STUDIES OF FOLATE METABOLISM IN MAN AND THE RAT

BY

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SUMMARY

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The metabolism of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid was studied in man and the rat. Similar observations were found in both species. Folic acid is metabolised to various compounds. Both intact folates and folate catabolites were excreted in the urine after administration and folate polyglutamates found in the tissues. The relative amounts of intact folates and folate catabolites varied with time and dose. The results presented support the existence of two metabolically distinct pools, a short-term pool with a half-life of approximately one day and a long-term pool with a half-life of 11 days and demonstrate that the breakdown of folate in vivo is a function of normal folate metabolism.

Patients with malignant disease and tumour-bearing rats excreted less of the dose in the urine, incorporated more into the reduced folate pool and showed decreased catabolism of folate when compared to controls.

The administration of methotrexate to rats increased the catabolic rate of folate and the excretion of radioactivity in the urine and caused a corresponding fall in the levels of radioactivity recovered in the tissues. Methotrexate also led to the excretion of 4 additional radioactive pterins not found in normal urine.

The induction of the hepatic enzymes by phenobarbitone failed to increase the catabolic rate of folate.

The possible mechanism of folate breakdown in the tissues has been elucidated to be a simple chemical oxidative cleavage of the C_9-N_{10} bond of labile folate derivatives produced during the normal metabolic pathways.

The metabolism of 10 formylfolate was also studied in man. It is excreted largely unchanged in the urine and appears not to be reduced by man. 10 Formylfolate enters the reduced folate pool extremely slowly via deformylation. Also, 10 formylfolate inhibits the reduction of folic acid in vivo.

KEY WORDS

Folate metabolism, Folate catabolism, Methotrexate, 10 formylfolate

malignant disease

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" My Lord bestow on them thy mercy

even as they cherished me in childhood "

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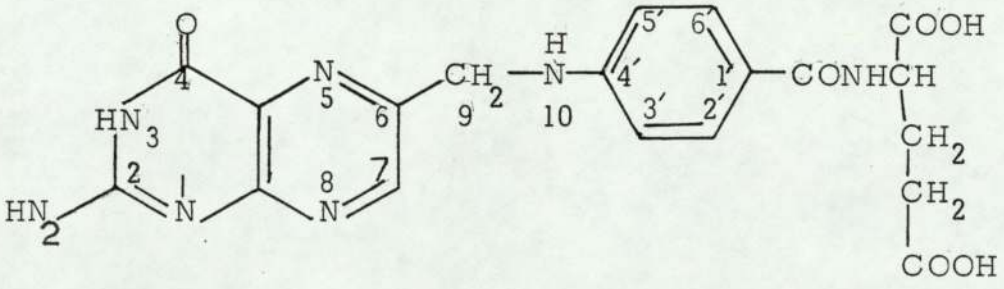
CHAPTER 1

INTRODUCTION

Folates are essential nutrients for the mammals and deficiency results in various forms of anaemia. The active compounds are reduced and substituted (N5 and N10 positions) derivatives of folic acid (I). Within the tissues folate conjugates occur. These are probably poly- γ-glutamate derivates. The folates are required for a number of one-carbon transfer reactions. The enzymatic reactions in which folate coenzymes are known to participate include certain aspects of purine and pyrimidine synthesis, formation of methionine from homocysteine, metabolism of other amino acids including the catabolism of histidine and the interconversion of serine and glycine. Formylation and oxidation - reduction of some folate derivatives also occurs (for reviews see Blakley, 1969: Hoffbrand, 1976: Rowe, 1978 and Chanarin 1979a). A scheme showing the major folate requiring reactions and interconversions in mammals is illustrated in Figure (1-1).

Folic acid (FA) enters the folate pool only after it has undergone reduction to 7,8-dihydrofolate (DHF) (II) and that is in turn reduced to 5,6,7,8-tetrahydrofolate (THF) (III). The enzyme responsible, dihydrofolate reductase 5,6,7,8 THF:$NADP^+$ oxidoreductase (EC 1.5.1.3)(DHFR) is widely distributed in most mammalian tissues. The highest levels of this cytoplasmic enzyme have been found in liver, kidney, embryonic tissues (Roberts and Hall, 1965) and in tumours (Blakley 1969).

Dihydrofolate reductase is strongly inhibited by methotrexate (MTX) (IV) (Bertino et al., 1964) and other folate analogues. Inhibition of



I Folic acid

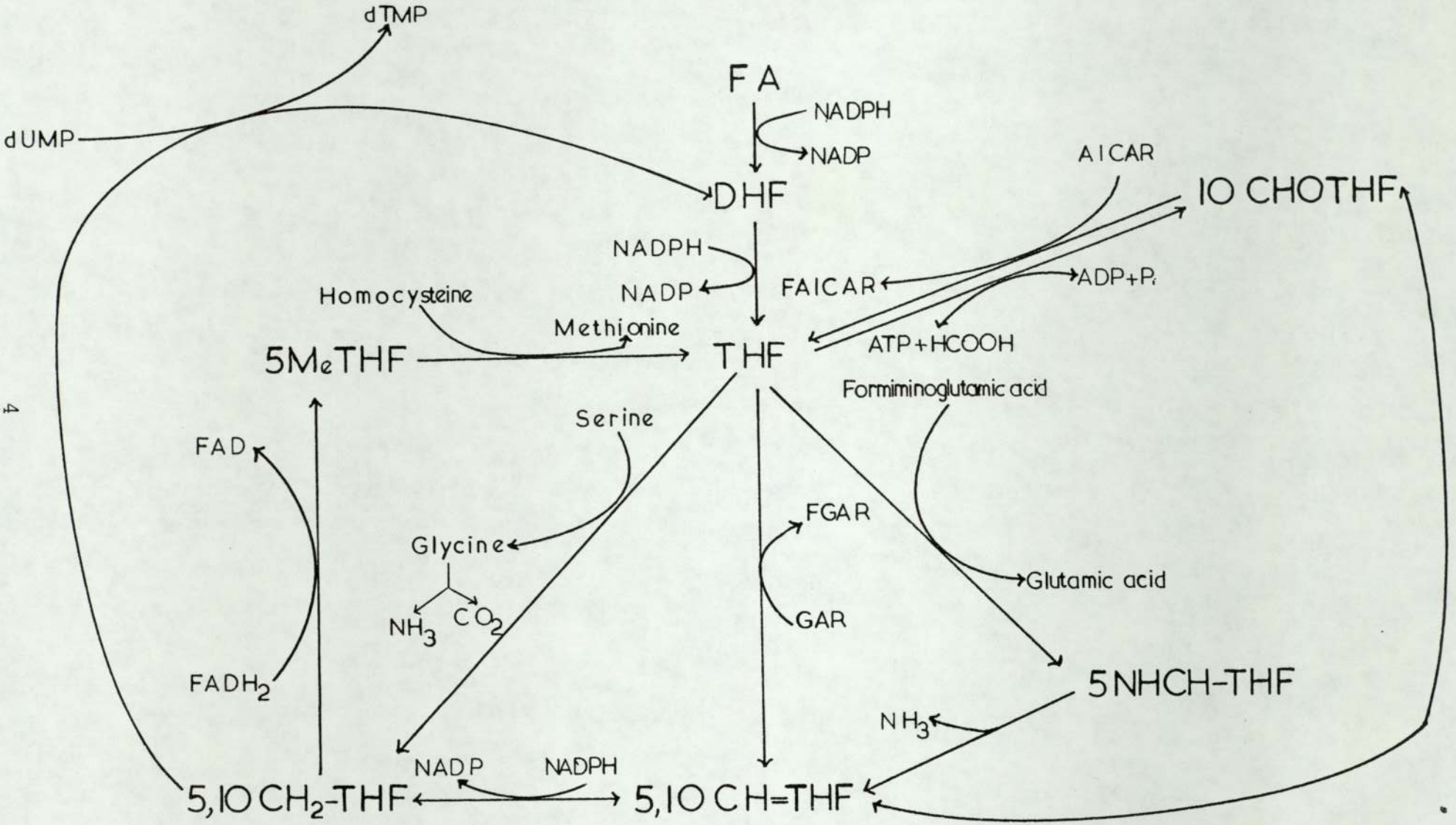
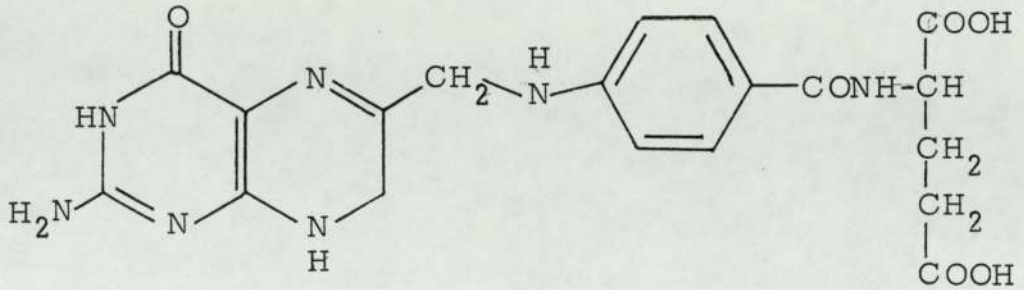
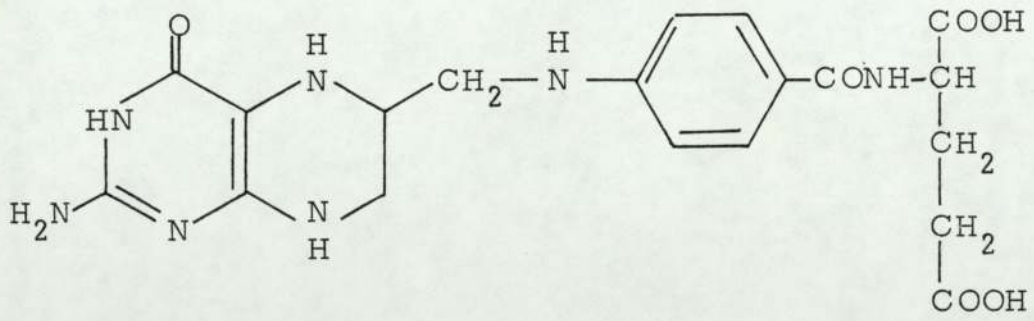


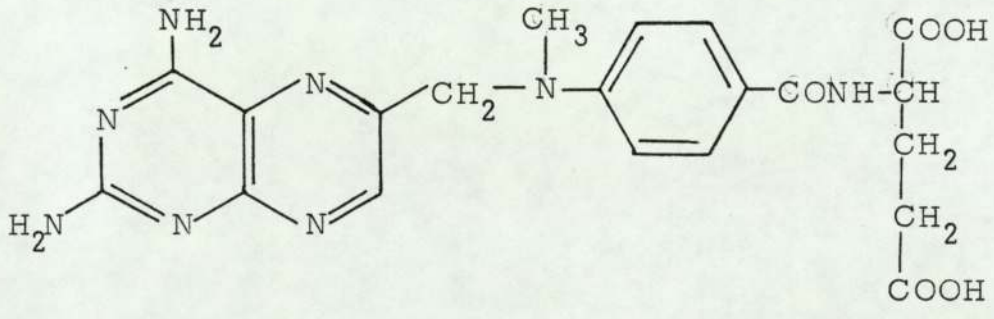
Figure (1-1) The major folate interconversion pathways



II 7,8 Dihydrofolic acid



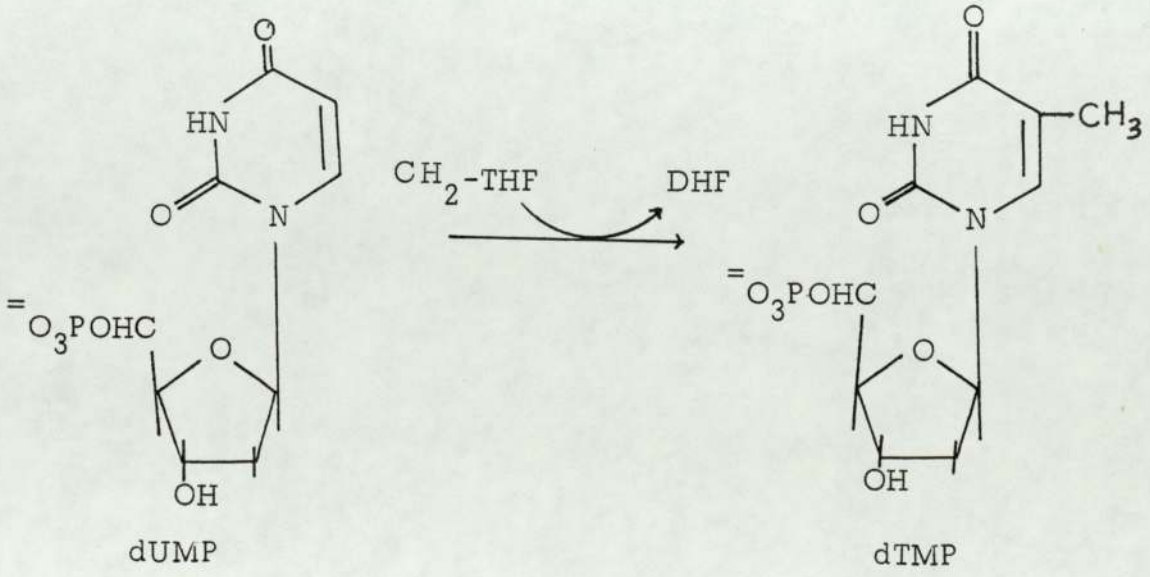
III 5,6,7,8 Tetrahydrofolic acid

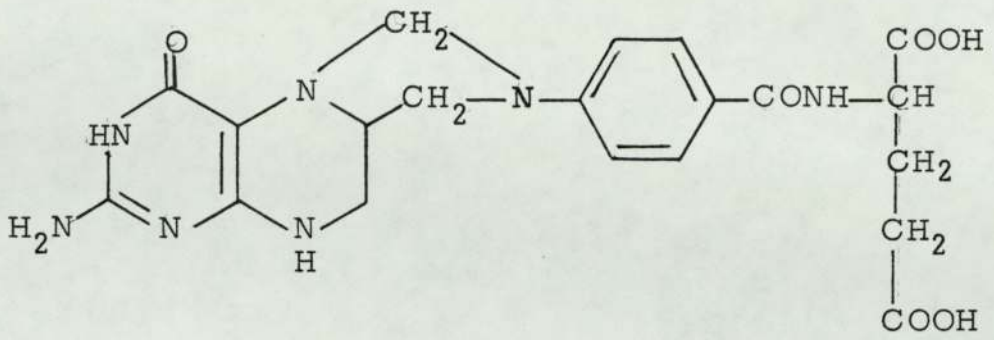


IV Methotrexate

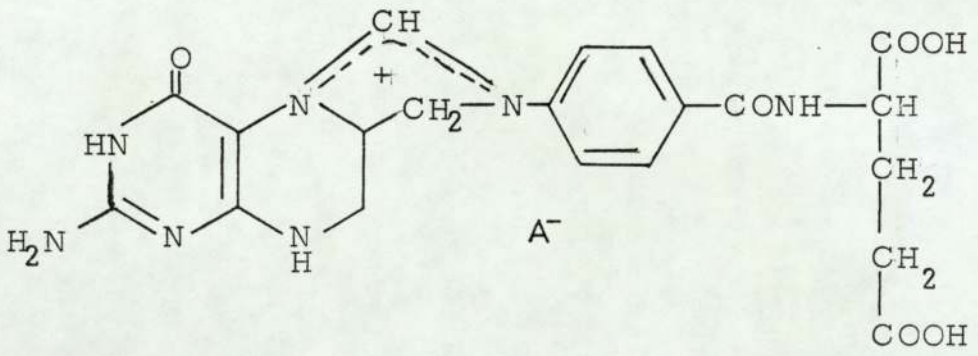
DHFR leads to depletion of the cellular pool of folate coenzymes and thus a lack of purines and pyrimidines and ultimately, cell death (Blakley, 1969). This observation has been exploited in cancer chemotherapy and will be discussed later.

5,10 Methylenetetrahydrofolate (5,10CH_2-THF) (V), 5,10 methenyltetrahydrofolate (5,10CH=THF) (VI) and 10 formyltetrahydrofolate (10CHO-THF) (VII) are the folate coenzymes that are involved in pyrimidine and purine biosynthesis. Transfer of the CH_2 group from 5,10 methylenetetrahydrofolate to deoxyuridine is accompanied by its simultaneous reduction to CH_3, and 5,10 methylenetetrahydrofolate is thereby oxidised to dihydrofolate. The enzyme responsible is thymidylate synthetase (EC 2.1.1.6).

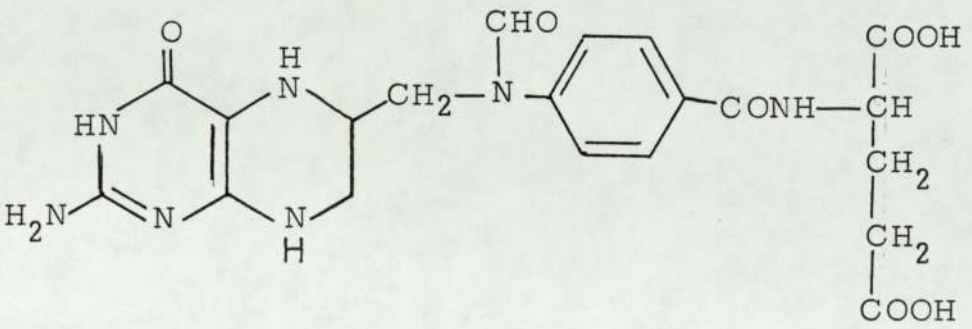




V 5,10 Methylenetetrahydrofolic acid



VI 5,10 Methenyltetrahydrofolic acid



VII 10 Formyltetrahydrofolic acid

Thymidylate synthetase is coupled with dihydrofolate reductase and these enzymes operate in sequence in the synthesis of thymidine and tetrahydrofolate which can re-accept one carbon units and be recycled Figure (1-2).

Tetrahydrofolate is also liberated by the reactions of purine biosynthesis. Carbon atoms 8 and 2 of the purine ring were thought to be derived by the donation of the carbon unit from the bridge carbon of 5,10 methenyltetrahydrofolate to 5', phosphoribosyl glycinamide and the donation of the one carbon group of 10 formyltetrahydrofolate to 5', phosphoribosyl-4-carboxamide-5 aminoimidazole respectively, Figure (1-3). However, Dev and Harvey (1978a) using a purified glycinamide ribotide transformylase (EC 2.1.2.2.) from Escherichia Coli found that the required coenzyme in both cases is 10 formyltetrahydrofolate. They suggested that during the earlier studies on pigeon liver and bacterial extracts (Goldthwait, et al., 1956, Westby and Gots, 1969) 5,10 methenyltetrahydrofolate was hydrolysed to 10 formyltetrahydrofolate either chemically or by the enzyme cyclohydrolase.

$$5,10\text{CH=THF} + H_2O \rightleftharpoons 10\text{ formyl-THF} + H^+$$

At physiological pH and temperature the ring cleavage reaction is the only one detectable: this raises a question about the role of 5,10 methenyltetrahydrofolate in purine biosynthesis and about the role of the cyclohydrolase enzyme in maintaining an equilibrium between these two tetrahydrofolate derivatives. It is interesting to note that in the last few years it has been reported that methylenetetrahydrofolate dehydrogenase

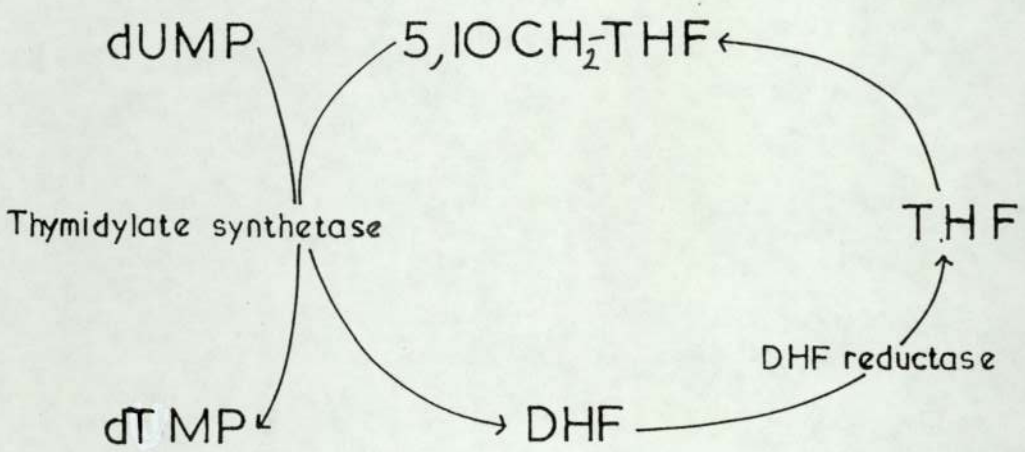
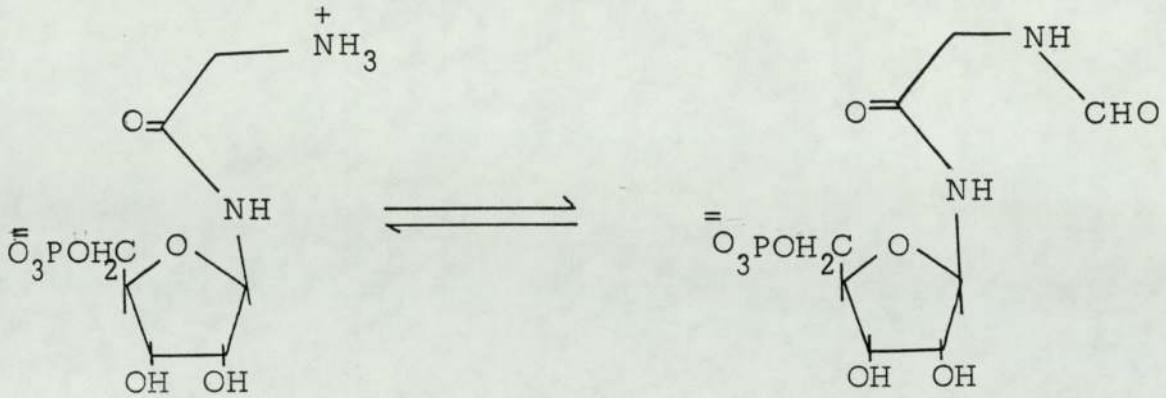


Figure (1.2) The methylation of deoxyuridine catalysed by thymidylate systhetase is accompanied by formation of dihydrofolate which in turn is reduced to THF by dihydrofolate reductase.



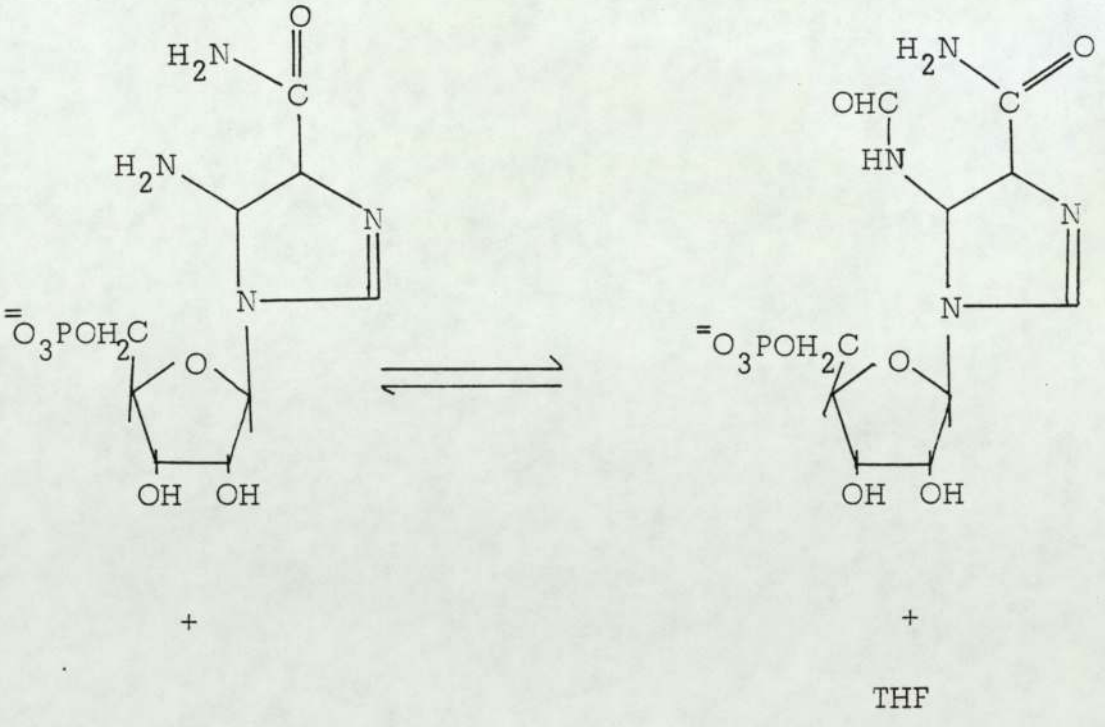


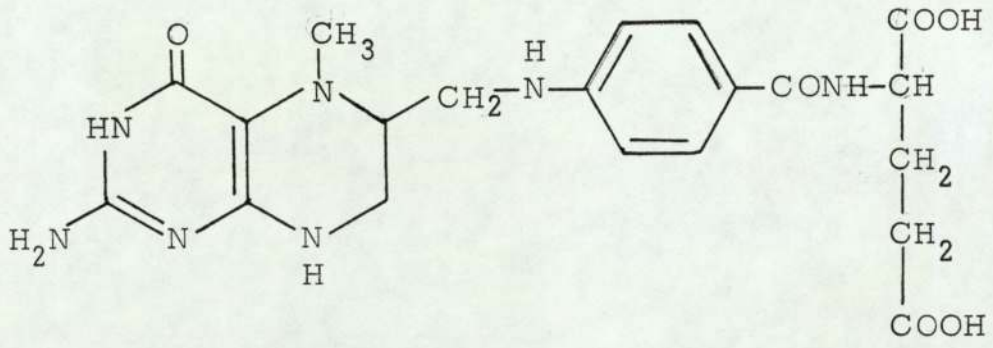
Figure (1-3) Folate dependent steps in the biosynthesis of purine ring.

and cyclohydrolase activities are present on a single protein together with 10 formyltetrahydrofolate synthetase. Such a multifunctional protein has been isolated from E. Coli (Dev and Harvey, 1978b), pig liver (MacKenzie, 1973, Tan et al., 1977 and MacKenzie and Baugh 1980), sheep liver (Paukert et al., 1976) porcine liver (MacKenzie and Tan 1980), chicken liver (Caperelli et al., 1980) and from yeast (Paukert et al., 1977). Hence, during the conversion of 5,10 methylenetetrahydrofolate to 10 formyltetrahydrofolate, 5,10 methenyltetrahydrofolate would have only a transient existence and would be unlikely to have a donor role in vivo for a transformylation reaction. This suggests that 10 formyltetrahydrofolate may be the exclusive formyldonor in mammalian purine biosynthesis.

The folate coenzymes are concerned with the formation of methionine from homocysteine. 5 Methyltetrahydrofolate (5MeTHF) (VIII) the major plasma folate (Ratanasthein, 1975) donates its one carbon group to homocysteine forming methionine. This reaction is catalyzed by a cobalamin containing enzyme 5 methyltetrafolate-homocysteine methyltransferase (EC 2.1.1.13) (Methionine synthetase) and requires catalytic levels of a reducing system and S-adenosylmethionine (SAM).

$$5\text{MeTHF} + \text{Homocysteine} \xrightarrow[\text{SAM} + FADH_2]{B_{12}} \text{THF} + \text{Methionine}$$

Many years ago it was observed that folic acid can correct much of the haematological damage due to deficiency of Vitamin B_{12}. The



VIII 5 Methyltetrahydrofolic acid

"methyl trap" hypothesis was put forward to account for this observation. The basis of the hypothesis was that in the absence of Vitamin B_{12}, 5MeTHF which is generated from 5,10CH_2-THF by the enzyme 5,10CH_2-THF reductase, (EC 1.1.1.68) is trapped since the latter reaction is considered to be irreversible in vivo. THF cannot be regenerated because the cobalamin dependent methionine synthetase reaction does not operate (Herbert and Zalusky, 1962, Noronha and Silverman, 1962a), Figure (1-4). The total body pool of folates becomes predominantly 5 MeTHF; the decreased availability of the other reduced folate derivatives slows the rates of reactions for which they are essential cofactors and megaloblastic anaemia develops. This is characterised by insufficient numbers of red blood cells and the formed blood cells are large and misshaped. For more details see Chanarin (1979a).

The methylfolate trap hypothesis is widely accepted as providing the best explanation of the events in B_{12} deficiency and evidence for it is as follows: 5MeTHF is elevated in the serum of pernicious anaemia patients (Thenen, et al., 1973), the levels of cellular folate polyglutamates are low (Perry et al., 1979), incorporation of ^{14}C activity from 5 $^{14}CH_3$ - THF into proteins is decreased and synthesis of thymidylate is decreased in cultured B_{12} deficient lymphocytes (Sakamoto et al., 1975). The observations on polyglutamate levels can be explained by the methyl trap hypothesis since B_{12} deficiency reduces the proportion of THF, a good substrate for polyglutamate synthesis in favour of 5MeTHF which is not incorporated into polyglutamates (Spronk, 1973). However, Rowe (1978)

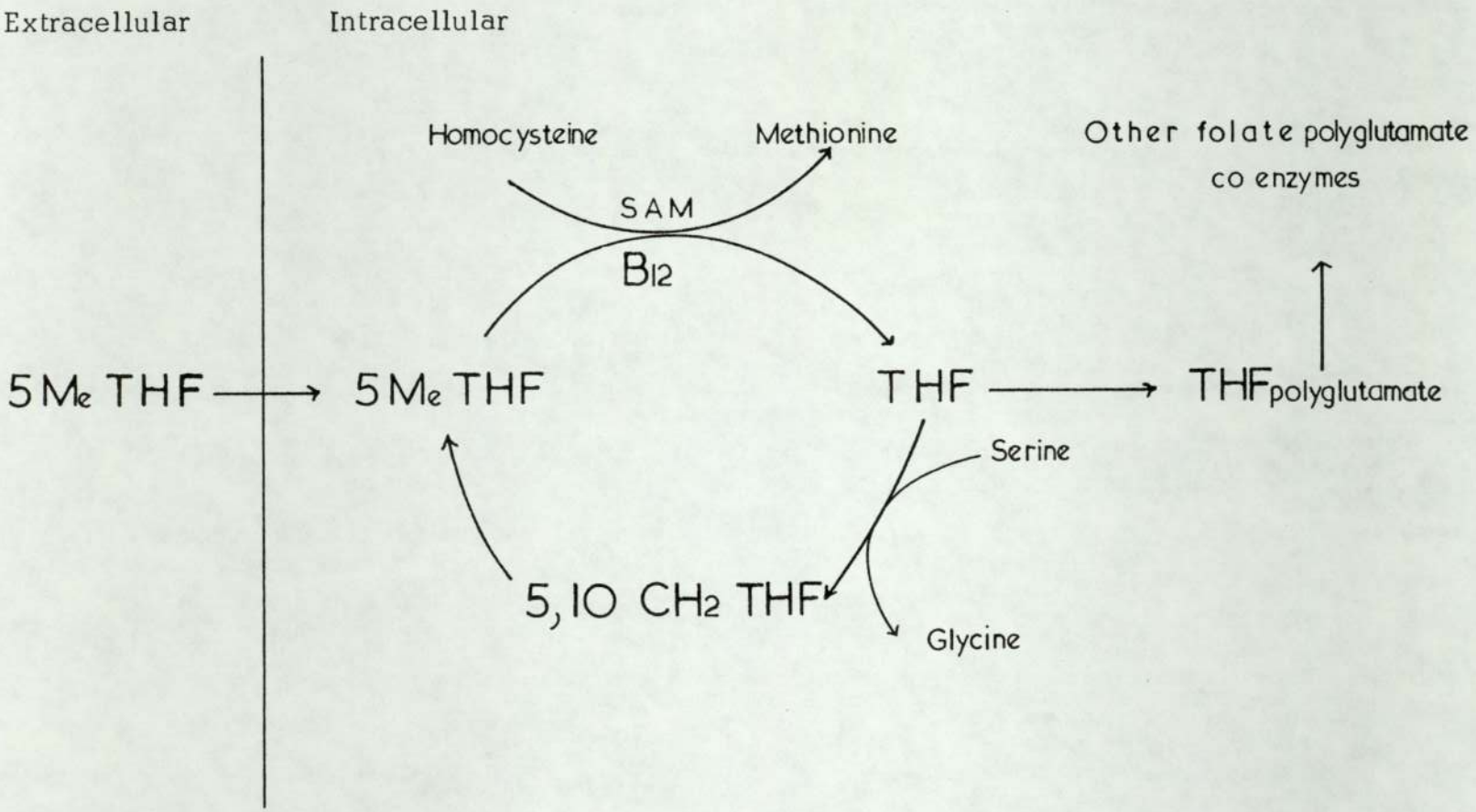


Figure (1-4) The interaction of B_{12} and folates

has suggested that the hypothesis is insufficient to explain why megaloblastic anaemia does not develop with congenital methylmalonicaciduria in spite of the defect of cobalamin metabolism and methionine synthetase activity (Dillon et al., 1974).

In the last few years Chanarin and co-workers and Scott and co-workers have reported that nitrous oxide (N_2O) inactivates methionine synthetase in vivo by oxidising B_{12} from the active reduced form Co(I) to the inactive oxidised form Co(III) and interrupts formation of the folate coenzymes. Therefore they have used rats exposed to N_2O as a model for B_{12} deficient animals and made several observations which indicated that B_{12} deficiency has another effect on folate metabolism as well as trapping 5MeTHF (Perry, et al., 1979, Deacon et al., 1980a,b, Lumb et al., 1980, McGing et al., 1978, Scott et al., 1979), and Chanarin et al., 1980 have proposed an alternative hypothesis to explain the role of Vitamin B_{12} on folate metabolism which invokes a role for B_{12} in the supply of formate for the formylation of folate. According to them formate arises from the oxidation of methyl groups, particularly the methyl group of methionine. In B_{12} deficiency the formation of methionine is decreased and this in turn leads to shortage of formate for the formylation of THF: formyltetrahydrofolate is the required substrate for the synthesis of folate polyglutamates (folate coenzymes) and impairment of this step compromises general folate metabolism. However their results can also be interpreted in terms of the methyl trap hypothesis.

Although methionine can be synthesised from homocysteine in the mammal, it is considered as an essential aminoacid. This reflects the relatively high requirement for methionine compared to its rate of synthesis Mudd and Pool (1975) calculate that human volunteers on a normal nitrogen diet synthesised approximately 50% of their methionine requirement de novo. It is required not only as a protein constituent, but also as a methyl donor for a large number of methylation reactions. The administration of an oral dose of methionine to humans causes a rapid fall in plasma folate, reversible by folate supplements (Connor, et al., 1978) and this is presumably due to repressed 5MeTHF synthesis. Buehring et al., (1972) found that an increase in the relative concentrations of formyltetrahydrofolate and free THF in livers of cobalamin-deficient rats on addition of methionine. Krebs et al., (1976) stresses the central role of the enzyme formyltetrahydrofolate dehydrogenase (EC 1.5.1.6) in the metabolism of folate and methionine. At high methionine concentration, the concentration of S-adenosyl-methionine (SAM) is raised leading to the inhibition of methylenetetrahydrofolate reductase (Kutzbach and Stokstad, 1971). This inhibition causes an increase in the concentration of THF derivatives in equilibrium with methylenetetrahydrofolate, including 10 formyltetrahydrofolate (Figure 1-5); the increased concentration of the latter accelerates the formyltetrahydrofolate dehydrogenase reaction because the normal concentration of the substrate is far below the K_m value of the enzyme for the substrate and thereby disposes the excess of C_1 units as CO_2. When the methionine concentration is low, C_1 units are preserved

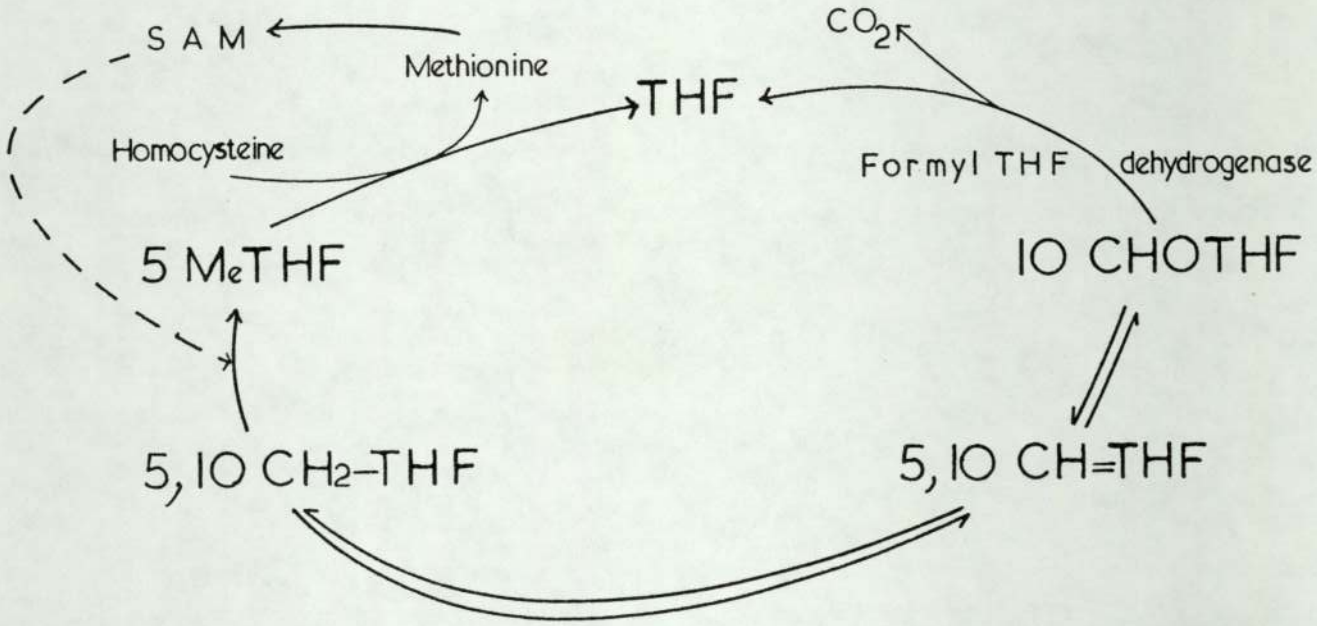
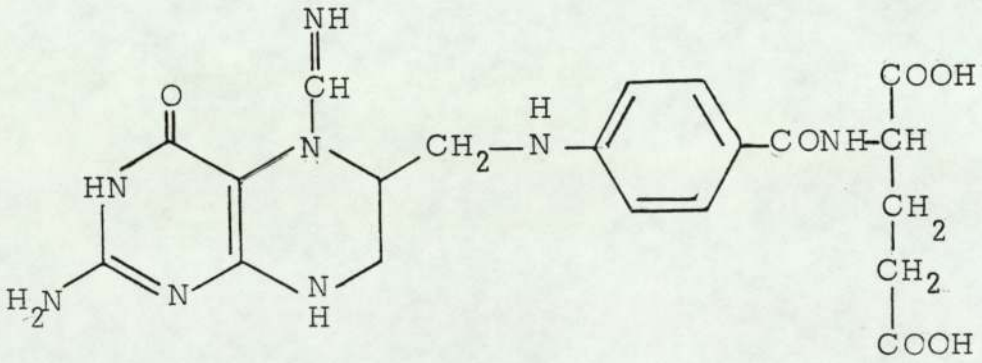


Figure (1-5) The role of the enzyme formyltetrahydrofolate dehydrogenase in the metabolism of folates and methionine



IX Formiminotetrahydrofolic acid

by the decreased activity of formyltetrahydrofolate dehydrogenase and are utilized for the synthesis of methionine purines and pyrimidines.

The interconversion of the aminoacids serine and glycine, is the principal single source of one carbon units (Blakley, 1969) for THF to form 5,10 CH_2-THF.

$$\text{Serine} + \text{THF} \rightleftharpoons 5,10\ CH_2\text{-THF} + \text{glycine}$$

This reaction is catalyzed by serine transhydroxymethylase (EC 2.1.2.1); an enzyme which is widely distributed in animals and plants (Blakley, 1969). The bulk of the enzyme activity is in the cytosol and it exists also in mitochondria (Rowe, 1978). In folate deficiency when there is a lack of free THF, serine enters other pathways leading to glucose synthesis or yielding energy by complete degradation. In mitochondria the glycine/serine levels play a role in regulating the production of 5,10 CH_2-THF, 10 formyl THF and THF via 10 formyl THF-dehydrogenase, Figure (1-6).

Formiminoglutamic acid derived from histidine catabolism is the other major source of one carbon units which are transferred to THF, in this case to form 5 formiminotetrahydrofolate (5CHNH-THF) (IX). This reaction is catalyzed by the enzyme formiminoglutamate tetrahydrofolate formiminotransferase (EC 2.1.2.5).

$$\text{THF} + \text{CHNH glutamic acid} \longrightarrow 5\ \text{CHNHTHF} + \text{glutamic acid}$$

and this is the normal route of histidine catabolism. Zalusky and

Figure 1-6 The role of glycine levels in regulating the production of $5,10CH_2$-THF, 10CHOTHF and THF

At high glycine levels, N-methylglycine is enhanced, the transfer of methyl group from N-methylglycine to $5,10CH_2$-THF increases the 10CHOTHF production and thus one carbon group oxidation to CO_2. Also because of the loss of SAM due to N-methylglycine biosynthesis, inhibition of $5,10CH_2$-THF reductase is reduced and methionine levels are maintained, the constant recycling occurring until equilibrium is reached.

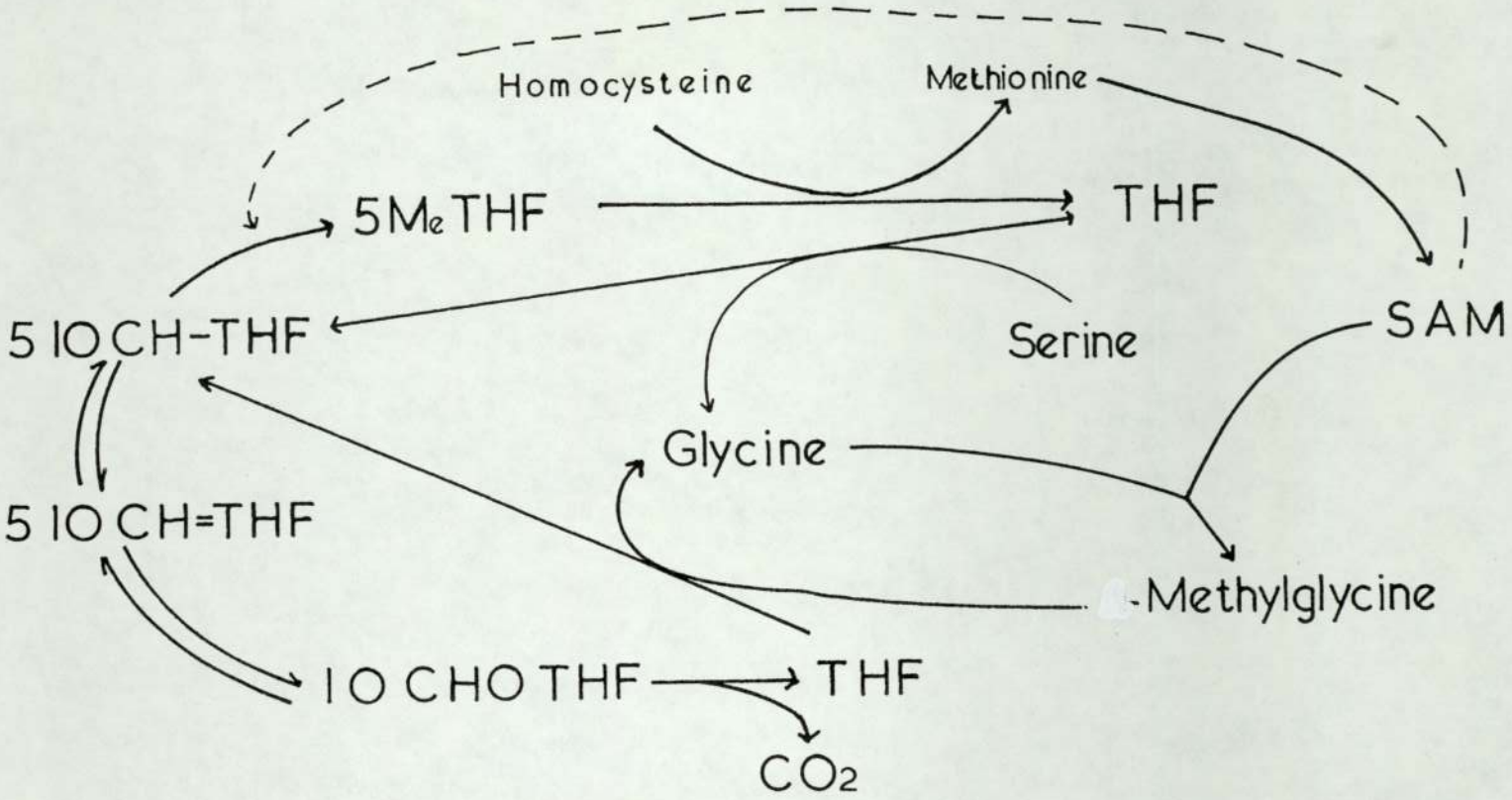


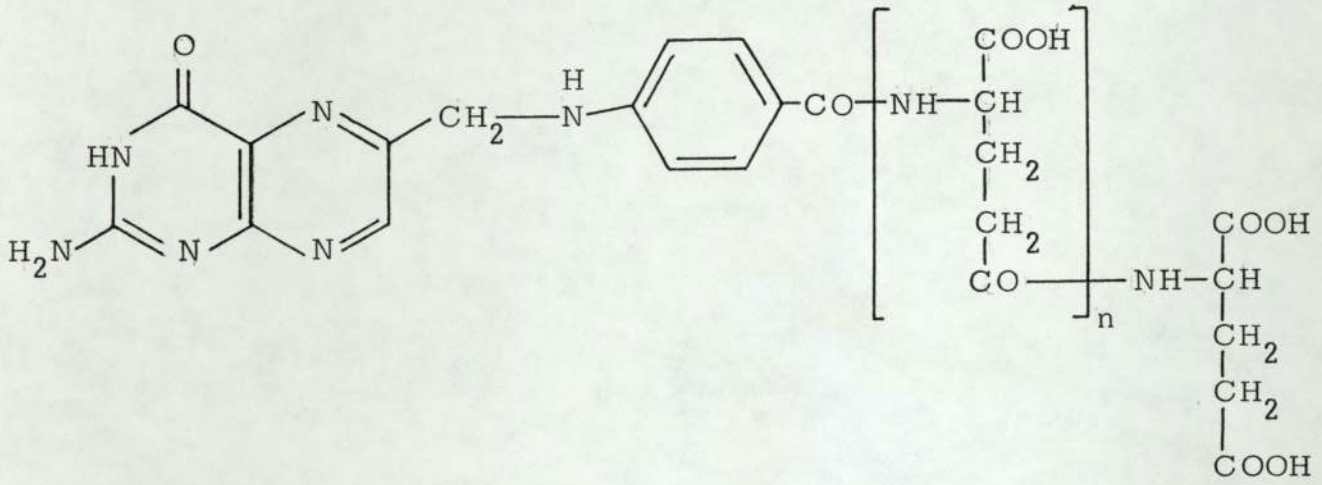
Figure 1-6 The role of glycine levels in regulating the production of 5,10CH_2-THF 10CHOTHF and THF

Herbert (1962) have exploited this pathway to diagnose folate deficiency by measuring the formiminoglutamate in the urine following a dose of histidine. 5CHNH-THF is unstable and hydrolysed to 5,10CH=THF by formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4).

$$5\,CHNH\text{-}THF \longrightarrow 5,10\,CH{=}THF + NH_3$$

MacKenzie and co-workers have isolated a multifunctional enzyme from pig liver that catalyzes the last two reactions(Beaudet and MacKenzie 1976, Drury and MacKenzie, 1977 and MacKenzie and Baugh 1980).

The bulk of tissue folates are in the form of polyglutamate derivatives (X) that have extraglutamate residues linked to the gamma carboxyl group of the glutamic acid residue (Shin et al., 1974 and 1976, Connor and Blair, 1980). Human cells rapidly take up folate monoglutamates whereas polyglutamates are not incorporated. Synthesis of polyglutamates appears to be intracellular mediated by one or more folate polyglutamate synthetases (Ligases). These enzymes are unevenly distributed among the intracellular compartments and the highest activity occurs in cytosol (Gawthorne,1980). Human fibroblasts cultured in vitro formed folate-polyglutamate from ^{3}H pteroylglutamic acid in the culture medium and intracellular polyglutamate could be detected within 4 h of incubation reaching a maximum after 10 days (Hilton et al., 1979). Studies in vivo showed that folate polyglutamates could be detected in rat tissues within 2 h of the administration of a mixture of ^{3}H and ^{14}C



X Folate polyglutamates

folic acid (Bates, et al., 1980). 5MeTHF and 10 formyl THF are the major folates in plasma. 10 Formyl THF is usually maintained at a constant level under normal circumstances whereas 5MeTHF acts as circulating storage form (Ratanasthein et al., 1974). Therefore 5MeTHF is probably the major form of folate entering the cells. Spronk (1973) demonstrated that THF and not 5MeTHF was the required substrate for polyglutamate synthesis in rat liver and a similar report was made in relation to Neurospora Crassa (Sakami et al., 1973) and although other folates may serve as substrates in vitro (Covey, 1980, Gawthorne, 1980, McGurine et al., 1980, Shane, 1980), THF appears to be the preferred substrate for polyglutamate synthesis. It was thought that folatepolyglutamates act as a storage form and that the monoglutamates which are rapidly interconverted in vivo function as coenzymes (Blair, 1976), but recently evidence has been accumulating that folatepolyglutamates are active folate coenzymes (Hoffbrand, 1976, Rowe 1978, Chanarin, 1979a) Covey 1980). Kinetic studies in vitro indicate that most, if not all, of the folate pathway enzymes can utilize the polyglutamate derivatives and folatepolyglutamates serve as better substrates in vitro for folate dependent enzymes than the corresponding monoglutamate derivatives (Whitfield and Weisbach, 1968; Coward, et al., 1974; Kisliuk, et al., 1974, Coward et al., 1975. Baggott and Krumdieck, 1979). In direct evidence also supports the view that folatepolyglutamates are the predominant coenzymes in the mammal. The high concentration and the slow turnover of folate polyglutamates (Whitehead, 1973, Barford et al., 1977) indicates that they could be the

active coenzymes for folate dependent enzymes and are unlikely to have a storage role. Other evidence for the importance of polyglutamate synthesis in folate metabolism is provided by a mutant strain of Chinese hamster ovary cells that lack the ligase enzyme. They fail to grow in the absence of glycine, adenosine and thymidine and the need of these compounds is presumably due to lack of the folate coenzymes required for their synthesis (McBurny and Whitmore, 1974 and Taylor and Hanna, 1977). However, this may simply be due to failure to keep folate in the cell and not due to an absolute requirement for folate polyglutamate *per se.*

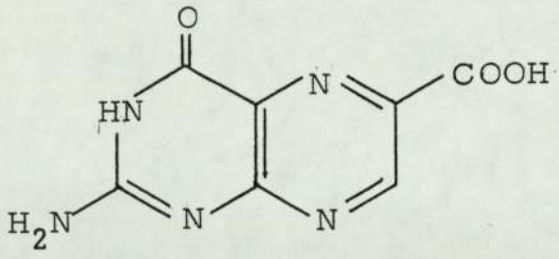
In addition to being active coenzymes the folate polyglutamates may have a role in the control of folate dependent enzyme activity with the cell. 10Formylpteroylpenta glutamate is a potent inhibitor of mouse leukaemia dihydrofolate reductase (Friedkin, *et al.*, 1975) and folatepolyglutamate, folatedihydropolyglutamate and 5 formyltetrahydrofolatetetraglutamate inhibit thymidylate synthetase (Kisliuk *et al.*, 1974 and Friedkin *et al.*, 1975).

The identification of folatepolyglutamates is hampered by their complexity. In earlier studies tissues were allowed to autolyse, endogenous conjugase liberating folates as monoglutamates and chromatography and microbiological assay were used to estimate the various folates produced. There is the possibility of folate interconversions during analysis using this method (Connor and Blair 1980). Separation of folate polyglutamates in relation to chain length by column chromatography (Shin, *et al.*, 1972)

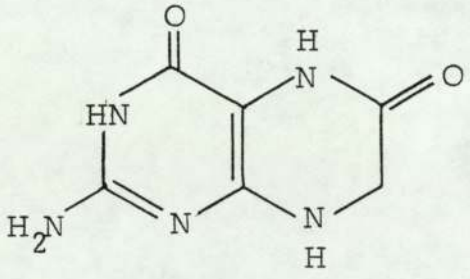
and high pressure liquid chromatography (Stout, et al., 1976), has been claimed. However, the elution position of polyglutamates is altered by the nature of the substituent and the oxidation state of the pterin ring. Therefore, splitting the molecule of folate polyglutamate at the C9-N10 position by oxidative scission techniques (Brown et al., 1974; Reed et al., 1977) has been used. These are claimed to produce a series of p-aminobenzoyl-polyglutamates which are separated chromatographically.

The number of glutamic acid residues present varies from 2-11 depending on the source of the folate-polyglutamate (Chanarin, 1979a) and also on folate concentrations in tissues. Cassady et al., (1980) have reported that the proportion of total folates in the form of longer chain polyglutamates, is greater in the livers of folate-deficient rats than in the livers of folate supplemented rats, whereas others have reported conflicting data (Barbiroli et al., 1980).

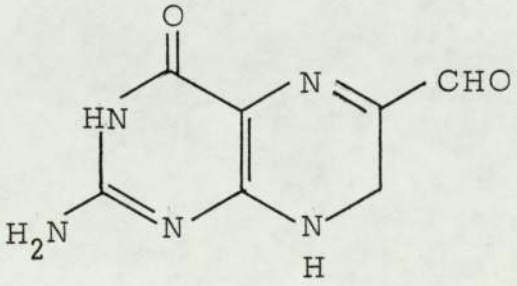
There are discrepancies between the reported nature of tissue folates depending on the methods of extraction and analysis. Norohana and Silverman (1962b), and Bird et al., (1965) reported that 5MeTHF polyglutamates were the major liver folates, while Shin et al., (1972) extracted a mixture of formyl and methylpolyglutamates. Recently Connor and Blair (1980) isolated 10 formylfolate tetraglutamate from rat liver and suggested that this is an oxidation product of 10 formyltetrahydro-tetraglutamate.



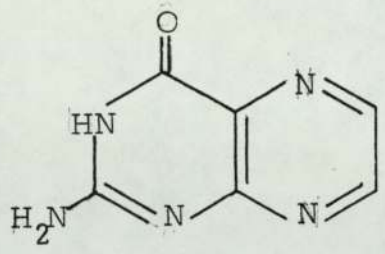
XI Pterin-6-Carboxylic acid



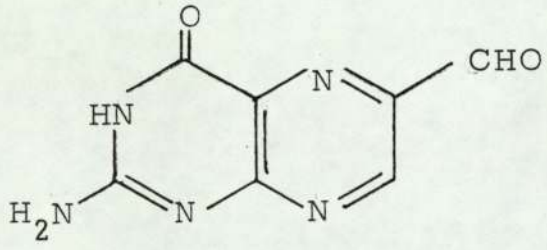
XII Dihydroxanthopterin



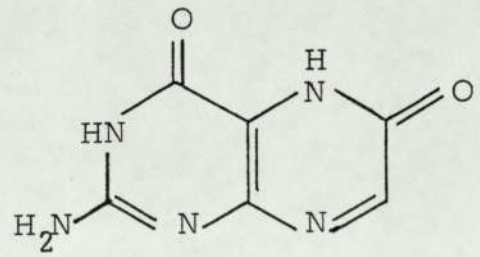
XIII Dihydropterin-6-aldehyde



XIV Pterin

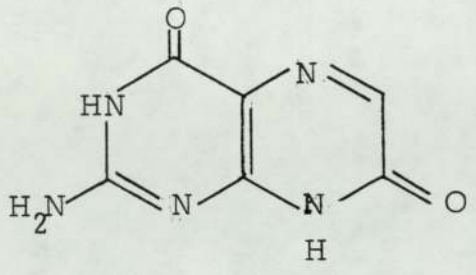


XV Pterin-6-aldehyde

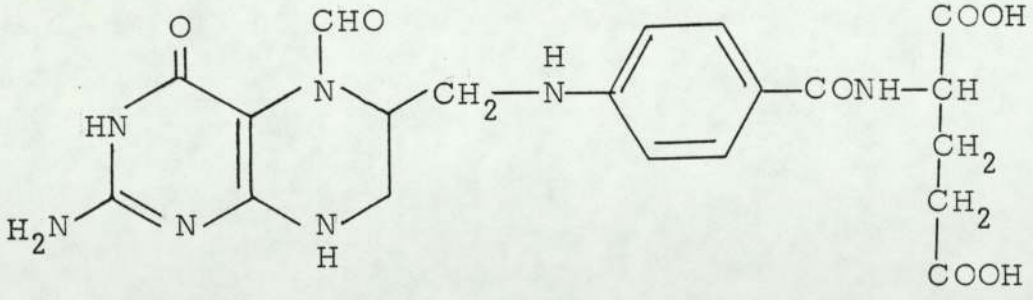


XVI Xanthopterin

labile folate, THF which cleaved spontaneously on exposure to air. However, the occurrence of folate catabolism *in vivo* was not considered since microbiological methods were used extensively to determine folate metabolites in the urine and tissues. Nevertheless, there have been several reports of pteridines appearing in the urine possibly as a result of folate degradation *in vivo* Blair (1958) and Fukushima and Shiota (1972) detected isoxanthopterin (XVII) in human urine. Labelled isoxanthopterin and pterin have both been isolated from the urine of a human subject dosed with $\left[2\text{-}^{14}C\right]$-folic acid (Krumdieck *et al.*, 1978). Pheasant and Blair (1979) reported an unidentified pteridine as a metabolite of ^{3}H and ^{14}C folic acid. Johns *et al.*, (1961) found ^{3}H -p-amino benzoyl-L-glutamate in human urine after i.p. administration of ^{3}H-folic acid. However, care needs to be taken in interpreting their studies since antioxidants were not used and considerable degradation could have occurred during collection. Dinning *et al.*, (1957) reported the presence of free and acetylated diazotizable amines in the urine of rats given large doses of folate and 5 formyl THF (XVIII). More recently ^{3}H -p-acetamidobenzoyl-L-glutamate has been identified in rat urine (Murphy *et al.*, 1976 and Connor *et al.*, 1979) in guinea pig (Choolun *et al.*, 1980) and in human urine (Saleh *et al.*, 1980) after oral doses of ^{3}H-folic acid, 10 formyl-folic acid, (Connor, 1979) or 5 MeTHF (Kennelly *et al.*, 1979b Kennelly, 1980). ^{3}H -p-Acetamidobenzoate has been also identified as a folate catabolite present in rat urine (Connor *et al.*, 1979) in guinea pig urine (Choolun *et al.*, 1980) and in human urine (Saleh *et al.*, 1980). These labelled acetylated products confirm that folate catabolism occurs *in vivo*



XVII Isoxanthopterin



XVIII 5 Formyltetrahydrofolic acid

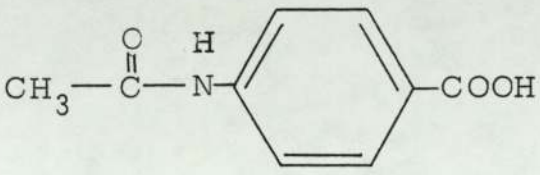
since acetylation must occur prior to excretion.

Blair (1958) and more recently Murphy et al., (1978) suggested that a possible metabolic route of folate catabolism would be via cleavage of C9-N10 bond in the tissues to give p-aminobenzoyl-L-glutamate and a pterin which would undergo further metabolism.

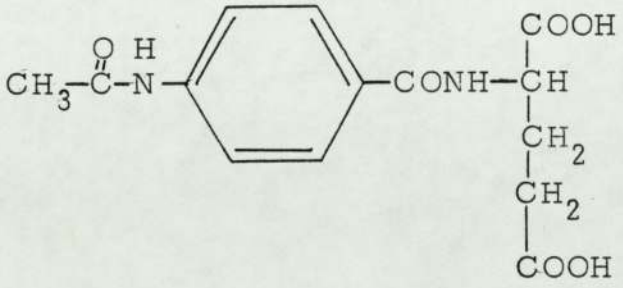
The appearance of p-acetamidobenoate (XIX) and p-acetamidobenzoyl-L-glutamate (XX) after the administration of folic acid, 10 formylfolic acid (Connor, 1979) or 5MeTHF (Kennelly, 1980) was a time dependent process p-Acetamidobenzoate was the major catabolite in the early urine samples whilst the relative amount of p-acetamidobenzoyl-L-glutamate increased with time until it dominated the later urine samples (Connor, 1979). A hypothesis has been put forward to explain the appearance of the two tritiated folate catabolites (Pheasant et al., 1981) stating that catabolite formation is regionalized within the body into distinct areas.

1) The generation of p-aminobenzoyl-L-glutamate within the tissues by either non-specific chemical oxidation or possibly enzymic degradation of retained labile folate polyglutamates is followed by acetylation and excretion of p-acetamidobenzoyl-L-glutamate in later urine samples.

2) Folate monoglutamates are secreted in bile, broken down chemically, enzymically or by the gut microflora to p-aminobenzoyl-L-glutamate in the gut and metabolised to p-acetamidobenzoate during



XIX p-Acetamidobenzoic acid



XX p-Acetamidobenzoyl-L-glutamic acid

reabsorption. Since the folate excreted in the bile is derived largely from the free (i.e. unconjugated) tissue and plasma folate (Hillman et al., 1977) the production of p-acetamidobenzoate from radiolabelled folate by this route would be reasonably fast and would fall off as the level of labelled free folate is reduced by excretion, catabolism and tissue uptake.

Thus oxidative degradation of folates is important in storage of food and in the loss of folate from the body.

There are many causes of folate deficiency (Blakley, 1969, Herbert, 1975 and Hoffbrand 1977). Nutritional folate deficiency is the commonest example of vitamin deficiency in man (Blakley 1969). However, the inadequate intake of folate may occur as a result of malabsorption of the vitamin (Rodriguez, 1978), e.g. coeliac disease or tropical spruce (Rosenberg et al., 1974) or increased demand for folate, e.g. in pregnancy (Chanarin, et al., 1968 and Cooper et al., 1970) and in malignant disease (Magnus 1967, Blakley, 1969, Poirier 1973). Metabolic defects of folate metabolism occur during vitamin B_{12} deficiency as in pernicious anaemia or during treatment with drugs inhibiting the folate dependent enzymes, e.g. MTX. Folate deficiency is frequently associated with anti-convulsant therapy (Chanarin, 1979b, Reynolds, 1976) and with alcoholism (Halsted, 1980) where there is much uncertainty as to whether alcohol and anti-convulsant drugs act directly or indirectly on folate coenzymes. However, it is likely that there are two general effects, firstly an effect on folate absorption and secondly, the effect on enzyme induction.

i) It is suggested that diphenylhydantoin inhibits folate absorption (Gerson et al., 1972) possibly by the inhibition of the conjugase enzymes that release folate from its conjugated form (Rosenberg et al., 1968, Hoffbrand and Necheles 1968). However, later studies failed to confirm this observation (Houlihan et al., 1972 and Fehling et al., 1973). Blair and Matty (1974) have emphasized the importance of the pH at the surface of the mucosal membrane for the absorption of folate. Folate absorption is inhibited when the pH of the acid microclimate (pH 5.5) is raised and there is evidence that diphenylhydantoin does cause this to occur (Benn et al., 1971). The effect of ethanol on intestinal absorption of folate is uncertain (see Rosenberg et al., 1979). However, Blair et al., (1979) showed that acute exposure of the rat intestine to alcohol caused a slight depression in folic acid transport and a considerable reduction in tissue fluid uptake (Swanston, 1978) and this probably due to a decrease in the acidity of microclimate in the alcohol treated tissues (Lucas et al., 1978). Nevertheless, it appears that ethanol induces folate malabsorption only when superimposed on the intestinal abnormalities associated with alcoholism and nutritional deprivation. Protein deprivation and folate deficiency in alcoholics can produce morphological and functional abnormalities of the intestine that probably contribute to the folate malabsorption that does occur in alcoholics.

ii) Maxwell et al., (1972) have suggested that anticonvulsants increase the activities of enzymes that catabolize folate. Kelly et al., (1979) have studied the effect of phenytoin and phenobarbitone on the catabolism

of ^{3}H-folic acid in mice and they reported that phenytoin but not phenobarbitone increased folate catabolism. Work on humans failed to find an effect of diphenylhydantoin on folate catabolism (Krumdieck et al., 1978) and the last authors reported that diphenylhydantoin increased folate urinary excretion. It is interesting to note that the anticonvulsant drug most frequently associated with megaloblastic aneamia is diphenyhydantoin either alone or in combination with phenobarbitone or primidone and deficiency is rare with phenobarbitone alone even in pregnant women (Gatenby, 1960). It is clear that alcohol decreases the serum folate levels (Eichner and Hillman, 1973). The exact mechanism of the effect of alcohol on folate metabolism is still unclear. It has been reported that ethanol impairs the enterohepatic cycle of folate (Steinberg et al., 1980) and hepatic secretion of 5MeTHF. Eichner et al., (1979) suggested that ethanol raised free fatty acids which could displace 5MeTHF bound to albumin. Steinberg et al., 1980, have reported that ethanol increased hepatic folate-polyglutamate and they suggested that "there is a possibility that the polyglutamate formed is altered and/or the cell is not able to use it"

As is mentioned above folates play an important role in cell division through their action in DNA synthesis. Folate requirement increases in malignant disease because cell division is accelerated and extra folate requiring tissue (the tumour mass) is present (Blakley, 1969, Poirier 1973). These facts have led to a number of studies being carried out on the role of folate coenzymes in tumour cells in order to observe any

metabolic variation from normal cells which may have diagnostic or therapeutic applications.

It has been suggested that folate metabolism is altered in malignancy (Hoffbrand et al., 1967) and that the pattern of folate coenzymes in malignant or normal rapidly dividing tissues is different from that resting tissues (Sotabayashi et al., 1966, Barbiroli et al., 1975).

A number of observations have also been made on folate dependent enzymes. Johnson et al., (1978) reported increased synthesis of dihydrofolate reductase in cells stimulated to re-enter the cell cycle and folate requiring enzymes involved with nucleic acid synthesis including dihydrofolate reductase show maximal activity in dividing cells (Rode et al., 1979, Rowe et al., 1979). Jackson and Neihammer (1979) have reported a 2,000 to 12,000 increase of thymidylate synthetase in various hepatoma cell lines compared to normal liver. These enzymatic changes would be expected to result in a displacement in the equilibrium between the various coenzymes.

Barford and Blair (1978) and Pheasant and Blair (1979) have observed changes in the ratios of urinary folates of rats bearing implanted tumours following oral doses of folic acid, and a reduced ratio of folic acid to 5MeTHF in the urine of patients with malignant disease has been reported (Pheasant et al., 1979, Saleh et al., 1980). Also Halpern and co-workers reported novel pterin cleavage products from tumour cells in culture and in the urine of cancer patients given large doses of folic acid (Halpern et al., 1977, Stea et al., 1978). However, this does not now appear to be a

specific test for cancer (Clynes and O'Neill 1980).

Rapidly dividing cells require an abundant supply of deoxythymidylate for the synthesis of DNA. MTX is a phase specific cancer chemotherapeutic agent stopping cell division in the S phase by DHFR inhibition. The faster the growth rate of a cell population, the greater is the susceptibility of that population to the cytocidal effects of antimetabolites. Krymuik et al., (1969) reported that the kill rate of MTX obtained with cell cultures in log growth phase is 6.7 times that obtained with resting cell cultures. Tumours are observed to have a variable fraction of cells in G_o (resting) with respect to mitotic cycle (Valeriote and Van Putten 1975) which are thus not subject to S phase inhibition by MTX. When this drug is used at high doses, all mitotically active cells will be destroyed. It is mandatory that an effective procedure is adopted to prevent toxic damage to normal proliferating tissues. This could be achieved with the provision of exogenous folate antidote, usually 5CHOTHF (Goldin et al., 1966).

MTX was found to be inactive against certain malignant cells such as sarcoma-180, murine leukaemic cells, Ehrlich ascites carcinoma, (Blakley 1969). This has been related to high levels of dihydrofolate reductase (Hakala , et al., 1961, Friedkin, et al., 1962). Recent studies have shown that the mechanism for this high production of dihydrofolate reductase appears due to the presence of multiple copies of the gene coding for this enyme (Kellems et al., 1976, Alt, et al., 1978).

Jaffe et al., (1974) have found that high-dose MTX therapy induced clinical responses in tumours previously considered to be resistant to MTX. Hence, a number of investigators have studied the metabolism and the mechanisms of toxicity of MTX. Goldman (1977) has stressed the importance of intracellular levels of MTX in excess of those required to bind to dihydrofolate reductase to achieve the cytotoxicity of MTX in vivo. This requirement for free intracellular MTX is needed to completely halt reduction of dihydrofolate reductase and probably to inhibit other reactions such as the incorporation of formate into DNA and THF-dependent thymidylate synthesis. Recently it was demonstrated that MTX undergoes metabolism in cells being converted to poly- γ -glutamate derivatives (Baugh et al., 1973, Jacobs, et al., 1977; Rosenblatt et al., 1978, Galivan 1979, Whitehead, et al., 1979) and studies have shown that MTX polyglutamates are as good or better inhibitors of DHFR (Jacobs et al., 1975) and thymidylate synthetase (Kisliuk et al., 1979). The synthesis of MTX polyglutamate is favoured by increasing MTX concentrations and longer incubation times and this may explain the need for high intracellular drugs.

The role of reduced folate to rescue the tissue was thought to act only by bypassing the block in folate reduction caused by MTX. However, it has been claimed that the effectiveness of reduced folate 'rescue' is also due to a stimulation of MTX efflux from cells (Sirotank et al., 1976). Therefore, as free intracellular MTX levels are reduced, polyglutamate synthesis should diminish and stop. Alternatively reduced folate may

promote MTX efflux by competing with MTX for transport into cells or/and by competing for conversion of MTX to the polyglutamate form (Whitehead and Rosenblatt 1979, Rosenblatt et al., 1981).

Harrap et al., (1977) have demonstrated that mice can be protected against the toxicity arising from lethal doses of MTX with purine and pyrimidine combinations and they claimed this treatment to be more effective than 5-formylTHF treatment.

Research Objectives and Methods

Due to the clinical importance of folates, there have been extensive investigations of these compounds. The minute amount of folates usually present in nature and their extreme susceptibility to destruction or denaturation by several factors have made their measurement and identification difficult. However, there are two major methods commonly used in studies of folate metabolism, microbiological assay and radiotracers. The three organisms frequently used for assay purposes are Lactobacillus casei, Streptococcus faecalis and Pediococcus cervisiae. These organisms respond differently to the various metabolic forms of folate and they are assumed to have no response to polyglutamates higher than the triglutamate. Their activity for the various metabolic forms of folate are summarised by Blakley (1969). This method was criticised by many investigators (Rodriguez 1978, Baril and Carmel 1978). Pollock and Kaufman (1978) found that different folates elicit varying growth responses from test organisms assumed to be equally sensitive to them. Many conjugated forms of folate are not detected unless first converted enzymatically to active forms and since the interconversion of folate monoglutamates is rapid (Nixon et al., 1973), tissue autolysis or the use of impure conjugase sources could lead to marked errors in interpretation of microbiological assay data. Also microbiological assay of folate in body fluids, after the administration of oral doses of folate cannot differentiate between folate derived from the administered dose and that derived by

displacement from the body pool (Johns and Plenderleith, 1963). However, it is still the most sensitive and often the only method available to determinate folates in natural materials.

Since radioactive folates became available, they have been widely used in folate metabolic studies. Both $[3',5',7,9\text{-}^{3}H]$ and $[2\text{-}^{14}C]$ labelled folic acid are commercially available, but their use has been criticised on the possibility of ^{3}H-exchange reactions (Blair, 1976) and the difficulties in distinguishing intact $[2\text{-}^{14}C]$ folates from their pterin scission products (Murphy et al., 1976). The use of mixed labels has been employed to overcome most of the problems associated with single labels. The combination of radio-assay and chromatographic techniques used for the separation of a mixture of folates has become the most widespread technique used at present. Handling of large volumes of fluid to be analysed makes column chromatography the preferred method in the identification of metabolites. DEAE-cellulose ion-exchange and Sephadex gel filtration are the techniques in most extensive use. High performance liquid chromatography (HPLC) has recently been applied to folate analysis however, the drawbacks of the incomplete separation and the relatively small sample sizes used has led to problems in detection and identification.

Folates are essential components in normal metabolism and are required for cell growth and propagation. The present study is concerned with the fate in vivo of administered folate in man and the rat, and the effect of the presence of tumours on this metabolism, hoping to observe

changes in folate metabolism which might be exploited diagnostically or therapeutically.

It has been suggested that folate catabolism is a major route for depleting tissues of folates (Murphy, et al., 1978, Connor, 1979), however, no detailed studies are available about the rate of folate degradation in vivo in health and disease or the mechanism by which folate catabolism occurs. Therefore, some experiments were undertaken to study folate catabolism in vivo and the effect of the presence of a tumour or the administration of compounds such as MTX and phenobarbitone on the catabolic rate of folate in an attempt to elucidate its mechanism.

10CHOFA is an important dietary folate (Santini et al., 1964) but little is known about its physiological and nutritional aspects, therefore the metabolism of 10CHOFA in man and its effect on folate metabolism are examined.

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

The following materials were obtained commercially as described: folic acid, xanthopterin, pterin and isoxanthopterin from Koch-Light Laboratories Limited, (Colnbrook, Bucks, U.K); p-aminobenzoyl-L-glutamate and dithiothreitol from the Sigma Chemical Co. Limited, (London, U.K.); p-acetamidobenzoic acid and p-aminobenzoic acid from the Aldrich Chemical Co. Limited, (Wembley, Middlesex, U.K.); 5MeTHF from Eprova Research Laboratories (Basle, Switzerland). $[2\text{-}^{14}C]$ -folic acid (specific activity 58 m Ci mM^{-1}) and $[3'\text{-}5',7,9\text{-}^{3}H]$ -folic acid (specific activity 500 m Ci mM^{-1}) were obtained from the Radiochemical Centre (Amersham, Bucks).

Other standard pteridines were gifts and donations from the following individuals and organisations; methotrexate and calcium leucovorin (5-formyl-THF) from Lederle Laboratories Division (Cyanamid of Great Britain Limited, London); 6-methylpterin, neopterin from Roche Products Limited (Welwyn Garden City, U.K.); pterin-6-aldehyde (Waller et al., 1950) and pterin 6-carboxylic acid (Zakrewski et al., 1970) were prepared by Dr. M. Connor, $5,10CH_2$-THF (Osborn et al., 1960) 2-acetamido-4-hydroxy-6-formyl-pteridine, 2-acetamido 4,6-dihydroxypteridine and 2-acetamido-4-hydroxy-pteridine were prepared by Dr. R. N. Mazher in this laboratory. Other chemicals and general laboratory reagents used were of Analar grade.

The following folate and pteridine derivatives were chemically

synthesised by methods described below.

10-formylfolic acid (10CHO-FA)

This was prepared by direct formylation of folic acid (Blakley, 1959). Folic acid (4 g) was dissolved in 160 ml of formic acid (90%) and the solution stored in the dark at room temperature for two days. The solution was then poured into an excess of diethylether (500 ml). The creamy precipitate was harvested by filtration, washed several times with ether and dried by suction. The product gave a single band on T.L.C. and gel filtration. U.V. spectroscopy gave λ_{max} = 254 nm, 327 nm (pH 1), λ_{max} = 259 nm, 370 nm (pH 13). λ_{max} = 245 nm, 269 nm, 350 nm (pH 7.0).

$[2\text{-}^{14}C]$ & $[3',5',7,9\text{-}^{3}H]$ -10 formyl-folic acid

This was prepared by a microscale version of previous method. A solution of $[2\text{-}^{14}C]$ folic acid (50μCi, 52.4 m Ci mM^{-1} Batch 27) and $[3',5',7,9\text{-}^{3}H]$-folic acid (250μ Ci, 500 m Ci mM^{-1} Batch 46) was made up in 0.8 ml of distilled formic acid (98%). After storing the solution in the dark at room temperature for 48 hours, the excess formic acid was removed by freeze drying. Chromatography of the product on Sephadex G15 gave a single radioactive peak at fraction 21 (folic acid elutes at fraction 36) and on DE-52 gave a single radioactive peak at 0.53 M NaCl (folic acid elutes at 0.96 M NaCl). It co-eluted from both columns with cold standard of 10 formyl-folic acid. The prepared material was stored in the deep-freeze (-20^{o}C) until used.

p-Acetamidobenzoyl-L-glutamate

This was prepared by the method of Baker et al., (1964). p-Aminobenzoyl-L-glutamate (1.0 g) was dissolved in 10 ml aqueous acetic acid (50% v/v) and acetic anhydride (1.5 ml) added. After standing in the dark at room temperature overnight, the reaction solution was diluted to 20 ml with distilled water, cooled (4°C) for several hours, and the precipitate recovered by centrifugation. After washing with ice-cold water, the product was recrystallized twice from boiling water yielding small white needles, (m.p. 206-208° C)

CHROMATOGRAPHY

i) Ion Exchange Chromatography

Diethylaminoethyl (DEAE)-cellulose (DE52, Whatman Limited, Maidstone, Kent, U.K.) (80-160 g) was washed with distilled water and equilibriated in 0.05 M-phosphate pH 7.0 containing dithiothreitol (5 mg % w/v) until the washings were of constant ionic strength and pH 7.0. After decanting off the fines and degassing, the prepared DE52 was packed into glass columns (normally 2 cm X 50 cm or 2 cm x 80 cm; occasionally small columns 1.5 cm x 30 cm or 1 cm x 30 m) plugged with glass wool. Samples (5-200 ml) and appropriate standards were diluted to the conductivity of the starting buffer with distilled water before loading onto columns. Standard linear gradients (0-1.2 M-NaCl in starting buffer) were eluted automatically using an LKB Ultra Grad attached to a peristaltic pump (LKB Instruments, Croydon, Surrey, U.K.). The eluant was passed

through two detectors in series, a purpose built scintillation flow cell for detecting radioactivity (Nuclear Enterprises Limited, Edinburgh, U.K.) and a U.V. monitor (LKB Uvicard II, LKB Instruments). Fractions (5 ml or 10 ml) were collected using an LKB Ultrarac fraction collector (LKB Instruments). When large volumes of samples were used, fractions (10 ml) were also collected during sample loading. Radioactivity in column effluents was determined and the salt gradient, usually eluted over eight hours was measured by determining the conductivity of every tenth fraction with a Mullard conductivity cell.

ii) Gel Filtration

Sephadex G15 was obtained from Pharmacia (Uppsala, Sweden). A slurry of the gel was prepared in phosphate buffer, pH 7.0 containing dithiothreitol (DTT) (5 mg % w/v) and left to swell for four hours. After degassing, the slurry was packed into 2 cm X 60 cm perspex column (Wright Scientific Limited, Surrey, U.K.) and allowed to pack under pressure. After loading samples and standards (5-25 ml) elution was achieved using 0.05 M-phosphate buffer pH 7.0. The eluant was monitored, collected and the radioactivity determined as above. A summary of the elution pattern of relevant folates, pteridines and p-aminobenzoic acid derivatives on DE52 and Sephadex G15 is given in Table (2.1).

iii) Paper Chromatography

Paper chromatography was performed by the descending method in a glass tank equilibriated with the relevant solvent using

Whatman 3 MM chromatography paper and chromatograms were developed overnight in the dark. Samples (^{3}H scission products) and standards (p-aminobenzoyl-L-glutamate, p-aminobenzoic acid and their acetyl derivatives) were applied as spots using glass micropipettes. Standards were observed as dark absorbing or fluorecing spots by viewing under U.V. light at 254 nm or 355 nm. The chromatography solvents used were

A - Propanol/aq. NH_3(Sp. gr. 0.88)/water (200:1:99 by vol.)

B - 1% (v/v) Acetic acid in water

C - Butanol/ethanol/aq NH_3(Sp. gr. 0.88)/water (10:10:1:4 by vol.)

D - n-Butanol/pyridine/water/glacial acetic acid (6:4:3:1 by vol.)

Table (2-2) shows the chromatographic behaviour of standard compounds on Whatman 3 MM paper.

ANIMALS

Experiments were conducted on male WAB/Not rats. Tumour bearing and control rats were obtained from Dr. M. Pimm (The University of Nottingham).

Both prior to and during experimentation, animals were kept at 21 ^{o}C in a sealed room having a fixed 12 hours dark and 12 hours light cycle, and allowed free access to food (Breeding Diet, Heygates Limited).

Metabolism studies were carried out by housing single rats in cages designed for the separate collection of faeces and urine (Jencons Metabowls; Jencons (Scientific) Limited; Hemel Hempstead, Herts, U.K.). Urine

samples were collected into flasks containing 10 ml of 0.05 M-sodium phosphate buffer pH 7.0 containing sodium ascorbate (2% w/v) and dithiothreitol (5 mg % w/v). To prevent light degradation of folates the flasks were surrounded by aluminium foil.

Rats were dosed orally by stomach intubation using specially prepared steel dosing needles. Administered compounds were dissolved in 0.05 M-sodium phosphate buffer at pH 7.0 containing sodium ascorbate (2% w/v). The volumes administered did not exceed 0.35 ml.

Rats were killed by cervical dislocation followed by surgical opening of the thorax and tissues were removed either for freeze-drying for direct determination of radioactivity or for extraction for qualitative examination of retained radioactivity.

PATIENTS

Experiments were carried out on human volunteers, hospital-inpatients at the General Hospital, Birmingham, U.K.

Two groups of patients were used, those suffering from malignant disease and control groups suffering from other disorders. Further details of these patients are given in the relevant chapter.

Patients were given oral doses of folates and urine was collected into polythene bottles containing 10 g sodium ascorbate. Urine volumes were measured and samples kept frozen at -20°C until analysed. In

some cases, faeces were collected in a plastic bag. The bag was closed with a rubber band and put into an air-tight screw-top container. The faeces were freeze-dried for direct determination of radioactivity.

Patients were allowed to eat and drink normally during the experiments.

MEASUREMENT OF RADIOACTIVITY

Prepared samples were counted in a Nuclear Enterprises Liquid Scintillation Counter Type 8310 (Nuclear Enterprises Limited, Edinburgh). Aqueous samples, e.g. column eluants and urine samples, were made up to one ml with water and 10 ml, a scintillation cocktail composed of toluene (1 litre) and Fisons emulsifier mix No. 1 (Fisons, Loughborough, Leics, U.K.) (500 ml) in which was dissolved 2,5-diphenyloxazole (PPO) (5 g) and 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) (0.1 g) was added. Radioactivity on paper chromatograms was counted directly by cutting up the strips of chromatography paper into 1 cm wide pieces and placing them into vials to which was added 10 ml of scintillation cocktail from which the emulsifier had been omitted.

Samples were counted for 10 min. or 10,000 counts. Appropriate corrections were made for background, quenching and overlap of ^{14}C into the ^{3}H channel using the external standard ratio.

Freeze-dried tissue and faecal samples were determined by combustion using a Beckman Biological Materials Oxidizer. Tritium was trapped

as tritiated water in a dry ice/methanol cold trap and counted in 10 ml of Fisons tritium absorber "H". Carbon-14-was trapped as carbon dioxide in 15 ml of Fisons absorber "p".

In the last few months, prepared samples were counted in a Beckman LS 7500 Liquid Scintillation Counter (Beckman Instruments Inc. Scientific Instruments Division, Irvine, California 92713). This is equipped with automatic quench compensation.

STATISTICAL ANALYSIS

Throughout the thesis all tests of statistical significance were performed using Student t Test and Wilcoxon's Sum of Ranks Test unless otherwise stated in the text.

Table 2-1. The elution properties of folates, p-aminobenzoic acid derivatives and the unknown metabolites on Sephadex -G15 gel filtration and DEAE-cellulose ion exchange chromatography.

Compound	Elution position	
	Sephadex-G 15 (fraction No.)	DEAE-cellulose molarity NaCl
Folic acid	37	0.96
pteroylheptaglutamate	11	0.60
10CHOFA	21	0.53
10CHOTHF	18	0.45
5CHOTHF	28	0.59
5MeTHF	37	0.67
$5,10CH_2$-THF	25	0.64
Methotrexate	60	-
Triazene	16	0.40
Folate (X)	36	0.70
pterin	35	0.30
pterin-6-CHO	43	0.70
pterin-6-COOH	30	0.60
* pterin-6-CH_3	44	-
* pterin-6-sulphonic acid	34	-
* D-neopterin	28	-
Xanthopterin	57	0.57
Isoxanthopterin	45	0.49

Continued..

Table 2-1 (continued...

Compound	Elution position	
	Sephadex-G 15 (fraction No.)	DEAE-cellulose molarity NaCl
dihydroxanthopterin	76	-
* 6-Hydroxymethylpterin	32	-
* 6-methyltetrahydropterin	36	-
* 6-COOH-lumazine	27	-
* 6-OXO-lumazine	34	-
* Lumazine	30	-
2-acetamido-4-hydroxypteridine	27	-
2-acetamido 4,6-hydroxypteridine	28	-
2-acetamido 4-hydroxy 6-formylpteridine	30	-
metabolite A	41	0.40
metabolite B	54	0.32
metabolite C	42	0.60
p-aminobenzoic acid	35	0.40
p-acetamidobenzoic acid	36	0.43
p-aminobenzoyl-L-glutamate	18	0.40
p-acetamidobenzoyl-L-glutamate	19	0.43
* p-aminohippuric acid	33	-
* p-acetamidohippuric acid	27	0.35
$^{3}H_2O$	21	0.0
Urea	21	0.0

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

Table (2-2) The chromatographic behaviour of p-aminobenzoic acid derivatives on Whatman 3 MM paper.

Compound	R_f Values in Solvent			
	A	B	C	D
p-Aminobenzoic acid	0.39	0.65	0.21	0.81
p-Acetamidobenzoic acid	0.54	0.56	0.36	0.84
p-Aminobenzoyl-L-glutamate	0.21	0.85	0.04	0.43
p-Acetamidobenzoyl-L-glutamate	0.29	0.85	0.09	0.58

CHAPTER 3

THE METABOLISM OF [2-^{14}C] AND [3',5',7,9-^{3}H] FOLIC ACID IN THE RAT

The study of folates began in 1931 when Wills discovered a factor from yeast or liver extracts that cured anaemia in pregnant women. Later there were several reports of the treatment of anaemia in monkeys and chicks with similar extracts (O'Dell and Hogan 1943). In 1943 Stokstad demonstrated that there are growth substances in liver and yeast extracts which are essential for lactic bacterium and Lactobacillus Casei and Mitchell et al., (1944) extracted a material needed for the growth of Streptococcus Lactis R from spinach leaves and termed it "folic acid". These substances and materials were later shown to belong to the same family of compounds. Pfiffner et al., (1943) isolated the crystalline form of this material from liver. This was followed by the synthesis of a pteridine identical in structure and biological function (Angier et al., 1945) that was termed pteroylglutamic acid (folic acid). Since then there have been numerous reports dealing with the fate of folates in different species and with their chemistry, enzymatic activity, clinical role and metabolism. (Reviewed by Stokstad and Koch 1967, Blakley 1969, Blair 1976, Hoffbrand 1976, Row 1978, Rodriguez, 1978 and Chanarin 1979a). However, the fate of folate in vivo is still far from clear and we have much to learn of its metabolic role.

In this Chapter some experiments were carried out using the rat as an experimental animal model which demonstrate the differences in handling a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ -folic acid in normal rats and tumour-bearing rats and the effect of methotrexate on tissue folates, with particular reference to folate catabolism, leading to the conclusion

that the rate at which this catabolic process occurs could have a significant effect on the folate status of the animal.

In order to investigate whether the mechanism of folate degradation is chemical or enzymatic, the effect of the hepatic enzyme inducer, phenobarbitone, on folate catabolism was studied.

3.1. Folic acid metabolism in normal rat

Several investigators have reported low urinary folate activity using microbiological assay (Cooperman, et al., 1970). Although little folate is lost in urine, the minimum amount of folate required to prevent the development of folate deficiency has been estimated to be 50 µg (Zaluzky and Herbert, 1961) to 100 µg (Hansen and Weinfield, 1962) daily. Thenen et al., (1973) reported that only 4% of the folate lost from the liver appeared in the urine and they suggested that the folate lost is degraded eventually either in the liver or in other tissues after redistribution. As early as 1947 Jukes et al., reached a similar conclusion. However, there was no evidence to support this conclusion despite the fact that some pteridines had been found in human urine (Blair, 1958, Fukushima and Shiota 1972) because the origin of these pteridines was not clear. Nevertheless the finding of labelled acetylated derivatives of p-aminobenzoate and p-aminobenzoyl-L-glutamate in the urine of rats and humans after the administration of labelled folates (see Chapter I for references) confirms that folate catabolism occurs in vivo since acetylation must occur before excretion. Thus, it is now realized that the catabolism of

folates in vivo is a normal phenomenon and can explain the low recovery of folate reported earlier as these folate scission products do not support the growth of the micro-organisms used to measure the urinary folate. This Chapter describes the metabolism of $[2\text{-}^{14}C]$-folic acid and $[3',5',7,9\text{-}^{3}H]$-folic acid in the rat.

Materials and Methods

15 Male syngeneic WAB/NOT rats (150-240 g body wt.) were dosed orally with a mixture of $[2\text{-}^{14}C]$ folic acid and $[3',5',7,9\text{-}^{3}H]$-folic acid (100 μg/Kg body wt.). Urine samples were collected from 5 animals. each time period as described (Chapter 2) for periods of 0-8 h, 8-24 h and 24-48 h after the administration of labelled folic acid. At the end of each time period, collection flasks of urine and faeces were changed and five animals were killed by cervical dislocation. Liver, kidney and gut tissues were removed, washed in 0.05 M phosphate buffer pH 7.0 and weighed before determination of radioactivity. The major portion of each liver was quickly chopped and dropped into 4 vol. of boiling 0.05 M-phosphate buffer pH 7.0 containing sodium ascorbate (2% w/v) for 10 minutes. The extract was cooled, homogenised, centrifuged and the supernatant recovered. Urine samples and liver extracts for each time period were pooled and stored frozen until required when they were examined by DE-52 ion exchange chromatography, Sephadex gel filtration and paper chromatography.

Results

The distribution of radioactivity recovered in the tissues, faeces and urine is summarised in Table (3.1.1). Quantitative analysis ot the urine and faeces revealed an imbalance in the excretion of the two isotopes. More ^{3}H than ^{14}C was excreted in the urine whereas an excess of ^{14}C over ^{3}H was present in the faeces. The differences in the recovery of ^{3}H and ^{14}C are statistically significant when compared in a paired t test ($p < 0.001$ for urine and faeces). However, similar percentages of the dose of ^{3}H and ^{14}C were found in the kidneys; the slight difference observed in the liver is not statistically significant but more ^{3}H than ^{14}C was found in 24 h and 48 h gut samples ($p < 0.01$).

Urinary metabolites

DE 52 chromatography of urine samples is illustrated in figures (3.1.1, 3.1.2 and 3.1.3). The 0-8 h urine sample showed a complex mixture of metabolites. Four of the metabolites detected retained the ^{3}H and ^{14}C label, had almost identical ratios of ^{3}H to ^{14}C to each other and were identified by co-chromatography with authentic standards in both column systems as I folic acid, II 5MeTHF, III 5,10CH_2-THF and IV 10 formylfolate. The ratio of ^{3}H:^{14}C in these folate derivatives was higher than in the folic acid administered. The other radioactive peaks had different ^{3}H:^{14}C ratios and appeared to be scission products. The radioactivity eluting at 0.4-0.5 M NaCl (peak V) was resolved into three components on Sephadex-G15 (Figure 3.1.4). One metabolite of

the three had a much reduced $^{3}H:^{14}C$ ratio, the second metabolite was labelled only with ^{3}H and the third peak (tube 20) had a higher $^{3}H:^{14}C$ ratio and appeared to be a mixture of two components, (a) 10 formyl-folate present due to overlap of peak IV with peak V on the DE52 column and due to the oxidation of 10CHO-THF associated with peak V and (b) a metabolite labelled only with tritium. The tritiated metabolites were eluted from Sephadex-G15 at tubes (20-21) and (36-37) with p-amino-benzoyl-L-glutamate and its acetamido derivative and p-aminobenzoate and its acetamido derivative respectively. Paper chromatography of these metabolites in different solvents (Solvents A,C and D) showe d them to be p-acetamidobenzoyl-L-glutamate and p-acetamidobenzoate. The peak eluting at tube 41 from Sephadex G15 remains unidentified (metabolite A). A great effort has been made in this laboratory to identify this metabolite (Connor, 1979, Pheasant et al., 1981). It has been run with different pteridines and also with three acetylated pterins (2-acetamido-4-hydroxy-6 formylpteridine, 2-acetamido 4,6-dihydroxy-pteridine and 2-acetamido-4-hydroxy-pteridine) on Sephadex-G15 and failed to chromatograph with any of them. However, it has been characterized (Connor, 1979, Pheasant et al., 1981) as a reduced pteridine which retained ^{3}H at C_7 position. It is not microbiologically active for L. Casei, S. Faecalis or C. Fasciculata, it is unaffected by treatment with phosphatase or B-glucuronidase and it decomposes to yield several products including xanthopterin (Connor, 1979).

The metabolite eluting at 0.25-0.35 M NaCl on DE 52 (peak VI) and

at tube 55 from Sephadex G15 (metabolite B) had a much reduced $^{3}H:^{14}C$ ratio, did not chromatograph with any of the simple pteridines used and because of its low concentration, no attempt at further identification was made. The remaining peak VII was eluted at the void volume on DE 52. The tritium was removed by evaporation or freeze drying and so was identified as tritiated water. The ^{14}C species was eluted at about fraction 21 on Sephadex-G15 and on both column systems it chromatographed in the position of urea (Connor et al., 1977).

The 8-24 h urine samples showed a decrease in the amount of intact folates relative to the scission products and the appearance of another metabolite (metabolite X) eluting immediately after 5 MeTHF from DE 52 (peak IIa). This had the same $^{3}H:^{14}C$ ratio as the intact folates. It was seen as a slight shoulder on 5 MeTHF peak of DE 52 of 0-8 h urine samples.

The pattern of DE 52-chromatography of 24-48 h urine samples was simpler than the early urine samples (Figure 3.1.3). It showed the disappearance of metabolite (X) and 5,10CH_2-THF, folic acid was seen in a trace amount only and 5 MeTHF and 10 formylfolate were present in small amounts. The scission products dominated this sample. Sephadex-G15 of the radioactivity eluting at 0.35-0.45 M NaCl (Figure 3.1.6) showed that p-AcBG was the major tritiated product and p-Ac BA decreased sharply compared to its amount in the early samples and relative to p-Ac BG.

Table (3.1.2) summarises the distribution of radioactivity between the metabolites appearing in the urine as a percentage of the dose. Large amounts of folatemonoglutamates appeared in 0-8 h urine samples and fell rapidly in the 8-24 h and 24-48 h urine samples whilst scission products dominated the later urine samples. In particular p-Ac BG and metabolite A represented about one-third of the ^{3}H and ^{14}C respectively recovered in the 24-48 h urine samples.

Liver extracts

Sephadex-G15 gel filtration of liver extracts are illustrated in Figures (3.1.7, 3.1.8 and 3.1.9). At all time periods the major radioactive peak eluted near to the void volume in the position of high molecular weight folate (folate polyglutamates). Small amounts of low molecular weight derivatives appeared at fractions (32-37). DE 52 chromatography of these fractions showed them to be single-labelled compounds including pterin. No folate monoglutamates were seen at any time.

Summary

1. Following the administration of ^{3}H + ^{14}C labelled folic acid to normal rats, radioactivity was recovered in the urine, faeces, liver, gut and kidneys.

2. Metabolites identified in the urine were as follows:- folic acid, 5 MeTHF, 10 formylfolates, 5,10 CH_2-THF, folate (X), p-Ac BG, p Ac BA, $^{3}H_2O$, urea and metabolites A and B.

3. The scission products dominate the late urine samples in particular pAc BG and metabolite A.

4. Hepatic folates were shown to be folylpolyglutamates from 8 h after administration of the dose.

3.2. Folic acid metabolism in tumour-bearing rats

Folate metabolism *in vivo* is known to be affected by the presence of a tumour. Low serum folates (Magnus, 1967), abnormal clearance of injected folic acid from the plasma (Hogan *et al.*, 1964) and higher excretion of formiminoglutamate after a histidine load (Mohamed and Roberts, 1966, Rose, 1966) have all been reported in patients with malignant diseases and in tumour-bearing rats (Poirier, 1973). A number of studies suggest that the pattern of folate coenzymes in malignant or normal rapidly dividing tissues is different from the resting tissues (Sotobayashi *et al.*, 1966, Barbiroli *et al.*, 1975) and changes in the levels of folate dependent enzymes in tumour cell lines (Johnson *et al.*, 1978, Jackson and Neihammer 1979) and in the folate species appearing in the urine of rats bearing implanted tumours (Barford and Blair, 1978, Pheasant and Blair, 1979) have been observed. Little information is available on changes in folate catabolism. Preliminary studies in this laboratory suggest that the catabolism of folate may be depressed in the presence of a tumour (Barford and Blair, 1978, Saleh *et al.*, 1980). This section describes the effect of Mc 103B sarcoma on the metabolism of a mixture of $\left[2\text{-}^{14}C\right]$-folic acid and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid in

the rat and the catabolism of tissue folates.

Materials and Methods

15 Male syngeneic WAB/NOT rats bearing Mc/103B sarcoma (260-320 g wt.) were used. The sarcoma Mc/103B was induced in Nottingham in an adult male WAB/NOT rat by subcutaneous injection of 1 mg 3-methylcholanthrene (Sigma, London) dissolved in 1 ml triactanoin (Eastman, Kodak, Rochester, New York, N.Y., U.S.A). A tumour transplant line was established and maintained in syngeneic male rats by subcutaneous tracer implantation of fragments of tumour tissue (Pimm et al., 1980). For this experiment the sarcoma was implanted subcutaneously on the right flank before the rats were supplied and allowed to grow for four weeks before the start of the experiment. Then they were dosed orally with a mixture of $\left[2\text{-}^{14}C\right]$ folic acid and $\left[3',5',7,9\text{-}^{3}H\right]$ -folic acid (100 µg/Kg body wt.) and treated exactly as described in the previous section (3.1). Urine and faeces were collected, tissues removed including tumour and liver and tumour extracts were prepared as previously described (Chapter 2 and Section 3.1).

Results

The distribution of radioactivity in the urine, faeces and tissues is summarised in Table (3.2.1). The imbalance in the excretion of the two isotopes in urine and faeces was also seen in tumour bearing rats. The differences in the recovery of ^{3}H and ^{14}C are again statistically signif-

icant when compared in a paired t test (more ^{3}H than ^{14}C in the urine in all time periods ($0.001 < p < 0.01$); less ^{3}H than ^{14}C in the faeces ($0.01 < p < 0.05$) for 8-24 h samples; not significant for 24-48 h samples). Urinary recovery of radioactivity was low compared to normal rats ($p < 0.05$) 16.9% of ^{3}H, 13% of ^{14}C and 28.9% of ^{3}H, 22.6% of ^{14}C being excreted in 48 h in the urine of tumour-bearing rats and normal rats respectively. Faecal recovery of radioactivity was also reduced in tumour-bearing rats but this could have been due to non-production of faeces by a number of rats.

The recovery of radioactivity in tissues showed that the radioactivity in the liver increased gradually with time as in normal rats. The radioactivity retained in control liver was higher than in host liver but not significantly so. Considerable amounts of radioactivity were present in tumour tissue. The radioactivity retained in the gut and kidney as a percentage of the dose was similar to that in normal rats, however, the total recovery of the radioactivity retained in the tissues examined was higher in the tumour-bearing rats than in normal rats due to the high proportion of radioactivity present in tumour tissue. No significant differences were observed between the recovery of ^{3}H and ^{14}C in the tissues except the gut where more ^{3}H than ^{14}C was observed in 8 and 24 h samples ($0.01 < p < 0.05$) and 48 h samples ($p < 0.001$) and the kidneys at 8 h ($^{3}H > ^{14}C$, $p < 0.05$).

Urinary metabolites

DE 52-chromatography of the urine samples is illustrated in Figures 3.2.1., 3.2.2 and 3.2.3. Qualitatively there is no differences between the metabolites appearing in the urine of tumour-bearing rats and normal rats. However, the relative amounts of urinary metabolites of tumour-bearing rats differed from those of normal rats. Table (3.2.2) summarises the distribution of radioactivity between the metabolites appearing in the urine as a percentage of the dose. Folic acid excretion was higher than that of normal rats, however, the overall excretion of folates was reduced in the urine of tumour-bearing rats. 5,10CH_2-THF which appeared in the first and second urine samples of normal rats, appeared only in the first urine sample of tumour-bearing rats and disappeared thereafter whereas folate (X) disappeared after 24 h. The 8-24 h and 24-48 h urine samples showed the same relationship between the intact folates and scission products in that the ratio of intact folates to scission products decreased with the time and the catabolites in particular p-acetamidobenzoyl-L-glutamate and metabolite A dominated the last urine samples. All scission products present in the three urine samples of tumour-bearing rats were depressed compared to normal rats.

Liver and tumour extracts

Sephadex-G15 chromatograms of liver extracts are illustrated in Figures 3.2.4., 3.2.5 and 3.2.6. In all time periods the major radioactive peak was eluted near to the void volume in the position of folate-

polyglutamates. Small amounts of low molecular weight derivatives were also present including pterin and, at 8 h only, a folatemonoglutamate (probably 5 MeTHF).

Sephadex-G15 chromatography of the tumour extracts (Figures 3.2.7., 3.2.8., 3.2.9) showed similar patterns to those of liver extracts in that the major peak was a high molecular weight product (folatepolyglutamate) and in the 8 h sample a small amount of folatemonoglutamate was present. In addition, a very small peak labelled solely with tritium eluting at fractions (22-24) on Sephadex-G15 was present in tumour extracts at all time periods but was not seen in liver extracts of tumour-bearing rats or normal rats.

Summary

Folic acid was administered to tumour-bearing rats. Qualitatively the handling of folic acid was similar to normal rats but the following differences were observed:

1. Lower recovery of radioactivity in urine and faeces.
2. Higher retention of radioactivity in tissues due principally to the radioactivity taken up into tumour tissue.
3. The pattern of excretion of $5,10CH_2$-THF and folate (X) was changed.
4. Intact folates excretion was lower.
5. Scission products excretion was depressed.

6. A small amount of 5MeTHF detected in tumour and liver tissues at 8 h after administration.

3.3. The effect of MTX on folic acid metabolism in the rat

Methotrexate is a folate antagonist which is used in cancer chemotherapy. It is a potent inhibitor of dihydrofolate reductase (Bertino et al., 1964) and is presumed to act by inhibiting this enzyme. However, the in vivo situation is complex and a number of interacting mechanisms may be involved in MTX cytotoxicity (Goldman, 1977). The drug also inhibits other folate dependent enzymes, e.g. thymidylate synthetase (Borsa and Whitmore, 1969) albeit at higher concentration, acts on dihydropteridine reductase (Craine et al., 1972) and Barford et al., (1980) have presented evidence that dihydrofolate reductase is not the sole target of MTX in vivo. However, previous studies have largely been concerned with the effect of MTX on the metabolism of radiolabelled folate monoglutamates. Administration of MTX with or before folic acid results in excretion of large amounts of unchanged folic acid with little or no radiolabel incorporated into the folate pool (Barford et al., 1980). Effects on the normal tissue folates are therefore difficult to establish. The experiment in this section is designed to study the effect of MTX on tissue folates particularly their breakdown. Therefore, the administration of MTX was delayed until 8 h after folic acid administration at which time the only labelled tissue folates are polyglutamate derivatives (see Section 3.1).

Materials and Methods

15 Male syngeneic WAB/NOT rats (190-280 g/body wt.) received an oral dose of a mixture of [2-^{14}C]-folic acid and [3',5',7,9-^{3}H]-folic acid (100 µg/Kg body wt.). Animals were then housed in metabolism cages as described previously (Chapter 2). Eight hours after the administration of labelled folic acid 5 animals were killed by cervical dislocation and the remaining animals were given an oral dose of MTX (100 mg/Kg wt.). At 24 h a further 5 animals were killed and the remainder received a second dose of MTX (100 mg/Kg wt.). The remaining animals were killed 24 h later. Animals were treated throughout the experiment exactly as described in (Section 3.1). The collection of urine and faeces, the removal of tissues, the preparation of liver extracts, the determination of radioactivity and column chromatography were carried out as described previously.

Results

Table (3.3.1) summarises the recovery of ^{3}H and ^{14}C in the urine, faeces and tissues. In all time periods the recovery of ^{3}H in the urine was significantly higher ($p < 0.001$) than ^{14}C. There was no significant difference in the recovery of radioactivity in 0 - 8 h urine samples between this group and the control group. However, after the administration of MTX the urinary recovery of radioactivity was significantly higher than in control rats ($p < 0.001$) in 8 - 24 h and 24 - 48 h urine samples. An excess of ^{14}C over ^{3}H was present in the faeces in the second time

periods which was statistically significant ($p<0.05$). In the third time period only two rats produced faeces therefore the mean faecal recovery of radioactivity was depressed compared to normal. The radioactivity recovered in the liver showed a reciprocal pattern to normal in that the radioactivity in the liver decreased gradually with time. Low radioactivity was also observed in the kidneys and gut and it appeared that MTX caused a fall in tissue radioactivity. There was no difference in the recovery of ^{3}H and ^{14}C in the liver, however, the recovery of ^{3}H was significantly higher than ^{14}C in gut and kidneys ($p<0.05$ in 8 h samples, $p< 0.01$ in 48 h samples).

Urinary Metabolites

As expected DE52-chromatography of 0 - 8 h urine sample showed an identical pattern to that seen in the control group. However, after the administration of MTX, the patterns of DE52 chromatograms of the second and third urine samples were altered. DE52-chromatography of 8-24 h urine samples (Figure 3.3.1) showed the higher production of folic acid, also showed the presence of an extra peak eluting between 10 formylfolate and 5 MeTHF. All radioactive peaks from DE52 chromatography at 8-24 h urine sample were re-chromatographed individually on Sephadex G15. This revealed a complex mixture of at least 14 metabolites. Four of them were the intact folates (I folic acid, II 5 MeTHF, III 5, $10CH_2$-THF and IV 10-formylfolate) that were present in control urine. Folate (X), detected in the urine of control and tumour bearing rats, was not seen here. All the remaining metabolites were scission products of which six were

detected in the urine of control and tumour-bearing rats also. These are p-acetamidobenzoate, p-acetamidobenzoyl-L-glutamate, metabolite A (Figure 3.3.2), metabolite B, tritiated water and urea. Paper chromatography showed no p-aminobenzoate or p-aminobenzoyl-L-glutamate associated with their acetylated derivatives. The other four catabolites were not detected either in the urine of control rats or tumour-bearing rats and had much reduced ratios of $^{3}H:^{14}C$ compared to intact folates which indicated that they may be pteridine derivatives. One of these catabolites eluted at fractions (23-37) (0.2-0.3 MNaCl peak VI) with metabolite (B): however, it eluted at fraction 35 on Sephadex G15 (Figure 3.3.3) well separated from metabolite B (Fraction 55) and it was identified as pterin by co-chromatography with an authentic standard in both column systems. The other catabolites eluted at 0.5-0.6 M NaCl (peaks IV and IVa) from DE52 columns and at tubes 30, 42, and 57 from Sephadex -G15. Two of them were identified as pterin 6-COOH and xanthopterin (see Table 2.1 for the elution position of pteridines derivatives from both column systems). The third catabolite has not been identified (metabolite C). It is not one of the known pteridines reported in Table 2.1 and because of its low radioactivity no further attempt at identification was made.

The third urine sample (24-48 h) was subjected to sequential chromatography on DE52 (Figure 3.3.4) and Sephadex G15 (Figures 3.3.5, 3.3.6 and 3.3.7). This revealed the same metabolites as were present in the second urine sample (8-24 h) except for the absence of $5,10CH_2$-THF.

The scission products dominated this sample in particular p-acetamido-benzoyl-L-glutamate which amounted to 45% of the tritiated urinary products. The amount of metabolite A (the major ^{14}C labelled product in the late urine sample of control rats; 30% of urinary ^{14}C) was depressed as a percentage of the urinary radioactivity (9% of urinary ^{14}C). However, the appearance of other ^{14}C labelled products in the urine of rats treated with MTX raised the total ^{14}C scission products to 55% of urinary ^{14}C.

Table 3.3.2 shows the relative distribution of each metabolite appearing in the urine of the rats treated with MTX. After the administration of MTX, the urinary excretion of folic acid increased sharply and represented an appreciable percentage of the total radioactivity (11% ^{3}H and 16% ^{14}C) appearing in the third urine sample whereas it existed only in a trace amount in the later urine samples of control rats. MTX decreased the amounts of other intact folates appearing in the urine as a percentage of urinary radioactivity and also in relation to urinary folic acid. This can be shown by 1) the disappearance of folate (X) 2) the decreased amount of 10 formyl folic acid as a percentage of urinary radioactivity 3) a similar decrease in 5 MeTHF as a percentage of urinary radioactivity although absolute excretion of 5 MeTHF was greater than normal in the second urine sample.

MTX caused a 3 fold increase in p-acetamidobenzoyl-L-glutamate excretion and the excretion of p-acetamidobenzoate was higher in the second urine sample but was similar to control rats in the third urine sample. In the case of the pteridine moiety about 41% and 55% of urinary ^{14}C were distributed between the six pteridines detected in 8 - 24 h and 24 - 48 h urine samples respectively. Among them the pterin-6-COOH was the major species and pterin and metabolite (C) were minor catabolites. The excretion of urea was similar to control rats but the excretion of tritiated water was almost twice normal.

Liver extracts

Sephadex G15 chromatography of liver extracts gave similar patterns to control rats in that the major radioactive peak eluted in the position of folypolyglutamates (close to the void volume). Also small amounts of single-labelled compounds including pterin were present and no folate monoglutamtes were observed.

Summary

The administration of MTX to normal rats caused several changes in the metabolism of folate.

1. Increased urinary excretion of radioactivity and loss of radioactivity from tissues.

2. Increased urinary excretion of folates particularly folic acid.

3. Increased excretion of all normal catabolites and the production of four additional radioactive pterins not found in normal urine.

3.4. The effect of phenobarbitone on folic acid metabolism in the rat

Previous sections of this Chapter have shown that folate degradation occurs in vivo and the rate of folate breakdown can be affected by several factors such as the presence of a tumour or the administration of MTX. However, the mechanism of folate degradation is not known but oxidative C_9-N_{10} cleavage is a possibility. This could be a chemical process or be catalysed by the microsomal enzymes. An attempt was made in this Section to answer this question by pretreating the animals with phenobarbitone to induce hepatic enzymes. If these enzymes are involved in folate breakdown an increase in the levels of folate scission products in urine would be expected.

Materials and Methods

14 Male syngeneic WAB/NOT rats (235-280 g body wt.) were dosed intraperitoneally with 20 mg phenobarbitone (sodium salt-BDH Chemicals Limited, Poole, England) per rat every 24 hours for three days. On the fourth day they were dosed orally with a mixture of $[2\text{-}^{14}C]$ folic acid and $[3',5',7,9\text{-}^{3}H]$ -folic acid (100 µg/Kg body wt.).

Animals were then housed in metabolism cages as described in Chapter 2. Urine and faeces were collected for periods of 0-8 h, 8-24 h, and 24-48 h as described previously. Five animals were killed by cervical dislocation 8 h and 24 h and 4 animals killed 48 h after the administration of folic acid. Animals were treated throughout the experiment exactly as described previously (Section 3.1.). The removal of tissues, the preparation of liver extracts, the determination of radioactivity and column chromatography were carried out as described in Section 3.1.

Results

Table 3.4.1. summarises the recovery of ^{3}H and ^{14}C in the urine, faeces and tissues. In all time periods the recovery of ^{3}H in the urine was significantly higher than ^{14}C ($p < 0.001$). The urinary recovery of radioactivity in the first 8 h was higher (30.2% of ^{3}H and 25.6% of ^{14}C of the dose) than control rats (19.8% of ^{3}H and 16.7% of ^{14}C of the dose). This difference is statistically significant by Student's t test ($0.01 < p < 0.05$) but not by Wilcoxon's sum of ranks test. Also there was no significant difference in the recovery of urinary radioactivity between control rats and rats treated with phenobarbitone in other time periods (8 - 24 h and 24 - 48 h) by the two statistical methods used. An excess of ^{14}C over ^{3}H was present in the faces which was

statistically significant ($0.01 < p < 0.05$) in the second time period (8-24h). The faecal recovery of radioactivity of rats treated with phenobarbitone was significantly lower than control rats ($p < 0.01$).

A high proportion of the dose was present in the liver which again increased gradually with time as in normal rats. Hepatic radioactivity was significantly higher than control rats ($0.01 < p < 0.05$) in 8 h and 24 h liver samples and $p < 0.05$ in 48 h samples. However, on a weight basis (% of the dose /g wet weight) no differences were observed. The radioactivity present in the gut and kidney decreased with time and was quantitatively similar to control rats. The recovery of ^{3}H was statistically similar to the recovery of ^{14}C in the gut, kidney and the 8 h liver samples and was significantly lower in the 24 h and 48 h liver samples ($0.01 < p < 0.05$).

Urinary metabolites

DE 52 chromatography of all urine samples (Figure 3.4.1., 3.4.2. and 3.4.3) showed similar patterns of metabolites to that of the controls. Although the same metabolites were detected in the 0 - 8 h urine of both groups, the relative amounts of the urinary labelled products differed from those of control rats. Folic acid and 5,10 CH_2-THF were higher as a percentage of urinary radioactivity than control rats whereas 10 formyl-folate and 5 MeTHF were similar. In the case of scission products, higher percentages of urinary radioactivity of p-AcBG and metabolite A and lower percentages of p-AcBA and metabolite B than control rats were

observed and only traces of $^{3}H_2O$ and urea detected. DE 52-chromatography of 8 - 24 h and 24 - 48 h urine samples again showed the same relationship between the intact folates and scission products in that the ratio of intact folates to scission products decreased with time and catabolites dominated the last urine sample in particular p-acetamidobenzoyl-L-glutamate and metabolite A. However, the intact folates fell more rapidly with time than in control rats. Folate (X) was not detected at any time and folic acid and 10 formylfolate disappeared from the third urine sample. The relative distribution of each metabolite appearing in the urine samples of rats pretreated with phenobarbitone as a percentage of the dose is given in Table (3.4.2). The amounts of all intact folates in 0-8 h urine sample were higher than those of control rats in particular folic acid and 5,10CH_2-THF. However, the amounts of intact folates dropped and became lower than those of control rats in the second and third urine samples. Higher amounts of p-AcBG and p-AcBA were present in the second urine samples and they were slightly higher than control in the third urine sample.

Liver extracts

Sephadex G15 chromatography of liver extracts showed again that the major folates in the liver were folate poly glutamates. No folatemonoglutamates were detected at any time in the liver extracts. Also small amounts of single-labelled compounds including pterin were present in 8 h and 48 h liver samples.

Summary

Pretreatment of normal rats with phenobarbitone caused the following changes in folic acid metabolism:

1. Higher urinary excretion of radioactivity in 0 - 8 h urine sample only due to higher excretion of folates.

2. Lower excretion of folates in 8 - 24 h and 24 -48 h urine samples.

3. Higher excretion of scission products in 8 - 24 h but not in 24 - 48 h sample.

4. 5,10CH_2-THF was present in the third urine sample but 10CHO-FA was not whereas in normal rats, 5,10CH_2THF was absent and 10CHO-FA was present.

3.5. Discussion

The administration of a mixture of ^{3}H and ^{14}C folic acid to rats gave rise to a variety of metabolites in the urine including intact folates and folate catabolites. Absorbed folic acid is reduced and enters the folate monoglutamate pool giving a rapid rise in serum folates which then falls due to renal and biliary excretion and uptake into tissues. Once the folate enters the cell it is converted to folatepolyglutamtes and studies in vivo have shown that folatepolyglutamates can be detected in the tissues within two hours of the administration of oral doses of labelled folic acid and reach maximum levels at 8 h (Bates et al., 1980). Sephadex G15 chromatography of liver extracts performed here showed the domination of

folate polyglutamates in the liver at 8 h and also in the following samples.

The identification of single labelled fragments in the urine demonstrated that degradation of the dosed folates was occurring *in vivo*. In all experiments reported in this Chapter significantly more ^{3}H than ^{14}C was recovered in the urine and an excess of ^{14}C over ^{3}H was present in the faeces. The cleavage of folate monoglutamates is suggested to occur within the gut lumen following biliary excretion (Pheasant *et al.*, 1981, Hillman *et al.*, 1977). The isotopic imbalances resulting from the pterin derived fragment being less well absorbed than ^{3}H labelled fragments. However, the excess of ^{3}H over ^{14}C in the urine which is due to tritiated scission products accounted for only 44%, 46%, 20% and 68% of the ^{3}H excess in the normal rat, tumour-bearing rat, rat treated with MTX and rat pretreated with phenobarbitone respectively, the remainder being due to the enhanced ^{3}H to ^{14}C ratio of the intact folates excreted in the urine compared to the administered folic acid. This is in agreement with the observation of a secondary isotope effect reported recently by Connor *et al.*, (1980) who found that ^{3}H-folate was absorbed from gut at a faster rate than ^{14}C-folate. Variable results were found from tissues but where differences were significant usually an excess of ^{3}H was found. Precisely the secondary isotope effect accounted for 40%, 44%, 42% and 17% of the tritium excess in the normal rat, tumour-bearing rat, rat treated with MTX and rat treated with phenobarbitone respectively and remaining excess of ^{3}H over ^{14}C was simply due to the attachment of ^{3}H at position 7 of the pterins excreted in the urine.

Over a 48 h period the relative proportion of the compounds appearing in the urine after the administration of folic acid changed with time. The excretion of folatemonoglutamates was high in early urine samples and this probably indicates that the level of the dose given was sufficient to exceed the renal threshold, and the scission products p-acetamidobenzoyl-L-glutamate and metabolite A increasingly dominate late urine samples. Similar observations have been reported previously after the administration of a mixture of ^{3}H and ^{14}C folic acid, 10 formylfolic acid, 10 formylfolatetetraglutamate (Pheasant et al., 1981) and 5 MeTHF (Kennelly 1980). 4a-Hydroxy-5 MeTHF, now described as a triazine derivative (Jongejan et al., 1979), which was reported to be the major metabolite in the late urine samples of rats dosed with $[2\text{-}^{14}C]$-folic acid (Barford and Blair 1976) was not detected and it is suggested that it was an artefact arising by oxidation of 5 Me THF (Blair et al., 1975) during urine collection.

The formation of the majority of folate catabolites can be explained by cleavage of C_9-N_{10} bond to give p-aminobenzoyl-L-glutamate and a pterin which undergoes further metabolism. p-Aminobenzoyl-L-glutamate has been shown to be metabolised to p-acetamidobenzoate and p-acetamidobenzoyl-L-glutamate in the rat and guinea pigs (Pheasant et al., 1981, Choolun et al., unpublished observations). The appearance of the two acetylated derivatives in the rat urine after the administration of labelled folates was time dependent and they are suggested to be formed by metabolically distinct routes (see Chapter 1).

Metabolite A showed similar chromatographic behaviour to pteridine (P) reported by Connor (1979). It has also been reported in rat urine following the administration of a mixture of ^{3}H and ^{14}C 10 formylfolate derivatives (Pheasant et al., 1981) and 5 MeTHF (Kennelly, 1980) and it seems to be a normal catabolite of folate degradation in vivo in rat. However, despite the numerous reports of the presence of pteridines in human urine (Blair, 1958, Fukushima and Shiota, 1972, Watson et al., 1977, Krumdieck et al., 1978) metabolite A does not correspond to any of these. Since pterins present in the gut appear to be poorly absorbed (Pheasant and Pearce, 1981, Knipe and McCorack, 1977) the amounts of metabolite A found in the urine may be attributed to the catabolism of the retained tissue folates, the corollary to the production of p-acetamidobenzoyl-L-glutamate, and this may explain the domination of these two catabolites in the late urine samples. A similar explanation can be put forward for the small amounts of metabolite B detected in the urine. However, the origin of these two catabolites, whether they are formed from different folates or whether one is a metabolite of the other is not known.

The tritiated water found was probably derived from the C_9 position during cleavage of the C_9-N_{10} bond. The only other derivative likely to be formed from C_9 position during chemical oxidation of THF and DHF is formaldehyde (Chippel and Scrimgeour, 1970). Moreover any formaldehyde generated in vivo would probably be oxidised to formate and water.

The detection of ^{14}C-urea indicates that degradation of the pterin

ring system occurs in vivo but the mechanism of this breakdown remains unclear. No attempt was made to collect CO_2 from the breath of rats. However, labelled urea and $^{14}CO_2$ were recovered from the urine and breath respectively of rats and guinea pigs following the administration of [2-^{14}C] -folates (Connor et al., 1977, Choolun, personal communication). Labelled urea was the major metabolite in the urine of rats dosed orally with [2-^{14}C] pterin 6-COOH (Pheasant and Pearce 1981) and this suggests that such a pterin may be an intermediate in the formation of urea and CO_2 from folate. Fukushima and Nixon (1980) reported that micro-organisms located within rat caecal contents catabolized [2-^{14}C] -pteridines under anaerobic conditions to $^{14}CO_2$ and it is possible that this may occur in vivo. No direct evidence is available to support this proposal; however, the work of Pheasant and Pearce (1981) suggested that the gut may be the main site of degradation of unconjugated pteridines. The contribution of the gut microflora to pteridine and folate degradation in vivo remains to be fully established.

The catabolites appearing after the initial 8 h period arise principally from the breakdown of folatepolyglutamates since only these forms were detected in the liver at this time. Therefore, the levels of p-acetamidobenzoyl-L-glutamate in urine after 8 h can be used as a measure of the breakdown of tissue polyglutamate. However, since the source of the tritium label at p-acetamidobenzoyl-L-glutamate was [3',5',7,9-^{3}H] folate with only 42.5% of the tritium at the 3',5' positions, the actual amount of folate cleaved is greater than that indicated by the quantity of

urinary p-acetamidobenzoyl-L-glutamate recovered and appropriate corrections are given (Table 3.5.1). It is possible to calculate a measure of folate polyglutamate breakdown. This is expressed as :- $\frac{X}{Y} \times 100$

where X = the percentage of the dose excreted in the late urine samples (8 - 48 h) as p-acetamidobenzoyl-L-glutamate

Y = the percentage of the dose retained in the body at 8 hour

Previous work has shown that tissues studied here are those which retain significant amounts of radioactivity therefore the radioactivity measured in these tissues can be used to express Y. The rate of folatepolyglutamate breakdown in normal rats was 6.9% (see Table 3.5.5.).

Effect of a tumour on ^{3}H and ^{14}C folic acid metabolism

The presence of an implanted tumour mass is observed to impose changes on the whole body metabolism of folic acid in the rat. Significantly less radioactivity was recovered in the urine of the tumour-bearing rats compred to normal rats. This may be due to the high folate requirement of the large tumour mass (approximately double of the host liver weight). Previous studies showed little or no effect on urinary radioactivity in tumour-bearing rats with a relatively small tumour mass (Pheasant and Blair 1979, Connor 1979). Indeed a high proportion of the radioactivity was taken up by the tumour tissue, thus increasing the total tissue uptake of radioactivity in tumour-bearing rats and rendering less available for excretion in urine or bile. The faecal radioactivity was also decreased in

tumour-bearing animals. The total excretion of intact folates was reduced in the urine of tumour-bearing rats compared to normal. This again is probably a reflection of uptake of folate and formation of folate-polyglutamates by the large tumour mass. Although these quantitative results are consistent with others reported for the Walker 256 tumour, the pattern of urinary folates observed here is different. Elevated 10 formyl-folates at the expense of 5 MeTHF were observed in the urine of rats-bearing the Walker 256 tumour after the admininistration of labelled folic acid, 10 formylfolic acid or 5 Me THF (Barford and Blair 1978, Connor, 1979, Kennelly 1980). The same trend was observed with folate (S) reported by Connor (1979) which has been identified in this thesis as 5,10-CH_2-THF. Rats bearing the Mc/103B sarcoma showed a lower production of 10 formylfolates and 5,10 CH_2-THF was detected only in the first urine sample and disappeared thereafter. Jackson and Niehammer (1979) reported that hepatomas show diminished 10 formyl-THF dehydrogenase activity compared to normal liver cells. This may be the case in Walker -256 but not in Mc/103B sarcoma. Since 10 formyl-THF dehydro-genase regulates the level of one-carbon unit in the folate pool (Krebs et al., 1976) it s deficiency results in a greater production of 10CHO-THF and subsequently, 5,10CH_2-THF which is in equilibrium with 10CHO-THF will increase.

All scission products recovered from the urine of tumour-bearing rats were reduced compared to normal rats and this demonstrates that despite the differences in the pattern of urinary folates recovered from the urine of

rats bearing different tumour lines, all are in agreement with the fact that folate scission products are depressed in the presence of a tumour mass (Barford and Blair, 1978, Kennelly, 1980). The reduced amounts of p-acetamidobenzoate and p-acetamidobenzoyl-L-glutamate in the urine of tumour-bearing rats indicates that the breakdown of both folate pools is diminished. This may be expected with folatemonoglutamate breakdown since lower levels of folates have been observed in the serum of tumour-bearing rats (Poirier, 1973). Therefore, probably lower levels of folates are passing through the gut during folate enterohepatic circulation. However, in the case of folate polyglutamates there is a higher retention of radioactivity in the body of tumour-bearing rats but, less scission products are excreted in the urine. This suggests that the rate of folatepolyglutamate breakdown in tumour-bearing rats is less (4.5%) than normal rats (6.9%) Table 3.5.5.

Effect of Methotexate

The administration of MTX significantly increased the urinary excretion of radioactivity. The increase in excretion of intact folates immediately following MTX administration could be due to displacement of the folates from circulating binding proteins and/or to the inhibition of uptake of folates into cells (Goldman, 1971); the level of intact folates other than folic acid dropped to normal in the following urine sample (24 - 48 h), MTX also increased the catabolism of folate. This is seen as an increase in urinary catabolites, particularly p-acetamidobenzoyl-L-glutamate which is increased to about 400% and 300% of control values

in 8 - 24 h and 24 - 48 h urine samples respectively. A corresponding fall was seen in the levels of radioactivity recovered in the tissues. This suggests that MTX increases the rate of folate polyglutamate breakdown (28%) (Table 3.5.5). The levels of p-acetamidobenzoate, metabolite A and urea were similar to those of control rats or slightly higher.

The detection of additional catabolites in the urine suggests that an abnormal route of breakdown also occurs. Trace amounts of labelled pterin and isoxanthopterin have been detected in the urine of a woman receiving labelled folic acid (Krumdieck et al., 1978) and Watson et al., (1977) have isolated dihydroxanthopterin from the urine of a patient on MTX therapy but these pterins have never been reported in rat urine (Murphy et al., 1976, Pheasant et al., 1981) and it seems that MTX has some effect on folate metabolism resulting in the presence of such pterins in the urine or at least increasing their amounts to detectable levels.

The effect of phenobarbitone

The administration of phenobarbitone caused some changes in folic acid metabolism in the rat. It increased the urinary excretion of radioactivity in the first urine sample only. The increase in excretion of folates could be due to the inhibition of uptake of folates into cells. Chen and Wagner (1975) found that phenobarbitone significantly inhibited the uptake of 5 MeTHF in isolated choroid plexus. The level of intact folates dropped sharply and became lower than normal in the third urine

sample. Phenobarbitone increases hepatic blood flow and bile flow (Fingle and Woodbury 1975) and thus could increase the folate entero-hepatic circulation which may result in a higher loss of folate than normal through breakdown in the gut. Higher excretion of p-acetamidobenzoate in the second urine sample may support this proposal, however, the recovery of radioactivity in the faeces, in particular, ^{14}C, does not. Nevertheless, micro-organisms are capable of degrading folate to scission products (Bacher and Rappold, 1980) and $[2\text{-}^{14}C]$-pteridines to $^{14}CO_2$ (Fukushima and Nixon 1980) and the rate of folate and pteridine degradation may be increased in these rats leading to increased loss of ^{14}C as $^{14}CO_2$. However, this has yet to be confirmed.

The disappearance of folate (X) and higher excretion of $5,10CH_2$-THF and its existence in the third urine sample suggest that anticonvulsant drugs alter the pattern of urinary folate. In rats pretreated with phenytoin the pattern of urinary folates was also different from controls (A. Guest, personal communication).

An increased level of p-acetamidobenzoyl-L-glutamate was seen in the second urine sample (8 - 24 h) but levels had returned to normal in the third urine sample. This indicates that the increase in the rate of folate-polyglutamate breakdown was only transient as the rates at 8-24 h and 24-48 h were 5.8% and 4.1% respectively in rats pretreated with phenobarbitone and 2.9% and 4.4% in control rats. This shows that the rate of tissue folate breakdown became normal by the second day despite the long

acting enzyme inducing effects of phenobarbitone and this may indicate that the induction of microsomal enzymes has little or no effect on the catabolism of folatepolyglutamate.

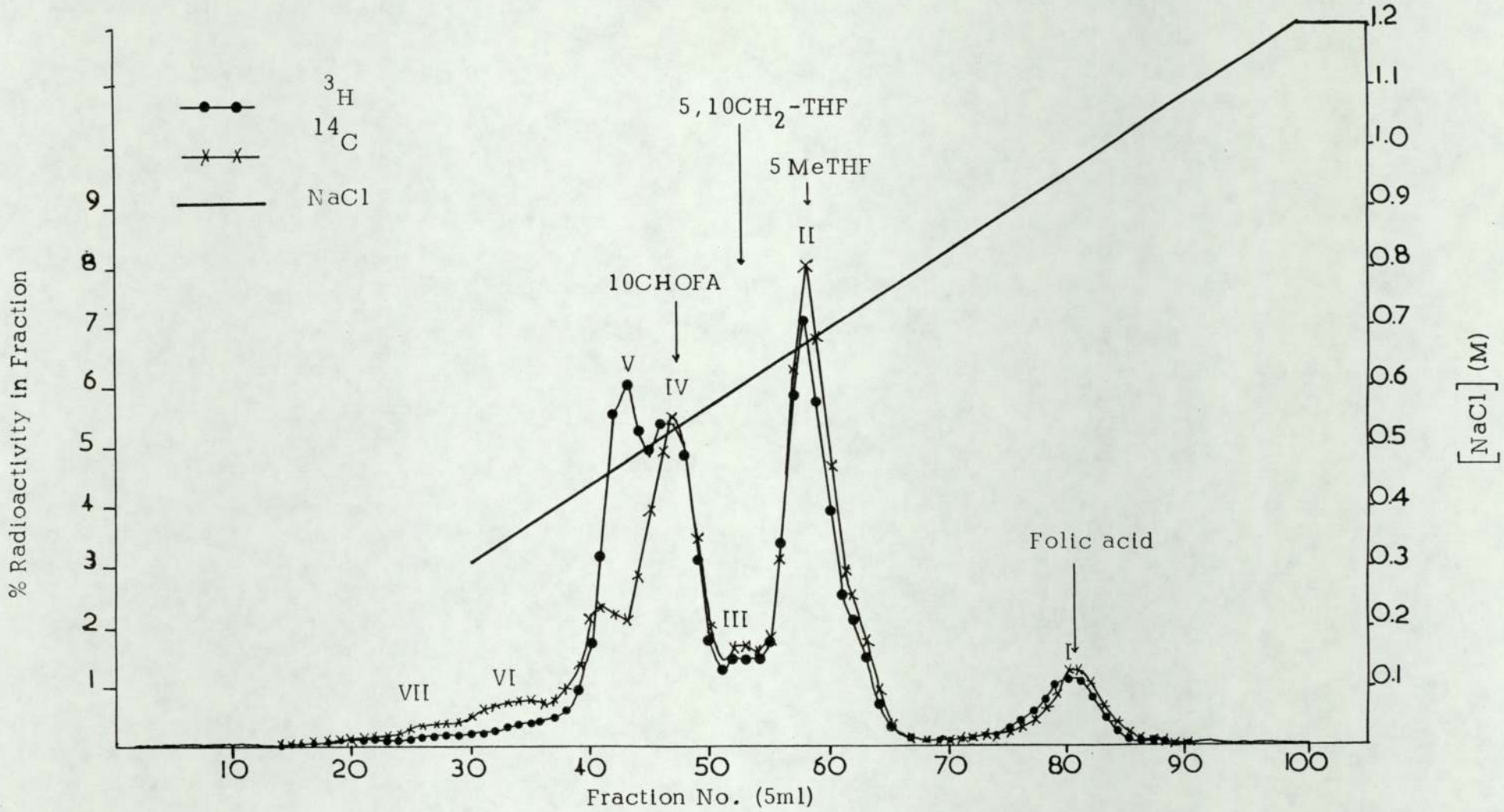


Fig. 3.1.1. DE52 Chromatography of normal rat urine samples collected 0-8h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}\ ^{3}H\right]$ folic acid.

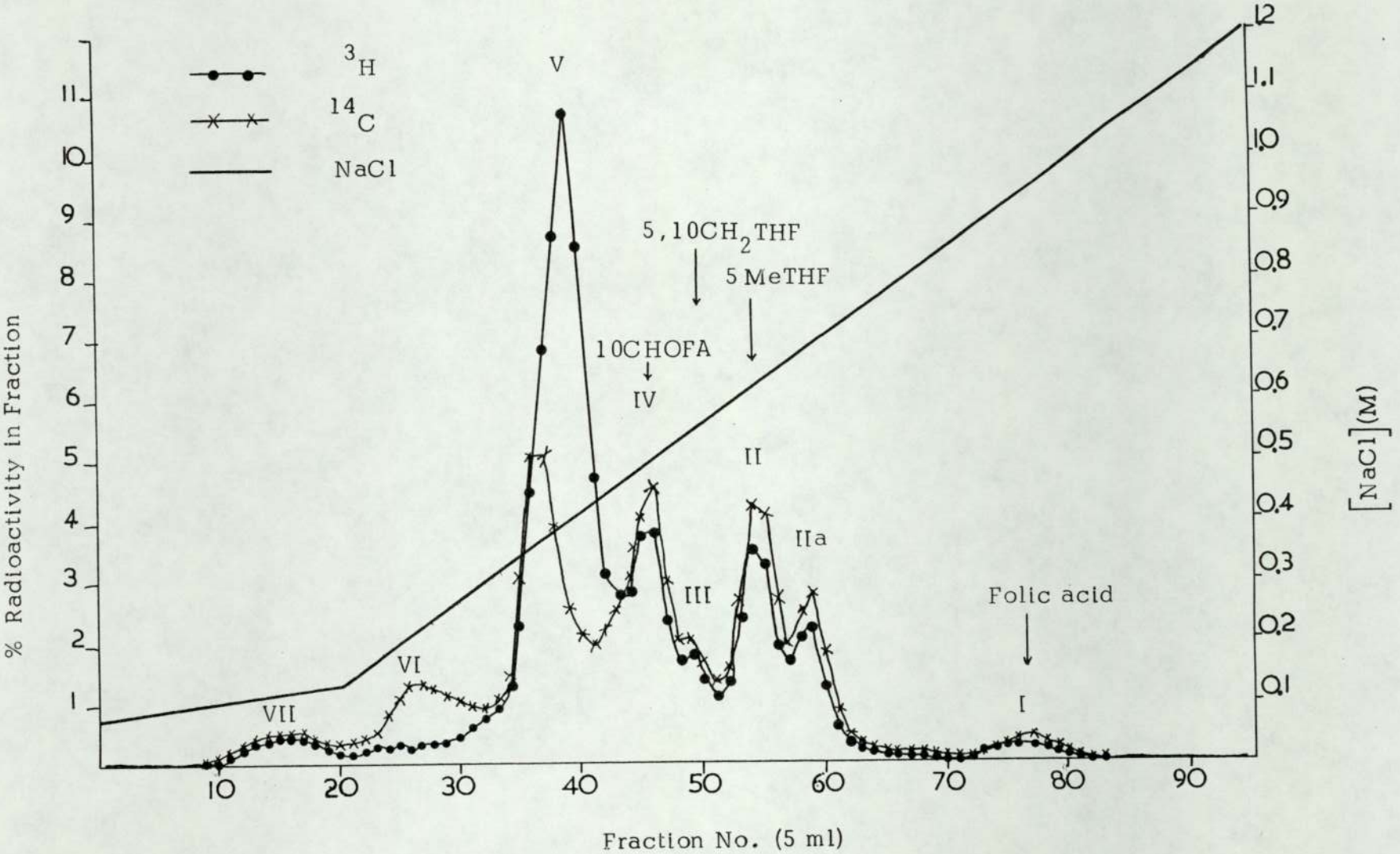


Fig. 3.1.2. DE52 Chromatography of normal rat urine samples collected 8-24h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid

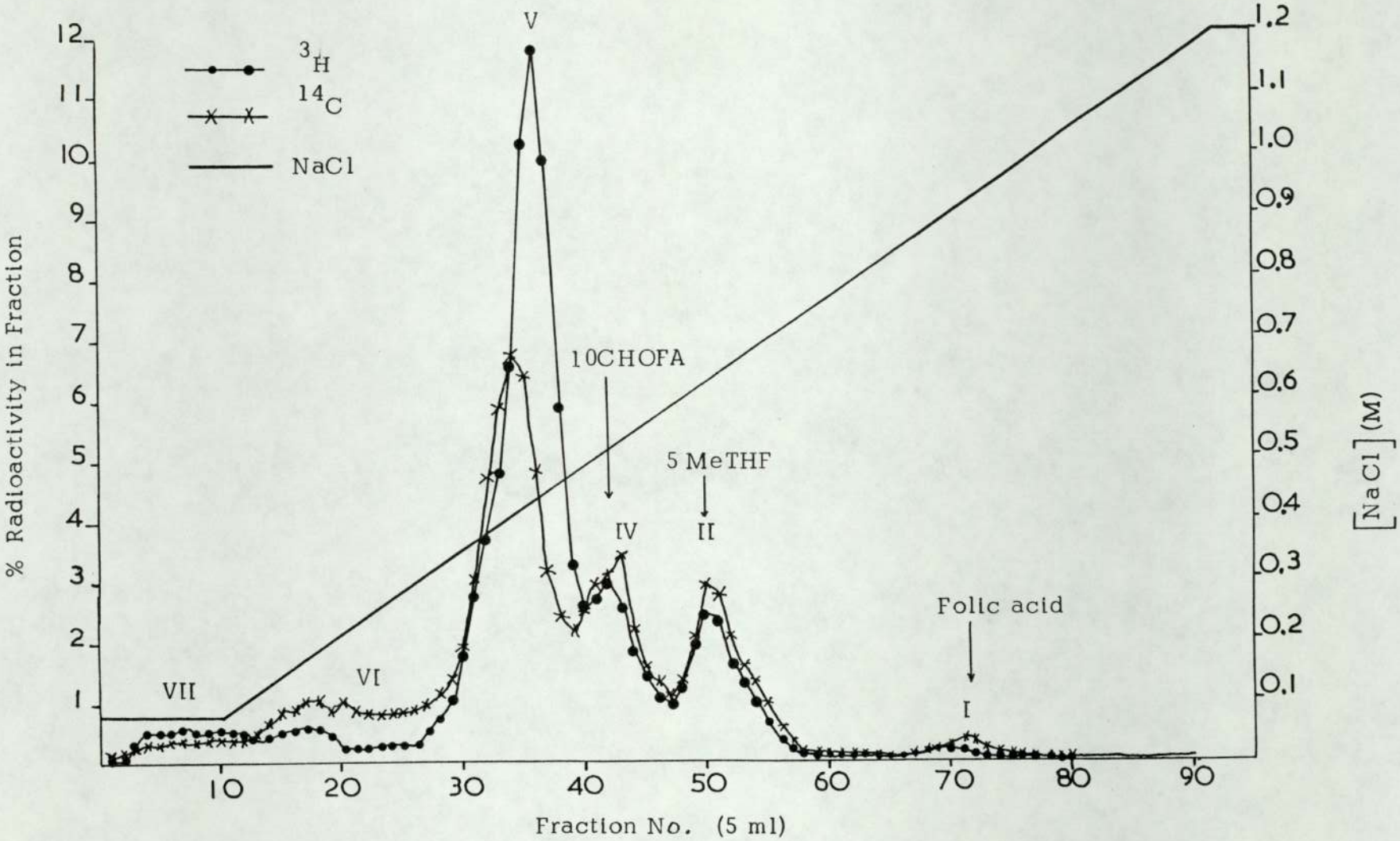


Fig. 3.1.3. DE52 Chromatography of normal rat urine samples collected 24-48h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid

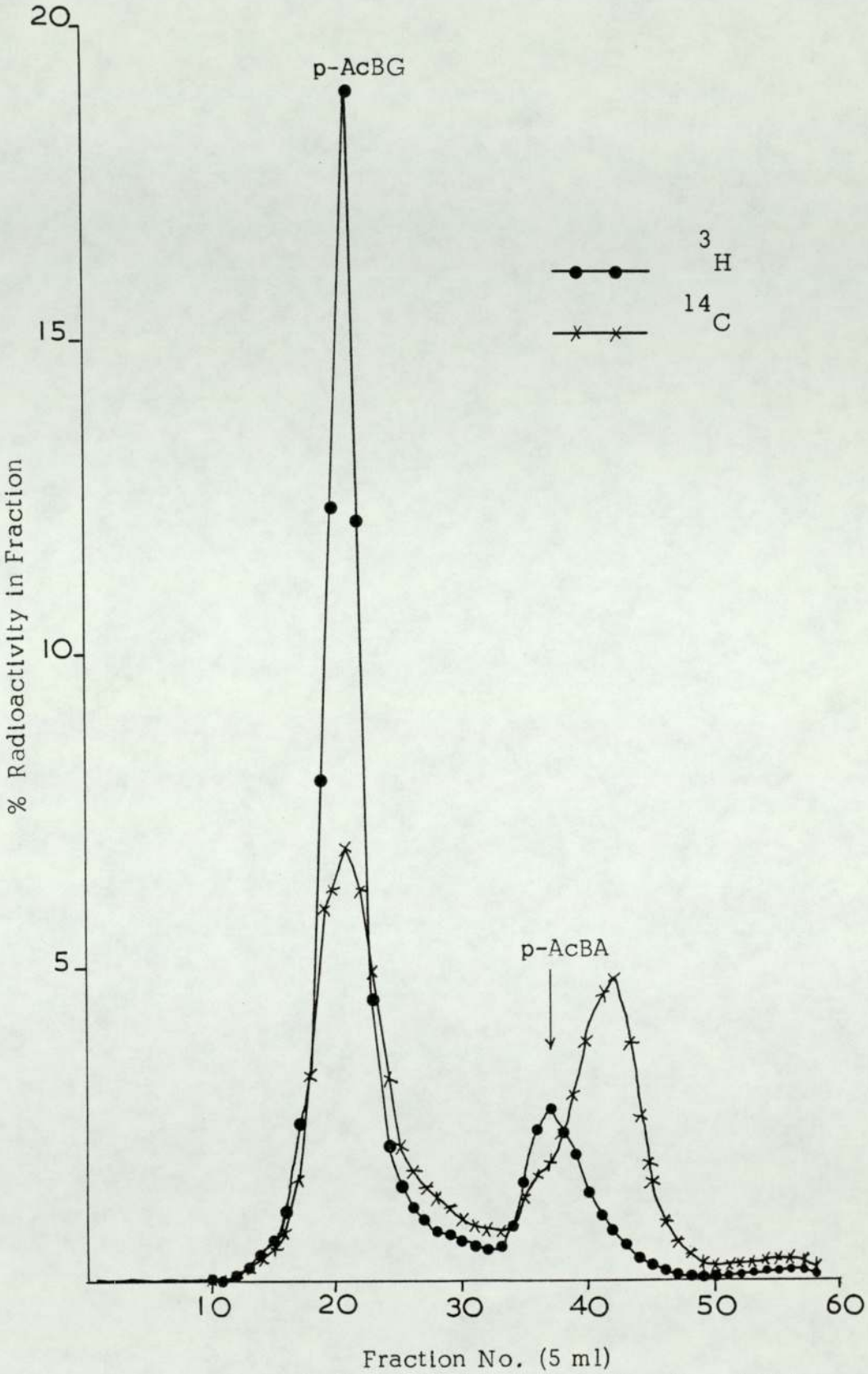


Fig. 3.1.4. Sephadex G15 chromatography of peak V from DE52 chromatography of normal urine samples collected 0-8h after the administration of labelled folic acid

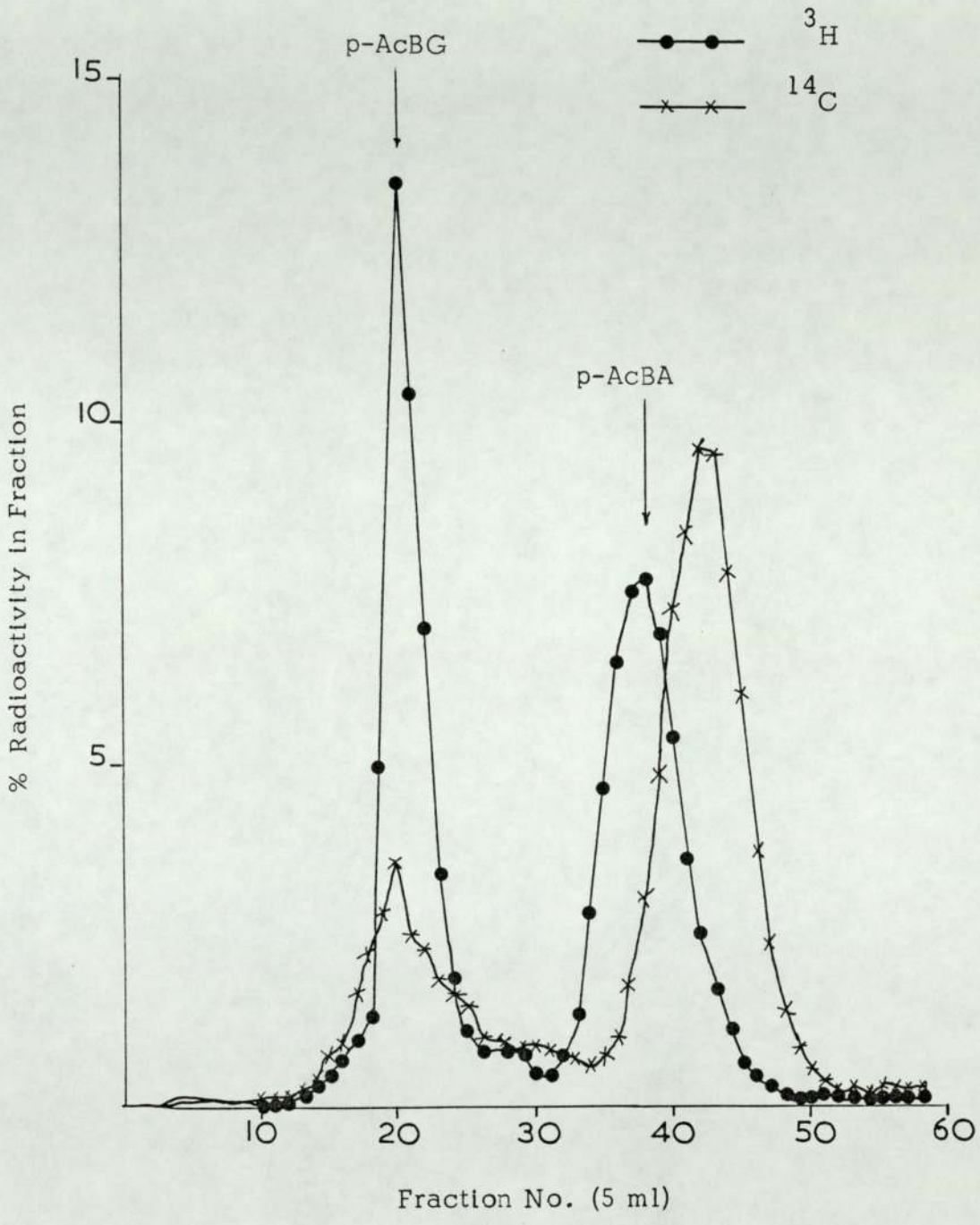


Fig. 3.1.5. Sephadex G15 chromatography of peak V from DE52 chromatography of normal urine samples collected 8-24h after the administration of labelled folic acid.

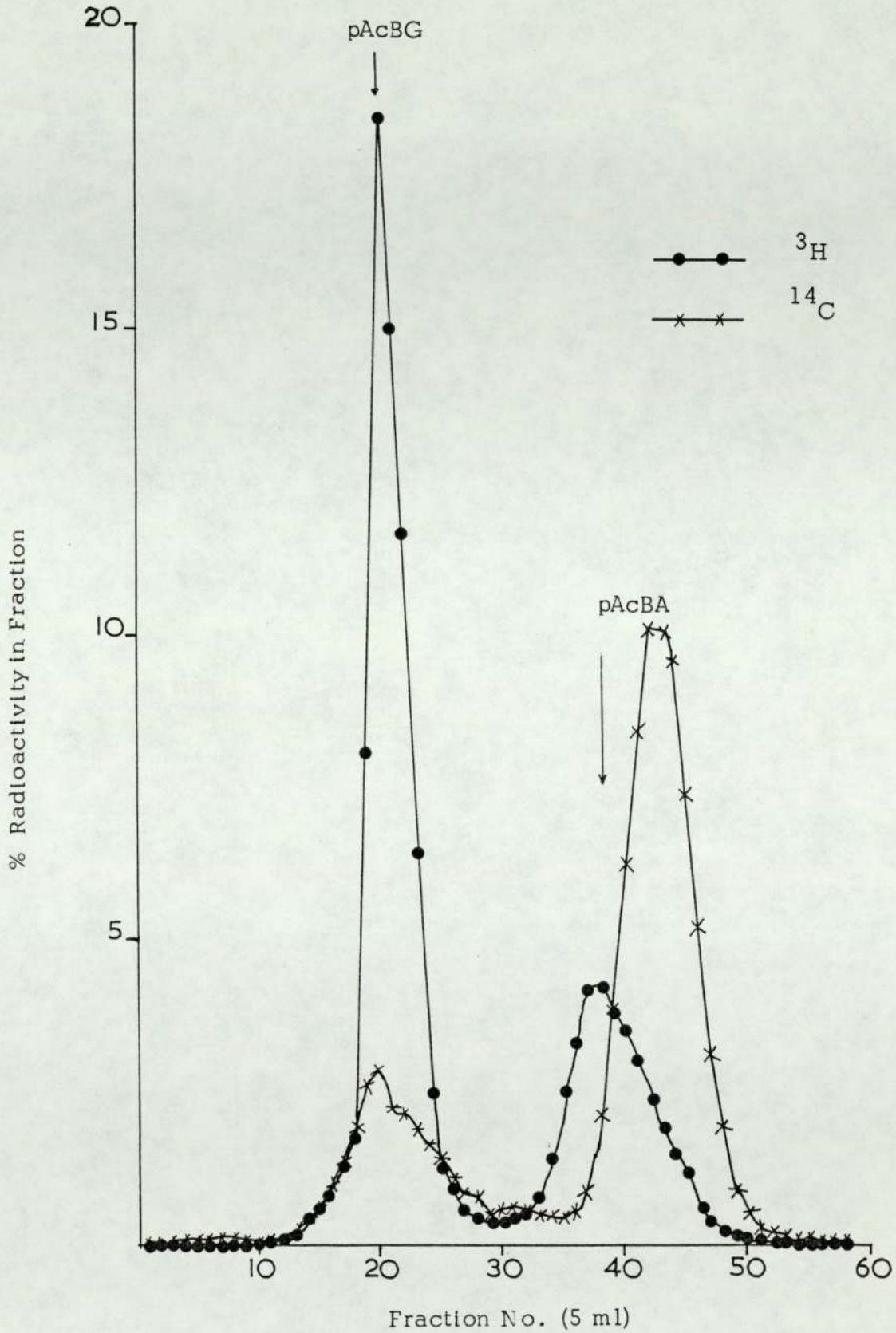


Fig. 3.1.6. Sephadex G15 chromatography of peak V of DE 52 chromatography of normal urine samples collected 24-48h after the administration of labelled folic acid.

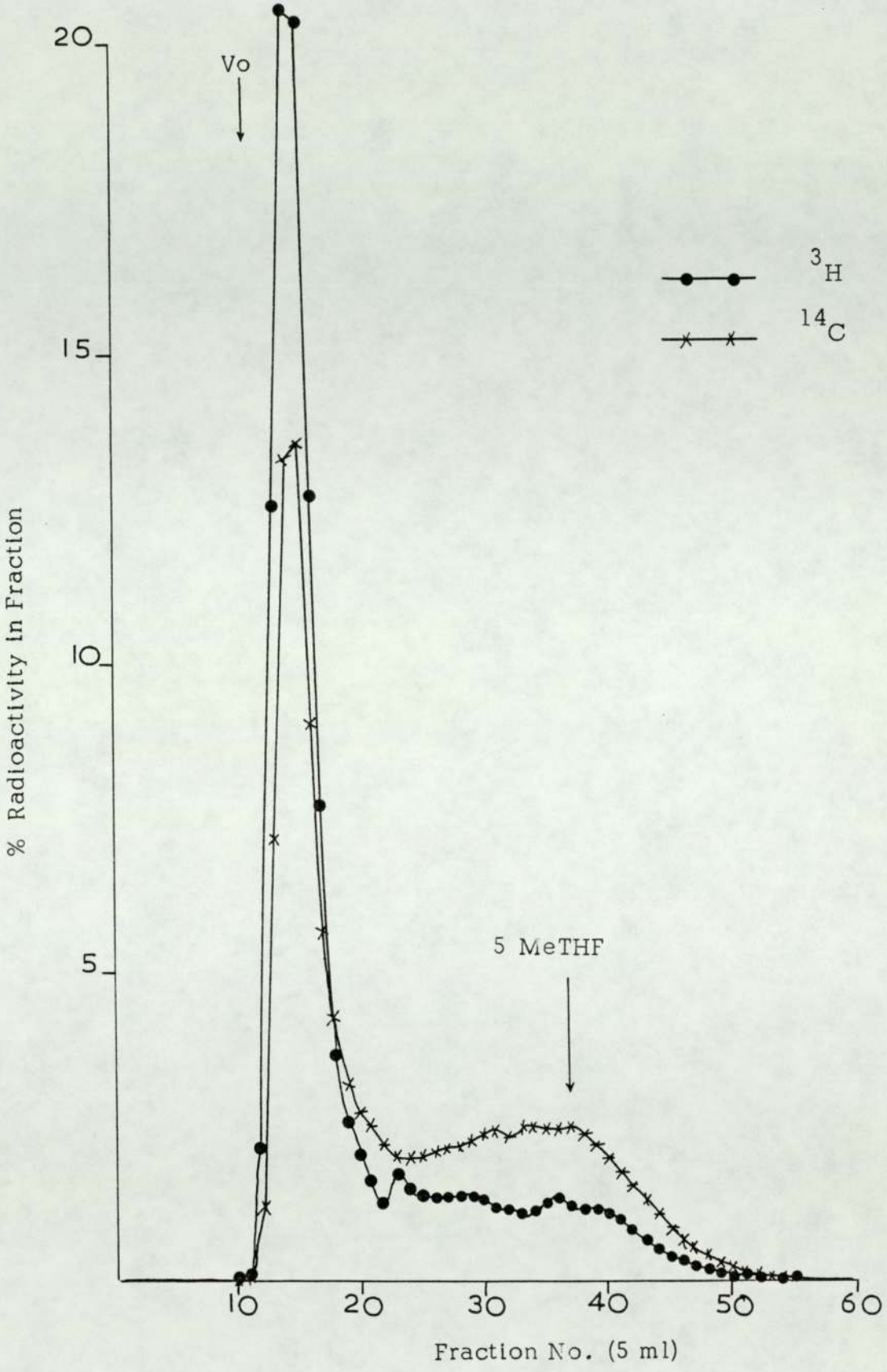


Fig. 3.1.7. Sephadex G15 chromatography of a hot extract of normal rat liver 8h after the administration of labelled folic acid

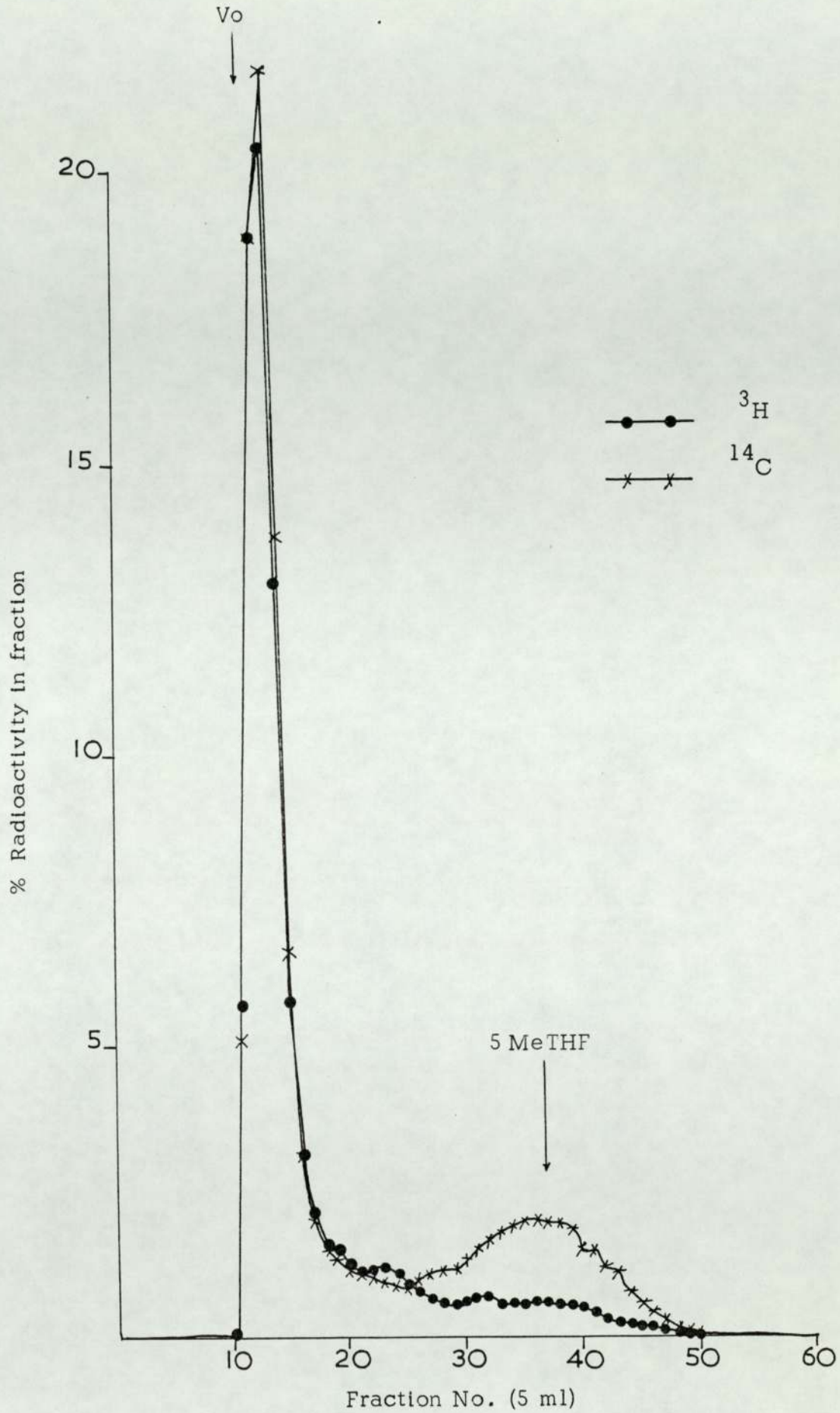


Fig. 3.1.8. Sephadex G15 chromatography of a hot extract of normal rat liver 24 h after the administration of labelled folic acid.

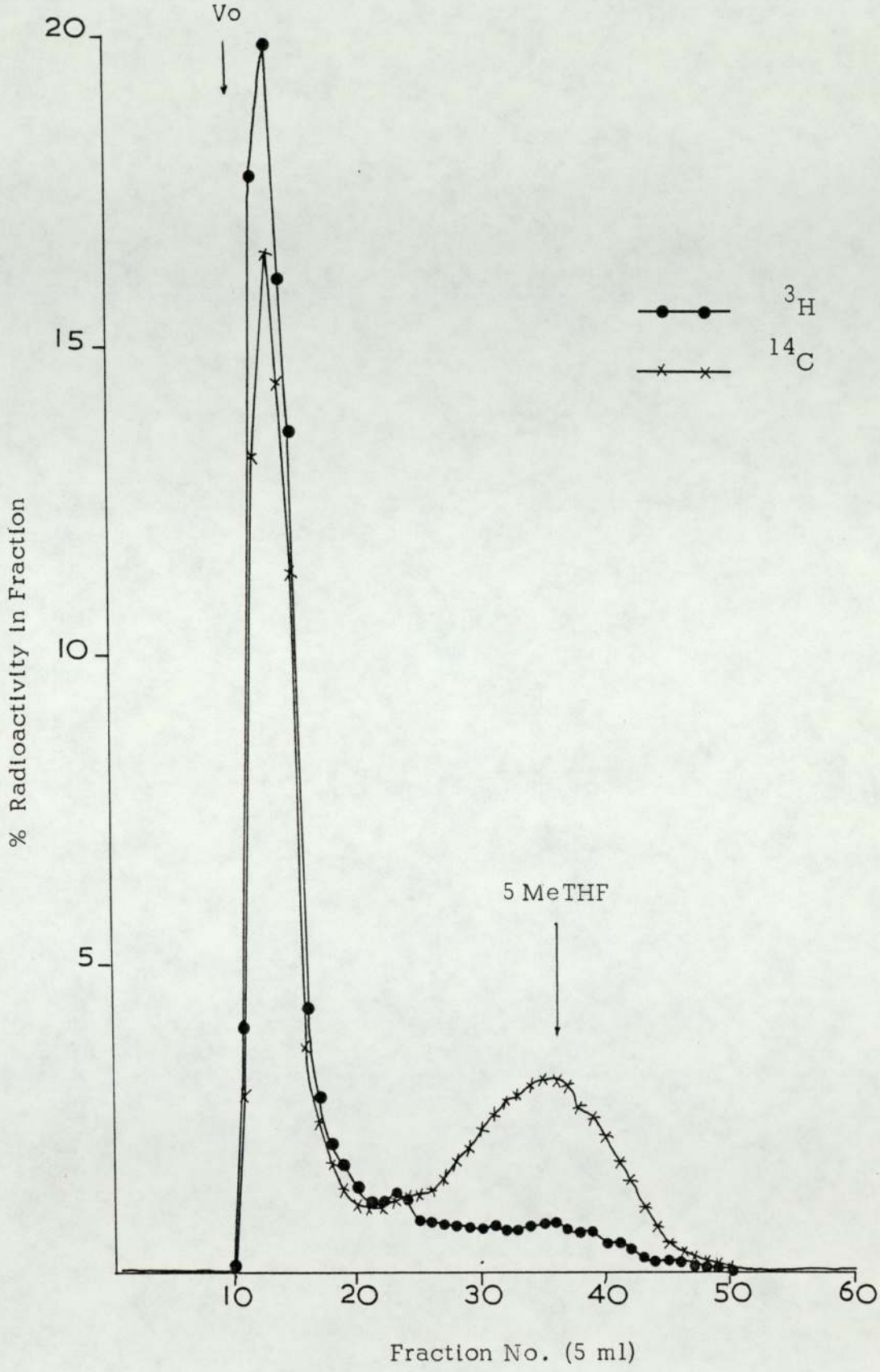


Fig. 3.1.9. Sephadex G15 chromatography of a hot extract of normal rat liver 48h after the administration of labelled folic acid.

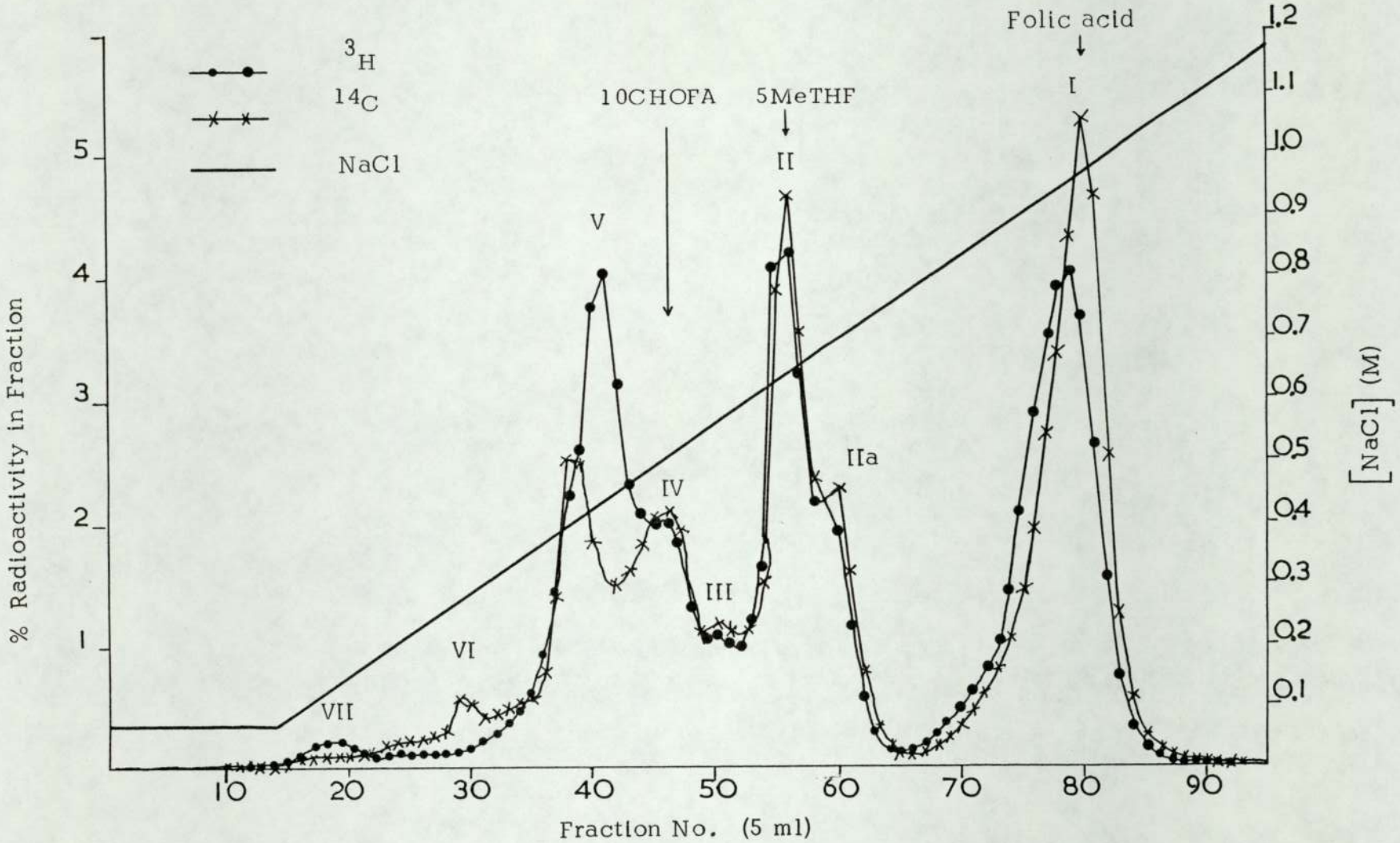


Fig. 3.2.1. DE52 chromatography of tumour-bearing rat urine samples collected 0-8h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid.

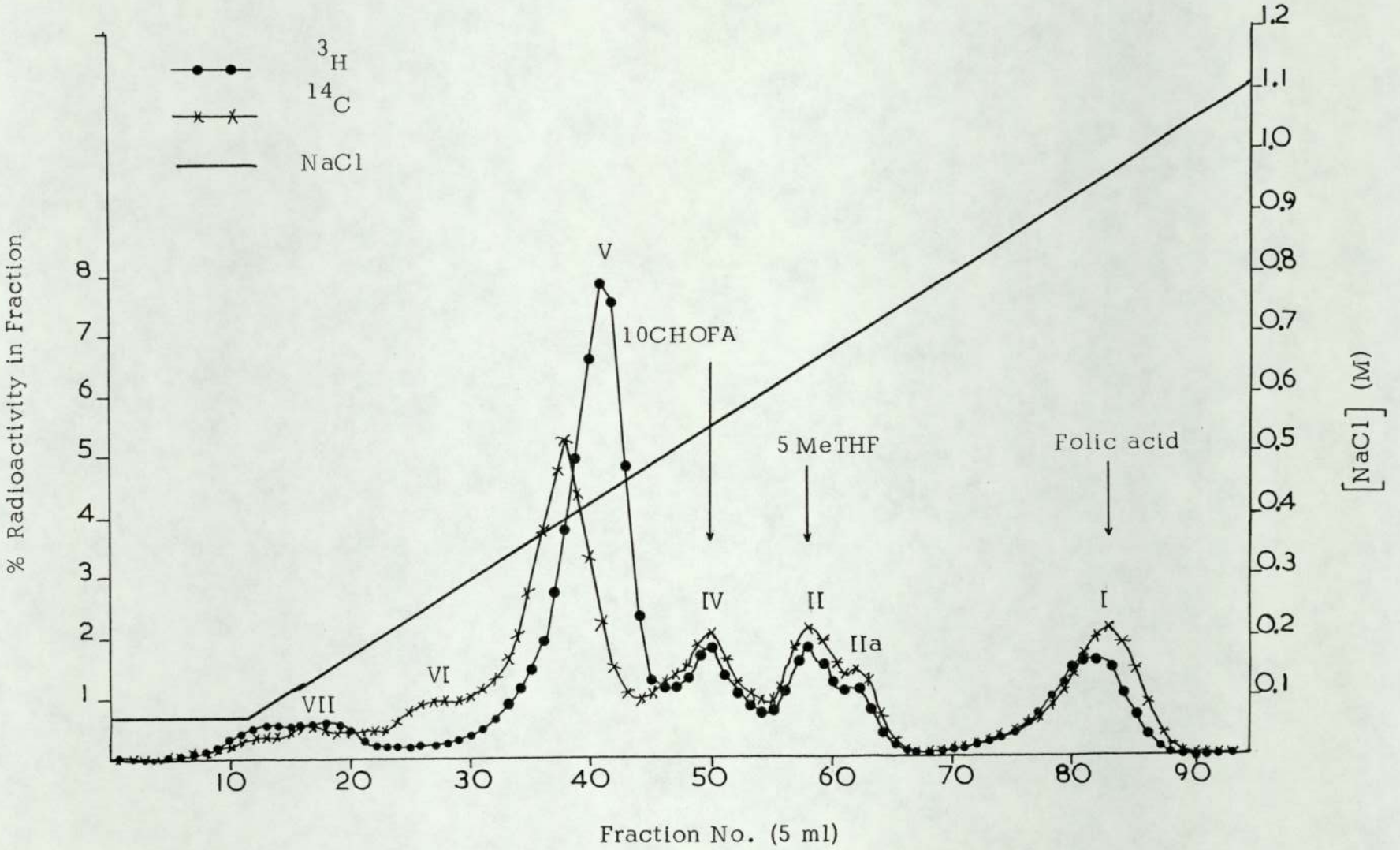


Fig. 3.2.2. DE52 chromatography of tumour-bearing rat urine samples collected 8-24h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid.

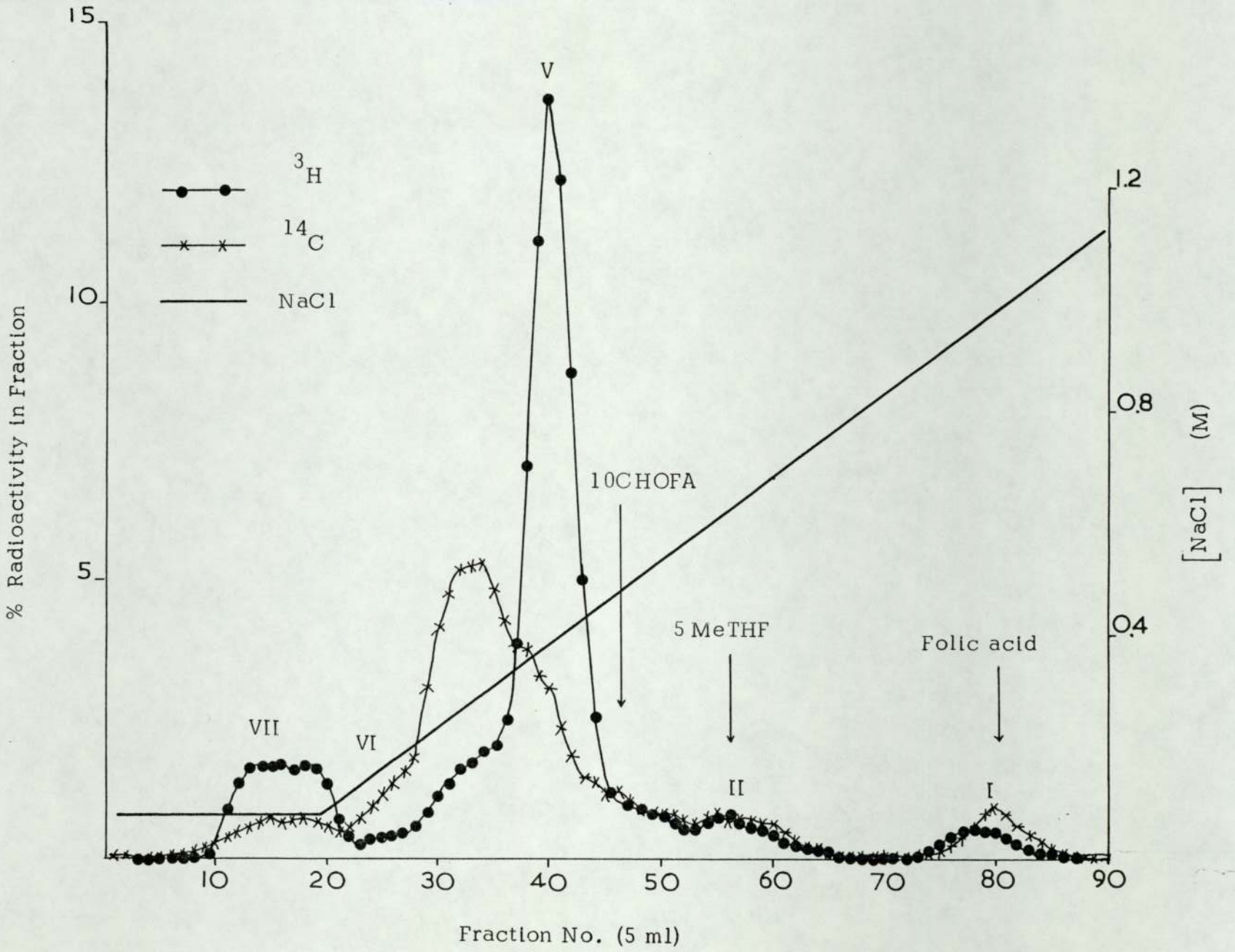


Fig. 3.2.3. DE52 chromatography of tumour-bearing rat urine samples collected 24-48 h after the administration of a mixture of ^{3}H and ^{14}C folic acid

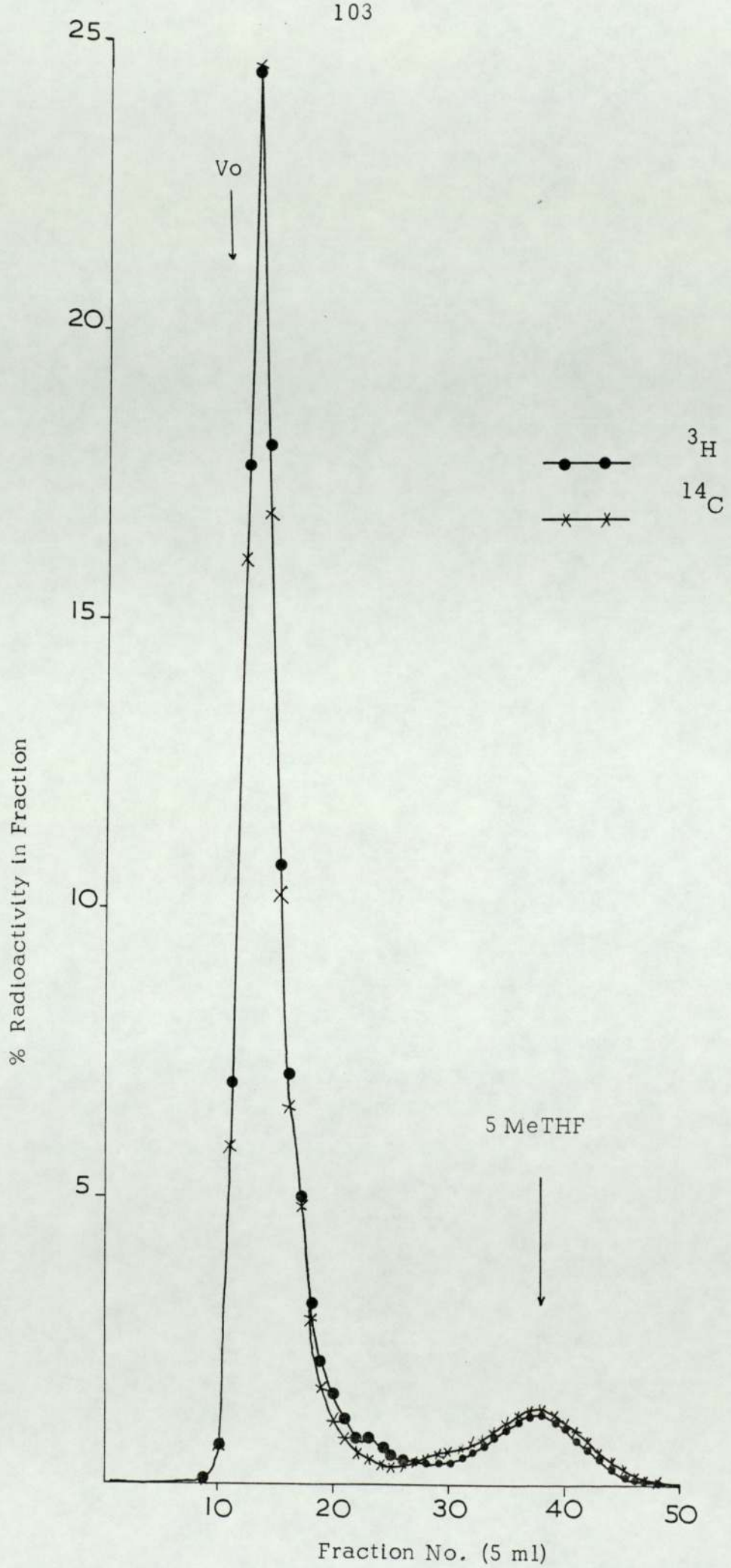


Fig. 3.2.4. Sephadex G15 chromatography of the liver extract of tumour-bearing rats 8h after the administration of labelled folic acid

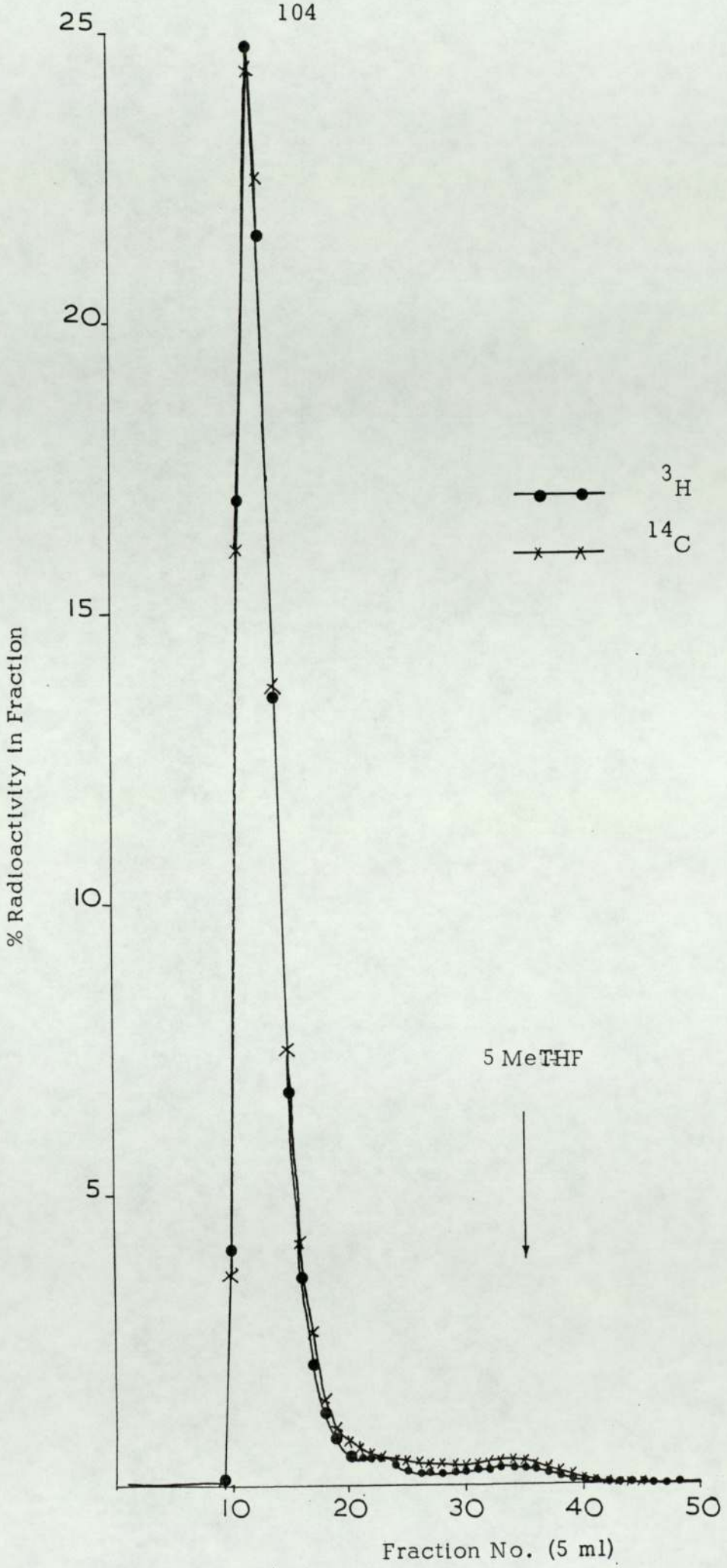


Fig. 3.2.5. Sephadex G15 chromatography of the liver extract of tumour-bearing rats 24h after the administration of labelled folic acid.

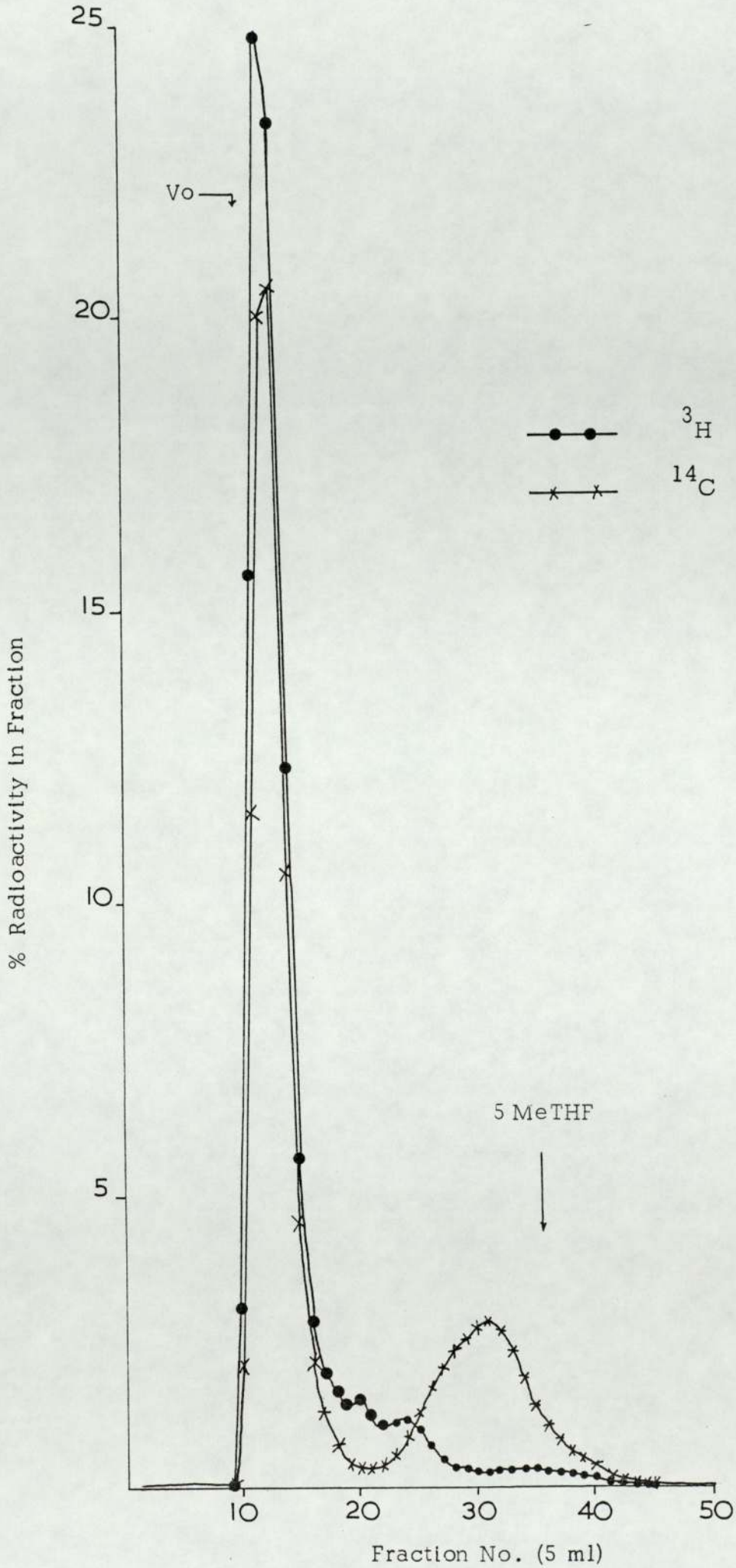


Fig. 3.2.6. Sephadex G15 chromatography of the liver extract of tumour-bearing rats 48h after the administration of labelled folic acid

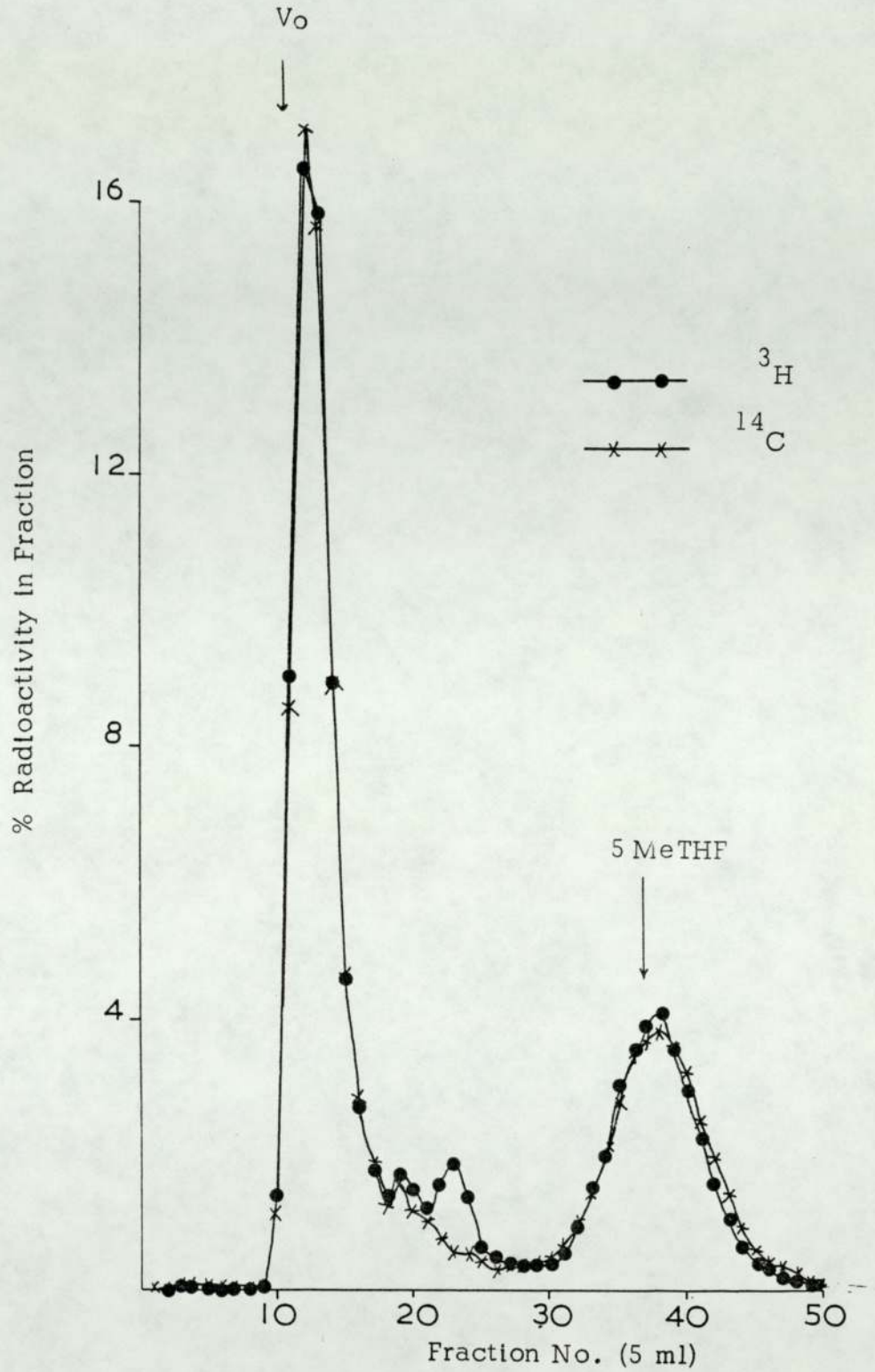


Fig. 3.2.7. Sephadex G15 chromatography of the tumour extract 8h after the administration of labelled folic acid.

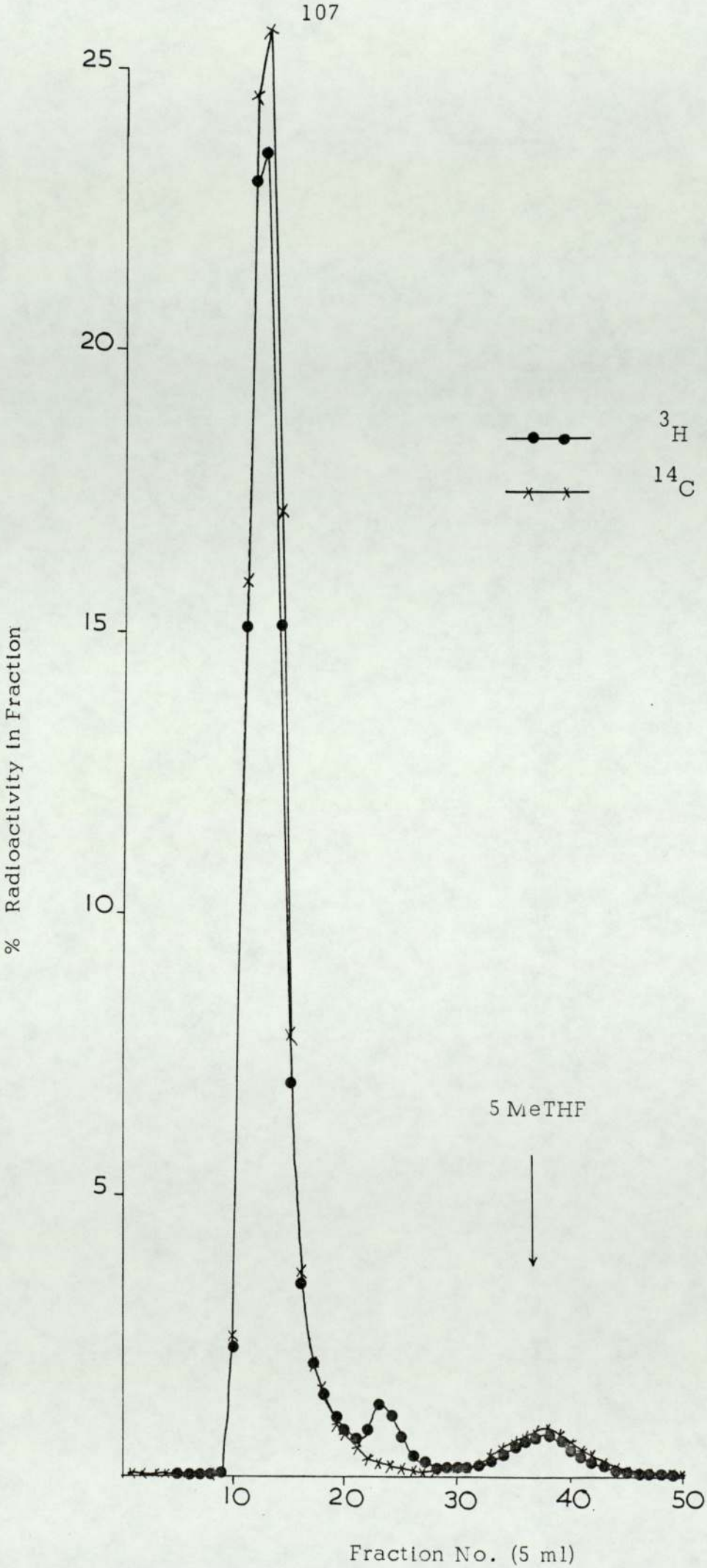


Fig. 3.2.8. Sephadex G15 chromatography of the tumour extract at 24h

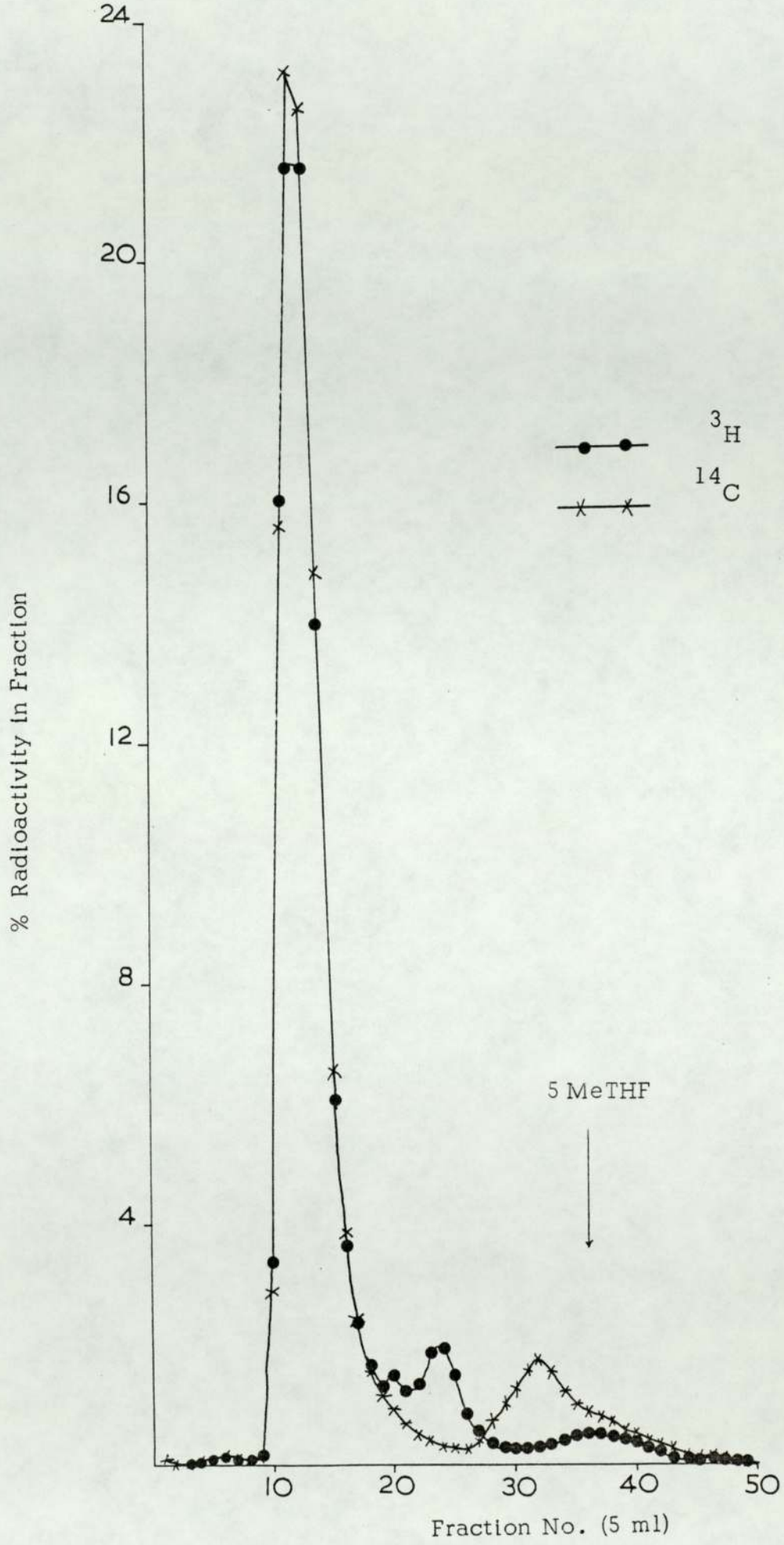


Figure 3.2.9. Sephadex G15 chromatography of the tumour extract at 48h

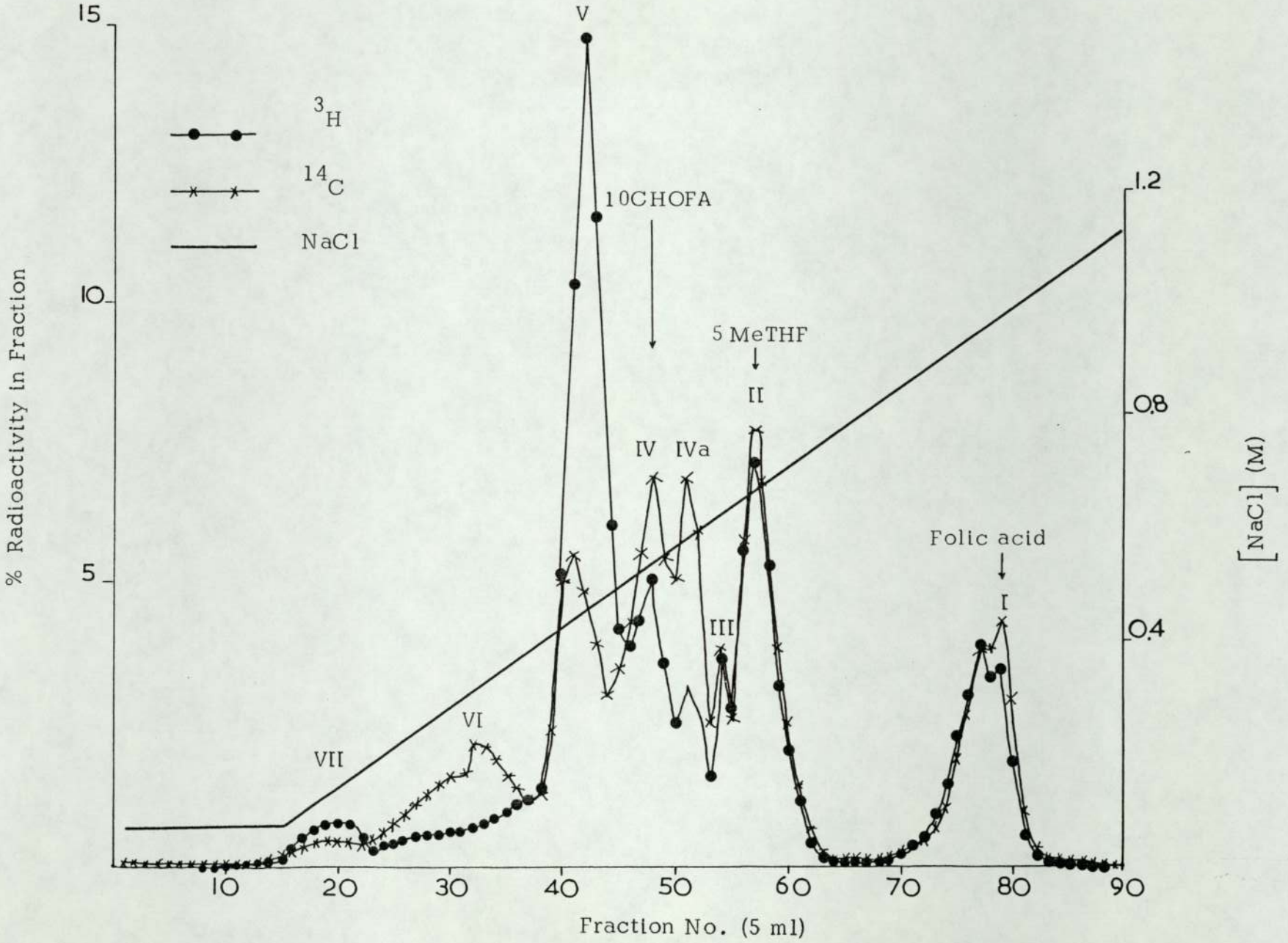


Fig. 3.3.1. DE52 chromatography of urine (8-24h) of normal rats treated with MTX.

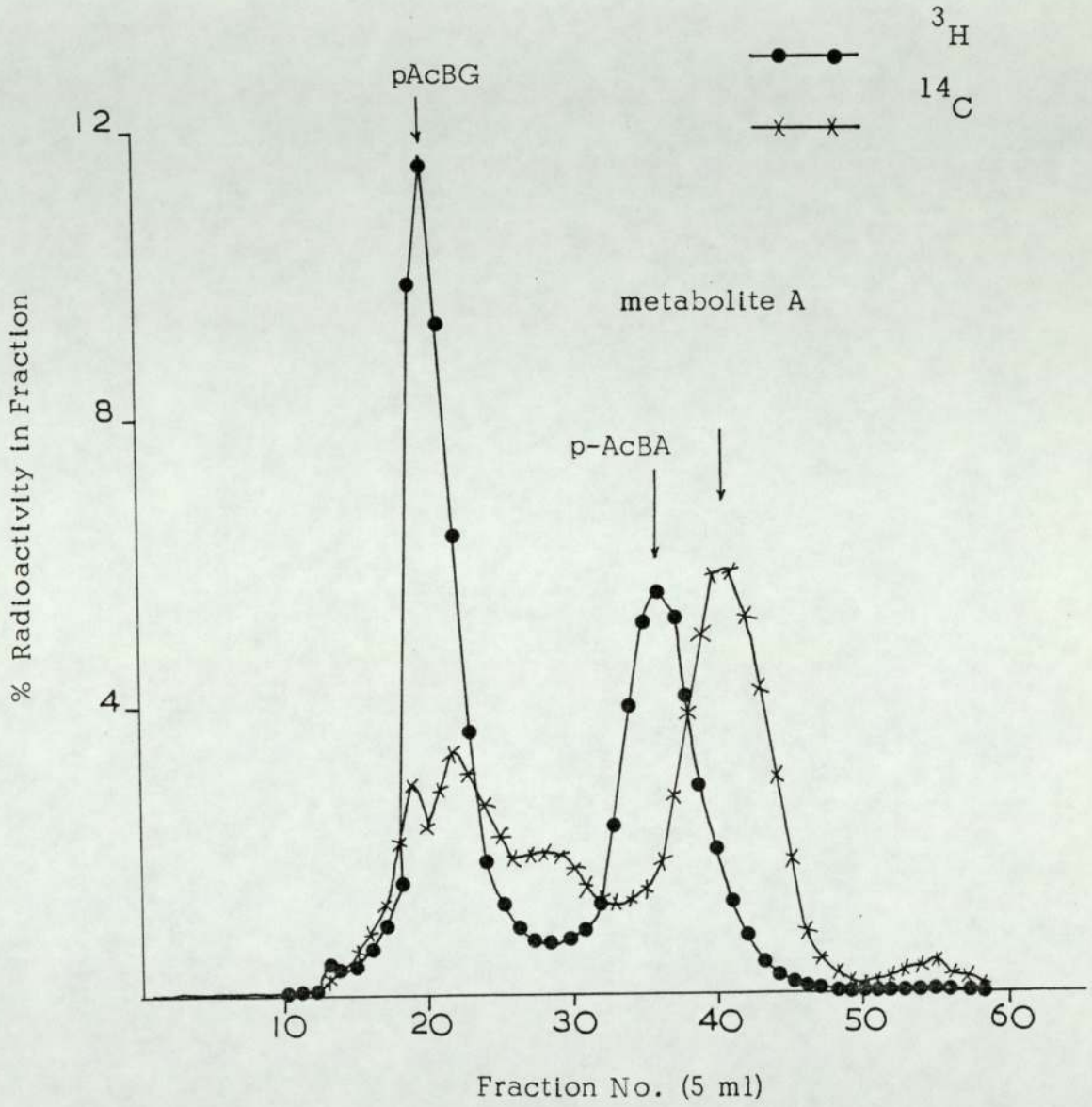


Fig. 3.3.2. Sephadex G15 chromatography of peak V from DE52 chromatography of urine (8-24h) of normal rats treated with MTX

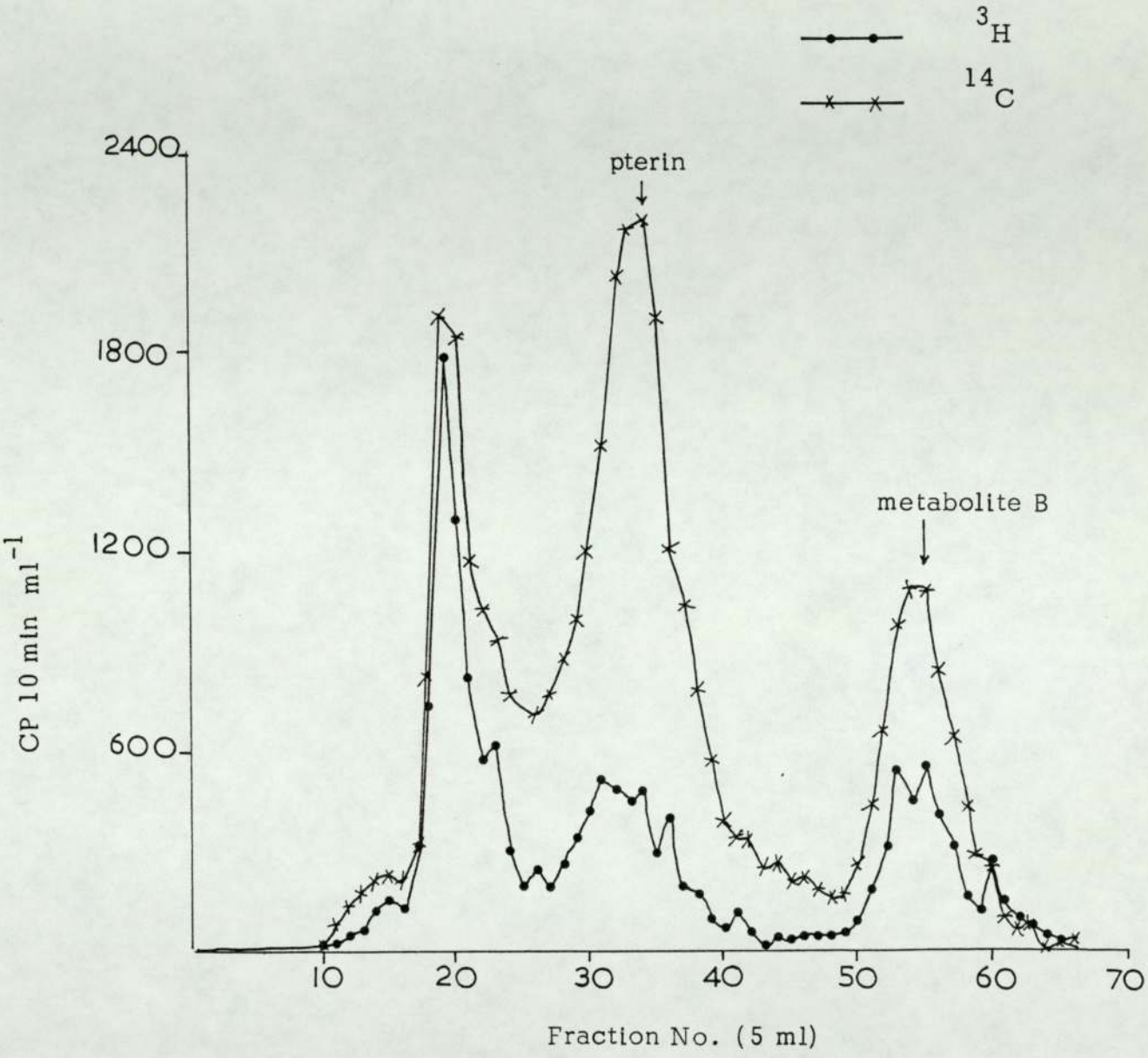


Fig. 3.3.3. Sephadex G15 chromatography of peak VI from DE52 chromatography of the urine (8-24h) of normal rats treated with MTX

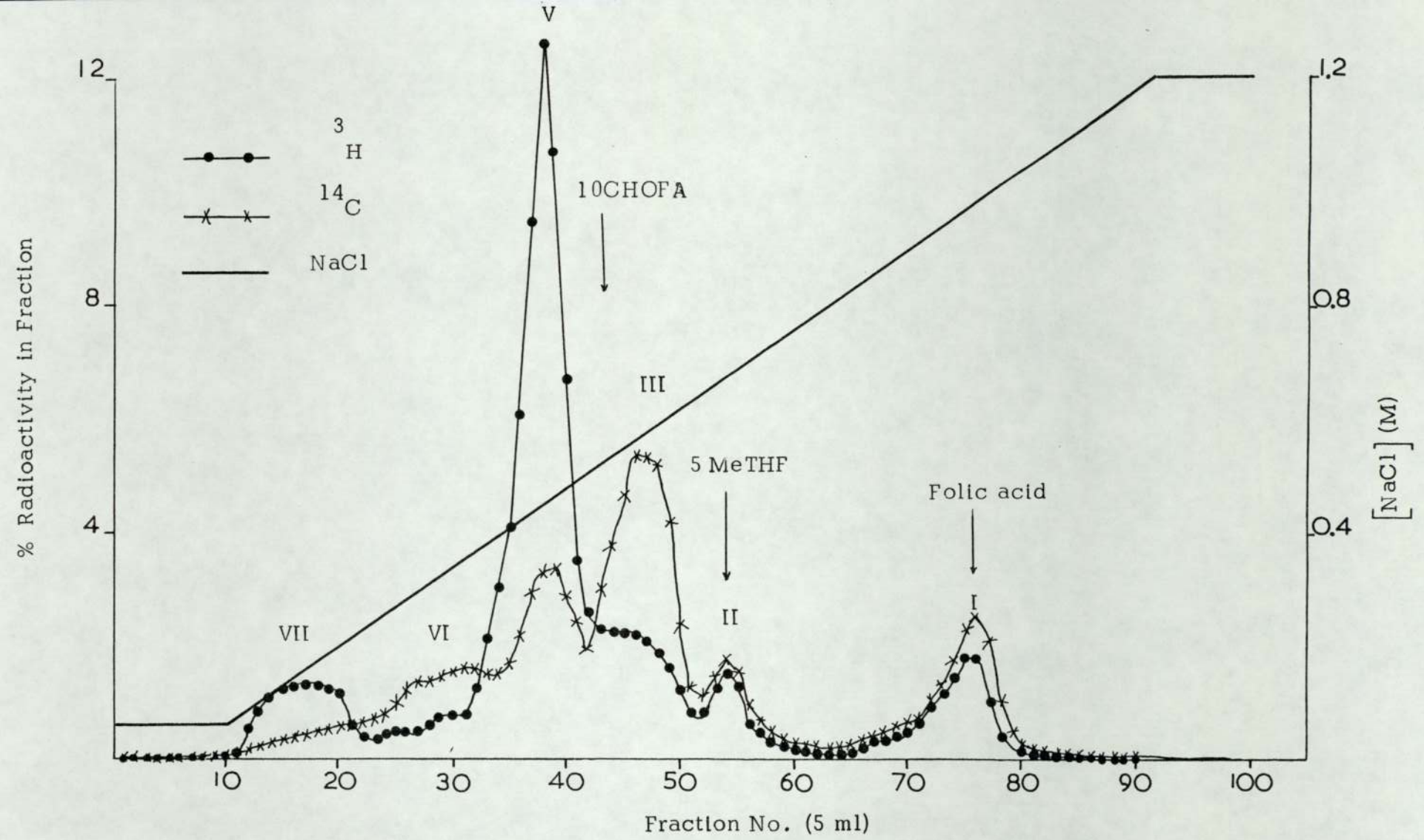


Fig. 3.3.4. DE52 chromatography of urine (24-48h) of normal rats treated with MTX

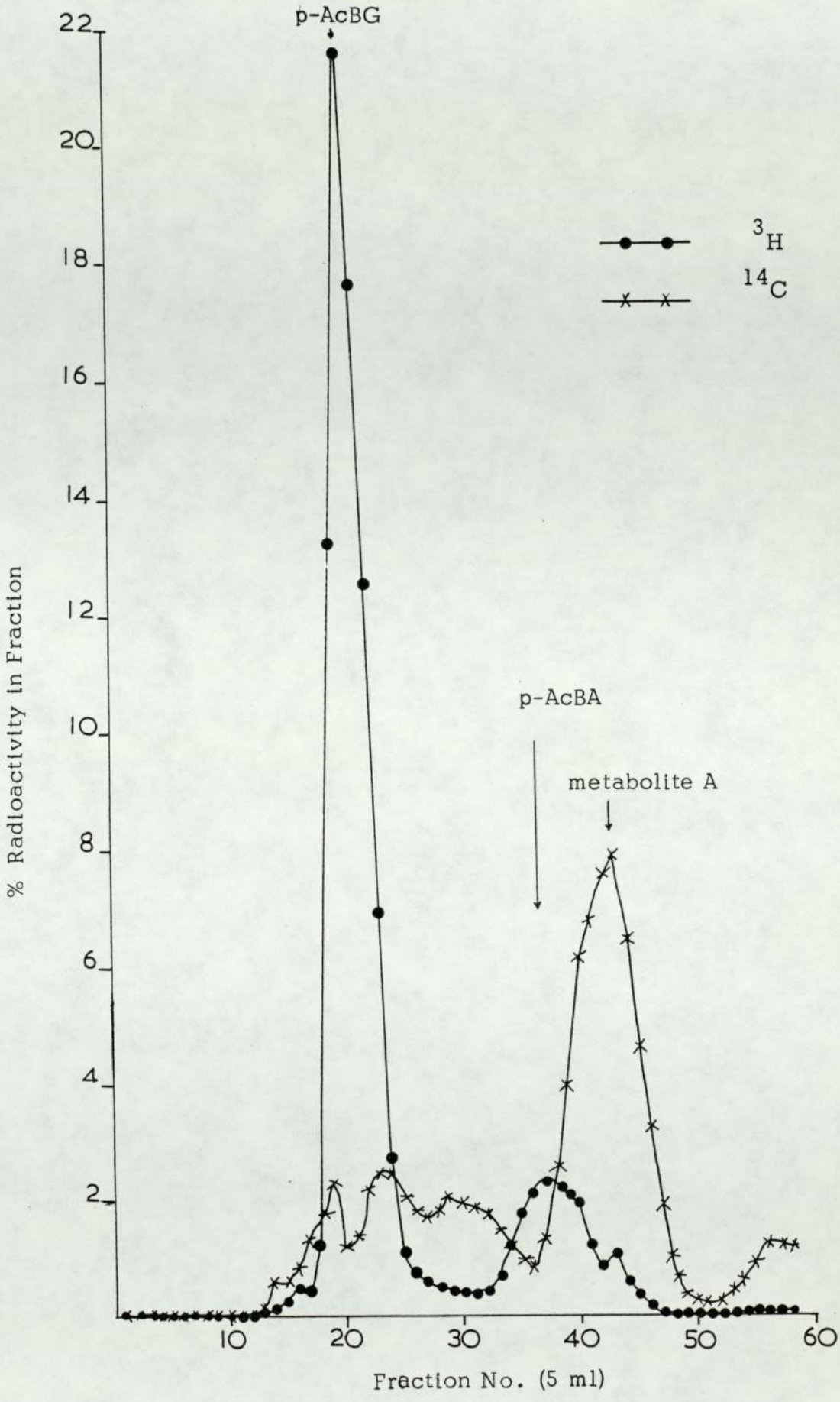


Fig. 3.3.5. Sephadex G15 chromatography of peak V from DE52 chromatography of urine (24-48h) of normal rats treated with MTX.

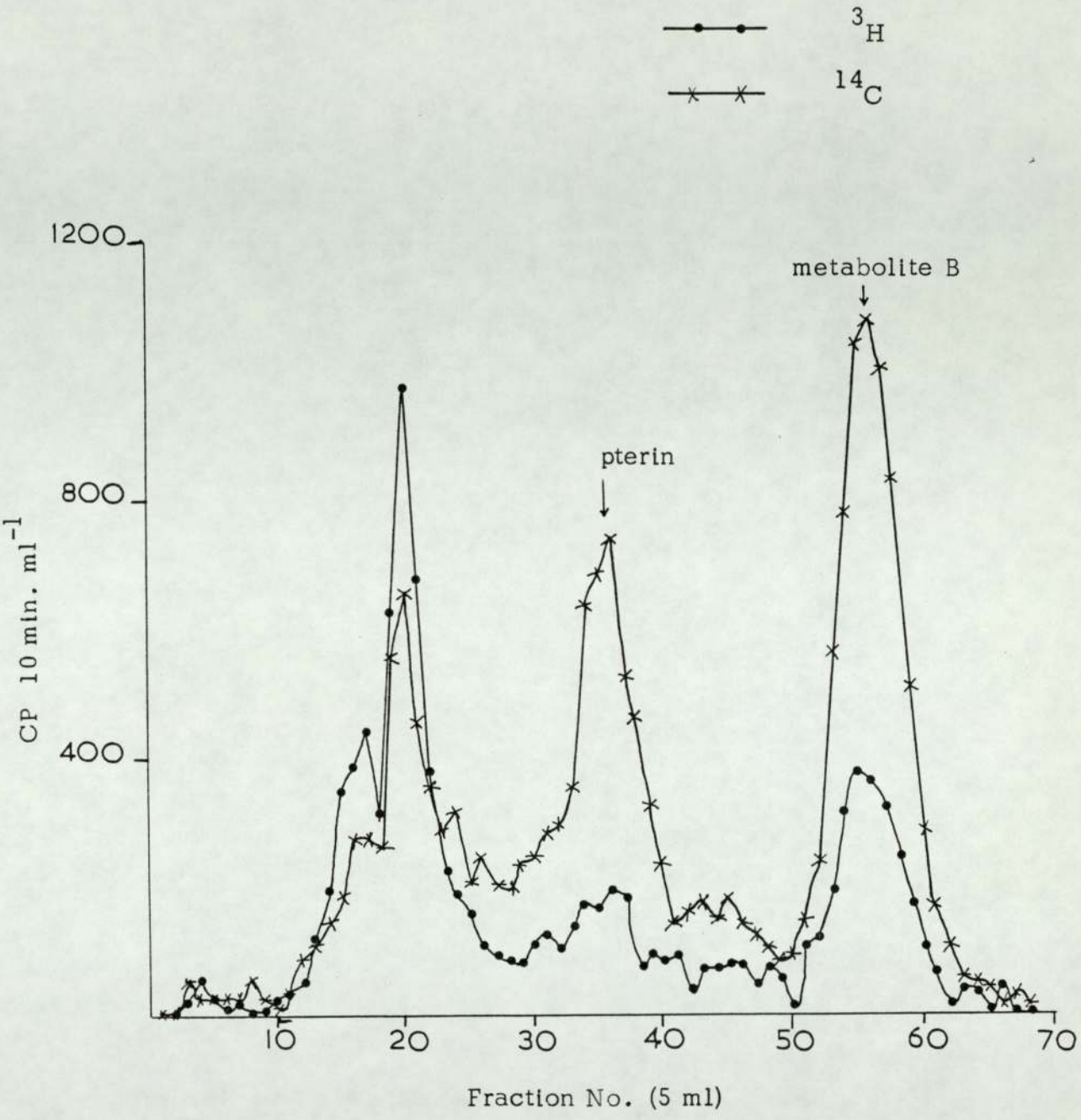


Fig. 3.3.6. Sephadex G15 chromatography of peak VI from DE52 chromatography of urine (24-48h) of normal rats treated with MTX.

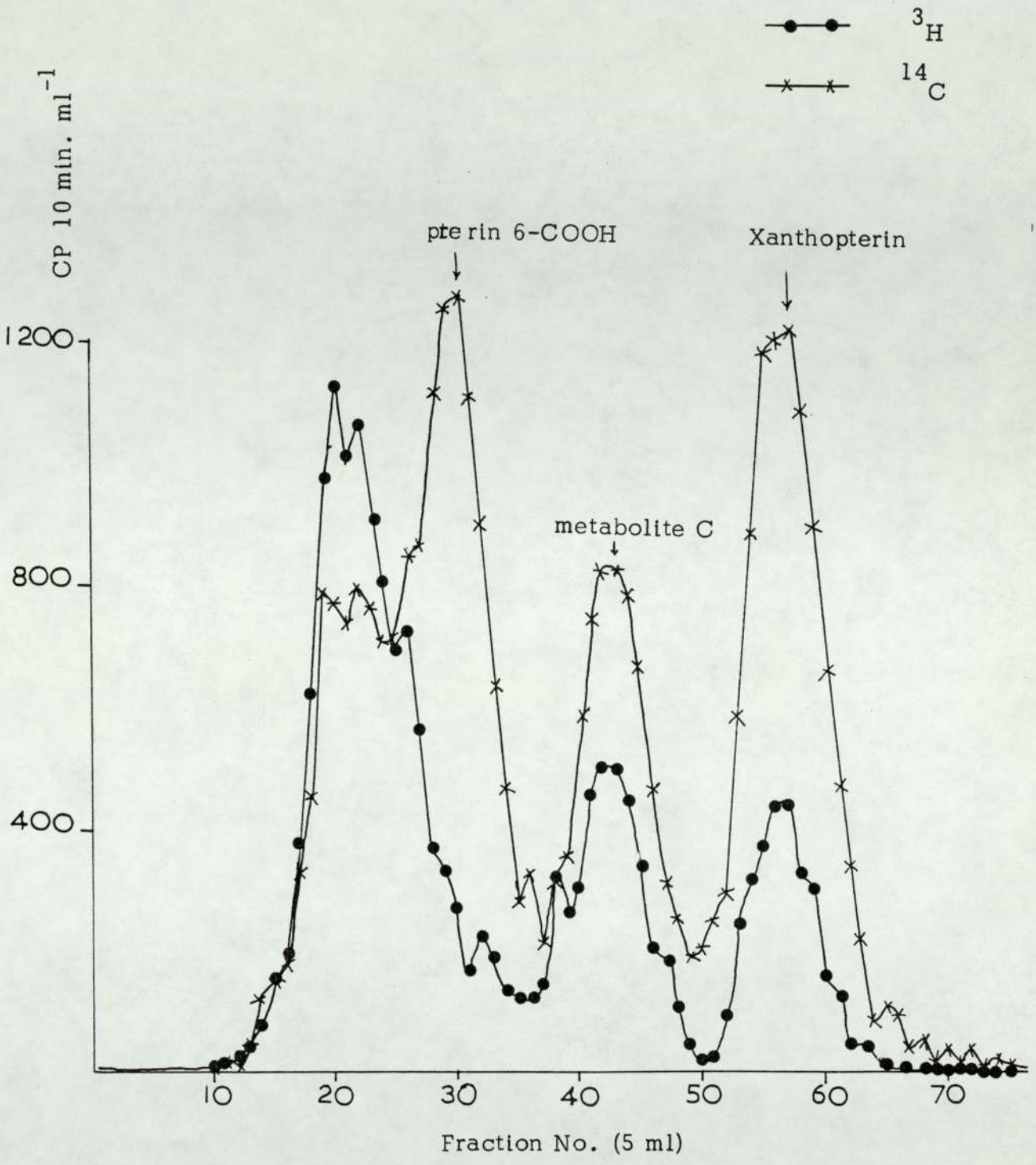


Fig. 3.3.7. Sephadex G15 chromatography of peak III from DE52 chromatography of the urine (24-48h) of normal rats treated with MTX.

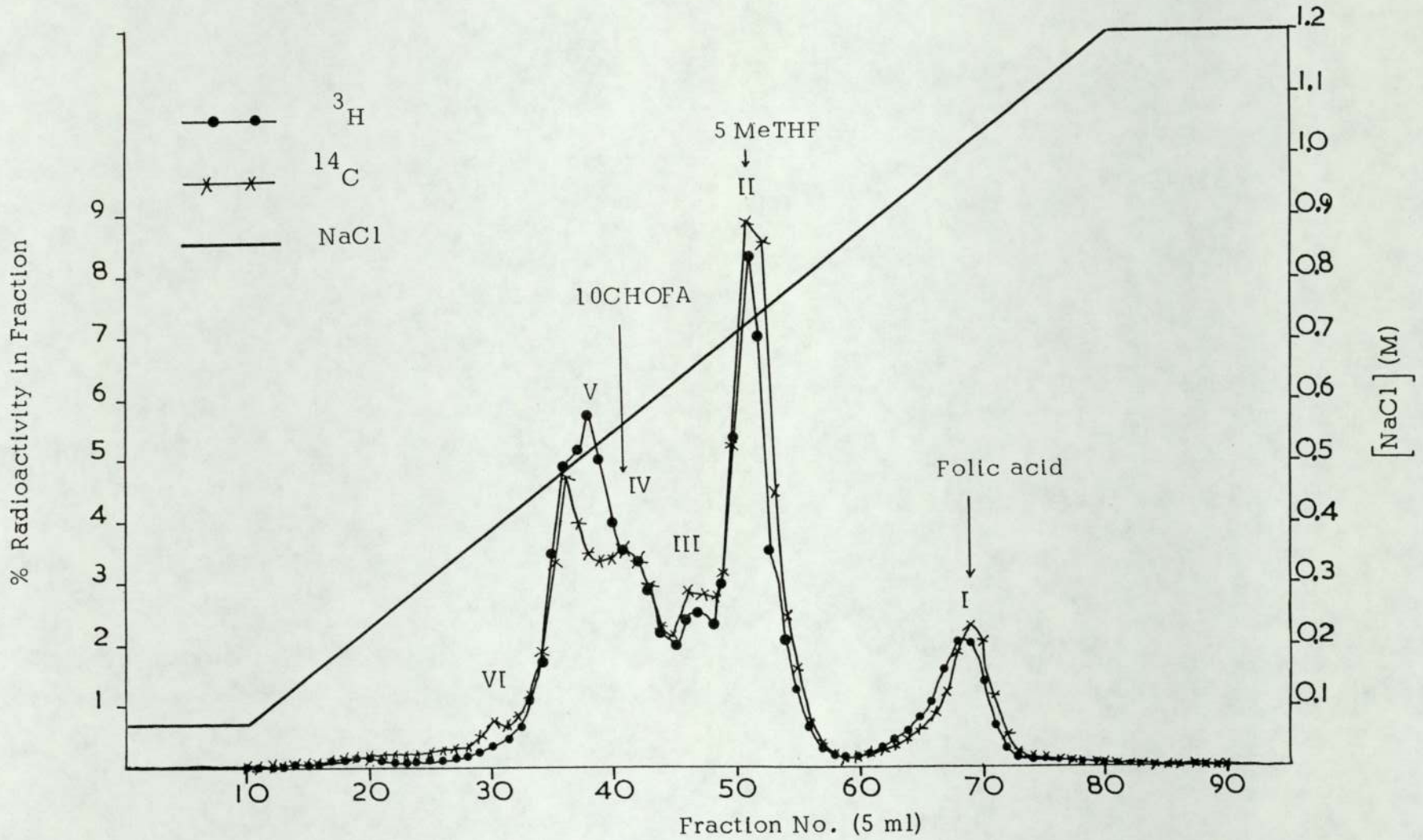


Fig. 3.4.1. DE52 chromatography of the (0-8h) urine sample of normal rats pre-treated with Phenobarbitone.

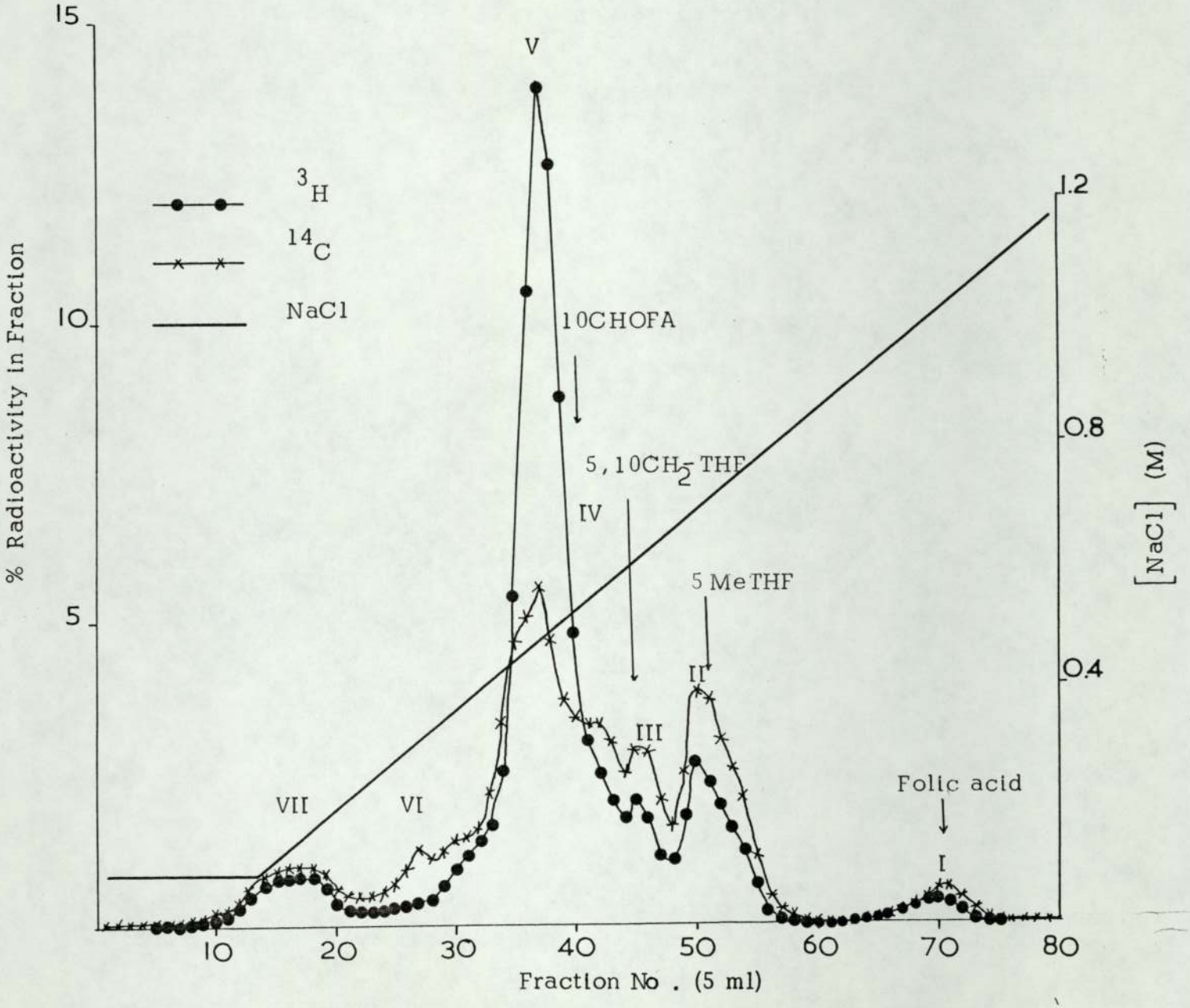


Fig. 3.4.2. DE52 chromatography of the (8-24h) urine sample of normal rat pre-treated with phenobarbitone.

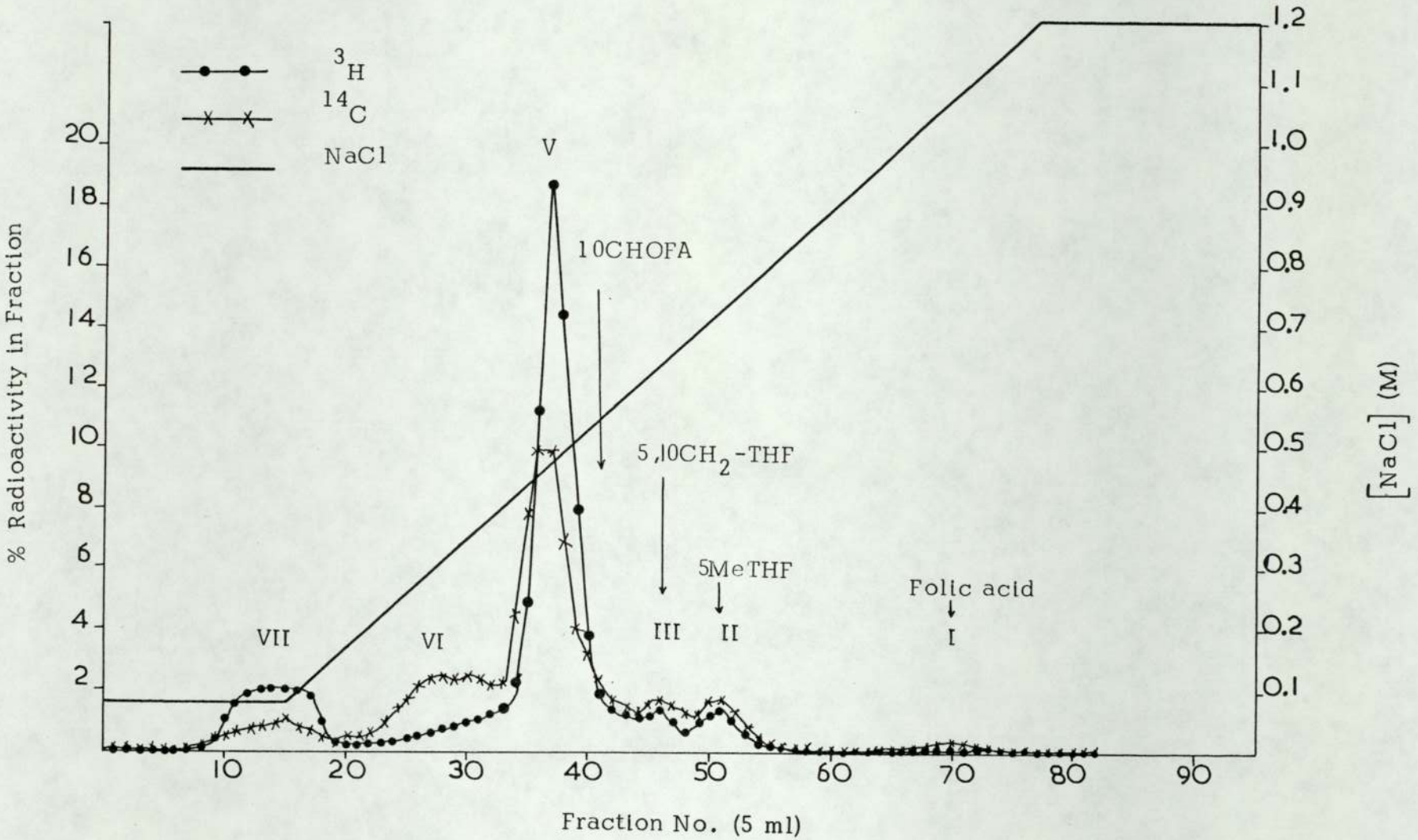


Fig. 3.4.3. DE52 chromatography of the (24-48h) urine sample of normal rat pretreated with phenobarbitone

Table (3.1.1.)

	Mean tissue weight (g)	% Dose radioactivity recovered 8 h ^{3}H	8 h ^{14}C	Mean tissue weight (g)	24 h ^{3}H	24 h ^{14}C	Mean tissue weight (g)	48 h ^{3}H	48 h ^{14}C	0 - 48 h ^{3}H	0 - 48 h ^{14}C
Urine		19.8 ± 2.6	16.7 ± 2.1		6.1 ± 0.9	3.8 ± 0.5		3.0 ± 0.3	2.1 ± 0.2	28.9	22.6
Faeces				2.7 ± 0.3	11.0 ± 0.9	22.1 ± 1.9	3.8 ± 0.4	4.8 ± 0.5	6.2 ± 0.9	15.8	28.3
Liver	9.4 ± 0.2	16.4 ± 0.7	17.5 ± 0.8	6.9 ± 0.3	18.1 ± 0.7	19.1 ± 0.7	7.1 ± 0.5	19.1 ± 0.6	20.7 ± 0.5	19.1	20.7
Gut	6.7 ± 0.3	4.8 ± 0.4	4.5 ± 0.5	4.8 ± 0.3	3.0 ± 0.2	2.7 ± 0.2	4.9 ± 0.3	2.3 ± 0.06	2.0 ± 0.07	2.3	2.0
Kidney	2.2 ± 0.03	3.3 ± 0.1	3.3 ± 0.2	1.7 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.6 ± 0.06	1.6 ± 0.07	1.6	1.6
Total										67.7	75.2

Recovery of radioactivity in the urine, faeces and tissues 8 h, 24 h and 48 h after the administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid to normal rats. The results are expressed as percentage of the dose (mean ± SEM n = 5)

Table 3.1.2.

Metabolite	0 - 8 h		8 - 24 h		24 - 48 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	1.4	1.3	0.1	0.1	0.03	0.04
5 MeTHF	6.9	6.5	0.9	0.6	0.4	0.4
10 CHO Folate	5.8	5.1	1.4	0.9	0.6	0.4
5,10 CH_2-THF	0.9	0.8	0.3	0.2	0.0	0.0
Folate (X)	0.0	0.0	0.5	0.4	0.0	0.0
p-AcABG	2.0	0.0	0.7	0.0	1.0	0.0
p-AcABA	1.7	0.0	1.5	0.0	0.6	0.0
Pterin (A)	0.0	1.4	0.0	0.7	0.0	0.7
Pterin (B)	0.2	1.0	0.1	0.5	0.1	0.3
Urea	0.0	0.6	0.0	0.2	0.0	0.2
$^{3}H_2O$	0.4	0.0	0.2	0.0	0.3	0.0
Total	19.5	16.7	5.7	3.6	3.0	2.0

Metabolites present in the urine of normal rats following the administration of ^{14}C + ^{3}H folic acid. The results are expressed as the percentage of the dose present as each metabolite (100 µg/Kg body wt.).

	Mean tissue Weight (g)	% Dose Radioactivity Recovered 8 h ^{3}H	8 h ^{14}C	Mean tissue weight (g)	24 h ^{3}H	24 h ^{14}C	Mean tissue weight (g)	48 h ^{3}H	48 h ^{14}C	0 - 48 h ^{3}H	0 - 48 h ^{14}C
Urine		$^{+}_{-}$ 12.7 2.4	$^{+}_{-}$ 9.9 1.9		$^{+}_{-}$ 3.0 0.5	$^{+}_{-}$ 2.1 0.3		$^{+}_{-}$ 1.2 0.1	$^{+}_{-}$ 1.0 0.1	16.9	13.0
Faeces				1.3 $^{+}_{-}$ 0.7	$^{+}_{-}$ 3.4 2.0	$^{+}_{-}$ 9.7 5.2	2.1 $^{+}_{-}$ 0.4	$^{+}_{-}$ 0.9 0.2	$^{+}_{-}$ 1.0 0.2	4.3	10.7
Liver	10.4 $^{+}_{-}$ 0.3	$^{+}_{-}$ 13.4 1.1	$^{+}_{-}$ 12.6 1.0	8.4 $^{+}_{-}$ 0.2	$^{+}_{-}$ 16.8 0.9	$^{+}_{-}$ 15.7 0.9	8.8 $^{+}_{-}$ 0.5	$^{+}_{-}$ 16.5 1.3	$^{+}_{-}$ 16.8 1.3	16.5	16.8
Tumour	17.2 $^{+}_{-}$ 1.7	$^{+}_{-}$ 7.9 0.9	$^{+}_{-}$ 8.6 1.2	19.4 $^{+}_{-}$ 3.0	$^{+}_{-}$ 9.3 1.1	$^{+}_{-}$ 9.3 1.0	20.0 $^{+}_{-}$ 2.8	$^{+}_{-}$ 7.0 1.0	$^{+}_{-}$ 7.6 1.2	7.0	7.6
Gut	6.3 $^{+}_{-}$ 0.3	$^{+}_{-}$ 2.8 0.3	$^{+}_{-}$ 2.7 0.3	5.4 $^{+}_{-}$ 0.2	$^{+}_{-}$ 2.7 0.3	$^{+}_{-}$ 2.5 0.2	5.5 $^{+}_{-}$ 0.2	$^{+}_{-}$ 2.0 0.2	$^{+}_{-}$ 1.7 0.2	2.0	1.7
Kidney	2.5 $^{+}_{-}$ 0.1	$^{+}_{-}$ 3.3 0.4	$^{+}_{-}$ 3.0 0.4	2.2 $^{+}_{-}$ 0.1	$^{+}_{-}$ 1.6 0.1	$^{+}_{-}$ 1.6 0.1	2.3 $^{+}_{-}$ 0.1	$^{+}_{-}$ 1.6 0.1	$^{+}_{-}$ 1.6 0.1	1.6	1.6
Total										48.3	51.4

Table (3.2.1) Recovery of radioactivity in the urine, faeces and tissues 8h, 24h and 48h after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ -folic acid to tumour-bearing rats. The results are expressed as percentage of the dose (mean $^{+}_{-}$ SEM, n = 5).

Table 3.2.2.

Metabolite	0 - 8 h		8 - 24 h		24 - 48 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	4.2	3.5	0.4	0.4	0.04	0.06
5 MeTHF	3.2	2.7	0.3	0.3	0.05	0.08
10 CHOFA	1.8	1.5	0.4	0.3	-	-
5,10 CH_2THF	0.6	0.5	-	-	-	-
Folate (X)	0.7	0.7	0.1	0.1	-	-
p-AcBA	0.7	-	0.7	-	0.25	-
p-AcBG	1.3	-	0.7	-	0.54	-
Pterin A		0.6		0.6		0.47
Pterin B	0.1	0.4	0.1	0.2	0.04	0.14
Urea	-	0.1	-	0.1		0.08
$^{3}H_2O$	0.1	-	0.2	-	0.17	
TOTAL	12.7	10.0	2.9	2.0	1.1	0.9

Metabolites present in the urine of tumour rats following the administration of ^{14}C + ^{3}H folic acid (100 ug/Kg body wt.). The results are expressed as the percentage of the dose present as each metabolite.

Table (3.3.1) Recovery of radioactivity in the urine, faeces and tissues 8 h, 24 h and 48 h after the administration of a mixture of $[2-^{14}C]$ and $[3',5',7,9-^{3}H]$ - folic acid and MTX (100 mg/Kg body wt.) 8h and 24 h after the administration of labelled folic acid to normal rats. The results are expressed as a percentage of the dose.

	Mean tissue Weight (g)	8 h		Mean tissue Weight (g)	24 h		Mean tissue Weight (g)	48 h		0 - 48 h	
		^{3}H	^{14}C		^{3}H	^{14}C		^{3}H	^{14}C	^{3}H	^{14}C
Urine		14.4 ±1.6	11.7 ±1.6		13.9 ±1.3	11.4 ±0.8		7.0 ±0.4	5.6 ±0.3	35.3	28.7
Faeces		-	-	*0.5±0.2	5.3 ±1.8	10.2 ±3.9	*0.4±0.2	0.7 ±0.4	0.8 ±0.5	6.0	11.0
Liver	9.2±0.6	14.9 ±1.2	13.9 ±1.3	7.3±0.6	13.6 ±0.9	13.9 ±0.9	7.3±0.5	12.0 ±0.7	12.4 ±0.9	12.0	12.4
Gut	6.1±0.1	3.7 ±0.3	3.5 ±0.3	5.2±0.4	2.4 ±0.1	1.9 ±0.1	4.3±0.1	1.0 ±0.1	0.8 ±0.1	1.0	0.8
Kidney	*0.7±0.06	2.5 ±0.2	2.1 ±0.2	*0.6±0.05	1.4 ±0.1	1.2 ±0.1	*0.6±0.05	1.1 ±0.1	1.0 ±0.1	1.1	1.0
Total										55.4	53.9

(mean ± SEM n = 5) * mean of dry weight ± SEM

Table 3.3.2. Metabolites present in the urine of normal rats following the administration of [2-^{14}C] + [3',5',7,9-^{3}H] folic acid (100 µg/Kg body wt) and methotrexate (100 mg/Kg body wt) after 8h and 24h. The results are expressed as the percentage of the dose present as each metabolite.

Metabolite	% dose recovered as each metabolite							
	0 - 8 h		8 - 24 h		24 - 48 h		Total	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	0.7	0.7	2.0	1.8	0.7	0.9	3.4	3.4
5MeTHF	6.0	5.1	2.6	2.3	0.4	0.5	9.0	7.9
10CHOFA	5.3	4.2	1.7	1.4	0.6	0.4	7.6	6.0
5,10CH$_2$THF	0.7	0.6	0.7	0.7	-	-	1.4	1.3
p-AcBA	0.5	-	2.2	-	0.7	-	3.4	-
p-AcBG	0.5	-	2.9	-	3.0	-	6.4	-
Metabolite A	+	0.9	+	1.0	+	0.5	-	2.4
Metabolite B	0.1	0.4	0.1	0.7	0.1	0.5	0.3	1.6
Metabolite C	-	-	0.1	0.2	0.2	0.5	0.3	0.7
Pterin	-	-	0.1	0.7	0.03	0.3	0.1	1.0
Pterin-6-COOH	-	-	+	1.4	+	0.7	+	2.1
Xanthopterin	-	-	0.2	0.7	0.1	0.6	0.3	1.3
$^{3}H_2O$	0.2	-	0.4	-	0.7	-	1.3	-
Urea	-	0.2	-	0.2	-	0.3	-	0.7

+ ^{3}H was associated with the metabolites but the precise recovery could not be calculated

	Mean tissue weight (g)	% Dose Radioactivity Recovered 8 h ^{3}H	8 h ^{14}C	Mean tissue weight (g)	24 h ^{3}H	24 h ^{14}C	Mean tissue weight (g)	48 h ^{3}H	48 h ^{14}C	0 - 48 h ^{3}H	0 - 48 h ^{14}C
Urine		30.2 ± 2.4	25.6 ± 2.1		7.3 ± 0.7	4.7 ± 0.5		2.9 ± 0.2	1.9 ± 0.1	40.4	32.2
Faeces				1.1±0.4	4.6 ± 1.5	6.9 ± 2.3	1.6±0.7	1.7 ± 0.9	1.8 ± 0.9	6.3	8.7
Liver	10.0±0.5	20.4 ± 0.9	21.3 ± 0.8	8.5±0.2	21.4 ± 1.6	22.9 ± 1.3	8.5±0.3	23.6 ± 1.4	25.6 ± 1.5	23.6	25.6
Gut	6.3±0.3	4.2 ± 0.3	4.3 ± 0.3	5.4±0.2	3.5 ± 0.3	3.4 ± 0 3	5.2±0.2	2.8 ± 0.06	2.8 ± 0.07	2.8	2.8
Kidney	2.3±0.1	3.2 ± 0.2	3.4 ± 0.2	2.0±0.08	2.1 ± 0.06	2.2 ± 0.07	1.9±0.01	2.1 ±0.02	2.1 ± 0.04	2.1	2.1
Total										75.2	71.4

Table (3.4.1) Recovery of radioactivity in the urine, faeces and tissues 8h, 24 h and 48h after the administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ - folic acid to normal rats initially pretreated with phenobarbitone (20 mg per rat) for three days.
The results are expressed as a percentage of the dose (mean ± SEM n = 5)

• n = 4

Metabolites present in the urine of rats pretreated with phenobarbitone following the administration of ^{14}C and ^{3}H folic acid (100 µg/Kg body wt.). The results are expressed as the percentage of the dose as each metabolite

Table 3.4.2.

Metabolite	0 - 8 h		8 - 24 h		24 - 48 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	3.6	3.2	0.2	0.2	-	-
5 MeTHF	9.9	9.3	1.0	1.0	0.2	0.2
10 CHOFA	8.1	7.4	0.7	0.6	-	-
5,10 CH_2THF	2.1	2.0	0.5	0.5	0.1	0.1
p-AcBG	3.6	-	1.6	-	1.1	-
p-AcBA	1.7	-	2.7	-	0.8	-
Pterin A		2.5		0.9		1.0
Pterin B	0.2	0.8	0.1	0.5	0.1	0.4
Urea	-	T	-	0.4		0.2
$^{3}H_2O$	T		0.4	-	0.4	-
TOTAL	29.0	25.2	7.1	4.1	2.6	1.9

T = trace amount

Table 3.5.1 The excretion of the tritiated catabolites (p-AcBG and p-AcBA) in the urine of rats dosed with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid. The results are expressed as a percentage of the dose. (Values in parenthesis refer to the estimated % of the dose catabolised)

Group	8 - 24 h		24 - 48 h		8 - 48 h	
	p-AcBG	p-AcBA	p-AcBG	p-AcBA	p-AcBG	p-AcBA
Control rat	0.7	1.5	1.0	0.6	1.7	2.1
	(1.6)	(3.5)	(2.4)	(1.4)	(4.0)	(4.9)
Tumour-bearing rat	0.7	0.7	0.5	0.3	1.2	1.0
	(1.6)	(1.6)	(1.2)	(0.7)	(2.8)	(2.3)
MTX treated rat	2.9	2.2	3.0	0.7	5.9	2.9
	(6.8)	(5.2)	(7.1)	(1.6)	(13.9)	(6.8)
Phenobarbitone treated rat	1.6	2.7	1.1	0.8	2.7	3.5
	(3.8)	(6.4)	(2.6)	(1.9)	(6.4)	(8.3)

Table (3.5.2) Radiolabelled metabolites excreted in the urine of rat with implanted Mc/103 sarcoma 0-8 h, 8-24 h and 24-48 h after the administration of ^{3}H and ^{14}C folic acid (100 µg/Kg body wt.). The results are expressed as a percentage of normal rat metabolite level.

	0 - 8 h		8 - 24 h		24 - 48 h		Total	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	300	269	400	400	T	T	306	286
5MeTHF	46	42	33	50	25	25	44	41
10CHO-folates	31	29	29	33	0.0	0.0	28	28
5,10CH_2-THF	67	63	0.0	0.0	n.d	n.d	50	50
Folate (X)	*	*	20	25	n.d	n.d	160	200
p-AcBA	41	-	47	-	50	-	45	-
p-AcBG	65	-	100	-	50	-	68	-
Metabolite A	-	43	-	86	-	71	-	61
Metabolite B	-	40	-	40	-	33	-	41
$^{3}H_2O$	25	-	100	-	67	-	56	-
Urea	-	17	-	50	-	50	-	30

n.d = not detected in both groups of rats

* = not detected in normal rat

T = trace amount

Table (3.5.3) Radiolabelled metabolites excreted in the urine of rats following the administration of labelled folic acid (100 µg/Kg body wt) and MTX (100 mg/Kg body wt) after 8 and 24 h. The results are expressed as a percentage of normal rat metabolite level.

	8 - 24 h		24 - 48 h		8 - 48 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	2000	1800	*	*	2700	2700
5MeTHF	289	383	100	125	230	280
10CHO folates	121	156	100	100	115	138
5,10CH_2-THF	233	350	n.d	n.d	233	350
p-AcBA	147	-	116	-	138	-
p-AcBG	414	-	300	-	347	-
Metabolite A	-	143	-	71	-	107
Metabolite B	-	140	-	167	-	150
Metabolite C	-	*	-	*	-	*
pterin	-	*	-	*	-	*
pterin-6-COOH	-	*	-	*	-	*
Xanthopterin	-	*	-	*	-	*
$^{3}H_2O$	200	-	233	-	220	-
Urea	-	100	-	150	-	125

n.d. = not detected in both groups

* = not detected in control rat

Table (3.5.4) Radiolabelled metabolites excreted in the urine of rats pretreated with phenobarbitone, collected 8 h, 24 h and 48 h after the administration of ^{3}H and ^{14}C folic acid (100 µg/Kg body wt). The results are expressed as a percentage of normal rat metabolite level.

	0 - 8 h		8 - 24 h		24 - 48 h		0 - 48 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	257	246	200	200	n.d	n.d	248	243
5MeTHF	143	143	111	167	50	50	135	140
10CHO folates	140	145	50	67	n.d	n.d	113	125
5,10CH_2-THF	233	250	167	250	*	*	225	260
p-AcBA	18	-	180	-	133	-	136	-
p-AcBG	180	-	229	-	110	-	170	-
Metabolite A	-	179	-	129	-	142	-	157
Metabolite B	100	125	100	100	100	133	100	94
$^{3}H_2O$	T	-	200	-	133	-	89	-
Urea	-	T	-	200	-	100	-	60

n.d = not detected in rat pretreated with phenobarbitone

* = not detected in control rat

T = trace amount

Table (3.5.5) Excretion of the catabolite of folate polyglutamates. The results are given as the percentage of tissue radioactivity excreted as p-AcBG

Animal Group	% ^3H of the dose found in tissues after 8h Y	% ^3H dose excreted as pAcBG at 8-24h X	% retained radio-activity excreted as pAcBG $\frac{X}{Y}$ x100	% ^3H of the dose found in tissues after 24 h Y_1	% ^3H dose excreted as pAcBG at 24-48 h X_1	% retained radio-activity excreted as pAcBG $\frac{X_1}{Y_1}$ x100	% ^3H of the dose excreted as pAcBG of 8-48h X_2	% retained radio-activity excreted as p-AcBG $\frac{X_2}{Y}$ x 100
Control rat	24.5	0.7	2.9	22.8	1.0	4.4	1.7	6.9
Tumour-bearing rat	27.4	0.7	2.6	30.4	0.5	1.6	1.2	4.5
MTX treated rat	21.1	2.9	13.7	17.4	3.0	17.2	5.9	28.0
Phenobarbitone treated rat	27.8	1.6	5.8	27.0	1.1	4.1	2.7	9.7

CHAPTER 4

LONG TERM METABOLISM OF [2-^{14}C] AND [3',5',7,9-^{3}H] FOLIC ACID IN THE RAT

The regulation and turnover of the folate co-enzymes are complex and can be affected by several factors. Little experimental data is available in this area because of the difficulty in setting up such a study. However, the relative maximal activities *in vitro* of a number of the folate pathway enzymes have been reported using different cell lines (Rowe, 1978; Nixon *et al.*, 1973, Jackson and Harrap, 1973). Nevertheless, the situation *in vivo* may differ and moreover these experiments were carried out over very short time periods (minutes or hours) and therefore they cannot be used to predict the long term fate of the retained folates.

The existence of two metabolically distinct folate pools has been reported by several investigators (Blair, 1976, Krumdieck *et al.*, 1978, Pheasant *et al.*, 1981), a short term pool corresponding to newly absorbed folate and a long term pool representing the retained tissue folates. Extensive studies have been carried out to elucidate the nature of the short term pool, however, only contradictory reports have been published concerning the long term pool (Barford *et al.*, 1977, 1978, Murphy *et al.*, 1976, Krumdieck *et al.*, 1978).

It has been assumed for a number of years that folate polyglutamates act as a storage form for the folate monoglutamates which function as coenzymes. When the catabolic route of folate was recognised the possibility arose that this was not so and that the incorporation of folate monoglutamates into folate polyglutamates was one way process (Connor,

1979).

The present chapter describes the metabolic fate of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid in the rat up to 22 days with more emphasis on the metabolites appearing in the urine up to 15 days since different catabolites have been suggested to be derived from each pool. Data on the biological half-life of retained radio-labelled folate and an assessment of the contributions of urinary and faecal losses are also obtained.

Materials and Methods

18 Male syngeneic WAB/NOT rats (190-225 g body wt.) were dosed orally with a mixture of 2μCi [2-^{14}C] and 5 μCi [3',5',7,9-^{3}H]- folic acid. Urine samples were collected from 5 animals daily as described in Chapter 2 for up to 13 days and from 4 animals at 14 and 15 days after the administration of labelled folic acid. Collection flasks of faeces were changed at 1, 3, 5, 7, 9 and 11 days after the administration of folic acid. Urine samples were pooled as follows: days 1, (2+3), (4+5), (6+7), (8+9) and (10+11) and stored frozen until required when they were examined by DE52-chromatography, Sephadex G15 and paper chromatography.

*Liver analysis

At 1, 3, 5, 7, 9, 11, 13, 15 and 22 days after the administration of labelled folic acid, two rats at each time period were killed and their livers

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* These experiments were performed at the same time by J. Bates.

removed and extracted as described in Chapter 3. Crude liver extracts were chromatographed on Sephadex G15 and the levels of folate polyglutamates measured.

Results

Table 4.1. summarises the urinary recovery of radioactivity. More ^{3}H than ^{14}C was excreted in the urine at all times except days 11 and 14 where the recovery of ^{3}H was equal to ^{14}C. The differences in the recovery of ^{3}H and ^{14}C were statistically significant for the first 6 days only, when compared in a paired t test ($p < 0.001$ for days 1, 2 and 3, $p < 0.05$ for days 4, 5 and 6). The maximal urinary excretion of radioactivity occurred in the first day, declined rapidly in the second day, decreased slowly up to day 7 and became fairly constant thereafter up to day 10 when there was a small decrease in the level of radioactivity which again became constant thereafter. Faecal recovery of radioactivity is shown in Table 4.2. At all time periods more ^{14}C than ^{3}H was present in the faeces. The difference between the recovery of ^{3}H and ^{14}C at all times was statistically significant when compared in a paired t test ($p < 0.001$). Table 4.3 shows the radioactivity retained in the body calculated by subtracting the percentage of the dose appearing in the urine and faeces from 100%. It shows a rapid decline in the radioactivity in the first day and a slow decrease thereafter. This suggests the decay of two forms of folates with markedly different biological half-lives, a half-life of approximately 11.3 days for the long-term component and about

one day for the short-lived component (figure 4.1).

The hepatic recovery of administered radioactivity is presented in Table 4.4. The hepatic levels of radioactivity were maximal on the third day and then declined with a half-life of approximately 7 days (Figure 4.2). No significant difference was observed in the recovery of ^{3}H and ^{14}C in the liver.

Urinary metabolites

Urine samples were subjected to sequential chromatography on DE52, Sephadex G15, paper chromatography and in some cases peaks from Sephadex G15 were re-chromatographed again on DE52. This revealed the same metabolites as seen in the urine of control rats (Chapter 3) including, folic acid, 5MeTHF, 5,10CH_2-THF, 10CHOFA, pAcBG, pAcBA metabolite A, metabolite B, urea and $^{3}H_2O$. DE52 chromatography of urine sample of day one (Figure 4.3) represented a complex mixture of all these metabolites as before. DE52 chromatography of urine samples of days (2+3) (Figure 4.4) showed, the disapperance of 5,10CH_2-THF, decreased amounts of other intact folates and the relative increased amounts of catabolites which dominated this sample.

Later urine samples had lower radioactivity therefore large volumes of urine samples were used for chromatographic analysis to compensate for the low radioactivity. In some cases, the sample volume exceeded the void volume of the column used (DE52 columns only) which resulted in loss

of some of the radioactivity from the column during loading of the sample and before starting the salt gradient. However, the eluant was collected during sample loading and counted for radioactivity. DE52 chromatography of the urine samples of days (4+5) (Figure 4.5) and (6+7) showed a very similar pattern, a small amount of 5 MeTHF and a very wide peak which was resolved into several peaks on Sephadex G15. The components of the major peak were identified by co-chromatography with authentic standards in both column systems as 10 CHOFA, p-AcBG and p-AcBA together with the unidentified metabolites A and B. A high proportion of urinary radioactivity was detected as $^{3}H_2O$. The patterns of DE52 chromatography of urine samples of days (8+9) (Figure 4.6) and days (10+11) were quite similar and showed that the most of the radioactivity in the urine eluted as a single peak. Sephadex G15 resolved this peak to several metabolites. These metabolites were largely scission products in particular p-AcBG and pterin A. 10 CHOFA and an oxidation product of 5 MeTHF (a Triazine derivative) were present also.

Table 4.5. summarises the relative distribution of each metabolite appearing in the urine up to day 11. In day one, intact folates dominated the urinary radioactivity in particular, 5 MeTHF and 10 CHOFA, however, they declined rapidly in the urine of days (2+3) and the scission products became the dominant components. No folic acid was detected in the urine after the third day and 5 MeTHF and 10 CHOFA were the only intact folates present in the urine. However from the 8th day after the administration

of labelled folic acid, 5 MeTHF was not present but its oxidation product (Triazine) was detected. The estimation of the individual amounts of 10CHOFA and triazine was difficult due to their overlapping peaks on both column systems used, therefore, the total amount of these was calculated instead. Their excretion remained fairly constant up to day 11. The amount of p-AcBA dropped sharply from 1.5% ^{3}H of the dose at days (2+3) to 0.3% ^{3}H of the dose at days (4+5) and remained constant at this level up to day 9. The amount of metabolite B decreased with time and was detected as a trace amount only in the late urine samples. Also the level of metabolite A decreased with time, however, it was still the major ^{14}C-catabolite at all time periods. In days (10+11) urine samples, the levels of all catabolites decreased except $^{3}H_2O$ which represented a high proportion of urinary radioactivity throughout in particular in the late urine samples.

Liver extracts

Sephadex G15 chromarography of crude tissue extracts demonstrated a similar pattern of radiolabelled derivatives at all times. Folate polyglutamates were the predominant radiolabelled derivatives eluting as a single peak close to void volume.

Discussion

The results presented in this Chapter showed that in the first three days approximately 70% of the administered radioactivity was recovered in the urine and faeces with more than 45% recovered only in the first day.

However, the loss of radioactivity in the urine and faeces dropped sharply thereafter and its level declined with time. The pattern of excretion of radioactivity in the urine and faeces of the rat suggests the decay of two forms of folate with markedly different biological half-lives as previously reported in man (Krumdieck et al., 1978), one short lived (t $\frac{1}{2}$ = one day) corresponding to newly absorbed folate and one long lived (t $\frac{1}{2}$ = 11 days) representing the decay of body folates.

A considerable amount of radioactivity was found in the liver reaching a maximum on the third day after the administration of labelled folic acid. At day 22 after administration, there is still a substantial amount of radioactivity retained in the liver and in another experiment, radioactivity was detectable in the livers of rats up to 70 days (Barford et al., 1977). The major proportion of the retained radioactivity was in the form of folate polyglutamates. Therefore, the metabolites appearing in the urine after day one in this experiment must be derived from the folatepolyglutamates. All metabolites appearing in the urine of day 1 were detected in the urine samples of the following days except folic acid and 5,10CH_2-THF. However, the relative distribution of these metabolites changed. The intact folates apparently dominanted the first urine sample whereas scission products dominated the other urine samples in particular p-AcBG and metabolite A. Murphy et al., (1976) reported that 10 days after administration of ^{3}H-folic acid to rat, the radioactive materials excreted in the urine eluted as a single peak from an ion-exchange column (Salt gradient)

which contained four metabolites and the major one was identified as p-AcBG, the other three metabolites were not identified and permanganate oxidation of these metabolites did not alter their position on the column used. From this the authors deduced that these metabolites were not intact folates. However, permanganate oxidation is a system which does not cleave 10 CHOFA or 5 MeTHF or its oxidation product (Maruyama et al., 1978, Lewis and Rowe 1979), which were found in the urine of the rats examined here. However, 5 MeTHF would be oxidised by permanganate and the absence of any change in elution position suggests that the reduced compound was not present at that stage.

Barford et al., (1978) claimed that 4a-OH-5 MeTHF (now described as a pyrazino-s-triazine derivative) was one of the major metabolites found after day one in the urine samples of rats dosed with labelled folic acid. This compound was found only 8 days after the administration of folic acid in the experiment reported here, however, since the handling time involved in the analysis of these late urine samples was longer because of their low radioactivity, it is more likely to be an artefactual product resulting from the oxidation of 5 MeTHF. However, the oxidation of Me THF-polyglutamates to triazine polyglutamates in vivo cannot be entirely excluded.

Thus, labelled folic acid has been shown to be incorporated into the reduced folate pool very quickly and folate polyglutamates represent the principle folate derivatives in the tissues. Several metabolites were found

in the urine and folate scission products dominanted the late urine samples in particular, p-AcBG and metabolite A. However, the persistence of p-AcBA and folate monoglutamates in the urine samples at all times albeit at low levels, suggests that they are derived from folate polyglutamates since the folate polyglutamates were the only detectable form of radioactive folate in the tissues.

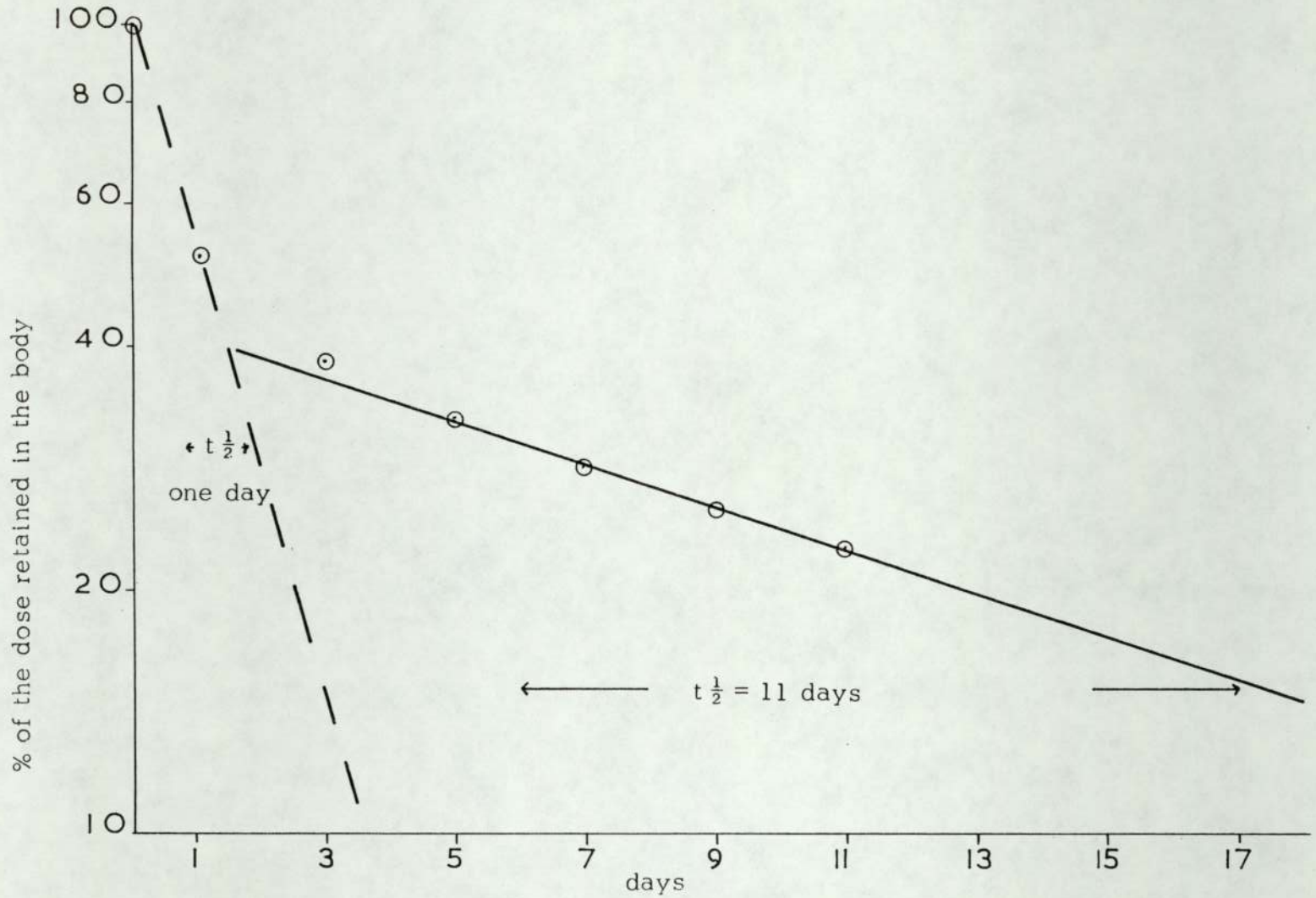


Figure 4.1. Semilogarithmic plot of the radioactivity retained in the body after the administration of a mixture of ^{3}H and ^{14}C folic acid to rats

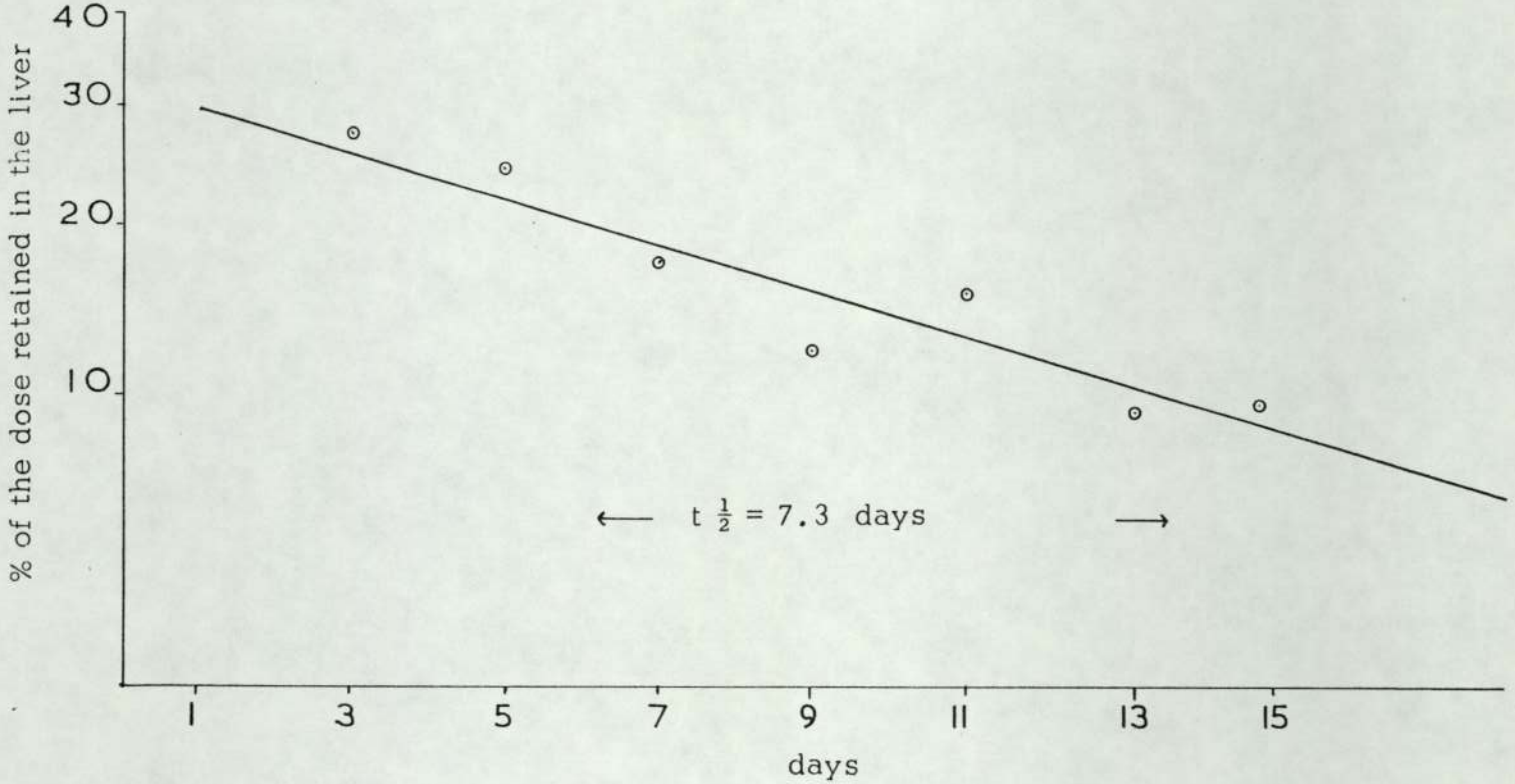


Figure 4.2. Semilogarithmic plot of the radioactivity retained in the liver after the administration of a mixture of ^{3}H and ^{14}C folic acid to rats

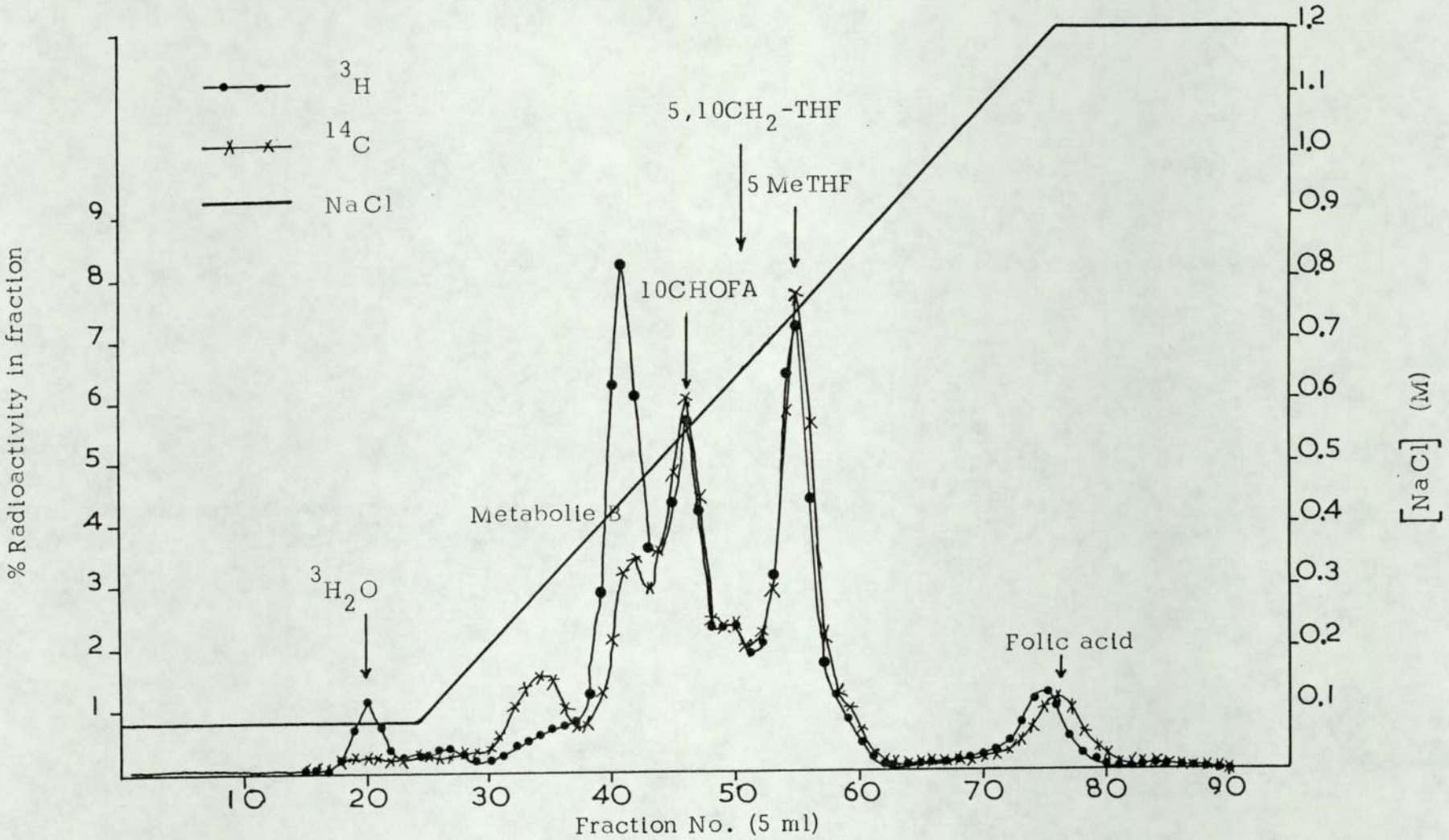


Figure 4.3 DE52 chromatography of normal rat urine samples collected (0-1day) after the administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid

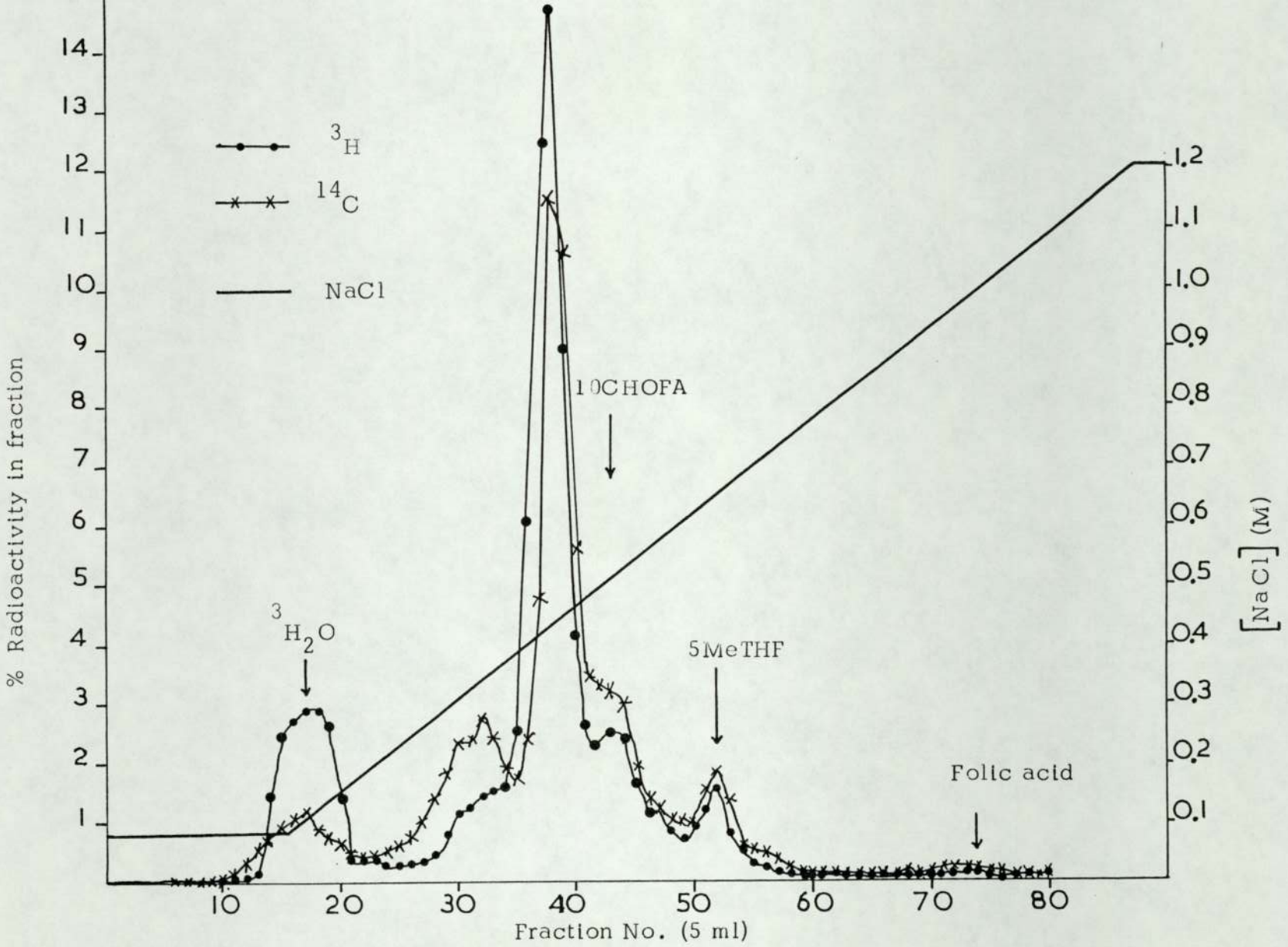


Figure 4.4. DE52chromatography of normal rat urine samples collected (2+3 days) after the administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid

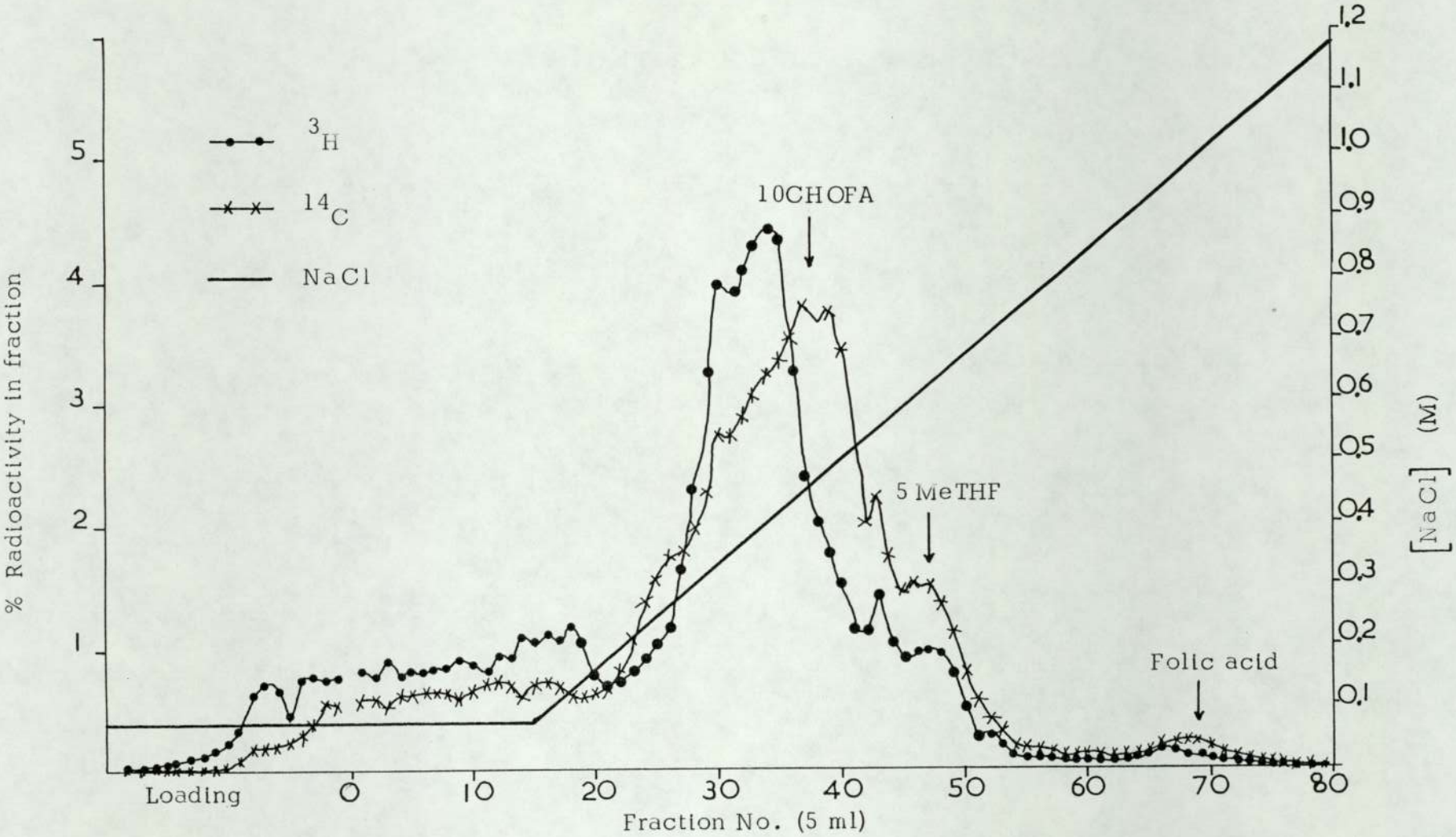


Figure 4.5. DE52 chromatography of normal rat urine samples collected (4+5 days) after the administration of a mixture of $[2-^{14}C]$ and $[3',5',7,9-^{3}H]$ folic acid

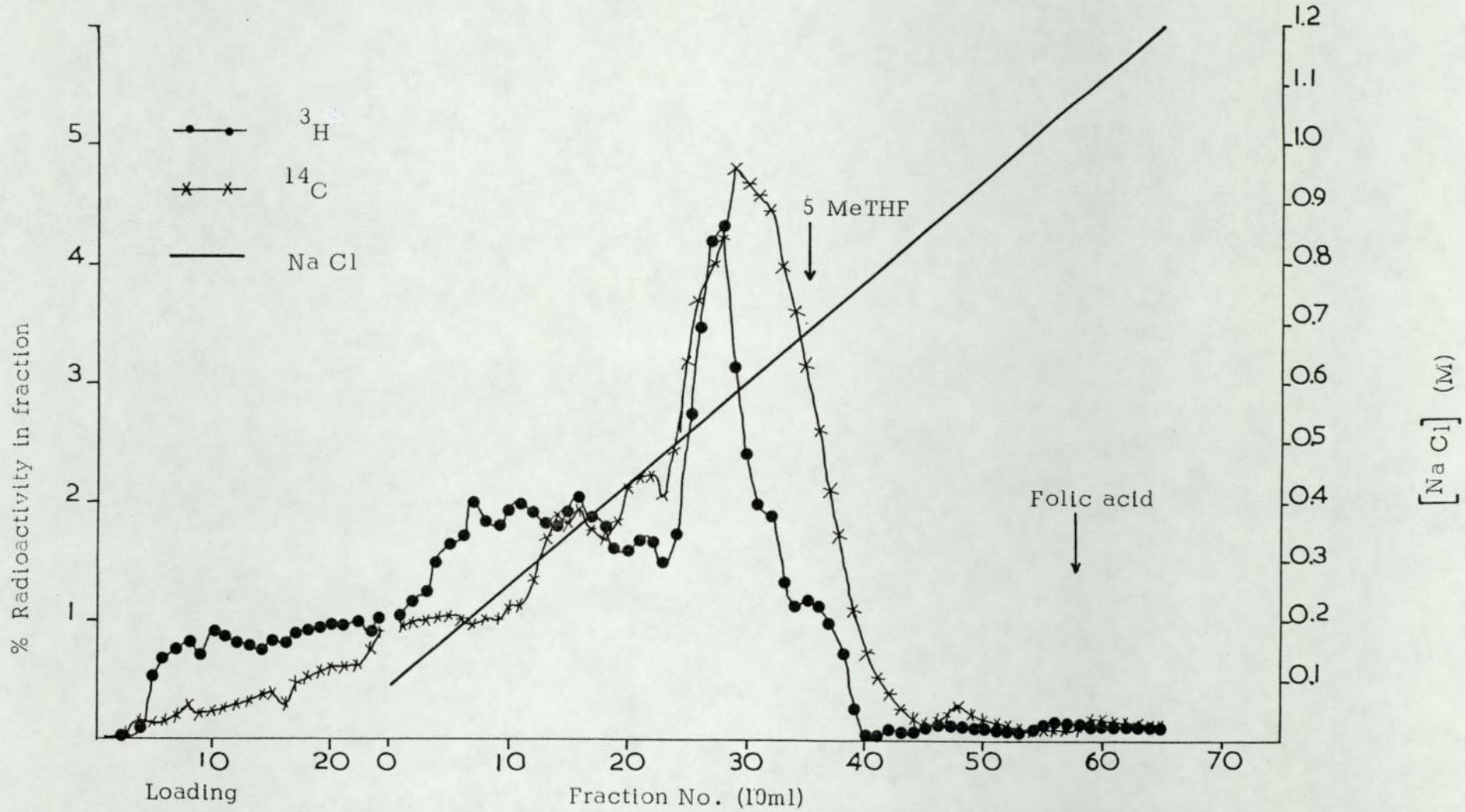


Figure 4.6 DE52 chromatography of normal rat urine samples collected (8+9 days) after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid

Days	% of the dose	
	% ^{3}H	% ^{14}C
1	42.8 ± 3.3	35.1 ± 2.5
2	3.9 ± 0.4	2.7 ± 0.2
3	2.7 ± 0.2	2.1 ± 0.1
4	1.8 ± 0.07	1.6 ± 0.1
5	1.9 ± 0.2	1.5 ± 0.2
6	1.6 ± 0.3	1.3 ± 0.2
7	1.1 ± 0.06	0.9 ± 0.09
8	1.1 ± 0.09	0.8 ± 0.07
9	1.1 ± 0.15	1.0 ± 0.10
10	0.9 ± 0.06	0.8 ± 0.06
11	0.76 ± 0.09	0.76 ± 0.09
12	0.70 ± 0.02	0.66 ± 0.04
13	0.77 ± 0.07	0.73 ± 0.03
14*	0.61 ± 0.06	0.61 ± 0.05
15*	0.75 ± 0.08	0.66 ± 0.07
Total	62.5	51.2

Table 4.1. Distribution of radioactivity in the urine collected 15 days after the administration of [2 - ^{14}C] and [3',5',7,9-^{3}H] folic acid to normal rats. The results are expressed as percentage of the dose (mean ± SEM n=5)

* Four rats were used

Days	% of the dose ^{3}H	^{14}C
1	3.8 ± 1.7	8.5 ± 3.8
2 + 3	8.5 ± 1.3	16.4 ± 2.7
4 + 5	2.3 ± 0.35	3.5 ± 0.9
6 + 7	1.7 ± 0.30	2.5 ± 0.6
8 + 9	1.0 ± 0.07	1.4 ± 0.08
10 + 11	0.8 ± 0.07	1.0 ± 0.10
Total	18.1	33.1

Table 4.2 Distribution of radioactivity in the faeces collected 11 days after the administration of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid to normal rats. The results are expressed as percentage of the dose (mean ± SEM n = 5)

Days	% of the dose			
	Radioactivity excreted in the urine and faeces		Radioactivity retained in the body	
	^{3}H	^{14}C	^{3}H	^{14}C
0	-	-	100	100
1	46.6	43.6	53.4	56.4
3	61.7	64.8	38.3	35.2
5	67.7	71.4	32.3	28.6
7	72.1	76.1	27.9	23.9
9	75.3	79.5	24.7	20.5
11	77.8	82.1	22.2	17.9

Table 4.3 The radioactivity retained in the body after the administration of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid. The results are calculated by subtracting the percentage of the dose appearing in the urine and faeces from 100%.

Time (days)	Percentage of dose radioactivity in the liver	
	^{14}C	^{3}H
1	24.6 (2.1)	25.8 (2.2)
3	27.8 (2.7)	28.4 (2.7)
5	24.6 (3.2)	23.0 (3.0)
7	17.7 (1.8)	15.0 (1.6)
9	12.2 (1.4)	11.8 (1.4)
11	15.9 (1.3)	17.2 (1.4)
13	9.4 (1.0)	8.2 (0.9)
15	9.8 (1.0)	9.8 (1.0)
22	4.0 (0.5)	4.3 (0.5)

Table 4.4. Recovery of radioactivity in the liver at 1-22 days after the oral administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid to normal rats

Units: % dose radioactivity in the whole tissue; values in parenthesis indicate % of dose radioactivity per gram (wet weight) of tissue.

Table 4.5. Metabolites present in the urine following the administration of ^{3}H and ^{14}C folic acid to normal rats. The results are expressed as the percentage of the dose present as each metabolite.

Metabolite	Day one		Days 2 + 3		Days 4 + 5	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Folic acid	3.2	2.9	0.05	0.08	-	-
5 MeTHF	12.0	11.9	0.43	0.48	0.3	0.4
10CHOTHF	8.7	8.1	1.0	1.0	0.5	0.9
5,10CH_2-THF	2.8	2.6	-	-	-	-
p-AcBA	4.4	-	1.46	-	0.3	-
p-AcBG	4.9	-	1.94	-	1.2	-
Metabolite A		3.1		1.7		0.9
Metabolite B		2.8		0.9		0.2
Urea	-	0.7	-	0.4		0.3
$^{3}H_2O$	1.5	-	1.2	-	0.8	-
	Day 6 + 7		Day 8 + 9		Day 10 + 11	
*Intact folates	0.7	0.7	0.5	0.7	0.5	0.5
p-AcBA	0.3	-	0.3	-	0.15	-
p-AcBG	1.0	-	1.0	-	0.5	-
Metabolite A		0.7		0.3		0.2
Metabolite B		0.1		T		T
$^{3}H_2O$	0.6	-	0.4	-	0.5	-

T Trace amounts

* Consists of 10CHOFA + 5 MeTHF oxidation product

CHAPTER 5

THE METABOLISM OF [2-^{14}C] AND [3',5',7,9-^{3}H] FOLIC ACID IN CONTROL AND CANCER PATIENTS

Previous studies on the metabolism of folic acid in man using ^{3}H-labelled tracers (Johns et al., 1961, Chanarin and McLean, 1967), ^{14}C-labelled tracers (Krumdieck et al., 1978) or microbiological assay (Ratanasthien, 1975) have given rise to conflicting results. Moreover, variation in urinary metabolites following the administration of different dose levels of labelled folic acid has been also reported (Pheasant et al., 1979). Although there have been extensive studies on folate metabolism in the rat (Vidal and Stokstad 1974, Beavon and Blair 1975, Pheasant et al., 1981), little is known about the metabolic fate of folic acid in man since most of the work on folate in man was more concerned with the absorption of folate (Anderson, et al., 1960, Klipstein, 1963, Butterworth et al., 1969) and the diagnosis of folate deficiency (Chanarin, et al., 1958).

Folates are of a great importance to cell division, hence there is considerable interest in the folate status during malignant disease. Although folate deficiency has long been known to be associated with malignant disease (Blakley, 1969, Chanarin, 1979a) no detailed comparative metabolic studies have been carried out in man. Catabolism of folate in the rat has been shown to be reduced in the presence of a tumour (Chapter 3). Therefore, the metabolism of a mixture of ^{14}C and ^{3}H labelled folic acid was studied in control patients and in patients with malignant disease in order to observe any metabolic variation which may have diagnostic or therapeutic applications and to resolve the apparent discrepancies arising from using different dose levels or using single

labelled tracers.

5.1. The metabolism of ^{3}H & ^{14}C folic acid (5 mg)

Materials and Methods

Two groups of patients were used

Group I : 11 Patients suffering from malignant disease.

Group II : 6 Patients suffering from other disorders as controls.

More details of the patients may be found in Table 5.1.1. All patients were given an oral dose of a mixture of 5 μCi $[2\text{-}^{14}C]$ folic acid and 20μCi $[3',5',7,9\text{-}^{3}H]$ -folic acid plus 5 mg unlabelled folic acid. Urine was collected onto sodium ascorbate for the following time periods, 0-6 h, 6-12 h and 12-24 h after the administration of the dose. In one case urine was collected for the second day and in another case urine was collected on the fifth day after the administration of the dose. Faeces were collected for 48 h from two subjects of each group. Determination of radioactivity, column chromatography and paper chromatography were performed as described in Chapter 2.

Results

The urinary recoveries of radioactivity after an oral dose of labelled folic acid (5 mg) are shown in Table 5.1.2. There is a discrepancy between the recovery of ^{3}H and ^{14}C in the urine samples of all patients

except the urine samples of (W.M.) where the recovery of ^{3}H and ^{14}C was equal. However, the urinary recovery of ^{3}H was significantly higher than ^{14}C in both groups of patients when determined by a paired t test ($p < 0.001$). Cancer patients excreted significantly less radioactivity in urine than control patients ($p < 0.001$); 14.1% ^{3}H, 12.4% ^{14}C and 32.0% ^{3}H and 26.2% ^{14}C of the dose being excreted in the urine of cancer patients and control patients respectively. Most of the radioactivity appearing in the urine was excreted in the first 12h from both groups of patients. Excreted radioactivity fell rapidly in the second day but was detected at low levels in the urine up to five days (0.1% ^{3}H and 0.03% ^{14}C of the dose (J.H.)). The urinary recovery of the individual cancer patients showed an inverse correlation between the total urinary recovery and the size of the tumour as judged by the co-operating clinician. The urinary recovery decreased as the approximate size and extent of the tumour mass increased indicating a larger requirement for folate in malignant disease (Table 5.1.3).

Table 5.1.4. shows the recovery of ^{3}H and ^{14}C radioactivity in the faeces. More ^{14}C than ^{3}H was present in the faeces. All the patients examined produced faeces only in the second day. No statistical test of significance can be used because of the small number of patients studied, however, no difference in faecal excretion was apparent from these investigations. The low recovery of radioactivity of patient (E.T.) might be due to the low production of faeces compared to other subjects.

Furthermore, no evidence in the literature suggests that malignant disease causes malabsorption of folate, (Chanarin 1979a) unless the tumour is located in the gut.

Urinary metabolites

Urine samples were sequentially chromatographed on DE 52 columns, Sephadex G15 and paper. In all cases this revealed a number of radioactive components. Examples of DE 52 chromatography of 0-6 h urine samples are illustrated in Figures 5.1.1., 5.1.2., 5.1.3 for control patients and 5.1.4., 5.1.5 and 5.1.6 for cancer patients. Three components detected in the urine retained the ^{3}H and ^{14}C label in the same ratio (however, this ratio may differ from one subject to another, see Table 5.1.5) and were identified by co-chromatography with authentic standards in both column systems as folic acid, 5 MeTHF and 10 formyl-folate. The ratio of ^{3}H:^{14}C in these folate derivatives was higher in all subjects than in the folic acid administered ($p < 0.05$). There was no difference in the ^{3}H:^{14}C ratios of folic acid excreted from the 0-6 h urine sample between control subjects and cancer patients.

Folic acid was the major radioactive component in the urine of control subjects and its amount varied in the urine of cancer patients. It appeared that in patients with large tumours, there was a relationship between the total urinary radioactivity recovered and the amount of folic acid and 5 MeTHF. The lower the urinary radioactivity recovered the lower the percentage of urinary radioactivity excreted as folic acid

and the higher the percentage of urinary radioactivity excreted as 5 MeTHF (see Figure 5.1.4., 5.1.5 and 5.1.6). The amount of 10 CHOFA was very small in both groups of patients and it was hardly seen in some subjects.

The remaining components detected in the urine of both groups were catabolites labelled solely with ^{3}H or principally with ^{14}C. The tritiated catabolites were identified as p-acetamidobenzoyl-L-glutamate and in some subjects, tritiated water was also detected. The ^{14}C labelled catabolite has not been identified, it eluted from both columns used in the position of metabolite B which was detected in rat urine (see Chapter 3) and in no urine sample was metabolite A detected.

DE 52 chromatograms of 6-12 h urine samples of control subjects showed similar patterns to 0-6 h urine samples with the exception that the percentage of the tritiated peak (p-acetamidobenzoyl-L-glutamate) increased and the peak became wider (Figures 5.1.7 and 5.1.8). The ratio of folic acid to 5 MeTHF was still constant or decreased slightly. However, DE 52-chromatograms of 6-12 h urine samples of cancer patients were different from 0-6 h urine samples in that the ratio of folic acid to 5 MeTHF apparently decreased and the major tritiated peak (p-acetamidobenzoyl-L-glutamate) became wider and in some cases partially splitting into two peaks (Figures 5.1.9, 5.1.10 and 5.1.11). Sephadex G 15 chromatography resolved the tritiated peak of most of the cancer patients and only one control patient (E.W.) into two peaks (Figure 5.1.12)

which were identified by paper chromatography as p-acetamidobenzoyl-L-glutamate and p-acetamidobenzoate. Other scission products such as $^{3}H_2O$ and metabolite B were still detected in some cases.

Some of the 12-24 h urine samples of both groups were not analysed due to the low radioactivity recovered and/or to the large volumes of urine which diluted the radioactivity. However, the other urine samples were analysed and DE 52 chromatograms of urine from control patients (Figure 5.1.13 and 5.1.14) showed that the ratio of folic acid to 5 MeTHF decreased and the amounts of scission products increased. Sephadex G15 chromatography of tritiated products from DE 52 chromatography of urine samples showed that the 12 - 24 h urine samples of all control patients examined contained p-acetamidobenzoyl-L-glutamate and p-acetamidobenzoate. In cancer patients DE 52-chromatograms (Figures 5.1.15 and 5.1.16) showed that tritiated scission products dominated the urinary metabolites and in some cases, little (e.g. L.J.) or no (e.g. J.H.) intact folates were detected. However, exceptionally, no scission products were detected at any time in the urine of patient (D.M.).

Table (5.1.6) summarises the relative distribution of the major metabolites appearing in the various urine samples of both groups of patients. Cancer patients excreted significantly less unchanged folic acid ($0.01 < p < 0.05$) than control patients. However, the apparent decrease in 5 MeTHF excretion was not statistically significant. Table

5.1.7 shows the individual ratios of folic acid to 5 MeTHF in all patients of both groups. This ratio decreased sharply in cancer patients with time where it was 5.2:1, 3.1:1 and 1.0:1 at 0-6 h, 6-12 and 12-24 h respectively whereas in control patients the decrease was slower (5.6:1, 5.1:1 and 2.8:1).

The ratios of $^{3}H:^{14}C$ in the folic acid excreted from the various urine samples of cancer and control patients were higher than the folic acid administered and these ratios increased with time in particular in the 12-24 h urine samples (Table 5.1.5).

Urinary scission product excretion relative to intact folates increased with time in both groups. 0.09:1, 0.20:1 and 0.83:1 were the ratios of scission products to intact folates excreted by cancer patients at 0-6 h, 6-12 h and 12-24 h respectively whereas in control patients the corresponding ratios were 0.12:1, 0.25:1 and 0.71:1. However, the total excretion of scission products was significantly depressed in cancer patients ($p < 0.05$). p-Acetamidobenzoate was not present in 0 - 6 h urine samples of either group and appeared in the 6 - 12 h urine samples of most of the cancer patients but only one control patient (E.W.) but there was no statistically significant difference in overall p-AcBA excretion between the two groups. The excretion of p-AcBA was maximal in the 12 - 24 h urine samples of both groups. p-Acetamidobenzoyl-L-glutamate showed a reciprocal pattern, it was maximal in the first urine samples (0-6 h) and decreased slowly as a

percentage of the dose thereafter. The excretion of p-AcBG from the urine samples was significantly depressed ($p < 0.05$) in cancer patients.

Summary

1. Oral doses of a mixture of 5 μCi [2-^{14}C] and 20 μCi [3',5',7,9-^{3}H] -folic acid plus 5 mg of unlabelled folic acid were given to patients with malignant diseases and to control patients.

2. Qualitatively there was no difference in the metabolites detected in the urine of both groups. Folic acid, 5 MeTHF 10CHOFA, p-AcBA, p-AcBG, $^{3}H_2O$ and unidentified ^{14}C-labelled species have been detected.

3. Cancer patients excreted significantly less of the dose in the urine than control patients and the urinary recovery of radioactivity decreased as the size and extent of the tumour increased.

4. Cancer patients excreted less unchanged folic acid.

5. The ratio of folic acid to 5 MeTHF decreased rapidly with time in cancer patients.

6. Cancer patients excreted significantly less scission products than control patients in particular p-AcBG.

7. No difference was observed in the faecal recoveries of radioactivity between the two groups.

5.2 The effect of the dose

Materials and Methods

9 Patients were used, 4 patients suffering from malignant diseases and 5 patients suffering from other disorders as controls. More details of the patients may be found in Table 5.2.1. The patients were given oral doses of a mixture of 5 μCi [2-^{14}C] and 20 μCi [3',5',7,9-^{3}H] - folic acid (0.057 mg, 0.5 mg or 1.0 mg folic acid). Urine and faeces were collected as described in Section 5.1. and their analysis performed as described in Chapter 2.

Results

The urinary recovery of radioactivity after an oral dose of labelled folic acid (1 mg, 0.5 mg or 0.057 mg folic acid) is shown in Table 5.2.2. At all doses, the urinary recovery of ^{3}H was higher than ^{14}C and cancer patients excreted less radioactivity in the urine than control patients:-

2.5% ^{3}H, 1.7% ^{14}C and 5.3% ^{3}H, 3.5% ^{14}C of the 1 mg dose

3.0% ^{3}H, 2.1% ^{14}C and 3.4% ^{3}H, 2.3% ^{14}C of the 0.5 mg dose

2.0% ^{3}H, 1.5% ^{14}C and 2.6% ^{3}H, 1.8% ^{14}C of the 0.057 mg dose

being excreted in the urine of cancer and control patients respectively.

It appeared that there was a relationship between the dose and the radioactivity excreted in the urine of control subjects, the lower the dose given the lower the percentage of the dose excreted in the urine. However, this relationship does not apply to cancer patients since the presence

of the tumour can affect the urinary recovery of radioactivity (see Section 5.1).

The radioactivity in the faeces was measured only in two control subjects given the lowest dose (0.057 mg). More ^{14}C than ^{3}H was present in the faeces of both subjects. 1.7% ^{14}C and 1.33% ^{3}H were present in the faeces of these subjects (see Table 5.2.3).

Urinary metabolites

5.2.A. The 1 mg dose

Urine samples were sequentially chromatographed on DE 52 columns, Sephadex G15 and paper. The radioactivity appearing in the 0 - 6 h urine sample of the cancer patient was too low to be chromatographed. However, DE 52 chromatography of the urine of a control patient receiving 1 mg folic acid is illustrated in Figure 5.2.A.1. It showed a similar pattern to 0-6 h urine samples of control patients given 5 mg dose (Section 5.1). Two major components detected in the urine retained the ^{3}H and ^{14}C label in the same ratio and were identified by co-chromatography with authentic standards in both column systems as folic acid and 5 MeTHF. The ratio ^{3}H:^{14}C in these folates was higher than in the folic acid administered. Sephadex G15 chromatography of the tritiated peak showed that it contained only one tritiated component associated with a very small amount of 10 CHOFA and the component was identified by paper chromatography as p-AcBG.

DE 52 chromatograms of 6-12 h urine samples of the control and cancer patients are illustrated in Figures 5.2.A.2. and 5.2.A.3 respectively. Again folic acid and 5 MeTHF were identified in the urine of both patients. The ratio of folic acid to 5 MeTHF decreased in control patient from 3.3:1 at 0 - 6 h urine sample to 1.3:1 at 6 - 12 h urine sample, however, it is still higher than that of cancer patient (0.25:1). Scission products dominated the 6 - 12 h urine samples of both patients and they were identified by the chromatographic methods used as p-AcBA, p-AcBG and tritiated water.

No intact folates were detected in the third urine sample of the cancer patient (A.L.) (see Figure 5.2.A.4), and only tritiated scission products were present in particular p-AcBA and p-AcBG. ^{14}C-Labelled species were too low in radioactivity to be detected.

Table 5.2.4. summarises the relative distribution of the major metabolites appearing in the various urine samples of both patients. Unchanged folic acid was the major compound in first urine sample of the control patient (0 - 6 h). However, in the second urine sample p-AcBG was the major metabolite and the amount of folic acid dropped from 1.3% of the dose at 0 - 6 h to 0.4% of the dose at 6 - 12 h but was still higher than its amount in the urine of the cancer patient. p-AcBA was excreted in the urine of both patients at 6 - 12 h sample and its amount increased with time so that p-AcBA and p-AcBG were almost the only ^{3}H-metabolites in the 12 - 24 h urine sample of the cancer patient. The 12 - 24 h urine

of the control patient was not examined.

5.2.B. The 0.5 mg dose

Urine samples (0 - 6 h) of a control patient and a cancer patient receiving an oral dose of ^{3}H and ^{14}C folic acid (0.5 mg) were sequentially chromatographed on DE 52 columns, Sephadex G15 and paper chromatography. This revealed the same metabolites appearing in the early urine samples of patients receiving higher doses of folic acid with the exception that p-AcBA was detected before 6 h. DE 52 chromatograms of 0 - 6 h urine samples of the control and cancer patients are shown in Figures 5.2.B.1. and 5.2.B.2 respectively. They were similar, however, the ratio of folic acid to 5 MeTHF again was depressed in the cancer patient (0.5:1) compared to the control patient (0.77:1). The other urine samples were not analysed. Table 5.2.4. shows the relative distribution of the metabolites appearing in the 0 - 6 h urine samples of both patients. The cancer patient excreted less unchanged folic acid, 5 MeTHF and p-AcBG than control.

5.2.C. The 0.057 mg dose

DE 52-chromatography of 0 - 6 h urine samples from patients receiving the low dose (Figures 5.2.C.1 and 5.2.C.2 for controls and 5.2.C.3 for cancer patient) separated the radioactivity almost completely into a ^{3}H only peak and ^{14}C-labelled species. No folic acid was detected in the urine of either group and little or no 5 MeTHF was present. The major urinary catabolite was identified as p-AcBG. p-AcBA was also

detected in small amounts.

5.3. Discussion

Following the administration of oral doses of labelled folic acid to man, several metabolites appeared in the urine including intact folates such as unmetabolised folic acid and a reduced folate, 5 MeTHF, together with scission products. This indicates that folic acid is incorporated into the reduced folate pool and confirms the existence of a breakdown process in man, similar to that elucidated in the rat (Pheasant et al., 1981). The folates present in urine had a higher ^{3}H:^{14}C ratio than the administered folic acid indicating that the secondary isotope effect observed in the handling of labelled folic acid by the rat (Connor et al., 1980) also occurs in man. However, this was not the only reason for the significant excess of ^{3}H over ^{14}C which is present in the urine. The excess is also due to the higher excretion of ^{3}H-labelled scission products over ^{14}C-labelled species. These presumably arise by degradation in the intestine of a labile folate excreted via the bile to produce a ^{3}H-species which is absorbed and excreted in the urine and a ^{14}C-species which is poorly absorbed and excreted in the faeces. The excess of ^{14}C over ^{3}H present in the faeces confirms this, since the isotope effect cannot account for all the excess of ^{14}C over ^{3}H in the faeces (Chapter 3). The continuous increase in the ^{3}H:^{14}C ratio of the excreted folic acid with time seen with both groups of patients raised a

question about the handling of the two isotopes of folic acid *in vivo.* This increase may be due to accumulative secondary isotope effect resulting from the re-absorption of labelled folate during the enterohepatic circulation and/or the incorporation of ^{14}C-folic acid into the reduced folate pool at a faster rate than ^{3}H-folic acid. Studies *in vitro* have shown that the reduction of $[9\text{-}^{2}H_2]$ - folic acid by either *L.casei* or bovine DHF reductase is slower than the unlabelled folic acid (A. Sahota, personal communication) and this could be the case for ^{3}H-folic acid *in vivo.* The increase in ratio of ^{3}H:^{14}C was more marked in cancer patients suggesting extra site of reduction in these patients (i.e. the tumour).

The excretion of folic acid continued throughout the experiment representing a large proportion of the dose unlike the rat. This indicates a slow reduction of folic acid in man relative to the rat. However, when folic acid has been reduced, it is largely converted to 5 MeTHF the major serum folate in man (Ratanasthien, *et al.,* 1974). The absolute amount of 5 MeTHF excreted from the urine increased when the level of the dose increased, but 10CHOFA which is probably an oxidised product of 10CHOTHF, was detected only in very small amounts in the urine of some patients after the administration of 5 mg folic acid and hardly detected at lower doses. This indicates that a high dose such as 5 mg folic acid does not effectively change the amounts of 10CHOFA in the serum (Ratanasthien *et al.,* 1977) or the urine.

The same ^{3}H-labelled catabolites were identified in the urine of man and the rat, however, the appearance of p-AcBA was delayed. It seems that there is a relationship between the level of the dose administered and the time of appearance of p-AcBA. The lower the level of the dose given, the earlier the appearance of p-AcBA. In control subjects when 0.5 mg, 1.0 mg or 5.0 mg of folic acid was given, p-AcBA started to appear in 0 - 6 h, 6 - 12 h or 12 - 24 h urine samples respectively. Several investigators reported that folate can be cleaved at the amide bond by various bacterial species (Levy and Goldman 1967, Pratt et al., 1968, Bertino et al., 1971). Two enzymes designated as carboxypeptidase have been isolated, carboxypeptidase G(CPDG) which is able to remove the terminal glutamate from oxidized folates (Levy and Goldman 1967) and carboxypeptidase G_1 ($CPDG_1$) that rapidly cleaves the terminal glutamate from reduced folates as well as oxidized folates. $CPDG_1$ was reported to inhibit the growth of leukaemia cells by hydrolysis the glutamate moiety from folates which resulted in depleting the cells of folates (Bertino et al., 1971). Moreover, it has been used to reduce the serum MTX concentration in a patient with leukaemia and renal failure (Howell et al., 1978). Studies in vivo showed that in the rat and mouse MTX underwent cleavage at the amide bond (Zaharko and Oliverio 1970, Valerino et al., 1972). These authors suggested that intestinal bacteria were responsible for this cleavage since the breakdown process was significantly less in germ-free mice and also in mice which had been pretreated with antibiotics. Little is known about the properties of

$CPDG_1$ and its existence in mammalian tissues. It has been reported that $CPDG_1$ is inhibited by 0.1 mM EDTA, however, the effect of folate analogues on its activity is not known. The results in this Chapter suggest that in man such an enzyme which catalyzed the removal of the glutamic acid moiety from p-aminobenzoyl-L-glutamate could be inhibited by folic acid. This may explain the early appearance of p-AcBA at low doses of folic acid and its delay at higher doses. On this hypothesis, at 5.0 mg level, the concentration of folic acid may be sufficient to inhibit the enzyme but this inhibition would decrease with time due to the decreased amount of folic acid that resulted from renal excretion and metabolism. However, 0.5 mg folic acid was not enough to inhibit the enzyme, therefore, p-AcBA appeared in the first urine samples of patients dosed at this level. This may explain also the earlier detection of p-AcBA in the urine of cancer patients than controls dosed with the same level of folic acid since folic acid clearance and metabolism are accelerated in malignant disease. However, this proposal has yet to be confirmed.

Unlike the rat the amount excreted of p-AcBA increased with time whereas p-AcBG fell suggesting that in man, p-AcBA is also derived from tissue folates. However, in the rat, the maximal amount of p-AcBA was observed in 6 - 24 h urine sample after the administration of labelled folates (Connor et al., 1979, Pheasant et al., 1981, Saleh et al., 1981) and dropped sharply in the second day. This could have happened in

man since the investigation time was only one day and the amounts of p-AcBA in the urine may not have reached their peak. Alternatively, the metabolism of p-aminobenzoyl-L-glutamate could be different in man and give p-AcBA as metabolite so that it dominates the urine sample as observed in the guinea pig after the administration of labelled folic acid or p-aminobenzoyl-L-glutamate (Choolun, et al., 1980, Choolun et al., 1981 unpublished observation).

The catabolites appearing in the late urine samples (6 - 24 h) are assumed to be formed largely from the breakdown of tissue folates by analogy with the rat and since studies in vitro showed that human fibroblasts formed folate polyglutamates from ^{3}H-folic acid in the culture medium within 4 h of incubation, Therefore, the rate of tissue folates breakdown can be measured as mentioned in Chapter 3 by the equation

$$\frac{X}{Y} \times 100$$

where X : The percentage of the dose excreted in the late urine samples (6 - 24 h) as p-AcBG or p-AcBG plus p-AcBA

Y : The percentage of the dose retained in the body at 6 h.

Table 5.3.1. shows the rate of tissue folates breakdown of individual subjects; 2.0 ± 0.3 was the rate of tissue folate breakdown in control subjects estimated as X represented by the excretion of p-AcBG only and 2.9 % $\pm$ 0.2 when it was taken as the total excretion of p-AcBG and p-AcBA.

Patients with malignant disease excreted less radioactivity in the urine. This decrease is not explained by poor absorption from gut and by analogy with the rat, the remaining radioactivity is presumably taken up into tumour tissue (Chapter 3, Saleh et al., 1981). This is supported by the inverse correlation observed between the approximate tumour mass and urinary radioactivity. The patients also incorporate more of the administered folic acid into the reduced folate pool as shown by the decreased excretion of folic acid and the reduced folic acid: 5 MeTHF ratio. Thus, these results demonstrate the great demand for folate in malignant disease.

Despite the increased incorporation of labelled folate into the reduced folate pool, lower levels of labelled scission products were excreted in the urine. This shows that the presence of the tumour in man decreases significantly ($p < 0.05$) the rate of tissue folates breakdown, 0.94% and 1.5% were the rates of tissue folate breakdown in cancer patients when X was represented by the excretion of p-AcBG and p-AcBG plus p-AcBA respectively (Table 5.3.1).

The metabolites appearing in the urine were dose dependent, this being most marked with unchanged folic acid. When the dose of folic acid was decreased to 1.0 mg or 0.5 mg, lower recovery of radioactivity in the urine was found with excretion of folic acid falling more than the other metabolites, but the same differences between the control and cancer patients were observed at these doses. However, when a dose

approximately equivalent to the daily intake of folate was given (0.057mg), little or no folate was excreted and the only detectable metabolites were scission products confirming the observations of Pheasant et al., (1979). The low recovery of radioactivity in the urine indicates that the renal threshold for folates has not been exceeded whereas the catabolites appear to have much lower threshold values.

Thus, the results presented here indicate that folic acid is metabolised in man in a similar manner to that elucidated in the rat and demonstrates the existence of the catabolic process of folate in man. The results also show that the presence of a tumour increases the incorporation of folic acid into the reduced folate pool and decreases the catabolic rate of tissue folate.

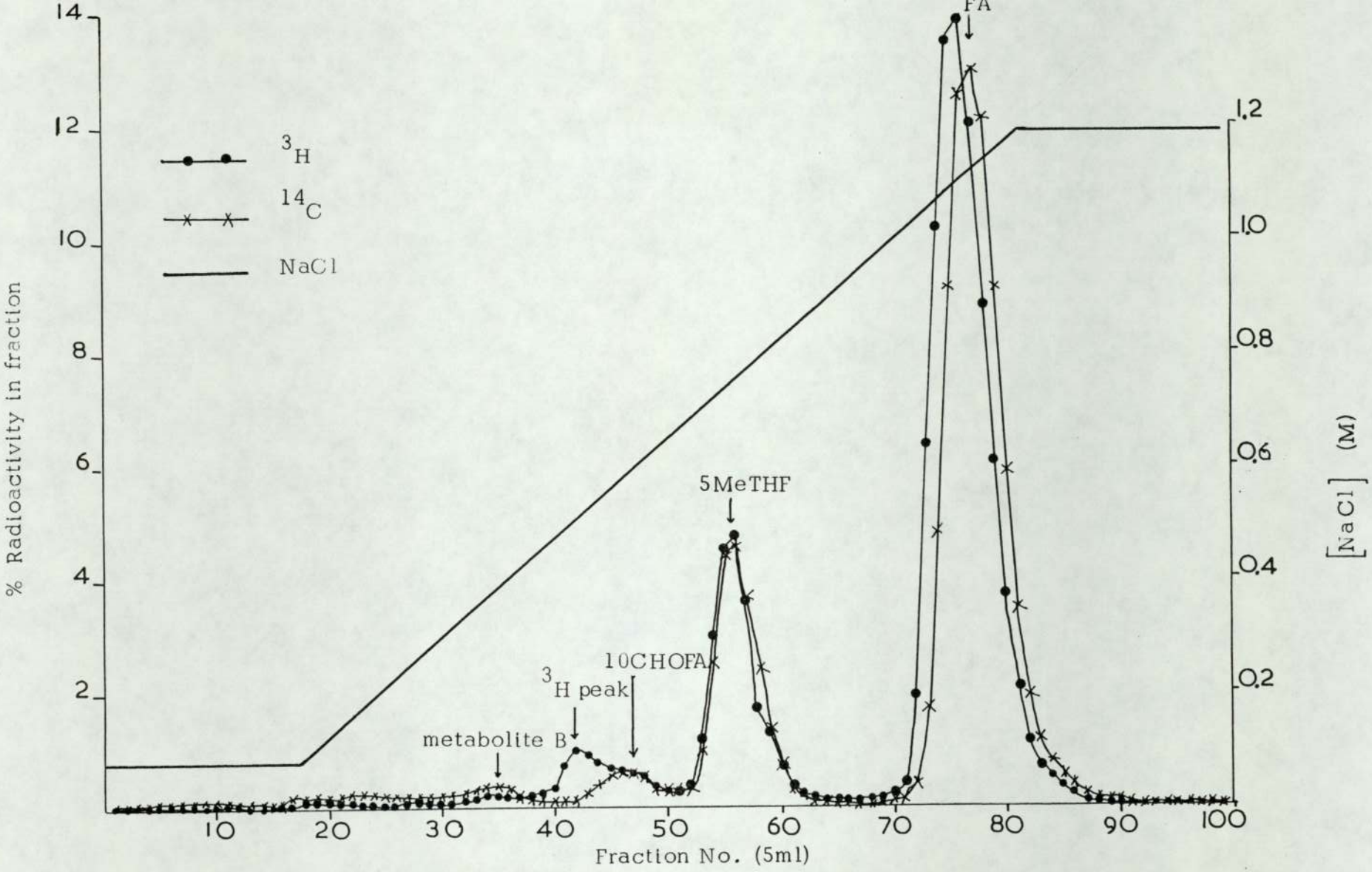


Figure 5.1.1. DE 52 Chromatography of 0-6 h urine sample of a control patient (S.R.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

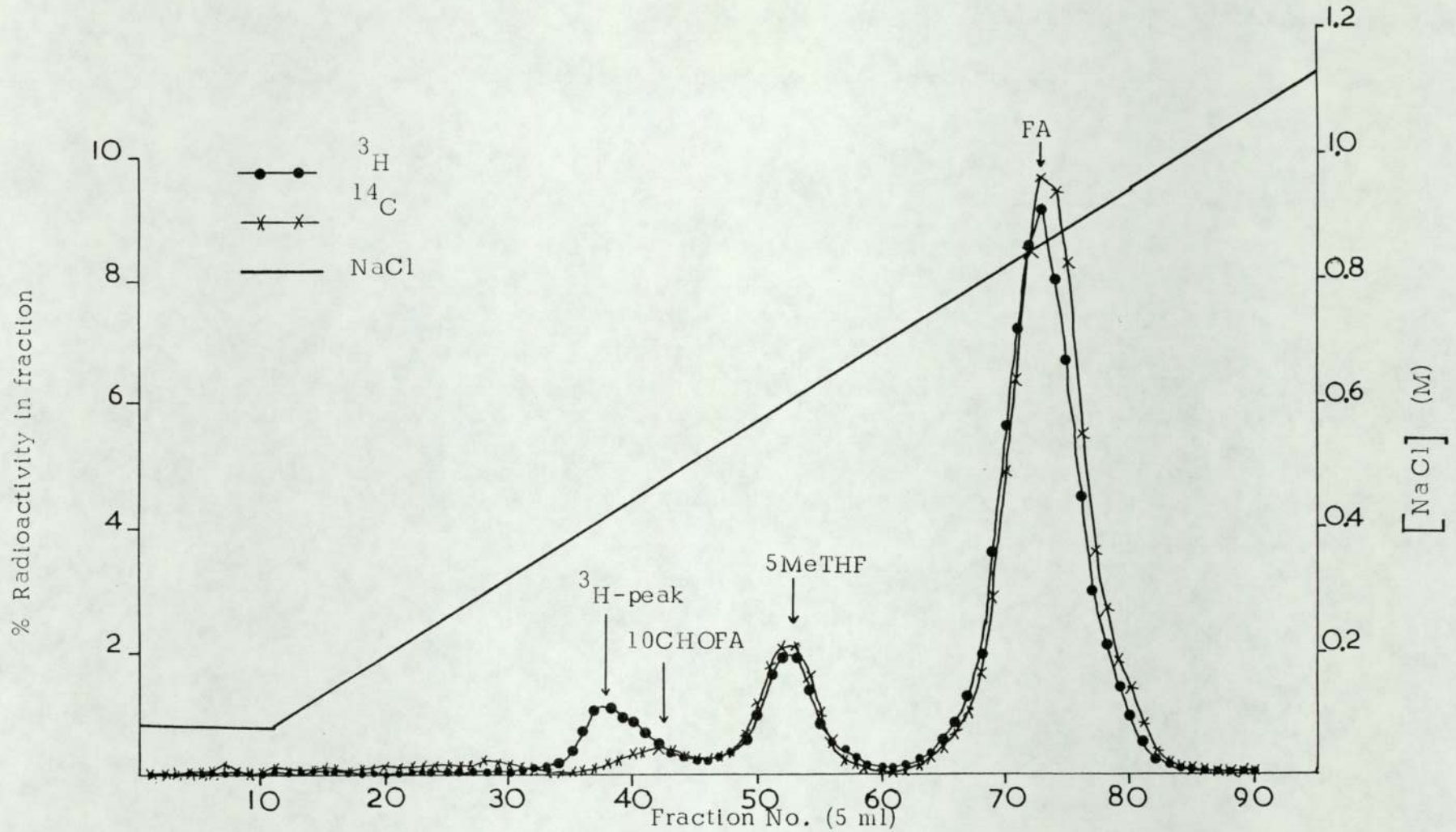


Figure 5.1.2. DE 52 Chromatography of 0-6 h urine sample of a control patient (E.W.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

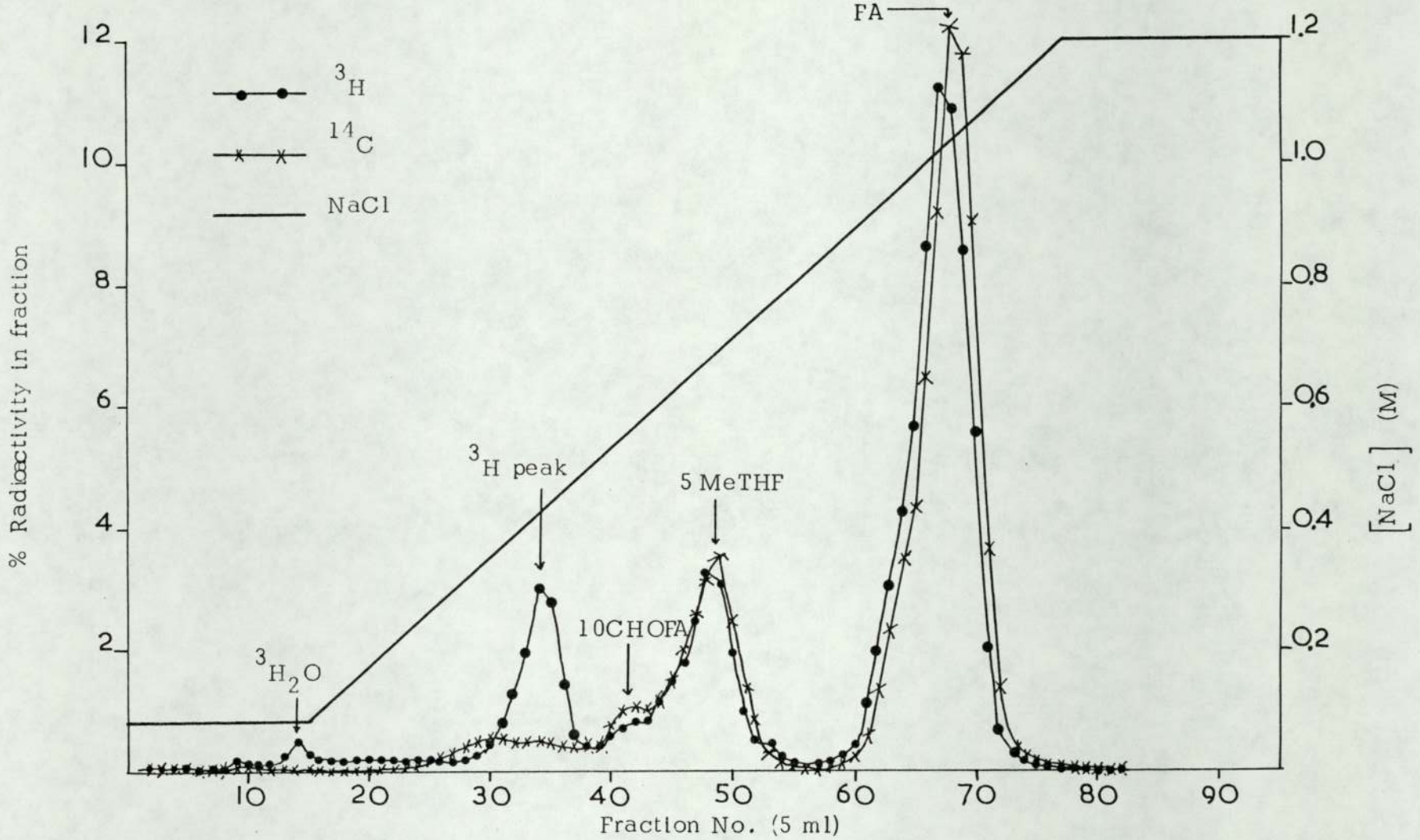


Figure 5.1.3. DE 52 Chromatography of 0-6 h urine sample of a control patient (E.T.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

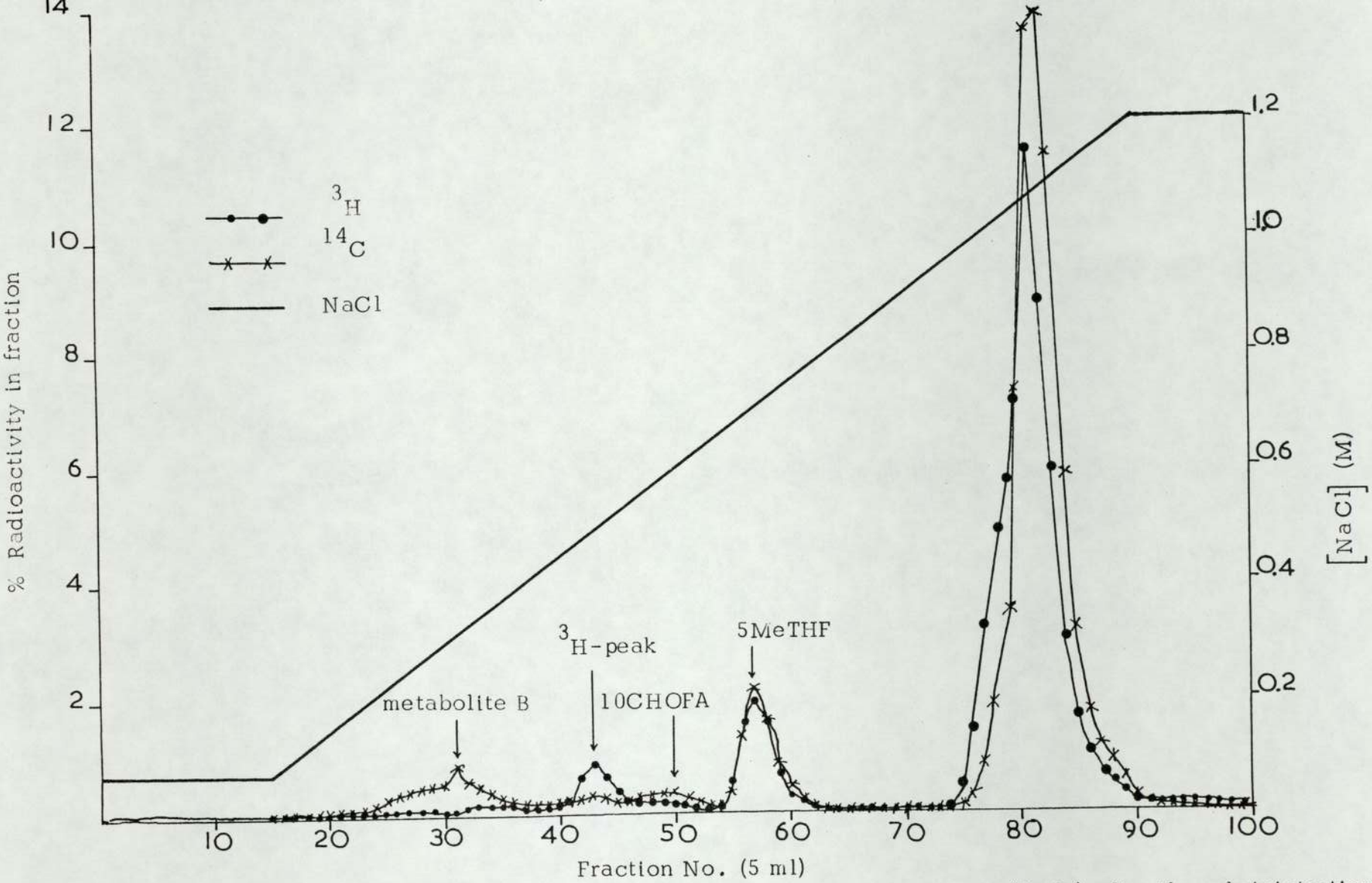


Figure 5.1.4. DE 52 Chromatography of 0-6 h urine sample of a cancer patient (H.P.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

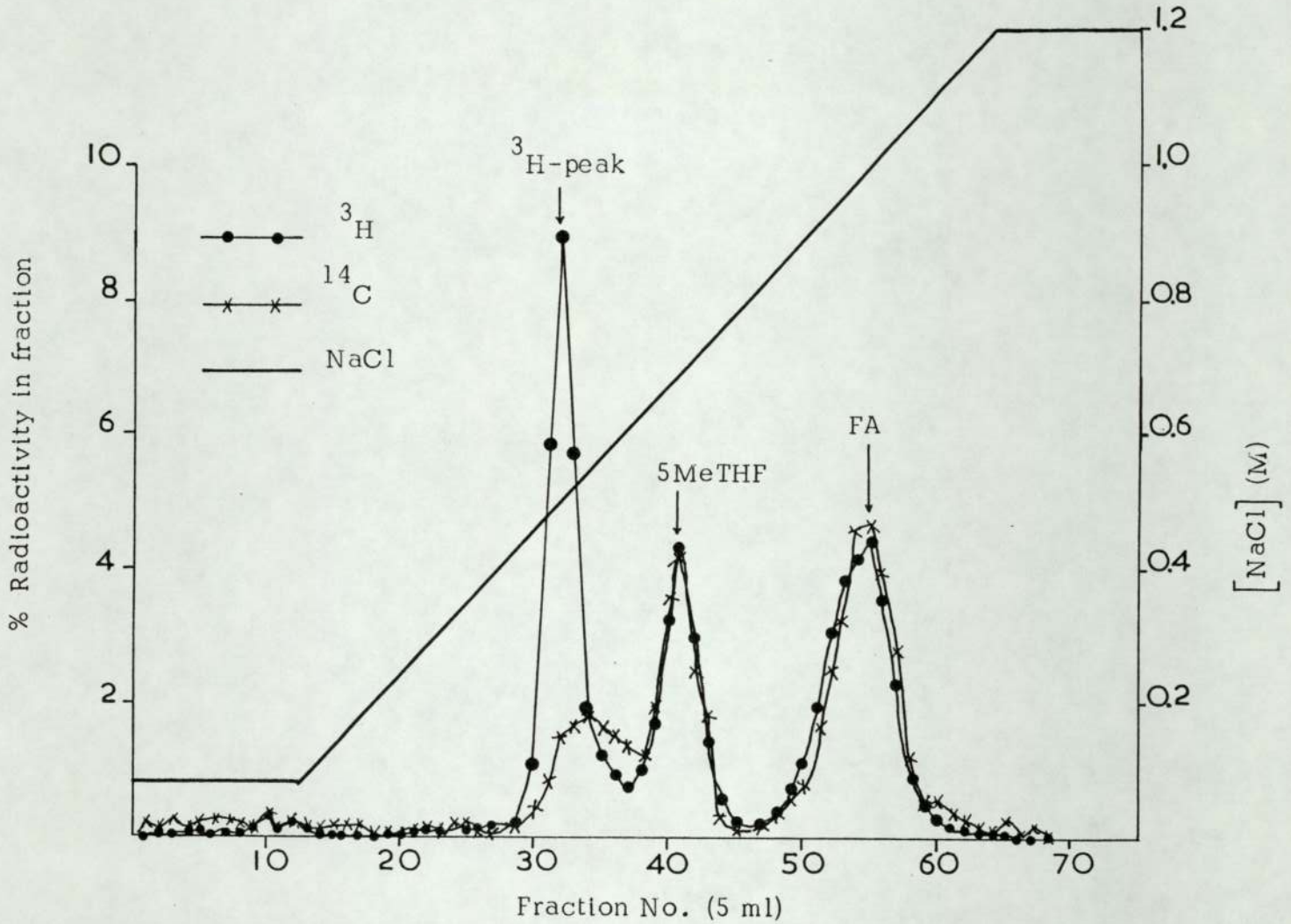


Figure 5.1.5. DE 52 Chromatography of 0-6 h urine of a cancer patient (L.J.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

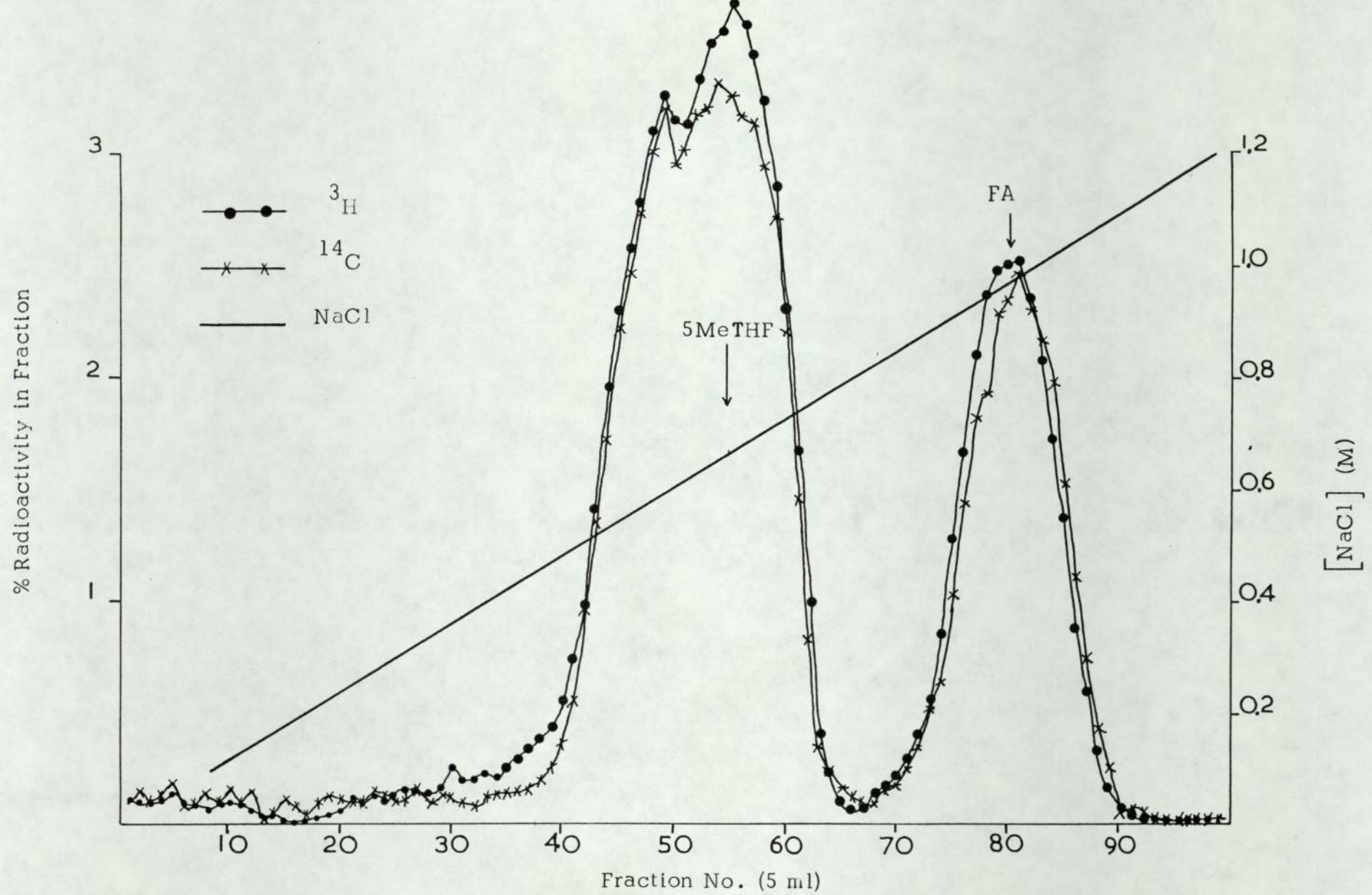


Figure 5.1.6. DE 52 Chromatography of 0-6 h urine sample of a cancer patient (D.M) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

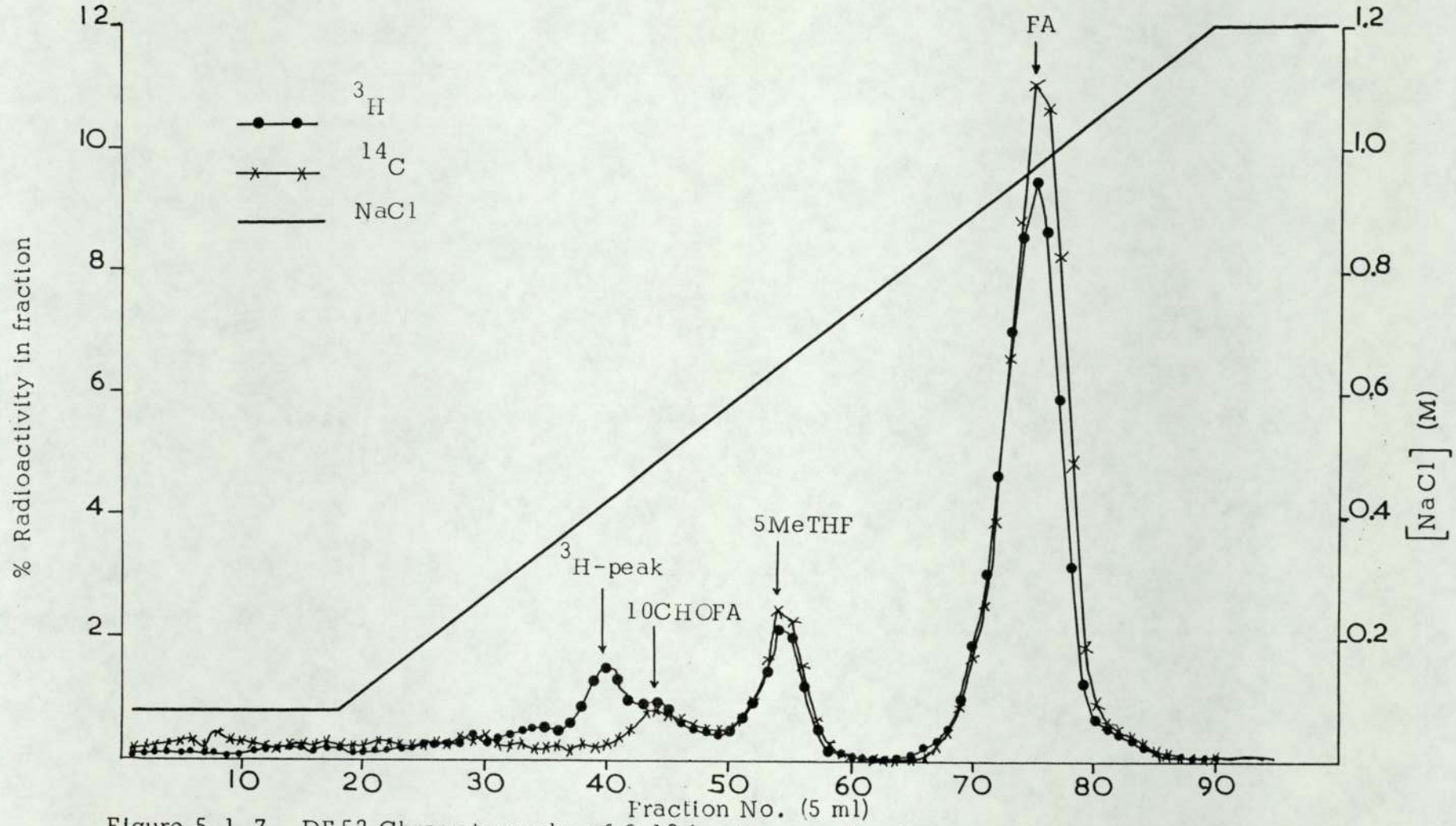


Figure 5.1.7. DE 52 Chromatography of 6-12 h urine sample of a control patient (E.W) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

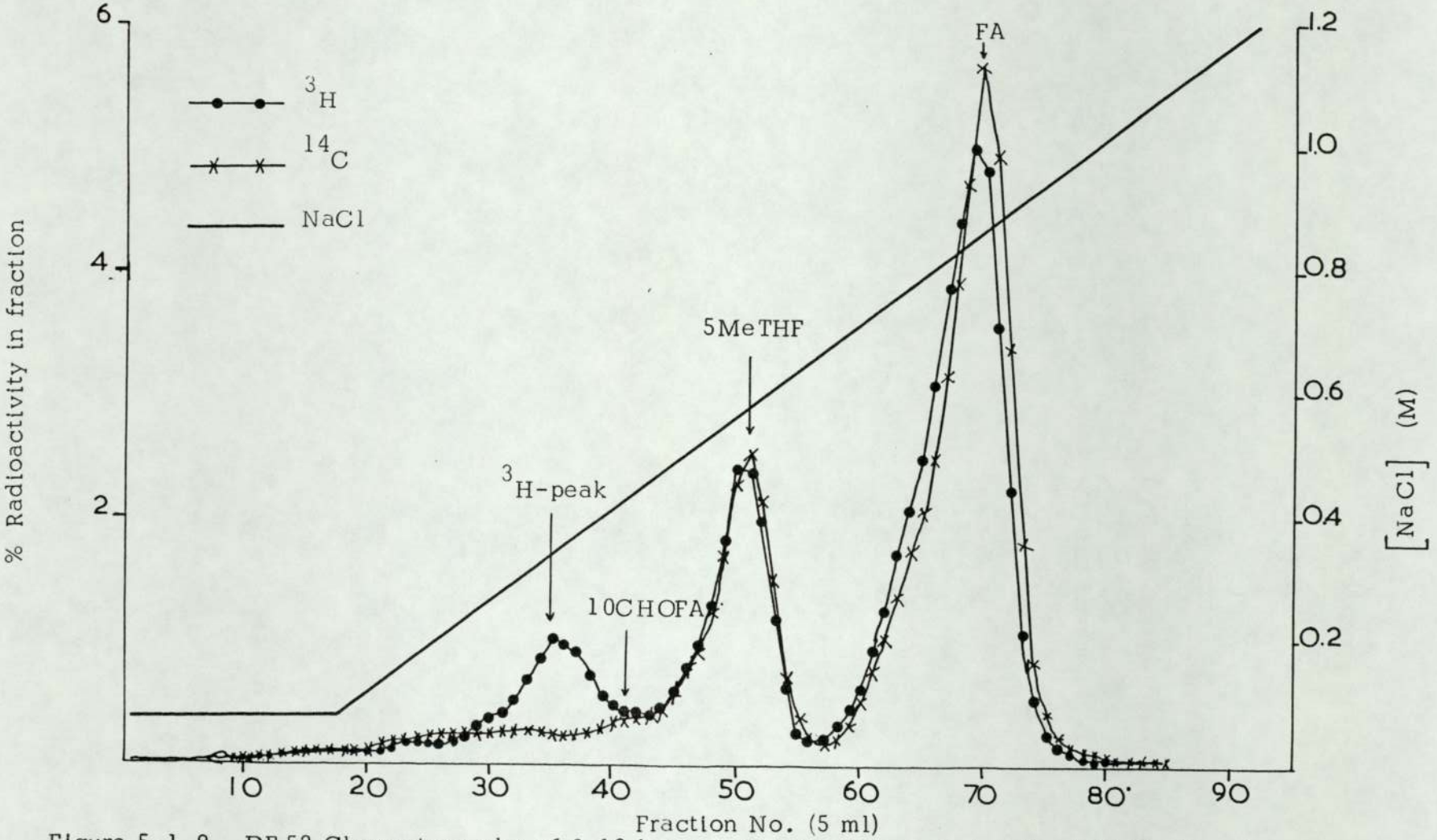


Figure 5.1.8. DE 52 Chromatography of 6-12 h urine sample of a control patient (E.T) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

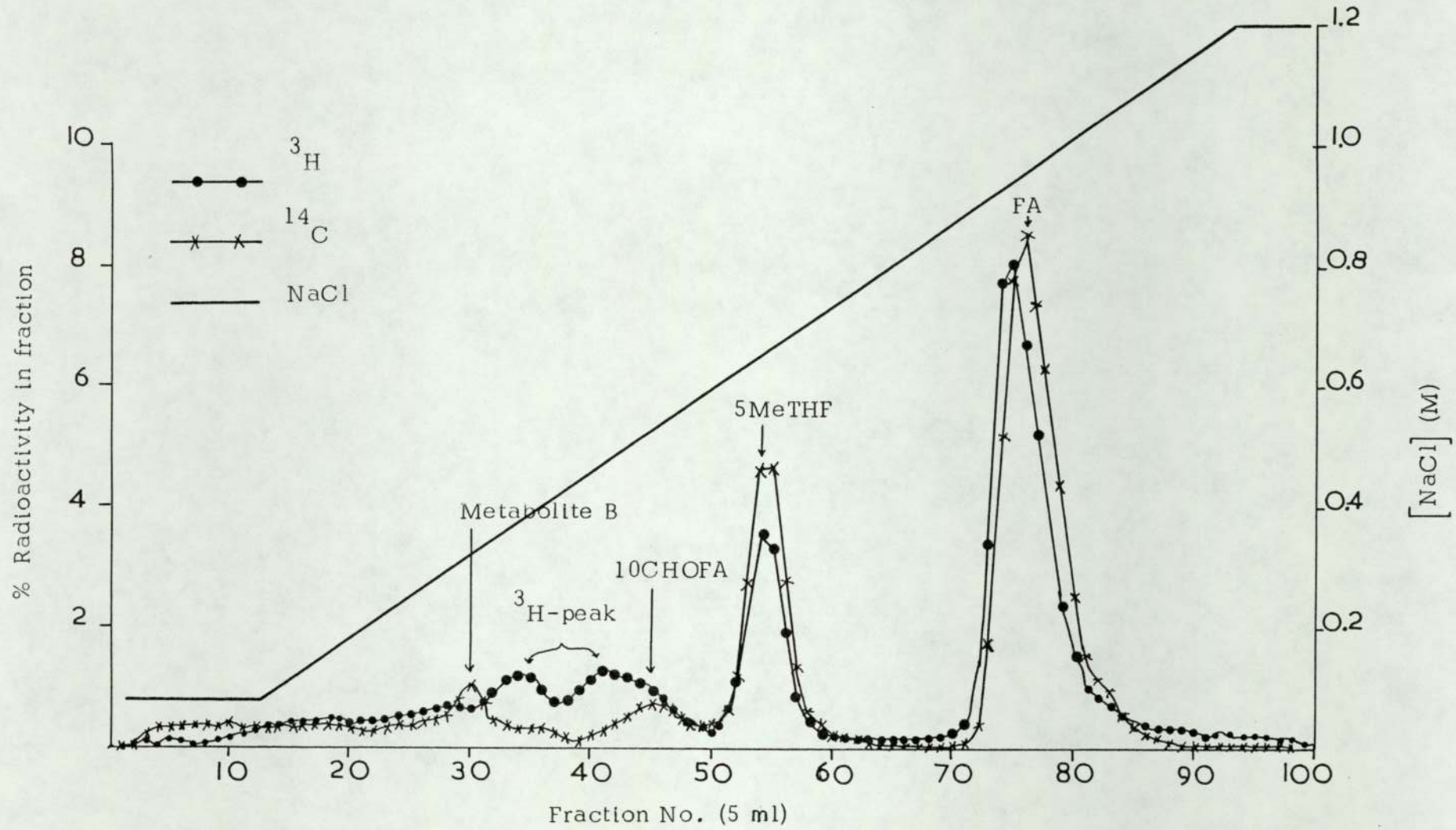


Figure 5.1.9. DE 52 Chromatography of 6-12 h urine sample of a cancer patient (H.P) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

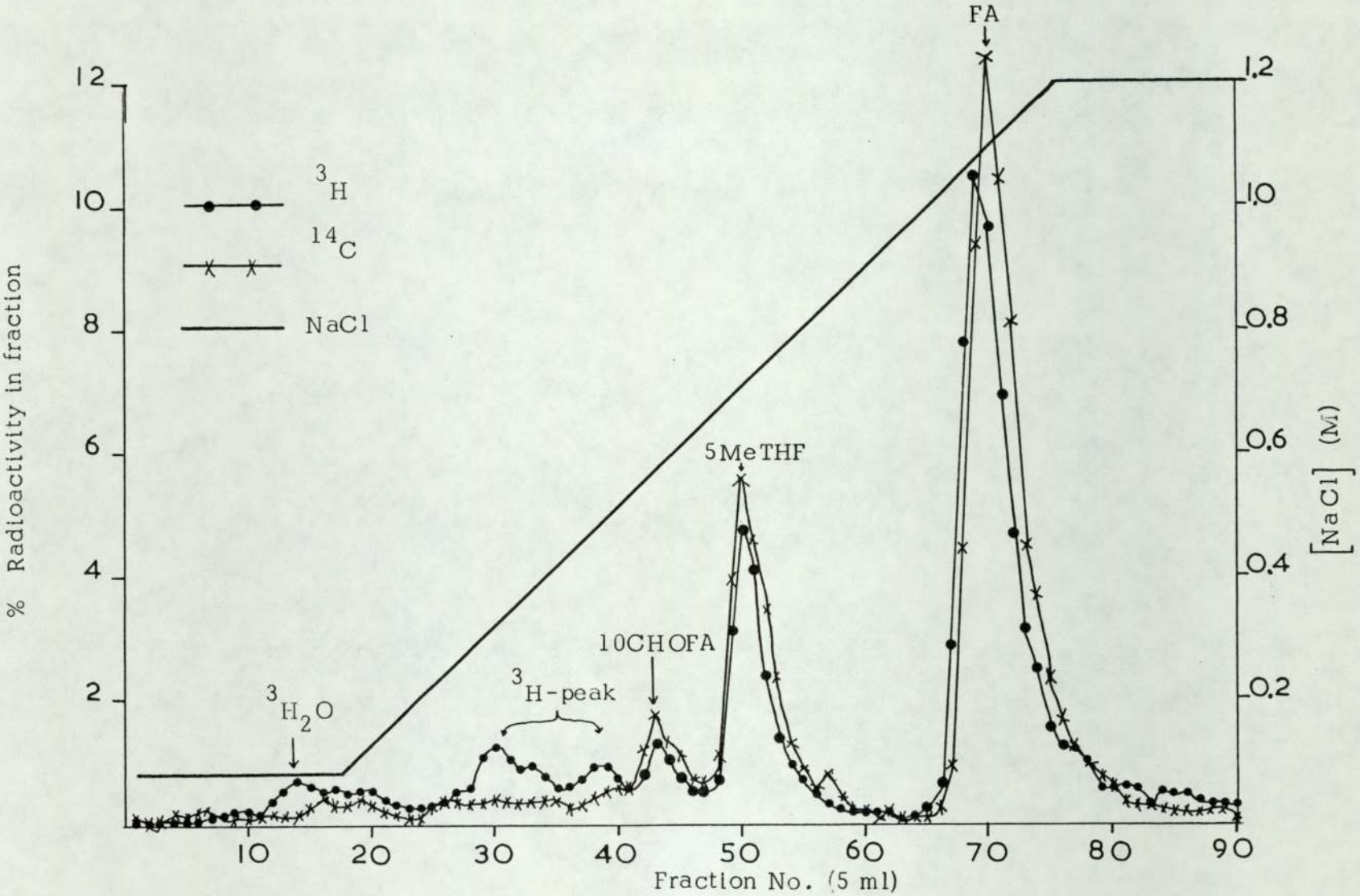


Figure 5.1.10 DE 52 Chromatography of 6-12 h urine sample of a cancer patient (N.C) after the administration of a mixture of ^3H and ^{14}C folic acid (5 mg)

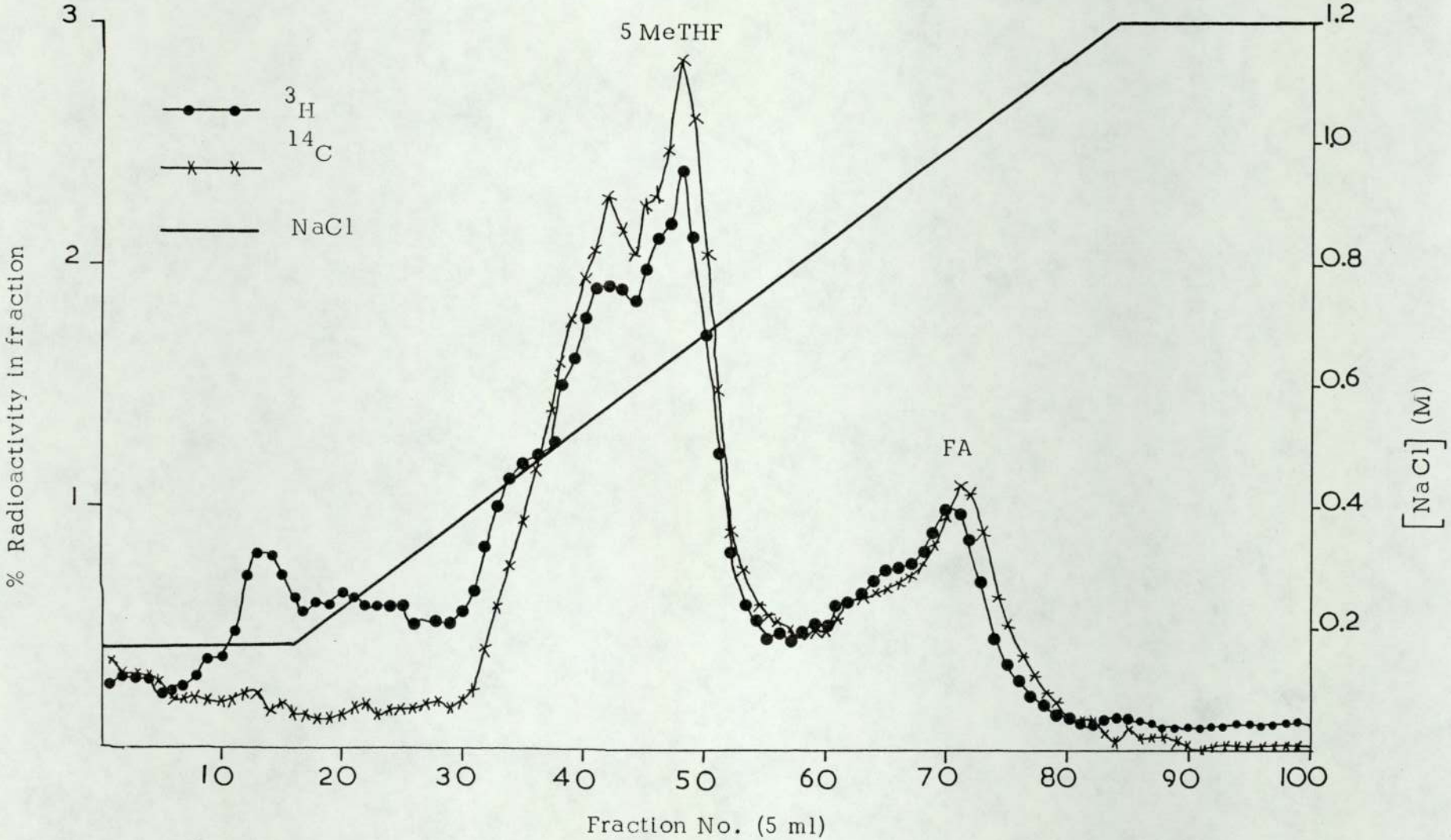


Figure 5.1.11 DE 52 chromatography of 6-12 h urine sample of a cancer patient (J.H) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

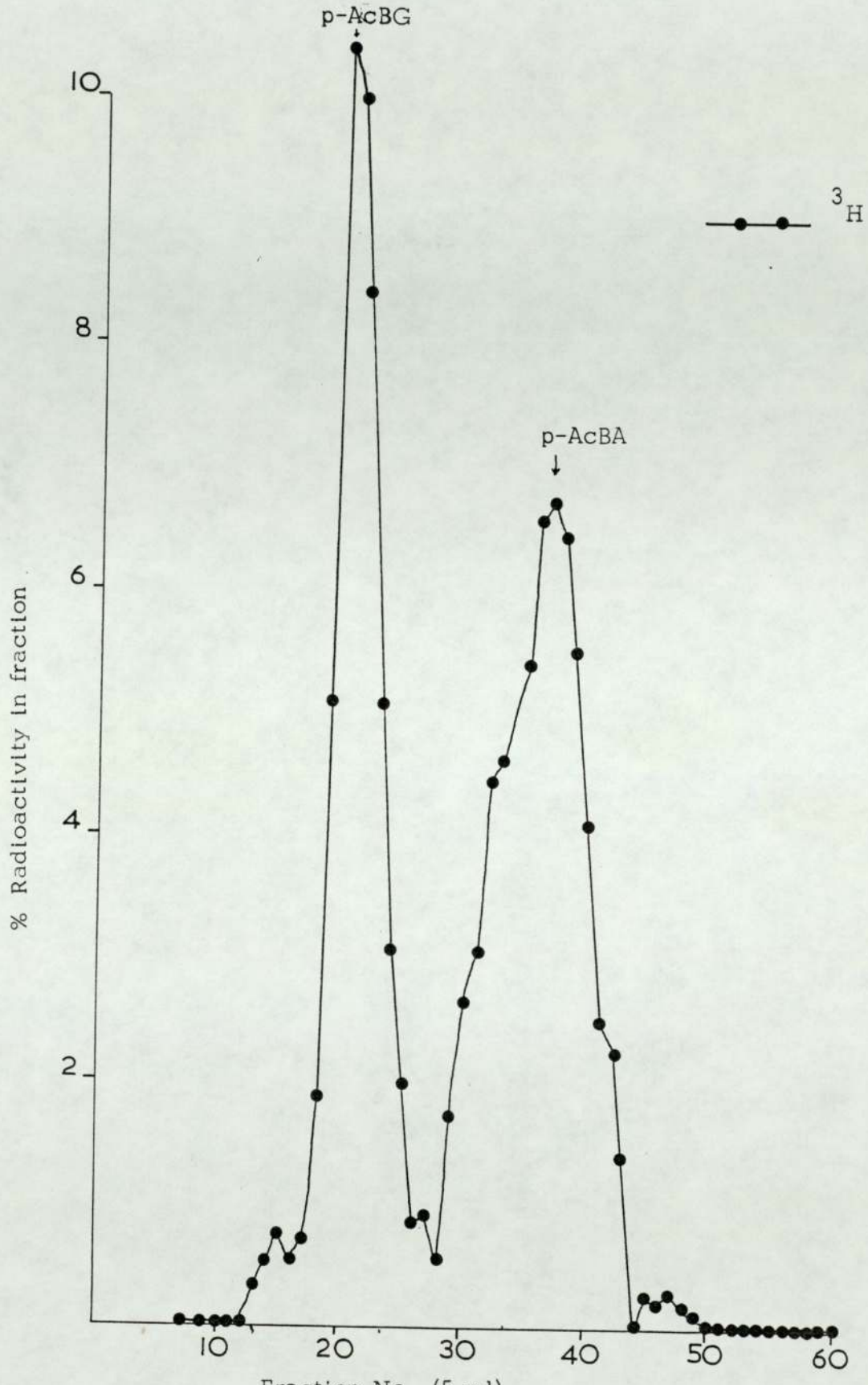


Figure 5.1.12 Sephadex G15 chromatography of the tritiated peak from DE52 chromatography of 6-12 h urine sample of a cancer patient (J.H)

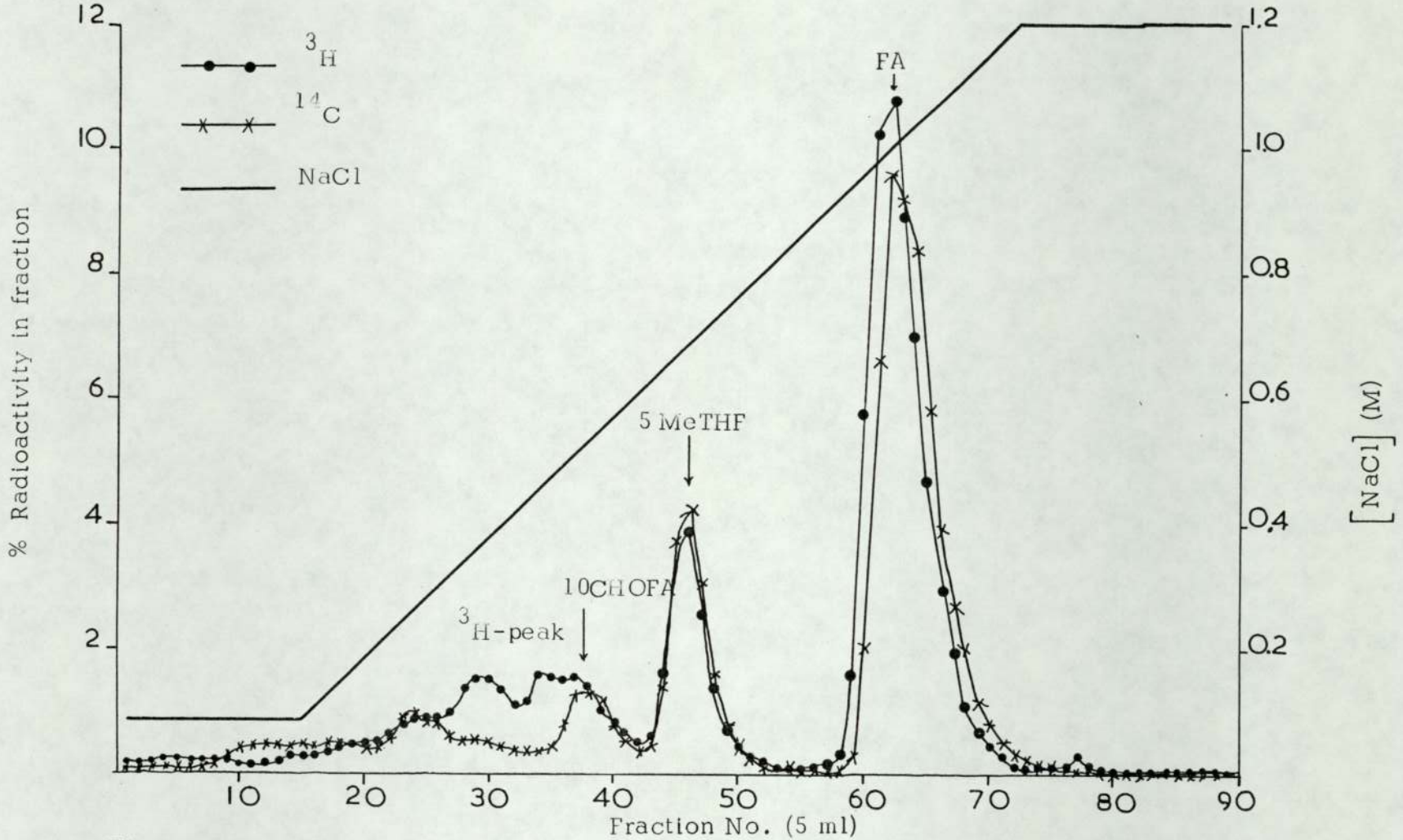


Figure 5.1.13 DE 52 chromatography of 12-24 h urine sample of a control patient (S.R.) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg)

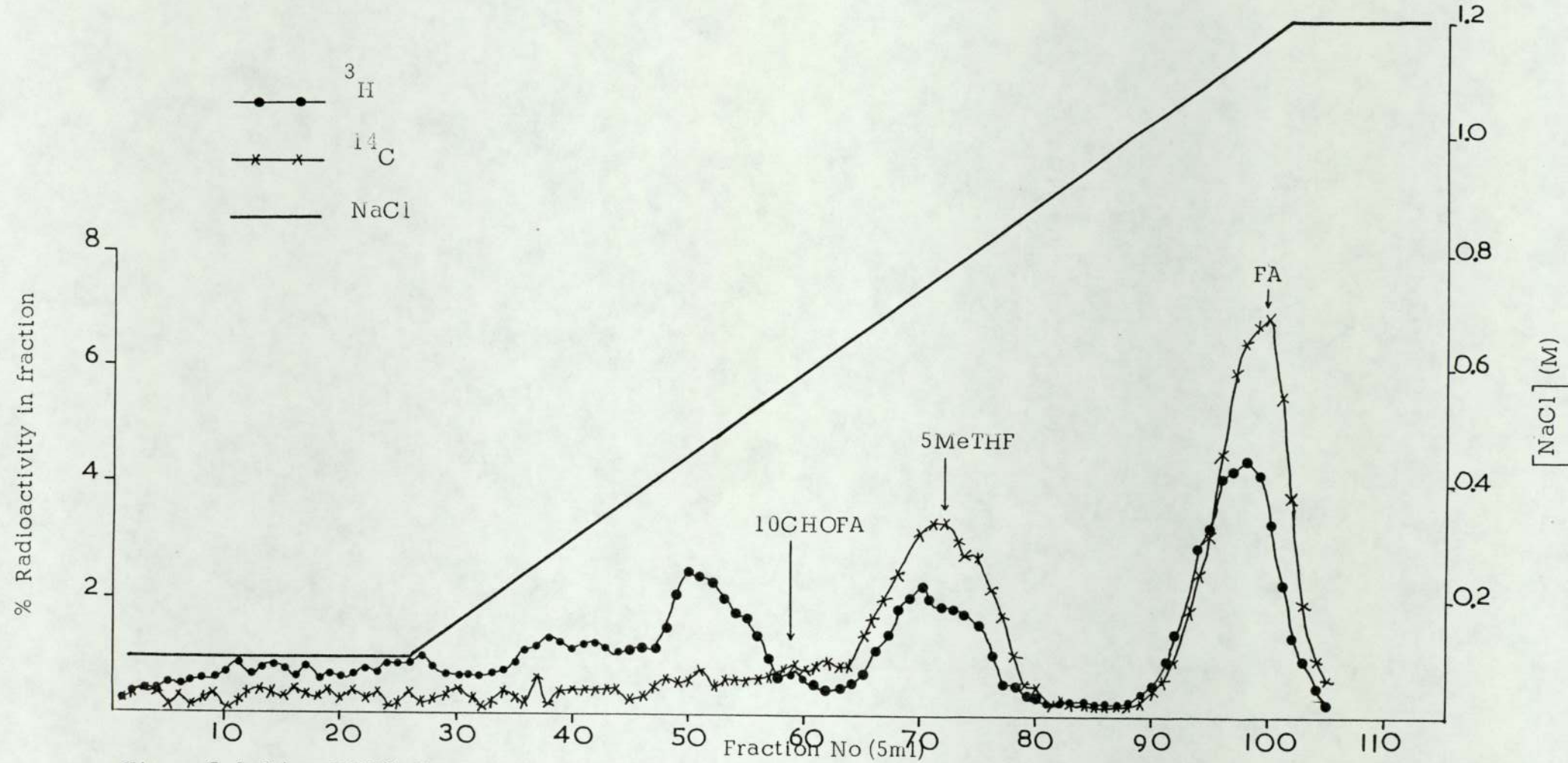


Figure 5.1.14. DE 52 chromatography of 12-24 h urine sample of a control patient (E.T) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

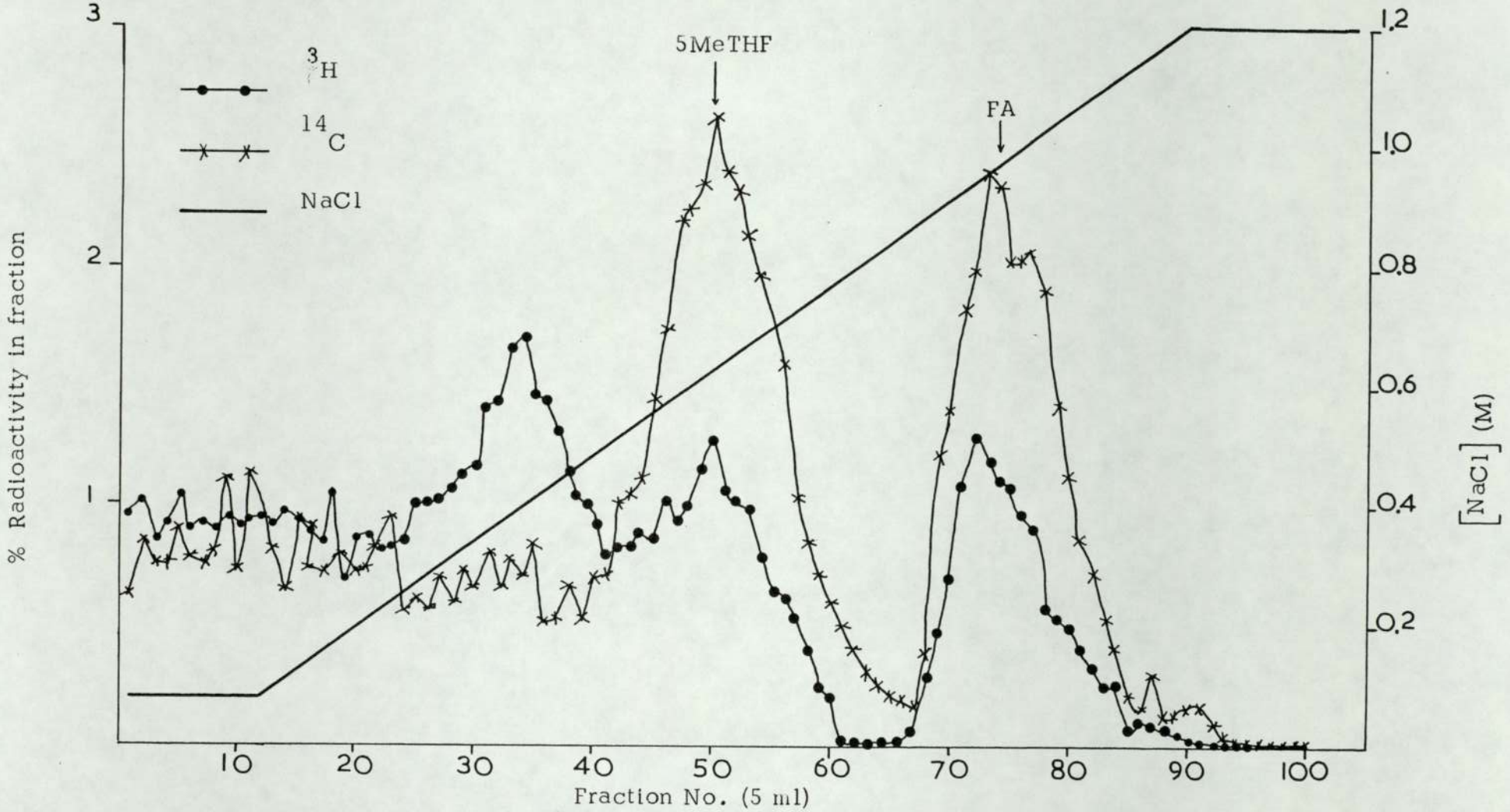


Figure 5.1.15. DE 52 chromatography of 12-24 h urine sample of a cancer patient (H.P) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

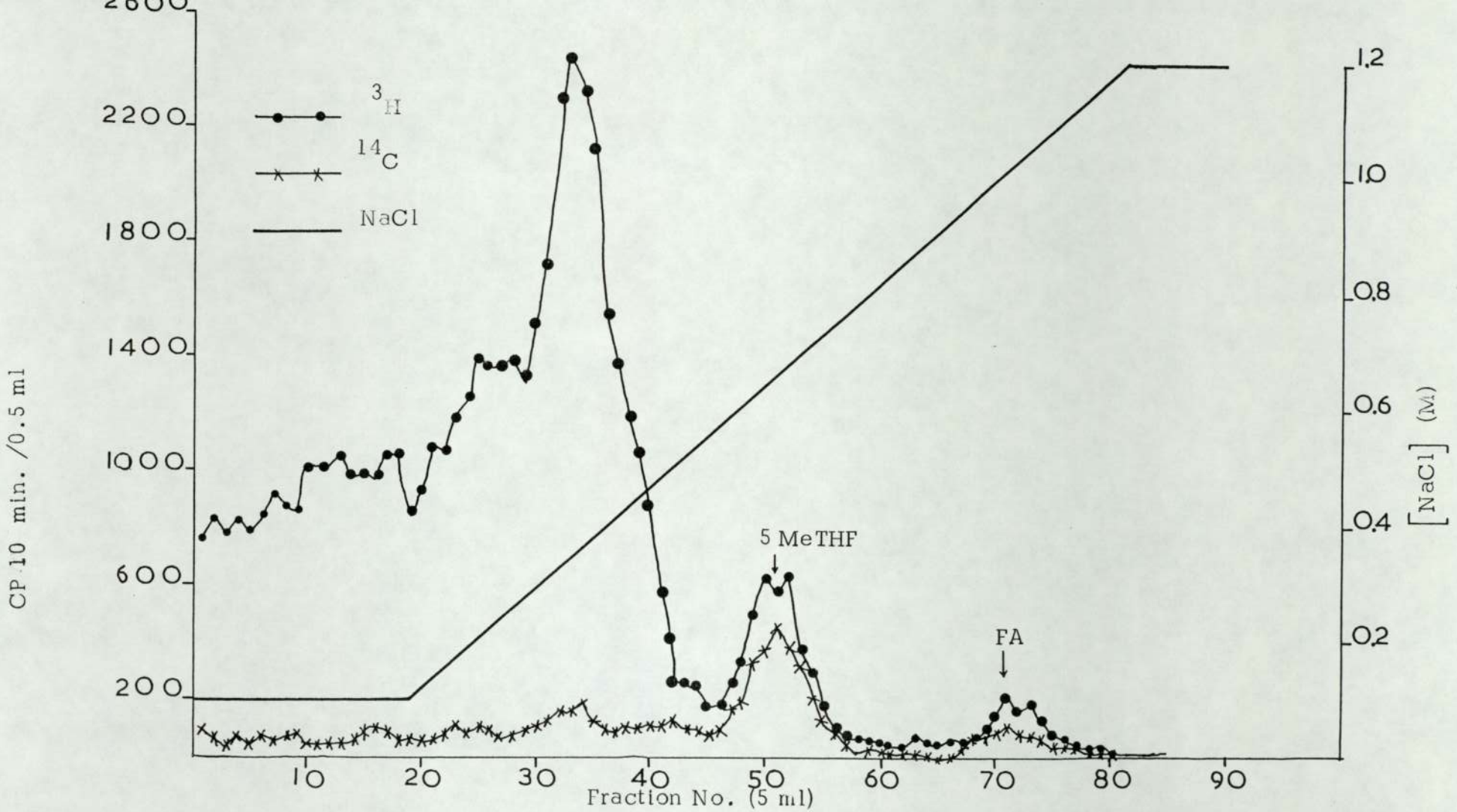


Figure 5.1.16 DE 52 chromatography of 12-24 h urine sample of a cancer patient (L.J) after the administration of a mixture of ^{3}H and ^{14}C folic acid (5 mg).

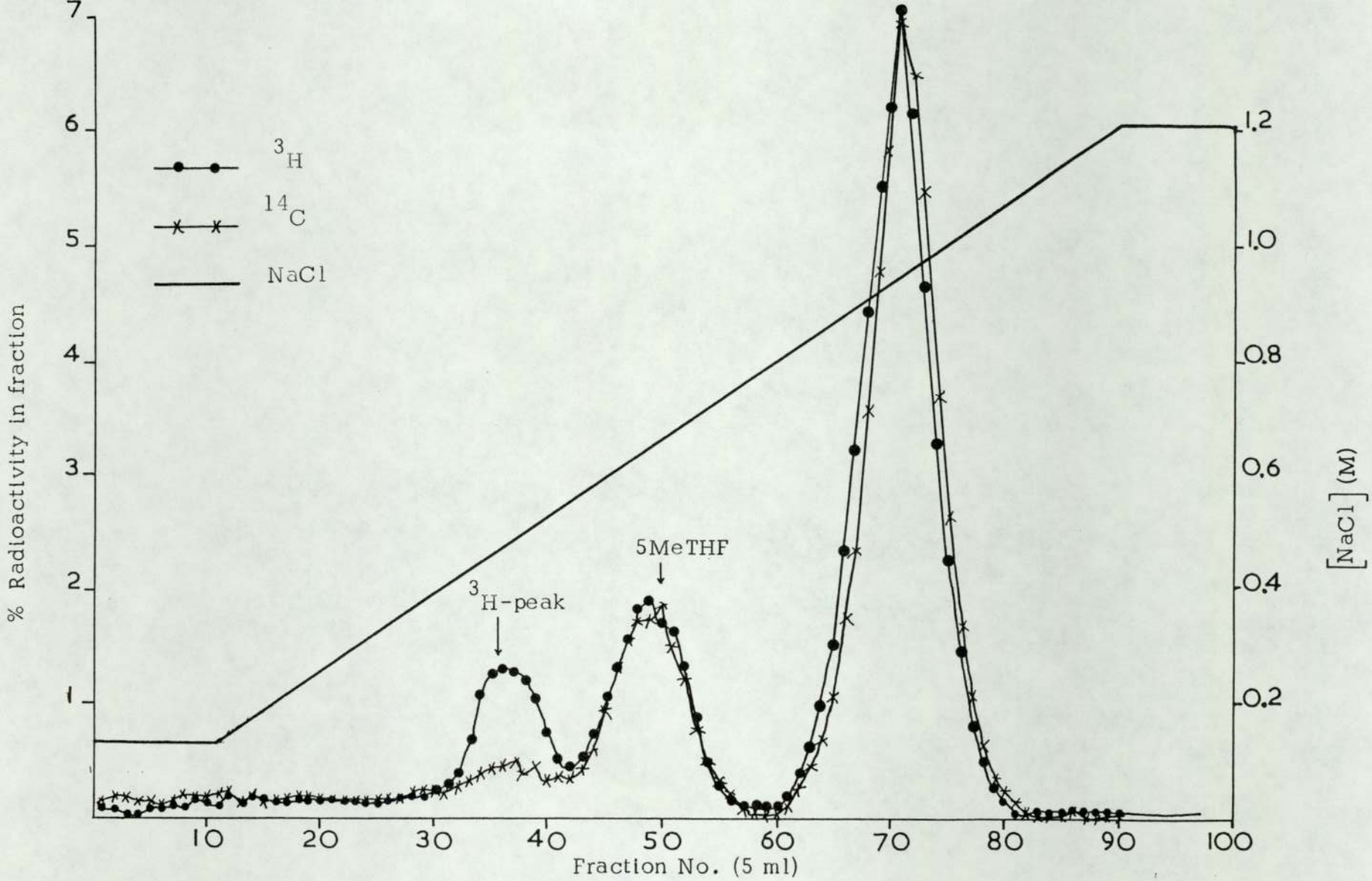


Figure 5.2.A.1. DE 52 chromatography of 0-6 h urine of the control patient (E.D$_1$) after the administration of a mixture of ^{3}H and ^{14}C folic acid (1 mg).

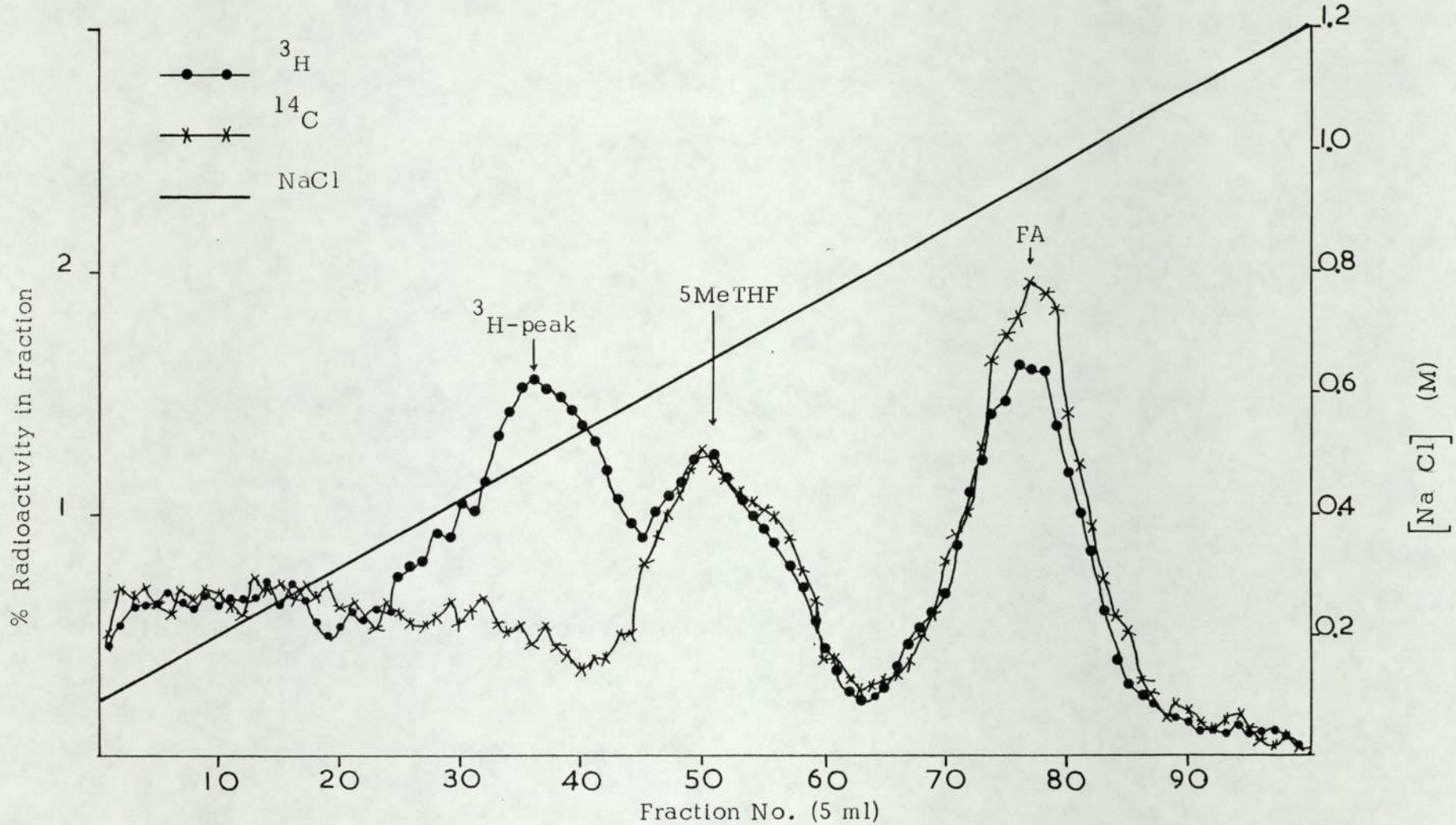


Figure 5.2.A.2. DE52 chromatography of 6-12 h urine sample of the control patient (E.D) after the administration of a mixture of ^{3}H and ^{14}C folic acid (1 mg).

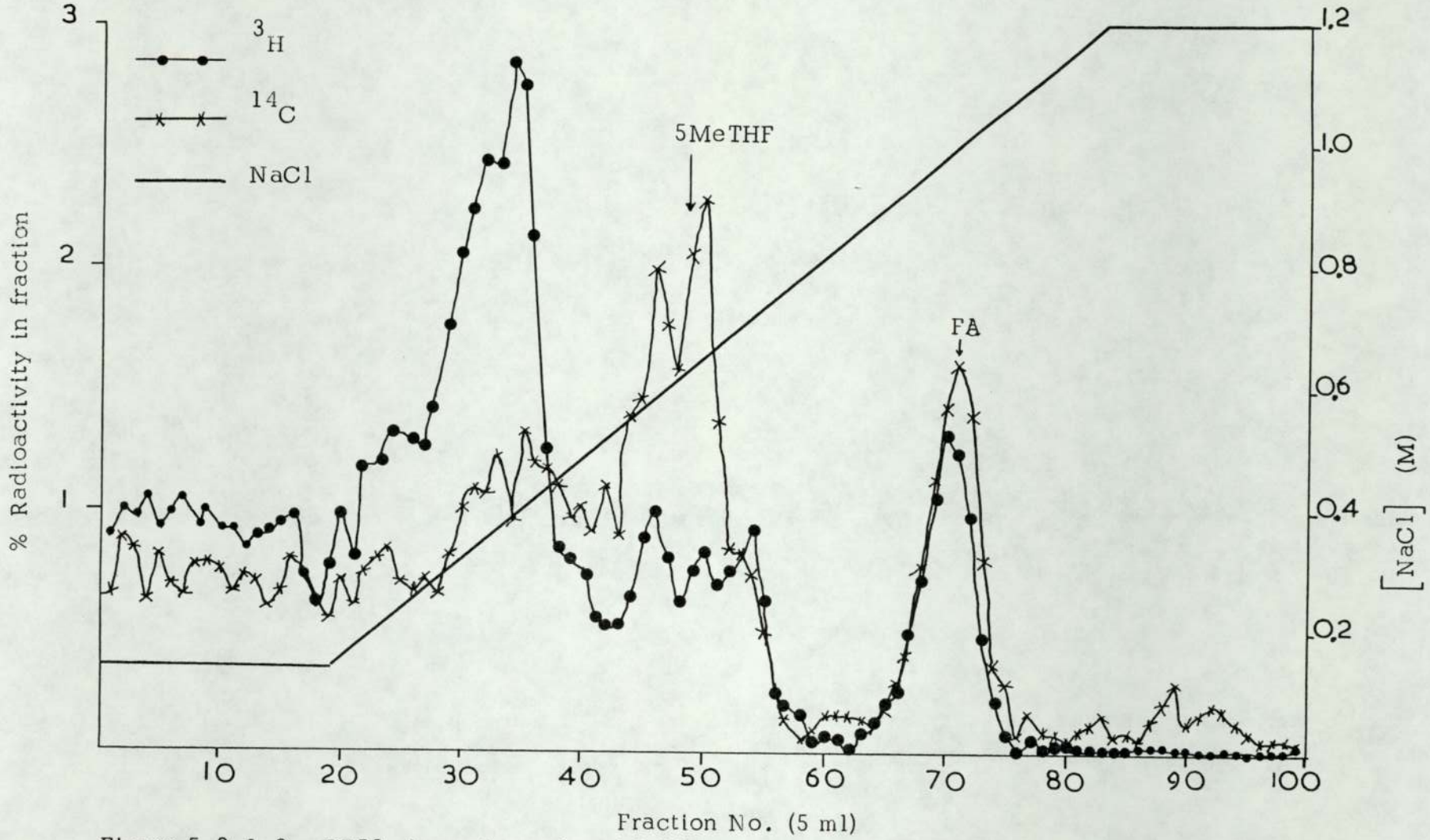


Figure 5.2.A.3. DE52 chromatography of 6-12 h urine sample of the cancer patient (A.L) after the administration of a mixture of ^{3}H and ^{14}C folic acid (1 mg)

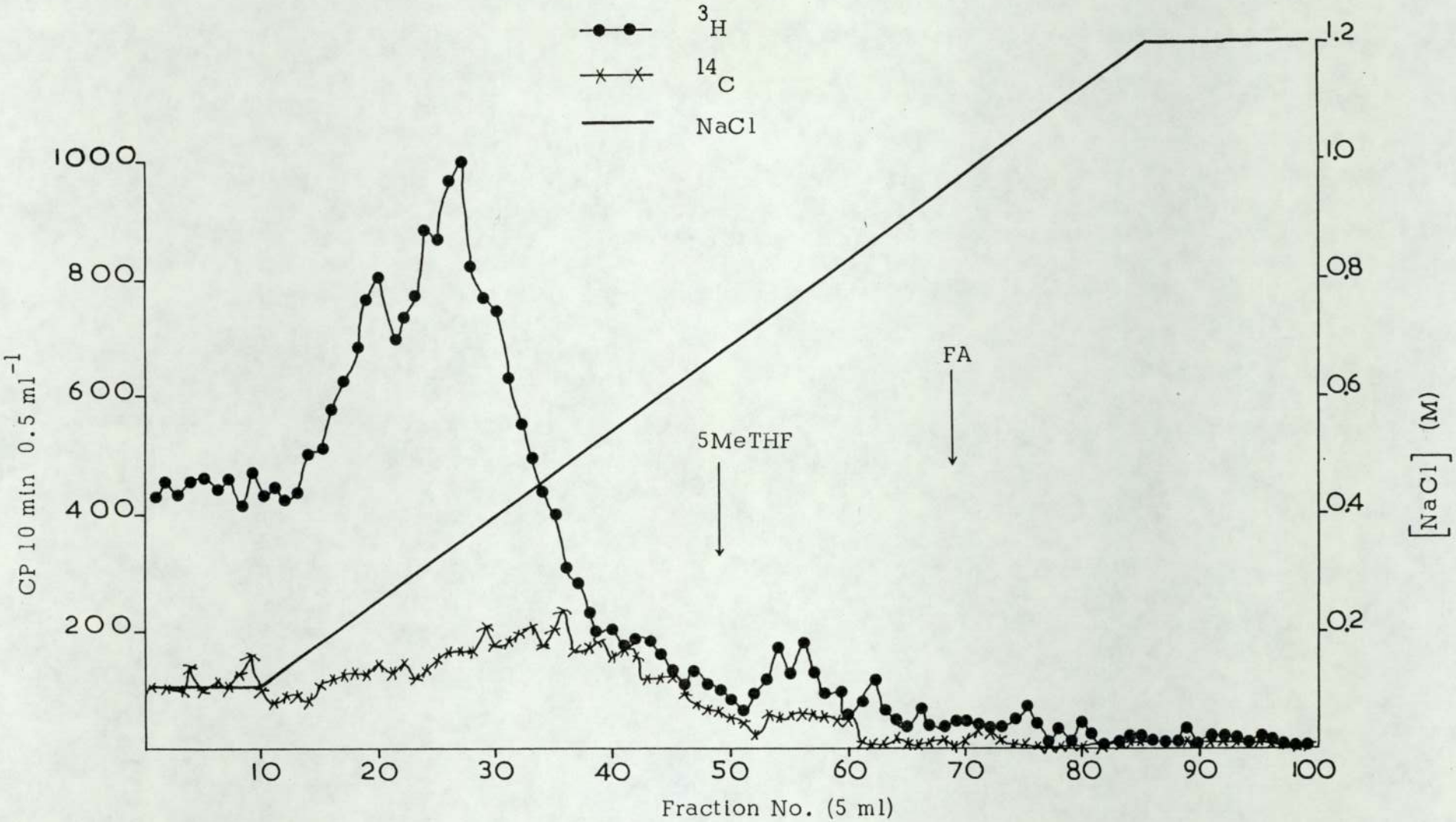


Figure 5.2.A.4. DE52 chromatography of 12-24 h urine sample of the cancer patient (A.L) after the administration of a mixture of ^{3}H and ^{14}C folic acid (1 mg)

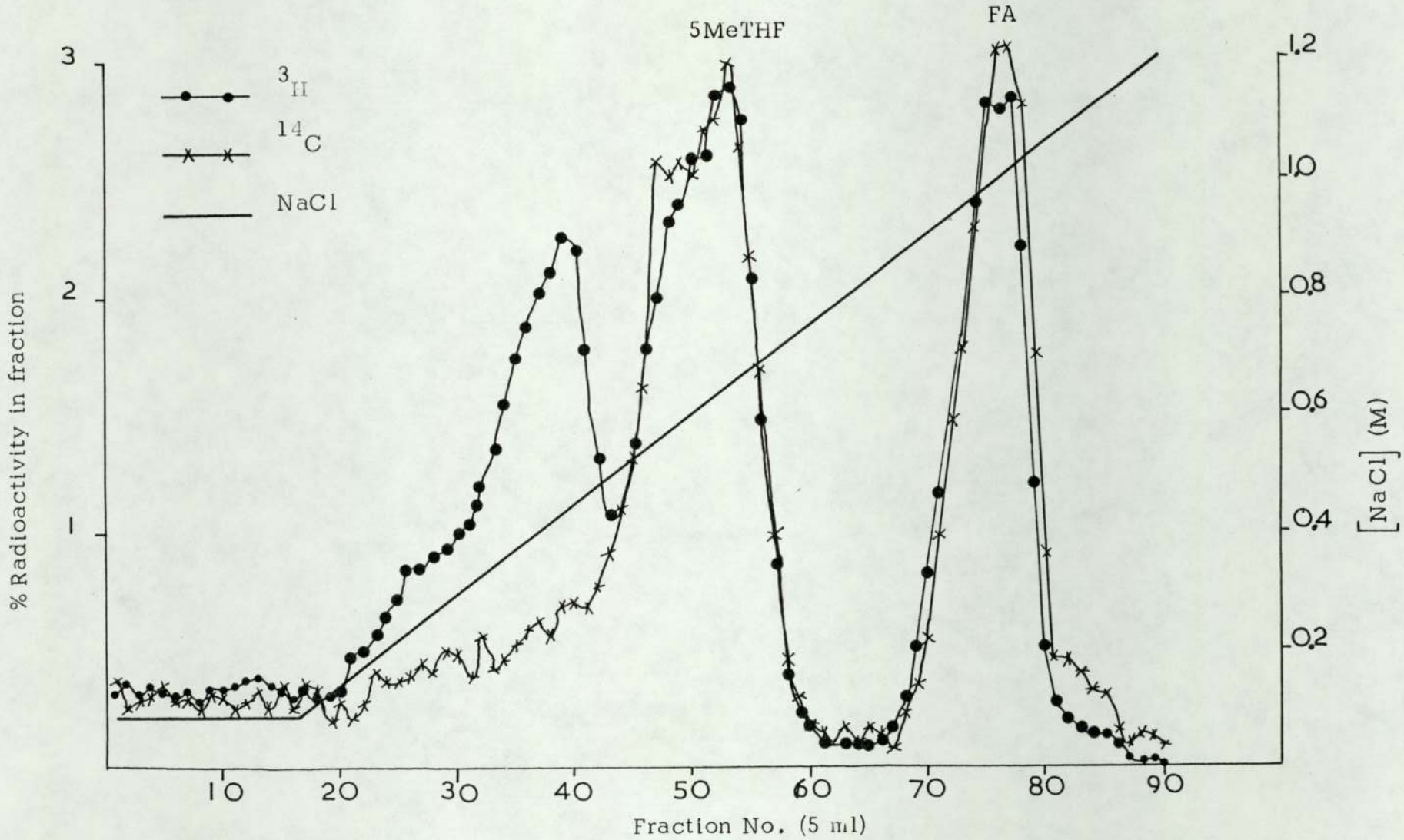


Figure 5.2.B.1. DE 52 chromatography of 0-6 h urine sample of the control patient (J.H) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.5 mg).

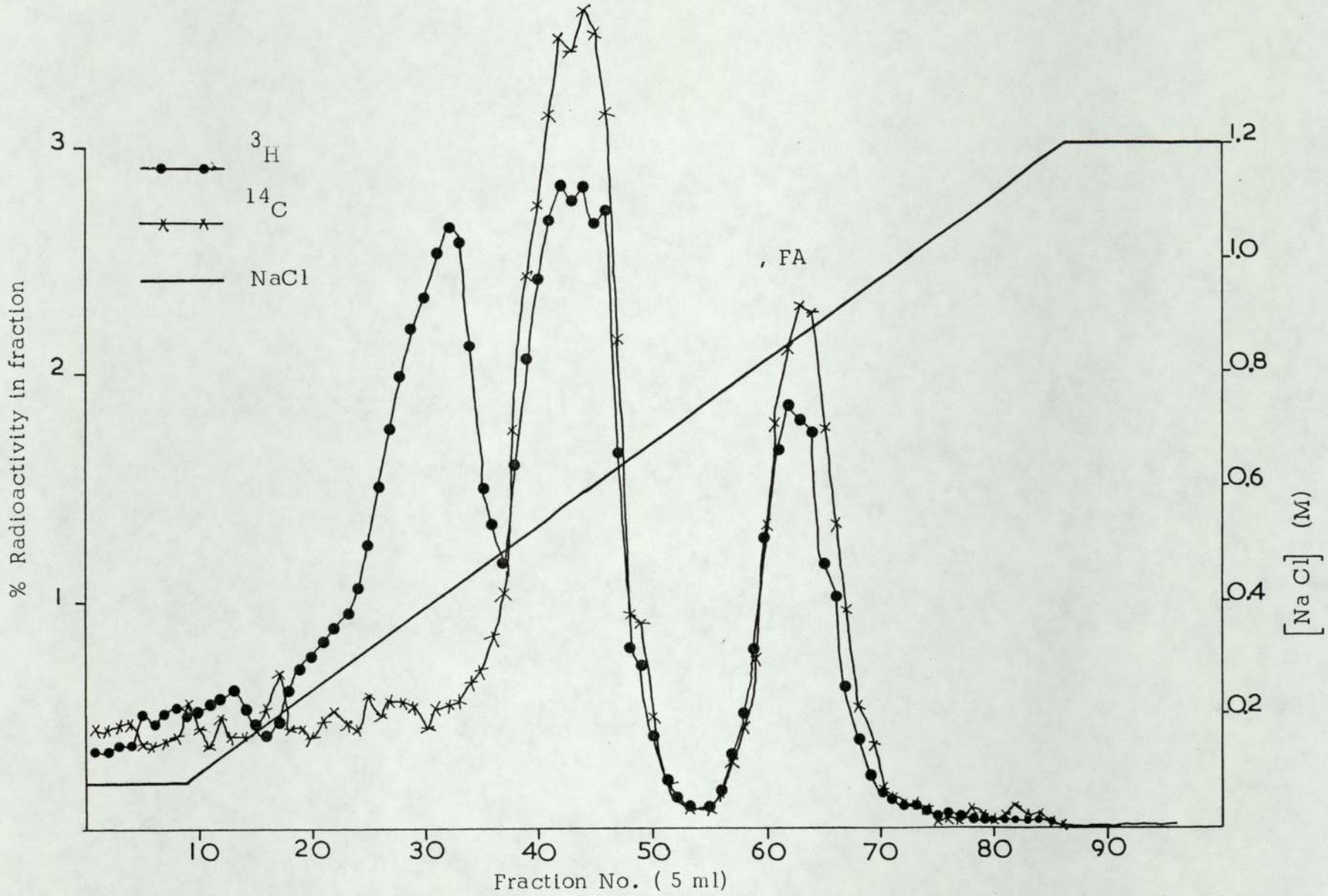


Figure 5.2.B.2. DE52 chromatography of 0-6 h urine sample of the cancer patient (S.F) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.5 mg)

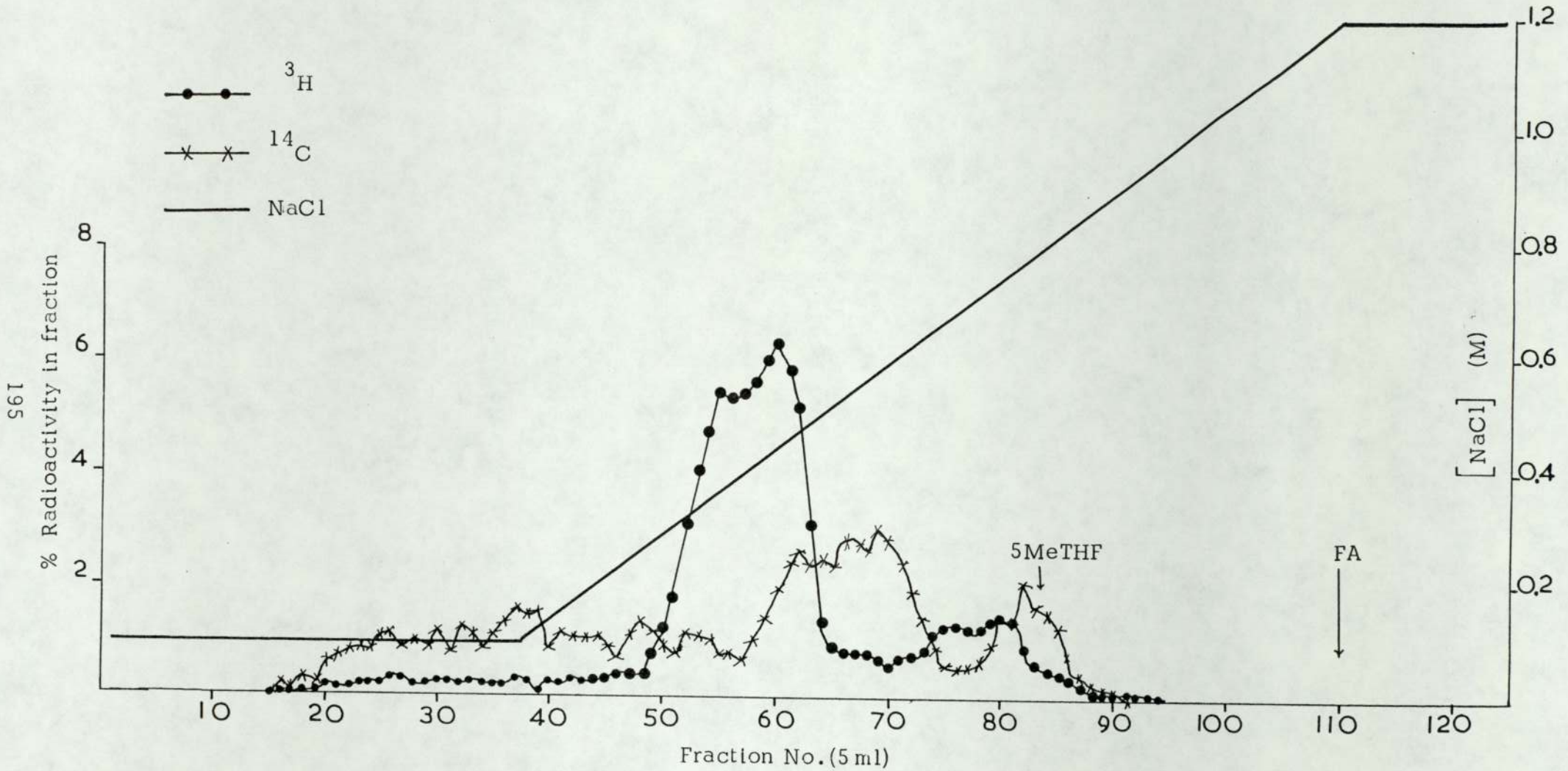


Figure 5.2.C.1. DE52 chromatography of 0-6 h urine sample of a control patient (P.H) after the admintstration of a mixture of ^{3}H and ^{14}C folic acid (0.057mg)

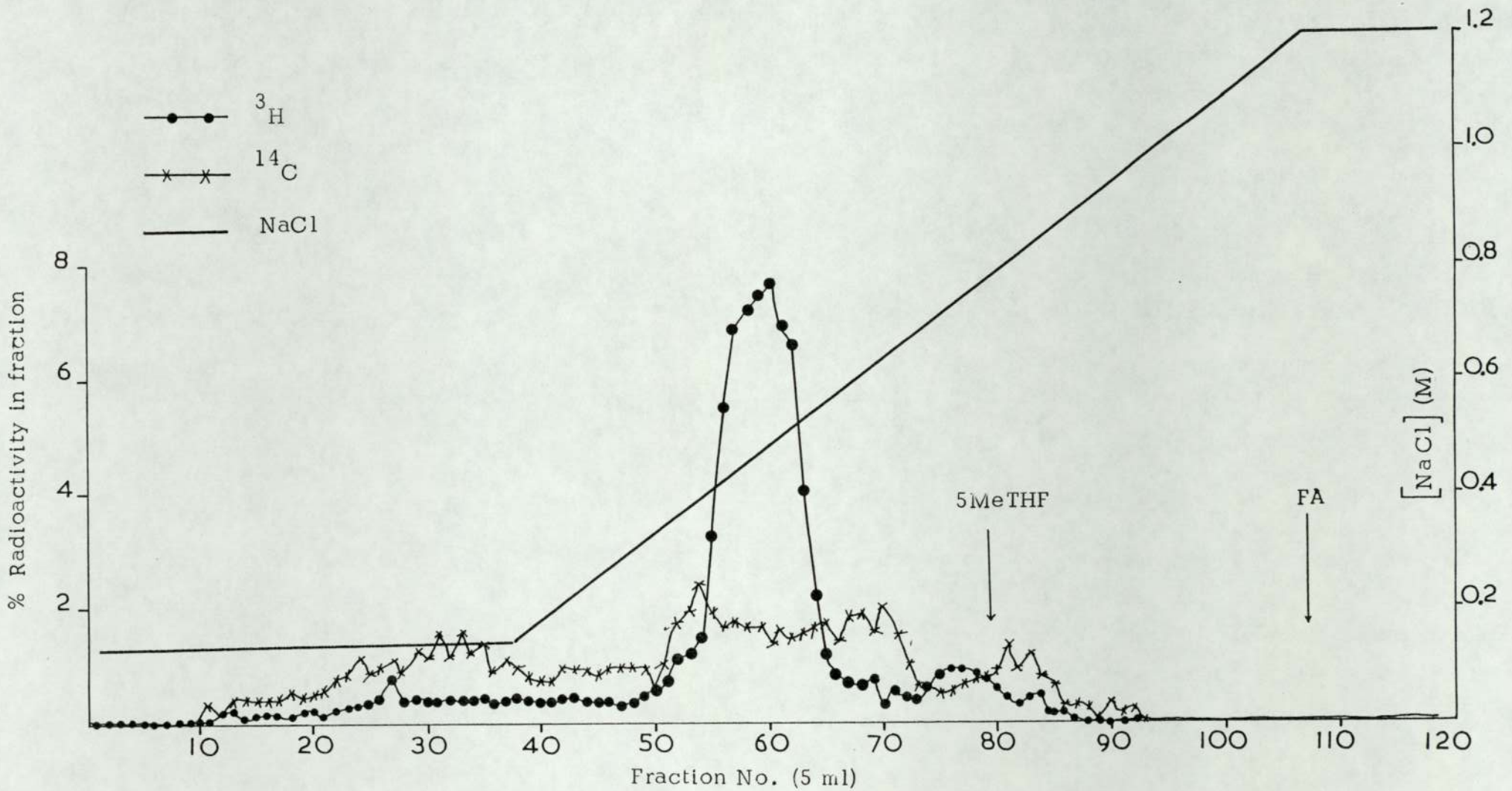


Figure 5.2.C.2. DE52 chromatography of 0-6 h urine sample of a control patient (A.G) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg)

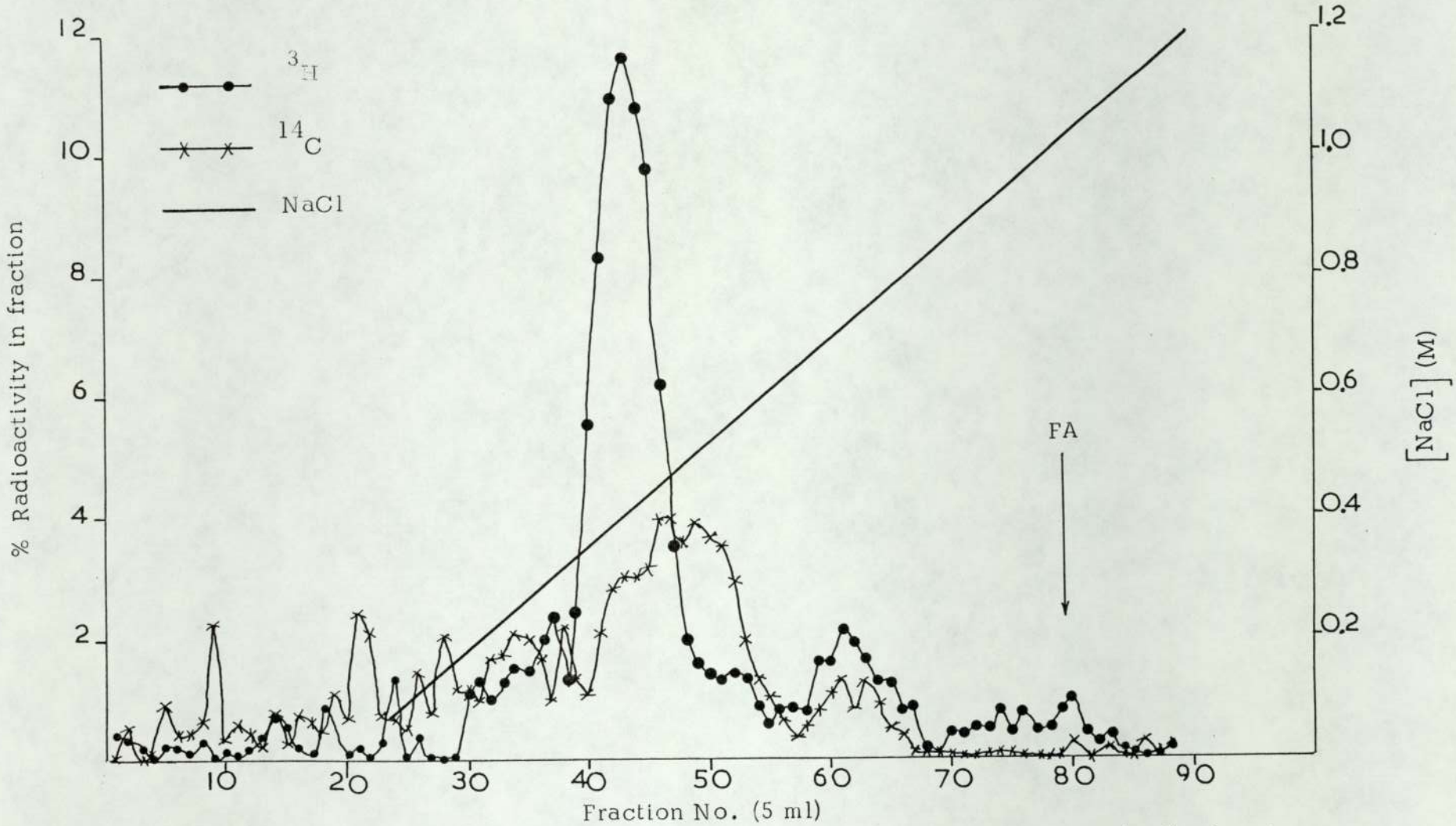


Figure 5.2.C.3. DE52 chromatography of 0-6 h urine sample of a cancer patient (R.S) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg).

Table 5.1.1. Clinical details of patients studied

Name	Age	Sex	Diagnosis	Therapy
N.C.	48	M	Cancer of Oesophagus	None
G.R.	60	M	Lymphosarcoma	Daunarubicin, Cytosine Arabinoside, Thioguanine
B.B.	63	F	Adeno-carcinoma of Ascending Colon	None
K.M.	68	F	Bronchial carcinoma	Cyclizine hydrochloride, Dihydro-codeine- Distalgestic
H.P.	67	M	Bronchial carcinoma	None
I.G.	75	F	Cancer of Breast-Diabetes	Morphine - Tomoxphen, Chloro-propamide
C.H.	68	M	Cancer of Lung	Radiotherapy
L.J.	64	M	Lymphosarcoma	Radiotherapy - Paracetamol
J.H.	73	M	Adenocarcinoma of the Stomach	None
D.M.	17	M	Lymphocytic Lymphoma	Prednisone - Potassium Supplement
W.M.	66	M	Non-Hodgkins Lymphoma	Prednisone - Pethidine

Continued...

Table 5.1.1. (Continued.....)

Name	Age	Sex	Diagnosis	Therapy
C.C.	66	M	Chronic bronchitis and Emphysema	Salbutamol-Diuretics
N.R.	67	M	Hypertension	Diuretics
S.R.	74	M	Myocardial infarction	Diuretics
E.W.	68	F	Cervical Spondylosis	Dihydrocodeine
E.T.	82	M	Deep vein thrombosis	Diuretics and Digoxin
L.W.	55	M	Hypertension	None

Table 5.1.2. Urinary recovery of radioactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]folic acid (5 mg). The results are expressed as the percentage of the dose recovered during the three collection periods

	% of dose recovered							
	0 - 6 h		6 - 12 h		12 - 24 h		0 - 24 h	
PATIENT	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Cancer								
N.C.	23.8	20.4	4.6	3.7	1.6	0.2	30.0	24.3
G.R.	15.1	14.6	8.4	8.0	2.1	2.2	25.6	24.8
B.B.	0.3	0.2	15.0	12.0	7.0	6.0	22.3	18.3
K.M.	12.3	10.4	7.0	5.9	2.4	1.7	21.7	18.0
H.P.	14.7	13.2	3.6	3.1	0.9	0.5	19.2	16.8
I.G.+	10.2	8.0						
C.H.	1.2	0.9	8.2	7.3	died			
L.J.	2.5	2.4	-	-	2.4	0.8	4.9	3.2
J.H.	-	-	1.7	1.4	3.0	1.4	4.7	2.8
D.M.	2.0	1.8	-	-	0.4	0.5	2.4	2.3
W.M.	0.1	0.1	0.7	0.7	0.3	0.3	1.1	1.1
Mean							14.1 ± 3.7*	12.4 ± 3.3

Table 5.1.2 (continued...)

	% of dose recovered							
	0 - 6 h		6 - 12 h		12 - 24 h		0 - 24 h	
PATIENT	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Control								
C.C.	29.0	23.3	9.0	7.4	1.2	1.1	39.2	31.8
N.R.$^{\Delta}$	8.6	8.4	24.0	22.2	4.7	3.1	37.3	33.7
S.R.	19.6	17.3	5.3	4.6	8.4	7.3	33.3	29.2
E.W.	16.5	12.3	6.6	4.9	2.9	1.6	26.0	18.8
E.T.	11.6	9.4	7.1	5.7	5.6	2.3	24.3	17.4
L.W. +	12.7	11.3						
Mean							32 ± 3.0*	26.2 ± 3.4*

* Mean $\pm$ SEM (n = 9, Group I, n = 5 Group II)

\+ One sample only collected

Δ Data supplied by A. E. Pheasant

Table 5.1.3. The relationship between the approximate size of the tumour as judged by the clinician and the urinary recovery of radioactivity 24 h after the administration of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid (5mg).

Patient	Size of tumour	% of dose recovered in 24 h ^{3}H	^{14}C
N.C.	small	30.0	14.3
G.R.	"	25.6	24.8
K.M.	moderate	21.7	18.0
H.P.	"	19.2	16.8
L.J.	large	4.9	3.2
J.H.	"	4.7	2.8
D.M.	"	2.4	2.3
W.M.	"	1.1	1.1

Table 5.1.4. Faecal recovery of radioactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid. The results are expressed as the percentage of the dose recovered in 48h.

Patient	Faecal dry wt.	% dose recovered ^{3}H	^{14}C
GROUP 1			
L.J.	87.3	10.8	28.0
G.R.	60.0	6.7	13.9
GROUP 2			
E.W.	38.1	10.3	26.6
E.T.	4.9	0.2	0.3

Group 1 - Patients with malignant disease

Group 2 - Control patients

Table 5.1.5. The ratios of ^{3}H:^{14}C in the administered folic acid and in folic acid excreted from various urine samples 24 h after the administration of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid

		^{3}H : ^{14}C ratio			% ^{3}H-enrichment		
Name	Dose	0 - 6h	6 - 12 h	12 - 24h	0-6h	6-12h	12-24h
Cancer							
N.C.	1.60:1	1.85:1	2.07:1	-	15.6	29.4	-
G.R.	1.46:1	1.67:1	1.54:1	-	14.4	5.5	-
B.B.	1.80:1	-	2.04:1	2.65:1	-	13.3	47.2
K.M.	1.43:1	1.80:1	1.88:1	1.69:1	25.9	31.5	18.2
H.P.	1.43:1	1.75:1	1.71:1	2.16:1	22.4	19.6	51.0
I.G.	1.79:1	2.22:1	-	-	24.0	-	-
C.H.	1.44:1	1.61:1	-	-	11.8	-	-
L.J.	1.45:1	1.66:1	-	2.14:1	14.5	-	47.6
J.H.	1.58:1	1.88:1	-	-	19.0	-	-
D.M.	1.79:1	2.22:1	-	1.91:1	24.0	-	6.7
Mean $\pm$ SEM	1.58:1$\pm$0.05	1.85:1$\pm$0.08	1.85:1$\pm$0.1	2.11:1$\pm$0.16	19.1$\pm$1.7	19.9$\pm$4.9	34.1$\pm$9.1

Table 5.1.5. (continued...)

		^{3}H : ^{14}C ratio			% ^{3}H - enrichment		
Name	Dose	0-6h	6-12h	12-24h	0-8h	6-12h	12-24h
Control							
C.C.	1.47:1	1.81:1	2.0:1	-	23.1	36.1	-
S.R.	1.54:1	1.81:1	1.61:1	1.61:1	17.5	4.5	4.5
E.W.	1.82:1	2.32:1	2.49:1	2.63:1	27.4	36.8	44.5
E.T.	1.18:1	1.34:1	1.30:1	1.44:1	13.6	10.2	22.0
L.W.	1.39:1	1.75:1	-	-	25.9	-	-
Mean $\pm$ SEM	1.48:1 $\pm$ 0.1	1.85:1 $\pm$ 0.16	1.85:1 $\pm$ 0.26	1.89:1 $\pm$ 0.37	21.5 $\pm$ 2.6	21.9 $\pm$ 8.5	23.7 $\pm$ 11.6

Table 5.1.6. The distribution of radioactivity amongst the labelled metabolites found in the urine 0-6 h, 6-12 h and 12 - 24 h after oral doses of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid (5 mg) to cancer and control patients. The results are expressed as percentage of the dose.

Patient	0 - 6 h					
	Folic acid		5 MeTHF		p-Ac BG	p-Ac BA
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
N.C.	16.5	14.9	2.9	2.9	0.6	0.0
G.R.	6.8	7.0	2.4	2.6	2.1	0.0
B.B.	0.0	0.0	0.0	0.0	0.0	0.0
K.M.	9.6	7.9	1.0	0.9	0.9	0.0
H.P.	11.3	10.4	1.4	1.1	0.7	0.0
I.G.	8.3	6.4	1.0	0.8	0.8	0.0
C.H.	0.8	0.7	0.1	0.1	0.2	0.0
L.J.	1.0	1.1	0.5	0.6	0.9	0.0
J.H.	0.0	0.0	0.0	0.0	0.0	0.0
D.M.	0.6	0.5	1.2	1.1	0.0	0.0
Mean $\pm$ SEM	5.5 ± 1.9	4.9 ± 1.7	1.1 ± 0.3	1.0 ± 0.3	0.6 ± 0.2	

Continued.....

Table 5.1.6 (continued...)

Patient	Folic acid		5 Me THF		p-AcBG	p-AcBA
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
	0 - 6 h					
C.C.	20.9	15.8	3.4	2.8	3.2	0.0
N.R.	5.8	5.4	0.7	0.7	n.d.	n.d.
S.R.	13.9	12.4	3.7	3.5	1.2	0.0
E.W.	12.5	9.7	2.1	1.7	1.3	0.0
E.T.	7.3	6.5	2.0	1.9	1.4	0.0
L.W.	7.4	7.0	3.3	3.1	1.5	0.0
Mean $\pm$ SEM	11.3$\pm$2.3	9.5$\pm$1.6	2.5$\pm$0.5	2.3$\pm$0.4	1.7$\pm$0.4	0.0
	6 - 12 h					
N.C.	2.3	2.1	0.9	0.8	0.3	0.1
G.R.	2.1	2.2	2.3	2.7	0.7	0.1
B.B.	8.1	6.3	4.2	3.2	1.0	0.0
K.M.	3.9	3.5	0.7	0.7	1.7	0.3
H.P.	1.7	1.7	0.6	0.7	0.3	0.3
*I.G.						
C.H.	5.0	5.2	1.0	0.9	1.4	0.0
L.J.	0.0	0.0	0.0	0.0	0.0	0.0
J.H.	0.1	0.1	0.2	0.2	0.4	0.6
D.M.	0.0	0.0	0.0	0.0	0.0	0.0
Mean $\pm$ SEM	2.6$\pm$0.9	2.3$\pm$0.8	1.1$\pm$0.5	1.0$\pm$0.4	0.6$\pm$0.2	0.15$\pm$0.07

Table 5.1.6 (continued....)

Patient	Folic acid		5 MeTHF		p-AcBG	p-AcBA
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
6 - 12 h						
C.C.	5.4	4.4	1.6	1.4	1.7	0.0
N.R.	18.3	16.1	2.1	2.1	n.d	n.d
S.R.	3.6	3.3	0.7	0.7	1.0	0.0
E.W.	4.4	3.5	0.8	0.6	0.4	0.3
E.T.	4.2	3.6	1.7	1.4	0.9	0.0
*L.W.						
Mean $\pm$SEM	7.2 ± 2.8	6.2 ± 2.5	1.4 ± 0.3	1.2 ± 0.3	1.0 ± 0.3	0.07 ± 0.07
12 - 24 h						
N.C.	n.d	n.d	n.d	n.d	n.d	n.d
G.R.	n.d	n.d	n.d	n.d	n.d	n.d
B.B.	3.5	3.2	2.1	1.7	0.7	0.0
K.M.	0.5	1.0	0.3	0.5	0.2	0.1
H.P.	0.1	0.1	0.2	0.1	0.2	0.1
C.H.	died					
L.J.	0.1	T	0.2	0.1	0.5	1.6
J.H.	0.0	0.0	0.0	0.0	1.2	1.6
D.M.	0.1	0.1	0.2	0.2	0.0	0.0
Mean $\pm$SEM	0.76 ± 0.6	0.7 ± 0.5	0.5 ± 0.3	0.4 ± 0.3	0.47 ± 0.18	0.57 ± 0.3

Table 5.1.6 (continued...)

Patient	Folic acid		5 Me THF		p-AcBG	p-AcBA
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
C.C.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
N.R.	2.1	1.7	0.5	0.4	n.d.	n.d.
S.R.	4.6	4.3	1.3	1.3	1.0	0.9
E.W.	1.2	0.9	0.7	0.4	0.4	1.1
E.T.	1.9	1.1	1.1	0.7	1.3	0.8
Mean	2.5 ± 0.7	2.0 ± 0.8	0.9 ± 0.2	0.7 ± 0.2	0.9 ± 0.3	0.9 ± 0.09

Table 5.1.6 (continued...)

Patient	0 - 24 h					
	Folic acid		5 MeTHF		p-AcBG	p-AcBA
	^{3}H	^{14}C	$^{3}H1$	^{14}C	^{3}H	^{14}C
N.C.	18.8	17.0	3.8	3.7	0.9	0.1
G.R.	8.9	9.2	4.7	5.3	2.8	0.1
B.B.	11.6	9.5	5.3	4.9	2.7	0.0
K.M.	14.1	12.8	2.0	2.1	2.8	0.4
H.P.	13.1	12.2	2.2	1.9	1.2	0.4
C.H.	5.8	5.9	1.1	1.0	1.6	0.0
L.J.	1.1	1.1	0.7	0.7	1.4	1.6
J.H.	0.1	0.1	0.2	0.2	1.6	2.2
D.M.	0.7	0.6	1.4	1.3	0.0	0.0
Mean $\pm$ SEM	8.2 ± 2.2	7.6 ± 2.0	2.4 ± 0.6	2.3 ± 0.6	1.66 ± 0.3	0.5 ± 0.27
C.C.	26.3	20.2	5.0	4.2	5.0	0.0
N.R.	26.2	23.2	3.3	3.3	n.d	n.d
S.R.	22.1	20.0	5.7	5.5	3.2	0.9
E.W.	18.2	14.1	3.6	2.7	2.2	1.4
E.T.	13.4	11.2	4.8	4.0	3.6	0.8
Mean $\pm$SEM	21.2 ± 2.5	17.7 ± 2.2	4.5 ± 0.4	3.9 ± 0.5	3.5 ± 0.6	0.8 ± 0.3

* One sample only collected

n.d. Not determined

T Trace amount

The ratio of Folic acid : 5 MeTHF

Patient	0 - 6 h	6 - 12 h	12 - 24 h
N.C.	5.7:1	2.5:1	n.d.
G.R.	2.8:1	0.9:1	n.d.
B.B.	-	1.9:1	1.7:1
K.M.	9.6:1	5.6:1	1.7:1
H.P.	8.1:1	2.8:1	0.5:1
C.H.	8.0:1	5.0:1	-
L.J.	2.0:1	-	0.5:1
J.H.	-	0.5:1	No intact folates
D.M.	0.5:1	-	0.5:1
Mean	5.2:1	3.1:1	0.98:1
C.C.	6.1:1	3.4:1	n.d.
N.R.	8.3:1	8.7:1	4.2:1
S.R.	3.8:1	5.3:1	3.5:1
E.W.	6.0:1	5.5:1	1.7:1
E.T.	3.7:1	2.5:1	1.7:1
Mean	5.6:1	5.1:1	2.8:1

Table 5.1.7. The ratios of folic acid to 5 MeTHF excreted from the urine of cancer and control patients given a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ - folic acid (5 mg).

n.d. Not determined

Table 5.2.1. Clinical details of patients studied

Name	Age	Sex	Diagnosis	Therapy
A.L.	74	F	Cerebro vascular accident + Cancer of liver	Frusemide - Slow K - Duphabe
E.D.	70	M	Heart failure	Frusemide - Slow K - Digoxin
S.F.	66	M	Cancer of lung	Radiotherapy - Aminophylline
J.H.	42	M	Pericardites	Digoxin
R.S. *	53	M	Massive right temporal neoplasm	None
C.H. *	69	F	Cancer of lung	Slow K
A.G.	59	M	Myocardial infarction	Moduretic
P.H.	63	M	Hypertension and possible Myocardial infarction	Thiazide - Triazolam - Distalgesic
T.C.	56	M	Myocardial infarction	None

Patient		Dose mg	% of the dose 0-6h ^{3}H	0-6h ^{14}C	6-12h ^{3}H	6-12h ^{14}C	12-24h ^{3}H	12-24h ^{14}C	0-24h ^{3}H	0-24h ^{14}C
A.L.	Cancer	1.0	0.4	0.5	0.8	0.6	1.3	0.6	2.5	1.7
E.D.	Control	1.0	2.1	1.7	1.6	1.1	1.6	0.7	5.3	3.5
S.F.	Cancer	0.5	1.9	1.4	1.1	0.7	-	-	3.0	2.1
J.H.	Control	0.5	2.6	1.8	0.3	0.3	0.5	0.2	3.4	2.3
*R.S.	Cancer	0.057	1.0	0.6	0.6	0.5	1.0	0.6	2.6	1.7
*C.H.	Cancer	0.057	0.8	0.7	0.2	0.2	0.3	0.3	1.3	1.2
Mean									2.0	1.5
A.G.	Control	0.057	1.0	0.6	0.9	0.6	0.3	0.2	2.2	1.4
P.H.	Control	0.057	1.8	1.1	0.6	0.5	0.7	0.5	3.1	2.1
T.C.	Control	0.057	0.8	0.6	1.2	0.7	0.5	0.6	2.5	1.8
Mean									2.6 ± 0.2	1.8 ± 0.2

Table 5.2.2. Urinary recovery of radiactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (1mg, 0.5 mg or 0.057 mg folic acid). The results are expressed as the percentage of the dose.

* Data supplied by A E Pheasant

Table 5.2.3. Faecal recovery of radioactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (0.057 mg).

The results are expressed as the percentage of the dose recovered in 48 h.

	% Dose recovered	
Patient	^{3}H	^{14}C
P.H.	2.6	3.1
T.C.	0.06	0.3
Mean	1.33	1.7

Patient	Dose	Sample	Folic acid		5 MeTHF		pAcBG	pAcBA
			^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{3}H
* E.D.	1.0mg	0-6h	1.3	1.1	0.4	0.3	0.4	-
		6-12h	0.4	0.3	0.3	0.2	0.7	0.2
+ A.L.	1.0mg	6-12h	0.05	0.05	0.1	0.1	0.3	0.1
		12-24h	-	-	-	-	0.5	0.7
* J.H.	0.5mg	0-6h	0.6	0.5	0.8	0.7	0.8	0.01
+ S.F.	0.5mg	0-6h	0.3	0.3	0.7	0.6	0.7	0.1

Table 5.2.4. The relative distribution of the major labelled metabolites appearing in various urine samples of control and cancer patients after the administration of a mixture of ^{3}H and ^{14}C folic acid (1 mg or 0.5 mg). The results are expressed as the percentage of the dose present as each metabolite.

* Control patient

\+ Cancer patient

Table 5.3.1. Excretion of the catabolites of tissue folates. The results are given as the percentage of tissue radioactivity at 6h excreted as pAcBG or (pAcBG + pAcBA) in the 6-24 h urine sample

Patient Cancer	% ^{3}H of the dose in the body after 6 h	% ^{3}H dose excreted as p-AcBG	% ^{3}H dose excreted as p-AcBG + pAcBA	% retained radio-activity excreted as pAcBG	% retained radio-activity excreted as pAcBG + pAcBA
N.C.	76.2	0.3	0.4	0.4	0.5
G.R.	84.9	0.7	0.8	0.8	0.9
B.B.	99.7	1.7	1.7	1.7	1.7
K.M.	87.7	1.2	2.3	1.4	2.6
H.P.	85.3	0.5	0.9	0.6	1.1
C.H.	98.8	1.4	1.4	1.4	1.4
L.J.	97.5	0.5	2.1	0.5	2.1
J.H.	100.0	1.6	3.8	1.6	3.8
D.M.	98.0	0.0	0.0	0.0	0.0
W.M.	99.9	<1.0	<1.0	<1.0	<1.0
Mean	92.8 ± 2.7	$* 0.89 \pm 0.2$	1.44 ± 0.3	$* 0.94 \pm 0.2$	$* 1.5 \pm 0.3$

Continued....

Table 5.3.1. (Continued...)

Patient Control	% ^{3}H of the dose in the body after 6 h	% ^{3}H dose excreted as p-AcBG	% ^{3}H dose excreted as p-AcBG + pAcBA	% retained radio-activity excreted as pAcBG	% retained radio-activity excreted as pAcBG + pAcBA
C.C.	71.0	1.7	1.7	2.4	2.4
S.R.	91.4	2.0	2.9	2.2	3.2
E.W.	83.5	0.8	2.2	1.0	2.6
E.T.	88.4	2.2	3.0	2.5	3.4
Mean	83.6 $\pm$ 4.5	* 1.7 $\pm$ 0.3	2.45 $\pm$ 0.3	* 2.0 $\pm$ 0.3	* 2.9 $\pm$ 0.2

* $p < 0.05$

CHAPTER 6

THE METABOLISM OF 10 FORMYLFOLATE IN MAN AND ITS EFFECT ON FOLATE METABOLISM

10-Formylfolate (10CHOFA), an oxidised folate, is an important food folate (Santini et al., 1964). However, the metabolism of 10CHOFA has rather limited coverage in the literature and some controversy exists over its handling and metabolic fate. Zakrewski (1960) using a partially purified chicken liver folic acid reductase at pH 5.0 found that 10CHOFA was not reduced and that it inhibited folic acid reduction. Studies in vitro using dihydrofolate reductase from various mammalian sources showed that 10CHOFA was not reduced by either DHF reductase from Erlich ascites cells (Bertino et al., 1965) or bovine DHF reductase (A. Sahota, personal communication). Moreover, it acts as a competitive inhibitor of mammalian DHF reductase with I_{50} values of 50 nM, 80 nM and 100 nM for a bovine liver reductase, Erlich ascites cells reductase and a mouse leukaemia reductase respectively (A. Sahota, personal communication, d'Urso-Scott et al., 1974 and Friedkin et al., 1975).

The only other report available concerns an L. casei DHF reductase (McIntyre and Harding 1977) where 10CHOFA was shown to be reduced at six times the rate of folic acid. However, comparison of the reductases from such diverse organisms should be viewed with caution and in fact Friedkin et al., (1975) found that 10CHOFA was 1,000 times less inhibitory towards an E. coli reductase compared to the mouse leukaemia enzyme.

Detailed in vivo studies of 10CHOFA are lacking. Beavon (1973) found that $\left[2\text{-}^{14}C\right]$-10CHOFA was not readily incorporated into the reduced folate pool of the rat. However, more recent evidence from

Pheasant et al., (1981) using radiolabelled 10CHOFA suggests that extensive metabolism of this compound occurs in the rat. Other studies performed in man (Ratanasthien, et al., 1974) using microbiological assay showed no metabolism of orally administered 10CHOFA.

In the present chapter the metabolism of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] -10CHOFA in man and the first in vivo study of the effect 10CHOFA on the metabolism of folic acid are reported.

MATERIALS AND METHODS

Two groups of patients were used. Each group consisted of three control patients (see Table 6-1 for details of the patients).

GROUP A: Patients were given a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] -10CHOFA plus 5 mg unlabelled 10CHOFA.

GROUP B: Patients were given a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (57μg) plus 5 mg unlabelled 10CHOFA.

Urine was collected for the following time periods, 0-6 h, 6-12 h and 12-24 h after the administration of the dose as described in Chapter 5. Determination of radioactivity and column chromatography were performed as described in Chapter 2.

RESULTS

GROUP A: The metabolism of 10CHOFA

The urinary recovery of the radioactivity in 24 h after the administration of [2-^{14}C] and [3',5',7,9-^{3}H] 10CHOFA acid is given in Table (6-2). 49.1% of ^{3}H and 45.2% of ^{14}C of the dose were recovered in 24 h in the urine. Excretion of radioactivity in the urine was maximal in the first time period and dropped sharply thereafter.

Urinary metabolites

Urine samples were sequentially chromatographed on DE52 and Sephadex G15 and this revealed a number of radioactive components. Examples of DE52 chromatography of 0-6 h urine samples are illustrated in Figures 6.A.1 and 6.A.2. More than 91% of the urinary radioactivity was identified as 10CHOFA. The other metabolites detected in the urine were folic acid, 5MeTHF and a very small amount of ^{3}H labelled catabolites. More folic acid was excreted than 5MeTHF, the ratio of 5MeTHF to folic acid in 0-6 h urine sample being 1:1.8, however, the total amount of both did not exceed 6% of the urinary radioactivity. No ^{14}C-labelled catabolites were detected and the ^{3}H-labelled species was identified as p-AcBG.

DE 52 chromatography of 6-12 h urine samples (Figures 6.A.3 and 6.A.4) showed that the amount of 10 formylfolic acid decreased as a percentage of the urinary radioactivity to about 80% of this sample, and the amount of folic acid and 5MeTHF increased as a percentage of urinary radioactivity. 12-24 h urine samples were not examined.

Table 6.3 summarises the relative distribution of the metabolites appearing in the 0-12 h urine samples. Approximately 1% and 1.6% of the dose were excreted as 5MeTHF and folic acid respectively in the first 12 h after the administration of the dose and most of the dose excreted in the urine was 10CHOFA.

GROUP B: The effect of 10CHOFA on folic acid metabolism

Subjects receiving oral doses of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid (57μg) plus 5 mg unlabelled 10-formylfolic acid, excreted in their urine 17.7% ^{3}H and 16.7% ^{14}C of the labelled dose (Table 6.4). The recovery of ^{3}H and ^{14}C in individual samples was quite similar particularly in the 0-6 h and 6-12 h urine samples.

Urinary metabolites

DE 52 chromatograms of 0-6 h urine samples (Figures 6.B.1 and 6.B.2) showed a similar pattern to those of control subjects receiving 5 mg folic acid (Chapter 5). Unmetabolised folic acid was the major metabolite in the urine representing about 80% ^{3}H and 80.5% ^{14}C of 0-6 h urinary radioactivity. The other metabolites detected were 5MeTHF, 10CHOFA and p-AcBG.

DE 52 chromatography of 6-12 h urine sample (examples: Figures 6.B.3 and 6.B.4) showed exactly the same pattern of metabolites as the 0-6 h urine samples with approximately the same distribution of urinary radioactivity between the metabolites appearing in the urine. The third

urine samples were not chromatographed due to their low radioactivity. Table 6.5 shows the distribution of radiolabelled folate derivatives in the urine 12 h after the administration of the dose. The major urinary radioactive product was unmetabolised folic acid.

Discussion

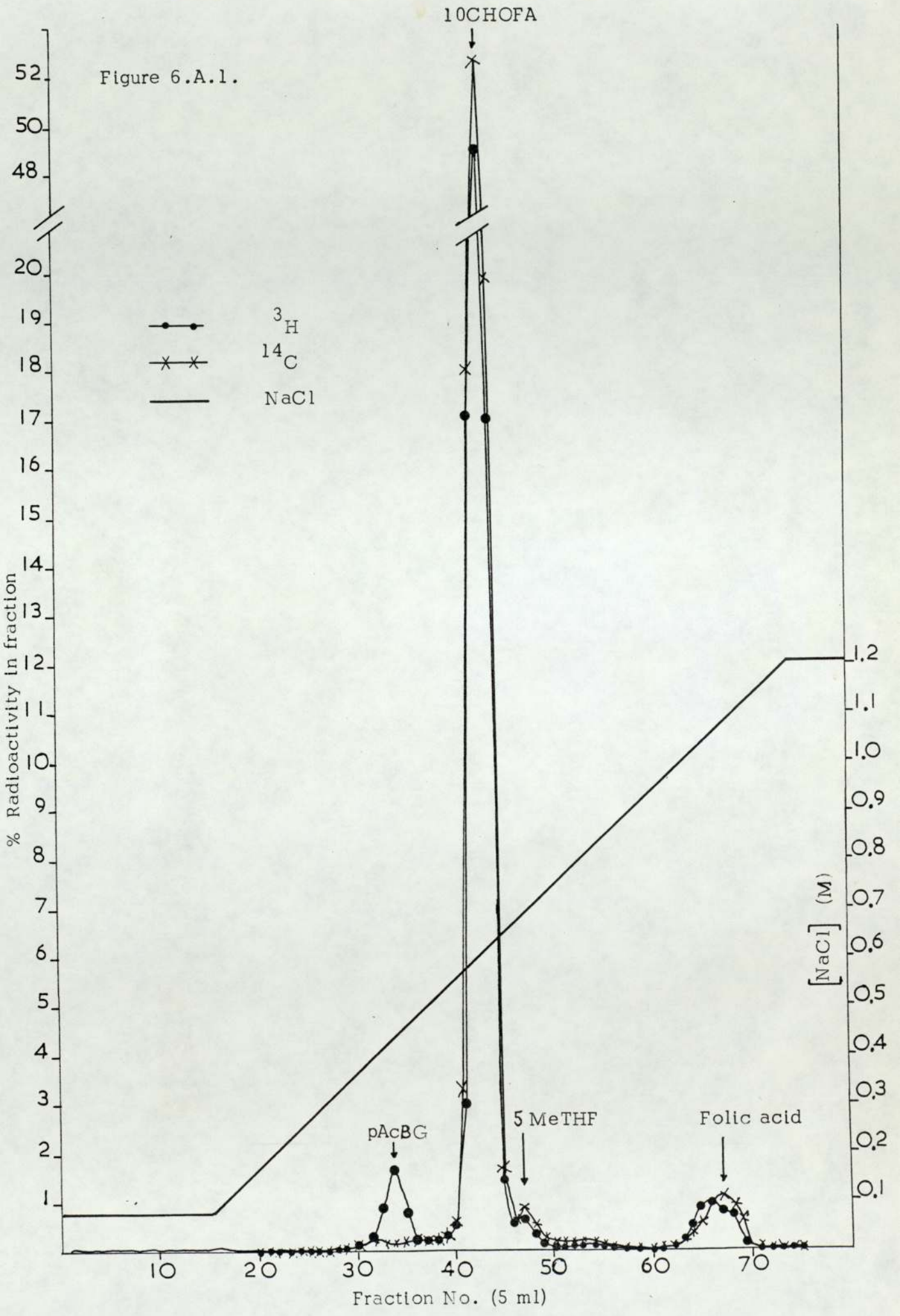
Administered 10-formylfolate (5 mg) was excreted largely unchanged. Earlier studies using microbiologically assay had shown that, at the same dose level, 10CHOFA was readily absorbed and peak serum levels were found between 1-1$\frac{1}{2}$ h after dosing (Ratanasthien, 1975). Bioautography revealed that the microbiological activity was entirely due to unmetabolised 10CHOFA indicating no intestinal metabolism occurred. However, in the present study approximately 1% of the dose was excreted as 5MeTHF and a small proportion was present as catabolites. This indicates very slow incorporation of 10CHOFA into the reduced folate pool. Folic acid was present also in the urine and its amount increased slowly as a percentage of urinary radioactivity in relation to 10CHOFA. This suggests that deformylation may preceed reduction. Indeed, the folic acid: 5MeTHF ratio was similar to that observed following the administration of folic acid to control subjects (Chapter 5). Since 10CHOFA absorption is probably rapid, the higher urinary recoveries in the first urine samples probably represent excretion of free plasma 10CHOFA above the renal threshold. A higher proportion of urinary radioactivity was excreted as unmetabolised 10CHOFA in the second urine samples, and work on the rat

dosed orally with a similar dose level showed that unmetabolised 10CHOFA was apparently found in 24-48 h urine samples although not at low doses (Connor, 1979, Pheasant et al., 1981). This suggests that some form of "storage" of unmetabolised 10CHOFA occurs which may be due to protein-binding in the plasma and possibly in the tissues. The presence of some radioactivity in the tissues is possible since approximately 50% of the dose was not recovered in the urine and it is unlikely that all of this was excreted in the faeces. Any radioactivity retained in the body may reflect the metabolism of that part of the dose deformylated to folic acid. Thus it appears that in contrast to the rat 10CHOFA is utilised only poorly by man and is probably not reduced to any significant extent in the human body.

When a low dose of labelled folic acid (57μg) was given orally to human patients (Chapter 5, Pheasant, et al., 1979), low recovery of radioactivity was observed in the urine. Little or no folate was excreted and the only detectable metabolites were scission products. However, the pattern of urinary metabolites altered dramatically when the same low dose of radiolabelled folic acid was given along with unlabelled 10CHOFA (5 mg). A substantial proportion of the folic acid was rapidly excreted unchanged and relatively little 5 MeTHF and scission products were formed. It is known that 10CHOFA inhibits the reduction of folic acid and dihydrofolate by dihydrofolate reductase in vitro (see the introduction of this Chapter for references). These observations suggest that 10CHOFA also effectively blocks the reduction of folic acid in vivo.

Figure 6.A.1. DE52 chromatography of 0-6h urine sample of a Control patient (A.M) after the administration of a mixture of ^{3}H and ^{14}C 10CHOFA (5 mg)

Figure 6.A.1.


10CHOFA
^{3}H
^{14}C
NaCl
pAcBG
5 MeTHF
Folic acid
% Radioactivity in fraction
[NaCl] (M)
Fraction No. (5 ml)

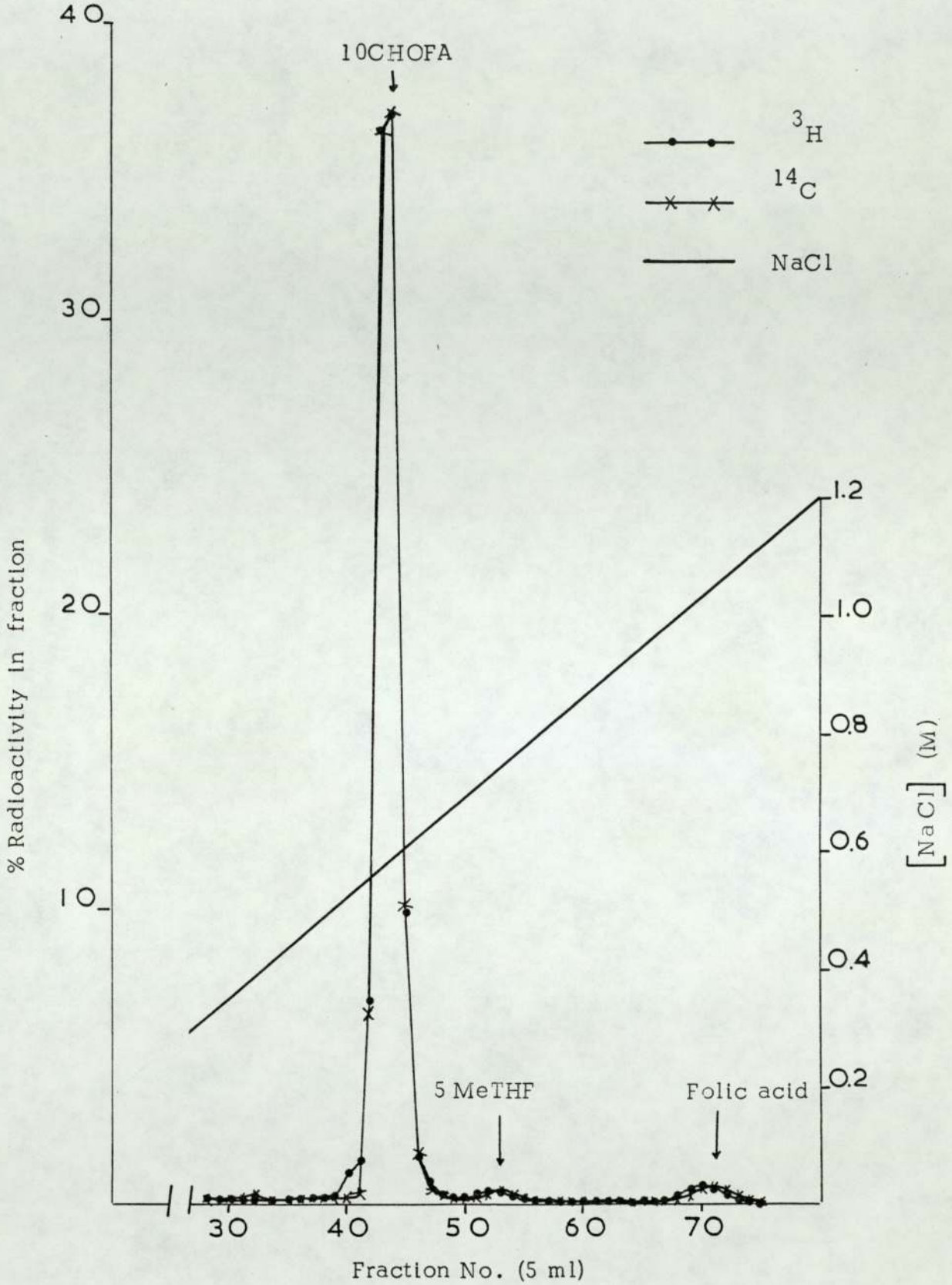


Fig. 6.A.2. DE52 chromatography of 0-6 h urine sample of a control patient (R.R.) after the administration of a mixture of ^{3}H and ^{14}C 10CHOFA (5 mg).

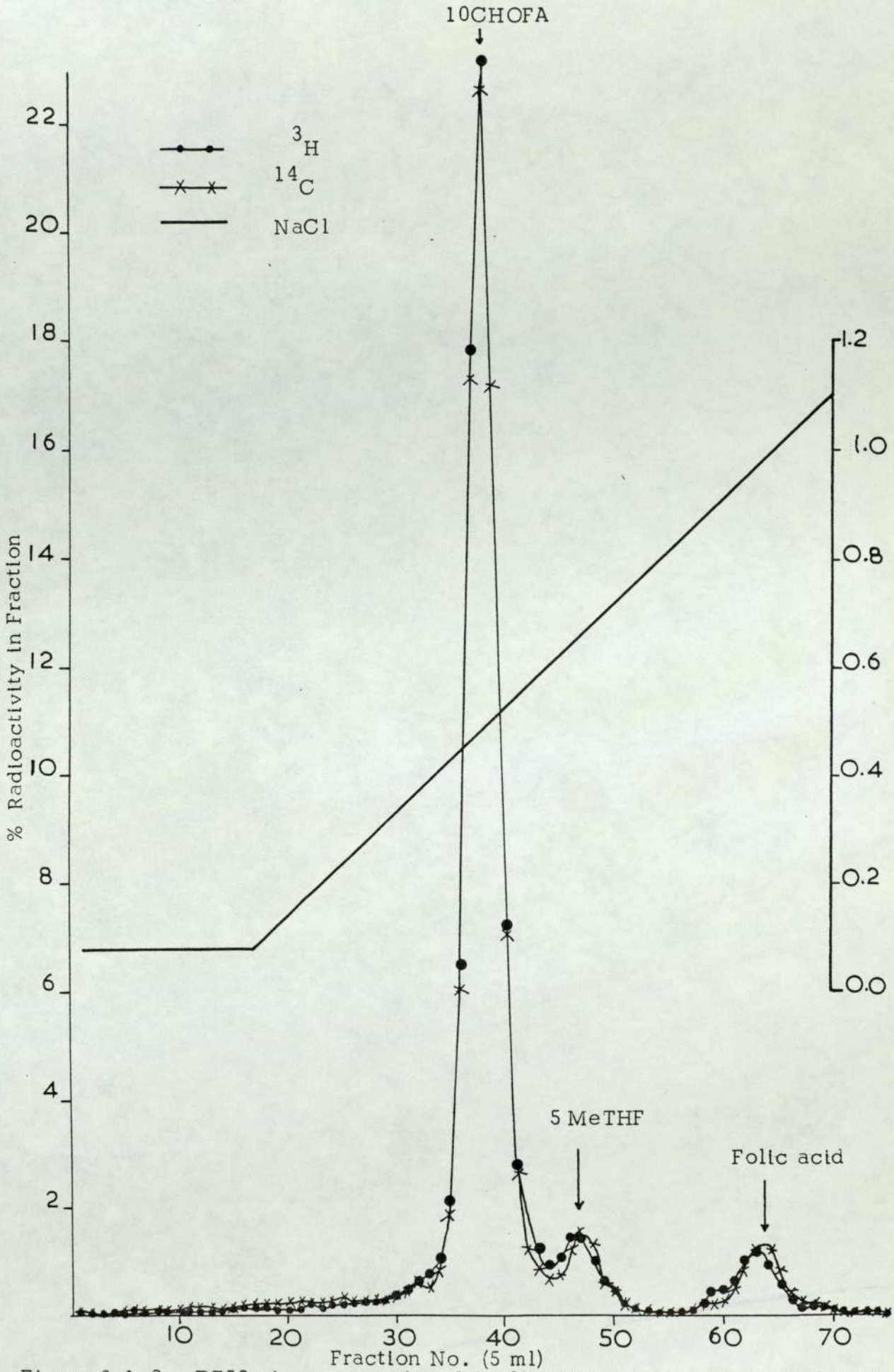


Figure 6.A.3. DE52 chromatography of 6-12h urine sample of a control patient (A.M) after the administration of a mixture of ^{3}H and ^{14}C 10CHOFA (5 mg)

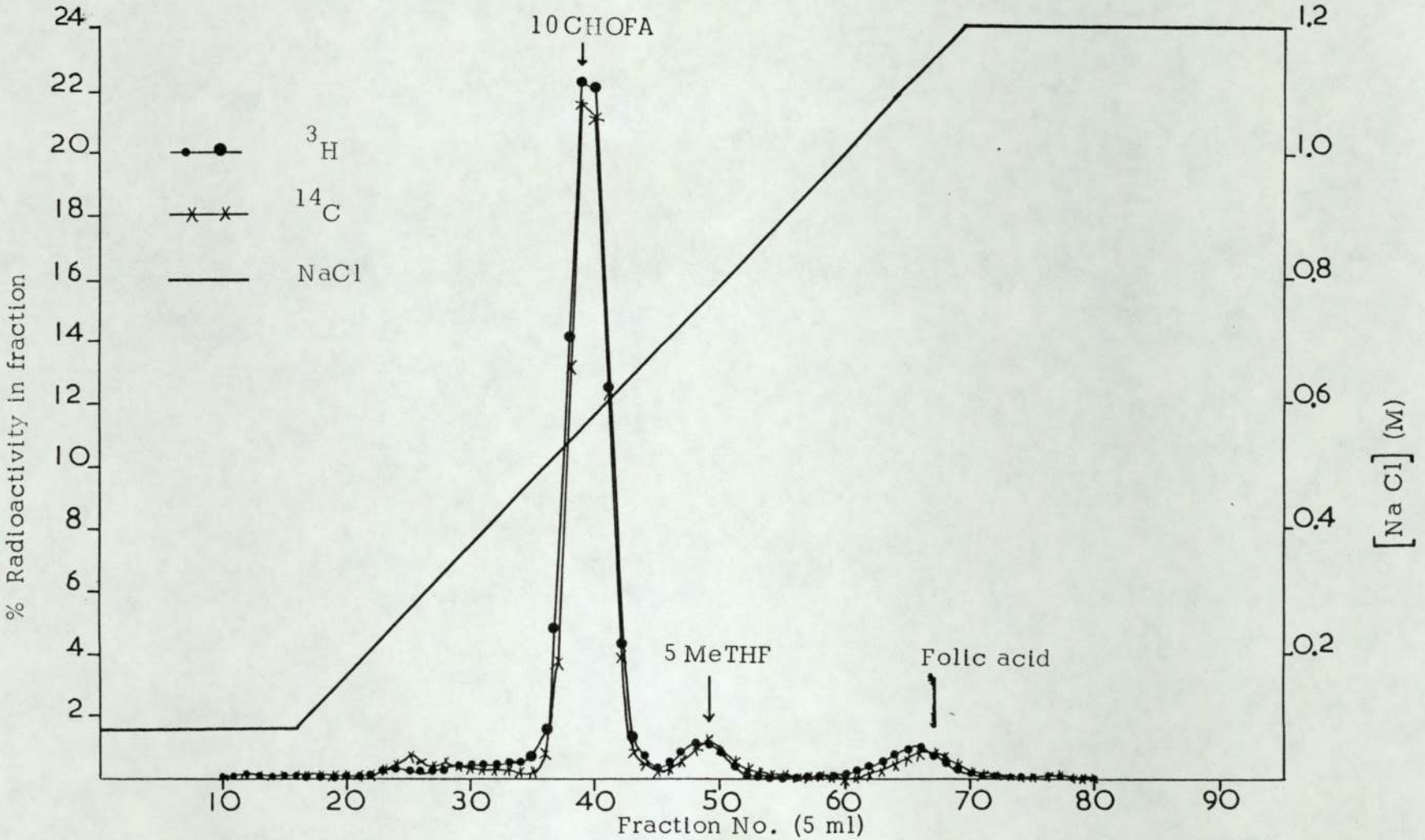


Figure 6.A.4. DE52 chromatography of 6-12 h urine sample of a control patient (R.R) after the administration of a mixture of ^{3}H and ^{14}C 10CHOFA (5 mg)

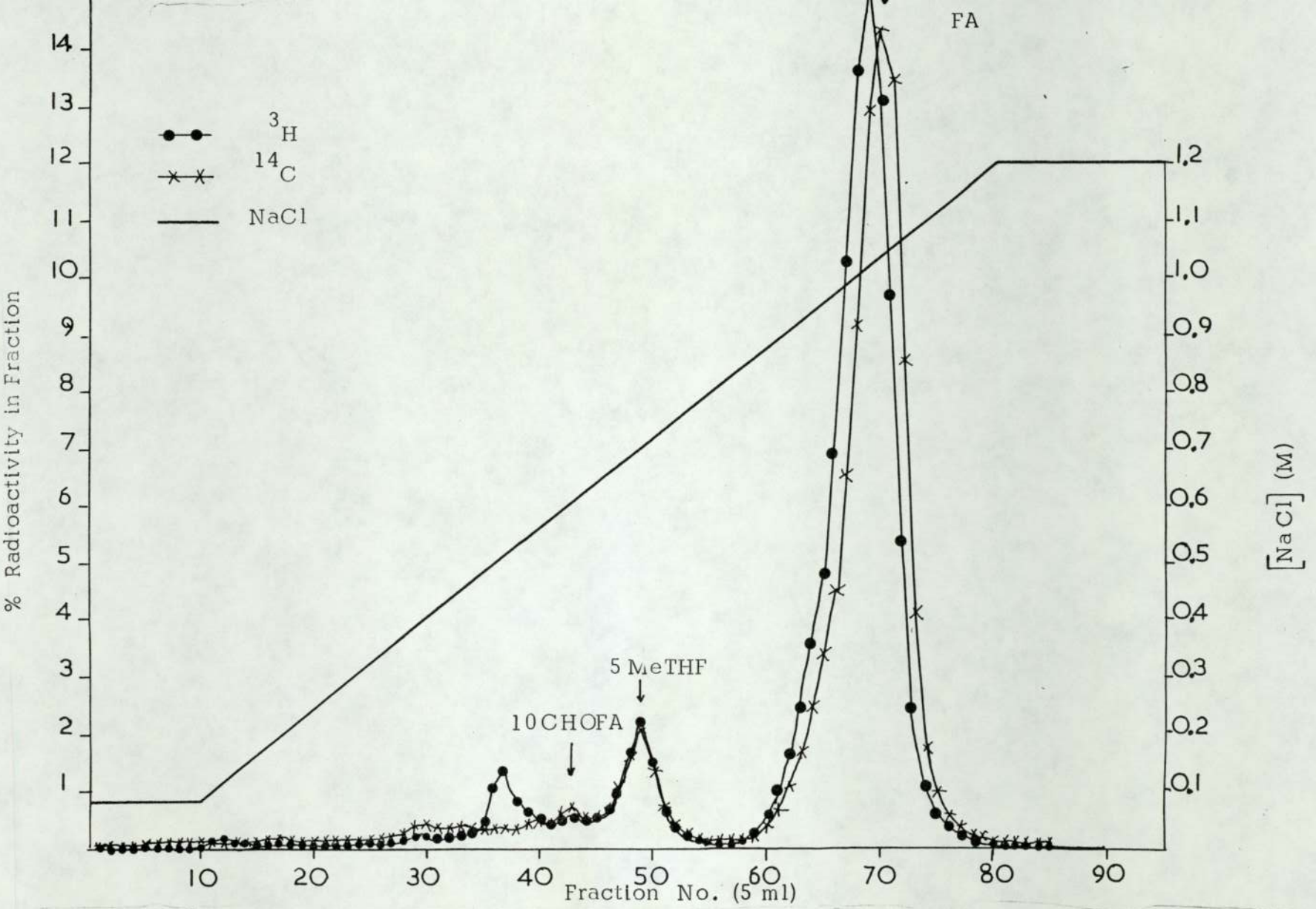


Figure 6.B.1. DE52 chromatography of 0-6h urine sample of a control patient (S.H) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg) plus 5 mg unlabelled 10CHOFA

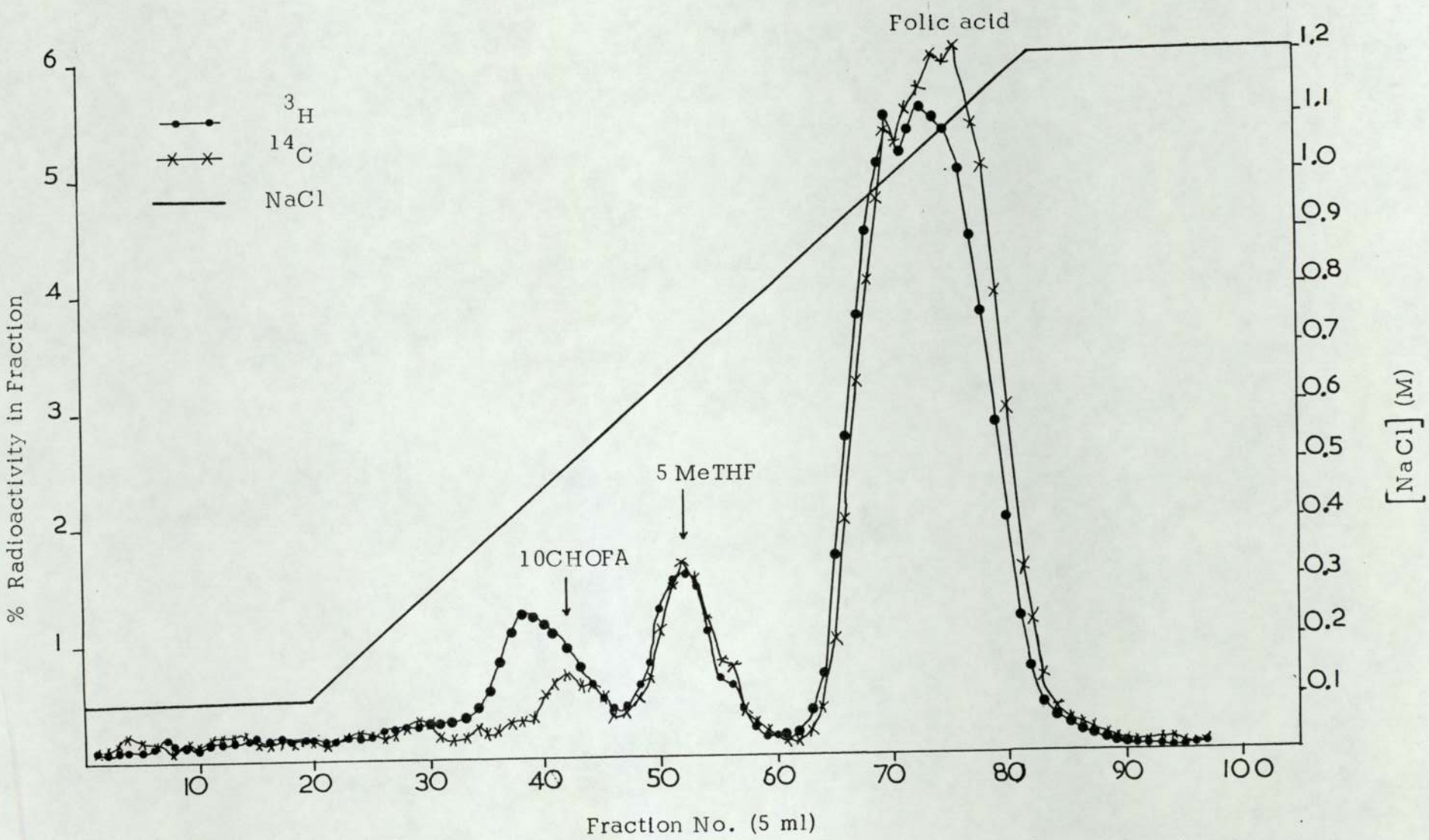


Figure 6.B.2. DE52 chromatography of 0-6h urine sample of a control patient (F.C) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg) plus 5 mg unlabelled 10CHOFA.

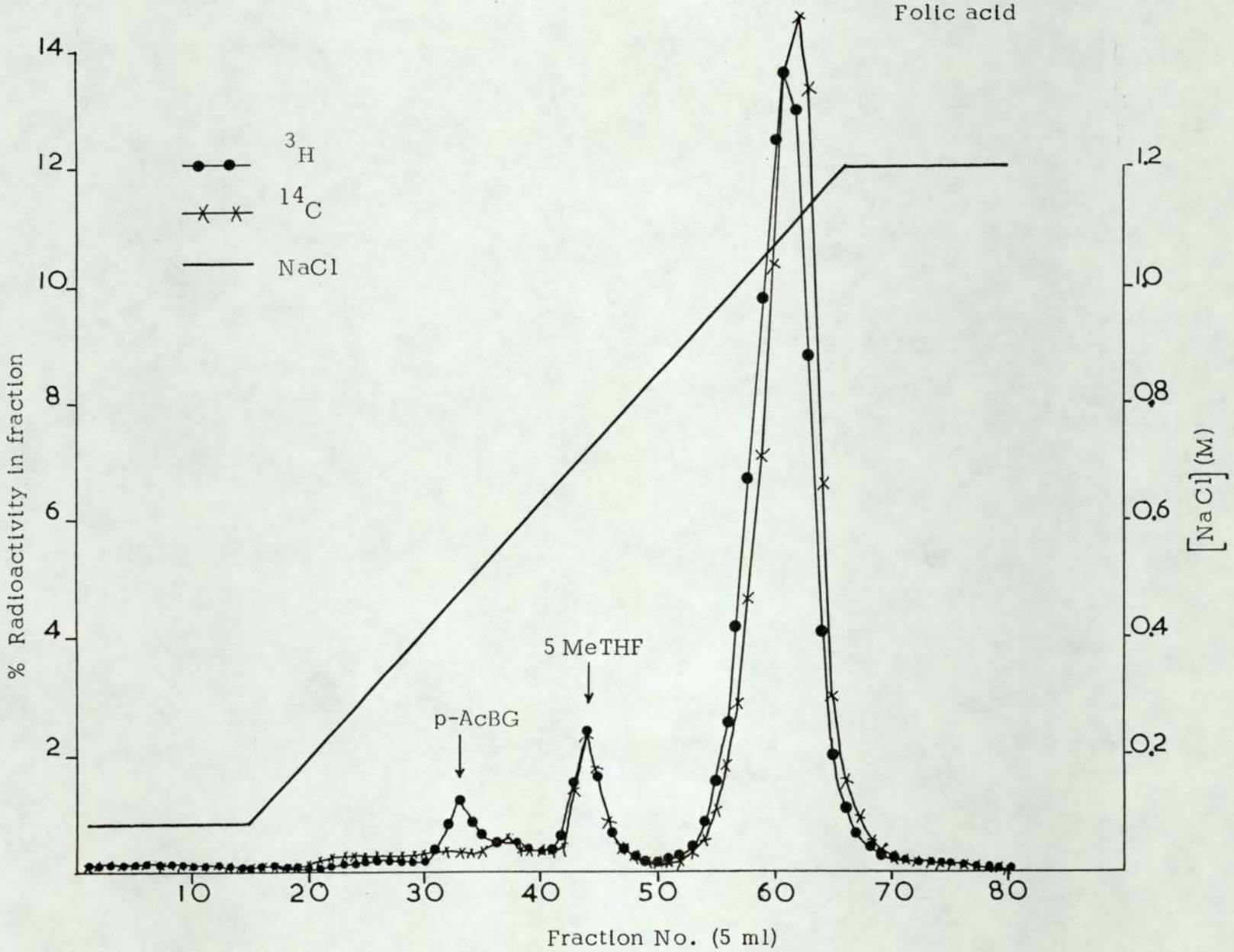


Figure 6.B.3 . DE52 chromatography of 6-12h urine sample of a control patient (S.H) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg) plus 5 mg unlabelled 10CHOFA

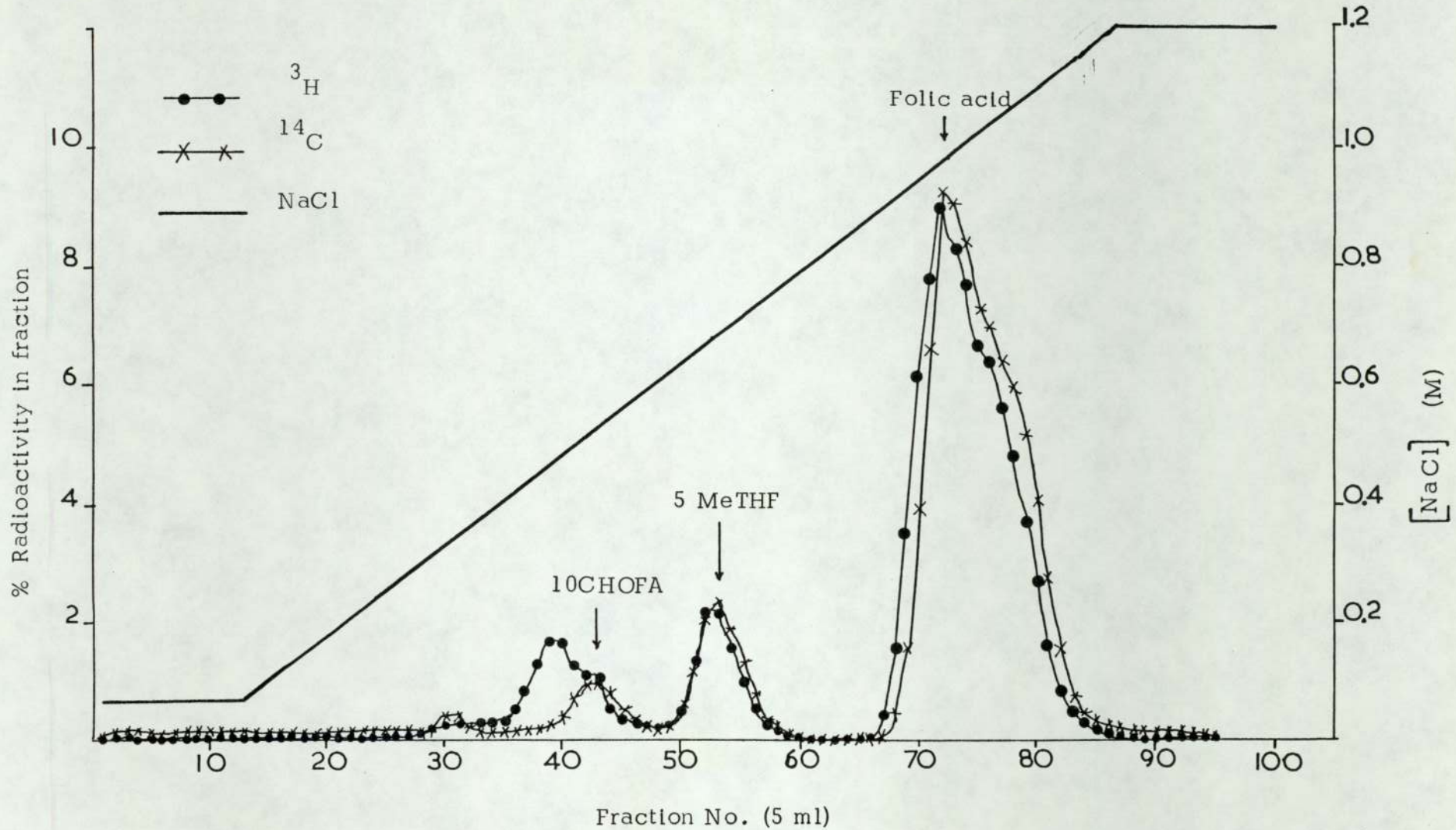


Figure 6.B.4. DE52 chromatography of 6-12h urine sample of a control patient (F.C) after the administration of a mixture of ^{3}H and ^{14}C folic acid (0.057 mg) plus 5 mg unlabelled 10CHOFA

Table 6-1 Clinical details of patients studied

Name	Age	Sex	Diagnosis	Therapy
R.S.	57	M	Myocardial infarction	Nitrazepam
A.M.	48	M	Retroperitonial fibrosis	Paracetamol
G.L.	75	M	Myocardial infarction	Triazolam-Aspirin
F.C.	67	F	Myocardial infarction	Frusemide-SlowK-Triazolam
S.H.	72	M	Bulbar palsy	None
M.F.	70	F	Myocardial infarction-Hypertension	Moduretic

Patient	% of the dose							
	0-6 h		6-12 h		12-24 h		O-24 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
R.S.	46.1	41.1	4.3	4.0	1.4	0.7	51.8	45.8
A.M.	40.1	38.2	5.8	5.7	0.4	0.7	46.3	44.6
G.L.	25.4	22.4	died					
Mean							49.1	45.2

Table 6.2 Urinary recovery of radioactivity after an oral dose of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ -10CHOFA (5 mg). The results are expressed as the percentage of the dose.

Patient	0 - 6 h						6 - 12 h					
	FA		5 MeTHF		10 CHOFA		FA		5 MeTHF		10CHOFA	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	14C
R.S.	1.3	1.2	0.7	0.7	42.8	38.1	0.2	0.2	0.2	0.2	3.6	3.2
A.M.	1.7	1.7	0.7	0.8	35.6	34.5	0.4	0.4	0.4	0.4	4.8	4.5
G.L.	0.9	0.8	0.5	0.5	23.7	20.7						
Mean	1.3 $\pm$0.2	1.2 $\pm$0.3	0.6 $\pm$0.07	0.7 $\pm$0.09	34.0 $\pm$5.6	31.1 $\pm$5.3	0.3	0.3	0.3	0.3	4.2	3.9

Table 6.3 The relative distribution of the labelled metabolites present in the urine collected 12 h after the administration of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ -10 CHOFA (5 mg)

The results are expressed as the percentage of the dose present as each metabolite.

Patient	% of the dose							
	0-6 h		6-12 h		12-24 h		0-24 h	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
F.C.	5.4	5.5	3.2	2.9	1.2	0.2	9.8	8.6
S.H.	23.1	22.4	5.8	5.9	1.8	1.2	30.7	29.4
M.F.	7.6	7.5	5.0	4.6	0.0	0.0	12.6	12.1
Mean							17.7	16.7

Table 6.4 Urinary recovery of radioactivity after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid (57 μ g) plus 5 mg unlabelled 10 CHOFA. The results are expressed as the percentage of the dose.

Table 6.5 The relative distribution of the labelled metabolites present in the urine collected 12 h after the administration of [2-^{14}C] and [3',5',7,9-^{3}H] -folic acid (57 μg) plus 5 mg unlabelled 10 CHOFA The results are expressed as the percentage of the dose present as each metabolite

Patient	0 - 6 h						6 - 12 h					
	FA		5 MeTHF		10CHOFA		FA		5 MeTHF		10CHOFA	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
M.F.	6.3	6.3	0.5	0.5	0.3	0.3	3.7	3.6	0.4	0.4	0.2	0.2
S.H.	19.0	18.3	1.8	1.8	0.4	0.5	4.8	4.9	0.5	0.5	0.2	0.2
F.C.	3.8	4.2	0.6	0.6	0.3	0.3	2.4	2.3	0.3	0.3	0.2	0.2
Mean	9.7 ±4.7	9.6 ±4.4	1.0 ±0.4	1.0 ±0.4	0.33 ±0.03	0.37 ±0.07	3.6 ±0.7	3.6 ±0.8	0.4 ±0.06	0.4 ±0.06	0.2 ±0.0	0.2 ±0.0

CHAPTER 7

GENERAL DISCUSSION

Experiments described in this thesis highlight the complexity of folate metabolism in man and the rat. In the rat, folic acid is metabolised to a variety of reduced folate monoglutamate derivatives, to high molecular weight polyglutamate forms in the tissues and several degradation products are found in the urine. Similar observations are found in man. Both reduced folate monoglutamates and folate catabolites are found in the urine and it is likely that folate polyglutamates are formed in the tissues by analogy with the rat and since almost all of the folate in human liver is present in the form of polyglutamates (Hoppner and Lampi, 1980).

The results obtained here indicate the existence of a breakdown process in man and the rat and support the hypothesis of the presence of two metabolically distinct folate pools (Blair 1976, Krumdieck et al., 1978).

Folic acid is rapidly absorbed across the intestine. The mechanism involved in the intestinal absorption of folate has been suggested to be a simple diffusion of the neutral compound formed in an acid microclimate (assumed to have a pH less than 4) at the mucosal membrane (Blair and Matty 1974). Active transport of folic acid has been reported also (Hepner, 1969) but not confirmed (Said, 1981). Once absorbed, unchanged folic acid and its metabolites (reduced folates arising from intestinal metabolism) are found in the plasma and appear quickly in the urine as shown here with a half life of approximately one day in the rat (Chapter 4) and 31.5 h in man (Krumdieck et al., 1978). This demonstrates the

short-term pool. Then, an equilibrium between protein binding, urinary excretion and subsequent tissue metabolism is established for the reduced monoglutamates and there are two additional routes available for further metabolism. 5MeTHF, 10CHOTHF and 5,10CH_2-THF can undergo biliary excretion in the rat (Pheasant et al., 1981) and in man (Lavoie and Cooper 1974) and studies have shown that the biliary folates are derived principally from the circulating monoglutamates (Strum et al., 1979). Once in the intestine, folate monoglutamates are available for re-absorption and it is believed that at least one of these folates can also undergo a cleavage reaction yielding p-aminobenzoyl-L-glutamate and pterins. The precursor of the cleavage products and the catabolic mechanism remain unknown and no attempt was made to determine these. However, it has been suggested that the deformylation of 10CHOTHF by host enzymes or by gut microfloral metabolism to yield the more unstable THF molecule which readily undergoes scission to p-aminobenzoyl-L-glutamate and pterins (Blair and Pearson, 1974), is the most likely route (Connor, 1979). Once formed in the intestine p-aminobenzoyl-L-glutamate is readily absorbed and undergoes intestinal metabolism to p-acetamidobenzoate in the rat (Pheasant et al., 1981). However, the metabolism of p-aminobenzoyl-L-glutamate in man may differ from the rat (Chapter 5) since the appearance of p-acetamidobenzoate in the urine of man was delayed.

The second route available to the reduced folate monoglutamates is conversion to folate polyglutamate forms in the tissues. Since after 8h, labelled folate retained in the rat tissues is largely incorporated into the

polyglutamate form (Chapter 3), depletion of folate monoglutamates by this route occurs on a considerable scale. 5 MeTHF is the major serum folate in man (Ratanasthien et al., 1977) and its level in serum and urine is dose dependent (Chapter 5). This, with its persistence in the rat urine for long periods (Chapter 4) and the limited routes available to it, i.e. protein binding, biliary excretion and metabolism to THF by methionine synthetase reaction (Chapter 1) supports its function as a major folate storage and transport form. 5 MeTHF and 10CHOTHF (or its oxidation product, 10CHOFA) persist in the rat urine for long time periods (Chapter 4) and are the normal serum folates in man (Ratanasthien et al., 1974). Therefore, they are probably the folates which enter the cell. Whether the mammalian cell uses only one substrate or a variety of substrates for polyglutamate synthesis in vivo is not known. Studies in vitro showed that 10CHOTHF and THF were the substrates for polyglutamate synthesis. (Spronk, 1973 and McGuire et al., 1979). 5 MeTHF does not enter the folate polyglutamate pool without prior loss of the methyl group. Nevertheless since mammalian cells in tissue culture are reported to transfer 81–85% of the methyl group of 5 MeTHF within 5 minutes of entry into the cell (Nixon et al., 1973), 5 MeTHF may be incorporated into the tissue folate polyglutamate after losing its methyl group. Polyglutamate synthesis was completed by 8 h after the administration as shown in Chapter 3. Once formed, this second pool of retained folate persists for a relatively long time period, the biological half-life having been estimated at 11 days in the whole body of the rat (Chapter 4) and 100 days in man (Krumdieck et al., 1978).

The fate of this second pool is complex. The urinary radioactivity of rats dosed with radio-labelled folates is associated predominantly with folate cleavage products, largely p-AcBG and metabolite A from day 1 up to 11 days, at which time the retained radioactivity is largely associated with polyglutamate derivatives (i.e. after 8 h and up to 22 days) and so catabolism would appear the most likely route. However, the existence of folate monoglutamates and p-AcBA, the major catabolite of the folate monoglutamates in the rat, albeit in small amounts, in all urine samples examined up to 11 days, suggests that folate polyglutamates are broken down also to folate monoglutamates although at low rate compared to the cleavage reactions. Similar conclusions can be put forward for the human since scission products dominated the late urine samples. However, long-term studies in man are needed to confirm this.

The metabolic function of the folate polyglutamate pool is a source of controversy. The slow turnover of folate polyglutamates and the increased retention of labelled folate observed in the tissues of tumour-bearing rats (Chapter 3) and in humans with malignant diseases (Chapter 5) when the demand for folate coenzymes is likely to be enhanced, supports the conclusion of numerous in vitro studies (reviewed by Hoffbrand 1976, Rowe, 1978, Chanarin, 1979a, Covey 1980) that conjugated folates have a primary coenzyme function rather than acting as a storage pool for the folate monoglutamates. Connor and Blair (1980) have isolated labelled 10-formyl-folatepolyglutamates from the livers of rats dosed with labelled folate and they were not able to detect any methylfolatepolyglutamates. Mutant

cell lines of Chinese hamster ovary cells, lacking folypolyglutamate synthetase, have an absolute requirement for adenosine, glycine and thymine for growth but no pronounced abnormal requirement for methionine (McBurney and Whitmore, 1974). This indicates that 5 MeTHF polyglutamates may not be required for the methionine synthetase reaction *in vivo*. Hence, these observations may suggest that folate coenzymes are divided into two pools, a monoglutamate pool required for methionine synthesis and the non-methylpolyglutamate pool for thymidylate and purine synthesis (Figure 7.1).

Regulation of folate concentrations and the level of folate metabolising enzymes is complex. The data available on mammalian enzyme regulation are still very limited and no overall study has been undertaken on a whole series of these enzymes within one tissue or cell type. Several folate polyglutamate derivatives have marked inhibitory properties at physiological concentrations *in vitro* and this may also be important *in vivo* such as the inhibition of thymidylate synthesis by DHF-polyglutamate derivatives (Kisliuck *et al.*, 1974). Another possible regulatory system for all the folate enzymes may depend upon the relative rates of enzyme action with monoglutamate and polyglutamate derivatives of each folate substrate and also the relative rates of conversion of polyglutamate to monoglutamate and *vice-versa*. In relation to methyl group synthesis, methylene THF reductase is subject to feedback inhibition by SAM (Kutzbach and Stokstad 1971). Furthermore the existence of an enzyme complex (see Chapter 1) will involve a different set of regulatory controls.

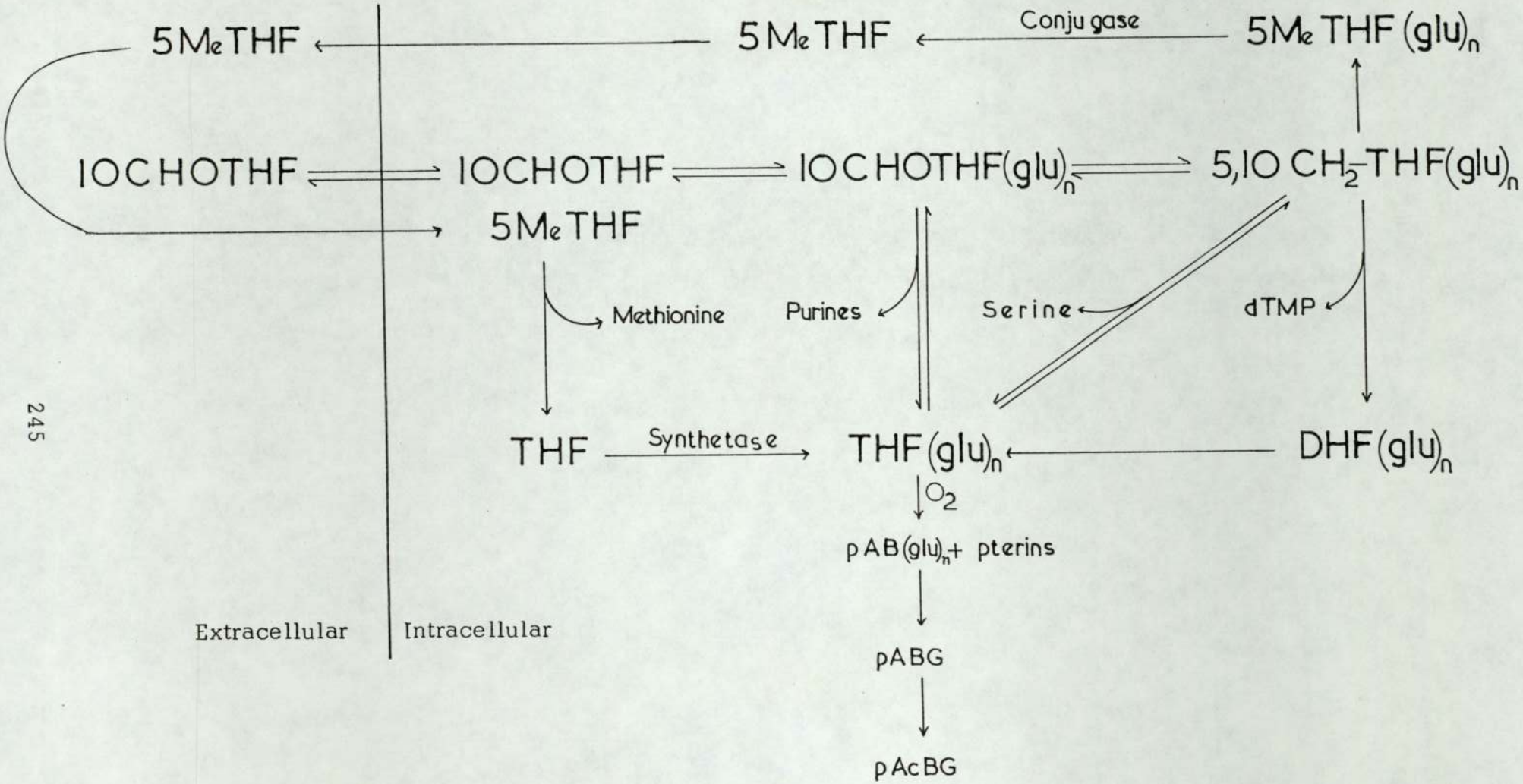


Fig. 7.1. The role of the retained tissue folate (second pool)

The loss of folate from the tissues *in vivo* by degradation to structurally less complex molecules is now well evident but the mechanism of the catabolic process is still controversial. Several investigators have suggested that the possible catabolic route of folate would be via cleavage of the C_9-N10 bond in the tissue to give p-aminobenzoyl-L-glutamate and a pterin (Blair, 1958, Weir, 1974, Murphy *et al.*, 1978). The identification of the metabolites of p-aminobenzoyl-L-glutamate (p-AcBA and p-AcBG) in the urine of both the rat and man confirms this type of reaction. However, whether the cleavage is enzymatic or a chemical process is not known.

Evidence *in vitro* of a specific folate cleaving enzyme in the mammal is lacking (see Chapter 1). The liver is the organ which contains the largest concentration of folate and studies *in vivo* have shown that there is a relationship between the amounts of radioactivity retained in the liver of rats (Pheasant *et al.*, 1981, Saleh *et al.*, 1981) dosed with labelled folates and the amounts of p-AcBG excreted in the urine. The higher the radioactivity retained in the liver the higher the amounts of pAcBG excreted in the urine (Table 7.1). This suggests that the liver is also a major site for folate catabolism *in vivo*. The induction of the hepatic microsomal enzymes by phenobarbitone failed to increase the rate of folate degradation (Chapter 3). Thus, following the failure by several groups of workers to demonstrate the existence of folate cleavage enzymes *per se* (see Chapter 1 for references) and failure of the induction of hepatic enzymes to increase

Type of folate	Dose (μg/Kg body wt)	Species	% of dose retained in the liver	% of dose excreted as pAcBG in the urine	Reference
Folic acid	100	Rat	19.1	3.7	Saleh et al., 1981
Folic acid	100	Rat	11.4	1.2	Pheasant et al 1981
10CHOFA	10	Rat	10.8	1.7	"
10CHOFA	50	Rat	7.6	1.1	"

Table 7.1. The excretion of labelled p-AcBG in the urine of rats dosed with labelled folates and the amounts of radioactivity in the liver 48 h after administration.

The results are expressed as a percentage of the dose

folate catabolism, spontaneous chemical scission of the folate molecule appears to be the simplest and the most probable mechanism bearing in mind the extreme lability of the reduced folate coenzymes. The folate derivative which undergoes scission has not yet been identified but THF and DHF derivatives are the most likely candidates due to their inherent chemical instability. However, the intracellular DHF level is very low (10^{-8}M) under normal circumstances (see below) since it is an excellent substrate for DHF reductase and is rapidly reduced to THF (Goldman, 1977). THF can also oxidise via q DHF. Pollock and Kaufman (1978) have proposed a mechanism whereby dihydropteridine reductase participates in maintaining tissue folate in a reduced state. This enzyme can reduce the quinonoid form of DHF to THF (Lind, 1972) (Figure 7.2).

Changes in the redox potential in the tissues could alter the rate of folate degradation. Human liver is reported to have more oxidative conditions than rat liver which is in turn more oxidising than guinea pig liver. This is shown by the hepatic NAD/NADH ratio which is reported to be 1871 in man (R. H. Harris, personal communication), 725 in the rat (Williamson et al., 1967) and 293 in guinea pigs (Garber and Hanson 1971). This is consistent with the suggestion quoted above that catabolism proceeds via oxidative cleavage of the C_9-N_{10} bond since the rate of tissue folate degradation decreases as follows; man $>$ rat $>$ guinea pig. (Table 7.2).

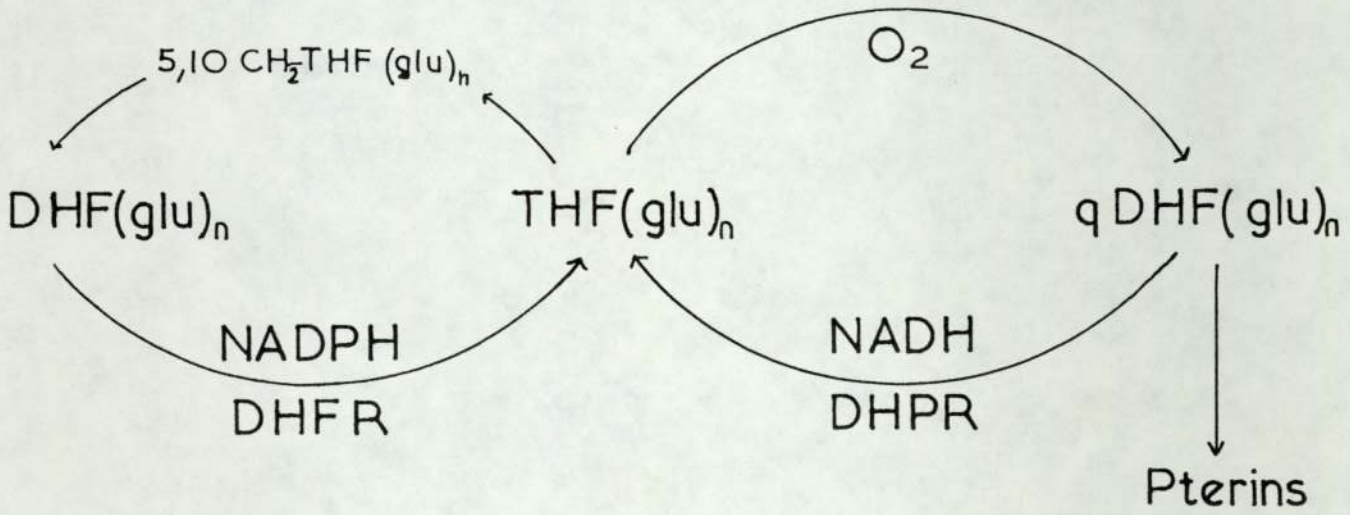


Figure 7.2. The role of DHPR in maintaining tissue folate in a reduced state

Species	NAD/NADH ratio	% of the body retained radio-activity excreted as p-AcBG
Man	1871	2.0
Rat	725	0.9
Guinea pig$^{\Delta}$	344$^{\bullet}$	0.4*
Guinea pig	293	0.2*

Table 7.2. The excretion of the catabolite of folate polyglutamate (p-AcBG) in the urine of man, rat and guinea pig 6 h after the administration of ^{3}H and ^{14}C folic acid. The results are given as the percentage of the body radioactivity at 6 h in man and 8 h in the rat excreted as pAcBG in the urine of 6-24h, 8-24h and 0-24h time period of man, the rat and guinea pig respectively.

* Data supplied by R. Choolun

Δ Scorbutic

$\bullet$ NAD/NADH ratio in Fasted Guinea Pig (96 h)

The metabolism of radiolabelled folic acid was disturbed in patients with malignant disease and in rats with implanted tumours. Similar changes were observed in man and the rat such as lower excretion of the administered radioactivity in the urine, higher incorporation of folic acid into the reduced folate pool and the higher retention of the labelled folate in the body as demonstrated by the inverse correlation between the approximate tumour mass and urinary radioactivity in cancer patients (Chapter 5) and the high radioactivity found in the tumour of the rat (Chapter 3). These results reflect the increased requirement for folate in malignant disease and in tumour-bearing rats and may suggest that analysis of urine and plasma samples from subjects with malignant disease does not accurately reflect the tissue folate levels. Indeed, Magnus (1967) reported that although 85% of patients with malignant diseases had low serum folate, the red blood cell folate level remained normal in 37 of 38 patients. However, the most striking feature of these studies is the observation of the significant depression of folate scission products excreted in the urine of cancer patients and tumour-bearing rats despite the enhanced tissue retention of radioactivity. The presence of larger amounts of radioactivity in the body would be expected to lead to an increase in the production of radio-labelled scission products if the catabolic rate remained unaltered. This suggests that the level of folate catabolites in the urine after an oral dose of folate may be a useful as a tool for diagnosis of such diseases.

Breakdown of the folate molecule most likely proceeds by oxidative scission of a labile folate derivative (probably THF) produced during the

normal metabolic pathways as quoted above. It is interesting to note that tumour cells are reported to exhibit more reducing conditions in their cytosol which are reflected in increased lactate/pyruvate ratios (Weber et al., 1971, Williamson et al., 1970) and a lower NAD/NADH ratios than normal (Schwartz et al., 1974). Excessive production of lactate by a tumour can also lead to increased lactate in blood and hence, may affect other tissues. Also hypoxia is common in solid animal and human tumours (Denekamp et al., 1980, Fowler and Denekamp 1979). Therefore the decrease in catabolism of folate in the presence of a tumour is most likely to be due to the stabilisation of the labile folate derivative by the more reducing conditions prevailing. The effect on NAD/NADH ratio increases as the rate of growth of the tumour line increases (Weber et al., 1971, Williamson et al., 1970, Schwartz et al., 1974) and the decrease in urinary folate catabolites also becomes more marked in advanced malignant disease up to the extreme case of (D.M.) when no scission products were excreted in the urine.

The administration of MTX with or before folic acid results in excretion of large amounts of folic acid which has not entered the reduced folate pool (Barford et al., 1980). The use of MTX in cancer chemotherapy is thought to act by blocking the pathways of folate coenzymes but the fate of these coenzymes has never been reported. Here the effect on tissue folates was investigated by labelling the tissue folate pool before the administration of MTX. The experiments presented in Chapter 3 have shown, following the administration of MTX, higher excretion of folic acid

and scission products particularly, p-AcBG, detection of additional catabolites in the urine including pterin, xanthopterin, pterin-6-COOH and metabolite C and a fall in the levels of tissue radioactivity.

As described earlier the labile folate derivatives likely to undergo scission are, DHF and THF. Inhibition of dihydrofolate reductase by MTX would lead to a build up of DHF polyglutamates. Chemical oxidation of DHF gives folic acid, formaldehyde, p-aminobenzoyl-L-glutamate, dihydroxanthopterin and 7,8 dihydropterin-6-carboxaldehyde (Chippel and Scrimgeour, 1970). The dihydro derivatives are likely to oxidise further giving xanthopterin and pterin-6-COOH respectively. Thus inhibition of dihydrofolate reductase leading to increased breakdown via DHF derivatives can account for a proportion of the increase in p-AcBG excretion and the appearance of xanthopterin, pterin-6-COOH, $^{3}H_2O$ and folic acid in the urine of rats treated with MTX but cannot explain the formation of pterin. Pterin is not produced by the metabolism in vivo of pterin-6-COOH (Pheasant and Pearce, 1981). However, oxidation of THF via quinonoid-DHF gives ultimately pterin and xanthopterin as the final products (Blair and Pearson, 1974). Thus the pterin derivatives found in urine following MTX administration are only consistent with the breakdown of both THF and DHF polyglutamates in the tissues. Quinonoid DHF is a substrate for dihydropteridine reductase (Lind 1972). MTX inhibits dihydropteridine reductase with a K_i of 3.8×10^{-5} M (Craine, et al., 1972) and this could cause increased folate breakdown from THF via quinonoid DHF. The effect of

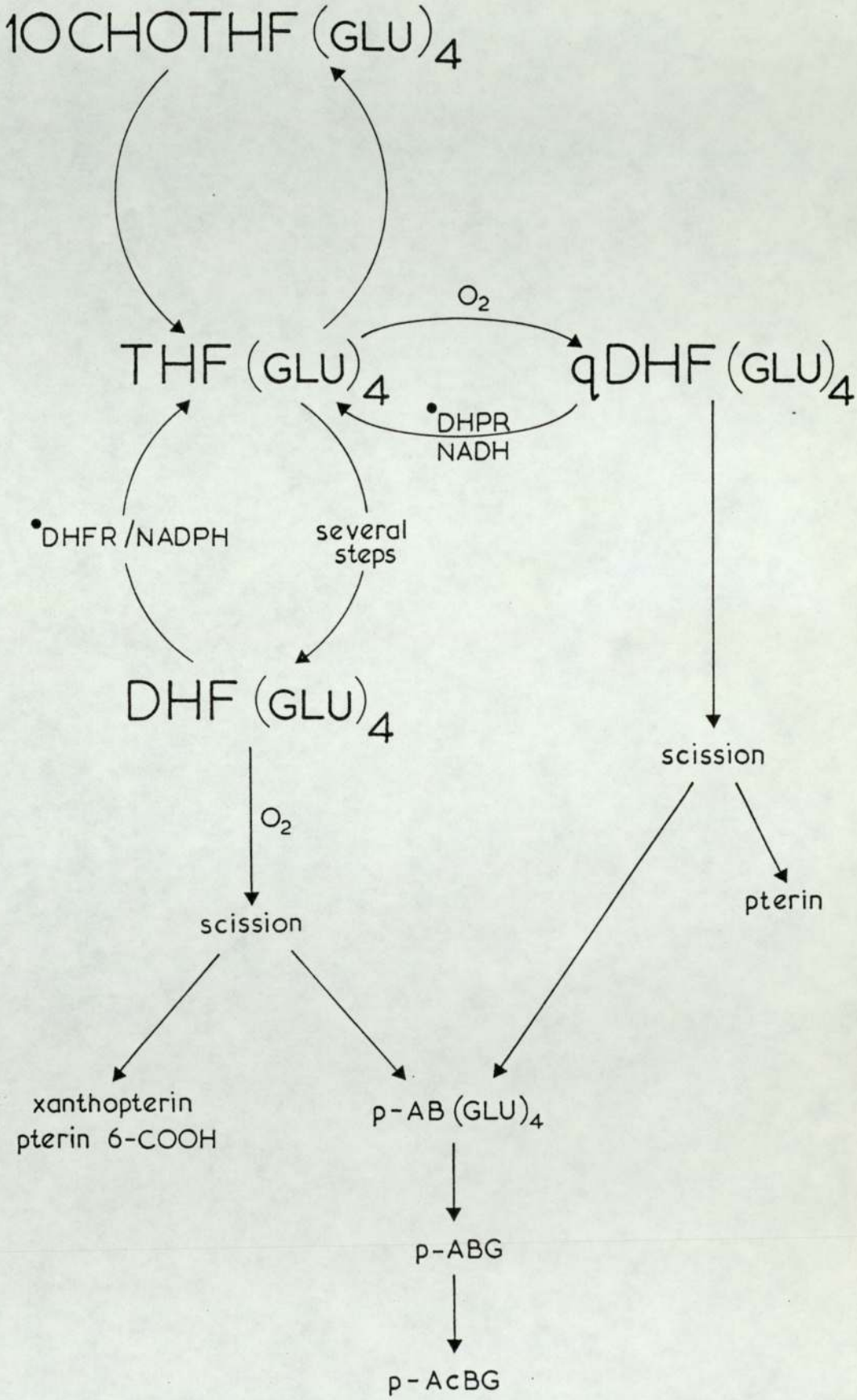
MTX on serum biopterin derivatives (Leeming et al., 1976) suggest that such inhibition of dihydropteridine reductase does occur *in vivo*. Thus the appearance of pterin in the urine following MTX administration is further evidence that dihydropteridine reductase does have a role in folate metabolism in vivo and that MTX interferes with this function (Figure 7.3).

Recently 5,10CH_2-THF reductase has also been shown to have dihydropteridine reductase activity and to reduce quinonoid DHF to THF (Matthews and Kaufman, 1980). This enzyme is weakly inhibited by MTX (Mangum *et al.*, 1979).

Thus these studies on the catabolism of the folate polyglutamates suggest that dihydrofolate reductase is not the sole enzyme affected by MTX *in vivo*. Other possible target enzymes include dihydropteridine reductase and 5,10CH_2-THF reductase. The design of specific anti-folate drugs which can attack certain tissues and maximise their effect on these enzymes could lead to a more effective chemotherapeutic agent which depletes the cell of folate by increasing breakdown. This could be particularly useful since the catabolic process appears to be decreased in a tumour.

Thus, the results presented in this thesis show that folate catabolism *in vivo* is a normal phenomenum. Also, they support the hypothesis of an oxidative breakdown process of tissue folates normally and under drug treatment, and suggest that the rate at which this breakdown process occurs could have a significant effect on the folate status in the animal.

Figure. 7.3. The effect of methotrexate on tissue folate



• The site of inhibition by MTX

As 10CHOFA is not reduced by mammalian dihydrofolate reductases *in vitro* (Bertino *et al.*, 1965, A. Sahota personal communication) and probably not reduced to any significant extent in the human body, it may therefore, enter the folate pool in man via deformylation (Chapter 6). 10CHOFA has been shown to be extensively metabolized in the rat (Pheasant *et al.*, 1981) but it is utilised only poorly by man (Ratanasthien *et al.*, 1974, Chapter 6). The difference in handling of 10CHOFA in man and the rat may reflect a difference in gut microflora since some micro-organisms have been reported to reduce 10CHOFA (McIntyre, and Harding, 1977).

A large proportion of folates in food presents in the form of 10CHOFA derivatives (Chararin, 1979a). This could have a significant effect on the folate status in the body and accelerate the appearance of folate deficiency not only because it is not metabolised to a great extent in man but also because of its effect on the activity of dihydrofolate reductase *in vivo* (Chapter 6). This may explain the megalobastic anaemia associated with vitamin C deficiency (Stokes *et al.*, 1975) since food lacking vitamin C would contain elevated levels of oxidized folates including 10CHOFA. Vitamin C deficiency may also increase the conversion of 10CHOTHF polyglutamate to 10CHOFA polyglutamate which is a potent inhibitor of dihydrofolate reductase (Friedkin *et al.*, 1975). The accumulated DHF-polyglutamate resulting from 10CHOFA-polyglutamate formation, could thus undergo degradation and result in loss of folate polyglutamate. In addition to this, 10CHOFA derivatives may have a role in

regulating DHF reductase activity in the normal cell.

Although most mammalian dihydrofolate reductases are inhibited by 10CHOFA with a K_i of 0.006-0.02 μM *in vitro* (Bertino *et al.*, 1965, Friedkin *et al.*, 1975, A. Sahota, personal communication) and *in vivo* by an oral dose of 5 mg to humans (Chapter 6), dihydrofolate reductase from human neoplastic intestinal cells is not inhibited by concentrations up to 10 μM of 10CHOFA *in vitro* (A. Sahota, personal communication). The presence of such a property in a malignant tissue may represent the breakdown of a normal regulatory mechanism in this tissue which contributes to the loss of control in cell growth and division.

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