FOLATE METABOLISM

IN

THE GUINEA PIG

A thesis submitted for the degree of

DOCTOR OF PHILSOPHY

by

RAJINDRANATH CHOOLUN

in the Department of Chemistry

THE UNIVERSITY OF ASTON IN BIRMINGHAM

June 1982

SUMMARY

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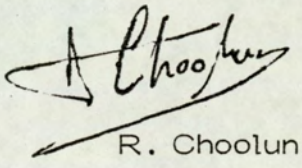
Following the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid to normal guinea pigs, p-acetamidobenzoate (major product) and p-acetamidobenzoyl-L-glutamate (minor product) were found in the urine. No radioactive folate was excreted except at very high doses when trace amounts of folic acid appeared, or after pre-treatment of the guinea pigs with antibiotics or methotrexate. Up to 20% of an oral dose of [2-^{14}C] folic acid was found in the expired air. Only small amounts of radioactive polyglutamates were synthesized in the liver. Scorbutic guinea pigs similarly dosed gave qualitatively similar results to normal guinea pigs but catabolized rather more of the liver polyglutamates.

p-Acetamidobenzoate (major product) and p-acetamidobenzoyl-L-glutamate (minor product) still remained the dominant metabolites following an oral dose of a mixture of either [2-^{14}C] and [3',5',7,9-^{3}H] 10-formyl-folic acid or of the biologically active mixture of [2-^{14}C] and [3',5',7,9-^{3}H] 5-methyltetrahydrofolate. Oral administration of the 50:50 diastereoisomeric mixture of [5-^{14}C] 5-methyltetrahydrofolate gave small amounts of 5-methyltetrahydrofolate in the urine but largely breakdown products.

These results contrast sharply with those described for the rat and man. They show that in the guinea pig all folates are poorly absorbed from the gut as would be expected from the nearly neutral acid microclimate. Folic acid and 10-formyl-folic acid are reduced to tetrahydrofolates in the gut. These tetrahydrofolates and 5-methyltetrahydrofolate acid are catabolized to p-acetamidobenzoate in the gut. Liver folate polyglutamates are broken more slowly to p-acetamidobenzoyl-L-glutamate than in the rat and man as would be expected from the greater cytosolic reducing capacity of the guinea pig. The more rapid liver folate breakdown in the scorbutic guinea pig suggests a steady depletion of folate would occur in man in scurvy and this will precipitate anaemia.

KEY WORDS: Folate metabolism, folate catabolism, 10 formyl folic acid , 5-methyltetrahydrofolate, scurvy.

This work was carried out from March 1979 to March 1982 in the Department of Chemistry at the University of Aston in Birmingham. It was undertaken independently and has not been submitted for any other degree.



R. Choolun.

ACKNOWLEDGEMENT

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To Sarojini

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1.1. FOLATE MONOGLUTAMATES AND POLYGLUTMATES

The folates consist of a large group of naturally-occurring molecules derived from the reduction of or substitution of, or addition to the parent compound, folic acid (reviewed by Blakley, 1969; Hoffbrand, 1976; Rowe, 1978; Chanarin 1979). The structural formula of folic acid is illustrated in Figure 1.1. The molecule is made up of a pterin moiety linked by a methylene bridge to p-amino-benzoic acid which itself is joined in amide linkage to L-glutamic acid. Folic acid is not normally found in food or in the human body in significant concentrations. It is not biochemically active but becomes so after reduction. In vivo, reduction is catalyzed by the enzyme dihydrofolate reductase (DHFR) [5,6,7,8 THF : NAD(P) oxidoreductase (EC 1.5.1.3)]. The pyrazine ring of the pterin moiety is easily reduced, first to 7,8 dihydrofolate (DHF) (Figure 1.2) then to 5,6,7,8 tetrahydrofolate (THF) (Figure 1.3).

A substituent group may be present at either the N_5 position e.g. 5-methyltetrahydrofolate (5-MeTHF) (Figure 1.4) and 5-formyl-tetrahydrofolate (5-CHOTHF) (Figure 1.19) or the N_{10} position, e.g. 10-formylfolic acid (10-CHO-FA) (Figure 1.10) or 10-formyltetra-hydrofolic acid (10-CHOTHF) (Figure 1.6). Additional glutamate residues may be added to the terminal glutamate, forming folate poly-glutamates. A typical polyglutamate, folic acid polyglutamate is illustrated in Figure 1.11. Polyglutamates are the most common

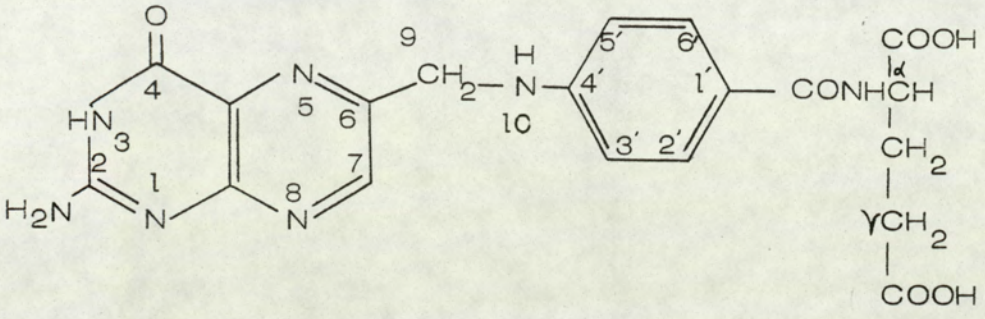


Figure 1.1. Folic acid

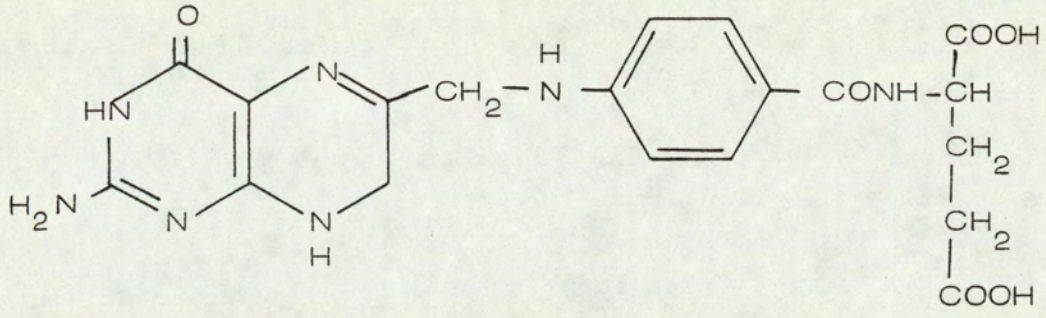


Figure 1.2. 7,8 Dihydrofolic acid (DHF)

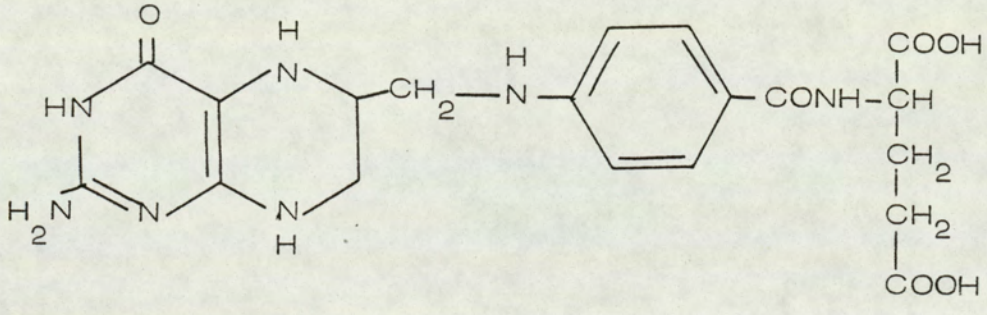


Figure 1.3. 5,6,7,8 Tetrahydrofolic acid (THF)

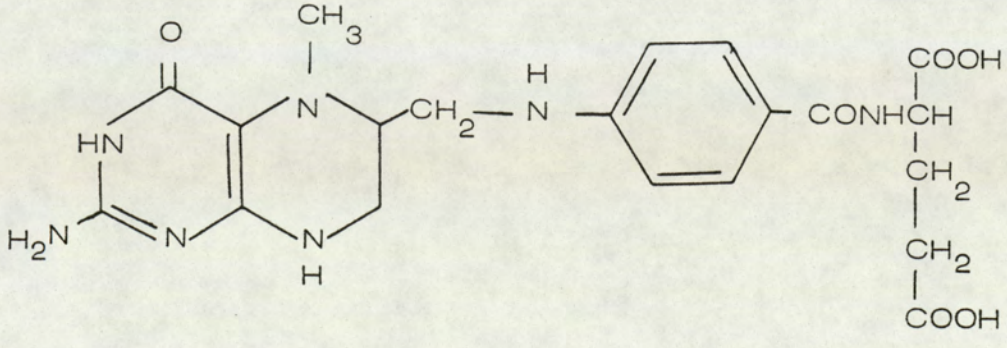


Figure 1.4. 5-Methyltetrahydrofolic acid (5-MeTHF)

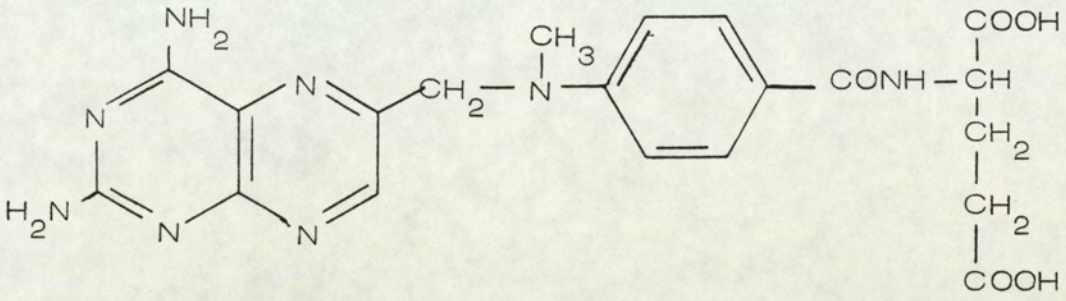


Figure 1.5. Methotrexate (MTX)

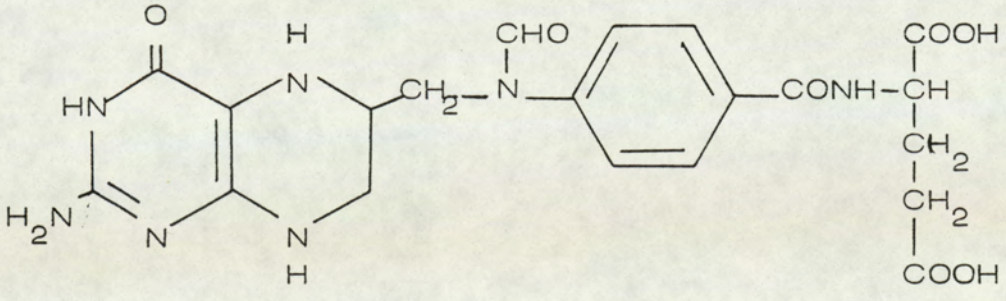


Figure 1.6 10-Formyltetrahydrofolic acid (10-CHOTHF)

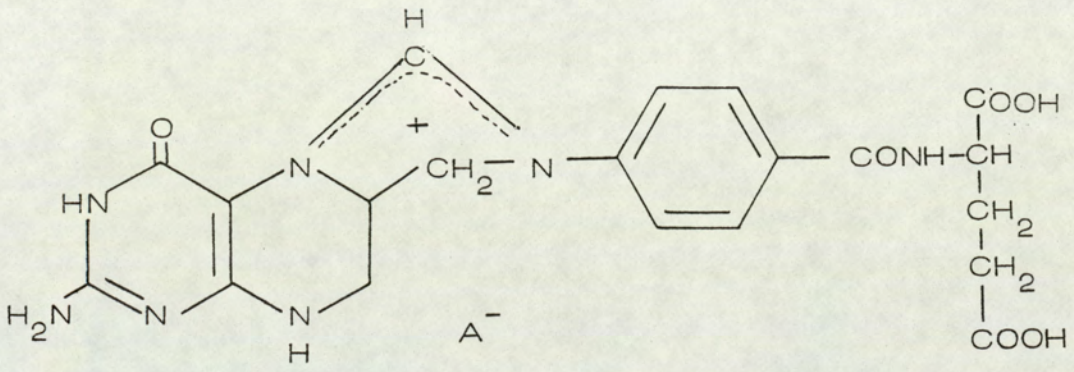


Figure 1.7. 5,10 Methenyltetrahydrofolic acid (5,10 CH=THF)

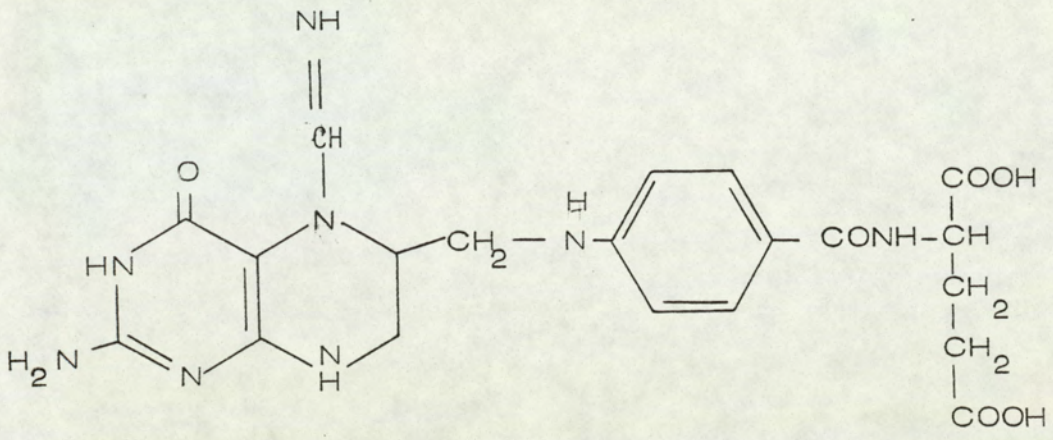


Figure 1.8 Formiminotetrahydrofolic acid

(5-NH =CHTHF)

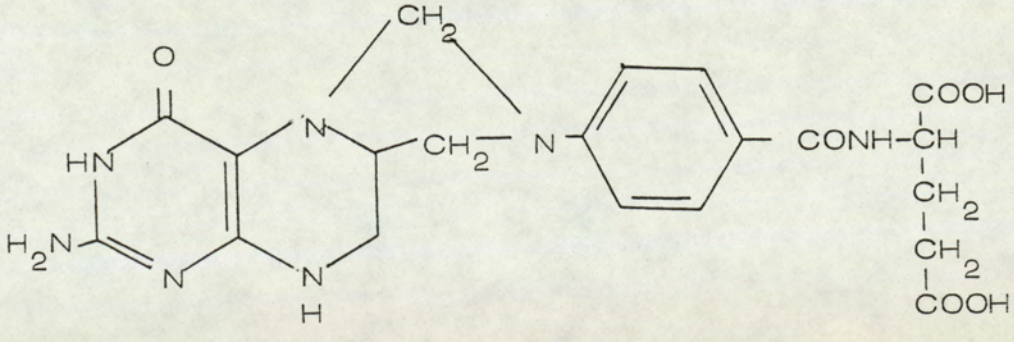


Figure 1.9 5,10 Methylenetetrahydrofolic acid

(5,10-CH_2THF)

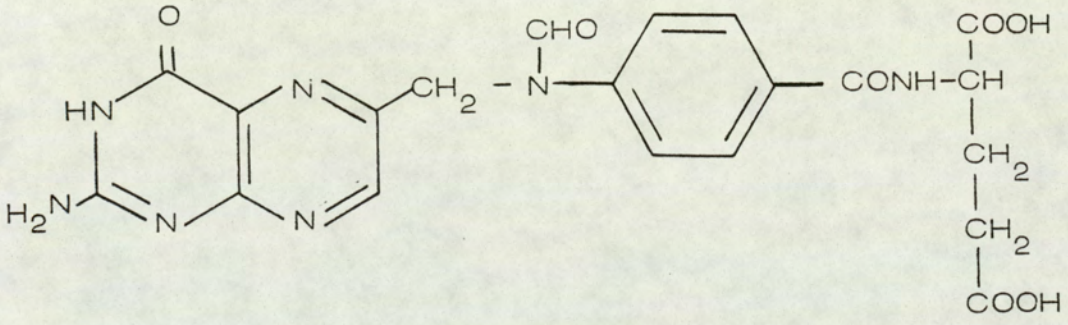
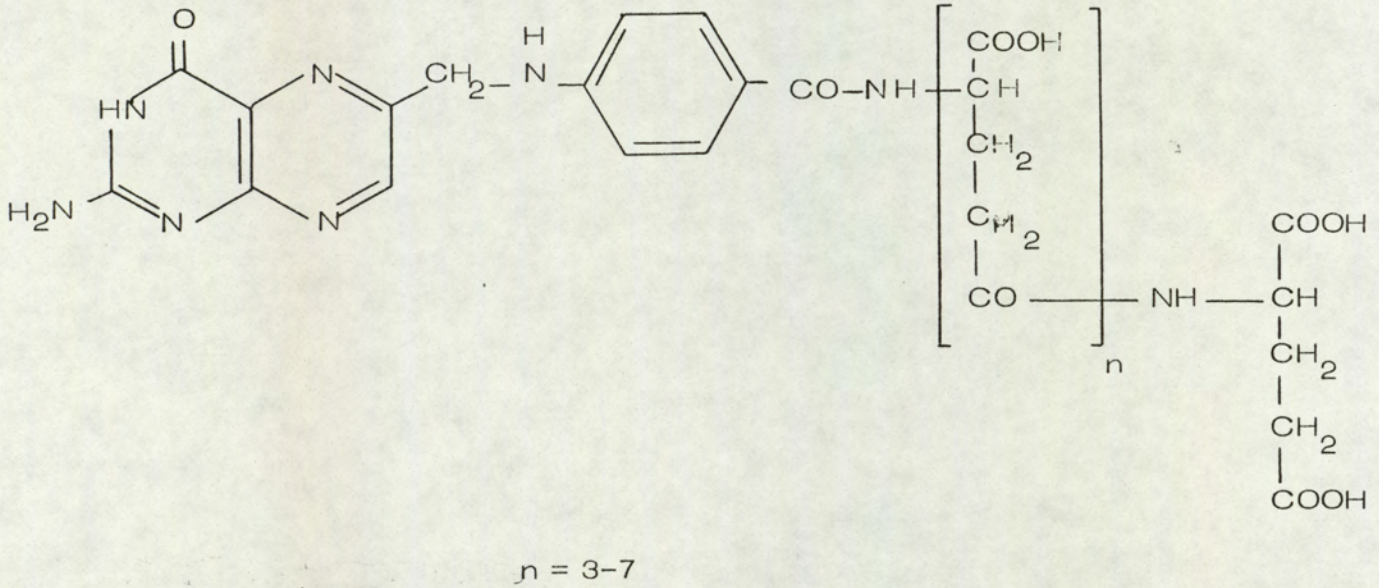


Figure 1.10 10-formylfolic acid

(10-CHOFA)



n = 3–7

Figure 1.11. Folic acid polyglutamates

naturally-occurring folates in foods (Leichter et al., 1979). The structure of the folate coenzymes (as folate monoglutamates) and some of their metabolised excretory products are illustrated in Figures 1.1 - 1.21 (except Figure 1.5). Folate coenzymes can be very readily interconverted. A simplified scheme showing the series of interrelated reactions is shown in Figure 1.22.

Folate polyglutamates are the major form of folate in mammalian cells, accounting for up to 90% of cell folate (Hoffbrand et al., 1977) whereas the monoglutamate which is concerned with transport of folate within the body, is the principal form in the extracellular fluid together with small amounts of 10-CHOTHF (Chanarin, 1979). Until recently, the view was held that folate polyglutamates act only as a storage form and that monoglutamates which are rapidly converted in vivo function as coenzymes (Blair, 1976). But although, no doubt, folate monoglutamates still have a role to play in functioning as coenzymes, recent evidence indicate that folate polyglutamates in addition to their storage functions, are, in general, the naturally-occurring folate coenzymes for the folate-mediated reactions in amino acid metabolism and in purine and pyrimidine synthesis in mammalian cells (Baugh and Krumdieck, 1969; Hoffbrand et al., 1977; Chanarin, 1979). Biochemical studies in vitro have shown that the reduced folate polyglutamates serve as better coenzymes than the corresponding folate monoglutamates in folate-mediated reactions

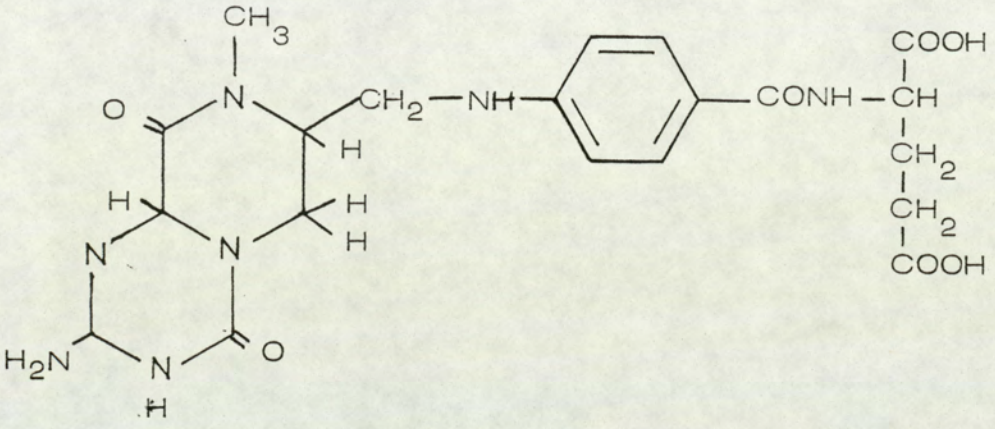


Figure 1.12 Pyrazine-S-triazene derivative

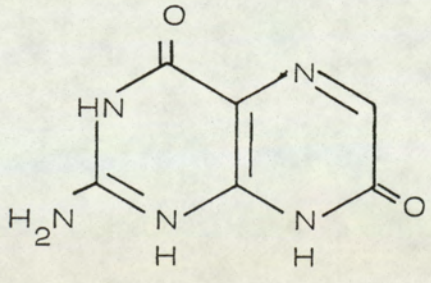


Figure 1.13 Isoxanthopterin

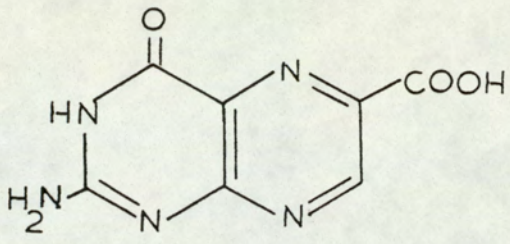


Fig. 1.14

Pterin-6-carboxylic acid

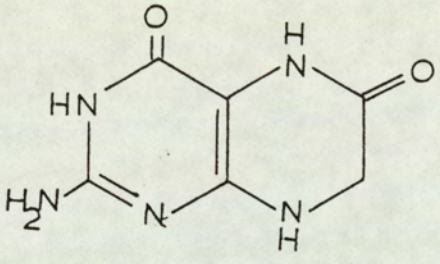


Fig. 1.15

Dihydroxanthopterin

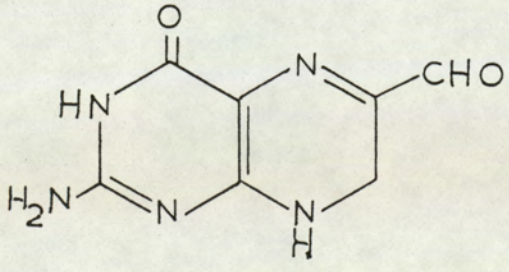


Fig. 1.16

Dihydropterin-6-aldehyde

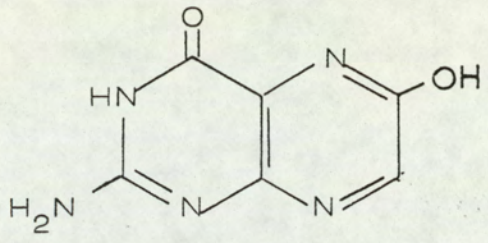


Figure 1.17 Xanthopterin

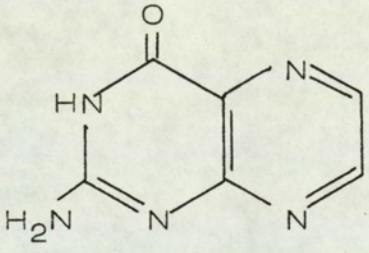


Figure 1.18 Pterin

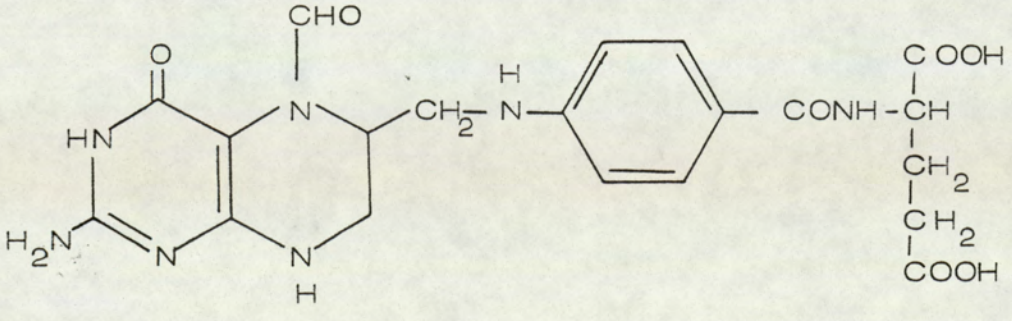


Fig. 1.19 5-Formyltetrahydrofolic acid (5-CHOTHF)

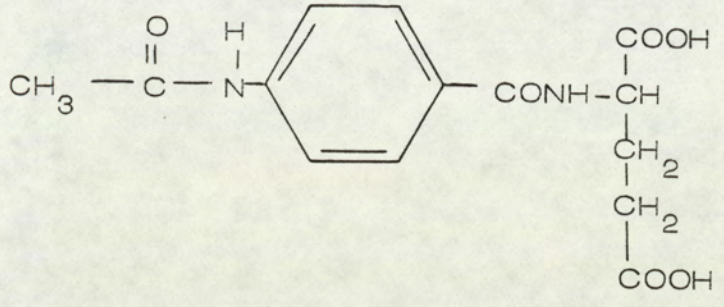


Fig. 1.20 p-Acetamidobenzoyl-L-glutamic acid

(pAABglu)

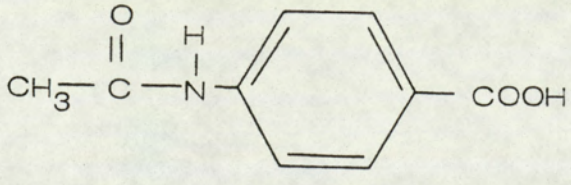


Fig. 1.21 p-Acetamidobenzoic acid

(pAAB)

(Hoffbrand et al., 1977), and are more active substrates for the reducing enzymes dihydrofolate reductase and 5,10-CH_2THF reductase (Hoffbrand et al., 1977). Moreover, mammalian cells with a genetic inability to synthesise folate polyglutamates require extra thymidine, adenosine and glycine, compounds whose de novo synthesis is known to require folate coenzymes in their culture medium for growth.• In addition to their newly found role as coenzymes, folate polyglutamates still have a function (even if minor) as a storage system in the liver (Hoppner and Lampi, 1980).

Since the major circulating form of folate in plasma is a monoglutamate, 5-methyltetrahydrofolate (5MeTHF) (Figure 1.4) (small amounts of 10-formyl-tetrahydrofolate (10-CHOTHF) (Figure 1.6) are also present), mammalian cells must each build up all their own folate polyglutamate coenzymes from this compound. In vitro studies of the synthesis of folate polyglutamate compounds in human lymphocytes using radioactively labelled folic acid, 5-formyl tetrahydrofolate or 5-MeTHF suggest that 10-CHOTHF and tetrahydrofolate rather than 5MeTHF are the preferred substrates in human tissues (Hoffbrand et al., 1977). This observation implies that vitamin B_{12} may have a key role in the synthesis of folate coenzymes since it is needed in the homocysteine-methionine reaction (Figure 1.27) by which 5 MeTHF (the form of folate which cells take up from plasma) is converted to THF. The role of vitamin B_{12} will be discussed later in this chapter.

• McBurney and Whitmore (1974)

The enzyme catalysing the synthesis of folate polyglutamate, folate polyglutamate synthetase or ligase, has been studied in extracts of E. coli (Griffin and Brown, 1964), Neurospora crassa (Sakami et al., 1973), rat liver (Spronk, 1973) and sheep liver (Gawthorne and Smith, 1973) and has been found to require ATP, Mg^{++}, K^{+} and a pH around neutral. These enzymes are unevenly distributed among the intracellular compartments, the highest activity occurring in the cytosol (Gawthorne, 1980).

1.2. ROLE OF FOLATE COENZYMES IN ONE-CARBON-UNIT TRANSFERS

The primary function of folates in mammalian metabolism is to transport one-carbon units. This one-carbon metabolism is a cyclic array of enzymatically catalyzed transformations involving folates and encompassing the uptake and disposition of methyl groups at different states of oxidation (Blakley, 1969). Folates are required in three reactions in DNA synthesis, one in pyrimidine synthesis (thymidylate synthesis), and two in purine synthesis (Hoffbrand, 1977). Thymidylate synthesis is a rate-limiting step in DNA synthesis (Herbert and Das, 1976; Hoffbrand, 1977) and because several such one-carbon unit transfers are crucial to DNA synthesis, folate deficiency would affect the most rapidly dividing cells and tissues of the body. The organs most affected are the bone marrow, the cells of the haematopoietic system, the epithelial cells of

the gastrointestinal and urinogenital tracts (Herbert, 1975). Other reactions involving one-carbon-unit transfers are in the methionine synthesis, serine-glycine interconversion and histidine degradation (Reviewed by Blakley, 1969; Hoffbrand, 1976; Rowe, 1978; Chanarin, 1979). The series of interrelated reactions are illustrated in Figure 1.22.

Folic acid enters the folate pool only after it has undergone reduction, first to dihydrofolate, then to tetrahydrofolate (see Section 1.1) which is the precursor of coenzymes acting in the transfer of one-carbon-units. The reaction, catalyzed by the enzyme dihydrofolate reductase (DHFR) can be inhibited by various folate antagonists which bind to it, notably methotrexate (MTX) (Figure 1.5), a non-metabolizable folate analogue. Other inhibitors include aminopterin, pyrimethamine and triamterene (Herbert and Das, 1976). Inhibition of DHFR leads to the depletion of the cellular pool of folate coenzymes, therefore, to a lack of purines and pyrimidines and ultimately cell death (Blakley, 1977).

10-formyl tetrahydrofolate (Figure 1.6) is the folate coenzyme involved in purine biosynthesis. Also thought to be involved was 5,10-methenyl tetrahydrofolate (5,10CH=THF) (Figure 1.7) but the view has now been abandoned. 5,10 Methylene tetrahydrofolate (5,10CH_2-THF) (Figure 1.9) is involved in pyrimidine synthesis (Herbert and Das, 1976; Rowe, 1978; Chanarin, 1979). Dev and Harvey (1978) using a purified glycinamide ribonucleotide transformylase

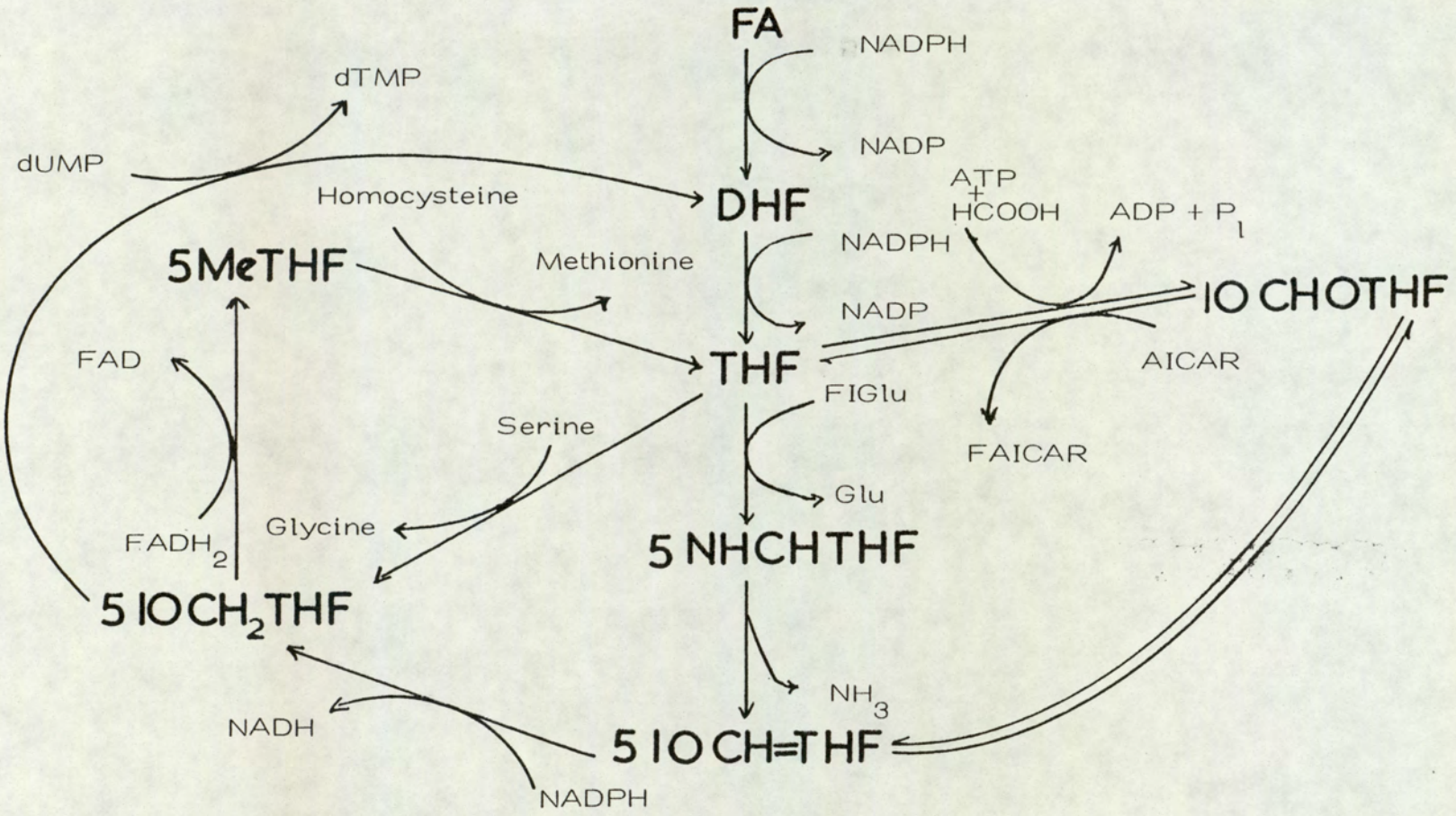


Fig. 1.22 Flow chart of folate metabolism in man

(EC 2.1.2.2) from E. coli found that the required coenzyme is 10-formyl tetrahydrofolate. They suggested that during earlier studies on pigeon liver and bacterial extracts (Goldthwait et al., 1956; Westby & Gots, 1969), 5,10 methenyl tetrahydrofolate was hydrolysed to 10-formyl THF either chemically or by the enzyme cyclohydrase.

The reactions described in the biosynthesis of the purine ring are those envisaged to happen (Herbert and Das, 1976; Rowe, 1978; Chanarin 1979) with 5,10 methylene THF, 5,10-methenyl THF and 10-formyl THF (Figure 1.24)

The methylation of uridylate to thymidylate being a rate-limiting step in cellular DNA synthesis (Herbert and Das, 1976) (Fig. 1.23) has already been mentioned. The enzyme thymidylate synthetase [5,10 methylene THF: dUMP c-methyltransferase (EC 2.1.1.6)] transfers a methylene group from 5,10-methylene tetrahydrofolate (Figure 1.9) to uridylate and the methylene group is simultaneously reduced to methyl group forming thymidylate. In the process, 5,10 methylene tetrahydrofolate is not only demethylated but also converted to dihydrofolate (Figure 1.2), which requires dihydrofolate reductase for reconversion to tetrahydrofolate for further participation in one-carbon transfer reactions (Friedkin, 1957) (Figure 1.23). Folate antagonists block this latter reductive process, thus interferring with thymidylate synthesis.

DE NOVO PATHWAY

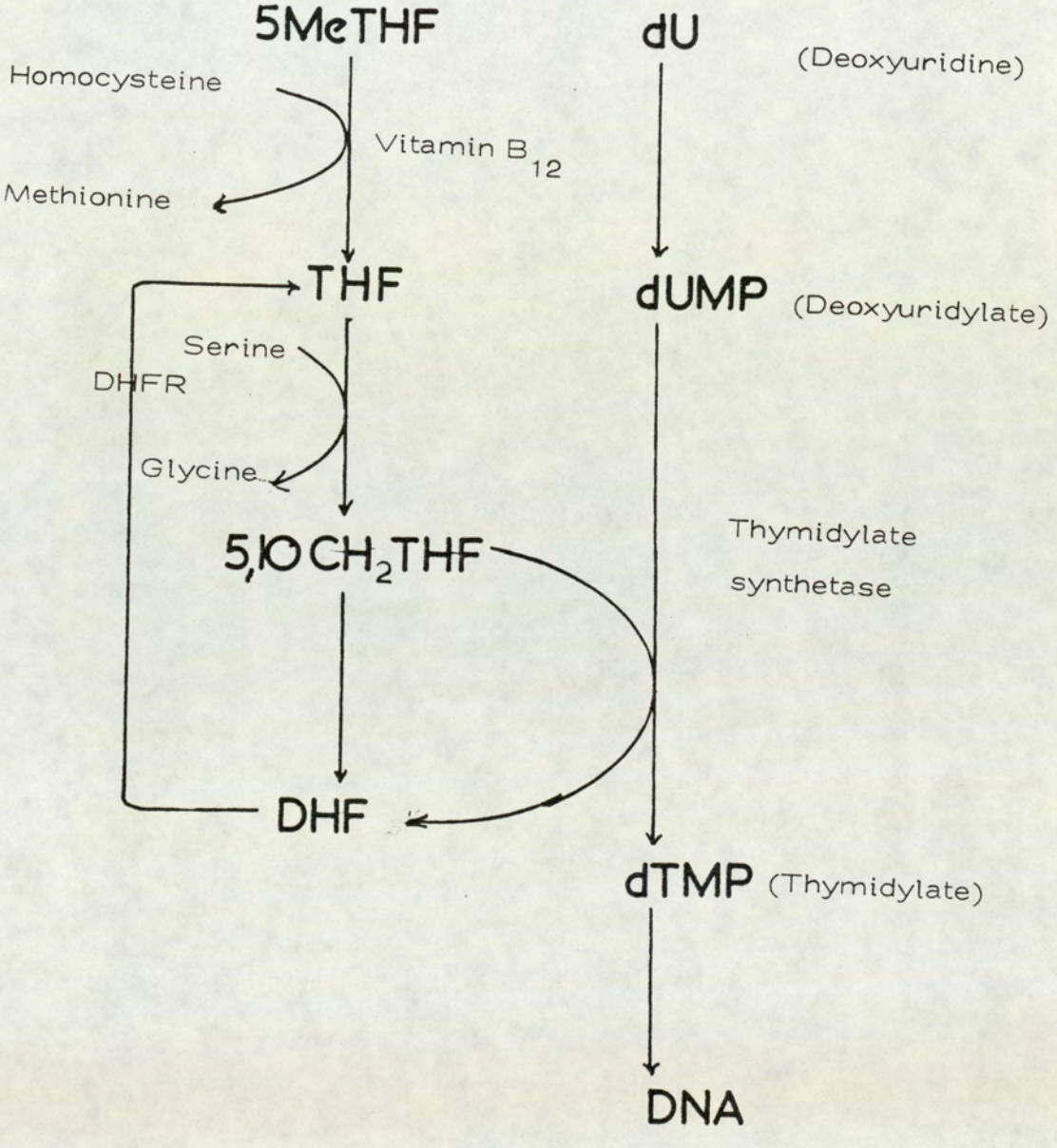
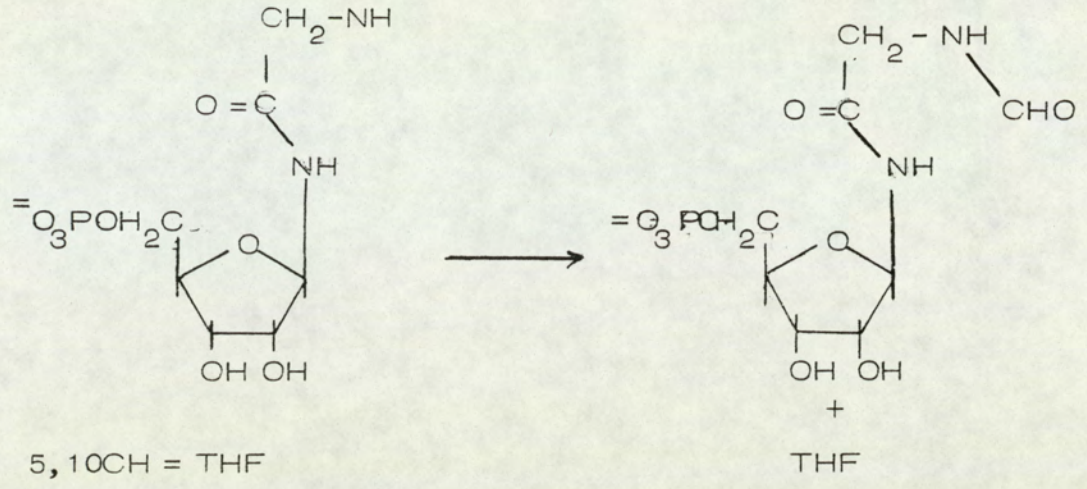


Figure 1.23 DNA synthetic pathways in man

Two reactions in the biosynthesis of the purine ring involve additional carbon units by tetrahydrofolate-dependent transformylases (illustrated in Figure 1.24). In the first reaction, formylation of glycinamide ribonucleotide by 5,10 methenyl tetrahydrofolate (Figure 1.7) occurs to form carbon-8 of the purine ring (reviewed by Rowe, 1978; Chanarin, 1979). The second reaction involves the formylation of 5-amino-4-imidazole carboxamide ribonucleotide (AICAR) by 10-formyl tetrahydrofolate to close the purine ring. This provides carbon-2 and the purine formed is inosinic acid (Figure 1.24).

Other major sources of one-carbon units are provided by the conversion of serine to glycine and by the degradation of histidine. The conversion of serine to glycine is catalyzed by the enzyme serine transhydroxymethylase [5,10-methylene tetrahydrofolate glycine hydroxymethyltransferase (EC 2.1.2.1] and requires as cofactor pyridoxal-5-phosphate, with tetrahydrofolate acting as a carbon-acceptor. The enzyme has been detected in the tissues of many species and high levels are found in vertebrate livers (Rowe, 1978). The bulk of the enzyme is in the cytosol but an enzyme with almost identical physical and kinetic properties has been found in rabbit liver mitochondria (Rowe, 1978). The reaction is reversible and may account for the reported toxicity of glycine in rats and chickens, which is corrected by folic acid (Dinning et al., 1949) and for the fact that glycine may make human megaloblastosis worse (Waxman et al., 1970).



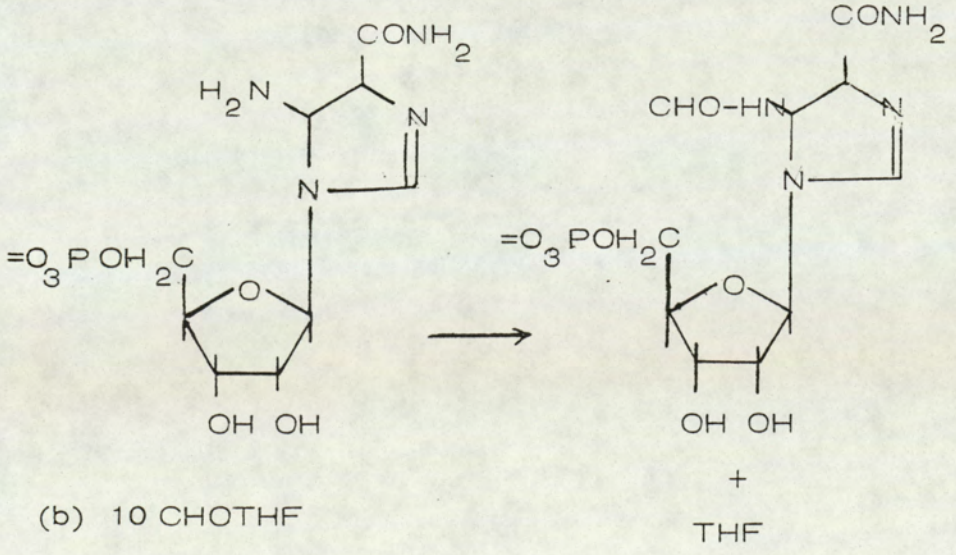
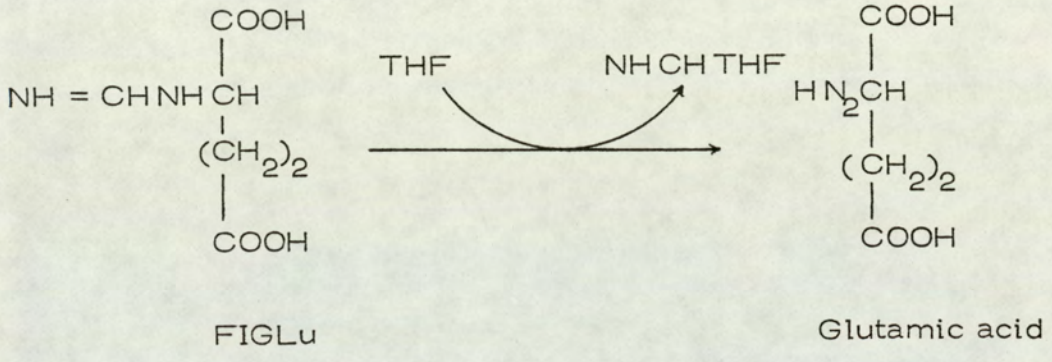


Figure 1.24 Folate dependent steps in the biosynthesis of the purine ring. (a) Generation of C-8 (b) Generation of C-2 of the purine ring.

Formiminoglutamic acid (FIGlu) is an intermediate enzymic degradation product of histidine, serving as another major source of one-carbon units. Formiminotransferase (EC 2.1.2.5) catalyzes the further breakdown of this product to glutamic acid (Figure 1.25) which is then excreted in the urine.



FIGLu Glutamic acid

Figure 1.25 Formation of formiminotetrahydrofolate (NHCHTHF)

Tetrahydrofolate acts as cofactor and accepts the formimino group, forming formiminotetrahydrofolate (Figure 1.8), a rather unstable product which is rapidly converted to 5,10 CH=THF (Figure 1.7). Folate deficiency or metabolic folate abnormality interferes with the removal of the formimino unit of FIGlu, which consequently is excreted unchanged in large amounts in the urine (Herbert and Das, 1976).

Another involvement of a folate coenzyme is in the formation of methionine from homocysteine (Figure 1.26). The reaction is

catalyzed by the enzyme 5-methyltetrahydrofolate homocysteine transmethylase (methionine synthetase) (EC 2.1.1.13) and requires 5MeTHF for the formation and transfer of a methyl group to homocysteine to form methionine. Other cofactors involved in the reaction are S-adenosyl methionine (SAM), reduced flavin adenine dinucleotide ($FADH_2$) and vitamin B_{12}

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

SAM is necessary to activate the enzyme and it does so through the methylation of the enzyme-bound cobalamin (Taylor and Weissbach, 1973). Once initiated, SAM is no longer required and the cobalamin is methylated by 5-MeTHF. The final requirement is a reducing system to keep cobalamin at the Cob(I)alamin state. The overall reaction is summarized in Figure 1.26.

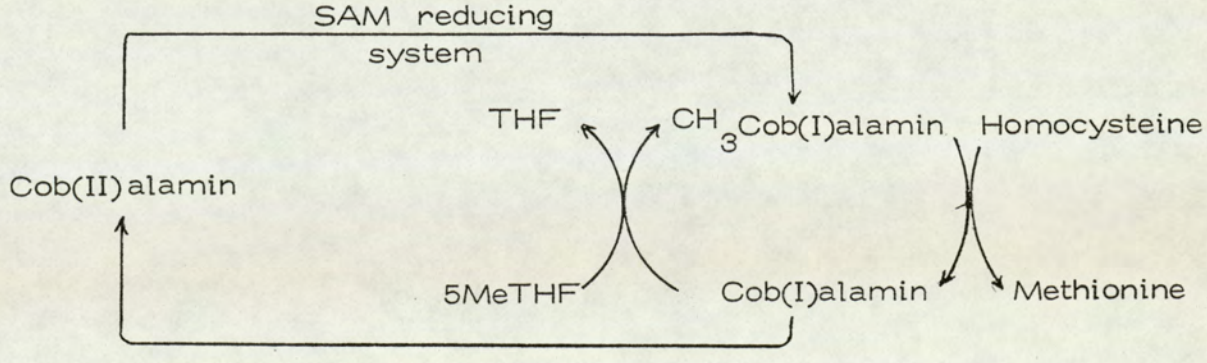


Figure 1.26 Methylation of homocysteine to methionine

The importance of this reaction lies not only in its regeneration of tetrahydrofolate from 5-MeTHF but in its generation as well of methionine, an amino acid required both as a protein constituent and as a methyl donor for a large number of methylation reactions. However, the amount required by the human body cannot be met by synthesis alone. Mudd and Pool (1975) calculated that human volunteers on a normal nitrogen diet synthesized approximately only 50% of their methionine requirements de novo.

The metabolic relationship between folate, vitamin B_{12} and methionine has been a subject of several investigations and reviews in recent years (Vidal and Stokstad, 1974; Herbert and Das, 1976; Krebs et al., 1976). 5MeTHF (Figure 1.4) the folate coenzyme required in the conversion of homocysteine to methionine is produced from 5,10-methylene THF by an irreversible reaction and the methylation of homocysteine is the only known way in which 5MeTHF may lose its methyl group to regenerate THF for one-carbon-unit transfer reactions (Katzen and Buchanan, 1965) (Figure 1.27).

The effects of methionine in Vitamin B_{12} deficiency will be discussed later in conjunction with the methyl-trap hypothesis.

1.3. FOLATE OXIDATION AND CATABOLISM

Numerous studies have been performed on the stability of

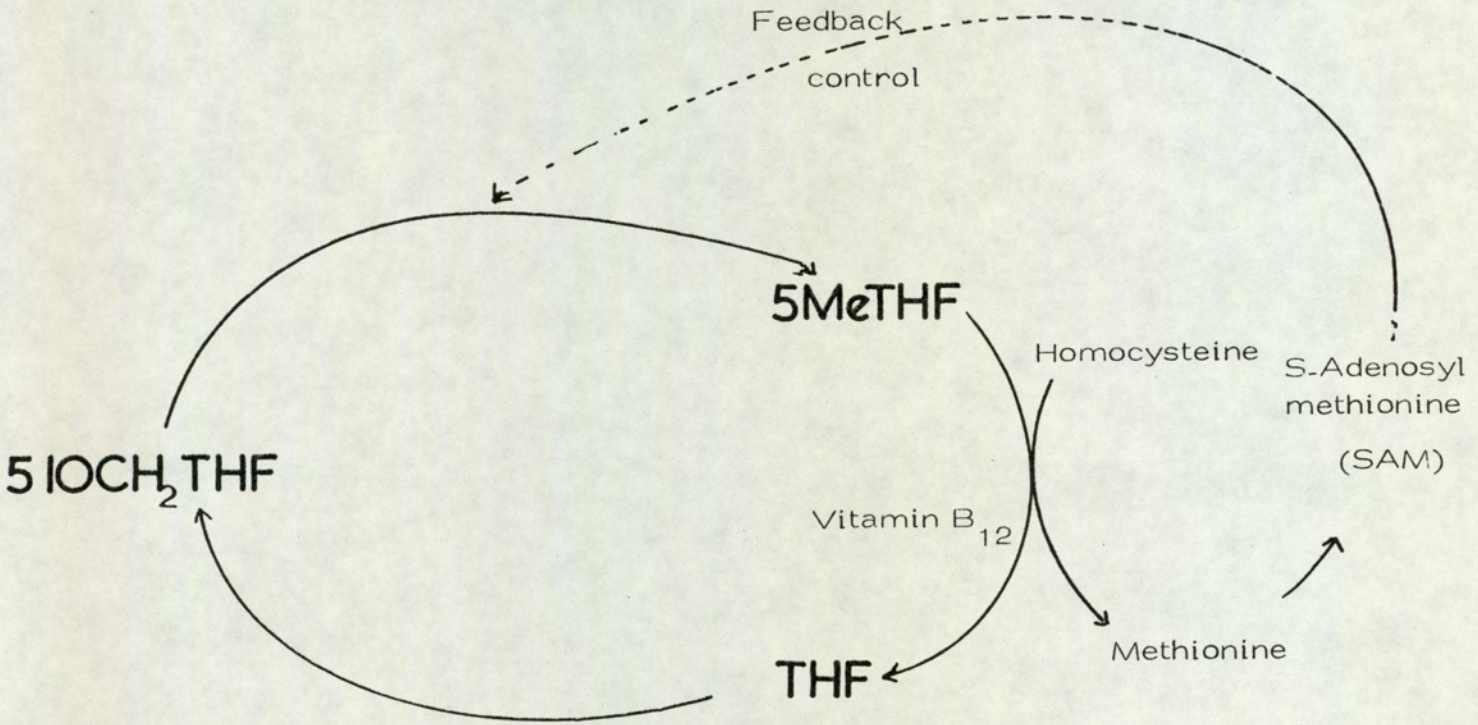


Fig. 1.27 Interrelationship between folic acid, vitamin B_{12} and methionine

naturally-occurring folates in food to identify and quantitate the products of folate oxidation (Blakley, 1969; Chippel and Scrimgeour, 1970; Blair and Pearson, 1974; Maruyama et al., 1978; Lewis and Rowe, 1979). 10-CHOTHF, 5,10-methenyl THF and 5,10-methylene THF are oxidized to 10-formylfolate, which is metabolized in the rat (Connor, 1979) probably by its gut flora (Anne Guest, personal communication), but appears to be excreted largely unchanged in man (Ratanasthien et al., 1974; Saleh, 1981). 5MeTHF is oxidized to 5Me-5,6 DHF and a pyrazino-s-triazene derivative (Figure 1.12) (Jongejan et al., 1979) which is not metabolized either in the rat (Kennelly et al., 1979a) or in man (Ratanasthien et al., 1974). Other folates give scission products as a result of their oxidation and these scission products do not act as coenzymes in folate-dependent reactions. Folic acid is oxidized to p-aminobenzoyl-L-glutamate and pterin-6-carboxylic acid (Figure 1.14) (Lewis and Rowe, 1979) whereas DHF gives folic acid, formaldehyde, p-aminobenzoyl-L-glutamate, dihydroxanthopterin (Figure 1.15) and 7,8-dihydropterin-6-aldehyde (Figure 1.16) (Chippel and Scrimgeour, 1970). The oxidation of THF gives p-aminobenzoyl-L-glutamate and different pterins depending on conditions: at low pH, oxidative cleavage gives rise to pterin (Figure 1.18) or dihydropterin whereas at high pH, xanthopterin (Figure 1.17) is formed (Blair and Pearson, 1974).

Several reports exist of the appearance of some pteridines in urine, possibly as a result of folate degradation in vivo (Blair,

1958, Fukushima and Shiota, 1972). Labelled pterin, xanthopterin (Figure 1.17) and isoxanthopterin (Figure 1.13) have also been isolated from the urine of a human subject dosed with [2-^{14}C]-folic acid (Krumdieck et al., 1978) Pheasant and Blair (1979) reported the presence of an unidentified pteridine as a metabolite of [^{3}H] and [^{14}C]-folic acid. Dinning et al., (1957) reported the presence of free and acetylated diozotizable amines in the urine of rats given large doses of folate and 5-formyl THF (Figure 1.19). Recently, [^{3}H]-p-acetamidobenzoyl-L-glutamate (Figure 1.20) has been identified in rat urine (Murphy et al., 1976; Connor et al., 1979), in guinea-pig urine (Choolun et al., 1980) and in human urine (Saleh et al., 1980), after oral doses of [^{3}H] -folic acid, 10-formyl FA (Connor, 1979) or 5-MeTHF (Kennelly et al., 1979 b; Kennelly, 1980). [^{3}H]p-acetamidobenzoate (Figure 1.21) has also been identified as a folate catabolite in rat urine (Connor et al., 1979), in guinea-pig urine (Choolun et al., 1980) and in human urine (Saleh et al., 1980). The identification of these tritiated catabolites confirm that catabolism occurs in vivo since acetylation must occur prior to excretion. Administration of MTX to rats increase the catabolic rate of folate and the excretion of radioactivity in the urine (Saleh et al., 1981).

Blair (1958) and more recently, Murphy et al., (1978) suggested that a possible route of folate catabolism would be via cleavage of the C_9-N_{10} bond in the tissues to give p-aminobenzoyl-L-

glutamate and a pterin which would undergo further metabolism.

An explanation for the appearance of the two tritiated catabolites, p-acetamidobenzoyl-L-glutamate (pAABglu) and p-acetamidobenzoate (pAAB) was put forward (Pheasant et al., 1981) by stating that catabolite formation is regionalized within the body into two 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, enzymatically or by the gut microflora to p-aminobenzoyl-L-glutamate in the gut and metabolized to p-acetamidobenzoate during reabsorption. Since the folate excreted in the bile is derived mainly from the free (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.

1.4. PATHOLOGY OF FOLATE DEFICIENCY AND THE METHYL TRAP HYPOTHESIS

Folate deficiency produces in man, a clinical disorder known as megaloblastic anaemia, characterized by increased size of erythrocytes and slowed DNA synthesis in all proliferating cells of the body (Herbert and Das, 1976). Three main types of deficiency may affect man. Most frequently, it arises because of inadequate intake of the vitamin, e.g. in a diet deficient in fresh greens, fruits, nuts, liver or in conditions of increased demand as in pregnancy, increased cell proliferation, e.g. malignancy and in malabsorption, e.g. coeliac and tropical sprue (Reviewed by Chanarin, 1979; Halsted, 1979).

A second type of folate deficiency may be produced by treatment with a drug inhibiting folate metabolism, most commonly, an inhibitor of the enzyme dihydrofolate reductase, e.g. methotrexate or pyrimethamine. These drugs deprive the body of the fully reduced (tetrahydro) folates which form the active folate coenzymes needed for a variety of different biochemical reactions (see Section 1.2). Rapidly dividing cells (e.g. in malignancy) require an abundant supply of deoxythymidylate for DNA synthesis. MTX is a phase-specific cancer chemotherapeutic agent stopping cell division in the S phase by DHFR inhibition (Krymuik et al., 1969).

A third type of deficiency is the metabolic defect of folate metabolism caused by lack of Vitamin B_{12}. Here, the 5MeTHF

homocysteine transmethylase activity is depressed. Because of the irreversibility of the reaction 5-MeTHF accumulates and much of the available folate becomes trapped and unavailable. This metabolic trapping of folate as 5-MeTHF, leading secondarily to folate deprivation was put forward as the "methyl folate trap hypothesis" (Herbert and Zalusky, 1962; Noronha and Silverman, 1962).

A large body of clinical and biochemical evidence provides substantial support to this hypothesis: 5MeTHF, the major folate component in human plasma (Herbert et al., 1962) is elevated in the serum of pernicious anaemia patients (Thenen et al., 1973), the levels of cellular folate polyglutamates are low (Perry et al., 1976), the uptake of 5-MeTHF by PHA-stimulated lymphocytes (Das and Hoffbrand, 1970; Lavoie et al., 1974) and bone marrow cells (Tisman and Herbert 1973) of Vitamin-B_{12}-deficient patients is impaired and can be corrected by the addition of B_{12}. These findings are consistent with the view that Vitamin B_{12} deficiency produces functional folate deficiency, which can be attributed to metabolic blockade of the utilization of 5-MeTHF. Rowe (1978), has, however, 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).

A study of several patients who excreted excessive amounts

of both homocystine and methylmalonic acid in their urine (Dillon et al., 1974) has revealed that the activities of at least two enzymes are affected in these patients

(i) a decrease of 5-MeTHF-homocysteine transferase activity leads to abnormalities of the metabolism of sulphur-containing amino acids, including a tendency to accumulate and excrete excessive amounts of homocysteine and an inability to maintain normal concentrations of methionine in plasma and tissues

(ii) a decrease in the activity of methyl malonyl CoA mutase leads to excessive urinary excretion of methyl malonic acid. The two enzymes in question are the only ones in mammals known to require vitamin B_{12} derivatives for catalytic activity. However, a case was described (Dillon et al., 1974) where the lack of two coenzyme forms of vitamin B_{12} caused reduced activity of the two enzymes, resulting in methylmalonic aciduria but failed to show megaloblastosis typical of vitamin B_{12} deficiency.

The administration of methionine to vitamin B_{12}-deficient animals appears to restore folate metabolism to normal or near-normal even in the absence of vitamin B_{12} (Shin et al., 1975, Connor et al., 1978). 5MeTHF accumulates in vitamin B_{12}-deficiency as the methylene tetrahydrofolate reductase reaction is essentially

irreversible under physiological conditions (Kutzbach and Stokstad, 1971) and S-adenosyl methionine (SAM) is a non-competitive inhibitor of the reductase (Kutzbach and Stokstad, 1971). Methionine can therefore, prevent folate from being trapped as 5-MeTHF by feedback control over the synthesis of 5MeTHF (Chiao and Stokstad, 1977) (Figure 1.27), which would generate more tetrahydrofolate and 10-formyl tetrahydrofolate, substrates for folate polyglutamate synthetase (Chiao and Stokstad, 1977), 5-MeTHF being a poor substrate for the enzyme. Experimentally, methionine supplementation in vitamin B_{12}-deficient rats caused an increase in the net hepatic uptake of labelled folate, an increase in the level of folate polyglutamates and decreased the proportion of 5MeTHF. Thus methionine serves as a key factor in the regulation of the 5-MeTHF concentration (Figure 1.27) (Chiao and Stokstad, 1977; Jagerstad et al., 1980).

More recently, Chanarin et al., (1980) have put forward the hypothesis that vitamin B_{12} regulates folate metabolism by the supply of formate. It was found that nitrous oxide (N_2O) inactivates methionine synthetase in vivo by oxidising B_{12} from the active reduced Co(I) form to the inactive Co(III) form and interrupts the formation of the folate coenzyme (folate polyglutamate). Using rats exposed to N_2O as a model for vitamin B_{12}-deficient animals, they made several observations which indicated that B_{12} deficiency has another effect on folate metabolism as well as trapping 5MeTHF (McGing et al., 1978,

Deacon et al., 1978; Scott et al., 1979). Chanarin et al (1980) 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. Formate is normally derived from the oxidation of methyl groups, methionine being an important source. In the N_2O-treated rat, the B_{12} coenzyme involved in methionine synthesis is inactivated and formation of folate coenzyme is interrupted. Chanarin et al (1980) suggested that failure of methionine synthesis leads to a paucity of formate and in turn to inadequate formylation of tetrahydrofolate. In the N_2O-treated rat, there is no formation of folate polyglutamate from tetrahydrofolate. Formyl tetrahydrofolate becomes the substrate for folate polyglutamate synthesis and impairment of formylation compromises general folate metabolism. Their results can, however, still be interpreted according to the methyl trap hypothesis.

1.5. FOLATE ANALYSIS

Folates in plants and animal tissues have been difficult to investigate since the study of this rather large family of related compounds is complicated by their existence in low concentrations in nature ($< 10^{-6}$[M]), the problems of their variable instability to heat, light, pH and oxygen and the fact that they are often present in biological materials containing enzymes, e.g. endogenous conjugase capable of modifying or degrading them during sample preparation. Chemical

assay of the small amounts of folate present in plants and animal tissues are too insensitive to be useful in biological studies (Shin et al., 1975).

Many studies on the folate content of foods failed up until the mid-1960's to provide adequate protection against photodecomposition and oxidation of folate derivatives during their extraction from tissues (Rowe, 1978) and both qualitative and quantitative data were subject to large variations. Limited chromatographic analytical techniques resulted in a reliance on the bioassays which utilized the folate auxotrophs Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae, which vary in their ability to grow on different folate derivatives (Reviewed by Rowe, 1978; Chanarin, 1979).

L. casei demonstrates a good growth response to folic acid and its reduced derivatives and can utilize any of these containing up to three glutamate residues. S. faecalis cannot use any of the polyglutamates or 5-MeTHF. P. cerevisiae responds only to tetrahydromonoglutamate derivatives of folic acid and can be used to measure 5-formyl THF by the selective oxidation of the other less stable derivatives (Rowe, 1978). This variable growth response of different organisms has been the basis of analytical folate studies for many years. The value of the data, though helpful, is uncertain as it is now recognized that during autolysis or conjugase treatment, the polyglutamate chain lengths are altered and enzymatic interconversion

of folate derivatives may occur (Rowe, 1978).

The availability of high specific-activity readioisotopes of folates coupled with various chromatographic techniques has offered an attractive alternative to bioassay in the isolation and characterization of folates and folate derivatives.

Commercially available radioactive folic acid is labelled either in the C-2 position as [2-^{14}C]-folic acid (Figure 1.1) or with ^{3}H, as the [3',5',7,9-^{3}H]-folic acid (Figure 1.1). However, in vivo cleavage of the folate molecule at the C_9-N_{10} position (Murphy et al., 1976; Connor et al., 1979) would result in the formation of [^{3}H]-derivatives of p-aminobenzoyl glutamate and an unlabelled pterin if [3',5',7,9-^{3}H]-folic acid is used or a [^{14}C]labelled pterin plus unlabelled p-aminobenzoyl glutamate derivatives if [2-^{14}C] folic acid is used.

The use of a mixture of the two radioactive species and the subsequent demonstration of dual labelled derivatives provide good evidence for the intact folate molecule. The combination of radioassay and chromatographic techniques has become the most widespread technique used at present to separate a mixture of folates. DEAE-cellulose ion-exchange and Sephadex gel filtration have proved to be very useful in the purification and identification of folate coenzymes (see Table 2.1). Even more sensitive than DEAE-cellulose column chromatography, is High Performance Liquid Chromatography (HPLC)

which has the added advantage of even better separation and yet using a smaller volume of sample (Allen and Newman, 1980). Other methods of folate analysis include thin layer chromatography (Scott, 1980; Brown et al., 1973), paper chromatography and electrophoresis (Connor and Blair, 1980). Radiolabelled metabolites are identified on different chromatographic systems by co-chromatography with authentic folates or folate derivatives as markers, thereby increasing the probability of identification.

There are discrepancies between the reported nature of tissue folates depending on methods of extraction and analysis. Many earlier studies suffered from the drawback of permitting endogenous conjugase to act on the tissue folate polyglutamates from the time the tissue is homogenized until the sample is autoclaved. (Reed et al., 1976). Hence, inactivation of endogenous conjugases is essential in folate analysis in tissues.

Noronha and Silverman (1962) separated chicken liver extracts which had been protected from endogenous conjugase action (by stirring acetone powdered liver for 30 minutes at 70^{o}C in 1 litre of 1% ascorbate solution) on DEAE-cellulose and used microbiological assay to analyse the fractions. They reported 5MeTHF polyglutamates to be the major liver folates but their work highlighted two main difficulties: (i) the need of synthetic standards to calibrate the elution positions of various folate polyglutamates (ii) the problem of resolution.

After having successfully synthesized a range of folate polyglutamate standards by the method of Krumdieck and Baugh (1969), Stokstad's group separated intact rat liver folates by a combination of ion-exchange chromatography with DEAE-cellulose and gel-filtration chromatography with Sephadex. On the basis of these studies, they concluded that rat liver contains exclusively folate pentaglutamate (Shin et al., 1972, 1974).

To minimise hydrolysis of the conjugates by endogenous conjugases animal (or plant) tissues are cut into thin slices and dropped into boiling phosphate buffer containing ascorbic acid (Bird et al., 1965) Boiling for about five minutes inactivate the conjugases. The tissue extracts can then be analyzed. Using this method, Connor and Blair (1980) isolated 10-formyl folate tetraglutamate from rat liver and suggested that it is an oxidation product of 10-formyl THF tetraglutamate. Other metabolites identified have been reviewed in Section 1.3.

1.6. VITAMIN C AND ITS POSSIBLE ROLE IN FOLATE METABOLISM

The importance of ascorbic acid (Vitamin C) has long been known, scurvy being the first recognized deficiency disease in man. A central feature of the classical description of scurvy was general lassitude (Woodall, 1639), pale and bloated complexion, aversion to any

sort of exercise, breathlessness or panting (Lind, 1753). However, it was not until the middle of the 18th Century that the role of citrus fruit in fighting scurvy was scientifically demonstrated (Lind 1752) and that scurvy was due to the lack of an essential food element, now recognized as Vitamin C. The vitamin has now been shown to be involved in biological hydroxylation reactions. It is essential for the formation of hydroxyproline and hydroxylysine in the collagen molecule (Udenfriend, 1966), is necessary for the catabolism of cholesterol to bile acids (Hornig and Weiser, 1976), is involved in the formation and protection from oxidation of the adrenal hormones adrenaline and nor-adrenaline (Park et al., 1956) and in detoxifying histamine in different stress conditions (Chatterjee et al., 1975).

Ascorbic acid is produced by nearly all living organisms, plants and animals. However, in the course of evolution, primates, guinea-pigs (Chatterjee et al., 1961) and bats (Birney et al., 1976) suffered a mutation, which has made them dependent on an exogenous source of the vitamin. As a result, they are susceptible to the deficiency disease, scurvy, if vitamin C is not supplied.

After elucidation of the biosynthetic pathway of ascorbic acid in mammals, it was shown that tissues of animals subject to scurvy (guinea pigs, bats and primates) contain no L-gulonolactone oxidase activity. Since all the other enzyme activities were present, it was concluded that the genetic defect in scurvy-prone animals was due to

a deficiency of the enzyme L-gulonolactone oxidase (EC 1.1.3.8). Chatterjee et al., (1961) speculated that animals developing scurvy might lack a second enzyme, D-glucuronolactone reductase but no evidence for this has been found (Sato and Udenfriend, 1978).

Although no evidence for L-gulonolactone oxidase has been found in man, monkeys or guinea-pigs, some reports have appeared that under certain circumstances, ascorbic acid synthesis does in fact occur. Odumosu and Wilson (1973) have claimed that a small proportion of female guinea-pigs possess the ability to synthesize ascorbic acid. Ginter (1973) reported finding three guinea-pigs out of several thousand, capable of synthesizing sufficient ascorbic acid for their needs. None of these findings has, however, been substantiated (Jones et al., 1973; Barnes et al., 1973; Sato and Udenfriend, 1978).

A number of metabolic lesions are associated with scurvy in the guinea-pig: pronounced cholesterol accumulation and decreased cholesterol catabolism (Hornig and Weiser, 1976), development of gall stones on a high cholesterol diet (Jenkins, 1977), changes in carbohydrate metabolism, fall in insulin content of the pancreas and degranulation of the β-cells (Banerjee, 1945) have been reported. Hughes et al., (1980) have suggested that muscle carnitine is a highly sensitive indicator of tissue ascorbic acid contents.

In recent years, ascorbate in megadoses, has been claimed to have prophylactic and therapeutic effects in many pathological conditions, including the common cold, burns, bone pain and cancer (Pauling 1970; Prasad et al., 1979). The results of studies relating to the efficacy of pharmacological doses of ascorbate in these conditions are, however, conflicting (Basu et al., 1979; Creagan et al., 1979).

Stokes et al., (1975) suggested that ascorbic acid has an important role in preventing the oxidation of tetrahyd rofolate, thus keeping the metabolic folate pool available. As the serum level of ascorbate falls to zero in scorbutics, the serum folate of scorbutics will be more rapidly oxidised than in normal subjects. 10-CHOTHF will be rapidly oxidized to the non-utilizable 10-CHOFA, causing a steady depletion of the folate pool, resulting in anaemia (Blair, 1976).

1.7. RATIONALE FOR THE PRESENT STUDY

Folate derivatives are essential components in normal metabolism and are required for cell growth and propagation. Deficiency in man is characterized by megaloblastic anaemia. Rats fed a folate-deficient diet manifest decreased growth and often develop a scruffy appearance, diarrhea, with rapid hepatic folate decline over a 3-week period (Chanarin et al., 1969). Folates have long been recognised as essential nutrients for the guinea-pig (Woolley and

Sprince, 1945). Subsequent reports characterized the growth failure, poor survival, anaemia and leukopenia with dietary folate deficiency in the guinea-pig (Woodruff et al., 1953; Reid et al., 1956; Slungaard et al., 1956). Thenen (1978) also reported significantly reduced growth in guinea-pigs fed a folate-deficient diet, with about 25% of the animals dying during the course of the experiment.

The metabolism of folates has been extensively studied in the rat (Connor, 1979; Saleh, 1981; Bates, 1981) but few studies have been carried out in the guinea-pig (Hoffbrand and Peters, 1969; Corrocher and Hoffbrand, 1972; Corrocher et al., 1972). Because of its dependence on an external source of vitamin C (like man) and hence, its ability to become scorbutic, the guinea-pig was thought to be a better model for the study of folate metabolism in man, than would be the rat.

The present study is directed towards further elucidation of the fate of folate derivatives in the normal and scorbutic mammal and where possible, by the application of more rigorous chemical and biological analysis to the identification of the different derivatives present in the tissues. Induced changes (if any) in whole body metabolism of folate by pretreatment of the animals with Methotrexate or antibiotics are investigated as are the ability of the guinea-pig to handle oral doses of the reduced folates 5-CH_3THF and 10-CHOTHF.

CHAPTER 2

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

The following materials were obtained commercially as described : folic acid, xanthopterin, pterin 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); 5-MeTHF from Eprova Research Laboratories (Basle, Switzerland). [2-^{14}C]-folic acid (specific activity 55 mCi mM^{-1}), [3',5',7,9-^{3}H]-folic acid (specific activity 500 mCi mM^{-1}) and [5-^{14}C]-5MeTHF (specific activity 58 mCi mM^{-1}) were obtained from the Radiochemical Centre (Amersham, Bucks, U.K). Terramycin was obtained from Pfizer Limited (Sandwich, England).

Other standard pterins were gifts or donations from the following individuals and organisations: methotrexate from Lederle Laboratories Division (Cyanamid of Great Britain Limited, London); L-neopterin, lumazine 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 in this laboratory. Other chemicals and general laboratory reagents used were of Analar grade or equivalent.

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

A solution of [2-^{14}C]-folic acid (50 μCi, 55 Ci mM^{-1}) and [3',5',7,9-^{3}H]-folic acid (250 μCi, 500 μCi mM^{-1}) was made up in 0.8 ml of distilled formic acid (98%). The solution was stored in the dark at room temperature for 48h, after which excess formic acid was removed by freeze-drying. Chromatography of the product on Sephadex G-15 gave a single radioactive peak at fraction 21 (folic acid elutes at fraction 36) and co-chromatographed with cold standard 10-formyl folic acid. The prepared material was stored at -20^{o}C until used.

10-Formyl folic acid (10-CHOFA)

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 filtered, 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 at pH 1, λ_{max} = 259 nm, 370 nm at pH 13 and λ_{max} = 245 nm, 269 nm and 350 nm at pH 7.0.

[3',5'-^{3}H] p-aminobenzoyl-L-glutamate [3,5-^{3}H]pABglu

This was prepared by the method of Maruyama et al., (1978) by oxidation of [3',5',7,9-^{3}H] folic acid with alkaline potassium permanganate. The reaction was stopped with 0.5 ml of 30% H_2O_2. The product was isolated by column chromatography, concentrated by freeze-drying and its purity checked on a Sephadex G-15 column.

p-Acetamidobenzoyl-L-glutamate (pAABglu)

This was prepared by the method of Baker et al., (1964). p-Aminobenzoyl-L-glutamate (1.0 g) was dissolved in 10 ml of aqueous acetic acid (50% v/v) and acetic anhydride was added. The solution was left standing in the dark at room temperature after which it was diluted to 20 ml with distilled water, cooled for several hours at 4^{o}C and the precipitate recovered by centrifugation. After washing with ice-cold water, the product was recrystallized twice from boiling water yielding small needle-like crystals (m.p. 206-208^{o}C).

[2-^{14}C] and [3',5',7,9-^{3}H]-5MeTHF

[2-^{14}C]-folic acid (55 mCi mM^{-1}) and [3',5',7,9-^{3}H]-folic acid (500 mCi mM^{-1}) were each dissolved in 0.05 M phosphate buffer, pH 7.0, containing 2% w/v sodium ascorbate and mixed with

cold folic acid to give a solution containing 10 μCi [^{14}C], 50 μCi [^{3}H] and 80 μg folic acid per ml. Three rats were dosed daily for two days with 0.2 ml of the folic acid. Urine was collected individually in 10 ml of 0.05M phosphate buffer containing 2% w/v sodium ascorbate to prevent oxidation, with the collection flasks shielded from light. The pooled urines were chromatographed on a DEAE-cellulose column. The 5-MeTHF fraction was pooled, desalted by passage through a Sephadex G-15 column and eluted with water. The 5-MeTHF fraction was pooled, concentrated by freeze-drying and its purity checked on a Sephadex G-15 column. It gave a single radioactive peak at fraction 37. The prepared material was stored at -20°C until required for use.

CHROMATOGRAPHY

1. Ion-exchange chromatography

Diethylaminoethyl cellulose (DEAE-cellulose) (DE-52, Whatman Limited, Maidstone, Kent, U.K.) (50-80 g) was washed with distilled water and equilibrated in 0.05M phosphate buffer, pH 7.0, containing dithiothreitol (5 mg % w/v) until the washings were of constant ionic strength and pH 7.0. After decanting the fine particles and degassing, the DE-52 was packed into a 2 cm x 50 cm glass column (occasionally 2 x 80 cm) plugged with glass wool. Samples (10-30 ml) were diluted to the conductivity of the starting buffer with distilled water before loading onto columns. Appropriate

standards were applied while loading. 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.). Fractions (generally 5 ml, sometimes 10 ml) were collected using an LKB Ultrarac fraction collector (LKB Instruments). 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 using a Mullard conductivity cell.

2. Gel Filtration

Sephadex G-15 was obtained from Pharmacia (Uppsala, Sweden). A slurry of the gel was prepared in 0.05 M 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 Ltd, Surrey, U.K.) and allowed to pack under pressure. After loading samples and standards (5-25 ml) elution was carried out with 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-amino-benzoic acid derivatives on DE-52 and Sephadex G-15 is given in Table 2.1.

3. Paper Chromatography

Paper chromatography was performed by the descending method in a glass tank equilibrated with the appropriate solvent using Whatman 3 MM chromatography paper and chromatograms were run overnight in the dark. Samples ([^{3}H]-scission products) and standards (p-aminobenzoyl-L-glutamate, p-aminobenzoate and their acetyl derivatives) were applied as spots using glass micro pipettes. Standards were observed as dark absorbing or fluorescing spots by viewing under UV light at 254 nm or 355 nm. The following chromatography solvents were used.

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 3MM paper.

ANIMALS

Male Dunkin-Hartley guinea-pigs (300 - 400 g wt) were used for experimentation, receiving either orally or by intra-peritoneal

(i.p.) injections, doses of [2-^{14}C]-folic acid or [3',5',7,9-^{3}H]-folic acid or a mixture of both. The animals were kept both prior to and during experimentation in a ventilated room having a free access to food and water. Guinea pigs were made scorbutic by keeping them for 14-21 days on a rat diet (which contains no vitamin C) to which no greens or hay was added. Composition of the rat diet is shown in Table 2.3.

Metabolic studies were carried out by housing individual guinea-pigs in metabolism cages designed for the separate collection of urine and faeces (Jencons Metabowls; Jencons (Scientific) Ltd., Hemel Hempstead, Herts, U.K.). To prevent oxidation of folates urine samples were collected in 10 ml of 0.05 M sodium phosphate buffer, pH 7.0, containing 2% (w/v) sodium ascorbate, and flasks were covered with aluminium foil to prevent light degradation of folates. The urine samples were collected every 24h for 48h (sometimes 72h) and faeces were collected as a single sample at the end of the experiment. At this point, the animals were killed and the livers removed. About half of the livers were chopped immediately after removal and plunged into a solution of boiling 0.05 M sodium phosphate buffer, pH 7.0, containing 2% w/v sodium ascorbate and 5 mg of dithiothreitol/100 ml, and maintained at 100°C for 5 min. The liver extracts were cooled, homogenized and centrifuged to remove precipitated protein and the supernatant stored at -18°C until required (Barford et al., 1977).

MEASUREMENT OF RADIOACTIVITY

The faeces and remaining livers were freeze-dried, then ground to a homogeneous powder. Samples (100 mg) of the powder were oxidized in a Beckman biological-material oxidizer. Tritium was trapped as [$^{3}H_2O$] in a dry ice / methanol cold trap and counted in 10 ml of Fisons tritium absorber. ^{14}C was trapped as $^{14}CO_2$ in 15 ml of Fisons absorber P (Fisons, Loughborough, Leics., U.K.), a scintillation cocktail designed for the collection of $^{14}CO_2$. Samples were counted in a Nuclear Enterprises liquid-scintillation counter type NE 8310 (Nuclear Enterprises Limited, Edinburgh). Samples for radioactivity counting e.g. column eluants and urine samples, were made up to 1 ml with distilled water to which was added 10 ml of a scintillation cocktail. The cocktail was made up of toluene (1 litre) and Fisons emulsifier mix No. 1 (500 ml) in which was dissolved 2,5-diphenyloxazole (PPO) (5 g) and 1,4-bis-2-(-5-phenyloxazolyl)-benzene (POPOP) (0.1 g). Samples were counted for 10 min. or 10,000 counts, whichever came first. Appropriate corrections were made for background, quenching and overlap of ^{14}C into the ^{3}H channel using the external standard ratio.

In the past few months, prepared samples were counted in a Beckman LS 7500 Liquid Scintillation counter (Beckman Instruments Inc., Scientific Instruments Division, Irvine, California,

92713). The instrument is equipped with automatic quench compensation.

TABLE 2.1.

The elution properties of folates, p-aminobenzoic acid derivatives and the unknown metabolites on Sephadex G-15 gel-filtration and DEAE-cellulose ion-exchange chromatography.

Compound	Elution Position		
	Sephadex G-15 (fraction No.	(Kav)	DEAE cellulose [M] NaCl
Folic acid	37	(1.79)	0.92
Pteroylheptaglutamate	11	(0.13)	0.60
10-CHO-FA	21	(0.77)	0.51
10-CHO-THF	18	(0.58)	0.45
5-CHO-THF	28	(1.22)	0.59
5-MeTHF	37	(1.79)	0.65
5,10-CH_2-THF	25		0.64
Methotrexate	60	(3.27)	
Triazene	16		0.40
Pterin	35	(1.67)	0.30
Pterin-6-CHO	43	(2.18)	0.70
Pterin-6-COOH	30	(1.67)	0.60
Xanthopterin	57	(0.77)	0.57
Isoxanthopterin	45	(2.31)	0.49
dihydroxanthopterin	76	(4.29)	
+ 6-COOH Lumazine	27	(1.15)	
+ 6-oxo-lumazine	34	(1.60)	
p-aminobenzoic acid	35	(1.67)	0.40
p-acetamidobenzoic acid	36	(1.73)	0.43
p-aminobenzoyl-L-glutamate	18	(0.58)	0.40

Continued..

Table 2.1. continued.....

p-acetamidobenzoyl-L-glutamate	19	(0.66)	0.43
+ p-aminohippuric acid	33	(1.51)	
+ p-acetamidohippuric acid	27	(1.15)	0.35
$^{3}H_2O$	21	(0.76)	0.0
Urea	21		0.0
^{14}C peak I	21		0.10

+ Elution positions taken from Connor (1979).

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume of solute

V_t = total volume of chromatography bed

V_o = void volume

Table 2.2

The chromatographic behaviour of p-aminobenzoic acid deriatives on Whatman 3 MM paper

	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

Table (2-3)

Factual/Calculated full analysis of laboratory animal diets

		Modified rat and mouse breeding diet cube
Crude Oil	%	3.26
Crude Protein	%	21.23
Crude Fibre	%	3.48
Digestible Crude Oil	%	2.48
Digestible Crude Protein	%	17.60
Digestible Crude Fibre	%	2.10
Digestible Carbohydrates	%	46.80
Gross Energy	Cal /kg	4073.00
Metabolisable Energy	Cal /kg	3666.00
Rabbit TDN	%	
Saturated Fatty Acids	%	0.73
Linoleic Acid	%	0.99
Other unsaturated Acids	%	1.54
Calcium	%	1.30
Phosphorus	%	1.00
Sodium Chloride	%	0.635
Magnesium	%	0.24
Potassium	%	0.80
Sulphur	%	0.23
Iron	mg/kg	171.60
Copper	mg/kg	14.50
Manganese	mg/kg	86.60
Cobalt	ug/kg	104.70
Zinc	mg/kg	39.60
Iodine	µg/kg	600.00

Continued..

Table (2-3) continued....

Arginine	%	1.31
Lysine	%	1.14
Methionine	%	0.36
Cystine	%	0.33
Tryptophan	%	0.23
Glycine	%	1.57
Histidine	%	0.51
Threonine	%	0.71
Isoleucine	%	0.84
Leucine	%	1.49
Phenylalanine	%	0.89
Valine	%	1.07
Tyrosine	%	0.69
Aspartic Acid	%	1.64
Glutamic Acid	%	3.96
Proline	%	1.55
Serine	%	1.00
Vitamin 'A'	i.u./kg	1,1587.00
Carotene	mg/kg	0.79
Vitamin B1 (Thiamine)	mg/kg	9.10
Vitamin B2 (Riboflavine)	mg/kg	9.90
Vitamin B6 (Pyridoxin)	mg/kg	10.10
Vitamin B12	µg/kg	17.00
Vitamin 'E'	mg/kg	77.00
Vitamin 'K'	mg/kg	3.00
Folic Acid	mg/kg	0.70
Nicotinic Acid	mg/kg	78.00
Pantothenic Acid	mg/kg	27.80
Choline Chloride	mg/kg	2.22

Table (2-3) continued.....

Biotin	mg/kg	0.12
Vitamin D3	i.u/kg	859.00

CHAPTER 3

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

This chapter describes the study of the metabolism of folic acid in the normal and scorbutic guinea pig. Normal male guinea pigs were dosed with either [2-^{14}C]- or [3',5',7,9-^{3}H]- folic acid or a mixture of both radioactive species at various dose levels (Table 3.1.1) and scorbutic guinea pigs received doses of the mixture at three dose levels (Table 3.2.1.).

The metabolism is compared of orally-dosed guinea pigs to intra-peritoneally-dosed ones and the effects on folic acid metabolism are investigated of methotrexate (MTX) and antibiotics.

MATERIALS AND METHODS

Guinea-pigs: Male Dunkin-Hartley guinea pigs, 300-400g wt. received doses of either [2-^{14}C] or [3',5',7,9-^{3}H]-folic acid or a mixture of both species, administered either orally or intra-peritoneally. Where scorbutic guinea pigs were used, the animals were made scorbutic by being kept strictly on a vitamin C-deficient diet, whose composition is given in Table 2.3. After about 18-20 days on the diet, the guinea pigs started manifesting signs and symptoms of scurvy, such as loss of appetite, loss of hair, paralysis of the hind limbs and general lassitude.

After dosing, the guinea pigs were kept individually in wired-bottom metabolic cages (Jencons Metabowls, see Chapter 2) designed for the separate collection of urine and faeces. Food and water were

provided ad libit um. The collection of urine and faeces, the preparation of hot ascorbate liver extracts, freeze-drying and burning of the liver and faecal samples were carried out as described in Chapter 2.

Administration of methotrexate (MTX) : Methotrexate (80 mg/kg body wt.) was orally administered to individual guinea-pigs either at the time of dosing or 8 hours before or 8 hours after dosing orally or intra-peritoneally with labelled folic acid.

Administration of antibiotics : Terramycin, commercially available as a soluble powder (Pfizer Limited, Sandwich, England) was dissolved (200 mg/litre) in drinking water and supplied to the guinea pigs as the only source of drinking water. The animals were kept on this regime for 10 days prior to experimentation and during the whole experiment.

CO_2 collection : Carbon dioxide was collected by pumping the expired air from the sealed Metabowl in which the guinea pigs were kept into a trap containing a ^{14}C absorber, Fisons absorber P (see Chapter 2). The absorber was changed every 2 hours for the first 12 hours, then collected as one sample in the same liquid for the next 12 hours. The radioactivity therein(as $^{14}CO_2$) was determined in a liquid scintillation counter.

Gut flushings : The entire small intestine from the

duodenum to the beginning of the colon was removed and the contents flushed with distilled water. After centrifugation, the clear supernatant was sequentially chromatographed on DE-52 and Sephadex G-15. Gut flushings were carried out at 1, 2, 4, 6, 8 and 12 h after oral dosing (400 µg/ kg body wt.) with folic acid.

Bile duct cannulation : Following oral dosing (400 µg/ kg body wt.) with a mixture of [^{3}H] and [^{14}C]-labelled folic acid, guinea pigs were anaesthesized with 10 ml of 25% ^{w}/v urethane (Sigma Chemical Co., London, UK) in physiological saline. Bile cannulations were carried out according to the method of James et al., (1968) and bile was collected in graduated 10 ml tubes onto sodium ascorbate (10 mg) for one-hour periods (a) for 5 hours immediately after dosing (b) for 5 hours, 2 h after dosing. Radioactivity was determined in the bile and in the liver, stomach wall and gut of the guinea pig.

Chromatography : Gut flushings, bile and pooled urine samples were sequentially chromatographed on DEAE-cellulose (Whatman DE-52) and by Sephadex gel-filtration. Folic acid, 5-methyltetrahydrofolic and 10-formyl folate were identified by co-chromatography (on both systems) with authentic folate markers and their elution positions determined by uv-spectroscopy, p-aminobenzoate (pAB) p-acetamidobenzoate (pAAB) and p-acetamidobenzoyl-L-glutamate (pAABglu) were identified by co-chromatography with authentic markers on Sephadex G-15 and on paper in three solvent systems, A, B and C

(see Chapter 2), $^{3}H_2O$ was characterized by its volatility and its chromatographic behaviour on a DE-52 column. Urea was identified by incubating the ^{14}C fraction with urease at the appropriate conditions and collecting any $^{14}CO_2$ evolved into a trap containing a [^{14}C] absorber (see Chapter 2) by acidifcation of the incubation mixture and displacement of any gas formed with nitrogen gas.

Statistical analysis : Wherever possible, figures given are the mean for the number of animals used, with the standard deviation shown in brackets. Tests of statistical significance using student t test or Wilcoxon's sum of ranks test were not performed because of the small number of animals used and because of the wide range in standard deviation.

RESULTS AND DISCUSSION

3.1. Folic acid metabolism in normal guinea pigs

Table 3.1.1. summarizes the distribution of radioactivity in the urine, liver and faeces of normal guinea pigs, following oral doses of either [3',5',7,9-^{3}H]- or [2-^{14}C]- folic acid or a mixture of both radioactive species.

The bulk of the excreted radioactivity appears in the 0 - 24 h urine, with only small amounts appearing on subsequent days. Following an oral dose of a mixture of [^{3}H] and [^{14}C] labelled folic acid, considerably more [^{3}H] than [^{14}C] is excreted in the

TABLE 3.1.1. Recovery of radioactivity in urine, liver and faeces from normal guinea pigs following the oral administration of doses of either [2-^{14}C] or [3',5',7,9-^{3}H] folic acid or a mixture of both radioactive species. Results are expressed as mean % of the administered dose ± standard deviation shown in brackets.

no. of animals	Dose (oral) µg/kg body wt	% recovery of radioactivity				
		URINE		LIVER	FAECES	TOTAL
		0-24h	24-48h	(48h)	(48h)	(48h)
6	160 [^{3}H]	30.0 (0.5)	1.1 (1.6)	1.7 (1.3)	2.2 (1.2)	35.0
4	400 [^{3}H]	30.0 (5.8)	4.1 (1.6)	1.3 (0.5)	7.7 (5.4)	43.1
6	400 [^{3}H]	23.4 (3.7)	2.0 (0.7)	1.2 (0.5)	16.8 (6.0)	43.4
	[^{14}C]	3.0 (0.9)	0.4 (0.2)	1.6 (0.5)	35.3 (9.5)	40.3
3	1050 [^{3}H]	24.1 (5.8)	N Dtm	N Dtm	NDtm	-
	[^{14}C]	3.5 (1.5)	"	"	"	-
5	1370 [^{14}C]	4.4 (0.5)	0.5 (0.1)	0.1 (0.3)	4.8 (1.1)	9.8
3	2330 [^{3}H]	16.9 (8.6)	N Dtm	N Dtm	N Dtm	-
	[^{14}C]	4.3 (1.8)	"	"	"	-

N Dtm = Not Determined

TABLE 3.1.2. Metabolites present in the pooled urine of normal guinea pigs following the oral administration of [^{14}C] and [^{3}H]- folic acid. Results expressed as % of administered dose

no. of animals	Dose (oral) µg/kg body wt.	Time period	Peak I [^{14}C]	$^{3}H_2O$	[^{3}H]pAABglu	[^{3}H]pAAB	Ratio pAABglu/pAAB	Folic acid ^{3}H	Folic acid ^{14}C
6	160 [^{3}H]	0 - 24h		3.0	0.9	24.5	1 : 27	N Dt	
		24-48h		0.7	0.2	0.2	1 : 1	N Dt	
4	400 [^{3}H]	0 - 24h		2.0	4.0	20.4	1 : 5	N Dt	
		24-48h		1.6	0.8	0.4	1 : 0.5	NDt	
6	400 [^{3}H] + [^{14}C]	0 -24h	0.4	1.9	1.4	17.5	1 : 12	0.003	0.002
		24-48h	0.1	0.7	0.04	0.2	1 : 4	0.0004	0.0004
3	1050[^{3}H] [^{14}C]	0 -24h	0.99	0.6	0.1	13.2	1 : 164	1.1	1.0

N Dt = Not Detected

Continued.....

TABLE 3.1.2. Metabolites present in the pooled urine of normal guinea pigs following the oral administration of [^{14}C] and [^{3}H]-folic acid. Results expressed as % of the administered dose

no. of animals	Dose (oral) µg/kg body wt	Time period	Peak I [^{14}C]	$^{3}H_2O$	[^{3}H] pAABglu	[^{3}H]pAAB	Ratio pAABglu/pAAB	Folic acid ^{3}H	Folic acid ^{14}C
5	1370 [^{14}C]	0- 24h	0.2	—	—	—	—		0.03
		24-48h	0.2						0.04
3	2330 [^{3}H] + [^{14}C]	0-24 h	0.8	0.8	0.3	9.8	1:32	0.3	0.5

N = None T = Trace NDt = Not Detected

urine and generally, total ^{14}C recovery is low. This is further emphasized when [^{14}C] only folic acid is administered. Whatever the dose administered the liver shows little retention of radioactivity.

The urine samples were sequentially chromatographed on DE-52 and Sephadex G-15 columns. The amounts of the various metabolites found in the urine are summarized in Table 3.1.2. DE-52 chromatography of the 0-24h urine sample, following an oral dose of 160 or 400 µg/k g body wt. of [^{3}H]- folic acid show the presence of only two tritiated metabolites (Figure 3.1.1). The first [^{3}H] peak eluting at 0.12 - 0.14 [M] NaCl was characterized as $^{3}H_2O$ and the second [^{3}H] peak eluting at 0.38 - 0.44 [M] NaCl was after rechromatography on Sephadex G-15, resolved into two peaks (Figure 3.1.2) which were identified as pAABglu and pAAB, by the criteria discussed earlier (see Materials and Methods, Chapter 3), both on Sephadex G-15 and on paper. Sequential chromatography of the 24-48h urine samples on DE-52 and Sephadex G-15 showed similar features to the 0-24h sample and the chromatograms are not shown. Chromatography of liver extracts are shown in Figure 3.1.3.

The amounts of radioactivity recovered in the urine, liver and faeces following a dose (400 µg/k g body wt.) of a mixture of [^{3}H]- and [^{14}C]- labelled folic acid are shown in Table 3.1.1. and the level of urinary metabolites in Table 3.1.2.

DE-52 chromatography of the 0-24 h and 24-48h urine

samples are shown in Figures 3.1.4., 3.1.5. Because of the similarity in the elution positions of the excreted metabolites, similar labels (I, II, III) have been assigned to the corresponding peaks in the three chromatograms. The ^{14}C peak (marked peak I), eluting at 0.10-0.12 [M] NaCl contains urea and will be discussed in further details, later in this Section. Peak II has been characterized as $^{3}H_2O$ and peak III, after rechromatography on Sephadex G-15 and paper, as pAABglu and pAAB (Figures 3.1.6., 3.1.7).

Liver extracts: Sephadex G-15 chromatography of the liver extracts is shown in Figure 3.1.8. A major radioactive peak containing both the [^{3}H] and [^{14}C] species eluted close to the void volume between fractions 10-17 in the position of the high molecular weight folate (folate polyglutamate). Small amounts of low molecular weight derivative appeared at fractions 21-26.

With no folate being found excreted intact in the urine of normal guinea pigs, higher oral doses of folic acid were administered to determine whether folate would be excreted intact at all. This was done first at a dose of 1050 µg/kg body wt., then at 2,330 µg/kg body wt. (Table 3.1.1.).

DE-52 chromatography of the 0-24h urine sample of the two doses show similar features and the one for the higher dose is illustrated (Figure 3.1.9). Peak I (^{14}C peak) contains urea. Peak II has been identified as $^{3}H_2O$. Peak III, after rechromatography on

Sephadex G-15 (Figure 3.1.10) and on paper has been identified as pAABglu and pAAB. A metabolite eluting at 0.84-0.88 [M] NaCl (Figure 3.1. 9) contained both radioactive species (peaks IV and V) and has been identified on DE-52 and Sephadex G-15 as folic acid.

The low recovery of ^{14}C radioactivity seen at a dose of 400 µg/ kg body wt. was further highlighted in the urine, liver and faeces following an oral dose (1370 µg/kg body wt) of [^{14}C]-only folic acid (Table 3.1.1). DE-52 chromatography of the 0-24h and 24-48h urine samples (Figures 3.1.11 and 3.1.12) shows only two radioactive peaks. The second peak eluting at 0.84-0.88 [M] NaCl has been identified as folic acid.

The other (main) ^{14}C peak eluting at 0.12-0.16 [M] NaCl also elutes at fraction 23 on a Sephadex G-15 column. It does not co-chromatograph with any of a number of markers tried, notably, pterin, xanthopterin, isoxanthopterin, pterin-6-CHO, pterin-6-COOH, methotrexate, lumazine or 7,8 dihydroxanthopterin. Incubation of the [^{14}C] peak with urease, under appropriate conditions, yields $^{14}CO_2$, representing 42-57% of the radioactivity in the peak and indicating urea to be a component of the peak.

Radioactivity, if any, was determined in certain other tissues in an effort to account for the rather low total recovery of [^{14}C] (Table 3.1.1.). However, little radioactivity was found to be

present in the spleen, heart and kidneys (Table 3.1.3).

TABLE 3.1.3.

Recovery of radioactivity in the heart, kidneys and spleen of normal male guinea pigs, following an oral dose of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid. Results expressed as mean % of dose administered. Dose 400 µg/kg body wt.

No. of animals used	Tissues	Radioactivity recovered ^{3}H	^{14}C
5	Heart	0.02	0.02
6	Kidney	0.03	0.01
6	Spleen	0.02	0.01

The air expired by the guinea pig was collected over a 24 hour period, following oral dosing by drawing the air from the sealed meta-bowl, using a peristaltic pump, into a trap containing a [^{14}C] absorber. Using 4 animals, it was found that between 16-20% of the administered dose was passed out as $^{14}CO_2$.

Gut contents : Table 3.1.4. summarizes the radioactivity recovered from flushed gut contents, 1, 2, 4, 6, 8 and 12 hours

TABLE 3.1.4.

Radioactivity recovered in flushed gut contents following an oral dose (400 μg/kg body wt) of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]- folic acid. Results are expressed as mean % of dose administered.

No. of animals	Time after dosing (hr)	Radioactivity recovered [^{3}H]	[^{14}C]
2	1	0.01	0.02
3	2	1.3	1.3
2	4	0.1	0.1
2	6	0.02	0.03
3	8	0.01	0.01

TABLE 3.1.5

Metabolites identified in gut flushings 2h after an oral dose (400 μg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Results expressed as % of administered dose.

	5-MeTHF	FOLIC ACID	SCISSION PRODUCTS
[^{3}H]	0.09	0.14	0.06
[^{14}C]	0.03	0.10	-

TABLE 3.1.6

Recovery of radioactivity in bile after an oral dose (400 µg/kg body wt) of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid.

Results expressed as % of administered dose

Time period	Dose administered at time of collection		Dose administered 2h before collection	
	^{3}H	^{14}C	^{3}H	^{14}C
0 - 1 h	0.02	0.01	0.16	0.09
1 - 2 h	0.02	0.01	0.22	0.21
2 - 3 h	0.03	0.03	0.22	0.50
3 - 4 h	0.01	0.02	0.34	0.11
4 - 5 h	0.02	0.01	0.23	0.09

following the oral administration (400 μg/kg body wt.) of a mixture of [^{3}H] and [^{14}C]-labelled folic acid. Except for the 2h gut flushings, radioactivity was too low for chromatographic analysis. DE-52 chromatography of the 2h flushed gut contents (Figure 3.1.13) showed the presence of two metabolites, each containing both radioactive species, co-chromatographing respectively with 5-MeTHF and folic acid, and a peak labelled with [^{3}H] only in the position of scission products. The amounts of 5-MeTHF, folic acid and scission products are shown in Table 3.1.5.

Bile duct cannulation : Bile was collected for one-hour periods (a) for 5h immediately after oral dosing, b) for 5h, 2 hours after dosing with a mixture of [^{3}H] and [^{14}C]-labelled folic acid (400 μg/kg body wt.) Very little radioactivity (Table 3.1.6) was found present in the bile samples collected. Chromatographic analysis was carried out on the sample with the highest radioactivity (2-3h) and DE-52 chromatography showed that 10-CHOFA, 5-MeTHF and folic acid were completely absent (Figure 3.1.14).

3.2. METABOLISM OF ORAL DOSES OF A MIXTURE OF [2-^{14}C] [3',5',7,9-^{3}H]-FOLIC ACID IN SCORBUTIC GUINEA PIGS

The radioactivity recovered in the urine, liver and faeces, following oral doses of 400, 930 and 2185 μg/kg body wt. of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to scorbutic guinea pigs is

summarized in Table 3.2.1. As is seen with normal guinea pigs, considerably more ^{3}H than ^{14}C is excreted in the urine. As the dose is increased in scorbutic guinea pigs, there is a decrease in the urinary excretion of radioactivity and decreased retention by the liver. Again, as is seen with normal guinea pigs, the overall recovery of ^{14}C radioactivity is rather low.

DE-52 chromatographic analysis of the urine samples (0-24h, 24-48h,) are shown in Figures 3.2.1., 3.2.2. The chromatograms all show similar features and those shown are taken as representative of the three different doses. The ^{14}C peak eluting at 0.10-0.14 [M] NaCl contains urea (see Section 3.1). Peak II [^{3}H] has been characterized as $^{3}H_2O$. The metabolite eluting at 0.84-0.88 [M] NaCl (peaks IV and V) contain both radioactive species and has been identified as folic acid on DE-52 and on Sephadex G-15. The [^{3}H]-only peak III eluting at 0.34-0.38 [M] NaCl was resolved into two tritiated peaks when rechromatographed on Sephadex G-15 (Figures 3.2.3(a) & (3.2.3 (b). eluting at fractions 18-20 and 35-37. These have been identified on Sephadex G-15 and on paper (see Chapter 2) as pAABglu and pAAB.

At the two higher doses of 930 and 2185 µg/ kg body wt. (but not at 400 µg/ kg body wt.), the [^{3}H] peak eluting at fractions 35-37 on Sephadex G-15 was resolved on paper into two [^{3}H] components, which are identified as p-aminobenzoate (pAB) and p-acetamidobenzoate

TABLE 3.2.1.

Recovery of radioactivity in the urine, liver and faeces of scorbutic guinea pigs following the oral administration of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid.

Results expressed as mean % of administered dose $\pm$ standard deviation shown in brackets.

No. of animals	Dose (oral) µg/kg body wt	% recovery of radioactivity				
		Urine		Liver	Faeces	Total
		0-24h	24-48h	48h	48h	48h
6	400 [^{3}H]	17.5 (8.7)	4.6 (1.5)	0.9 (0.8)	12.7 (5.1)	35.7
	[^{14}C]	4.7 (1.9)	2.0 (1.3)	1.5 (1.6)	23.3 (7.3)	31.5
3	932 [^{3}H]	11.8 (3.5)	2.2 (0.8)	0.6 (0.1)	7.2 (1.7)	21.8
	[^{14}C]	2.0 (0.6)	0.7 (0.1)	0.9 (0)	14.1 (3.6)	17.7
3	2185 [^{3}H]	5.7 (1.4)	3.0 (2.2)	0.3 (0.1)	5.7 (0.9)	14.7
	[^{14}C]	1.2 (0.5)	0.7 (0.5)	0.5 (0.3)	8.0 (0.2)	10.4

TABLE 3.2.2. Metabolites found present in pooled urine samples from scorbutic guinea pigs following the oral administration of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid.

Results expressed as % of administered dose.

No. of animals	Dose µg/kg body wt.	Time period	[^{14}C] Peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	Folic acid ^{14}C
6	400	0-24h	1.7	0.7	0.7	7.7	0.5	0.2
		24-48h	1.0	1.2	0.7	1.0	0.4	0.4
3	932	0-24h	1.3	0.6	0.2	9.0 *	0.2	0.4
		24-48h	0.3	0.4	0.1	1.0	0.0002	0.1
3	2185	0-24h	0.7	0.4	0.1	3.8 *	0.1	0.2
		24-48h	0.2	1.5	0.1	0.5	0.1	0.1

* p-aminobenzoate (pAB) found as an additional urinary metabolite at these dbses

respectively, indicating incomplete acetylation in the scorbutic guinea pig (see Chapter 2 for R_f values). The relative amounts of the different urinary metabolites excreted are shown in Table 3.2.2.

Liver extracts : Sephadex G-15 chromatography of the hot ascorbate extracts of the livers of scorbutic guinea pigs is shown in Figure 3.2.4. which is representative of all three chromatograms at the three dose levels mentioned. The chromatogram is similar to that seen for the normal guinea pig (Figure 3.1.8), with a radioactive peak containing both [^{3}H] and [^{14}C] species eluting close to the void volume between fractions 10-15 in the position of high molecular weight folate (folate polyglutamate) and a small peak containing [^{3}H] only appearing at fractions 21-26.

3.3. FOLIC ACID METABOLISM IN THE NORMAL GUINEA PIG FOLLOWING i.p. ADMINISTRATION

Table 3.3.1. summarizes the recovery of radioactivity in urine, liver and faeces following on intra-peritoneal dose of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid (400 µg/ kg body wt.), to 3 normal male guinea pigs. Compared to the same dose, orally administered, (Table 3.1.1.), there is increased excretion of radioactivity in the urine, increased uptake by the liver, decreased excretion via faeces and overall, greater accountability of the radioactivity input, especially as far as [^{14}C] is concerned.

TABLE 3.3.1. Recovery of radioactivity in urine, liver and faeces following an intra-peritoneal dose (400 μg/kg body wt) of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid. Results are expressed as mean % of the administered dose ± standard deviation shown in brackets.

No. of animals		Urine 0–24 h	Urine 24–48 h	Liver (48 h)	Faeces (48 h)	Total (48 h)
3	[^{3}H]	32.4 (1.4)	3.2 (2.9)	30.9 (1.3)	5.2	71.7
	[^{14}C]	28.2 (1.7)	0.5 (0)	14.8 (0.1)	5.8	49.3

TABLE 3.3.2.

Metabolites present in pooled urine from 3 normal male guinea pigs following an i.p. dose of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]- folic acid (400 µg/kg body wt) Results expressed as % of administered dose.

	Peak I [^{14}C]	$^{3}H_2O$	[^{3}H pAABglu]	[^{3}H pAAB]	pAABglu/pAAB	Folic acid ^{3}H	Folic acid ^{14}C
0 – 24 h	0.9	0.9	4.4	7.4	1 : 1.6	12.1	20.2
24–48 h	0.1	1.7	0.1	0.4	1 : 4	N.Dt	N.Dt

N.Dt = Not Detected

The amounts of the different metabolites excreted in the urine are shown in Table 3.3.2. DE-52 chromatography of the 0-24h and 24-48h urine samples are shown in Figures 3.3.1 and 3.3.2. Peak I [^{14}C] contains urea. (see Section 3.1.). Peak II [^{3}H] has been identified as $^{3}H_2O$ and the metabolite containing both radioactive species (denoted as peaks IV and V), as folic acid by the criteria discussed earlier. Folic acid is excreted here in large amounts (12% of the dose as [^{3}H] and 20% as [^{14}C] but is hardly found in the urine at this dose, following oral administration. Peak III, eluting at 0.34-0.38 [M] NaCl was found to contain pAABglu (minor amount) and pAAB (major metabolite) after rechromatography on Sephadex G-15 and on paper(Fig.3.3.3 However, the pAABglu:pAAB ratio is much higher following an i.p. dose (Table 3.3.2) than following a similar oral dose (Table 3.1.2).

Liver extracts: Much more radioactivity (31% of [^{3}H] dose, 15% of [^{14}C] dose) was incorporated in the liver following an i.p. dose of folic acid (Table 3.3.1) than following an oral dose (Table 3.1.1). Sephadex G-15 chromatography of the hot ascorbate liver extracts (Figure 3.3.4) gave similar patterns to those following oral administration (Section 3.1) in that the major radioactive peak eluted close to the void volume in the position of folate polyglutamate (fractions 11-16) with small amounts of singly-labelled compounds at fractions 21-26.

3.4. THE EFFECTS OF METHOTREXATE ON FOLATE METABOLISM

Table 3.4.1. summarizes the recovery of radioactivity in the urine, liver and faeces from 3 normal guinea pigs, after an i.p. dose (400 μg/kg body wt.) of a mixture of [2-^{14}C], and [3',5',7,9-^{3}H]-folic acid, with MTX (80 mg/kg body wt.) administered orally at the time of dosing. Little difference is shown in the urinary output of radioactivity compared to the case in the absence of MTX (Table 3.3.1) but there is a rather drastic decrease in liver uptake in the presence of MTX and excretion via faeces is lower.

Sequential chromatography of the 0-24 h and 24-48 h urine samples on DE-52 (Figures 3.4.1, 3.4.2) and on Sephadex G-15 (Figures 3.4.3 (a), 3.4.3. (b)) and subsequent confirmation by paper chromatography showed the following metabolites to be present : urea (peak I), $^{3}H_2O$ (peak II), pAABglu and pAAB (peak III), and folic acid (peaks IV and V). The amount of intact folate excreted as folic acid was fairly high (18% of the [^{3}H] dose, 16% of the [^{14}C] dose) (Table 3.4.2), comparable to the case without MTX (Table 3.3.2). There was, however, a change in the level of urinary scission products excreted: considerably more pAAB was produced in the presence of MTX (Table 3.4.2) with a concomitant decrease in the level of pAABglu but no new metabolite was induced by the MTX, nor was there any substantial increase in urinary output of radioactivity.

TABLE 3.4.1.

Recovery of radioactivity in urine, liver and faeces from normal male guinea pigs following an i.p. dose (400 μg/kg body wt) of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid with methotrexate (80 mg/kg body wt) orally administered at the time of dosing. Results are expressed as mean % of the dose administered ± standard deviation shown in brackets.

	URINE		LIVER	FAECES	TOTAL
	0-24h	24-48h	(48h)	(48h)	(48h)
[^{3}H]	36.4 (8.6)	2.2 (0.9)	4.1 (3.2)	0.6 (0.5)	43.3
[^{14}C]	27.1 (21.0)	0.6 (0.2)	5.8 (3.9)	2.9 (0.1)	36.4

TABLE 3.4.2.

Metabolites found in pooled normal guinea pig urine (from 3 animals), following the i.p. administration (400 μg/kg body wt) of [^{3}H] and [^{14}C] labelled folic acid in the presence of MTX (80 mg/kg body wt).

Results expressed as % of administered dose

Time period	Peak I [^{14}C]	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Ratio pAABglu/pAAB
0-24h	1.4	2.4	1.3	10.7	1 : 8
24-48h	0.3	0.8	0.1	1.1	1 :11

Time period	Folic acid [^{3}H]	[^{14}C]
0-24h	18.4	16.1
24-48h	0.1	0.1

TABLE 3.4.3

Recovery of radioactivity after an i.p. dose of [^{3}H] and [^{14}C] labelled folic acid (400 µg/kg body wt.) with MTX (80 mg/kg body wt) administered 8 h after dosing, to 3-guinea pigs.

Results expressed as mean % of administered dose $\pm$ standard deviation shown in brackets

	URINE			LIVER	FAECES	TOTAL
	0 – 8 h before MTX	8 – 24 h with MTX	24 – 48 h with MTX	48 h	48 h	48 h
[^{3}H]	23.2 (13.6)	9.2 (10.5)	2.1 (0.2)	13.5 (1.4)	0.2 (0)	48.2
[^{14}C]	23.9 (13.5)	8.4 (10 .6)	1.3 (0.1)	17.8 (3.0)	0.6 (0.1)	52.0

Liver extracts : Sephadex G-15 chromatography of hot ascorbate liver extracts (Figure 3.4.4) gave similar patterns to that of i.p. administration of labelled folic acid without MTX (Figure 3.3.4), with the major radioactive peak eluting close to the void volume in the position of folate polyglutamate (fractions 11-16). Small amounts of singly labelled compounds were also observed (Fractions 21-25).

An additional experiment carried out with MTX administered 8 h after an i.p. dose of [^{3}H] and [^{14}C]- labelled folic acid, showed little excretion of radioactivity after MTX administration but a high incorporation in the liver 48 h after dosing (Table 3.4.3). This is presumably because the bulk of the radioactivity had already been excreted in the 0-8 h urine before MTX was administered. The high level of liver incorporation of radioactivity was probably due to the fact that MTX had less effect on folate already incorporated in the liver. Chromatographic analysis of the urine samples showed that no new metabolite was induced by the administered MTX (Table 3.4.4).

The effects of methotrexate on the induction of new metabolites in guinea pig urine were studied by orally dosing 4 normal guinea pigs (400 µg/kg body wt.) with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid after pre-treatment with MTX (80 mg/kg body wt., administered 8h before folate dosing). Urinary radioactivity recovered is illustrated in Table 3.4.5. Chromatographic analysis showed no new metabolite present. However, the level of intact

Table 3.4.4

Metabolites found in pooled guinea pig urine following an i.p. dose (400 µg/kg body wt) of ^{3}H and ^{14}C labelled folic acid to animals treated with MTX (80 mg/kg body wt) administered orally 8 h after dosing.

Results expressed as % of administered dose

Time period	Peak I ^{14}C	$^{3}H_2O$	^{3}H pAABglu	^{3}H pAAB	Folic acid ^{3}H	Folic acid ^{14}C
0-8h	0.8	0.7	3.3	5.4	9.1	15.2
8-24h	0.9	0.8	1.2	4.7	1.4	1.3
24-48h	0.2	0.7	0.1	1.2	0.2	0.1

Table 3.4.5

Urinary recovery of radioactivity following an oral dose (400 µg/kg body wt) of labelled folic acid to normal guinea pigs pre-treated with MTX (80 mg/kg body wt) orally administered 8 h before dosing.

Results expressed as mean % of administered dose ± standard deviation shown in brackets.

No. of		Urine		
animals	label	0-24 h	24-48 h	Total (48 h)
4	^{3}H	19.2 (3.8)	4.4 (2.4)	23.6
	^{14}C	4.5 (0.7)	1.0 (0.2)	5.5

TABLE 3.4.6.

Metabolites found in the pooled urine from 4 normal guinea pigs following an oral dose (400 µg/kg body wt) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid after 8h pretreatment with methotrexate (80 mg/kg body wt) also orally administered. Results expressed as % of administered dose.

Urinary radioactivity recovered		Time period	[^{14}C] peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid	
^{3}H	^{14}C						^{3}H	^{14}C
19.2	4.5	0-24h	2.6	0.4	2.1	10.6	1.1	1.4
4.4	1.0	24-48h	0.3	1.9	0.3	1.6	0.16	0.22

folate excreted as folic acid (1.1% of the dose as [^{3}H] and 1.4% as [^{14}C]) is slightly higher than without the use of MTX (Table 3.1.1., 3.4.6).

3.5. THE EFFECTS OF ANTIBIOTICS ON FOLIC ACID METABOLISM

The recovery of radioactivity in the urine, liver and faeces of normal guinea pigs orally administered with [^{3}H] and [^{14}C] labelled folic acid (400 and 800 µg/ kg body wt. respectively) after a 10-day pretreatment period with antibiotics (see Chapter 2) is summarized in Table 3.5.1. As without antibiotics (Section 3.1.), considerably more [^{3}H] than [^{14}C] is excreted in the urine and the liver uptake of radioactivity is very low, both features now characteristic of oral doses. Urinary excretion of radioactivity decreases at the higher dose (800 µg/ kg body wt.) but there is increased excretion via faeces and higher retention by the liver.

Sequential chromatography of the 0-24h and 24-48h urine samples on DE-52 and Sephadex G-15 show the chromatograms to have similar features at both dose levels and those shown (Figures 3.5.1 and 3.5.2) are taken as representative of either dose. The metabolites identified were urea (peak I), $^{3}H_2O$ (peak II), intact folate (peaks IV and V) and scission products (peak III). The intact folate was identified as folic acid on DE-52 and Sephadex G-15. The scission products (peak III) were identified as pAABglu and pAAB by

TABLE 3.5.1.

Recovery of radioactivity in urine, liver and faeces following the oral administration of a dose (400 or 800 µg/kg body wt) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to normal guinea pigs pre-treated with antibiotics. Results expressed as mean % of administered dose ± standard deviation shown in brackets.

No. of animals	Dose (µg/kg body wt)	Label	Urine 0 -24 h	Urine 24-48 h	Liver (48h)	Faeces (48 h)	Total (48h)
5	400	[^{3}H]	23.9 (1.9)	2.3 (0.9)	0.2 (0)	0.4 (0.3)	26.8
		[^{14}C]	3.1 (0.3)	0.4 (0.2)	0.6 (0)	0.9 (1.0)	5.0
5	800	[^{3}H]	16.4 (4.2)	3.3 (2.8)	1.4 (0.3)	5.4 (2.5)	26.5
		[^{14}C]	3.3 (0.4)	1.3 (0.8)	3.0 (0.4)	8.0 (3.9)	15.6

TABLE 3.5.2.

Metabolites found in pooled guinea-pig urine following the oral administration (400 or 800 µg/kg body wt) of labelled folic acid to normal guinea pigs pre-treated with antibiotics. Results expressed as % of administered dose.

No. of animals	Dose µg/kg body wt.	Time	[^{14}C] Peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	Folic acid ^{14}C
5	400	0-24h	1.9	3.4	T	19.9	0.2	0.3
		24-48h	0.1	2.3	0.04	0.76	0.06	0.1
5	800	0-24h	2.2	0.2	0.14	12.85	0.2	0.3
		24-48h	0.5	1.3	0.04	1.65	0.04	0.08

T = Trace

rechromatography of peak III on Sephadex G-15 (Figure 3.5.3) and on paper. pAABglu is practically non-existent in the 0-24 h urine at the lower dose (400 µg/kg body wt) appearing only as a trace but increasing to a higher level over the following 24 hours (Table 3.5.2). Still, no reduced folate was found as a metabolite in the urine; pAAB was still present as the major urinary metabolite.

Liver extracts : Sephadex G-15 chromatography of the hot ascorbate extracts of the livers (Figure 3.5.4) is similar to those previously described, with a radioactive peak containing both [^{3}H] and [^{14}C] radioactive species eluting near the void volume (fractions 11-15) in the position of folate polyglutamate and a peak containing [^{3}H] only af fractions 23-27.

Gut flushings : DE-52 chromatography (Figure 3.5.5) of gut flushings 2h after an oral dose (400 µg/kg body wt) of labelled folic acid showed the presence of intact folic acid and 5MeTHF.

SUMMARY

1. Following the oral administration of [^{3}H] and [^{14}C] labelled folic acid, radioactivity was recovered in the urine, liver, faeces and expired air but extremely little, if any, in the heart, spleen, kidneys, gut wall or stomach wall.

2. The following metabolites were identified in the urine:

pAABglu, pAAB, $^{3}H_2O$, urea from the ^{14}C peak I, and folic acid. All urine samples were dominated by scission products, notably pAAB.

3. No reduced folate was found present in any of the urine samples. However, 5 MeTHF was identified in the DE-52 chromatography of gut flushings 2h after an oral dose of labelled folic acid.

4. Folic acid metabolism in the scorbutic guinea pig was very slightly different to that in the normal, in that at very high doses, incomplete acetylation occurs, producing p-amino-benzoate (pAB) as an additional urinary metabolite and rather more intact folic acid.

5. Intra-peritoneal administration of folic acid caused several changes over folic acid orally administered, notably, increased excretion of radioactivity in the urine, increased excretion of folic acid and increased uptake by the liver.

6. MTX depressed the level of urinary pAABglu with a concomitant increase in pAAB.

7. Antibiotics also depressed urinary pAABglu level making pAAB the most predominant metabolite.

8. In all cases, hepatic folates were mainly folate polyglutamates.

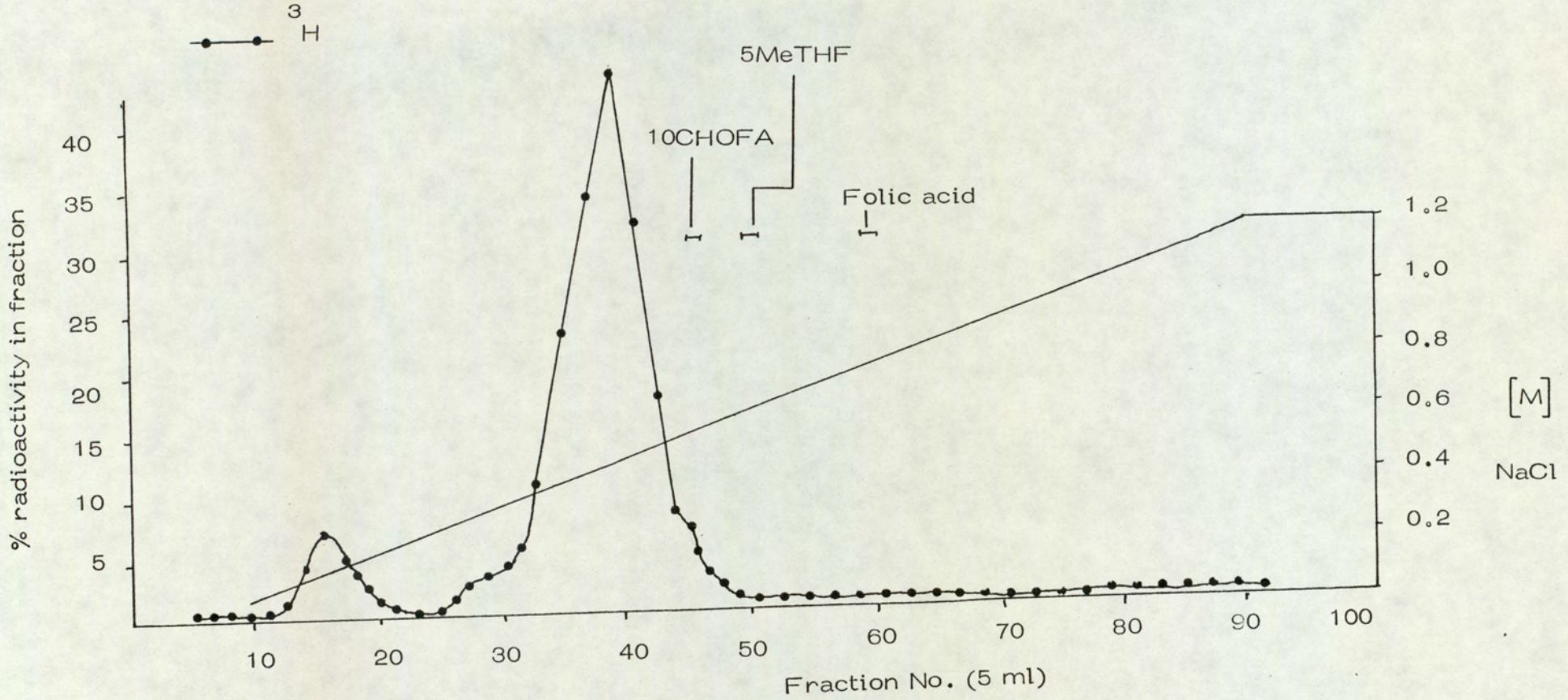


Fig. 3.1.1.

Typical DE-52 chromatogram of 0-24h or 24-48h normal guinea-pig urine after an oral dose of $[^{3}H]$ only folic acid at 160 or 400 µg/kg body wt.

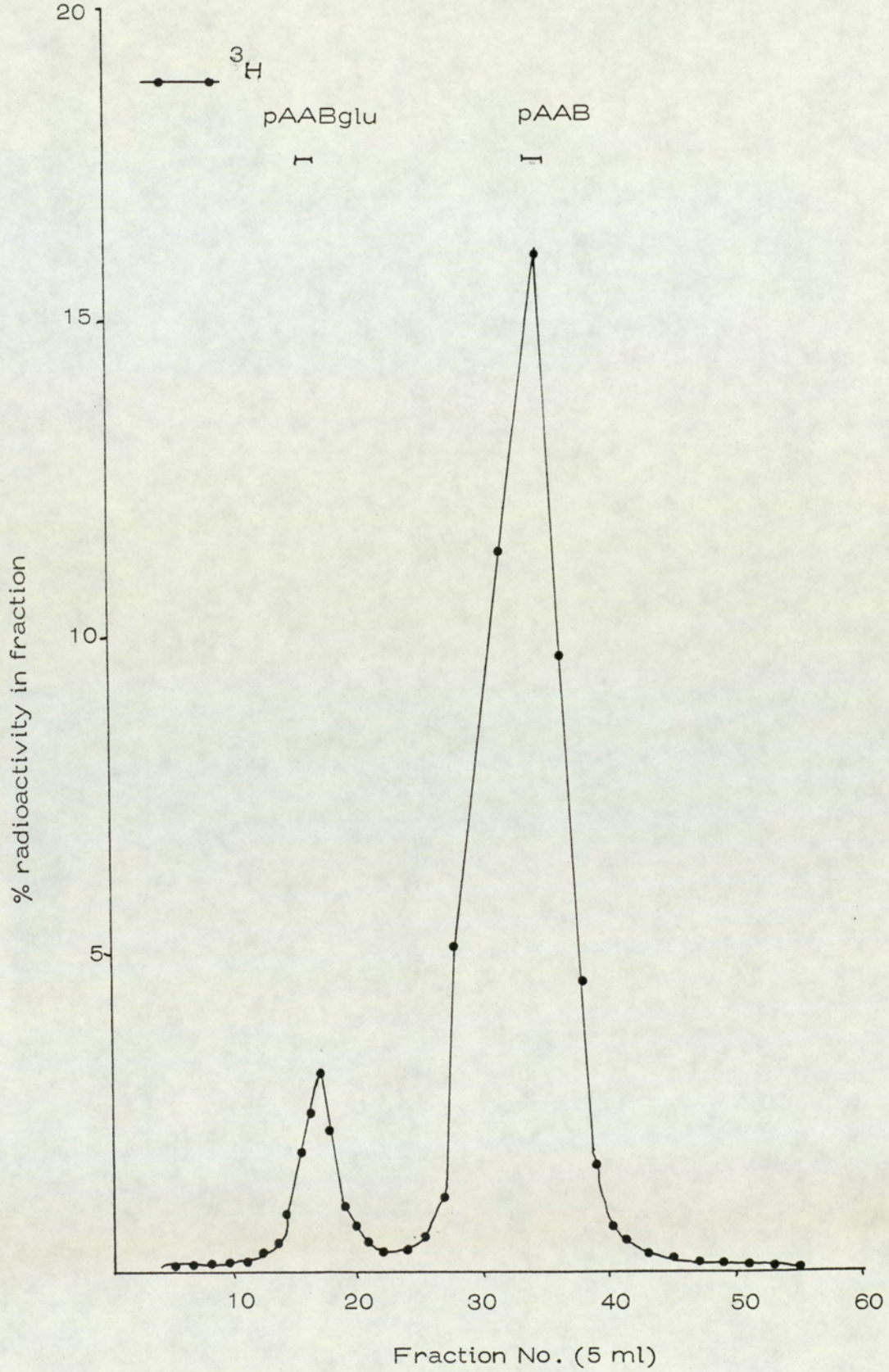


Fig. 3.1.2. Typical Sephadex G.15 chromatogram of ^3H peak II from DE.52 chromatography of normal guinea pig urine after oral administration of $\left[^3H\right]$ only folic acid.

Dose 160 or 400 µg/kg body wt.

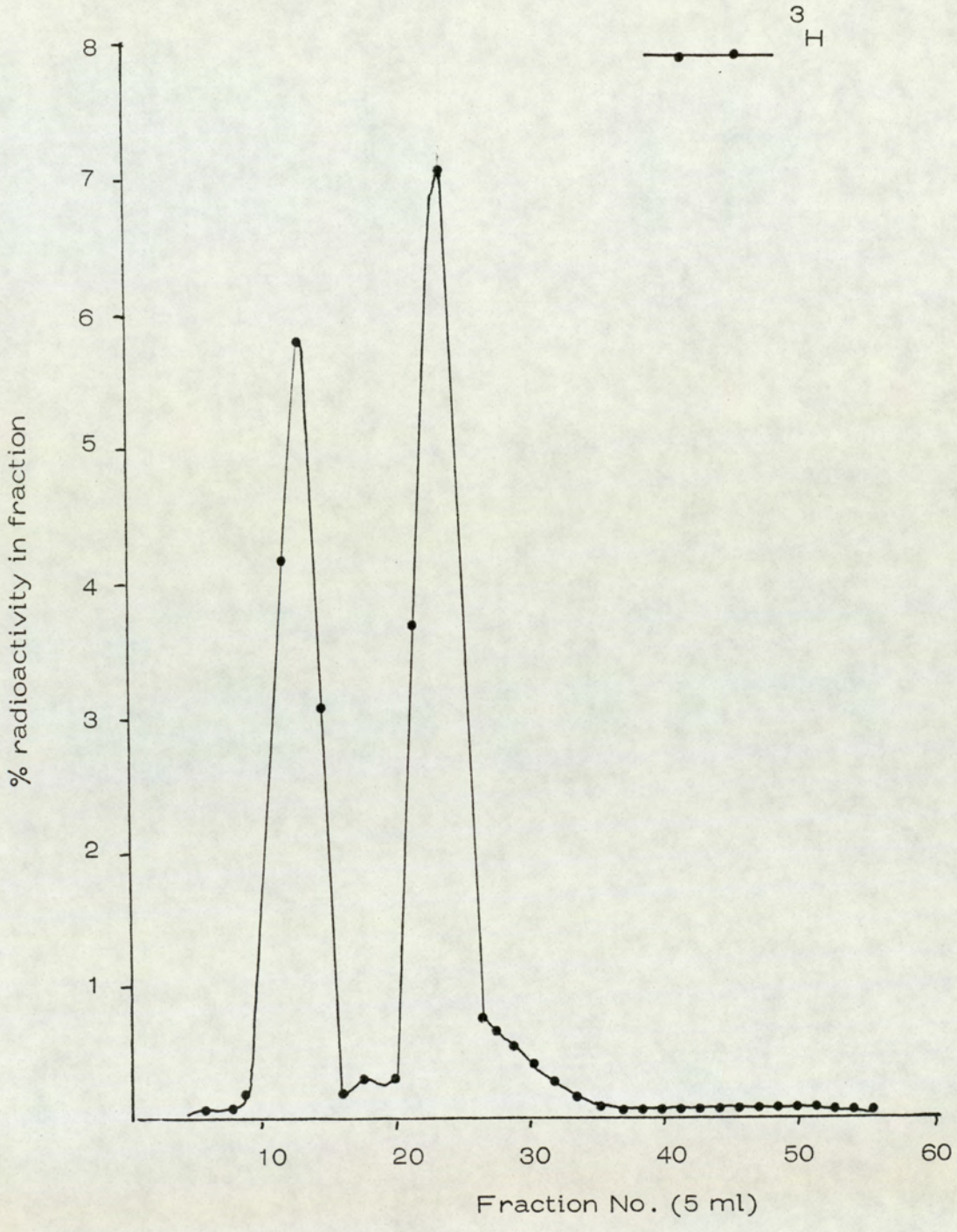


Fig. 3.1.3. Sephadex G.15 chromatography of hot ascorbate liver extract 48h after an oral dose of [3',5',7,9-^{3}H] - folic acid

Dose 160 or 400 µg/kg body wt.

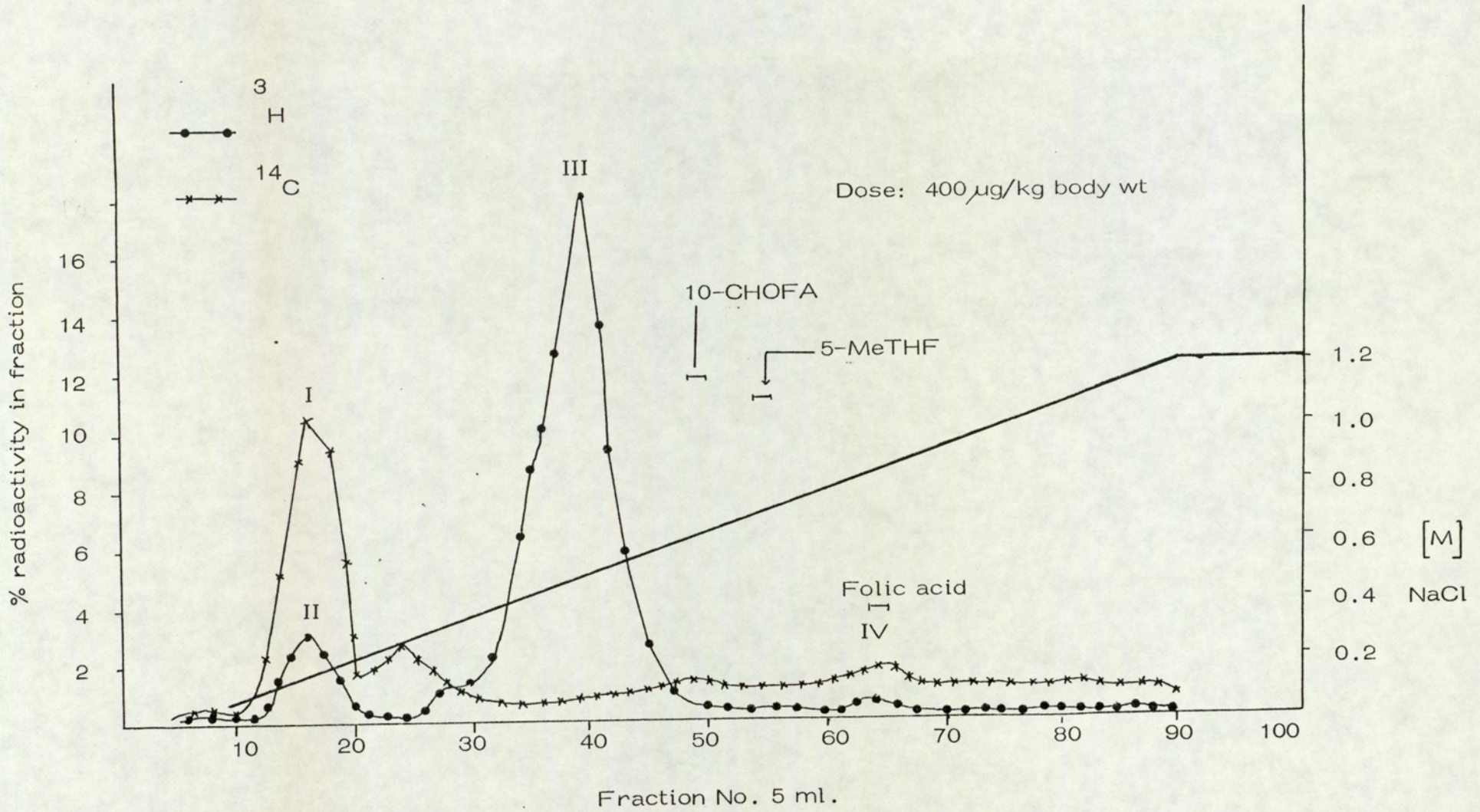


Fig. 3.1.4. DE.52 chromatography of normal guinea-pig urine collected 0-24h after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid

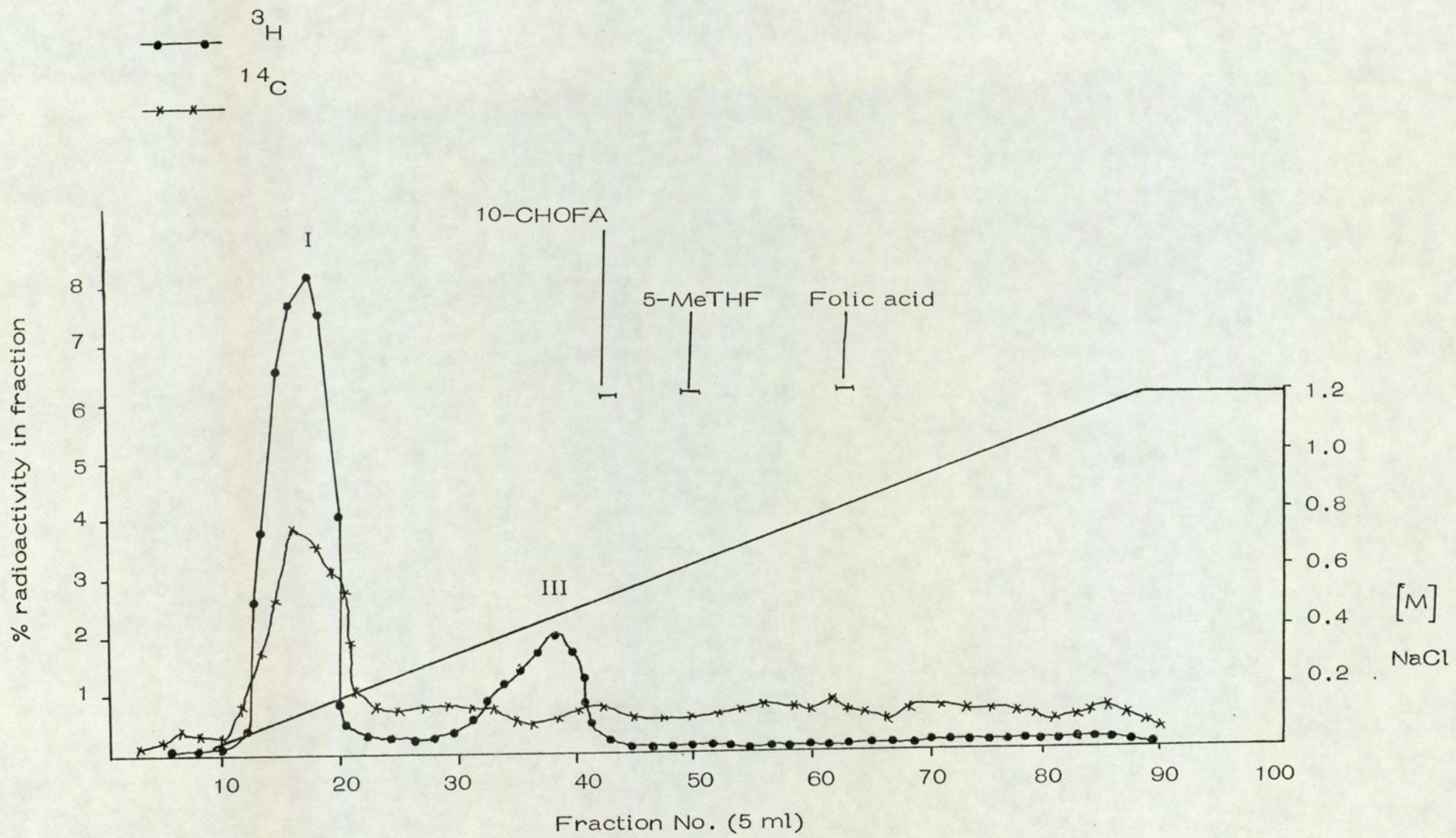


Fig. 3.1.5. DE.52 chromatography of normal guinea-pig urine 24–48h after the oral administration of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid Dose 400 µg/kg body wt.

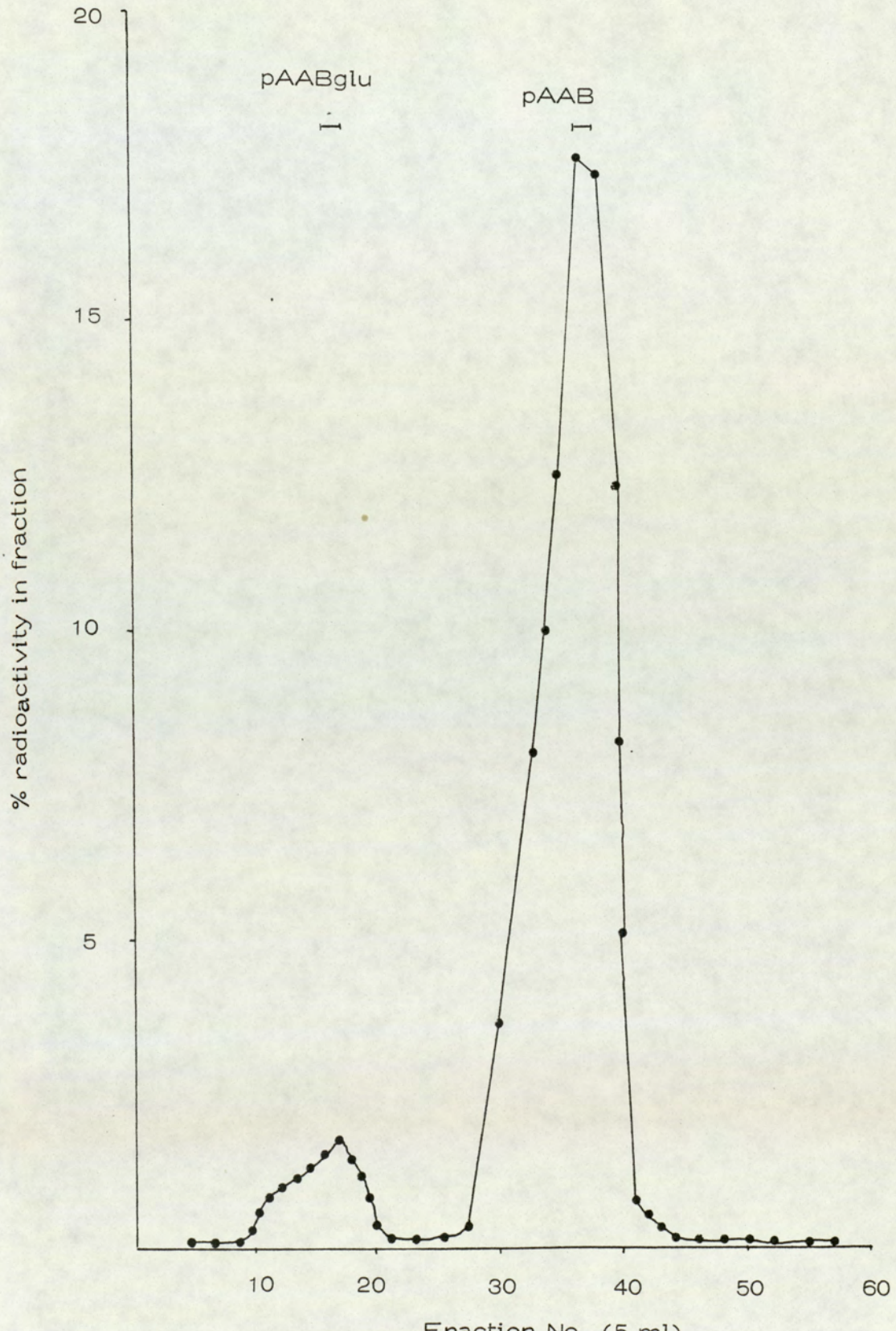


Fig. 3.1.8. Sephadex G.15 chromatography of peak III of DE.52 chromatography of normal urine samples collected 0-24h after the administration of labelled folic acid. Dose 400 µg/kg body wt.

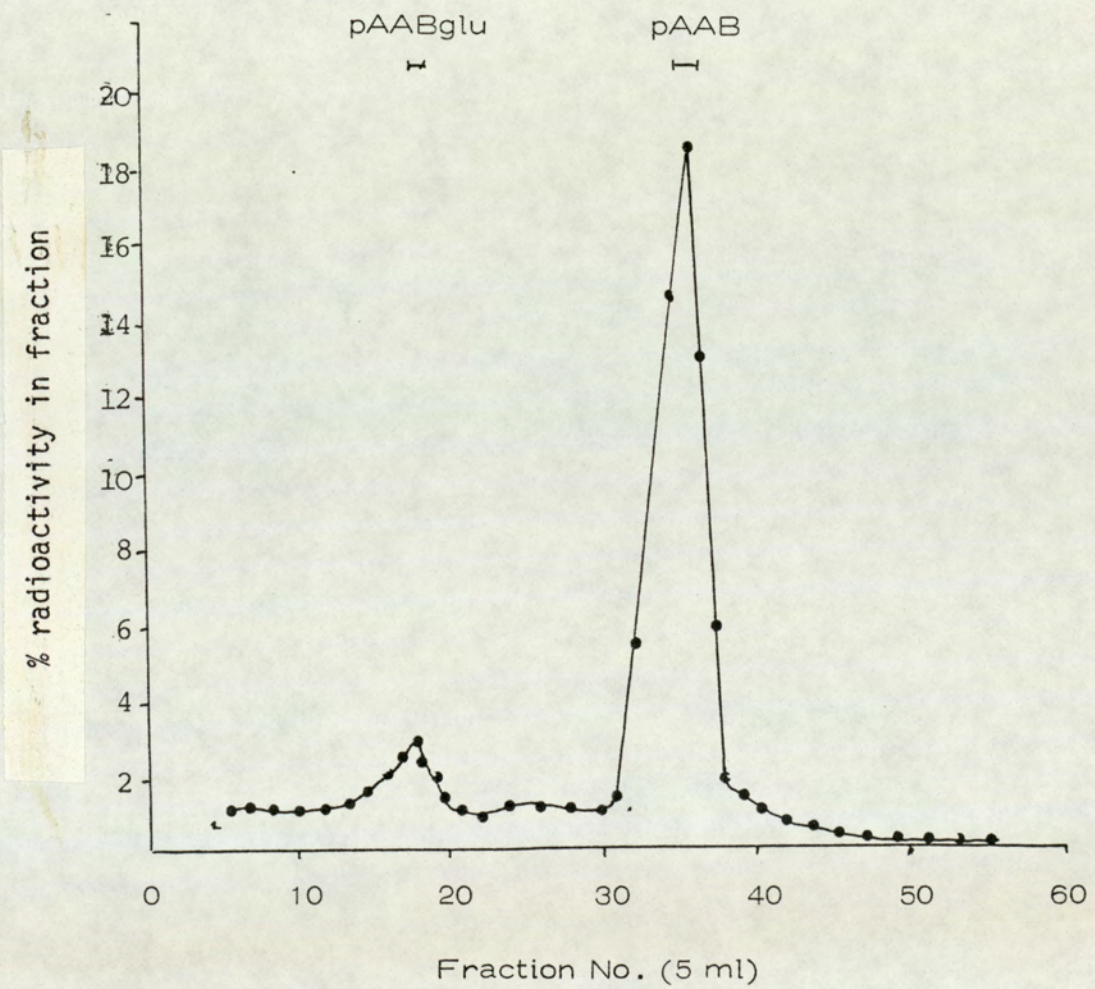


Figure 3.1.7. Seph adex G-15 chromatography of peak III from DE-52 chromatography of 24-48h normal guinea pig urine following an oral dose (400 µg/kg body wt) of labelled folic acid.

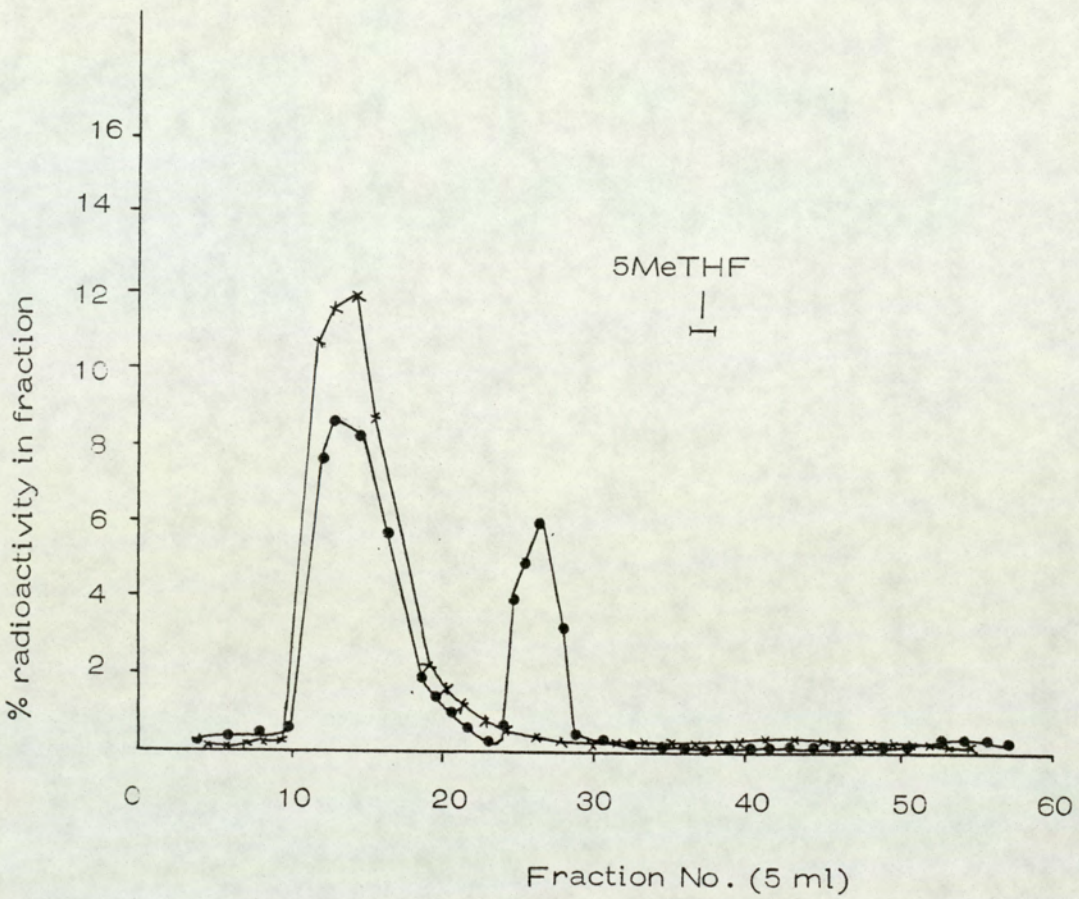


Fig. 3.1.8. Sephadex G.15 chromatography of a hot extract of normal guinea-pig liver 48 h after the administration of an oral dose of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid Dose 400 µg/kg body wt.

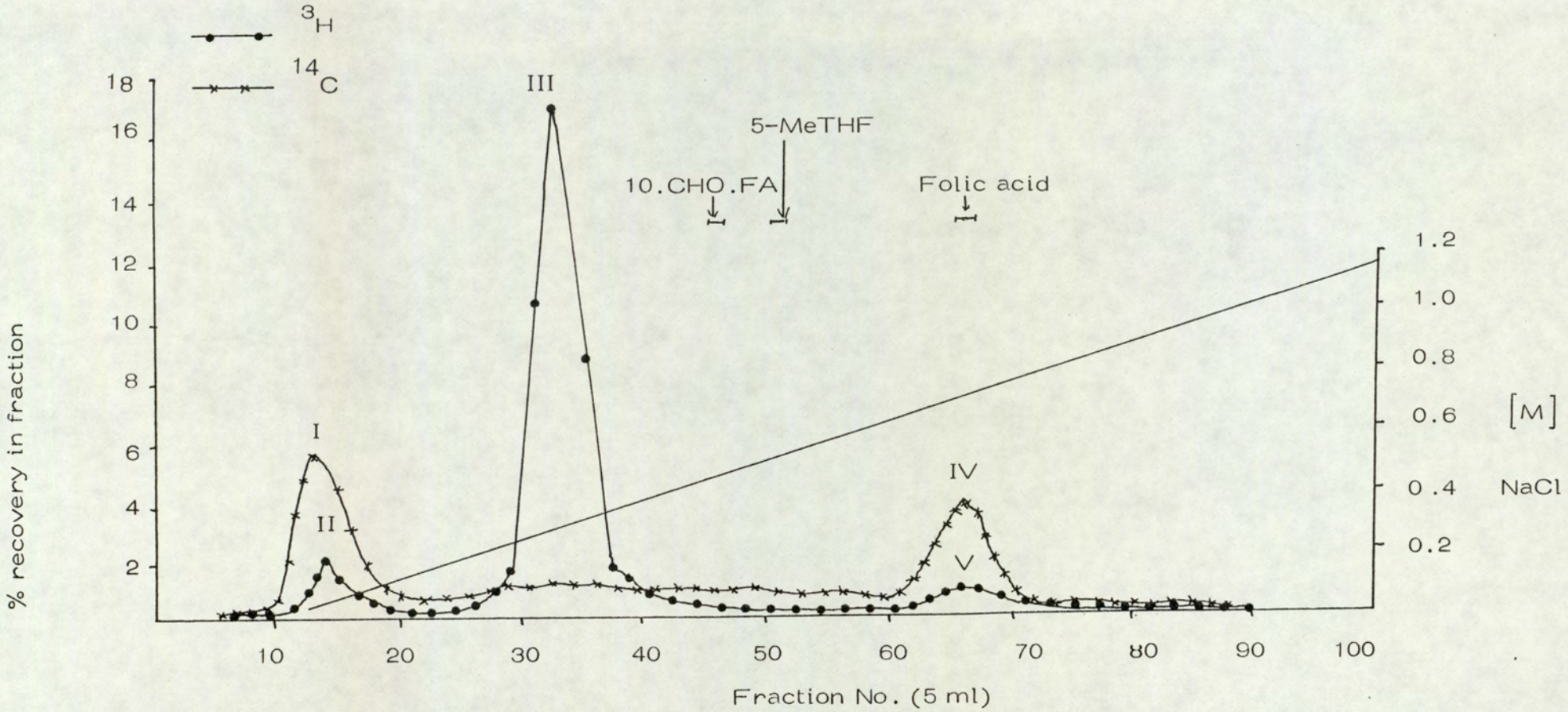


Fig. 3.1.9 DE.52 chromatography of 0-24h normal guinea pig urine, following an oral dose of $\left[^{3}H\right]$ and $\left[^{14}C\right]$- folic acid.
Dose: 2,330 µg/kg body wt.

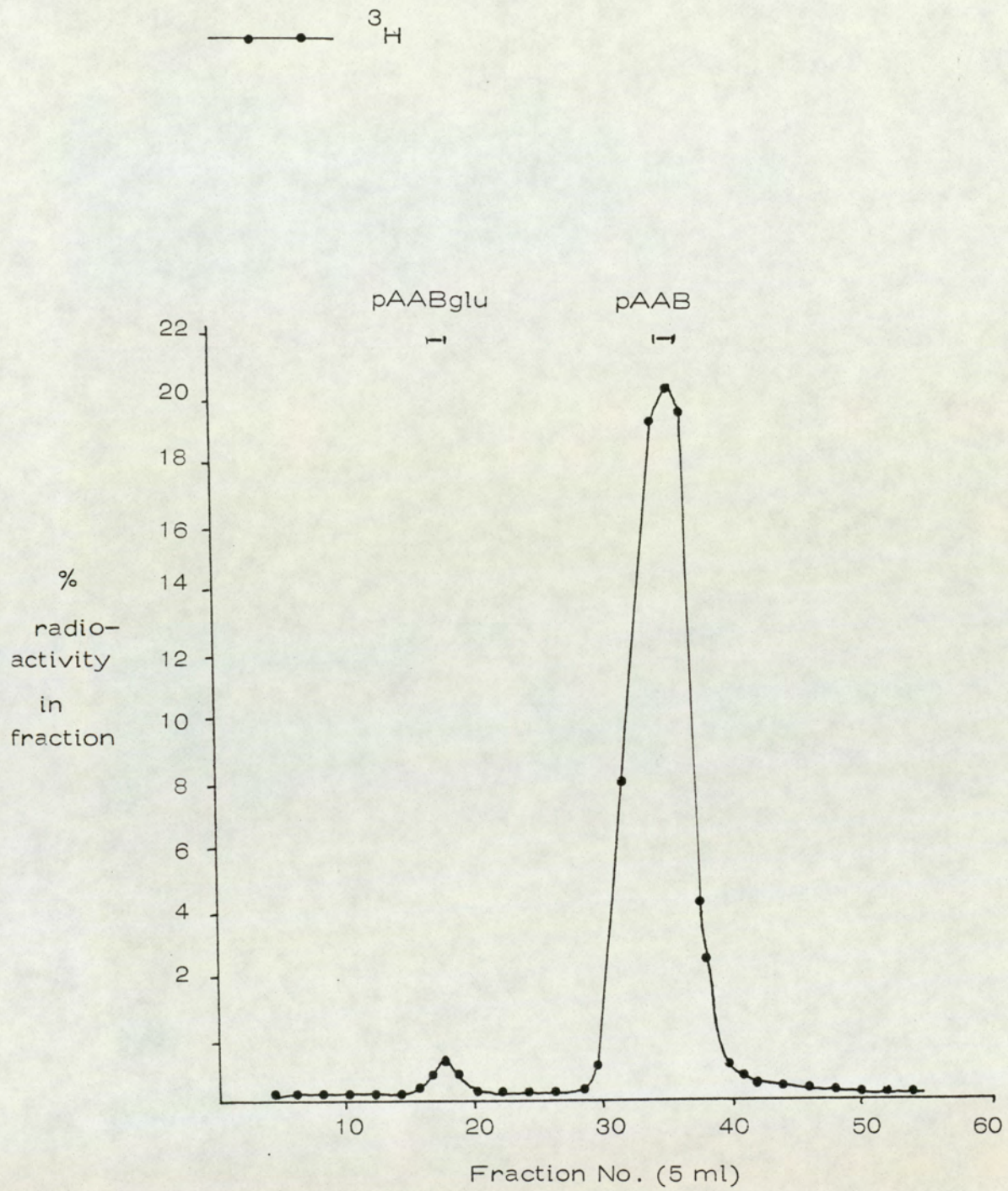


Fig. 3.1.10. Sephadex G.15 chromatography of peak III from DE.52 of 0-24 h normal guinea-pig urine following an oral dose of labelled folic acid

Dose: 2,330 µg/kg body wt.

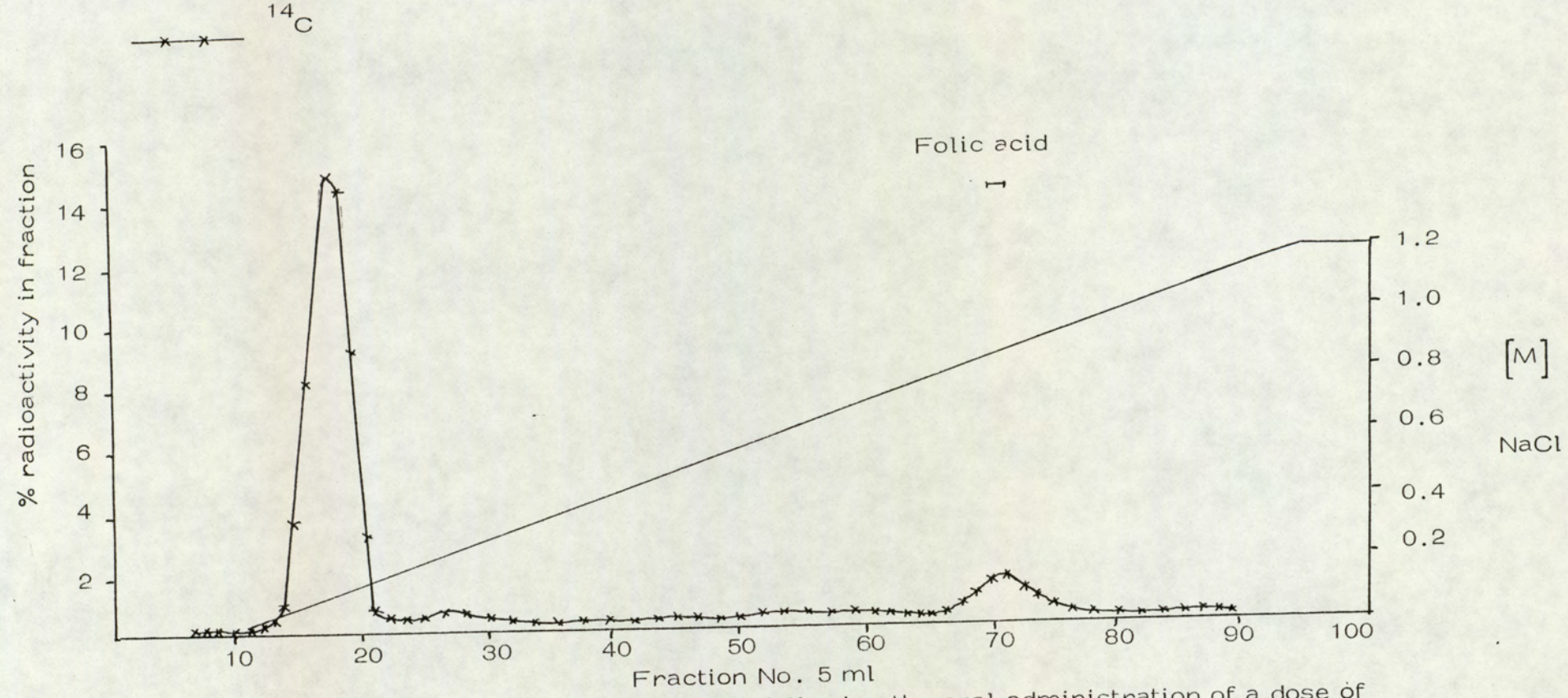


Fig. 3.1.11 DE.52 of 0–24h normal guinea-pig urine following the oral administration of a dose of $[2\text{-}^{14}C]$ only folic acid. Dose: 1370 µg/kg body wt.

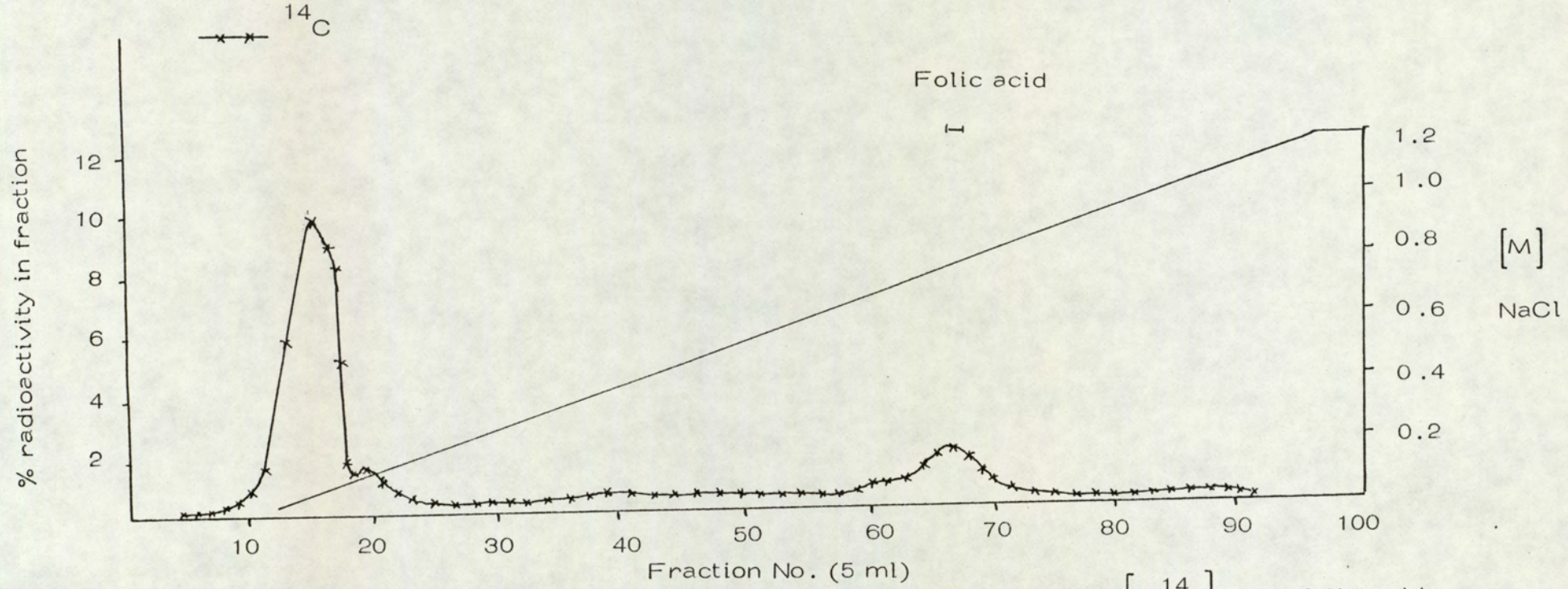


Figure 3.1.12 DE.52 of 24-48h normal guinea pig urine following an oral dose of $\left[2\text{-}^{14}C\right]$ only folic acid.

Dose: 1370 µg/kg body wt.

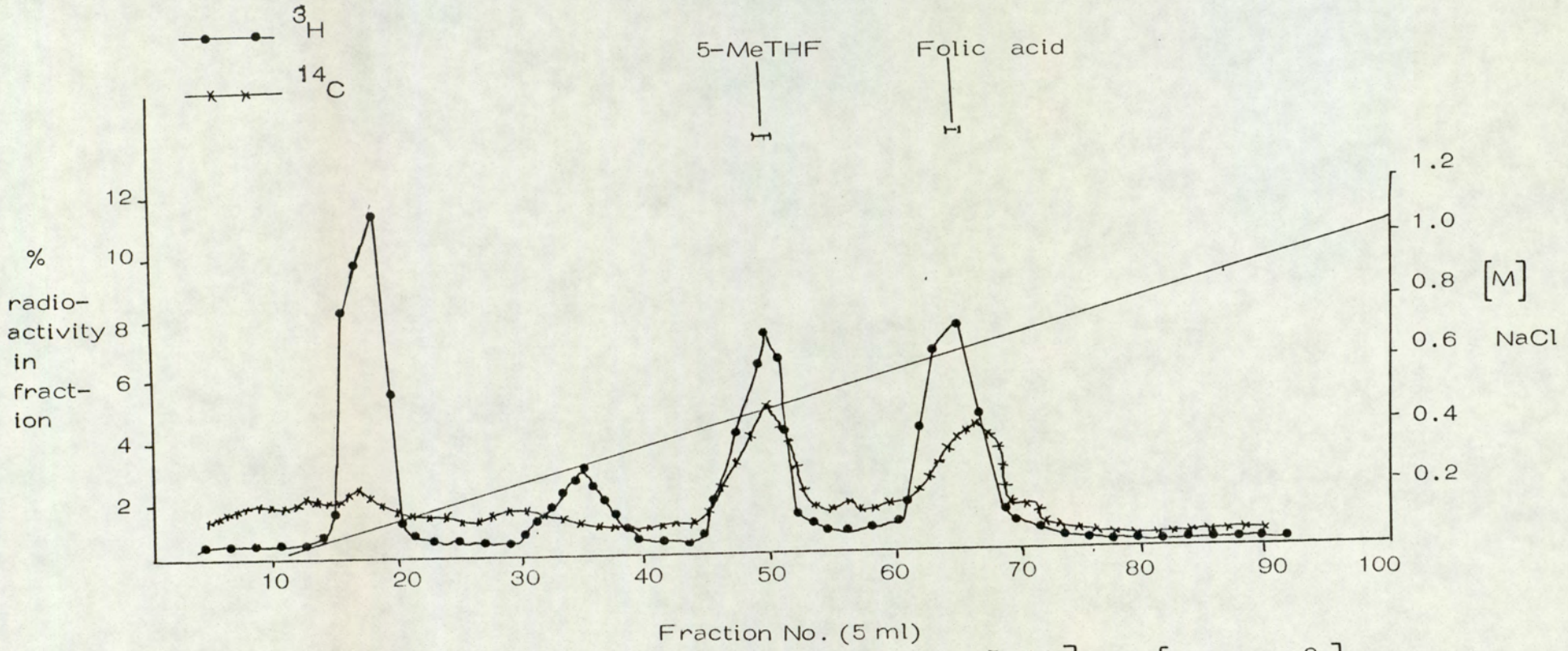


Fig. 3.1.13 DE.52 of gut flushings 2h after a dose of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$-folic acid. Dose: 400 µg/kg body wt.

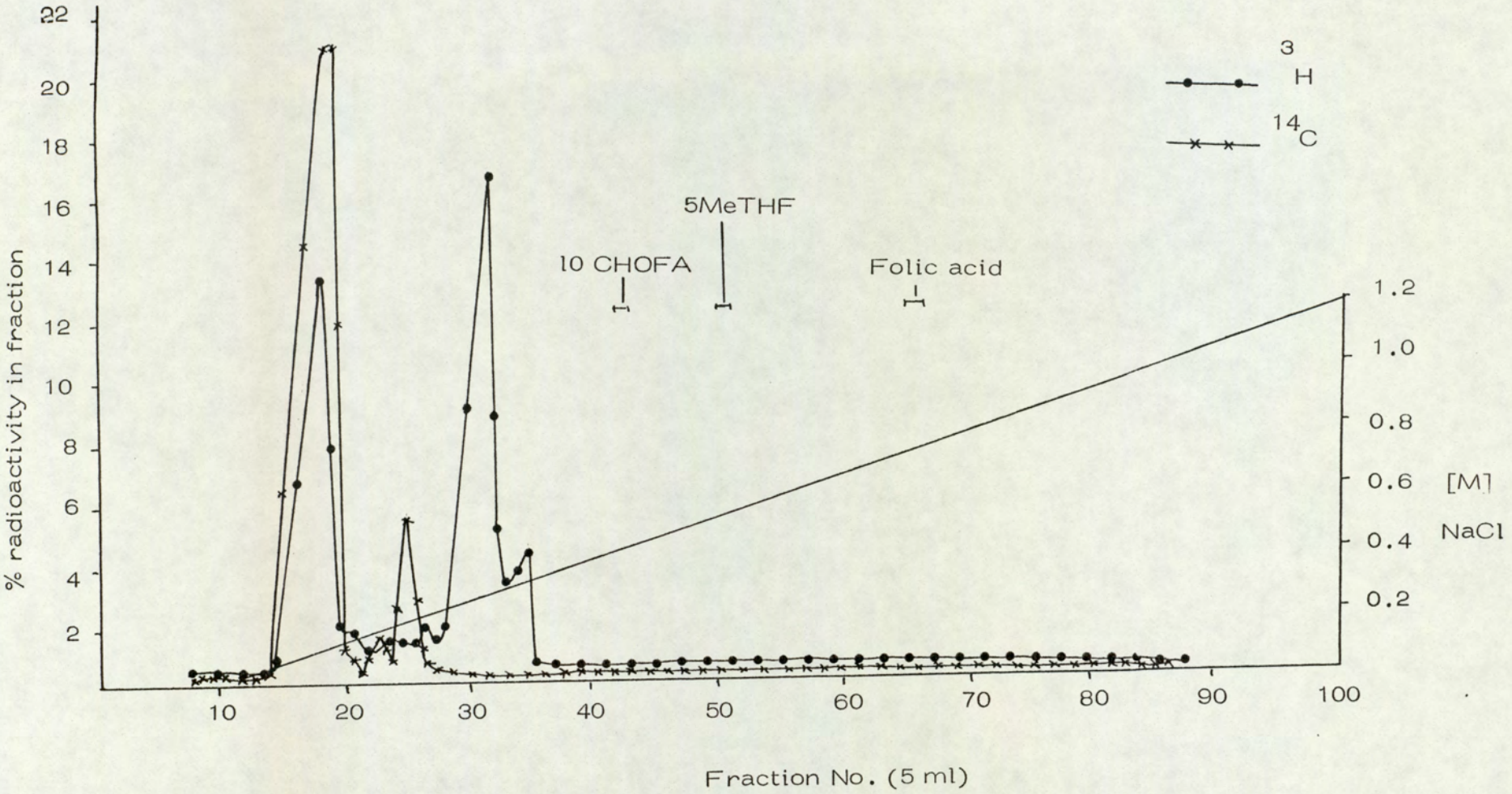


Figure 3.1.14. DE-52 chromatography of bile collected after an oral dose of a mixture of [2-^{14}C] and [3^{r},5',7,9-^{3}H]-folic acid. Dose 400 µg/kg body wt.

Time of collection: 2-3 h after dosing

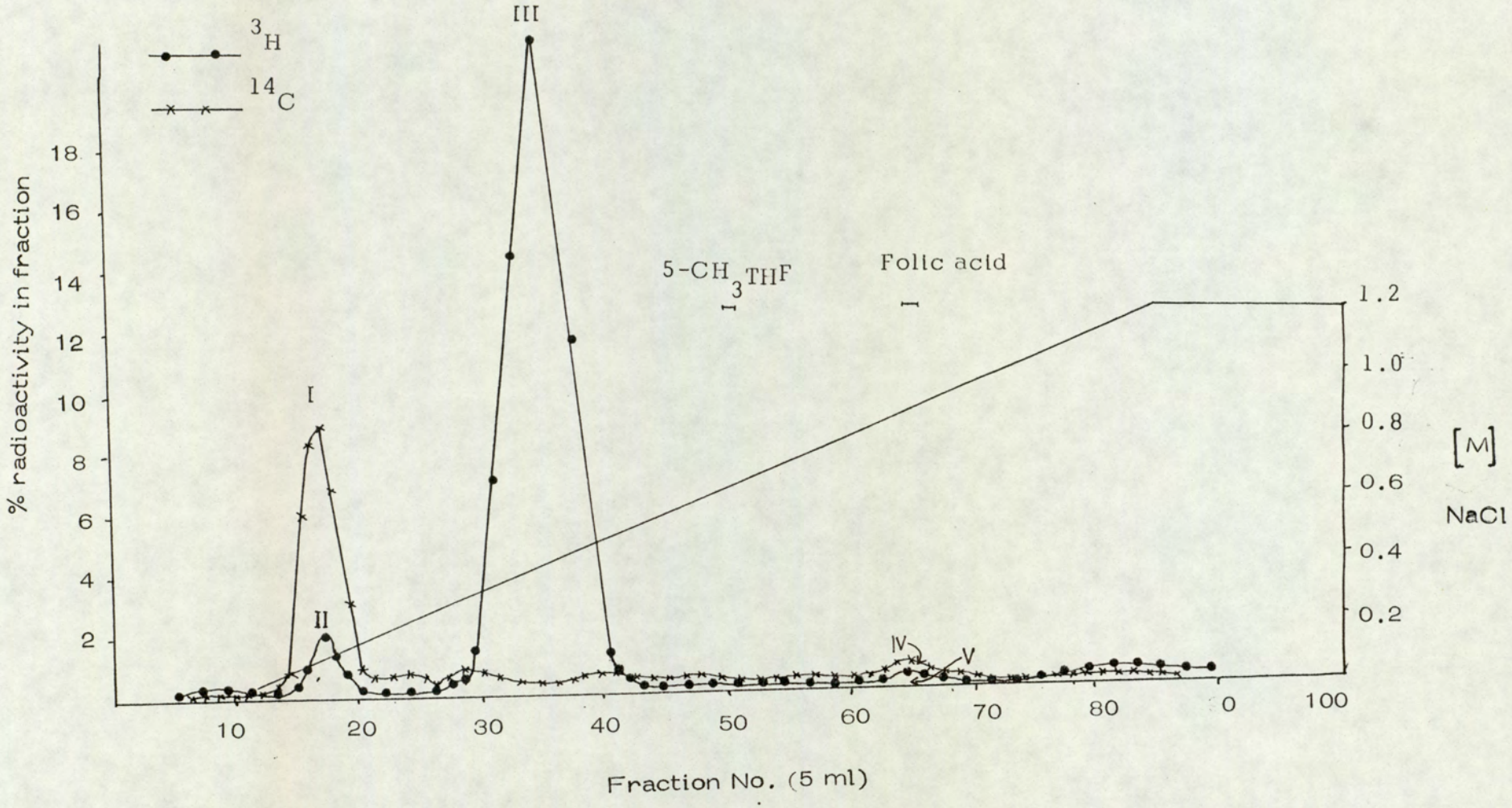


Fig. 3.2.1. DE-52 chromatography of 0-24h scorbutic guinea-pig urine after the administration of a mixture of [2-^{14}C] and [3',5',7,9 ^{3}H] - folic acid
Dose 400, 930 or 2185 µg/kg body wt.

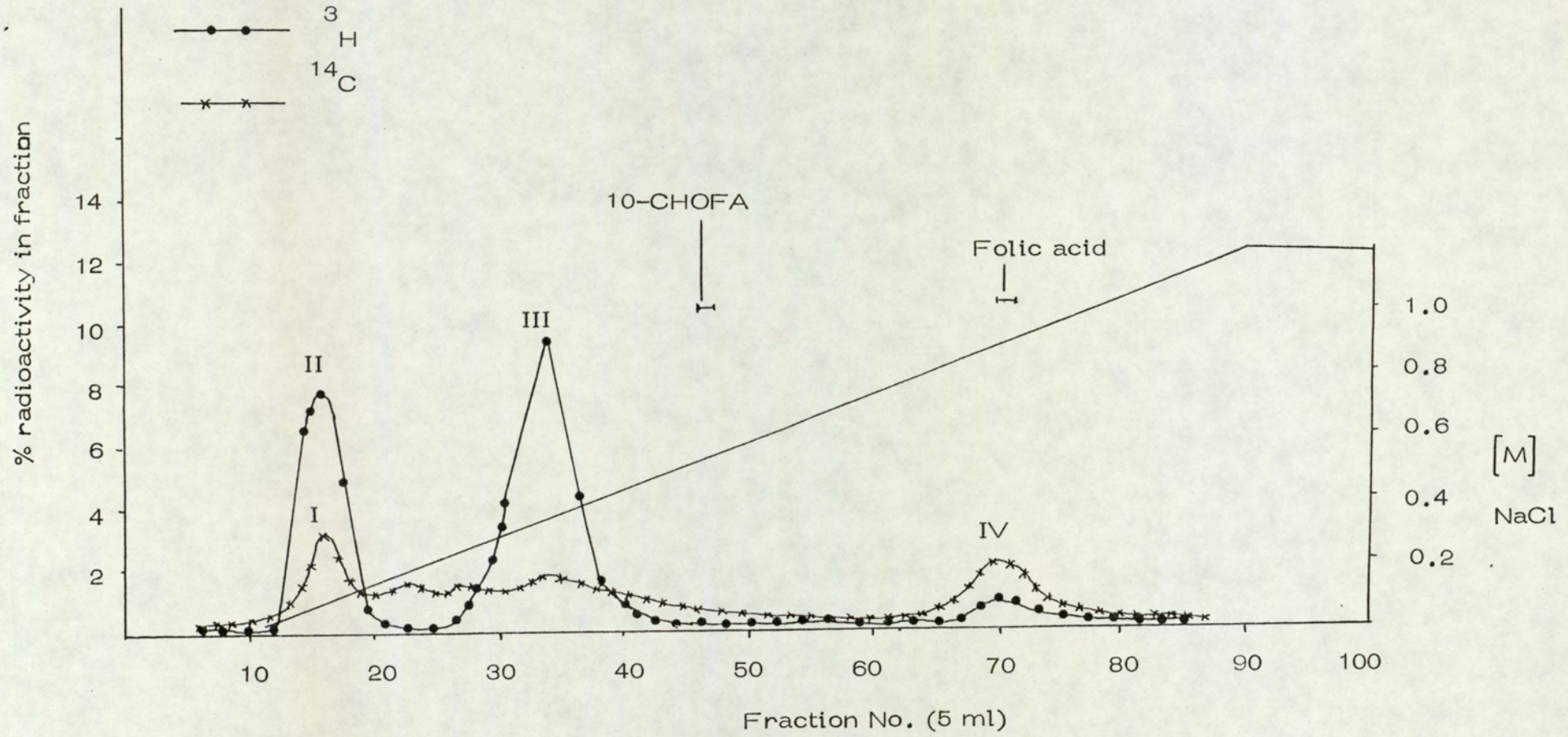


Fig. 3.2.2. DE-52 chromatography of 24-48h scorbutic guinea-pig urine after an oral dose of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ folic acid

Dose: 400, 930 or 2185 µg/kg body wt.

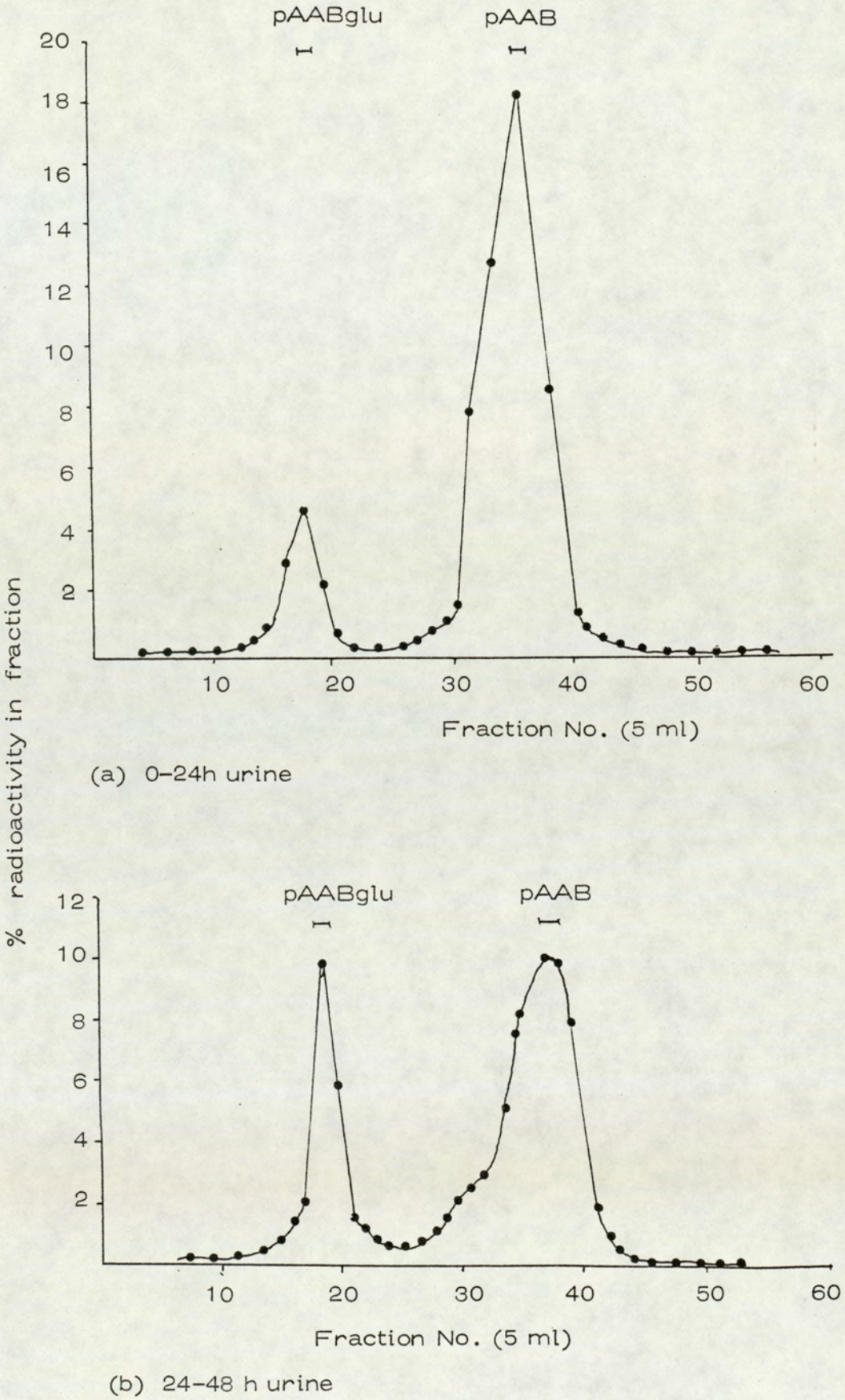


Fig. 3.2.3 Sephadex G-15 chromatography of (a) peak III from DE-52 chromatography of 0-24h scorbutic urine sample (b) peak III from DE-52 chromatography of 24-48h scorbutic urine after the oral administration of labelled folic acid

Dose 400, 930 or 2185 µg/kg body wt.

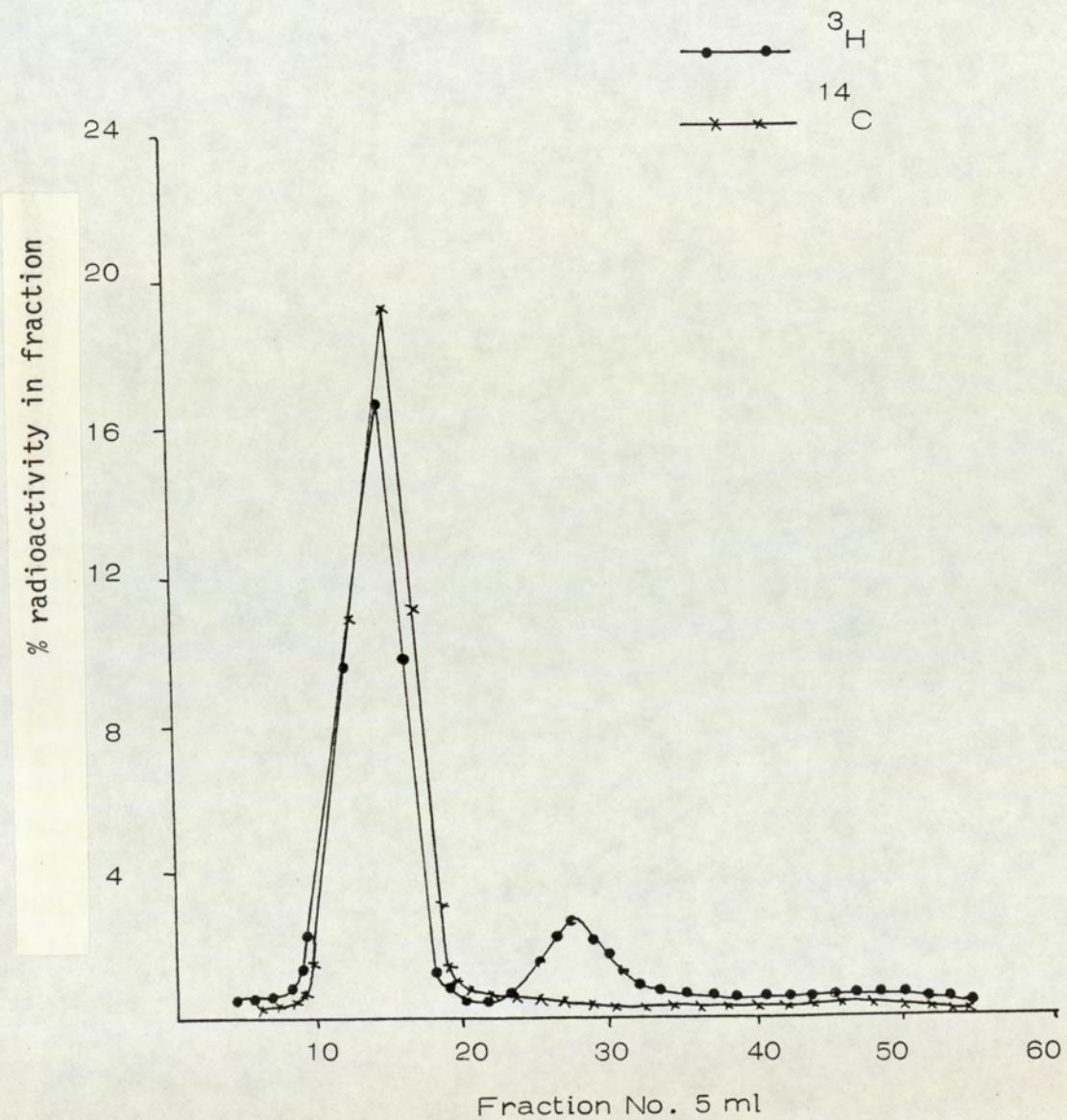


Figure 3.2.4. Sephadex G-15 chromatography of hot ascorbate liver extracts from scorbutic guinea pigs 48h after an oral dose of labelled folic acid. Dose : 400, 930 or 2185 µg/kg body wt.

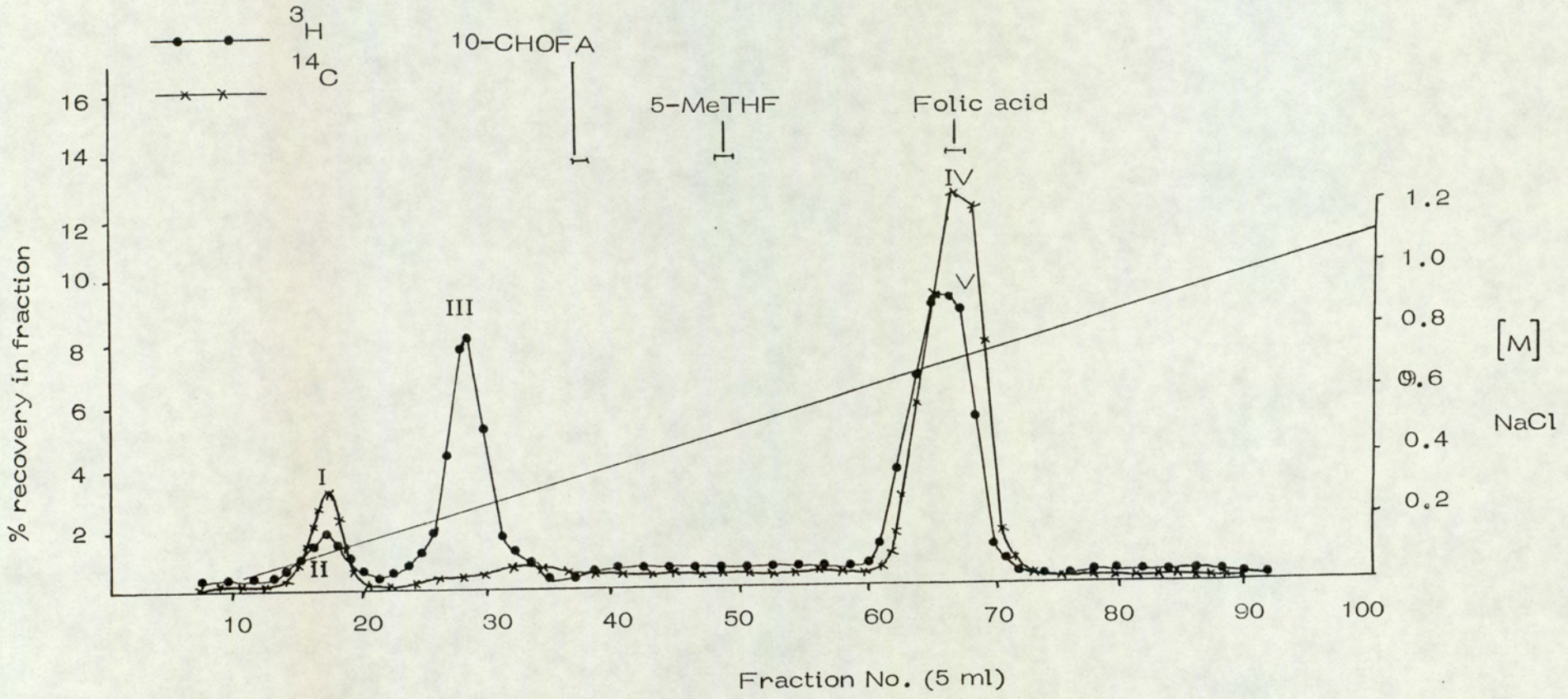


Fig. 3.3.1. DE-52 chromatography of 0-24h normal guinea-pig urine after an i.p. dose of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ - folic acid Dose- 400 µg/kg body wt.

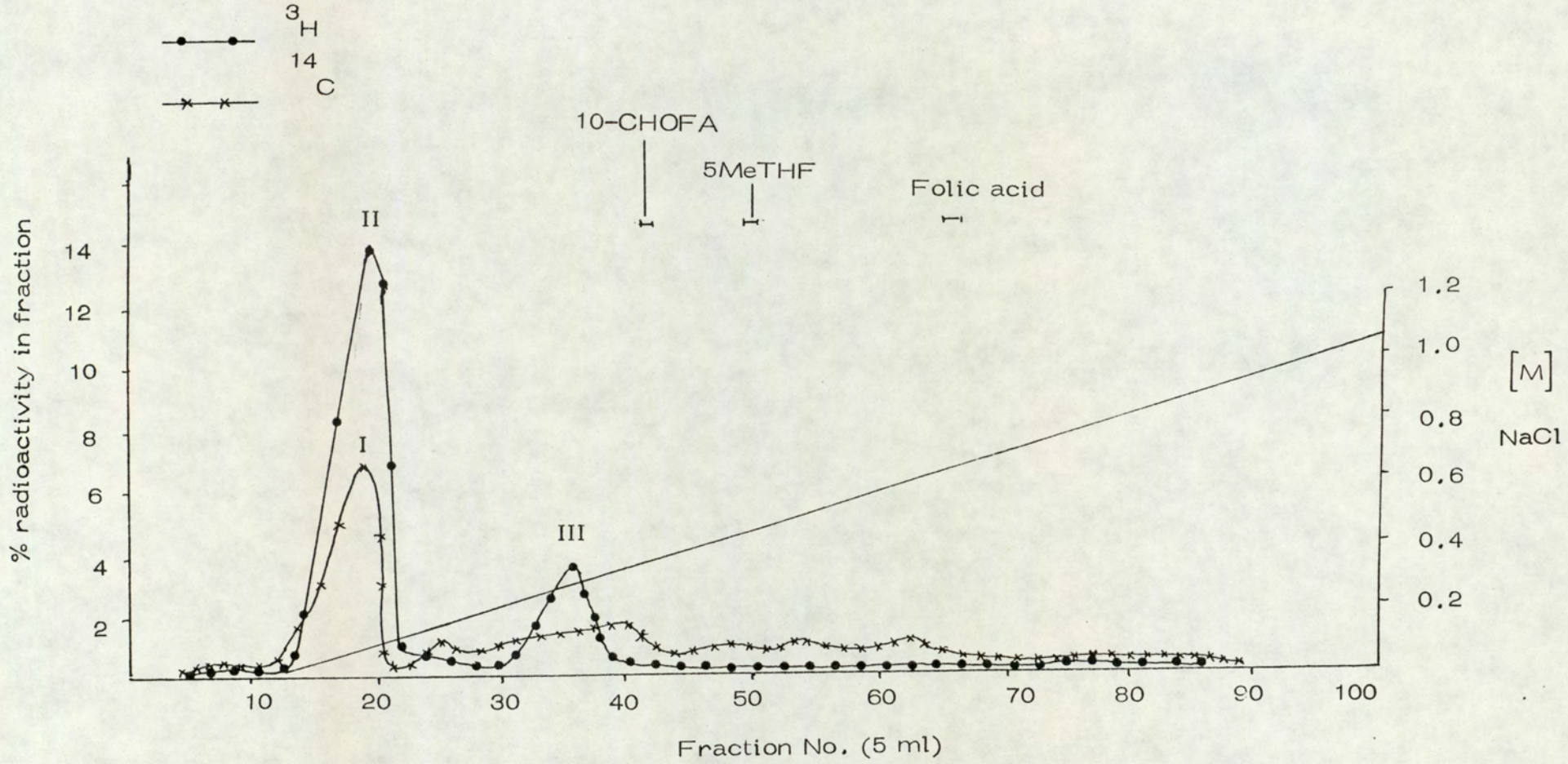


Fig. 3.3.2. DE-52 chromatography of 24-48h normal guinea-pig urine following an i.p dose of a mixture of $[2-^{14}C]$ and $[3',5',7,9-^{3}H]$ folic acid Dose: 400 µg/kg body wt.

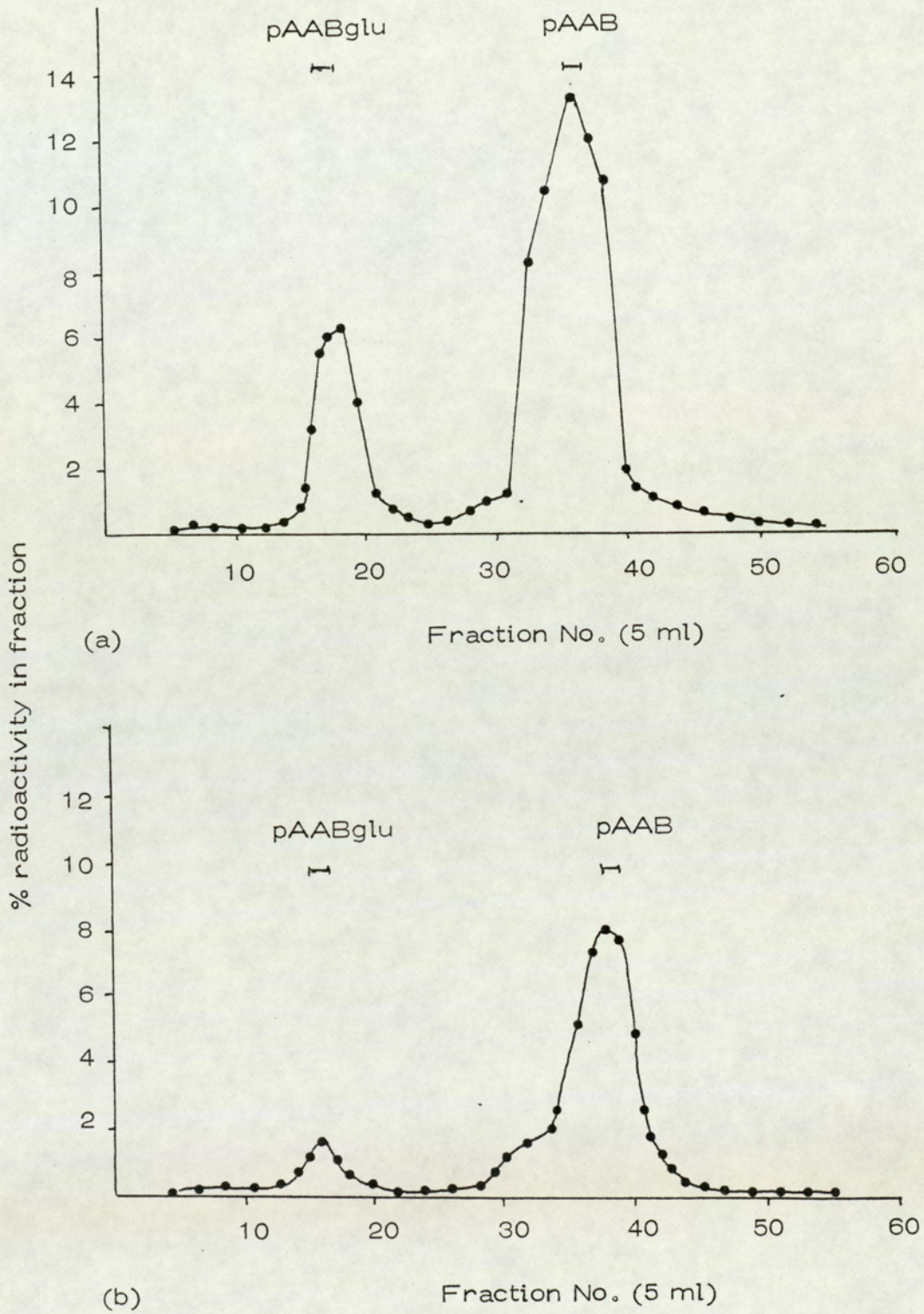


Fig. 3.3.3.

Sephadex G-15 chromatography of (a) peak III from DE-52 chromatography of 0-24 h urine (b) peak III from DE-52 chromatography of 24-48h urine, following an i.p. dose of labelled folic acid. Dose 400 µg/kg body wt.

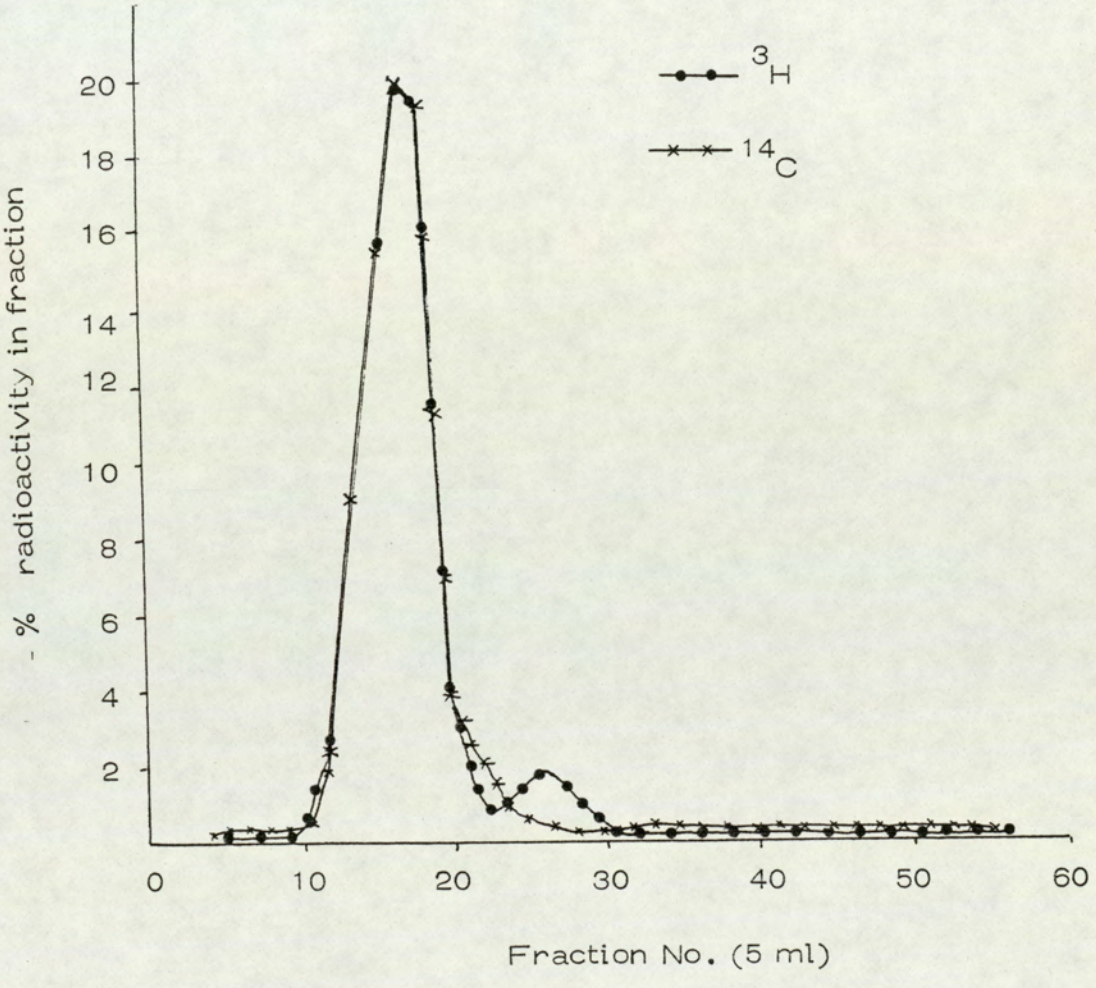


Fig. 3.3.4.

Sephadex G-15 chromatography of a hot ascorbate extract of normal guinea-pig liver, 48h after an i.p. dose of a mixture of $\left[^{3}H\right]$- and $\left[^{14}C\right]$ - labelled folic acid.

Dose: 400 µg/kg body wt.

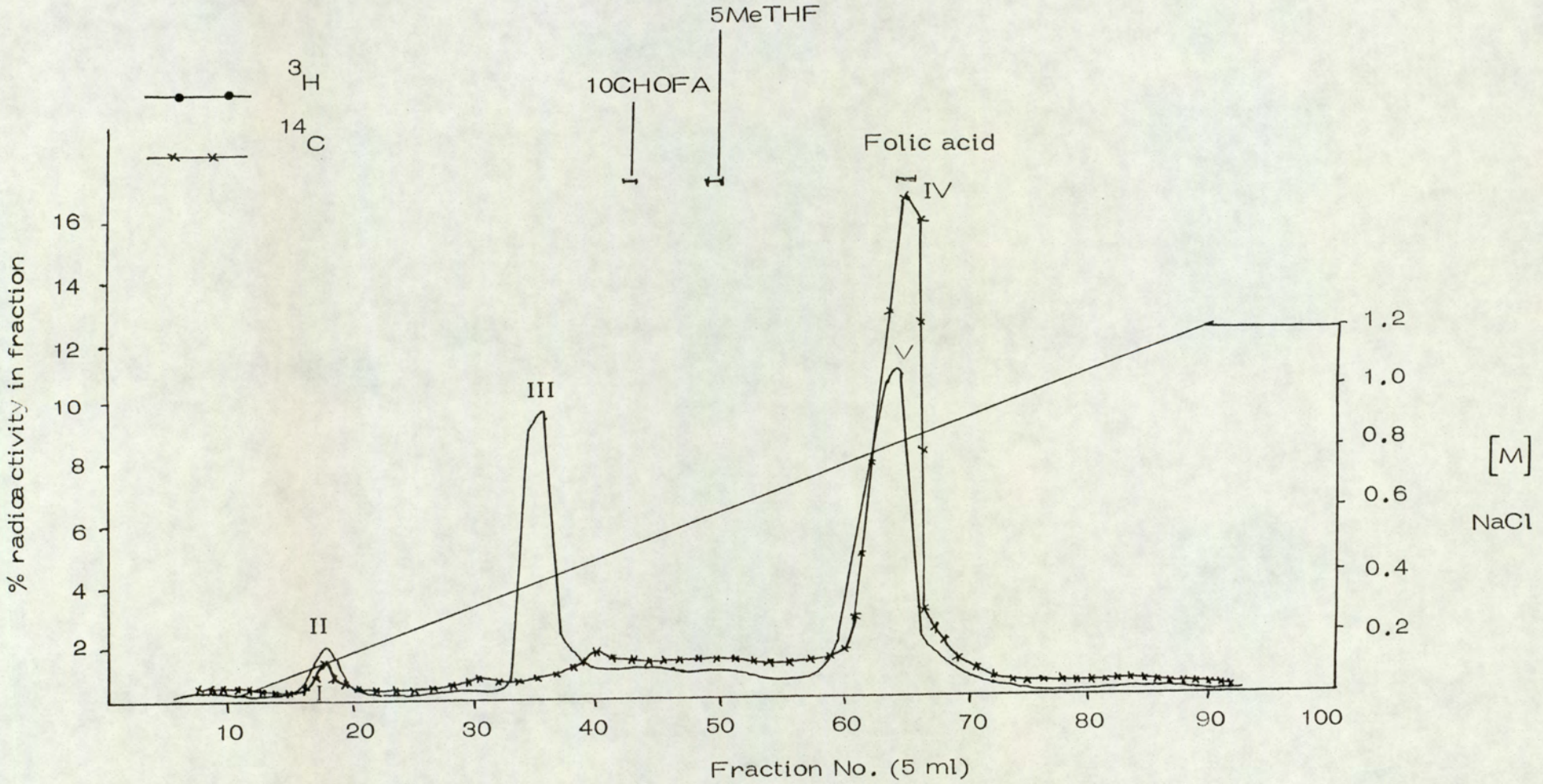


Fig. 3.4.1. DE.52 chromatography of 0-24h normal guinea-pig urine after a dose of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ folic acid in the presence of MTX at the time of dosing

Dose 400 µg/kg body wt.

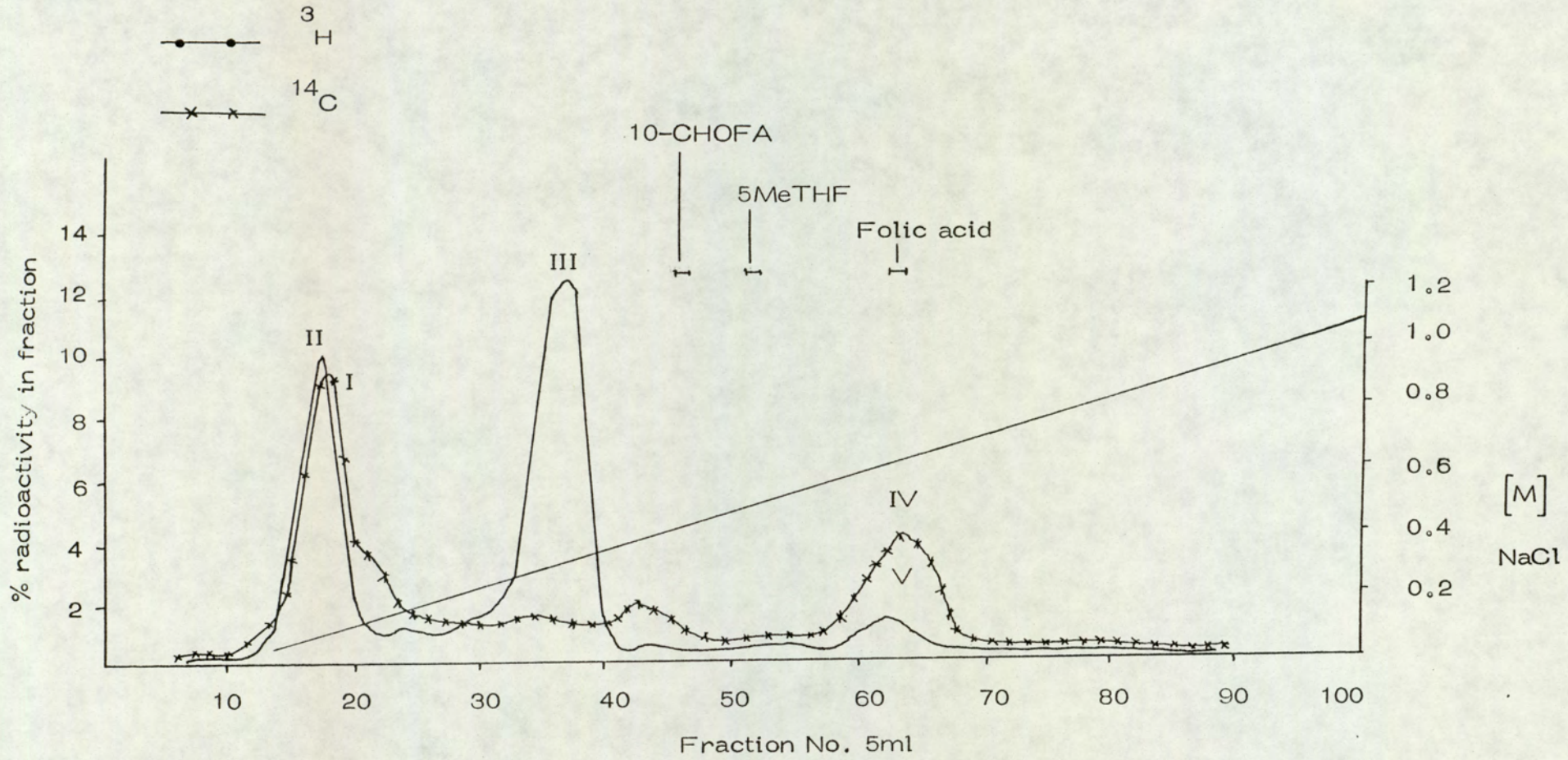


Fig. 3.4.2.

DE-52 chromatography of 24-48h normal guinea-pig urine after an i.p. dose of $[^{14}C]$ and $[^{3}H]$ folic acid in the presence of MTX administered orally at the time of dosing. Dose 400 µg/kg body wt.

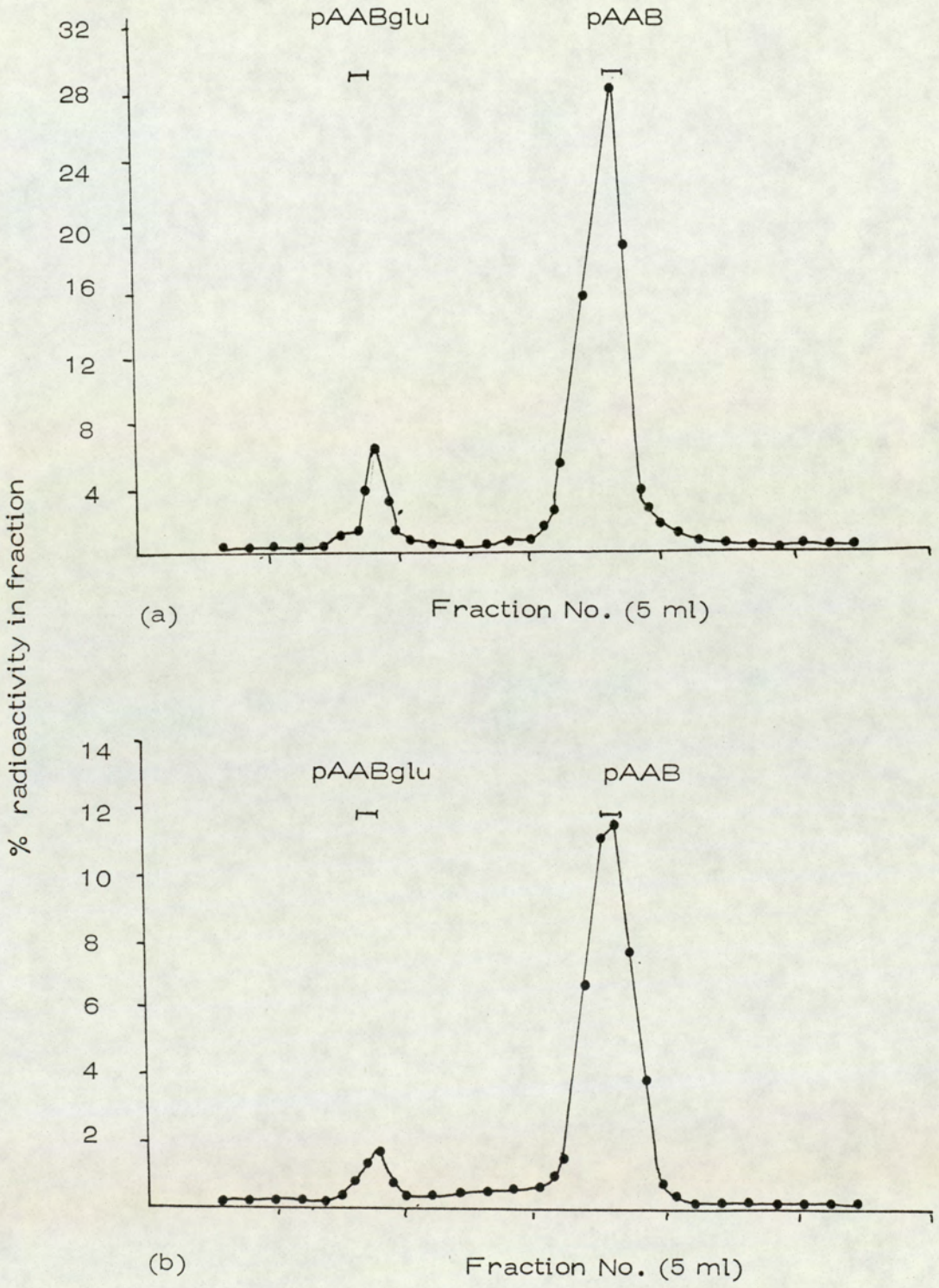


Fig. 3.4.3.

Sephadex G-15 chromatography of (a) peak III from DE-52 of 0-24h normal guinea-pig urine (b) peak III from DE-52 of 24-48h normal guinea-pig urine, following an i.p. dose of labelled folic acid in the presence of MTX

Dose 400 µg/kg body wt.

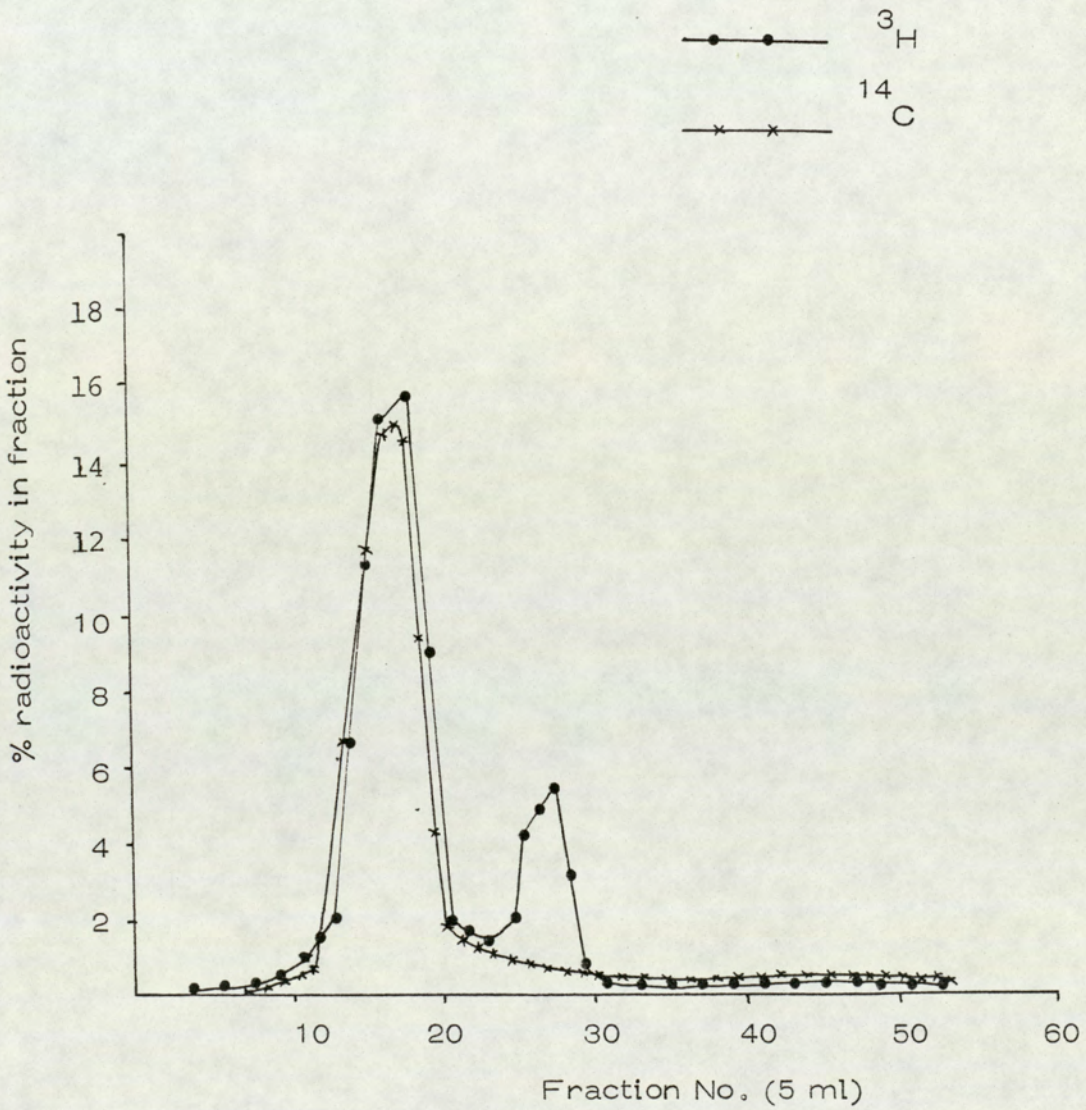


Fig. 3.4.4.

Sephadex G-15 chromatography of hot ascorbate liver extracts from normal guinea-pigs 48h after an i.p. administration of labelled folic acid in the presence of MTX

Dose 400 µg/kg body wt.

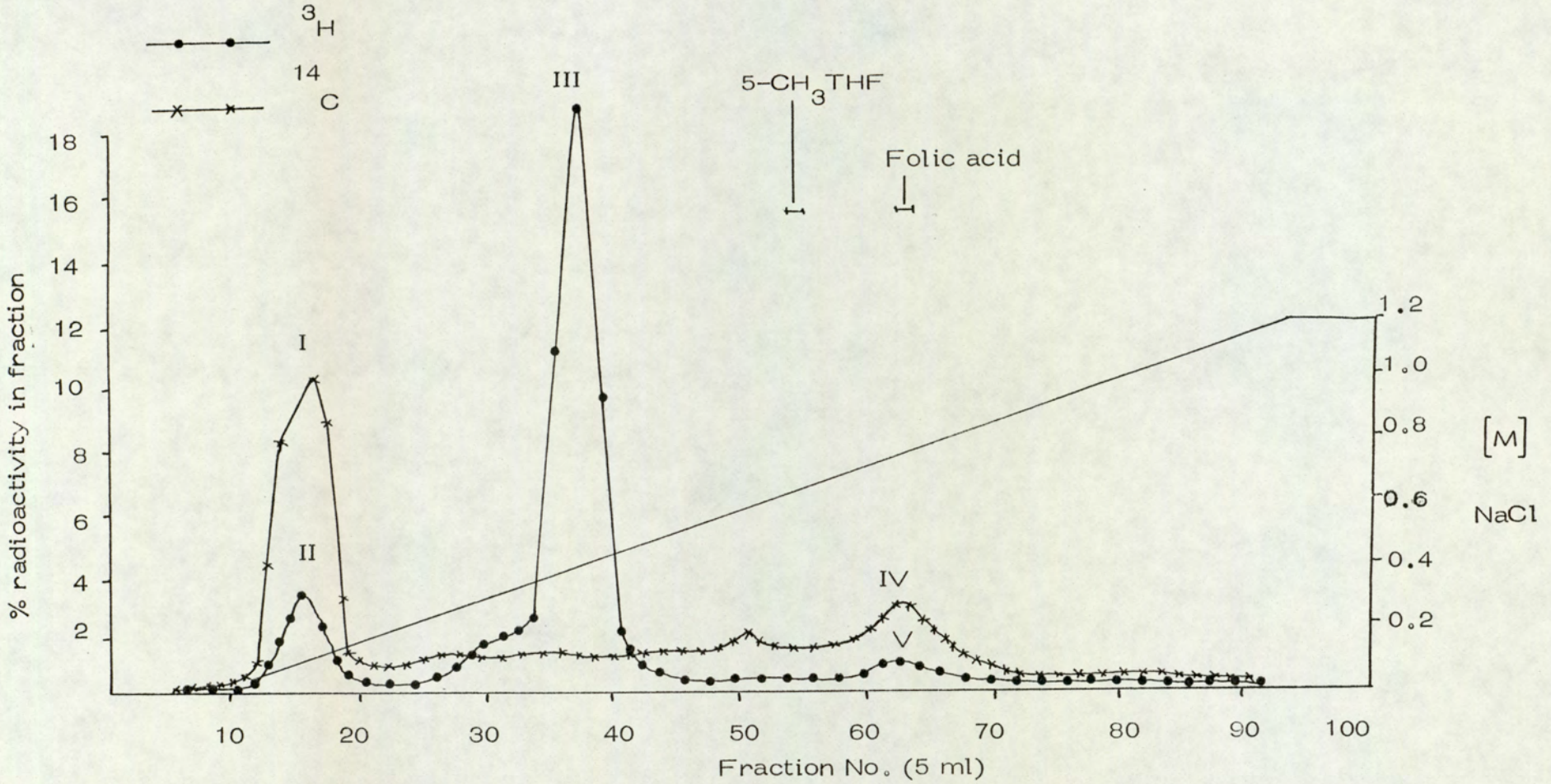


Fig. 3.5.1. DE-52 chromatography of 0-24h normal guinea-pig urine following oral administration of a dose of a mixture of $\left[^{3}H\right]$- and $\left[^{14}C\right]$-folic acid in the presence of antibiotics

Dose 400 or 800 µg/kg body wt.

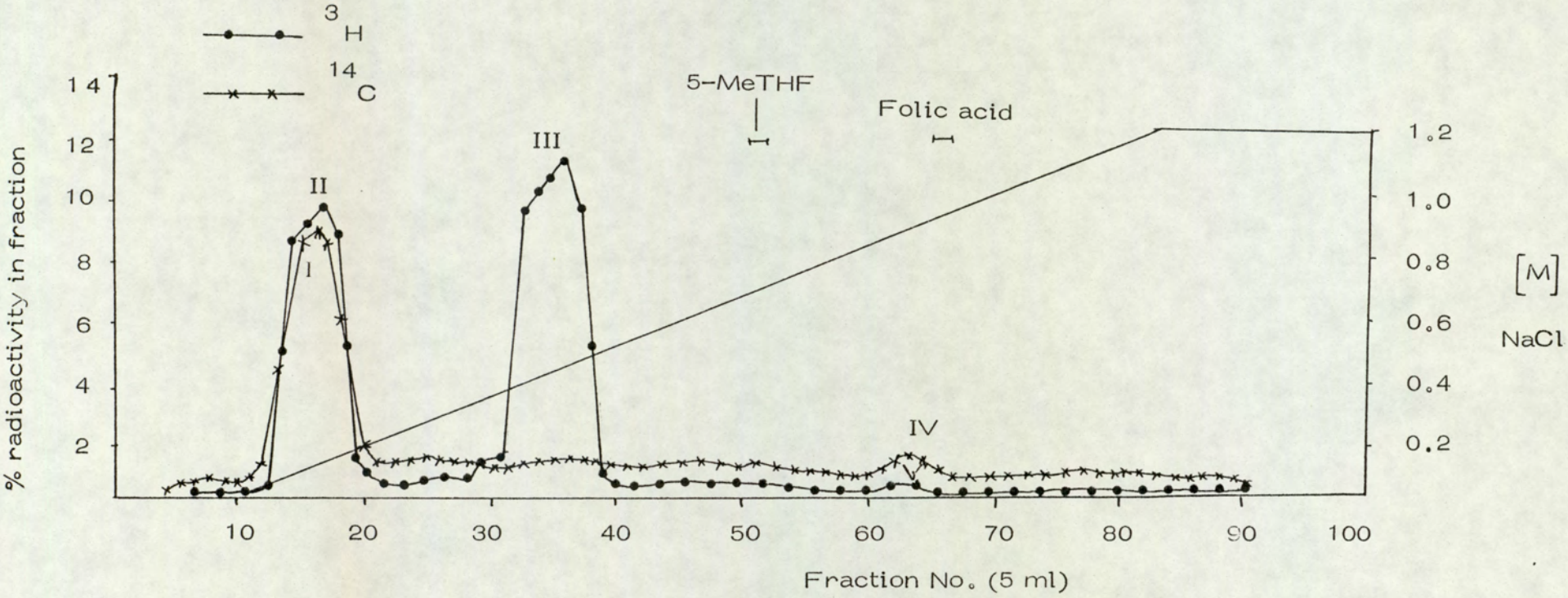


Fig. 3.5.2. DE-52 chromatography of 24-48h normal guinea-pig urine following an oral dose of $[^{3}H]$ and $[^{14}C]$ labelled folic acid in the presence of antibiotics

Dose 400 or 800 µg/kg body wt.

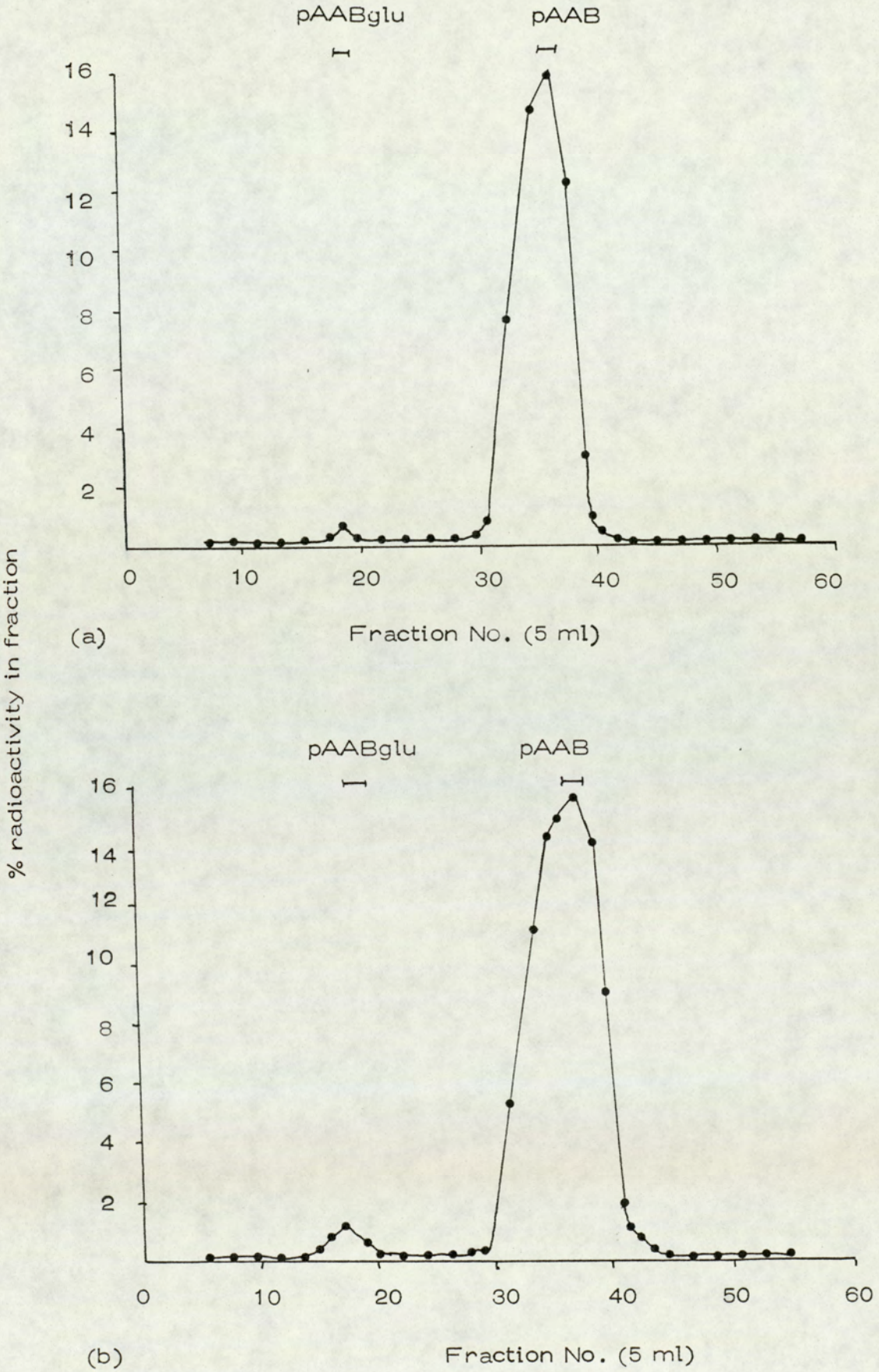


Fig. 3.5.3. Sephadex G.15 chromatography of (a) ^{3}H peak III from DE.52 of 0-24 h urine (b) ^{3}H peak III from DE.52 of 24-48h urine following an oral administration of labelled folic acid to normal guinea pigs in the presence of antibiotics.

Dose 400 or 800 µg/kg body wt.

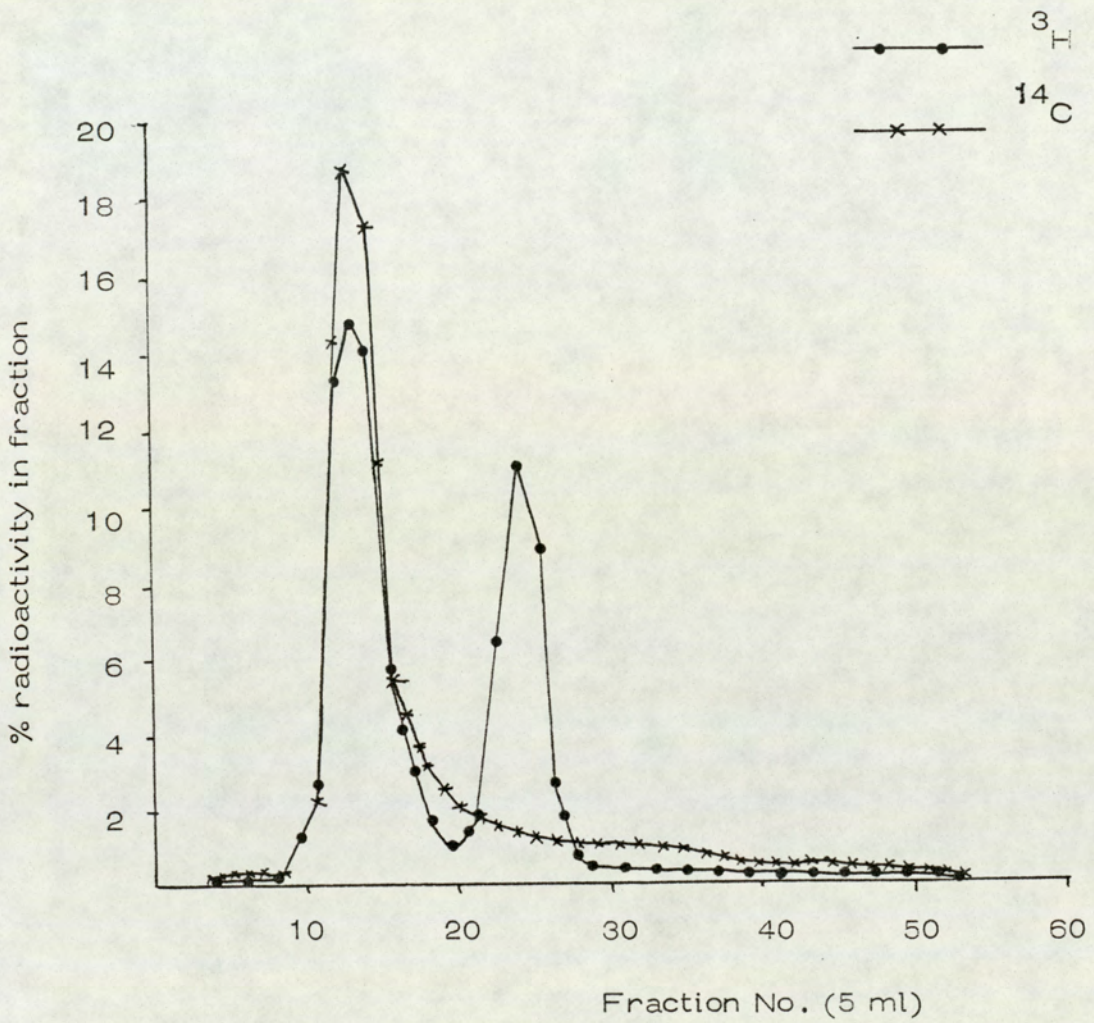


Fig. 3.5.4. Sephadex G.15 chromatography of hot ascorbate liver extracts 48h after an oral dose of a mixture of ^{3}H and ^{14}C - labelled folic acid in the presence of antibiotics.

Dose 400 or 800 µg/kg body wt.

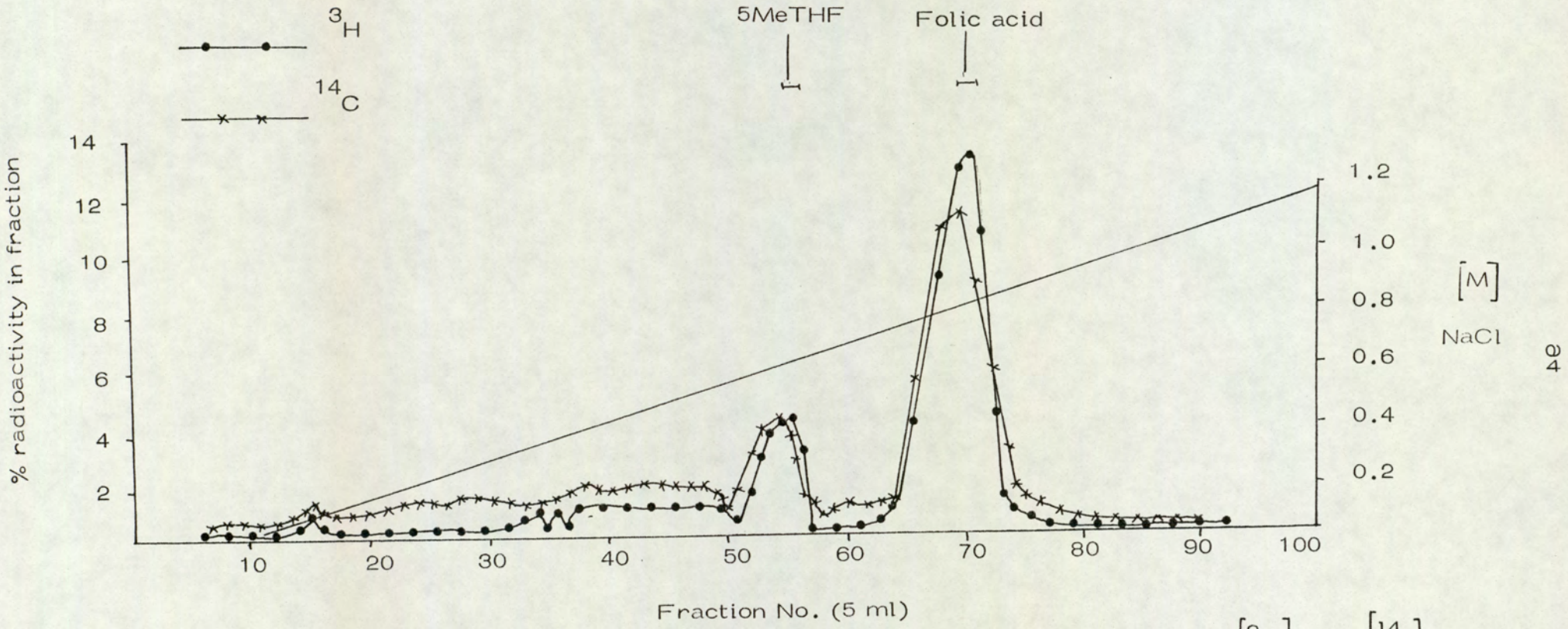


Fig. 3.5.5. DE-52 chromatography of gut flushings 2h after an oral dose of a mixture of $[^{3}H]$ and $[^{14}C]$ folic acid in the presence of antibiotics.

Dose 400 or 800 µg/kg body wt.

CHAPTER 4

THE METABOLISM OF [3,5-^{3}H] p-AMINOBENZOYL-L GLUTAMATE IN THE NORMAL AND SCORBUTIC GUINEA PIG

Results from previous experiments have shown that the normal and scorbutic guinea pig are able to cleave folic acid 'in vivo' at the $C_9 - N_{10}$ bond, forming a pterin and p-aminobenzoyl-L-glutamate, compounds which are further metabolized. This chapter describes the study of the metabolism and handling of oral and intra-peritoneal doses of p-aminobenzoyl-L-glutamate by both normal and scorbutic guinea pigs.

MATERIALS AND METHODS

[3',5-^{3}H] p-aminobenzoyl-L-glutamate was prepared as described in Chapter 2. Guinea pigs were made scorbutic by being kept strictly on a diet lacking vitamin C (see Chapter 2). 6 Male normal and 6 male scorbutic guinea pigs (2 groups of 3 animals each) received either oral (1 normal group, 1 scorbutic) or i.p. (1 group normal, 1 scorbutic) doses of pABglu to which was added a small amount of the radioactive [3',5-^{3}H] pABglu prepared. The dose administered, 242 µg/k g body wt. was equivalent to the amount of p-aminobenzoyl-L-glutamate as contained in the 400 µg/k g body wt. now used as standard amount for dosing guinea pigs. The collection of urine and faeces, hot ascorbate extraction of the livers and the freeze-drying and burning of the liver and faeces were carried out as described in Chapter 2. Figures quoted in Tables 4.1 and 4.3 are the average for groups of 3 animals used and figures in Tables 4.2 and 4.4. are those for pooled urine samples.

RESULTS AND DISCUSSION

The recovery of radioactivity in the urine, liver and faeces from normal guinea pigs is summarized in Table 4.1. and for scorbutics, Table 4.3. Results show tremendous differences in the handling of a dose of [^{3}H] p ABglu not only between the normal and the scorbutic state, but also within a given state depending on the mode of administration of the compound. In the normal guinea pig, more radioactivity is recovered in the urine after oral administration (56.7%) than after an i.p. administration (33.2%) (Table 4.1) but this situation is reversed in the scorbutic animal, where 56.4% is recovered after an oral dose and 73.8%, following an i.p. dose (Table 4.3). Chromatographic analysis of the pooled urine samples on Sephadex G-15 and on paper showed the presence of only two tritiated metabolites, p-acetamidobenzoyl-L-glutamate (pAABglu) and p-acetamidobenzoate (pAAB). Their proportions, however, differ according to the mode of administration and according to the vitamin C status of the animal.

Following the administration of pABglu, normal guinea pigs excreted approximately equal amounts of the two metabolites (22.2% pAABglu and 27.2% pAAB) when the compound was orally administered but about 50 times more pAABglu (30.1%) than pAAB (0.6%) when the compound was administered i.p. (Table 4.2). This contrasted sharply with recoveries in the scorbutic guinea pig, which produced more pAAB (47.9%) than pAABglu (5.5%) after an oral dose (Table 4.4).

TABLE 4.1.

Recovery of radioactivity in urine, liver and faeces, following the administration of [3 ,5 - ^{3}H] p-aminobenzoyl-L-glutamate (242 µg/kg body wt) to normal guinea pigs. Results expressed as mean % of the dose $\pm$ standard deviation (shown in brackets).

No. of animals	Mode of administration	Urine 0 - 24h	Urine 24-48 h	Liver (48 h)	Faeces (48h)	Total (48h)
3	Oral	56.7 (4.6)	3.5 (1.7)	0.3	19.0 (4.0)	79.5
3	i.p.	33.2 (14.3)	3.4 (0.8)	NDt	1.0 (0.5)	37.6

NDt = Not Detected

TABLE 4.2.

Metabolites found in pooled normal guinea pig urine following a dose of [3 ,5- ^{3}H] p ABglu (242 µg/kg body wt). Results expressed as % of the administered dose.

	Oral (3 animals)			i.p. (3 animals)		
Time	pAABglu	pAAB	Ratio	pAABglu	pAAB	Ratio
0 - 24h	22.2	27.2	1:1.2	30.1	0.6	1:0.02
24-48h	1.3	1.5	1:1.2	3.2	0.4	1:0.1

However, although the scorbutic guinea pig produced more pAABglu (50.5%) than pAAB (17.1%) (Tables 4.2., 4.4), the pAABglu level was only 3 times higher than the pAAB level in scorbutics whereas it was 50 times higher in the normal. No unacetylated metabolite (either p-aminobenzoyl-L-glutamate was found excreted in any of the urine samples.

The differences in handling the compound are further highlighted in the way the normal and scorbutic guinea pigs handle (a) oral dose (b) i.p. doses of the pABglu. After an oral dose, normal guinea pigs excreted approximately equal amounts of pAABglu (22.2%) and pAAB (27.2%) (Table 4.2) but in scorbutics, the pAAB amount was 9 times higher than the urinary pAABglu level. But following an i.p. administration, although normals excrete 50 times more pAABglu (30.5%) than pAAB (0.6%), in scorbutics, pAABglu level (50.5%) was only 3 times the level of pAAB (17.1%) (Tables 4.2., 4.4.). pAABglu still remains the dominant urinary metabolite when administered i.p. both in normals and scorbutics and pAAB is dominant only in scorbutics following oral administration. More pAABglu is excreted when administered i.p. to the guinea pig whether in the normal or scorbutic state.

The livers of all the guinea pigs (normal and scorbutic) showed very low incorporation of radioactivity, if at all, irrespective of the mode of administration of [^{3}H] pABglu(Tables 4.1. and 4.3).

TABLE 4.3

Recovery of radioactivity in urine, liver and faeces following the administration of a dose (242 µg/kg body wt) of [3,5-^{3}H]p-aminobenzoyl-L-glutamate to scorbutic guinea pigs.

Results expressed as % of administered dose $\pm$ standard deviation shown in brackets.

Mode of administration	No. of animals	URINE 0 - 24h	URINE 24-48 h	LIVER (48 h)	FAECES (48 h)	TOTAL (48 h)
Oral	3	56.4 (4.8)	2.3 (0.8)	0.08	12.8 (9.2)	71.5
i.p.	3	73.8 (17.9)	1.6 (1.1)	1.5	1.7 (1.8)	78.6

TABLE 4.4.

Metabolites found in pooled scorbutic guinea pig urine following oral or i.p. doses (242 µg/kg body wt) of [^{3}H] p-aminobenzoyl-L-glutamate. Results expressed as % of administered dose.

	Oral administration (3 animals)			i.p. administration (3 animals)		
Time	pAABglu	pAAB	ratio	pAABglu	pAAB	ratio
0-24h	5.5	47.9	1:8.7	50.5	17.1	1:0.3
24-48h	0.5	0.9	1:1.8	0.7	0.9	1:1.3

Excretion via faeces was higher when the [^{3}H] pABglu was orally administered (normal 19.0%, scorbutic 12.8%) than when administered i.p. (normal 1.0%, scorbutic 1.7%) (Tables 4.1. and 4.3.).

Liver extracts : Sephadex G-15 chromatography of the hot ascorbate liver extracts were carried out but the liver content of radioactivity (Tables 4.1. and 4.3) was too low for any of the chromatograms to be meaningful.

This is in sharp contrast to that seen earlier with folic acid (see Chapter 3), especially where i.p. administration is concerned, when the liver incorporates a fair amount of radioactivity (Section 3.3).

SUMMARY

1. Following the administration (oral or i.p) of labelled [^{3}H] pABglu to the guinea pig (normal or scorbutic), radioactivity is recovered mainly in the urine and faeces but extremely little, if any, in the liver.

2. Orally administered [^{3}H]pABglu results in fairly similar amounts of urinary radioactivity being excreted in normal guinea pigs, but, following i.p. administration, scorbutics excrete more than twice the amounts of normals.

3. Following the oral administration of pABglu, pAAB is the dominant urinary metabolite, especially in scorbutics, where-

as, following i.p. administration, pAABglu becomes the dominant one in both normals and scorbutics.

4. No unacetylated metabolite was found in any of the urine samples.

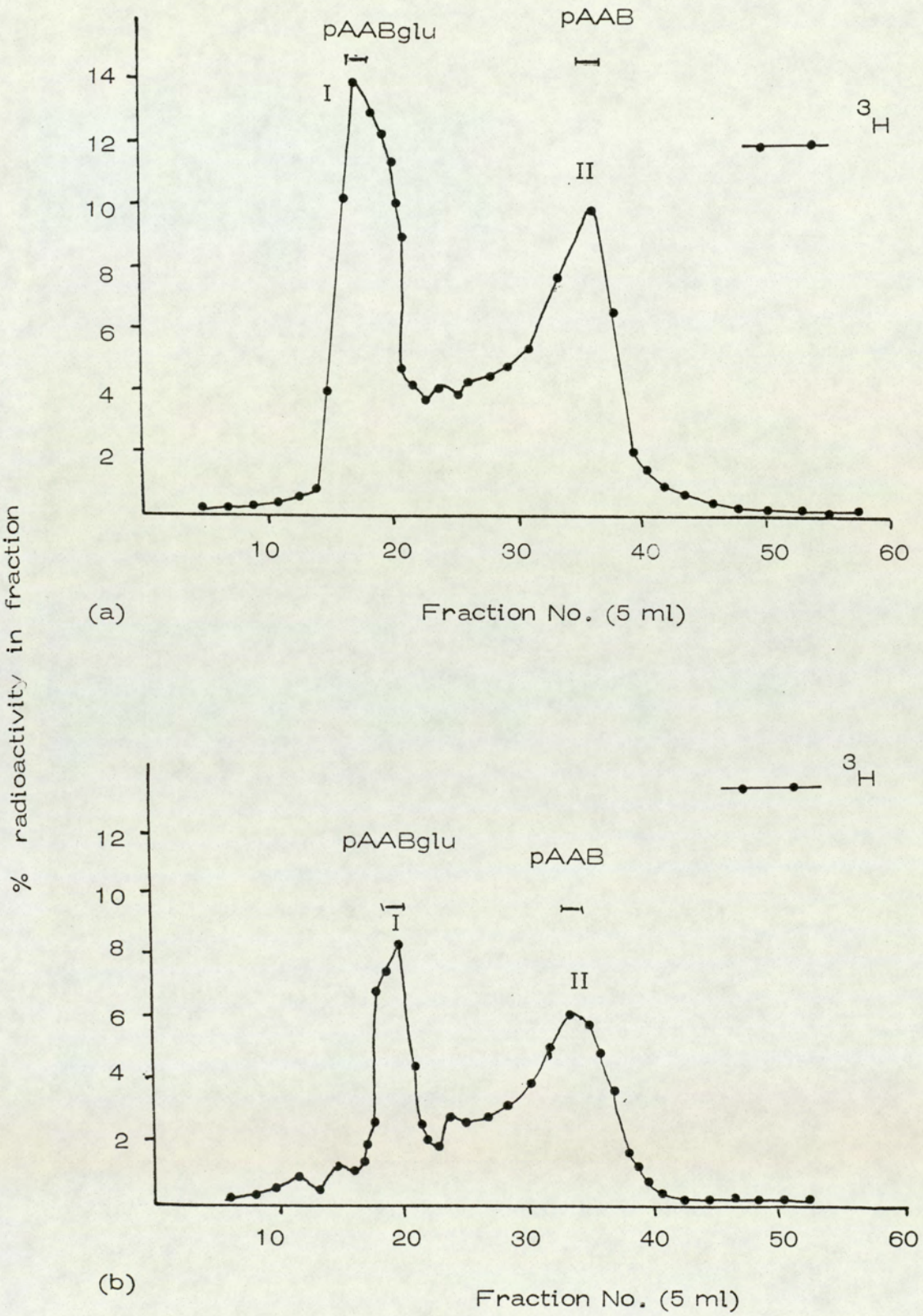


Fig. 4.1. Sephadex G-15 chromatography of (a) 0-24h (b) 24-48h normal guinea-pig urine samples after an oral dose of [3 ,5 ,- ^3H] p-aminobenzoyl-L-glutamic acid

Dose 242 µg/kg body wt.

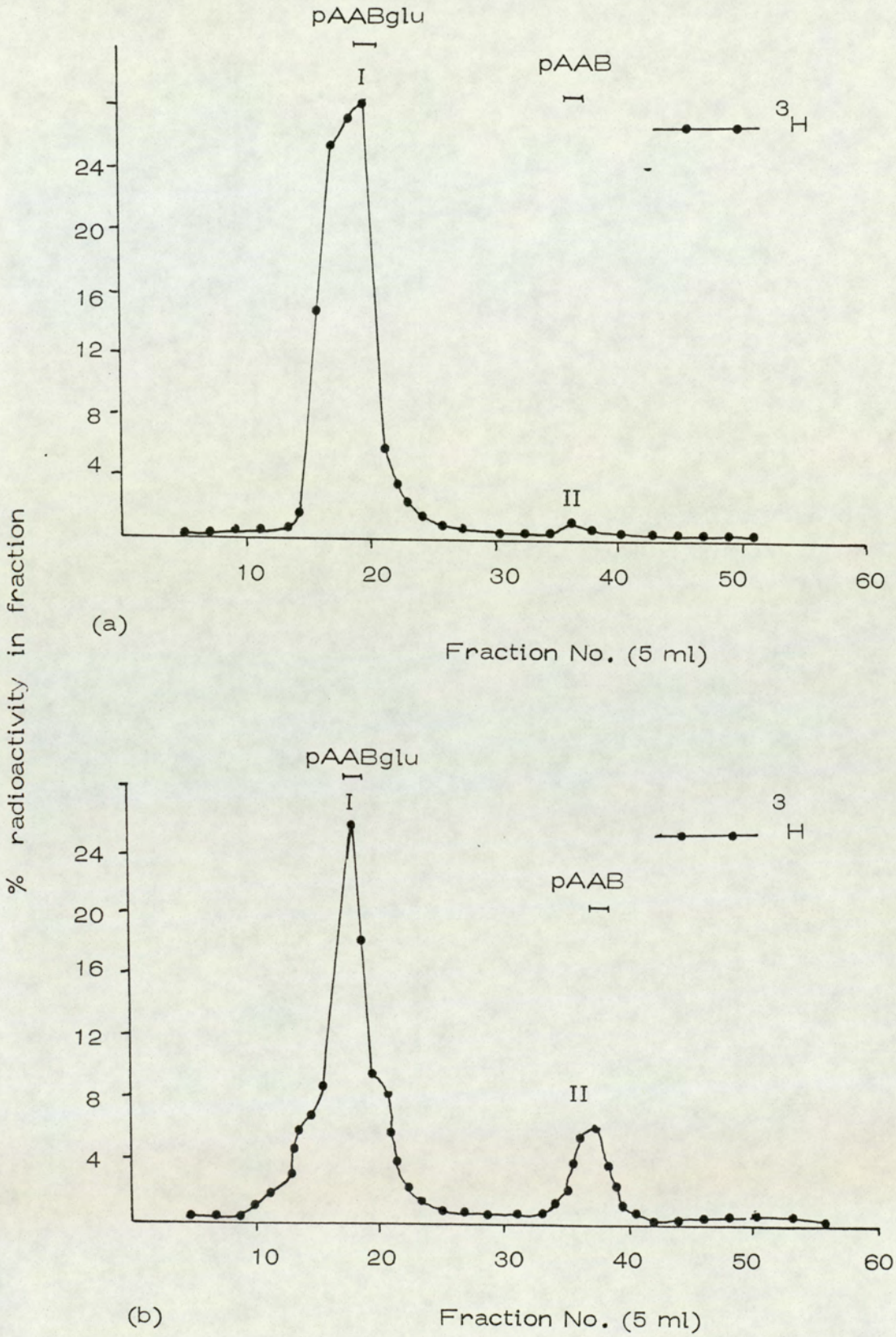


Fig. 4.2. Sephadex G-15 chromatography of (a) 0-24h (b) 24-48 h urine samples following an i.p. dose of [3',5'-^{3}H] p-amino-benzoyl-L-glutamate to normal guinea-pigs

Dose: 242 µg/kg body wt.

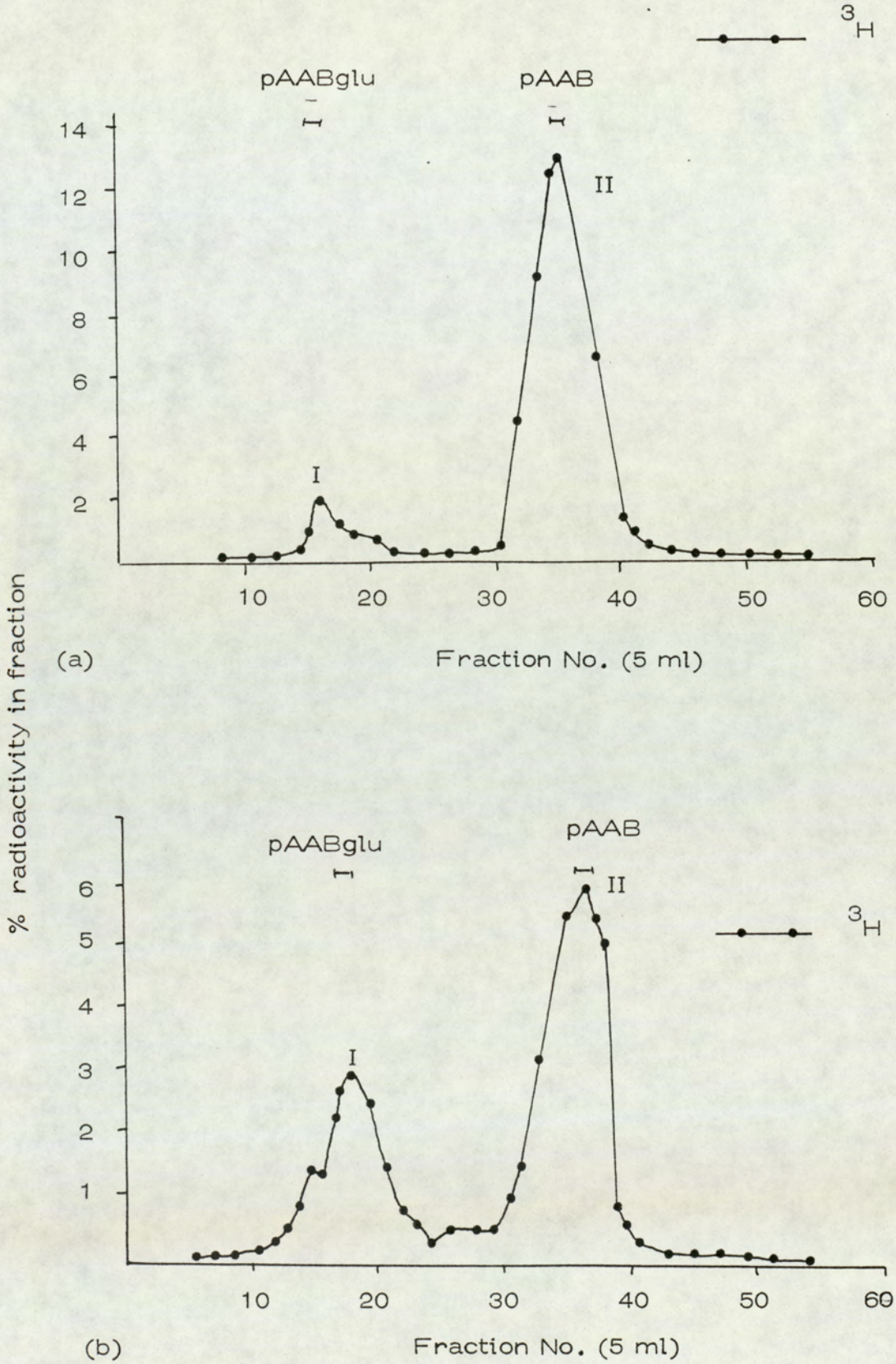


Fig. 4.3. Sephadex G-15 chromatography of (a) 0-24h (b) 24-48h urine samples from scorbutic guinea-pigs, following an oral dose of p-aminobenzoyl-L-glutamate

Dose: 242 µg/kg body wt.

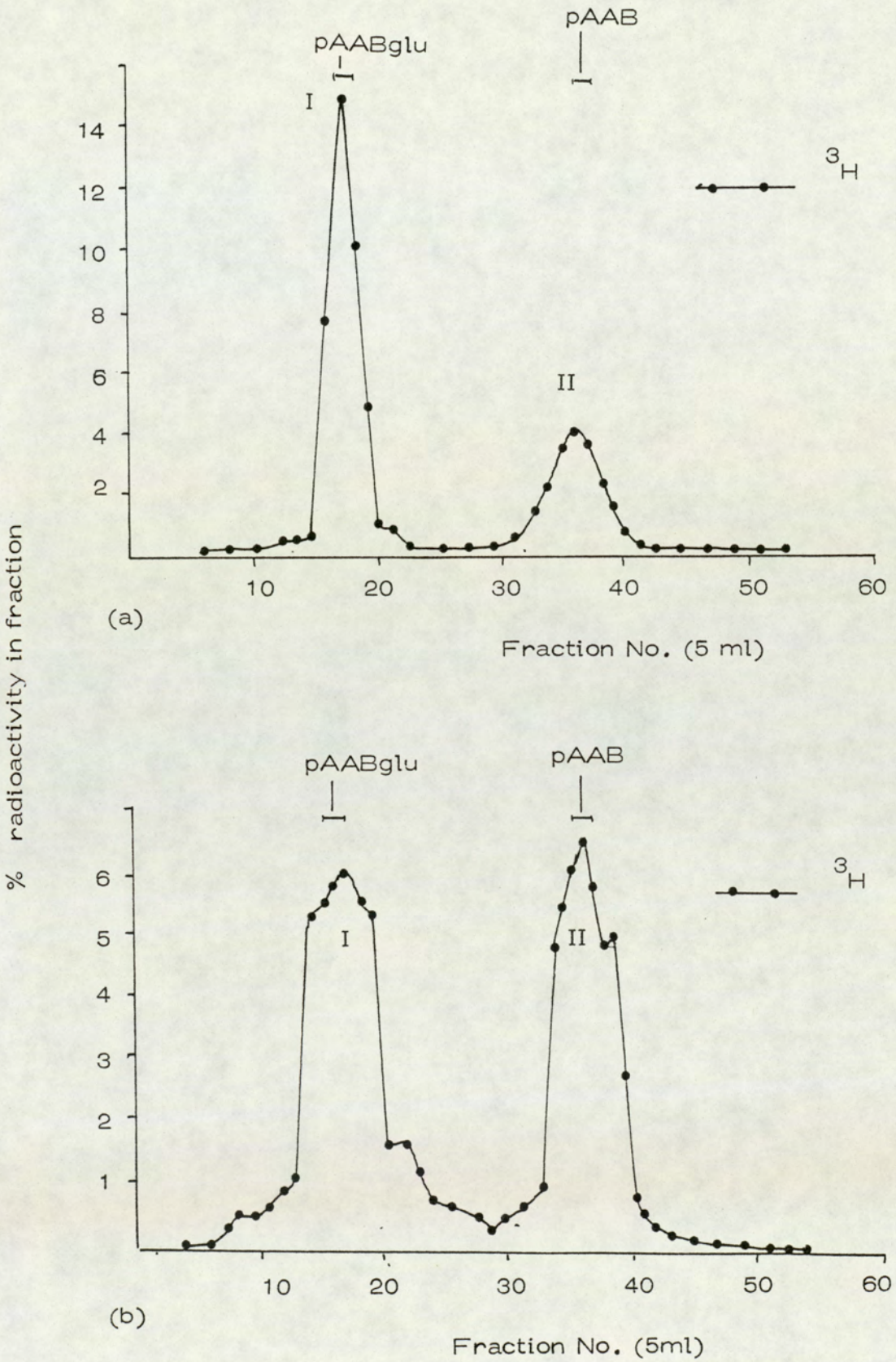


Fig. 4.4.

Sephadex G-15 chromatography of (a) 0-24h and (b) 24-48h urine samples following an i.p. dose of p-aminobenzoyl-L-glutamic acid to scorbutic guinea-pigs

Dose 242 μg/kg body wt.

CHAPTER 5

THE METABOLISM OF 5-METHYL TETRAHYDROFOLATE IN THE NORMAL GUINEA-PIG

This chapter describes the study of the metabolism in normal guinea pigs, of the monoglutamate, 5-methyltetrahydrofolate, a compound occurring widely in many foods (Shin et al., 1974; Chanarin, 1979) and also identified as a major circulating folate in the serum of mammals (Herbert et al., 1962; Ratanasthien et al., 1974). The compound is concerned with many biochemical reactions in the body, particularly, the synthesis of methionine, which requires the presence of Vitamin B_{12} as coenzyme.

In this experiment, the metabolic fate of the different parts of the 5-methyltetrahydrofolate molecule are followed by using the compound labelled either in the methyl position (as [5-^{14}C]-5MeTHF) or by using a mixture of the two radioisotopes, [2-^{14}C] and [3',5', 7,9-^{3}H]-5MeTHF.

MATERIALS AND METHODS

3 Normal Dunkin-Hartley guinea pigs (300-400 g wt.) received oral doses (400 µg/kg body wt.) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] 5MeTHF, prepared as described in Chapter 2. Another group of 4 normal guinea pigs were orally dosed with 400 µg/ kg body wt. of [5-^{14}C]5MeTHF (2 µCi per animal), commercially obtained. The prepared [2-^{14}C] and [3',5',7,9-^{3}H]-5MeTHF is biologically active but caution is required over the use of the [5-^{14}C]-5 MeTHF species, which is a 50:50 mixture of the two diastereoisomers, only one of which is

biologically active. The animals were then individually kept in metabolic cages designed for the separate collection of urine and faeces (see Chapter 2). The collection of urine and faeces, preparation of hot ascorbate liver extracts, freeze-drying and burning of the liver and faecal samples and chromatography on column and paper were carried out as described in Chapter 2. Creatinine was identified by co-chromatography with the authentic compound on Sephadex G-15 and its elution position determined by spectrophotometry. Figures presented in Table 5.1. are the average for the number of animals used and those in Table 5.2 represent the amounts for the pooled urine samples.

RESULTS AND DISCUSSION

The recovery of radioactivity in the urine, liver and faeces, following the oral administration of labelled 5-MeTHF to normal guinea-pigs is summarized in Table 5.1. Slightly more [^{14}C] (12.2% of the dose) than ^{3}H (10.8% of the dose) is excreted in the 0-24h urine and much more [^{14}C] in the 0-24h urine is excreted with [2-^{14}C] 5MeTHF than with similarly labelled [2-^{14}C] folic acid (see Section 3.1). With the mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-5MeTHF, the liver incorporates 14% of the administered [^{14}C] dose, which contrasts sharply with the 5-MeTHF compound labelled in the 5-methyl position, where there is very little (1.2%) incorporation of radioactivity. Following the oral administration of [5-^{14}C]-5MeTHF, 6% of the

TABLE 5.1.

Recovery of radioactivity in urine, liver and faeces, following the oral administration of either a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] 5 MeTHF or [5-^{14}C] 5 MeTHF to normal guinea pigs (400 µg/kg body wt.). Results are expressed as mean % of the dose ± standard deviation shown in brackets.

Radioactive Species	No. of animals		Urine 0 - 24h	Urine 24-48 h	$^{14}CO_2$	Liver (48h)	Faeces) (48h)	Total 48h
[^{3}H] + [^{14}C] 5 MeTHF	3	[^{3}H]	10.8 (0.7)	3.8 (2.0)		2.5 (1.2)	18.2 (3.1)	35.3
		[^{14}C]	12.2 (0.9)	2.9 (1.1)	N Dtm	14.0 (9.5)	33.3 (9.3)	62.4
[5-^{14}C] 5 MeTHF	4	[^{14}C]	4.0 (1.4)	1.3 (0.2)	6.0	1.2 (0.5)	24.0 (2.6)	36.5

N Dtm = Not Determined

TABLE 5.2.

Metabolites recovered in pooled normal guinea pig urine (from 3 animals) following the oral administration (400 µg/kg body wt.) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-5MeTHF.

Results expressed as % of the administered dose.

Time Period	Peak I [^{14}C]	$^{3}H_2O$	[^{3}H] p AABglu	[^{3}H] pAAB	[^{3}H] pAABglu/ [^{3}H] pAAB ratio	5 MeTHF ^{3}H	5 MeTHF ^{14}C
0 - 24h	0.5	1.3	0.04	7.6	1 : 189	0.7	0.6
24-48h	0.5	0.6	0.7	0.3	1 : 0.5	0.2	0.3

TABLE 5.3.

Metabolites present in pooled normal guinea pig urine (from 4 animals), following the oral administration of a dose (400 µg/kg body wt.) of [5-^{14}C] 5 MeTHF.

Results expressed as % of the administered dose.

Time	Peak I	Creatinine	5 MeTHF
0 - 24h	1.1	0.5	1.0
24-48h	0.3	0.3	0.2

administered [^{14}C] dose was recovered in the expired air in the first 24h.

The 0-24h and 24-48h pooled urine samples from guinea-pigs fed a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] 5 MeTHF were sequentially chromatographed on DE-52 (Figures 5.1 and 5.2) and on Sephadex G-15 (Figures 5.3 (a) and 5.3(b)). The two DE-52 chromatograms (Figures 5.1., 5.2) show fairly similar features and the radioactive peaks have been assigned similar labels. Peak I [^{14}C] eluting at about 0.10-0.12 [M] NaCl contains urea (see Section 3.1). Peak II has been characterized as $^{3}H_2O$ by the identification criteria discussed in Chapter 3. The metabolite eluting at about 0.62-0.64 [M] Na Cl contained both radioactive species, [^{3}H] and [^{14}C] (peaks IV and V of Figures 5.1, 5.2) and has been identified on both DE-52 and Sephadex G-15, and spectrophotometrically as 5-MeTHF. Peak III containing only one radioactive species [^{3}H] has been identified by rechromatography on Sephadex G-15 (Figures 5.3 (a), 5.3 (b)) and on paper as pAABglu and pAAB. A higher amount of the pAABglu metabolite was found in the 24-48 h urine (pAABglu/pAAB ratio 1:0.5) than in the 0-24h urine (pAABglu/pAAB ratio 1 : 189).

Liver extracts : Sephadex G-15 chromatography of the hot ascorbate extracts of the liver 48h after the oral administration of a [2-^{14}C]- and [3',5',7,9-^{3}H]- 5 MeTHF dose is shown in Figure 5.4. The major radioactive peak eluted close to the void volume in the position

of high molecular weight folate (folate polyglutamate). Small amounts of a metabolite containing both radioactive species appeared at fractions 35-39 and has been identified as 5-MeTHF.

The recovery of radioactivity in urine, liver, faeces and expired air after an oral dose of [5-^{14}C]-5MeTHF is summarized in Table 5.1. and has been discussed earlier. DE-52 chromatography of the 0-24h and 24-48h urine samples are illustrated in Figures 5.5. and 5.6. Peak III has been identified as 5-MeTHF and peak II as creatinine. Peak I remains unidentified, but is not methionine (see Table 5.3).

Liver extracts : Sephadex G-15 gel filtration of the hot ascorbate liver extracts is shown in Figure 5.7. It shows a high peak of radioactivity eluting at a position (Fractions 10-15) close to the void volume.

SUMMARY

1. Following the oral administration of either [5-^{14}C]-5MeTHF or a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H] 5MeTHF, radioactivity is recovered in the urine, liver, faeces, and expired air.

2. The liver was found to incorporate a higher amount of [^{14}C] radioactivity after oral [2-^{14}C] 5MeTHF than after oral [2-^{14}C]-folic acid.

3. Following an oral dose of [2-^{14}C]- and [3',5',7,9-^{3}H]-5MeTHF the urine is dominated by scission products, notably pAAB.

4. Following an oral dose of [5-^{14}C]-5MeTHF, the urinary metabolites identified were 5MeTHF and creatinine. Methionine was absent.

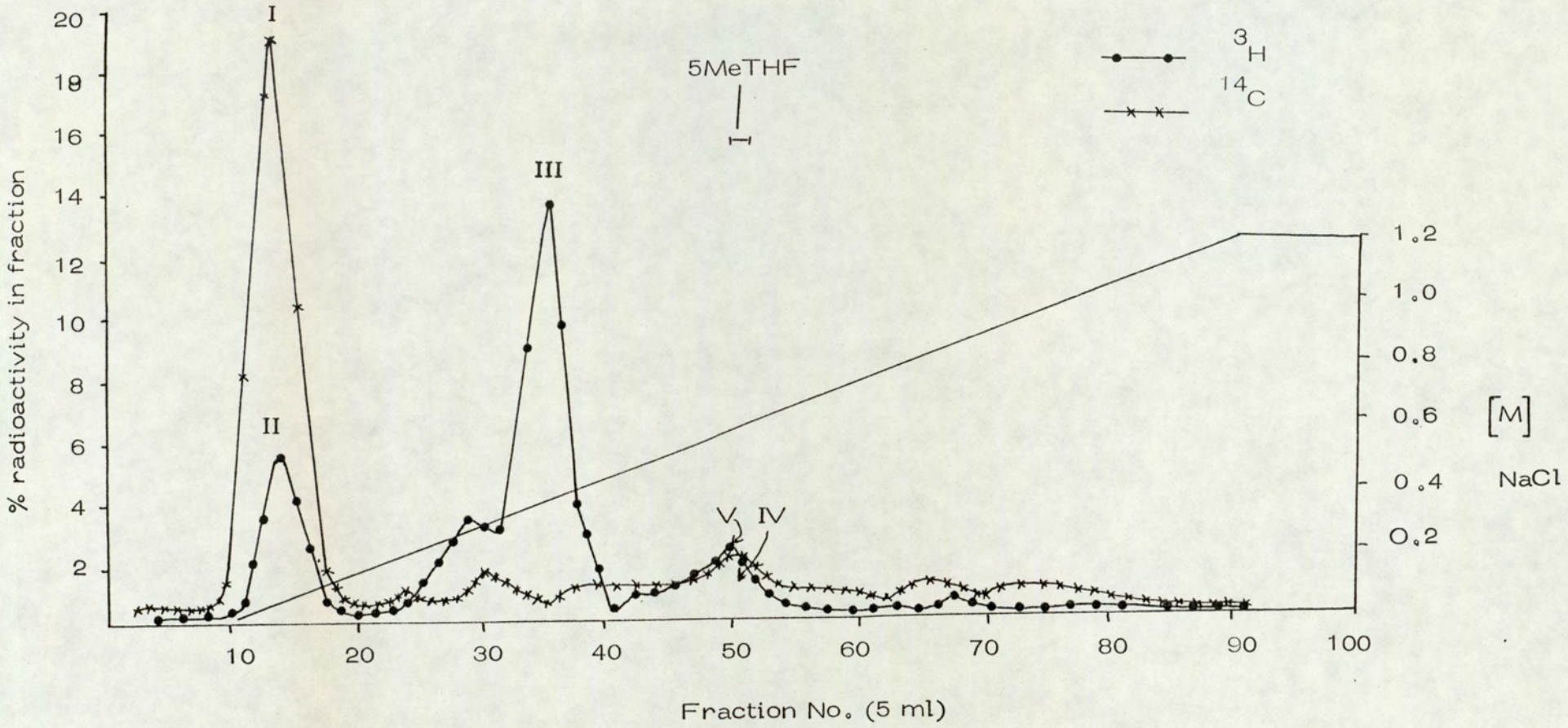


Fig. 5.1

DE-52 chromatography of 0-24h normal guinea-pig urine following an oral administration of a mixture of $[2-^{14}C]$ and $[3',5',7,9-^{3}H]$ - 5MeTHF Dose 400 µg/kg body wt.

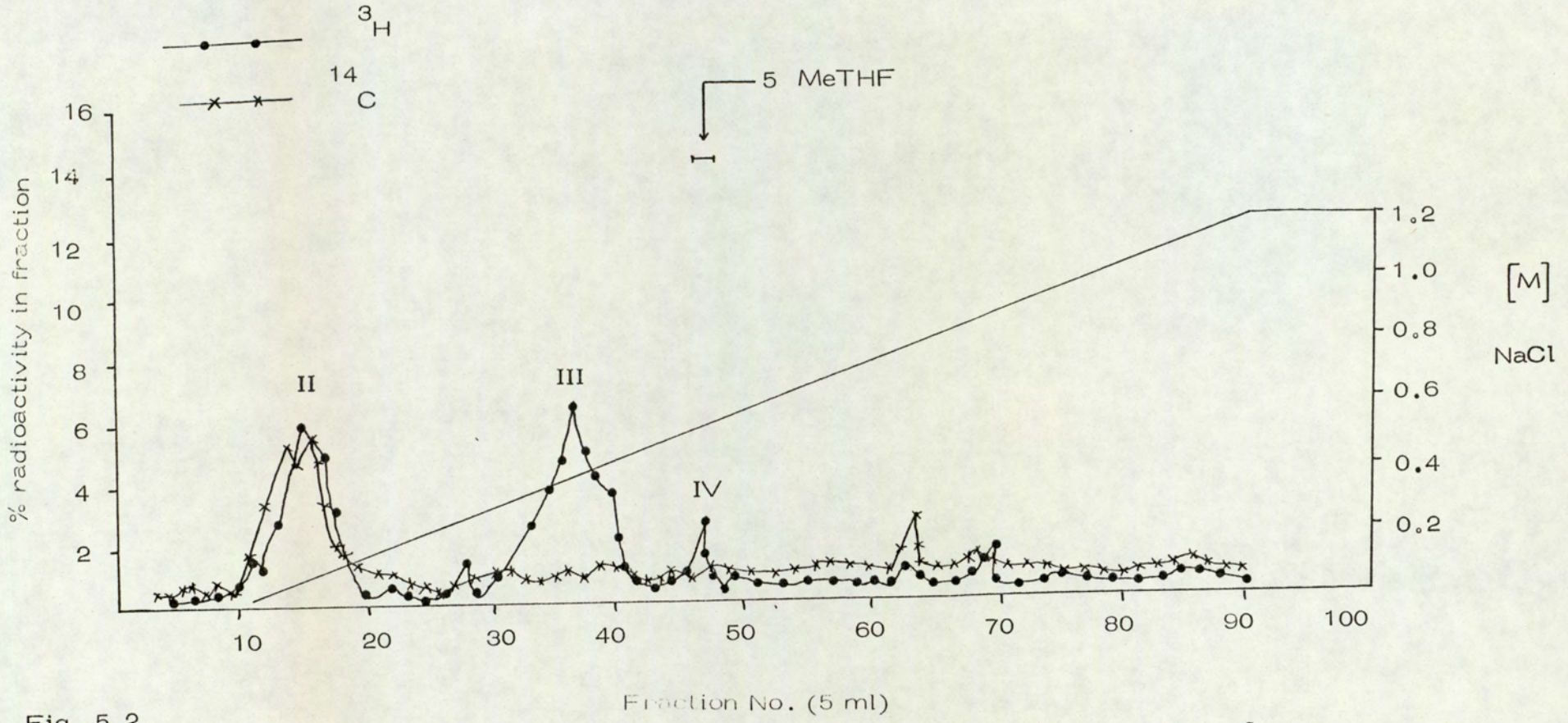


Fig. 5.2.

DE-52 chromatography of normal 24-48h guinea-pig urine following an oral dose of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ - 5MeTHF Dose · 400 µg/kg body wt.

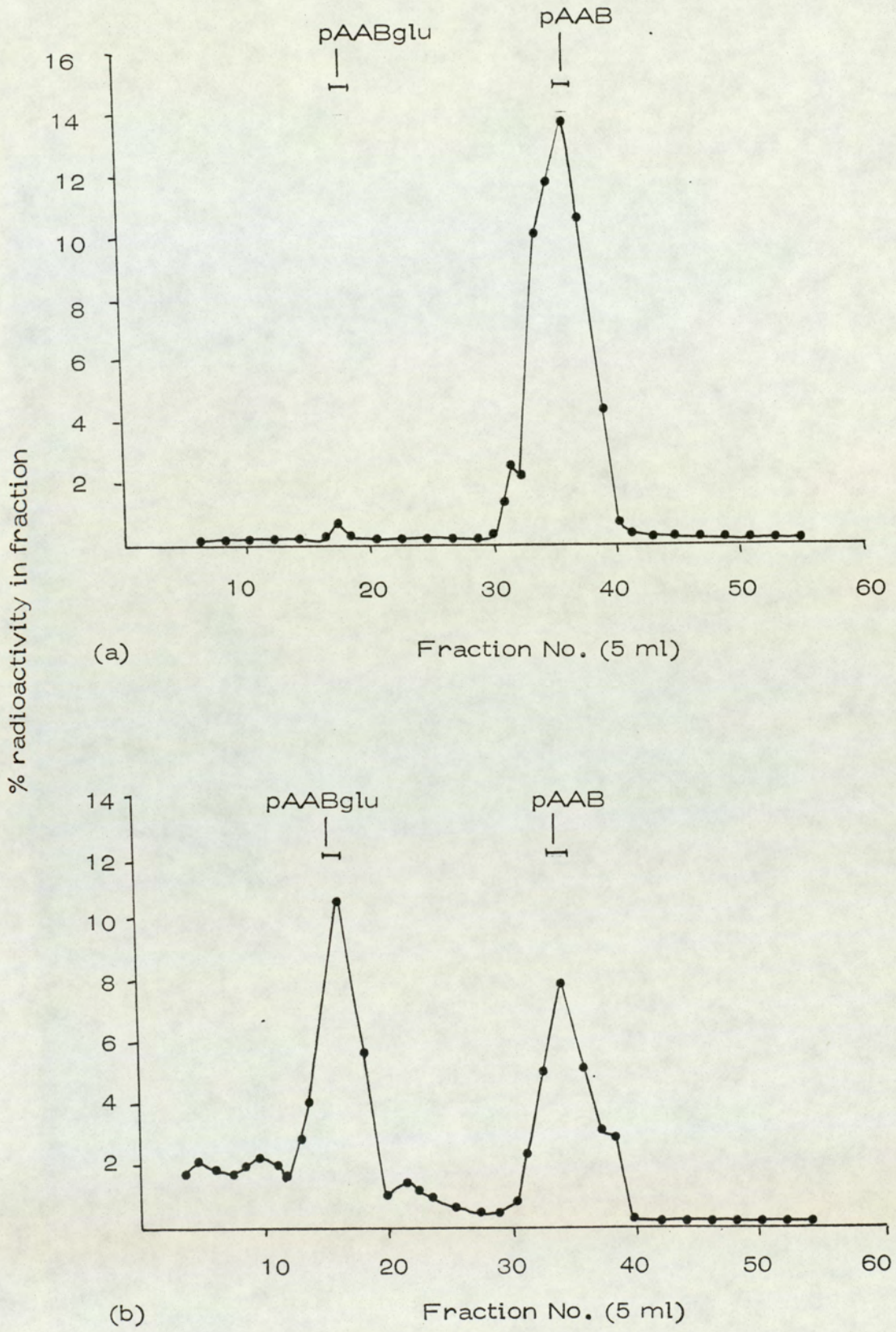


Fig. 5.3. Sephadex G.15 chromatography of (a) peak III from DE-52 chromatography of 0-24h guinea-pig urine (b) peak III of DE-52 chromatography of 24-48h guinea-pig urine, after an oral dose of $[^{3}H]$ and $[^{14}C]$ labelled 5 MeTHF Dose: 400 µg/kg body wt

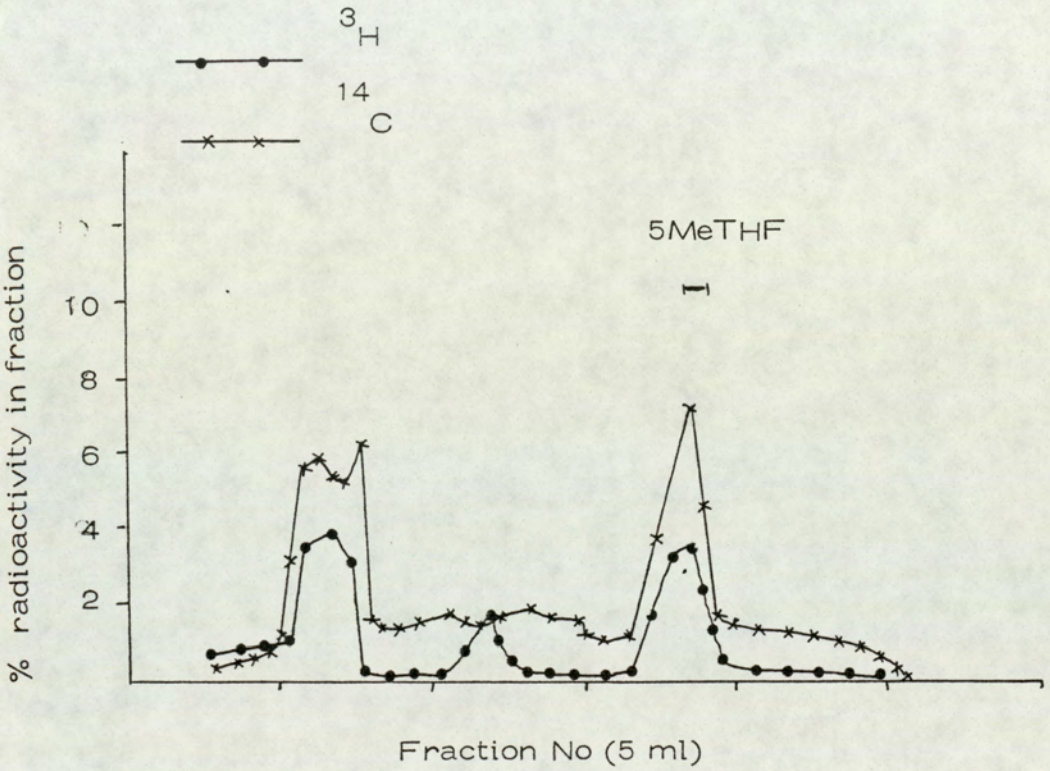


Fig. 5.4.

Sephadex G-15 chromatography of hot ascorbate liver extracts 48h after an oral dose of a mixture of $\left[2\text{-}^{14}C\right]$ and $\left[3',5',7,9\text{-}^{3}H\right]$ -5 Me-THF

Dose 400 µg/kg body wt.

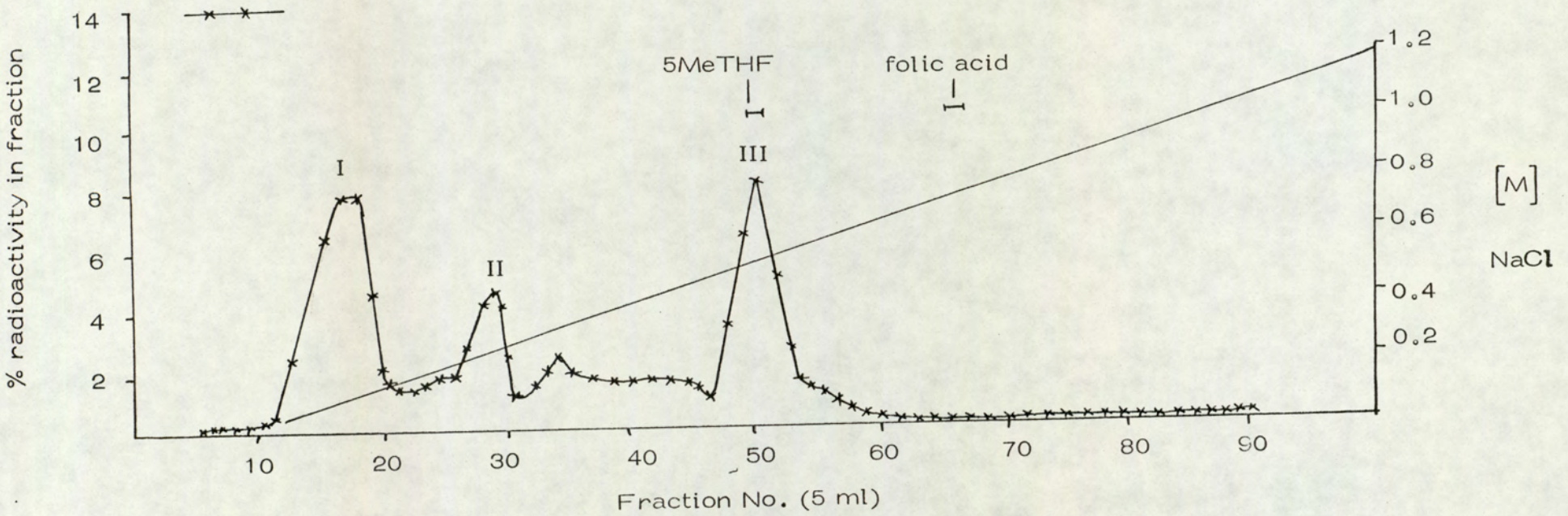


Fig. 5.5. DE-52 chromatography of 0-24h normal guinea-pig urine after oral administration of $\left[5\text{-}^{14}C\right]$ - 5 MeTHF Dose: 400 µg/kg body wt.

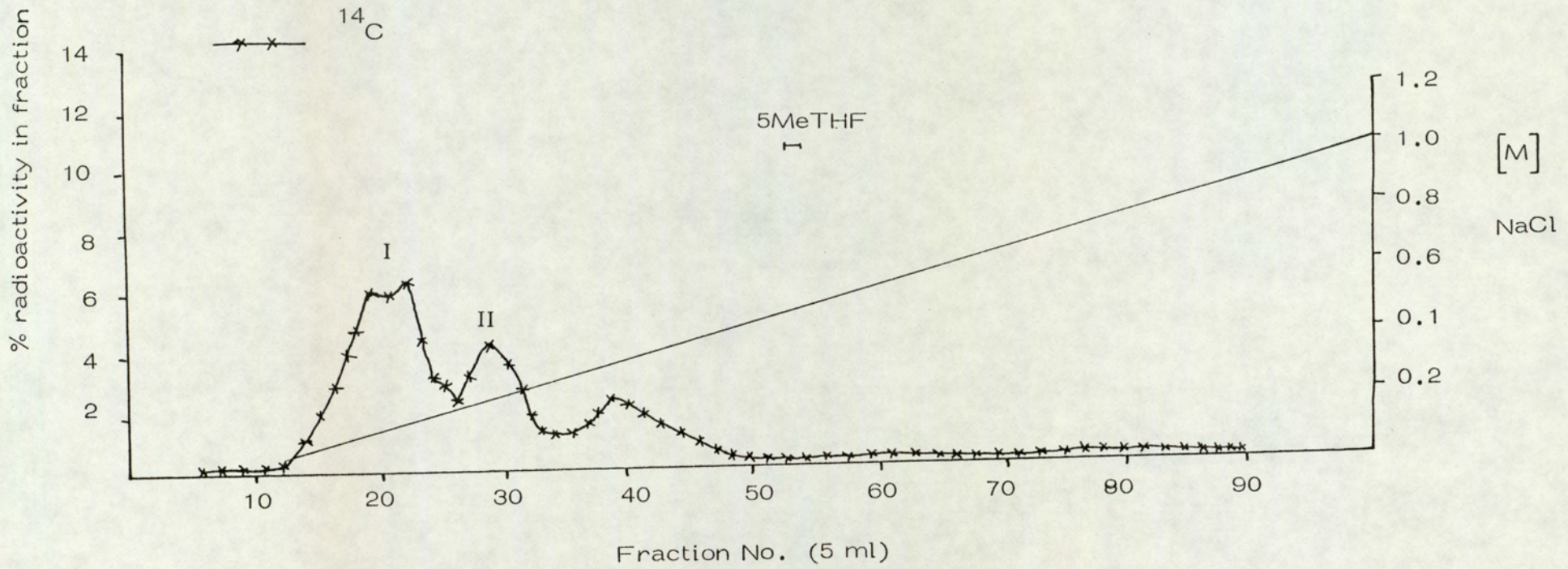


Fig. 5.6 DE-52 chromatography of 24-48h normal guinea-pig urine after an oral administration of $\left[5\text{-}^{14}C\right]$-5 MeTHF Dose 400 µg/kg body wt.

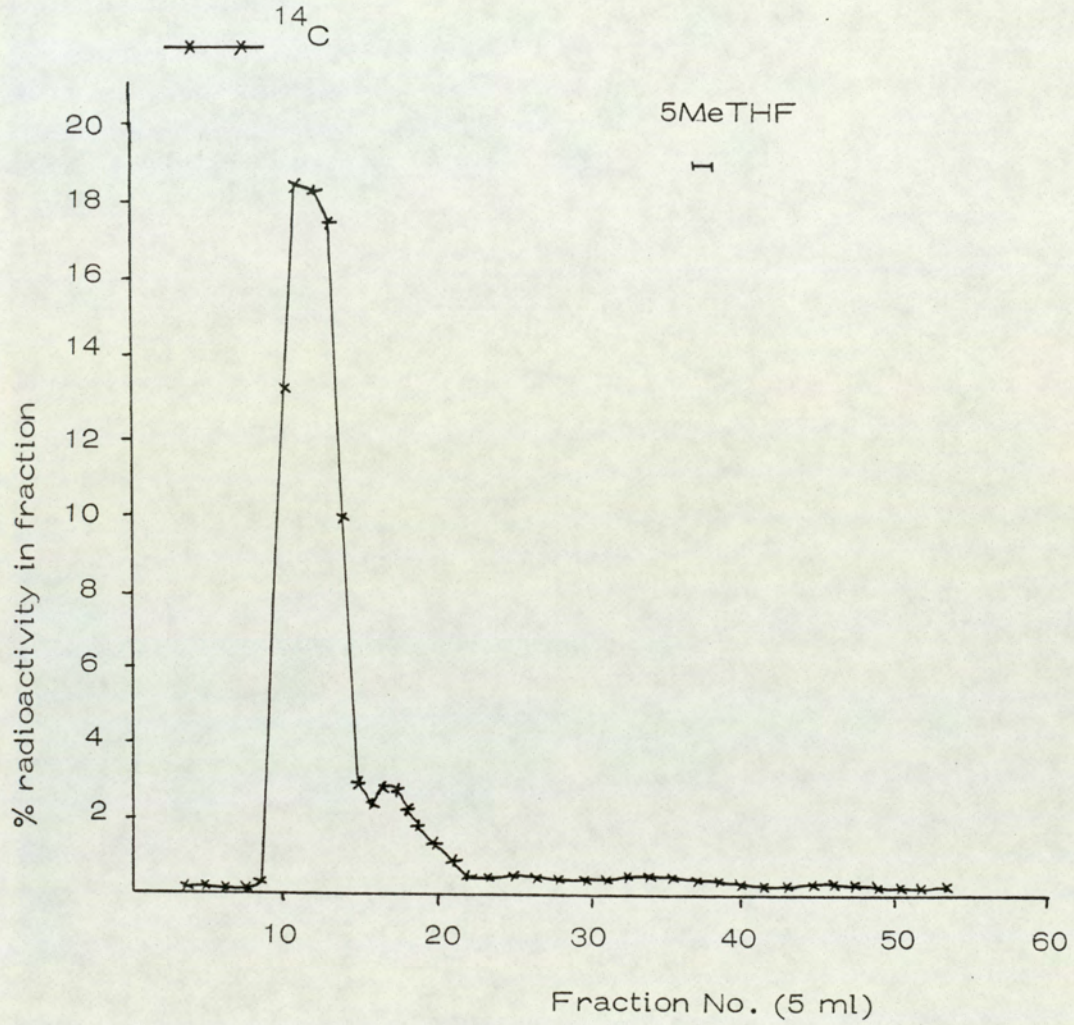


Fig. 5.7.

Sephadex G-15 of hot ascorbate liver extracts 48h after an oral administration of $\left[5-^{14}C\right]$-5-MeTHF to normal guinea-pigs

Dose 400 µg/kg body wt.

CHAPTER 6

THE METABOLISM OF 10-FORMYL FOLIC ACID IN NORMAL GUINEA PIGS

This Chapter describes the metabolism of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folic acid in normal guinea pigs.

MATERIALS AND METHODS

10-Formyl folic acid (both radioactive and 'cold') were prepared as described in Chapter 2. 5 Normal male guinea pigs were orally dosed (400 µg/kg body wt) with a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folic acid. The animals were then kept in individual metabolic cages designed for the separate collection of urine and faeces, food and water being provided ad libitum. The collection of urine and faeces, extraction of livers with hot ascorbate and the freeze-drying and burning of the liver and faecal samples were carried out as described in Chapter 2. Identification of metabolites in urine and analysis of gut flushings were carried out as described in Chapter 3.

RESULTS AND DISCUSSION

The recovery of radioactivity in urine, liver and faeces are summarized in Table 6.1. Considerably more [^{3}H] than [^{14}C] is excreted in the urine but excretion via faeces show nearly comparable figures for the two isotopes. Incorporation of radioactivity in the liver is very low (1%), now characteristic of oral doses. Althaugh 43.5% of the administered [^{3}H] radioactivity can be accounted for in the samples analysed, the recovery of [^{14}C] radio-

TABLE 6.1

Recovery of radioactivity in urine, liver and faeces of 5 normal guinea pigs, following an oral dose (400 μg/k g body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folic acid. Results are expressed as the mean % of the administered dose ± standard deviation (shown in brackets)

	Urine		Liver	Faeces	Total
	0-24h	24-48h	(48h)	48h	48h
[^{3}H]	35.1(4.6)	2.3 (0.5)	0.6 (0.2)	5.5 (1.0)	43.5
[^{14}C]	4.2(2.0)	0.5 (0.3)	0.9 (0.1)	6.2 (1.3)	11.8

TABLE 6.2

Metabolites present in guinea pig urine following on oral dose of [^{3}H] and [^{14}C] labelled 10-formyl folic acid (400 μg/k g body wt)

Results expressed as % of administered dose

	^{14}C peak I	$^{3}H_2O$	[^{3}H]pAABglu	[^{3}H]pAAB	10-CHOFA
0-24h	2.2	1.4	1.0	25.3	1.1 [as ^{14}C]
24-48h	1.4	0.2	0.3	1.3	0.5 [as ^{14}C]

activity (11.8%) still remains very low.

The 0-24h and 24-48h urine samples were sequentially chromatographed on DE-52 (Figures 6.1 and 6.2) and Sephadex G-15 (Figures 6.3, 6.4 and 6.5). Because of the similarity in features of the two DE-52 chromatograms (Figures 6.1, 6.2), similar labels have been assigned. The ^{14}C peak I has already been discussed (see Section 3.1). Peak II [^{3}H] has been characterized as $^{3}H_2O$ on the criteria earlier discussed (see Chapter 3). Peak III ([^{3}H]-only), on rechromatography on Sephadex G-15 (Figures 6.3, 6.4) was resolved into two [^{3}H] components and identified respectively as pAABglu (minor metabolite) and pAAB (major metabolite) on both Sephadex G-15 and paper. Peak IV [^{14}C] (which overlaps slightly with [^{3}H] peak III) on rechromatography on Sephadex G-15 (Figure 6.5) was also resolved into two components eluting at fractions 18-22 and 32-38 respectively. Fraction 18-22 contained both [^{3}H] and [^{14}C] radioactive species and was identified as 10-formyl folic acid while fractions 32-38 contained only [^{3}H] and was identified as pAAB.

Liver extracts : Sephadex G-15 chromatography of hot ascorbate liver extracts, 48h after dosing is illustrated in Figure 6.5. A very small peak containing both radioactive species elutes at a position close to the void volume (fractions 10-14) and another small peak with both species at fractions 16-19. A major peak containing [^{3}H] only elutes at fractions 21-26.

Gut flushings: De-52 chromatography of gut flushings 2h after an oral dose of [^{3}H] and [^{14}C] labelled 10-formyl folic acid show 10-formylfolate to be the major compound present, with small amounts of $^{3}H_2O$. No 5-MeTHF or folic acid was present. (Figure 6.6).

SUMMARY

1. Following the oral administration of [^{3}H] and [^{14}C] labelled 10-formyl folic acid to normal male guinea pigs, radioactivity was recovered in the urine, liver and faeces.

2. Chromatographic analysis of the urine samples showed very little of the administered 10-formyl folic acid to be excreted intact.

3. The urine samples were dominated by scission products, notably pAAB.

4. 2h after dosing, gut flushings contained mostly 10-CHOFA with small amounts of $^{3}H_2O$.

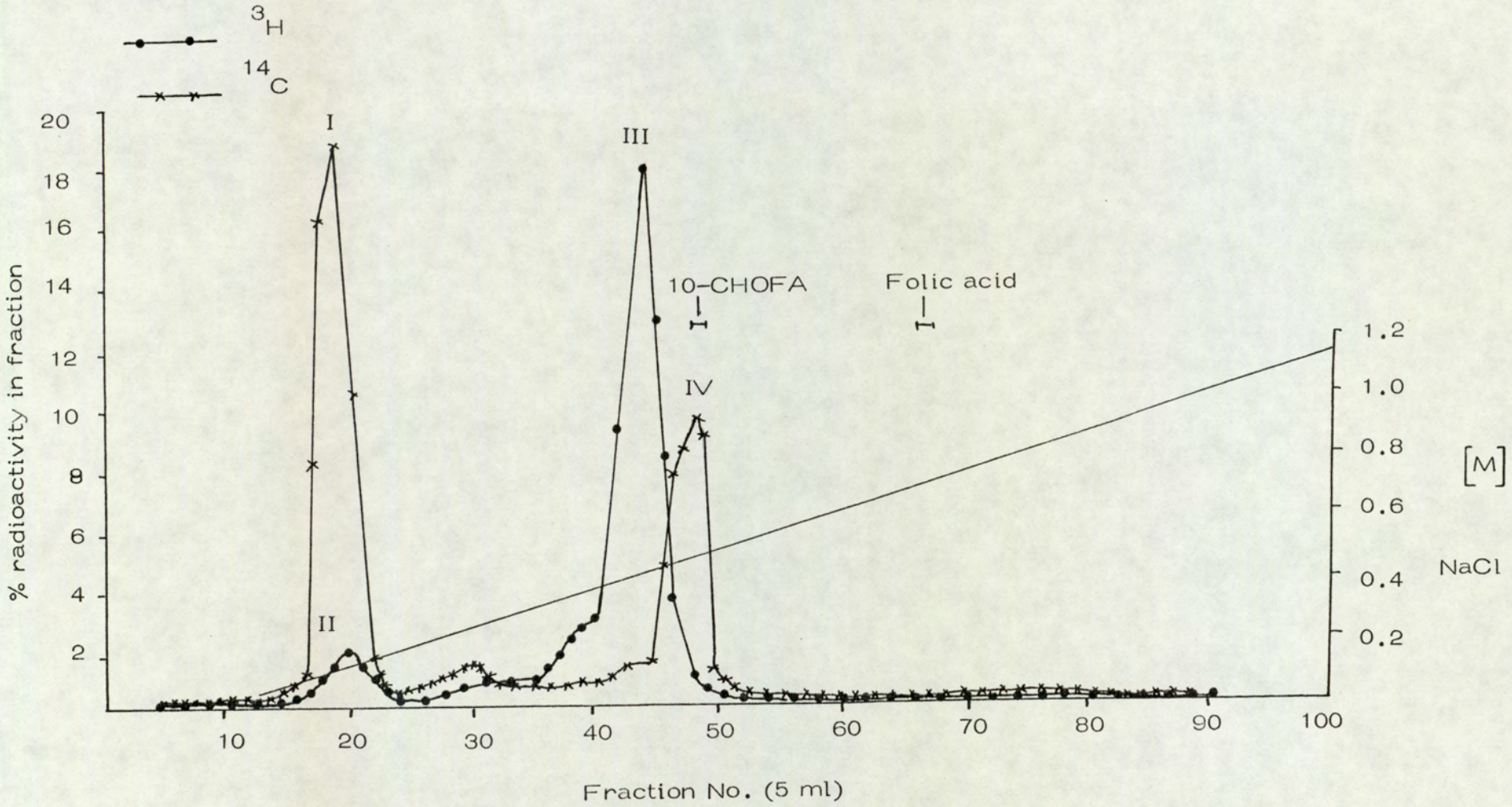


Fig. 6.1. DE-52 chromatography of normal 0-24h guinea-pig urine after oral administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ - 10 formyl folic acid

Dose· 400 µg/kg body wt.

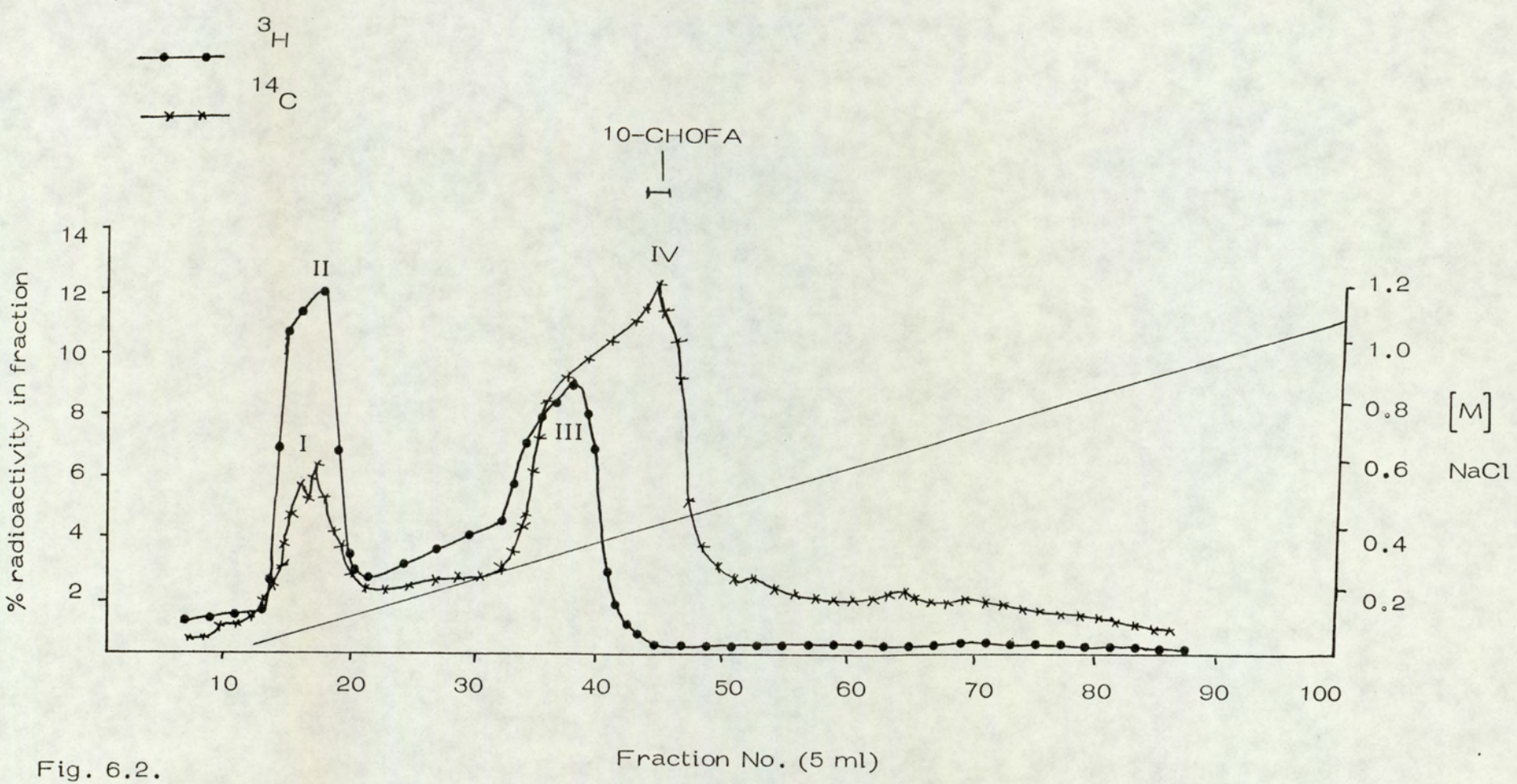


Fig. 6.2.

DE-52 chromatography of 24-48h normal guinea-pig urine after oral administration of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$-10 formyl folic acid Dose: 400 µg/kg body wt.

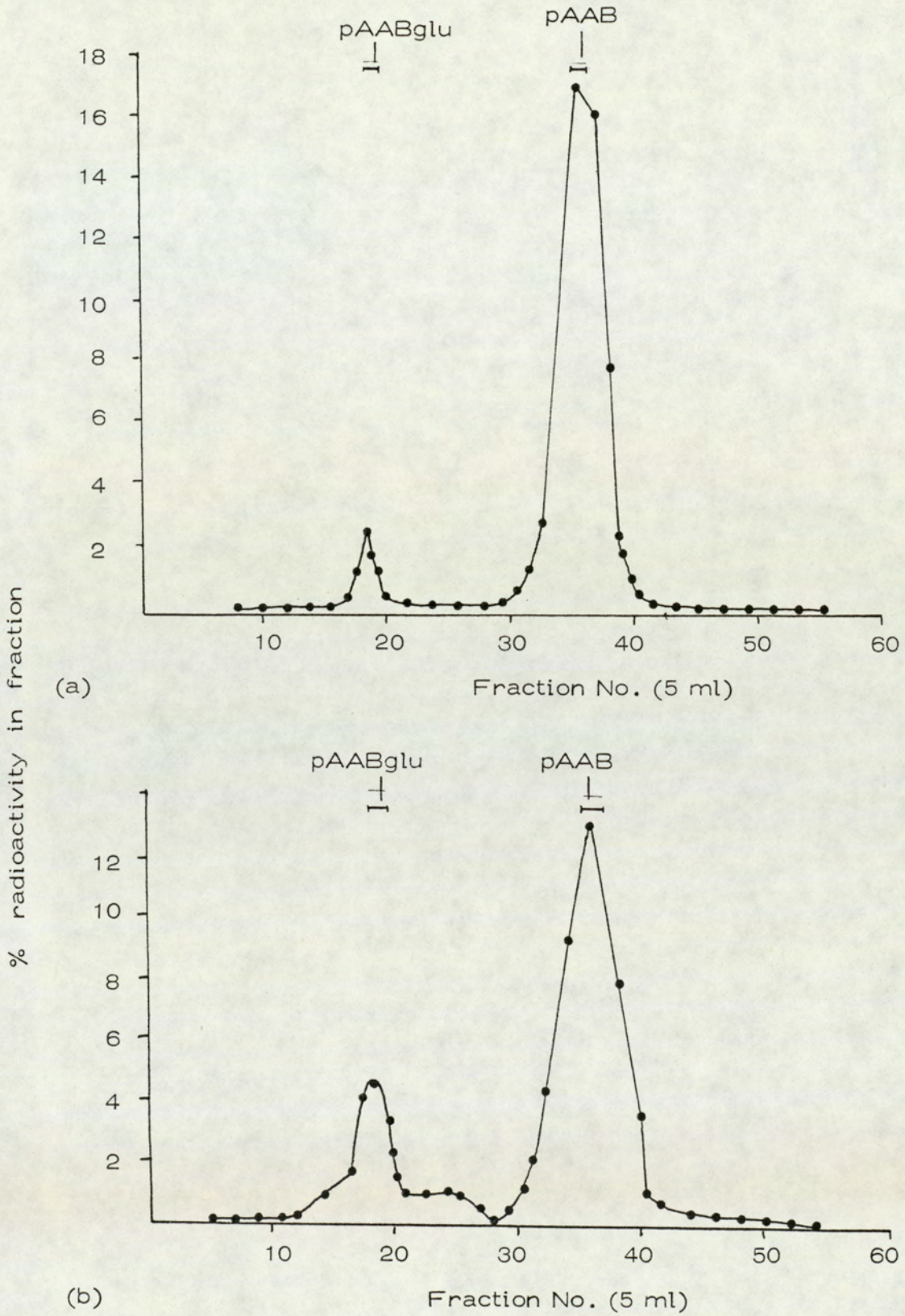


Fig. 6.3. Sephadex G-15 chromatography of (a) ^{3}H peak III from DE-52 of 0-24h urine (b) ^{3}H peak III from DE-52 of 24-48h urine, after an oral dose of labelled 10-CHOFA Dose· 400 µg/kg body wt.

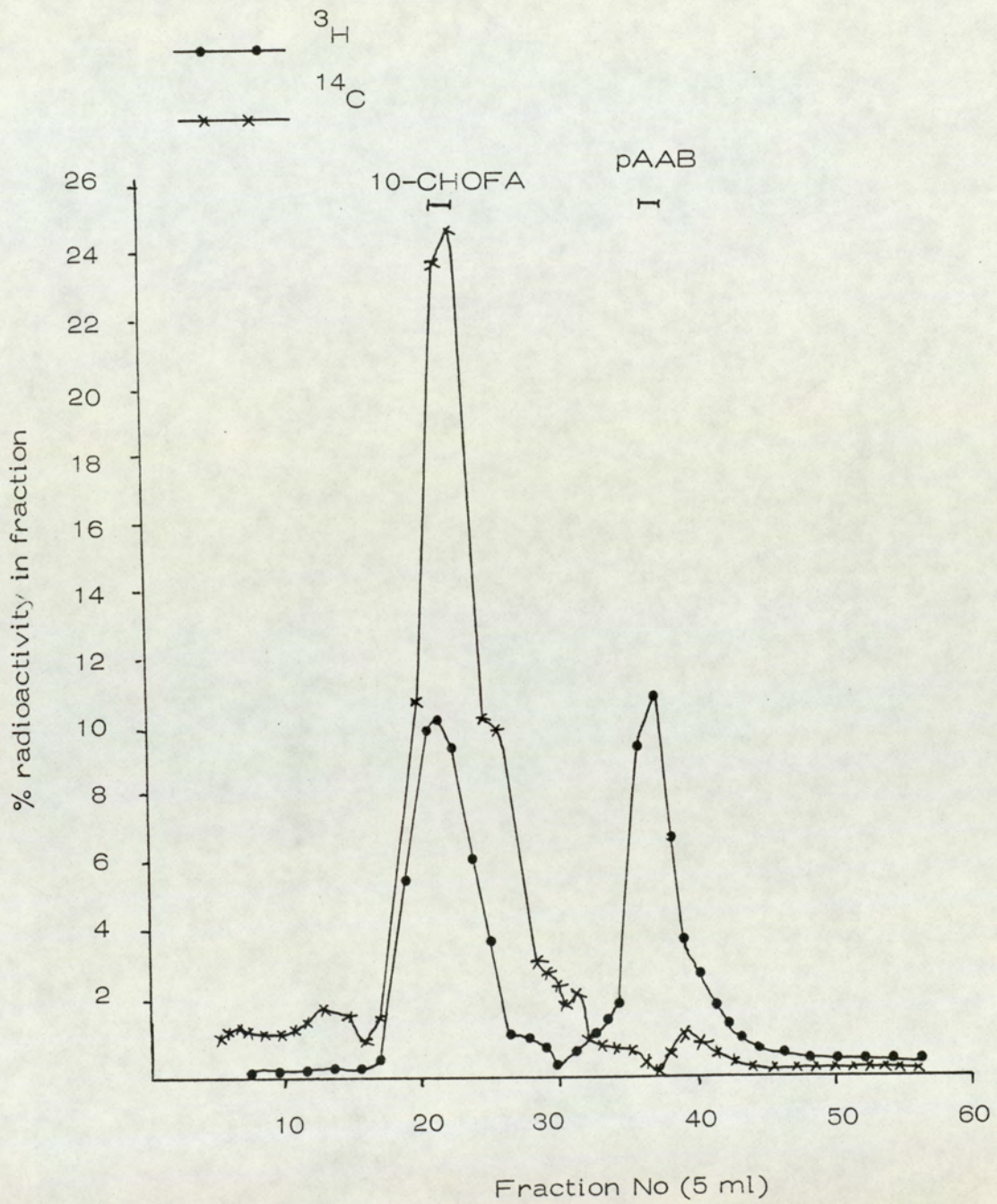


Fig. 6.4. Sephadex G-15 chromatography of peak IV from DE-52 chromatography of 0-24h guinea-pig urine following an oral dose of labelled 10-CHO-FA

Dose· 400 µg/kg body wt.

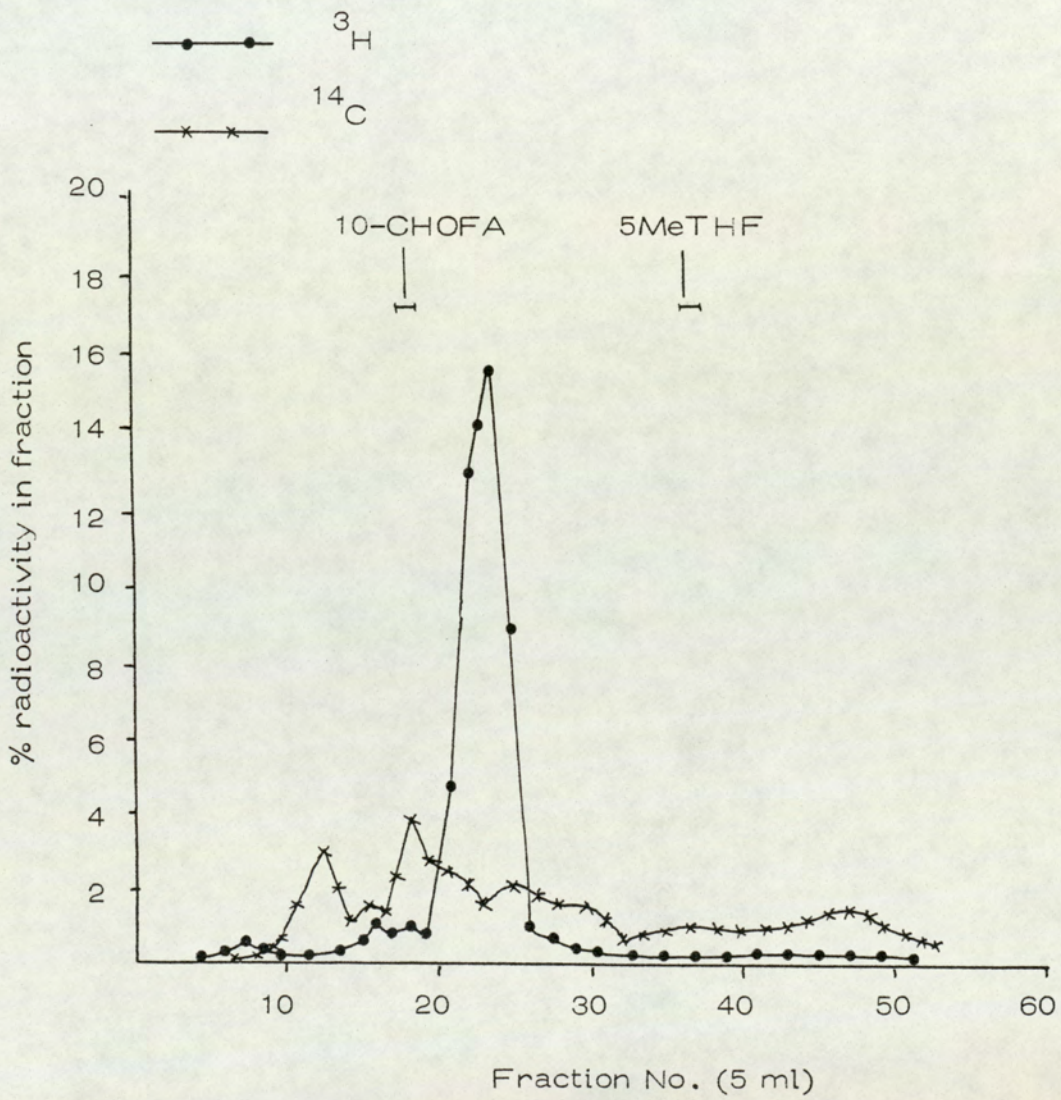


Fig. 6.5. Sephadex G-15 chromatography of hot ascorbate liver extracts after an oral dose of labelled 10-CHOFA

Dose· 400 µg/kg body wt.

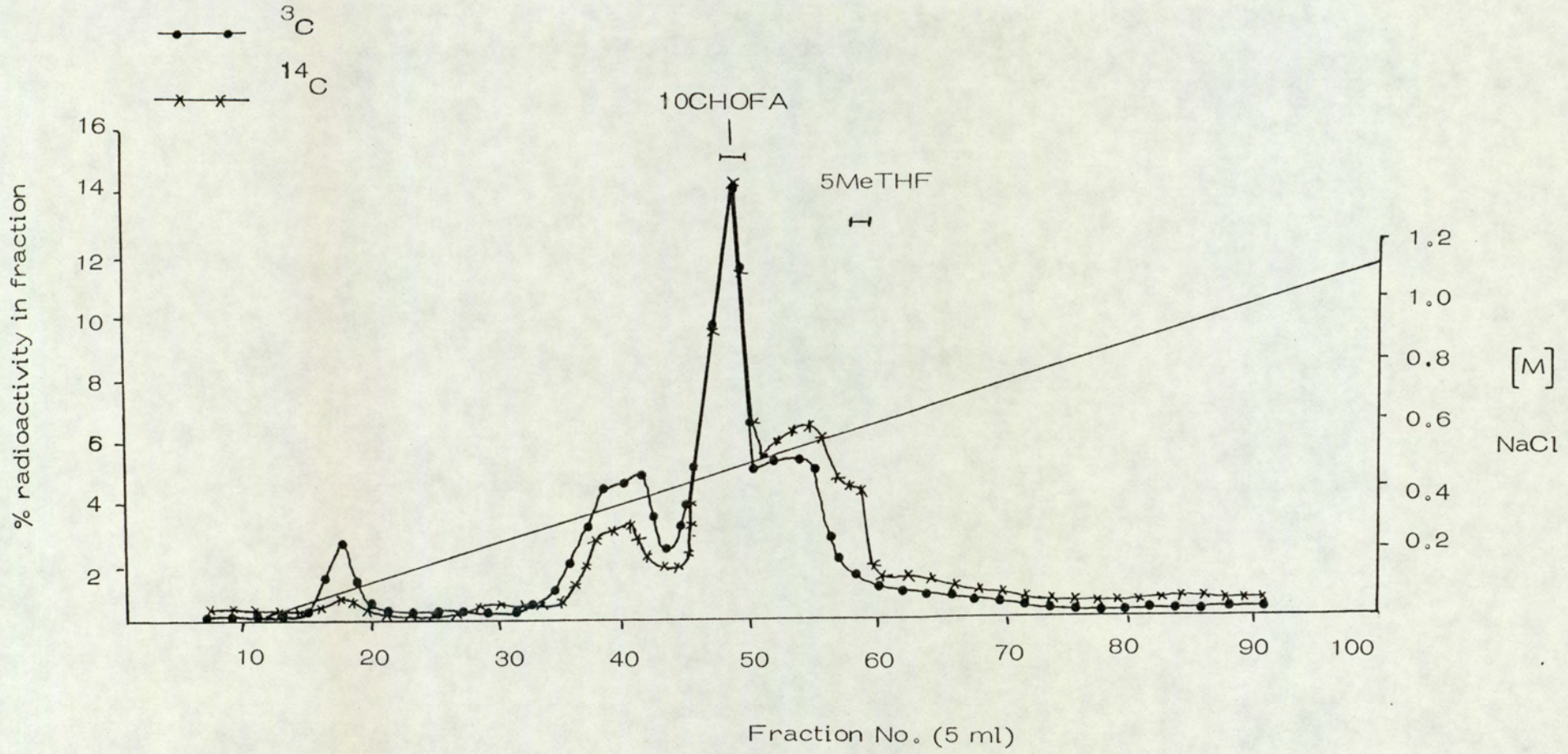


Fig. 6.6. DE-52 Chromatography of gut flushings 2h after an oral dose of a mixture of $[2\text{-}^{14}C]$ and $[3',5',7,9\text{-}^{3}H]$ 10-formyl-folic acid

Dose 400 µg/kg body wt.

CHAPTER 7

POLYGLUTAMATE FORMATION IN THE LIVERS OF NORMAL GUINEA PIGS

This Chapter describes the study of the biosynthesis of polyglutamate in the liver of normal guinea pigs following oral doses of labelled folic acid.

MATERIALS AND METHODS

Normal male guinea pigs were orally administered with a dose (400 μg/kg body wt.) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Pairs of guinea pigs were killed 1, 2, 4, 6, 8 and 12 h after dosing, the livers immediately removed and dropped onto boiling phosphate buffer containing ascorbate. Liver extracts were prepared as described earlier (see Chapter 2). Radioactivity in each of the extracts was measured before they were chromatographed on Sephadex G-15.

RESULTS AND DISCUSSION

The amount of radioactivity recovered in the livers of the guinea pigs are summarized in Table 7.1. All the livers up to 12 h after oral dosing (in this experiment), and up to 48 and 72 h after oral dosing in previous experiments (see Chapter 3) show very little incorporation of radioactivity.

Sephadex G-15 chromatography of the respective hot ascorbate liver extracts are illustrated in Figures 7.1. - 7.6. All the chromatograms show a metabolite containing both [^{3}H] and [^{14}C]

radioactive species eluting at a position close to the void volume (in the position of folate polyglutamate) at fractions 10 - 15 and a peak labelled with [^{3}H] only at fractions 20 - 25.

TABLE 7.1.

Recovery of radioactivity in the livers of normal male guinea pigs following oral doses (400 µg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid.

Results expressed as % of administered dose.

Time after dosing	Liver radioactivity		Liver polyglutamate	
	^{3}H	^{14}C	^{3}H	^{14}C
1 h	0.1	0.1	0.03	0.07
2 h	0.1	0.1	0.02	0.08
4 h	0.1	0.2	0.02	0.1
6h	1.1	0.5	0.4	0.4
8 h	0.6	0.4	0.3	0.3
12h	0.6	0.4	0.02	0.2

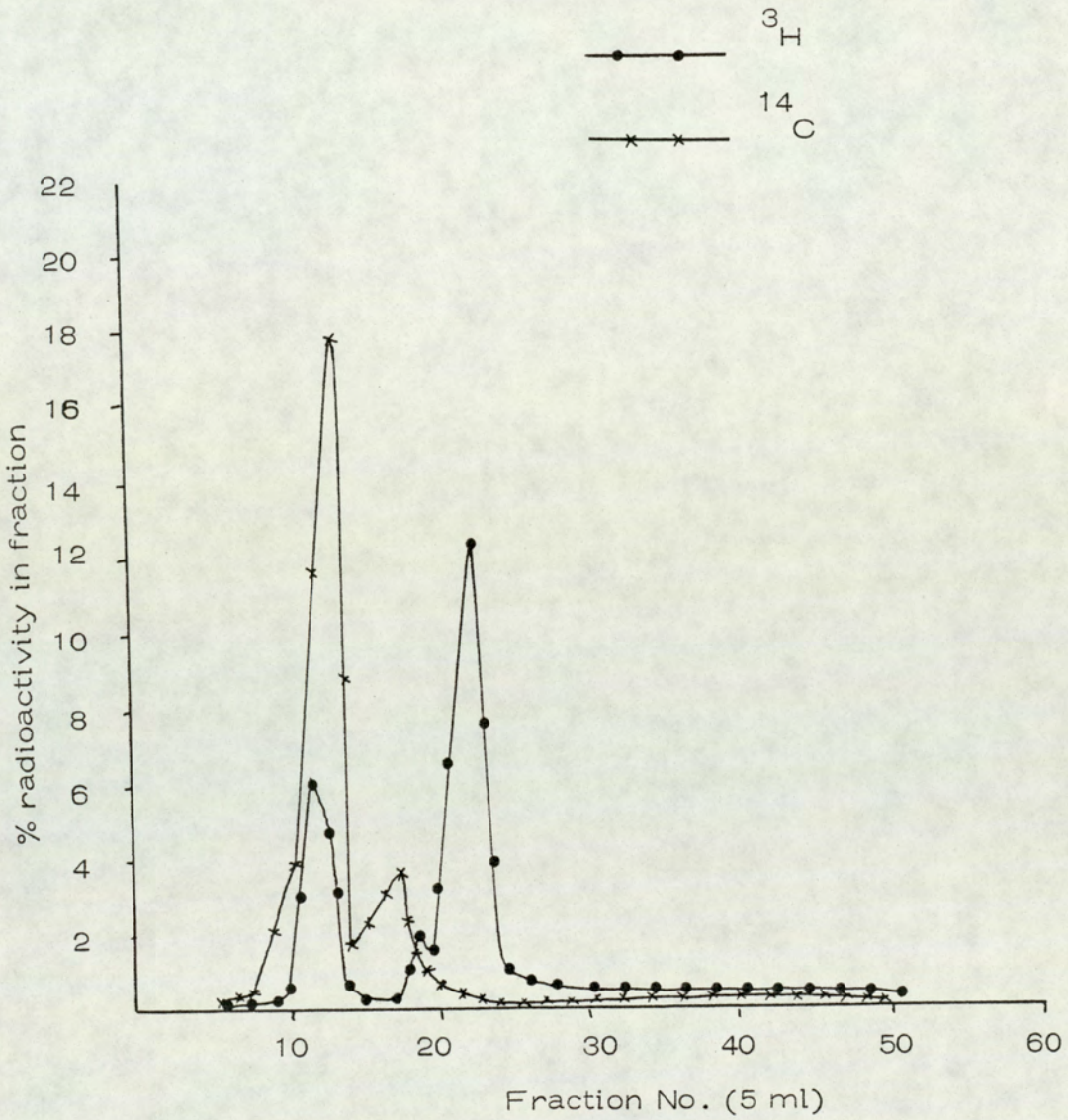


Figure 7.1. Sephadex G-15 chromatography of hot ascorbate liver extracts 1h after an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid to normal guinea pigs.

Dose: 400 µg/kg body wt.

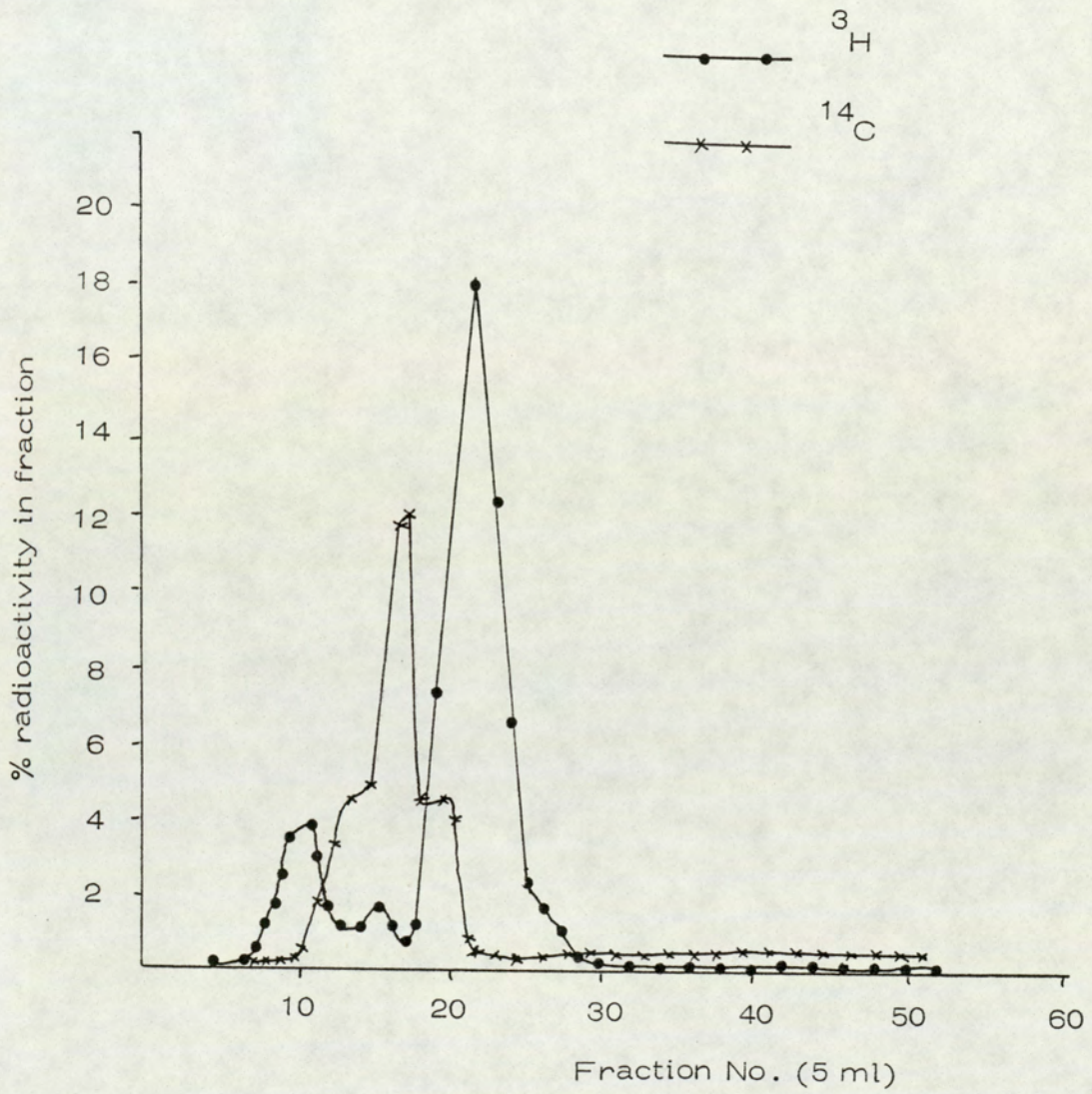


Figure 7.2.

Sephadex G-15 chromatography of hot ascorbate extracts of normal guinea pig livers 2h after an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Dose: 400 µg/kg body wt.

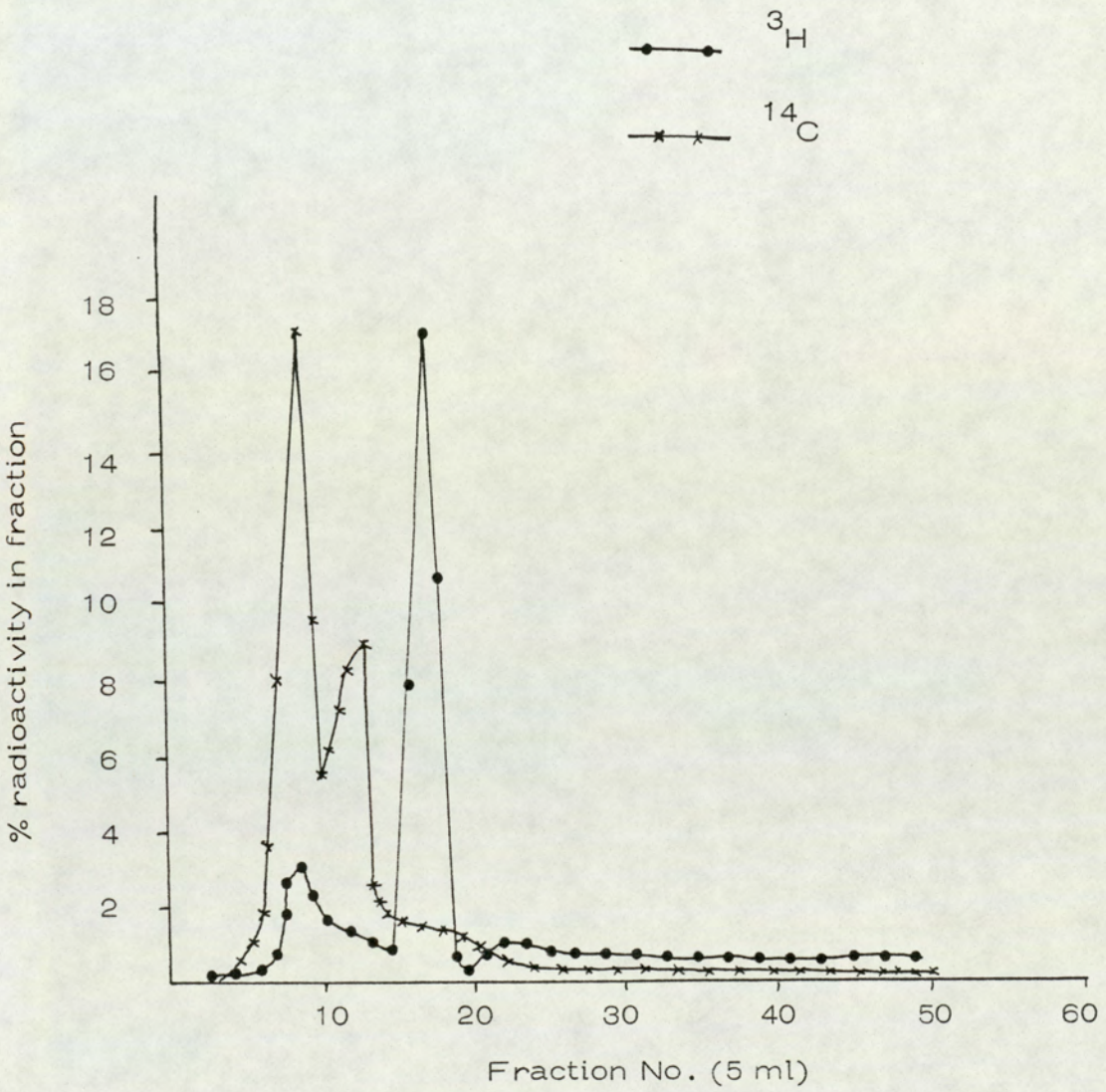


Figure 7.3.

Sephadex G-15 chromatography of hot ascorbate extracts of livers from normal guinea-pigs 4h after oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Dose 400 µg/kg body wt.

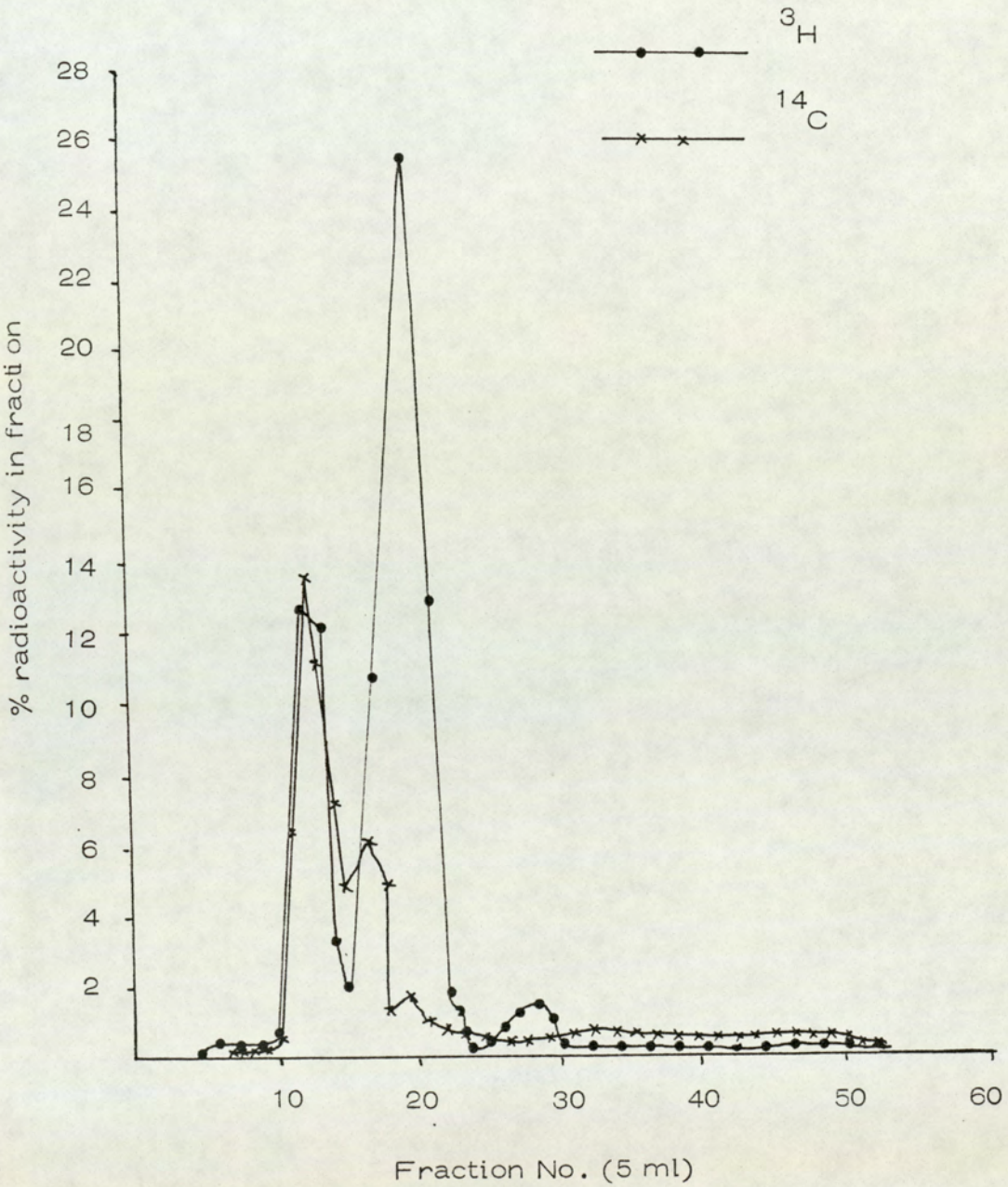


Figure 7.4. Sephadex G-15 chromatography of hot ascorbate extracts of livers from normal guinea pigs 6h after an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid.

Dose: 400 µg/kg body wt.

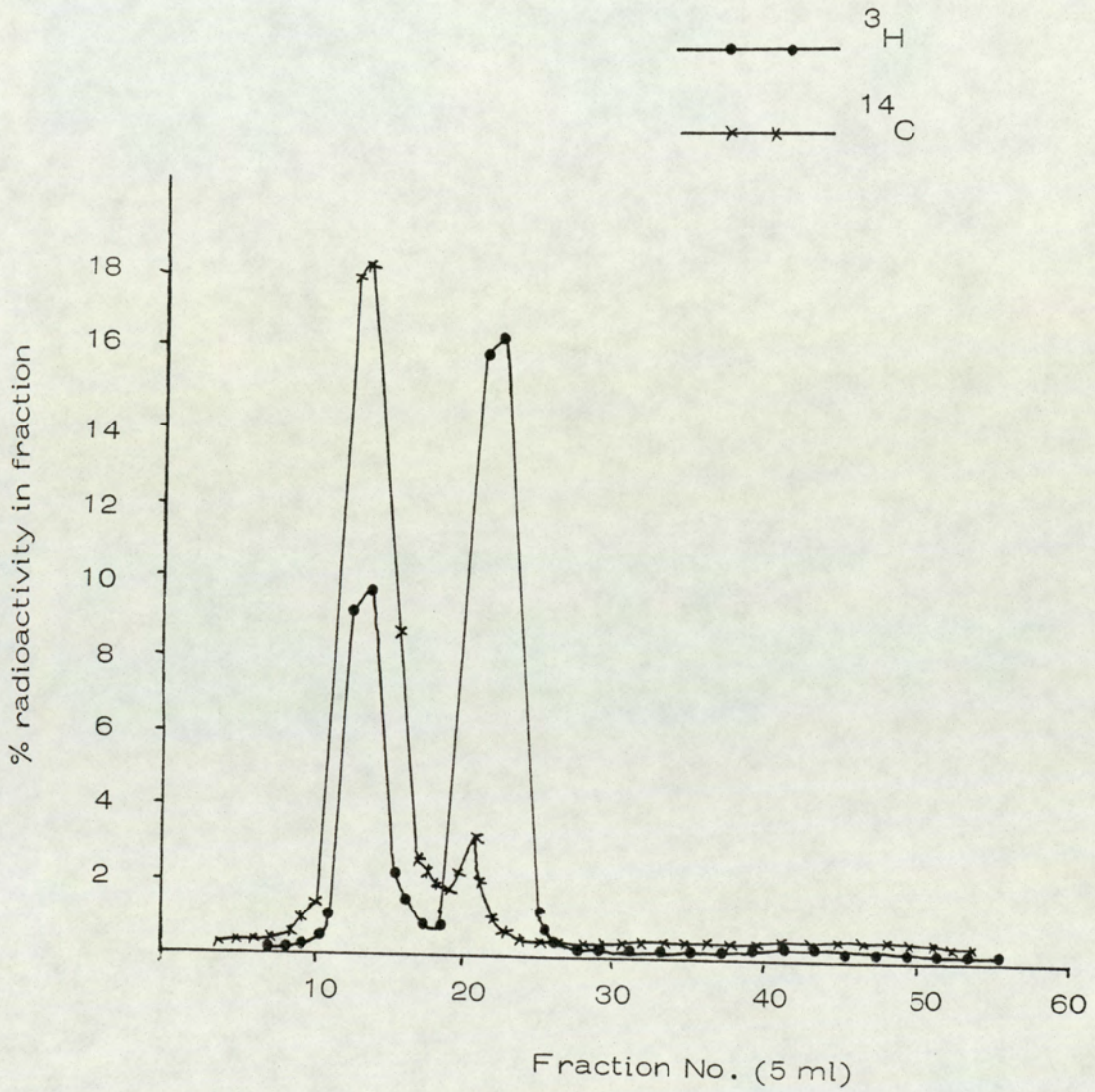


Figure 7.5. Sephadex G-15 chromatography of hot ascorbate extracts of livers from normal guinea-pigs 8h after a dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid.

Dose : 400 µg/kg body wt.

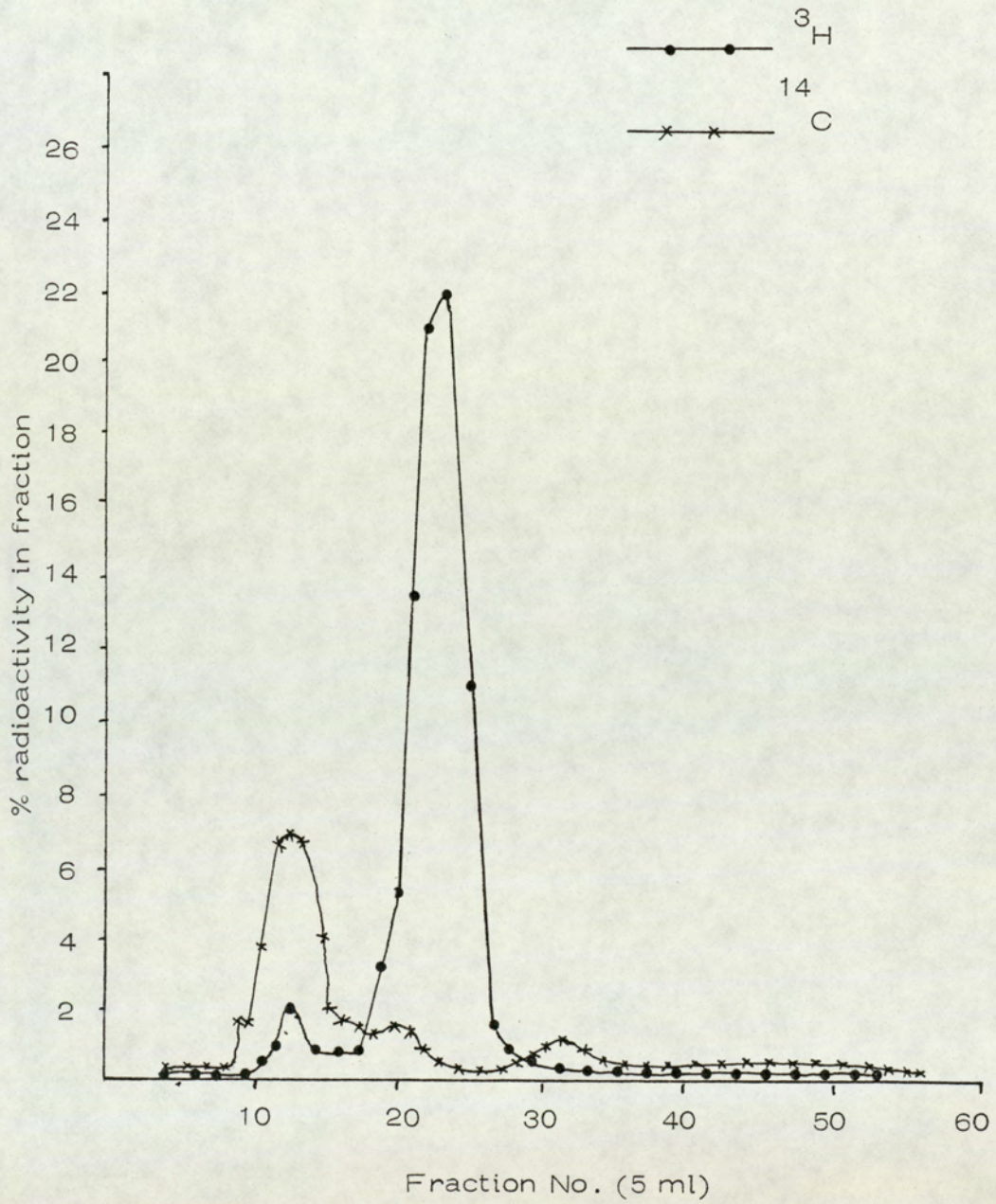


Figure 7.6. Sephadex G-15 chromatography of hot ascorbate extracts from livers of normal guinea-pigs 12h after an oral dose of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid.

Dose 400 µg/kg body wt.

CHAPTER 8

DISCUSSION

The experiments described in the previous chapters bring into focus the complexity of folate metabolism not only in the guinea pig but in mammals in general. Marked species variation in the extent of urinary excretion of folate catabolites have been found to occur in the guinea pig, in the rat and in man.

Following oral doses (Table 8.1) of either [2-^{14}C]- or [3',5',-7,9-^{3}H]-folic acid or a mixture of both radioactive species, radioactivity was recovered in the urine, liver and faeces but little in the kidneys, heart, spleen, gut wall, stomach wall or in bile (Table 8.2). The major portion of the urinary radioactivity was excreted in the first 24 hours immediately following oral dosing after which, there was a sharp decline in the 24-48h sample (Table 8.1). At oral doses of 160 and 400 µg/kg body wt of [^{3}H]-only labelled folic acid, the major portion of the radioactivity was excreted in the urine and a smaller amount via the faeces, while the liver incorporated very little of the administered dose (Table 8.1).

Following an oral dose of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid (400 µg/kg body wt), there was an imbalance in the radioactivity contained in the urine and faeces. More [^{3}H] (25.4% of the dose) than [^{14}C] (3.4% of the dose) (Table 8.1) was excreted in the total urine (0-48h) whereas an excess of [^{14}C] (35.3% of the dose) over [^{3}H] (16.8% of the dose) was found in the faeces. The differences in the recovery of [^{3}H] and [^{14}C] are statistically significant when compared

TABLE 8.1 Distribution of recovered radioactivity in the urine, liver and faeces of normal guinea pigs following the oral or intra-peritoneal administration of doses of either [2-^{14}C]- or [3',5',7-^{3}H] folic acid or a mixture of both species. Results expressed as mean % of administered dose $\pm$ standard deviation shown in brackets.

No. of animals	Dose administered (µg/kg body wt)	Mode of administration	Label	% recovery of radioactivity				
				Urine 0-24h	24-48h	Liver 48h	Faeces 48h	Total 48h
6	160	oral	[^{3}H]	30.0 (0.5)	1.1 (1.6)	1.7 (1.3)	2.2 (1.2)	35.0
4	400	oral	[^{3}H]	30.0 (5.8)	4.1 (1.6)	1.3 (0.5)	7.7 (5.4)	43.1
6	400 *	oral	[^{3}H]	23.4 (3.7)	2.0 (0.7)	1.2 (0.5)	16.8 (6.0)	43.4
			[^{14}C]	3.0 (0.9)	0.4 (0.2)	1.6 (0.5)	35.3 (9.5)	40.3
3	400	i.p.	[^{3}H]	32.4 (1.4)	3.2 (2.9)	30.9(1.3)	5.2 (3.2)	71.7
			[^{14}C]	28.2 (1.7)	0.5 (0)	14.8 (0.1)	5.8 (3.5)	49.3
3	1050	oral	[^{3}H]	24.1 (5.8)	N Dtm	NDtm	N Dtm	-
			[^{14}C]	3.5 (1.5)	"	"	"	-
5	1370	oral	[^{14}C]	4.4 (0.5)	0.5 (0.1)	0.1 (0.3)	4.8 (1.1)	10.0
3	2330	oral	[^{3}H]	16.9 (8.6)	N Dtm	N Dtm	N Dtm	-
			[^{14}C]	4.3 (1.8)	"	"	"	-

N Dtm = Not Determined

* = Very low levels of radioactivity found at this dose in the heart, kidneys, spleen, gut wall, stomach wall and bile

TABLE 8.2

Recovery of radioactivity in tissues and fluids following oral doses (400 μg/kg body wt) of a mixture of [2-^{14}C] - and [3',5',7,9-^{3}H]- folic acid.

Results expressed as mean % of administered dose

No. of animals	Tissues/fluids	Radioactivity recovered	
		^{3}H	^{14}C
5	Heart [1]	0.02	0.02
6	Kidneys [1]	0.03	0.01
6	Spleen [1]	0.02	0.01
2	Bile [2]	0.2	0.3
2	Gut wall [3]	0.2	0.3
2	Stomach wall [3]	0.1	0.1

1 48 h after dosing

2 2-3 h after dosing

3 7 h after dosing

in a paired 't' test in the urine and faeces ($p < 0.02$) but not significant in the liver, where the uptake of radioactivity remains very low (1.2% of the [^{3}H] dose, 1.6% of the [^{14}C] dose (Table 8.1). A similar significant excess of [^{3}H] over [^{14}C] is observed in the urine of the rat and man and of [^{14}C] over [^{3}H] in their faeces (Table 8.3). Following the administration of an oral dose of 100 µg/kg body wt of [^{3}H] and [^{14}C]-labelled folic acid to the rat, and of 71 µg/kg body wt in man, the rat urine accounted for 28.9% of the [^{3}H] dose and 22.6% of the [^{14}C] dose while in man, the recovery figures were 32.0% of the [^{3}H] dose and 22.6% of the [^{14}C] dose (Table 8.3).

However, in contrast to the guinea pig, the rat liver incorporates a higher amount of the administered folate (19.1% of the [^{3}H] dose, 20.7% of the [^{14}C] dose) (Table 8.3) than does the guinea pig liver (1.2% of the [^{3}H] dose, 1.6% of the [^{14}C] dose). These figures could, however, give a misleading picture of liver synthesis of polyglutamates in the two species since the dose administered to the guinea pig (400 µg/kg body wt) was four times the amount of that administered to the rat (Table 8.3). But, even if calculated on a weight to weight basis, the rat liver is found to incorporate more of the administered folate (2.5 µg [^{3}H] / g liver, 2.7 µg [^{14}C] / g liver) than the guinea pig liver (0.20 µg [^{3}H] / g liver, 0.27 µg [^{14}C] / g liver (Table 8.4). The total folate content of the entire liver amounts to 19.5 µg for the [^{3}H] dose and 21.1 µg for the [^{14}C] dose in the rat and 4.8 µg for the [^{3}H] dose

TABLE 8.3.

Species variation in the distribution of recovered radioactivity in man, the rat and the guinea pig following the oral administration of a dose of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid. Results expressed as % of administered dose.

	Rat △*		Man △ ○		Guinea pig □	
Dose	100 µg/kg body wt		71 µg/kg body wt		400 µg/kg body wt	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Urine	28.9	22.6	32.0	26.2	25.4	3.4
Faeces	15.8	28.3	10.3	26.6	16.8	35.3
Liver (total)	19.1	20.7	NDm	NDm	1.2	1.6
Gut	2.3	2.0	"	"	0.2	0.3
Kidney	1.6	1.6	"	"	0.03	0.01
Total	55.4	53.9	42.3	52.8	43.6	40.6

△ : From Saleh (1981)

N Dm : Not Determined

□ : Total 0-48 h except gut, 0-7h

* : 0 - 48 h Total

○ : Urine 0-24 h
Faeces 0-48 h

TABLE 8.4

Liver folate status in the rat and the guinea pig following oral doses of [^{3}H] and [^{14}C]-labelled folic acid

	RAT 100 µg/kg body wt		GUINEA PIG Dose 400 µg/kg body wt	
	^{3}H	^{14}C	^{3}H	^{14}C
As % of dose	19.1	20.7	1.2	1.6
As µg/g liver	2.5	2.7	0.20	0.27
As µg/total liver	19.5	21.1	4.8	6.4

and 6.4 μg for the [^{14}C] dose in the guinea pig. Given that the guinea pig liver incorporates about 2% of the administered dose and that this is equivalent to 10% compared to the rat, for a 4-fold increase in the administered dose (100 μg/kg body wt in the rat, 400 μg/kg body wt in the guinea pig) (Table 8.4), effectively, the uptake by the guinea pig liver is one-fortieth that of the rat liver, demonstrating poorer recovery in the guinea pig, although bearing in mind that liver uptake does not increase linearly with dose.

Chromatographic analysis of the pooled urine samples at various dose levels revealed the presence of a number of metabolites: urea, tritiated water, p-acetamidobenzoyl-L-glutamate, p-acetamidobenzoate and folic acid (Table 8.5). No reduced folate or folic acid could be detected in the urine at the lower dose levels of 160 or 400 μg/kg body wt of [3',5',7,9-^{3}H]-folic acid. Scission products were the only metabolites present. There was, however, a trace amount of folic acid (Table 8.5) excreted when a mixture of [^{3}H] and [^{14}C]-labelled folic acid was orally administered at 400 μg/kg body wt and this amount increased to about 1% of the dose when the orally-administered dose was increased to 1050 μg/kg body wt (Table 8.5). Although folic acid was excreted intact at higher doses in the normal guinea pig urine, there was still no reduced folate present, either as a function of time or dose.

The dominant metabolite in the urine of normal guinea pigs

after oral dosing was p-acetamidobenzoate (Table 8.5). Its percentage however, decreased in the 0-24h urine sample as the level of the administered dose was increased (Table 8.5). The level of tritiated water followed likewise, decreasing with increasing dose levels (Table 8.5).

The underlying trends in the guinea pig of very small amounts of liver folate incorporated, the absence of any reduced folate in the urine and the presence therein of large amounts of scission products point to the fact that either (a) the folates are not reduced or, (b) polyglutamates are poorly synthesized by the liver or (c) there is slow absorption of folic acid.

Poor reduction can be ruled out as analysis of bile and various body tissues (heart, kidneys, spleen, gut wall, and stomach wall) have failed to show any accumulation of folic acid anywhere in the guinea pig; nor is there any accumulation in the urine.

The low incorporation of radioactivity by the guinea pig liver, following an oral dose of labelled folic acid has already been emphasized earlier in this Chapter. The results obtained (Table 8.1) therefore suggests that the guinea pig liver is unable to synthesize folate polyglutamates. However, when the same dose of labelled folic acid (400 µg/kg body wt) is administered intra-peritoneally instead of orally, the guinea pig liver is found to accumulate an increased amount

TABLE 8.5 Metabolites present in the urine of normal guinea pigs following the administration of either [3',5',7,9-^{3}H] folic acid or a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]- folic acid. Results expressed as % of dose administered

No. of animals	Dose admin. µg/kg bodywt.	% of dose [^{3}H] recovered	Time period	Admin.	Label	Peak I [^{14}C]	$^{3}H_2O$	[^{3}H]p-AABglu	[^{3}H]p-AAB	Folic acid ^{3}H	Folic acid ^{14}C
6	160	30.0	0-24h	Oral	[^{3}H]		3.0	0.9	24.5	N Dt	
		1.1	24-48h				0.7	0.2	0.5	N Dt	
4	400	30.0	0-24h	Oral	[^{3}H]		2.0	4.0	20.4	N Dt	
		4.1	24-48h				1.6	0.8	0.4	N Dt	
6	400	23.1	0-24h	Oral	[^{3}H]+	0.4	1.9	1.4	17.5	0.003	0.002
		2.0	24-48h		[^{14}C]	0.1	0.7	0.04	0.2	0.004	0.004
4	400	32.4	0-24h	i.p.	[^{3}H] +	0.9	0.9	4.4	7.4	12.1	20.2
		3.2	24-48h		[^{14}C]	0.1	1.7	0.1	0.4	NDt	NDt
3	1050	24.1	0-24h	Oral	[^{3}H]+[^{14}C]	0.9	0.6	0.1	13.2	1.1	1.0
3	2330	16.9	0-24h	Oral	[^{3}H]+[^{14}C]	0.8	0.8	0.3	9.8	0.3	0.5

N Dt = Not Detected

of radioactivity (30.9% of the [^{3}H] dose, 14.8% of the [^{14}C] dose) (Table 8.1). This demonstrates that the guinea pig liver can and does synthesize polyglutamates if the folate monoglutamates are available as evidenced by the high liver content of radioactivity incorporated without the obligatory intervention of the gut.

The amount of [^{14}C] radioactivity incorporated in the liver and hence, the amount entering the animal suggests poor folic acid absorption in the guinea pig and certainly much slower absorption than is seen in the rat due mainly to the less acid microclimate of the guinea pig gut (Lucas, M.H., and Mankornthong, P., personal communication). Guinea pig urine is dominated by folic acid breakdown products, some p-acetamidobenzoyl-L-glutamate and mainly p-acetamidobenzoate. The most likely explanation is of cleavage of the C_9-N_{10} bond in the guinea pig gut occurring relatively faster than the absorption process across the gut wall.

C_9-N_{10} bond cleavage has already been suggested as a possible metabolic route to folate catabolism (Blair, 1958; Murphy et al., 1978) in the rat, to give p-aminobenzoyl-L-glutamate and a pterin, which would undergo further metabolism. The presence in guinea pig urine of the metabolites p-acetamidobenzoyl-L-glutamate, p-acetamidobenzoate and urea and of $^{14}CO_2$ in the exhaled air lends support to the view that C_9- N_{10} bond cleavage of the folic acid molecule occurs in the guinea pig and the products are further metabolized. Results so far obtained suggest that p-acetamidobenzoate is formed in the gut while

p-acetamidobenzoyl-L-glutamate originates from the tissues (Blair, 1976; Connor, 1979; Pheasant et al., 1981 ; Saleh, 1981). A similar metabolic process is believed to occur in man (Saleh, 1981).

Following the oral dosing of normal guinea pigs with 242 μg/kg body wt of [3,5-^{3}H] p-aminobenzoyl-L-glutamate (the equivalent contained in 400 μg of folic acid), a total recovery of 79.5% was obtained in the urine, liver and faeces (Table 8.6) while a similar dose administered intraperitoneally gave a total recovery of approximately half (37.6%) that amount (Table 8.6). Chromatographic analysis of the pooled urine samples showed in both cases, the presence of only two tritiated metabolites, p-acetamidobenzoate and p-acetamidobenzoyl-L-glutamate. The amounts recovered, however, varied according to the mode of administration.

When the compound was administered intraperitoneally, 33.3% of of the dose (Table 8.7) was recovered as p-acetamidobenzoyl-L-glutamate but only 1.0% as p-acetamidobenzoate. In contrast, following oral administration, 23.5% of the dose was recovered as p-acetamidobenzoyl-L-glutamate and 28.7% as p-acetamidobenzoate (Table 8.7), indicating the formation of p-acetamidobenzoate to be a function of the guinea pig small intestine.

A similar observation of orally administered p-aminobenzoyl-L-glutamate being metabolized to p-acetamidobenzoyl-L-glutamate and

TABLE 8.6.

Recovery of radioactivity in urine, liver and faeces, following the oral and i.p. administration (242 μg/kg body wt) of [3,5-^{3}H] p-aminobenzoyl-L-glutamate to normal guinea pigs

Results expressed as mean % of the dose ± standard deviation shown in brackets.

No. of animals	Mode of administration	Urine 0-24 h	Urine 24-48 h	Liver (48 h)	Faeces (48 h)	Total (48 h)
3	oral	56.7 (4.6)	3.5 (1.7)	0.3	19.0 (4.0)	79.5
3	i.p.	33.2 (14.3)	3.4 (0.8)	N.Dt.	1.0 (0.5)	37.6

N.Dt = Not Detected

TABLE 8.7

Metabolites identified in pooled normal guinea pig urine following oral and i.p. doses (242 μg/kg body wt) of [3,5-^{3}H]- p-aminobenzoyl-L-glutamate. Results expressed as % of dose administered.

Mode of administration	Time period	pAABglu	pAAB
Oral	0-24 h	22.2	27.2
	24-48 h	1.3	1.5
i.p.	0-24 h	30.1	0.6
	24-48 h	3.2	0.4

to p-acetamidobenzoate in the rat and in everted sacs of rat proximal jejunum has been reported by Pheasant et al., (1981). They also noted that more p-acetamidobenzoate was formed when p-aminobenzoyl-L-glutamate was administered orally than intra-peritoneally and suggested that the formation of p-acetamidobenzoate is a property of the rat small intestine (Pheasant et al., 1981).

The figures for total recovery of radioactivity (43.4% of the [^{3}H] dose, 40.3% of the [^{14}C] dose) (Table 8.1) following the oral administration of [^{3}H]- and [^{14}C]-labelled folic acid to normal guinea pigs, account for rather little of the administered level of folate. However, about 20% of the administered [^{14}C] radioactive dose was found present as $^{14}CO_2$ in the expired air, within the first 24 hours following oral dosing. This amount of [^{14}C] radioactivity together with that recovered in the urine, liver and faeces would therefore account for approximately 60.3% of the orally-administered [^{14}C] dose in the normal guinea pig.

The tritiated folic acid used in this series of experiments is labelled in the 3', 5', 7, and 9 positions with the tritium label distributed as follows : 3',5'-position: 42.5% ; 7-position: 25.5% and 9-position: 32% (Radiochemical Batch Analysis sheet H/2015/2, Amersham International Limited, Amersham, Bucks., U.K). Since a fairly high proportion of the radioactivity (57.5%) is distributed outside the p-aminobenzoyl-L-glutamate moiety, in the pterin portion of the

ring (positions 7 and 9), a correction is required for total folate recovery. At an oral dose of 400 μg/kg body wt of a mixture of [^{3}H] and [^{14}C]-labelled folic acid, the proper values for folate recovery after adjustment are for p-aminobenzoate, 41.6% and p-acetamidobenzoyl-L-glutamate, 3.4%. These new figures added to the recovery of 1.2% in the liver and 16.8% in the faeces would give a total folate recovery value of 63.0% based on the recovery of the [^{3}H] compound.

The formation of p-acetamidobenzoyl-L-glutamate and of p-acetamidobenzoate through C_9-N_{10} bond cleavage in the gut followed by further metabolism has already been mentioned. There are various ways by which folic acid may be broken down in the gut: it may be broken down itself or, reduced in the gut andbroken down by gut tissue, or gut bacteria; or, it may be reduced, then absorbed and the reduced folates broken down while being transported within bile to the gut. However, bile collected in cannulation experiments show very low levels of radioactivity present up to 7h after oral dosing (Table 3.1.6), with no intact folate and some scission products, making this route of folate breakdown unlikely to occur. But, analysis of gut contents flushed 2h after the oral administration (400 μg/kg body wt) of [^{3}H] and [^{14}C]-labelled folic acid show a reduced folate to be present, 5-methyltetrahydrofolate, together with scission products. As there exists little possibility of back diffusion should the 5-methyl-tetrahydrofolate have been formed within the gut tissue the most likely

source of this compound would be its formation by reduction by bacteria present in the gut of the guinea pig.

These gut bacteria would either break down folic acid as it appears in the gut, or, would reduce the folic acid first, then break it down afterwards. The chemical species brokden down could be folic acid, dihydrofolate, tetrahydrofolate or 5-methyltetrahydrofolate. Given the fact that tetrahydrofolate is the least stable of these compounds, it is the one which is most likely to undergo breakdown (Blair and Pearson, 1974).

Normal guinea pigs were orally dosed (400 µg/kg body wt) with a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-5 MeTHF, consisting only of the natural biologically-active isomer. The recovery of radioactivity in the urine, liver and faeces are summarized in Table 8.8 and show similar trends as seen with oral folic acid administration (Table 8.1) except for a few differences:

(i) a greater amount of [^{14}C] radioactivity was excreted in the urine

(ii) the liver retained a higher dose of the administered radioactivity (Table 8.8) and

(iii) a greater amount of the intact 5-methyltetrahydrofolate (0.9% of [^{3}H] dose, 0.9% of [^{14}C] dose) (Table 8.9) than intact folic acid excreted (trace amounts) in the urine following a similar dose of oral folic acid (Table 8.1).

TABLE 8.8

Recovery of radioactivity in the urine, liver and faeces following the oral administration (400 µg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]- 5MeTHF * to normal guinea pigs.

Results expressed as mean % of administered dose ± standard deviation shown in brackets.

No. of animals	Radioactive species	Urine 0 - 24h	Urine 24 - 48h	Liver (48)	Faeces (48h)	Total (48h)
3	[^{3}H]	10.8 (0.7)	3.8 (2.0)	2.5 (1.2)	18.2 (3.1)	35.3
	[^{14}C]	12.2 (0.9)	2.9 (1.1)	14.0 (9.5)	33.3 (9.3)	62.4

* The mixture contains only the natural biologically-active diastereoisomer

TABLE 8.9.

Metabolites recovered in the pooled urine samples from 3 normal guinea pigs following the oral administration (400 µg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] - 5 MeTHF *

Results expressed as % of the dose

Time period	[^{14}C] peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	5 MeTHF ^{3}H	5 MeTHF ^{14}C
0 - 24h	0.5	1.3	0.04	7.6	0.7	0.6
24-48 h	0.5	0.6	0.7	0.3	0.2	0.3

* This compound consists only of the natural biologically active diastereoisomer

The greater liver incorporation of labelled 5-methyltetrahydrofolate, especially of [^{14}C], suggests that the compound is more readily available to the guinea pig than folic acid.

Although some 5-methyltetrahydrofolate appears as scission products, as evidenced by the urinary metabolites (Table 8.9), some 5-methyltetrahydrofolate still passes across the gut intact. The compound therefore does not seem to be a strong candidate for scission. It can, however, be converted to a scissionable product by enzymic demethylation, e.g. tetrahydrofolate, which is then oxidized. The lability of tetrahydrofolate (Pearson, 1974) readily undergoing scission was mentioned earlier. Another source of scissionable products is the compound 5Me-5,6-dihydrofolate, identified in man (Ratanasthien et al., 1977) and formed by conversion of 5-methyltetrahydrofolate by transition metals (Gapski et al., 1971; Blair et al., 1975). 5-Me-5,6-dihydrofolate can readily undergo scission in the acid microclimate of the gut (Lucas, 1979).

The recovery of radioactivity in the urine, liver and faeces from normal guinea pigs following the oral administration of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-10 formylfolic acid is summarized in Table 8.10 and compared to that of normal guinea pigs similarly dosed with labelled folic acid. The following differences are seen in relation to the folic acid:

(i) a lower amount is excreted via the faeces and

TABLE 8.10

Comparative recovery of radioactivity in the urine, liver and faeces of normal guinea pigs, following an oral dose (400 μg/kg body wt) of either a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid or [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folic acid. Results are expressed as mean % of administered dose ± standard deviation shown in brackets.

No. of animals	Folate	Label	Urine		Liver	Faeces	Total	Corrected
			0 - 24h	24-48h	48 h	48 h	48 h	Total △
6	Folic acid	[^{3}H]	23.4 (3.7)	2.0 (0.7)	1.2 (0.5)	16.8 (6.0)	43.4	63.0
		[^{14}C]	3.0 (0.9)	0.4 (0.2)	1.6 (0.5)	35.3 (9.5)	40.3	60.3
5	10-formyl folic acid	[^{3}H]	35.1 (4.6)	2.3 (0.5)	0.6 (0.2)	5.5 (1.0)	43.5	83.7
		[^{14}C]	4.2 (2.0)	0.5 (0.3)	0.9 (0.1)	6.2 (1.3)	11.3	

△ Figures corrected for total folate and $^{14}CO_2$ in expired air

(ii) a higher amount is excreted in the urine and

(iii) liver incorporation is lower, amounting to only half the values of the folic acid.

Chromatographic analysis of the pooled urine samples following oral dosing with 10-formyl folic acid show the presence of a small amount of the administered compound but with large amounts of scission products, mostly p-acetamidobenzoate (Table 8.11). There is no evidence to suggest that the administered 10-formyl folic acid is first deformylated, then cleaved. It is most likely that the compound is first reduced, then deformylated and cleaved (evidence from gut washings, Chapter 6).

Table 8.12 summarizes the radioactivity recovered from urine, liver and faeces, following an oral dose (400 μg/kg body wt) of a mixture of [^{3}H] and [^{14}C]-labelled folic acid to guinea pigs pretreated for 10 days with the antibiotic 'Terramycin' (Kucers and Bennett, 1979). This antibiotic, however, may not clear the gut completely of bacteria. Although more potent bactericidal agents are known, their use is rather restricted as they are lethal to the guinea pig. Comparison of the guinea pig pretreated with antibiotics show the following differences in metabolism over their untreated counter-parts:

(i) the liver retention of radioactivity is lower

(ii) excretion of radioactivity via faeces is considerably reduced (Table 8.12)

(iii) large amounts of scission products appear in the urine and

TABLE 8.11 Metabolites found in the pooled urine samples from 5 guinea pigs following an oral dose (400 μg/kg body wt) of [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folic acid.

Results expressed as % of administered dose

	[^{14}C] peak I	[$^{3}H_2O$	^{3}HpAABglu *	^{3}HpAAB **	10-CHOFA
0 - 24 h	2.2	1.4	1.0	25.3	1.1 as [^{14}C]
24-48 h	1.4	0.2	0.3	1.3	0.5 as [^{14}C]

* correction for total folate 3.1% (0-48 h)

** correction for total folate 62.6% (0-48h)

TABLE 8.12 Recovery of radioactivity in the urine, liver and faeces from normal guinea pigs following the oral administration (400 μg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid in the absence and presence of antibiotics.

Results expressed as mean % of administered dose ± standard deviation shown in brackets.

No. of animals	Pre-treatment with antibiotics		Urine 0-24 h	Urine 24-48 h	Liver 48 h	Faeces 48 h	Total 48 h	Total * corrected
6	None	[^{3}H]	23.4 (3.7)	2.0 (0.7)	1.2 (0.5)	16.8 (6.0)	43.4	63.0
		[^{14}C]	3.0 (0.9)	0.4 (0.2)	1.6 (0.5)	35.3 (9.5)	40.3	60.3
5	Yes	[^{3}H]	23.9 (1.9)	2.3 (0.9)	0.2 (0)	0.4 (0.3)	26.8	49.6
		[^{14}C]	3.1 (0.3)	0.4 (0.2)	0.6 (0)	0.9 (1.0)	5.0	

* Figures corrected to total folate for [^{3}H] and for [$^{14}CO_2$] for [^{14}C]

(iv) a much larger amount of intact folic acid is excreted in the urine as opposed to a trace amount in the untreated guinea pigs (Table 8.13).

The presence of intact folic acid in the urine (0.3% of the [^{3}H] dose, 0.4% of the [^{14}C] dose) (Table 8.13) gives an indication of the possible role of gut bacteria in the conversion of folic acid to tetrahydrofolate.

Normal guinea pigs, orally pre-treated with methotrexate 8h before dosing, also produced large amounts of scission products (Table 8.14) following the oral administration (400 μg/kg body wt) of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid. Compared to the untreated guinea pigs (Table 8.14), methotrexate pre-treated guinea pigs produced in their urine a higher level of p-acetamidobenzoyl-L-glutamate, a lower level of p-acetamidobenzoate and a higher amount of intact folic acid (only a trace amount is excreted in the absence of methotrexate) (Table 8.14). This again points to the possible role of bacteria in the reduction of folic acid to tetrahydrofolate in the gut as MTX will stop this reduction stage. The significant reduction of p-AAB again suggests gut breakdown.

The process of scission, which was found to occur with folic acid, is now extended to two more compounds, 5-methyltetrahydrofolate and 10-formyl folate. Results, so far obtained, confirm that folic acid is not the only species for scission as other folates can be

TABLE 8.13

Changes in metabolites in pooled guinea pig urine following the administration of an oral dose of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid (400 µg/kg body wt) in normal guinea pigs and in guinea pigs pretreated with antibiotics. Results expressed as % of administered dose

No. of animals	Pre-treatment with antibiotics	Time period	Peak I [^{14}C]	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	Folic acid ^{14}C
6	None	0-24h	0.4	1.9	1.4	17.5	0.003	0.002
		24-48h	0.1	0.7	0.04	0.2	0.0004	0.0004
5	Yes	0-24h	1.9	3.4	NDt	19.9	0.2	0.3
		24-48h	0.1	2.3	0.04	0.76	0.06	0.1

N Dt = Not Detected

TABLE 8.14 Changes in metabolites in pooled guinea pig urine following the administration of an oral dose (400 µg/kg body wt) of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to normal guinea pigs and to guinea pigs pre-treated for 8h with methotrexate (80mg/kg body wt) also orally administered

Results expressed as % of administered dose

No. of animals	Pre-treatment with MTX	Urinary radioactivity recovered ^{3}H	^{14}C	Time period	[^{14}C] peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	^{14}C
6	None	23.4	2.0	0 -24h	0.4	1.9	1.4	17.5	0.003	0.002
		3.0	0.4	24-48h	0.1	0.7	0.04	0.2	0.0004	0.0004
4	Yes	19.2	4.5	0-24h	2.6	0.4	2.1	10.6	1.1	1.4
		4.4	1.0	24-48h	0.3	1.9	0.3	1.6	0.16	0.22

converted to scissionable products, then cleaved.

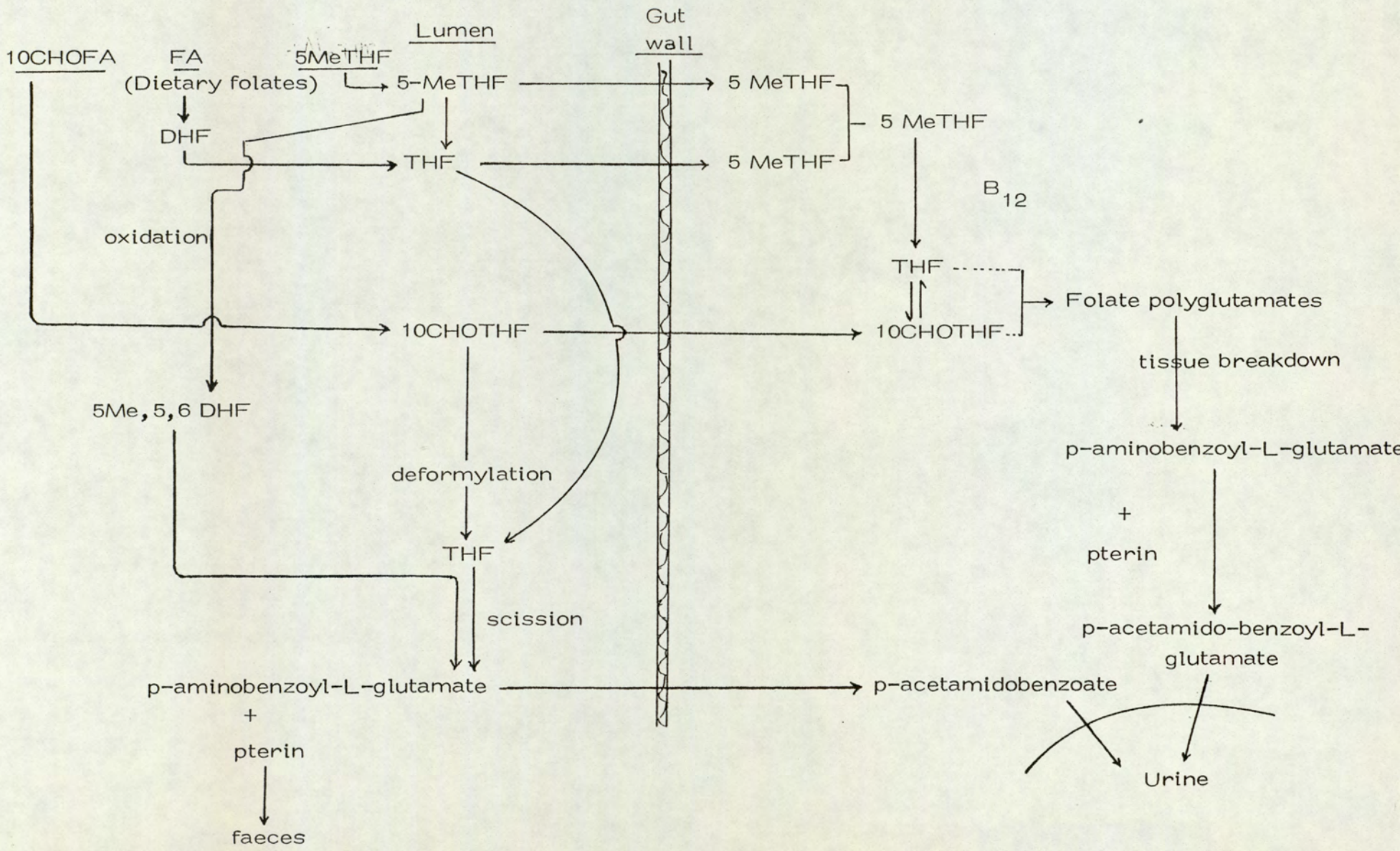
The routes shown in Figure 8.1 are the catabolic routes envisaged to occur in the guinea pig. Both folic acid and 10-formyl folic acid may be reduced in the guinea pig gut and enter the gut folate pools as THF or 10-formyl THF respectively.

The surface pH of the guinea pig gut being more alkaline than that of the rat or man (Lucas, M.H., and Mankornthong, P., personal communication) causes movement of folates across the guinea pig gut to be relatively slower than in the two other species.

Any folate surviving scission is probably converted to 5-MeTHF in transport across the gut wall (Figure 8.1) and goes via the portal vein to the liver to be incorporated into polyglutamates. The movement of 5-MeTHF, the major serum folate in man (Ratanasthien et al., 1977) across the gut wall does not involve prior demethylation, according to our present knowledge (Cooper, 1977; Kennelly, 1980).

Because of the slower rate of transport across the gut wall in the rather alkaline microclimate of the guinea pig gut, scission occurs at a relatively faster rate. As no enzymic evidence of scission is available, the process is likely to occur by chemical oxidation. Scission is decreased in the presence of phenytoin (A. Guest, personal communication) but no increase was observed in the presence of phenobarbitone (Saleh, 1981). 5-MeTHF is an unlikely candidate to

FIGURE 8.1. Suggested routes of folate catabolism in vivo in the guinea pig



undergo scission since some 5-MeTHF passes intact through the gut-wall. Likely species appear to be DHF or THF, due to their inherent chemical instability. As DHF is an excellent substrate for DHF reductase and is rapidly converted to THF (Goldman, 1977), the latter is now the most likely candidate for scission. Another species likely to undergo scission is the compound 5-Me-5,6-dihydrofolate formed by the oxidation of 5MeTHF in the gut (Ratanasthien et al., 1977) which can undergo C_9- N_{10} cleavage (Deits et al., 1976) under acidic conditions.

Several investigations have suggested that the possible catabolic route of folate would be via cleavage of the C_9-N_{10} bond to give p-aminobenzoyl-L-glutamate and a pterin (Blair, 1958; Weir, 1974; Murphy et al., 1978). The identification of the derivatives of p-aminobenzoyl-L-glutamate (pAAB and pAABglu) confirms that C_9-N_{10} bond cleavage does take place.

It is suggested that the folates are reduced by gut bacteria and the reduced folates cleaved by chemical oxidation. This is supported by the fact that a higher dose of folic acid is excreted as a urinary metabolite when the guinea pigs are pre-treated with methotrexate or with antibiotics. The p-aminobenzoyl-L-glutamate formed by cleavage in the guinea pig gut is metabolised to p-acetamidobenzoate, a function of the small intestine. This is supported by studies on the administration of oral and i.p. doses of labelled p-aminobenzoyl-L-glutamate to normal

guinea pigs (see Chapter 4). Results indicate that when orally administered, nearly all the excreted urinary radioactivity is p-acetamidobenzoate (Table 8.6, 8.7). As bile contains extremely low doses of radioactivity (Table 8.2) and no folic acid or reduced folate (Chapter 3), biliary excretion of radioactivity followed by scission seems to be an unlikely route.

Of the two processes occurring in the guinea pig gut, scission and transport across the gut wall, the latter appears to be relatively slower in the rather alkaline microclimate. In vitro studies showed that 10-CHOTHF and THF are the substrates for the synthesis of polyglutamates (Spronk, 1973; McGuire et al., 1979). 5-MeTHF may be incorporated into the tissue folate polyglutamate only after demethylation, as occurs in the methylation of homocysteine to methionine.

Following the oral administration of a labelled dose of [^{3}H] and [^{14}C]-folic acid, low levels of radioactivity were found in the livers of guinea pigs at 1, 2, 4, 6, 8 and 12 h (Table 7.1) after dosing (400 µg/kg body wt) and also at 48h after dosing (Table 8.15). This radioactivity, associated mainly with polyglutamates, shows that folates through the obligatory function of the gut, are not available to the guinea pig liver for polyglutamate synthesis, rather than the liver is incapable of synthesising folate polyglutamates, since a greater incorporation of radioactivity is achieved (Table 8.1) following a similar dose of folic acid administered intra-peritoneally. The

TABLE 8.15 Recovery of radioactivity in the livers of guinea pigs following oral or i.p. doses (400 μg/kg body wt) of a mixture of either. [2-^{14}C] and [3',5',7,9-^{3}H]-5MeTHF or [2-^{14}C] and [3',5',7,9-^{3}H]-10 formyl folate or [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Results expressed as % of administered dose.

Tye of folate	Mode of administration	State of guinea pigs	Liver radioactivity (48 h) ^{3}H	^{14}C
Folic acid	oral	normal	1.2	1.6
"	i.p.	normal	30.9	14.8
"	i.p.	Normal, MTX oral at time of dosing	4.1	5.6
"	i.p.	Normal, MTX oral after 8 h	13.5	17.8
"	oral	Normal, pretreated for 10 days with antibiotics	0.2	0.6
5MeTHF	oral	normal	2.5	14.0
10-CHOFA	oral	normal	0.6	0.9

presence of very low levels of p-acetamidobenzoyl-L-glutamate in the urine and its persistence on Day 2 again at a low level suggests that it arises by a metabolically distinct route from p-acetamidobenzoate which occurs in the urine in much larger amounts, especially on Day 1. p-Acetamidobenzoyl-L-glutamate is believed to arise from the breakdown of tissue (liver) folate polyglutamate producing p-aminobenzoyl-L-glutamate and a pterin both of which are metabolized further. The detection of urea and $^{14}CO_2$ indicates that considerable degradation of the pterin ring system occurs 'in vivo' in the guinea pig. A similar degradation of the pterin ring system also occurs in the rat producing $^{14}CO_2$ and urea, but as in the guinea pig, the mechanism of breakdown in the rat is still unclear (Pheasant et al., 1981).

The scission products in the 0-24h urine are thought to occur mainly by scission of the gut folates but the 24-48h pAABglu value may represent scission outside the gut from folates incorporated in the tissues. Thus the 24-48h urinary pAABglu value may give a clue as to the extent of the tissue folate breakdown process and this can be calculated as

$$\% \text{ breakdown} = \frac{x}{y} \times 100$$

where x = % pAABglu in the 24-48h urine, corrected to total folate and y = % of administered [^{3}H] folate dose retained in the body at 24h. The results are illustrated in Table 8.16 as % breakdown I.

An alternative way of calculating the % breakdown would be:

$$\% \text{ breakdown} = \frac{x}{z} \times 100$$

where x = % pAABglu in 24–48h urine, corrected to total folate and z = % [^{3}H] dose in the liver at 48h.

Results calculated by this method are shown in Table 8.16 as % breakdown II.

Again the results can be misleading because of certain assumptions made: x represents scission from tissue folate in the 24–48h period whereas z represents the amount of [^{3}H] radioactivity in the liver after 48h but not after 24 h. A correction factor is required and it was thought that $(x/x+z) \times 100$ might give a better indication of percentage breakdown of tissue folates. This method of calculation is represented in Table 8.16 as % breakdown III.

These percentage breakdown values, calculated in three different ways (Table 8.16), need to be interpreted with care because of the following assumptions made:

(a) the calculations made are based on the premise that the 24–48h pAABglu (after correction to total folate), represents breakdown of body folates

(b) that the error introduced is disregarded while counting pAABglu in the 24–48h urine but measuring liver folate at 48 h

instead of at 24h (% breakdown II, Table 8.16) and

(c) the total liver folate is not measured in man unlike in other species and hence, the late pAABglu in man will represent the whole body pAABglu, which is the only measurable pAABglu in man.

In spite of these assumptions, certain trends can still be discerned in the table (Table 8.16) in that there is some agreement with one another in the three methods of calculations. It appears that retained folates (Table 8.16) are broken down at a much faster rate when folic acid is administered orally than intra-peritoneally. The table also indicates that the liver burden is reduced with folate administered intra-peritoneally in the presence of methotrexate than in its absence, while scission is meaningfully increased. This increase in the rate of scission in the guinea pig has also been found to occur in the rat (Saleh, 1981), where MTX alters the normal metabolic processes by causing increased loss of radioactivity from tissues and the increased excretion of all normal catabolites. The greater percentage breakdown of tissue folates that appears to occur (Table 8.16) in the presence of antibiotics need, however, to be investigated further as a greater dose of intact folate is excreted in the urine in the absence of antibiotics (Table 8.13). The administration of antibiotics to the guinea pigs before (10 days) and during (2 days) the experiment (Chapter 3) may interfere with the gut processes and cause liver damage as the pro-

TABLE 8.16 Percentage breakdown of folates from body tissues following oral and i.p. doses (400µg/kg body wt) of a mixture of [^{3}H] and [^{14}C]-labelled folic acid, 5MeTHF or 10-CHOFA

Type of Folate	Mode of admin	Guinea pigs	% dose[1] (x) radioactivity as pAABglu	% [^{3}H][2] (y) retained in body	% [^{3}H][3] (z) retained in liver	% breakdown I[4] x/y x 100	% breakdown II[5] x/z x 100	% breakdown III[6] $\frac{x}{x+z}$ x100	[^{3}H] in liver Total (µg)	(48h) µg/g Liver
10-CHO-FA	Oral	Normal	0.70	57.1	0.6	1.23	116.7	53.8	2.4	0.13
Folic acid	Oral	Normal + Antibiotics[9]	0.10	73.4	0.4	0.14	25.0	20.0	1.6	0.06
5MeTHF	Oral	Normal	0.16	67.2	0.7	0.24	22.9	18.6	2.8	0.12
Folic acid	Oral	Normal	0.094	56.3	1.2	0.17	7.8	7.3	4.8	0.20
Folic acid	i.p.	Normal[7] + MTX 0h	0.24	60.6	4.1	0.40	5.8	5.5	16.4	0.6
Folic acid	i.p.	Normal + MTX + 8h[8]	0.34	65.3	13.5	0.5	2.5	2.5	54.0	2.5
Folic	i.p.	Normal	0.24	59.2	30.9	0.41	0.8	0.8	123.6	3.5

(1) % pAABglu excreted in 24–48 h urine corrected to total folate (denoted by x)

(2) [100% – sum of radioactivity excreted in urine and faeces for 48 h] denoted by y

(3) % of [^{3}H] administered dose incorporated in liver in 0–48 h (denoted by z)

(4) % breakdown of tissue folates calculated as $x/y \times 100$

(5) % breakdown of folates calculated as $x/z \times 100$

(6) % breakdown of folates calculated as $x/x+z \times 100$

(7) MTX (80 mg/kg body wt) administered orally at the time of dosing

(8) MTX (80 mg/kg body wt) administered orally 8h after dosing

(9) Guinea pigs pre-treated with 'Terramycin" for 10 days prior to experimentation and during the experiment

duction of p-AABglu is virtually stopped. pAABglu is still produced (pAABglu:pAAB ratio, approx. 50:50) when [3,5-^{3}H]pABglu is orally administered to normal guinea pigs in the urine (Table 8.7), showing oral pABglu escaping scission. Antibiotics (Table 8.16) bring about a substantial amount of scission in the liver, as indicated by the % breakdown figures. The trends in the table also indicate a greater amount of breakdown when 5-MeTHF is orally administered and massive breakdown of tissue folates following the administration of 10-CHOFA. The compound is a known inhibitor of the enzyme dihydrofolate reductase both in vitro (Zakrewski, 1960; Bertino et al., 1965) and in vivo (Saleh, 1981). Inhibition will therefore lead to a build-up of dihydrofolate which would therefore lead to an increase in scission and hence, an increase in the amount of scission products in the urine.

Urinary scission products were different to those previously seen when an oral dose (400 µg/kg body wt) of [5-^{14}C] 5 MeTHF was administered to normal guinea-pigs. The compound which consists of an equimolar mixture of the biologically active and biologically inactive diastereoisomers, indicates the fate of the methyl position of the molecule. The recovery of radioactivity in urine, liver, faeces and expired air is summarized in Table 8.17 and compared to the recovery of [2-^{14}C] 5MeTHF, the latter being orally given at a similar dose mixed with [3',5',7,9-^{3}H] 5MeTHF (Table 8.17). The major portion of the radioactivity (24.0%) is excreted via the faeces and a

TABLE 8.17

Recovery of radioactivity in urine, liver and faeces following the oral administration of either a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] 5 MeTHF or [5-^{14}C] 5MeTHF to normal guinea pigs (400 µg/kg body wt). Results are expressed as mean % of the dose ± standard deviation shown in brackets.

Radioactive species	No. of animals		Urine 0-24h	Urine 24-48h	$^{14}CO_2$	Liver (48 h)	Faeces (48h)	Total 48h
[^{3}H] + [^{14}C]	3	[^{3}H]	10.8 (0.7)	3.8 (2.0)	-	2.5 (1.2)	18.2 (3.1)	35.3
5 MeTHF		[^{14}C]	12.2 (0.9)	2.9 (1.1)	N Dtm	14.0 (9.5)	33.3 (9.3)	62.4
[5-^{14}C]5 MeTHF	4	[^{14}C]	4.0 (1.4)	1.3 (0.2)	6.2	1.2 (0.5)	24.0 (2.6)	36.7

N Dtm = Not determined

substantial amount (6.2%) is passed out in the exhaled air in the first 24 hours following oral dosing. Urinary excretion of radioactivity is very low (5.3% total for 48h) (Table 8.17) compared to the [2-^{14}C] label (15.1% for 48h) and a very low level of radioactivity is incorporated in the liver (Table 8.17).

Urinary excretion of radioactivity following an oral dose (80 µg/kg body wt) of [5-^{14}C] 5MeTHF is much higher (54.0%) in the rat (Kennelly, 1980). It is associated mainly with 5MeTHF and is thought to be the biologically inactive isomer. Radioactivity in the urine is found to be associated with creatinine (Table 8.18), but more confirmation is required. Some 5-MeTHF (1.2%) is also excreted and this is most likely to be the inactive biological isomer. This lower recovery of 5MeTHF in the guinea pig urine suggests intensive breakdown of the compound in the guinea pig.

Folic acid metabolic studies in the scorbutic guinea pig shows a gradual decrease in the total recovery of radioactivity with increasing orally-administered dose levels (Table 8.19). As with normal guinea pigs, there is an imbalance of radioisotopes with a considerable excess of [^{3}H] over [^{14}C] in the urine but more [^{14}C] than [^{3}H] in the faeces and in the liver (Table 8.19) at all three dose levels.

The urinary metabolites were mainly scission products dominated by p-acetamidobenzoate, with a fair amount of intact folate excreted as folic acid (Table 8.20). However, an additional metabolite is found

TABLE 8.18

Metabolites present in pooled normal guinea pig urine (from 4 animals), following the oral administration of a dose (400 µg/kg body wt) of [5-^{14}C] 5 MeTHF

Results expressed as % of the administered dose

Time	Peak I	Creatinine	5 MeTHF
0 - 24h	1.1	0.5	1.0
24-48h	0.3	0.3	0.2

TABLE 8.19 Recovery of radioactivity in the urine, liver and faeces of scorbutic guinea pigs following the oral administration of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid. Results expressed as mean % of administered dose $\pm$ standard deviation shown in brackets.

No. of animals	Dose (oral) µg/kg body wt.	% recovery of radioactivity					
		Urine		Liver	Faeces	Total	Corrected*
		0-24h	24-48h	48h	48h	48h	total
6	400 [^{3}H]	17.5 (8.7)	4.6 (1.5)	0.9 (0.8)	12.7 (5.1)	35.7	38.3
	[^{14}C]	4.7 (1.9)	2.0 (1.3)	1.5 (1.6)	23.3 (7.3)	31.5	
3	932 [^{3}H]	11.8 (3.5)	2.2 (0.8)	0.6 (0.1)	7.2 (1.7)	21.8	32.2
	[^{14}C]	2.0 (0.6)	0.7 (0.1)	0.9 (0)	14.1 (3.6)	17.7	
3	2185 [^{3}H]	5.7 (1.4)	3.0 (2.2)	0.3 (0.1)	5.7 (0.9)	14.7	16.8
	[^{14}C]	1.2 (0.5)	0.7 (0.5)	0.5 (0.3)	8.0 (0.2)	10.4	

* Total recovery corrected to total folate for [^{3}H]

TABLE 8.20 Metabolites present in the pooled urine of scorbutic guinea pigs following oral administration of a mixture of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid.

No. of animals	Dose µg/kg bodywt.	Time period	[^{14}C] peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	Folic acid ^{14}C
6	400	0 -24h	1.7	0.7	0.7	7.7	0.5	0.2
		24-48h	1.0	1.2	0.7	1.0	0.4	0.4
3	932	0 -24h	1.3	0.6	0.2	9.0 *	0.2	0.4
		24-48h	0.3	0.4	0.1	1.0	0.0002	0.1
3	2185	0 -24h	0.7	0.4	0.1	3.8 *	0.1	0.2
		24-48h	0.2	1.5	0.1	0.5	0.1	0.1

* p-aminobenzoate (pAB) found as an additional urinary metabolite at these doses

in the urine at the two higher doses and is not seen in the urine from normal guinea pigs at comparatively similar dose levels. This metabolite, p-aminobenzoate, shows that the scorbutic animal is unable to acetylate completely p-aminobenzoate. A similar finding was made by Belavady and Banerjee (1954) who noted that scorbutic guinea pigs acetylated a lesser amount of injected p-aminobenzoic acid. Following an oral dose (242 µg/kg body wt) of p-aminobenzoyl-L-glutamate to the scorbutic guinea pig, (the amount as contained in 400 µg of folic acid), 56.4% (Table 8.23) were recovered in the urine and 12.8% in the faeces (Table 8.23). The urinary radioactivity was associated mainly with p-acetamidobenzoate (Table 8.24) demonstrating, as in the normal guinea pig, the function of the small intestine in metabolising p-aminobenzoyl-L-glutamate to p-acetamidobenzoate. A similar dose level of p-aminobenzoyl-L-glutamate given intra-peritoneally to scorbutic guinea pigs produced a urinary excretion value of 73.8% (Table 8.23) which was found to be associated mainly with p-acetamidobenzoyl-L-glutamate (50.5%) and a small amount of p-acetamidobenzoate (12.1%) (Table 8.24).

In scorbutic guinea pigs, a smaller total recovery figure (38.3% of [^{3}H] dose, 51.5% of [^{14}C] dose) is obtained following the oral administration of folic acid than with normal guinea pigs (63.0% of [^{3}H] dose, 60.3% of [^{14}C] dose) (Table 8.21). Fairly little radioactivity is incorporated in the liver in either state but excretion via

TABLE 8.21 Comparison of recoveries of radioactivity from the urine, liver and faeces of normal and scorbutic guinea pigs following an oral dose (400 μg/kg body wt) of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H] folic acid. Results expressed as mean % of dose administered ± standard deviation shown in brackets.

No. of animals	Guinea pigs	% RECOVERY OF RADIOACTIVITY					
		Urine		Liver	Faeces	Total	Corrected
		0-24h	24-48 h	48 h	48 h	48 h	Total *
6	Normal [^{3}H]	23.4 (3.7)	2.0 (0.7)	1.2 (0.5)	16.8 (6.0)	43.4	63.0
	[^{14}C]	3.0 (0.9)	0.4 (0.2)	1.6 (0.5)	35.3 (9.5)	40.3	60.3
6	Scorbutic [^{3}H]	17.5 (8.7)	4.6 (1.5)	0.9 (0.8)	12.7 (5.1)	35.7	38.3
	[^{14}C]	4.7 (1.9)	2.0 (1.3)	1.5 (1.6)	23.3 (7.3)	31.5	

* Corrected for total folate [^{3}H] radioactivity and for [^{14}C] radioactivity as $^{14}CO_2$

TABLE 8.22

Comparison of urinary metabolites in pooled guinea pig urine from normal and scorbutic animals following the oral administration of a mixture of [^{3}H]- and [^{14}C] labelled folic acid (400 µg/kg body wt)

No. of animals	Guinea pigs	Time period	[^{14}C] peak I	$^{3}H_2O$	^{3}HpAABglu	^{3}HpAAB	Folic acid ^{3}H	Folic acid ^{14}C
6	Normal	0–24h	0.4	1.9	1.4	17.5	0.003	0.002
		24–48h	0.1	0.7	0.04	0.2	0.004	0.004
6	Scorbutic	0 –24h	1.7	0.7	0.7	7.7	0.5	0.2
		24–48h	1.0	1.2	0.7	1.0	0.4	0.4

TABLE 8.23

Recovery of radioactivity in urine, liver and faeces following the oral and i.p. administration (242 µg/kg body wt) of [3,5-^{3}H] p-aminobenzoyl-L-glutamate to scorbutic guinea pigs.

Results expressed as mean % of dose ± standard deviation shown in brackets

No. of animals	Admin.	Urine		Liver	Faeces	Total
		0 - 24h	24-48h	(48h)	(48h)	(48h)
3	Oral	56.4 (4.8)	2.3 (0.8)	0.08	12.8 (9.2)	71.5
3	i.p.	73.8 (17.9)	1.6 (1.1)	1.5	1.7 (1.8)	78.6

TABLE 8.24

Metabolites found in pooled scorbutic guinea pig urine following a dose (242 μg/kg body wt) of [3,5-^{3}H] p-aminobenzoyl-L-glutamate.

Results expressed as % of administered dose.

	Oral administration		i.p. administration	
Time	pAABglu	pAAB	pAABglu	pAAB
0 - 24h	5.5	47.9	50.5	17.1
24-48h	0.5	0.9	0.7	0.9

faeces is much higher in normal guinea pigs (16.8% of [^{3}H] dose, 35.3% of [^{14}C] dose) than in the scorbutics (12.7 % of [^{3}H] dose, 23.3% of [^{14}C] dose) (Table 8.21).

A comparison of the urinary metabolites (Table 8.22) shows that a greater amount of scission occurs in the normal guinea pig, producing increased amounts (17.9%) of p-acetamidobenzoate, with trace amounts of folic acid. Comparatively, the scorbutic guinea pig urine contains a smaller amount of scission products, still dominated by p-acetamidobenzoate but a much larger amount of folic acid (0.9% [^{3}H] dose, 0.6% [^{14}C] dose) is excreted intact (Table 8.22). This may be due to decreased reduction in the gut and therefore, less material being available for scission. The poor recovery of $^{3}H_2O$ may be due to its distribution over the whole body.

Table 8.25 gives comparative data of the urinary metabolites recovered when a dose (242 µg/kg body wt) of [3, 5-^{3}H] - p-Aminobenzoyl-L-glutamate is orally administered to normal and scorbutic guinea pigs and Table 8.26 summarizes the case for a similar dose administered intra-peritoneally. Both the normal and the scorbutic guinea pigs produce substantial amounts of p-AAB when the original compound was orally administered (Table 8.25) than when administered intra-peritoneally (Table 8.26), demonstrating that in the scorbutic guinea pig too, removal of the glutamate moiety is a function of the small intestine.

TABLE 8.25

Comparison of urinary metabolites following the oral administration of a dose of [3,5-^{3}H] pABglu (242 µg/kg body wt) to 3 normal and 3 scorbutic guinea pigs.

Results expressed as % of the administered dose from the pooled urine samples.

	pAABglu		pAAB	
Time period	Normal	Scorbutic	Normal	Scorbutic
0 - 24 h	22.2	5.5	27.2	47.9
24-48 h	1.3	0.5	1.5	0.9
Total	23.5	6.0	28.7	48.8

TABLE 8.26

Comparison of urinary metabolites following the i.p. administration of a dose (242 μg/kg body wt) of [3,5-^{3}H]pABglu to 3 normal and 3 scorbutic guinea pigs.

Results expressed as % of the administered dose from the pooled urine samples.

	pAABglu		pAAB	
Time period	Normal	Scorbutic	Normal	Scorbutic
0 - 24h	30.1	50.5	0.6	17.1
24 - 48h	3.2	0.7	0.4	0.9
Total	33.3	51.2	1.0	18.0

The rates of breakdown of tissue folates in the scorbutic guinea pig for the three oral doses administered have been worked out in a similar way to those of the normal and making similar assumptions (Table 8.16). Results, so obtained, together with the liver burden of folates, are summarized in Table 8.27 and again, a great deal of care needs to be exercised in the interpretation of these results because of the assumptions.

No clear trend can, however, be seen in the Table (Table 8.27) whereby the percentages obtained by the three methods of calculation agree with one another. Based on earlier assumptions, and also from evidence from the raw data, it would appear (Table 8.28) that a greater rate of liver folate breakdown occurs in the scorbutic guinea pig at whatever dose compared to the normal, although the scorbutic liver has a diminished capacity of incorporating orally-administered folate (Table 8.21) than the normal.

The model put forward for folate catabolism in the normal guinea pig (Figure 8.1) can now be extended to cover the scorbutic animal. Results indicate that in the absence of vitamin C and hence in scurvy, the guinea pig's reducing capacity is diminished, as evidenced by the much larger excretion of intact folic acid in the urine of the scorbutic animal (Table 8.22) as opposed to the normal. As the reduced folates are more prone to spontaneous chemical scission, failure to reduce totally the folic acid would result in the urinary

TABLE 8.27

Percentage breakdown of folates from body tissues following oral doses of a mixture of [2-^{14}C] and [3',5',7,9-^{3}H]-folic acid to scorbutic guinea pigs

Dose µg/kg body weight	% dose (1) radioactivity excreted as pAABglu (x)	% ^{3}H (2) retained in body (y)	% ^{3}H (3) in liver (z)	% breakdown I (4) $\frac{x}{y} \times 100$	% breakdown II (5) $\frac{x}{z} \times 100$	% breakdown III (6) $\frac{x}{x+z} \times 100$	Liver [^{3}H] at 48 h	
							Total (µg)	µg/g Liver
400	1.65	65.2	0.9	2.5	183	64.7	3.6	0.10
932	0.24	71.5	0.6	0.34	40	28.6	5.6	0.20
2185	0.24	83.7	0.3	0.29	80	44.4	6.6	0.37

(1) 24-48 h urinary pAABglu corrected to total folate - denoted as x

(2) [100% - (% dose excreted in urine (24h) and faeces (48h)-denoted as y)

(3) [^{3}H] liver radioactivity at 48h

(4) % breakdown (I) calculated as x/y x 100

(5) % breakdown II calculated as x/z x 100

(6) % breakdown III calculated as (x/x+z) x 100

TABLE 8.28

Comparison of percentage breakdown of tissue folates following an oral dose (400 μg/kg body wt) of [2-^{14}C]- and [3',5',7,9-^{3}H]-folic acid to the normal and scorbutic guinea pig.

No. of animals	Guinea pigs	% dose (1) radioactivity as pAABglu (x)	% [^{3}H] (2) retained in body (y)	% [^{3}H] (3) in liver (z)	% breakdown I (4) $\frac{x}{y} \times 100$	% breakdown II (5) $\frac{x}{z} \times 100$	% breakdown III (6) $\frac{x}{x+z} \times 100$	Liver [^{3}H] at 48 h	
								Total (μg)	μg/g Liver
6	Normal	0.094	56.3	1.2	0.17	7.8	7.3	4.8	0.20
6	Scorbutic	1.65	65.2	0.9	2.5	183	64.7	3.6	0.10

(1) 24-48 h pAABglu corrected to total folate (denoted as x)

(2) [100% - (% dose excreted in urine (0-24h) and faeces (0-48h)] - denoted as y

(3) [^{3}H] liver radioactivity at 48h

(4) % breakdown I calculated as (x/y x 100)

(5) % breakdown II calculated as (x/z x 100)

(6) % breakdown III calculated as (x/x+z) x 100

excretion of intact folic acid.

The greater rate of tissue folate breakdown may be brought about by changes in the cytosolic redox potential in the liver. The scorbutic guinea pig liver has a more oxidising environment than that of the normal (as shown by the NAD/NADH ratio) (Table 8.29) and would therefore result in a greater rate of folate degradation. When extended to other species, it is seen that human liver has more oxidative conditions than rat liver, with a NAD/NADH ratio of 1871 (Saleh, 1981), which in turn is more oxidising than guinea pig liver (Williamson et al., 1967) with a NAD/NADH ratio of 725 (rat) and guinea pig liver (Garber and Hanson, 1971), ratio 293. The reduced cytosolic environment of the guinea pig might explain the greater rate of folate breakdown in the guinea pig and the lower rate in the rat and man. Although based on different time scales (8-24h urinary pAABglu in the rat and 24-48h in the guinea pig), the % liver folate scission calculated still show a greater rate of scission in the scorbutic guinea pig where the cytosolic capacity is considerably reduced (Table 8.29).

Folic acid has been recognized as an essential nutrient in the guinea pig (Woolley and Sprince, 1945) and dietary deficiency is characterized by growth failure, poor survival and anaemia (Woodruff et al., 1953). Because of its requirement for folates like man, and because, like man, the guinea pig is prone to scurvy if the supply of vitaminc C is inadequate, the animal can provide some insight into folic

TABLE 8.29

Species variation in liver cytosolic [NAD]/[NADH] ratio in conjunction with the excretion of the catabolite of folate polyglutamate (pAABglu)

Species	[NAD] / [NADH] ratio	% scission of body folates (C)			% dose excreted as pAABglu
		I	II	III	
Man	1871 (1)			NA	4.7 (2) Δ
Rat	725 (2)			19.3 (5)	2.1 (2) ○
Guinea pig	344 (3)	2.5	18.3	64.7	1.65 (6)
Guinea pig	299 (4)	0.17	7.8	7.3	0.09 (6)

N.A. = Data not available

(1) R.H. Harris, personal communication

(2) From Saleh, A.M., 1981

(3) Data from Garber and Hanson (1971) from fasted guinea pigs

(4) Data from Garber and Hanson (1971) from normal guinea pigs

(5) From Saleh et al., 1980

(6) See Table 8.28

Δ Value for 6-24h urine

○ Value for 8-24h urine

acid metabolism in man, using the animal model.

1 The principal urinary folate in scurvy in man is 10-formyl folate (Cox et al., 1966; Stokes et al., 1975). Subsequent daily administration of ascorbic acid to a patient suffering from megaloblastic anaemia in scurvy caused a change in the main urinary folate from 10-formyl folate to 5-MeTHF. As 10-formyl THF (a normal serum folate component) is readily oxidized in the body to 10-formyl folate and excreted essentially unchanged, the process will steadily withdraw folate from the serum folate pool, ultimately resulting in body depletion of folates and large urinary excretion of 10-formyl folates (Stokes et al., 1975). A function postulated for ascorbic acid in man would be to reduce the rate of oxidation of 10-formyl THF and thereby keep the metabolic pool available (Stokes et al., 1975).

In the scorbutic guinea pig, no change in the nature of urinary metabolites is observed except at very high doses when p-aminobenzoate is excreted in the unacetylated form. There is increased excretion of scission products (Table 8.22) and increased excretion of intact folic acid, presumably due to decreased reduction in the gut and hence, less material becoming available for scission. The scorbutic guinea pig liver also shows a greater rate of folate breakdown (Table 8.28).

SUGGESTIONS FOR FURTHER WORK :

The experiments carried out and described in this thesis leave many questions unanswered. 10-Formyl folate is excreted in man, largely unchanged (Saleh, 1981) but recent evidence (Pheasant et al., 1981) suggests that there is extensive metabolism of this compound in the rat. In the guinea pig, the compound is metabolised to give largely scission products while the liver shows extensive folate breakdown to occur. The study of the metabolism of its reduced form, 10-formyl THF, in both the normal and the scorbutic guinea pig would contribute substantially to the model of folate metabolism in the animal. The results of the breakdown of tissue folates following a dose of labelled folic acid to guinea pigs pretreated with antibiotics also needs to be further investigated because of the fairly high percentage of liver folate breakdown that appears to take place and also because the level of pAABglu produced is extremely low.

Fukushima and Nixon (1980) have reported that micro-organisms located within rat caecal contents catabolised [2-^{14}C]-pteridines under anaerobic conditions to $^{14}CO_2$ and it is possible that this may occur 'in vivo'. This emphasizes the need to measure [^{14}C] radioactivity in the exhaled air from both normal and scorbutic guinea pigs, using different folates in order to account for more of the [^{14}C] radioactivity which so far has been rather low.

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