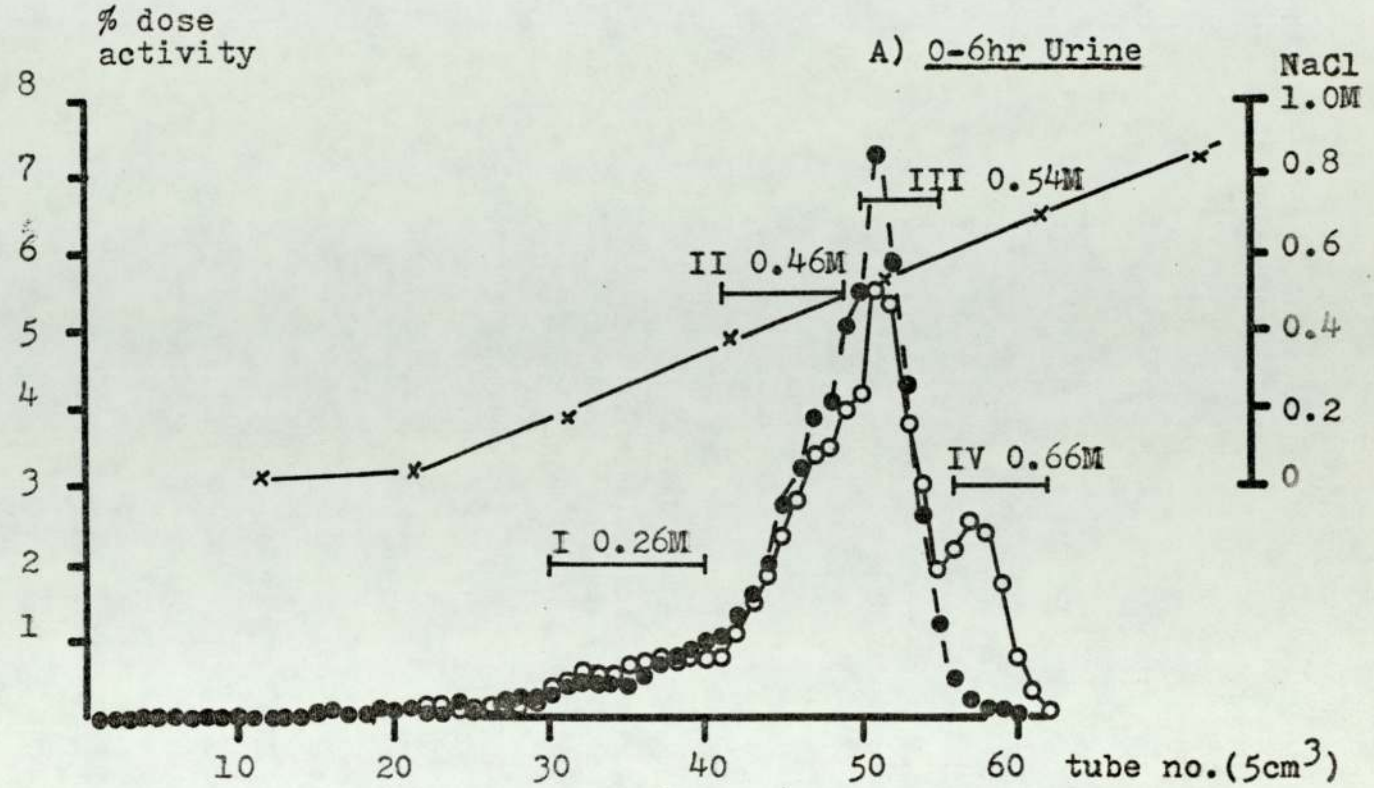
Table 6(6)

Tissue retention after 3 days of a dose of ($2^{14}C$)(3',5',7,9,^{3}H) 5MeTHF

Values as % dose radioactivity 8 µg/Kg dose Normals, 6 µg/Kg dose W-256 implant rats.

Tissue	Normals		W-256	
	^{14}C	^{3}H	^{14}C	^{3}H
Spleen	0.14	0.08	-	-
Gut	1.12	0.84		
Kidney	0.8	0.3	1.1	1.3
Muscle	0	0	-	-
Brain	0	0	-	-
Liver	6.8	ND	7.2	5.7
Tumour	-	-	5.1	5.1

Fig.6.4. DE.52 Chromatographs of urine from Walker-256 implanted rats dosed 6μg/Kg ($2^{14}C$)(3;5;7,9,^{3}H)5MeTHF



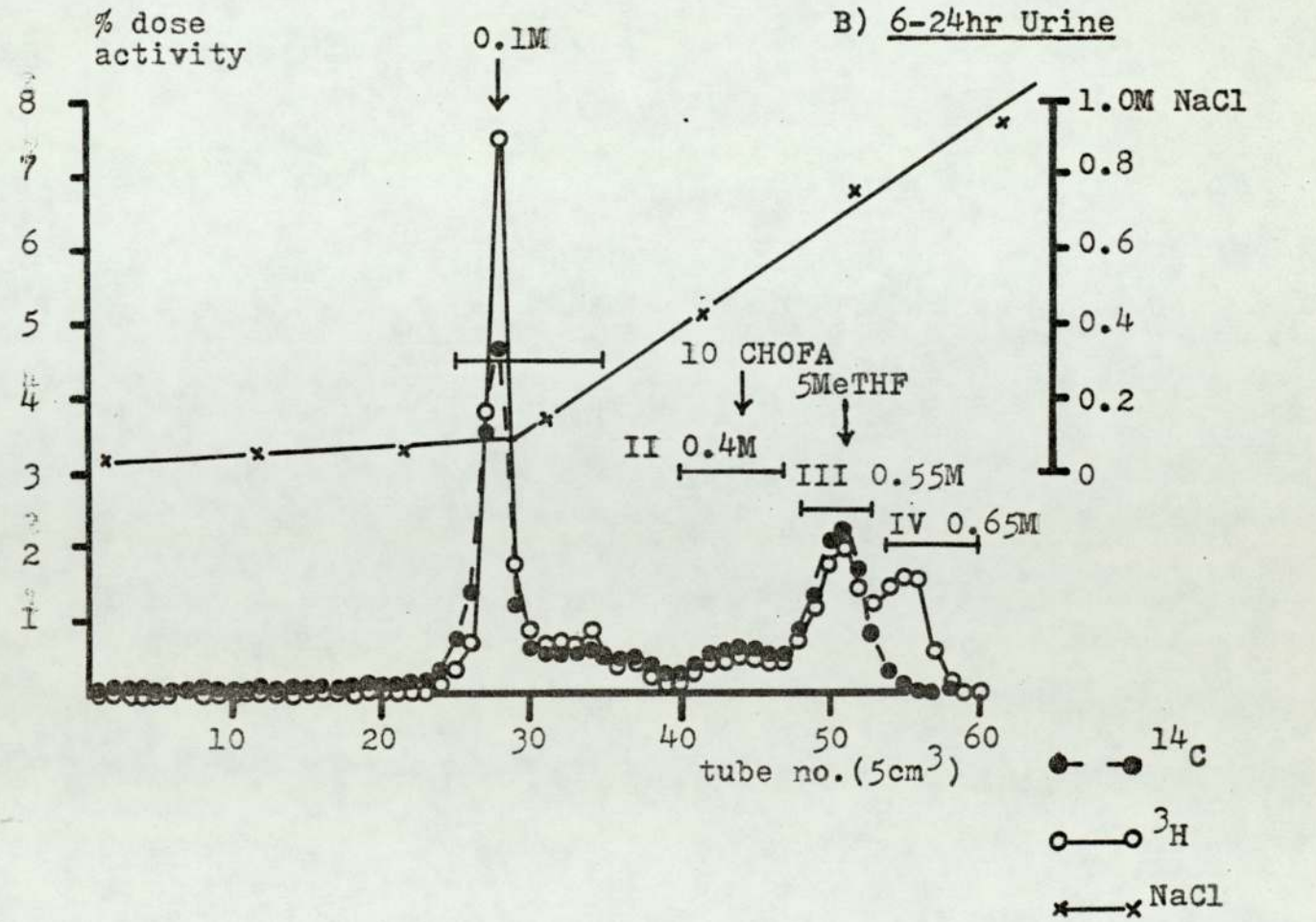


table 4(3), with the exception of that peak eluting after 5MeTHF on DE52 (Compound D) which is only labelled with ^{3}H in W256 animals. This may be due to a difference in labelling between the batches of 2^{14}C folic acid used for 5MeTHF preparation, the batch used for the W-256 was only 88% labelled in the C_2 position. (Radiochemical Centre, Amersham). There is a proportional decrease in the excretion of intact folates in the W-256 implated rats of the 0-24 hr urines 76% of the urinary ^{3}H is present in intact folates (10CHOFA, 5MeTHF, Compound 'D') while for normals the value is 80.4%. Although the proportion of scission products in the 0-24hr is increased, the total folate cleavage as measured by the excretion of pAcetABA and pAcetABglu is decreased in Walker 256 implanted rats. From table 4(3) 16% of 0-24hr urinary ^{3}H is excreted as pAcetABA + pAcetABglu, equivalent to 38.1% of the folate dose as 42.5% of the ^{3}H label is present in the 3',5', positions, while for W-256 bearing rats the values are 6.5% (table 6(7) fractions I 0-6, 6-24hr) dose ^{3}H and 15.3% dose folate.

G15 chromatography of the 6-24hr urine cleavage product fraction (fig 6.5) shows that the tumour bearing animals display two ^{14}C labelled products not resolved by similar chromatographs of urine from normal rats termed x and y.

The ratio of folate types in the urine differs between the two groups of animals, in normal rats the fraction of intact folate excreted as 10 formyl folate falls from the 0-6 to the 6-24 hr urine; 29% ^{14}C, 25.7^{3}H in the 0-6hr, 18.4% ^{14}C, 15.4%^{3}H in the 6-24hr, while with the W-256 implanted animals the fraction rises; 0-6hr 24.2% ^{14}C

Table 6(7)

Chromatographic fraction of 1st day urines from W256 implanted rats orally dosed with (2^{14}C)(3',5',7,9,)^{3}H 5MeTHF (6 µg/Kg)

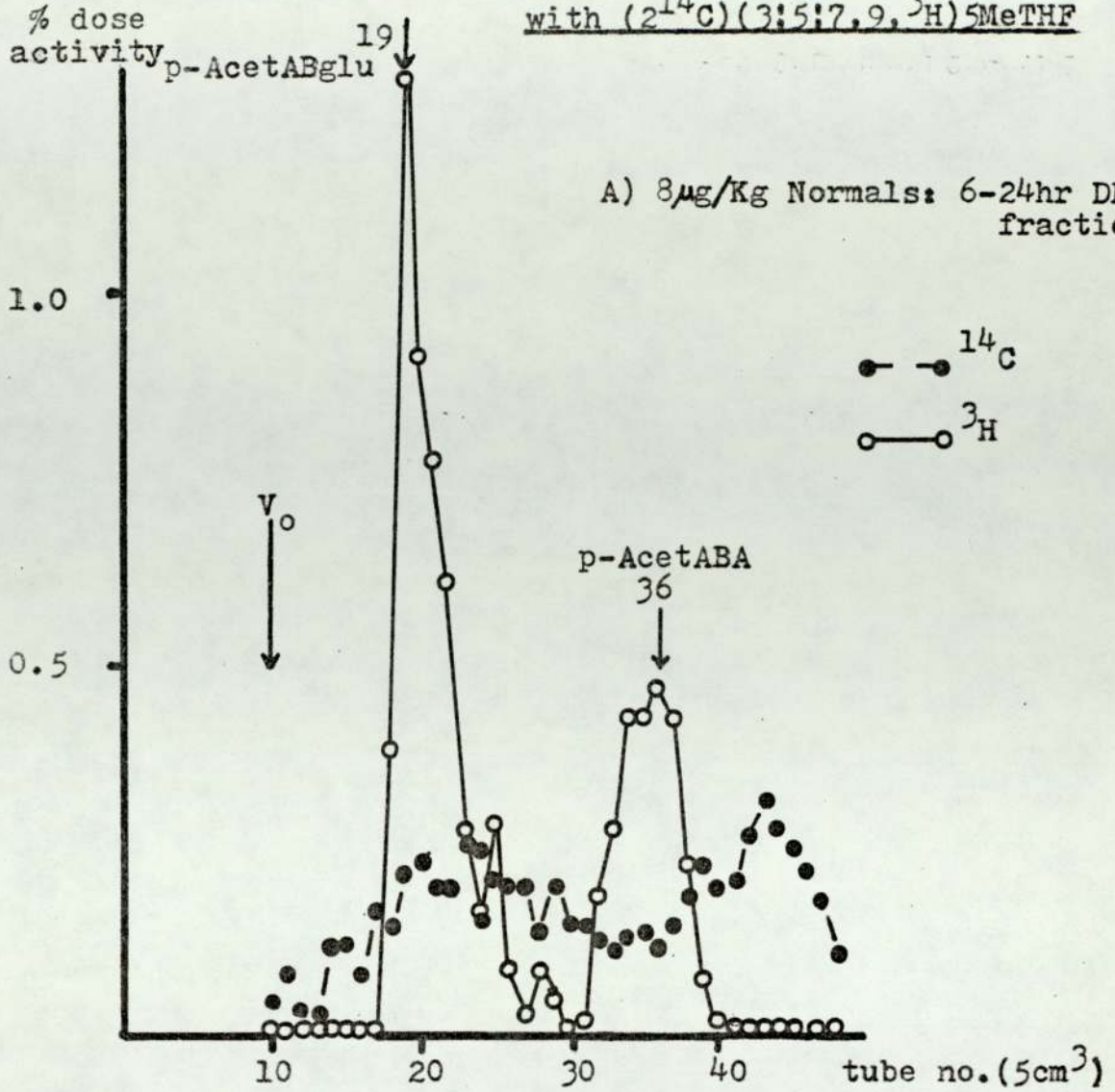
0-6 hr Urine: 16.1% ^{14}C, 18.4% ^{3}H dose

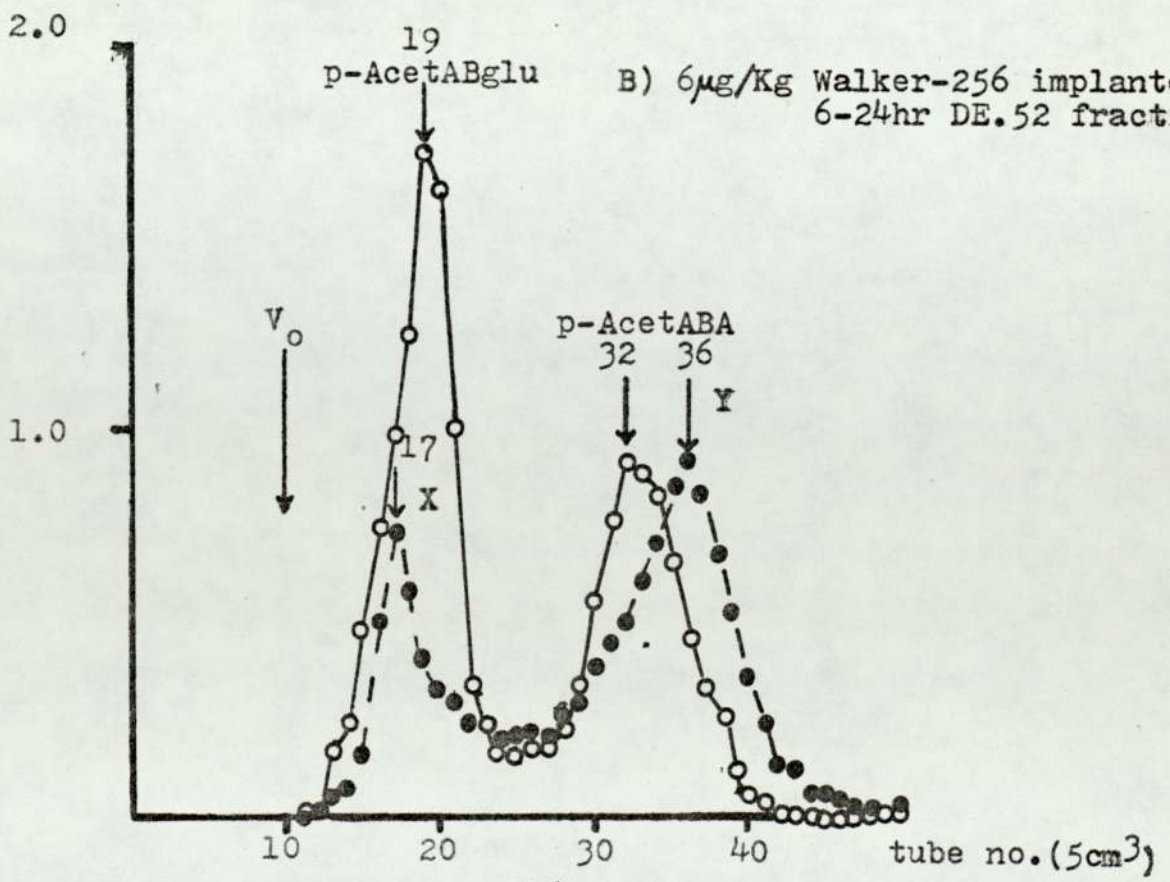
	%urine activity ^{14}C	%urine activity ^{3}H	%dose activity ^{14}C	%dose activity ^{3}H	^{14}C/^{3}H
scission products	10.8	11.6	1.71	2.1	0.81
10CHOFA	20.6	17.2	3.2	3.1	1.03
5MeTHF	61.3	52.8	9.7	9.6	1.01
Compound 'D'	1.9	15,7	0.3	2.9	0.10
Total	94.6	97.3	14.9	17.7	

6-24hr Urine: 7.3% ^{14}C, 8.8% ^{3}H dose

	%urine activity ^{14}C	%urine activity ^{3}H	%dose activity ^{14}C	%dose activity ^{3}H	^{14}C/^{3}H
scission products	47.6	51.2	3.4	4.4	0.77
10CHOFA	13.6	8.6	1.0	0.7	1.43
5MeTHF	27.6	20.1	2.0	1.7	1.12
Compound 'D'	2.0	14.3	0.1	1.2	12
Total	90.8	94.3	6.5	8.0	

Fig.6.5. G.15 of urinary scission products from rats orally dosed with ($2^{14}C$)(3;5;7,9,^{3}H)5MeTHF





19.8% ^{3}H, 6-24hr 32.3% ^{14}C, 19.4% ^{3}H. This change may be analagous to the changes in urinary folate types observed with the FA dosed W-256 implanted rat where there is increased excretion of formyl folates (Barford and Blair 1978).

Notably the 5MeTHF and 10CHOFA fractions do not show the ^{3}H excess in W-256 implanted rats observed with normals (table 4(3)), the $^{14}C/^{3}H$ ratios in W-256 implanted animals approaching 1 or showing a ^{14}C excess (table 6(7)).

6.5 Discussion

The presence of an implanted tumour mass is observed to impose changes on the whole body metabolism of 5MeTHF in the rat. In the W-256 implanted animals there is an increased rate of 5MeTHF demethylation and there is an observed diversion of methyl groups from muscle, the major recipient in normal rats, to the liver and tumour. This may be as a consequence of the Walker 256 carcinosarcoma lacking the ability to synthesise methionine or that the added mass of dividing tissue, 5% body weight, results in disturbance of the normal distribution of methyl groups in the rat. It remains to be seen whether other tumour cell lines which do not display an absolute methionine reuirement would cause a similar change in host methyl metabolism.

The W-256 implanted animal also shows a different handling of mixed label 5MeTHF. In the implanted animal folate urinary excretion and biliary circulation, as indicated by faecal radioactivity, are markedly depressed. The total and proportional excretion of intact folates in urine, and total urinary excretion of scission products is decreased. The normals show an excretion of 38% dose folate as catabolites after 24hrs, compared with only 15.3% cleavage by W256. In normals the ratio of dose cleavage to liver retention is 5.6, while for W-256 implanted rats the value is 2.1. This

decrease in folate cleavage may result from the smaller flow of folate into bile thence to the gut lumen, a site of scission reactions (chapter 4). Another possible mechanism would be a decrease in the rate of tissue folate oxidation (see chapter 7). This difference in scission reactions is also shown by the presence of two W-256 associated ^{14}C labelled catabolites x and y which are not resolved in chromatographs of normal urine (fig 6.5). At first sight these would appear to be tumour specific, however ^{14}C metabolites of similar chromatographic behaviour are observed after dosing normal rats with ($2^{14}C$)(3',5',7,9,^{3}H) mixed label FA (Connor 1979), and these compounds may be quantitatively increased, which enables their more ready detection. However, these results suggest there are tumour associated changes in folate catabolism which might have application in cancer diagnosis.

7. DISCUSSION

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7.1 The normal metabolism of 5MeTHF in the rat

5Methyltetrahydrofolic acid is an important dietary source of folate (Rodriguez 1978) and performs as a methyl donor in methionine synthesis.

Comparison of faecal recovery from IP and orally dosed rats indicates complete absence of 5MeTHF, as 5(^{14}C)MeTHF, from the gut. As this radiotracer is an equimolar mixture of C_6 epimers, this indicates there is no discrimination between the diastereoisomers as would be expected should there be a specific carrier mechanism. This would provide indirect evidence for the proposal that folate absorbtion takes place as the electrically neutral species, neutralised by an acid microclimate generated at the wall of the small intestine (Blair and Matty 1974, Coleman et al 1979) as the epimers would not differ in pKa, thus would not be distinguished by such a mechanism.

5MeTHF is utilised in the tissue and utilised in the formation of methionine (chapter 3). 5MeTHF is reported to be a poor substrate for polyglutamate synthetase in vitro (Spronk 1973, McGuire et al 1979) while the rate of intracellular demethylation is rapid (Nixon et al 1973) thus there was no observed incorporation of ^{14}C from 5(^{14}C)MeTHF into polyglutamates, although there was polyglutamate synthesis from simultaneously dosed ^{3}H folic acid (chapter 3), and suggests the primary demethylation of exogenous 5MeTHF takes place via the monoglutamate. However, the removal of the CH_3 group produces THF the most effective substrate for rat polyglutamate synthetase (Spronk 1973, McGuire et al 1979) thus there ought to be similar, if not greater incorporation of (2^{14}C)(3',5',7,9,^{3}H)5MeTHF compared to similarly labelled FA, especially bearing in mind the slow reduction of

folic acid to DHF, the rate limiting step of folic acid incorporation in to the folate pool (chapter 1). With mixed label 5MeTHF at 80 μg/Kg 2.5% of the dose is retained in the liver after three days while with similarly labelled 100 μg/Kg folic acid after two days the liver shows a retention of 11% (Pheasant et al 1980). Steinberg et al (1979) have also observed a diminished tissue incorporation of 5MeTHF.

When one compares the first day urines of rats dosed 2 μg/Kg with 5(^{14}C)MeTHF with those dosed 8 μg/Kg with mixed label (2^{14}C) (3',5',7,9,^{3}H)5MeTHF, 8% of the ^{14}C methyl activity is present as 5MeTHF (thus the same percentage as the active diastereoisomer) while at 8 μg/Kg 24% ^{14}C and 32% ^{3}H is excreted as 5 MeTHF. This indicates that the mixed label 5MeTHF is between 67 and 78% demethylated and subsequently remethylated after 24 hrs, while Nixon et al (1973) reports that within the L 1210 cell 82-89% of the methyl group is lost within 60 minutes while 87% remains as 5MeTHF, a demethylation an remethylation rate of 79-82%. At these dose levels the 5MeTHF excretion is unlikely to be due to the renal overloading and suggests the rate of whole body demethylation is slower than the apparent value given by cell culture. This slower rate may arise from 5MeTHF being taken up into short term storage, possibly protein binding prior to metabolism (chapter 4).

The role of tissue polyglutamates is a source of controversy. M.J. Connor in a rigorous investigation of retained tissue folate (Connor et al 1977, Connor and Blair 1980) was only able to demonstrate the presence of formyl folate polyglutamate types. He suggests (Connor 1979) this results in coenzyme functions being divided into two pools,

monoglutamate. 5MeTHF required for methionine synthesis and the non-methyl folate polyglutamates for thymidylate and purine synthesis. These observations that 5MeTHF is slowly demethylated and is held as the monoglutamate for a longer time than FA would tend to support the proposal of methionine synthesis proceeding via monoglutamate 5MeTHF.

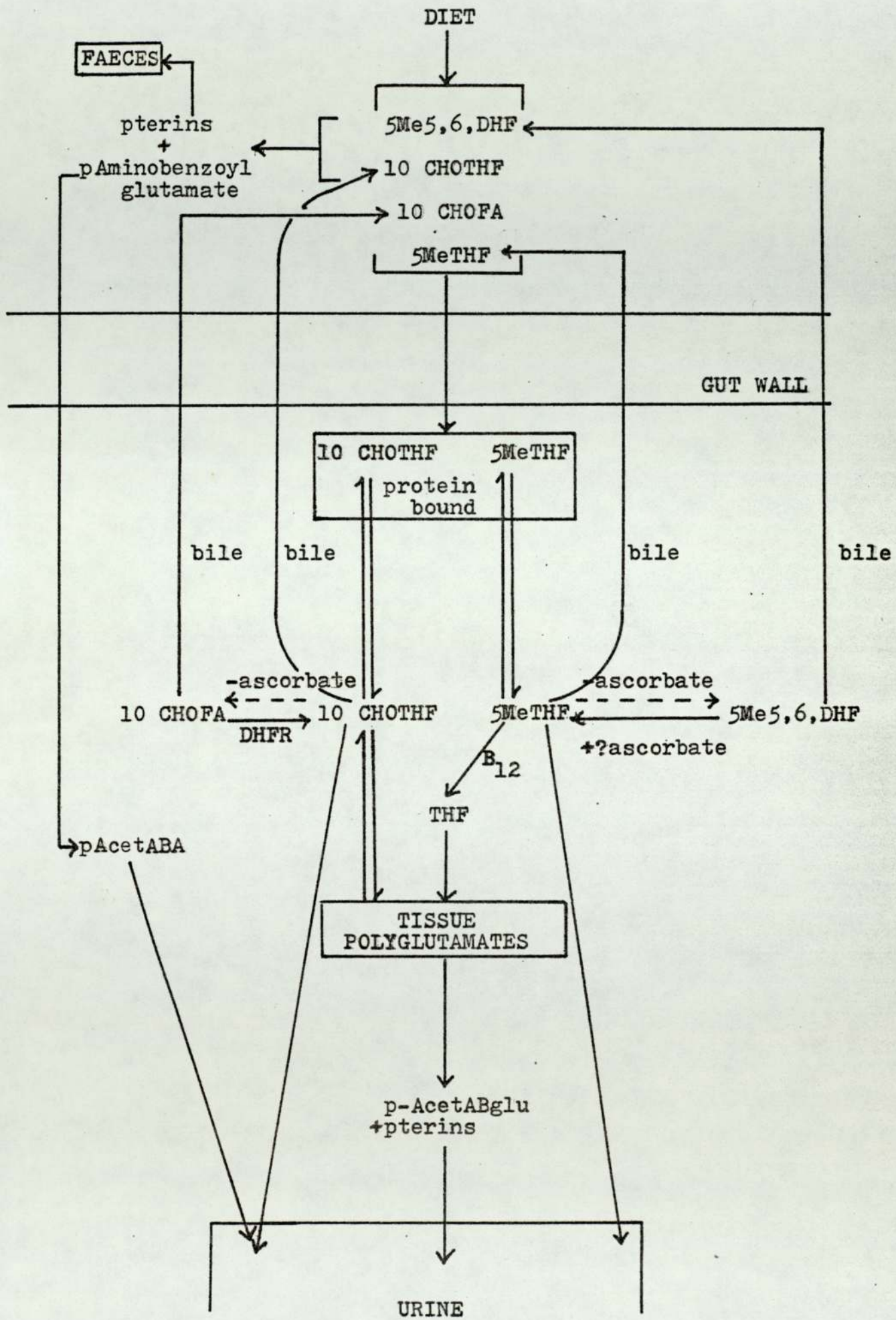
The involvement of the polyglutamate folates with nucleotide synthesis is evidenced by the increased retention of labelled folate in tissues when the animal has the load of a rapidly growing Walker 256 carcinosarcoma. Hilton et al(1979) have investigated polyglutamate synthesis from FA in cultured human fibroblasts. At folic acid concentrations which supported maximal logarithmic growth they observed that those cells in log phase, in addition to high intracellular folate, showed a higher ratio of polyglutamate to monoglutamate than confluent cells (2.6 polyglu: monoglu for log cells, 1.4 for confluent cells). Notably this ratio remains constant with rising external folic acid concentration for log cells, while with confluent cells the ratio increases with increased FA. Unfortunately the method used could not assay the chain length of the retained polyglutamate. As the polyglutamate folates are reported to be of different substrate and inhibitor activities according to chain length, it remains to be seen whether there is a corresponding change in polyglutamate chain length ratios with the demands made upon the folate pool during cell division. As polyglutamate synthesis is reported as the addition of a single glutamate residue at a time (Shin et al 1972) on occasions when there is an increased rate of polyglutamate synthesis, there would be expected to result a greater proportion of short chained

polyglutamate species within the cell which may have different enzyme substrate activities from the higher chain length forms.

The studies using ^{14}C methyl labelled compounds showed the fate of the methyl group of 5MeTHF and methionine was quantitatively the same (chaper 3). The experiments provided no evidence for the proposal that there is reversal of the methylene THF reductase reaction so that one carbon units from 5MeTHF could be used for nucleotide synthesis. Krebs et al (1976) and Scrutton and Beis (1979) report that the level of 10CHOTHF is normally depressed by the action of 10 formyl THF dehydrogenase, such that demands for 10CHOTHF and the folates in equilibrium with it will result in a decrease in CO_2 production rather than depressing the absolute level of 10CHOTHF. If the level of 10CHOTHF is maintained relatively constant this would also tend to keep the equilibrium between methylene and methyl THF constant. Also the intracellular turnover of one carbon units is rapid at least for 5MeTHF, the labelling of 5,10 CH_2THF may not be high enough to detect as subsequent production of (^{14}CHO) formyl urinary folates.

Mixed label (2^{14}C)(3',5',7,9,^{3}H)5MeTHF undergoes C9 - N^{10} scission in the rat to produce solely ^{3}H labelled urinary metabolites. Such scission had been previously observed with folic acid (Murphy et al 1976, Connor et al 1979) and 10CHOFA (Connor et al 1979), however, these experiments showed that such reactions were a part of the normal metabolic activity of the reduced folate pool. The scission products were observed to cochromatograph with pacetamidobenzoyl glutamate (pAcetABglu) and pacetamidobenzoate (pAcetABA) as observed after doses of FA and 10CHOFA (Connor et al 1979)

Fig.7.1. Distibution of folate monoglutamate



It also suggests (Pheasant et al 1980) that pacetABglu is associated with long term breakdown of folate, i.e. from the polyglutamate store, and that pacetABA is a short term metabolite derived from monoglutamates. In these studies with mixed label 5MeTHF the amount of dosed radioactivity is low and prevented resolution of metabolites after 24 hrs, however, comparison of the excretion of pacetABA and pacetABglu at 8 and 80 μg/Kg shows that the low dose with a proportionally greater retention in tissue shows a greater excretion of the proposed polyglutamate pool metabolite pacetABglu (8 μg/Kg pacetABglu: pacetABA = 1.9:1 at 80 μg/Kg ratio = 0.9:1). The short term scission products arise from the intraluminal breakdown of folate in the gut, as only intact folates are observed in the cannulated bile (Pheasant and Blair 1979), with the differential resorbtion of the pterin and pABglu fragments resulting in the ^{14}C and ^{3}H imbalance observed in the faeces (chapter 4). As 5MeTHF is absorbed completely from the gut (chapter 3) the substrate for scission is likely to be 10CHOTHF or THF, probably the latter as it is the more labile, readily undergoing scission in vitro (Pearson 1974). In humans another possible source of scission products is 5Me,5,6,DHF; Ratanasthein et al (1977) report normal human serum contains 0.75 μg/l (15% of 5MeTHF + 5Me,5,6,DHF) when fasting. This compound is observed to be unstable in acid conditions and it would be expected to be lost during enterohepatic circulation (fig 7.1).

7.2. The significance of the oxidation products of 5MeTHF

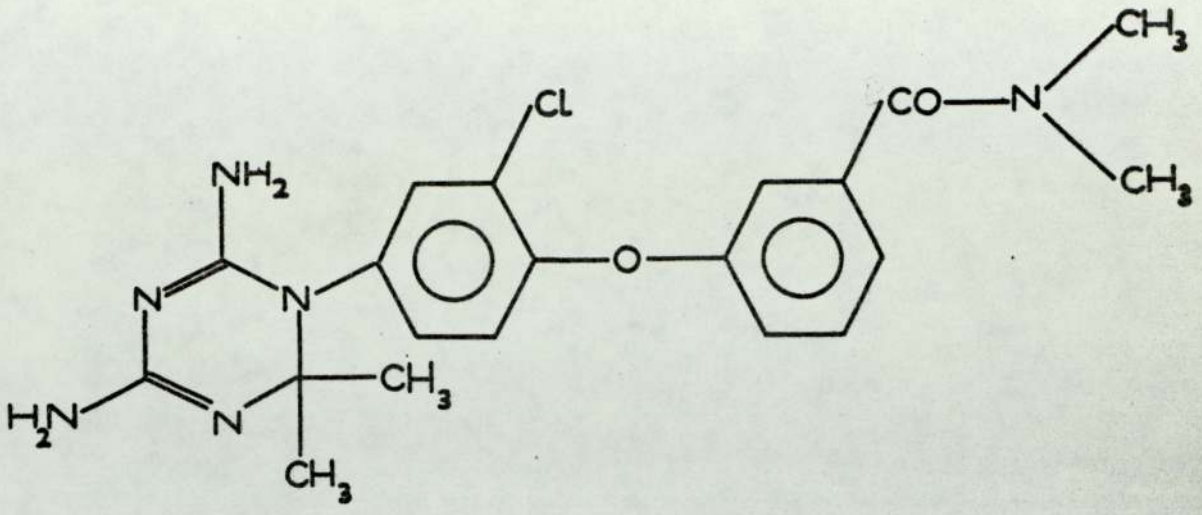
5MeTHF in storage has been observed to be oxidised to a variety of compounds; the triazine produced by peroxidation (Jongejan et al 1979) has been observed to be produced in

stored 5MeTHF at -20°C (Kennelly et al 1979, chapter 5), possibly via aerial oxidation or by the action of H_2O_2 produced during the oxidation of the ascorbate present. This compound was found to be biologically inactive in the rat, as would be expected from its non-folate structure. In fact triazine compounds such as those produced by Baker (1971) have potent antifolate activity fig 7.2.

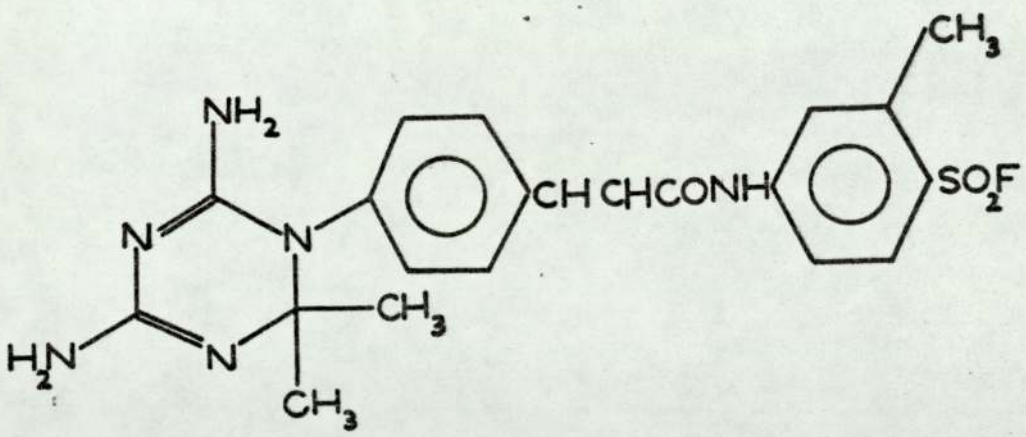
However, this triazine oxidation product of 5MeTHF is also inactive in promoting the growth of the common folate microbiological assay organisms (Gapski et al 1971, Ratanasthein et al 1977) thus its presence in foodstuffs would not result in specious estimation of folate content.

The other characterised oxidation product of 5MeTHF, 5methyl 5,6 dihydrofolate (Donaldson and Keretsky 1962) is fully active for L.casei in the presence of ascorbate (Ford et al 1978), however the acid conditions of the human stomach result in considerable loss of folate activity. (Ratanasthein et al 1977) Similarily with rats (chapter 5) while 5Me 5,6, DHF is readily assimulated into the folate pool, when given IP, there is no excretion of intact folates following an oral dose. Deits et al (1976) suggests that acid conditions result in C9 -N10 scission of 5Me 5,6,DHF, however, in vitro acidification of the labelled compound was observed to produce some pABglu;this represented only 26% cleavage at 22°C, 30% at 37°C, the remainder of products remaining unidentified. These compounds presumably have no folate activity. Ford et al (1978) reported there is considerable oxidation of milk folate (measured by L.casei) if oxygen entry occurs, the work of Hanson and Blair (chapter 5) showed that at least some of the L.casei folate activity of several foods

Fig.7.2. Triazine Antifolates



NSC 139105 "Baker's Antifol"



NSC 113423 "Insoluble Baker's Antifol"

was in fact due to 5Me 5,6,DHF. These results suggest that the available free folate of foodstuffs as estimated by L.casei may be high due to the prescence of labile 5Me5,6,DHF especially after prolonged storage.

Stokes et al (1975) proposed that the megaloblastic anaemia associated with Vitamin C deficiency may result from the conversion of 10CHOTHF to 10CHOFA under such conditions. However, Connor (1979) reported that 10CHOFA is metabolised in a manner similar to FA, suggesting the 10CHOFA enters the folate pool, albeit slowly, via dihydrofolate reductase action. Thus in a scorbutic subject although 10CHOFA may be formed there remains a pathway for its salvage. 5Me 5,6,DHF is probably formed in greater amounts than 10CHOFA during scurvey, as 5MeTHF is the major serum folate, and is prone to scission. It has not been reported to be salvaged by DHFR and is not demethylated by methionine synthetase (Deits et al 1976) to form DHF, thus 5Me 5,6,DHF will be likely to undergo scission reactions and result in loss of folate. This scheme cannot be investigated in the rat which does not require Vitamin C, however, studies on the scorbutic guinea pig with 5MeTHF would be interesting.

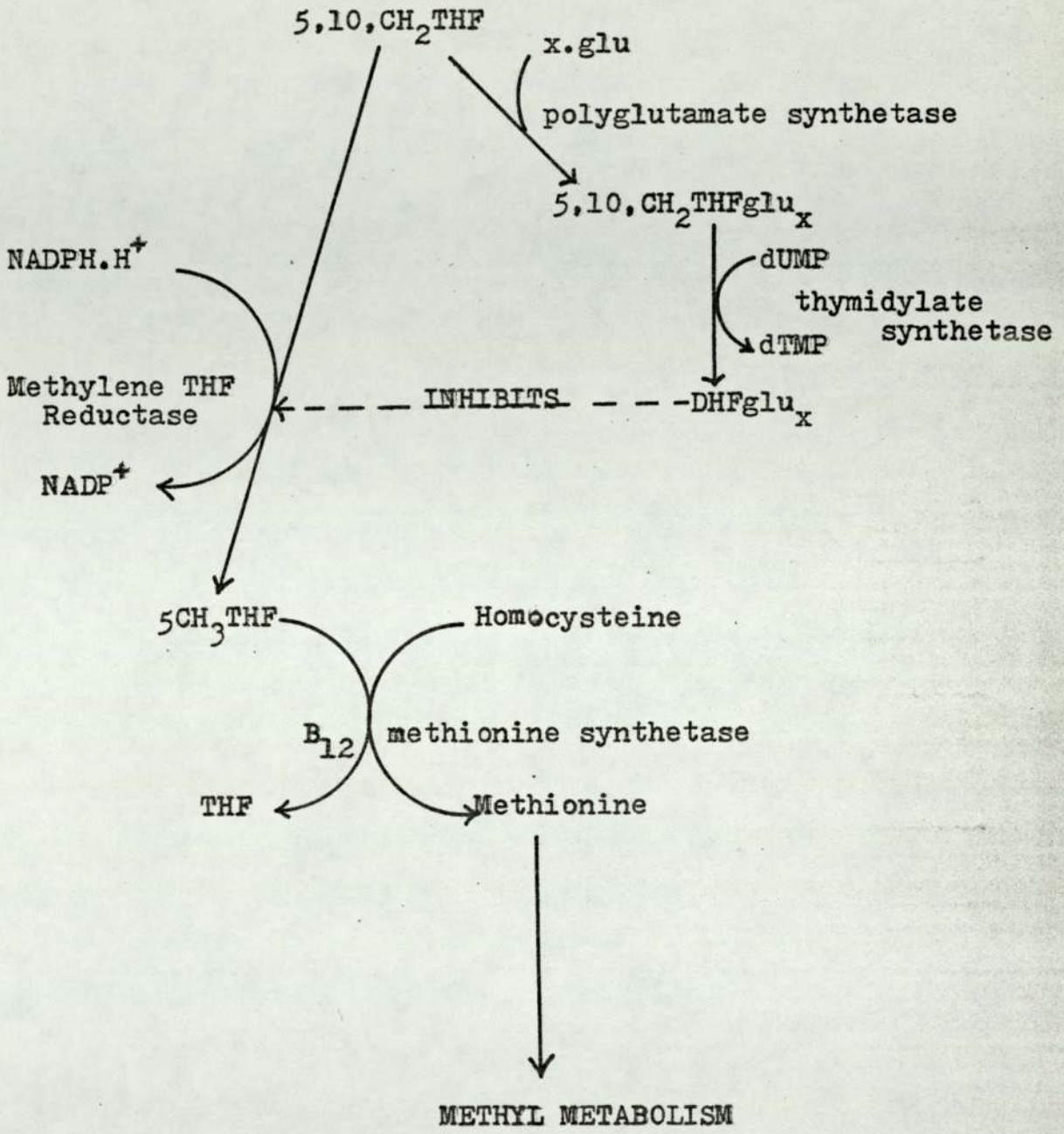
7.3 The Metabolism of 5MeTHF in Walker 256 Implanted Animals

The experiments in which W-256 bearing rats were dosed with 5(^{14}C)MeTHF were of especial interest as the tumour has an absolute requirement for preformed methionine in cell culture (Halpern et al 1974) although this phenomenum is not specific to or general amongst cancer cells (Chapter 1). The Walker 256 bearing animals showed a greater rate of demethylation of 5MeTHF as evidenced by the excretion of

^{14}C labelled non-folate compounds. The distribution of retained methyl activity was changed, the proportion retained by the muscle reduced from 13% to 3% and the production of $^{14}CO_2$ increased indicating a re-routing of methyl metabolism in the tumour implanted animals. This together with the increased retention of folate, observed with mixed label 5MeTHF, result in the increase in NFF and decrease in 5MeTHF excretion. Hoffman and Erbe (1976) found that although several tumour cell lines show a methionine requirement in cell culture they take up 5MeTHF and synthesise methionine at rates similar to, or greater than normal cells. Matthews and Haywood (1979) report that DHF is the most effective inhibitor of methylene THF reductase amongst naturally occuring folates; the effect of this inbition is to switch 5,10 CH_2THF from 5MeTHF formation to the completing pathway for thymidylate synthesis (fig 7.3).

Thus during the growth phase of a cell when there is an increase in TS activity(Rosenblatt and Erbe 1973) there would be an increase in the steady state concentration of DHF, the inhibitory effect of which would cause a concomittant decrease in 5MeTHF. Jackson and Niehammer (1979) report that hepatomas show a marked decrease of 10CHOTHF dehydrogenase compared to normal liver. As this enzyme depresses the level of 10CHOTHF in the folate pool, the decrease in the level of this enzyme would permit an increased steady state concentration of 10CHOTHF, possibly at the expense of 5MeTHF. Lack of intracellular 5MeTHF would have profound effects on tumour cells as they are observed to have abnormal requirements for methyl groups in nucleic acid synthesis [illegible], for example mouse mammary and S180 ascites tumours have a higher proportion of methylated purines in tRNA than normal mouse tissue (Berquist and Matthews 1962). Urinary excretions of methylated purines

Fig.7.3. Interaction of DHF with Methylene THF Reductase



is approximately twice that of normals in tumour bearing rats and mice (Mandel et al 1966), leukaemic tumours (Park et al 1962) and hamsters with adenovirus -12 induced tumours where the tumours consisted of 1-5% body weight (McFarlane and Shaw 1968). In a rapidly growing tumour such as Walker 256 these factors may combine to produce an absolute requirement for exogenous methionine.

The experiments with a mixture of 5(^{14}C)MeTHF and (^{3}H) folic acid provided no evidence of a substantial reversal of methylene THF reductase activity in W256 implanted animals, with the reservations noted in section 7.1 of this technique being able to detect such an event. The reversal reaction is unlikely as the redox potential of tumour cells is more reducing than normal (Schwartz and Passoneau 1974, Schwartz et al 1976), the data of Kutzbach and Stokstad predicting a more oxidising potential than normal is required to displace the equilibrium towards 5,10CH_2THF formation.

The handling of mixed label 5MeTHF by W256 implanted rats resembled that observed with FA by Barford and Blair (1978) in that there is a shift in the pattern of urinary metabolites with a proportionally greater urinary excretion of formyl folate. This may be a result of the induced enzyme changes as a result of the demands of the growing tumour mass. This folate demand is observed to increase the retention of folate in the tissue and decreased the total excretion of intact folate and scission products in urine and faeces. Rapid clearance of folate into the tissues would reduce the folate enterohepatic circulation and thus decrease scission in the gut.

Scission products also may result from the long term breakdown of tissue folate. However scission is diminished in W256 implanted rats, although there is a similar or increased amount of retained tissue folate (chapter 6). If the scission is as a result of oxidation, as with 5MeTHF (chapter 5) and THF, this would suggest reduced folate oxidation in the tumour cell. Pollock and Kaufman (1978) proposed a mechanism where dihydropteridine reductase (DHPR) participates in maintaining tissue folate in a reduced state. This enzyme can reduce the quinonoid form of DHF to THF(Lund 1972). Tissue DHPR distribution does not parallel that of the biopterin requiring hydroxylase enzymes, nor does DHPR activity increase when the level of catecholamines in the brain is depleted (Turner et al 1974). This suggests DHPR has a function other than BH_4 regeneration, Pollock and Kaufman suggest this is folate reduction. This enzyme has a Km for NADH of 2.10^{-5} in brain and 3.10^{-5} in liver (Cheema et al 1973), an order of magnitude higher than that for NADPH for DHFR($1.36.\ 10^{-6}$M, McCullough et al 1971), thus will show a greater increase in reaction rate with increasing reduced coenzyme concentration than DHFR. It is of note that tumour cells show a higher NADH/NAD ratio than normal (Schwartz et al 1974), resulting from the lactate/pyruvate increase which in hepatomas is 4 to 7 times that of normal liver cells (Weber et al 1971).

In these experiments it is observed the scission products from W256 rats differ from those of normals, which suggests that folate catabolites may be useful as a source of diagnostic tumour markers.

7.4 The use of folic acid in metabolic studies

This project has indicated that 5MeTHF undergoes similar qualitative handling to that observed with folic acid. The essential difference is that a pulse of FA on reduction to THF is more readily incorporated into tissue polyglutamates than 5 MeTHF. However, 10CHOTHF is a naturally occuring folate and is superior to 5MeTHF as a substrate for polyglutamate synthesis and thus would show a more rapid tissue incorporation. Thus conclusions drawn from experiments with folic acid are likely to be as valid as those using 5MeTHF.

7.5 Further Research

The technique used in this project would be improved should the chromatographic techniques enable examination of the folate output of individual animals and the routine examination of tissue polyglutamates. This may become possible with HPLC methods which have been applied to serum folate analysis.

From the studies of W256 implanted animals there has been observed differential handling of folate in cancer. However, it is necessary to distinguish whether this is as a result of tumour specific effects or due to the abnormally high growth rate of this particular tumour. Possibly cell culture studies with cell lines of graded growth rates such as the Morris hepatomas would furnish such information.

With the recognition of the importance of folate polyglutamates as intracellular coenzymes it is necessary to gain information of the in vivo metabolic relationships of the tissue folate types especially at different stages of cell growth. The mutant CHO line of McBurney and Whitmore (1974) may have further uses in showing the role of folate in the absence of polyglutamate synthesis. However, further research is required to produce an unambiguous polyglutamate assay technique.

8. REFERENCES

Albrecht A.M.(1979) in "Chemistry and Biology of Pteridines" Developments in Biochemistry Vol.4 eds. Kisliuk R.L. and Brown G.M. p.635-640 Elsevier/North Holland

Amstein H.R.V and Neuberger A.(1953) Biochem J. 55 259-271

Andreoli V.M. and Maffeu T. (1975) Lancet 2 922

Andrews J.(1977) Proc.Roy.Soc.Med. 70 84-86

Arakawa T.(1970) Am.J.Med. 48 594-598

Ashe H.,Clarke B.R.,Chu F.,Hardy D.N.,Halpern B.C., Halpern R.M. and Smith R.A. (1974) Biochem.Biophys.Res.Comm. 57 417-425

Axelrod A.E.(1971) Am.J.Clin.Nutr. 24 265-271

Axelrod J.(1957) Science 126 400-401

Baggot J.E. and Krumdiek C.L.(1979) Biochemistry 18 1036-1041

Baker B.R.(1971) Ann.N.Y.Acad.Sci. 186 214-226

Balinska M. and Grezelakowska-Sztabert B.(1978) Biochem.Biophys. Res.Comm. 85 1165-1172

Banerjee D.K.,Maitra A.,Basu A.K.,and Chatterjee J.B.(1975) Ind.J.Med.Res. 63 45-53

Banerjee S.P. and Snyder S.H.(1973) Science 182 74-75

Banerjee S.P. and Snyder S.H.(1974) Adv.Biochem.Psychopharmacol. 11 85-93

Barford P.A. and Blair J.A.(1976) in"Chemistry and Biology of Pteridines" ed. Pfleiderer W. p.413-427 Walter de Gruyer

Barford P.A.,Staff F.J, and Blair J.A(1977) Biochem.J. 164 601-605

Bauch C.M. and Krumdieck C.L.(1971) Ann.N.Y.Acad.Sci. 186 7-28

Beaton J.M.,Smithies J.R. and Bradly R.J.(1975) Biol.Psychiatr. 10 45-52

Beavon J.R.G.(1973) Ph.D. thesis Univ. Aston in Birmingham

Berquist P.L. and Matthews R.E.F.(1962) Biochem.J. 85 305-313

Bird O.D.,McGlohan V.A. and Vaitkus J.W.(1965) Anal.Biochem. 12 18-35

Blair J.A.(1976) in "Chemistry and Biology of Pteridines" ed. Pleiderer W. p.373-405 Walter de Gruyer

Blair J.A. and Dransfield E.(1971) Biochem.J. 123 907-914

Blair J.A. and Matty A.J. (1974) Clin. in Gastroent. 3 183-197

Blair J.A. and Pearson A.J. (1974) J. Chem. Soc. Perkin II 80-88

Blair J.A.,Pearson A.J., and Robb A.J. (1975) J. Chem. Soc. Perkin II 18-21

Blair J.A., Staff F.J., and Barford P.A. (1976) Biochem. Soc. Trans. 4 910-912

Blakley R.L. (1969) "The Biochemistry of Folic Acid and Related Pteridines" North Holland

Blumenstein J. and Williams G.R.(1960) Biochim. Biophys. Res. Comm. 3 259-263

Botez M.I., Young S.N., Bachevalier J. and Gauthier S. (1979) Nature 278 5700 182-183

Botez M.I., Fontaine F., Botez T., and Bachevalier J. (1978) Eur. Neurol. 16 230-246

Botsford J.L. and Parks L.W. (1967) J. Bacteriol. 94 966- 971

Butterfield S. and Calloway D.H. (1972) J. Am. Diet. Ass. 60 310-314

Butterworth C.E.Jnr., Santini R.Jnr.& Frommeyer W.B.Jnr.(1963) J. Clin. Inves. 42 1929-1939

Braumslag N., Edelstein T., and Metz J. (1970) Br. Med. J. 1 16-17

Brown J.P., Davidson G.E. and Scott J.M. (1974) Biochim. Biophys. Acta. 342 78-83

Brown G.M. (1962) J. Biol. Chem. 237 536-540

Cantoni G.L.(1975) Ann. Rev. Biochem. 435-451

Cantoni G.L.(1977) in "The Biochemistry of Adenosylmethionine" eds. Salvatore F., Borek E., Zappia V. Williams - Ashman H.G. and Schlenk F. p. 557-577 (Columbia Univ. Press.)

Carl G.F., Crews E.L., Carmichael S.M., Benesh F.C., and Smythies J.R. (1978) Biol. Psychiat. 13 773-776

Cauthen S.E., Foster M.A. and Woods D.P. (1966) Biochem. J. 98 630-635

Chanarin I., MacGibbon B.M., O'Sullivan W.J. and Mollin D.C. (1959) Lancet 2 634-639

Chandra R.K. (1975) Science 190 289

Cheema S., Soldin S.J., Knapp A., Hoffman T. and Scrimgeour K.G. (1973) Can. J. Biochem. 51 1229-1239

Chheda G.B.(1975) in"CRC Handbook of Biochemistry and Molecular Biology" 3rd Ed ed. Fasman G.D. p252-270

Ch'ien L.T.,Krumdiek C.L., Scott C.W. and Butterworth C.E.(1975) Am.J.Clin.Nutr. 28 51-58

Cohen S.S.(1971) Ann.N.Y.Acad.Sci. 186-292-301

Coleman I.P.L.,Hilburn M.E.H. and Blair J.A.(1979) Biochem. Soc. Trans. 7 675-676

Connor M.J.(1979) Ph.D. thesis, University of Aston in Birmingham

Connor M.J. and Blair J.A.(1980) Biochem.J. 186 235-242

Connor M.J., Blair J.A. and Barford P.A.(1977) Biochem.Soc.Trans 5 1319-1320

Connor M.J.,Pheasant A,E. and Blair J.A.(1979) Biochem.J. 178 795-7

Cooper B.A., Cantlie G.S.D. and Bruton M.(1970) Am.J.Clin.Nutr. 23 848-854

Costa E. and Meck J.L.(1974) Ann.Rev.Pharmacol. 491-511

Coward J.K.,Parameswaran K.N.,Cashmore A.R. and Bertino J.R. Biochemistry 13 3899-3903

Coward J.K.,Chello P.L.,Cashmore A.R.,Parameswaran K.N.,De Angelis L.M., and Bertino J.R.(1975) Biochemistry 14 1548-1552

Diets T.L.,Russel A.,Fujii K. and Whitely J.M.(1976) in"Chemistry and Biology of Pteridines" ed. Pleiderer W. p.525-534 W. de Gruyer

Dev I.K. and Harvey R.J(1978a) J.Biol.Chem. 253 4242-4244

Dev I.K. and Harvey R.J.(1978b) J.Biol.Chem. 253 4245-4255

Dickerman H.,Redfield B.G., Bieri J.G. and Weissbach (1964) J.Biol.Chem. 239 2545

Dillon M.J.,England J.M.,Gompertes D.,Goodey P.A.,Grant D.B., Hussein H.A-A.,Linnel J.C.,Matthews D.M.,Mudd S.H.,Newns G.H., Seakins J.W.T.,Uhlendorf B.W. and Wise J.K. (1974) Clin.Sci.Mol.Med. 47 43-64

Donaldson K.O. and Keresztesy J.C.(1959) J.Biol.Chem. 234 3235-3240

Donaldson K.O. and Keresztesy J.C.(1962) J.Biol.Chem.237 3815-3819

Dransfield E.(1972) Ph.D. thesis, Univ. Aston in Birmingham

Finkelstein J.D. Kyle.W.Z and Martin J.J.(1975) Biochem. Biophys.Res.Comm. 66 1491-1497

Finkelstein J.D.,Kyle W.Z. and Harris B.J.(1971) Arch.Biochem.Biophys. 146 76-83

Finkelstein J.D., Harris B.J.,Grossman M.R. and Morris H.P. (1978) Proc.Soc.Exp.Biol.Med. 159 313-316

Ford J.E.,Scott K.J. and Blair J.A.(1978) Inter. Dairy Congress 1069-1071

Freeman J.M., Finkelstein J.D. and Mudd S.H.(1975)
N. Eng. Med. J. 292 491-496

Friedkin M. (1973) Adv.Enzymol. 38 235-295

Friedkin M., Plante L.T., Crawford E.J. and Crumm M.(1975)
J. Biol. Chem. 250 5614-5621

Fuller R.W. (1976) Life Sci. 19 625-628

Gapski G.R.,Whitely J.M. and Huennekens F.M.(1971)
Biochemistry 10 2930-2934

Gatenby P.B.B. and Lillie E.W. (1960) Br. J. Med. 2 1111-1114

Giles C.(1966) J. Clin. Path. 19 1-11

Goff J.P. and Blakley R.L. (1978) Cancer Res. 38 3847-3853

Gready J.E. (1979) Nature 282 674-675

Gross R.L.,Reid J.V.O.,Newberne P.M.,Burgess B.,Marstm R. and Hift W.(1975) Am. J. Clin. Nutr. 28 225-232

Guest J.R.,Freidman S.,Dilworth M.J. and Woods P.P. (1964)
Ann. N.Y. Acad. Sci. 112 774-790

Gupta V.S. and Huennekens F.M.(1967) Arch.Biochem.Biophys 120 712-8

Halpern B.C.,Clark B.R.,Hardy D.N.,Halpern R.M. and Smith R.A(1974
Proc. Nat. Acad. Sci.(USA) 71 1133-1136

Halpern R.M.,Halpern B.C., Clark B.R., Ashe H., Hardy D.N. Jenkinson P.Y. and Smith R.A.(1975) Proc.Nat.Acad.Sci(USA) 72
4018-4022

Halpern R.,Halpern B.C.,Stea B.,Dunlap A.,Conkin K., Clark B., Ashe H.,Sperling L.,Halpern J.A.,Hardy D. and Smith R.A.(1977)
Proc.Nat. Acad. Sci. (USA) 74 587-591

Halstead C.H. (1979) Am. J. Clin. Nutr.32 846-855

Heady J.E. and Kerr S.J.(1973) J. Biol. Chem. 248 69-72

Herbert V. (1961) J. Clin. Inves. 40 81

Herbert V. and Zalusky R,J.(1962) J. Clin. Inves. 41 1263-1275

Hibbard B.M. and Hibbard E.D. (1968) Br. Med. Bull. 24 10-12

Hillman R.S.,McGuffin R.& Campbell C.(1977)
Trans. Ass. Am. Phys. 90 145-156

Hilton J.G.,Cooper B.A. & Rosenblatt D.(1979) J.Biol.Chem 254
8398-8403

Hoffbrand A.V. (1976) Progress. Haematol. 9 85-105

Hoffbrand A.V. (1977) Proc. Roy. Soc. Med. 70 82-84

Hoffman R.M. and Erbe R.W.(1976) Proc.Nat.Acad.Sci(USA) 73
1523-1527

Hoffman R.M. Jacobson S.I. and Erbe R.W. (1979)
Proc. Nat. Acad. Sci. (U.S.A.) 76 1313-1317

Hooton J.W.L. and Hoffbrand A.V. (1977)
Biochim. Biophys. Acta 477 250-263

Hsu L.L. and Mandell A.J. (1973) Life Sci. 13 847-858

Hunter R. Barnes J.; Oakley H.F. and Matthews R.M. (1970)
Lancet 1 61-63

Hunter R., Barnes J., Curzon G., Kantamanei B.D. and Duncan C. (1971)
J. Neurol. Neurosurg. Psychiat. 34 571-575

Hrynuik W.M., Fischer G.A. and Bertino J.R. (1969)
Mol. Pharmacol. 5 557-564

Jackson R.C. and Niehammer B. (1979) in "Chemistry and Biology of Pteridines" eds. Kisliuk R.L. and Brown G.M. p.665-670
(Eisevier/North Holland)

Johnson L.F., Fuhrman C.L. and Abelson H.T. (1978a)
Cancer Res. 38 2408-2412

Johnson L.F., Fuhrman C.L. and Wiederman L.M. (1978b)
J. Cell Physiol. 97 397-406

Jongejan J.A., Mager H.I.X., and Berends W. (1979)
in "Chemistry and Biology of Pteridines" eds. Kisliuk R.L. and Brown G.M. p. 241-246 (Elsevier/North Holland)

Kamely D., Littlefield J.W. and Erbe R.W. (1973)
Proc. Nat. Acad. Sci. (USA) 70 2585-2589

Kennelly J.C., Blair J.A. and Pheasant A.E. (1979)
Biochem. Soc. Trans. 7 646-648

Keresztesy J.C., and Donaldson K.O. (1961)

Kisliuk R.L., Gaumont G. and Baugh C.M. (1974)
J. Biol. Chem. 249 4100-4103

Klipstein F.A. (1964) Blood 23 68-86

Krebs H.A., Hems R. and Tyler B. (1976) Biochem. J. 158 341-353

Kreis W. and Goodenow M. (1978) Cancer Res. 38 2259-2262

Krumdiek C.L., Fukushima K., Fukushima T., Shiota T. and Butterworth C.E. Jnr. (1978) Am. J. Clin. Nutr. 31 88-93

Kutzbach C.A., Galloway E., and Stokstad E.L.R. (1967)
Biochim. Biophys. Acta 139 217-220

Kutzbach C.A. and Stokstad E.L.R.(1971)Biochim.Biophys. Acta 250 459-477

Laduron P.M.(1972) Nature(New Biol.) 238 212-213

Lankowski P.,Erlandson M.E.,and Bezan A.L.(1969)Blood 34 452-465

Laster L.,Mudd S.H. Finkelstein J.D. and Irreverre F. (1965) J. Clin. Inves. 44 1708-1719

Latife I.,Sargent T.,Dobson E.L. and Polycore M.(1978) Biol. Psychiat. 13 649-659

Lavoie A.,Tripp E., and Hoffbrand A,V.(1974) Clin. Sci. Mol. Med. 47 617-630

Lepage R., Poirier L.A.,Poirier M.C. and Morris H.P.(1972) Cancer Res. 32 1099-1103

Levi R.W. and Waxman S.(1975) Lancet 2 11-13

Levitt M.,Nixon P.F.,Pincus J.H. and Bertino J.R.(1971) J. Clin. Inves. 50 1301-1308

Lewis G.F and Rowe P. B. (1979) Anal. Biochem 93 91-97

Lewis K.F.,Randolf V.M.,Nemeth E. and Frisell W.R.(1978) Arch. Biochem. Biophys.185 443-449

Lovenberg W.,Jequier E.,Sjoersma A.(1967) Science 155 217-219

Lowenstein P.,Bruton L. and Hseih Y.S.(1966) Can. Med. Ass. J. 94 636-645

Lund K.E.(1972) Eu. J. Biochem. 25 560-562

McBurney M.W. and Whitmore (1974) Cell 2. 173-182.

McCullough J.L.,Nixon P.F.,and Bertino J.R.(1971) Ann. N.Y. Acad. Sci. 186 131-142

McFarlane E.S. and Shaw G.L.(1968) Can.J.Microbiol. 14 185-187

McGuire J.J.,Kitamoto V.,Hseih P.,Coward J.K.and Bertino J.R.(1979) in "Chemistry and Biology of Pteridines" eds. Kisliuk R.L. and Brown G.M. p.471-476 (Elsevier/North Holland)

MacKenzie C.G.,Chandler J.P.,Keller E.B.Rachele J.R.,Cross N., and du Vigneaud V.(1949) J. Biol. Chem. 180 99-111

MacKenzie C.G. and du Vigneaud V.(1952) J.Biol.Chem. 195 487-491

Magnum J.H.,Murray B.K.& North J.A.(1969) Biochemistry 8 3496-3499

Mahoney M.J.,Rosenberg L.E.,Mudd S.H. and Uhlendorf B.W.(1971) Biochem. Biophys. Res. Comm. 44 375-381

Maley F.and Maley G.F.(1960) Fed. Proc. 19 310

Mandel L.R., Scrinivasan P.R. and Borek E.(1966) Nature 209 586-8

Manzoor M. and Runcie J.(1976) Br. Med. J. 1 1176-1178

Maruyama T.,Shiota T. and Krumdiek C.L.(1978) Anal. Biochem. 84 277-295

Matthews R.G. and Haywood J.(1972) Biochemistry 18 4845-4850

Meller E.,Rosengarten H.,Friedhoff A.J.,Stebbins., and Silber R.(1975) Science 187 171-173

Meslson M.,Yuan R.,and Haywood J.(1972) Ann.Rev.Biochem.41 447-466

Morningstar J.F.Jnr. and Kisliuk R.L.(1965) J. Gen. Microbiol. 39 43-51

Mudd S.H. and Poole J.R.(1975) Metabolism 24 721-735

Murphy M.,Keating M.,Boyle P.,Weir D.G. and Scott J.M.(1976) Biochem. Biophys. Res. Comm. 71 1017-1024

Nau F.(1976) Biochemie(Paris) 58 629-645

Newberne P.M.(1977) in"Malnutrition and the Immune Response" ed. Suskind R.M. p.375-387(Raven Press,New York)

Newman E.B.,Miller b.,and Kapoor V.(1974) Biochim. Biophys, Acta 338 529-539

Nixon P.F.,and Bertino J.R.(1972) J.Clin.Inves. 51 1431-1439

Nixon P.F.,Slutsky G.,Natias A. and Bertino J.R.(1973) J. Biol. Chem. 248 5932-5936

Norohana J.M. and Aboobaker V.S(1963) Arch.Biochem.Biophys. 101 445-477

Norohana J.M. & Silverman M.(1962) J.Biol.Chem. 237 3299-3302

Osifio B.O.A.(1978) Enzyme 23 116-120

Osmond H. and Smythies J.(1952) J. Ment. Sci. 98 20

Park R.W.,Holland J.F. and Jenkins (1962) Cancer Res. 22 469-477

Paubert J.L.,Strauss L.D. & Rabinowitz(1976) J.Biol.Chem. 251 5104-5111

Pearson A.J.(1974) Chem. and Ind. 233-239

Perry J.(1971) Br. J. Haematol. 21 435-441

Perry J. & Chanarin I.(1977) Br. J. Haematol. 35 397-402

Perry J., Chanarin I.,Deacon R. and Lumb (1979a) Biochem. Biophys. Res. Comm. 91 678-684

Perry J.,Lumb M., Van der Westhuyzen J., Fernandez-Costa F. Metz J. and Chanarin I.(1979b) in "Chemistry and Biology of Pteridines" eds. Kisliuk R.L. and Brown R.M. p.315-320 (Eisevier/North Holland)

Petrymann R., Thorndike J. and Beck W.S.(1975) J. Clin. Inves. 56 1293-1301

Pheasant A.E. and Blair J.A. (1979) in 'Chemistry and Biology of Pteridines' eds Kisluik R.L. and Brown G.M. p. 577 -579 (Elsevier/North Holland)

Pheasant A.E., Connor M.J. and Blair J.A. (1980) Biochem J. (in press)

Pfiffner J.J., Calkins D.G, Bloom E.S. and O'Dell B.L. (1946) J. Am. Chem Soc. 68 1392

Poirier L.A. (1973) Cancer Res 33 2109 - 2113

Pollock R.J. and Kaufman S. (1978) J. Neurochem. 31 115-123

Pritchard J.A. Scott D.E. Walley P.J., and Halling R.F. (1970) J. Am. Med. Ass. 211 1982 -1984

Rao K.N. and Norohena J.M. (1977) Biochim Biophys Acta 481 594-607

Ratanasthein K, Blair J.A. Leeming R.J., Cooke W.T., and Melikan V. (1974) J. Clin. Path. 27 875-879

Ratanasthein K, Blair J.A., Leeming R.J., Cooke W.T., and Melikan V. (1977) J. Clin. Path 30 438-448

Reynolds E.H. (1968a) Lancet 1 398-401

Reynolds E.H. (1968b) Brain 91 197-214

Robinson D.R. (1971) Meth. Enzymol 18B 716-725

Rodriguez M.S.(1978) J. Nutr. 108 1983-2075

Rosenberg R.N., Van der Venter L., De Francesco L, and Friedkin M.E. (1971) Proc. Nat. Acad, Sci. (USA) 68 1436-1440

Rosenblatt D.S. and Erbe R.W. (1973) Biochem. Biophys Res. Comm. 54 1627-1633

Rosengarten S.P. and Friedhoff A.J. (1976) Schizophrenia Bull. 2 90-105

Rowe P.B. (1978) in "The metabolic basis of inherited disease" eds Stanbury, Wyngarden J.B. and Friedrickson D.S. 4th ed. p. 430-457 (McGraw-Hill)

Sakami W. Ritan S.J, Black C.W., and Rzepka J. (1973) Fed. Proc. 32 471 Abstract 1400

Sakami W. and Uskins 1 (1961) J.Biol. Chem. 236 PC 50-PC51

Sakamoto S., Niiana M., and Tamaku T., (1975) Blood 46 699-704

Salem A.R. and Foster M.A., (1972) Biochem. J. 127 845-853

Salvatore, Borek E, Zappia V., Williams -Ashtiman H.G and Schienk F., (1977) eds 'Biochemistry of adensoyl methionine (Columbia Univ. Press)

Scarano E., Tosi L., and Granien A., (1977) in 'The Biochemistry of Adensoyl Methionine' eds Salvatore F., Borek E., Zappia V., Williams-Ashman H.G. AND Schlenk F., p 369-382 (Columbia Univ. Press)

Schnell M., and Ordonez F., (1977) J. Neurochem. 29 121-126

Schwartz J.P. and Johnson G.S. (1976) Arch Biochem. Biophys. 173 237-245

Schwartz J.P., Passonneau J.V., Johnson G.S. and Pastan I. (1974) J. Biol. Chem. 249 4138-4143

Scrimigeour K.G. and Vitrois K.S. (1966) Biochemistry 5 1438-1444

Scrutton M.C. and Beis I.S (1979) Bichem J. 177 833-846

Selgison D. (1961) in "Methods in Clinical Chemistry" 3rd. Ed. (Academic Press)

Selhub B., Gay A.C., and Rosenberg I.H. (1979) Biochim. Biophys. Acta. 557 372-384

Shane B., Watson J.E. and Stokstad E.L.R. (1977) Biochim. Biophys. Acta 497 241-252

Shin Y.S., Williams M.A. and Stokstad E.L.R. (1972) Biochem. Biophys. Res. Comm. 47 35-43

Smythies J.R (1975) in 'New Perspectives in Schizophrenia' Eds. Forrest A., and Affleck J. (Churchill-Livingstone)

Smythies J.R. (1976) Lancet 2 136-139

Spector R., Fosbury M., Levy P., and Abelson M.T., (1978) J. Neurochem. 30 899-901

Spronk A.M. (1973) Fed. Proc. 32 471 abstract 1398

Stea B., Backund P.S., Berkey P.B., Cho A.K., Halpern B., Halpern R.M., and Smith R.A., (1978) Cancer Res. 38 2378-2384

Stebbins R.D. Meller E., Rosengarten H., Friedhoff A., Silber R., (1976) Arch. Biochem. Biophys. 173 673-679

Steinberg S.E., Campbell C.L., and Hillman R.S. (1979) J. Clin. Inves. 64 83-88

Stokes P.L., Melikan V., Leeming R.J., Portman-Graham H., Blair J.A., and Cooke W.T., (1975) Am.J.Clin. Nutr. 28 126-129

Sugimura T., Birnbaum S.M., Winitz M. and Greenstein J.P., (1959) Arch. Biochem. Biophys 81 448-456

Tamura T, Shin Y.S., Williams M.A., and Stokstad E.L.R, (1972) Anal. Biochem. 49 517-521.

Tan L.U.L., Drury E.J. and Mackenzie R.E. (1977) J. Biol. Chem. 252 1117-1122

Tan L.U.L. and MacKenzie R.E. (1979) Can. J. Biochem. 57 806-812

Taylor R.T., and Hanna M.L. (1975) Life Sci. 17 11-119

Taylor R.T. and Hanna M.L. (1977) Arch. Biochem. Biophys 181 331-344

Thenen S.E., Shin Y.S. and Stokstad E.L.R. (1978) Proc. Soc. Exp. Biol. Med. 142 638-641

Thomson D., Baker H., and Leevy C (1971) Gastroent. 60 756 (Abstract)

Thorndike J., and Beck W.S., (1977) Cancer Res. 37 1125-1132

Tisdale M.J. (1979) Br. J. Cancer 40 303 (Abstract)

Turner A.J. Ponzio F., and Algen S., (1974) Brain Res. 553-558

Tyerman M.J., Watson J.E., Shane B., Schutz D.E., and Stokstad E.L.R., (1977) Biochem. Biophys. Acta 497 234-240

d'Urso-Scott M., and Maluki D.R. (1973) Fed Proc. 32 471 abstract 1399

Valenote F., and Van Putten (1975) Cancer Res. 35 2619-2630

Varadi S., Abbot D., and Elwis A. (1966) J. Clin. Path. 19 33-36

Waxman S., (1976) in 'Chemistry and Biology of Pteridines' ed Pfleiderer W. p165-178 (Walter de Gruyer).

Waxman S., Carcino J.J., and Herbert V., (1970) Am. J. Med 48 599-608

Weber G., Stubbs M., and Morris H.P., (1971) Cancer Res. 31 2177-2183

Weir D.G., Brown J.P., Freedman D.S., and Scott J.M., (1973) Clin Sci Mol. Med. 45 625-631

Williams E.A.J., Gebhardt B.M., Morton B., and Newberne P.M. (1979) Am.J.Clin. Nutr. 32 1214-1223

Williamson D.H., Lund P., and Krebs H.A., (1967) Biochem. J. 103 514-527

Williamson J.R. and Corkey B.E. (1969) Methods in Enzymology 13 434 -

Wills L. (1931) Br. Med. J. 1 1059

Zalusky R.J. and Herbert V. (1961) N. Engl. Med. J. 1033-1038